Appendix CV

June A. Munford
Curriculum Vitae

Education

University of Wisconsin-Milwaukee - MS, Library & Information Science, 2009
Milwaukee, WI

- Coursework included cataloging, metadata, data analysis, library systems, management strategies and collection development.
- Specialized in library advocacy, cataloging and public administration.

Grand Valley State University - BA, English Language & Literature, 2008
Allendale, MI

- Coursework included linguistics, documentation and literary analysis.
- Minor in political science with a focus in local-level economics and government.

Professional Experience

Researcher / Expert Witness, October 2017 – present
Freelance • Pittsburgh, Pennsylvania & Grand Rapids, Michigan

- Material authentication and public accessibility determination. Declarations of authenticity and/or public accessibility provided upon research completion. Experienced with appeals and deposition process.

- Research provided on topics of public library operations, material publication history, digital database services and legacy web resources.

- Past clients include Alston & Bird, Arnold & Porter, Baker Botts, Fish & Richardson, Erise IP, Irell & Manella, O'Melveny & Myers, Perkins-Coie, Pillsbury Winthrop Shaw Pittman and Slayden Grubert Beard.

Library Director, February 2013 - March 2015
Dowagiac District Library • Dowagiac, Michigan

- Executive administrator of the Dowagiac District Library. Located in

Southwest Michigan, this library has a service area of 13,000, an annual operating budget of over $400,000 and total assets of approximately $1,300,000.

- Developed careful budgeting guidelines to produce a 15% surplus during the 2013-2014 & 2014-2015 fiscal years while being audited.

- Using this budget surplus, oversaw significant library investments including the purchase of property for a future building site, demolition of existing buildings and building renovation projects on the current facility.

- Led the organization and digitization of the library's archival records.

- Served as the public representative for the library, developing business relationships with local school, museum and tribal government entities.

- Developed an objective-based analysis system for measuring library services - including a full collection analysis of the library's 50,000+ circulating items and their records.

November 2010 - January 2013
Librarian & Branch Manager, Anchorage Public Library • Anchorage, Alaska

- Headed the 2013 Anchorage Reads community reading campaign including event planning, staging public performances and creating marketing materials for mass distribution.

- Co-led the social media department of the library's marketing team, drafting social media guidelines, creating original content and instituting long-term planning via content calendars.

- Developed business relationships with The Boys & Girls Club, Anchorage School District and the US Army to establish summer reading programs for children.

June 2004 - September 2005, September 2006 - October 2013
Library Assistant, Hart Area Public Library
Hart, MI

- Responsible for verifying imported MARC records and original MARC

cataloging for the local-level collection as well as the Michigan Electronic Library.

- Handled OCLC Worldcat interlibrary loan requests & fulfillment via ongoing communication with lending libraries.

Professional Involvement

Alaska Library Association - Anchorage Chapter
- Treasurer, 2012

Library Of Michigan
- Level VII Certification, 2008
- Level II Certification, 2013

Michigan Library Association Annual Conference 2014
- New Directors Conference Panel Member

Southwest Michigan Library Cooperative
- Represented the Dowagiac District Library, 2013-2015

Professional Development

Library Of Michigan Beginning Workshop, May 2008
Petoskey, MI
- Received training in cataloging, local history, collection management, children's literacy and reference service.

Public Library Association Intensive Library Management Training, October 2011
Nashville, TN
- Attended a five-day workshop focused on strategic planning, staff management, statistical analysis, collections and cataloging theory.

Alaska Library Association Annual Conference 2012 - Fairbanks, February 2012
Fairbanks, AK
- Attended seminars on EBSCO advanced search methods, budgeting, cataloging, database usage and marketing.

Depositions

2019 • Fish & Richardson

Apple v. Qualcomm (IPR2018-001281, 39521-00421IP, IPR2018-01282 and 39521-00421IP2)

2019 • Erise IP

Implicit, LLC v. Netscout Systems, Inc (Civil Action No. 2:18-cv-53-JRG)

2019 • Perkins-Coie

Adobe Inc. v. RAH Color Technologies LLC (Cases IPR2019-00627, IPR2019-00628, IPR2019-00629 and IPR2019-00646)

2020 • O'Melveny & Myers

Maxell, Ltd. v. Apple Inc. (Case No. 5:19-cv-00036-RWS)

2021 • Pillsbury Winthrop Shaw Pittman LLP

Intel v. SRC (IPR2020-1449)

2022 • Perkins-Coie

Realtek v. Future Link (IPR2021-01182)

2023 • Fish & Richardson

Neuroderm Ltd. v. Abbvie, Inc (Case No. PGR2022-00040)

2023 • Fish & Richardson

Nearmap US Inc. v. Pictometry International Corp. (IPR2022-00735)

2023 • Fish & Richardson

Samsung Electronics v. MemoryWeb LLC (Case No. 39843-0136PS1)

2023 • Pillsbury Winthrop Shaw Whitman LLP

Gravel Rating Systems v. Costco Wholesale Corp. (Civil Action No. 4:21-cv-149-ALM)

2024 • Willkie-Farr

Netflix, Inc. v. VideoLabs. Inc. (IPR2023-00628)

Limited Case and Clientele History

Alston & Bird

- Ericsson

  v. Collision Communications (IPR2022-01233)

- Nokia

  v. Neptune Subsea, Xtera (Case No. 1:17-cv-01876)

- Universal Electronics Inc

  v. Roku Inc (IPR2022-00818)

Arnold & Porter

- Ivantis

  v. Glaukos (Case No. 8:18-cv-00620)

- Samsung

  v. Jawbone (Case No. 2:21-cv-00186)

Benesch Friedlander Coplan & Aronoff

- Voyis

  v. Cathx (Case No. 5:21-cv-00077-RWS)

Deschert LLP

- Smaxtec Animal Care

  v. ST Reproductive Technologies, LLC (IPR2024-00885)

Erise I.P.

- Apple

  v. Ericsson Inc. (IPR2022-00715)

  v. Future Link Systems (IPRs 6317804, 6622108, 6807505, and 7917680)

  v. INVT (Case No. 20-1881)

  v. Navblazer LLC (IPR2020-01253)

  v. Qualcomm (IPR2018-001281, 39521-00421IP, IPR2018-01282, 39521-00421IP2)

  v. Quest Nettech Corp (Case No. 2:19-cv-00118-JRG)

  v. Telefonaktiebolaget LM Ericsson (IPR2022-00275)

  v. Theta IP, LLC (IPR2024-00818)

- Fanduel

  v. CGT (Case No. 19-1393)

- Garmin

  v. Phillips North America LLC (Case No. 2:19-cv-6301-AB-KS)

- Netscout
  - v. Longhorn HD LLC (Case No. 2:20-cv-00349)
  - v. Implicit, LLC (Civil Action No. 2:18-cv-53-JRG)

- Sony Interactive Entertainment LLC
  - v. Bot M8 LLC (IPR2020-01288)
  - v. Infernal Technology LLC (Case No. 2:19-CV-00248-JRG)

- Unified Patents
  - v. GE Video Compression (Civil Action No. 2:19-cv-248)

Fish & Richardson

- Apple
  - v. AliveCor (3:21-cv-03958)
  - v. LBS Innovations (Case No. 2:19-cv-00119-JRG-RSP)
  - v. Koss Corporation (IPR2021-00305)
  - v. Masimo (IPR 50095-0012IP1, 50095-0012IP2, 50095-0013IP1, 50095-0013IP2, 50095-0006IP1, 50095-0135IP1)
  - v. Neonode (Case No. 21-cv-08872-EMC)
  - v. Qualcomm (IPR2018-001281, 39521-00421IP, IPR2018-01282, 39521-00421IP2)

- Dell
  - v. Neo Wireless (IPR2022-00616)

- Dish Network
  - v. Entropic Communications, LLC (Case No. 2:2023-CV-01043)

v. Realtime Adaptive Streaming (Case No. 1:17-CV-02097-RBJ)

v. TQ Delta LLC (Case No. 18-1798)

- Evapco Dry Cooling

v. SPG Dry Cooling (IPR2021-00688)

- Genetec

v. Sensormatic Electronics (Case No. 1:20-CV-00760)

- Huawei

v. Bell Northern Research LLC (IPR2019-01174)

- Kianxis

v. Blue Yonder (Case No. 3:20-cv-03636)

- LG Electronics

v. Bell Northern Research LLC (Case No. 3:18-cv-2864-CAB-BLM)

- Metaswitch

v. Sonus Networks (IPR2018-01719)

- Microsoft

v. Throughputer Inc (IPR2022-00757)

- Mom Enterprises

v. Ddrops Company (Case No. 1:22-cv-00332)

- MLC Intellectual Property

v. MicronTech (Case No. 3:14-cv-03657-SI)

- Nearmap Inc

  v. EagleView Technologies (IPR2022-01009)

- Neuroderm Ltd.

  v. Abbvie, Inc (Case No. PGR2022-00040)

- Realtek Semiconductor

  v. Future Link (IPR2021-01182)

- Quectel

  v. Koninklijke Philips (Case No. 1:20-cv-01710)

- Samsung

  v. Aire Technology (IPR2022-00877)

  v. Bell Northern Research (Case No. 2:19-cv-00286-JRG)

  v. Communication Technologies Inc (IPR2022-01221)

  v. Jawbone Innovations (IPR2022-00865)

  v. MemoryWeb LLC (IPR2022-00885)

  v. Dodots Licensing Solutions (IPR2023-00701)

  v. Telefonaktiebolaget LM Ericsson (IPR2021-00615)

- Texas Instruments

  v. Vantage Micro LLC (IPR2020-01261)

- Xilinx

  v. Sentient Sensors LCC (Case No. 1:22-cv-00173)

Groombridge Wu

- Nearmap

  v. Eagle View Technologies Inc. and Pictometry International Corp. (Case No. 2:21-cv-00283-TS-DAO)

Irell & Manella

- Curium

O'Melveny & Myers

- Apple

  v. Maxell (Case No. 5:19-cv-00036-RWS)

- Disney Media & Entertainment Distribution LLC

  v. Digital Media Technology Holdings, LLC (Case No. 2:22-cv-01642)

- Micron Technology Inc.

  v. Besang Inc (IPR2023-00900)

- Samsung

  v. Daedalus Prime LLC (1335 Investigation)

Perkins-Coie

- Amazon

  v. Nokia (IPR2024-00847 and IPR2024-00848)

- Heru Industries

  v. The UAB Research Foundation (IPR2022-01148)

- Intel Corporation

  v. BeSang Inc. (IPR2023-00991)

  v. BitMICRO (Case No. 5:23-cv-00625)

  v. Telefonaktiebolaget L M Ericsson (IPR2024-00610)

- Realtek Semiconductor

  v. Future Link (IPR2021-01182)

- Twitter Inc

  v. VOIP-Pal.com (Case No. 3:20-cv-02397-JD)

- TCL Industries

  v. Koninklijke Philips NV (IPR2021-00495, IPR2021-00496 and IPR2021-00497)

Pillsbury Winthrop Shaw Pittman

- Intel

  v. FG SRC LLC (Case No. 6:20-cv-00315)

- Gravel Rating Systems

  v. Costco (Case No. 4:21-cv-149)
  v. Lowe's Home Centers (Case No. 4:21-cv-150)
  v. T-Mobile USA (Case No. 4:21-cv-152)
  v. Kohl's Inc. (Case No. 4:21-cv-258)
  v. Under Armor (Case No. 4:21-cv-356)

Quinn Emanuel

- ServiceNow, Inc.

  v. InQuisient, Inc. (Case No. 22-900-CJB)

- Wolfspeed

  v. The Trustees of Purdue University (Case No. 1:2021-cv-00840)

Sheppard, Mullin, Richter & Hampton LLP

- Cadence Design Systems Inc.

  v. Semiconductor Design Technologies (Case No. 3:23-cv-01001)

Stern, Kessler, Goldstein & Fox

- Preliminary Research

Willkie, Farr & Gallagher LLP

- Neurent

  v. The Foundry, LLC (IPR2024-00669)

- Netflix, Inc.

  v. VideoLabs, Inc. (IPR2023-00628)

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European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V

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Related titles	Earlier title : Acta pharmaceutica technologica
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Physical Description	volumes : illustrations
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Review article

Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect

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ABSTRACT

For over half a century extensive research has been undertaken for the control of cancer. However, success has been limited to certain malignancies, and surgical intervention is potentially curative for early stage patients. For the majority of patients with advanced stage of cancer, the treatment is limited to chemotherapy or radiation. Chemotherapy in particular has limitations due to the lack of selectivity with severe toxicity. Under these circumstances tumor-targeted delivery of anticancer drugs is perhaps one of the most important steps for cancer chemotherapy. We reported such a drug for the first time, styrene-maleic acid copolymer-conjugated neocarzinostatin (SMANCS) in 1979, and it eventually led to formulate the concept of the enhanced permeability and retention (EPR) effect of solid tumors in 1986. Monoclonal antibody conjugates are another direction, of which interest is increasing recently though with limited success. The EPR-effect appears as a universal phenomenon in solid tumors which warrants the development of other polymeric drugs or nanomedicine.

EPR-effect is applicable for any biocompatible macromolecular compounds above 40 kDa, even larger than 800 kDa, or of the size of bacteria; thus complexed molecules like micelles and liposomes containing anticancer drugs are hallmark examples. The drug concentration in tumor compared to that of the blood (T/B ratio) can be usually as high as 10–30 times. In case of SMANCS/Lipiodol given via tumor feeding artery, the T/B ratio can be as high as 2000, a real pin-point targeting. EPR-effect is not just passive targeting for momentary tumor delivery, but it means prolonged drug retention for more than several weeks or longer.

This review describes the pathophysiological mechanisms of the EPR-effect, architectural difference of tumor blood vessel, various factors involved and artificial augmentation of EPR-effect with respect to tumor-selective delivery, and then advantages and problems of macromolecular drugs.

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1. Introduction

The field of drug delivery systems (DDS) utilizing synthetic polymers either by covalent conjugation or by composite of micellar drugs has become a new domain for new drug development for numerous diseases. Synthetic polymers become an indispensable component for micellar or stealth liposome drugs and protein–polymer conjugates [1–3]. These polymer-based new drug entities are called "polymer therapeutics" [2,3] or macromolecular drugs, and they overlap with nanomedicine that becomes popular in recent years [4]. The polymer therapeutics or nanomedicines are designed to improve drug performance by utilizing pathophysiological uniqueness of solid tumor, of which conventional low molecular weight drugs are incapable. Macromolecular drugs or nanomedicines show improved tumor-selective targeting; the improved therapeutic efficacy and fewer side effects are their primary benefits, in which prolonged circulation time plays a crucial role [4–9].

Most conventional low molecular weight anticancer drugs have inherent character to traverse in and out of blood vessels freely, unless the drug is linked with a tumor-specific molecular ligand having high binding constant. For instance, low molecular weight

Abbreviations: AT-II, angiotensin-II; AUC, area under the concentration curve (vs time); CML, chronic myeloid leukemia; EPR, enhanced permeability and retention effect (of macromolecular drugs in solid tumor); HPMA, poly(hydroxypropyl methacrylic acid); HCC, hepatocellular carcinoma (hepatoma); i.v., intravenously; i.a., intra-arterially; MDR, multidrug resistance; NCS, neocarzinostatin; NO, nitric oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; PEG, polyethylene glycol (also called polyoxyethylene); PGs, prostaglandins; PEG-poly(Asp), block copolymer (polyethylene glycol) linked to poly (aspartic acid-benzyl ester); SMA, copolymer of styrene-maleic acid; SMANCS, copoly (styrene-maleic acid) conjugated neocarzinostatin; SOD, superoxide dismutase; T/B, tumor to blood ratio of drug (delivered concentration); VPF, vascular permeability factor; VEGF, vascular endothelial growth factor.

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drugs injected intramuscularly reach a distant site of the body in 10 min. Consequently, their undesirable indiscriminatory distribution in normal tissues causes severe systemic side effects in case of anticancer agents. Namely, free diffusion of toxic drugs in a non-selective manner in the body, and their inability to accumulate selectively in tumor tissues make them disastrous to patients.

Experiences in antibiotic research tell us that selective toxicity is possible in prokaryotic kingdom, where different types of biochemical machinery are used from the animal kingdom. For instance, machinery for protein-synthesis (ribosomes) in prokaryotes is different from eukaryotic cells. Similarly, the cell-wall synthesis of peptidoglycan in bacteria (a target for penicillin) does not exist in eukaryotes. On the contrary, it is difficult to get tumor-selective toxicity because the biological events taking place in cancer cells are essentially the same as that of the host cells. Namely, there is little difference in biochemical or molecular machinery between cancer and normal cells at a cellular or molecular level. Therefore, to target tumor cells more selectively, active targeting based on antibodies or the receptor-mediated targeting with cancer-specific ligands are developed. However, recent clinical results of molecular target-based drugs were somewhat disappointing, if not completely.

Tumor cells have inherent heterogeneity and epitopic diversification as a result of great magnitude of mutation frequency even amongst the same cancer patient [10,12]. The recent results of cancer genomics showed that most human solid tumors were not only single gene-based events, but also multiple genomic alterations. Namely, there were no specific alterations or gene mutations common among individual patients if not name p53, a cancer suppressor gene. Extensive genomic studies of 11 colon cancer and 11 breast cancer patients revealed numerous genetic variants arising from a single solid malignancy. On average about 90 or so such variants were found in a patient. This makes the task of specific antibodies for each of these diverse epitopic targets inefficient if not unrealistic from antibody therapy [10,11].

Furthermore, according to recent reports in the annual meeting of *American Society of Clinical Oncology (ASCO)*, efficacy of molecular target drugs exhibited only 4–5% of response rate despite very high expectation and very high cost of manufacturing. It is generally thought this much efficacy is only useful as adjuvant or supplementary. Although it is beyond this review, the costs of antibody drugs are so expensive that public and national insurance systems may be at risk aside from the low response rate achieved with these therapies. In this regard, for example, Avastin was not recommended in the UK for reimbursement of national insurance [13]. Thus, 'the cost-benefit' will be considered more than ever for drug approval. Recently, the editorial in the Lancet criticized these issues one step further for drug appraisal [14].

Under these circumstances, a more universal and efficient strategy for anticancer drug design having high selectivity to tumor tissues must be developed. To solve this problem, the phenomenon of "enhanced permeability and retention (EPR)-effect" discovered by Maeda and Matsumura is now becoming the gold-standard in cancer-targeting drug designing that is based on macromolecular, micellar and lipidic particles [5–9], all utilizing EPR-effect as a guiding principle, and the EPR-effect is applicable for almost all rapidly growing solid tumors [7–9,15–18].

Most importantly, EPR-effect can be observed in almost all human cancers with the exception of hypovascular tumors such as prostate cancer or pancreatic cancer. As clinical examples for this, we have experienced that SMANCS/Lipiodol given via the hepatic artery accumulated selectively in hepatocellular carcinoma distinctively [7,17,19–22]. A similar result in clinical setting was also reported for Doxil, a liposomal type of doxorubicin. Namely, Doxil mimic was prepared for radio scintigraphy, and clear tumor accumulation was seen in the whole body scintigram [23]. Another clinical example of EPR-effect can be demonstrated in the traditional tumor imaging in the clinic that utilizes (γ)-emitting gallium scintigraphy based on the selective accumulation of radioactive gallium (used as citrate) in the tumor. ^{65}Gallium ion as injected i.v. will bind to plasma protein transferrin (90 kDa) in the blood, thus radioactive transferrin will accumulate in the tumor by EPR-effect, which will take more than 24 h. Usually radio-scintigram is obtained 2–3 days after intravenous injection of ^{65}Ga when signal/noise ratio is improved; while its clearance from the normal tissues will take place in a day or so via the lymphatic system. The tumor, however, retains this ^{65}Ga-transferrin for several days at high levels by EPR-effect.

Another case of EPR-effect observed in human tumor is the selective accumulation of Lipiodol in the tumor after intra-arterial infusion of Lipiodol which is visualized by X-ray CT-scan [19–21]. This tumor detection method by use of Lipiodol staining after our report [19–22] is now becoming a routine examination before hepatic tumor resection.

Conventional angiography for tumor detection uses water-soluble low molecular weight radio contrast agent, and its high electron density yields staining of tumor as it is infused intra-arterially. This means increased uptake (staining) of this contrast agent by the tumor mass, which is a part of EPR-effect though it is washed out rapidly by diffusion due to the small molecular size (i.e. no retention). Thus, the tumor staining in this angiography is only transitory, less than a few minutes or so. This is a passive delivery of drug, but not EPR-effect which requires long time retention.

Based on EPR-effect, many polymeric drugs are being developed as a new class of antitumor agents [1,24–28], including nanoparticles [16], polymer micelles [27,28] and liposomes [28–31]. Further, EPR-effect is not only limited to these nanoparticles, but it is also valid for tumor-imaging contrast agent Lipiodol as described above, where Lipiodol shows virtually pin-point targeted delivery to the tumor, i.e. tumor/blood (T/B) ratio of more than 2000 can be obtained [19–22,32]. Further, bacterial cells as well as quantum dots (QDs) as ultra-sensitive imaging probe showed more selective accumulation into tumor tissue, which can be explained by EPR-effect [33–35].

In this review, we will describe current problems in cancer chemotherapy, the mechanism of EPR-effect and factors involved, artificial augmentation of EPR-effect for polymeric or macromolecular drugs under the angiotensin-II (AT-II) induced hypertension, and advantages of macromolecular drugs are also discussed.

2. How good is cancer chemotherapy; status quo

In the past 40–50 years, low molecular weight anticancer drugs have been the main treatment modality for many cancers of advanced stage, but have offered no improvement in the cure rate [36,37]. The biggest limitation of these therapeutic agents is overwhelming toxicity due to lack of selectivity. Scientists realized this fact finally towards the end of 20th century and thus cancer-selective targeting became one of the most important goals.

Theoretically, in drug development, molecular target drug is considered more ideal not only in cancer but also in other diseases such as inflammation, primarily to avoid side effects. In this context, *Meares*, editor in chief of *Bioconjugate Chemistry*, a journal of *American Chemical Society*, commented that drug targeting was the most popular subject recently in this journal [38]. In any event, cancer still remains a major cause of death in most developed countries, and the lack of effective control of many cancers is becoming increasingly burdensome on the health care system [36].

As discussed in above references [36,37], the death rate of major cancers such as lung, breast, colon, prostate, and pancreas at advanced stage, has not changed much in the last half century. Further, the clinical benefit of chemotherapy in the most common cancers, for instance the breast cancer and the prostate cancer, is

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only marginal when the stage of cancer is advanced. Among the breast cancer patients who underwent surgery only, or who had surgery plus chemotherapy, the benefit of chemotherapy is only 2–3%. Namely, survival extension was 2 months in 60 months, while extensive medical cares including blood transfusion, platelets and leukocytes transfusion, injection of hematogenic factors (EPO, GCSF, etc.), antibiotics, ambulatory care or hospitalization are required for chemotherapy patients, which requires a huge expense. In addition, severe loss of appetite, hair loss, and poor quality of life (QOL) are more frequent in chemotherapy [39,40].

There have substantial increases in survival rate in some malignancies such as early stage gastric cancer by surgical intervention. Clinical results in lymphocytic leukemia and Hodgkin's disease have also been improved more effectively with chemotherapy than before. Early stage cervical cancer has responded well to surgery, radiation, and chemotherapy or a combination approach. Another most recent successful agent is *Gleevec®* (Imanitib) against chronic myeloid leukemia (CML) which has demonstrated 90% regression at least for 6 months. CML is one of the few cancers caused by the activation of *ABL/BCR* oncogene. However, at 6 months when leukemic cells began to propagate (called blastic crisis), drug-resistant mutants concurrently emerge in many or most, if not all patients, making a cure for CML far from reality. Life expectancy of CML patients is about five years or longer if patients remain stable and if it does not turn to blastic crisis.

Prostate cancer is one of the most common cancers amongst males in developed countries. Among the prostate cancer patients, 10–20% have metastatic diseases, most commonly to the bone. These metastatic tumors are less frequently treated by radiotherapy or by surgery, and chemotherapy is used as the next best alternative. In one recent study with more than one thousand prostate cancer patients with hormone refractory metastasis, clinical efficacy of docetaxel plus prednisolone, or mitoxantrone plus prednisolone, was evaluated [41]. The median survival was 17.4 months and 18.9 months, respectively. Although PSA levels decreased by more than 50% in 30–50% of the patients and the QOL was improved, adverse side effects remain a severe problem compared with the slight survival benefit. Similar studies without steroid resulted in 10–21 months of median survival span. An earlier study with mitoxantrone plus prednisone or prednisone alone showed no difference in overall survival benefit [42]. In any event about 65–70% of all patients died within three years, which means a not so remarkable cure rate, and also PSA is not a so accurate marker.

A few kinases, growth factors, DNA polymerases, transporters, etc. are also believed to be unique molecular targets, but the problems are that therapeutic windows are too narrow due to dose limiting toxicity, followed by emerging resistance. One such protein kinase inhibitor trastuzumab (Herceptin®) is an antibody against epidermal growth factor receptor (HER-2) and is used for breast cancer. Herceptin® targets to oncogene product "HER-2", it is thus only suitable for HER-2 positive patients, which comprises only 20% ~ 30% of the total breast cancer patients. Furthermore, among this group, only 20–30% will respond to Herceptin. Therefore, the value will end up at about 4–6% as an overall response rate.

Whatever the chemotherapeutic agents currently available, their excessive and prolonged exposure will ultimately damage normal cells. More serious of all is emergence of drug resistance, even to Gleevec®. Therefore, importance of more universal tumor-targeted drug delivery becomes obvious under these circumstances.

3. Phenomenon and mechanism of the EPR-effect

3.1. Historical background

During the study of in vivo behavior for antitumor protein antibiotic, neocarzinostatin, and its polymer conjugate SMANCS, Maeda et al. discovered a great difference in tumor uptake between low molecular weight drugs and biocompatible macromolecular drug SMANCS in solid tumors [17,24,43–46]. In a series of studies, the EPR-effect was discovered using the most biocompatible macromolecule drug candidates such as albumin (65 kDa), transferrin (90 kDa), IgG (immunoglobulin, 150 kDa), α_2-macroglobulin (240 kDa), ovomucoid of chicken eggwhite (29 kDa, highly glycosylated protein), SMANCS (16 kDa, with albumin binding character becomes 80 kDa) and neocarzinostatin (12 kDa) [6,7]. Macromolecules such as albumin, transferrin, IgG, SMANCS and lipid particles (Lipiodol) accumulated in the solid tumor so remarkably, and they were retained for a very extended period up to 3 months or longer [5–9,17,22].

In general, they found the areas under the concentration curve vs time (AUC) of a drug and its tumor uptake are positively related, whereas the rate of urinary clearance is inversely related to tumor uptake [5,7,46,47]. In collaboration with Prof. K. Ulbrich and Prof. R. Duncan, a series of biocompatible synthetic polymers, HPMA (4-hydroxypropylmethacrylate copolymer) ranging from 20 to 800 kDa in various tumor models (S-180, meth A, colon 38, VX-2 of rabbit, Walkar-256 carcinomas and AH66 of rats) were investigated, and the findings are consistent with previous studies [5–7,46,47]. These polymers displayed remarkable tumor accumulation in mouse and human xenograft tumors up to ~20% dose/g in some case, and 1–5% dose/g usually in small tumors. However, in larger tumors, i.e. more than 0.5 g, the tumor uptake of Evans-blue albumin became lower [46,47,64]. From these results we concluded that, in order to exhibit features of the EPR-effect, a drug must have a higher molecular weight than the renal excretion threshold (typically > 40 kDa) [5–9,17,25–27,46,47].

3.2. Factors involved in EPR-effect

Maeda et al. had previously demonstrated that bacterial proteinases initiated bradykinin generation via factor XII (Hageman factor) activation followed by activation of prekallikrein to kallikrein, which releases bradykinin from kininogen. Bradykinin is known to induce vascular permeability and plays an important role in the EPR-effect. Thus the site of infection/inflammation where excess of bradykinin is generated also exhibits EPR-effect [50–53]. This study was extended to identify the cause of ascitic and pleural effusions of carcinomatosis in peritoneal and pleural compartments. In these studies Maeda et al. were able to identify that bradykinin formation was responsible for the extravasation of ascetic fluid [54–59]. The only difference between infection-induced EPR-effect and that of cancer is duration of retention period; the retention in normal tissue, where inflammation occurs, is shorter than cancer because the lymphatic drainage system is still operative, thus swelling may dissipate in a matter of a few days. In contrast, macromolecular or lipidic drug retention in cancer tissue can be as long as weeks, a great contrast to that of infection-induced inflammation [7,17,19–22]. Under these circumstances, it was realized that vascular phenomenon was important not only because of its critical role in tumor growth and metastasis, but also for tumor-selective delivery of anticancer agents, on which Maeda's group had been working [19–22]. Then, they demonstrated the EPR-effect [5]. Namely, EPR-effect is a phenomenon of enhanced extravasations of macromolecules from tumor blood vessels, and their retention in tumor tissues, which is not observed in normal vasculature.

We then investigated vascular mediators involved in EPR-effect and found that nitric oxide (NO) also facilitates this effect [58,59,64,65] in addition to bradykinin. Oxidized product of NO (peroxynitrite) also shows this effect with or without activation of collagenase [66,67]. A similar phenomenon was observed with other proinflammatory mediators such as prostaglandins [58,66,68] and vascular endothelial growth factor (VEGF) [54,58,

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69–71] which was formerly identified by Dvorak et al. as vascular permeability factor (VPF) [71]. In these connections, dual function of VEGF, namely, angiogenesis and enhancement of vascular permeability, both facilitate and sustain rapid growth of tumor. Furthermore, NO is also involved in tumor growth [72], VEGF and bradykinin are also known to be potent activators of NOS which generates NO from L-arginine. Following is the summary of factors facilitating EPR-effect:

(1) Vascular endothelial growth factor (VEGF/VPF).
(2) Bradykinin/3-hydroxyprolyl bradykinin (BK).
(3) Nitric oxide (NO).
(4) Peroxynitrite ($ONOO^-$) (reaction product of superoxide anion radical and NO).
(5) Prostaglandins (PGs).
(6) Matrix metalloproteinases (MMPs); proMMP is activated to MMP by $ONOO^-$.
(7) Other proteinases (e.g. kinin/kallikrein system) involving various protease cascade.
(8) Other cytokines (e.g. tumor necrosis factor, IL-2, and TNF-α) also facilitating EPR-effect.

3.3. Anatomical and pathophysiological difference of tumor vasculature

As to the anatomical or architectural difference between normal and tumor vasculature, O'Brien's group has demonstrated remarkably unique difference using scanning electron microscope (SEM) [60,61]. Namely, a water-soluble acrylic monomer that forms polymeric resin in the blood vessels was injected into the blood vessel of tumor-bearing mice, thus the cast of the blood vessels of tumor was obtained, which is made of plastic resin. As shown in Fig. 1(C and D), plastic resin is extravasculated extensively outside of blood vessels in the metastatic tumor nodule; more importantly these features are not seen in normal capillary (Fig. 1A and B).

These photographs (Fig. 1C and D) clearly show completely different morphology of tumor blood vessels in microtumor nodules (Fig. 1C) compared with that in normal blood capillary (Fig. 1A and B). More recently Hashizume et al. also observed this phenomenon in mouse mammary carcinoma using scanning electron microscopy [62] and identified structural abnormalities in the endothelium of tumor blood vessels, whose intercellular openings were found to be as large as 4.7 μm (mean size 1.7 μm) in diameter. In other studies Yuan et al. have measured the pore size of tumor vessel in LS-147 human colon adenocarcinoma implanted in SCID mouse and found that it could be as large as 0.4 μm in diameter [63]. All these studies demonstrate that the abnormal vascular architecture plays a major role in excessive vascular permeability of tumor, i.e. EPR-effect in tumor for selective macromolecular drug targeting at tissue level, that can be summarized as follows:

(1) Extensive angiogenesis and high vascular density (hypervasculature).
(2) Lack of smooth-muscle layer; pericytes; no AT-II-induced vasoconstriction; more leakage at tumor vessels under hypertensive state.
(3) Defective vascular architecture; irregular vasculature networks, large openings, fenestration.
(4) Whimsical blood flow; no constant blood flow and direction.
(5) Meager lymphatic clearance that leads to enhanced retention of macromolecular drugs and lipidic particles in the interstitium of tumors.
(6) Slow venous return that leads to accumulation of macromolecular drugs and lipidic particles from the interstitium of tumor.

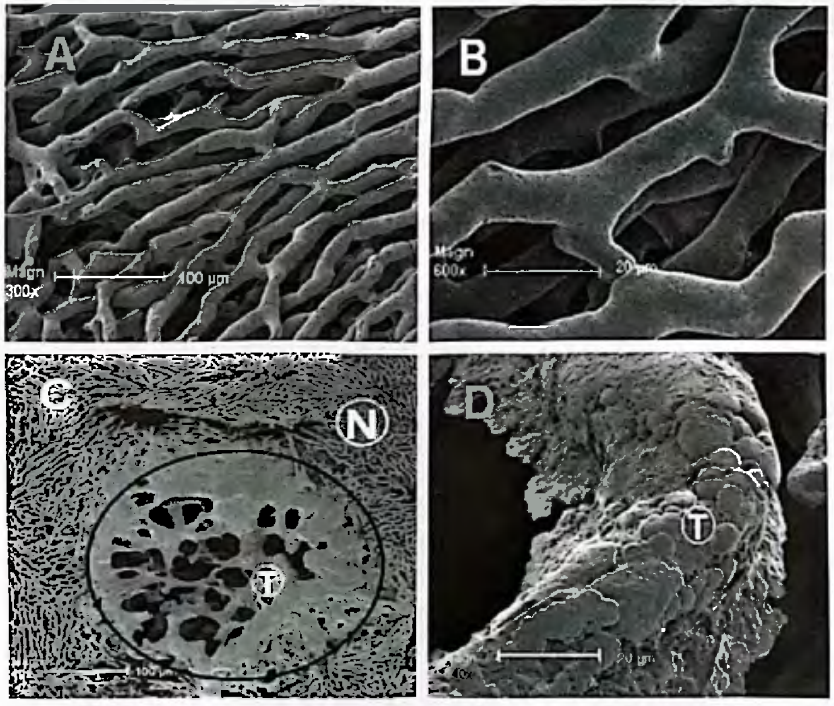


Fig. 1. Scanning electron micrograms of blood vessels at capillary level in normal tissue [liver] (A and B) and blood vessels of tumor nodules (C and D) in the liver. Enlarged image of capillary network of (A) is shown in (B). No leakage of the polymer is seen in the normal liver as indicated by ⓝ whereas tumor-selective extravasation of polymer (by EPR-effect) in tumor nodule (ⓣ) is seen, but it is not observed in normal vascular area ⓝ, or in (A) or (B). (D) Tumor vessels in which extravasation of acrylic polymer is seen. This will lead to selective delivery of polymeric drug in tumor as seen ⓣ in (C) and (D) [58,61].

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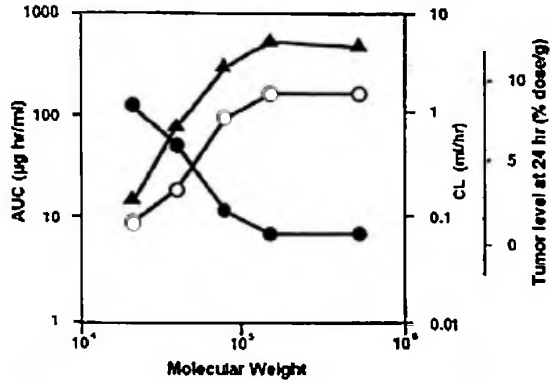


Fig. 2. Relationships between molecular weights and tumor uptake and clearance of ^{125}I-tyr-HPMA-polymer of varied molecular weight drugs. Mice bearing S-180 solid tumor received about 1.8×10^6 cpm/mouse by i.v. injection. ●, CL = renal clearance rate; ▲, AUC = area under the concentration curve for different Mw polymers in plasma. Both values are based on 72 h after injection; ○, tumor uptake values are based on 24 h data [18].

Demonstration of the EPR-effect in metastatic micronodules of tumor in the liver is seen in (Fig. 1C) by using SEM, and blood capillary of normal liver is shown in (Fig. 1A and B). Tumor blood vessels illustrated here show that monomer of plastic resin penetrated the vascular wall during polymerization (Fig. 1C and D), and the vascular network of tumor vessels in the nodule looks abnormal and shows appearance of leaked resin; these are clearly different from the blood capillary of normal liver tissue. Namely, the tumor nodule is selectively almost filled with plastic resin (Fig. 1C).

In the case of SMANCS/Lipiodol, it is administered via the tumor feeding artery. This arterial infusion will result in direct drug capturing, that is known as the first path effect, in which EPR-effect works additively [20–22]. Remarkable success of the SMANCS/Lipiodol therapy can be attributed to the hypervascular nature as well as EPR-effect due to excessive vascular mediators in the hepatoma. This trend of efficient uptake of SMANCS/Lipiodol is also seen for renal cancer. Most water-soluble polymers can accomplish EPR-effect by i.v. infusion as seen by T/B ratio ranging 5–30 [5,7,15–18,64] and EPR-effect becomes obvious even 10 min after i.v. injection [46]. The tumor/normal tissue ratio is as high as 30- to 300-folds at 6 h or later, of which the values depend largely on time of quantification. Most of the macromolecular drugs exhibit prolonged plasma $t_{1/2}$ (>several hours) and very low renal clearance as seen in Fig. 2.

4. Recovery of macromolecules from the tissues interstitium: the lymphotropism and lymphatic metastasis

When lipids and lipidic particles or macromolecules are injected into the interstitial space of normal tissue, they are known to be recovered via the lymphatic systems. Now it should be noted that this is the route of clearance of lipidic or macromolecular drugs from the normal tissue, but it is also the route of lymphatic metastasis of cancer cells. Initially, Maeda et al. found that when NCS (12 kDa) or SMANCS (16 kDa, by albumin binding it becomes 80 kDa) was injected into normal tissue subcutaneously, they accumulated most effectively in the regional lymph nodes, and very slowly traversed into the general circulation [73–76]. The lymphotropic property is more clearly seen for lipids in general. Namely, the lymphographic contrast agent Lipiodol® (Laboratoire Guerbet, France), which is iodinated poppy seed oil, has been used for lymphography because it moves into the lymphatics and its exclusive presence in the lymphatic duct can be detected under the X-ray system. It should be worthwhile to count these lymphotropic characters in the drug designing aiming at antimetastatic property. The control of lymphatic metastasis is so important because lymphatic metastasis of tumor is the more frequent cause of therapeutic failure. Therefore, suppression of lymphatic metastasis by lymphatic drug delivery with the use of liposomes or nanoparticles may be of critical importance [24,73–75].

Antitumor efficacy is usually judged by the effect on the major or primary cancer, which is the target of current chemotherapy or surgery, and concomitant effect on micrometastasis in the lymph nodes or elsewhere will become ultimately very important, which can be more effectively achieved by lymphotropic drugs. Further, definition of the regression (response) is traditionally defined as size reduction to less than half of the original size. For instance, metastatic micronodules as shown in Fig. 1C are invisible under conventional methods of cancer detection. Thus, evaluation of response in these microtumor by tumor size is not possible. More recently, prolongation of survival time span is being considered as an important parameter. However, it is often difficult to control the lymphatic metastasis. If one could control lymphatic metastasis by the lymphotropic drug delivery, one would contribute survival span. In the SMANCS therapy, we experienced that there are many patients who become tumor stabilized (but not regression) after treatment without further disease progression, nor emergence of new tumor. As stated above, lymphotropic nature of macromolecular drugs will contribute on this point and it will be a highly preferred character. In this connection, a recent report of PEG-interferon 2b treatment resulted in prolonged tumor-free survival of patients with malignant melanoma, which is interesting [77].

5. Augmentation of EPR-effect and drug delivery by angiotensin-II induced hypertension

The vascular density of many tumors, if not all, is higher than that of normal tissues. Furthermore, tumor blood vessels frequently lack the smooth-muscle layer, which plays a vital role in regulating blood pressure and flow volume. In normal blood vessels, the smooth-muscle layer responds to vascular mediators such as bradykinins, acetylcholine and NO via receptor binding on vascular smooth-muscle cells, which modulate intracellular calcium levels, helping to maintain constant blood-flow volume. In normal tissues, when hypertension is induced by infusing angiotensin-II (AT-II) intravenously, vasoconstriction of the smooth-muscle layer will result in higher blood pressure and higher flow velocity. Interestingly, however, the blood-flow volume remains constant [78]. In contrast, in tumor tissues under the AT-II-induced hypertensive state, the blood-flow volume does not remain constant. Tumor blood-flow volume actually increases in response to elevated blood pressure. In tumor-bearing rats, raising the systolic blood pressure from 90 to 160 mm Hg resulted in nearly 2–6 times increase in tumor blood-flow volume depending on the blood pressure attained, whilst blood-flow volume in the normal tissues such as the kidney and the bone marrow remained constant [78]. Therefore, we anticipated that the induction of the hypertensive state would augment the EPR-effect, and hence, the delivery of macromolecular drugs would increase. In fact, when ^{51}Cr-labeled SMANCS or ^{51}Cr-labeled albumin was injected i.v. in rats bearing Walkar-256 carcinoma and when the blood pressure was raised from 90 to 150 mm Hg only for 15 min, there was a 1.3- to 3.0-fold increase in accumulation of these macromolecular drugs in the tumor tissue [79]. At the same time, a significant reduction of the drug delivered to healthy organs (e.g. intestine, kidney, liver and bone marrow), and thereby lesser toxicity, was observed because of vasoconstriction and tightening of endothelial gap junctions, which suppressed the extravasation of polymeric drugs.

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Similar results were obtained with SMANCS/Lipiodol administered under the AT-II-induced hypertensive state, in many patients with solid tumors including hepatocellular carcinoma (HCC), metastatic liver cancer, cholangiocarcinoma, pancreatic cancer, and renal cell carcinoma [7,9,18,80 – and unpublished data]. It is known that in common cancer chemotherapy with low molecular weight anticancer agents, the anticancer agents should be administered no more than the recommended dose, because of severe dose limiting toxicity. However, under the hypertensive state, using macromolecular drugs in animal models as well as in the clinical setting, it was possible to achieve more than 1.3- to 5-fold higher tumor concentration in the tumor with the same dosage. More importantly, sufficient augmentation of tumor-targeting effect could be achieved even when hypertension was maintained for only 20 min, meanwhile no apparent toxicity was seen [9,80]. Having these obvious advantages and evidences, we warrant AT-II-induced hypertension chemotherapy should be applied for macromolecular anticancer chemotherapy. This method is approved in Japan for conventional drugs but not yet for nanomedicines.

6. Consideration in designing of macromolecular anticancer drugs: for utilization of tumor targeting mechanism by EPR-effect

6.1. Release rate and pharmacokinetics

In recent years, many efforts were directed towards designing of macromolecular drugs utilizing EPR-effect for tumor-seeking anticancer agents. The method involves conjugates or encapsulation of low molecular weight anticancer drugs, or proteins by chemical conjugation or micellar or liposomal encasement, respectively, to water-soluble, biocompatible, biodegradable polymers, or nanoparticles such as encapsulated liposomes or polymeric micelles [1–4,25–28,81]. The crucial point of all is the optimally sustained rate of drug release from these nanoparticles to attain the required drug level.

In this context, when liposomes are too stable and have slower release rate than the optimum rate for drug release, the drug concentration cannot reach the required concentration within the adequate time span. Doxil, adriamycin encapsulated liposome, belongs to this category, and it is used for first line of defence against Kaposi's sarcoma and second line drug for breast cancer and others, but not universal for solid tumor in general. On the other hand, such liposomes also need to be stable enough for storage period, and on the shelf, or refrigerator to the bed side. As an example, adriamycin-encapsulated block copolymer micelles (PEG-polyAsp), designated NK-911, on the contrary showed initial rate of drug release too fast, it thus could not release adriamycin in a sustained manner for adequate time span of few days. Therefore, it cannot attain adequate pharmacological drug concentration inside tumors in human. As 50% of the drug was released less than an hour after i.v. injection, this micellar formulation has no advantage (Fig. 3A-(b)) [25,26,28,81]; also the results in phase I discouraged for further development.

In Fig. 3B, plasma concentration of another synthetic polymer micelle SMA-doxorubicin is shown, which exhibits longer residence time than NK-911. Further, when one compares between SMA-micelles of doxorubicin and pirarubicin, the latter micelle showed higher AUC and very higher tumor uptake than SMA-doxorubicin. Namely, SMA-pirarubicin micelles showed 100 × higher plasma AUC and 27 × higher tumor concentration than parental-free pirarubicin [25,26]. This means a combination of a drug and chemical constituent of the micelles is an important factor to have preferred characteristics.

6.2. General advantages of polymer conjugates or nanomedicine

6.2.1. Prolongation of half-life, stealth character and reduced antigenicity

One of the important advantages of polymer conjugation is the stabilization and prolongation of the plasma half-life of the low molecular weight drugs or proteins. SMANCS, a conjugate of SMA and neocarzinostatin (NCS), showed this character as well as polyethylene glycol (PEG)-conjugated bilirubin oxidase and others [24,76,82]. PEG-conjugated interferon, PEG-INFα2a (Pegasys®), showed increase in plasma half-life to about 10-fold of native INFα2a (8 h vs 80 h) when injected s.c. in human [83]. Second advantage is solubilization of insoluble low MW compounds as discussed below. Further, polymer conjugates or micellar formulation under specific condition also confers 'stealth' characteristics and diminishes uptake by the reticuloendothelial system (RES) or macrophages, and hence the half-life in blood circulation can be extended [29–31,82,83]. These merits cannot only improve the chemotherapeutic efficacy, but also reduce the time and cost of frequent injections to a great extent. This had been observed in SMANCS/Lipiodol, of which arterial injection is required at most once a month, usually once in 2–3 months or less [7].

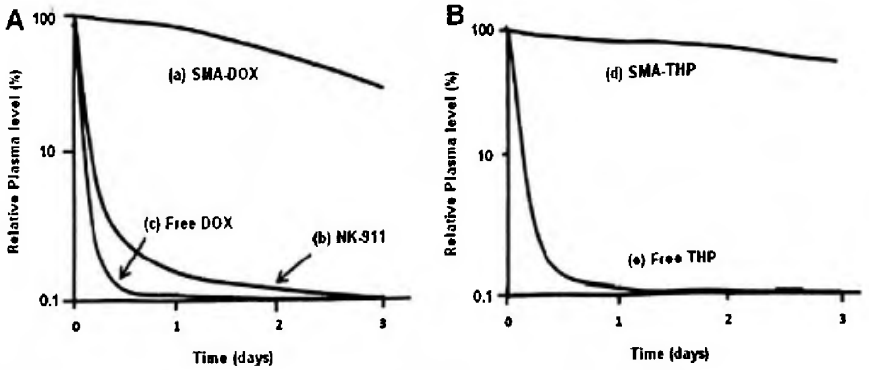


Fig. 3. Improved plasma half-life of anthracyclines by micellar encapsulation, and different micelle materials and different drugs are shown. (A) Plasma half-life of free doxorubicin (DOX) and that of SMA or PEG-Asp block copolymer micelles. SMA-micelles of doxorubicin shows much prolonged plasma residence time [25], while PEG-Asp block copolymer (NK-911) bursted very rapidly within short time after injection. The data values were simplified from published literature [28]. Higher stability of SMA-DOX micelle is apparent. (B) SMA-THP (4-O-tetrahydropyranyladriamycin) micelles are compared with that of free THP in human cancer patients. Advantage of SMA-THP micellar formulation in the plasma residence time over free THP is apparent as well as higher tumor targeting by EPR-effect [25,26]. Free THP is more rapidly taken up by cancer cells than free doxorubicin. THP has higher affinity to SMA than DOX so that SMA-THP micelles in plasma stayed longer and more stable than SMA-DOX (A vs B).

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6.2.2. Biological response modification or immunopotentiation by polymers

Some polymer conjugates, such as SMANCS, for instance, displayed various immunomodulating properties, including macrophage (Mϕ) activation, induction of interferon-γ production [84–87], and augmentation of splenic and peritoneal natural killer (NK) cells [86,87]; thus it stimulates non-specific cytotoxicity to tumors in mice bearing syngeneic tumors. Similar findings were also reported for pyran copolymer and other anionic polymers, which have the capacity to induce interferons and other cytokines [86].

6.2.3. Resistance against free radicals, proteolytic degradation, addition of metal chelating capacity and surface lubrication

The pathological and inflammatory lesions including cancer are constantly under the exposure of endogenously formed free radicals such as superoxide anion radical $O_2^{\cdot-}$, hydrogen peroxide (H_2O_2), NO, $ONOO^-$ and hypochlorite (HClO) [88–91]. Many proteins, enzymes, nucleic acids or active drugs, when exposed to these radicals, may be readily inactivated or modified. However, polymer conjugation or micellar shield will confer better stability against these radicals. To clarify this point, we studied the effect of oxygen radicals (ROS) to functional proteins in model systems [92,93]. One example is superoxide dismutase (SOD), which catalyzes the dismutation of superoxide anion radical $O_2^{\cdot-}$ to H_2O_2. Subsequently, if catalase is not available in the milieu, H_2O_2 will accumulate in the lesion. We previously showed that Cu, Zn-SOD was disintegrated by exposure to H_2O_2 (the product of SOD), and then free Cu^{2+} ion was liberated, which will serve to catalyze ·OH generation from H_2O_2 effectively by Fenton type reaction [92,93]. However, polymer conjugates of SOD with pyran copolymer {DiV-EMA, poly(divinylether-maleic acid)} remarkably reduced this ·OH generation, suggesting the protective and stabilizing effect of DiV-EMA modification to SOD against free radicals. Further, we extended the study to an acute phase plasma protein, α_1-protease inhibitor (α_1-PI) as the target using various polymers *per se* to suppress inactivation by ·OH. Among the polymers tested, pyran copolymer showed the most potent inhibitory activity against ·OH generation and greatest suppressive effect against α_1-PI inactivation, whereas BuSMA (butylated-SMA) or PEG alone suppressed α_1-PI inactivation only weakly (Table 1 and Fig. 4). The effect of pyran copolymer may be attributed to the chelating activity of Cu^{2+} by dicarboxylate ion in pyran copolymer, which was found to exhibit high activity comparable to the potent chelating agent, DTPA, whereas not all polymers have bicarboxylic groups thus showing no chelating effect, but shielding effect.

Along this line, we further examined the time course of H_2O_2-induced inactivation of native SOD and various polymer-conjugated SOD, and those of α_1-PI by ·OH radical similarly. Namely, assuming the metal (Cu^{2+}) would catalyze Fenton reaction, through which H_2O_2 will become hydroxyl radical in the presence of various SODs and α_1-PI. We found that polymers having paired carboxyl groups and OH groups such as pyran copolymer and PVA effectively suppressed the inactivation of SOD as well as α_1-PI. It is probably attributed either by direct scavenging of ·OH (by PVA) or by chelating transition metal ion Cu^{2+} [88,92,94].

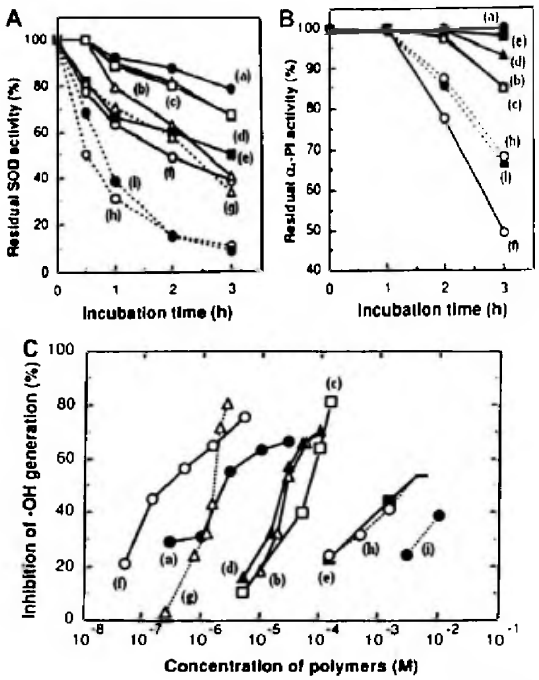


Fig. 4. Protective effect of enzyme activity of SOD by various polymer conjugation (A), and (B) inactivation of α_1-protease inhibitor (α_1-PI) both by exogenously adding H_2O_2 [94]. (C) The extent of ·OH generation by SOD or various polymer-conjugated SODs upon addition of H_2O_2. Concentration of SOD and each polymer-SOD conjugates (3.7 μM) were incubated with 0.1 mM H_2O_2 in 25 mM sodium phosphate buffer (pH 7.4) for the indicated time at 37 °C. In (A), (B) and (C), (a) -●-, high molecular weight pyran(H)-SOD; (b) -△-, low molecular weight pyran (L)-SOD; (c) -□-, PVA-(L)-SOD; (d) -▲-, high molecular weight PVA(H)-SOD; (e) -■-, succinylated-gelatin SOD; (f) -○-, native Cu, Zn-SOD; (g) --△--, PEG-SOD; (h) --○--, carboxyl HSMA-SOD; (i) --●--, BuSMA-SOD. The [·OH] radical was quantified by electron spin resonance spectroscopy by using of spin trapping agent [See text for details and Ref. [92,94]]. (For abbreviation, PVA, etc. see Table 1).

Another advantage of polymer conjugations is that they also confer resistance against proteolytic degradation without much affect on biological activity. We previously showed PEG-conjugated bilirubin oxidase becomes more resistant against proteolytic degradation than native enzyme [73,95].

Viscosity of high molecular weight polymers for injection-drug at high concentration may impede blood flow. We investigated previously the effect of PVA, PEG and gelatin on the lubricating

Table 1
Inhibitory potentials of various free polymers and DTPA against ·OH generation, and their protective against inactivation of α_1-protease inhibitor (α_1-PI) by ·OH[a].

Polymers (abbrev.)	Approx. Mw.	Concentration of 50% inactivation [μM]
Pyran copolymer; divinylether-maleic acid (DIVEMA)	30,000	0.48
N-ethylsuccinimidyl-S-poly(vinylalcohol) (PVA)	11,000	37.0
Succinylated-gelatin fragment (gelatin)	23,000	16.0
Poly(styrene-co-maleic acid) half-butyl ester (SMA)	1600	>1000
Monomethoxypoly(ethylene glycol) (PEG)	5000	> 100
Diethylenetriaminopenta acetic acid (DTPA, chelating agent)[b]	393	1.6

[a] Hydroxyl radical (·OH) was generated in the presence of 7.4 μM Cu^{+2} and 3 mM H_2O_2. Concentration of α_1-PI, 20 μg/ml [data are from Ref. [92]].
[b] DTPA is one of the best chelating agent; it is not a polymer. Potent suppression of ·DH generation by DTPA is due to removal of Cu^{+2} from the ·OH generating system, whereas all the polymers will scavenge ·OH, or chelating Cu^{+2} (e.g. DIVEMA) and suppress ·OH generation.

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effect of the erythrocyte passage through micropore up to 5 μm in pore size [96,97,93]. Gelatin and PEG showed preventive effect of red blood cell from bursting (hemolysis) and facilitated the passage through the micropore. Modification of SOD with succinyl gelatin also conferred this beneficial effect in addition to the enhancement of pharmaceutical effect [93].

6.2.4. Enhancement of solubility, cellular uptake, intestinal absorption, and physical stability

Besides the increase in $t_{1/2}$ and EPR-effect, we can confer different characteristics and thus drug formulations from the parent drugs by polymer conjugation or micellar encapsulation. It is well known that many promising drug candidates fail to become therapeutic agents because of their poor water-solubility. The conjugation or encapsulation of such drugs to water-soluble nanoparticles confers remarkable solubility. For instance, Taxol®, caplitaxel, KRN 5500, SN38, Zn-protoporphyrin are converted to highly water-soluble drugs [25–28,98–100]. Moreover, many proteins and drugs which have poor intestinal absorption may be rendered orally active by conjugation with appropriate polymers in combination with oily formulation [101]. One such formulation is SMANCS in medium chain triglyceride (MCT) solution (oily SMANCS); when it was orally administered to mice, it was more efficiently absorbed than aqueous solution (PBS) from the intestine. Plasma concentration or AUC of oily SMANCS given orally was about 11 times greater than that of aqueous SMANCS. In comparison to parent NCS, total AUC could be improved by more than 100-folds. Further the stability (and half-life) of SMANCS was remarkably improved in oily formulation with Lipiodol®, not to mention of its 20 times increased stability at 37 °C in vitro [102], which could be given orally, and enhancement in peak plasma level was 9-fold higher than aqueous formulation of SMANCS.

Further, enhanced intracellular uptake to tumor cells without any known specific receptor was observed for SMANCS than for NCS. Interestingly, it was more efficient in acidic pH (5.5–6.5) than neutral pH [103,104]. However, receptor-mediated internalization with tagged ligand following the EPR-effect-based vascular crossing delivery is considered efficient and important steps for this type of drugs. One such example is galactose-containing HPMA copolymer-doxorubicin targeted to the liver [48,49,105]. Another probably more promising receptor is transferrin receptor which is known to be over expressed in tumor cells. Consequently, the transferrin–methotrexate conjugate was developed, as well as liposomes [106,107]. More recently, a similar result was seen by the use of folate receptor, where folate-mitomycin C is cross-linked on polymer conjugates [108]. There are several other examples of receptor-mediated drug delivery reported elsewhere [16,109–112].

A naive question is how transferrin conjugates be so effective despite excessive presence of free transferrin in the circulation that would compete with the binding of the transferrin-conjugated drugs. The same is true, while antibody–drug conjugates are reaching the tumor-associated antigen on tumor cells, free tumor-associated antigen being produced by the tumor cells is in circulation. Then, how can it effectively reach the tumor cells despite large amount of tumor antigens in circulation, which will also bind to the target; like α-feto protein of hepatoma or CEA of colon cancer present in circulation. The answer may be that these conjugates utilize EPR-effect more than free antigens. Consequently, even though antibody function of antigen binding may be nullified by free antigens in circulation, the antigen-antibody complexes in circulation may exert EPR-effect and reach the target because of their large size, and thus they are capable of easy access to tumor cells followed by endocytosis. In any event, dividing tumor cells are known to have higher endocytotic uptake capacity than non-dividing normal cells [112], which makes another unique advantage of macromolecular drugs.

6.2.5. Advantage of polymeric drugs over p-glycoprotein-dependent multidrug resistance

To overcome the multidrug resistance (MDR), we worked with SMANCS, and found that p-glycoprotein-resistant MDR cells are sensitive to SMANCS, probably by preventing the binding of the polymeric drug to p-glycoprotein that leads to suppression of the efflux of the drug, because the macromolecular size of the drug does not fit to the efflux transporter system [43,44]. Minko et al. reported that HPMA copolymer-linked doxorubicin affected the signal transduction system of MDR cells and induced apoptosis which was not seen in the parent drug [113–115].

6.2.6. Patient compliance and quality of life (QOL)

The polymer-conjugated drugs or nanomedicines require less frequent administrations due to the increased tissue and blood half-life ($t_{1/2}$) which is usually 10 times or more than the free drugs, that is of great benefit to patients [1,8,97,93]. For instance, for the treatment of lymphocytic leukemia the use of native enzyme L-asparagine having a short $t_{1/2}$ (8–30 h) necessitates daily administration for 4 weeks, whereas the PEG-L-asparaginase conjugate (Oncaspar®, $t_{1/2}$ approx 14 days) requires only one infusion every 2 weeks [116–118]. The same is true for native interferon-α vs pegylated interferon-α (Pegasys®, MW 50 kDa), the former requires more than 2–3 injections per week, while the latter requires only once a week intramuscular injection [83].

6.3. Disadvantages and limitations of polymer conjugates or unsolved issues

In view of clearance from the body, a size or unit of polymer being used should be preferably smaller than the size of renal excretion threshold (40 kDa), or biodegradable, or evidence of clearance via fecal route; in this case molecular weight may be of secondary importance. Further, the biocompatibility of polymers to be conjugated has to be well established. Hydrophobic polymers, if resistant to degradation in vivo, may tend to localize eventually in the skin or lipid-rich tissues, although mostly they may be eliminated via the bile. Therefore, the total amount of polymers to be administered may become important if the dose becomes large (e.g. 10 g). Some polymers may elicit immune response or allergic reactions, more likely in the subsequent injections, although it may be controlled by corticosteroid and antihistamine. There were reports in earlier days on the so called "macromolecular syndrome", or more lately on the infusion-related reaction that is observed at first usage even with antibody drugs or liposomal drugs, that is not observed in the second administration [119].

7. Species difference and optimization of release rate from the drug complex

The rate of initial burst of free drugs in vivo is frequently a problem in many polymeric drug conjugates, micellar, or liposomal drugs. However, the release rate from the given polymer micelles or from liposomes is purely physical matter exhibiting zero order rate kinetics. In contrast, species difference in the renal or the hepatic functions or metabolic rate and their time scale in different animal species may need to be considered in terms of time course of drug release. For instance mice may tolerate toxicity well because of the more rapid metabolic rate or urinary clearance compared to human time scale. The metabolic rate particularly in the liver or urinary clearance rate in mouse may be far faster than that in human. We may not see effective therapeutic effect if the conjugates were not designed for human metabolic rate. Therefore, ideal experimental data in mice or rats do not guarantee good therapeutic response in human. We also encountered that SMA copolymer has little binding to rabbit albumin, in contrast to mouse or human

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albumin (unpublished data). This means that some animal models may not reflect the actual drug behavior for optimization in the human situation. Therefore optimization of the parameters has to be targeted for human setting, but not for mice.

Particular notion in this regard should be addressed to the nature of chemical bonding between the drug and polymers. For instance, chemical bond in the conjugates either linked by ester- or amide-bond makes a great difference. Namely, cleavage of ester bond could be 10- to 30-folds faster than that of amide bond in mice than in humans [119,120].

Another discrepancy in animal model vs real human cancer treatment is the tumor model using ascetic form (i.p.) tumor and the drug given i.p. or i.v. route. Frequently one injects the drug i.p. within one day after tumor inoculation into the peritoneal cavity, and hence the drug will be accessible to the tumor cells directly at a very early stage and it will be so effective. However, this protocol is an unrealistic model to develop a drug for solid tumor. In this system of the i.p. tumor/i.p. drug, there is no solid tumor being developed yet, thus no EPR-effect. Also, there are no human patients with 10^6 tumor cells (very small inoculums size) to be diagnosed to have cancer at that early stage. If this is a case, cancer cells are easy to eradicate by helping immune surveillance. When both tumor cells and drug were administered i.p., easy access of drug to the tumor is guaranteed. Thus it is not a good model because the peritoneal compartment becomes a type of test tube, and the drug concentration in the compartment is very high and effective, resulting in more chance to eradicate tumor cells.

8. Conclusion

For the tumor-targeted drug delivery system, EPR-effect is now widely accepted as a guiding principle (see recent review) [121]. The EPR-effect is observed in most macromolecular drugs that include polymer conjugates, micellar drugs, and liposomal drugs; all exhibiting tumor-targeting characteristics. Once these macromolecular drugs are delivered to the tumor, they remain in the tumor tissues for extended period (days to weeks), which is different from just passive targeting and is different from the behavior in the normal tissue. Even cancer-targeting drugs that are conjugated with monoclonal antibodies will rely on this EPR-effect at first crossing of vascular barriers to be accessible to tumor tissue. In this connection, it was shown previously that normal IgG exhibited tumor-selective accumulation by EPR-effect [5]. Various factors that mediate EPR-effect such as bradykinin, NO, VEGF, and prostaglandins are reviewed and discussed.

EPR-effect can be further augmented by elevating the systemic blood pressure artificially by infusing AT-II [79], and also by prostaglandin I_2 agonist [67,68]. Consequently, the delivery of macromolecular drugs is facilitated significantly even to metastatic tumor which has low vascular density [122]. Thereby therapeutic effect of macromolecular drugs will be enhanced while side effect will be lesser. Whilst most micellar nanoparticle drugs and liposome formulations require proper rate of drug release in order to exert optimal therapeutic effect, these formulations need special attention involving stability and modulation of release kinetics in vivo. This involves meticulous adjustments in the formulation, or selection of materials, or chemistry during development of drugs for human use, not for mice.

We discussed here the advantages of polymer therapeutics or nanomedicine including tumoritropic and lymphotropic nature; enhanced residence time in circulation and in tumor; lesser side effect than parent drugs; and ways to augment the delivery of polymeric or macromolecular drugs, e.g. by elevating blood pressure. Further, benefits of macromolecular drugs are also found against multidrug resistance. All these improved characteristics warrant the promising future of macromolecular therapeutics for many diseases, especially for cancer.

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Review article

Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect

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ABSTRACT

For over half a century extensive research has been undertaken for the control of cancer. However, success has been limited to certain malignancies, and surgical intervention is potentially curative for early stage patients. For the majority of patients with advanced stage of cancer, the treatment is limited to chemotherapy or radiation. Chemotherapy in particular has limitations due to the lack of selectivity with severe toxicity. Under these circumstances tumor-targeted delivery of anticancer drugs is perhaps one of the most important steps for cancer chemotherapy. We reported such a drug for the first time, styrene-maleic acid copolymer-conjugated neocarzinostatin (SMANCS) in 1979, and it eventually led to formulate the concept of the enhanced permeability and retention (EPR) effect of solid tumors in 1986. Monoclonal antibody conjugates are another direction, of which interest is increasing recently though with limited success. The EPR-effect appears as a universal phenomenon in solid tumors which warrants the development of other polymeric drugs or nanomedicine.

EPR-effect is applicable for any biocompatible macromolecular compounds above 40 kDa, even larger than 800 kDa, or of the size of bacteria; thus complexed molecules like micelles and liposomes containing anticancer drugs are hallmark examples. The drug concentration in tumor compared to that of the blood (T/B ratio) can be usually as high as 10–30 times. In case of SMANCS/Lipiodol given via tumor feeding artery, the T/B ratio can be as high as 2000, a real pin-point targeting. EPR-effect is not just passive targeting for momentary tumor delivery, but it means prolonged drug retention for more than several weeks or longer.

This review describes the pathophysiological mechanisms of the EPR-effect, architectural difference of tumor blood vessel, various factors involved and artificial augmentation of EPR-effect with respect to tumor-selective delivery, and then advantages and problems of macromolecular drugs.

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1. Introduction

The field of drug delivery systems (DDS) utilizing synthetic polymers either by covalent conjugation or by composite of micellar drugs has become a new domain for new drug development for numerous diseases. Synthetic polymers become an indispensable component for micellar or stealth liposome drugs and protein–polymer conjugates [1–3]. These polymer-based new drug entities are called "polymer therapeutics" [2,3] or macromolecular drugs, and they overlap with nanomedicine that becomes popular in recent years [4]. The polymer therapeutics or nanomedicines are designed to improve drug performance by utilizing pathophysiological uniqueness of solid tumor, of which conventional low molecular weight drugs are incapable. Macromolecular drugs or nanomedicines show improved tumor-selective targeting; the improved therapeutic efficacy and fewer side effects are their primary benefits, in which prolonged circulation time plays a crucial role [4–9].

Most conventional low molecular weight anticancer drugs have inherent character to traverse in and out of blood vessels freely, unless the drug is linked with a tumor-specific molecular ligand having high binding constant. For instance, low molecular weight

Abbreviations: AT-II, angiotensin-II; AUC, area under the concentration curve (vs time); CML, chronic myeloid leukemia; EPR, enhanced permeability and retention effect (of macromolecular drugs in solid tumor); HPMA, poly(hydroxypropyl methacrylic acid); HCC, hepatocellular carcinoma (hepatoma); i.v., intravenously; i.a., intra-arterially; MDR, multidrug resistance; NCS, neocarzinostatin; NO, nitric oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; PEG, polyethylene glycol (also called polyoxyethylene); PGs, prostaglandins; PEG-poly(Asp), block copolymer (polyethylene glycol) linked to poly (aspartic acid-benzyl ester); SMA, copolymer of styrene-maleic acid; SMANCS, copoly (styrene-maleic acid) conjugated neocarzinostatin; SOD, superoxide dismutase; T/B, tumor to blood ratio of drug (delivered concentration); VPF, vascular permeability factor; VEGF, vascular endothelial growth factor.

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drugs injected intramuscularly reach a distant site of the body in 10 min. Consequently, their undesirable indiscriminatory distribution in normal tissues causes severe systemic side effects in case of anticancer agents. Namely, free diffusion of toxic drugs in a non-selective manner in the body, and their inability to accumulate selectively in tumor tissues make them disastrous to patients.

Experiences in antibiotic research tell us that selective toxicity is possible in prokaryotic kingdom, where different types of biochemical machinery are used from the animal kingdom. For instance, machinery for protein-synthesis (ribosomes) in prokaryotes is different from eukaryotic cells. Similarly, the cell-wall synthesis of peptidoglycan in bacteria (a target for penicillin) does not exist in eukaryotes. On the contrary, it is difficult to get tumor-selective toxicity because the biological events taking place in cancer cells are essentially the same as that of the host cells. Namely, there is little difference in biochemical or molecular machinery between cancer and normal cells at a cellular or molecular level. Therefore, to target tumor cells more selectively, active targeting based on antibodies or the receptor-mediated targeting with cancer-specific ligands are developed. However, recent clinical results of molecular target-based drugs were somewhat disappointing, if not completely.

Tumor cells have inherent heterogeneity and epitopic diversification as a result of great magnitude of mutation frequency even amongst the same cancer patient [10,12]. The recent results of cancer genomics showed that most human solid tumors were not only single gene-based events, but also multiple genomic alterations. Namely, there were no specific alterations or gene mutations common among individual patients if not name p53, a cancer suppressor gene. Extensive genomic studies of 11 colon cancer and 11 breast cancer patients revealed numerous genetic variants arising from a single solid malignancy. On average about 90 or so such variants were found in a patient. This makes the task of specific antibodies for each of these diverse epitopic targets inefficient if not unrealistic from antibody therapy [10,11].

Furthermore, according to recent reports in the annual meeting of *American Society of Clinical Oncology (ASCO)*, efficacy of molecular target drugs exhibited only 4–5% of response rate despite very high expectation and very high cost of manufacturing. It is generally thought this much efficacy is only useful as adjuvant or supplementary. Although it is beyond this review, the costs of antibody drugs are so expensive that public and national insurance systems may be at risk aside from the low response rate achieved with these therapies. In this regard, for example, Avastin was not recommended in the UK for reimbursement of national insurance [13]. Thus, 'the cost-benefit' will be considered more than ever for drug approval. Recently, the editorial in the Lancet criticized these issues one step further for drug appraisal [14].

Under these circumstances, a more universal and efficient strategy for anticancer drug design having high selectivity to tumor tissues must be developed. To solve this problem, the phenomenon of "enhanced permeability and retention (EPR)-effect" discovered by Maeda and Matsumura is now becoming the gold-standard in cancer-targeting drug designing that is based on macromolecular, micellar and lipidic particles [5–9], all utilizing EPR-effect as a guiding principle, and the EPR-effect is applicable for almost all rapidly growing solid tumors [7–9,15–18].

Most importantly, EPR-effect can be observed in almost all human cancers with the exception of hypovascular tumors such as prostate cancer or pancreatic cancer. As clinical examples for this, we have experienced that SMANCS/Lipiodol given via the hepatic artery accumulated selectively in hepatocellular carcinoma distinctively [7,17,19–22]. A similar result in clinical setting was also reported for Doxil, a liposomal type of doxorubicin. Namely, Doxil mimic was prepared for radio scintigraphy, and clear tumor accumulation was seen in the whole body scintigram [23]. Another clinical example of EPR-effect can be demonstrated in the traditional tumor imaging in the clinic that utilizes (γ)-emitting gallium scintigraphy based on the selective accumulation of radioactive gallium (used as citrate) in the tumor. ^{65}Gallium ion as injected i.v. will bind to plasma protein transferrin (90 kDa) in the blood, thus radioactive transferrin will accumulate in the tumor by EPR-effect, which will take more than 24 h. Usually radio-scintigram is obtained 2–3 days after intravenous injection of ^{65}Ga when signal/noise ratio is improved; while its clearance from the normal tissues will take place in a day or so via the lymphatic system. The tumor, however, retains this ^{65}Ga-transferrin for several days at high levels by EPR-effect.

Another case of EPR-effect observed in human tumor is the selective accumulation of Lipiodol in the tumor after intra-arterial infusion of Lipiodol which is visualized by X-ray CT-scan [19–21]. This tumor detection method by use of Lipiodol staining after our report [19–22] is now becoming a routine examination before hepatic tumor resection.

Conventional angiography for tumor detection uses water-soluble low molecular weight radio contrast agent, and its high electron density yields staining of tumor as it is infused intra-arterially. This means increased uptake (staining) of this contrast agent by the tumor mass, which is a part of EPR-effect though it is washed out rapidly by diffusion due to the small molecular size (i.e. no retention). Thus, the tumor staining in this angiography is only transitory, less than a few minutes or so. This is a passive delivery of drug, but not EPR-effect which requires long time retention.

Based on EPR-effect, many polymeric drugs are being developed as a new class of antitumor agents [1,24–28], including nanoparticles [16], polymer micelles [27,28] and liposomes [28–31]. Further, EPR-effect is not only limited to these nanoparticles, but it is also valid for tumor-imaging contrast agent Lipiodol as described above, where Lipiodol shows virtually pin-point targeted delivery to the tumor, i.e. tumor/blood (T/B) ratio of more than 2000 can be obtained [19–22,32]. Further, bacterial cells as well as quantum dots (QDs) as ultra-sensitive imaging probe showed more selective accumulation into tumor tissue, which can be explained by EPR-effect [33–35].

In this review, we will describe current problems in cancer chemotherapy, the mechanism of EPR-effect and factors involved, artificial augmentation of EPR-effect for polymeric or macromolecular drugs under the angiotensin-II (AT-II) induced hypertension, and advantages of macromolecular drugs are also discussed.

2. How good is cancer chemotherapy; status quo

In the past 40–50 years, low molecular weight anticancer drugs have been the main treatment modality for many cancers of advanced stage, but have offered no improvement in the cure rate [36,37]. The biggest limitation of these therapeutic agents is overwhelming toxicity due to lack of selectivity. Scientists realized this fact finally towards the end of 20th century and thus cancer-selective targeting became one of the most important goals.

Theoretically, in drug development, molecular target drug is considered more ideal not only in cancer but also in other diseases such as inflammation, primarily to avoid side effects. In this context, *Meares*, editor in chief of *Bioconjugate Chemistry*, a journal of *American Chemical Society*, commented that drug targeting was the most popular subject recently in this journal [38]. In any event, cancer still remains a major cause of death in most developed countries, and the lack of effective control of many cancers is becoming increasingly burdensome on the health care system [36].

As discussed in above references [36,37], the death rate of major cancers such as lung, breast, colon, prostate, and pancreas at advanced stage, has not changed much in the last half century. Further, the clinical benefit of chemotherapy in the most common cancers, for instance the breast cancer and the prostate cancer, is

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only marginal when the stage of cancer is advanced. Among the breast cancer patients who underwent surgery only, or who had surgery plus chemotherapy, the benefit of chemotherapy is only 2–3%. Namely, survival extension was 2 months in 60 months, while extensive medical cares including blood transfusion, platelets and leukocytes transfusion, injection of hematogenic factors (EPO, GCSF, etc.), antibiotics, ambulatory care or hospitalization are required for chemotherapy patients, which requires a huge expense. In addition, severe loss of appetite, hair loss, and poor quality of life (QOL) are more frequent in chemotherapy [39,40].

There have substantial increases in survival rate in some malignancies such as early stage gastric cancer by surgical intervention. Clinical results in lymphocytic leukemia and Hodgkin's disease have also been improved more effectively with chemotherapy than before. Early stage cervical cancer has responded well to surgery, radiation, and chemotherapy or a combination approach. Another most recent successful agent is *Gleevec*® (Imanitib) against chronic myeloid leukemia (CML) which has demonstrated 90% regression at least for 6 months. CML is one of the few cancers caused by the activation of *ABL/BCR* oncogene. However, at 6 months when leukemic cells began to propagate (called blastic crisis), drug-resistant mutants concurrently emerge in many or most, if not all patients, making a cure for CML far from reality. Life expectancy of CML patients is about five years or longer if patients remain stable and if it does not turn to blastic crisis.

Prostate cancer is one of the most common cancers amongst males in developed countries. Among the prostate cancer patients, 10–20% have metastatic diseases, most commonly to the bone. These metastatic tumors are less frequently treated by radiotherapy or by surgery, and chemotherapy is used as the next best alternative. In one recent study with more than one thousand prostate cancer patients with hormone refractory metastasis, clinical efficacy of docetaxel plus prednisolone, or mitoxantrone plus prednisolone, was evaluated [41]. The median survival was 17.4 months and 18.9 months, respectively. Although PSA levels decreased by more than 50% in 30–50% of the patients and the QOL was improved, adverse side effects remain a severe problem compared with the slight survival benefit. Similar studies without steroid resulted in 10–21 months of median survival span. An earlier study with mitoxantrone plus prednisone or prednisone alone showed no difference in overall survival benefit [42]. In any event about 65–70% of all patients died within three years, which means a not so remarkable cure rate, and also PSA is not a so accurate marker.

A few kinases, growth factors, DNA polymerases, transporters, etc. are also believed to be unique molecular targets, but the problems are that therapeutic windows are too narrow due to dose limiting toxicity, followed by emerging resistance. One such protein kinase inhibitor trastuzumab (Herceptin®) is an antibody against epidermal growth factor receptor (HER-2) and is used for breast cancer. Herceptin® targets to oncogene product "HER-2", it is thus only suitable for HER-2 positive patients, which comprises only 20% ~ 30% of the total breast cancer patients. Furthermore, among this group, only 20–30% will respond to Herceptin. Therefore, the value will end up at about 4–6% as an overall response rate.

Whatever the chemotherapeutic agents currently available, their excessive and prolonged exposure will ultimately damage normal cells. More serious of all is emergence of drug resistance, even to Gleevec®. Therefore, importance of more universal tumor-targeted drug delivery becomes obvious under these circumstances.

3. Phenomenon and mechanism of the EPR-effect

3.1. Historical background

During the study of in vivo behavior for antitumor protein antibiotic, neocarzinostatin, and its polymer conjugate SMANCS, Maeda et al. discovered a great difference in tumor uptake between low molecular weight drugs and biocompatible macromolecular drug SMANCS in solid tumors [17,24,43–46]. In a series of studies, the EPR-effect was discovered using the most biocompatible macromolecule drug candidates such as albumin (65 kDa), transferrin (90 kDa), IgG (immunoglobulin, 150 kDa), α_2-macroglobulin (240 kDa), ovomucoid of chicken eggwhite (29 kDa, highly glycosylated protein), SMANCS (16 kDa, with albumin binding character becomes 80 kDa) and neocarzinostatin (12 kDa) [6,7]. Macromolecules such as albumin, transferrin, IgG, SMANCS and lipid particles (Lipiodol) accumulated in the solid tumor so remarkably, and they were retained for a very extended period up to 3 months or longer [5–9,17,22].

In general, they found the areas under the concentration curve vs time (AUC) of a drug and its tumor uptake are positively related, whereas the rate of urinary clearance is inversely related to tumor uptake [5,7,46,47]. In collaboration with Prof. K. Ulbrich and Prof. R. Duncan, a series of biocompatible synthetic polymers, HPMA (4-hydroxypropylmethacrylate copolymer) ranging from 20 to 800 kDa in various tumor models (S-180, meth A, colon 38, VX-2 of rabbit, Walkar-256 carcinomas and AH66 of rats) were investigated, and the findings are consistent with previous studies [5–7,46,47]. These polymers displayed remarkable tumor accumulation in mouse and human xenograft tumors up to ~20% dose/g in some case, and 1–5% dose/g usually in small tumors. However, in larger tumors, i.e. more than 0.5 g, the tumor uptake of Evans-blue albumin became lower [46,47,64]. From these results we concluded that, in order to exhibit features of the EPR-effect, a drug must have a higher molecular weight than the renal excretion threshold (typically > 40 kDa) [5–9,17,25–27,46,47].

3.2. Factors involved in EPR-effect

Maeda et al. had previously demonstrated that bacterial proteinases initiated bradykinin generation via factor XII (Hageman factor) activation followed by activation of prekallikrein to kallikrein, which releases bradykinin from kininogen. Bradykinin is known to induce vascular permeability and plays an important role in the EPR-effect. Thus the site of infection/inflammation where excess of bradykinin is generated also exhibits EPR-effect [50–53]. This study was extended to identify the cause of ascitic and pleural effusions of carcinomatosis in peritoneal and pleural compartments. In these studies Maeda et al. were able to identify that bradykinin formation was responsible for the extravasation of ascetic fluid [54–59]. The only difference between infection-induced EPR-effect and that of cancer is duration of retention period; the retention in normal tissue, where inflammation occurs, is shorter than cancer because the lymphatic drainage system is still operative, thus swelling may dissipate in a matter of a few days. In contrast, macromolecular or lipidic drug retention in cancer tissue can be as long as weeks, a great contrast to that of infection-induced inflammation [7,17,19–22]. Under these circumstances, it was realized that vascular phenomenon was important not only because of its critical role in tumor growth and metastasis, but also for tumor-selective delivery of anticancer agents, on which Maeda's group had been working [19–22]. Then, they demonstrated the EPR-effect [5]. Namely, EPR-effect is a phenomenon of enhanced extravasations of macromolecules from tumor blood vessels, and their retention in tumor tissues, which is not observed in normal vasculature.

We then investigated vascular mediators involved in EPR-effect and found that nitric oxide (NO) also facilitates this effect [58,59,64,65] in addition to bradykinin. Oxidized product of NO (peroxynitrite) also shows this effect with or without activation of collagenase [66,67]. A similar phenomenon was observed with other proinflammatory mediators such as prostaglandins [58,66,68] and vascular endothelial growth factor (VEGF) [54,58,

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69–71] which was formerly identified by Dvorak et al. as vascular permeability factor (VPF) [71]. In these connections, dual function of VEGF, namely, angiogenesis and enhancement of vascular permeability, both facilitate and sustain rapid growth of tumor. Furthermore, NO is also involved in tumor growth [72], VEGF and bradykinin are also known to be potent activators of NOS which generates NO from L-arginine. Following is the summary of factors facilitating EPR-effect:

(1) Vascular endothelial growth factor (VEGF/VPF).
(2) Bradykinin/3-hydroxyprolyl bradykinin (BK).
(3) Nitric oxide (NO).
(4) Peroxynitrite ($ONOO^-$) (reaction product of superoxide anion radical and NO).
(5) Prostaglandins (PGs).
(6) Matrix metalloproteinases (MMPs); proMMP is activated to MMP by $ONOO^-$.
(7) Other proteinases (e.g. kinin/kallikrein system) involving various protease cascade.
(8) Other cytokines (e.g. tumor necrosis factor, IL-2, and TNF-α.) also facilitating EPR-effect.

3.3. Anatomical and pathophysiological difference of tumor vasculature

As to the anatomical or architectural difference between normal and tumor vasculature, O'Brien's group has demonstrated remarkably unique difference using scanning electron microscope (SEM) [60,61]. Namely, a water-soluble acrylic monomer that forms polymeric resin in the blood vessels was injected into the blood vessel of tumor-bearing mice, thus the cast of the blood vessels of tumor was obtained, which is made of plastic resin. As shown in Fig. 1(C and D), plastic resin is extravasculated extensively outside of blood vessels in the metastatic tumor nodule; more importantly these features are not seen in normal capillary (Fig. 1A and B).

These photographs (Fig. 1C and D) clearly show completely different morphology of tumor blood vessels in microtumor nodules (Fig. 1C) compared with that in normal blood capillary (Fig. 1A and B). More recently Hashizume et al. also observed this phenomenon in mouse mammary carcinoma using scanning electron microscopy [62] and identified structural abnormalities in the endothelium of tumor blood vessels, whose intercellular openings were found to be as large as 4.7 μm (mean size 1.7 μm) in diameter. In other studies Yuan et al. have measured the pore size of tumor vessel in LS-147 human colon adenocarcinoma implanted in SCID mouse and found that it could be as large as 0.4 μm in diameter [63]. All these studies demonstrate that the abnormal vascular architecture plays a major role in excessive vascular permeability of tumor, i.e. EPR-effect in tumor for selective macromolecular drug targeting at tissue level, that can be summarized as follows:

(1) Extensive angiogenesis and high vascular density (hypervasculature).
(2) Lack of smooth-muscle layer; pericytes; no AT-II-induced vasoconstriction; more leakage at tumor vessels under hypertensive state.
(3) Defective vascular architecture; irregular vasculature networks, large openings, fenestration.
(4) Whimsical blood flow; no constant blood flow and direction.
(5) Meager lymphatic clearance that leads to enhanced retention of macromolecular drugs and lipidic particles in the interstitium of tumors.
(6) Slow venous return that leads to accumulation of macromolecular drugs and lipidic particles from the interstitium of tumor.

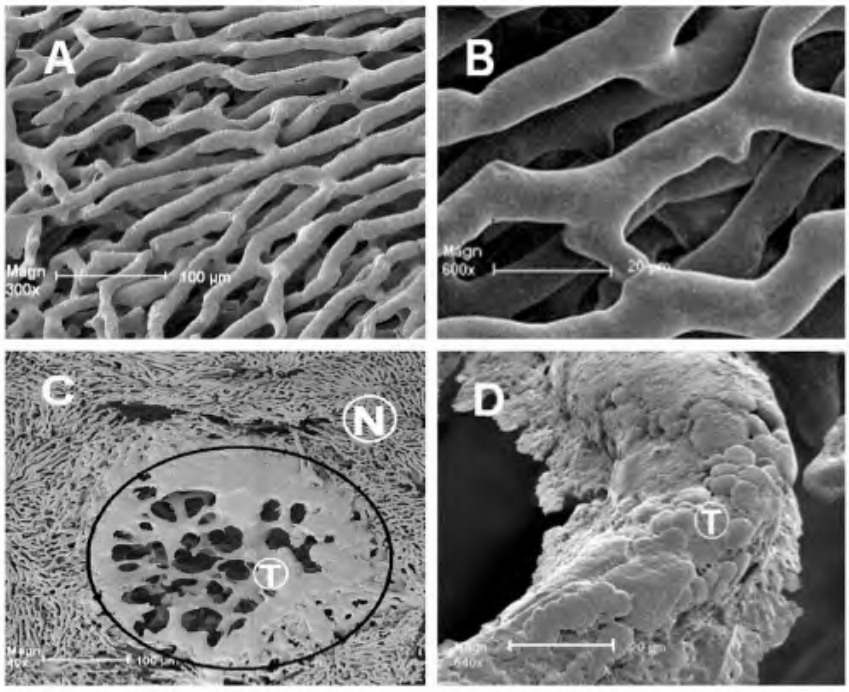


Fig. 1. Scanning electron micrograms of blood vessels at capillary level in normal tissue [liver] (A and B) and blood vessels of tumor nodules (C and D) in the liver. Enlarged image of capillary network of (A) is shown in (B). No leakage of the polymer is seen in the normal liver as indicated by N whereas tumor-selective extravasation of polymer (by EPR-effect) in tumor nodule (T) is seen, but it is not observed in normal vascular area N, or in (A) or (B). (D) Tumor vessels in which extravasation of acrylic polymer is seen. This will lead to selective delivery of polymeric drug in tumor as seen T in (C) and (D) [58,61].

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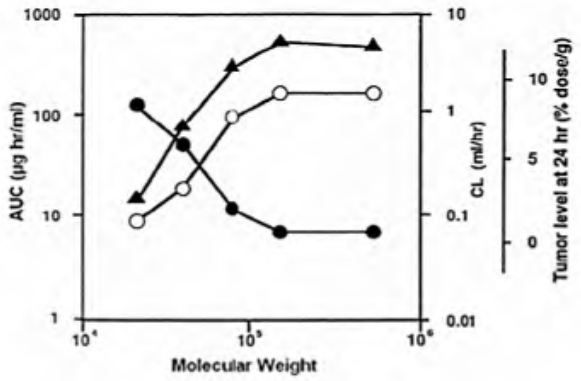


Fig. 2. Relationships between molecular weights and tumor uptake and clearance of ^{125}I-tyr-HPMA-polymer of varied molecular weight drugs. Mice bearing S-180 solid tumor received about 1.8×10^6 cpm/mouse by i.v. injection. ●, CL = renal clearance rate; ▲, AUC = area under the concentration curve for different Mw polymers in plasma. Both values are based on 72 h after injection; ○, tumor uptake values are based on 24 h data [18].

Demonstration of the EPR-effect in metastatic micronodules of tumor in the liver is seen in (Fig. 1C) by using SEM, and blood capillary of normal liver is shown in (Fig. 1A and B). Tumor blood vessels illustrated here show that monomer of plastic resin penetrated the vascular wall during polymerization (Fig. 1C and D), and the vascular network of tumor vessels in the nodule looks abnormal and shows appearance of leaked resin; these are clearly different from the blood capillary of normal liver tissue. Namely, the tumor nodule is selectively almost filled with plastic resin (Fig. 1C).

In the case of SMANCS/Lipiodol, it is administered via the tumor feeding artery. This arterial infusion will result in direct drug capturing, that is known as the first path effect, in which EPR-effect works additively [20–22]. Remarkable success of the SMANCS/Lipiodol therapy can be attributed to the hypervascular nature as well as EPR-effect due to excessive vascular mediators in the hepatoma. This trend of efficient uptake of SMANCS/Lipiodol is also seen for renal cancer. Most water-soluble polymers can accomplish EPR-effect by i.v. infusion as seen by T/B ratio ranging 5–30 [5,7,15–18,64] and EPR-effect becomes obvious even 10 min after i.v. injection [46]. The tumor/normal tissue ratio is as high as 30- to 300-folds at 6 h or later, of which the values depend largely on time of quantification. Most of the macromolecular drugs exhibit prolonged plasma $t_{½}$ (>several hours) and very low renal clearance as seen in Fig. 2.

4. Recovery of macromolecules from the tissues interstitium: the lymphotropism and lymphatic metastasis

When lipids and lipidic particles or macromolecules are injected into the interstitial space of normal tissue, they are known to be recovered via the lymphatic systems. Now it should be noted that this is the route of clearance of lipidic or macromolecular drugs from the normal tissue, but it is also the route of lymphatic metastasis of cancer cells. Initially, Maeda et al. found that when NCS (12 kDa) or SMANCS (16 kDa, by albumin binding it becomes 80 kDa) was injected into normal tissue subcutaneously, they accumulated most effectively in the regional lymph nodes, and very slowly traversed into the general circulation [73–76]. The lymphotropic property is more clearly seen for lipids in general. Namely, the lymphographic contrast agent Lipiodol® (Laboratoire Guerbet, France), which is iodinated poppy seed oil, has been used for lymphography because it moves into the lymphatics and its exclusive presence in the lymphatic duct can be detected under the X-ray system. It should be worthwhile to count these lymphotropic characters in the drug designing aiming at antimetastatic property. The control of lymphatic metastasis is so important because lymphatic metastasis of tumor is the more frequent cause of therapeutic failure. Therefore, suppression of lymphatic metastasis by lymphatic drug delivery with the use of liposomes or nanoparticles may be of critical importance [24,73–75].

Antitumor efficacy is usually judged by the effect on the major or primary cancer, which is the target of current chemotherapy or surgery, and concomitant effect on micrometastasis in the lymph nodes or elsewhere will become ultimately very important, which can be more effectively achieved by lymphotropic drugs. Further, definition of the regression (response) is traditionally defined as size reduction to less than half of the original size. For instance, metastatic micronodules as shown in Fig. 1C are invisible under conventional methods of cancer detection. Thus, evaluation of response in these microtumor by tumor size is not possible. More recently, prolongation of survival time span is being considered as an important parameter. However, it is often difficult to control the lymphatic metastasis. If one could control lymphatic metastasis by the lymphotropic drug delivery, one would contribute survival span. In the SMANCS therapy, we experienced that there are many patients who become tumor stabilized (but not regression) after treatment without further disease progression, nor emergence of new tumor. As stated above, lymphotropic nature of macromolecular drugs will contribute on this point and it will be a highly preferred character. In this connection, a recent report of PEG-interferon 2b treatment resulted in prolonged tumor-free survival of patients with malignant melanoma, which is interesting [77].

5. Augmentation of EPR-effect and drug delivery by angiotensin-II induced hypertension

The vascular density of many tumors, if not all, is higher than that of normal tissues. Furthermore, tumor blood vessels frequently lack the smooth-muscle layer, which plays a vital role in regulating blood pressure and flow volume. In normal blood vessels, the smooth-muscle layer responds to vascular mediators such as bradykinins, acetylcholine and NO via receptor binding on vascular smooth-muscle cells, which modulate intracellular calcium levels, helping to maintain constant blood-flow volume. In normal tissues, when hypertension is induced by infusing angiotensin-II (AT-II) intravenously, vasoconstriction of the smooth-muscle layer will result in higher blood pressure and higher flow velocity. Interestingly, however, the blood-flow volume remains constant [78]. In contrast, in tumor tissues under the AT-II-induced hypertensive state, the blood-flow volume does not remain constant. Tumor blood-flow volume actually increases in response to elevated blood pressure. In tumor-bearing rats, raising the systolic blood pressure from 90 to 160 mm Hg resulted in nearly 2–6 times increase in tumor blood-flow volume depending on the blood pressure attained, whilst blood-flow volume in the normal tissues such as the kidney and the bone marrow remained constant [78]. Therefore, we anticipated that the induction of the hypertensive state would augment the EPR-effect, and hence, the delivery of macromolecular drugs would increase. In fact, when ^{51}Cr-labeled SMANCS or ^{51}Cr-labeled albumin was injected i.v. in rats bearing Walkar-256 carcinoma and when the blood pressure was raised from 90 to 150 mm Hg only for 15 min, there was a 1.3- to 3.0-fold increase in accumulation of these macromolecular drugs in the tumor tissue [79]. At the same time, a significant reduction of the drug delivered to healthy organs (e.g. intestine, kidney, liver and bone marrow), and thereby lesser toxicity, was observed because of vasoconstriction and tightening of endothelial gap junctions, which suppressed the extravasation of polymeric drugs.

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Similar results were obtained with SMANCS/Lipiodol administered under the AT-II-induced hypertensive state, in many patients with solid tumors including hepatocellular carcinoma (HCC), metastatic liver cancer, cholangiocarcinoma, pancreatic cancer, and renal cell carcinoma [7,9,18,80 – and unpublished data]. It is known that in common cancer chemotherapy with low molecular weight anticancer agents, the anticancer agents should be administered no more than the recommended dose, because of severe dose limiting toxicity. However, under the hypertensive state, using macromolecular drugs in animal models as well as in the clinical setting, it was possible to achieve more than 1.3- to 5-fold higher tumor concentration in the tumor with the same dosage. More importantly, sufficient augmentation of tumor-targeting effect could be achieved even when hypertension was maintained for only 20 min, meanwhile no apparent toxicity was seen [9,80]. Having these obvious advantages and evidences, we warrant AT-II-induced hypertension chemotherapy should be applied for macromolecular anticancer chemotherapy. This method is approved in Japan for conventional drugs but not yet for nanomedicines.

6. Consideration in designing of macromolecular anticancer drugs: for utilization of tumor targeting mechanism by EPR-effect

6.1. Release rate and pharmacokinetics

In recent years, many efforts were directed towards designing of macromolecular drugs utilizing EPR-effect for tumor-seeking anticancer agents. The method involves conjugates or encapsulation of low molecular weight anticancer drugs, or proteins by chemical conjugation or micellar or liposomal encasement, respectively, to water-soluble, biocompatible, biodegradable polymers, or nanoparticles such as encapsulated liposomes or polymeric micelles [1–4,25–28,81]. The crucial point of all is the optimally sustained rate of drug release from these nanoparticles to attain the required drug level.

In this context, when liposomes are too stable and have slower release rate than the optimum rate for drug release, the drug concentration cannot reach the required concentration within the adequate time span. Doxil, adriamycin encapsulated liposome, belongs to this category, and it is used for first line of defence against Kaposi's sarcoma and second line drug for breast cancer and others, but not universal for solid tumor in general. On the other hand, such liposomes also need to be stable enough for storage period, and on the shelf, or refrigerator to the bed side. As an example, adriamycin-encapsulated block copolymer micelles (PEG-polyAsp), designated NK-911, on the contrary showed initial rate of drug release too fast, it thus could not release adriamycin in a sustained manner for adequate time span of few days. Therefore, it cannot attain adequate pharmacological drug concentration inside tumors in human. As 50% of the drug was released less than an hour after i.v. injection, this micellar formulation has no advantage (Fig. 3A-(b)) [25,26,28,81], also the results in phase I discouraged for further development.

In Fig. 3B, plasma concentration of another synthetic polymer micelle SMA-doxorubicin is shown, which exhibits longer residence time than NK-911. Further, when one compares between SMA-micelles of doxorubicin and pirarubicin, the latter micelle showed higher AUC and very higher tumor uptake than SMA-doxorubicin. Namely, SMA-pirarubicin micelles showed 100 × higher plasma AUC and 27 × higher tumor concentration than parental-free pirarubicin [25,26]. This means a combination of a drug and chemical constituent of the micelles is an important factor to have preferred characteristics.

6.2. General advantages of polymer conjugates or nanomedicine

6.2.1. Prolongation of half-life, stealth character and reduced antigenicity

One of the important advantages of polymer conjugation is the stabilization and prolongation of the plasma half-life of the low molecular weight drugs or proteins. SMANCS, a conjugate of SMA and neocarzinostatin (NCS), showed this character as well as polyethylene glycol (PEG)-conjugated bilirubin oxidase and others [24,76,82]. PEG-conjugated interferon, PEG-INFα2a (Pegasys®), showed increase in plasma half-life to about 10-fold of native INFα2a (8 h vs 80 h) when injected s.c. in human [83]. Second advantage is solubilization of insoluble low MW compounds as discussed below. Further, polymer conjugates or micellar formulation under specific condition also confers 'stealth' characteristics and diminishes uptake by the reticuloendothelial system (RES) or macrophages, and hence the half-life in blood circulation can be extended [29–31,82,83]. These merits cannot only improve the chemotherapeutic efficacy, but also reduce the time and cost of frequent injections to a great extent. This had been observed in SMANCS/Lipiodol, of which arterial injection is required at most once a month, usually once in 2–3 months or less [7].

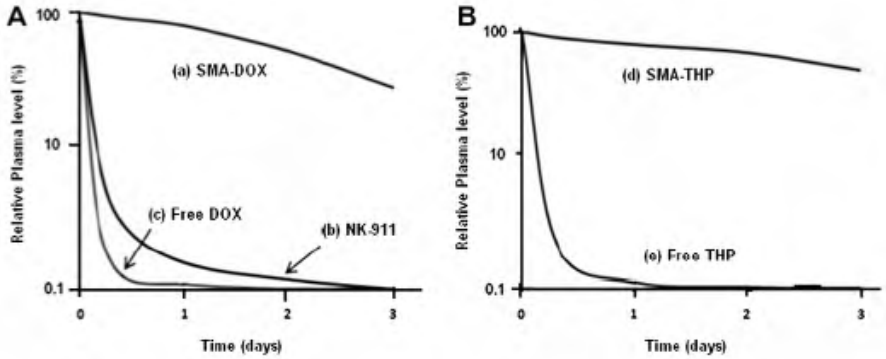


Fig. 3. Improved plasma half-life of anthracyclines by micellar encapsulation, and different micelle materials and different drugs are shown. (A) Plasma half-life of free doxorubicin (DOX) and that of SMA or PEG-Asp block copolymer micelles. SMA-micelles of doxorubicin shows much prolonged plasma residence time [25], while PEG-Asp block copolymer (NK-911) bursted very rapidly within short time after injection. The data values were simplified from published literature [28]. Higher stability of SMA-DOX micelle is apparent. (B) SMA-THP (4-O-tetrahydropyranyladriamycin) micelles are compared with that of free THP in human cancer patients. Advantage of SMA-THP micellar formulation in the plasma residence time over free THP is apparent as well as higher tumor targeting by EPR-effect [25,26]. Free THP is more rapidly taken up by cancer cells than free doxorubicin. THP has higher affinity to SMA than DOX so that SMA-THP micelles in plasma stayed longer and more stable than SMA-DOX (A vs B).

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6.2.2. Biological response modification or immunopotentiation by polymers

Some polymer conjugates, such as SMANCS, for instance, displayed various immunomodulating properties, including macrophage (MΦ) activation, induction of interferon-γ production [84–87], and augmentation of splenic and peritoneal natural killer (NK) cells [86,87], thus it stimulates non-specific cytotoxicity to tumors in mice bearing syngeneic tumors. Similar findings were also reported for pyran copolymer and other anionic polymers, which have the capacity to induce interferons and other cytokines [86].

6.2.3. Resistance against free radicals, proteolytic degradation, addition of metal chelating capacity and surface lubrication

The pathological and inflammatory lesions including cancer are constantly under the exposure of endogenously formed free radicals such as superoxide anion radical $O_2^{\cdot -}$, hydrogen peroxide (H_2O_2), NO, $ONOO^-$ and hypochlorite (HClO) [88–91]. Many proteins, enzymes, nucleic acids or active drugs, when exposed to these radicals, may be readily inactivated or modified. However, polymer conjugation or micellar shield will confer better stability against these radicals. To clarify this point, we studied the effect of oxygen radicals (ROS) to functional proteins in model systems [92,93]. One example is superoxide dismutase (SOD), which catalyzes the dismutation of superoxide anion radical $O_2^{\cdot -}$ to H_2O_2. Subsequently, if catalase is not available in the milieu, H_2O_2 will accumulate in the lesion. We previously showed that Cu, Zn-SOD was disintegrated by exposure to H_2O_2 (the product of SOD), and then free Cu^{2+} ion was liberated, which will serve to catalyze ·OH generation from H_2O_2 effectively by Fenton type reaction [92,93]. However, polymer conjugates of SOD with pyran copolymer {DiVEMA, poly(divinylether-maleic acid)} remarkably reduced this ·OH generation, suggesting the protective and stabilizing effect of DiVEMA modification to SOD against free radicals. Further, we extended the study to an acute phase plasma protein, α_1-protease inhibitor (α_1-PI) as the target using various polymers *per se* to suppress inactivation by ·OH. Among the polymers tested, pyran copolymer showed the most potent inhibitory activity against ·OH generation and greatest suppressive effect against α_1-PI inactivation, whereas BuSMA (butylated-SMA) or PEG alone suppressed α_1-PI inactivation only weakly (Table 1 and Fig. 4) The effect of pyran copolymer may be attributed to the chelating activity of Cu^{2+} by dicarboxylate ion in pyran copolymer, which was found to exhibit high activity comparable to the potent chelating agent, DTPA, whereas not all polymers have bicarboxylic groups thus showing no chelating effect, but shielding effect.

Along this line, we further examined the time course of H_2O_2-induced inactivation of native SOD and various polymer-conjugated SOD, and those of α_1-PI by ·OH radical similarly. Namely, assuming the metal (Cu^{2+}) would catalyze Fenton reaction, through which H_2O_2 will become hydroxyl radical in the presence of various SODs and α_1-PI. We found that polymers having paired carboxyl groups and OH groups such as pyran copolymer and PVA effectively suppressed the inactivation of SOD as well as α_1-PI. It is probably attributed either by direct scavenging of ·OH (by PVA) or by chelating transition metal ion Cu^{2+} [88,92,94].

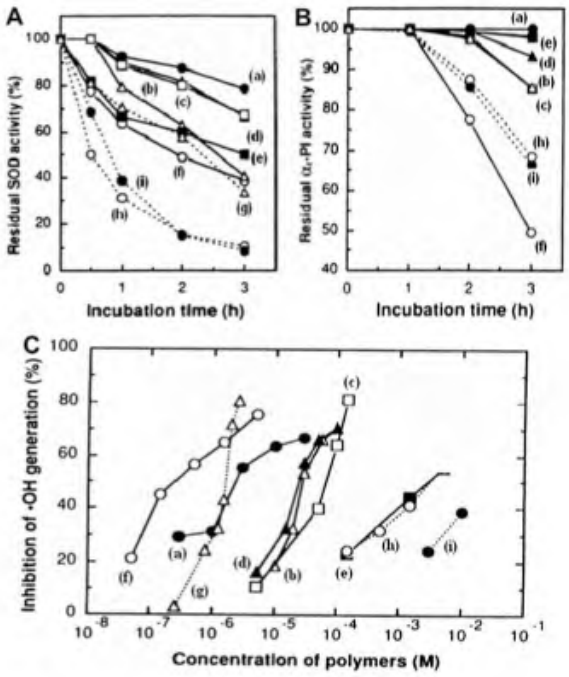


Fig. 4. Protective effect of enzyme activity of SOD by various polymer conjugation (A), and (B) inactivation of α_1-protease inhibitor (α_1-PI) both by exogenously adding H_2O_2 [94]. (C) The extent of ·OH generation by SOD or various polymer-conjugated SODs upon addition of H_2O_2. Concentration of SOD and each polymer-SOD conjugates (3.7 μM) were incubated with 0.1 mM H_2O_2 in 25 mM sodium phosphate buffer (pH 7.4) for the indicated time at 37 °C. In (A), (B) and (C), (a) -●-, high molecular weight pyran(H)-SOD; (b) -△-, low molecular weight pyran (L)-SOD; (c) -□-, PVA-(L)-SOD; (d) -▲-, high molecular weight PVA(H)-SOD; (e) -■-, succinylated-gelatin SOD; (f) -○-, native Cu, Zn-SOD; (g) --△--, PEG-SOD; (h) --○--, carboxyl HSMA-SOD; (i) --●--, BuSMA-SOD. The [·OH] radical was quantified by electron spin resonance spectroscopy by using of spin trapping agent [See text for details and Ref. [92,94]] (For abbreviation, PVA, etc. see Table 1)

Another advantage of polymer conjugations is that they also confer resistance against proteolytic degradation without much affect on biological activity. We previously showed PEG-conjugated bilirubin oxidase becomes more resistant against proteolytic degradation than native enzyme [73,95].

Viscosity of high molecular weight polymers for injection-drug at high concentration may impede blood flow. We investigated previously the effect of PVA, PEG and gelatin on the lubricating

Table 1
Inhibitory potentials of various free polymers and DTPA against ·OH generation, and their protective against inactivation of α_1-protease inhibitor (α_1-PI) by ·OH[a]

Polymers (abbrev.)	Approx. Mw.	Concentration of 50% inactivation [μM]
Pyran copolymer; divinylether-maleic acid (DIVEMA)	30,000	0.48
N-ethylsuccinimidyl-S-poly(vinylalcohol) (PVA)	11,000	37.0
Succinylated-gelatin fragment (gelatin)	23,000	16.0
Poly(styrene-co-maleic acid) half-butyl ester (SMA)	1600	>1000
Monomethoxypoly(ethylene glycol) (PEG)	5000	> 100
Diethylenetriaminopenta acetic acid (DTPA, chelating agent)[b]	393	1.6

[a] Hydroxyl radical (·OH) was generated in the presence of 7.4 μM Cu^{+2} and 3 mM H_2O_2. Concentration of α_1-PI, 20 μg/ml [data are from Ref. [92]]

[b] DTPA is one of the best chelating agent; it is not a polymer. Potent suppression of ·OH generation by DTPA is due to removal of Cu^{+2} from the ·OH generating system, whereas all the polymers will scavenge ·OH, or chelating Cu^{+2} (e.g. DIVEMA) and suppress ·OH generation.

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effect of the erythrocyte passage through micropore up to 5 μm in pore size [96,97,93]. Gelatin and PEG showed preventive effect of red blood cell from bursting (hemolysis) and facilitated the passage through the micropore. Modification of SOD with succinyl gelatin also conferred this beneficial effect in addition to the enhancement of pharmaceutical effect [93].

6.2.4. Enhancement of solubility, cellular uptake, intestinal absorption, and physical stability

Besides the increase in $t_{1/2}$ and EPR-effect, we can confer different characteristics and thus drug formulations from the parent drugs by polymer conjugation or micellar encapsulation. It is well known that many promising drug candidates fail to become therapeutic agents because of their poor water-solubility. The conjugation or encapsulation of such drugs to water-soluble nanoparticles confers remarkable solubility. For instance, Taxol®, caplitaxel, KRN 5500, SN38, Zn-protoporphyrin are converted to highly water-soluble drugs [25–28,98–100]. Moreover, many proteins and drugs which have poor intestinal absorption may be rendered orally active by conjugation with appropriate polymers in combination with oily formulation [101]. One such formulation is SMANCS in medium chain triglyceride (MCT) solution (oily SMANCS); when it was orally administered to mice, it was more efficiently absorbed than aqueous solution (PBS) from the intestine. Plasma concentration or AUC of oily SMANCS given orally was about 11 times greater than that of aqueous SMANCS. In comparison to parent NCS, total AUC could be improved by more than 100-folds. Further the stability (and half-life) of SMANCS was remarkably improved in oily formulation with Lipiodol®, not to mention of its 20 times increased stability at 37 °C in vitro [102], which could be given orally, and enhancement in peak plasma level was 9-fold higher than aqueous formulation of SMANCS.

Further, enhanced intracellular uptake to tumor cells without any known specific receptor was observed for SMANCS than for NCS. Interestingly, it was more efficient in acidic pH (5.5–6.5) than neutral pH [103,104]. However, receptor-mediated internalization with tagged ligand following the EPR-effect-based vascular crossing delivery is considered efficient and important steps for this type of drugs. One such example is galactose-containing HPMA copolymer-doxorubicin targeted to the liver [48,49,105]. Another probably more promising receptor is transferrin receptor which is known to be over expressed in tumor cells. Consequently, the transferrin–methotrexate conjugate was developed, as well as liposomes [106,107]. More recently, a similar result was seen by the use of folate receptor, where folate-mitomycin C is cross-linked on polymer conjugates [108]. There are several other examples of receptor-mediated drug delivery reported elsewhere [16,109–112].

A naive question is how transferrin conjugates be so effective despite excessive presence of free transferrin in the circulation that would compete with the binding of the transferrin-conjugated drugs. The same is true, while antibody–drug conjugates are reaching the tumor-associated antigen on tumor cells, free tumor-associated antigen being produced by the tumor cells is in circulation. Then, how can it effectively reach the tumor cells despite large amount of tumor antigens in circulation, which will also bind to the target; like α-feto protein of hepatoma or CEA of colon cancer present in circulation. The answer may be that these conjugates utilize EPR-effect more than free antigens. Consequently, even though antibody function of antigen binding may be nullified by free antigens in circulation, the antigen-antibody complexes in circulation may exert EPR-effect and reach the target because of their large size, and thus they are capable of easy access to tumor cells followed by endocytosis. In any event, dividing tumor cells are known to have higher endocytotic uptake capacity than non-dividing normal cells [112], which makes another unique advantage of macromolecular drugs.

6.2.5. Advantage of polymeric drugs over p-glycoprotein-dependent multidrug resistance

To overcome the multidrug resistance (MDR), we worked with SMANCS, and found that p-glycoprotein-resistant MDR cells are sensitive to SMANCS, probably by preventing the binding of the polymeric drug to p-glycoprotein that leads to suppression of the efflux of the drug, because the macromolecular size of the drug does not fit to the efflux transporter system [43,44]. Minko et al. reported that HPMA copolymer-linked doxorubicin affected the signal transduction system of MDR cells and induced apoptosis which was not seen in the parent drug [113–115].

6.2.6. Patient compliance and quality of life (QOL)

The polymer-conjugated drugs or nanomedicines require less frequent administrations due to the increased tissue and blood half-life ($t_{1/2}$) which is usually 10 times or more than the free drugs, that is of great benefit to patients [1,8,97,93]. For instance, for the treatment of lymphocytic leukemia the use of native enzyme L-asparagine having a short $t_{1/2}$ (8–30 h) necessitates daily administration for 4 weeks, whereas the PEG-L-asparaginase conjugate (Oncaspar®, $t_{1/2}$ approx 14 days) requires only one infusion every 2 weeks [116–118]. The same is true for native interferon-α vs pegylated interferon-α (Pegasys®, MW 50 kDa), the former requires more than 2–3 injections per week, while the latter requires only once a week intramuscular injection [83].

6.3. Disadvantages and limitations of polymer conjugates or unsolved issues

In view of clearance from the body, a size or unit of polymer being used should be preferably smaller than the size of renal excretion threshold (40 kDa), or biodegradable, or evidence of clearance via fecal route; in this case molecular weight may be of secondary importance. Further, the biocompatibility of polymers to be conjugated has to be well established. Hydrophobic polymers, if resistant to degradation in vivo, may tend to localize eventually in the skin or lipid-rich tissues, although mostly they may be eliminated via the bile. Therefore, the total amount of polymers to be administered may become important if the dose becomes large (e.g. 10 g). Some polymers may elicit immune response or allergic reactions, more likely in the subsequent injections, although it may be controlled by corticosteroid and antihistamine. There were reports in earlier days on the so called "macromolecular syndrome", or more lately on the infusion-related reaction that is observed at first usage even with antibody drugs or liposomal drugs, that is not observed in the second administration [119].

7. Species difference and optimization of release rate from the drug complex

The rate of initial burst of free drugs in vivo is frequently a problem in many polymeric drug conjugates, micellar, or liposomal drugs. However, the release rate from the given polymer micelles or from liposomes is purely physical matter exhibiting zero order rate kinetics. In contrast, species difference in the renal or the hepatic functions or metabolic rate and their time scale in different animal species may need to be considered in terms of time course of drug release. For instance mice may tolerate toxicity well because of the more rapid metabolic rate or urinary clearance compared to human time scale. The metabolic rate particularly in the liver or urinary clearance rate in mouse may be far faster than that in human. We may not see effective therapeutic effect if the conjugates were not designed for human metabolic rate. Therefore, ideal experimental data in mice or rats do not guarantee good therapeutic response in human. We also encountered that SMA copolymer has little binding to rabbit albumin, in contrast to mouse or human

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albumin (unpublished data). This means that some animal models may not reflect the actual drug behavior for optimization in the human situation. Therefore optimization of the parameters has to be targeted for human setting, but not for mice.

Particular notion in this regard should be addressed to the nature of chemical bonding between the drug and polymers. For instance, chemical bond in the conjugates either linked by ester- or amide-bond makes a great difference. Namely, cleavage of ester bond could be 10- to 30-folds faster than that of amide bond in mice than in humans [119,120].

Another discrepancy in animal model vs real human cancer treatment is the tumor model using ascetic form (i.p.) tumor and the drug given i.p. or i.v. route. Frequently one injects the drug i.p. within one day after tumor inoculation into the peritoneal cavity, and hence the drug will be accessible to the tumor cells directly at a very early stage and it will be so effective. However, this protocol is an unrealistic model to develop a drug for solid tumor. In this system of the i.p. tumor/i.p. drug, there is no solid tumor being developed yet, thus no EPR-effect. Also, there are no human patients with 10^6 tumor cells (very small inoculums size) to be diagnosed to have cancer at that early stage. If this is a case, cancer cells are easy to eradicate by helping immune surveillance. When both tumor cells and drug were administered i.p., easy access of drug to the tumor is guaranteed. Thus it is not a good model because the peritoneal compartment becomes a type of test tube, and the drug concentration in the compartment is very high and effective, resulting in more chance to eradicate tumor cells.

8. Conclusion

For the tumor-targeted drug delivery system, EPR-effect is now widely accepted as a guiding principle (see recent review) [121]. The EPR-effect is observed in most macromolecular drugs that include polymer conjugates, micellar drugs, and liposomal drugs; all exhibiting tumor-targeting characteristics. Once these macromolecular drugs are delivered to the tumor, they remain in the tumor tissues for extended period (days to weeks), which is different from just passive targeting and is different from the behavior in the normal tissue. Even cancer-targeting drugs that are conjugated with monoclonal antibodies will rely on this EPR-effect at first crossing of vascular barriers to be accessible to tumor tissue. In this connection, it was shown previously that normal IgG exhibited tumor-selective accumulation by EPR-effect [5]. Various factors that mediate EPR-effect such as bradykinin, NO, VEGF, and prostaglandins are reviewed and discussed.

EPR-effect can be further augmented by elevating the systemic blood pressure artificially by infusing AT-II [79], and also by prostaglandin I_2 agonist [67,68]. Consequently, the delivery of macromolecular drugs is facilitated significantly even to metastatic tumor which has low vascular density [122]. Thereby therapeutic effect of macromolecular drugs will be enhanced while side effect will be lesser. Whilst most micellar nanoparticle drugs and liposome formulations require proper rate of drug release in order to exert optimal therapeutic effect, these formulations need special attention involving stability and modulation of release kinetics in vivo. This involves meticulous adjustments in the formulation, or selection of materials, or chemistry during development of drugs for human use, not for mice.

We discussed here the advantages of polymer therapeutics or nanomedicine including tumoritropic and lymphotropic nature; enhanced residence time in circulation and in tumor; lesser side effect than parent drugs; and ways to augment the delivery of polymeric or macromolecular drugs, e.g. by elevating blood pressure. Further, benefits of macromolecular drugs are also found against multidrug resistance. All these improved characteristics warrant the promising future of macromolecular therapeutics for many diseases, especially for cancer.

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Appendix ADAIR02

EXPERT OPINION

1. Introduction
2. Composition of ADCs
3. Drug targets
4. Manufacture
5. Commercial development of clinical pipeline
6. Expert opinion

Antibody–drug conjugates – a perfect synergy

John R Adair, Philip W Howard, John A Hartley, David G Williams† & Kerry A Chester
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Introduction: There is a great unmet need for effective new treatments in cancer, which continues to be a major cause of death. Antibody–drug conjugates (ADCs) are emerging, after a long gestation, as a class of biopharmaceuticals with the potential to address this need by directing highly potent cytotoxic drugs to their point of action. There is increasing interest in ADCs by major pharmaceutical companies and a growing pipeline of candidates for clinical use. This review summarises progress with development of this new class of drugs.

Areas covered: The authors describe separately the antibody and drug elements of ADCs and then examine the technology and consequences of linkage. The work is presented in the light of recent developments in the design, using clinical examples where possible.

Expert opinion: Since their emergence as independent drugs, antibodies and chemotherapy are being brought together in effective synergy. The conjunction is timely: many of the technical challenges in preparing antibodies have been addressed; potent new drugs are available and linker technology is advancing apace. ADCs however are not just a sum of their individual parts. The current challenge is in understanding the holistic nature of this exciting class of drugs that promise a new avenue for cancer treatment. Target selection, the interaction of ADC with tumour and off-tumour targets and the internalisation of ADCs, are critical to the effective maturation of ADC technology. Ongoing recent developments in attachment sites and linker chemistry can provide fine-tuning of drug loading, elements of ADC PK and off-target ADC toxicity.

Keywords: ADC, antibody, antibody–drug conjugate, cancer, chemotherapy, clinical trial, oncology

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1. Introduction

Paul Ehrlich visualised the future of antibodies as 'magic bullets' and pioneered the use of chemotherapy. Yet, in over 100 years of research since their birth, these two classes of agents largely have been developed independently. Antibodies, due to their specificity and multi-functionality, have met with remarkable success as cancer drugs and are now market leaders in the field of targeted therapies. However, there is still great clinical need; even when using antibodies in combination with chemotherapy, long-term control of tumours is unusual and the majority of advanced solid tumours are incurable.

Antibodies can be synergistically improved by arming them with a toxic chemical warhead, to create a new class of drugs: antibody–drug conjugates (ADCs) that target drugs, often too powerful to be used on their own. The molecular combination provides an extremely effective means to achieve potent and selective target cell killing. The concept of ADCs has been around for many years [1] but they are now

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Article highlights.

- Antibody–drug conjugates (ADCs) are disease-targeted drug constructs that combine the specificity of antibodies (or another type of targeting agent) with the potent toxicity of novel chemical 'warheads'.
- The discovery and design of new warheads/linkers has allowed ADCs to become one of the most rapidly growing and exciting new classes of anti-cancer therapeutics.
- ADCs are complex drugs. Antibody type, target antigen, linker technology and warhead mechanism/potency must be considered.
- The linker between each antibody and the drug is a unique biochemical entity and reductions in unwanted toxicity are most likely to be achieved by close attention to linking technologies.
- Knowing the requirement for each part is essential but an individual ADC is more than a sum of its individual parts and understanding the holistic nature of each ADC combination is a key factor in achieving success.
- The promising ADC efficacy data generated by pharmaceutical companies and the strong pipeline of potential new agents indicate that the next few years will deliver a number of effective new ADC drugs.

This box summarises key points contained in the article.

rapidly becoming lead agents in the antibody therapeutic field. The reduction to practice for ADCs is timely; recombinant antibody technologies and advanced biological manufacturing have addressed many of the practical issues associated with antibody–drug development, and a plethora of potent drugs and linkers, synthesised specifically for attachment to antibodies, are becoming available. As more ADCs are being examined in preclinical models and clinical trials, the parameters required for success are becoming elucidated. Here the authors review those parameters, with a focus on the means and potential consequences of joining antibodies to drugs and in light of recent advances in translation of ADCs for clinical use in patients with cancer.

2. Composition of ADCs

ADCs are complex entities comprising three components: the targeting element, the drug and a linker (**Figure 1**). These components are detailed separately below but it should be borne in mind that they are interdependent on one another and on the nature of the target. Thus, the ADC is greater than the sum of its parts and its mode of action is environmentally and biologically determined.

2.1 Targeting element

Although any entity with high affinity for a tumour-associated cell surface antigen (e.g., peptides, vitamins, aptamers) might be considered as the targeting element for an ADC, the most common and established targeting element used, to date, is an antibody. The development of antibodies as independent drugs has been instrumental in this respect. While the early history of therapeutic antibody development was dominated by the issue of patient immune responses to murine and chimeric antibodies [2], this has largely been addressed [3] by the development of humanised, deimmunised and 'fully human' antibodies (the latter derived from phage display or transgenic mice technologies) [4]. Some companies that are developers of ADCs have their own proprietary antibody engineering technologies to produce 'humanised' or human antibodies. Thus, ADCs in the current clinical pipeline have been created using antibodies engineered to have reduced, or negligible, immunogenicity (**Table 1**). Furthermore, the commercial success of antibodies has fuelled an extremely active research field generating new antibodies and knowledge applicable to ADC optimisation.

There are, as yet, insufficient clinical data to direct the choice of targeting agent or even the best antibody format for an optimal ADC because clinically tested ADCs to date have used whole antibodies, mostly of IgG1 isotype, although there are several that are IgG2 or IgG4 (**Table 1**). Each of these isotypes potentially confers long *in vivo* half-life to the ADC via engagement with the neonatal Fc receptor, FcRn [5,6]. In the case of IgG1, Fc engagement with immune cells or complement components could add additional immune effector functions to the ADC. It remains to be seen, however, whether antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, or Fc-mediated complement-dependent cytotoxicity (CDC) provides significant additional efficacy to an ADC. Conversely, FcR binding could cause internalisation of ADC into innocent FcR-expressing immune cells such as endothelium (FcRn) and reticuloendothelial system monocytes and macrophages (FcγR). It is possible that internal release of active cytotoxic agent from an ADC in these innocent cells might lead to unwanted systemic side effects. Although such effects have not yet been reported, the potential for their existence suggests that design of the non-antigen-binding portion of an antibody is a matter of some note.

Despite the predominant use of IgGs for clinical ADCs, it is not known whether full-sized IgGs are the optimum structure to be used: a long serum half-life may have unwanted consequences if there are any issues of linker instability leading to release of free drug and a potential for non-specific toxicity. Furthermore, it has been noted, from studies with radiolabelled antibodies, that IgGs generally achieve < 0.1% injected dose/g of solid tumour [7] indicating that there is significant room for improvement in overall format of the antibody element to confer optimal pharmacokinetics (PK). Factors affecting antibody penetration and retention in tumour have been reviewed by others [8,9] and recent data indicate that most efficient tumour uptake can be obtained with large proteins such as IgG, which exceed the size limit for renal clearance, or with very small proteins with high binding affinity (long off-rate) [10,11]. Thus, it may be feasible to utilise high affinity antibody fragments or non-antibody scaffolds as favourable carrier platforms for drugs.

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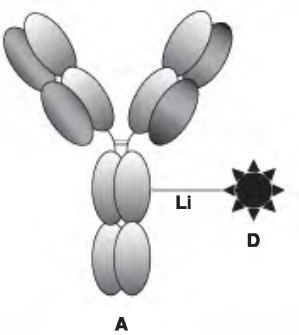


Figure 1. Diagrammatic representation of an ADC. Antibody (A) is attached by a chemical linker (Li) to drug (D).

2.2 Drug

Of the many drugs that have been evaluated for specific targeting [12,13], only a few are consistently used in clinical-stage drug conjugates. The leading candidates are either tubulin inhibitors, which act on dividing cells, or those that target the cellular DNA. There is a relatively small number of companies leading the field in developing these as drugs and prodrugs for ADCs (**Table 2**).

2.2.1 Tubulin inhibitors

There are two major groups of tubulin inhibitors being developed for ADCs: auristatins and maytansine derivatives. Monomethyl auristatin E (MMAE) and auristatin F are the two forms of auristatins that are in clinical development. MMAE is a synthetic pentapeptide based on the naturally occurring tubulin polymerase inhibitor dolastatin-10 with a 2-amino-1-phenylpropan-1-ol unit at its C-terminus. The MMAE molecule exhibits nanomolar to high sub-nanomolar activity in human tumour cell lines [14]. Auristatin F (with a phenylalanine C-terminus) is at least 100-fold less potent than MMAE as a stand-alone agent as the ionisable phenylalanine carboxyl group presumably hinders cellular absorption. Interestingly, Monomethyl auristatin F (MMAFs) exhibit similar potencies to their MMAE analogues when delivered to cells in an ADC format [15], highlighting the importance of taking a holistic rather than reductionist approach to ADC design.

The other class of tubulin binding drugs employed in ADCs are maytansine derivatives. Maytansine was originally isolated from the bark of the African shrub *Maytenus ovatus* and found to be a potent inhibitor of microtubulin assembly leading to the apoptosis of tumour cells. A series of clinical trials failed to establish a sufficient therapeutic window for maytansine [16] and its picomolar activity *in vitro* suggested a more suitable role as an ADC warhead. Maytansine is a structurally complex polyketide that can be accessed synthetically by derivatizing ansamitocins obtained by fermentation. Maytansine bears a C3 *N*-acyl-*N*-methyl-L-alanyl side chain, which, although essential for activity, can be modified to carry a disulphide 'handle' that can be incorporated in linkers designed to be cleaved by thiols inside target cells.

2.2.2 DNA interacting agents

The other major group of drugs used as ADC warheads are DNA interacting agents. These include calicheamicins, duocarmycins and, more recently, pyrrolobenzodiazepines (PBDs). Calicheamicins are naturally occurring anti-tumour antibiotics found in the soil microorganism *Micromonospora echinospora* ssp. *calichensis*. They associate with the minor groove of DNA and when activated by glutathione undergo a Bergman cyclisation to generate a diradical species which cleaves the DNA backbone. Due to their potency, a number of calicheamicins have undergone preclinical evaluation as stand-alone agents but they have not been progressed due to their delayed toxicity and narrow therapeutic index. However, as for maytansine, their potency recommended them as ADC warheads.

The duocarmycins (including CC-1065) are a family of naturally occurring anti-tumour antibiotics found in *Streptomyces* species. Although CC-1065 is highly potent, these molecules have not been used clinically because of serious delayed hepatoxicity. Similarly CC-1065 duocarmycin dimer, biselezin was found to induce severe myelosuppression in man. The duocarmycins bind sequence-selectively in the minor groove of DNA forming a covalent linkage to the N3 of adenine. The duocarmycins comprise cyclopropylindole (CPI) tricyclic core attached via an amide bond to a second indole unit. Naturally occurring duocarmycins possess a pyrrole ring (fully aromatic or reduced) fused to the benzo ring of the indole but this has been replaced with a second benzene ring in most ADC warheads. The strained cyclopropyl group allows the formation of the covalent linkage to adenine while rotation of the second indole unit, once in the minor groove can help to activate the molecule. The cyclopropyl group can exist as a ring-open chloromethyl precursor in which case the benzo ring of the indole is in a phenolic, as opposed to cyclohexadienone, form. This allows the duocarmycins to function as a pro-drug if the phenolic form is locked-in by functionalisation of the phenolic hydroxyl group.

The use of methyl substituted cyclopropylbenzoindole (CBI) duocarmycin warheads has also been investigated. These warheads are locked in their cyclopropyl ring-open *seco* forms, by protecting the phenolic hydroxy group as a carbamate. This carbamate forms part of a cathepsin-labile linker, ending in a caproyl maleimide connecting group. Hydrophilic polyethylene glycol (PEG) chains can be incorporated in this linker and *in vivo* studies showed that shorter PEGs were associated with better activity (growth delay at 12 mg/kg) [17]. Alternatively, the CBI unit can be attached to the antibody via the auxiliary indolecarboxamide moiety using cathepsin-labile linkers. In this case, a water-solubilising *N*-methylpiperazino carbamate is used to protect the phenolic group of the CBI moiety [18]. A similar strategy has been

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Table 1. Clinical stage ADCs.

Product international non-proprietary Name, synonyms	Antibody type	Linker/ linker type	Drug conjugate	Drug mode of action	Target antigen	Tumour type	Application or indication	Approval year or latest clinical phase*	Company/Licensee
Mylotarg® Gemtuzumab ozogamicin	Humanised IgG4P	Ac-but acid cleavable linker	Calicheamicin	DNA cleavage	CD33	Haematological tumours	Treatment of patients 60 years and older in first relapse with CD33+ AML who are not considered candidates for cytotoxic chemotherapy	US 2000 (accelerated approval process) Approval withdrawn June 2010 Several open Phase III and II/III studies are in progress	Pfizer/UCB Pharma
Adcetris™ brentuximab vedotin, SGN-35	Chimeric IgG1	Peptide cleavable Val-Cit linker	MMAE	Tubulin inhibitor	CD30	Haematological tumours	The treatment of patients with Hodgkin's lymphoma after failure of ASCT or after failure of at least two prior multi-agent chemotherapy regimens in patients who are not ASCT candidates. The treatment of patients with systemic ALCL after failure of at least one prior multi-agent chemotherapy regimen	US August 2011 (accelerated approval process) Phase III AETHERA study and further Phase II and I studies are in progress	Seattle Genetics/ Millennium
Trastuzumab-DM1 trastuzumab emtansine, T-DM1, RG3502	Humanised IgG1	MCC, thioether non-cleavable linker	Maytansinoid DM1	Tubulin inhibitor	CD340 p185HER2	Solid tumours	HER2+ breast cancer and metastatic breast cancer	Phase III (including MARIANNE and EMILIA studies) and several other open studies in progress	Genentech/Roche/ Immunogen
CMC-544 inotuzumab ozogamicin	Humanised IgG4P	Ac-but cleavable linker	Calicheamicin	DNA cleavage	CD22	Haematological tumours	Non-Hodgkin's lymphoma	Phase III and several Phase I studies in progress	Pfizer/UCB Pharma
CDX-011 glembatumumab vedotin (CR011-vcMMAE)	Human IgG2 (Abgenix Xenmouse transgenic mouse)	Peptide cleavable Val-Cit linker	MMAE	Tubulin inhibitor	GPNMB	Solid tumours	Advanced GPNMB-expressing breast cancer Melanoma	Phase II (EMERGE study) Phase I/II completed May 2011	Celldex/Seattle Genetics
SAR3419	Humanised IgG1 (Immunogen resurfacing)	SPDB; cleavable linker	Maytansinoid DM4	Tubulin inhibitor	CD19	Haematological tumours	Diffuse large B-cell lymphoma (Single agent and separate trial with rituximab), Acute lymphoblastic lymphoma, non-Hodgkin's lymphoma	Phase I	Sanofi-aventis/ Immunogen
BT-062	Chimeric IgG4	SPDB cleavable linker	DM4	Tubulin inhibitor	CD138 (syndecan-1)	Haematological tumours	Relapsed or refractory multiple myeloma	Phase I/II	Biotest AG/ Immunogen
IMGN901 (orvotuzumab mertansine, BB-10901; huN901-DM1	Humanised IgG1 (Immunogen resurfacing)	SPP cleavable linker	DM1	Tubulin inhibitor	CD56	Solid tumours/ haematological tumours	Small cell lung cancer, multiple myeloma	Phase I/II	Immunogen
Milatuzumab-DOX IMMU-110	Humanised IgG1	MCC	Doxorubicin	DNA intercalator	CD74	Haematological tumours	Multiple myeloma	Phase I/II	Immunomedics, Inc.

*Information on clinical status was obtained from Company websites and/or www.clinicaltrials.gov between 20 December 2011 and 18 March 2012.

Ac-but: 4-(4′-Acetylphenoxy) butanoic acid; AML: Acute myeloid leukaemia; ALCL: Anaplastic large cell lymphoma; ASCT: Autologous stem cell transplant; ND: Investigational New Drug; MC: Maleimidocaproyl; MCC: *N*-maleimidomethylcyclohexane-1-carboxylate; MMAE: Monomethyl auristatin E; MMAF: Monomethyl auristatin F; n/a: Not available, not disclosed at present; PSMA: Prostate-specific membrane antigen; SPDB: *N*-succinimidyl 4-(2-pyridyldithio) butyrate; SPP: *N*-succinimydyl 4-(2-pyridyldithio)-pentanoate; Val-Cit: Valine–citrulline.

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Table 1. Clinical stage ADCs (continued).

Product international non-proprietary Name, synonyms	Antibody type	Linker/ linker type	Drug conjugate	Drug mode of action	Target antigen	Tumour type	Application or indication	Approval year or latest clinical phase*	Company/Licensee
AGS-16M8F	Human IgG2 (Abgenix Xeno-mouse transgenic mouse)	MC non-cleavable linker	MMAF	Tubulin inhibitor	Ectonucleotide pyrophosphatase/ phosphodiesterase 3 (ENPP3) (AGS-16)	Solid tumours	Renal cell carcinoma	Phase I	Astellas Pharma/ Seattle Genetics
AMG 172	n/a	n/a	n/a	Tubulin inhibitor	n/a	Solid tumours	Renal cell adenocarcinoma Clear cell renal carcinoma Clear cell renal cell carcinoma Renal cell carcinoma	Phase I	Amgen/Immunogen
AMG 595	Human IgG (Abgenix Xenomouse transgenic mouse)	Non-cleavable linker	DM1	Tubulin inhibitor	EGFR variant III	Solid tumours	Glioma	Phase I	Amgen/Immunogen
ASG-5ME AGS-5ME	Human IgG2 (Abgenix Xenomouse transgenic mouse)	Peptide cleavable Val-Cit linker	MMAE	Tubulin inhibitor	SLC44A4 (AGS-5)	Solid tumours	Pancreatic, prostate cancers	Phase I	Astellas Pharma/ Seattle Genetics
ASG-22ME AGS-22M6E ASG-22M6E	Human IgG (Abgenix Xenomouse trans-genic mouse)	Peptide cleavable Val-Cit linker	MMAE	Tubulin inhibitor	Nectin-4	Solid tumours	Multiple solid tumours to include breast, bladder, lung and pancreatic cancers	Phase I	Astellas Pharma/ Seattle Genetics
BAY 94-9343	Fully human IgG1 (Morphosys phage display)	Immunogen 'engineered linker'	DM4	Tubulin inhibitor	Mesothelin	Solid tumours	Advanced solid tumours	Phase I	Bayer-Schering/ Immunogen
IMGN529 K7153A-SMCC-DM1	Humanised IgG1 (Immunogen resurfacing)	MCC thioether non-cleavable linker	DM1	Tubulin inhibitor	CD37	Haematological tumours	Non-Hodgkin's lymphoma	Phase I	Immunogen
IMGN853	Humanised antibody	n/a	DM4	Tubulin inhibitor	Folate receptor 1	Solid tumours	Ovarian cancer, non-small cell lung cancer and FOLR1 positive epithelial malignancies	Phase I	Immunogen
MDX-1203	Human IgG (Medarex UltiMab transgenic mouse technology)	Peptide cleavable Val-Cit linker	CC-1065 (rachelmycin)	DNA minor groove alkylator	CD70	Solid tumours	Advanced/recurrent clear cell renal cell carcinoma or relapsed/refractory B-cell non-Hodgkin's lymphoma	Phase I	Bristol Myers-Squibb

*Information on clinical status was obtained from Company websites and/or www.clinicaltrials.gov between 20 December 2011 and 18 March 2012.

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Table 1. Clinical stage ADCs (continued).

Product international non-proprietary Name, synonyms	Antibody type	Linker/ linker type	Drug conjugate	Drug mode of action	Target antigen	Tumour type	Application or indication	Approval year or latest clinical phase*	Company/Licensee
PSMA ADC	Human IgG (Abgenix Xenmouse transgenic mouse)	Peptide cleavable Val-Cit linker	MMAE	Tubulin inhibitor	PSMA	Solid tumours	Prostate cancer	Phase I	Progenics/Seattle Genetics
RG7450 DSTP3086S	n/a	n/a	n/a	Tubulin inhibitor	Not disclosed	Solid tumours	Prostate cancer	Phase I	Genentech/Seattle Genetics
RG7458 DMUC5754A	n/a	n/a	n/a	Tubulin inhibitor	Not disclosed	Solid tumours	Ovarian cancer	Phase I	Genentech/Seattle Genetics
RG7593 DCDT2980S	Humanised IgG1	Peptide cleavable Val-Cit linker	MMAE	Tubulin inhibitor	CD22	Haematological tumours	Haematological malignancies	Phase I	Genentech/Seattle Genetics
RG7596 DCDS4501A	n/a	n/a	MMAE	Tubulin inhibitor	CD79b	Haematological tumours	Haematological malignancies	Phase I	Genentech/Seattle Genetics
RG7598	n/a	n/a	n/a	Tubulin inhibitor	Not disclosed	Solid tumours	Multiple myeloma	Phase I	Genentech/Seattle Genetics
RG7599 DNIB0600A	n/a	n/a	n/a	Tubulin inhibitor	Not disclosed	Solid tumours	Non-small cell lung cancer, ovarian cancer	Phase I	Genentech/Seattle Genetics
RG7600	n/a	n/a	n/a	Tubulin inhibitor		Solid tumours	Pancreatic, ovarian cancers	Phase I	Genentech/Seattle Genetics
SAR566658 huDS6-DM4	Humanised IgG (immunogen resurfacing)	n/a	DM4	Tubulin inhibitor	DS6 (MUC1 analogue)	Solid tumours	DS6-positive and refractory solid tumours	Phase I	Sanofi-aventis/ Immunogen
SGN-75	Humanised IgG1	MC non-cleavable linker	MMAF	Tubulin inhibitor	CD70	Haematological tumours	CD70+ non-Hodgkin's lymphoma or renal cell carcinoma	Phase I	Seattle Genetics
Undisclosed	n/a	n/a	n/a	Tubulin inhibitor	n/a	Solid tumours	n/a	IND submitted	Genentech/Seattle Genetics

*Information on clinical status was obtained from Company websites and/or www.clinicaltrials.gov between 20 December 2011 and 18 March 2012.

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Table 2. Major developers of drugs for ADCs.

Developer	Warhead	Mode of action
Seattle Genetics	Auristatins	Tubulin polymerase inhibitor
Immunogen	Maytansines	Tubulin depolymerisation
Pfizer	Calicheamicins	DNA cleavage
Bristol-Myers Squibb	Duocarmycins	DNA minor groove alkylating agent
Syntarga (Synthon)	Duocarmycins	DNA minor groove alkylating agent
Spirogen Ltd.	PBD dimers	DNA minor groove cross-linker
Wilex (Heidelberg Pharma)	α-Amanitin	RNA polymerase II inhibitor

PBD: Pyrrolobenzodiazepine.

pursued with CBI duocarmycins, with comparable results of tumour growth delay at 15 mg/kg [19].

PBDs are a class of naturally occurring anti-tumour antibiotic found, like the duocarmycins, in *Streptomyces*. They were first isolated, structurally elucidated and synthesised by Leimbruber and co-workers in the late 1960s. The molecules bind sequence-selectively in the minor groove of DNA at purine–guanine–purine motifs forming a covalent aminal linkage to the N2 of guanine. Joining two PBD units together via a flexible polymethylene tether allows the PBD dimers to cross-link opposing DNA strands producing highly lethal lesions. The non-distortive nature of PBD adducts requires them to be repaired via a homologous recombination repair mechanism, which is frequently impaired in human tumours as opposed to normal tissue. The potency of PBD dimers can be further enhanced by introducing unsaturation about the C2-position of the PBD C-ring and installing substituents that are directed along the floor of the DNA minor groove. Several PBD dimers have exhibited sub-nanomolar/picomolar activity against a range of human tumour cell lines and have demonstrated curative action in human tumour xenograft models. A lead PBD dimer, SG2000, is currently undergoing Phase II clinical [20] trial for the treatment of cisplatin refractory ovarian carcinoma and Phase II leukemia trial is scheduled for later in 2012.

2.2.3 *Other drug targets*

α-Amanitin, a bicyclic octapeptide RNA polymerase II inhibitor is being investigated as an alternative ADC warhead. Conjugation of α-amanitin to lysines on antibodies, via a stable proprietary linker system, prevents the liver toxicity associated with hepatocyte uptake of free α-amanitin. The α-amanitin-based ADC, Her-DSC-30.0134, where drug is attached to the antibody trastuzumab, with a drug:antibody ratio (DAR) of 4, has exhibited picomolar activity against SKOV33 and SK-Br-3 cell lines. The ADC appears promising as a single 5.7 mg/kg dose of Her-DSC-30.0134 demonstrated curative activity against the herceptin/lapatinib-resistant JIMT-1 xenograft model.

2.3 Antibody–drug linkage

Optimisation of antibody–drug linkage is a pivotal and often understated part of ADC design and development. Sophisticated function is required for the linker itself. Moreover, the chemistry of linkage to the drug and the means of release when the ADC reaches its target cell are all vitally important for success. First, the linker must be capable of attachment to both the targeting element and the drug without affecting the function of either; ideally resulting in a homogeneous ADC in terms of DAR and biophysical properties. Second, it is essential that the linker is stable during storage and in blood during treatment. Third, there must be a strategy for separating the drug from antibody after internalisation in the target cell.

2.3.1 *Antibody conjugation site*

The antigen binding site of an antibody has been considered as a carrier for derivatized drugs, taking advantage of the high affinity for small haptens that can be achieved in some circumstances [21]. Recombinant DNA technology allows a wide range of antibody-based structural formats and so introducing a number of such binding sites into an ADC is not a major obstacle. More importantly, the drug should not dissociate from the targeting element before it reaches the target cell. Perhaps for these reasons (and historically to obtain high DARs), chemical conjugation has, to date, been the more popular and clinically validated approach.

For chemical conjugation, the linker–drug moieties are generally attached to the targeting antibody either at lysines or thiols. Alternative linking sites may include carbohydrate, glutamine [22] and SNAP-tag [23]. However, as asparagine197-linked carbohydrate is critical to FcγR binding (but not to FcRn binding), this has been avoided as an attachment site to Fc. Reactive surface lysine residues are abundant in antibodies (e.g., there are 88 lysines in trastuzumab) [24]. Indeed, lysine has been a common point of conjugation for radioisotope attachment to antibodies using chelating agents. For example, 90Y-ibritumomab tiuxetan (Zevalin) is generated by covalently attaching the chelator, MX-DTPA, to surface lysine residues of the anti-CD20 antibody [25]. Here a low attachment ratio is optimal because conjugate clearance to the liver and spleen and the loss of the positive charge associated with the reactive amine group is minimised [26,27].

In the case of trastuzumab–emtansine [28,29] (trastuzumab-DM1, T-DM1, **Table 1**) TDM-1, antibody lysine side chains are functionalised with the *N*-maleimidomethylcyclohexane-1-carboxylate (MCC) linker. The maleimide group then reacts with a thiol group attached to the maytansinoid warhead. Alternatively, in gemtuzumab ozogamicin (Mylotarg®, **Table 1**) and inotuzumab ozogamicin (CMC-544, **Table 1**) the calicheamicin warhead is attached directly to the antibody lysine via an amide bond. Drug conjugation via surface lysines in T-DM1 results in a DAR of 3.5 but the process results in partial modification of at least 47% of lysine residues in regions of high solvent accessibility and structural

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flexibility [30]. This light smear of chemical perturbation over the antibody surface will ensure that any loss of function at a particular conjugation site will not destroy overall ADC functionality. At pH 6 (when lysine side chains are protonated), amine coupling is focused at the α-amino group of the N-terminal residue [31]. This technique has not been described for ADCs, possibly because N-terminal adducts may be too close to the antigen-binding region of IgG.

The use of free thiols for ADC attachment offers possibilities for more controlled and site-specific attachment. Free thiols may be generated on antibodies in a number of ways:

- Modification of surface lysines [32] using:
  - Traut's reagent (2-iminothiolane)
  - *N*-succinimidyl *S*-acetylthioacetate (SATA)
  - *S*-acetylmercaptosuccinic anhydride (SAMSA)

- Reduction [33] of inter-chain disulphides with:
  - dithiothreitol (DTT)
  - 2-aminoethylisothiouronium bromide (AET)
  - tris(2-carboxyethyl)phosphine (TCEP)

- Genetic modifications to:
  - introduce an unpaired cysteine at a specific surface location [33-41]
  - to introduce an unpaired selenocysteine whose reduction is compatible with retention of native disulphide bonds [34]

- Incorporation of unnatural amino acids as sites of drug attachment.

Conjugation to reduced inter-chain disulphides (compared with lysine conjugation) focuses drug attachment on the (H-H) and (H-L) inter-chain disulphides. ADC chemical heterogeneity is thereby reduced, but not eliminated, because the four reducible disulphide bonds (in IgG1) give rise to eight potential free thiols for conjugation. It is possible that this heterogeneity could be reduced further. The H-L disulphide bond is more readily reduced by DTT than the H-H bond [42] and, vice-versa, the reduced H-L disulphide is more readily re-oxidised by 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) [33]. Partial reduction will place drug preferentially on the H-L axis, whereas reduction/reoxidation will focus drug on the H-H axis. However, little difference in maximum tolerated dose (MTD) was noted in the one example where attachment to these two axes was compared [33]. It should be noted that removal of the hinge region inter-chain disulphide bonds from an IgG1 has been reported to greatly impair ADCC, consistent with impaired Fc–Fcγ receptor interactions, and also to reduce CDC [37,43]. Therefore, options for inter-chain disulphide reduction need to be considered carefully.

The anti-tumour efficacy of the anti-CD30 antibody cA10 (brentuximab) conjugated to MMAE with a DAR of 4, was as effective as the ADC with DAR of 8. However, the MTD was doubled and the clearance rate was one-third [44]. Thus, a lower DAR can improve the PK of ADCs, without loss of efficacy, by reducing clearance rate and increasing *in vivo* exposure. Interestingly, the cysteine-coupling location of the drug moiety had little bearing on this pharmacodynamic effect [45]. In recent ADCs, DAR refinement to improve ADC PK has prompted design of novel conjugation technologies to avoid a significant component of naked antibody [35,46].

The question of whether antibody stability is compromised by disulphide reduction remains. Antibodies are likely to differ in this respect. Each of the IgG isotypes is bridged between the heavy and light chains (two S-S bridges per IgG, four cysteines in total), however, the number of cysteines in the hinge region that may contribute to inter-heavy chain bridging varies. IgG1 has 2 inter-heavy chain disulphide bonds, IgG2 has 4, IgG3 has 11 and IgG4 has 2. It is known that loss of the single disulphide bond can destabilise a recombinant Fab [47]. In the absence of such a bond, the affinity of reduced, iodoacetylated heavy and light chains may be dependent on the variable region sequences and their contribution to binding affinity [48]. Thus, there will be a case-by-case variation in the biophysical stability of an ADC based on inter-chain disulphide bond reduction and alkylation. This must be taken into account in antibody selection for ADC construction. Inter-heavy chain disulphide shuffling and its possible effects on antibody function are an additional risk specific to IgG2 [49]. The formation of monovalent half molecules by disulphide bond scrambling in IgG4 is another risk of the disulphide bond reduction strategy of ADC construction [49,50]. However, it is possible that this can be addressed, because a reduced disulphide bond and re-stabilised by alkylation with a bis(thiol)-specific reagent, generating a 3-carbon bridge [49,51].

One of the consequences of drug–linker attachment onto random reactive groups on the surface of an antibody, or by partial reduction of inter-domain thiols is that it can lead to heterogeneity of loading stoichiometry, with some antibody not having any drug loaded at all. There are several examples: in the case of Mylotarg (**Table 1**) conjugation via surface lysines gives an average DAR of 2 – 3 but approximately 50% of the antibody is loaded with 4 – 6 moles calicheamicin per mole of antibody so 50% of Mylotarg is unconjugated antibody [52]. CMC-544 (**Table 1**) has a DAR of 5 – 7 mol of calicheamicin dimethyl hydrazide per mol antibody but less than 10% of the antibody is unconjugated [53]. T-DM1 has an average DAR of 3.5 with a heterogeneity of 1 – 7 modified lysine residues/antibody [24]. Conjugation at inter-domain thiols can also lead to heterogeneity of loading. Adcetris™ (brentuximab vedotin) has a DAR of approximately 4 [54] but each antibody has a drug–linker distribution from 0 to 8 [55].

Further focusing of the position of drug attachment via thiols has been achieved by reducing the number of cysteines that

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normally form the inter-domain disulphide bridges, or by genetic insertion of an unpaired cysteine, or introduction of C-terminal selenocysteine. For example, McDonagh *et al.* [45] used Cys-Ser replacement at inter-chain disulphide bonds to generate variants of brentuximab. Cysteines that normally form inter-chain disulphides were reduced from 8 to 4 or 2 and ADCs were generated with near quantitative, homogenous conjugation at the remaining cysteines. Retention of Fc effector function however, requires the inter-heavy chain disulphide bonds [43], suggesting that FcR-dependent effector functions could be affected by saturation reduction and alkylation at this location.

McDonagh *et al.* noted that other groups had previously attempted to introduce unpaired thiols on antibodies for site-specific conjugation [36,38]. This approach has been pursued since by others [39-41] but developed furthest for ADC conjugation in THIOMAB technology. Here, several locations on the surface of IgG have been defined where cysteine residues might be introduced at the genetic level to allow site-specific attachment of drug without affecting structural integrity [35,46,56]. Attachment at one or more locations has been tested with a number of antibodies with interesting results that highlight the sensitivity of ADCs to conjugation differences. Comparison of three different engineered cysteines on herceptin/trastuzumab has exposed significant differences in the chemical stability of conjugates. Unpaired cysteine was introduced to replace kappa chain valine 205, heavy chain alanine 114 or heavy chain serine 396. Maleimide conjugation at C205 was found to be stable, whereas conjugation at C396 was least stable in serum, with apparent exchange onto other plasma protein free thiols including albumin. The lower stability correlated with increased liver toxicity, presumably by cytotoxin–albumin conjugate uptake. This appears to underline the importance of the reversibility of the maleimide reaction, and the increased susceptibility to reversal *in vivo* in highly solvent-exposed conjugation sites.

An elegant alternative site-specific labelling technology employs incorporation of selenocysteine (Sec) at the C-terminus of an IgG Fc region by introducing a Sec insertion sequence (SECIS) element into the mRNA 3′-untranslated region leading to the stop codon being used as a Sec insertion codon. The nucleophilic selenol group of Sec (pKa 5.2) can then be utilised as a specific attachment location [34]. Another interesting approach to site-specific drug attachment is the incorporation of unnatural amino acids. For example, using Ambrx technology [57], p-acetylphenylalanine has been incorporated into Herceptin Fab at lysine169 and serine202 encoded by an amber codon, facilitating attachment of hydrazine or aminooxy groups to produce a hydrazone or oxime adduct, respectively. However, while much effort is being spent to direct conjugation to specific locations and provide more homogeneous conjugation, it has been shown that the stoichiometry of drug:antibody loading can be more important for efficacy than the loading positions [42], although the latter may be more relevant for manufacturing and product homogeneity.

2.3.2 Mechanism of conjugation:linker chemistry

Drug warheads can be linked to the antibody by various means. For attachment to antibody thiols, the N-terminus of MMAE has been functionalised with a self-immolative para aminobenzyl (PAB) group which in turn is attached to a cathepsin-labile valine–citrulline dipeptide. The N-terminus of the valine group is coupled to a caproic maleimide group to complete the MMAE–linker molecule. The MMAE–PAB–valine–citrulline caproic maleimide unit comprises a flexible building block for attachment. MMAE has been conjugated in this fashion to brentuximab to produce the recently Food and Drug Administration (FDA)-approved brentuximab vedotin (Adcetris). Alternatively, MMAF-based ADCs are still active, even in the absence of the cleavable valine–citrulline and PAB components of the linker, and the drug can be linked to antibody directly using the caproic maleimide unit (MC-MMAF). This is being used for a number of ADC conjugates (**Table 1**) and provides useful properties to the ADC (see later below).

As noted earlier, the C3 *N*-acyl-*N*-methyl-L-alanyl side chain of maytansine can be modified to carry a disulphide 'handle', that is, a disulphide that can be incorporated in linkers designed to be cleaved by thiols inside target cells. Addition of alkyl groups adjacent to the disulphide has been shown to increase the half-life of maytansine–ADCs in circulation [58]. Alternatively, maytansines can be linked to antibody by thioether linkages. For example, in T-DM1 the maytansine DM1 is linked to antibody via a bifunctional MCC linker that attaches to lysine residues on the antibody surface. The thiol group in DM1 is then reacted with the maleimide group of the linker to form the thioether bond.

For PBDs, the dimer warheads (e.g., SG2000, **Figure 2**) have been functionalised with linkers designed to facilitate conjugation to antibodies. For example, linkers have been attached at the C2-anilino substituents (e.g., SG3211, **Figure 2**) terminating in an antibody-reactive group, for example, maleimide.

2.3.3 Release of drug:linker chemistry

Once the drug is linked to antibody, the next challenge is to ensure active drug release following internalisation into the target cell. This can be achieved using both cleavable and non-cleavable linkers but the mechanisms are different. With cleavable linkers, drug is released from the antibody carrier as a consequence of the linker chemistry and the cell microenvironment. For example, the relatively acidic conditions in the lysosome act to cleave the linker in Mylotarg at a pH sensitive hydrazone. In other cases, there may be a reduction of a disulphide bridge as occurs with the SPP-DM1, SPDB-DM4 linkers (**Table 1**) due to the relatively high levels of glutathione inside cells compared with serum [13] or, as in the MC-vc-PAB linker in Adcetris, after cleavage of

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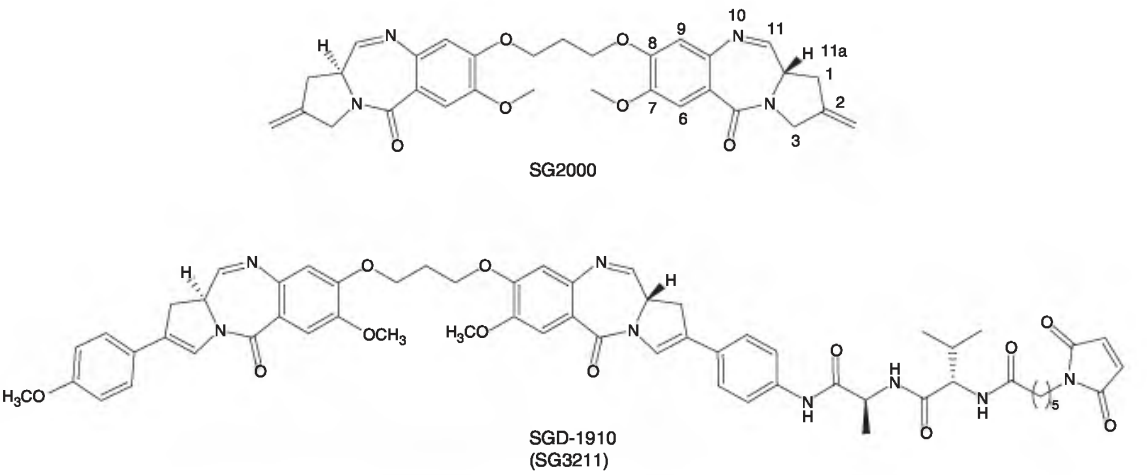


Figure 2. Pyrrolobenzodiazepine warhead SG2000 and C2 pyrrolobenzodiazepine–linker SGD-1910 (SG3211).

the valine–citrulline di-peptide that forms part of the linker. In some cases, there may be two release events: the first acts to detach the drug from the antibody and the second leads to activation of the drug from a prodrug form. In Mylotarg, for example, pH-dependent cleavage of the hydrazone is followed by reduction of a disulphide bridge in the calicheamicin warhead [59]. In the duocarmycin ADC MDX-1203, release occurs by the separate actions of a lysosomal protease then a carboxylesterase [60,61].

In the linker class described as 'uncleavable', separation of antibody and drug occurs by degradation of the antibody protein in the lysosome leaving the drug attached to the conjugated amino acid. For example, lysine in the case of T-DM1; and cysteine in the case of the MC-MMAF-based ADCs such as SGN75 and AGS-16M8F.

The more recent PBD ADCs can be formatted in either cleavable or uncleavable linker formats. Linkers at the N10 position and located on C2-anilino substituents must be fully cleavable in order to liberate an active PBD, while the aromatic tether can accommodate both cleavable and non-cleavable linkers.

The nature of the linker will influence the potential for extracellular drug release from ADC leading to off-target toxicity. While extracellular drug release prior to antibody localisation is clearly undesirable, drug release following localisation and internalisation into the target cell can lead to desirable bystander effects. In some cases, the drug, once separated from the antibody, is thought to be released from the targeted cell by active efflux mechanisms via p-glycoprotein. The subsequent killing of neighbouring cells is believed to be a factor in the pharmacodynamics and efficacy of many drugs being considered as ADC warheads [62]. In other situations, the chemical nature of the drug itself, or its metabolites, may allow drugs to diffuse from the targeted cell after release from the antibody carrier. This is believed to be a factor in DM1 release when conjugated to a disulphide-containing SPDB linker [63]. In other cases, the nature of the drug and the cleavage mechanism means the drug cannot be cleaved outside the cell or cannot escape from the cell (e.g., MC-linked MMAF and MCC-linked DM1). Where extracellular dissociation or drug efflux does occur, this could lead to drug uptake by adjacent tumour and non-tumour cells as well as leakage back into circulation. Thus, there is a range of considerations to be addressed when choosing appropriate drug–linker combinations and the choice will be affected by the nature of target antigen and tumour (see below).

3. Drug targets

Key issues in selecting antigen targets for ADC efficacy are: i) appropriate antigen density on the tumour cell surface and lack of biologically available target on crucial tissue elsewhere, providing desired specificity; ii) appropriate rate of internalisation after antibody binding followed by mechanisms of trafficking that promote active drug release from the antibody; iii) ability to address tumour heterogeneity by presence throughout the tumour or on areas of most concern in terms of malignant progression. The issue of antigen heterogeneity, coupled with the factors involved in solid tumour penetration, suggests that lymphoid tumours will be more tractable for treatment with ADCs (e.g., Mylotarg, Adcetris, CMC-544) but several solid tumour targets are also being assessed (**Table 1**) [64,65]. If ADCs are to be successful, there is a need to identify further useful tumour targets with the features mentioned above [66].

A common feature of cell surface targets for therapeutic antibodies in oncology is that they are not strictly tumour-specific. Targets may be expressed on cells in other tissues, which may

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lead to unwanted effects. A search for tumour-specific targets continues but an alternative approach to greater tumour specificity may be made by targeting two different antigens on the tumour cell using bispecific targeting platforms. Together with ADCs, bispecific antibodies are undergoing a renaissance and there are now a range of new formats. Many bispecifics are being developed for immune effector cell recruitment via CD3 or CD16 specificity but there is an opportunity to use molecules with specificity for two tumour-associated antigens and provide superior targeting specificity while reducing off-target effects. Depending on the targets there may also be additional benefits in blocking ligand binding at two targets on the tumour cell. Whatever format of targeting element is used, the need for a high specificity index is essential.

4. Manufacture

ADCs are relatively complicated products which require careful assembly. In some cases, preparation of antibody and drug–linker and the final conjugation are done at different sites by specialist companies. Production of antibody at large scale and at reasonable cost of goods can now be achieved [67]. It might be expected that the major pharmaceutical companies that are now interested in antibodies as products and that have begun to enter into supply deals with the ADC drug providers would have facilities for the assembly and formulation of ADCs.

5. Commercial development of clinical pipeline

Over the last 18 months (to March 2012), there have been a number of significant developments with ADCs. In 2010, Mylotarg [68-70], the first ADC product approved, was voluntarily withdrawn from the market. Mylotarg was approved in the USA by the FDA in 2000 under the accelerated approval procedure for the treatment of patients 60 years and older in first relapse with $CD33^+$ acute myeloid leukaemia (AML) who are not considered candidates for cytotoxic chemotherapy [71]. Statements by Mylotarg's developer Pfizer [72,73] noted '*The approval of single agent Mylotarg in the U.S. was granted under FDA's accelerated approval regulations based on overall response rate in three non-comparative studies and required submission of additional data to confirm clinical benefit. The required post-approval study (SWOG S0106)* [74] *combining chemotherapy and Mylotarg did not demonstrate improved survival compared with chemotherapy alone in patients with previously untreated AML*'. Very recently, however, a conference report [75,76] has identified that Mylotarg may be beneficial in new studies. Pfizer has noted [77] that it is discussing, with FDA, future courses of action for Mylotarg.

In 2010, Genentech submitted a Biologics Licence Application (BLA) to the US FDA for trastuzumab-DM1 for treatment of patients with advanced HER2-positive breast cancer who have previously received multiple HER2-targeted medicines and chemotherapies [78]. However, the FDA issued a 'Refuse to File' letter for accelerated approval for the company's T-DM1 ADC [78]. Genentech noted [79] that in its review of the BLA, the FDA stated the T-DM1 trials did not meet the standard for accelerated approval because all available treatment choices approved for metastatic breast cancer, regardless of HER2 status, had not been exhausted in the study population. Genentech are now performing additional studies with T-DM1 and have noted results from a Phase II study in people with previously untreated HER2-positive metastatic breast cancer, as well as noting other Phase III studies. Most recently, good initial results for the EMELIA Phase III study have been reported [80]. This international randomised open-label Phase III study, comparing T-DM1 alone with lapatinib plus Xeloda® (capecitabine), showed significant improvement in progression-free survival. Genentech plan to submit a BLA for T-DM1 in 2012 based on the EMELIA [81] study.

Recently, Seattle Genetics' Adcetris [54,82,83] gained accelerated approval for two indications: the treatment of patients with Hodgkin's lymphoma after failure of autologous stem cell transplant (ASCT) or after failure of at least two prior multi-agent chemotherapy regimens in patients who are not ASCT candidates [55], and the treatment of patients with systemic anaplastic large cell lymphoma (ALCL) after failure of at least one prior multi-agent chemotherapy regimen [84]. The approval was based on a study in Hodgkin's lymphoma of 102 patients that showed a 75% overall response (OR) rate (34% complete remission (CR); 40% partial remission (PR)) and a 58 patient study in ALCL showing an 87% OR (57% CR; 30% PR) [85]. As with Mylotarg, further studies will be needed to obtain full approval. Adcetris comprises a chimeric antibody and there have been indications of transient immune reaction to the antibody element [54]. Whether this becomes an issue for Adcetris remains to be seen. Several other recombinant antibodies stimulate some level of immune reaction with no obvious severe impediment to function [86,87].

Following along behind these product candidates there is now a relatively long clinical pipeline of other ADCs (**Table 1**) of which Pfizer's CMC-544 [70,88] is now in Phase III studies [89]. This list is rapidly enlarging with companies such as Genentech [90] and Seattle Genetics [91] noting substantial preclinical products and collaborations. It is expected that several more candidates will enter clinical development during 2012 and the near future, although as with most areas of pharmaceutical development some candidates will not be continued (e.g., recent notices of discontinuation of clinical development of the ADCs IMGN388 [92] and BAY79-4620 [93]).

In addition to drug development progress, there have been a number of business activities indicating general interest in this product group. Recently, Heidelberg Pharma and Syntarga, both developing new drugs and linkers, have been acquired by Wilex [94] and Synthon [95], respectively. Spirogen

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Ltd. recently announced a collaboration with Genentech on the discovery and development of ADCs using Spirogen's PBD drugs and associated linker technology [96]. A single Spirogen PBD has recently been positively reported by Seattle Genetics as a potent anti-CD70 ADC in models of renal cell carcinoma [97]. Both Seattle Genetics and Immunogen have announced further collaborations, providing large Pharma with access to their technologies. Recently, Seattle Genetics and Oxford Biotherapeutics entered an agreement [98] to jointly discover novel ADCs. Under the collaboration, Oxford Biotherapeutics is to generate panels of monoclonal antibodies against novel tumour-specific antigens identified using its Oxford Genome Anatomy Project (OGAP®) database which will then be screened for activity using Seattle Genetics' ADC technology. The OGAP proteomic database includes information on over 7500 cancer membrane proteins.

6. Expert opinion

After decades of independent development, antibodies and small chemical toxins are being successfully conjugated to create a new class of targeted anti-cancer missiles. The concept of synergistically combining the specificity of antibodies with the lethality of ultra-toxic chemicals is not new but a key development is that the utility of ADCs has now been exemplified in the clinic. Clinical feasibility, combined with the discovery and design of new warheads and linkers, has fuelled the research field leading to many promising new ADC combinations. Despite the market withdrawal of Mylotarg in 2010, the principle of highly potent armed antibodies for tumour therapy has been well made and has opened up the antibody arena for a new harvest of effective therapeutic modalities.

The ultimate goal must be successful treatments for a broad patient group, either as personalised medicines, based on individual tumour expression of the ADC target antigen or by identification of tumour-associated targets with broad patient group distribution. Target selection is a key factor in achieving these goals. Targets should not be down-regulated during therapy and, for ADCs, a further limitation in antigen selection is the requirement for internalisation; a feature of some, but by no means all plasma membrane targets.

Knowledge of how an individual ADC behaves *in vivo* is critical for success. Information on how the ADC accesses and interacts with the target antigen on the tumour, how it internalises, how soluble target interacts and competes and how off-target binding and non-tumour ADC uptake translates into off-target toxicity is vitally important. Antibody selection is crucial here. While technical developments in antibody technology in the last 20 years have enabled generation of high affinity, highly selective, humanised antibodies, identifying the most appropriate target for these antibodies when used as ADCs remains a challenge. Nonetheless, clinically proven and internalising antibodies, such as trastuzumab, provide an excellent starting point for research in this area.

New ADC candidates are being rapidly generated as more novel ultra-cytotoxic drugs are emerging along with superior methods of attaching the drug to the targeting agent in order to optimise drug antibody ratios, improve product homogeneity and reduce undesired dissociation from the antibody prior to targeting. The linker between each antibody and the drug is a unique biochemical entity. Understanding how this interface will impact on ADC biology and utility is fundamental for success. Significant reductions in non-specific or off-target toxicity are most likely to be achieved by close attention to these linking technologies. Therefore, it is possible that the next advances will be made by utilising recent knowledge on the chemistry of conjugation and linker design. The avoidance of linker cleavage until the ADC has entered the acidic endosome is now a possibility. However, the reversibility of the maleimide reaction suggests that further scope for toxicity reduction may be found in selection of the protein-reactive moiety on the linker. Recent research into the effect of drug linkage sites on linker stability and the effect of drug attachment stoichiometry on PK of the ADC, shows that basic chemical studies of the ADC and the understanding gleaned from them has an important translational impact on therapeutic success.

The growing body of preclinical and clinical data for these new products will guide further design to improve specificity and reduce off-target effects. Improvements could be achieved by better initial targeting, improved linker design to avoid detachment of drug prior to targeting, better retention of drug within the targeted cell and an increased understanding of the catabolic routes of ADCs, which may involve further consideration of FcR interactions.

In the short to medium term, it is expected to see more ADC candidates entering clinical trials and increased adoption of this class of products among the major pharmaceutical companies, whose long experience in drug design and development can lead to new generations of drug and linker design. The current late-stage candidates should provide further product approvals in the next 2 – 3 years with the earlier-stage clinical pipeline providing several further products in the ensuing years, further validating the approach. Late-stage clinical trials for some of these products will take several years but while there may be a period of consolidation as the pipeline develops and new product formats are tested in the clinic, the potential returns for the industry in this area of therapy should provide the impetus for continued development. The significant level of ADC development combined with the strong pipeline of drug candidates and growing engagement by large Pharma give promise that the next few years will deliver a number of exciting new approved ADCs to treat a range of cancers.

Declaration of interest

JR Adair is a consultant at Ithaka Life Sciences, which provides consultancy services to Spirogen Ltd. PW Howard is a

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founding scientists and head of targeted therapeutics at Spirogen Ltd., which is involved in research and development of pyrrolobenzodiazepines and related antibody–drug conjugates. DG Williams is an employee of Spirogen Ltd. JA Hartley is a founder scientist of Spirogen Ltd., has an equity interest in the company and is paid as a consultant. In addition, this author has received grants from Cancer research UK, the Department of Health, MRC and Cephalon Ltd. KA Chester is a recipient of grants from European commission FP7, EPSRC, Cancer Research UK, NIHR, Breast Cancer Campaign, MRC, Debbie Fund, Mologic Ltd. and Cogent Ltd. This author is a paid consultant for Spirogen Ltd. and an advisor to ThioLogics, whose technology is applicable to the assembly of homogeneous antibody conjugates.

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Appendix KOVTUN01

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222			0	a| Cancer research b| (Baltimore)
245	1		0	a| Cancer research : b| the official organ of the American Association for Cancer Research, Inc.
246	3		0	a| Cancer research
264			1	a| Baltimore, Md. : b| Waverly Press.
300				a| volumes : b| illustrations ; c| 27 cm
310				a| Semimonthly, b| <Jan. 1, 1987->
321				a| Monthly, b| <Mar. 1980>-198
336				a| text b| txt 2| rdacontent
337				a| unmediated b| n 2| rdamedia
338				a| volume b| nc 2| rdacarrier
362	1			a| Began with: vol. 1 in Jan. 1941.
500				a| Imprint varies: Chicago, Ill. : University of Chicago Press, 1941- ; Baltimore, Md. : Cancer Research, Inc., <Jan. 1, 1987->; Philadelphia, PA : American Association for Cancer Research, <Nov. 1, 1994->
500				a| Some issues accompanied by DVD-ROM version.
500				a| Description based on: Vol. 40, no. 3 (Mar. 1980); title from cover.
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510	2			a| Chemical abstracts x| 0009-2258
515				a| Issues for Sept. 1958-Dec. 1967 have a Pt. 2 called Cancer chemotherapy screening data which was incorporated into Cancer chemotherapy reports. Pt. 2 as of Jan. 1968.
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650			0	a| Cancer x| Research v| Periodicals.
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655			2	a| Periodicals.
710	2			a| American Association for Cancer Research.
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770 0 t| Cancer research. Supplement x| 0576-6656 w| (OCoLC)2258973 g| 1953-56

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993 a| 700000010274 n| Paterno Libraries Endowment

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949 a| RC261.A274 v.61 no.5-6 2001 w| LCPER c| 1 i| 000047647657 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/12/2001

949 a| RC261.A274 v.61 no.7-8 2001 w| LCPER c| 1 i| 000047647664 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/12/2001

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949 a| RC261.A274 v.66 no.2 2006 w| LCPER c| 1 i| 000055116312 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/2/2006
949 a| RC261.A274 v.66 no.3 2006 w| LCPER c| 1 i| 000055117227 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2006
949 a| RC261.A274 v.66 no.4 2006 w| LCPER c| 1 i| 000055119214 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/16/2006
949 a| RC261.A274 v.66 no.5 2006 w| LCPER c| 1 i| 000055131681 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 7/21/2006
949 a| RC261.A274 v.66 no.6 2006 w| LCPER c| 1 i| 000055130790 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 7/21/2006
949 a| RC261.A274 v.66 no.8 2006 w| LCPER c| 1 i| 000055061872 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 8/7/2006
949 a| RC261.A274 v.66 no.7 2006 w| LCPER c| 1 i| 000055062282 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 8/7/2006
949 a| RC261.A274 v.66 no.10 2006 w| LCPER c| 1 i| 000055067256 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/15/2006
949 a| RC261.A274 v.66 no.9 2006 w| LCPER c| 1 i| 000055067249 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/15/2006
949 a| RC261.A274 v.66 no.12 2006 w| LCPER c| 1 i| 000055076364 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 10/11/2006
949 a| RC261.A274 v.66 no.11 2006 w| LCPER c| 1 i| 000055076371 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 10/11/2006
949 a| RC261.A274 v.66 no.14 2006 w| LCPER c| 1 i| 000055089531 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 12/11/2006
949 a| RC261.A274 v.66 no.13 2006 w| LCPER c| 1 i| 000055089616 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 12/11/2006
949 a| RC261.A274 v.66 no.16 2006 w| LCPER c| 1 i| 000055094962 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 1/11/2007
949 a| RC261.A274 v.66 no.15 2006 w| LCPER c| 1 i| 000055094887 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 1/11/2007
949 a| RC261.A274 v.66 no.18 2006 w| LCPER c| 1 i| 000055193207 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/26/2007
949 a| RC261.A274 v.66 no.17 2006 w| LCPER c| 1 i| 000055193122 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/26/2007
949 a| RC261.A274 v.66 no.20 2006 w| LCPER c| 1 i| 000055211512 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/1/2007
949 a| RC261.A274 v.66 no.19 2006 w| LCPER c| 1 i| 000055211130 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/1/2007
949 a| RC261.A274 v.66 no.24 2006 w| LCPER c| 1 i| 000061726963 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 8/20/2007
949 a| RC261.A274 v.66 no.22 2006 w| LCPER c| 1 i| 000061726857 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 8/20/2007
949 a| RC261.A274 v.66 no.21 2006 w| LCPER c| 1 i| 000061726864 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 8/20/2007
949 a| RC261.A274 v.66 no.23 2006 w| LCPER c| 1 i| 000061730229 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/14/2007
949 a| RC261.A274 v.67 no.3-4 2007 w| LCPER c| 1 i| 000061739345 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 10/31/2007
949 a| RC261.A274 v.67 no.1-2 2007 w| LCPER c| 1 i| 000061739338 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 10/31/2007
949 a| RC261.A274 v.67 no.5-6 2007 w| LCPER c| 1 i| 000061743724 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 11/15/2007
949 a| RC261.A274 v.67 no.7-8 2007 w| LCPER c| 1 i| 000061752627 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 1/3/2008
949 a| RC261.A274 v.67 no.9-10 2007 w| LCPER c| 1 i| 000061757639 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 1/9/2008
949 a| RC261.A274 v.67 no.11-12 2007 w| LCPER c| 1 i| 000061759565 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 1/25/2008
949 a| RC261.A274 v.67 no.13-14 2007 w| LCPER c| 1 i| 000059710646 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/11/2008
949 a| RC261.A274 v.67 no.15-16 2007 w| LCPER c| 1 i| 000059630432 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 4/18/2008
949 a| RC261.A274 v.67 no.17-18 2007 w| LCPER c| 1 i| 000059643272 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/21/2008
949 a| RC261.A274 v.67 no.19-20 2007 w| LCPER c| 1 i| 000059648307 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/6/2008
949 a| RC261.A274 v.67 no.21-22 2007 w| LCPER c| 1 i| 000059658986 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 7/14/2008
949 a| RC261.A274 v.67 no.23-24 2007 w| LCPER c| 1 i| 000059658979 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 7/14/2008
949 a| RC261.A274 v.68 no.1-2 2008 w| LCPER c| 1 i| 000059672852 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/19/2008
949 a| RC261.A274 v.68 no.3-4 2008 w| LCPER c| 1 i| 000055222785 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 10/20/2008
949 a| RC261.A274 v.68 no.5-6 2008 w| LCPER c| 1 i| 000055230315 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 11/5/2008
949 a| RC261.A274 v.68 no.7-8 2008 w| LCPER c| 1 i| 000055229555 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 11/19/2008
949 a| RC261.A274 v.68 no.9-10 2008 w| LCPER c| 1 i| 000059736516 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/17/2009
949 a| RC261.A274 v.68 no.11-12 2008 w| LCPER c| 1 i| 000059743446 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 3/24/2009
949 a| RC261.A274 v.68 no.13-14 2008 w| LCPER c| 1 i| 000059752936 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/4/2009
949 a| RC261.A274 v.68 no.15-16 2008 w| LCPER c| 1 i| 000059757191 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/15/2009
949 a| RC261.A274 v.68 no.17-18 2008 w| LCPER c| 1 i| 000059757207 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/15/2009
949 a| RC261.A274 v.68 no.19-20 2008 w| LCPER c| 1 i| 000059804918 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/29/2009
949 a| RC261.A274 v.68 no.21-22 2008 w| LCPER c| 1 i| 000059807889 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/5/2009
949 a| RC261.A274 v.68 no.23-24 2008 w| LCPER c| 1 i| 000059807896 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/8/2009
949 a| RC261.A274 v.69 no.1-2 2009 w| LCPER c| 1 i| 000059809975 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/19/2009
949 a| RC261.A274 v.69 suppl.1 2009 w| LCPER c| 1 i| 000059809968 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/19/2009
949 a| RC261.A274 v.69 no.3-4 2009 w| LCPER c| 1 i| 000059812166 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 7/2/2009
949 a| RC261.A274 v.69 no.5-6 2009 w| LCPER c| 1 i| 000059816461 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 7/2/2009
949 a| RC261.A274 v.69 no.7 2009 w| LCPER c| 1 i| 000059772415 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 8/24/2009
949 a| RC261.A274 v.69 no.8 2009 w| LCPER c| 1 i| 000059772057 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 8/24/2009
949 a| RC261.A274 v.69 no.9 2009 w| LCPER c| 1 i| 000059778806 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/29/2009
949 a| RC261.A274 v.69 no.10 2009 w| LCPER c| 1 i| 000059778653 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/29/2009
949 a| RC261.A274 v.69 no.11 2009 w| LCPER c| 1 i| 000059786269 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 10/20/2009
949 a| RC261.A274 v.69 no.12 2009 w| LCPER c| 1 i| 000059786290 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 10/20/2009
949 a| RC261.A274 v.69 no.13 2009 w| LCPER c| 1 i| 000061840614 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 12/23/2009
949 a| RC261.A274 v.69 no.14 2009 w| LCPER c| 1 i| 000061840621 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 12/23/2009
949 a| RC261.A274 v.69 no.15 2009 w| LCPER c| 1 i| 000061846197 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/5/2010
949 a| RC261.A274 v.69 no.16 2009 w| LCPER c| 1 i| 000061845640 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/5/2010
949 a| RC261.A274 v.69 no.17 2009 w| LCPER c| 1 i| 000061852457 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/25/2010
949 a| RC261.A274 v.69 no.18 2009 w| LCPER c| 1 i| 000061852440 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/25/2010
949 a| RC261.A274 v.69 no.20 2009 w| LCPER c| 1 i| 000061867123 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/7/2010

949 a| RC261.A274 v.69 no.21 2009 w| LCPER c| 1 i| 000061872479 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/10/2010
949 a| RC261.A274 v.69 no.22 2009 w| LCPER c| 1 i| 000061872486 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/10/2010
949 a| RC261.A274 v.69 no.19 2009 w| LCPER c| 1 i| 000061866904 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/17/2010
949 a| RC261.A274 v.69 no.23 2009 w| LCPER c| 1 i| 000061886926 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 7/6/2010
949 a| RC261.A274 v.69 no.24 2009 w| LCPER c| 1 i| 000061886858 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 7/6/2010
949 a| RC261.A274 v.69 suppl.3 2009 w| LCPER c| 1 i| 000061886841 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 7/6/2010
949 a| RC261.A274 v.70 no.5 2010 w| LCPER c| 1 i| 000061889477 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 7/14/2010
949 a| RC261.A274 v.70 no.4 2010 w| LCPER c| 1 i| 000061889460 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 7/14/2010
949 a| RC261.A274 v.70 no.7 2010 w| LCPER c| 1 i| 000061897038 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 8/16/2010
949 a| RC261.A274 v.70 no.8 2010 w| LCPER c| 1 i| 000061897021 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 8/16/2010
949 a| RC261.A274 v.70 no.9 2010 w| LCPER c| 1 i| 000061903319 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/20/2010
949 a| RC261.A274 v.70 no.10 2010 w| LCPER c| 1 i| 000061903272 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/20/2010
949 a| RC261.A274 v.70 no.11 2010 w| LCPER c| 1 i| 000061907829 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 10/5/2010
949 a| RC261.A274 v.70 no.6 2010 w| LCPER c| 1 i| 000061907836 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 10/11/2010
949 a| RC261.A274 v.70 no.12 2010 w| LCPER c| 1 i| 000061915978 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 11/4/2010
949 a| RC261.A274 v.70 no.13 2010 w| LCPER c| 1 i| 000061918108 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 11/29/2010
949 a| RC261.A274 v.70 no.14 2010 w| LCPER c| 1 i| 000061918115 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 11/29/2010
949 a| RC261.A274 v.70 no.17 2010 w| LCPER c| 1 i| 000061820159 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 3/7/2011
949 a| RC261.A274 v.70 no.18 2010 w| LCPER c| 1 i| 000061820142 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 3/7/2011
949 a| RC261.A274 v.70 no.16 2010 w| LCPER c| 1 i| 000061820173 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 3/8/2011
949 a| RC261.A274 v.70 no.15 2010 w| LCPER c| 1 i| 000061820166 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 3/8/2011
949 a| RC261.A274 v.70 no.19 2010 w| LCPER c| 1 i| 000061834408 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 3/31/2011
949 a| RC261.A274 v.70 no.20 2010 w| LCPER c| 1 i| 000059401001 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 4/11/2011
949 a| RC261.A274 v.70 no.21 2010 w| LCPER c| 1 i| 000059407645 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/5/2011
949 a| RC261.A274 v.70 no.22 2010 w| LCPER c| 1 i| 000059407638 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/5/2011
949 a| RC261.A274 v.70 no.23 2010 w| LCPER c| 1 i| 000059410638 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/31/2011
949 a| RC261.A274 v.70 no.24 2010 w| LCPER c| 1 i| 000059410652 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/31/2011
949 a| RC261.A274 v.70 no.24 suppl.2 2010 w| LCPER c| 1 i| 000059410645 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 5/31/2011
949 a| RC261.A274 v.71 no.1 2011 w| LCPER c| 1 i| 000059418559 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/16/2011
949 a| RC261.A274 v.71 no.2 2011 w| LCPER c| 1 i| 000059418542 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/16/2011
949 a| RC261.A274 v.71 no.3 2011 w| LCPER c| 1 i| 000059421078 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 7/8/2011
949 a| RC261.A274 v.71 no.4 2011 w| LCPER c| 1 i| 000059421061 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 7/8/2011
949 a| RC261.A274 v.71 no.5 2011 w| LCPER c| 1 i| 000059424833 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 8/5/2011
949 a| RC261.A274 v.71 no.6 2011 w| LCPER c| 1 i| 000059424826 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 8/5/2011
949 a| RC261.A274 v.71 no.7 2011 w| LCPER c| 1 i| 000059428459 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 8/24/2011
949 a| RC261.A274 v.71 no.8 2011 w| LCPER c| 1 i| 000059428435 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 8/24/2011
949 a| RC261.A274 v.71 no.9 2011 w| LCPER c| 1 i| 000059428428 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/1/2011
949 a| RC261.A274 v.71 no.10 2011 w| LCPER c| 1 i| 000059428442 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/1/2011
949 a| RC261.A274 v.71 no.11 2011 w| LCPER c| 1 i| 000059433002 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/22/2011
949 a| RC261.A274 v.71 no.12 2011 w| LCPER c| 1 i| 000059433019 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 9/22/2011
949 a| RC261.A274 v.71 no.13 2011 w| LCPER c| 1 i| 000059439691 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 11/11/2011
949 a| RC261.A274 v.71 no.14 2011 w| LCPER c| 1 i| 000059440000 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 11/11/2011
949 a| RC261.A274 v.71 no.15 2011 w| LCPER c| 1 i| 000059366072 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 12/21/2011
949 a| RC261.A274 v.71 no.16 2011 w| LCPER c| 1 i| 000059366065 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 12/21/2011
949 a| RC261.A274 v.71 no.17 2011 w| LCPER c| 1 i| 000059370024 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 1/24/2012
949 a| RC261.A274 v.71 no.18 2011 w| LCPER c| 1 i| 000059370000 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 1/24/2012
949 a| RC261.A274 v.71 no.23 2011 w| LCPER c| 1 i| 000059373148 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/3/2012
949 a| RC261.A274 v.71 no.21 2011 w| LCPER c| 1 i| 000059372455 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/3/2012
949 a| RC261.A274 v.71 no.22 2011 w| LCPER c| 1 i| 000059373131 d| 11/9/2012 l| PATERNO-4 m| UP-PAT q| 1 r| Y s| Y t| PERIODICAL u| 2/3/2012
949 a| RC261.A274 v.71 no.20 2011 w| LCPER c| 1 i| 000059372479 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/7/2012
949 a| RC261.A274 v.71 no.19 2011 w| LCPER c| 1 i| 000059372486 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/7/2012
949 a| RC261.A274 v.71 no.24 2011 suppl. w| LCPER c| 1 i| 000059372448 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/7/2012
949 a| RC261.A274 v.71 no.24 2011 w| LCPER c| 1 i| 000059372462 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 2/21/2012
949 a| RC261.A274 v.7 1947 w| LCPER c| 1 i| 000073966326 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013
949 a| RC261.A274 v.8 1948 w| LCPER c| 1 i| 000073966319 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013
949 a| RC261.A274 v.9 1949 w| LCPER c| 1 i| 000073966302 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013
949 a| RC261.A274 v.10 1950 w| LCPER c| 1 i| 000073966296 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013
949 a| RC261.A274 v.1-10 1941-50 index w| LCPER c| 1 i| 000073966289 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013
949 a| RC261.A274 v.11 1951 w| LCPER c| 1 i| 000073966272 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013
949 a| RC261.A274 v.12 1952 w| LCPER c| 1 i| 000073966265 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013
949 a| RC261.A274 v.13 1953 w| LCPER c| 1 i| 000073966258 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013
949 a| RC261.A274 1953 suppl.1 w| LCPER c| 1 i| 000073966241 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013
949 a| RC261.A274 v.14 1954 w| LCPER c| 1 i| 000073966227 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013
949 a| RC261.A274 v.15 1955 w| LCPER c| 1 i| 000073966234 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 1955 suppl.2 w| LCPER c| 1 i| 000073966210 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 1955 suppl.3 w| LCPER c| 1 i| 000073966203 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.16 1956 w| LCPER c| 1 i| 000073966197 d| 12/6/2018 l| PATERNO-4 m| UP-PAT q| 1 r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.17 no.1-5 1957 w| LCPER c| 1 i| 000073966180 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.17 no.6-11 1957 w| LCPER c| 1 i| 000073966173 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.18 no.1-6 1958 w| LCPER c| 1 i| 000073966166 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.19 no.1-5 1959 w| LCPER c| 1 i| 000073966142 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.20 no.1-5 1960 w| LCPER c| 1 i| 000073966128 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.21 no.1-6 1961 w| LCPER c| 1 i| 000073966104 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.22 1962 w| LCPER c| 1 i| 000073966081 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.23 no.1-5 1963 w| LCPER c| 1 i| 000073966074 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.24 no.1-5 1964 w| LCPER c| 1 i| 000073966050 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.25 no.1-4 1965 w| LCPER c| 1 i| 000073966029 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.26 no.1-4 1966 w| LCPER c| 1 i| 000073966890 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.27 no.1-4 1967 w| LCPER c| 1 i| 000073966869 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.18 no.7-11 1958 w| LCPER c| 1 i| 000073966159 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.19 no.6-11 1959 w| LCPER c| 1 i| 000073966135 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.20 no.6-11 1960 w| LCPER c| 1 i| 000073966111 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.21 no.7-11 1961 w| LCPER c| 1 i| 000073966098 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.23 no.6-11 1963 w| LCPER c| 1 i| 000073966067 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.24 no.6-8 1964 w| LCPER c| 1 i| 000073966043 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.24 no.9-11 1964 w| LCPER c| 1 i| 000073966036 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.25 no.5-8 1965 w| LCPER c| 1 i| 000073966012 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013 o| .PUBLIC. No v.7

949 a| RC261.A274 v.25 no.9-11 1965 w| LCPER c| 1 i| 000073966906 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.26 no.5-8 1966 w| LCPER c| 1 i| 000073966883 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.26 no.9-12 1966 w| LCPER c| 1 i| 000073966876 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.27 no.5-9 1967 w| LCPER c| 1 i| 000073966852 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.27 no.10-12 1967 w| LCPER c| 1 i| 000073966845 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.28 no.1-4 1968 w| LCPER c| 1 i| 000073966838 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.29 no.1-3 1969 w| LCPER c| 1 i| 000073966807 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.30 no.1-3 1970 w| LCPER c| 1 i| 000073966760 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.26-30 1966-70 index w| LCPER c| 1 i| 000073966722 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.31 no.1-3 1971 w| LCPER c| 1 i| 000073966715 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.32 no.1-3 1972 w| LCPER c| 1 i| 000073966678 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.33 no.1-3 1973 w| LCPER c| 1 i| 000073966630 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.28 no.5-8 1968 w| LCPER c| 1 i| 000073966821 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.28 no.9-12 1968 w| LCPER c| 1 i| 000073966814 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.29 no.4-6 1969 w| LCPER c| 1 i| 000073966791 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.29 no.7-9 1969 w| LCPER c| 1 i| 000073966784 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.29 no.10-12 1969 w| LCPER c| 1 i| 000073966777 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.30 no.4-6 1970 w| LCPER c| 1 i| 000073966753 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.30 no.7-9 1970 w| LCPER c| 1 i| 000073966746 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.30 no.10-12 1970 w| LCPER c| 1 i| 000073966739 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.31 no.4-6 1971 w| LCPER c| 1 i| 000073966708 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.31 no.7-10 1971 w| LCPER c| 1 i| 000073966692 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.31 no.11-12 1971 w| LCPER c| 1 i| 000073966685 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.32 no.4-6 1972 w| LCPER c| 1 i| 000073966661 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.32 no.7-9 1972 w| LCPER c| 1 i| 000073966654 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.32 no.10-12 1972 w| LCPER c| 1 i| 000073966647 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.33 no.4-5 1973 w| LCPER c| 1 i| 000073966623 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.33 no.6-7 1973 w| LCPER c| 1 i| 000073966616 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.33 no.8-10 1973 w| LCPER c| 1 i| 000073966609 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.33 no.11-12 1973 w| LCPER c| 1 i| 000073966593 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.34 no.1-2 1974 w| LCPER c| 1 i| 000073966586 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.35 no.1-2 1975 w| LCPER c| 1 i| 000073966524 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.36 no.1-2 1976 w| LCPER c| 1 i| 000073966463 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.37 no.1-2 1977 w| LCPER c| 1 i| 000073967309 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.34 no.3-4 1974 w| LCPER c| 1 i| 000073966579 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.34 no.5-6 1974 w| LCPER c| 1 i| 000073966562 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.34 no.7-8 1974 w| LCPER c| 1 i| 000073966555 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.34 no.9-10 1974 w| LCPER c| 1 i| 000073966548 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.34 no.11-12 1974 w| LCPER c| 1 i| 000073966531 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.35 no.3-4 1975 w| LCPER c| 1 i| 000073966517 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.35 no.5-6 1975 w| LCPER c| 1 i| 000073966500 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.35 no.7-8 1975 w| LCPER c| 1 i| 000073966494 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013

949 a| RC261.A274 v.35 no.9-10 1975 w| LCPER c| 1 i| 000073966487 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013
949 a| RC261.A274 v.35 no.11-12 1975 w| LCPER c| 1 i| 000073966470 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/7/2013
949 a| RC261.A274 v.36 no.3-4 1976 w| LCPER c| 1 i| 000073967354 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.36 no.5-6 1976 w| LCPER c| 1 i| 000073967347 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.36 no.7-8 1976 w| LCPER c| 1 i| 000073967330 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.36 no.9-10 1976 w| LCPER c| 1 i| 000073967323 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.36 no.11-12 1976 w| LCPER c| 1 i| 000073967316 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.37 no.3-4 1977 w| LCPER c| 1 i| 000073967293 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.37 no.5-6 1977 w| LCPER c| 1 i| 000073967286 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.37 no.7-8 1977 w| LCPER c| 1 i| 000073967279 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.37 no.9-10 1977 w| LCPER c| 1 i| 000073967262 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.37 no.11-12 1977 w| LCPER c| 1 i| 000073967255 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.38 no.1-2 1978 w| LCPER c| 1 i| 000073967248 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.39 no.1-2 1979 w| LCPER c| 1 i| 000073967163 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.40 no.1-2 1980 w| LCPER c| 1 i| 000073967095 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.38 no.3-4 1978 w| LCPER c| 1 i| 000073967231 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.38 no.5-6 1978 w| LCPER c| 1 i| 000073967224 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.38 no.7-8 1978 w| LCPER c| 1 i| 000073967217 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.38 no.9-10 1978 w| LCPER c| 1 i| 000073967200 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.38 no.11 pt.1 1978 w| LCPER c| 1 i| 000073967194 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.38 no.11 pt.2 1978 w| LCPER c| 1 i| 000073967187 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.38 no.12 1978 w| LCPER c| 1 i| 000073967170 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.39 no.3-4 1979 w| LCPER c| 1 i| 000073967156 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.39 no.5-6 1979 w| LCPER c| 1 i| 000073967149 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.39 no.7-8 1979 w| LCPER c| 1 i| 000073967132 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.39 no.9-10 1979 w| LCPER c| 1 i| 000073967125 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.39 no.11 1979 w| LCPER c| 1 i| 000073967118 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.39 no.12 1979 w| LCPER c| 1 i| 000073967101 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.40 no.3-4 1980 w| LCPER c| 1 i| 000073967088 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.40 no.5-6 1980 w| LCPER c| 1 i| 000073967071 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.40 no.7-8 1980 w| LCPER c| 1 i| 000073967064 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.40 no.9-10 1980 w| LCPER c| 1 i| 000073967057 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.40 no.11-12 1980 w| LCPER c| 1 i| 000073967040 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.41 no.1-2 1981 w| LCPER c| 1 i| 000073967033 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.41 no.3-4 1981 w| LCPER c| 1 i| 000073967026 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.41 no.5-6 1981 w| LCPER c| 1 i| 000073967019 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.41 no.7-8 1981 w| LCPER c| 1 i| 000073967002 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.41 no.9 1981 w| LCPER c| 1 i| 000073966999 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.41 no.10 1981 w| LCPER c| 1 i| 000073966982 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.41 no.11 1981 w| LCPER c| 1 i| 000073966975 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.41 no.12 1981 w| LCPER c| 1 i| 000073966968 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.42 no.1-2 1982 & suppl. w| LCPER c| 1 i| 000073966951 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.43 no.1 1983 w| LCPER c| 1 i| 000073967774 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.42 no.3-4 1982 w| LCPER c| 1 i| 000073966944 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.42 no.5-6 1982 w| LCPER c| 1 i| 000073966937 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.42 no.7-8 1982 & suppl. w| LCPER c| 1 i| 000073966920 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.42 no.9-10 1982 w| LCPER c| 1 i| 000073966913 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.42 no.11 1982 w| LCPER c| 1 i| 000073967804 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.42 no.12 1982 w| LCPER c| 1 i| 000073967798 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.43 no.2 1983 w| LCPER c| 1 i| 000073967767 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.43 no.3 1983 w| LCPER c| 1 i| 000073967750 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.43 no.4 1983 w| LCPER c| 1 i| 000073967743 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.43 no.5 1983 & suppl. w| LCPER c| 1 i| 000073967736 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.43 no.6 1983 w| LCPER c| 1 i| 000073967729 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.42 1982 index w| LCPER c| 1 i| 000073967781 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.44 no.1 1984 w| LCPER c| 1 i| 000073967651 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.45 no.1 1985 w| LCPER c| 1 i| 000073967538 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.43 no.7 1983 w| LCPER c| 1 i| 000073967712 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.43 no.8 1983 w| LCPER c| 1 i| 000073967705 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.43 no.9 1983 w| LCPER c| 1 i| 000073967699 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.43 no.10 1983 w| LCPER c| 1 i| 000073967682 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.43 no.11 1983 w| LCPER c| 1 i| 000073967675 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.43 no.12 1983 w| LCPER c| 1 i| 000073967668 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.44 no.2 1984 w| LCPER c| 1 i| 000073967644 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013

949 a| RC261.A274 v.44 no.3 1984 w| LCPER c| 1 i| 000073967637 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.44 no.4 1984 w| LCPER c| 1 i| 000073967620 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.44 no.5 1984 w| LCPER c| 1 i| 000073967613 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.44 no.6 1984 w| LCPER c| 1 i| 000073967606 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.44 no.7 1984 w| LCPER c| 1 i| 000073967590 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.44 no.8 1984 w| LCPER c| 1 i| 000073967583 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.44 no.9 1984 w| LCPER c| 1 i| 000073967576 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.44 no.10 1984 & suppl. w| LCPER c| 1 i| 000073967569 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.44 no.11 1984 w| LCPER c| 1 i| 000073967552 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.44 no.12 1984 w| LCPER c| 1 i| 000073967545 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.45 no.2 1985 w| LCPER c| 1 i| 000073967521 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.45 no.3 1985 w| LCPER c| 1 i| 000073967514 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.45 no.4 1985 w| LCPER c| 1 i| 000073967507 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.46 no.1 1986 w| LCPER c| 1 i| 000073967415 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.47 no.1-2 1987 w| LCPER c| 1 i| 000073968191 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.45 no.5 1985 w| LCPER c| 1 i| 000073967491 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.45 no.6 1985 w| LCPER c| 1 i| 000073967484 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.45 no.7 1985 w| LCPER c| 1 i| 000073967477 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.45 no.8 1985 w| LCPER c| 1 i| 000073967460 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.45 no.9 1985 & suppl. w| LCPER c| 1 i| 000073967453 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.45 no.10 1985 w| LCPER c| 1 i| 000073967446 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.45 no.11 1985 w| LCPER c| 1 i| 000073967439 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.45 no.12 1985 & index w| LCPER c| 1 i| 000073967422 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.46 no.2 1986 w| LCPER c| 1 i| 000073967408 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.46 no.3 1986 w| LCPER c| 1 i| 000073967392 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.46 no.4 1986 w| LCPER c| 1 i| 000073967385 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.46 no.5 1986 w| LCPER c| 1 i| 000073967378 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.46 no.6 1986 w| LCPER c| 1 i| 000073967361 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.46 no.7 1986 w| LCPER c| 1 i| 000073968252 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.46 no.8 1986 & suppl. w| LCPER c| 1 i| 000073968245 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.46 no.9 1986 w| LCPER c| 1 i| 000073968238 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.46 no.10 1986 w| LCPER c| 1 i| 000073968221 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.46 no.11 1986 w| LCPER c| 1 i| 000073968214 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.46 no.12 & index w| LCPER c| 1 i| 000073968207 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.47 no.3-4 1987 w| LCPER c| 1 i| 000073968184 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.47 no.5-6 1987 w| LCPER c| 1 i| 000073968177 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.48 no.1-2 1988 w| LCPER c| 1 i| 000073968078 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.47 no.7-8 1987 w| LCPER c| 1 i| 000073968160 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.47 no.9-10 1987 w| LCPER c| 1 i| 000073968153 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.47 no.11-12 1987 w| LCPER c| 1 i| 000073968146 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.47 no.13-14 1987 w| LCPER c| 1 i| 000073968139 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.47 no.15-16 1987 w| LCPER c| 1 i| 000073968122 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.47 no.17-18 1987 w| LCPER c| 1 i| 000073968115 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.47 no.19-20 1987 w| LCPER c| 1 i| 000073968108 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.47 no.21-22 1987 w| LCPER c| 1 i| 000073968092 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.47 no.23-24 1987 & index w| LCPER c| 1 i| 000073968085 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.48 no.3-4 1988 w| LCPER c| 1 i| 000073968061 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.48 no.5-6 1988 w| LCPER c| 1 i| 000073968054 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.48 no.7-8 1988 w| LCPER c| 1 i| 000073968047 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.48 no.9-10 1988 w| LCPER c| 1 i| 000073968030 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.48 no.11-12 1988 w| LCPER c| 1 i| 000073968023 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.48 no.13-14 1988 w| LCPER c| 1 i| 000073968016 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.48 no.15-16 1988 w| LCPER c| 1 i| 000073968009 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.48 no.17-18 1988 w| LCPER c| 1 i| 000073967996 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.48 no.19-20 1988 w| LCPER c| 1 i| 000073967989 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.48 no.21-22 1988 w| LCPER c| 1 i| 000073967972 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.48 no.23-24 1988 & index w| LCPER c| 1 i| 000073967965 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.49 no.1-2 1988 w| LCPER c| 1 i| 000073967958 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.49 no.3-4 1988 w| LCPER c| 1 i| 000073967941 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.49 no.5-6 1988 w| LCPER c| 1 i| 000073967934 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.49 no.7-8 1988 & suppl. w| LCPER c| 1 i| 000073967927 l| PATERNO-4 m| UP PAT r| Y s| Y t| PERIODICAL u| 6/12/2013

949 a| RC261.A274 v.49 no.9-10 1988 w| LCPER c| 1 i| 000073967910 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.49 no.11-12 1988 w| LCPER c| 1 i| 000073967903 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.49 no.13-14 1988 w| LCPER c| 1 i| 000073967897 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.49 no.15-16 1988 w| LCPER c| 1 i| 000073967880 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.49 no.17-18 1988 w| LCPER c| 1 i| 000073967873 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.49 no.19-20 1988 w| LCPER c| 1 i| 000073967866 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.49 no.21-22 1988 w| LCPER c| 1 i| 000073967859 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.49 no.23 1988 w| LCPER c| 1 i| 000073967842 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.49 no.24 1988 & index w| LCPER c| 1 i| 000073967835 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 1989 suppl. w| LCPER c| 1 i| 000073968702 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.1-2 1989 w| LCPER c| 1 i| 000073967828 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.3-4 1989 w| LCPER c| 1 i| 000073967811 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.5-6 1989 w| LCPER c| 1 i| 000073968696 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.7-8 1989 w| LCPER c| 1 i| 000073968689 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.9-10 1989 w| LCPER c| 1 i| 000073968672 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.11-12 1989 w| LCPER c| 1 i| 000073968665 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.13-14 1989 w| LCPER c| 1 i| 000073968658 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.15-16 1989 w| LCPER c| 1 i| 000073968641 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.17 1989 & suppl. w| LCPER c| 1 i| 000073968634 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.18 1990 w| LCPER c| 1 i| 000073968627 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.19-20 1990 w| LCPER c| 1 i| 000073968610 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.21-22 1990 w| LCPER c| 1 i| 000073968603 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.23 1990 w| LCPER c| 1 i| 000073968597 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.50 no.24 1990 & suppl. w| LCPER c| 1 i| 000073968580 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.51 no.1-2 1991 w| LCPER c| 1 i| 000073968573 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.51 no.3-4 1991 w| LCPER c| 1 i| 000073968566 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.51 no.5-6 1991 w| LCPER c| 1 i| 000073968559 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.51 no.7-8 1991 w| LCPER c| 1 i| 000073968542 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.51 no.9-10 1991 w| LCPER c| 1 i| 000073968535 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.51 no.11-12 1991 w| LCPER c| 1 i| 000073968528 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.51 no.13-14 1991 w| LCPER c| 1 i| 000073968511 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.51 no.15-16 1991 w| LCPER c| 1 i| 000073968504 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.51 no.17-18 1991 & suppl. w| LCPER c| 1 i| 000073968498 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.51 no.19-20 1991 w| LCPER c| 1 i| 000073968481 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.51 no.21-22 1991 w| LCPER c| 1 i| 000073968474 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.51 no.23-24 1991 & index w| LCPER c| 1 i| 000073968467 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.52 1992 suppl. w| LCPER c| 1 i| 000073968405 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.52 no.1-2 1992 w| LCPER c| 1 i| 000073968450 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.52 no.3-4 1992 w| LCPER c| 1 i| 000073968443 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.52 no.5-6 1992 w| LCPER c| 1 i| 000073968436 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.52 no.7-8 1992 & suppl. w| LCPER c| 1 i| 000073968429 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.52 no.9-10 1992 w| LCPER c| 1 i| 000073968412 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/12/2013
949 a| RC261.A274 v.52 no.11-12 1992 w| LCPER c| 1 i| 000073968399 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.52 no.13-14 1992 w| LCPER c| 1 i| 000073968382 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.52 no.15-16 1992 w| LCPER c| 1 i| 000073968375 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.52 no.17-18 1992 w| LCPER c| 1 i| 000073968368 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.52 no.19-20 1992 & suppl w| LCPER c| 1 i| 000073968351 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.52 no.21-22 1992 w| LCPER c| 1 i| 000073968344 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.52 no.23-24 1992 & suppl. w| LCPER c| 1 i| 000073968337 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.53 no.1-2 1993 w| LCPER c| 1 i| 000073968320 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.53 no.3-4 1993 w| LCPER c| 1 i| 000073968313 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.53 no.5-6 1993 w| LCPER c| 1 i| 000073968306 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.53 no.7-8 1993 w| LCPER c| 1 i| 000073968290 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.53 no.9-10 1993 w| LCPER c| 1 i| 000073968283 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.53 no.11-12 1993 w| LCPER c| 1 i| 000073968276 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.53 no.13-14 1993 w| LCPER c| 1 i| 000073968269 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.53 no.15-16 1993 w| LCPER c| 1 i| 000073969150 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.53 no.17-18 1993 w| LCPER c| 1 i| 000073969143 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013
949 a| RC261.A274 v.53 no.19-20 1993 w| LCPER c| 1 i| 000073969136 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.53 no.21-22 1993 w| LCPER c| 1 i| 000073969129 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.53 no.23-24 1993 & index w| LCPER c| 1 i| 000073969112 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.54 1994 index w| LCPER c| 1 i| 000073968986 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.54 no.1-2 1994 w| LCPER c| 1 i| 000073969105 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.54 no.3-4 1994 w| LCPER c| 1 i| 000073969099 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.54 no.5-6 1994 w| LCPER c| 1 i| 000073969082 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.54 no.7-8 1994 & suppl. w| LCPER c| 1 i| 000073969075 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.54 no.9-10 1994 w| LCPER c| 1 i| 000073969068 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.54 no.11-12 1994 w| LCPER c| 1 i| 000073969051 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.54 no.13-14 1994 w| LCPER c| 1 i| 000073969044 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.54 no.15-16 1994 w| LCPER c| 1 i| 000073969037 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.54 no.17-18 1994 w| LCPER c| 1 i| 000073969020 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.54 no.19-20 1994 w| LCPER c| 1 i| 000073969013 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.54 no.21-22 1994 w| LCPER c| 1 i| 000073969006 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.54 no.23-24 1994 w| LCPER c| 1 i| 000073968993 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.1-2 1995 w| LCPER c| 1 i| 000073968979 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.3-4 1995 w| LCPER c| 1 i| 000073968962 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.5-6 1995 w| LCPER c| 1 i| 000073968955 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.7-8 1995 w| LCPER c| 1 i| 000073968948 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.9-10 1995 w| LCPER c| 1 i| 000073968931 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.11-12 1995 w| LCPER c| 1 i| 000073968924 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.13-14 1995 w| LCPER c| 1 i| 000073968917 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.15-16 1995 w| LCPER c| 1 i| 000073968900 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.17-18 1995 w| LCPER c| 1 i| 000073968894 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.19-20 1995 w| LCPER c| 1 i| 000073968887 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.21 1995 w| LCPER c| 1 i| 000073968870 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.22 1995 w| LCPER c| 1 i| 000073968863 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.23 1995 & suppl. w| LCPER c| 1 i| 000073968856 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.55 no.24 1995 & index w| LCPER c| 1 i| 000073968849 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.56 no.1-2 1996 w| LCPER c| 1 i| 000073968832 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.56 no.3-4 1996 w| LCPER c| 1 i| 000073968825 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.56 no.5-6 1996 w| LCPER c| 1 i| 000073968818 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.56 no.7-8 1996 w| LCPER c| 1 i| 000073968801 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.56 no.9-10 1996 w| LCPER c| 1 i| 000073968795 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.56 no.11-12 1996 w| LCPER c| 1 i| 000073968788 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.56 no.13-14 1996 w| LCPER c| 1 i| 000073968771 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.56 no.15-16 1996 w| LCPER c| 1 i| 000073968764 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.56 no.17-18 1996 w| LCPER c| 1 i| 000073968757 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.56 no.19-20 1996 w| LCPER c| 1 i| 000073968740 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.56 no.21-22 1996 w| LCPER c| 1 i| 000073968733 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.56 no.23-24 1996 & index w| LCPER c| 1 i| 000073968726 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.57 no.1-2 1997 w| LCPER c| 1 i| 000073968719 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.57 no.3-4 1997 w| LCPER c| 1 i| 000073969600 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.57 no.5-6 1997 w| LCPER c| 1 i| 000073969594 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.57 no.7-8 1997 w| LCPER c| 1 i| 000073969587 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.57 no.9-10 1997 w| LCPER c| 1 i| 000073969570 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.57 no.11-12 1997 w| LCPER c| 1 i| 000073969563 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.57 no.13-14 1997 w| LCPER c| 1 i| 000073969556 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.57 no.15-16 1997 w| LCPER c| 1 i| 000073969549 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.57 no.17-18 1997 w| LCPER c| 1 i| 000073969532 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.57 no.19-20 1997 w| LCPER c| 1 i| 000073969525 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/13/2013

949 a| RC261.A274 v.57 no.21-22 1997 w| LCPER c| 1 i| 000073969518 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013

949 a| RC261.A274 v.57 no.23-24 1997 & index w| LCPER c| 1 i| 000073969501 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013

949 a| RC261.A274 v.58 no.1-2 1998 w| LCPER c| 1 i| 000073969495 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013

949 a| RC261.A274 v.58 no.3-4 1998 w| LCPER c| 1 i| 000073969488 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013

949 a| RC261.A274 v.58 no.5-6 1998 w| LCPER c| 1 i| 000073969471 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013

949 a| RC261.A274 v.58 no.7-8 1998 w| LCPER c| 1 i| 000073969464 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013

949 a| RC261.A274 v.58 no.9-10 1998 w| LCPER c| 1 i| 000073969457 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013

949 a| RC261.A274 v.58 no.11-12 1998 w| LCPER c| 1 i| 000073969440 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013

949 a| RC261.A274 v.58 no.13-14 1998 w| LCPER c| 1 i| 000073969433 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013

949 a| RC261.A274 v.58 no.15-16 1998 w| LCPER c| 1 i| 000073969426 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013

949			a| RC261.A274 v.58 no.17-18 1998 w| LCPER c| 1 i| 000073969419 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.58 no.19-20 1998 w| LCPER c| 1 i| 000073969402 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.58 no.21-22 1998 w| LCPER c| 1 i| 000073969396 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.58 no.23-24 1998 & index w| LCPER c| 1 i| 000073969389 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.59 no.1-2 1999 w| LCPER c| 1 i| 000073969372 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.59 no.3-4 1999 w| LCPER c| 1 i| 000073969365 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.59 no.5-6 1999 w| LCPER c| 1 i| 000073969358 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.59 no.7-8 1999 & suppl. w| LCPER c| 1 i| 000073969341 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.59 no.9-10 1999 w| LCPER c| 1 i| 000073969334 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.59 no.11-12 1999 w| LCPER c| 1 i| 000073969327 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.59 no.13-14 1999 w| LCPER c| 1 i| 000073969310 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.59 no.15-16 1999 w| LCPER c| 1 i| 000073969303 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.59 no.17-18 1999 w| LCPER c| 1 i| 000073969297 d| 6/20/2022 l| PATERNO-4 m| UP-PAT q| 1 r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.59 no.19-20 1999 w| LCPER c| 1 i| 000073969280 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.59 no.21-22 1999 w| LCPER c| 1 i| 000073969273 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.59 no.23-24 1999 & suppl. w| LCPER c| 1 i| 000073969266 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.60 no.1-2 2000 w| LCPER c| 1 i| 000073969259 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.60 no.3-4 2000 w| LCPER c| 1 i| 000073969242 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.60 no.5-6 2000 w| LCPER c| 1 i| 000073969235 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.60 no.7-8 2000 w| LCPER c| 1 i| 000073969228 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.60 no.9-10 2000 w| LCPER c| 1 i| 000073969211 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.60 no.11-12 2000 w| LCPER c| 1 i| 000073969204 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.60 no.13-14 2000 w| LCPER c| 1 i| 000073969198 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.60 no.15-16 2000 w| LCPER c| 1 i| 000073969181 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.60 no.17-18 2000 w| LCPER c| 1 i| 000073969174 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.60 no.19-20 2000 w| LCPER c| 1 i| 000073969167 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.60 no.21-22 2000 w| LCPER c| 1 i| 000073970057 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| RC261.A274 v.60 no.23-24 2000 & index w| LCPER c| 1 i| 000047186057 l| PATERNO-4 m| UP-PAT r| Y s| Y t| PERIODICAL u| 6/14/2013
949			a| Periodical 1941 1 w| ASIS c| 1 i| 32239000883344 l| CATO-2 m| UP-ANNEX r| Y s| Y t| BNDSER-HY u| 12/13/2001 z| 7086
949			a| Periodical 1942 2 w| ASIS c| 1 i| 32239000883351 d| 11/17/2003 e| 11/13/2003 l| CATO-2 m| UP-ANNEX n| 1 q| 2 r| Y s| Y t| BNDSER-HY u| 12/13/2001 z| 7086
949			a| Periodical 1943 3 w| ASIS c| 1 i| 32239000883369 l| CATO-2 m| UP-ANNEX r| Y s| Y t| BNDSER-HY u| 12/13/2001 z| 7086
949			a| Periodical 1944 4 w| ASIS c| 1 i| 32239000883377 l| CATO-2 m| UP-ANNEX r| Y s| Y t| BNDSER-HY u| 12/13/2001 z| 7086
949			a| Periodical 1945 5 w| ASIS c| 1 i| 32239000883385 l| CATO-2 m| UP-ANNEX r| Y s| Y t| BNDSER-HY u| 12/13/2001 z| 7086
949			a| Periodical 1946 6 w| ASIS c| 1 i| 32239000883393 l| CATO-2 m| UP-ANNEX r| Y s| Y t| BNDSER-HY u| 12/13/2001 z| 7086
949			a| Periodical 1947 7 w| ASIS c| 1 i| 32239000883401 l| CATO-2 m| UP-ANNEX r| Y s| Y t| BNDSER-HY u| 12/13/2001 z| 7086
852	8	1	a| PSt b| UP-ANNEX c| CATO-2 h| Periodical
866		0	8| 0 a| v.1(1941)-v.7(1947)
852	0		a| PSt b| UP-PAT c| PATERNO-4 h| RC261 i| .A274
866		0	8| 0 a| v.7(1947)-v.26(1966), v.27(May 1967)-v.71(2011)

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RC261.A274 v.70 no.15 2010	Bound Journal	Paterno - 4th Floor
RC261.A274 v.70 no.14 2010	Bound Journal	Paterno - 4th Floor
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RC261.A274 v.70 no.5 2010	Bound Journal	Paterno - 4th Floor
RC261.A274 v.70 no.4 2010	Bound Journal	Paterno - 4th Floor
RC261.A274 v.69 suppl.3 2009	Bound Journal	Paterno - 4th Floor
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RC261.A274 v.69 no.19 2009	Bound Journal	Paterno - 4th Floor
RC261.A274 v.69 no.18 2009	Bound Journal	Paterno - 4th Floor
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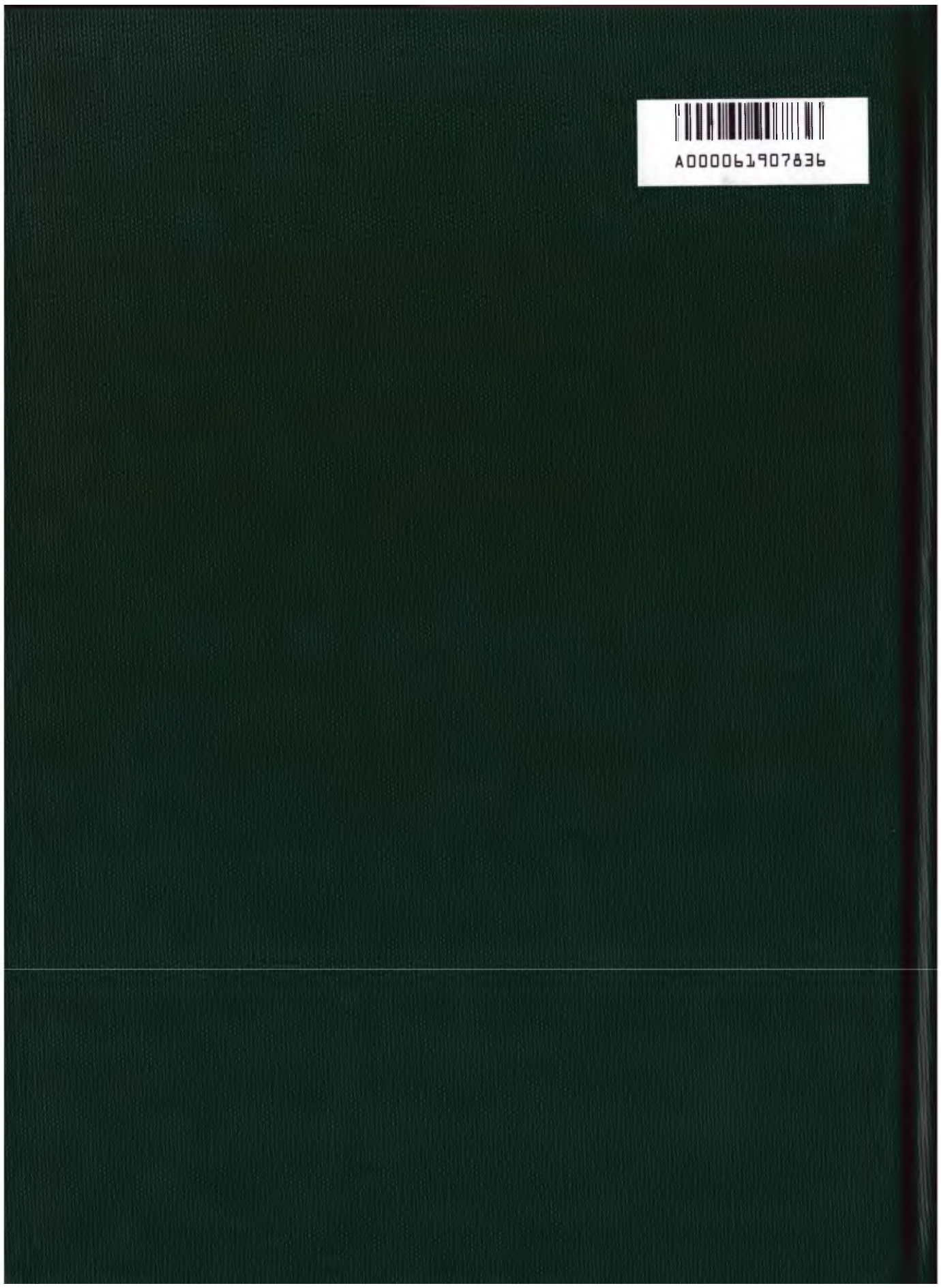
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2568 **Correction: α7-Nicotinic Acetylcholine Receptors Affect Growth Regulation of Human Mesothelioma Cells: Role of Mitogen-Activated Protein Kinase Pathway**

2569 **Correction: CCL2 Blockade Augments Cancer Immunotherapy**

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Cancer Research

Antibody-Maytansinoid Conjugates Designed to Bypass Multidrug Resistance

Yelena V. Kovtun, Charlene A. Audette, Michele F. Mayo, Gregory E. Jones, Heather Doherty, Erin K. Maloney, Hans K. Erickson, Xiuxia Sun, Sharon Wilhelm, Olga Ab, Katharine C. Lai, Wayne C. Widdison, Brenda Kellogg, Holly Johnson, Jan Pinkas, Robert J. Lutz, Rajeeva Singh, Victor S. Goldmacher, and Ravi V.J. Chari

Abstract

Conjugation of cytotoxic compounds to antibodies that bind to cancer-specific antigens makes these drugs selective in killing cancer cells. However, many of the compounds used in such antibody-drug conjugates (ADC) are substrates for the multidrug transporter MDR1. To evade the MDR1-mediated resistance, we conjugated the highly cytotoxic maytansinoid DM1 to antibodies via the maleimidyl-based hydrophilic linker PEG_4Mal. Following uptake into target cells, conjugates made with the PEG_4Mal linker were processed to a cytotoxic metabolite that was retained by MDR1-expressing cells better than a metabolite of similar conjugates prepared with the nonpolar linker *N*-succinimidyl-4-(maleimidomethyl)cyclohexane-1-carboxylate (SMCC). In accord, PEG_4Mal-linked conjugates were more potent in killing MDR1-expressing cells in culture. In addition, PEG_4Mal-linked conjugates were markedly more effective in eradicating MDR1-expressing human xenograft tumors than SMCC-linked conjugates while being tolerated similarly, thus showing an improved therapeutic index. This study points the way to the development of ADCs that bypass multidrug resistance.
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Introduction

Treatment of cancer patients with chemotherapeutic drugs often leads to the emergence of tumors with a multidrug-resistant (MDR) phenotype (1, 2). Many mechanisms of MDR have been described, such as the overexpression of MDR1 (also known as P-glycoprotein or ABCB1) or other ATP-dependent transporters that pump drugs out of the cancer cells, amplification of drug-inactivating enzymes, mutations or modifications of drug targets, alterations in DNA repair machinery, and increased resistance to apoptosis (1–3). The MDR1-mediated efflux of anticancer drugs is the most commonly observed MDR phenotype clinically, and correlations between the transporter expression and poor response to chemotherapy have been documented for many cancer types (4, 5). To overcome MDR1-mediated resistance, numerous MDR inhibitors or modulators have been developed and tested in clinical trials in combination with approved anticancer drugs. However, high systemic toxicity was observed in these trials, possibly because these inhibitors also affect the MDR1 transporter in normal tissue, giving rise to increased accumulation of cytotoxic anticancer drugs in normal tissue as well as in tumors (1, 4).

A promising approach to achieve targeted delivery of cytotoxic drugs to tumor cells, thereby reducing their systemic toxicity, is to use antibodies as specific delivery vehicles. Several highly potent cytotoxic compounds have been conjugated to tumor-targeting antibodies. A number of such antibody-drug conjugates (ADC) are currently undergoing clinical trials, and one conjugate, gemtuzumab ozogamicin, has been approved for the treatment of acute myeloid leukemia (6, 7). However, the majority of cytotoxic compounds that have been used in ADCs, such as calicheamicin (8–10), doxorubicin, taxanes (1, 4), maytansinoids (11), analogues of dolastatin 10 (12), and CC-1065 (13, 14), are substrates for the MDR1 transporter, and the activity of many ADCs is poor in MDR1-expressing cells (8–11, 15, 16).

MDR1 is believed to confer resistance to cytotoxic compounds via two mechanisms: first, by mediating the efflux of drugs that diffuse into the plasma membrane from the extracellular space, thereby preventing the compound from reaching the cytoplasm, and second, by effluxing any compound that does enter the cytoplasm back to the outside of the cell (3, 17). ADCs deliver their cytotoxic payload inside cells via antigen-mediated endocytosis, thus bypassing the first MDR1 mechanism (16, 18). Upon intracellular processing, conjugates are processed into cytotoxic metabolites, whose structures depend on the chemistry of the linker between the cytotoxic compound and the antibody (19, 20). These metabolites may be MDR1 substrates and therefore susceptible to the second resistance mechanism, MDR1-mediated efflux from the cytoplasm to the exterior of the cell.

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Maytansinoids, highly potent antimicrotubule agents, are the payload of several ADCs in clinical development (7, 21). A maytansine-resistant phenotype, linked to the expression of MDR1, has been previously described (11, 22, 23). Building on this observation, we first confirmed that MDR1-expressing cells were resistant to killing by maytansinoids and maytansinoid conjugates. We then reengineered the antibody-maytansinoid conjugates to bypass this MDR1-mediated resistance. Although MDR1 has been shown to recognize and transport a great variety of compounds sharing little if any structural similarities, most of the reported MDR1 substrates are hydrophobic (24). We hypothesized that if maytansinoids were attached to an antibody via a hydrophilic linker, the resulting metabolite would be hydrophilic and, therefore, might be a poor substrate of MDR1, thus avoiding MDR1-mediated resistance. In this report, we describe novel antibody-maytansinoid conjugates that, upon internalization and processing, generate a cytotoxic metabolite that seems to be a poor MDR1 substrate. We found that these conjugates bypass MDR1-mediated resistance both in cultured human cell lines and in xenograft tumors in immunodeficient mice, resulting in an increase in the therapeutic index of the conjugates.

Materials and Methods

Antibody-maytansinoid conjugates. The anti–epidermal growth factor receptor (EGFR) antibody (rat IgG2a developed at ImmunoGen, Inc.), the anti-EpCAM monoclonal antibody B38.1 [murine IgG1, HB 8110; American Type Culture Collection (ATCC)], and the anti-CanAg antibody huC242 (humanized IgG1; refs. 25, 26) were modified with either *N*-succinimidyl-4-(maleimidomethyl)cyclohexane-1-carboxylate (SMCC; Sigma-Aldrich) or Mal-dPEG4-NHS (QuantaBiodesign) before conjugation with DM1 (23) as described previously (27). The conjugates used in this study contained, on average, 3.5 molecules of DM1 per antibody molecule.

Generation of COLO 205^{MDR} cell line. The coding sequence of the human *MDR1* (*ABCB1*) gene was cloned into pQCXIN plasmid (BD Biosciences) and packaged using the pantropic retroviral expression system (BD Biosciences). COLO 205 cells (ATCC CCL-222) were transduced with *MDR1* using this vector in accordance with the manufacturer's recommendations, and a COLO 205 clone expressing a functional MDR1 pump, denoted COLO 205^{MDR}, was selected by culturing the cells in medium containing paclitaxel (5 nmol/L) with subsequent subcloning.

Binding studies. Binding of antibodies and antibody-maytansinoid conjugates to cells was evaluated by indirect immunofluorescence using flow cytometry as described previously (28).

Rhodamine-123 accumulation and retention assays. Cultures of HCT-15 (ATCC CCL-225), UO-31 (National Cancer Institute-Frederick Cancer Research & Development Center, Frederick, MD), COLO 205, and COLO 205^{MDR} were maintained in RPMI 1640 (Invitrogen, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and 50 μg/mL gentamicin sulfate (Invitrogen) at 37°C in 6% CO_2. Cell samples (2.5×10^5 cells/mL) were treated with rhodamine-123 (1.3 μmol/L; Sigma-Aldrich) in the presence or absence of cyclosporin A (5 μmol/L; Sigma-Aldrich) for 90 min at 37°C. The cells were then washed twice with HBSS containing 10% (v/v) fetal bovine serum and analyzed by flow cytometry. The cells evaluated for rhodamine-123 retention were resuspended in culture medium with or without cyclosporin A (5 μmol/L), incubated for an additional 90 min at 37°C, washed, and analyzed by flow cytometry.

In vitro *cytotoxicity assays.* Cells in 96-well plates (2×10^3 per well) were treated with test compounds at various concentrations in appropriate cell culture medium (total volume, 0.2 mL). Control wells containing cells and the medium but lacking test compounds and wells containing medium only were included in each assay plate. The plates were incubated for 4 to 6 d at 37°C in a humidified atmosphere containing 6% CO_2. WST-8 reagent (10%, v/v; Dojindo Molecular Technologies) was then added to the wells, and the plates were incubated at 37°C for 2 to 6 h depending on the cell line. Then, the absorbance was measured on a plate reader spectrophotometer in the dual-wavelength mode 450 nm/650 nm, and the absorbance at the 650 nm (nonspecific light scattering by cells) was subtracted. The apparent surviving fraction of cells in each well was calculated by first correcting for the medium background absorbance and then dividing each value by the average of the values in the control wells (nontreated cells). Each cytotoxicity experiment was repeated at least twice. The data shown in the figures represent a typical experiment.

Intracellular metabolism. COLO 205^{MDR} cells (2×10^6/mL) were incubated in culture medium containing a conjugate (10^{-8} mol/L) for 24 h at 37°C. Cells were then washed in medium, and metabolites were extracted with acetone and analyzed by reverse-phase high-performance liquid chromatography (HPLC) and by electrospray mass spectrometry as described previously (19).

In vivo *efficacy study.* COLO 205, COLO 205^{MDR}, or HCT-15 cells (1×10^7 per animal) were injected s.c. in the area under the right shoulder of female CB.17 severe combined immunodeficient (SCID) mice (Charles River Laboratories). When the tumor volume reached average size of 130 to 140 mm^3, mice were randomized into treatment groups with similar mean tumor volumes ($n = 6$ animals per group). Groups were treated with a single i.v. bolus injection of PBS (vehicle control) or an antibody-maytansinoid conjugate. Tumor growth was monitored and tumor volume was calculated by the following formula: length × width × height × 1/2.

Cell cycle analysis. Cells (2.5×10^5/mL) were exposed to conjugate (4 nmol/L of conjugated DM1) for 3 h at 37°C, washed, and incubated in conjugate-free medium for an additional 18 h at 37°C. Cells were then fixed with 1 mL of 70% ethanol, and DNA content was determined after staining with propidium iodide by flow cytometry as previously described (19).

Quantification of maytansinoid metabolites. The cells were collected by centrifugation and resuspended in 50 μL of 0.05% Tween 20 in TBS (pH 7.5). Cold acetone (200 μL) was added to each sample, and the samples were then incubated on ice for 30 min, which led to protein precipitation.

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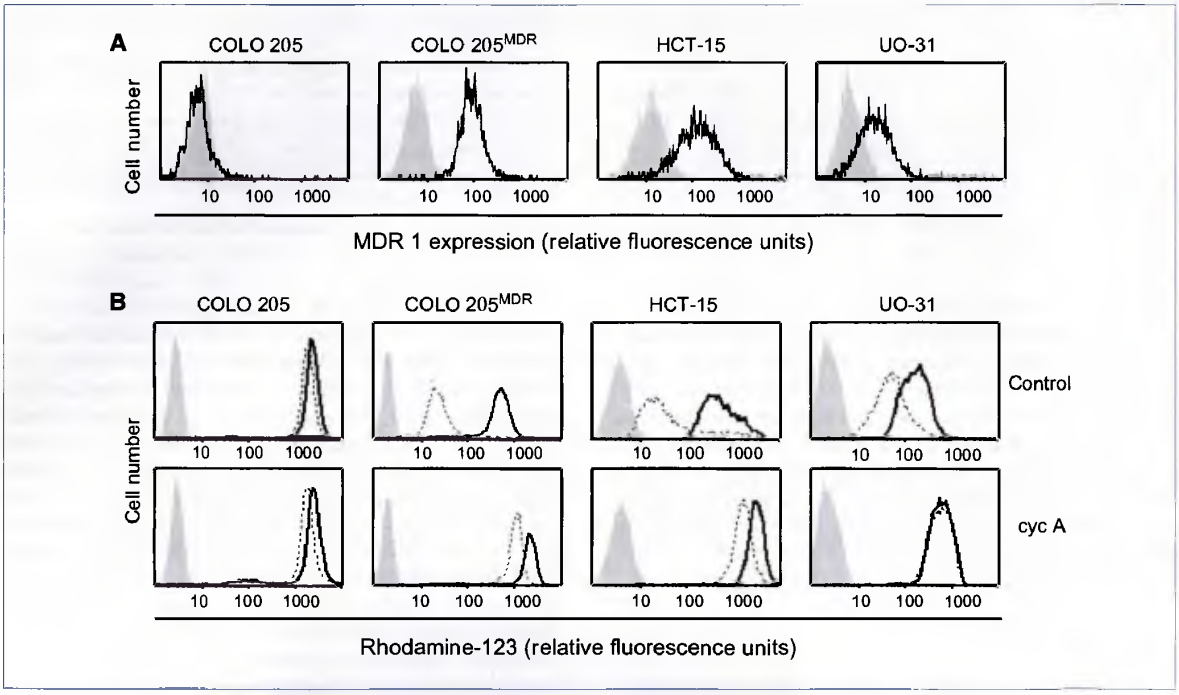


Figure 1. COLO 205^{MDR}, HCT-15, and UO-31 cells express functionally active MDR1 protein on their surface. A, cell surface expression of MDR1. Cells were incubated with the anti-MDR1 antibody (solid lines) or binding buffer (filled histograms) and then with anti-IgG–FITC and analyzed on a flow cytometer. B, uptake (solid lines) and retention (dotted lines) of rhodamine-123 in the absence (Control) or the presence of cyclosporin A (cyc A). Filled histograms show the cell autofluorescence.

The samples were then centrifuged (16,100 × *g*, 30 min) and ^{3}H levels of supernatants were determined using a liquid scintillation analyzer.

Results

Human tumor cell lines that express active MDR1 show decreased sensitivity to maytansine and to some antibody-maytansinoid conjugates. Three MDR human cell lines were used in this study to evaluate the effect of the MDR1 transporter on maytansinoid-dependent cytotoxicity: a clone of the colon adenocarcinoma cell line COLO 205 that has been retrovirally transduced with MDR1 (COLO 205^{MDR}) and two naturally evolved MDR cell lines (the colon adenocarcinoma HCT-15 and the renal adenocarcinoma UO-31). The expression of MDR1 on the plasma membrane of these cells was confirmed by immunostaining with an anti-MDR1 antibody (Fig. 1A). The parental MDR1-negative COLO 205 cells were used as a negative control (Fig. 1A). The functional integrity of the MDR1 pump was examined by the ability of the cells to accumulate and retain the fluorescent dye rhodamine-123, a well-established MDR1 substrate (29). The exposure of COLO 205^{MDR} cells to rhodamine-123 resulted in a 4-fold lower accumulation of the dye compared with that in the parental COLO 205 cells (mean fluorescence of 700 versus 2,800) and a 100-fold reduction in the amount of cell-associated dye retained by the cells after incubation in dye-free medium (mean fluorescence of 28 versus 2,800; Fig. 1B). Importantly, in the presence of an inhibitor of MDR1, cyclosporin A (1, 3), the COLO 205^{MDR} cells accumulated and retained as much rhodamine-123 as COLO 205 cells (Fig. 1B), indicating that the reduced accumulation and retention in COLO 205^{MDR} cells was due to the activity of the MDR1 pump. Similar results were obtained with other MDR1-expressing cell lines, HCT-15 and UO-31, where cyclosporin A caused a substantial increase in the accumulation and retention of rhodamine-123 (Fig. 1B). These results show that COLO 205^{MDR}, HCT-15, and UO-31 cells express active MDR1 transporter on their plasma membrane.

To evaluate the effect of MDR1 expression on maytansinoid-induced cell killing, we compared the sensitivities of COLO 205^{MDR} cells and the parental cell line COLO 205 to maytansine and its derivative DM1SMe (23). We also tested the activity of two other antimicrotubule cytotoxic compounds, paclitaxel and vinblastine, which have previously been shown to be MDR1 substrates (1, 3). As expected, paclitaxel and vinblastine were less potent in killing COLO 205^{MDR} cells than in killing COLO 205 cells (13- and 17-fold, respectively; Table 1). Similarly, sensitivity of cells to maytansine and DM1SMe was also affected by MDR1 expression: COLO 205 ^{MDR} cells were 8-fold less sensitive to the maytansinoids than COLO 205 cells. We also compared the cytotoxicity of

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Table 1. *In vitro* cytotoxicity of paclitaxel, vinblastine, maytansine, and DM1SMe for COLO 205, COLO 205^{MDR}, HCT-15, and UO-31 cells in the presence or absence of 1 μmol/L cyclosporin A

Cytotoxic agent	COLO 205^{MDR}	COLO 205	IC_{50} COLO 205^{MDR}/IC_{50} COLO 205	HCT-15			UO-31		
	IC_{50} (nmol/L)			IC_{50} (nmol/L)		IC_{50} (−cycA)/ IC_{50} (+cycA)	IC_{50} (nmol/L)		IC_{50} (−cycA)/ IC_{50} (+cycA)
				−cycA	+cycA		−cycA	+cycA	
Paclitaxel	53 ± 5	4.0 ± 1.6	13	70 ± 29	4 ± 2	18	105 ± 40	6 ± 3	18
Vinblastine	24 ± 11	1.4 ± 0.4	17	5 ± 2	0.5 ± 0.2	10	65 ± 21	6.5 ± 2.1	10
Maytansine	0.6 ± 0.1	0.08 ± 0.01	8	0.4 ± 0.2	0.05 ± 0.03	8	0.7 ± 0.2	0.12 ± 0.06	6
DM1SMe	0.42	0.05	8	0.14	0.02	7	0.4	0.07	6

NOTE: The data are presented as the mean IC_{50} ± SEM (n = 3).
Abbreviation: cycA, cyclosporin A.

maytansine, DM1SMe, paclitaxel, and vinblastine to MDR1-positive HCT-15 and UO-31 cells in the presence or absence of the MDR1 inhibitor cyclosporin A. Maytansinoid-induced cytotoxicity against HCT-15 and UO-31 cells was enhanced 6- to 8-fold in the presence cyclosporin A, and that of paclitaxel and vinblastine was enhanced 10- and 18-fold, respectively (Table 1). Cyclosporin A alone at 1 μmol/L was not cytotoxic to the cells (data not shown). These results indicate that MDR1 activity limits the potency of these cytotoxic compounds.

To evaluate whether expression of MDR1 affects the cytotoxicity of an antibody-maytansinoid conjugate, we evaluated the activity of an anti-EpCAM antibody-maytansinoid conjugate against the EpCAM-positive cell lines HCT-15, COLO 205^{MDR}, and COLO 205 (Supplementary Fig. S1; Supplementary Table S1). The conjugate anti-EpCAM–SMCC–DM1 was composed of the anti-EpCAM antibody B38.1 conjugated with the maytansinoid DM1 using the SMCC linker (Fig. 2A). The conjugation did not affect the ability of the antibody to bind to the EpCAM-positive cells (Supplementary Fig. S2). The anti-EpCAM–SMCC–DM1 conjugate showed antigen-dependent cytotoxicity against the MDR1-positive HCT-15 and COLO 205^{MDR} cells, and the MDR1-negative COLO 205 cells, as shown by the ability of excess unconjugated anti-EpCAM antibody to block the cell-killing activity (Fig. 3A, top row). Cyclosporin A (Fig. 3A, top row) or another MDR1-specific inhibitor, PGP4008 (data not shown; ref. 30), significantly enhanced the potency of anti-EpCAM–SMCC–DM1 against the MDR1-positive HCT-15 and COLO 205^{MDR} cells, but not against the MDR1-negative COLO 205 cells, indicating that MDR1 activity limits the potency of this conjugate.

In addition to the MDR1 transporter, we evaluated expression of two other ATP-dependent transporters that are

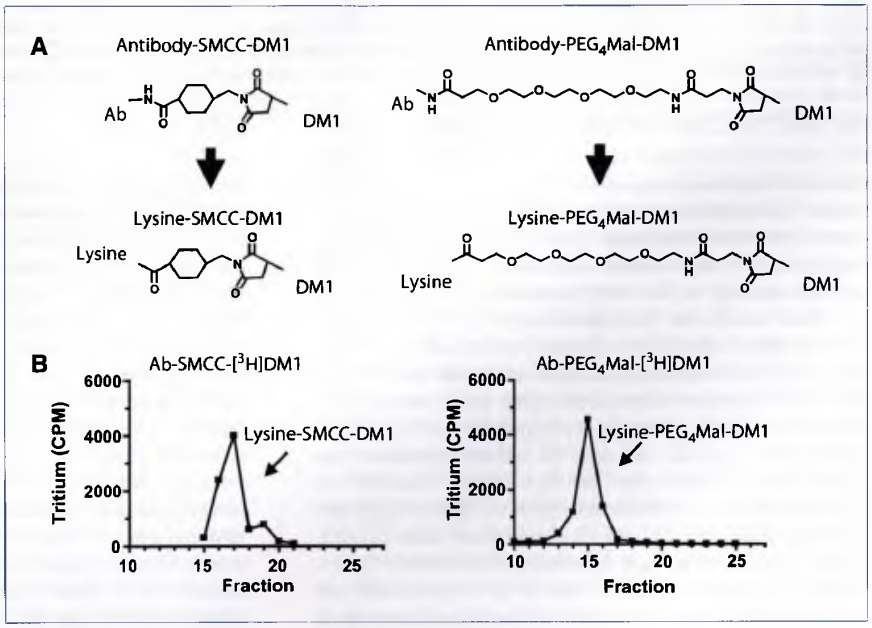


Figure 2. Metabolism of antibody-maytansinoid conjugates. A, chemical structures of antibody (Ab)-maytansinoid conjugates and their metabolites. B, HPLC analysis of [^{3}H]maytansinoid metabolites of the anti-EpCAM–DM1conjugates. COLO 205^{MDR} cells were exposed to anti-EpCAM–SMCC–[^{3}H]DM1 and anti-EpCAM–PEG$_4$Mal–[^{3}H] DM1. The [^{3}H]maytansinoid metabolites were extracted from the cells with acetone as described in Materials and Methods and analyzed by reverse-phase HPLC and liquid scintillation counting.

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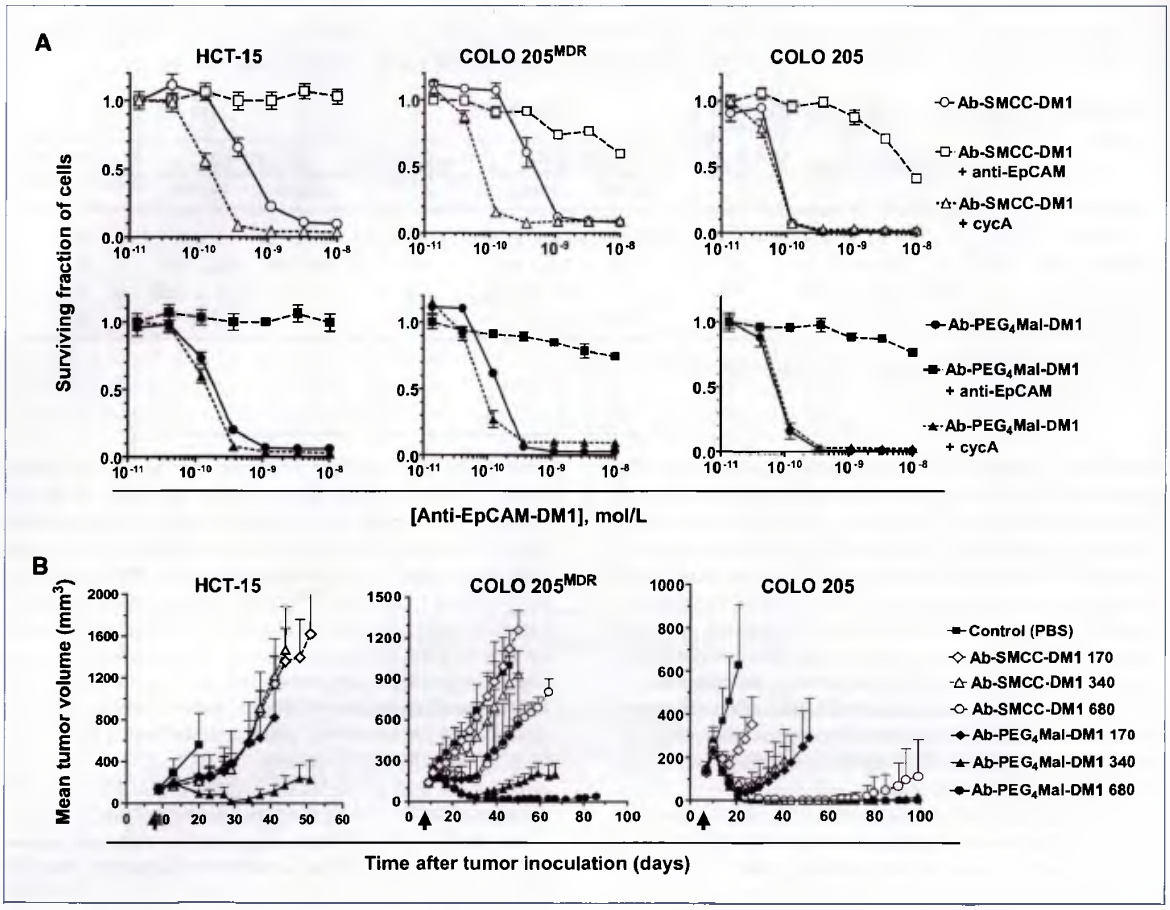


Figure 3. The anti-EpCAM–DM1 conjugate with PEG_4Mal linker shows improved potency against MDR1-positive cells and tumors. A, *in vitro* cytotoxicity of anti-EpCAM–SMCC–DM1 (top row) and anti-EpCAM–PEG_4Mal–DM1 (bottom row) toward HCT-15, COLO 205^{MDR}, or COLO 205 cells in the presence of cyclosporin A (1 μmol/L; Δ and ▲), unconjugated anti-EpCAM antibody (1 μmol/L; □ and ■), or neither (○ and ●). The data are presented as the mean ± SEM ($n = 3$). B, *in vivo* efficacy of anti-EpCAM–DM1 conjugate in subcutaneous xenograft tumor models. Established xenografts of human colon carcinoma HCT-15, COLO 205^{MDR}, and COLO 205 cells were treated with a single i.v. dose (arrow) of anti-EpCAM–SMCC–DM1 or anti-EpCAM–PEG_4Mal–DM1 conjugate (170, 340, 680 μg/kg of conjugated DM1). Data are presented as the mean ± SEM of tumor volume ($n = 6$).

commonly expressed in tumors: multidrug resistance protein 1 (MRP1; also known as ABCC1) and breast cancer resistance protein (BCRP; also known as ABCG2; refs. 1–3, 5). COLO 205, COLO 205^{MDR}, HCT-15, and UO-31 cells seem to express significant amounts of the MRP1 transporter but very little of the BCRP transporter (Supplementary Fig. S3). To evaluate whether MRP1 reduced the sensitivity of the cells to maytansinoids and antibody-maytansinoid conjugates, we tested if MK571, a selective MRP inhibitor (31), would enhance the cytotoxicity of DM1SMe and anti-EpCAM–SMCC–DM1 toward COLO 205 cells. Although the inhibitor enhanced the cytotoxicity of the known MRP1 substrate doxorubicin (1, 3), it did not alter the sensitivity of the cells to DM1SMe or anti-EpCAM–SMCC–DM1 (Supplementary Table S2), suggesting that maytansinoids are not MRP1 substrates. To test if BCRP could reduce the cytotoxicity of maytansinoids, we used RPMI-8226 cells, which express significant amounts of the transporter (Supplementary Fig. S3; ref. 32) as well as EpCAM (Supplementary Fig. S1; Supplementary Table S1). A specific inhibitor of BCRP transporter, fumitremorgin C (33), enhanced the cytotoxicity of a known BCRP substrate mitoxantrone (1, 3), but not of DM1SMe or anti-EpCAM–SMCC–DM1 (Supplementary Table S2), indicating that maytansinoids and antibody-maytansinoid conjugates are not BCRP substrates. These observations agreed with a recent report that MDR1, but not MRP1 and BCRP, mediates resistance to maytansinoids and antibody-maytansinoid conjugates (11).

An anti-EpCAM–DM1 conjugate linked via PEG_4Mal shows improved potency against MDR1-expressing cells and tumors. Previously, it was shown that an antibody-SMCC-DM1 conjugate is processed in the lysosomes of target cells to yield a sole cytotoxic metabolite, lysine-SMCC-DM1 (Fig. 2A; ref. 19). Therefore, we speculated that

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an antibody-maytansinoid conjugate comprising a more polar linker moiety might yield a hydrophilic maytansinoid metabolite that would be a poor MDR1 substrate and thereby would show improved potency against MDR1-expressing cells. To test this hypothesis, we prepared an anti-EpCAM–DM1 conjugate using a PEG_4Mal linker (Fig. 2A). We expected that incubation of this conjugate with EpCAM-expressing cells would result in the formation of a hydrophilic metabolite, lysine-PEG_4Mal-DM1 (Fig. 2A). Indeed, incubation of EpCAM-positive COLO 205^{MDR} cells with either anti-EpCAM–SMCC–[^{3}H]DM1 or anti-EpCAM–PEG_4Mal–[^{3}H]DM1 for 24 hours yielded acetone-extractable radiolabeled compounds with retention times on HPLC that were identical to those of synthetic lysine-SMCC-DM1 or lysine-PEG_4Mal-DM1, respectively (Fig. 2B). The identities of the metabolites as lysine-SMCC-DM1 and lysine-PEG_4Mal-DM1 were confirmed in a separate experiment by electrospray mass spectrometry.

The anti-EpCAM–PEG_4Mal–DM1 conjugate showed antigen-selective cell killing *in vitro* (Fig. 3A, bottom row), and although its potency toward COLO 205 cells was equal to that of the SMCC-linked conjugate (Fig. 3A, top row), it was significantly higher on two MDR1-expressing cell lines: HCT-15 and COLO 205^{MDR}. The MDR1 inhibitor cyclosporin A had only a marginal effect on the potency of anti-EpCAM–PEG_4Mal–DM1 against the MDR1-expressing cell lines (Fig. 3A, bottom row) but significantly enhanced the potency of SMCC-linked conjugate (Fig. 3A, top row), suggesting that MDR1 activity limits the potency of the SMCC-linked but not of the PEG_4Mal-linked conjugate.

The improved antitumor activity of the PEG_4Mal-linked conjugate against MDR1-positive tumor cells was also apparent in human tumor xenograft studies in mice. SCID mice bearing established HCT-15, COLO 205^{MDR}, and COLO 205 xenografts were treated with a single i.v. injection of either anti-EpCAM–PEG_4Mal–DM1 or anti-EpCAM–SMCC–DM1 at 170, 340, or 680 μg/kg of conjugated DM1. Both conjugates showed antigen-dependent antitumor activity in the HCT-15 tumor xenograft model (Fig. 3B) because control conjugates that do not bind to HCT-15 cells did not affect the tumor growth (Supplementary Fig. S4). The anti-EpCAM–PEG_4Mal–DM1 conjugate was ~2-fold more active than the SMCC-linked conjugate (in Fig. 3B, compare the antitumor activity of the anti-EpCAM–PEG_4Mal–DM1 conjugate dosed at 340 μg/kg DM1 with that of the anti-EpCAM–SMCC–DM1 conjugate dosed at 680 μg/kg DM1). The difference in the activity of the two conjugates was even more pronounced in the COLO 205^{MDR} tumor xenograft model, with the anti-EpCAM–PEG_4Mal–DM1 conjugate being ~4-fold more efficacious than the anti-EpCAM–SMCC–DM1 conjugate, as the activity of the 170 μg/kg DM1 dose of the PEG_4Mal conjugate was comparable with that of the 680 μg/kg DM1 dose of the SMCC conjugate (Fig. 3B). In addition, a single treatment with anti-EpCAM–PEG_4Mal–DM1 at 680 μg/kg DM1 resulted in complete regressions in all five treated mice in this MDR1-positive tumor model, whereas no complete regressions were observed at this dose with anti-EpCAM–SMCC–DM1. These data agree with the *in vitro* cytotoxicity data showing that anti-EpCAM–PEG_4Mal–DM1 is more active than anti-EpCAM–SMCC–DM1 against MDR1-positive HCT-15 and COLO 205^{MDR} tumor cell lines. The anti-EpCAM–PEG_4Mal–DM1 conjugate was also more efficacious than the conjugate with SMCC linker in the MDR1-negative COLO 205 model (Fig. 3B), albeit the advantage of the PEG_4Mal linker was greater in COLO 205^{MDR} model (4-fold versus 2-fold).

The improved cell-killing activity of PEG_4Mal-linked conjugates toward MDR1-expressing cells is not limited to anti-EpCAM. To test whether the enhanced activity of anti-EpCAM–PEG_4Mal–DM1 against MDR1-expressing cells is a general property of antibody-PEG_4Mal-DM1 conjugates and not limited to the anti-EpCAM antibody and/or target antigen, we constructed PEG_4Mal- and SMCC-linked DM1 conjugates with antibodies targeting EGFR and CanAg. The human renal adenocarcinoma cell line UO-31, a MDR cell line that expresses both MDR1 transporter (Fig. 1A) and EGFR (Supplementary Fig. S1; Supplementary Table S1), was used to test the activity of the anti-EGFR conjugates *in vitro*. Although the two conjugates bound to the UO-31 cells with similar affinity (Supplementary Fig. S2), anti-EGFR–PEG_4Mal–DM1 was 7-fold more potent in killing UO-31 cells than anti-EGFR–SMCC–DM1 (Fig. 4A). Cyclosporin A (Fig. 4A) or another MDR1-specific inhibitor, PGP4008 (data not shown), restored the potency of the SMCC-linked conjugate to the level of the PEG_4Mal-linked conjugate but failed to enhance the already high cytotoxicity of PEG_4Mal-linked conjugate, suggesting that the latter is capable of evading the MDR1-mediated resistance of these cells.

COLO 205 and COLO 205^{MDR} cells, which express similar levels of CanAg (Supplementary Fig. S1; Supplementary Table S1), were used to test the cytotoxicity of anti-CanAg–DM1 conjugates linked via PEG_4Mal and SMCC. Although the two conjugates were equally potent toward COLO 205 cells (Fig. 4B), the PEG_4Mal-linked conjugate was 5-fold more potent toward COLO 205^{MDR} cells (Fig. 4B). Consistent with the *in vitro* results, anti-CanAg–PEG_4Mal–DM1 was more efficacious than anti-CanAg–SMCC–DM1 against COLO 205^{MDR} tumors (Fig. 4C).

The maximum tolerated dose for a PEG_4Mal-linked conjugate is similar to that of an analogous SMCC-linked conjugate in mice. The tolerability of anti-CanAg–SMCC–DM1 and anti-CanAg–PEG_4Mal–DM1 was tested in CD1 mice. This antibody has no cross-reactivity with mouse tissues. Toxicity was monitored by frequent body weight measurements in groups of mice treated with a single i.v. bolus of either conjugate at approximately 1.8 and 2.7 mg/kg of conjugated DM1. Similar changes in body weight compared with control were observed for each conjugate, with some mortality observed in the high-dose group for both conjugates, suggesting that the maximum tolerated doses for the two conjugates are similar, ~2.2 mg/kg (Fig. 4D).

A PEG_4Mal-linked conjugate is more effective than an analogous SMCC-linked conjugate in arresting COLO 205^{MDR} cells in the G_2-M phase. Maytansinoids and antibody-maytansinoid conjugates arrest dividing cells in the G_2-M phase of the cell cycle (19), an event that ultimately leads to cell death. We compared the antimitotic activities

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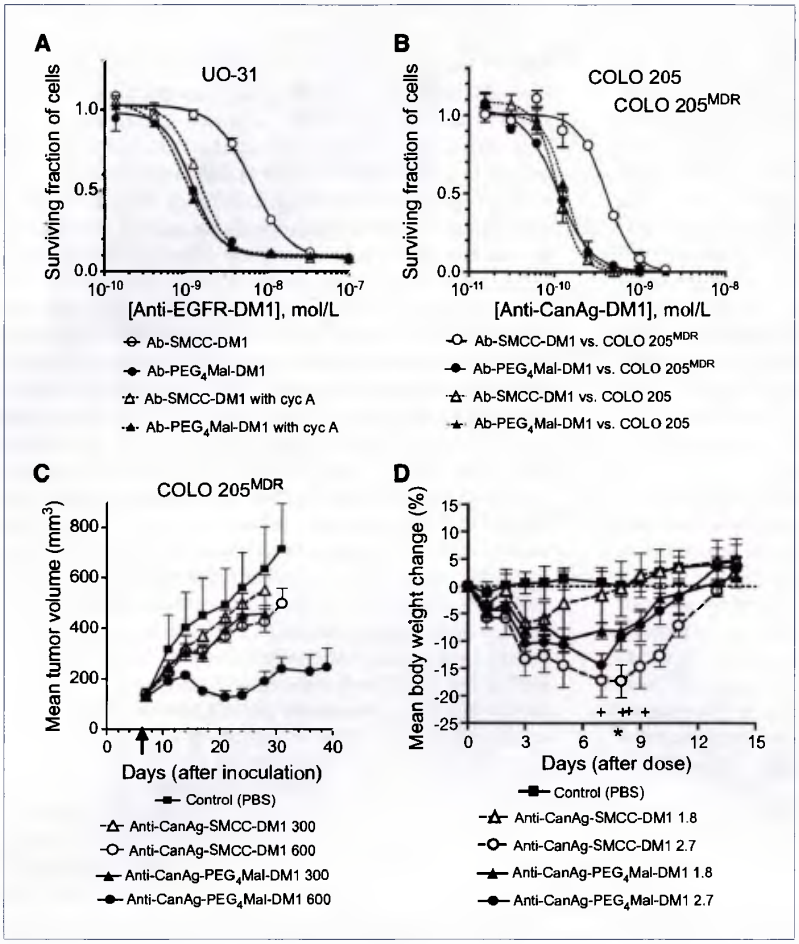


Figure 4. PEG_4Mal-linked conjugates of anti-EGFR and anti-CanAg display improved cytotoxicity over the SMCC-linked versions while being tolerated similarly. A, cytotoxicity of anti-EGFR–SMCC–DM1 (○ and Δ) and anti-EGFR–PEG_4Mal–DM1 (● and ▲) to UO-31 cells in the presence (Δ and ▲) or absence (○ and ●) of cyclosporin A (1 μmol/L). B, cytotoxicity of anti-CanAg–SMCC–DM1 (○ and Δ) and anti-CanAg–PEG_4Mal–DM1 (● and ▲) to COLO 205 (Δ and ▲) and COLO 205^{MDR} cells (○ and ●). C, *in vivo* efficacy of anti-CanAg–SMCC–DM1 and anti-CanAg–PEG_4Mal–DM1 conjugates (300 and 600 μg/kg of conjugated DM1) in established subcutaneous COLO 205^{MDR} xenograft tumor models. Data are presented as the mean ± SEM of tumor volume ($n = 6$). D, tolerability assessment of anti-CanAg–SMCC–DM1 and anti-CanAg–PEG_4Mal–DM1 conjugates in mice. CD1 mice were randomized based on body weight and treated on day 0 with a single i.v. dose of a conjugate (~1.8 and ~2.7 mg/kg of conjugated DM1). Body weights are represented as the change from a pretreatment measurement ($n = 8$). Four animals treated with the higher dose of anti-CanAg–SMCC–DM1 (+) and one animal treated with anti-CanAg–PEG_4Mal–DM1 (*) were sacrificed as indicated due to >20% body weight loss.

of anti-CanAg–SMCC–DM1 and anti-CanAg–PEG_4Mal–DM1 in COLO 205 and COLO 205^{MDR} cells. The cells were incubated with either conjugate for 3 hours, then washed to remove unbound conjugate, and incubated for an additional 18 hours in the conjugate-free medium. The percentage of cells in the G_2-M phase was then determined by flow cytometry. COLO 205 cells were equally sensitive to the two conjugates, with an increase in cells in the G_2-M phase after treatment from 27% to 85% and 88% (Fig. 5A). In contrast, COLO 205^{MDR} cells were less sensitive to anti-CanAg–SMCC–DM1 than to the PEG_4Mal-linked conjugate. The percentage of cells in the G_2-M phase went up from 15% to 37% in the anti-CanAg–SMCC–DM1–treated culture and from 15% to 67% in the anti-CanAg–PEG_4Mal–DM1–treated culture (Fig. 5A).

MDR1-expressing cells accumulate a higher level of DM1-containing metabolite when treated with a PEG_4-Mal-linked conjugate than with an analogous SMCC-linked conjugate. To determine whether the enhanced potency of PEG_4Mal-linked conjugates against MDR1-positive cells could be attributed to increased retention of active metabolites within the cells, COLO 205 and COLO 205^{MDR} cells were incubated with either anti-CanAg–PEG_4Mal–[^{3}H]DM1 or anti-CanAg–SMCC–[^{3}H]DM1 under conditions identical to those in the cell cycle experiments. The metabolites lysine-PEG_4Mal-[^{3}H]DM1 and lysine-SMCC-[^{3}H]DM1 produced during the incubation and retained in the cells were then extracted as in Fig. 2B, and their amounts were determined by liquid scintillation counting. Similar levels of metabolites were detected in COLO 205 cells treated with either conjugate. However, the level of maytansinoid metabolites in COLO 205^{MDR} cells incubated with the SMCC-linked conjugate was ~50% lower than that in the cells treated with the PEG_4Mal-linked conjugate (Fig. 5B). Therefore, the enhanced cytotoxicity and cell cycle arrest activity of the PEG_4-Mal-linked conjugates against MDR1-positive cells can be explained by increased intracellular retention of the active maytansinoid metabolite.

MDR1 transporter activity is not affected in the cells treated with PEG_4Mal-linked conjugates. It has been reported previously that polyethylene glycol can inhibit the transporter activity of MDR1 *in vitro* (34). To test whether the metabolite of PEG_4Mal-linked conjugates, lysine-PEG_4-

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Mal-DM1, inhibits the transporter activity, COLO 205^{MDR} cells were incubated with either anti-EpCAM–PEG$_4$Mal–DM1 or anti-EpCAM–SMCC–DM1 for 3 hours at a concentration that can cause substantial killing followed by 18 hours in conjugate-free medium to allow for accumulation of the metabolites within the cells. The activity of MDR1 transporter was then tested using the rhodamine-123 accumulation and retention assay as described in Fig. 1. In contrast to that observed upon treatment with cyclosporin A (Fig. 1B), the treatment of cells with either conjugate did not alter the levels of rhodamine-123 accumulation and retention (Fig. 5C). In addition, incubation of the cells with lysine-PEG$_4$Mal-DM1 directly did not inhibit MDR1 activity (data not shown). Therefore, inhibition of the transporter by the PEG$_4$Mal-containing metabolite is unlikely.

Discussion

Several antibody-maytansinoid conjugates are presently in clinical trials for patients with multiple myeloma, non–Hodgkin's lymphoma, breast cancer, gastric cancer, and other solid tumors (6, 7, 35, 36). Patients with these cancers often develop MDR1-mediated multidrug resistance. Similar to other cytotoxic compounds used in ADCs, such as calicheamicin, dolastatin 10, and CC-1065 analogues (4, 8, 10, 12–14), maytansine is a substrate for the MDR1 transporter, and consequently, the transporter can reduce the potency of some antibody-maytansinoid conjugates. Because preferred substrates for the MDR1 transporter are hydrophobic compounds (24), we reasoned that the potency of antibody-maytansinoid conjugates toward MDR1-positive cells could be improved by increasing the hydrophilicity of the maytansinoid metabolites released from conjugates inside the cell, thereby avoiding MDR1-mediated efflux. To achieve this, we used an ethylene glycol tetramer (PEG$_4$)–containing linker. The observed metabolite, lysine-PEG$_4$Mal-DM1, was retained inside MDR1-expressing cells better than the lysine-SMCC-DM1 metabolite from an analogous SMCC-linked conjugate. The increased retention of the metabolite of PEG$_4$Mal-linked conjugate correlated with the greater antimitotic and cytotoxic potency of the conjugate toward MDR1-expressing cells *in vitro* and with its increased efficacy against MDR1-positive xenograft tumors.

We showed that PEG$_4$Mal linkers can bestow the ability to bypass MDR1-mediated resistance to any antibody-maytansinoid conjugate. Antibody-maytansinoid conjugates with this

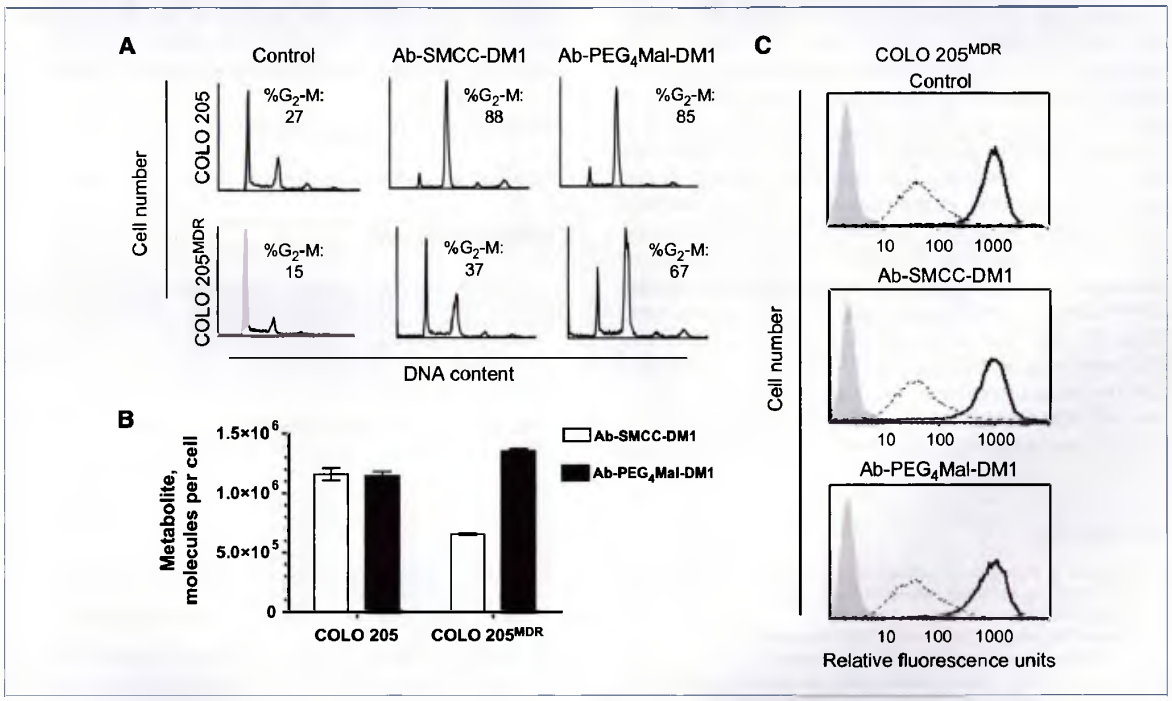


Figure 5. The enhanced potency of PEG$_4$Mal-linked conjugate for MDR cells correlates with increased intracellular retention of maytansinoid metabolite. A, cell cycle distribution in COLO 205 (top row) and COLO 205^{MDR} (bottom row) cell populations exposed to either anti-CanAg–SMCC–DM1 or anti-CanAg–PEG$_4$Mal–DM1 compared with nontreated exponentially growing cells (Control). Cells were exposed to each conjugate for 3 h at 37°C, washed, and incubated in conjugate-free medium for an additional 18 h at 37°C. The DNA content of the cells was evaluated by flow cytometry as described in Materials and Methods. B, intracellular concentrations of metabolites retained by COLO 205 and COLO 205^{MDR} cells. The cells were exposed to anti-CanAg–SMCC–[^{3}H]DM1 or anti-CanAg–PEG$_4$Mal–[^{3}H]DM1 as described in A. The metabolites were then extracted from the cells and quantified as described in Materials and Methods. Data are presented as the mean ± SEM ($n = 3$). C, rhodamine-123 uptake (solid lines) and retention (dotted lines) by COLO 205^{MDR} cells preexposed to either anti-EpCAM–SMCC–DM1 or anti-EpCAM–PEG$_4$Mal–DM1 compared with that of the nontreated cells (Control). Filled histograms show the cell autofluorescence.

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linker were as cytotoxic to MDR1-expressing cells as they were to MDR1-negative cells or to MDR cells whose MDR1 activity was inhibited, suggesting that the PEG_4Mal-linked conjugates were capable of evading the MDR1-mediated resistance. Significantly, the advantage of the linker was observed for the conjugates of antibodies that recognize three different antigens: anti-EpCAM, anti-EGFR, and anti-CanAg. Although the PEG_4Mal-linked conjugate was more efficacious than the SMCC-linked conjugate in MDR tumor models, the two conjugates were equally well tolerated in mice. Thus, incorporation of a short PEG oligomer within the conjugate linker improved the therapeutic index of the antibody-maytansinoid conjugate against the MDR1-expressing tumors.

There are several possible explanations for the enhanced potency of the PEG_4Mal-linked conjugates in MDR1-expressing cells. First, PEG_4Mal-linked conjugates or their metabolites might inhibit MDR1-mediated efflux. The unaltered activity of the transporter in the cells pretreated with cytotoxic concentrations of a PEG_4Mal-linked conjugate (Fig. 5C) or its cellular metabolite, lysine-PEG_4Mal-DM1, argues against this hypothesis. Second, the resulting lysine-PEG_4Mal-DM1 metabolite may bind tighter to its intracellular target, β-tubulin, than maytansine or lysine-SMCC-DM1, thereby reducing the amount of active metabolite needed to kill the cell and/or reducing the intracellular free metabolite pool available to interact with the MDR1 pump. Although we cannot rule out this possibility at present, the similar potencies of the conjugates with the PEG_4Mal and SMCC linkers in non-MDR cells suggest that this hypothesis is unlikely. Another possible explanation is that the lysine-PEG_4Mal-DM1 metabolite may be a poor MDR1 substrate due to its low affinity for the transporter. Finally, the PEG_4Mal-containing metabolite may simply have limited accessibility to the transporter. According to several reports, the binding of a substrate to MDR1 occurs only after diffusion of the substrate into the lipid bilayer of the plasma membrane (37–39). The increased hydrophilicity of the PEG_4Mal-containing metabolite may diminish its partitioning into the plasma membrane and, therefore, prevent interaction with the transporter. Indeed, lysine-PEG_4Mal-DM1 is ~8-fold less cytotoxic than lysine-SMCC-DM1 (data not shown), suggesting that the former has limited ability to penetrate the plasma membrane.

Hydrophilic (or polar) linkers have not been used to bypass multidrug resistance previously. However, our analysis of two prior studies suggests that the polarity of a released metabolite may have enhanced the cytotoxicity of two non-maytansinoid ADCs to MDR cells. Hamann and colleagues (16) reported that substitution of a hydrazide group by an amide in the linker of an antibody-calicheamicin conjugate rendered this ADC more efficacious against MDR cells. Although the authors did not explain the mechanism of this phenomenon, we speculate that the conjugate with the pH-sensitive hydrazide linker was reduced to a hydrophobic calicheamicin, whereas the noncleavable amide-linked conjugate was processed to a polar amino acid–containing derivative that might be a poor MDR1 substrate. In another study, ADC of a polar version of the cytotoxic compound auristatin was able to kill MDR cells (20). However, it is not clear if the potency of this conjugate could be attributed to the polarity of the drug because the potency of ADC of the original non-polar auristatin was not reported.

In conclusion, our finding that the antitumor potency of antibody-maytansinoid conjugates can be improved by rational linker design suggests that this strategy may be also effective in improving the potency of immunoconjugates with other cytotoxic molecules, paving the way for a new generation of ADCs that are more active against MDR tumors.

Disclosure of Potential Conflicts of Interest

All authors are employed by and have ownership interest in ImmmunoGen, Inc.

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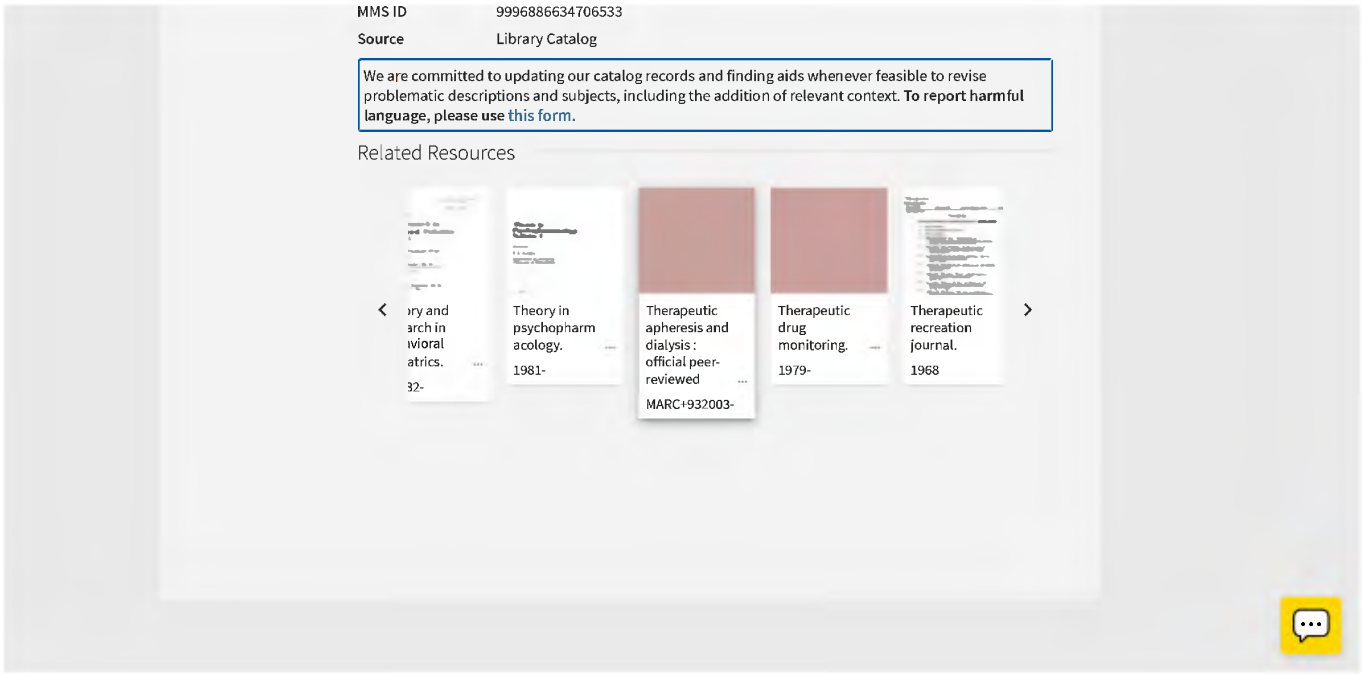
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Appendix GOLDMACHER02

REVIEW
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Antibody–drug conjugates: using monoclonal antibodies for delivery of cytotoxic payloads to cancer cells

One approach to improving activity of anticancer drugs is to conjugate them to antibodies that recognize tumor-associated, cell-surface antigens. The antibody–drug conjugate concept evolved following major advances, first, in the development of humanized and fully human antibodies; second, in the discoveries of highly cytotoxic compounds ('drugs') linkable to antibodies; and finally, in the optimization of linkers that couple the drug to the antibody and provide sufficient stability of the antibody–drug conjugate in the circulation, optimal activation of the drug in the tumor, and the ability of the activated drug to overcome multidrug resistance. In this article, we will review the considerations for selecting a target antigen, the design of the conjugate, and the pre-clinical and clinical experiences with the current generation of antibody–drug conjugates.

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Rationale for delivery of cytotoxic drugs with monoclonal antibodies

Cytotoxic chemotherapeutic drugs and therapeutic monoclonal antibodies (mAbs) represent two major classes of agents currently used for cancer treatment. The therapeutic activity of cytotoxic chemotherapeutic drugs is, in general, limited by their narrow therapeutic window, which may be attributed to their indiscriminate cytotoxicity, and/or poor pharmacokinetics and organ distribution [1]. Historically, most of the tested cytotoxic compounds have failed in clinical trials due to their excessive toxicity, and, accordingly, lack of sufficient therapeutic activity at the maximal tolerated dose (MTD).

Therapeutic mAbs display better pharmacokinetics than small-molecule drugs (longer circulation times: days vs minutes), various degrees of antigen-selective tumor accumulation, and often only moderate or no systemic toxicity [2]. Yet only a few of the large number of tested antibodies against various tumor-associated antigens, such as rituximab, trastuzumab, cetuximab and panitumumab, have made it into clinical practice [3], while the majority of the antibodies were found to lack sufficient therapeutic activity in clinical trials [4] or in preclinical studies.

The rationale for the development of **antibody–drug conjugates** (ADCs), as defined in a number of previous reviews [5–12], is to combine the selectivity, favorable pharmacokinetics, biodistribution and, when present, functional activity of antibodies with the high cytotoxic potency of a drug. An ideal ADC should: retain the favorable pharmacokinetic and functional properties of antibodies; remain intact and nontoxic in the compartment of its systemic delivery (typically blood); become active in the tumor compartment, with the active form of drug released in a sufficient amount to kill tumor cells; and combine the cytotoxic activity of the payload with the intrinsic anti-tumor activities of the antibody, including natural humoral immune responses such as the **antibody-dependent cellular cytotoxicity** (ADCC), **complement-dependent cytotoxicity** (CDC), antibody-dependent cellular phagocytosis and any direct functional activities of the antibody, such as blocking of a receptor signal transduction or engaging pro-apoptotic pathways.

Target antigen selection

The vast majority of antigens that are expressed on cancer cells have also been found to some degree on at least some normal tissues, and for this reason the term 'tumor-associated antigen' is more accurate than 'tumor-specific antigen'. The expression of an antigen of interest in some normal tissues does not necessarily preclude the development of an ADC targeting this antigen, if the normal tissue is either nonessential, or insensitive to the action of the drug (e.g., non-proliferating cells towards antimitotic agents such as **maytansinoids** or **auristatin**). Prostate-specific membrane antigen (PSMA) and HER2 are two examples of such target antigens. PSMA is expressed on normal prostate as well as on prostate cancer cells and has been the focus of

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Key Terms

Antibody–drug conjugate: A cytotoxic drug covalently linked to a monoclonal antibody that recognizes a tumor-associated antigen.

Antibody-dependent cellular cytotoxicity: Natural killer cells and neutrophils bind to the antibody Fc portion, thus making a contact with the antigen-expressing target cell. The effector cells then use the perforin-granzyme pathway and Fas ligand to kill the target cells. In addition to antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis may also contribute to the target cell eradication. Phagocytes such as monocytes/macrophages and dendritic cells take up and eliminate IgG-coated target cells.

Complement-dependent cytotoxicity: Some (but not all) human or humanized monoclonal antibodies of IgG1, IgG3, and (to a lesser degree) IgG2 isotypes trigger activation of complement pathways, which may lead to the destruction of the target cells.

Maytansinoids: Derivatives of the semi-synthetic microtubule-targeting agent maytansine.

Auristatin: Synthetic derivative of the microtubule-targeting agent dolastatin 10.

Human antimouse antibodies: Polyclonal antibodies that are produced by the human immune system against therapeutic murine monoclonal antibodies.

several ADC programs [13–16]. Targeting of normal prostate tissue may be of no safety concern and, in fact, most patients may have had their prostate surgically removed prior to ADC therapy. Trastuzumab emtansine (T–DM1) is an ADC consisting of the anti-HER2 mAb trastuzumab (Herceptin®) and the maytansinoid DM1, and has been administered safely at therapeutically effective doses, despite HER2 being expressed on some normal tissues [17–20]. Properties of a target antigen that are generally assumed to be favorable for an ADC approach include: strong expression on the surface of tumor cells; either good penetrance (percentage of tumor samples that are positive), or expression on tumors of cancer patients with high unmet medical need (a life-threatening disease with limited treatment choices); and, at the same time, little or no expression on essential normal tissues [8,11,21–23]. A requirement for the antigen of interest to be expressed homogeneously throughout a tumor tissue is probably not absolute, due to the ability of some ADCs to mediate bystander cell killing [24,25].

Accumulating, albeit still fragmentary, evidence suggests that a combination of several antigen-related factors determines the cytotoxic and anti-tumor activities of ADCs. These include, but are probably not limited to, the following parameters: the degree of the cell surface expression of the antigen; the rate of internalization of antigen–ADC complexes, and the rate and direction of their intracellular trafficking; the rate and/or degree of antigen–ADC complex processing leading to the release of the drug in its active form (proteolytic degradation of the antibody and/or cleavage of the linker). For some targets, the dynamics or efficiency of ADC internalization/trafficking may vary depending on the specific epitope on the antigen that is bound by a particular antibody. In addition, phosphorylation or other post-translational modification of the target protein or expression of cellular proteins that physically or functionally interact with the antigen may affect ADC internalization and/or its intracellular trafficking [10,11,26–31]. Available experimental evidence suggests that correlation between antigen expression and potency of an ADC is not a general phenomenon and is evident only for some antigens [12]. In one example, in an experiment evaluating the *in vitro* sensitivity of 18 non-Hodgkin lymphoma cell lines to an anti-CD22–DM1 conjugate, it was found that sensitivity to the maytansinoid component of the ADC was a more predictive indicator of sensitivity than was either the level of CD22 expression or the amount of internalized DM1 [32,33]. Therefore, selection of patients based solely on high tumor antigen expression may not guarantee clinical efficacy.

A potentially attractive property for a prospective ADC target antigen is a biological role in a pathway essential for tumor growth or survival. In principle, the intrinsic anti-tumor activity of the antibody that blocks such a pathway and the cytotoxic payload provided by the ADC format may produce a potent combined anti-tumor activity. T–DM1 [17–19] and SGN-35 [34] are examples where ADC clinical evaluation was preceded by clinical experience with the respective naked antibodies, trastuzumab and SGN-30. Trastuzumab (Herceptin) targets the HER2-receptor tyrosine kinase and suppresses the oncogenic function of this receptor, which is overexpressed on tumor cells. Trastuzumab was approved for the treatment of HER2-positive metastatic breast cancer [35]. Genentech/Roche are currently developing the ADC T–DM1 for patients with advanced HER2-positive breast cancer who have previously received multiple HER2-targeted medicines and chemotherapies [36]. Phase III studies are underway that could lead to approval of this agent in second-line and first-line therapy of HER2-positive metastatic breast cancer. Results of a Phase II study showed that T–DM1 has impressive anti-tumor activity in patients who have progressed on prior HER2-targeted therapies (including trastuzumab) [37]. The chimeric antibody SGN-30 targets CD30, a member of the TNF-R superfamily of immunoregulatory molecules, and was found to promote growth arrest and DNA fragmentation in cultured CD30-positive cells, and to have activity against Hodgkin lymphoma xenografts in immunodeficient mice [38]. Evidence of clinical activity has been reported for both SGN-30 and its conjugate with auristatin, brentuximab vedotin (SGN-35), and advanced trials are underway [34,39,40].

Apart from the biological role of the target antigen, there are two additional key considerations when developing an ADC that potentially combines anti-tumor activities provided by the cytotoxic payload with the intrinsic anti-tumor activity of its antibody component. First, the modification, conjugation, linker and payload chemistries employed must yield an ADC that retains the full biological properties of the functional antibody. T–DM1, an ADC created by conjugation of maytansinoids to trastuzumab through lysine residues, retains

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all of trastuzumab's anti-tumor mechanisms of action, including HER2 binding affinity, inhibition of HER2 signaling and Fc-mediated immune-effector activities, such as ADCC [41]. Similar combination of the antibody functional activity and the effector drug cytotoxicity may be expected for the anti-tumor activity of the SGN-30–auristatin conjugate SGN-35.

Second, to achieve the combined effect, such ADCs may have to be administered at high enough doses, since optimal anti-tumor activity of functional antibodies in cancer patients requires relatively high doses, typically several mg/kg or greater every 3 weeks (e.g., rastuzumab, cetuximab, rituximab or panitumumab). ADCs that employ calicheamicin as a payload, a highly cytoxic DNA minor groove-binding agent, achieve an MTD of only 9 mg/m^2 or lower [42–44], doses wherein the antibody component (<0.3 mg/kg) would be unlikely to contribute to the efficacy of the ADC. By contrast, maytansinoid conjugates incorporating multiple linker formats (both cleavable and noncleavable) can achieve MTDs greater than 3 mg/kg in clinical testing [5]. In Phase I studies, the reported MTD for T–DM1 is 3.6 mg/kg when given every 3 weeks, and 2.4 mg/kg when administered weekly [19,45], which is similar to the trastuzumab dose adminstered to patients with metastatic breast cancer (e.g., 2 mg/kg weekly, after the first dose of 4 mg/kg). Therefore, maytansinoid ADC formats can produce compounds that have the prospect of retaining anti-tumor activities of the antibody component, with the added cytotoxic potency provided by the maytansinoid payload.

A wide variety of tumor-associated antigens have been explored to date as potential targets for ADCs, including various cell-surface proteins and glycoproteins, gangliosides and extracellular proteins [12,46]. In addition, in recent years, there has been increasing interest in antibody-based targeting of the tumor vasculature and stromal tissue that often play a supporting role in tumor growth [3,47–51]. ADCs that target neovasculature may act by destroying the architecture of the tumor blood supply, leading to hypoxia and nutrient deprivation [47,52], while ADCs that target the tumor stroma may reduce the level of tumor growth-supporting factors [53,54]. IMGN388 is an ADC that consists of the maytansinoid DM4 attached to an antibody against αV integrin, which is expressed on the cells of many solid tumors and also on endothelial cells during angiogenesis [55]. IMGN388 is currently in a Phase I clinical trial, and has demonstrated favorable tolerability at the dose levels evaluated to date, and evidence of activity [56]. Since it targets both tumor cells and cells that form new blood vessels that support tumor growth, its activity may exceed the clinical benefit achievable by targeting either component separately, especially with well-vascularized tumors. FAP5–DM1 is an ADC that targets fibroblast activation protein-α, an antigen found on the surface of tumor stromal fibroblasts, and it was shown to have anti-tumor activity in tumor xenografts and models of lung, pancreas, head and neck cancers in immunodeficient mice [57]. Another potential ADC stromal target is Ptk7, a pseudokinase that is highly expressed on many cancer types either in the cancer cells themselves or in the tumor stroma [58].

Targeting cancer stem cell (CSC)-specific antigens with ADCs has also been explored recently [59,60]. If the CSC hypothesis is correct then, following the elimination of the majority of cancer cells, the tumor may regrow if the CSCs are not also eradicated [61–64]. In addition to being implicated in initiating and propagating tumors, CSCs may also contribute to angiogenesis and drug resistance [65–68]. Several CSC-associated antigens are potential targets of ADC therapeutics [29,69,70]. Targeting stromal cells surrounding tumors may eliminate a source of inhibitors of bone morphogenic proteins, allowing bone morphogenic proteins to induce differentiation and inhibition of CSC proliferation [71–74].

Antibody selection

The first mAbs to be examined in the clinic were murine. In most instances, even a single injection of a murine mAbs elicits the generation of **human antimouse antibodies** within 2 weeks of antibody administration, limiting the therapeutic efficacy of murine mAbs to the first and at most, the second dose administration [2]. To date, most antibodies for use in ADCs were generated as mouse mAbs that were subsequently converted to human IgGs [3,75,76]. The initial goal for antibody chimerization (substitution of human sequences into the Fc regions) and humanization (retention of the murine complementarity-determining regions only) was to minimize the immunogenicity of antibodies when administered to human patients [76–80], and both approaches have achieved this goal [80]. Beyond reduced immunogenicity, human IgGs have two other

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advantages over murine IgGs, first, they are retained longer in human circulation (because of their recognition by the human Fc neonatal receptor [FcRn], which regulates IgG circulation half-life [81]); and second, they are more effective than murine antibodies in eliciting ADCC with human effector cells [2]. Other approaches to generating human IgGs are to either directly raise human mAbs in transgenic mice in which the murine antibody-producing genes have been inactivated and replaced with human antibody-producing genes [82], or to select antibodies from large recombinant antibody libraries, such as those generated by phage display [76,83–87].

In general, desirable properties of antibodies for use in ADCs include: specificity; high binding affinity; long-circulation times; immune effector functions such as CDC, ADCC and antibody-dependent cellular phagocytosis; tumor-suppressing modulation of antigen's biological activity when possible; and minimal loss of the above-listed properties upon its conjugation to the drug of choice. To date, there are no clinical data available that would compare the anti-tumor activities of ADCs differing in one or more of these antibody-dependent properties (but made with the same drug, targeting the same antigen), and it remains unknown how much any of these properties contribute to (or interfere with) the anti-tumor efficacy of ADCs. Thus, the above-mentioned criteria are assumptions based largely on the available clinical and preclinical results obtained with naked therapeutic antibodies, and on preclinical results with ADCs in animal models, mostly immunodeficient mice. Significant differences exist between the human and murine immune systems [88,89], and between human patients' tumors and xenografts of human tumor cell lines in immunodeficient mice, and thus these criteria need further validation.

One of these factors, affinity, deserves a more detailed consideration. Typically, antibodies with the highest affinity are selected for use in ADCs on the tacit assumption that the higher affinity of an antibody will lead to the higher anti-tumor activity of its ADC. Apart from well documented *in vitro* correlation between ADC affinity and its cytotoxicity, there is no conclusive evidence of any *in vivo* correlation between affinity of an ADC and its efficacy in either preclinical models [32] or in the clinic, and it may well be that there is no such correlation because of the following considerations:

- ADCs are administered intravenously, at doses that can translate into an initial blood concentration of 0.1 μM or higher, which is much higher than the typical K_d values for antibodies used in ADCs (nM range or lower). If the ADC concentration at the tumor sites is as high as that in the blood, the ADC would be expected to saturate the antigen irrespective of its K_d;
- An exceedingly high affinity of an ADC may lead to its fast binding to the perivascular regions of the tumor, thus limiting its diffusion inside the tumor [90,91];
- A recently developed computational model of tumor targeting predicts that similar amounts of antibodies (and, by extention, ADCs) with affinities of 10^{-8} M or higher would be retained at the antigen-expressing tumor site [92].

Similarly, there are varying views on the desirability of the immune-effector functions in mAbs used in ADCs. The ADCC and CDC activities of antibodies are dependent on the antibody isotype. Human IgG1 and IgG3 isotypes elicit strong ADCC and CDC immune-effector responses, while IgG4 and IgG2 antibodies typically induce weak immune-effector responses [89,93,94]. It is generally assumed that CDC and ADCC may contribute to the anti-tumor activity of ADCs [93,94]. On the other hand, effector functions may interfere with target-cell localization or internalization of the ADC, and therefore, antibody isotypes with reduced effector functions, IgG2 and IgG4, were chosen for some ADCs [94–98].

Humanized IgG1 has been the most common isotype chosen for ADC antibodies in clinical development, with few instances of humanized IgG4 and IgG2 being selected to date (**Table 1**). The circulation half-life of human IgG3 antibodies is shorter than that of IgG1, IgG2 and IgG4 isotypes, making them less desirable for therapeutic use [89,93]. One potential disadvantage of the IgG4 subclass is the propensity of IgG4 antibodies to exchange half-molecules with other IgG4 molecules *in vivo* and hence to become bi-specific, but this can be overcome by replacing the third constant domain of the IgG4 molecule with that of human IgG1 [99]. Only one ADC that underwent clinical testing, CRO11–valine–citrilline monomethylauristatin E (vcMMAE), was of an IgG2 type [100]. IgG2 antibodies are capable of forming covalent dimers [101], which conceivably may enhance solid-phase homotypic antibody–antibody

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Table 1. Antibody–cytotoxic compound conjugates in clinical development.

Antibody–cytotoxic compound	Target antigen	Linker–cytotoxic compound, class	Antibody	Tumor type(s)	Developer	Status
Gemtuzumab ozogamicin (Mylotarg®)	CD33 (Siglec-3)	Hydrazone, AcBu, *N*-acetyl-γ calicheamicin, calicheamicin	hP67.6 humanized IgG4	Acute myeloid leukaemia	Pfizer	US FDA conditionally approved, recently withdrawn
Inotuzumab ozogamicin (CMC-544)	CD22 (Siglec-2)	Hydrazone, AcBu, *N*-acetyl-γ calicheamicin, calicheamicin	G5/44 humanized IgG4	B-cell lymphomas	Pfizer	Phase I and I/II Phase III combination
Trastuzumab–DM1 (T–DM1)	HER2 (ErbB2)	Thioether, SMCC–DM1, maytansinoid	Transtuzumab humanized IgG1	Metastatic breast cancer	Roche	Phase II and III
Lorvotuzumab mertansine (IMGN901, huN901–DM1, BB10901)	CD56 (NCAM)	Hindered disulfide, SPP–DM1, maytansinoid	huN901 humanized IgG1	Solid tumors, multiple myeloma	ImmunoGen	Phase I and II
IMGN388 (CNTO365)	Integrin	Highly hindered disulfide, SPDB–DM4, maytansinoid	Anti-integrin human IgG1	Solid tumors	ImmunoGen	Phase I
SAR3419 (huB4–DM4)	CD19	Highly hindered disulfide, SPDB–DM4, maytansinoid	huB4 humanized IgG1	Non-Hodgkin lymphoma	Sanofi–Aventis	Phase I
BIIB015	Cripto	Highly hindered disulfide SPDB–DM4, maytansinoid	Anti-Cripto humanized IgG1	Cripto-positive solid tumors	Biogen Idec	Phase I
BT062	CD138	Highly hindered disulfide SPDB–DM4, maytansinoid	Anti-CD138 chimeric IgG4	Multiple myeloma	Biotest	Phase I
Brentuximab vedotin (SGN-35)	CD30	Dipeptide, vc-MMAE, auristatin	SGN-30 chimeric IgG1	Relapsed/refractory Hodgkin lymphoma, systemic anaplastic large-cell lymphoma	Seattle Genetics, Millenium	Phase II and III
Glembatumumab vedotin (CR011–vcMMAE, CDX-011)	Glycoprotein NMB (osteoactivin)	Dipeptide, vc-MMAE, auristatin	CR011 fully human IgG2	Metastatic breast cancer and melanoma	Celldex Therapeutics	Phase I/II
SGN-75 (h1F6–mcMMAF)	CD70	Noncleavable, mc-MMAF, auristatin	SGN-70 humanized IgG1	Non-Hodgkin lymphoma and renal carcinoma	Seattle Genetics	Phase I
PSMA ADC	Prostate-specific membrane antigen	Dipeptide, vc-MMAE, auristatin	Anti-PSMA	Progressive, metastatic, hormone-refractory prostate cancer following prior taxane chemotherapy	Progenics Pharmaceuticals	Phase I
ASG–5ME	SLC44A4 (AGS-5)	Dipeptide, vc-MMAE, auristatin	Anti-AGS-5 human IgG2	Pancreatic cancer	Agensys, Seattle Genetics	Phase I
MDX–1203	CD70	Dipeptide, MGBA	Anti-CD-70 human	Non-Hodgkin lymphoma and advanced/recurrent clear-cell renal cell carcinoma	Bristol–Myers Squibb	Phase I

MMAE: Monomethylauristatin E; MMAF: Monomethylauristatin F; NCAM: Neural cell-adhesion molecule; PSMA: Prostate-specific membrane antigen; SPDB: N-succinimidyl-3-(2-pyridyldithio)butyrate; SPP: N-succinimidyl 4-(2-pyridyldithio)pentanoate.

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associations to boost affinity and/or internalization, but may also contribute to aggregation issues. Antibody engineering can enhance the immune-effector functions and extend the circulation half-life of antibodies [85,102], with current technologies focusing on modifying the antibody amino acid sequence or on its defucosylation to enhance affinity for IgG Fc-γ receptor IIIa (FcγRIIIa) [103–107]. The effect of isotype and FcγR binding on the properties of monomethylauristatin F (MMAF) conjugates of an anti-CD70 antibody have been recently reported [88]. Conjugates were prepared from variant antibodies of the parental anti-CD70 IgG1, which included an IgG2, an IgG4 and an IgG1v1, the latter engineered for impaired binding to huFcγ receptors, but having only modest impairment of binding to FcRn, which regulates retention of IgG in circulation. All the conjugates bound well to antigen, had good cytotoxicity activity *in vitro* and were well-tolerated *in vivo* in mice. The IgG1v1 conjugate was found to have an increased anti-tumor activity in xenograft models, and a longer circulation half-life than the parental IgG1 conjugate. Interpretation of these results and their implications for ADC clinical therapy is difficult, however, because of the above-mentioned differences between the human and murine systems, it remains to be seen how much ADCC and CDC contribute to the overall activity of ADCs.

Cytotoxic drugs used in ADCs

■ Considerations for ADC drugs

One important requirement for a drug to be considered as an ADC effector is that this drug should be sufficiently cytotoxic at concentrations that can be achieved by antibody delivery. Early immunoconjugates developed for oncology incorporated the standard anticancer drugs in use at the time, such as methotrexate, doxorubicin or the vinca alkaloids, and yielded disappointing results, with little or no anti-tumor activity observed in clinical trials [7,8,108,109]. Delivery of the cytotoxic agent to a tumor cell via an ADC is limited by the amount of the target antigen on the cell surface, and by the access of the ADC to the cell within the tumor. It was later proposed that one of the reasons these early ADCs failed, and had a narrow (if any) therapeutic window, was insufficient potency of the conjugated drugs [7,25,108,110,111]. Cytotoxic drugs used in the current generation of ADCs are at least 100-fold more potent than those used in the early conjugates [7]. On the other hand, more potent ADCs may be more systemically toxic, which may narrow their therapeutic window. Another common feature of effectors utilized in the current generation of ADCs is their selectivity towards killing proliferating cells. An effector that preferentially kills proliferating cells may, in principle, improve the selectivity of the ADC since the incidence of proliferating cancer cells in tumors is higher than that in most normal tissues. Other preferable characteristics for a drug include low molecular weight, and the related lack of immunogenicity, a reasonably low cost of production, availability in a linkable form and sufficient solubility and stability in water to facilitate preparation and storage of the antibody conjugates [112].

■ Types of ADC drugs

The drugs used in ADCs currently in the clinic or preclinical testing can be divided into two categories, those that target DNA, and those that target microtubules. In addition, several potent ADC drugs have also been reported that have not yet reached the clinic. These include the microtubule-targeting taxol derivatives (taxanes) [7,8,113–116], the DNA-targeting streptonigrin, the HSP90-targeting geldanamycin and the topoisomerase-inhibiting camptothecin analogs [117,118]. These preclinical-stage ADCs will not be discussed here further.

■ DNA-damaging drugs

N-acetyl-γ-calicheamicin dimethyl hydrazide, a derivative of calicheamicin, is the cytotoxic component of Mylotarg® gemtuzumab ozogamicin, which targets CD33 [96,119], and was conditionally approved in May 2000 under the US FDA's accelerated-approval program, but was recently withdrawn from the USA market by Pfizer due to its failing to show inproved efficacy in a Phase III combination trial. Calicheamicin is a highly cytotoxic enediyne antibiotic that binds to the minor groove of DNA and induces double-strand DNA breaks that result in cell death [98,120]. The ADC showed higher *in vitro* potency than unconjugated calicheamicin against CD33-positive cells, and eradicated CD33-positive HL-60 xenografts in nude mouse models [96]. Several other ADCs comprised of antibodies linked to calicheamicin are being evaluated, including inotuzumab ozogamicin (CMC-544), which targets CD22 and is in Phase III clinical testing against non-Hodgkin lymphoma [98,121,122]. Calicheamicin conjugates of several other antibodies, the anti-CD20 antibody rituximab [123], the H8 antibody directed against

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the oncofetal antigen 5T4 expressed on various carcinomas [124], and an anti-MUC1 antibody targeting various solid tumors [125], are currently in preclinical evaluation.

Analogs of CC-1065 and the duocarmycins are highly cytotoxic DNA minor-groove binding alkylating agents (MGBA) [126]. The first MGBA-based ADCs were made with the CC-1065 analog DC1 [127], which turned out to be nearly insoluble, making conjugation reactions inefficient, and with DC4, a water-soluble phosphate prodrug of DC1 [8]. Several other conjugates have been reported more recently [16,128–132]. MDX-1203, a conjugate of the fully human anti-CD70 antibody and a duocarmycin analog has been developed at Medarex (now Bristol–Myers Squibb). In this conjugate, the cytotoxic effector is attached to the antibody in prodrug form, linked to it via a protease-cleavable dipeptide linker, and a maleimide attachment site, requiring not only release from the antibody but also esterase/protease cleavage to release the active cytotoxic drug [131]. MDX-1203 showed efficacy in renal cancer xenograft models, was well tolerated in cynomolgus monkey safety studies [131,132] and is currently in a Phase I clinical study in patients with advanced/recurrent clear-cell renal-cell carcinoma or relapsed/refractory B-cell non-Hodgkin lymphoma. Two other MGBA-based ADCs are being explored as potential prostate cancer therapeutics, one targeting PSMA, which is found on the surface of the prostate tumor cells, and another directed against the extracellular protein mindin/RG-1 which is thought to be retained in the extracellular matrix of human prostate tumors [16].

■ Microtubule-targeting drugs

Among ADCs based on microtubule-targeting cytotoxic drugs, two categories have reached the stage of clinical development, those utilizing derivatives of maytansine (maytansinoids), and those utilizing auristatins [5,6,8,9,110,133]. Maytansine, with IC_{50} values in the range of 10 to 100 pM depending upon the cell type [110,133], is significantly more cytotoxic against cell lines *in vitro* than clinically used microtubule-targeting drugs such as vinca alkaloids or taxol. Maytansine and maytansinoids appear to act by interfering with microtubule dynamics [134–136], thereby inducing cell-cycle arrest at G2/M, which eventually leads to cell death. This mode of cytotoxic action may explain why proliferating cells are more sensitive to maytansine than are nonproliferating cells [137]. Two maytansinoids are being used in ADCs currently in clinical development, DM1 and DM4, which differ in the degree of hindrance of the disulfide bond they afford in the linker between the drug and the antibody. The first antibody–maytansinoid conjugate to undergo clinical testing was cantuzumab mertansine [25,110], which was comprised of the cytotoxic maytansinoid compound DM1 conjugated by a disulfide linkage to the huC242 antibody, a humanized IgG1 antibody that targets the extracellular domain of the tumor-associated carbohydrate antigen CanAg, expressed on most colorectal, biliary and pancreatic cancers, as well as on many non-small-cell lung, gastric, uterine and bladder cancers [138–140]. Cantuzumab mertansine was shown to have specific and highly efficacious anti-tumor activity in preclinical xenograft models [25]. It was well-tolerated in Phase I clinical studies of CanAg-expressing solid tumors and showed evidence of biological activity in terms of tumor shrinkage and stable disease [139,141]. The development of this conjugate was stopped because its activity was considered insufficient. The evaluation of this initial antibody–maytansinoid conjugate has been followed by the development of an expanding number of antibody–maytansinoid conjugates that incorporate a growing portfolio of maytansinoid compounds and linker technologies [8,133]. At this time, six of these conjugates are undergoing clinical evaluation in a variety of tumor indications (discussed below), and many more are undergoing preclinical evaluation, including an ADC that targets CD79 on non-Hodgkin lymphoma [142]; an ADC that targets CD22, another B-cell antigen [143]; and SAR566658 (huDS6-DM4), which targets a Muc1 sialoglycotope on solid tumors [144,145]. Antibody–maytansinoid conjugates are discussed in greater detail in a recent review [5].

The auristatins, another group of antimicrotubule agents with potent cell-killing activity, are fully synthetic analogs of the pentapeptide natural product dolastatin 10 [146,147]. Auristatin E and MMAE are 50- to 200-fold more potent than the vinca alkaloids against a diverse panel of tumor cell lines, and ADCs prepared with these compounds were highly effective in preclinical studies [147]. An auristatin derivative with a negatively charged C-terminal phenylalanine residue, MMAF, was shown to have much lower cytotoxic activity as an unconjugated compound than MMAE, probably due to its reduced plasma membrane permeability

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and intracellular access [148]. Nonetheless, antibody–MMAF conjugates were found to be comparable with antibody–MMAE conjugates in their cytotoxic potency towards target antigen-positive cell lines, and over three orders of magnitude more potent than free MMAF, presumably because of the different route of drug delivery by ADCs, traversing the plasma membrane [148]. It is still an open question if the low cytotoxicity of the unconjugated MMAF will result in lower toxic side-effects of its respective ADCs compared with similar MMAE conjugates. Currently, five antibody–auristatin conjugates are undergoing clinical evaluation (**Table 1**). Pre-clinical studies have been reported for several other antibody–auristatin conjugates, such as ADCs that target the B-cell antigens CD22 [143], CD19 [149,150] and CD79 [151], as well as EphA2, an antigen that is overexpressed in many solid tumors [152,153].

Linkers used in ADCs

The third ADC component is the linker used to covalently attach the drug to the antibody. Several recent reviews have focused on emerging linker technologies [8,9,11,112,154,155]. There are several important considerations regarding the linker component, including: the site of attachment on the antibody; the average number of attachment sites per antibody molecule; the cleavability of the linker, that is, its ability to chemically disintegrate releasing the drug; and the polarity of the linker.

Most drugs have been linked to antibodies at the amino groups of lysine residues or thiol groups of cysteine residues [11,112,156]. New approaches to engineer specific sites of modification are also being evaluated [157]. The relative benefit of the different approaches to the function of the resulting ADC is still largely unknown.

The number of cytotoxic molecules attached to the antibody is an important consideration. A low drug per antibody ratio (DAR) may result in low potency of the ADC, while a too-high degree of antibody modification may adversely affect its affinity towards the target antigen, FcγR, or FcRn, and may also result in aggregation and precipitation of the antibody and/or a decrease in its stability. The optimal DAR is determined empirically for each antibody on the basis of a variety of tests such as the feasibility of conjugate synthesis and solubility of the resulting conjugate, impact on antigen-binding affinity, antigen-specific as well as nontargeted cytotoxicity of the ADC, and its behavior in animal models such as anti-tumor activity, pharmacokinetics and systemic toxicity. Most of the conjugates currently in clinical testing (regardless of which cytotoxic compound, antibody or linker was used) have three to four cytotoxic molecules per antibody molecule. Gemtuzumab ozogamicin, prepared with a strategy designed to minimize aggregation, consists of an approximate 1:1 mixture of unconjugated antibody and conjugated antibody having four to six calicheamicin molecules per antibody [96,119], and inotuzumab ozogamicin has an average calicheamicin:antibody loading of five:seven per antibody [98].

The anti-tumor activity of antibody–auristatin conjugates with different molar ratios of auristatin to antibody was evaluated by Hamblett *et al.*, who separated a sample of an anti-CD30 antibody–MMAE conjugate into fractions having approximately two, four or eight MMAE molecules per antibody [158]. Although the potency of the resulting conjugates was found to correlate with auristatin load when tested *in vitro*, the therapeutic index in xenograft tumor models in mice did not improve with increasing DAR. The discrepancy between the *in vitro* and *in vivo* results was consistent with the observations that higher MMAE load resulted in faster clearance of the conjugate from circulation and increased toxicity in mice [158]. Whether the enhanced clearance is a direct effect of the increased load of MMAE on the antibody, or an effect on the antibody of progressively decreasing the number of inter-chain disulfide bonds that are used in linking MMAE, is unknown. Subsequently, the anti-CD30 antibody was engineered to generate conjugates with defined stoichiometries of either two or four auristatin molecules per antibody [159].

Since a key advantage of antibody-based therapeutics over most chemotherapeutic drugs is their long retention in circulation, an optimal conjugate linkage must be stable during circulation in the patient yet allow for efficient release of the cytotoxic compound in active form upon internalization into the tumor cell. A variety of linkers have been designed to take advantage of differences between the extracellular and intracellular environments to allow release of an active cytotoxic molecule only after antigen-specific, antibody-mediated internalization of the ADC into tumor cells has occurred. Linkers containing disulfide bonds were used to take advantage of the different concentrations of free thiols (free thiols are the main physiological substances

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that can cleave these bonds) in the intracellular and extracellular space. The most abundant intracellular thiol, reduced glutathione, is present in cells in the concentration range of 0.5 to 10 mM [160] compared with a significantly lower concentration of the most abundant low-molecular-weight thiol in circulation, cysteine, approximately 5 μM [161]. In addition, the intracellular enzyme protein disulfide isomerase, or similar enzymes that are also capable of cleaving disulfide bonds [162], may also contribute to the preferential cleavage of disulfide bonds inside cells. Acid-labile linkers were used because of the low pH in the late endosomes and lysosomes, compared with near-neutral pH in circulation and other extracellular environments. Peptide-based linkers were used because such linkers could be cleaved by lysosomal and endosomal proteolytic enzymes. In principle, these linkers may also be cleaved by cytoplasmic proteases as well [163].

Most ADCs currently undergoing clinical evaluation contain linkers that fall into two broad categories: 'cleavable', that is, those that can disintegrate inside the cell, such as hydrazone, disulfide or protease-sensitive peptide linkers; and 'noncleavable', which require proteolytic cleavage of the antibody portion of the ADC for release of the cytotoxic molecule, still attached to the linker and the single remaining amino acid derived from the antibody. For more details the reader can refer to the recent review by Singh and Erickson [112].

Calicheamicin-containing ADCs utilize a hydrazide functionality introduced into calicheamicin γ1 to enable its linkage to an antibody via an acid-hydrolizable bond [164]. In gemtuzumab ozogamicin, *N*-acetyl-γ-calicheamicin dimethyl hydrazide is conjugated to lysines in the antibody using a 4-(4'-acetylphenoxy) butanoic acid linker, which is stable in physiological buffers (pH 7.4), but hydrolyses at the pH of lysosomes (pH ~4) [164]. Gemtuzumab ozogamicin was found to be reasonably stable in blood in animal studies [95,165] and in acute myeloid leukaemia patients [166]. In mouse circulation, the amount of calicheamicin conjugated to the hP67.6 decreased twofold in approximately a 2-day period, and the presence of a CD33-expressing xenograft did not influence the rate of this apparent decomposition, although the method used in the study could not distinguish between cleavage of calicheamicin on all the conjugate molecules and the generation of a fraction of unconjugated antibody, and between conjugate decomposition and preferential clearance of a fraction of the conjugate with a higher DAR [165].

Antibody–maytansinoid conjugates currently in clinical testing utilize either cleavable disulfide or noncleavable thioether linkages. Both are formed from a heterobifunctional crosslinking reagent that reacts with a lysine residue on an antibody, and then maytansine derivatives containing thiol groups are reacted with the other end of the heterobifunctional reagent [110,133,167]. This method of conjugation results in a random distribution of the maytansinoid molecules randomly linked to a small subset of approximately 40 different sites [156]. Newer thiol-containing maytansinoids have been synthesized with one or more methyl groups (substituting hydrogen) on the carbon atoms adjacent to the disulfide linkage, to create linkers having varying degrees of steric hindrance around the disulfide bond [8,133]. These hindering modifications were designed to minimize cleavage of the conjugates during circulation in the blood, without interfering with efficient release of the cytotoxic agents once internalized into tumor cells. Preclinical studies comparing huC242–DM4 (IMGN242), a conjugate utilizing a more-hindered maytansinoid, with huC242–DM1 (cantuzumab mertansine), a conjugate having a less-hindered maytansinoid, revealed decreased plasma clearance for huC242–DM4 and also greater anti-tumor activity in xenograft models [133,168]. Several maytansinoid conjugates currently undergoing clinical testing, IMGN388, SAR3419, BIIB015 and BT062, utilize this more-hindered disulfide linkage. One ADC in clinical testing, T–DM1, utilizes a noncleavable thioether linkage to attach the maytansinoid molecule to the antibody [18].

The intracellular processing of disulfide- or thioether-linked maytansinoid conjugates has been examined [169,170]. Both types of maytansinoid conjugates were found to degrade in lysosomes to yield metabolites consisting of a lysine adduct of the maytansinoid and linker, and this lysosomal processing was required for their cytotoxic activity. The disulfide-containing metabolites were processed further via reduction of the disulfide bond and subsequent *S*-methylation, to produce *S*-methyl-maytansinoids. These lipophilic *S*-methyl-maytansinoid metabolites were highly cytotoxic towards cell lines when added exogenously (comparable to maytansine), whereas the more hydrophilic lysine-containing

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Key Term

P-glycoprotein: A transmembrane ATP-dependent drug transporter. Overexpression of P-glycoprotein often contributes to the emergence of multidrug resistance in tumors following chemotherapy.

maytansinoid metabolites were much less active. These observations may help explain both the phenomenon of target-cell-activated killing of bystander cells and the superior efficacy of disulfide-linked conjugates over noncleavable conjugates seen in some xenograft models [10,24,169,170]. Antigen-positive cells treated with a disulfide-linked conjugate, but not those treated with a thioether-linked conjugate, release the highly cytotoxic *S*-methyl-maytansinoid capable of killing nearby antigen-negative cells [24]. This additional indirect killing of adjacent tumor cells expressing little or no surface antigen may contribute to the activity of disulfide-linked antibody–maytansinoid conjugates against tumors having heterogeneous antigen expression. In addition, such bystander killing could potentially impact tumor-supporting neovasculature and proliferating stromal cells while causing minimal collateral toxicity to adjacent normal cells, as proliferating cells are much more sensitive to the cytotoxic effects of maytansine and its derivatives than are nonproliferating cells [10,24].

To prepare the auristatin conjugates, antibody disulfide bonds are reduced to generate two to eight cysteine residues that are subsequently linked to an auristatin derivative, either maleimide–MMAE or maleimide–MMAF, via a dipeptide linker [147,148,158,171]. One of the dipeptide linkers used, valine–citrulline, was designed to be cleaved by cathepsin B upon intracellular delivery of the conjugates into the lysosomal compartment [147,148]. Intracellular processing of brentuximab vedotin (anti-CD30–MMAE containing this dipeptide linker) was examined in detail [172]. Exposure of CD30-expressing cells to this conjugate resulted in its lysosomal degradation and intracellular release of chemically unmodified MMAE. Efflux of MMAE led to killing of cocultivated CD30-negative cells, a bystander killing effect similar to that observed with disulfide-linked maytansinoid ADCs.

In pre-clinical studies, the valine–citrulline peptide linkage appeared to be quite stable in circulation, with a half-life of auristatin release of 6 days in mice and 9.6 days in cynomolgus macaques [173], similar to that of *N*-succinimidyl 3-[2-pyridyldithio]-propionate (SPDB)-linked maytansinoid conjugates. New auristatin ADC linkers have been generated by replacing the maleimide with a halo-acetamide and have demonstrated improved *in vivo* stability in preclinical studies [154]. In another recent development, anti-CD70–auristatin conjugates have been synthesized utilizing novel dipeptide linkers and new auristatins linked through the C-terminus, and some of these new conjugates showed improved therapeutic windows over the original 1F6-Val-Cit-PABC–MMAF conjugate [155].

A method for site-specific antibody conjugation has been developed to reduce the heterogeneity of ADCs [157]. The 'ThiomAb' technology involves the engineering of reactive cysteine residues into specific sites of the antibody, to allow drug conjugation with defined stoichiometry and without disruption of interchain disulfide bonds [157]. A ThiomAb–drug conjugate comprised of MMAE conjugated to an anti-MUC16 antibody was found to be as efficacious as a conventional MMAE conjugate in mouse xenograft models and was better tolerated in rat and cynomolgus monkeys, possibly due to the absence of species carrying overly high MMAE loads [157].

Multidrug resistance (MDR) is a major obstacle to successful cancer chemotherapy and potentially to ADC therapy as well [27,57,133,174,175]. MDR1 (also known as **P-glycoprotein** or ABCB1), the best-characterized member of the ABC family of transporters, is expressed in a variety of cancers with evidence of increased incidence following chemotherapy, and its expression has been found to correlate with poor responses to chemotherapeutic compounds [176–178]. The majority of drugs used in ADCs are MDR1 substrates [167], and it is expected that improved intracellular retention of ADC's active metabolites would result in increased anti-tumor efficacy in cancers expressing MDR1. Efforts to evaluate cytotoxic compounds as substrates of the various MDR efflux pumps and to develop strategies to circumvent resistance mechanisms have become a priority in ADC research [125,167,179–181]. We have recently developed a new generation of hydrophilic linkers that enable antibody–maytansinoid conjugates to overcome MDR1-mediated resistance. Maytansinoid conjugates prepared with one of these linkers, maleimidyl-based PEG_4Mal, were processed by target cells to a yield a polar cytotoxic metabolite that appears to be a poor MDR substrate. The increased retention of this metabolite correlated with greater antimitotic and cytotoxic potency of the conjugates toward MDR1-expressing cells *in vitro* and with increased efficacy against MDR1-positive xenograft tumors [167,181].

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Clinical experience with ADCs

■ ADCs in clinical development

A list of ADCs in clinical development at the time of preparation of this review is presented in TABLE 1.

Gemtuzumab ozogamicin (Mylotarg) was conditionally approved by the FDA (accelerated approval) for treatment of acute myeloid leukemia, but was recently withdrawn from the US market. A confirmatory, postapproval trial was stopped early when no improvement in clinical benefit was observed, combined with serious drug-related toxicities. A second calicheamicin conjugate, inotuzumab ozogamicin (CMC-544), targets CD22 and is undergoing clinical testing against B-cell lymphomas [98,121,182].

Maytansinoid-containing conjugates are undergoing clinical testing in a variety of tumor indications. T–DM1, comprised of Roche's anti-HER2 antibody trastuzumab linked by a noncleavable thioether linkage to the maytansinoid DM1, is in broad clinical development for HER2-positive breast cancer. Results of a Phase II study, showed that T–DM1 has impressive anti-tumor activity in patients who have progressed on prior HER2-targeted therapies (including trastuzumab) [37]. One could speculate that residual intrinsic activity of trastuzumab, insufficient to suppress tumor growth on its own in these patients, may be contributing to the efficacy observed with T–DM1. Alternatively, the efficacy of T–DM1 observed in clinical trials could be a result of the high expression of HER2 and the ability of Herceptin to stimulate endocytosis of the T–DM1 complex with HER2 and deliver the maytansinoid into the cancer cells [183]. The FDA denied accelerated approval of T–DM1 Biologics License Application (BLA) for metastatic breast cancer. The BLA was submitted in July 2010 on the basis of the results of a single-arm, Phase II study, which showed that T–DM1 shrank tumors in one-third of women with advanced HER2-positive breast cancer who had received, on average, seven prior medicines, including two HER2-targeted medicines. In its review of the BLA, the FDA stated the T–DM1 trials did not meet the standard for accelerated approval because all available treatment choices approved for metastatic breast cancer, regardless of HER2 status, had not been exhausted in the study population. Roche stated that it will submit data from the amended Phase III randomized study to the FDA to support a new T–DM1 BLA in mid 2012. This study compares T–DM1 to lapatinib in combination with capecitabine in people with advanced HER2-positive breast cancer whose disease has worsened after receiving initial treatment.

Lorvotuzumab mertansine (IMGN901, BB-10901 or huN901-DM1) [184], which targets CD56 (neural cell adhesion molecule 1) comprises a humanized version of the N901 antibody [78] conjugated to DM1 via a hindered disulfide linker. CD56 is found on a variety of tumor types of hematopoietic and neuroendocrine origin, including multiple myeloma, certain types of leukemia, neuroblastoma, astrocytoma, glioma, most cases of small-cell lung cancer and many cases of ovarian cancer [184–186]. Its normal tissue expression is restricted to natural killer cells and a subset of T cells, as well as in nerve tissue and heart muscle [187]. Encouraging anti-tumor activity of lorvotuzumab mertansine was reported in clinical studies in multiple myeloma and Merkel-cell carcinoma [188,189] (see [5] for more detail). Its clinical evaluation in patients with solid tumors, in particular small-cell lung cancer and Merkel-cell carcinoma, and multiple myeloma is continuing.

IMGN388 is a maytansinoid ADC comprised of a humanized anti-integrin antibody conjugated to DM4 [55]. The target antigen is strongly expressed on a wide variety of solid tumors, including melanomas, sarcomas and a large number of carcinomas, as well as on endothelial cells in the process of forming new blood vessels. The targeting of neovasculature and/or stromal components supporting tumor growth, in addition to the tumor cells themselves, may afford greater efficacy than targeting the tumor cells alone. The initial clinical testing of IMGN388 showed favorable tolerability and evidence of activity [190]. Several other antibody–maytansinoid conjugates (utilizing conjugate technology from ImmunoGen) are in clinical development. SAR3419 (sanofi-aventis) targets the CD19 antigen and is in clinical testing in patients with relapsed or refractory B-cell non-Hodgkin lymphoma [191]. BIIB015 (Biogen Idec), a humanized IgG1 anti-Cripto antibody conjugated to DM4, is in clinical testing for treatment of relapsed or refractory Cripto-positive solid tumors. BT062 (Biotest AG), comprised of a chimerized anti-CD138 antibody and DM4 [192], is in Phase I clinical testing for multiple myeloma [193].

A total of five auristatin conjugates are in clinical evaluation; promising Phase I/II results have been reported for two

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of these conjugates (TABLE 1). SGN-35, which consists of a CD30-targeting antibody conjugated to MMAE [171,194] is undergoing clinical evaluation in Phase II and Phase III trials in patients with Hodgkin lymphoma and systemic anaplastic large-cell lymphoma. CD30 is a member of the tumor necrosis factor receptor superfamily, a transmembrane glycoprotein receptor that is highly expressed on Hodgkin lymphoma, anaplastic large cell lymphoma, and some other T-cell lymphoproliferative disorders, but is restricted on normal tissues primarily to expression on activated leukocytes [39,158]. Seattle Genetics expects to file a BLA submission to the FDA in the first half of 2011. CR011–vcMMAE is being developed by Celldex Therapeutics for the treatment of metastatic breast cancer and advanced melanoma [100,195,196]. The targeted antigen is the glycoprotein NMB (GPNMB), which is highly expressed in most metastatic melanoma samples [197], and is also expressed in liver carcinomas, squamous-cell lung cancers, breast cancers, soft-tissue tumors and gliomas [11,195,197]. CR011–vcMMAE is comprised of a fully human IgG2 antibody conjugated to MMAE via a valine–citrulline dipeptide linker, and is currently being evaluated in two Phase I/II clinical trials, for unresectable stage III or IV melanoma and for locally advanced or metastatic breast cancer, and in Phase IIb for GPNMB-expressing breast cancers.

Several auristatin conjugates have recently entered clinics. SGN-75, a conjugate of a humanized anti-CD70 mAb with MMAF, is being developed by Seattle Genetics and is currently in a Phase I clinical trial for relapsed or refractory non-Hodgkin lymphoma or metastatic renal cell carcinoma. CD70 is expressed on several types of non-Hodgkin lymphoma, multiple myeloma, renal-cell carcinoma, pancreatic, ovarian and lung cancer, while having limited expression in normal tissues. In preclinical studies, SGN-75 demonstrated potent antitumor activity in models of CD70-positive renal-cell carcinoma and hematologic malignancies. A conjugate of a fully human anti-PSMA mAb with MMAE [14] is being developed by Progenics Pharmaceuticals, and is in a Phase I trial in patients with progressive, metastatic and hormone-refractory prostate cancer [198]. PSMA is expressed on the surface of prostate cancer cells and on the neovasculature of many types of solid tumors, but has limited expression in normal tissues. PSMA expression is increased in high-grade, metastatic and hormone-refractory prostate cancer. Recently ASG–5ME, which consists of an anti-ASG-5 antibody attached via a valine–citrulline dipeptide linker to the auristatin derivative MMAE, has entered Phase I trials for pancreatic cancer patients.

■ Toxicity of ADCs

While ADCs retain antibody-like pharmacokinetics and biodistribution, these molecules ultimately are metabolized and eliminated *in vivo* by the same organ systems that normally metabolize circulating antibodies and/or chemotherapeutic drugs. Unlike 'naked' antibodies, ADCs will have inherent dose-limiting toxicities related to the systemic metabolism of the conjugate and its cytotoxic payload. The nature and degree of such 'nontargeted toxicity' of ADCs vary significantly depending on the cytotoxic payload and its metabolites; generally, one might expect that cytotoxic payloads with increased potency would yield ADCs with decreased MTDs. Similar to naked antibody therapeutics, ADCs also have the potential to cause toxicity in patients if they target a tumor antigen that is also expressed on normal tissues ('targeted toxicity').

Many ADCs tested to date, even those with some normal tissue crossreactivity, have been well-tolerated in patients, causing only minimal toxicities that were manageable and reversible. Examples of maytansinoid ADCs with favorable tolerability reported in clinical testing include lorvotuzumab mertansine [199–201], T–DM1 [17–19], cantuzumab mertansine (huC242–DM1) [139,141], MLN2704 [15] and AVE9633 [202]. However, sometimes significant ADC-related toxicities may only become apparent in the clinic. For example, treatment with gemtuzumab ozogamicin, which received conditional approval (accelerated approval) in 2000 based on single-arm Phase II trials [119,203] was associated with the serious, potentially fatal liver condition veno-occlusive disease [204], and the incidence of this disease increased in the confirmatory Phase III trial [301].

The clinical experience with bivatuzumab mertansine (Boehringer Ingelheim), an anti-CD44v6-directed maytansinoid ADC, provides an example of 'targeted toxicity' of an ADC. This compound showed evidence of efficacy in multiple early-stage clinical trials, but was discontinued due to skin-related adverse effects [205–208]. Since the observed toxicity was most likely due to abundant expression of the CD44v6 antigen in dividing epithelial cell layers of normal skin, as well as its high expression

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on head and neck cancer and other epithelial cancers [209], these clinical results testify to the specificity and potency of antibody-delivered cytotoxic agents.

■ Soluble antigen in circulation

The presence of target antigen in patient plasma, due to cleavage of the antigen from the surface of tumor cells ('shed' antigen), or from secretion of a soluble form of the antigen, can sometimes influence the pharmacokinetics and the efficacy of an ADC. Soluble antigen can bind the circulating ADC, potentially enhancing clearance of the latter and/or interfering with targeting of the antigen on tumor cells. A correlation between high peripheral blood antigen load and decreased efficacy has been suggested for gemtuzumab ozogamicin in acute myeloid leukaemia [210] and for an anti-MUC1 (hCTM01)–calicheamicin conjugate in ovarian cancer [125,211]. For other antibodies and ADCs, such as trastuzumab and IMGN242, high peripheral blood antigen levels have been correlated with altered pharmacokinetics, but have not been predictive of clinical outcome or associated with decreased efficacy (or did not reach statistical significance with the number of patients treated) [212–214]. The half-life of trastuzumab was reported to be 2 ± 1 days for patients with circulating levels of HER2 extracellular domain ≥500 ng/ml, as compared with a half-life of 9 ± 5 days for patients with <500 ng/ml serum HER2 [212]. However, the potential for soluble antigen to influence the pharmacokinetics of trastuzumab appears not to have precluded delivery of clinical benefit in early testing of T–DM1 for metastatic breast cancer patients, where the average terminal half-life of T–DM1 was reported to be 3.5 days at the MTD of 3.6 mg/kg given every 3 weeks [17–19].

Future perspective

The field of antibody-targeted drug delivery has been fueled by the development of mAb technology in 1975 and subsequent success in identification of many tumor-associated antigens, first with the help of mAbs and later also with a variety of other methods, most notably mRNA-expression profiles. It took many years to gradually develop the current (yet

Executive summary

- Antibody–drug conjugates (ADCs) combine the selectivity, favorable pharmacokinetics, biodistribution and, when present, functional activity of antibodies with the high cytotoxic potency of a small drug.
- Factors that determine the anti-tumor activity of ADCs include antigen expression, the rate of internalization and direction of intracellular trafficking of antigen–ADC complexes, the rate and/or degree of the intracellular release of the active drug and the ability of the antibody to interfere with tumor growth or tumor cell survival.
- A wide variety of tumor-associated cell-surface antigens have been explored as targets for ADCs. Other currently explored targets for ADCs include neovasculature, tumor stroma and cancer stem cells.
- Chimerization and humanization of murine IgGs or use of fully human antibodies minimized or eliminated their immunogenicity in humans, led to their longer retention in human circulation and increased their antibody-dependent cellular cytotoxicity activity.
- At present, there is no conclusive evidence of any *in vivo* correlation between the affinity of an ADC for its target and its efficacy in either preclinical models or in the clinic. A recently developed computational model of tumor targeting predicts that antigen-selective tumor uptake of ADCs depends on the antibody affinity in the range of K_d values of 10^{-8} M to 10^{-7} M or worse, but may not improve with the higher affinity antibodies.
- The desirability of the immune-effector functions in monoclonal antibodies used in ADCs is, at present, unclear.
- The drugs currently used in ADCs in the clinic or in preclinical testing are at least 100-fold more potent than those used in the early conjugates, and can be divided into two categories, those that target DNA and those that target microtubules. Two categories of ADCs based on microtubule-targeting cytotoxic drugs have reached the clinical development stage, those utilizing derivatives of maytansine and those utilizing auristatins.
- Most ADCs currently in clinical development contain linkers that fall into two categories: 'cleavable', that is, those that can disintegrate inside the cell; and 'noncleavable', which require proteolytic cleavage of the antibody portion of the ADC for release of the cytotoxic molecule. Antibody–maytansinoid conjugates utilize either cleavable disulfide or noncleavable thioether linkages. New linkers that enable maytansinoid ADCs to overcome P-glycoprotein-mediated resistance have been developed.
- Many ADCs tested to date, even those with some normal tissue crossreactivity, have been well-tolerated in patients, causing only minimal toxicities that were manageable and reversible.
- The chances for clinical success of ADCs have increased due to the continued improvements in their design: improved understanding of target-selection criteria, advances in antibody engineering, development of more potent cytotoxic drugs, and optimization of antibody–cytotoxic agent links.

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incomplete) knowledge of how to make ADCs that are potent, well-tolerated, nonimmunogenic in the clinic and feasible to develop from both an economic and logistical perspective. The chances for clinical success have increased due to the continued improvements in the design of ADCs: improved understanding of target-selection criteria, advances in antibody engineering, development of more potent cytotoxic drugs, and optimization of antibody–cytotoxic agent linkers. Future clinical studies with ADCs and therapeutic antibodies will give us further insight into the contribution of various factors, both in ADC design (the potency of the drug, the affinity of the antibody, the cleavability, polarity and solubility of the linker, and the roles of antibody-dependent humoral responses) and in the nature of the target tumors (antigen density and heterogeneity of expression, accessibility of the tumor to the ADC, MDR status, soluble antigen levels, FcγR genotype and tumor vascularization). ADCs are poised to provide a new generation of effective oncology treatment options in the near future.

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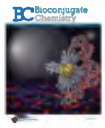
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Recent advances have resulted in linkers having increased stability in the bloodstream while allowing efficient payload release within the tumor cell.

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Subjects

Biopolymers Immunology Peptides And Proteins Pharmaceuticals Toxicity

Introduction

After cardiovascular diseases, cancer represents the second most common cause of death in the Western world and is a major area of focus for the pharmaceutical industry. Surgery and radiotherapy are generally used when a tumor is localized to a certain tissue, but chemotherapy is needed when metastasis has occurred. Despite extensive research, most anticancer drugs have nonspecific toxicity. By targeting the cell cycle and thereby killing rapidly proliferating cells, they do not explicitly discriminate between healthy and tumor tissues and only gain a limited selectivity for malignant cells. Such cytotoxic drugs have a narrow therapeutic window, which limits their efficacy and results in severe side effects. Due to a lack of selectivity, drug concentrations that would eradicate the tumor can often not be used. In addition, tumors can develop resistance against anticancer drugs after prolonged treatment. Therefore, achieving improved tumor selectivity through targeting of cytotoxic drugs to the cancer cells is needed.

A promising approach to achieving a more selective treatment is targeted prodrug therapy (1). Antibody−drug conjugates (ADCs) are ideal candidates for such prodrugs. ADCs are monoclonal antibodies (mAbs) linked to cell-killing drugs. Thanks to their high binding specificity for tumor-specific antigens, mAbs can be used as vehicles to target cell-killing payloads to tumor cells. Unique or overexpressed, tumor-specific antigens can be found in a wide range of human tumor cells (2). Some mAbs have the ability to recognize and specifically bind to these tumor-associated antigens. They can be used as single agents for the treatment of cancer through binding to cancer-cell-specific antigens and induction of an immunological response against the target cancer cell (3). However, therapeutic efficacy is often limited by the extent to which the antibody leads to cell death. Monoclonal antibodies are extremely discriminating for their targets but sometimes therapeutically ineffective on their own. The insufficient efficiency of most naked mAbs in cancer therapy has been

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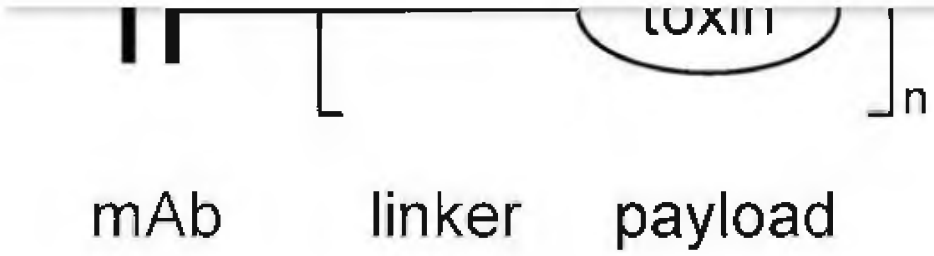


Figure 1. Schematic representation of an antibody–drug conjugate.

ADCs can be viewed as sophisticated delivery systems for antitumor cytotoxic drugs. The mAb guides the toxin precursor to the target cancer cell, where the prodrug can be converted chemically and/or enzymatically to the parent drug and unfold its cytotoxic activity. The ADC is exposed to different conditions on its journey from the blood vessels to the molecular target in the tumor tissue. The mode of action at cellular or molecular level is complex, with each step bringing its own challenges:

a Circulation

The ADC must behave like a naked antibody when circulating in the plasma. In particular, the linker must be stable in the bloodstream to limit the damage to healthy tissue. Decomposition or decay would release the cytotoxin before being delivered to the target site.

b Antigen Binding

It is necessary that the conjugated mAb retains high immunoaffinity. Attaching the cytotoxic compound to the mAb must thus not disturb its binding specificity.

c Internalization

A sufficient intracellular concentration of the drug must be achieved. This is challenging because antigen targets on cell surfaces are often present in limited numbers and the internalization process for antigen–antibody complexes is frequently inefficient.

d Drug Release

Once internalized, the ADC has to efficiently release the original cytotoxic drug in its active form inside the tumor cell.

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The molar loading of the drug on the mAb can be tuned to achieve the desired potency. Because of points (c) and (d), linkage of several drugs is generally necessary to achieve significant cytotoxicity. However, if too many cytotoxic molecules are attached to the antibody, the body may recognize the conjugate as a damaged form of the protein and quickly clears it from the system, proving points (a) and/or (b) to be problematic. The extent of drug substitution may also affect pharmacokinetics, so that most of the time 2–4 drugs per antibody give the best therapeutic window (8, 9). To avoid the interference with the antigen recognition, the drugs are ideally linked at the Fc or constant region of the mAb that does not participate in binding to the antigen (point (b)). To regain the full cytotoxic potential of the drugs, cleavable linkers releasing the drug molecule at the target site in unmodified form were developed to address point (d), although these also affect the stability of the construct. Therefore, the right balance between plasma stability and efficient drug release at the target cell has to be found.

The insights gained during ADC development within the past decade have resulted in a number of new strategies and drugs (4-7). This review focuses on linker technologies used to attach low molecular weight cytotoxic drugs to mAbs. The linker is a small but central part of ADCs and as such accounts for stability in circulation, favorable pharmacokinetic properties, and efficient release of toxic agent at the tumor site.

Chemically Labile Linkers

Selective linker cleavage and payload release is based upon the differential properties between the plasma and some cytoplasmic compartments. Linkers were chosen that were stable in the blood's neutral pH environment but were getting cleaved once the ADC entered the lower pH environment found inside a cell. The focus in the early days of ADC development was on acid-cleavable hydrazone linkers that were relatively stable at neutral pH (bloodstream pH 7.3–7.5), but were undergoing hydrolysis once internalized into the mildly acidic endosomes (pH 5.0–6.5) and lysosomes (pH 4.5–5.0).

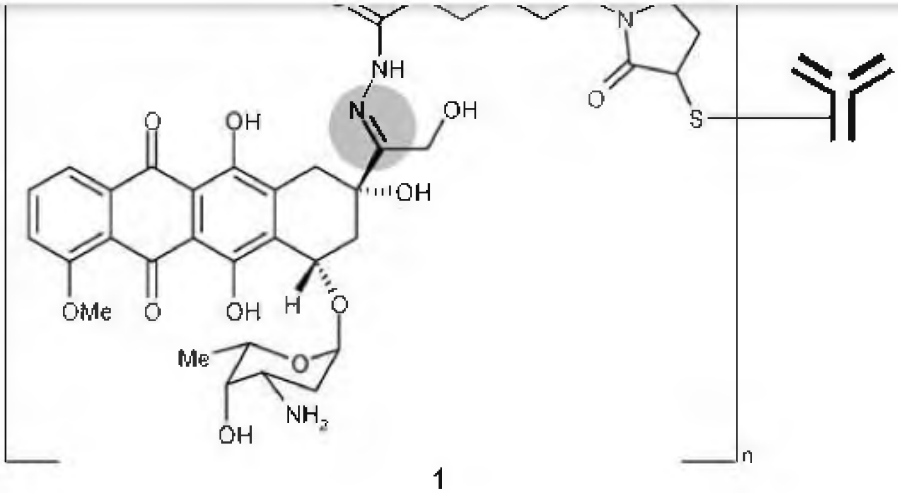
In the 1990s, Bristol-Myers Squibb scientists covalently linked doxorubicin, an intercalating agent that blocks DNA replication and is used as chemotherapeutic agent, to the humanized mAb BR96 (10). Doxorubicin was connected over a (6-maleimidocaproyl)hydrazone linker to cysteine residues of the Lewis-Y specific mAb. The preclinical activities of BR96-doxorubicin (**1**, Chart 1) were remarkable, but despite the use of eight drugs per mAb molecule, the amount of conjugate needed to achieve these effects *in vivo* were high (>100 mg/kg). This was presumably due to the relatively low potency of doxorubicin (IC_{50} of 0.1–0.2 μM for human carcinoma lines (11), whereas

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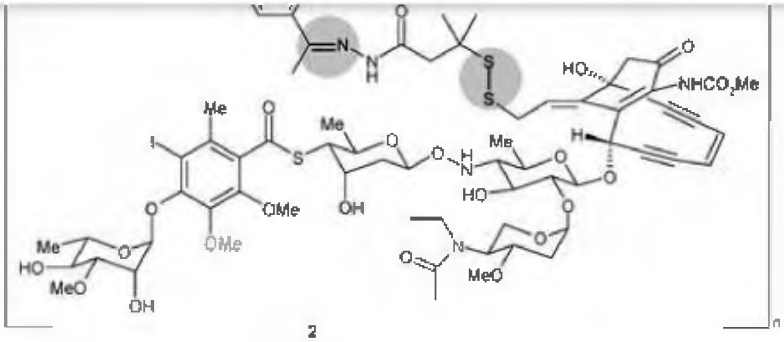
The first and so far sole mAb linked to a cytotoxic payload that has been given regulatory approval is Wyeth's gemtuzumab ozogamicin (**2**, Mylotarg; Chart 2) (12-14). The drug was approved by the US Food and Drug Administration (FDA) in 2000 for use in patients over 60 suffering from relapsed acute myelocytic leukemia (AML), the most common form of leukemia in adults. Gemtuzumab ozogamicin (**2**) consists of *N*-acetyl-γ-calicheamicin (an enediyne antibiotic that binds to the minor groove of DNA and causes double-strand breaks leading to apoptosis) covalently attached to humanized anti-CD33 IgG4 κ antibody (hP67.6) via a bifunctional linker. The 4-(4-acetylphenoxy)butanoic acid moiety allows for attachment to surface-exposed lysines of the antibody over an amide bond and forms an acyl hydrazone linkage with *N*-acetyl-γ-calicheamicin dimethyl hydrazide. Upon internalization of the ADC, the calicheamicin prodrug is released by hydrolysis of the hydrazone in lysosomes of the CD33-positive target cells. The hydrolyzability of an unconjugated intermediate at 37 °C over 24 h increased from 6% at pH 7.4 to 97% at pH 4.5 (15). The enediyne drug then gets activated by reductive cleavage of the disulfide bond. This disulfide linkage has been stabilized by two methyl groups to prevent premature release of calicheamicin by circulating reduced thiols, such as glutathione. In most preclinical models, the hydrazone linkage produced ADCs with higher potency than the corresponding amide-bearing conjugate **3**, providing evidence that with this mAb the disulfide alone is insufficient for efficient release of the drug in the target cell. Interestingly, with the murine mAb CTM01, ADC **3** showed activities equal to or even greater than that of the corresponding hydrazone conjugate **2** in several *in vitro* and *in vivo* tumor models, revealing that this conclusion is not general (16). In clinical trials, however, compound **3** had only limited evidence of activity.

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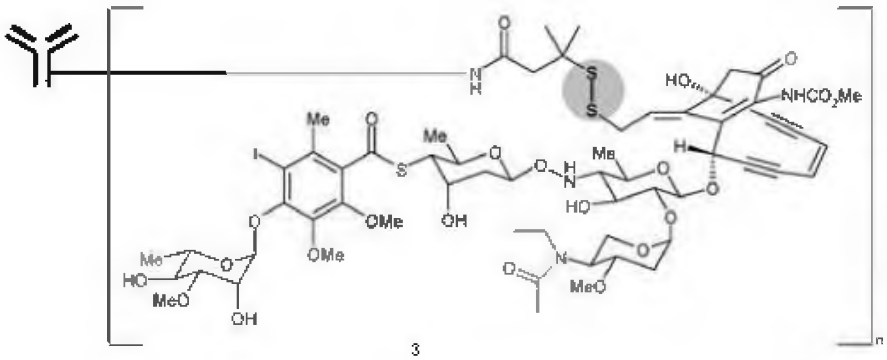
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2



3

Mylotarg has met limited successes due to a narrow therapeutic window. The linker technology based on pH-dependent release mechanism is possibly not sufficiently stable and too much of the drug is being released in the bloodstream, as pharmacokinetic data have shown that the mean half-life of Mylotarg is 72 h (15). Nonetheless, the development of CMC-544 (inotuzumab ozogamicin), which is a humanized anti-CD22 mAb identically attached to *N*-acetyl-γ-calicheamicin dimethyl hydrazide via the acid-labile 4-(4'-acetylphenoxy)butanoic acid linker is ongoing at Wyeth. Although this ADC is closely related to Mylotarg, good stability was shown in both human plasma and serum (rate of hydrolysis of 1.5–2%/day over 4 days) (17, 18).

Disulfide bonds can be an alternative to acid-labile hydrazone linkers. In this case, the release is attributed to the high intracellular concentration of glutathione. This thiol-containing tripeptide is present in micromolar concentrations in the blood, whereas its concentration in the cytoplasma is in the millimolar range (up to 1000-fold higher). This is especially true for tumor cells, where irregular blood flow leads to hypoxic state (decreased oxygen level), resulting in enhanced activity of reductive enzymes and therefore even higher glutathione concentrations. In the absence of sulfhydryl groups, disulfide bonds are thermodynamically stable and provide reasonably good

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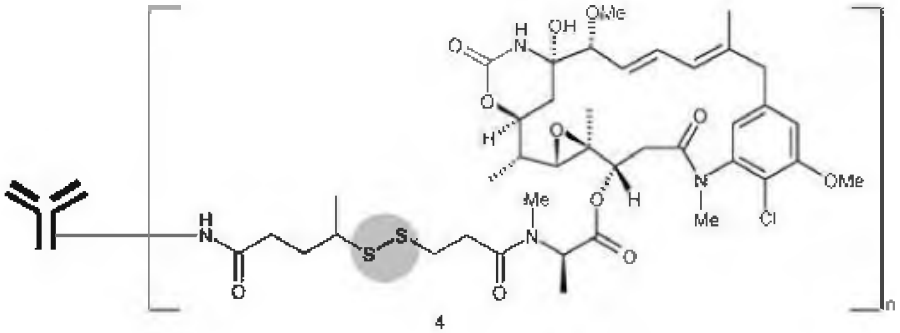
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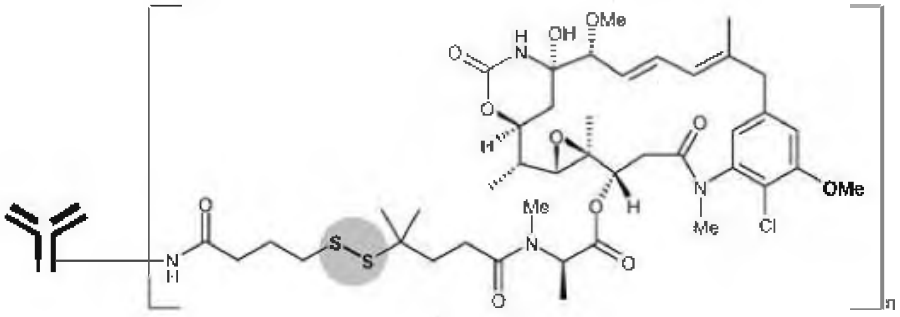
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vesicular trafficking, the mAb is believed to be degraded, affording lysine derivatives linked to the toxin (22). Further intracellular modifications like cleavage of the disulfide linker through a disulfide exchange, and finally thiol methylation presumably catalyzed by intracellular methyltransferases, generate potent derivatives of DM1 or DM4 (22). These methylated drugs are uncharged, enabling them to pass back out through cell membranes to broaden their attack; the drug not only kills from inside the cell, but also knocks out adjacent cells within the tumor that are not targeted by the antibody (23). The linker of compound **4** (Chart 3) nevertheless proved to be relatively unstable *in vivo* (half-life of 47 h in mice) (24). Replacement of DM1 by DM4 structurally leads to the introduction of two methyl substituents on the carbon atom geminal to the disulfide bond, thereby limiting the accessibility for reducing agents. Consistent with these considerations, ADC **5** exhibits an increased half-life of 102 h in mice.

Chart 3



4



5

Enzyme-Labile Linkers

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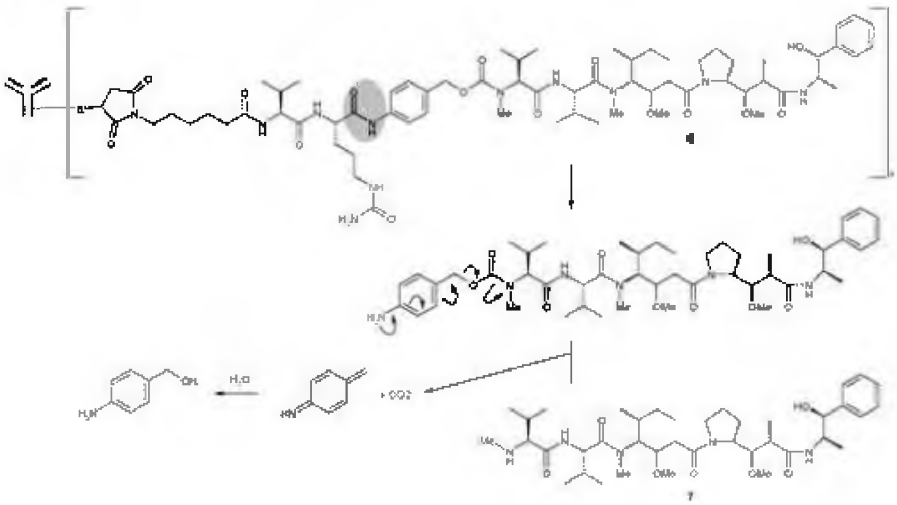
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mAbs with monomethyl auristatin E (MMAE, **7**) and F (MMAF), two auristatin derivatives that bind to tubulin and inhibit microtubule assembly. A valine-citrulline linker was designed to provide high plasma stability (half-lives in mice and monkey of 6.0 and 9.6 days, respectively) as well as a cleavage site for proteases (26). Efficient hydrolysis of the dipeptide linker by cathepsin B has been established *in vitro*. After enzymatic cleavage, 1,6-elimination of the strongly electron-donating 4-aminobenzyl group will occur, releasing MMAE in its active form (Scheme 1) This self-eliminating spacer is needed to spatially separate the drug from the site of enzymatic cleavage (27). Otherwise, the bulky payload has a negative influence on the kinetics of the peptide hydrolysis, as the access to the enzyme's active site can be affected.

Scheme 1



Scheme 1. Presumed Release Mechanism of MMAE (**7**) from ADC 6 Incorporating a Valine-Citrulline Peptidic Linker

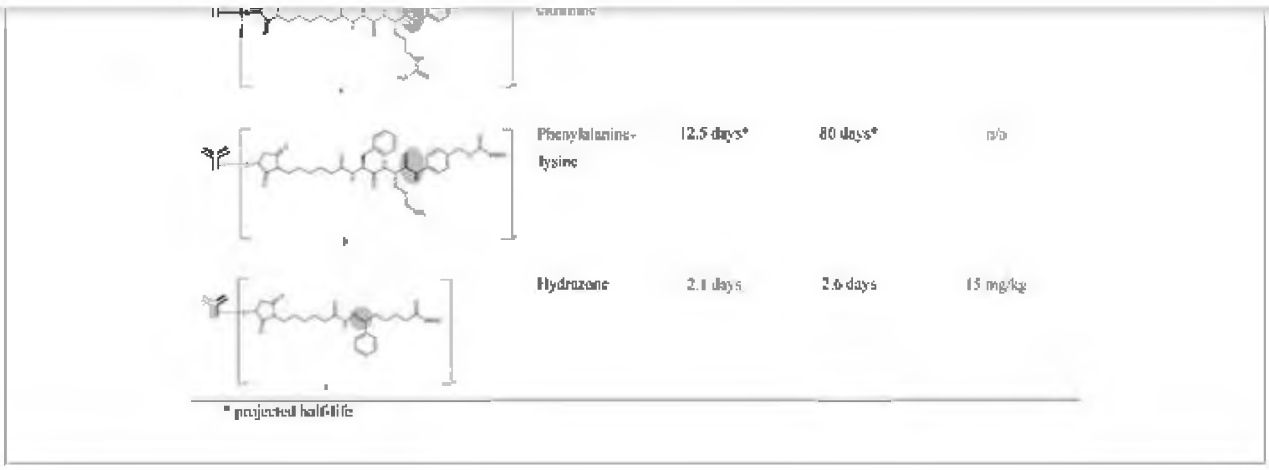
Comparison of plasma stabilities of ADCs bearing enzyme-labile valine-citrulline or phenylalanine-lysine linkers (**6** and **8**, respectively) with ADC **9** equipped with a chemically labile hydrazone linker connecting MMAE with the cBR96 mAb showed impressively the advantage of the peptide linkage (Table 1) in human plasma, the ADC **6**, featuring a peptidic valine-citrulline-derived linker, exhibits an almost 100-fold increased stability compared to its hydrazone relative **9** and the *in vitro* specificity was increased (26). The peptidic linker has a half-life of 6 days in mice compared to 2 days for the hydrazone linker (3-fold improvement), resulting in a lower *in vivo* toxicity (28). Several ADCs incorporating a peptidic linker are in clinical development, with the most advanced being cAC10-

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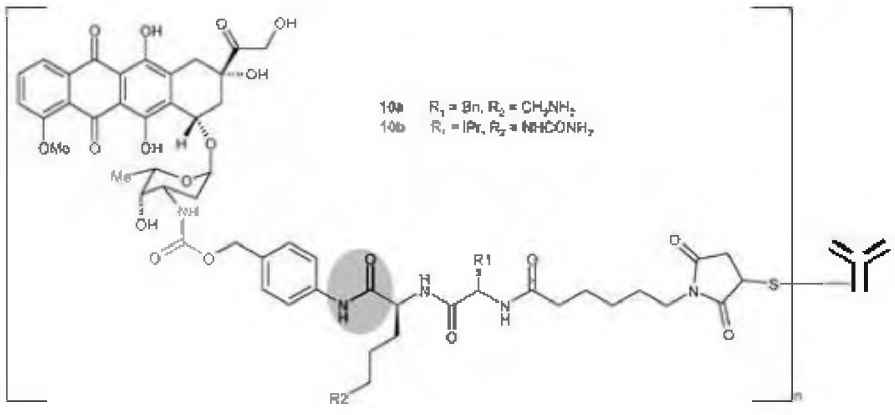
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In addition to *N*-terminus linked MMAE and MMAF, attachment of dipeptide linkers at the *C*-terminus was studied as a way to vary the rate of drug release and thus ADC potency and tolerability (26, 31). For MMAF, it could be shown that linking the mAb over a dipeptide linker to the C-terminus of the drug can lead to further improvement of the therapeutic window.

The superiority of enzyme-labile linkers was also shown for doxorubicin derivatives conjugated to mAbs (11, 32). With this strategy, the immunological specificity of BR96 mAb–peptide–doxorubicin conjugates **10a** and **10b** (Chart 4) was significantly improved compared to that of the corresponding hydrazone-based conjugate **1** (11), demonstrating again the importance of the linker structure for the ADC properties.

Chart 4



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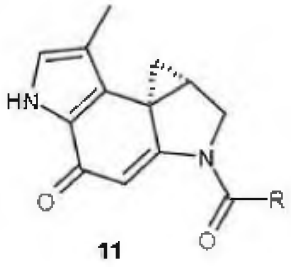
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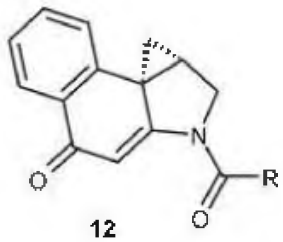
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while increasing its water solubility by introduction of an additional basic nitrogen (36).

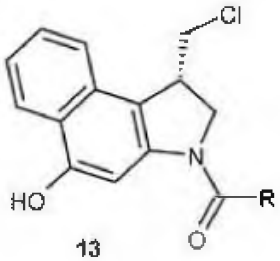
Chart 5



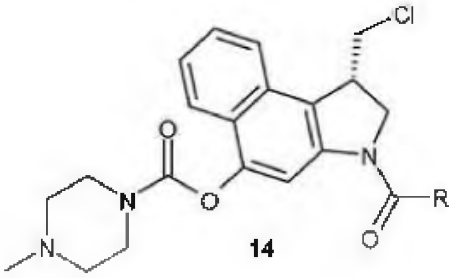
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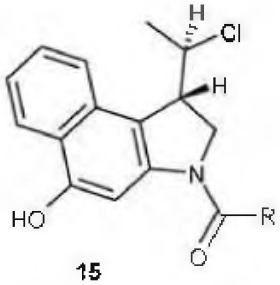
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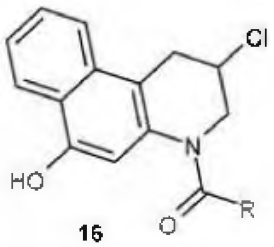
13



14



15



16

Using their UltiMAb technology, Medarex conjugated compound **14** to a mAb via a peptidic linker (37). Conceptually, this approach is a double prodrug strategy. It does not only improve the drug's therapeutic efficacy by conjugating it to a tumor-specific mAb, but additionally employs a prodrug of the cytotoxic agent that has reduced potency and thus minimizes side effects before being released intracellularly by carboxyesterases. The two activating steps needed to trigger the release of the active drug (Scheme 2) may lead to an enlarged therapeutic window.

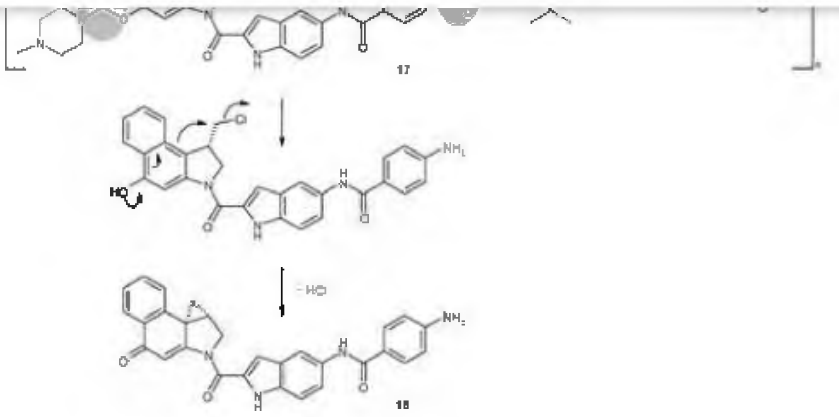
Scheme 2

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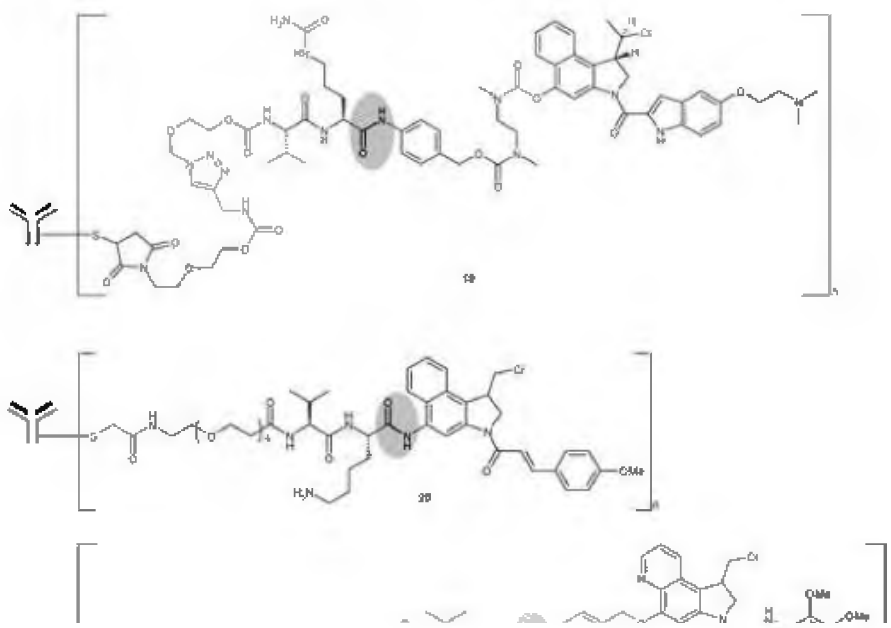
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Scheme 2. Presumed Release Mechanism of CBI-Containing Drug **18** from ADC **17**

Tietze et al. have reported that the CBI prodrugs **15** and **16** exhibit lower toxicities due to the reduced reactivity of the secondary chloride compared to the primary chloride present in **13** and **14** (39-41). Such derivatives with secondary chlorides (see, for example, ADC **19**; Chart 6) are used in Syntarga's SpaceLink conjugation technology (42). To our knowledge, no data are yet published on the linker stability.

Chart 6



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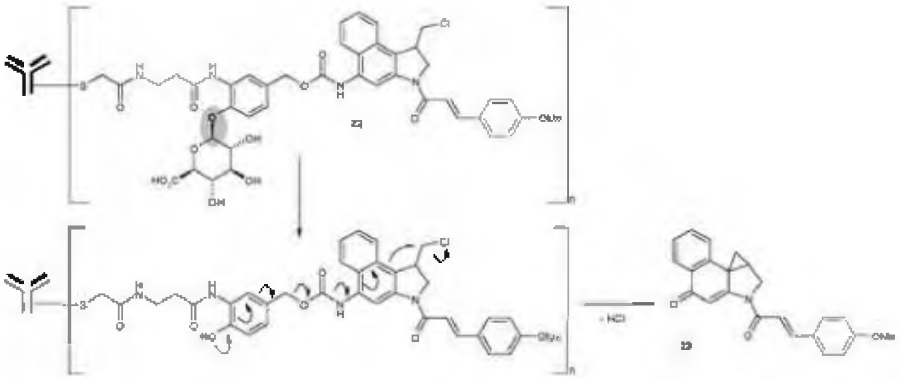
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by use of a more hydrophilic valine-lysine sequence (compared to valine-citrulline as in ADC **6**) and incorporating a tetra(ethylene glycol) unit between the mAb and the peptide linker. The direct attachment of the linker to the amine of the CBI building block (as in **20**) or over a self-immolative *p*-aminobenzyl spacer to the corresponding hydroxyl group of the aza-CBI toxin (as in **21**) prohibits the spontaneous formation of the active toxin in the plasma. Only the enzymatic cleavage of the linker after internalization of the ADC into the cancer cell triggers the release of the prodrug that can now be transformed into the DNA-alkylating cyclopropyl derivative via a Winstein cyclization.

Another type of enzyme-labile linker was recently reported by Seattle Genetics (44). In this case, a carbohydrate moiety serves as the cleavage site. Its hydrophilic nature should limit aggregation. Interestingly, the β-glucoronide moiety in ADC **22** is not directly linking the ADC with the payload. Nevertheless, cleavage by β-glucuronidase triggers the 1,6-elimination of the spacer liberating the free drug **23** (Scheme 3) This carbohydrate motif was also used in ADCs bearing Camptothecin analogues (45).

Scheme 3



Scheme 3. Postulated Release Mechanism of CBI-Containing Drug **23** from ADC **22**

Noncleavable Linkers

ImmunoGen has reported the fortuitous discovery of a noncleavable thioether bond to link DM1 to mAbs (22, 46). Derivative **24** was actually prepared as a control experiment, but surprisingly, this noncleavable thioether linked ADC also proved very potent. The release mechanism is believed to occur via internalization of the ADC followed by degradation of the mAb component in the lysosome, resulting in the release of the maytansinoid drug **25** still attached via the linker to a lysine

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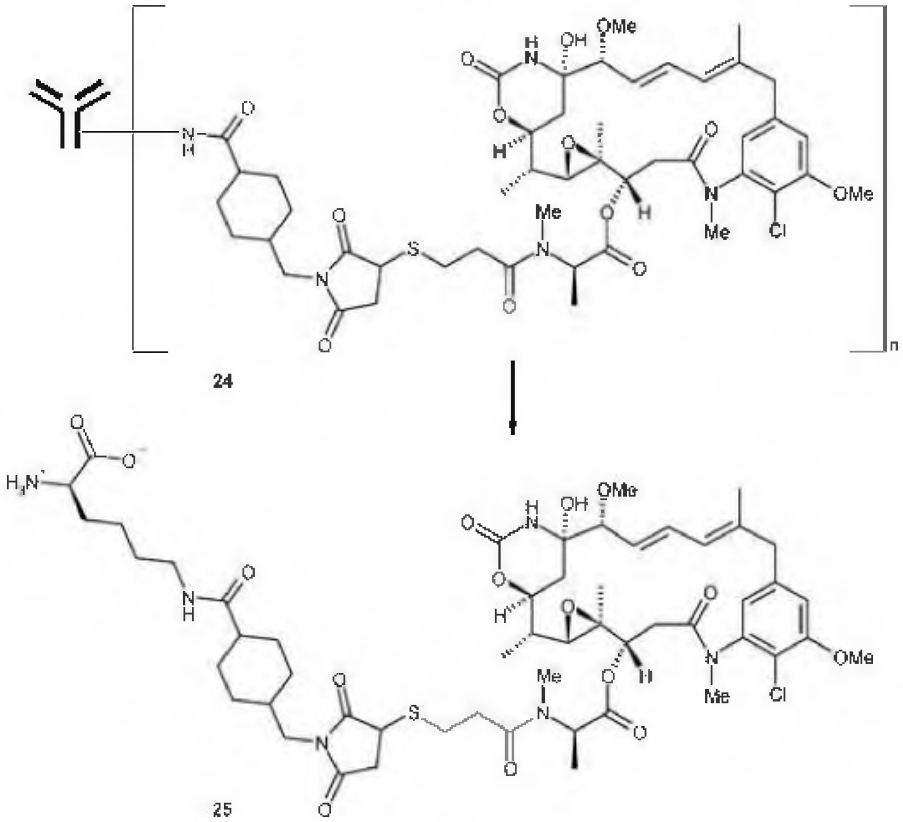
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internalized and degraded within the cell to become active. They are thus dependent on the biology of the target cell.

The *in vivo* stability of the thioether-linked ADC **24** proved, with a half-life of 134 h in mice, superior to that of compounds **4** and **5** (47 and 102 h, respectively). ADCs with noncleavable linkers were found to be better tolerated (47), presumably resulting in an improved therapeutic index. The use of noncleavable linkers has thus become an important feature of ImmunoGen TAP (tumor-activated prodrug) conjugation technology. This is best exemplified by the promising clinical results obtained by Genentech's trastuzumab-DM1 (T-DM1) for HER2-positive metastatic breast cancer (48).

Scheme 4



Scheme 4. Release of Maytansine Drug-Derivative **25** through Lysosomal Degradation of ADC **24**

Seattle Genetics made the same finding with MMAF, namely, that the cleavable dipeptide linker (as

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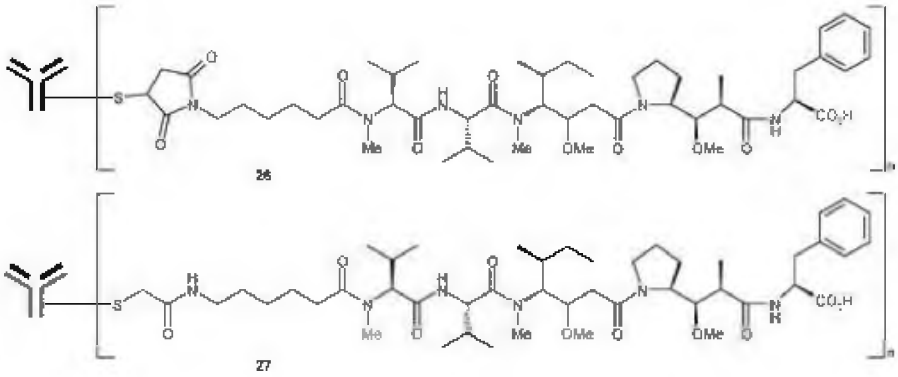
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up to 14 days.

Chart 7



Conclusion

A promising approach to improve the potency of antibody-based cancer therapies is to conjugate the mAb with cytotoxic chemotherapeutic drugs. In the foreseeable future, ADCs will eventually fulfill the promise of specific delivery of cytotoxic drugs to tumor cells (targeted therapy), thus avoiding the dose-limiting toxicity of chemotherapy that occurs as a result of its effects on normal cells. The linker technology profoundly impacts ADC potency, specificity, and safety. Early ADC linkers were commonly derived from acid-labile hydrazones, which were designed to be cleaved inside target cancer cells, but inevitably underwent cleavage at nontarget sites. Newer linkers feature hindered disulfides and peptidic moieties that have achieved greater *in vivo* stability. To spare nontarget tissues from chemotherapeutic damage, noncleavable linkers were recently developed. They are thus far the most stable linkers used in ADCs. This recent development may shift some of the future challenge in ADC development from the linker technology toward finding cytotoxic drugs that retain their potency when bearing a covalently attached linker. The improved linker stability and reduced toxicity, however, come together with greater target dependence than in the case of cleavable linkers. ADCs with noncleavable linkers must be internalized and degraded within the cell, whereas compounds with cleavable linkers may be active against targets that are poorly internalized through extracellular drug release and drug entry into tumor cells. Similarly, killing of bystander antigen-negative cells through targeting of antigen-positive cells (collateral toxicity) is presumably only possible with cleavable linkers. The Lonza ADC-team thus believes that no general linker design exists for all ADCs or cancer types. Despite significant progress, each mAb

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Appendix DUCRY02

REVIEWS

Antibody–Drug Conjugates: Linking Cytotoxic Payloads to Monoclonal Antibodies

Laurent Ducry* and Bernhard Stump

Lonza Ltd, CH-3930 Visp, Switzerland. Received May 4, 2009; Revised Manuscript Received July 7, 2009

Antibody–drug conjugates (ADCs) combine the specificity of monoclonal antibodies (mAbs) with the potency of cytotoxic molecules, thereby taking advantage of the best characteristics of both components. Along with the development of the mAbs and cytotoxins, the design of chemical linkers to covalently bind these building blocks is making rapid progress but remains challenging. Recent advances have resulted in linkers having increased stability in the bloodstream while allowing efficient payload release within the tumor cell.

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INTRODUCTION

After cardiovascular diseases, cancer represents the second most common cause of death in the Western world and is a major area of focus for the pharmaceutical industry. Surgery and radiotherapy are generally used when a tumor is localized to a certain tissue, but chemotherapy is needed when metastasis has occurred. Despite extensive research, most anticancer drugs have nonspecific toxicity. By targeting the cell cycle and thereby killing rapidly proliferating cells, they do not explicitly discriminate between healthy and tumor tissues and only gain a limited selectivity for malignant cells. Such cytotoxic drugs have a narrow therapeutic window, which limits their efficacy and results in severe side effects. Due to a lack of selectivity, drug concentrations that would eradicate the tumor can often not be used. In addition, tumors can develop resistance against anticancer drugs after prolonged treatment. Therefore, achieving improved tumor selectivity through targeting of cytotoxic drugs to the cancer cells is needed.

A promising approach to achieving a more selective treatment is targeted prodrug therapy (*1*). Antibody–drug conjugates (ADCs) are ideal candidates for such prodrugs. ADCs are monoclonal antibodies (mAbs) linked to cell-killing drugs. Thanks to their high binding specificity for tumor-specific antigens, mAbs can be used as vehicles to target cell-killing payloads to tumor cells. Unique or overexpressed, tumor-specific antigens can be found in a wide range of human tumor cells (*2*). Some mAbs have the ability to recognize and specifically bind to these tumor-associated antigens. They can be used as single agents for the treatment of cancer through binding to cancer-cell-specific antigens and induction of an immunological response against the target cancer cell (*3*). However, therapeutic efficacy is often limited by the extent to which the antibody leads to cell death. Monoclonal antibodies are extremely discriminating for their targets but sometimes therapeutically ineffective on their own. The insufficient efficiency of most naked mAbs in cancer therapy has been circumvented by arming the immunoglobulin with radioactive isotopes (*4*) or cytotoxic drugs (*5*–*7*) (Figure 1), yielding highly specific ADCs.

ADCs can be viewed as sophisticated delivery systems for antitumor cytotoxic drugs. The mAb guides the toxin precursor to the target cancer cell, where the prodrug can be converted chemically and/or enzymatically to the parent drug and unfold its cytotoxic activity. The ADC is exposed to different conditions on its journey from the blood vessels to the molecular target in the tumor tissue. The mode of action at cellular or molecular level is complex, with each step bringing its own challenges:

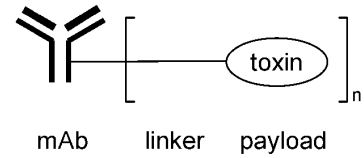


Figure 1. Schematic representation of an antibody–drug conjugate.

a. Circulation. The ADC must behave like a naked antibody when circulating in the plasma. In particular, the linker must be stable in the bloodstream to limit the damage to healthy tissue. Decomposition or decay would release the cytotoxin before being delivered to the target site.

b. Antigen Binding. It is necessary that the conjugated mAb retains high immunoaffinity. Attaching the cytotoxic compound to the mAb must thus not disturb its binding specificity.

c. Internalization. A sufficient intracellular concentration of the drug must be achieved. This is challenging because antigen targets on cell surfaces are often present in limited numbers and the internalization process for antigen–antibody complexes is frequently inefficient.

d. Drug Release. Once internalized, the ADC has to efficiently release the original cytotoxic drug in its active form inside the tumor cell.

e. Drug Action. The inherent potency of the released drug must be sufficient to kill the tumor cell, even at low concentration. To achieve significant cytotoxicity, use of very potent drugs with subnanomolar IC_{50} (as free drug) becomes necessary. It has been shown that compounds found to be too toxic when tested as a stand-alone chemotherapy agent are suitable candidates for ADC payloads. These toxins can be 100 to 1000 times more cytotoxic than traditional anticancer agents.

The molar loading of the drug on the mAb can be tuned to achieve the desired potency. Because of points (c) and (d), linkage of several drugs is generally necessary to achieve significant cytotoxicity. However, if too many cytotoxic molecules are attached

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Chart 1

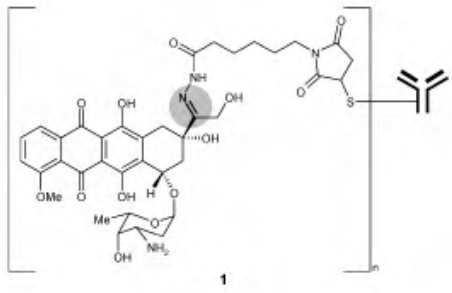


Chart 2

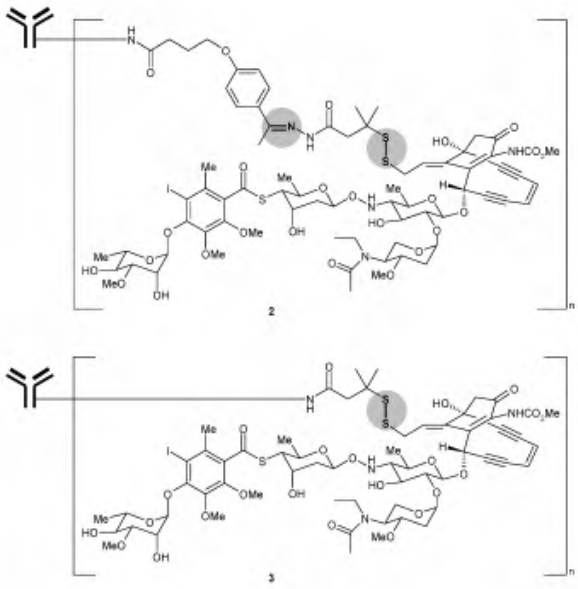
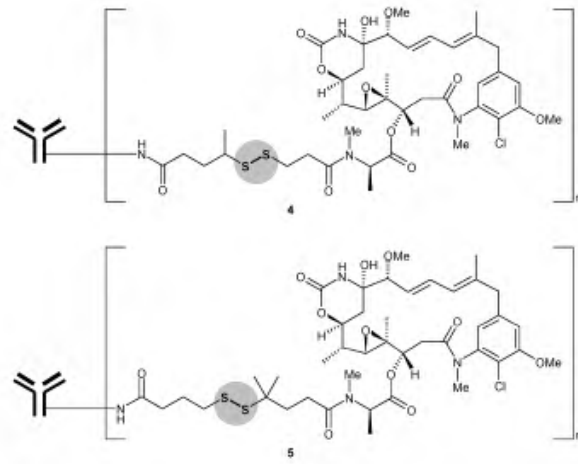


Chart 3



to the antibody, the body may recognize the conjugate as a damaged form of the protein and quickly clears it from the system, proving points (a) and/or (b) to be problematic. The extent of drug substitution may also affect pharmacokinetics, so that most of the time 2−4 drugs per antibody give the best therapeutic window (*8, 9*). To avoid the interference with the antigen recognition, the drugs are ideally linked at the Fc or constant region of the mAb that does not participate in binding to the antigen (point (b)). To regain the full cytotoxic potential of the drugs, cleavable linkers releasing the drug molecule at the target site in unmodified form were developed to address point (d), although these also affect the stability of the construct. Therefore, the right balance between plasma stability and efficient drug release at the target cell has to be found.

The insights gained during ADC development within the past decade have resulted in a number of new strategies and drugs (*4*−*7*). This review focuses on linker technologies used to attach low molecular weight cytotoxic drugs to mAbs. The linker is a small but central part of ADCs and as such accounts for stability in circulation, favorable pharmacokinetic properties, and efficient release of toxic agent at the tumor site.

CHEMICALLY LABILE LINKERS

Selective linker cleavage and payload release is based upon the differential properties between the plasma and some cytoplasmic compartments. Linkers were chosen that were stable in the blood's neutral pH environment but were getting cleaved once the ADC entered the lower pH environment found inside a cell. The focus in the early days of ADC development was on acid-cleavable hydrazone linkers that were relatively stable at neutral pH (bloodstream pH 7.3−7.5), but were undergoing hydrolysis once internalized into the mildly acidic endosomes (pH 5.0−6.5) and lysosomes (pH 4.5−5.0).

In the 1990s, Bristol-Myers Squibb scientists covalently linked doxorubicin, an intercalating agent that blocks DNA replication and is used as chemotherapeutic agent, to the humanized mAb BR96 (*10*). Doxorubicin was connected over a (6-maleimidocaproyl)hydrazone linker to cysteine residues of the Lewis-Y specific mAb. The preclinical activities of BR96-doxorubicin (**1**, Chart 1) were remarkable, but despite the use of eight drugs per mAb molecule, the amount of conjugate needed to achieve these effects *in vivo* were high (>100 mg/kg). This was presumably due to the relatively low potency of doxorubicin (IC_{50} of 0.1−0.2 μM for human carcinoma lines (*11*), whereas subnanomolar activities are now typically seen for ADC payloads). Moreover, the half-life of the drug in the blood was approximately 43 h, which is short compared to the half-lives of several days to weeks of the naked BR96 mAb in humans. The compound later failed to show sufficient clinical efficacy.

The first and so far sole mAb linked to a cytotoxic payload that has been given regulatory approval is Wyeth's gemtuzumab ozogamicin (**2**, Mylotarg; Chart 2) (*12*−*14*). The drug was approved by the US Food and Drug Administration (FDA) in 2000 for use in patients over 60 suffering from relapsed acute myelocytic leukemia (AML), the most common form of leukemia in adults. Gemtuzumab ozogamicin (**2**) consists of *N*-acetyl-γ-calicheamicin (an enediyne antibiotic that binds to the minor groove of DNA and causes double-strand breaks leading to apoptosis) covalently attached to humanized anti-CD33 IgG4 κ antibody (hP67.6) via a bifunctional linker. The 4-(4-acetylphenoxy)butanoic acid moiety allows for attachment to surface-exposed lysines of the antibody over an amide bond and forms an acyl hydrazone linkage with *N*-acetyl-γ-calicheamicin dimethyl hydrazide. Upon internalization of the ADC, the calicheamicin prodrug is released by hydrolysis of the hydrazone in lysosomes of the CD33-positive target cells. The hydrolyzability of an unconjugated intermediate at 37 °C over 24 h increased from 6% at pH 7.4 to 97% at pH 4.5 (*15*). The enediyne drug then gets activated by reductive cleavage of the disulfide bond. This disulfide linkage has been stabilized by two methyl groups to prevent premature release of calicheamicin by circulating reduced thiols, such as glutathione. In most preclinical models, the hydrazone linkage produced ADCs with higher potency than the corresponding amide-bearing conjugate **3**, providing evidence that with this mAb the disulfide alone is insufficient for

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Scheme 1. Presumed Release Mechanism of MMAE (7) from ADC 6 Incorporating a Valine-Citrulline Peptidic Linker

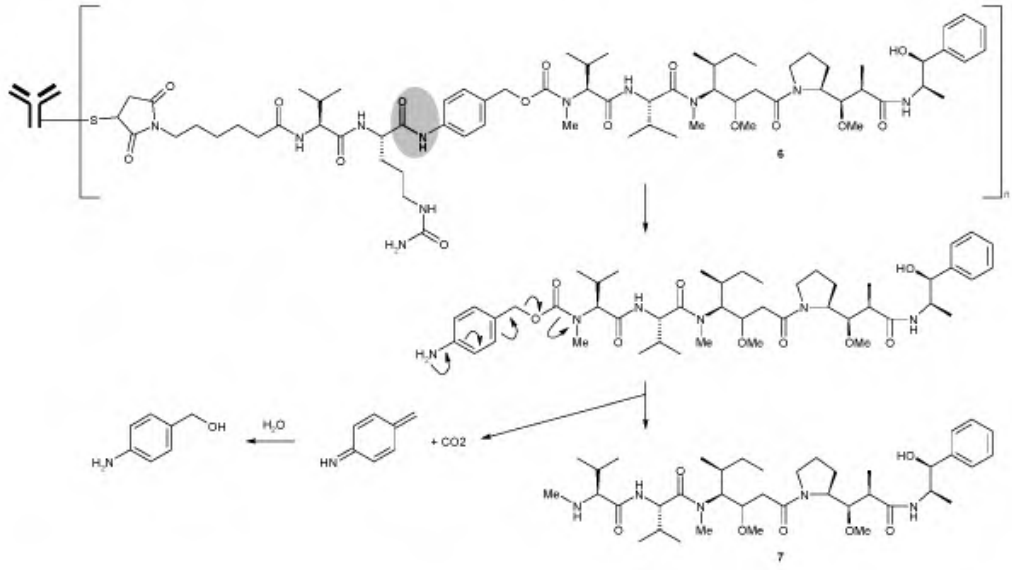
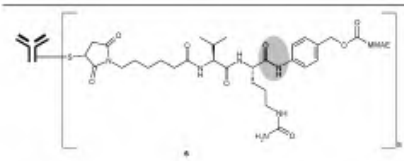
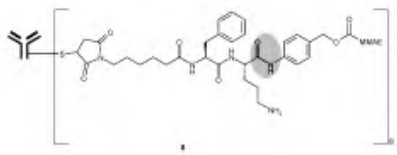
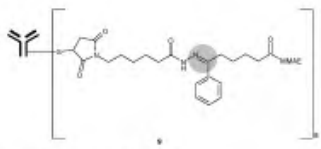


Table 1. Compared Stabilities of Enzyme-Labile and Chemically Labile Linkers in ADCs with MMAE Conjugated to cBR96 mAb

ADC	Linker	Half-live mouse plasma	Half-live human plasma	Maximum tolerated dose
	Valine-citrulline	30 days*	230 days*	30 mg/kg
	Phenylalanine-lysine	12.5 days*	80 days*	n/a
	Hydrazone	2.1 days	2.6 days	15 mg/kg

* projected half-life

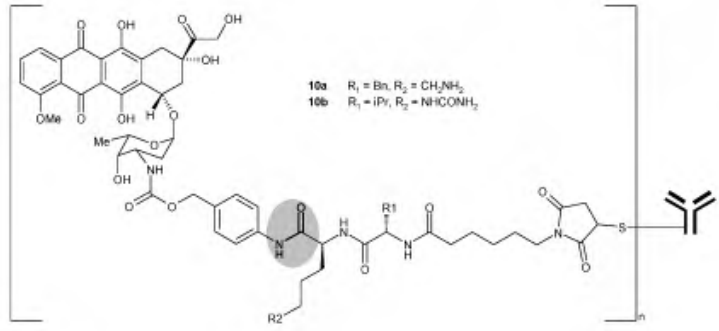
efficient release of the drug in the target cell. Interestingly, with the murine mAb CTM01, ADC **3** showed activities equal to or even greater than that of the corresponding hydrazone conjugate **2** in several *in vitro* and *in vivo* tumor models, revealing that this conclusion is not general (*16*). In clinical trials, however, compound **3** had only limited evidence of activity.

Mylotarg has met limited successes due to a narrow therapeutic window. The linker technology based on pH-dependent release mechanism is possibly not sufficiently stable and too much of the drug is being released in the bloodstream, as pharmacokinetic data have shown that the mean half-life of Mylotarg is 72 h (*15*). Nonetheless, the development of CMC-544 (inotuzumab ozogamicin), which is a humanized anti-CD22 mAb identically attached to *N*-acetyl-γ-calicheamicin dimethyl hydrazide via the acid-labile 4-(4′-acetylphenoxy)butanoic acid linker is ongoing at Wyeth. Although this ADC is closely related to Mylotarg, good stability was shown in both human plasma and serum (rate of hydrolysis of 1.5−2%/day over 4 days) (*17, 18*).

Disulfide bonds can be an alternative to acid-labile hydrazone linkers. In this case, the release is attributed to the high intracellular concentration of glutathione. This thiol-containing tripeptide is present in micromolar concentrations in the blood, whereas its concentration in the cytoplasma is in the millimolar range (up to 1000-fold higher). This is especially true for tumor cells, where irregular blood flow leads to hypoxic state (decreased oxygen level), resulting in enhanced activity of reductive enzymes and therefore even higher glutathione concentrations. In the absence of sulfhydryl groups, disulfide

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Chart 4



bonds are thermodynamically stable and provide reasonably good stability in the bloodstream.

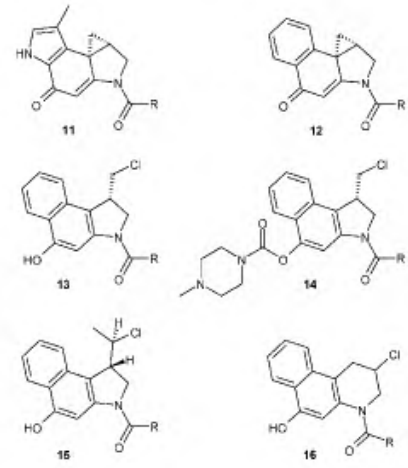
An example of an intracellularly cleavable disulfide-based linker was already discussed above (see "amide" conjugate **3**). Other examples are taxoid ADC payloads, conjugated via a disulfide-bearing 4-mercaptopentanoate linker to mAbs directed against the epidermal growth factor receptor (*19*). However, the most significant examples are ADCs where the immunoglobulin is connected over the same type of linker to ImmunoGen's drugs DM1 or DM4 (*20*−*22*). These potent thiol-containing maytansine analogues are antimitotic agents that bind to tubulin and inhibit microtubule assembly. After internalization of the ADC via antigen-mediated endocytosis and delivery to lysosomes by vesicular trafficking, the mAb is believed to be degraded, affording lysine derivatives linked to the toxin (*22*). Further intracellular modifications like cleavage of the disulfide linker through a disulfide exchange, and finally thiol methylation presumably catalyzed by intracellular methyltransferases, generate potent derivatives of DM1 or DM4 (*22*). These methylated drugs are uncharged, enabling them to pass back out through cell membranes to broaden their attack; the drug not only kills from inside the cell, but also knocks out adjacent cells within the tumor that are not targeted by the antibody (*23*). The linker of compound **4** (Chart 3) nevertheless proved to be relatively unstable *in vivo* (half-life of 47 h in mice) (*24*). Replacement of DM1 by DM4 structurally leads to the introduction of two methyl substituents on the carbon atom geminal to the disulfide bond, thereby limiting the accessibility for reducing agents. Consistent with these considerations, ADC **5** exhibits an increased half-life of 102 h in mice.

ENZYME-LABILE LINKERS

Chemically labile linkers often suffer from limited plasma stability. An alternative approach to achieve better control of the drug release involves linking a drug to an antibody via a peptide linkage. The free drug can be specifically cleaved from the carrier by the action of lysosomal proteases (e.g., cathepsin or plasmin) present at elevated levels in certain tumor tissues (*25*). Peptidic bonds are expected to have good serum stability, as proteases are normally not active outside cells because of unfavorable pH conditions and inhibition by serum protease inhibitors.

Seattle Genetics is using a maleimido-containing dipeptide linker to conjugate cysteine residues of mAbs with monomethyl auristatin E (MMAE, **7**) and F (MMAF), two auristatin derivatives that bind to tubulin and inhibit microtubule assembly. A valine-citrulline linker was designed to provide high plasma stability (half-lives in mice and monkey of 6.0 and 9.6 days, respectively) as well as a cleavage site for proteases (*26*). Efficient hydrolysis of the dipeptide linker by cathepsin B has been established *in vitro*. After enzymatic

Chart 5



cleavage, 1,6-elimination of the strongly electron-donating 4-aminobenzyl group will occur, releasing MMAE in its active form (Scheme 1). This self-eliminating spacer is needed to spatially separate the drug from the site of enzymatic cleavage (*27*). Otherwise, the bulky payload has a negative influence on the kinetics of the peptide hydrolysis, as the access to the enzyme's active site can be affected.

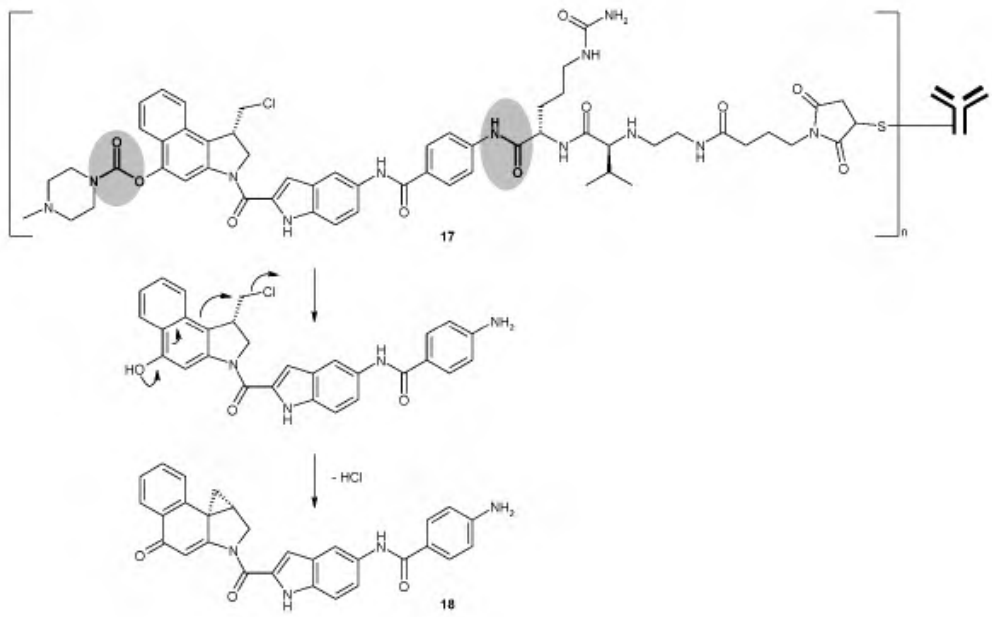
Comparison of plasma stabilities of ADCs bearing enzyme-labile valine-citrulline or phenylalanine-lysine linkers (**6** and **8**, respectively) with ADC **9** equipped with a chemically labile hydrazone linker connecting MMAE with the cBR96 mAb showed impressively the advantage of the peptide linkage (Table 1): in human plasma, the ADC **6**, featuring a peptidic valine-citrulline-derived linker, exhibits an almost 100-fold increased stability compared to its hydrazone relative **9** and the *in vitro* specificity was increased (*26*). The peptidic linker has a half-life of 6 days in mice compared to 2 days for the hydrazone linker (3-fold improvement), resulting in a lower *in vivo* toxicity (*28*). Several ADCs incorporating a peptidic linker are in clinical development, with the most advanced being cAC10-vcMMAE (SGN-35) for CD30-positive hematologic malignancies (*26, 29, 30*).

In addition to *N*-terminus linked MMAE and MMAF, attachment of dipeptide linkers at the *C*-terminus was studied as a way to vary the rate of drug release and thus ADC potency and tolerability (*26, 31*). For MMAF, it could be shown that linking the mAb over a dipeptide linker to the C-terminus of the drug can lead to further improvement of the therapeutic window.

The superiority of enzyme-labile linkers was also shown for doxorubicin derivatives conjugated to mAbs (*11, 32*). With this

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Scheme 2. Presumed Release Mechanism of CBI-Containing Drug 18 from ADC 17



strategy, the immunological specificity of BR96 mAb−peptide−doxorubicin conjugates **10a** and **10b** (Chart 4) was significantly improved compared to that of the corresponding hydrazone-based conjugate **1** (*11*), demonstrating again the importance of the linker structure for the ADC properties.

Peptide-containing linkers have been successfully used with CC-1065-derived toxins. CC-1065, duocarmycins, and other cyclopropapyrroloind-4-one (CPI, **11**; Chart 5) derivatives are potent minor-groove binding DNA alkylating agents. According to Boger et al., cyclopropabenzindol-4-one analogues (CBI, **12**) are chemically more stable, biologically more potent, and synthetically more accessible than their parent compounds incorporating the natural CPI alkylation subunit (*33*). In analogy to a duocarmycin prodrug (*34*), the seco-CBI precursor **13** was developed. This compound, which was postulated to show improved pharmacokinetics, will provide the CBI analogue **12** via Winstein cyclization (*35*). A further improvement was achieved by protecting the phenolic hydroxyl group with an enzyme-labile carbamate, as in derivative **14**, decreasing the toxicity of the prodrug while increasing its water solubility by introduction of an additional basic nitrogen (*36*).

Using their UltiMAb technology, Medarex conjugated compound **14** to a mAb via a peptidic linker (*37*). Conceptually, this approach is a double prodrug strategy. It does not only improve the drug's therapeutic efficacy by conjugating it to a tumor-specific mAb, but additionally employs a prodrug of the cytotoxic agent that has reduced potency and thus minimizes side effects before being released intracellularly by carboxyesterases. The two activating steps needed to trigger the release of the active drug (Scheme 2) may lead to an enlarged therapeutic window.

Tietze et al. have reported that the CBI prodrugs **15** and **16** exhibit lower toxicities due to the reduced reactivity of the secondary chloride compared to the primary chloride present in **13** and **14** (*39*−*41*). Such derivatives with secondary chlorides (see, for example, ADC **19**; Chart 6) are used in Syntarga's SpaceLink conjugation technology (*42*). To our knowledge, no data are yet published on the linker stability.

To extend its ADC technology to drugs with a complementary mode of action, Seattle Genetics has developed ADCs **20** and **21** (Chart 6) containing an amino-CBI and a hydroxy aza-CBI payload, respectively (*43*). Because of the hydrophobicity of this class of molecules, attention was focused on developing hydrophilic peptide-linker derivatives that prevented aggregation. This was achieved by use of a more hydrophilic valine-lysine sequence (compared to valine-citrulline as in ADC **6**) and incorporating a tetra(ethylene glycol) unit between the mAb and the peptide linker. The direct attachment of the linker to the amine of the CBI building block (as in **20**) or over a self-immolative *p*-aminobenzyl spacer to the corresponding hydroxyl group of the aza-CBI toxin (as in **21**) prohibits the spontaneous formation of the active toxin in the plasma. Only the enzymatic cleavage of the linker after internalization of the ADC into the cancer cell triggers the release of the prodrug that can now be transformed into the DNA-alkylating cyclopropyl derivative via a Winstein cyclization.

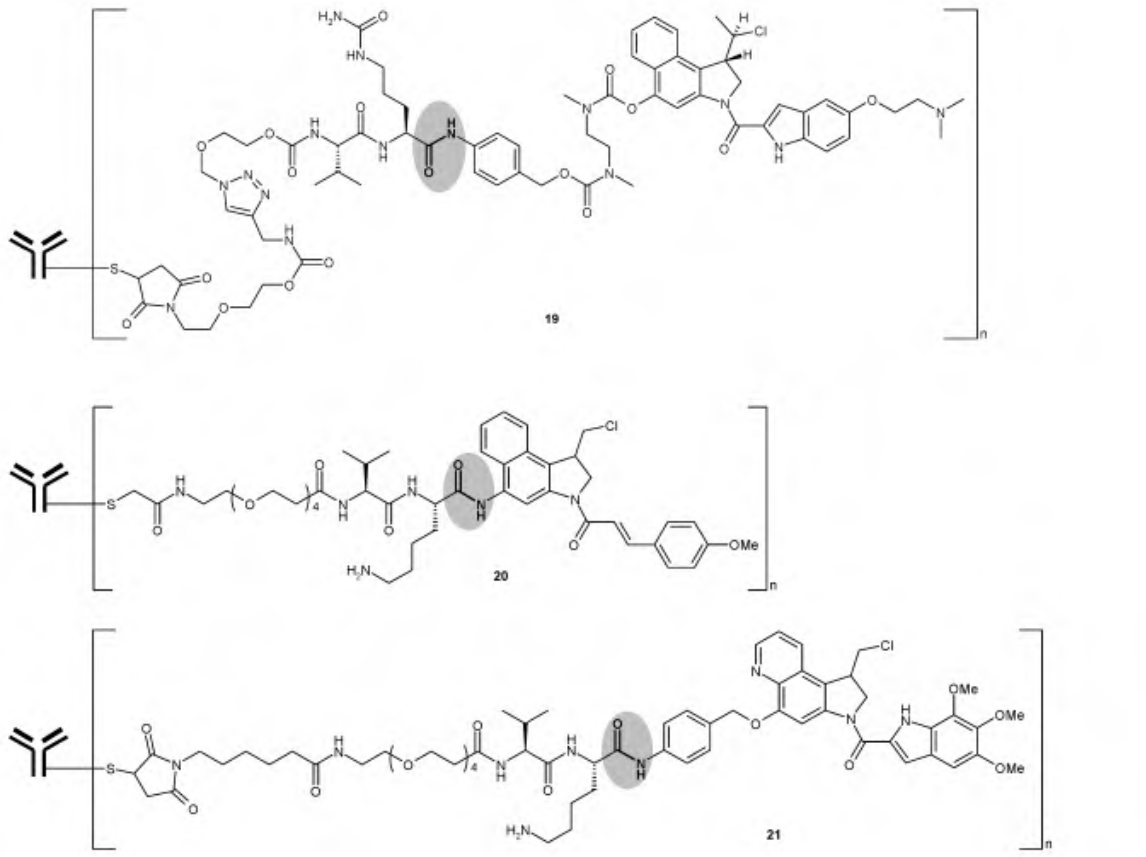
Another type of enzyme-labile linker was recently reported by Seattle Genetics (*44*). In this case, a carbohydrate moiety serves as the cleavage site. Its hydrophilic nature should limit aggregation. Interestingly, the β-glucoronide moiety in ADC **22** is not directly linking the ADC with the payload. Nevertheless, cleavage by β-glucuronidase triggers the 1,6-elimination of the spacer liberating the free drug **23** (Scheme 3). This carbohydrate motif was also used in ADCs bearing Camptothecin analogues (*45*).

NONCLEAVABLE LINKERS

ImmunoGen has reported the fortuitous discovery of a noncleavable thioether bond to link DM1 to mAbs (*22, 46*). Derivative **24** was actually prepared as a control experiment, but surprisingly, this noncleavable thioether linked ADC also proved very potent. The release mechanism is believed to occur via internalization of the ADC followed by degradation of the mAb component in the lysosome, resulting in the release of the maytansinoid drug **25** still attached via the linker to a lysine residue. This chemical modification of the drug did not diminish its cytotoxic potential. This form of the drug is, however, charged and presumably not able to diffuse into neighboring cells. Hence, it cannot kill adjacent tumor cells

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Chart 6

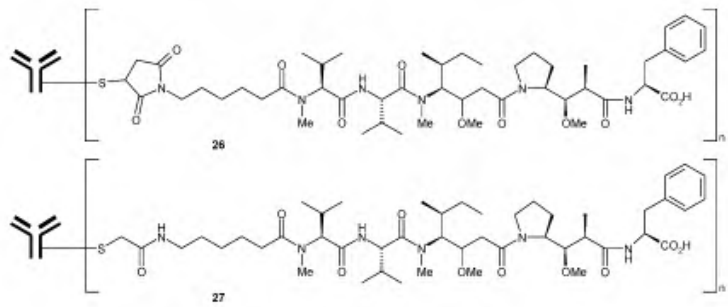


that do not express the target antigen (antigen-negative cells). Besides this by-stander effect (*23*), there is an additional reason why cleavable linkers may have a broader efficacy. Although internalization is often the common initial activation process for both cleavable and noncleavable linkers (*22*), ADCs with cleavable linkers may also be active even when they are poorly internalized (*47*). One possible explanation is that, when the linker is cleaved in extracellular space, the free drug subsequently permeates the cell to reach its target. This mechanism is not operating for ADCs incorporating noncleavable linkers, which must be internalized and degraded within the cell to become active. They are thus dependent on the biology of the target cell.

The *in vivo* stability of the thioether-linked ADC **24** proved, with a half-life of 134 h in mice, superior to that of compounds **4** and **5** (47 and 102 h, respectively). ADCs with noncleavable linkers were found to be better tolerated (*47*), presumably resulting in an improved therapeutic index. The use of noncleavable linkers has thus become an important feature of ImmunoGen TAP (tumor-activated prodrug) conjugation technology. This is best exemplified by the promising clinical results obtained by Genentech's trastuzumab-DM1 (T-DM1) for HER2-positive metastatic breast cancer (*48*).

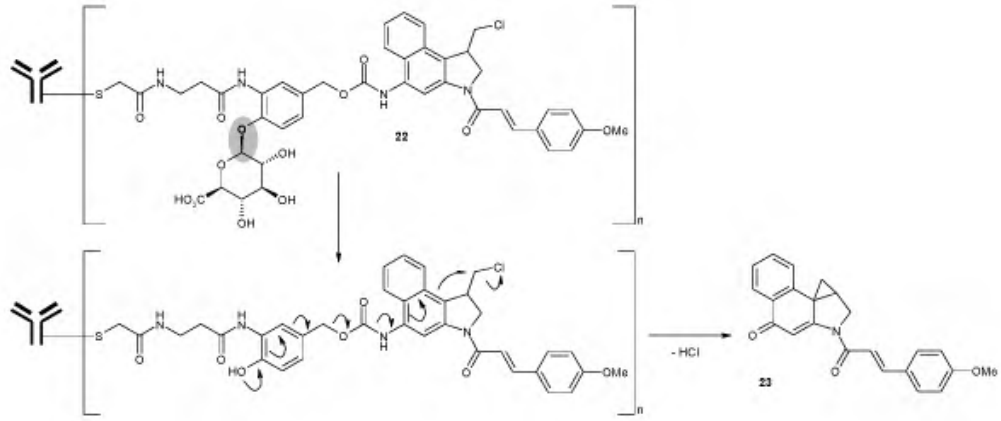
Seattle Genetics made the same finding with MMAF, namely, that the cleavable dipeptide linker (as in **6**) could be replaced by a noncleavable thioether bond (as in ADC **26**; Chart 7) while retaining the *in vivo* activity (*49*). Mass spectrometry showed that the released drug was a cysteine-adduct of MMAF, presumably resulting from mAb degradation within lysosomes as proposed for maytansinoid conjugate **24**. MMAE, although closely related structurally, was not

Chart 7

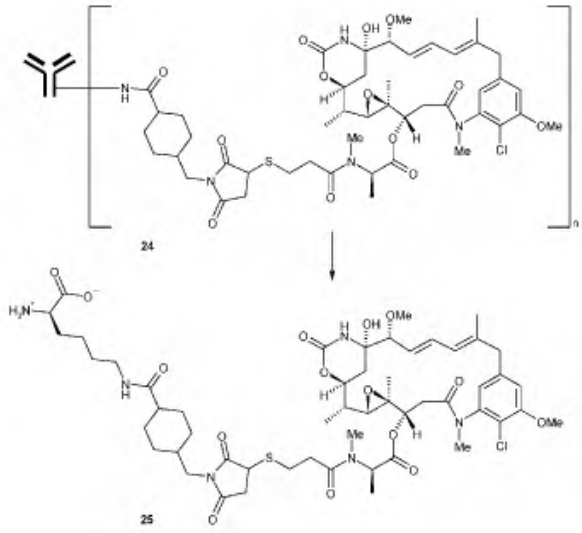


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Scheme 3. Postulated Release Mechanism of CBI-Containing Drug 23 from ADC 22



Scheme 4. Release of Maytansine Drug-Derivative 25 through Lysosomal Degradation of ADC 24



active when attached in this manner. Only MMAF can sustain significant modification to the *N*-terminal position and still retain its potency. Recently, it was shown that a slow transfer of the drug from the ADC to albumin cysteines in the plasma occurs with alkyl-maleimide ADCs (*50*). To counteract this effect, the linker stability was enhanced by replacing the maleimide (as in ADC **26**) with an acetamide functionality (as in ADC **27**). This type of ADC led to no apparent degradation in the bloodstream for up to 14 days.

CONCLUSION

A promising approach to improve the potency of antibody-based cancer therapies is to conjugate the mAb with cytotoxic chemotherapeutic drugs. In the foreseeable future, ADCs will eventually fulfill the promise of specific delivery of cytotoxic drugs to tumor cells (targeted therapy), thus avoiding the dose-limiting toxicity of chemotherapy that occurs as a result of its effects on normal cells. The linker technology profoundly impacts ADC potency, specificity, and safety. Early ADC linkers were commonly derived from acid-labile hydrazones, which were designed to be cleaved inside target cancer cells, but inevitably underwent cleavage at nontarget sites. Newer linkers feature hindered disulfides and peptidic moieties that have achieved greater *in vivo* stability. To spare nontarget tissues from chemotherapeutic damage, noncleavable linkers were recently developed. They are thus far the most stable linkers used in ADCs. This recent development may shift some of the future challenge in ADC development from the linker technology toward finding cytotoxic drugs that retain their potency when bearing a covalently attached linker. The improved linker stability and reduced toxicity, however, come together with greater target dependence than in the case of cleavable linkers. ADCs with noncleavable linkers must be internalized and degraded within the cell, whereas compounds with cleavable linkers may be active against targets that are poorly internalized through extracellular drug release and drug entry into tumor cells. Similarly, killing of bystander antigen-negative cells through targeting of antigen-positive cells (collateral toxicity) is presumably only possible with cleavable linkers. The Lonza ADC-team thus believes that no general linker design exists for all ADCs or cancer types. Despite significant progress, each mAb must be examined separately in order to optimize a specific delivery system.

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Antibody-Drug Conjugates for Cancer Therapy

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Abstract:

The antibody-drug conjugate (ADC) concept is to use an antibody to deliver a cytotoxic drug selectively to a target such as a tumor-associated antigen. Such conjugates represent a broadly applicable approach to enhance the antitumor activity of antibodies and improve the tumor-to-normal tissue selectivity of chemotherapy. Critical parameters for ADC development include target antigen selection, conjugate internalization by tumor cells, drug potency and stability of the linker between drug and antibody. Other important considerations include the conjugation methods, drug-to-antibody ratio, and the effects of drug conjugation on antibody properties. Highly potent drugs with more stable linkers have been attached to a new generation of antibodies to create conjugates with pronounced antitumor activities in preclinical studies and encouraging results in early stage clinical trials. This review details these advances, discusses some of the remaining challenges, and overviews ADCs currently in clinical trials for cancer therapy.

Antibody-based therapeutics are of growing significance for cancer therapy as evidenced by 12 such drugs approved for

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The concept of arming antibodies by conjugation to protein toxins dates back to 1970,[8] and was followed a few years later by antibody conjugates with cytotoxic drugs. Antibody therapeutics have come of age in the intervening decades with the advent of hybridoma technology to develop murine monoclonal antibodies (MAb),[9] chimerization and humanization to address the shortcomings of murine MAb as therapeutics, and more recently with direct routes to human antibodies using phage display or transgenic mice.[2] The clinical potential of ADCs has been greatly enhanced by improved choices of targets,[10] more potent drugs in conjunction with linkers of improved stability.[6,11] and greatly expanded knowledge of ADC cell biology.[32] and pharmacology.[13] ADCs are reviewed here from a design perspective from target selection, through ADC lead identification, optimization, and preclinical development to clinical experience (Fig. 2) Other payloading strategies for antibodies, as well as for alternative delivery vehicles, have been extensively reviewed elsewhere.[3,11,12,14] and are outside the scope of this article. Of these, radioimmunoconjugates stand out, with the approval of 2 different anti-CD20 radioimmunoconjugates for the treatment of non-Hodgkin lymphoma: ibritumomab tiuxetan (Zevalin, ^{90}Y conjugate) and ^{131}I tositumomab (Bexxar).[15]



Figure 2

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TARGET SELECTION

The de novo identification and validation of cell surface targets as candidates for ADCs is beyond the scope of this article and is reviewed elsewhere.[10] Target selection from a candidate pool is a critical first step in generating ADCs (Fig. 2) Expression profiling is a key aspect of target selection in that tumor-associated antigen expression is presumed to be one of the necessary criteria for successful targeting with an ADC, whereas little or no normal tissue expression is desirable and may be necessary to achieve an adequate therapeutic index.

Immunohistochemistry allows semi-quantitative analysis of protein expression in tissue samples and is often the most direct and consequently preferred method for evaluating potential ADC targets. The advent of tissue microarrays.[16] facilitates high-throughput expression profiling-hundreds to thousands of samples-with greater standardization.[17] A limitation of immunohistochemistry is the high variability of data with different reagents, protocols and samples.[17]- resolvable if concordant data are obtained using multiple antibodies. An additional challenge with immunohistochemistry is that data may be difficult to interpret if the antigen is located inside the cell as well as on the cell surface. Flow cytometry is also used for protein expression profiling, particularly for hematologic tumors. This method provides more quantitative information than immunohistochemistry and can demonstrate the accessibility of a target to an antibody. Limitations of flow cytometry include the limited availability of suitable patient samples, and the need to disaggregate solid tumor samples, that may potentially compromise antigen levels.

Expression profiling at the mRNA level is widely used for target assessment and can be a valuable, albeit indirect adjunct, to protein expression profiling.[10] It is important to verify the concordance of mRNA and protein levels, as demonstrated in at least a minority of cases.[18] Techniques available for mRNA expression profiling in clinical samples include microarrays, serial analysis of gene expression, quantitative real-time PCR, cDNA hybridizations using multiple tissue expression arrays/cancer profiling arrays, and RNA in situ hybridizations using frozen tissue samples.[10]

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Tumor Expression of Antigen

ADCs to date have been used almost exclusively to target antigens located on the surface of tumor cells. These targets are almost always normal antigens either expressed at abnormal times in development, at aberrantly high levels on cell

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High level and homogeneous antigen expression on the external surface of all tumor cells seems desirable to facilitate efficient targeting with ADCs. Elevated antigen expression can lead to greater ADC potency, as judged by in vitro and in vivo experiments with transfected cell lines.[127] However, such high level antigen expression is not a prerequisite for clinical benefit by ADCs as demonstrated by gemtuzumab ozogamicin approved for the treatment of AML with only approximately 5,000-10,000 copies of the cognate antigen, CD33, per cell.[23] Homogeneous antigen expression may be beneficial for targeting with ADCs, but is not a requirement, as demonstrated by in vivo xenograft studies with a heterogeneously expressed antigen, CanAg.[24] Successful targeting of heterogeneously expressed antigens may benefit from the use of ADCs with bystander killing activity [25,26] or by combining an ADC with another therapeutic modality such as cytotoxic chemotherapy.[27]

Optimally, the target antigen is expressed in most, if not all, patients with a particular tumor type. If not, a diagnostic test for antigen expression may be required for patient selection as used for HER2 expression profiling before trastuzumab therapy, and for EGFR profiling before treatment with cetuximab or panitumumab.[37] Flow cytometry is used widely for identifying antigen-positive hematologic tumors.[28] In addition, the target antigen should be expressed throughout the course of disease. Minimally, the antigen should be expressed in late stage disease-ie, patient populations commonly available for evaluating experimental drugs.

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Normal Tissue Expression of Antigen

The target antigen should ideally be absent from normal tissue, but this is seldom, if ever, achieved. Tumor-associated antigens are commonly selected on the basis of higher expression levels in tumors and limited normal tissue expression. Some normal tissue expression of antigen is anticipated and may be acceptable. Unfortunately, there is currently no reliable way to predict the significance of normal tissue expression of an antigen on ADC tolerability. Therefore, it is highly desirable to develop ADCs that cross-react to the corresponding antigen from other species-so called species cross-reactivity-to allow preclinical assessment of antigen-dependent toxicity, also known as "on-target" toxicity.

Antigen expression on normal tissue can lead to on-target toxicity with ADCs in patients,[29,30] but does not necessarily do so. For example, inefficient ADC localization to antigen-positive normal tissue is presumed to lower the risk of on-target toxicity. ADC localization may be particularly inefficient if the level of antigen expression is low, the antigenic sites are located in the central nervous system beyond the blood brain barrier or if the antigen is restricted to the apical surface of cells. Nonproliferating cells have reduced sensitivity to many cytotoxic drugs as compared with proliferating cells,[31] an attribute that that seems likely to apply to corresponding ADCs [32] and may reduce on-target toxicity. Extensive normal tissue expression of antigen may create a "sink" that soaks up the ADC and accelerate ADC clearance.[33]

Targeting may be possible for an antigen whose expression is restricted to a nonvital organ or cell population, even if the antigen is not differentially expressed between normal tissue and tumors. For example, CD20, CD33, and CD52 are cell surface markers for B cells, myeloid progenitors plus monocytes, and leukocytes, respectively-cell lineages whose elimination by antibody therapeutics can be tolerated on a temporary basis.

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Antigen Shedding

Shedding of target antigen from the surface of tumor cells into circulation may increase the risk of toxicity because of immune complex formation and deposition in kidney, accelerated clearance of ADC and impaired tumor localization. Shed antigen can sometimes be problematic for ADCs as suggested by a phase II trial for an anti-MUC1-calicheamicin conjugate in ovarian cancer.[34] However, shed antigen can sometimes be tolerated as preliminary reports of a phase I study with an anti-HER2 ADC, trastuzumab-DM1, in metastatic breast cancer.[35,36]

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ADC internalization (Fig. 1) is desirable and often necessary for efficient drug release, depending upon the drug and linker. Nevertheless, antigen internalization per se is not a useful criterion for target selection. This reflects the fact that antigen internalization may be impacted by many ADC attributes including choice of antibody epitope, as well as the drug and linker (see below). Moreover, inefficiently internalizing antigens such as CD20, a tetra-spanning membrane protein, have been successfully targeted with ADCs as judged by efficacy in xenograft experiments in vivo.[37,38] Antigen internalization can potentially be impacted by the presence of other membrane proteins, eg, CD21 expression was recently reported to impair the internalization of ADCs directed to its binding partner, CD19.[39]

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ANTIBODY LEAD IDENTIFICATION

Lead generation for antibody therapeutics has evolved in recent years to the pursuit of large antibody panels-tens to hundreds of antibodies-to increase the likelihood of identifying leads that satisfy multiple design criteria.[2] This large-scale antibody screening is now being evaluated as a paradigm for ADC development. Important antibody attributes for use as conjugates include binding to the human antigen on tumor cells, cytotoxic activity as ADCs, and other desirable attributes such as cross-reactivity with species that can be used in toxicology. These important and desired antibody attributes for ADCs are discussed below, together with a comparison of the main alternative antibody generation technologies.

Antibodies that bind the target antigen on tumor cells can be conjugated to one or more specific drug-linkers (see ADC Optimization section) and screened for in vitro cytotoxic and/or antiproliferative activity against tumor cell line panels. ADCs provide the most direct assessment of antibodies as drug-delivery vehicles and are therefore recommended as an initial in vitro screening strategy. Anti-IgG (secondary) drug conjugates have also been used for antibody screening.[40] Internalization assays are not recommended for screening of ADCs or unconjugated antibodies as they are indirect and poorly predictive surrogates for targeted toxicity. The in vitro potency of ADCs is commonly compared for a panel of antigen positive cell lines versus antigen negative cell lines. The selectivity of ADCs for antigen-positive over antigen-negative lines is reflected in differences in IC_{50} values for cytotoxicity that are typically >=100-fold. A highly desirable attribute of antibodies for ADCs is species cross-reactivity to the corresponding nonhuman primate, and ideally rodent antigens, to facilitate preclinical assessment of on-target toxicity of ADCs.

Large human antibody panels can be obtained from immunization of transgenic mice in conjunction with hybridoma technology [41] or by selection from phage display libraries.[42,43] Human antibodies can also be generated using emerging technologies such as yeast, ribosome or mRNA display.[42,43] Murine MAb identified using hybridoma technology,[9] can also provide a useful starting point for ADC lead generation. Murine MAb have several well-known shortcomings as therapeutics-immunogenicity, short terminal half-life, and commonly inefficient effector functions with human immune cells. However, these limitations of murine MAb have been largely overcome by chimerization and humanization.[2,44] These antibody technologies each have strengths and limitation for ADC lead generation as summarized below.

An attractive feature of phage and other display technologies is that they allow direct selection of species cross-reactive antibodies. Commonly, antibodies can be identified that cross-react with nonhuman primates, whereas cross-reactivity to rodents is less common. Phage display libraries also allow direct selection for efficiently internalizing antibodies,[45] that warrant exploration for use as ADCs. Phage-derived antibodies require reformatting of the displayed antibody fragment-commonly Fab or scFv-as an IgG for drug conjugation and functional comparison as ADCs. The advent of reformatting high-throughput methods has mitigated what was previously a significant bottleneck in deriving human IgG from phage display libraries.[42] It may be necessary or desirable to increase the antigen-binding affinity of phage-derived antibodies for therapeutic applications-affinity maturation. This suboptimal affinity issue has been addressed by larger and higher

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ANTIBODY LEAD OPTIMIZATION

The next step in ADC development is lead optimization, in which the antibody delivery vehicle and/or the drug payload are optimized. Permuting improvements in the antibody delivery vehicle with those in drug and linker payload is a potentially cumbersome and time-consuming task. ADC lead optimization can be streamlined in our experience by parallel optimization of the antibody and drug-linker and then combining the improvements obtained. Antibodies are commonly engineered to optimize their utility for specific therapeutic applications [2]-a paradigm that is now being extended to ADCs, as reviewed in this section.

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IgG Isotype Choice for ADCs


Table 1

A key issue for antibody therapeutics, and potentially also for ADCs, is that of isotype selection.[2,50,51] Most ADCs in clinical development use IgG1 (7/10), although IgG4 (2/10), and IgG2 (1/10) have also been used (Table 1) Isotype impacts the ability of IgG to support the secondary immune functions known as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC). For example, IgG1 can potentially bind to Fc[gamma] receptors and support ADCC and ADCP. Additionally, IgG1 can potentially bind to the complement component, C1q, and support CDC. In contrast, IgG2 and IgG4 are typically inefficient in their effector functions.[52] For ADCs, effector functions could be advantageous by providing additional antitumor activities. Conversely, effector cell binding could impair ADC localization to tumors, ADC internalization by target cells or give rise to off-target toxicities. The impact of antibody isotype on ADC function is challenging to study preclinically because of numerous differences between human and mouse immune systems including Fc[gamma] receptors and FcRn, as well the absence of endogenous IgG in immune-compromised mice typically used for xenograft studies.


Table 2

IgG2 can form covalent dimers,[53] an intriguing observation whose generality and significance for IgG2-based therapeutics remain to be determined. Nevertheless, dimerization does not seem to be a major limitation for IgG2-based drugs as judged by one IgG2 that is approved for therapeutic use, namely panitumumab,[54] and encouraging progress with several other IgG2 in clinical development [51] including one IgG2-based ADC (Tables 1 and 2)

IgG3 have been largely avoided for therapeutic applications including as ADCs for reasons that are not entirely clear but presumably relate to the differences between IgG3 and other IgG isotypes: longer hinge and greater susceptibility to proteolysis, more extensive polymorphism (13 allotypes), potentially immunogenicity, and a shorter terminal half-life (7 days vs 21 days for other IgG isotypes [55]) [2,50,51]

IgG4 have been used successfully as ADCs as demonstrated by the FDA-approved drug, gemtuzumab ozogamicin.[7] Nevertheless, IgG4 molecules may be nonideal for drug delivery in that they can exchange Fab arms in vivo to become functionally monovalent, a hypothesis [56] now supported by significant experimental evidence.[57] This apparent limitation of IgG4s can be overcome by replacing the C_H3 domain with the corresponding domain from IgG1.[57] Preclinical studies in mice may underestimate the clinical potential of IgG4 ADCs in that IgG4 have shorter terminal half-lives than their IgG1 and IgG2 counterparts.[58] In contrast, IgG4 have similar terminal half-lives as IgG1 and IgG2 in man.[55]

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Currently, there are limited data available regarding the impact of drug conjugation on IgG binding to either Fc[gamma] receptors or FcRn. Drugs conjugated through solvent accessible cysteine residues are distant from the binding sites for Fc[gamma] receptors 59,68 and FcRn 68,69 and unlikely to provide a major steric impediment to receptor binding. However, disruption of both hinge disulfides in a chimeric IgG1 by Cys->Ser mutations can greatly diminish ADCC activity, presumably via impaired binding to Fc[gamma] receptors.70 In contrast, maintaining at least one of the interheavy chain hinge disulfide bonds in a humanized IgG1 preserves efficient binding to Fc[gamma]RIIIA as judged by Cys->Ser mutations.71 For lysine conjugation chemistry, as exemplified by huN901-DM1, the drug is distributed among ~40 different sites.72 Thus, interference with binding to FcRn and Fc[gamma] receptors is likely minimized by the low occupancy of individual lysine sites in the population of lysine-conjugated ADCs.

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Antigen-Binding Affinity

One of the most intensively studied properties of antibodies is that of antigen-binding affinity.2 Antibody conjugation to drugs is undertaken so as to have little or no impact on antigen-binding affinity.73 This is accomplished by carefully controlling the number of drugs attached to each antibody, as discussed below. Limited data are available on the relationship between antigen-binding affinity and antitumor activity of ADCs. Important and as yet unanswered questions are whether or not there is an optimal affinity for ADC activity in vitro and in vivo, and if the antigen binding affinity impacts the therapeutic index.

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ADC OPTIMIZATION

Many different antibodies, drugs, and linkers have been combined to create ADCs. Cytotoxic drugs being explored for ADCs generally have potencies several orders of magnitude greater than for conventional chemotherapeutics, making them too potent for systemic delivery. Linkers used to attach the drugs to the antibody delivery vehicles have been designed to exploit intracellular conditions for drug release including the acidic environment of endosomes (pH 5.5-6.2) and lysosomes (pH 4.5-5.0), high thiol concentrations in the cytosol, and proteolytic enzymes in lysosomes. These linkers have provided half-lives of drug retention on circulating ADCs in clinical studies of ~43 hours for the hydrazone in BR96-doxorubicin,74 and ~41 hours for the sterically-hindered disulfide in huC242-DM1.75 Peptide linkers offer the promise of even greater stability as judged by half-lives of 7-10 days in preclinical in vivo studies.76

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Drug Potency and ADC Activity



Figure 3

Early ADCs used clinically approved chemotherapeutic drugs because of their ready availability, amenability to chemical manipulation and their well known toxicological properties.77 Much about the strengths and pitfalls of antibody-mediated drug delivery was learned from BR96-doxorubicin (Fig. 3), an ADC directed against the Lewis^Y tetra-saccharide antigen on human carcinomas.78,79 Doxorubicin was attached to a chimeric version of the BR96 antibody, through an acid-labile hydrazone linker. Upon binding to the antigen, the antibody was internalized and doxorubicin released within acidic endosomal and lysosomal vesicles. Treatment of tumor-bearing mice and rats with BR96-doxorubicin led to immunologically specific tumor cures, albeit at high (>100 mg/kg) doses, reflecting the low potency of the targeted drug.79 In a phase I clinical trial, the maximum-tolerated dose of BR96-doxorubicin was ~700 mg/m^2, with dose-limiting gastrointestinal toxicities.74 In a

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Calicheamicins are a family of enediyne antibiotics derived from the soil microorganisim, *Micromonspora echinospora* ssp. *calichensis*.[88] These drugs bind to the minor groove of DNA and induce double-stranded DNA breaks, resulting in cell death with >100-fold the potency of most standard chemotherapeutics.[84] As with BR96-doxorubicin, an acid-labile hydrazone linker was used to link calicheamicin to antibody delivery vehicles (Fig. 3), with a similar half-life for drug release from the ADC of ~48 hours.[89] Pronounced antitumor activities have been obtained with calicheamicin-containing ADCs targeting MUC1.[82,90] This calicheamicin ADC technology has also been successfully applied to antibodies recognizing a range of tumor antigens including: CD22,[92-94] CD33,[91,95] Lewis^{Y}[96] and oncofetal protein, 5T4.[97] ADCs utilizing a related enediyene, calicheamicin [theta]$^{I}_{1}$, have been used successfully to target tumors expressing ganglioside GD2 [98] and [gamma]-glutamyltransferase.[99] The doses required for activity were much lower than that required for a doxorubicin ADC, reflecting the greatly increased potency of the targeted drug. The sole clinically approved ADC to date is the calicheamicin-containing conjugate, gemtuzumab ozogamicin.

The effects of calicheamicin ADCs are in many cases antigen-specific.[90-97,100] In contrast, gemtuzumab ozogamicin can elicit potent antitumor activity in an antigen-independent manner on solid tumors in vivo.[101] These effects were attributed to passive targeting, and required the use of an acid-labile hydrazone linker. Nonspecific drug release through linker hydrolysis may contribute to the activity of gemtuzumab ozogamicin in these models. Passive targeting offers a potential explanation of the activity of gemtuzumab ozogamicin in some AML patients where the cognate antigen, CD33, was not detected [101,102] and is potentially extendable to other tumor types.

Maytansinoids [85] and auristatins [11] represent other classes of highly potent drugs that have been widely used for ADC development. These chemically unrelated drugs (Fig. 3) are 100-1,000 fold more cytotoxic than most cancer chemotherapeutics.[85,103] They act by binding to tubulin at the same site as the vinca alkaloids and inhibiting tubulin polymerization. A variety of maytansinoids have been derived semi-synthetically from ansamitocin isolated from the microorganism, *Actinosynema pretiosum* spp. *pretiosum*.[104] In contrast, auristatins [103,105] are fully synthetic analogs of the natural product dolastatin 10, which was originally isolated from the sea mollusk, *Dolabela auricularia*.[106]

One of the first maytansinoid-containing ADCs was C242-DM1, which targets CanAg, a tumor-selective carbohydrate epitope.[24] C242-DM1 was generated by modifying antibody lysine residues with a bifunctional cross-linking reagent, followed by the addition of the thiol-containing maytansinoid, DM1 (Fig. 3) The resultant ADC, C242-DM1, contained ~4 drugs/antibody and had potent and immunologically specific antitumor activity. Indeed, C242-DM1 eradicated established tumors in one xenograft model and gave complete responses in 2 other models with heterogeneous antigen expression.[24] The clinical potential of C242-DM1 was enhanced by humanization of the antibody component to create, huC242-DM1 (cantuzumab mertansine). The huC242-DM1 conjugate, like C242-DM1, had robust activity against tumors with heterogeneous antigen expression, reflecting its ability to kill bystander antigen negative tumor cells.[25] In vivo studies revealed that conjugation of DM1 to huC242 had no significant impact upon the biodistribution of the antibody in tumor-bearing mice.[17] The antibody component of huC242-DM1 has a terminal half-life of ~100 hours in mice, whereas terminal half-life of DM1 was about 4-fold shorter,[24] suggesting slow release of DM1 from the ADC in circulation. Similarly, in a subsequent phase I clinical study, the terminal half-lives of the huC242-DM1 ADC and DM1 were ~100 hours and ~24 hours, respectively.[75] The most likely mechanism for DM1 release from huC242-DM1 is by disulfide exchange with other sulfhydryls.[171] Analysis of human plasma indicates the presence of up to ~500 [mu]M free sulfhydryls that are almost entirely from albumin.[107] The huC242-DM1 conjugate was generally well tolerated in 2 phase I clinical trials with evidence for antitumor activity.[75,108]

A series of maytansinoid-disulfide linker derivatives with varying degrees of steric hindrance were subsequently developed and leads such as DM4 selected for antibody attachment.[109] The huC242-DM4 conjugate displays improved linker stability over huC242-DM1 resulting from increased steric hindrance around the disulfide bond [109] (Fig. 3)

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The auristatin, monomethyl auristatin E (MMAE), was modified with dipeptide linkers, and the resulting drug derivatives were linked to solvent-accessible antibody thiol groups,[105] generating conjugates of uniform composition (Fig. 3) The conjugates target CD30 on Hodgkin lymphoma and Lewis^Y on carcinomas. In vitro studies demonstrated that the MMAE peptide-linked ADCs were highly potent with 10-100-fold greater immunologically dependent cell kill compared with corresponding hydrazone-based ADCs. The half-lives of drug release from ADCs in vivo were 6 and 10 days in mice and cynomolgus macaques, respectively,[76] longer than previously described linkers. Furthermore, the peptide-linked MMAE ADCs were less toxic than corresponding hydrazone-linked ADCs.[103]

As shown first for antibodies targeting CD30 and Lewis^Y, auristatin-based ADCs with peptide linkers had pronounced antitumor activity in xenograft models, leading to cures of established tumors at very small fractions of the maximum tolerated doses.[103] Subsequently, auristatin ADC technology has been successfully applied to antibodies recognizing a wide range of tumor antigens, including BCMA,[123] CD19,[124] CD20,[37] CD70,[125] CD79,[114] E selectin,[126] EphB2,[127] glycoprotein NMB,[128] melanotransferrin/p97,[168] MUC16,[129] PSMA,[22] and TMEFF2.[130]

Because auristatins are synthetic, integral structural modifications can be made that significantly alter the properties of the drug. One such auristatin, MMAF, terminates with phenylalanine, a negatively charged residue that impairs cell membrane permeability.[105] Consequently, ADCs containing MMAF that facilitate drug uptake by antigen-positive cells are >2,000-fold more potent than the free drug itself. MMAF linked to an anti-CD70 antibody through a peptide linker was active against renal cell carcinoma xenografts and also circumvented multidrug resistance mechanisms.[125]

A surprising finding with MMAF ADCs was that the cleavable peptide linker could be eliminated, and highly active ADCs were obtained by direct attachment of the drug to anti-CD30 and anti-Lewis^Y antibodies through thioether adducts (Fig. 3)[105] Mass spectrometry showed that the released drug was a cysteine-adduct of the linker-MMAF derivative, presumably resulting from antibody degradation within lysosomes. The closely related auristatin, MMAE, was not active when attached in this manner, indicating that ADCs requiring antibody degradation for drug release are highly dependent on the nature of the drug for activity. MMAF can sustain significant modification to the N-terminal position and still remain active, while most other drugs are inactivated when modified in such an extensive manner. Similar results to that of MMAF were obtained with a thioether adduct of DM1, which was appended to lysine residues on the humanized antibody, huC242, (Fig. 3) In this case, the released drug upon cell incubation was a lysine-DM1 derivative in which the lysine residue was the attachment residue on the antibody delivery vehicle.[131] The requirements for effective cell kill using linkers that require antibody degradation for drug release remain to be elucidated. Although there was little potency loss with thioether adducts of MMAF in hematologic malignancies targeted with an anti-CD30 antibody (cAC10), a thioether adducts of DM1 had approximately one hundredth the in vitro potency of a corresponding disulfide-linked ADC [81] together with greatly diminished in vivo efficacy and potency.[131] The efficacy of ADCs requiring antibody degradation for drug release are likely to be antibody, drug and tumor dependent, in contrast to ADCs with cleavable linkers.

In a mouse preclinical model, an anti-CD70 (h1F6) ADC with a thioether linkage to MMAF had a half-life of 7 days for drug release,[132] which is similar to the 6-10 day half-life for drug release from the corresponding peptide-linked ADC.[76] Furthermore, an ADC with acetamide-thiol adduct in place of the maleimide-thioether (Fig. 3) did not lose any detectable drug from circulating ADC over 2 weeks in mice. The tolerability, efficacy, and intratumoral drug concentrations of the maleimide and acetamide-linked ADCs were similar.[132] Thus, extending the half-life of the ADCs beyond that of 6-10 days did not lead to activity improvements in preclinical models.

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Conjugation Technologies

ADCs described thus far were formed by reacting drugs or chemical crosslinking reagents with solvent accessible reactive

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The first auristatin-containing ADCs were generated by reducing all of the interchain disulfide bonds in the antibody, and then attaching the drugs to the resulting cysteine residues.[103,122] These ADCs were close to homogeneous with 8 drugs/antibody, attached at the predetermined cysteine residues. The binding properties of the antibody were unaffected, because the drug was conjugated in the hinge region, which is distant from the antigen binding sites. However, the pharmacokinetic properties of the antibody delivery vehicle were adversely affected, and the 8-loaded ADC cleared much more rapidly than the parent antibody.[135] This prompted investigation of anti-CD30 ADCs (cAC10-MMAE) with varied drug/antibody ratios to assess the impact of drug stoichiometry on therapeutic potential. An ADC with a mean loading of 4 drugs/antibody was fractionated by hydrophobic interaction chromatography to yield purified ADCs with 2, 4, or 8 MMAE drugs/antibody, designated E2, E4, and E8, respectively.[135] ADC potency in vitro correlated with drug loading in that the order of potency was E8 > E4 > E2. In contrast, the in vivo antitumor activity of E4 was comparable with E8 at equal antibody doses, although the E4 contained only half the amount of drug per antibody. These efficacy differences correlated with increased exposure to the E4 conjugate, since slower clearance resulted in a greater pharmacokinetic area-under-the-curve. E2 also had antitumor activity in vivo, albeit less potent than for E4. The maximum-tolerated dose increased inversely with respect to drug loading. Thus, the drug/antibody ratio is an important parameter in the design of ADCs as evidenced by >=2-fold increase in therapeutic index by reducing the loading from 8 to 4 drugs/antibody.[135] An optimized anti-CD30 ADC with a mean loading of 4 MMAE drugs/antibody, now known as SGN-35,[26,136] is well tolerated and has elicited multiple partial responses in a phase I clinical trial (Tables 1 and 2,[137])

Antibodies can be engineered for site-specific drug conjugation to enable the production of homogeneous ADCs with defined sites and stoichiometries of drug attachment, eg, a panel of anti-CD30 antibodies was generated in which solvent accessible cysteines were systemically replaced with serines. These Cys->Ser antibody variants were used to create homogeneous ADCs with 2 or 4 MMAE drugs/antibody [73] that were compared with those derived from the parent anti-CD30 IgG1. Surprisingly, the most homogenous ADCs with 4 drugs/antibody at defined sites had comparable, rather than improved, in vitro and in vivo properties as the most heterogeneous conjugate having 0-8 drugs/antibody with a mean of 4 drugs/antibody and variable sites of drug attachment. Thus, the mean drug/antibody ratio seems to be a more significant determinant of ADC potency and tolerability than conjugate homogeneity or sites of drug attachment.[73,135,138]

Antibody engineering involving the addition of accessible cysteines has also been used for site-specific drug conjugation [139,140] or to create novel intermolecular [141,142] or intramolecular [143] disulfide bonds. Such site-specific conjugation to IgG was first demonstrated by Lyons et al who engineered an extra cysteine residue at various locations within the C_H1 domain of an IgG4.[139] Subsequently, Stimmel et al engineered a cysteine residue into the C_H3 domain of an IgG4.[140] Although the engineered cysteine was blocked, mild and selective reduction conditions were devised thereby site-specific labeling with a radionuclide chelator. Recently, the efficiency of site-specific conjugation to IgG has been improved by better choices of cysteine replacement [144]-"thiomabs"-combined with refinements in conjugation methodology that reduce and then restore native interchain disulfide bonds.[170] A thiomab-drug conjugate with 1.6 auristatin drugs/antibody was compared with a conventional ADC with 3.1 auristatin drugs/antibody. The thiomab-drug conjugate showed comparable efficacy in a xenograft study, improved tolerability in rats and nonhuman primates and thus an increased therapeutic index (Junutula et al, unpublished). This improved therapeutic index may be due to thiomabs per se and/or reduction in the drug/antibody ratio.[135] The potential broad applicability of thiomab-drug conjugates provides a strong impetus to assess the reproducibility and transferability of the increased therapeutic index between different xenograft models, antibodies, drugs, and linkers.

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CLINICAL EXPERIENCE WITH GEMTUZUMAB OZOGAMICIN

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Gemtuzumab ozogamicin was approved by the US Food and Drug Administration in 2000 for the treatment of CD33 positive AML in first relapse in patients of at least 60 years of age and considered unsuitable candidates for cytotoxic chemotherapy.7 Gemtuzumab ozogamicin approval was based upon interim data analysis of 3 open label single-arm phase II studies in AML at first relapse. Patients received intravenous gemtuzumab ozogamicin at 9 mg/m^2 at 2 weekly intervals for 2 doses. Thirty percent of the patients (42 of 142) achieved a complete remission: <=5% blasts in the marrow, recovery of neutrophils to >=1,500/[mu]L and erythrocyte and platelet transfusion independence. Final analysis of these 3 gemtuzumab ozogamicin phase II studies with a larger number of patients (*n* = 277) demonstrated a 26% remission rate and a median overall survival of 4.9 months.149

Gemtuzumab ozogamicin induced severe myelosuppression in all patients including grade 3 or 4 thrombocytopenia (99%), neutropenia (98%), and anemia (52%), as anticipated from the CD33 expression on normal hematopoietic precursors.149 The myelosuppression was reversible as expected, since hematopoietic stem cells are CD33 negative and presumably spared. Gemtuzumab ozogamicin, as for other antibody-based therapeutics, can also give rise to acute infusion-related adverse events. Grade 3 or 4 infusion-related adverse events were much more common for the first dose (30%) than the second dose (10%) and included chills (8%), fever (6%), hypotension (4%), nausea (3%), and hypertension (2%). The incidence of hepatotoxicity associated with gemtuzumab ozogamicin included grade 3 or 4 hyperbilirubinemia (29%), elevations of aspartate aminotransferase (18%) or alanine aminotransferase (9%) that were generally transient and reversible without medical intervention. None of the patients developed an antibody response to either the antibody or drug-linker components of gemtuzumab ozogamicin. Additional grade 3 and 4 adverse events associated with gemtuzumab ozogamicin include sepsis and dyspnea.

Severe veno-occlusive disease (sinusoidal obstruction) has been associated with the use of gemtuzumab ozogamicin at incidences that vary greatly with the clinical setting.149-152 A low incidence (~1%) of veno-occlusive disease was observed following gemtuzumab ozogamicin monotherapy in patients without prior or subsequent hematopoietic stem cell transplants.149 In contrast, a higher incidence (22%) of veno-occlusive disease, sometimes fatal, was observed in patients who had received hematopoeitic stem cell transplantation before gemtuzumab ozogamicin. An intermediate rate (15%) of veno-occlusive disease was observed in patients transplanted after gemtuzumab ozogamicin therapy.

In contrast to the US Food and Drug Administration, the European Medicines Agency denied marketing authorization for gemtuzumab ozogamicin in Europe in 2007, citing concerns that the benefits of gemtuzumab ozogamicin in the treatment of AML had not been established and did not outweigh its risks. Several randomized and controlled trials are in progress to assess the benefit of gemtuzumab ozogamicin. Moreover, preliminary analysis of one such randomized clinical trial, MRC AML 15 (*n* = 1,113), revealed that the addition of gemtuzumab ozogamicin to induction chemotherapy for AML improves disease free survival without extra toxicity.153

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ADCs in Clinical Development

There are a growing number of ADCs in clinical development as discussed below-10 as of April 2008 including gemtuzumab ozogamicin (Tables 1 and 2) Below is a brief description of many of these ADCs and their performance in early stage clinical trials.

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SGN-35

SGN-35 is a CD30-targeting ADC with a protease-cleavable linker (valine-citrulline) between the antibody and the auristatin drug, MMAE, with a mean loading of ~4 drugs/antibody.26,136 CD30 is a member of the TNF receptor family with highly restricted normal tissue expression that includes activated, but not resting, T and B cells. CD30 expression by malignant

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CR011-vcMMAE is an ADC targeting glycoprotein NMB that, like SGN-35, includes a valine-citrulline protease cleavable linker between the antibody and the auristatin drug, MMAE, with a mean loading of ~3.8 drugs/antibody.[128,154] Glycoprotein NMB is a 560 amino acid glycoprotein of unknown function that is highly expressed in melanoma tissue, albeit predominantly intracellularly with a small cell surface component. Glycoprotein NMB expression has also been associated with glioma, liver carcinoma, squamous cell lung cancer, and soft tissue tumors, whereas normal tissue expression is restricted.[128]
A phase I/II trial of CR011-vcMMAE has been initiated in patients with unresectable stage III or IV melanoma (Table 2) Preliminary data have been reported on 25 patients who received escalating doses of CR011-vcMMAE from 0.03 to 1.88 mg/kg administered intravenously every 3 weeks for 4 cycles.[155] No objective responses were observed, although 7 of 22 evaluable patients demonstrated stable disease. Overall, CR011-vcMMAE has been well tolerated with no dose-limiting toxicities. Most drug-related adverse events were grade 1 and 2, with grade 3 or 4 neutropenia and leukopenia reported for 2 patients.

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Trastuzumab-DM1

The ADC, trastuzumab-DM1,[35,36,116] contains the humanized anti-HER2 IgG1 antibody, trastuzumab (formerly known as humAb4D5-8,[156]) conjugated to the maytansinoid, DM1, via a thioether linkage.[25,131] HER2 is a member of the EGFR family of growth factor receptors. HER2 is overexpressed as a result of gene amplification in ~25% of primary breast cancers and is associated with poor prognosis.[157] HER2 is expressed at lower levels in several normal tissues including gastrointestinal tract epithelia, tonsil, kidney tubules, and endometrium as described in the package insert for the diagnostic, HercepTest(TM). Trastuzumab-DM1 is unique among the ADCs in clinical development in that the parent antibody, trastuzumab (Herceptin) is a clinically and commercially successful therapeutic in its own right. Indeed, trastuzumab in combination with cytotoxic chemotherapy prolongs overall survival in patients with HER2-positive breast cancer in both the metastatic [158] and adjuvant [159] settings.
In a phase I study in HER2-positive metastatic breast cancer trastuzumab-DM1 was administered at 6 dose levels of 0.3-4.8 mg/kg every 3 weeks.[35,36] Partial responses were observed in 6 of 24 patients treated at or at below the maximum-tolerated dose of 3.6 mg/kg. Drug-related adverse events of grade 2 or higher have been infrequent and manageable. Dose-limiting grade 4 thrombocytopenia was observed in 2 of 3 patients treated at 4.8 mg/kg. Platelet nadirs were observed on about day 8 with recovery by day 15. No cardiotoxicity has been observed with trastuzumab-DM1, in contrast to trastuzumab used in conjunction with anthracyclines.[158] A phase II trial of trastuzumab-DM1 has been initiated in metastatic breast cancer.

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AVE9633

AVE9633 is a humanized antibody-maytansinoid (DM4) conjugate [112] that targets CD33-the tumor antigen validated by gemtuzumab ozogamicin. A phase I study has been initiated with AVE9633 in patients with relapsed or refractory CD33-positive AML (Table 2) AVE9633 was administered intravenously at dose levels of 30-150 mg/m^2 on 2 consecutive weeks every 4 weeks.[112] One complete response with incomplete platelet recovery and one partial response were observed from the first 17 patients treated. Two dose-limiting toxicities were seen at the dose of 150 mg/m^2 x 2 consisting of liver toxicity and keratitis. The most frequent grade 1 or 2 adverse events were infusion reactions-fevers, chills, hypotension, and musculoskeletal pain. Saturation and down-regulation of CD33 on the surface of blood and bone marrow blasts was observed from the 50 mg/m^2 dose. No human antibody response has been detected to either the ADC or DM4.

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HuC242-DM4

As previously described, huC242-DM4 is a humanized antibody-maytansinoid conjugate targeting CanAg,[109,131] a tumor-selective carbohydrate epitope. CanAg is highly expressed in most pancreatic, biliary and colorectal cancers as well as a significant proportion of gastric (55%), uterine (45%), nonsmall cell lung cancer (40%), and bladder cancers,[161,162] whereas only minimal immunostaining of CanAg in normal tissue has been reported.[162]

A phase I trial of HuC242-DM4 has been initiated in patients with metastatic or inoperable colorectal, pancreatic, and other CanAg positive cancers (Table 2) HuC242-DM4 was administered by intravenous infusion at dose levels of 18-297 mg/m^2-every 3 weeks.[163] No objective responses have been reported from the first 30 patients treated. Dose-limiting toxicity of decreased visual acuity, corneal deposits, and keratitis were experienced by 2 of 6 patients treated at the 223 mg/m^2 dose level. Both patients responded favorably to treatment with lubricating eye drops. HuC242-DM4 was well tolerated at the 168 mg/m^2 with 1 of the 8 patients experiencing a dose-limiting toxicity consisting of grade 3 diarrhea and dehydration that improved with intravenous fluids. Preliminary pharmacokinetic data revealed that huC242-DM4 had a half-life of ~5 days in patients with low plasma levels of antigen. Clearance of huC242-DM4 was increased in patients with high levels of shed antigen (>900 units/mL). No clinically significant myelosuppression has been observed, and antibody generation to the humanized antibody or DM4 has not been detected.

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HuN901-DM1

HuN901-DM1 is a humanized antibody-maytansinoid conjugate targeting CD56,[113] the neuronal cell adhesion molecule that is expressed in virtually all small cell lung carcinomas, neuroblastomas, gliomas, astrocytomas, and rhabdomyosarcomas, as well as ~78% of multiple myelomas and ~53% of AML.[164] As for normal tissue, CD56 is expressed on natural killer cells, neuroendocrine glands, the central and peripheral nervous systems and also cardiomyocytes.[164]

In a phase II study, patients with relapsed small-cell lung cancer and other CD56-positive small cell cancers were treated with huN901-DM1 intravenously at 60 mg/m^2/wk for 4 consecutive weeks every 6 weeks (Table 2) Two partial responses and 5 stable diseases were observed in the first 30 patients treated.[165] Transient severe headaches were observed in a few patients, but did not occur in subsequent patients premedicated with steroids. HuN901-DM1 was generally well tolerated, with no evidence of clinically significant myelosuppression and no serious infusion reactions. No human antibody response has been detected to either the antibody or drug components of the ADC. Additionally, a phase I study of huN901-DM1 is underway in patients with relapsed or refractory CD56-positive multiple myeloma (Table 2) Patients received escalating doses of huN901-DM1 of 40-90 mg/m^2/wk by intravenous infusion on 2 consecutive weeks every 3 weeks.[166] One minimal objective response has been observed for the first 12 patients treated. HuN901-DM1 has been well tolerated with no serious drug-related adverse events and no dose-limiting toxicity observed for the first 12 patients treated. Immunohistochemistry performed on bone marrow aspirates 24 hours after huN901-DM1 infusion at 40 mg/m^2 confirmed localization of ADC to myeloma cells. Preliminary pharmacokinetic analysis revealed a ~20 hour terminal half-life for huN901-DM1.

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CONCLUSIONS AND PERSPECTIVES

Significant advances have been made with ADCs over the last 3 decades. Building on the success of gemtuzumab ozogamicin, highly potent drugs have been attached to antibodies recognizing numerous different antigens expressed on a broad array of hematologic and solid tumors. The activities of these new ADCs underscore the importance of good target

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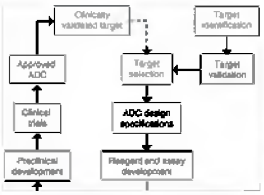
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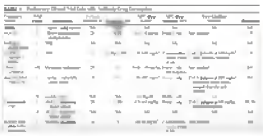
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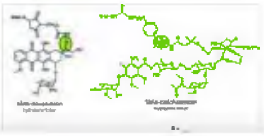


☐ Figure 2



☐ Table 1





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Appendix CARTER02

Antibody-Drug Conjugates for Cancer Therapy

Paul J. Carter, PhD, and Peter D. Senter, PhD

Abstract: The antibody-drug conjugate (ADC) concept is to use an antibody to deliver a cytotoxic drug selectively to a target such as a tumor-associated antigen. Such conjugates represent a broadly applicable approach to enhance the antitumor activity of antibodies and improve the tumor-to-normal tissue selectivity of chemotherapy. Critical parameters for ADC development include target antigen selection, conjugate internalization by tumor cells, drug potency and stability of the linker between drug and antibody. Other important considerations include the conjugation methods, drug-to-antibody ratio, and the effects of drug conjugation on antibody properties. Highly potent drugs with more stable linkers have been attached to a new generation of antibodies to create conjugates with pronounced antitumor activities in preclinical studies and encouraging results in early stage clinical trials. This review details these advances, discusses some of the remaining challenges, and overviews ADCs currently in clinical trials for cancer therapy.

Key Words: antibody, immunoconjugate, targeted therapy

(*Cancer J* 2008;14: 154–169)

Antibody-based therapeutics are of growing significance for cancer therapy as evidenced by 12 such drugs approved for oncologic indications since 1995, including 9 in the United States.[1] However, these antibody therapeutics are seldom curative, encouraging numerous approaches to enhance efficacy.[2–5] Two of the most promising strategies to enhance the antitumor activity of antibodies are antibody-drug conjugates (ADCs)—the focus of this review—and Fc optimization to augment secondary immune functions.[5]

ADCs comprise an antibody, usually in IgG format, conjugated to a cytotoxic drug via a chemical linker (Fig. 1). The therapeutic concept of ADCs is to use an antibody as a vehicle to deliver a cytotoxic drug to a tumor cell by means of binding to a target cell surface antigen (Fig. 1). ADCs are prodrugs requiring drug release for activation, commonly after ADC internalization into the target cell. Numerous preclinical efficacy studies show that ADCs have significant potential for enhancing the antitumor activity of "naked" antibodies and reducing the systemic toxicity of the conjugated drugs.[3,6] Moreover, clinical demonstration of the ADC concept has been provided by gemtuzumab ozogamicin (Mylotarg), a humanized anti-CD33 antibody conjugated to calicheamicin approved in the United States for the treatment of acute myeloid leukemia (AML).[7]

The concept of arming antibodies by conjugation to protein toxins dates back to 1970,[8] and was followed a few years later by antibody conjugates with cytotoxic drugs. Antibody therapeutics have come of age in the intervening decades with the advent of hybridoma technology to develop murine monoclonal antibodies (MAb),[9] chimerization and humanization to address the shortcomings of murine MAb as therapeutics, and more recently with direct routes to human antibodies using phage display or transgenic mice.[2] The clinical potential of ADCs has been greatly enhanced by improved choices of targets,[10] more potent drugs in conjunction with linkers of improved stability[6,11] and greatly expanded knowledge of ADC cell biology[32] and pharmacology.[13] ADCs are reviewed here from a design perspective from target selection, through ADC lead identification, optimization, and preclinical development to clinical experience (Fig. 2). Other payloading strategies for antibodies, as well as for alternative delivery vehicles, have been extensively reviewed elsewhere[3,11,12,14] and are outside the scope of this article. Of these, radioimmunoconjugates stand out, with the approval of 2 different anti-CD20 radioimmunoconjugates for the treatment of non-Hodgkin lymphoma: ibritumomab tiuxetan (Zevalin, ^{90}Y conjugate) and ^{131}I tositumomab (Bexxar).[15]

TARGET SELECTION

The de novo identification and validation of cell surface targets as candidates for ADCs is beyond the scope of this article and is reviewed elsewhere.[10] Target selection from a candidate pool is a critical first step in generating ADCs (Fig. 2). Expression profiling is a key aspect of target selection in that tumor-associated antigen expression is presumed to be one of the necessary criteria for successful targeting with an ADC, whereas little or no normal tissue expression is desirable and may be necessary to achieve an adequate therapeutic index.

Immunohistochemistry allows semi-quantitative analysis of protein expression in tissue samples and is often the most direct and consequently preferred method for evaluating potential ADC targets. The advent of tissue microarrays[16] facilitates high-throughput expression profiling—hundreds to thousands of samples—with greater standardization.[17] A limitation of immunohistochemistry is the high variability of data with different reagents, protocols and samples[17]—resolvable if concordant data are obtained using multiple antibodies. An additional challenge with immunohistochemistry is that data may be difficult to interpret if the antigen is

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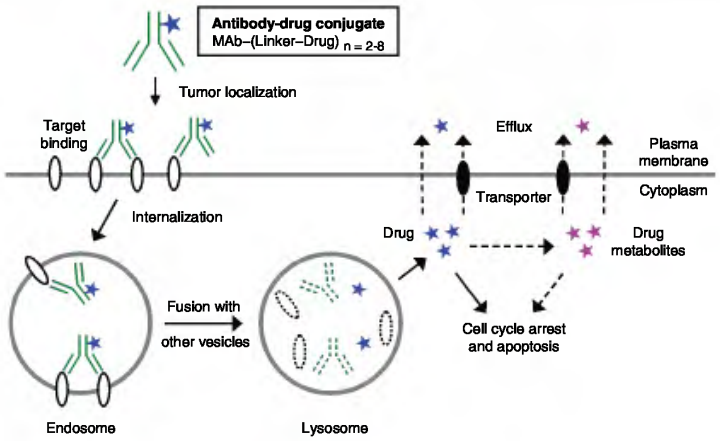


FIGURE 1. Antibody-drug conjugate concept. An ADC comprises an antibody conjugated via a linker to a cytotoxic drug. ADCs are commonly in IgG format with 2–8 drugs/antibody. Some portion of an ADC delivered intravenously localizes to a tumor and binds to a target antigen on the surface of the tumor cell. Internalization of the ADC commonly proceeds via a clathrin-coated pit mechanism[167] although other mechanisms such as calveolae[168] and pinocytosis sometimes occur.[32] Internalized vesicles are stripped of clathrin, fuse with other vesicles and enter the endosome-lysosome pathway. Proteases in the acidic environment of the endosomes digest the antibody, and potentially also the linker, to release free drug. The drug then crosses the membrane to enter the cytoplasm where it binds to its molecular target, commonly leading to cell cycle arrest and apoptosis. A portion of the drug is effluxed from the cell, either by passive diffusion, active transport, or leakage from dying cells. If the effluxed drug is cell permeable it may enter neighboring cells and give rise to so-called bystander cell killing.[25,131] In some cases, the drug is metabolized in the cell and these metabolites may differ from the parent drug in their ability to kill the tumor cells as well as in the propensity to be effluxed.[131]

located inside the cell as well as on the cell surface. Flow cytometry is also used for protein expression profiling, particularly for hematologic tumors. This method provides more quantitative information than immunohistochemistry and can demonstrate the accessibility of a target to an antibody. Limitations of flow cytometry include the limited availability of suitable patient samples, and the need to disaggregate solid tumor samples, that may potentially compromise antigen levels.

Expression profiling at the mRNA level is widely used for target assessment and can be a valuable, albeit indirect adjunct, to protein expression profiling.[10] It is important to verify the concordance of mRNA and protein levels, as demonstrated in at least a minority of cases.[18] Techniques available for mRNA expression profiling in clinical samples include microarrays, serial analysis of gene expression, quantitative real-time PCR, cDNA hybridizations using multiple tissue expression arrays/cancer profiling arrays, and RNA in situ hybridizations using frozen tissue samples.[10]

Tumor Expression of Antigen

ADCs to date have been used almost exclusively to target antigens located on the surface of tumor cells. These targets are almost always normal antigens either expressed at abnormal times in development, at aberrantly high levels on cell surfaces, or with such limited normal tissue expression as to render them potential suitable for targeted therapy. Beyond malignant cells, ADCs could potentially be used to target antigens on normal cells recruited to the tumor such as endothelial cell or tumor-associated neovasculature cells.[19,20] Indeed, at least 2 different ADCs have been developed to target prostate specific membrane antigen (PSMA),[21,22] an antigen associated with prostate cancer and more recently found to be expressed in the neovascular endothelium of a variety of solid tumors and essentially absent from normal vasculature.[19] The anti-PSMA ADC, MLN2704 (J591-DM1,[21]), has been evaluated in prostate cancer and showed antitumor activity at well-tolerated doses,[6] although clinical development of MLN2704 has subsequently been discontinued. Tumor-associated extracellular matrix protein variants such as the extradomain B of fibronectin domain and the long isoform of tenascin C have been successfully targeted with a panoply of experimental antibody-based drugs.[19] Targeting such extracellular matrix variants with ADCs would require drug and linker selections that facilitate extracellular drug release and drug entry into tumor cells.

High level and homogeneous antigen expression on the external surface of all tumor cells seems desirable to facilitate efficient targeting with ADCs. Elevated antigen expression can lead to greater ADC potency, as judged by in vitro and in vivo experiments with transfected cell lines.[127] However, such high level antigen expression is not a prerequisite for clinical benefit by ADCs as demonstrated by gemtuzumab ozogamicin approved for the treatment of AML with only approximately 5,000–10,000 copies of the cognate antigen, CD33, per cell.[23] Homogeneous antigen expression may be beneficial for targeting with ADCs, but is not a requirement, as demonstrated by in vivo xenograft studies with a heterogeneously expressed antigen, CanAg.[24] Successful targeting of heterogeneously expressed antigens may benefit from the

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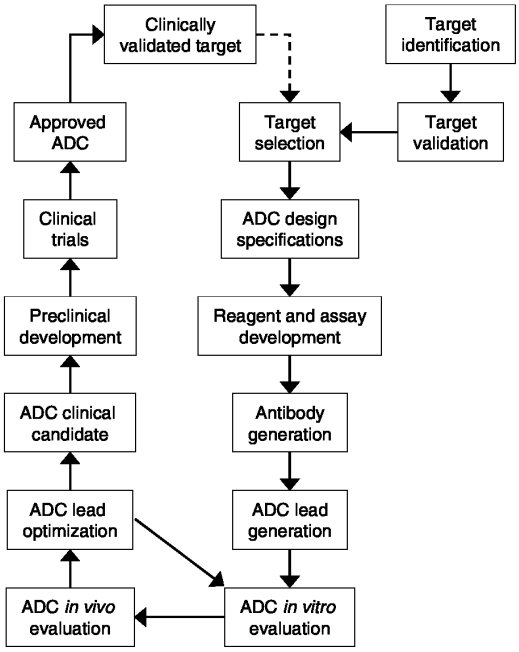


FIGURE 2. Antibody-drug conjugate development cycle. The development of ADCs, as for other antibody therapeutics,[2] can be conceptualized as a design-driven process that begins with target selection. The next step is to formulate the ADC design specification, which is a list of critical or desirable properties predicted to facilitate preclinical development of the ADC and to achieve the desired clinical outcome. The primary goal with an ADC is to deliver a cytotoxic drug selectively to a target within a tumor. An ADC can potentially be designed with additional mechanisms of antitumor activity, eg, by interfering with the biologic function of a target that is involved in oncogenesis, angiogenesis or metastasis. The design specification guides the method of antibody generation, plus the reagents and assays needed to identify ADC leads with the desired properties. Panels of ADCs are evaluated in vitro for cytotoxic activity against tumor cell lines and then in vivo for antitumor activity, pharmacokinetics and safety. Optimization of the antibody delivery vehicle and/or the drug-linker payload may be necessary or desirable, eg, to increase the therapeutic index of the ADC. The ADC clinical candidate is then subjected to further preclinical and, if warranted, clinical development. ADC clinical trial data—particularly if they validate the target through to drug approval—may provide the impetus to iterate the design cycle to create an improved ADC.

use of ADCs with bystander killing activity[25,26] or by combining an ADC with another therapeutic modality such as cytotoxic chemotherapy.[27]

Optimally, the target antigen is expressed in most, if not all, patients with a particular tumor type. If not, a diagnostic test for antigen expression may be required for patient selection as used for HER2 expression profiling before trastuzumab therapy, and for EGFR profiling before treatment with cetuximab or panitumumab.[17] Flow cytometry is used widely for identifying antigen-positive hematologic tumors.[28] In addition, the target antigen should be expressed throughout the course of disease. Minimally, the antigen should be expressed in late stage disease—ie, patient populations commonly available for evaluating experimental drugs.

Normal Tissue Expression of Antigen

The target antigen should ideally be absent from normal tissue, but this is seldom, if ever, achieved. Tumor-associated antigens are commonly selected on the basis of higher expression levels in tumors and limited normal tissue expression. Some normal tissue expression of antigen is anticipated and may be acceptable. Unfortunately, there is currently no reliable way to predict the significance of normal tissue expression of an antigen on ADC tolerability. Therefore, it is highly desirable to develop ADCs that cross-react to the corresponding antigen from other species—so called species cross-reactivity—to allow preclinical assessment of antigen-dependent toxicity, also known as "on-target" toxicity.

Antigen expression on normal tissue can lead to on-target toxicity with ADCs in patients,[29,30] but does not necessarily do so. For example, inefficient ADC localization to antigen-positive normal tissue is presumed to lower the risk of on-target toxicity. ADC localization may be particularly inefficient if the level of antigen expression is low, the antigenic sites are located in the central nervous system beyond the blood brain barrier or if the antigen is restricted to the apical surface of cells. Nonproliferating cells have reduced sensitivity to many cytotoxic drugs as compared with proliferating cells,[31] an attribute that that seems likely to apply to corresponding ADCs[32] and may reduce on-target toxicity. Extensive normal tissue expression of antigen may create a "sink" that soaks up the ADC and accelerate ADC clearance.[33]

Targeting may be possible for an antigen whose expression is restricted to a nonvital organ or cell population, even if the antigen is not differentially expressed between normal tissue and tumors. For example, CD20, CD33, and CD52 are cell surface markers for B cells, myeloid progenitors plus monocytes, and leukocytes, respectively—cell lineages whose elimination by antibody therapeutics can be tolerated on a temporary basis.

Antigen Shedding

Shedding of target antigen from the surface of tumor cells into circulation may increase the risk of toxicity because of immune complex formation and deposition in kidney, accelerated clearance of ADC and impaired tumor localization. Shed antigen can sometimes be problematic for ADCs as suggested by a phase II trial for an anti-MUC1-calicheamicin conjugate in ovarian cancer.[34] However, shed antigen can sometimes be tolerated as preliminary reports of a phase I study with an anti-HER2 ADC, trastuzumab-DM1, in metastatic breast cancer.[35,36]

Target Biology

The selection of target antigens that are causally involved in tumor pathogenesis, eg, HER2 and EGFR, is not a prerequisite for success with ADCs as suggested by targeting of CD33 with gemtuzumab ozogamicin. However the choice of such "functional antigens" may be advantageous for ADCs

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by lowering the risk for tumor "escape" therapy by the reduction or elimination of antigen expression—down modulation. Additionally, the ADC may be screened for the ability to interfere with the function of the target, eg, by growth factor blockade for a growth factor receptor, to provide additional mechanisms of antitumor activity beyond delivery of a cytotoxic drug.

Antigen Internalization

ADC internalization (Fig. 1) is desirable and often necessary for efficient drug release, depending upon the drug and linker. Nevertheless, antigen internalization per se is not a useful criterion for target selection. This reflects the fact that antigen internalization may be impacted by many ADC attributes including choice of antibody epitope, as well as the drug and linker (see below). Moreover, inefficiently internalizing antigens such as CD20, a tetra-spanning membrane protein, have been successfully targeted with ADCs as judged by efficacy in xenograft experiments in vivo.[37,38] Antigen internalization can potentially be impacted by the presence of other membrane proteins, eg, CD21 expression was recently reported to impair the internalization of ADCs directed to its binding partner, CD19.[39]

ANTIBODY LEAD IDENTIFICATION

Lead generation for antibody therapeutics has evolved in recent years to the pursuit of large antibody panels—tens to hundreds of antibodies—to increase the likelihood of identifying leads that satisfy multiple design criteria.[2] This large-scale antibody screening is now being evaluated as a paradigm for ADC development. Important antibody attributes for use as conjugates include binding to the human antigen on tumor cells, cytotoxic activity as ADCs, and other desirable attributes such as cross-reactivity with species that can be used in toxicology. These important and desired antibody attributes for ADCs are discussed below, together with a comparison of the main alternative antibody generation technologies.

Antibodies that bind the target antigen on tumor cells can be conjugated to one or more specific drug-linkers (see ADC Optimization section) and screened for in vitro cytotoxic and/or antiproliferative activity against tumor cell line panels. ADCs provide the most direct assessment of antibodies as drug-delivery vehicles and are therefore recommended as an initial in vitro screening strategy. Anti-IgG (secondary) drug conjugates have also been used for antibody screening.[40] Internalization assays are not recommended for screening of ADCs or unconjugated antibodies as they are indirect and poorly predictive surrogates for targeted toxicity. The in vitro potency of ADCs is commonly compared for a panel of antigen positive cell lines versus antigen negative cell lines. The selectivity of ADCs for antigen-positive over antigen-negative lines is reflected in differences in IC_{50} values for cytotoxicity that are typically $\geq$100-fold. A highly desirable attribute of antibodies for ADCs is species cross-reactivity to the corresponding nonhuman primate, and ideally rodent antigens, to facilitate preclinical assessment of on-target toxicity of ADCs.

Large human antibody panels can be obtained from immunization of transgenic mice in conjunction with hybridoma technology[41] or by selection from phage display libraries.[42,43] Human antibodies can also be generated using emerging technologies such as yeast, ribosome or mRNA display.[42,43] Murine MAb identified using hybridoma technology,[9] can also provide a useful starting point for ADC lead generation. Murine MAb have several well-known shortcomings as therapeutics—immunogenicity, short terminal half-life, and commonly inefficient effector functions with human immune cells. However, these limitations of murine MAb have been largely overcome by chimerization and humanization.[2,44] These antibody technologies each have strengths and limitation for ADC lead generation as summarized below.

An attractive feature of phage and other display technologies is that they allow direct selection of species cross-reactive antibodies. Commonly, antibodies can be identified that cross-react with nonhuman primates, whereas cross-reactivity to rodents is less common. Phage display libraries also allow direct selection for efficiently internalizing antibodies,[45] that warrant exploration for use as ADCs. Phage-derived antibodies require reformatting of the displayed antibody fragment—commonly Fab or scFv—as an IgG for drug conjugation and functional comparison as ADCs. The advent of reformatting high-throughput methods has mitigated what was previously a significant bottleneck in deriving human IgG from phage display libraries.[42] It may be necessary or desirable to increase the antigen-binding affinity of phage-derived antibodies for therapeutic applications—affinity maturation. This suboptimal affinity issue has been addressed by larger and higher quality phage libraries that have facilitated the identification of higher affinity antibodies, as well by streamlined methods for affinity maturation using phage, yeast, ribosome, or mRNA display technologies.[42,43]

Murine hybridoma technology is more widely available than human hybridoma technology and provides a potentially more facile route to generating large antibody panels. However these advantages are offset by the need for subsequent humanization of murine MAb. A favorable attribute of hybridoma technologies is that the resultant IgG can be conjugated to cytotoxic drugs and rapidly screened for ADC activity. Species cross-reactive MAb can sometimes be obtained from hybridoma technology, including transgenic mice making human MAb. Immunization strategies that include antigen from different species and methods to break immunologic tolerance to self-antigens may be helpful. "Knockout" mice can be used to generate antibodies against virtually any self protein that is not essential for survival or immune system function.[46] Immune tolerance can also be broken by coupling the protein to an immunogenic carrier through cross-linking,[47] or expressing the self protein with a foreign antigen[48] or by inserting a T cell epitope.[49] The generation of species cross-reactive antibodies from hybridoma technology relies on screening rather than the more facile selection possible with display technologies. Surrogate antibodies that react to the antigen from the species used for toxicology offer an alternative, albeit less desirable strategy, than using a species cross-reactive antibody.

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ANTIBODY LEAD OPTIMIZATION

The next step in ADC development is lead optimization, in which the antibody delivery vehicle and/or the drug payload are optimized. Permuting improvements in the antibody delivery vehicle with those in drug and linker payload is a potentially cumbersome and time-consuming task. ADC lead optimization can be streamlined in our experience by parallel optimization of the antibody and drug-linker and then combining the improvements obtained. Antibodies are commonly engineered to optimize their utility for specific therapeutic applications[2]—a paradigm that is now being extended to ADCs, as reviewed in this section.

IgG Isotype Choice for ADCs

A key issue for antibody therapeutics, and potentially also for ADCs, is that of isotype selection.[2,50,51] Most ADCs in clinical development use IgG1 (7/10), although IgG4 (2/10), and IgG2 (1/10) have also been used (Table 1). Isotype impacts the ability of IgG to support the secondary immune functions known as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC). For example, IgG1 can potentially bind to Fcγ receptors and support ADCC and ADCP. Additionally, IgG1 can potentially bind to the complement component, C1q, and support CDC. In contrast, IgG2 and IgG4 are typically inefficient in their effector functions.[52] For ADCs, effector functions could be advantageous by providing additional antitumor activities. Conversely, effector cell binding could impair ADC localization to tumors, ADC internalization by target cells or give rise to off-target toxicities. The impact of antibody isotype on ADC function is challenging to study preclinically because of numerous differences between human and mouse immune systems including Fcγ receptors and FcRn, as well the absence of endogenous IgG in immune-compromised mice typically used for xenograft studies.

IgG2 can form covalent dimers,[53] an intriguing observation whose generality and significance for IgG2-based therapeutics remain to be determined. Nevertheless, dimerization does not seem to be a major limitation for IgG2-based drugs as judged by one IgG2 that is approved for therapeutic use, namely panitumumab,[54] and encouraging progress with several other IgG2 in clinical development[51] including one IgG2-based ADC (Tables 1 and 2).

IgG3 have been largely avoided for therapeutic applications including as ADCs for reasons that are not entirely clear but presumably relate to the differences between IgG3 and other IgG isotypes: longer hinge and greater susceptibility to proteolysis, more extensive polymorphism (13 allotypes), potentially immunogenicity, and a shorter terminal half-life (7 days vs 21 days for other IgG isotypes[55]).[2,50,51]

IgG4 have been used successfully as ADCs as demonstrated by the FDA-approved drug, gemtuzumab ozogami-

TABLE 1. Antibody-Drug Conjugates in Clinical Development

Conjugate Names	Developer	Target Antigen Names	Antigen Class	Antibody	Drug Class, Drug	Linker Class, Linker	Mean Drug/ Antibody Ratio	References
BIIB015	Biogen-Idec	Cripto	EGF-CFC family	Hz IgG1	Maytansinoid, DM4	Sterically-hindered disulfide	NA	NA
SGN-35	Seattle Genetics	CD30 (TNFRSF8)	TNF receptor superfamily	Ch IgG1	Auristatin, MMAE	Dipeptide, valine-citrulline	4	26, 136
SAR3419 (huB4-DM4)	Sanofi-Aventis	CD19	IgG superfamily	Hz IgG1	Maytansinoid, DM4	Sterically-hindered disulfide	4	111
AVE9633 (huMy9-6-DM4)	Sanofi-Aventis	CD33 (Siglec-3)	Siglec	Hz IgG1	Maytansinoid, DM4	Sterically-hindered disulfide	3.5	112
CR011-vcMMAE	CuraGen	Glycoprotein NMB	Transmembrane glycoprotein	Hu IgG2	Auristatin, MMAE	Dipeptide, valine-citrulline	3.8	128, 154
HuC242-DM4 (IMGN242)	ImmunoGen	CanAg	Carbohydrate	Hz IgG1	Maytansinoid, DM4	Sterically-hindered disulfide	3.5	109, 131
Trastuzumab-DM1	Genentech	HER2 (ErbB2, HER2/*neu*)	Growth factor receptor, EGF receptor family	Hz IgG1	Maytansinoid, DM1	Thioether: SMCC	3.5	35, 36
huN901-DM1 (IMGN901, BB10901)	ImmunoGen	CD56 (NCAM)	Cellular adhesion molecule, IgG superfamily	Hz IgG1	Maytansinoid, DM1	Sterically-hindered disulfide	3.5	113
Inotuzumab ozogamicin (CMC-544)	Wyeth	CD22 (Siglec-2)	Siglec	Hz IgG4	Calicheamicin, *N*-acetyl-γ calicheamicin,	Hydrazone, AcBut	5–7	92, 93, 160, 169
Gemtuzumab ozogamicin (Mylotarg)	Wyeth	CD33 (Siglec-3)	Siglec	Hz IgG4	Calicheamicin, *N*-acetyl-γ calicheamicin	Hydrazone, AcBut	2–3*	95, 149, 152

*Gemtuzumab ozogamicin has a mean loading of 2–3 drugs/antibody and comprises a mixture of 50% ADC with 4–6 drugs/antibody and 50% unconjugated antibody.[95]

AcBut, 4-(4′-acetylphenoxy) butanoic acid; Ch, chimeric; EGF, epidermal growth factor; Hu, human; Hz, humanized; NA, not available; NCAM, neural cell adhesion molecule; siglec, sialic acid-binding immunoglobulin-like lectin; SMCC, *N*-succinimidyl-4-(*N*-maleidomethyl)cyclohexane-1-carboxylate and TNFRSF, tumor necrosis factor receptor superfamily.

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TABLE 2. Preliminary Clinical Trial Data with Antibody-Drug Conjugates

Conjugate Names	Trial Phase	Indications	Patients Treated (*n*)	Responses (*n*)	ADC Dose Level*	ADC Dose Schedule (IV)	Dose-Limiting Toxicity	Reference
BIIB015	I	Cripto+ solid tumors	NA	NA	NA	NA	NA	NA
SGN-35	I	CD30+ hematologic malignancies	23	4 PR	0.1–1.8 mg/kg	Every 3 wk	Not reached	137
SAR3419 (huB4-DM4)	I	NHL	NA	NA	NA	NA	NA	NA
AVE9633 (huMy9–6-DM4)	I	AML	17	1 CRp, 1 PR	30–150 mg/m^2	2 consecutive weeks every 4 wk	2 of 3 patients at 150 mg/m^2; liver toxicity, keratitis	112
CR011-vcMMAE	I/II	Metastatic melanoma	25	0	0.03–1.88 mg/kg	Every 3 wk for 4 cycles	Not reached	155
HuC242-DM4 (IMGN242)	I	CanAg+ metastatic solid tumors	30	0	18–297 mg/m^2	Every 3 wk	2 of 6 patients at 223 mg/m^2, decreased visual acuity, corneal deposits and keratitis	163
	II	Gastric cancer	NA	NA	168 mg/m^2	Every 3 wk	NA	
Trastuzumab-DM1	I	HER2+ metastatic breast cancer	24	6 PR	0.3–4.8 mg/kg	Every 3 wk	2 of 3 patients at 4.8 mg/kg, thrombocytopenia	35, 36
	II	HER2+ metastatic breast cancer	NA	NA	NA	NA	NA	NA
huN901-DM1 (IMGN901, BB10901)	I	Multiple myeloma	12	0	40–90 mg/m^2	2 consecutive weeks every 3 wk	Not reached	166
	II	CD56+ small cell cancers	30	2 PR	60 mg/m^2	4 consecutive weeks every 6 wk	NA	165
Inotuzumab ozogamicin (CMC-544)	I	NHL	NA	NA	NA	NA	NA	NA
	I/II	NHL	NA	NA	NA	NA	NA	NA
	III	NHL	NA	NA	1.8 mg/m^2	Every 4 wk	NA	NA

Sources include company web sites and www.clinicaltrials.gov.

*Interconversion of surface area-based and weight-based dosing units requires knowledge of the patient weight and height, eg, a dose of 1.0 mg/kg for a 6 foot (1.83 m) tall patient is equivalent to 38 mg/m^2 using the dosing calculator on the FDA web site (www.fda.gov/cder/cancer/animalframe.htm).

AML, acute myeloid leukemia; CR, complete response: CRp, complete response with incomplete platelet recovery; NA, not available; NHL, non-Hodgkin lymphoma; NR, not reached; and PR, partial response.

cin.[7] Nevertheless, IgG4 molecules may be nonideal for drug delivery in that they can exchange Fab arms in vivo to become functionally monovalent, a hypothesis[56] now supported by significant experimental evidence.[57] This apparent limitation of IgG4s can be overcome by replacing the C_H3 domain with the corresponding domain from IgG1.[57] Preclinical studies in mice may underestimate the clinical potential of IgG4 ADCs in that IgG4 have shorter terminal half-lives than their IgG1 and IgG2 counterparts.[58] In contrast, IgG4 have similar terminal half-lives as IgG1 and IgG2 in man.[55]

ADC Binding to Fcγ Receptors and FcRn

Binding interactions between IgG Fc and Fcγ receptors have been determined structurally by x-ray crystallography[59] and functionally by mutational analysis.[68] Tailoring the interaction between IgG and Fcγ receptors by modifying the Fc glycan or protein sequence can be used to increase or decrease IgG effector functions.[2,5] Combining such IgG Fc modifications with ADCs warrants exploration as a strategy to enhance the clinical potential of ADCs.

The interaction between IgG and its salvage receptor, FcRn, is critical to the recycling and long serum persistence of IgG.[61] Proteins in circulation, including IgG, are subject to fluid phase uptake—pinocytosis—by some cells including vascular endothelia. IgG can then bind to FcRn in endosomes under slightly acidic conditions (pH 6.0–6.5) and recycle to the cell surface, where they are released under near neutral conditions (pH 7.0–7.4).[62,63] The binding interaction between IgG Fc and FcRn has been elucidated structurally by x-ray crystallography[64] and functionally by mutational analysis.[60,61,68] Tailoring the interaction between IgG and FcRn can be used to increase or decrease the terminal half-life of IgG.[2,61] Indeed, the terminal half-life of human IgG has been extended in rhesus[65,66] and cynomolgus[67] macaques. These data suggest the feasibility of extending the plasma half-life of antibody therapeutics by engineering the interaction of Fc with FcRn and are potentially applicable to ADCs.

Currently, there are limited data available regarding the impact of drug conjugation on IgG binding to either Fcγ receptors or FcRn. Drugs conjugated through solvent accessible cysteine residues are distant from the binding sites for Fcγ receptors[59,68] and FcRn[68,69] and unlikely to provide a major steric impediment to receptor binding. However, dis-

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ruption of both hinge disulfides in a chimeric IgG1 by Cys→Ser mutations can greatly diminish ADCC activity, presumably via impaired binding to Fcγ receptors.[70] In contrast, maintaining at least one of the interheavy chain hinge disulfide bonds in a humanized IgG1 preserves efficient binding to FcγRIIIA as judged by Cys→Ser mutations.[71] For lysine conjugation chemistry, as exemplified by huN901-DM1, the drug is distributed among ~40 different sites.[72] Thus, interference with binding to FcRn and Fcγ receptors is likely minimized by the low occupancy of individual lysine sites in the population of lysine-conjugated ADCs.

Antigen-Binding Affinity

One of the most intensively studied properties of antibodies is that of antigen-binding affinity.[2] Antibody conjugation to drugs is undertaken so as to have little or no impact on antigen-binding affinity.[73] This is accomplished by carefully controlling the number of drugs attached to each antibody, as discussed below. Limited data are available on the relationship between antigen-binding affinity and antitumor activity of ADCs. Important and as yet unanswered questions are whether or not there is an optimal affinity for ADC activity in vitro and in vivo, and if the antigen binding affinity impacts the therapeutic index.

ADC OPTIMIZATION

Many different antibodies, drugs, and linkers have been combined to create ADCs. Cytotoxic drugs being explored for ADCs generally have potencies several orders of magnitude greater than for conventional chemotherapeutics, making them too potent for systemic delivery. Linkers used to attach the drugs to the antibody delivery vehicles have been designed to exploit intracellular conditions for drug release including the acidic environment of endosomes (pH 5.5–6.2) and lysosomes (pH 4.5–5.0), high thiol concentrations in the cytosol, and proteolytic enzymes in lysosomes. These linkers have provided half-lives of drug retention on circulating ADCs in clinical studies of ~43 hours for the hydrazone in BR96-doxorubicin,[74] and ~41 hours for the sterically-hindered disulfide in huC242-DM1.[75] Peptide linkers offer the promise of even greater stability as judged by half-lives of 7–10 days in preclinical in vivo studies.[76]

Drug Potency and ADC Activity

Early ADCs used clinically approved chemotherapeutic drugs because of their ready availability, amenability to chemical manipulation and their well known toxicological properties.[77] Much about the strengths and pitfalls of antibody-mediated drug delivery was learned from BR96-doxorubicin (Fig. 3), an ADC directed against the Lewis^Y tetra-saccharide antigen on human carcinomas.[78,79] Doxorubicin was attached to a chimeric version of the BR96 antibody, through an acid-labile hydrazone linker. Upon binding to the antigen, the antibody was internalized and doxorubicin released within acidic endosomal and lysosomal vesicles. Treatment of tumor-bearing mice and rats with BR96-doxorubicin led to immunologically specific tumor cures, albeit at high (>100 mg/kg) doses, reflecting the low potency of the targeted drug.[79] In a phase I clinical trial, the maximum-tolerated dose of BR96-doxorubicin was ~700 mg/m^2, with dose-limiting gastrointestinal toxicities.[74] In a subsequent phase II trial, the toxicities were attributed to normal gut expression of Lewis^Y.[30] In both trials, limited antitumor activity was obtained. Thus, the limitations of BR96-doxorubicin include low molar potency of the cytotoxic drug and antigen expression on sensitive normal tissue. Additionally, the hydrazone linker had a half-life of ~43 hours that was ~7-fold lower than the pharmacokinetic terminal half-life of the BR96 antibody.[74]

Several groups[24,80–83] hypothesized that it may be necessary to construct ADCs with drugs of much higher molar potencies than conventional chemotherapeutics to achieve therapeutically effective levels of active drug within tumors in patients. A major advance in ADC technology came with the utilization of such highly potent drugs as calicheamicins[84], maytansinoids,[85] auristatins,[11] and CC1065 analogs.[80,86] This progress reflects an appreciation that the localization of antibodies to tumors is inefficient in man: typically 0.0003%–0.08% injected dose per gram of tumor.[87]

Calicheamicins are a family of enediyne antibiotics derived from the soil microorganisim, *Micromonspora echinospora* ssp. *calichensis*.[88] These drugs bind to the minor groove of DNA and induce double-stranded DNA breaks, resulting in cell death with >100-fold the potency of most standard chemotherapeutics.[84] As with BR96-doxorubicin, an acid-labile hydrazone linker was used to link calicheamicin to antibody delivery vehicles (Fig. 3), with a similar half-life for drug release from the ADC of ~48 hours.[89] Pronounced antitumor activities have been obtained with calicheamicin-containing ADCs targeting MUC1.[82,90] This calicheamicin ADC technology has also been successfully applied to antibodies recognizing a range of tumor antigens including: CD22,[92–94] CD33,[91,95] Lewis^Y[96] and oncofetal protein, 5T4.[97] ADCs utilizing a related enediyene, calicheamicin θ^I_1, have been used successfully to target tumors expressing ganglioside GD2[98] and γ-glutamyltransferase.[99] The doses required for activity were much lower than that required for a doxorubicin ADC, reflecting the greatly increased potency of the targeted drug. The sole clinically approved ADC to date is the calicheamicin-containing conjugate, gemtuzumab ozogamicin.

The effects of calicheamicin ADCs are in many cases antigen-specific.[90–97,100] In contrast, gemtuzumab ozogamicin can elicit potent antitumor activity in an antigen-independent manner on solid tumors in vivo.[101] These effects were attributed to passive targeting, and required the use of an acid-labile hydrazone linker. Nonspecific drug release through linker hydrolysis may contribute to the activity of gemtuzumab ozogamicin in these models. Passive targeting offers a potential explanation of the activity of gemtuzumab ozogamicin in some AML patients where the cognate antigen, CD33, was not detected[101,102] and is potentially extendable to other tumor types.

Maytansinoids[85] and auristatins[11] represent other classes of highly potent drugs that have been widely used for ADC development. These chemically unrelated drugs (Fig. 3) are 100–1,000 fold more cytotoxic than most cancer chemotherapeutics.[85,103] They act by binding to tubulin at the same site

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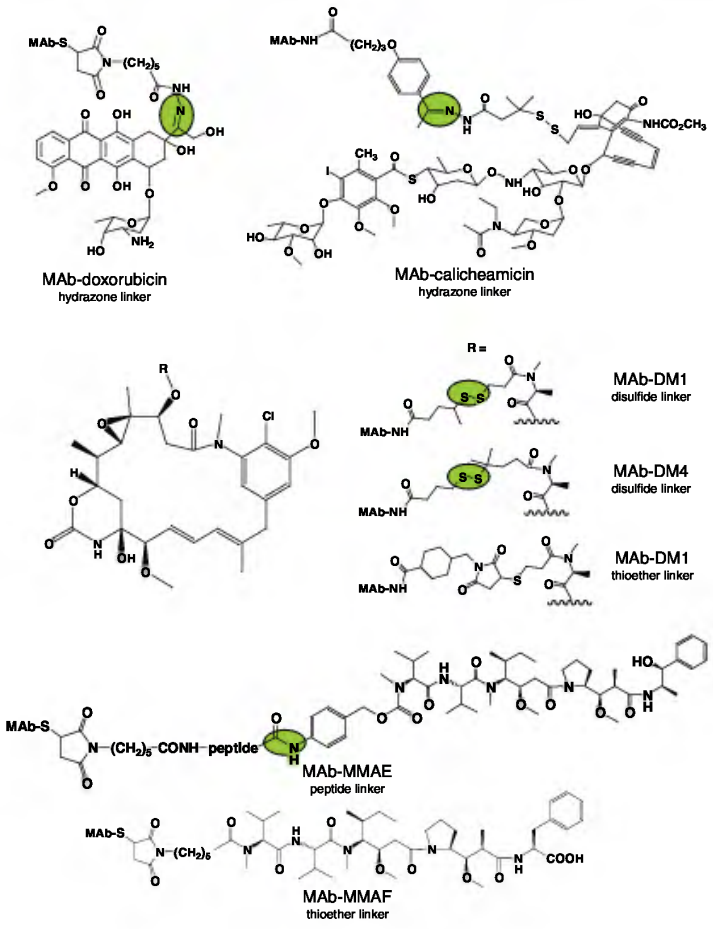


FIGURE 3. Drugs and linkers commonly used in ADCs. Conventional chemotherapeutics such as doxorubicin were once used for ADCs but have been superseded for this purpose by more potent drugs including calicheamicins, maytansinoids (DM1 and DM4) and auristatins (MMAE and MMAF). Commonly used linkers in ADCs include hydrazones, disulfides, and peptides that are cleaved (highlighted region) when exposed to acidic conditions, reducing agents or proteases, respectively. Thioether linkers have also been used for ADCs and drug release likely requires antibody proteolysis within lysosomes.

as the vinca alkaloids and inhibiting tubulin polymerization. A variety of maytansinoids have been derived semi-synthetically from ansamitocin isolated from the microorganism, *Actinosynema pretiosum* spp. *pretiosum*.[104] In contrast, auristatins[103,105] are fully synthetic analogs of the natural product dolastatin 10, which was originally isolated from the sea mollusk, *Dolabela auricularia*.[106]

One of the first maytansinoid-containing ADCs was C242-DM1, which targets CanAg, a tumor-selective carbohydrate epitope.[24] C242-DM1 was generated by modifying antibody lysine residues with a bifunctional cross-linking reagent, followed by the addition of the thiol-containing maytansinoid, DM1 (Fig. 3). The resultant ADC, C242-DM1, contained ~4 drugs/antibody and had potent and immunologically specific antitumor activity. Indeed, C242-DM1 eradicated established tumors in one xenograft model and gave complete responses in 2 other models with heterogeneous antigen expression.[24] The clinical potential of C242-DM1 was enhanced by humanization of the antibody component to create, huC242-DM1 (cantuzumab mertansine). The huC242-DM1 conjugate, like C242-DM1, had robust activity against tumors with heterogeneous antigen expression, reflecting its ability to kill bystander antigen negative tumor cells.[25] In vivo studies revealed that conjugation of DM1 to huC242 had no significant impact upon the biodistribution of the antibody in tumor-bearing mice.[17] The antibody component of huC242-DM1 has a terminal half-life of ~100 hours in mice, whereas terminal half-life of DM1 was about 4-fold shorter,[24] suggesting slow release of DM1 from the ADC in circulation. Similarly, in a subsequent phase I clinical study, the terminal half-lives of the huC242-DM1 ADC and DM1 were ~100 hours and ~24 hours, respectively.[75] The most likely mechanism for DM1 release from huC242-DM1 is by disulfide exchange with other sulfhydryls.[171] Analysis of human plasma indicates the presence of up to ~500 μM free sulfhydryls that are almost entirely from albumin.[107] The huC242-DM1 conjugate was generally well tolerated in 2 phase I clinical trials with evidence for antitumor activity.[75,108]

A series of maytansinoid-disulfide linker derivatives with varying degrees of steric hindrance were subsequently developed and leads such as DM4 selected for antibody attachment.[109] The huC242-DM4 conjugate displays improved linker stability over huC242-DM1 resulting from increased steric hindrance around the disulfide bond[109] (Fig. 3). Additionally, huC242-DM4 shows improved efficacy over huC242-DM1 in

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some xenograft models.[85,109] The huC242-DM4 conjugate has now superseded huC242-DM1 in clinical development (Table 2). Similarly, an anti-integrin-α_V-DM4 ADC showed superior antitumor activity in vivo to its DM1 counterpart.[110] Beyond the examples discussed above, maytansinoid ADC technology has been successfully applied to antibodies recognizing a wide range of tumor antigens, including CD19,[111] CD33,[112] CD56,[113] CD79,[114] CD138,[115] HER2,[116] PSCA,[117] and PSMA.[21]

Use of peptide-based linker technologies may present significant advantages over those that are hydrolytically or reductively labile, since hydrolysis is enzymatic, and enzymes can be selected for preferential expression within tumors. ADCs comprises drugs such as doxorubicin,[118] mitomycin C,[119] camptothecin,[120] tallisomycin,[121] and auristatins[103,105,122] have been prepared using peptide linkers for intracellular drug release. Auristatins are of particular interest, because they are highly potent, synthetic, stable, and amenable to chemical modification for linker attachment.

The auristatin, monomethyl auristatin E (MMAE), was modified with dipeptide linkers, and the resulting drug derivatives were linked to solvent-accessible antibody thiol groups,[105] generating conjugates of uniform composition (Fig. 3). The conjugates target CD30 on Hodgkin lymphoma and Lewis^Y on carcinomas. In vitro studies demonstrated that the MMAE peptide-linked ADCs were highly potent with 10–100-fold greater immunologically dependent cell kill compared with corresponding hydrazone-based ADCs. The half-lives of drug release from ADCs in vivo were 6 and 10 days in mice and cynomolgus macaques, respectively,[76] longer than previously described linkers. Furthermore, the peptide-linked MMAE ADCs were less toxic than corresponding hydrazone-linked ADCs.[103]

As shown first for antibodies targeting CD30 and Lewis^Y, auristatin-based ADCs with peptide linkers had pronounced antitumor activity in xenograft models, leading to cures of established tumors at very small fractions of the maximum tolerated doses.[103] Subsequently, auristatin ADC technology has been successfully applied to antibodies recognizing a wide range of tumor antigens, including BCMA,[123] CD19,[124] CD20,[37] CD70,[125] CD79,[114] E selectin,[126] EphB2,[127] glycoprotein NMB,[128] melanotransferrin/p97,[168] MUC16,[129] PSMA,[22] and TMEFF2.[130]

Because auristatins are synthetic, integral structural modifications can be made that significantly alter the properties of the drug. One such auristatin, MMAF, terminates with phenylalanine, a negatively charged residue that impairs cell membrane permeability.[105] Consequently, ADCs containing MMAF that facilitate drug uptake by antigen-positive cells are >2,000-fold more potent than the free drug itself. MMAF linked to an anti-CD70 antibody through a peptide linker was active against renal cell carcinoma xenografts and also circumvented multidrug resistance mechanisms.[125]

A surprising finding with MMAF ADCs was that the cleavable peptide linker could be eliminated, and highly active ADCs were obtained by direct attachment of the drug to anti-CD30 and anti-Lewis^Y antibodies through thioether adducts (Fig. 3).[105] Mass spectrometry showed that the released drug was a cysteine-adduct of the linker-MMAF derivative, presumably resulting from antibody degradation within lysosomes. The closely related auristatin, MMAE, was not active when attached in this manner, indicating that ADCs requiring antibody degradation for drug release are highly dependent on the nature of the drug for activity. MMAF can sustain significant modification to the N-terminal position and still remain active, while most other drugs are inactivated when modified in such an extensive manner. Similar results to that of MMAF were obtained with a thioether adduct of DM1, which was appended to lysine residues on the humanized antibody, huC242, (Fig. 3). In this case, the released drug upon cell incubation was a lysine-DM1 derivative in which the lysine residue was the attachment residue on the antibody delivery vehicle.[131] The requirements for effective cell kill using linkers that require antibody degradation for drug release remain to be elucidated. Although there was little potency loss with thioether adducts of MMAF in hematologic malignancies targeted with an anti-CD30 antibody (cAC10), a thioether adducts of DM1 had approximately one hundredth the in vitro potency of a corresponding disulfide-linked ADC[81] together with greatly diminished in vivo efficacy and potency.[131] The efficacy of ADCs requiring antibody degradation for drug release are likely to be antibody, drug and tumor dependent, in contrast to ADCs with cleavable linkers.

In a mouse preclinical model, an anti-CD70 (h1F6) ADC with a thioether linkage to MMAF had a half-life of 7 days for drug release,[132] which is similar to the 6–10 day half-life for drug release from the corresponding peptide-linked ADC.[76] Furthermore, an ADC with acetamide-thiol adduct in place of the maleimide-thioether (Fig. 3) did not lose any detectable drug from circulating ADC over 2 weeks in mice. The tolerability, efficacy, and intratumoral drug concentrations of the maleimide and acetamide-linked ADCs were similar.[132] Thus, extending the half-life of the ADCs beyond that of 6–10 days did not lead to activity improvements in preclinical models.

Conjugation Technologies

ADCs described thus far were formed by reacting drugs or chemical crosslinking reagents with solvent accessible reactive amino acids, such as lysine and cysteine. The process used to covalently attach the drug to the antibody delivery vehicle can sometimes adversely affect activity, eg, linker technologies have been developed to attach as many as 16 doxorubicin molecules/antibody, but many of the resulting ADCs were largely aggregated and had impaired antigen binding.[133] Some drugs are prone to self-associate when attached to antibodies and may cause aggregation. Such aggregation of gemtuzumab ozogamicin was minimized by limiting the loading to a mean of 2–3 drugs/antibody: 50% ADC with 4–6 drugs/antibody and 50% unconjugated antibody.[95] The heterogeneity of calicheamicin containing ADCs has recently been reduced by additives in the conjugation process.[134] Conjugation technology is a critical aspect in generating effective ADCs, and optimization strategies can vary with the drug, linker, and the antibody used.

The first auristatin-containing ADCs were generated by reducing all of the interchain disulfide bonds in the antibody,

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and then attaching the drugs to the resulting cysteine residues.[103,122] These ADCs were close to homogeneous with 8 drugs/antibody, attached at the predetermined cysteine residues. The binding properties of the antibody were unaffected, because the drug was conjugated in the hinge region, which is distant from the antigen binding sites. However, the pharmacokinetic properties of the antibody delivery vehicle were adversely affected, and the 8-loaded ADC cleared much more rapidly than the parent antibody.[135] This prompted investigation of anti-CD30 ADCs (cAC10-MMAE) with varied drug/antibody ratios to assess the impact of drug stoichiometry on therapeutic potential. An ADC with a mean loading of 4 drugs/antibody was fractionated by hydrophobic interaction chromatography to yield purified ADCs with 2, 4, or 8 MMAE drugs/antibody, designated E2, E4, and E8, respectively.[135] ADC potency in vitro correlated with drug loading in that the order of potency was E8 > E4 > E2. In contrast, the in vivo antitumor activity of E4 was comparable with E8 at equal antibody doses, although the E4 contained only half the amount of drug per antibody. These efficacy differences correlated with increased exposure to the E4 conjugate, since slower clearance resulted in a greater pharmacokinetic area-under-the-curve. E2 also had antitumor activity in vivo, albeit less potent than for E4. The maximum-tolerated dose increased inversely with respect to drug loading. Thus, the drug/antibody ratio is an important parameter in the design of ADCs as evidenced by ≥2-fold increase in therapeutic index by reducing the loading from 8 to 4 drugs/antibody.[135] An optimized anti-CD30 ADC with a mean loading of 4 MMAE drugs/antibody, now known as SGN-35,[26,136] is well tolerated and has elicited multiple partial responses in a phase I clinical trial (Tables 1 and 2,[137]).

Antibodies can be engineered for site-specific drug conjugation to enable the production of homogeneous ADCs with defined sites and stoichiometries of drug attachment, eg, a panel of anti-CD30 antibodies was generated in which solvent accessible cysteines were systemically replaced with serines. These Cys→Ser antibody variants were used to create homogeneous ADCs with 2 or 4 MMAE drugs/antibody[73] that were compared with those derived from the parent anti-CD30 IgG1. Surprisingly, the most homogenous ADCs with 4 drugs/antibody at defined sites had comparable, rather than improved, in vitro and in vivo properties as the most heterogeneous conjugate having 0–8 drugs/antibody with a mean of 4 drugs/antibody and variable sites of drug attachment. Thus, the mean drug/antibody ratio seems to be a more significant determinant of ADC potency and tolerability than conjugate homogeneity or sites of drug attachment.[73,135,138]

Antibody engineering involving the addition of accessible cysteines has also been used for site-specific drug conjugation[139,140] or to create novel intermolecular[141,142] or intramolecular[143] disulfide bonds. Such site-specific conjugation to IgG was first demonstrated by Lyons et al who engineered an extra cysteine residue at various locations within the C_H1 domain of an IgG4.[139] Subsequently, Stimmel et al engineered a cysteine residue into the C_H3 domain of an IgG4.[140] Although the engineered cysteine was blocked, mild and selective reduction conditions were devised thereby site-specific labeling with a radionuclide chelator. Recently, the efficiency of site-specific conjugation to IgG has been improved by better choices of cysteine replacement[144]—"thiomabs"—combined with refinements in conjugation methodology that reduce and then restore native interchain disulfide bonds.[170] A thiomab-drug conjugate with 1.6 auristatin drugs/antibody was compared with a conventional ADC with 3.1 auristatin drugs/antibody. The thiomab-drug conjugate showed comparable efficacy in a xenograft study, improved tolerability in rats and nonhuman primates and thus an increased therapeutic index (Junutula et al, unpublished). This improved therapeutic index may be due to thiomabs per se and/or reduction in the drug/antibody ratio.[135] The potential broad applicability of thiomab-drug conjugates provides a strong impetus to assess the reproducibility and transferability of the increased therapeutic index between different xenograft models, antibodies, drugs, and linkers.

CLINICAL EXPERIENCE WITH GEMTUZUMAB OZOGAMICIN

Gemtuzumab ozogamicin represents a major milestone as the first, and currently sole, ADC approved for clinical use. Here we review the clinical data that led to the approval of gemtuzumab ozogamicin for AML, whereas subsequent clinical trials with this drug are reviewed elsewhere.[145–147] Gemtuzumab ozogamicin comprises a humanized anti-CD33 IgG4 antibody conjugated to *N*-acetyl-γ-calicheamicin dimethyl hydrazide via the acid labile hydrazone, 4-(4′-acetylphenoxy) butanoic acid, known as "AcBut."[95] CD33 (SIGLEC-3) is a sialic-acid dependent adhesion protein expressed on the surface of ~90% of leukemic blasts in AML[148] and immature normal cells of the myelomonocytic lineage, but absent from normal hematopoietic stem cells.

Gemtuzumab ozogamicin was approved by the US Food and Drug Administration in 2000 for the treatment of CD33 positive AML in first relapse in patients of at least 60 years of age and considered unsuitable candidates for cytotoxic chemotherapy.[7] Gemtuzumab ozogamicin approval was based upon interim data analysis of 3 open label single-arm phase II studies in AML at first relapse. Patients received intravenous gemtuzumab ozogamicin at 9 mg/m^2 at 2 weekly intervals for 2 doses. Thirty percent of the patients (42 of 142) achieved a complete remission: ≤5% blasts in the marrow, recovery of neutrophils to ≥1,500/μL and erythrocyte and platelet transfusion independence. Final analysis of these 3 gemtuzumab ozogamicin phase II studies with a larger number of patients (n = 277) demonstrated a 26% remission rate and a median overall survival of 4.9 months.[149]

Gemtuzumab ozogamicin induced severe myelosuppression in all patients including grade 3 or 4 thrombocytopenia (99%), neutropenia (98%), and anemia (52%), as anticipated from the CD33 expression on normal hematopoietic precursors.[149] The myelosuppression was reversible as expected, since hematopoietic stem cells are CD33 negative and presumably spared. Gemtuzumab ozogamicin, as for other antibody-based therapeutics, can also give rise to acute infusion-related adverse events. Grade 3 or 4 infusion-related adverse events were much more common for the first dose

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(30%) than the second dose (10%) and included chills (8%), fever (6%), hypotension (4%), nausea (3%), and hypertension (2%). The incidence of hepatotoxicity associated with gemtuzumab ozogamicin included grade 3 or 4 hyperbilirubinemia (29%), elevations of aspartate aminotransferase (18%) or alanine aminotransferase (9%) that were generally transient and reversible without medical intervention. None of the patients developed an antibody response to either the antibody or drug-linker components of gemtuzumab ozogamicin. Additional grade 3 and 4 adverse events associated with gemtuzumab ozogamicin include sepsis and dyspnea.

Severe veno-occlusive disease (sinusoidal obstruction) has been associated with the use of gemtuzumab ozogamicin at incidences that vary greatly with the clinical setting.[149–152] A low incidence (~1%) of veno-occlusive disease was observed following gemtuzumab ozogamicin monotherapy in patients without prior or subsequent hematopoietic stem cell transplants.[149] In contrast, a higher incidence (22%) of veno-occlusive disease, sometimes fatal, was observed in patients who had received hematopoeitic stem cell transplantation before gemtuzumab ozogamicin. An intermediate rate (15%) of veno-occlusive disease was observed in patients transplanted after gemtuzumab ozogamicin therapy.

In contrast to the US Food and Drug Administration, the European Medicines Agency denied marketing authorization for gemtuzumab ozogamicin in Europe in 2007, citing concerns that the benefits of gemtuzumab ozogamicin in the treatment of AML had not been established and did not outweigh its risks. Several randomized and controlled trials are in progress to assess the benefit of gemtuzumab ozogamicin. Moreover, preliminary analysis of one such randomized clinical trial, MRC AML 15 (n = 1,113), revealed that the addition of gemtuzumab ozogamicin to induction chemotherapy for AML improves disease free survival without extra toxicity.[153]

ADCs in Clinical Development

There are a growing number of ADCs in clinical development as discussed below—10 as of April 2008 including gemtuzumab ozogamicin (Tables 1 and 2). Below is a brief description of many of these ADCs and their performance in early stage clinical trials.

SGN-35

SGN-35 is a CD30-targeting ADC with a protease-cleavable linker (valine-citrulline) between the antibody and the auristatin drug, MMAE, with a mean loading of ~4 drugs/antibody.[26,136] CD30 is a member of the TNF receptor family with highly restricted normal tissue expression that includes activated, but not resting, T and B cells. CD30 expression by malignant Reed-Sternberg cells is a defining feature of Hodgkin lymphoma. CD30 is also expressed in other hematologic malignancies including diffuse large B cell, anaplastic large cell, and cutaneous T cell lymphomas as well as Kaposi sarcoma.

A phase I trial of SGN-35 is ongoing in CD30 positive hematologic malignancies (Table 2). Preliminary data have been reported on the first 23 patients, including 21 with Hodgkin lymphoma, who received escalating doses of SGN-35 from 0.1 to 1.8 mg/kg administered intravenously every 3 weeks.[137] Tumor reduction was observed in over 75% of the patients treated, including 4 partial responses. SGN-35 was reasonably well tolerated, with most observed adverse events of grade 1 and 2.

CR011-vcMMAE

CR011-vcMMAE is an ADC targeting glycoprotein NMB that, like SGN-35, includes a valine-citrulline protease cleavable linker between the antibody and the auristatin drug, MMAE, with a mean loading of ~3.8 drugs/antibody.[128,154] Glycoprotein NMB is a 560 amino acid glycoprotein of unknown function that is highly expressed in melanoma tissue, albeit predominantly intracellularly with a small cell surface component. Glycoprotein NMB expression has also been associated with glioma, liver carcinoma, squamous cell lung cancer, and soft tissue tumors, whereas normal tissue expression is restricted.[128]

A phase I/II trial of CR011-vcMMAE has been initiated in patients with unresectable stage III or IV melanoma (Table 2). Preliminary data have been reported on 25 patients who received escalating doses of CR011-vcMMAE from 0.03 to 1.88 mg/kg administered intravenously every 3 weeks for 4 cycles.[155] No objective responses were observed, although 7 of 22 evaluable patients demonstrated stable disease. Overall, CR011-vcMMAE has been well tolerated with no dose-limiting toxicities. Most drug-related adverse events were grade 1 and 2, with grade 3 or 4 neutropenia and leukopenia reported for 2 patients.

Trastuzumab-DM1

The ADC, trastuzumab-DM1,[35,36,116] contains the humanized anti-HER2 IgG1 antibody, trastuzumab (formerly known as humAb4D5–8,[156]) conjugated to the maytansinoid, DM1, via a thioether linkage.[25,131] HER2 is a member of the EGFR family of growth factor receptors. HER2 is overexpressed as a result of gene amplification in ~25% of primary breast cancers and is associated with poor prognosis.[157] HER2 is expressed at lower levels in several normal tissues including gastrointestinal tract epithelia, tonsil, kidney tubules, and endometrium as described in the package insert for the diagnostic, HercepTest™. Trastuzumab-DM1 is unique among the ADCs in clinical development in that the parent antibody, trastuzumab (Herceptin) is a clinically and commercially successful therapeutic in its own right. Indeed, trastuzumab in combination with cytotoxic chemotherapy prolongs overall survival in patients with HER2-positive breast cancer in both the metastatic[158] and adjuvant[159] settings.

In a phase I study in HER2-positive metastatic breast cancer trastuzumab-DM1 was administered at 6 dose levels of 0.3–4.8 mg/kg every 3 weeks.[35,36] Partial responses were observed in 6 of 24 patients treated at or at below the maximum-tolerated dose of 3.6 mg/kg. Drug-related adverse events of grade 2 or higher have been infrequent and manageable. Dose-limiting grade 4 thrombocytopenia was observed in 2 of 3 patients treated at 4.8 mg/kg. Platelet nadirs were observed on about day 8 with recovery by day 15. No cardiotoxicity has been observed with trastuzumab-DM1, in contrast to trastuzumab used in conjunction with anthracy-

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clines.[158] A phase II trial of trastuzumab-DM1 has been initiated in metastatic breast cancer.

AVE9633

AVE9633 is a humanized antibody-maytansinoid (DM4) conjugate[112] that targets CD33—the tumor antigen validated by gemtuzumab ozogamicin. A phase I study has been initiated with AVE9633 in patients with relapsed or refractory CD33-positive AML (Table 2). AVE9633 was administered intravenously at dose levels of 30–150 mg/m^2 on 2 consecutive weeks every 4 weeks.[112] One complete response with incomplete platelet recovery and one partial response were observed from the first 17 patients treated. Two dose-limiting toxicities were seen at the dose of 150 mg/m^2 × 2 consisting of liver toxicity and keratitis. The most frequent grade 1 or 2 adverse events were infusion reactions—fevers, chills, hypotension, and musculoskeletal pain. Saturation and down-regulation of CD33 on the surface of blood and bone marrow blasts was observed from the 50 mg/m^2 dose. No human antibody response has been detected to either the ADC or DM4.

Inotuzumab Ozogamicin

Inotuzumab ozogamicin is an ADC targeting CD22 that shares some attributes of gemtuzumab ozogamicin: humanized IgG4 linked to *N*-acetyl-γ-calicheamicin dimethyl hydrazide via an acid labile, AcBut, linker.[92] CD22 (SIGLEC-2) is a sialic-acid dependent adhesion protein of the immunoglobulin superfamily that is expressed in B cell malignancies such as non-Hodgkin B cell lymphoma, and acute lymphoblastic leukemia. Normal tissue expression of CD22 is highly restricted to mature B cells and it is absent from other hematopoietic cells including hematopoietic stem cells.

Interim data from on-going phase I and I/II trials with inotuzumab ozogamicin are not readily available, but presumably were sufficiently favorable to provide a rationale for the recent initiation of a pivotal trial in follicular non-Hodgkin lymphoma. Specifically, a 2-arm, randomized, open-label phase III trial was initiated in late 2007 to compare rituximab in combination with inotuzumab ozogamicin to rituximab in combination with the investigator's choice of cytotoxic chemotherapy. Preclinically, the antitumor activity of inotuzumab ozogamicin in combination with rituximab against non-Hodgkin B-cell lymphoma was superior to either inotuzumab ozogamicin or rituximab alone.[160]

HuC242-DM4

As previously described, huC242-DM4 is a humanized antibody-maytansinoid conjugate targeting CanAg,[109,131] a tumor-selective carbohydrate epitope. CanAg is highly expressed in most pancreatic, biliary and colorectal cancers as well as a significant proportion of gastric (55%), uterine (45%), nonsmall cell lung cancer (40%), and bladder cancers,[161,162] whereas only minimal immunostaining of CanAg in normal tissue has been reported.[162]

A phase I trial of HuC242-DM4 has been initiated in patients with metastatic or inoperable colorectal, pancreatic, and other CanAg positive cancers (Table 2). HuC242-DM4 was administered by intravenous infusion at dose levels of 18–297 mg/m^2—every 3 weeks.[163] No objective responses have been reported from the first 30 patients treated. Dose-limiting toxicity of decreased visual acuity, corneal deposits, and keratitis were experienced by 2 of 6 patients treated at the 223 mg/m^2 dose level. Both patients responded favorably to treatment with lubricating eye drops. HuC242-DM4 was well tolerated at the 168 mg/m^2 with 1 of the 8 patients experiencing a dose-limiting toxicity consisting of grade 3 diarrhea and dehydration that improved with intravenous fluids. Preliminary pharmacokinetic data revealed that huC242-DM4 had a half-life of ~5 days in patients with low plasma levels of antigen. Clearance of huC242-DM4 was increased in patients with high levels of shed antigen (>900 units/mL). No clinically significant myelosuppression has been observed, and antibody generation to the humanized antibody or DM4 has not been detected.

HuN901-DM1

HuN901-DM1 is a humanized antibody-maytansinoid conjugate targeting CD56,[113] the neuronal cell adhesion molecule that is expressed in virtually all small cell lung carcinomas, neuroblastomas, gliomas, astrocytomas, and rhabdomyosarcomas, as well as ~78% of multiple myelomas and ~53% of AML.[164] As for normal tissue, CD56 is expressed on natural killer cells, neuroendocrine glands, the central and peripheral nervous systems and also cardiomyocytes.[164]

In a phase II study, patients with relapsed small-cell lung cancer and other CD56-positive small cell cancers were treated with huN901-DM1 intravenously at 60 mg/m^2/wk for 4 consecutive weeks every 6 weeks (Table 2). Two partial responses and 5 stable diseases were observed in the first 30 patients treated.[165] Transient severe headaches were observed in a few patients, but did not occur in subsequent patients premedicated with steroids. HuN901-DM1 was generally well tolerated, with no evidence of clinically significant myelosuppression and no serious infusion reactions. No human antibody response has been detected to either the antibody or drug components of the ADC. Additionally, a phase I study of huN901-DM1 is underway in patients with relapsed or refractory CD56-positive multiple myeloma (Table 2). Patients received escalating doses of huN901-DM1 of 40–90 mg/m^2/wk by intravenous infusion on 2 consecutive weeks every 3 weeks.[166] One minimal objective response has been observed for the first 12 patients treated. HuN901-DM1 has been well tolerated with no serious drug-related adverse events and no dose-limiting toxicity observed for the first 12 patients treated. Immunohistochemistry performed on bone marrow aspirates 24 hours after huN901-DM1 infusion at 40 mg/m^2 confirmed localization of ADC to myeloma cells. Preliminary pharmacokinetic analysis revealed a ~20 hour terminal half-life for huN901-DM1.

CONCLUSIONS AND PERSPECTIVES

Significant advances have been made with ADCs over the last 3 decades. Building on the success of gemtuzumab ozogamicin, highly potent drugs have been attached to antibodies recognizing numerous different antigens expressed on a broad array of hematologic and solid tumors. The activities of these new ADCs underscore the importance of good target

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selection as well as drug potency and high stability linkers for drug attachment. Early ADC linkers were primarily hydrazones or minimally hindered disulfides, whereas newer linkers include more hindered disulfides, thioethers, and peptides that have achieved greater in vivo stability.

As for the antibody delivery vehicle, much remains to be learned about the importance of antigen binding affinity, antibody isotype, binding to Fcγ receptors and FcRn, and pharmacokinetics in optimizing ADCs for cancer therapy. In addition, the roles that antigen density, internalization rates and recycling play in ADC activity remain to be elucidated. Two major paradigms established with antibody therapeutics[2] are now being explored in the context of ADCs: large panels of antibodies are being developed to increase the likelihood of identifying leads that satisfy multiple design criteria, and antibodies are being engineered to optimize their utility as ADCs.

Insights from many early ADCs have led to a promising new generation of experimental drugs. These include SGN-35[26,136,137] and trastuzumab-DM1,[35,36,116] which are demonstrating pronounced antitumor responses in CD30-positive hematologic malignancies and HER2-positive metastatic breast cancer, respectively. The technologies developed for these ADCs are being broadly applied to other antibody delivery vehicles, to generate potent ADCs that are, or soon will be, in clinical trials. ADCs now seemed poised to realize their potential to play a significant role in cancer therapy.

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Formulation and delivery issues for monoclonal antibody therapeutics ☆

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Abstract

Antibodies can have exquisite specificity of target recognition and thus generate highly selective outcomes following their systemic administration. While antibodies can have high specificity, the doses required to treat patients, particularly for a chronic condition, are typically large. Fortunately, advances in production and purification capacities have allowed for the exceptionally large amounts of highly purified monoclonal antibodies to be produced. Additionally, genetic engineering of antibodies has provided a stable of antibody-like proteins that can be easier to prepare. Together, these advances have made antibody-based therapies one of the most commonly pursued pharmaceuticals in biotechnology pipelines. With this success, however, has come a series of technical challenges in the formulation of antibody-based materials to maintain sufficient stability in a variety of configurations and sometimes at particularly high concentrations. This review focuses on issues related to identifying and verifying stable antibody-based formulations.

Previous Next

Keywords

Antibody; Stability; Delivery; Formulation

1. Introduction

Therapeutic and diagnostic antibodies have become the fastest growing area of biopharmaceutical applications. There are now 18 monoclonal antibodies on the market and over 100 in clinical trials, which

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highlights the significance of these therapeutics and the advances made in antibody engineering. Further, by 2008, engineered antibodies are projected to be the source of over a third of the revenues from biotechnology [1], [2].

A few seminal events have led to the current and projected prominence of antibodies as biopharmaceuticals include the identification of methods to generate murine monoclonal versions of antibodies [3], the cloning of human antibody sequences to allow for humanization of murine monoclonal antibodies through complementary-determining region (CDR) grafting [4] and even the establishment of fully humanized systems to generate monoclonal antibodies [5]. With the sequential identification of these technological advances, antibodies for therapeutic and prophylactic indications have moved from fully murine, to partially murine (mostly human) to completely human constructions. Added to these events, dramatic advances in production have led to the ability to prepare monoclonal antibodies at scales that can provide sufficient material at costs that make this area appealing to pharmaceutical companies. One important outcome of these various advances is a greater potential to use antibody drugs in chronic settings, tremendously expanding their biopharmaceutical applications.

The majority of currently approved antibody drugs address previously unmet medical needs in the areas of cancer and inflammation. These indications came from practical considerations related to earlier forms of murine and partially murine antibody therapeutics; the first monoclonal to be approved (OKT-3®, muromonab-CD3) was approved in 1986 for a single treatment regimen to protect from life-threatening tissue rejection following kidney transplant [6]. Adverse reactions due to acute immunogenicity problems due to delivery of a non-self protein limited the use of OKT-3® to only acute application [7], [8]. Subsequent partially murine antibodies such as Panorex® (17-1A), Zevalin® (ibritumomab tiuxetan) and Bexxar® (tositumomab) were approved for a limited number of administrations to cancer patients who had failed frontline therapies [2]. With the advent of fully humanized antibodies, chronic therapeutic or prophylactic applications for infectious agents have become a realistic clinical option [2]. Another critical aspect to the success of antibodies as therapeutic agents involves improved methods to express, purify and characterize these proteins [9]. In general, antibody therapeutics are large (typically >150 kDa), complex in nature (most are glycosylated) and must be administered in stoichiometric rather than catalytic quantities (nearly a gram per dose is required for many antibodies to be effective). Production and purification scales have thus reached levels of production that were previously assumed impossible in biotech companies [10]. Novel concerns then related to the development of stable formulations and delivery strategies for such large amounts of a complex molecule. Understanding product instability with regard to physicochemical and thermodynamic events becomes more and more critical to ensure market success [11]. The required high concentrations of antibodies in these formulations reduced the need to add a carrier protein, as is the case where serum albumin is commonly added in formulations of highly potent proteins such as erythropoietin alpha (Epogen®) and Factor VIII (Kogenate®) to enhance stability and reduce loss to vial surfaces. Simultaneously, the large amounts of an antibody protein used in these formulations allowed degradation products to be more readily detectable particularly when using the highly sensitive methods designed for evaluating hormone biotherapeutics.

Finally, the ability to prepare large quantities of an antibody that could be administered for chronic therapy resulted in a plethora of new and novel applications [9], [10] involving the delivery of chemotherapeutics, radionuclides and imaging agents — to name a few. New issues, related to identifying stable formulations such as high concentration formulations that might reduce the volume of antibody administrations, arose in

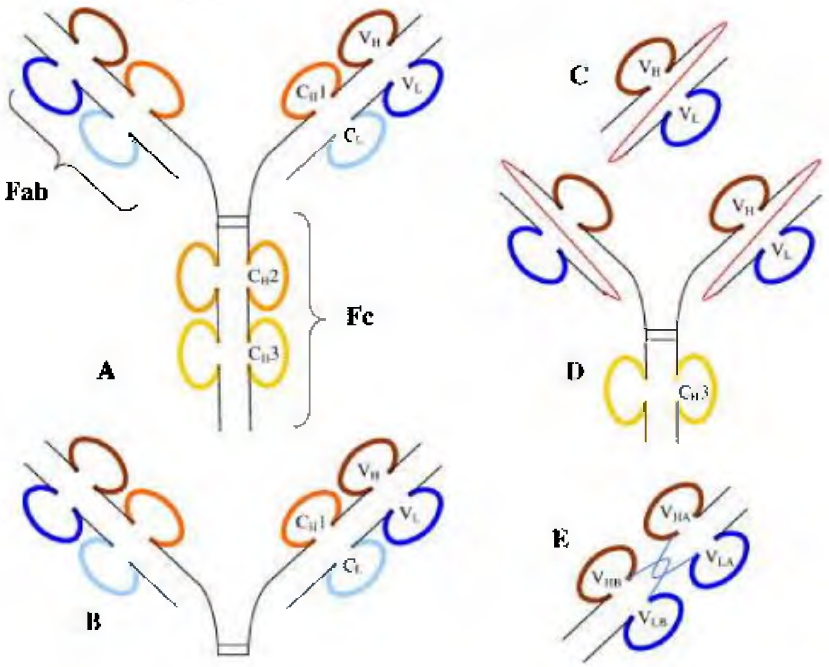
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the move from intravenous infusions to the realm of subcutaneous injection strategies. In some cases the combination of agents with antibodies produced conflicting stability profiles. Additionally, potential new indications for antibody drugs increased the desire to find novel methods for sustained release, intracellular targeting [12] or delivery via methods not involving an injection. Thus, as is the case in most scientific advances, technological advances opened up possibilities for therapeutic opportunities that were not possible previously as well as a totally new set of problems that required solving. In this review we have attempted to identify the major issues associated with formulating and delivering antibody drugs, to recognize challenges already identified accompanied when possible by solutions, as well as to discuss concerns that will need to be addressed in the future as the use of antibody drugs continues to evolve.

2. Antibody characteristics relevant for formulation and delivery

The human body produces several classes of antibodies that appear to perform specific functions under different conditions and at discrete anatomic sites. Most of the currently approved antibody-based drugs are of the IgG1 class [13] and are glycosylated on the Fc (fragment crystallizable) domain. Glycosylation has been shown to participate in specific cell response events mediated by antibodies [14], [15], [16]: antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cell-mediated cytotoxicity (CDCC) through receptor recognition events. Individual glycoforms of antibodies can provide improved efficacies for certain clinical outcomes [17]. In general, IgG1 and IgG3 antibodies are effective at initiating ADCC while IgG2 readily fixes complement but is poor at inducing ADCC. IgG4 appears to be inefficient at both ADCC and CDCC [13]. Interestingly, antibodies can be re-engineered to increase their capacity to recruit complement [18] or to adjust their affinities to the various classes of Fc receptors and thus ADCC function through mutation of specific amino acids within the Fc domain [19]. Molecular engineering has also provided a spectrum of new antibodies (Fig. 1) Other chapters in this issue delve deeply into the many characteristics of antibodies that make them remarkable and diversely applicable treatment agents: ligand binding properties, effector functions and potential for modification in their design. Other chapters will also address applications of newly engineered antibody-like molecules that move beyond the realm of intact, full-length IgG antibodies. In the current review, attention will be focused primarily on IgG1 since this is where the majority of published information exists for antibody drug formulation and delivery.

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Fig. 1. General antibody structure and some antibody-based protein constructions. A) Human antibodies have a common framework that can be described as Fc (fragment crystallizable) and Fab (fragment antigen binding) domains that are derived from the folded structure of heavy (H) and light (L) chain proteins. Multiple constant (C) regions on both the H and L chain proteins are donated as are the variable (V) segments involved in antigen-specific recognition. B) Removal of the Fc domain produces a F(ab)2 structure. C) Single chain structures of the antigen binding region (scFv) can be prepared by themselves or D) in a construct that includes Fc domain elements. E) Bi-specific antibody elements (diabodies) can be prepared to bring two different antigens into close proximity. C_H, heavy chain constant regions; C_L, light chain constant region; V_H, heavy chain variable region; V_L, light chain variable region.

Antibodies, due to their large size (being greater than the filtration cut-off size of the kidney) and their ability to be sequestered for periods of time from the blood, via interactions with FcRn receptors present on endothelial cells, can have extended serum profiles in the range of weeks. A number of engineered antibody-like molecules are now being examined for therapeutic applications [20]. Some engineered fragments, such as diabodies and minibodies, are much smaller than intact, full-length IgG1 and have very different properties of systemic distribution and clearance. Additionally, methods to prepare other antibody types such as dimeric secretory IgA [21] and methods to prepare antibodies using non-mammalian cell systems, such as plants [22], from the eggs of chimeric chickens [23] and from transgenic animals [24], are now being explored by academic and industrial groups. As these new approaches gain prominence, more information will be acquired about their unique requirements for successful formulation and delivery. At present, however, there is little information on formulation approaches for antibodies produced using these production processes.

3. General concerns for antibody formulations

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Due to their proteinacous nature, antibodies present generic formulation issues that are similar for most protein therapeutics [11]. Proteins, in general, can be degraded under conditions when they are exposed to heat, freezing, light, pH extremes, agitation, sheer-stress, some metals and organic solvents. With ever advancing analytical technologies, the ability to assess the physicochemical and thermodynamic instability of antibody drugs has also led to the identification of several events that are more specific to the unique nature of this particular class of proteins: variations in Fc glycosylation, partial heavy chain C-terminal Lys processing, Fc methionine oxidation, hinge-region cleavage and glycation of Lys residues [25]. In addition to these issues is the difficulty of preparing dosing materials that might have protein concentrations in the range of 100mg/ml or greater. Since early preclinical assessment is so critical to successful identification of a new biotherapeutic product, general pre-formulation strategies are usually employed for initial studies with new antibody drugs. An initial formulation effort will likely employ an isotonic, non-hemolytic, slightly acidic solution that can be administered by intravenous infusion or subcutaneous injection in studies employing a relevant animal model. In some cases, some stability information for the antibody drug can be obtained from initial in silico and in vivo testing during this pre-clinical phase that aids in the selection of a formulation that is used in clinical trials [26].

Since most early pre-clinical studies attempt to emulate the delivery strategy that will be used in the clinic, initial studies frequently identify problems with either the initial pre-formulation approach and/or stability issues with the antibody drug itself. In the case of the formulation, protein precipitation can occur with refrigerated storage or with repeated freeze–thaw cycles; events that may be facilitated by an inappropriate pH or buffer selection. Analysis of antibody drugs stored in this initial pre-formulation system may also identify unstable sites within the protein or its linkage to attached molecules (chemotherapeutics, imagining agents, etc.). In the case of the antibody, this information is frequently evaluated to determine if re-engineering of the protein could be performed, for example, to provide greater thermodynamic stability or folding efficiency [27]. Such changes in the Fc region are typically conservative, but might act to modify Fc receptor binding properties to provide a different biological outcome [28], [29]. Amino acid substitutions can also be made within the Fv region to improve thermal stability [30], but such changes might also alter antigen binding specificity [31].

4. Stability issues for antibody formulations

If one examines the amino acid sequences for currently approved IgG1-based antibody drugs, it becomes quite clear that only a small segment of these proteins are dramatically different from one another, being the Fv segments that are involved in antigen binding. It should be pointed out, however, that there are some minor differences in conserved regions as well. A priori, one might assume that by finding a stable formulation for one of these antibody drugs, that such a formulation would be good for most if not all, similar antibodies. If this were borne out by experience, there would be no need for a review such as this. Instead, each antibody seems to have a unique personality related to its requirements for stability; a phenomenon derived from the fact that the small differences between these antibodies are focused on surface-exposed amino acid differences that stipulate antigen specificity. Thus, the interfacial surface of each antibody drug is unique and thus requires specific formulation components to provide maximal stability and retention of activity.

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Probably the most important aspect of identifying a successful antibody drug formulation is in the identification of truly detrimental alterations rather than all of the modifications that might occur. Although, from a regulatory standpoint, it may be beneficial to understand all of the potential degradation events associated with an antibody formulation, it is the recognition of a stability-indicating parameter (or several parameters) that allows the formulation scientist to focus in on what is critical to development of a successful product. Here, a series of physicochemical and bioanalytical assays can be used to identify these parameters [32]. Although in vivo bioassays may ultimately be required to validate a stability-indicating formulation parameter, the most critical tools at the disposal of the formulation scientist are rapid and reproducible assays to measure protein changes due to oxidation, deamidation, aggregation, fragmentation and other forms of chemical modification that could lead to a loss of function of the antibody drug.

4.1. Oxidation

Methionine and cysteine residues are frequently a site of oxidation in protein drugs and this is also the case for antibody drugs [33]. In the case of antibody-based drugs, specific methionine residues within the Fc domain may be prone to oxidation, resulting in the production of methionine sulfoxide [25]. While cysteines are present in the Fc framework as disulfide pairs, unpaired cysteines in the variable region may also be sites of oxidation. Besides methionine and cysteine residues, oxidation of histidine, tyrosine, tryptophan and phenylalanine residues can also occur [34]. A number of methods can be used to monitor oxidation of amino acid residues. As a starting point, the number of reactive protein thiols can be monitored using reagents such as 5,5′-dithionitrobenzoic acid or iodoacethyl dansylcadaverine [35], providing a simple readout on the number of protein thiols per protein. Total amino acid analysis can also be used to monitor reductions in methionine, tyrosine and phenylalanine residues. Tryptic digestion and peptide separation by liquid chromatography followed by mass spectroscopy analysis (LC-MS) can be used to identify specific sites of amino acid oxidation.

A careful study of methionine oxidation has been performed for the humanized monoclonal antibody that recognizes the her2/neu gene product. For this protein it was determined that a methionine at position 255 in at heavy chain of the Fc region was the primary site of oxidation and that a methionine at position 431 could also oxidize under more aggressive conditions. In this study, a chromatography assay was used to detect oxidation events following papain digestion to separate Fab and Fc segments of the antibody [36]. Another study on the stability of this same protein provides a valuable template for dealing with antibody oxidation: identification of a critical oxidation event, validation of a robust assay for monitoring that event and comparison of formulation parameters to determine conditions that facilitate oxidation. Addition of antioxidants or stoichiometric levels of free methionine or thiosulfate were found to reduce the extent of oxidation in formulations of this antibody [37].

Issues associated with administration of oxidized forms of an antibody may extend beyond concerns of reduced efficacy. Systemic lupus erythematosus (SLE) is a disease characterized by the induction of anti-double-stranded DNA antibodies. Studies have shown that increased levels of markers of protein oxidation, including those of methionine sulfoxide and 3-nitrotyrosine, correlated with SLE disease activity [38]. Additionally, oxidative modifications of antibodies isolated from synovial fluid samples collected from patients with rheumatoid arthritis suggested alteration of histidine, methionine, tyrosine and cysteine residues [39]. While no direct causal association is shown or implied, these observations bring attention to the issue that we do not fully understand the clinical impact of antibody oxidation events.

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4.2. Deamidation

Glutamine and asparagine residues show a propensity for deamidation [40]. Both light and heavy chains of antibodies can undergo deamidation of these amino acids [41] and deamidation events are considered to be one of the major sources of charge heterogeneity of monoclonal antibodies [42]. Deamidation events appear to be highly selective events for individual antibodies. For example, analysis of several monoclonal antibodies that bind the her2/neu gene product demonstrated asparagine 30 deamidation on one or both light chains and asparagine 55 on the heavy chain [43]. Deamidation events lead to more acidic forms of the antibody through the acquisition of additional carboxylic acid groups. Conversely, aspartic acid residues can also undergo modification to a succinimide moiety that results in a basic antibody isoform of the antibody by removal of a carboxylic acid group [43]. In general, deamidation events appear to be relatively slow but are accelerated by unfolding of the antibody [44]. Initial detection of deamidation in antibody preparations is typically identified by differences in charge distribution or content using methods such as isoelectric focusing (IEF) or high-performance cation-exchange chromatography [43].

High performance chromatography can then be used to separate succinimide, isoaspartic and aspartic acid isoforms of tryptic digest fragments of the antibody which can be unambiguously characterized by tandem mass spectroscopy (MS). Correlation between tryptic digest tandem MS results and some other more readily accessible method, such as IEF, can provide a rapid format to follow antibody deamidation events. Using these techniques, asparagine deamidation events residues appears to occur more frequently than glutamine deamidation and specific asparagine residues appear to be selectively targeted for this event [33]. Deamidation of asparagine is frequently associated with rearrangement events that lead to the formation of isoaspartic acid [42] and this outcome is correlated to amino acids adjacent to the asparagine prone for deamidation [44] that provide a method for prediction of potential deamidation sites (BOX). In particular, asparagine–glycine sequences can demonstrate accelerated deamidation rates since glycine residues lack a side-chain structure that would otherwise act to decreased conformation interference for backbone-associated isoaspartyl formation [45].

Box 1

Amino acid* sequences predicted to deamidate

SNG, LNG, LNN and ENN

(Note these have a following amino acid of G or N)

and those that are less prone to deamidation

GNT, GNS, GNV, TNY, SNY, SNF, SNT, SNK, SNL,

WNS, WNS, FNW, FNS, FNR HNA, HNH, LNW,

CNV, NNF, DNA, and YNP

*standard single amino acid letter nomenclature.

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Non-enzymatic parameters of asparagine and glutamine deamidation in proteins have been defined in detail for factors such as pH, temperature and ionic strength [46], [47]. The primary sequence dependence of these deamidation events can be explained by a simple steric and catalytic model [46], [47]. Prediction of deamidation events based upon three-dimensional structures have also been described but the data set for these studies is not yet sufficient to produce a general model [48]. That asparagine and glutamine deamidation appear to be a constant phenomenon of proteins and correlate with their turnover provides the basis for the hypothesis that the presence and distribution of these amino acids within a protein can act as a molecular clock for its biological turnover [49], [50]. For the formulation scientist, empiric assessment of factors such as pH and ionic strength provide the best strategy to identify a formulation that minimizes deamidation events. In general, deamidation does not result in a decrease in potency and deamidation may be hard to detect as the charge difference is small. At times, however, there can be a detrimental effect on potency through the introduction of an unfavorable negative charge that can have further ramification on biological function, particularly when deamidation occurs in the binding regions [51]. As particularly reactive residues are identified, it may be possible to have the antibody engineered to remove the problematic amino acid or to change adjacent residues to reduce its reactivity.

4.3. Aggregation

Liquid formulations of protein therapeutics frequently exhibit a decrease in concentration due to adsorptivity to container walls at low concentration and can show aggregation events at high concentrations. Initial preparations of antibody drugs were administered in acute situations by IV infusion that could allow for medium protein concentration (1–10mg/ml) formulations not dramatically affected by adherence or aggregation losses. As antibody-based drugs became more widely used in chronic care, high concentration formulations that would allow for clinically acceptable SC injection volumes became desirable and aggregation became an important concern for designing formulations. Along with aggregation events, protein solutions at concentrations>50mg/ml may become sufficiently viscous as to no longer readily pass through the gauge of a needle that would be comfortable for patients' use. Some antibodies demonstrate high concentration viscosity issues while others do not. For example, the anti-respiratory syncytial viral antibody Synagis® is formulated at 100mg/ml protein and is administered successfully by IM injection. Thus, addressing viscosity, adsorptive loss and aggregation in any specific antibody formulation will likely be performed on a case-by-case basis.

As protein–protein contact frequency increases at high concentrations, the opportunity for stable aggregate formation increases proportionally. Proteins are folded in such a way as to internalize hydrophobic domains and surface-expose more hydrophilic domains in a thermodynamically stable situation. As folded proteins undergo normal molecular motion transient surface exposure of hydrophobic domains can lead to protein–protein contacts that hide these surfaces in a more energetically favorable manner. The outcome of these events is the stabilization of these protein–protein contacts with the ultimate outcome of dimerization, trimerization and so on — leading to extended aggregation events [52]. As protein concentration and temperature goes up, so does the probability of energetically favorable contacts leading to aggregation with increased frequency and duration of reversible protein–protein contacts leading to a greater extent of stable (even irreversible) aggregation events. Aggregation (covalent, non-covalent, dissociable, non-dissociable) must be considered a likely degradation event for high concentration antibody formulations.

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For some antibodies reduced temperatures can incite reversible self-associations; these molecules are known as cryoimmunoglobulins [53]. IgG3 cryoimmunoglobulins have been shown to self-associate through sugar structures of the Fc domain [54]. Self-association events involving IgG1 antibodies seem to occur primarily through weak ionic and hydrophobic interactions [55]. More recent studies have examined kinetic and thermodynamic aspects of antibody solution dimerization using a recombinant human monoclonal antibody that recognizes vascular endothelial growth factor (rhMab-VEGF) as a model [56]. It was found that for this IgG1 class antibody, aggregation rates were greater in slightly alkaline (pH 7.5–8.5) compared to slightly acidic (pH 6.5–7.5) conditions. A high salt environment (1 M NaCl) also enhanced dimerization. Not surprisingly, self-association events were accelerated as the storage temperature was increased. One of the most striking aspects of these dimerization studies on rhMab-VEGF is that trastuzumab, another genetically engineered antibody with the same IgG1 framework and a 92% sequence homology with rhMab-VEGF, fails to demonstrate aggregation events under the same conditions.

A number of additives have been identified that can reduce the rate of protein aggregation [52], [57]. A rather wide spectrum of agents can reduce protein aggregation rates: urea, guanidinium chloride, amino acids (in particular glycine and arginine), various sugars, polyalcohols, polymers (including polyethylene glycol and dextrans), surfactants and even antibodies themselves. Studies have shown that different proteins benefit from different types of anti-aggregating factors, consistent with the notion that the interfacial surfaces that drive interactions leading to aggregation are protein-specific. Conceptually, anti-aggregating agents might fall into one of two categories: small agents that fit into channels or grooves and large agents that might interact with lower-curvature surfaces of the protein. In essence, the small agents would act to stabilize a protein from acquiring a conformation that might be more reactive toward aggregation while a large agent might act to reduce the frequency of surface contacts that lead to an aggregation event. In the case of antibody formulation, either or both may be important. As we will discuss, agents found to effectively protect an antibody from aggregation may differ for various stress conditions; antibodies in solution that are undergoing agitation and thermal stress may differ from those of the same antibody as it undergoes lyophilization and reconstitution.

Protection from aggregation can be limited in antibody formulations by the addition of surface-active agents such as detergents, both charged and uncharged. As an interesting aside, such surface-active agents can help reduce protein loss due to adherence to surfaces that can occur with low concentration protein formulations. Polysorbate 20 and 80 (now available from a vegetal source) are examples of detergents that are widely used to reduce the rate of protein aggregation in high concentration antibody drug formulations. A precautionary note must be sounded, however, as detergents perform this function optimally at low concentrations (0.01% to 0.05%) and can act detrimentally to participate in protein unfolding at higher concentrations, e.g., 1%.

That antibody aggregation can be accelerated by several physical conditions provides the potential for multiple methods to determine if a particular antibody drug is prone to aggregation and to rapidly test formulations for their ability to impede aggregation. Agitation studies are done, in addition to thermal stability studies, to determine if aggregation can occur in a given formulation. Different test methods are used, employing a shaker or a stirring apparatus. In one study done on an IgG1 antibody found sizes of aggregates produced by the two methods were comparable [58]. They concluded that a higher throughput test could be constructed using stirring analyzed by absorbance measurement. In their work, the stirred samples exhibited much higher absorbance and second particle species in DLS, indicating that stirring stress produces a higher amount of smaller protein aggregates. While Mahler found polysorbate 80 protects

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against aggregation, the stirred samples had an increase in turbidity and more aggregate products were detected by DLS as compared to a surfactant-free formulation. Polysorbate 80 stabilizes small aggregates and prevents further progress in the aggregation process.

4.4. Fragmentation

Intact, full-length antibodies are composed of several segments that have natural flexure sites. One site, in particular, exists at the flexure point between the Fc and Fv domain. Cleavage at this hinge region is a common concern when assessing antibody drug stability [25]. Molecular sizing methods such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion high performance liquid chromatography (SE-HPLC) can be used to rapidly identify fragmentation events in antibody drugs [59]. Fragmentation of full-length antibody drugs is a common occurrence and invariably affects their function. In general, cleaved forms are present in such low amounts that it is likely that little change in efficacy would be seen [60]. At the very least, the antibody drug may have some small reduction in efficacy following fragmentation or may be more prone to proteases, degradation and clearance events that would limit the duration of its efficacy. At the worst, a fragmented antibody preparation may result in a different biodistribution and thus a different safety profile. This can be of particular concern if the antibody is being used to delivery a cytotoxic chemotherapeutic agent. Fortunately, the extent of fragmentation in an antibody can be easily limited in the formulated product.

5. Strategies to stabilize antibody formulations

While liquid antibody formulations are less expensive, faster to develop and generally easier to prepare for administration than alternative formulation approaches, liquid antibody formulations are prone to oxidation, deamidation, aggregation and fragmentation as discussed above. In each of these events, water is the common culprit: water mediates electron transfer during oxidation and deamidation events and its required addition across a peptide bond can be a critical step in fragmentation. The thermodynamic stresses that lead to protein aggregation are caused by hydrophobic protein surfaces being exposed to water and trying to find a lower energy state by non-native protein–protein interactions. Thus, water is a big problem for stabilizing antibody-based drugs. Lyophilization or introduction into hydrophobic polymer systems can reduce the impact of water on antibody drug formulations.

5.1. Lyophilization

Although some antibodies have demonstrated a propensity to undergo precipitation as they are cooled from 37°C [53], antibodies are typically robust to freeze/thaw cycles [61]. Early studies reported that antibodies were stable to the freeze-drying (lyophilization) process in the absence of excipients that function as cryoprotectants [62], however, with the technical advancement of certain analytical methodologies, became evident that antibodies are generally damaged in some way as a result of lyophilization as demonstrated by extensive formation of insoluble aggregates [61]. More recently, studies have shown that freeze-drying antibodies to a specific percent of residual water (usually between 1 and 8%) allows for optimal stabilization in the dry state and stability during reconstitution [63]. Higher moisture content in lyophilized antibody preparations correlates with increased aggregation (as seen by SEC) and asparagine deamidation and isomerization (as measured by HIC). Studies have also been performed to identify optimized lyophilization schedules where the secondary drying phase is performed at an elevated temperature [64]. This technique

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allows the lyophylized cake to achieve optimum moisture content, allowing the final product to be conformationally stable (as measured by DSC) during extended storage. Solid-state structural studies can be used to monitor conformational changes and aggregation events during the storage of a lyophilized monoclonal antibody preparation [65]. Improved native-like structure and a reduction in aggregation can be obtained by incorporation of a carbohydrate excipient in sufficient quantities to fulfill the hydrogen bonding requirements on the protein surface, suggesting a critical role for non-water molecules to act as placeholders for water molecules during drying and in the dry state. That the addition of excess carbohydrate failed to improve the stabilizing function of this excipient, reaffirms the conclusion that antibodies have a limited number of sites that must be protected in this fashion [65]. A variety of carbohydrates or polyol compounds such as sucrose, trehalose and mannitol have been shown to provide this stabilizing function [66], [67].

The rate of rehydration when water is reintroduced during reconstitution of lyophilized formulations is a critical parameter. As water is removed during the different drying stages of the lyophilization cycle and replaced by a shell of non-water excipients, the antibody drug can undergo structural modifications that result in a non-native conformation as may be seen by differential scanning calorimetry [63]. If the rate of rehydration is sufficiently slow to allow recovery of native conformation as water replaces non-water excipient molecules, reconstitution will typically provide a satisfactory outcome for the antibody. If, however, the antibody does not have sufficient time or capacity to recover its native state during reconstitution, extensive aggregation can result. Efforts to protect monoclonal antibodies from the rigors of freeze-drying and reconstitution have been aided by a number of analytical methods that can examine protein modifications. In the solid state, circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy may be used to evaluate the protein; after reconstitution in the liquid phase, SEC or SDS-PAGE have been utilized to gather information from lyophilization studies [68], [69]. A microscopic apparatus to visually monitor freeze-drying events has even been described [70]. Excipients that have been reported to slow the reconstitution rate and thereby preventing such changes in the rehydrated antibody are glycerol [71], [72] and polymeric materials such as polyethylene glycol [73].

Pharmaceutical scientists frequently use increased temperatures for accelerated formulation stability studies. Although such studies can be used to assess formulation parameters such as pH and ionic strength, antibodies lyophilized in the presence of a cryoprotective sugar excipient provide a more complicated picture. Lyophilized amorphous solids composed of a monoclonal antibody and a sugar cryoprotectant material such as sucrose or trehalose form glassy structures, liquids that are too viscous to flow. That antibody-excipient complexes exhibit a glass transition temperature (Tg) provides the basis for observations showing that these materials have unique properties above and below this value. Thus, in predicting shelf life in accelerated degradation studies, it is important to use testing temperatures that do not exceed the Tg threshold of the complex [67]. In general, lyophilized formulations containing trehalose may likely show less tendency toward antibody aggregation than those with sucrose, since trehalose preparations have a higher Tg [67]. With this in mind, a promising approach to assess secondary structural parameters in evaluating antibody formulation stability is the use of amide I band Raman spectroscopy. This method demonstrates a good correlation between structural protein perturbations immediately after lyophilization and the rate of aggregation during long-term storage under accelerated conditions [74].

5.2. Polymer delivery systems

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Removal of water from antibody preparations, either by lyophilization or spray freeze-drying, provides a provides a starting point for incorporation of this dried material into a hydrophobic polymer matrix such as polylactide-co-glycolide (PLGA) that can be made into microspheres generated through a solid-in-oil-in-water (S/O/W) encapsulation process [75]. Although both mannitol and trehalose can stabilize human IgG during spray freeze-drying, the double-emulsion solvent evaporation method used to load spray freeze-dried human IgG into PLGA microspheres has been shown to produce extensive antibody aggregation [75]. Such an outcome is likely the result of thermodynamic stress experienced at the water/organic solvent interface and a variety of process methods and excipients can be use to minimize this concern [76].

One feature of PLGA microparticle preparations is their recognition by immune elements and their capacity to stimulate immunization against incorporated proteins [77], [78], [79]. While this has clear advantages for vaccines, it poses a serious concern for therapeutic protein delivery. Hydrophobic microparticles have been reported to incite local inflammation at subcutaneous injection sites [80], events that may be partially mediated through macrophage recruitment [81]. Not only is the potential for antibody formation increased with local inflammation, the resulting injection site reaction may be clinically unacceptable.

In spite of some serious caveats, polymeric microspheres and delivery systems can be successful antibody formulations if administered to the appropriate sites, using certain types of polymers or preparing microspheres or polymeric formulations by methods that are more protective of the antibody. An example of a certain delivery site that may have less proclivity towards an inflammatory response is reported, where PLGA microspheres loaded with an anti-VEGF antibody were injected into the intravitreal humor of the eye, an immune-privileged site, for the treatment of age related macular degeneration (AMD) [82]. Resorbable polymers that show less potential to incite inflammation, e.g., have smoother surface characteristics, might be used to deliver antibodies in a sustained manner. Direct, local delivery of antibodies using carboxymethylcellulose aqueous gels can be an effective post-surgical anti-infective strategy [83]. Antibodies covalently coupled to a biodegradable hyaluronic acid hydrogel through a labile hydrazone linkage have been used for the sustained delivery of antibodies to sites within the central nervous system [84]. Introduction of dry powder IgG into ethylene–vinyl acetate copolymer (EVAc) has also been examined as a potential polymer-based antibody delivery system [85]. Similar to PLGA systems, EVAc microspheres can extend the time course of IgG release. This property makes polymers loaded with antibodies interesting opportunities for injectable sustained release delivery formats but also for topical application at mucosal surfaces. For example, long-term vaginal delivery of an antibody can be achieved in a mouse model using polymer vaginal rings [86], [87]. Formulations of a biomedical grade polyurethane hydrogel with an antibody coating have also been prepared and shown to release bioactive protein [88]. It is important to note that one complicating aspect of many of these approaches is the requirement of a polymer solubilization step using a solvent, such as methylene chloride or isopropanol, which can compromise protein drug stability. Sustained release formulation approaches such as microspheres prepared using water, such as the PROMAXX microspheres (Epic Therapeutics) or crystallization of proteins [89] can bypass the use of solvents that are incompatible with proteins and antibodies.

6. Formulation issues for alternative forms of antibody delivery

To date, antibody development has focused primarily on injectable routes of administration: intravenous (IV), intramuscular (IM) and subcutaneous (SC). A long history of IV antibody infusions exists [90] and the

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advantages are clear for antibody-based drugs that are used in cancer treatments in order to reach tumor sites that may be diffuse or not otherwise accessible. In fact, the majority of the approved antibody-based drugs are administered by IV infusion (Table 1) with the total dose administered approaching one gram of protein per treatment in some cases. For many antibody-based therapies a critical safety concern involves the onset of acute or delayed systemic reactions following infusion [91], [92]. Parameters and characteristics of the "infusion reaction" as well as treatment protocols have been well described with acute events commonly occurring within 10 min to 4 h and delayed events occurring 24 h to 4 days post dosing [93]. Although formation of antibodies to the administered antibody drug appear to be a risk factor for infusion reaction events [94], infusion reactions can occur following the initial exposure [93] and these reactions do not appear to be due to IgE responses or mast cell activation [95]. Most importantly for the patient, there appear to be treatment protocols to provide re-treatment of patients even after an infusion reaction event [93] with concomitant administration of immunomodulation molecules [94]. There are many unanswered questions about the mechanism and prevention of adverse infusion reaction events associated with IV infusion of antibody drugs. Certainly, one method is to explore IM and SC delivery when possible, although these delivery approaches may also have confounding aspects of tissue response and volume limitations.

Table 1. Formulations of approved products

Antibody product; company	Generic name; description	Delivery route	Formulation concentration	Buffer components; pH[a]	*Excipients*
Othroclone OKT3®; J & J-Ortho Biotech	Muromonab-CD3; Murine IgG2a, anti-CD3	IV	1 mg/ml	Na,K phosphate; pH 7	NaCl, polysorbate 80
Reopro®; Centocor-Lilly	Abciximab; Chimeric IgG1, anti-GPIIb/IIIa, Fab	IV	2 mg/ml	Na phosphate; pH 7.2	NaCl, polysorbate 80
Rituxan®; Idec-Genentech	Rituximab; Chimeric IgG1, anti-CD20	IV	10 mg/ml	Na citrate; pH 6.5	NaCl, polysorbate 80
Zenapax®; Roche	Daclizimab; Humanized IgG1, anti-CD25	IV	5 mg/ml	Na phosphate; pH 6.9	NaCl, polysorbate 80
Simulect; Novartis[b]	Basilixamab; Chimeric IgG, anti-CD25	IV	4 mg/ml	Na,K phosphate, glycine	NaCl, sucrose, mannitol, polysorbate 80
Synagis®; MedImmune[b]	Palivizumab; Humanized IgG1, anti-RSV	IM	100 mg/ml	Histidine, glycine	Mannitol
Remicade®; Centocor[b]	Infliximab; Chimeric IgG1, anti-TNFα	IV	10 mg/ml	Na phosphate; pH 7.2	Sucrose, polysorbate 80
Herceptin®; Genectech[b]	Trastuzumab; Humanized IgG1, anti-HER2	IV	21 mg/ml	Histidine; pH 6	Trehalose, polysorbate 20, benzyl alcohol[c]
Mylotarg®; Wyeth[b]	Gemtuzumab ozogamicin; Humanized IgG4, anti-CD33,	IV	4 mg/ml	Na phosphate	NaCl, sucrose, dextran 40

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Antibody product; company	Generic name; description	Delivery route	Formulation concentration	Buffer components; pH[a]	*Excipients*
	immunotoxin				
Campath®-1H; Millennium-ILEX	Alemtuzumab; Humanized IgG1, anti-CD52	IV	Unclear — 30mg /vial	Na,K-phosphate pH 7	NaCl, KCl, NaEDTA, polysorbate 80
Zevalin®; Biogen Idec	Ibritumomab tiuxetan; Murine IgG1, anti-CD20; radiolabeled (^{90}Y or ^{111}In)	IV[d]	1.6mg/ml	Na Acetate, Na,K-phosphate pH 7	NaCl, human albumin
Humira®; Abbott	Adalimumab; Human IgG1, anti-TNFα	IV	50mg/ml	Na phosphate, Na Citrate; pH 5.2	Mannitol, polysorbate 80
Xolair®; Genentech[b]	Omalizumab; Humanized IgG1, anti-IgE	SC	125mg/ml	Histidine	Sucrose, polysorbate 20
Bexxar®; Corixam-GSK	Tositumomab-I131; Murine IgG2a, anti-CD20, radiolabeled (^{131}I)	IV	14mg/ml	Na phosphate; pH 7.2	NaCl, maltose
Raptiva®; Genentech	Efalizumab; Humanized IgG1, anti-CD11a	SC	100mg/ml	Histidine; pH 6.2	Sucrose, polysorbate 20
Erbitux®; Imclone Systems	Cetuximab; Chimeric IgG1, anti-EGFR	IV	2mg/ml	Na phosphate; pH 7.2	NaCl
Avasatin®; Genentech	Bevacizumab; Humanized IgG1, anti-VEGF	IV	25mg/ml	Na phosphate; pH 6.4	Trehalose, polysorbate 20
Tysabri; Biogen Idec	Natalizumab; Humanized IgG4, anti-4α-integrin	IV	20g/ml	Na phosphate; pH 6.1	NaCl; polysorbate 80

Ordered by approval dates in the US.

Abbreviations: RSV=respiratory syncytial virus; TNFα=tumor necrosis factor alpha; EGFR=epidermal growth factor receptor; VEGF=vascular endothelial growth factor; NaEDTA=sodium edentate.

a
Some lyophilized formulations do not stipulate a pH (or range) following reconstitution.

b
Lyophlized product, concentration, pH, etc. values are obtained after reconstitution.

c
Added as a preservative to generate a multi-dose vial configuration.

d
Kit where components are mixed just prior to administation.

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Other routes are also amenable to antibody delivery. The increased number of antibody therapeutics on the market and the greater exploration of non-life threatening indications being treated by antibodies, drive efforts to deliver these agents by routes other than IV infusion. These routes may be more effective, less invasive, provide easier delivery, permit self-administration and thereby be more convenient and possibly have lower costs if less active agent can be used, as in the case where the alternate route of administration delivers the therapeutic agent to the intended site directly. Interestingly, some studies have suggested that antibodies administered by IV infusion are more susceptible to proteolysis than via IM injection [59] and sucrose-stabilized immunoglobulins have been associated with acute renal failure following IV infusion [96]. The antibody used to protect neonates from respiratory syncytial viral (RSV) infection (Synagis®) is one example of an antibody administered by IM injection. For reasons discussed above, the majority of antibody-based drugs have moved toward SC delivery with formulations efforts being focused to this end. Based upon the desired actions for a number of antibody-based drugs, however, routes of administration other than IV, IM or SC may be desirable. These alternative routes can provide unique formulation and stability issues.

6.1. Oral

Uptake of antibodies from the intestinal lumen can occur through fetal Fc receptors (FcRn) expressed at the apical surface of enterocytes [97]. This natural antibody uptake mechanism was initially identified in the gastrointestinal (GI) tract of neonatal rodents and its expression shown to vanish following “closure” of the intestine after weaning. It was only recently that the continued expression of this receptor in the adult intestine was demonstrated and this finding has prompted efforts to examine this receptor system as a means of delivery antibodies after oral administration. Although this pathway appears to be a viable route for the delivery of therapeutic antibodies, issues associated with antibody stability in the GI tract [98] and competition from endogenous antibodies can dramatically reduce the efficiency of uptake after oral delivery. Outside of this specific mechanism, no other transcellular mechanism of efficient antibody transport across epithelial cell of the GI tract has been demonstrated to date. Topical application of antibodies to produce a desired clinical outcome have been shown, however, with the oral delivery of a chicken-derived (IgY) antibodies for treating and preventing infectious diseases of the GI tract [98], [99], [100]. It should be noted that IgY antibodies have been shown to not bind to (or be transported by) FcRn [97].

6.2. Respiratory tract

Intranasal and pulmonary administration of protein therapeutics has shown more promising clinical outcomes than oral delivery. While the surfaces of both the respiratory tract and the gastrointestinal tracts are readily accessible, the respiratory tract is a far less hostile environment for an administered protein therapeutic. Nonetheless there are physical clearance mechanisms in place to remove foreign particles which act to reduce residence time dramatically following administration. In the case of nasal administration the use of a cream emulsion and a polymer delivery system has been shown to significantly decrease the clearance rate for a monoclonal antibody when applied to the nasal mucosa [101]. This study provides one approach for the topical application of a therapeutic antibody designed to provide clinical benefit at the mucosal surface. It does not, however, suggest that an appreciable amount of an antibody might be absorbed into the systemic circulation from this site. The nasal mucosa affords only a small absorptive surface area compared to the epithelial linings of the lung.

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Aerosol delivery of antibodies to the lung has been explored as either a liquid or dry powder formulation, where in both cases the droplets or particles must be of sufficiently small size that they are respirable perhaps to the level of the deep lung but large enough that they are not readily exhaled or just impact the back of the throat [102]. The sizes of particles that provide this performance have a mass mean aerodynamic diameter and density such that they enter the alveoli and remain in the lung. This size window has been reported to be between 1 to 5μm depending on the formulation used and in one case, far larger, 20μm where the particles are dense (<0.4g/cm^3) and highly porous [102].

Formulation efforts were made for producing fine dry powder particles of an anti-IgE monoclonal antibody (E25) in the size range desired for delivery to the lung [103]. These studies showed that sodium phosphate was capable of extending the usefulness of mannitol as a protective excipient in spray-dried antibody formulations and that mannitol can play a role both in maintaining protein stability as well as producing a suitable aerosol preparation. Further studies to assess carbohydrate excipients for spray-dried aerosol E25 formulations showed that excipient to protein ratios were critical. Degradation issues involved not only protein aggregation but also protein glycation or lactosylation, a protein modification event where a reducing sugar can add covalently at a free amine [104]. The ability to formulate and delivery an antibody-based drug to the lung provides the potential for systemic delivery of that protein via uptake through the FcRn expressed at this mucosal surface. Elegant studies showing the distribution and function as well as the application of this transport route for systemic deliver of an Fc-containing protein chimera has been demonstrated [105], [106]. Thus, the potential or systemic delivery of antibody-based drugs via the respiratory tract looks promising.

6.3. Miscellaneous delivery sites of the body

Unique aspects of antibody–polymer formulations designed for intravitreal injection [82] or topical vaginal delivery [86], [87] have been discussed above. Additionally, antibody formulations using hyaluronic acid hydrogels for sustained brain delivery have been noted [84]. These methods focus on strategies to administer large amounts of an antibody at a specific site in order to provide a sustained level to improve efficacy and/or to minimize injection frequency. Other strategies have been described to deliver antibodies to miscellaneous sites throughout the body, For example, an anti-transferrin antibody (OX-26) has been described as a carrier for delivery of covalently attached therapeutic molecules across the blood–brain barrier following IV injection [107]. An anti-tumor necrosis factor antibody has been adsorbed onto cardiovascular stent wires for local neutralization of this cytokine in order to limit restenosis events following stent placement [108]. A CMC gel containing antibodies to prevent post-surgical infections has been administered to the intraperitoneal cavity [83].

6.4. Intracellular targeting

Some promising antibody drug targets are intracellular. Although techniques exist where a cell can synthesize an antibody and secrete it into the cell cytoplasm, successful use of this technology requires gene therapy. Instead, it is possible to chemically couple an antibody to a pH-sensitive polymer that functioned to disrupt membranes at acidic pH. Many antibodies are internalized by cells through a receptor-mediated uptake process that leads to their delivery into early and then late endosomes. During endosomal maturation after receptor–antibody internalization the pH of these vesicles will drop from near neutral to below pH 6. This approach was demonstrated in a format where the pH-responsive polymer

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poly(propylacrylic acid) was covalently attached to a monoclonal antibody to facilitate its delivery to the cytoplasm of target cells [12].

7. Other delivery issues

A wide range of novel drugs and drug conjugates are being developed using monoclonal antibodies and engineered antibody fragments [20]. For some of these novel therapeutic approaches to be successful, they require additional or non-traditional delivery strategies. A cursory discussion of some of these approaches as they relate to intracellular targeting, site placement and engineered antibodies fragments follows.

7.1. Antibody fragments

An entire spectrum of antibody fragments have been identified through protein engineering studies with many of these showing remarkable potential for unique and novel clinical opportunities [109]. These smaller recombinant antibody fragments, the monovalent antibody fragments, Fab and single chain Fv and the engineered variants, diabodies, triabodies, minibodies and single-domain antibodies are an emerging form of antibody therapy that are less costly to manufacture while still retaining the targeting specificity of full length antibodies. In fact some may have greater efficacy and more applications than whole monoclonal antibodies. It may be anticipated that this new subset of antibodies are posing new formulation challenges, however, to date, publications detailing stability studies and formulation development for these molecules are rare in the scientific literature so formulation data on antibody fragments is scant. The single V domains have been found to be poorly soluble and have a tendency to aggregate [109], but these problems are being overcome by the identification of mutants that minimize the hydrophobic interface and selected by phage display [110]. Five Fab molecules have been approved as of this writing with the one humanized Fab (ranibizumab rhFab) awaiting approval in the very near future, but many are in early clinical trials and most in preclinical development. One study has been described that focused on Fab fragments. In general, the binding of an antibody to an effector protein does not necessarily inactivate that target's function [111]. When an antibody is effectively inactivating, however, the Fab fragments in the absence of an Fc domain can frequently function as highly potent binding agents capable of masking binding sites or enzymatic elements on macromolecules. For example, Fab fragments prepared from sheep anti-snake venom antibodies (Digibind, from GSK) can be an effective anti-venom. Storage of liquid formulations of such anti-venoms in sub-tropical countries where refrigeration may not be possible provides a significant formulation challenge. Although lyophilized products can perform well under these conditions, a pH 4.0 (acetate buffer) formulation was found to also be stable and potent for at least one year at room temperature [112], presumably room temperatures characteristic of the tropical and subtropical regions where the material would be used. Strikingly, this stable formulation did not require the presence of a protective carbohydrate excipient. To improve drug delivery, antibody fragments have been fused to many types of molecules, such as radionuclides, cytotoxic drugs, toxins, peptides and proteins, enzymes and liposomes [113]. An immunoliposome made with a Mab fragment is reported to deliver drugs to the brain as such a molecule is able to cross the formidable barrier of the blood brain barrier [114].

7.2. Immunoconjugates

Arming antibodies with materials useful for detection or cell killing has become a prominent approach to increase the utility of these proteins, reviewed by Wu and Senter, [20]. Antibodies provide a useful format

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for the production of radiopharmaceuticals for site- or tissue-specific localization using gamma scintigraphy [10], [115] or for enhanced cell killing. There are currently three approved antibody–radionuclide drugs: two are murine radiolabeled Ab for B cell lymphomas ibritumomab tuixetan (^{90}Y, Zevalin) and tositumomab (^{131}I Bexxar) and the third is a humanized, gemtuzumab ozogamicin (calicheamicin, Mylotarg) for leukemia. The presence of a high energy emitting radionuclide, however, will compromise long-term stability of any antibody formulation; not only would the released energies act to damage the protein but there is strong preference to use short half-life isotopes for these procedures [116]. Thus, in these instances, a stable formulation based upon the principles discussed above can provide a product that can be radiolabeled and then used soon after this step. One example of this approach is a lyophilized antibody formulation that is reconstituted and prepared by a kit method prior to patient dosing [117].

Antibodies coupled with cytotoxins for targeted cell killing [118] are particularly useful in antibody-based cancer therapies [119]. Beyond the identification of a stable formulation for the antibody, such materials have several additional problems for the formulation scientist to solve: stability issues of the attached agent and stability of the linker used to join the attached agent to the antibody. In the case of a monoclonal antibody-vinca alkaloid conjugate, a pH range of 4.5–5.5 in a phosphate buffer showed improved solution stability compared to material stored at pH 6.5–7.4 as evidenced by antibody aggregation and hydrolysis of the chemical linker used to conjoin the alkaloid to the antibody [120]. In most cases, cytotoxic materials are coupled to antibodies using reversible chemistries to provide a release mechanism for maximal activity at the target site. Many of these antibody conjugates use a disulfide bond linkage that can be reduced by a novel redox enzyme present in endosomes and lysosomes of target cells [121]. Slightly oxidizing conditions that would provide added stability of this linker in the formulation could, over time, act to cause detrimental oxidation events in the antibody and/or the linked compound. Other conjugates use an acid-labile linker [122]. In this case, formulation pH must be restricted to either neutral or alkaline conditions. Enzymatic separation of antibody and a cytotoxic material could also be achieved by coupling the two through an amino acid linker that is recognized as a substrate by a specific protease or peptidase [123]. Formulation instability of this linker sequences can be corrected to some extent by selective changes of the amino acids used.

Thus, the challenges of immunoconjugates include maintaining both drug and antibody potency and stability. While some antibodies are efficacious by themselves, many are much more therapeutically active after being armed with a toxin, drug and radionuclide [124]. These changes make the resulting product more difficult to prepare, stabilize and deliver. Yet, there is tremendous enthusiasm for these approaches as they provide the promise for some companies to transform their antibody into a potentially curative drug. For this reason many antibodies are combined with a generic delivery system, such as a liposome, that can be used to delivery a variety or even multiple agents simultaneously through antibody-directed targeting [125]. Examples of such an approach include studies performed using an antibody directed against the Her2/neu antigen to delivery liposomes containing a chemotherapeutic have been described [126], antibodies used to deliver cationic liposomes for the administration of nucleic acid material for gene therapy [127] and antibodies used in the targeted delivery of modified gelatin nanoparticles [128] that can be coupled through a disulfide linkage [129].

8. Conclusions — challenges and opportunities

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As antibody-based drugs come to the forefront of therapeutics derived from biotechnology, they provide a range of new and exciting opportunities to treat a number of currently unmet medical needs. Successful clinical application of these novel agents requires the development of stable formulations that can be used for specific delivery methods. Antibodies, because of their endogenous nature, have built-in features that may pose problems for stability as biotherapeutics, that function in the normal clearance of these proteins from the body [40]. Thus, despite our best efforts to prepare stable antibody-based drug formulations, the ultimate stability of these products may be limited to inherent mechanisms of degradation pre-designed for the rate of clearance optimal for their function and turnover in the body. Protein engineering has led to significant advances in improving some of these parameters and the trend would predict that additional advances will be made in the near future (reviewed in [109]) Protein modifications can be engineered such that stability and efficacy can be improved in ways that cannot be achieved solely, if at all, with formulation alterations. While it is clear that more research is needed in the relatively new area of engineered antibody fragments for clinical use [109], they have great potential for diagnostic and therapeutic application.

In general, successful antibody formulation designs are simply the best compromise of minimizing several competing degradation pathways. One approach is to find a pH where the minimal amount of degradation events such as deamidation and oxidation will occur. Another is to identify a buffer system that can stabilize the formulation at that pH without damaging the antibody. One common tactic taken to reduce the rate of many of these chemical degradation events is to prepare lyophilized or freeze-dried antibody preparation but even these can be compromised by aggregation issues due to thermodynamic stress that occurs during freezing, drying and reconstitution. A number of protective excipients, mostly carbohydrates, can reduce the propensity of antibodies to aggregate during these stressful events. Another issue is to produce a formulation that is compatible with delivery — neither hypotonic — nor hypertonic solutions are well-tolerated upon injection. Added to this is the potential use of a surface active agent to help with liquid stability such as a non-ionic detergent at a low concentration [58] as well as the possible addition of preservatives required for multi-use systems [130].

The future of antibody based drugs is likely to include more complex systems than those currently approved for use in man. Possibly the most critical aspect of generating a pharmaceutically successful formulation for antibody based drugs is an understanding the critical aspects of stability for the protein or the materials associated with the protein. It has been known for some time that even a single amino acid change in the Fv region of an antibody can result in dramatic differences in antigen-binding specificity [31]. Also, alterations at specific amino acids in the Fc domain can dramatically change functional aspects of an antibody. Thus, these kinds of changes must be considered more important in the search for a successful formulation than those changes that do not appear to affect function. In the case of antibody or antibody-like agents being used to delivery a payload such as a toxin or chemotherapeutic, the stability of the material to be delivered and its continued association with the delivery antibody is crucial.

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of references cited were selected to highlight specific aspects within the scope of this review. We regret that many excellent publications could not be cited that have made important contributions to this field.

Special issue articles Recommended articles

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Formulation and delivery issues for monoclonal antibody therapeutics☆

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Abstract

Antibodies can have exquisite specificity of target recognition and thus generate highly selective outcomes following their systemic administration. While antibodies can have high specificity, the doses required to treat patients, particularly for a chronic condition, are typically large. Fortunately, advances in production and purification capacities have allowed for the exceptionally large amounts of highly purified monoclonal antibodies to be produced. Additionally, genetic engineering of antibodies has provided a stable of antibody-like proteins that can be easier to prepare. Together, these advances have made antibody-based therapies one of the most commonly pursued pharmaceuticals in biotechnology pipelines. With this success, however, has come a series of technical challenges in the formulation of antibody-based materials to maintain sufficient stability in a variety of configurations and sometimes at particularly high concentrations. This review focuses on issues related to identifying and verifying stable antibody-based formulations.
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Keywords: Antibody; Stability; Delivery; Formulation

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1. Introduction

Therapeutic and diagnostic antibodies have become the fastest growing area of biopharmaceutical applications. There are now 18 monoclonal antibodies on the market and over 100 in clinical trials, which highlights the significance of these therapeutics and the advances made in antibody engineering. Further, by 2008, engineered antibodies are projected to be the source of over a third of the revenues from biotechnology [1,2].

A few seminal events have led to the current and projected prominence of antibodies as biopharmaceuticals include the identification of methods to generate murine monoclonal versions of antibodies [3], the cloning of human antibody sequences to allow for humanization of murine monoclonal antibodies through complementary-determining region (CDR) grafting [4] and even the establishment of fully humanized systems to generate monoclonal antibodies [5]. With the sequential identification of these technological advances, antibodies for therapeutic and prophylactic indications have moved from fully murine, to partially murine (mostly human) to completely human constructions. Added to these events, dramatic advances in production have led to the ability to prepare monoclonal antibodies at scales that can provide sufficient material at costs that make this area appealing to pharmaceutical companies. One important outcome of these various advances is a greater potential to use antibody drugs in chronic settings, tremendously expanding their biopharmaceutical applications.

The majority of currently approved antibody drugs address previously unmet medical needs in the areas of cancer and inflammation. These indications came from practical considerations related to earlier forms of murine and partially murine antibody therapeutics; the first monoclonal to be approved (OKT-3®, muromonab-CD3) was approved in 1986 for a single treatment regimen to protect from life-threatening tissue rejection following kidney transplant [6]. Adverse reactions due to acute immunogenicity problems due to delivery of a non-self protein limited the use of OKT-3® to only acute application [7,8]. Subsequent partially murine antibodies such as Panorex® (17-1A), Zevalin® (ibritumomab tiuxetan) and Bexxar® (tositumomab) were approved for a limited number of administrations to cancer patients who had failed frontline therapies [2]. With the advent of fully humanized antibodies, chronic therapeutic or prophylactic applications for infectious agents have become a realistic clinical option [2]. Another critical aspect to the success of antibodies as therapeutic agents involves improved methods to express, purify and characterize these proteins [9]. In general, antibody therapeutics are

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large (typically >150 kDa), complex in nature (most are glycosylated) and must be administered in stoichiometric rather than catalytic quantities (nearly a gram per dose is required for many antibodies to be effective). Production and purification scales have thus reached levels of production that were previously assumed impossible in biotech companies [10]. Novel concerns then related to the development of stable formulations and delivery strategies for such large amounts of a complex molecule. Understanding product instability with regard to physicochemical and thermodynamic events becomes more and more critical to ensure market success [11]. The required high concentrations of antibodies in these formulations reduced the need to add a carrier protein, as is the case where serum albumin is commonly added in formulations of highly potent proteins such as erythropoietin alpha (Epogen®) and Factor VIII (Kogenate®) to enhance stability and reduce loss to vial surfaces. Simultaneously, the large amounts of an antibody protein used in these formulations allowed degradation products to be more readily detectable particularly when using the highly sensitive methods designed for evaluating hormone biotherapeutics.

Finally, the ability to prepare large quantities of an antibody that could be administered for chronic therapy resulted in a plethora of new and novel applications [9,10] involving the delivery of chemotherapeutics, radionuclides and imaging agents — to name a few. New issues, related to identifying stable formulations such as high concentration formulations that might reduce the volume of antibody administrations, arose in the move from intravenous infusions to the realm of subcutaneous injection strategies. In some cases the combination of agents with antibodies produced conflicting stability profiles. Additionally, potential new indications for antibody drugs increased the desire to find novel methods for sustained release, intracellular targeting [12] or delivery via methods not involving an injection. Thus, as is the case in most scientific advances, technological advances opened up possibilities for therapeutic opportunities that were not possible previously as well as a totally new set of problems that required solving. In this review we have attempted to identify the major issues associated with formulating and delivering antibody drugs, to recognize challenges already identified accompanied when possible by solutions, as well as to discuss concerns that will need to be addressed in the future as the use of antibody drugs continues to evolve.

2. Antibody characteristics relevant for formulation and delivery

The human body produces several classes of antibodies that appear to perform specific functions under different conditions and at discrete anatomic sites. Most of the currently approved antibody-based drugs are of the IgG1 class [13] and are glycosylated on the Fc (fragment crystallizable) domain. Glycosylation has been shown to participate in specific cell response events mediated by antibodies [14–16]: antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cell-mediated cytotoxicity (CDCC) through receptor recognition events. Individual glycoforms of antibodies can provide improved efficacies for certain clinical outcomes [17]. In general, IgG1 and IgG3 antibodies are effective at initiating ADCC while IgG2 readily fixes complement but is poor at inducing ADCC. IgG4 appears to be inefficient at both ADCC and CDCC [13]. Interestingly, antibodies can be re-engineered to increase their capacity to recruit complement [18] or to adjust their affinities to the various classes of Fc receptors and thus ADCC function through mutation of specific amino acids within the Fc domain [19]. Molecular engineering has also provided a spectrum of new antibodies (Fig. 1) Other chapters in this issue delve deeply into the many characteristics of antibodies that make them remarkable and diversely applicable treatment agents: ligand binding properties, effector functions and potential for modification in their design. Other chapters will also address applications of newly engineered antibody-like molecules that move beyond the realm of intact, full-length IgG antibodies. In the current review, attention will be focused primarily on IgG1 since this is where the majority of published information exists for antibody drug formulation and delivery.

Antibodies, due to their large size (being greater than the filtration cut-off size of the kidney) and their ability to be sequestered for periods of time from the blood, via interactions with FcRn receptors present on

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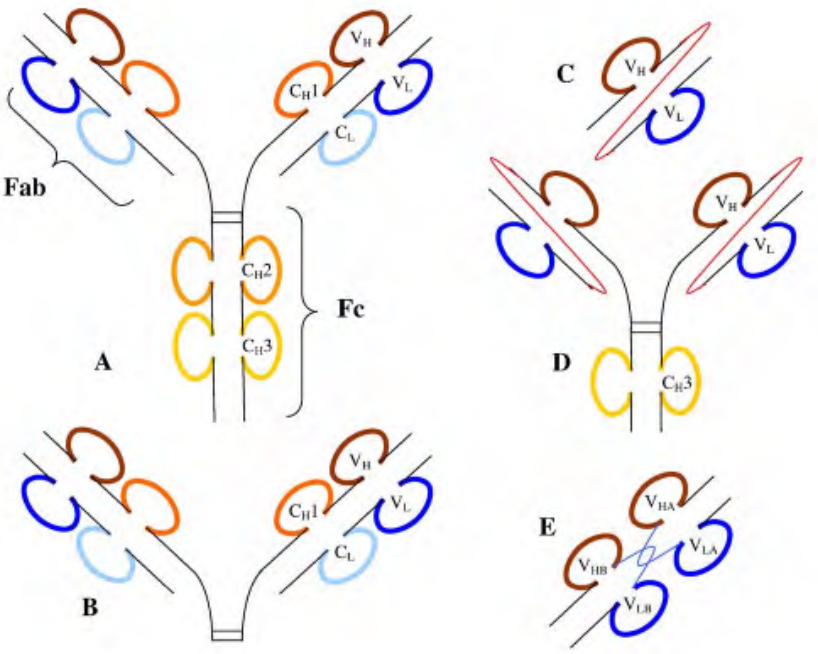


Fig. 1. General antibody structure and some antibody-based protein constructions. A) Human antibodies have a common framework that can be described as Fc (fragment crystallizable) and Fab (fragment antigen binding) domains that are derived from the folded structure of heavy (H) and light (L) chain proteins. Multiple constant (C) regions on both the H and L chain proteins are donated as are the variable (V) segments involved in antigen-specific recognition. B) Removal of the Fc domain produces a F(ab)2 structure. C) Single chain structures of the antigen binding region (scFv) can be prepared by themselves or D) in a construct that includes Fc domain elements. E) Bi-specific antibody elements (diabodies) can be prepared to bring two different antigens into close proximity. C_H, heavy chain constant regions; C_L, light chain constant region; V_H, heavy chain variable region; V_L, light chain variable region.

endothelial cells, can have extended serum profiles in the range of weeks. A number of engineered antibody-like molecules are now being examined for therapeutic applications [20]. Some engineered fragments, such as diabodies and minibodies, are much smaller than intact, full-length IgG1 and have very different properties of systemic distribution and clearance. Additionally, methods to prepare other antibody types such as dimeric secretory IgA [21] and methods to prepare antibodies using non-mammalian cell systems, such as plants [22], from the eggs of chimeric chickens [23] and from transgenic animals [24], are now being explored by academic and industrial groups. As these new approaches gain prominence, more information will be acquired about their unique requirements for successful formulation and delivery. At present, however, there is little information on formulation approaches for antibodies produced using these production processes.

3. General concerns for antibody formulations

Due to their proteinacous nature, antibodies present generic formulation issues that are similar for most protein therapeutics [11]. Proteins, in general, can be degraded under conditions when they are exposed to heat, freezing, light, pH extremes, agitation, sheer-stress, some metals and organic solvents. With ever advancing analytical technologies, the ability to assess the physicochemical and thermodynamic instability of antibody drugs has also led to the identification of several events that are more specific to the unique nature of this particular class of proteins: variations in

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Fc glycosylation, partial heavy chain C-terminal Lys processing, Fc methionine oxidation, hinge-region cleavage and glycation of Lys residues [25]. In addition to these issues is the difficulty of preparing dosing materials that might have protein concentrations in the range of 100 mg/ml or greater. Since early preclinical assessment is so critical to successful identification of a new biotherapeutic product, general pre-formulation strategies are usually employed for initial studies with new antibody drugs. An initial formulation effort will likely employ an isotonic, non-hemolytic, slightly acidic solution that can be administered by intravenous infusion or subcutaneous injection in studies employing a relevant animal model. In some cases, some stability information for the antibody drug can be obtained from initial in silico and in vivo testing during this pre-clinical phase that aids in the selection of a formulation that is used in clinical trials [26].

Since most early pre-clinical studies attempt to emulate the delivery strategy that will be used in the clinic, initial studies frequently identify problems with either the initial pre-formulation approach and/or stability issues with the antibody drug itself. In the case of the formulation, protein precipitation can occur with refrigerated storage or with repeated freeze–thaw cycles; events that may be facilitated by an inappropriate pH or buffer selection. Analysis of antibody drugs stored in this initial pre-formulation system may also identify unstable sites within the protein or its linkage to attached molecules (chemotherapeutics, imagining agents, etc.). In the case of the antibody, this information is frequently evaluated to determine if re-engineering of the protein could be performed, for example, to provide greater thermodynamic stability or folding efficiency [27]. Such changes in the Fc region are typically conservative, but might act to modify Fc receptor binding properties to provide a different biological outcome [28,29]. Amino acid substitutions can also be made within the Fv region to improve thermal stability [30], but such changes might also alter antigen binding specificity [31].

4. Stability issues for antibody formulations

If one examines the amino acid sequences for currently approved IgG1-based antibody drugs, it becomes quite clear that only a small segment of these proteins are dramatically different from one another, being the Fv segments that are involved in antigen binding. It should be pointed out, however, that there are some minor differences in conserved regions as well. A priori, one might assume that by finding a stable formulation for one of these antibody drugs, that such a formulation would be good for most if not all, similar antibodies. If this were borne out by experience, there would be no need for a review such as this. Instead, each antibody seems to have a unique personality related to its requirements for stability; a phenomenon derived from the fact that the small differences between these antibodies are focused on surface-exposed amino acid differences that stipulate antigen specificity. Thus, the interfacial surface of each antibody drug is unique and thus requires specific formulation components to provide maximal stability and retention of activity.

Probably the most important aspect of identifying a successful antibody drug formulation is in the identification of truly detrimental alterations rather than all of the modifications that might occur. Although, from a regulatory standpoint, it may be beneficial to understand all of the potential degradation events associated with an antibody formulation, it is the recognition of a stability-indicating parameter (or several parameters) that allows the formulation scientist to focus in on what is critical to development of a successful product. Here, a series of physicochemical and bioanalytical assays can be used to identify these parameters [32]. Although in vivo bioassays may ultimately be required to validate a stability-indicating formulation parameter, the most critical tools at the disposal of the formulation scientist are rapid and reproducible assays to measure protein changes due to oxidation, deamidation, aggregation, fragmentation and other forms of chemical modification that could lead to a loss of function of the antibody drug.

4.1. Oxidation

Methionine and cysteine residues are frequently a site of oxidation in protein drugs and this is also the case for antibody drugs [33]. In the case of antibody-based drugs, specific methionine residues within the Fc domain may be prone to oxidation, resulting in the

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production of methionine sulfoxide [25]. While cysteines are present in the Fc framework as disulfide pairs, unpaired cysteines in the variable region may also be sites of oxidation. Besides methionine and cysteine residues, oxidation of histidine, tyrosine, tryptophan and phenylalanine residues can also occur [34]. A number of methods can be used to monitor oxidation of amino acid residues. As a starting point, the number of reactive protein thiols can be monitored using reagents such as 5,5′-dithionitrobenzoic acid or iodoacethyl dansylcadaverine [35], providing a simple readout on the number of protein thiols per protein. Total amino acid analysis can also be used to monitor reductions in methionine, tyrosine and phenylalanine residues. Tryptic digestion and peptide separation by liquid chromatography followed by mass spectroscopy analysis (LC-MS) can be used to identify specific sites of amino acid oxidation.

A careful study of methionine oxidation has been performed for the humanized monoclonal antibody that recognizes the her2/neu gene product. For this protein it was determined that a methionine at position 255 in at heavy chain of the Fc region was the primary site of oxidation and that a methionine at position 431 could also oxidize under more aggressive conditions. In this study, a chromatography assay was used to detect oxidation events following papain digestion to separate Fab and Fc segments of the antibody [36]. Another study on the stability of this same protein provides a valuable template for dealing with antibody oxidation: identification of a critical oxidation event, validation of a robust assay for monitoring that event and comparison of formulation parameters to determine conditions that facilitate oxidation. Addition of antioxidants or stoichiometric levels of free methionine or thiosulfate were found to reduce the extent of oxidation in formulations of this antibody [37].

Issues associated with administration of oxidized forms of an antibody may extend beyond concerns of reduced efficacy. Systemic lupus erythematosus (SLE) is a disease characterized by the induction of anti-double-stranded DNA antibodies. Studies have shown that increased levels of markers of protein oxidation, including those of methionine sulfoxide and 3-nitrotyrosine, correlated with SLE disease activity [38]. Additionally, oxidative modifications of antibodies isolated from synovial fluid samples collected from patients with rheumatoid arthritis suggested alteration of histidine, methionine, tyrosine and cysteine residues [39]. While no direct causal association is shown or implied, these observations bring attention to the issue that we do not fully understand the clinical impact of antibody oxidation events.

4.2. Deamidation

Glutamine and asparagine residues show a propensity for deamidation [40]. Both light and heavy chains of antibodies can undergo deamidation of these amino acids [41] and deamidation events are considered to be one of the major sources of charge heterogeneity of monoclonal antibodies [42]. Deamidation events appear to be highly selective events for individual antibodies. For example, analysis of several monoclonal antibodies that bind the her2/neu gene product demonstrated asparagine 30 deamidation on one or both light chains and asparagine 55 on the heavy chain [43]. Deamidation events lead to more acidic forms of the antibody through the acquisition of additional carboxylic acid groups. Conversely, aspartic acid residues can also undergo modification to a succinimide moiety that results in a basic antibody isoform of the antibody by removal of a carboxylic acid group [43]. In general, deamidation events appear to be relatively slow but are accelerated by unfolding of the antibody [44]. Initial detection of deamidation in antibody preparations is typically identified by differences in charge distribution or content using methods such as isoelectric focusing (IEF) or high-performance cation-exchange chromatography [43].

High performance chromatography can then be used to separate succinimide, isoaspartic and aspartic acid isoforms of tryptic digest fragments of the antibody which can be unambiguously characterized by tandem mass spectroscopy (MS). Correlation between tryptic digest tandem MS results and some other more readily accessible method, such as IEF, can provide a rapid format to follow antibody deamidation events. Using these techniques, asparagine deamidation events residues appears to occur more frequently than glutamine deamidation and specific asparagine residues appear to be selectively targeted for this event [33]. Deamidation of asparagine is frequently associated with rearrangement

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events that lead to the formation of isoaspartic acid [42] and this outcome is correlated to amino acids adjacent to the asparagine prone for deamidation [44] that provide a method for prediction of potential deamidation sites (BOX). In particular, asparagine–glycine sequences can demonstrate accelerated deamidation rates since glycine residues lack a side-chain structure that would otherwise act to decreased conformation interference for backbone-associated isoaspartyl formation [45].

Box 1

Amino acid* sequences predicted to deamidate
SNG, LNG, LNN and ENN
(Note these have a following amino acid of G or N)
and those that are less prone to deamidation
GNT, GNS, GNV, TNY, SNY, SNF, SNT, SNK, SNL,
WNS, WNS, FNW, FNS, FNR HNA, HNH, LNW,
CNV, NNF, DNA, and YNP
*standard single amino acid letter nomenclature.

Non-enzymatic parameters of asparagine and glutamine deamidation in proteins have been defined in detail for factors such as pH, temperature and ionic strength [46,47]. The primary sequence dependence of these deamidation events can be explained by a simple steric and catalytic model [46,47]. Prediction of deamidation events based upon three-dimensional structures have also been described but the data set for these studies is not yet sufficient to produce a general model [48]. That asparagine and glutamine deamidation appear to be a constant phenomenon of proteins and correlate with their turnover provides the basis for the hypothesis that the presence and distribution of these amino acids within a protein can act as a molecular clock for its biological turnover [49,50]. For the formulation scientist, empiric assessment of factors such as pH and ionic strength provide the best strategy to identify a formulation that minimizes deamidation events. In general, deamidation does not result in a decrease in potency and deamidation may be hard to detect as the charge difference is small. At times, however, there can be a detrimental effect on potency through the introduction of an unfavorable negative charge that can have further ramification on biological function, particularly when deamidation occurs in the binding regions [51]. As particularly reactive residues are identified, it may be possible to have the antibody engineered to remove the problematic amino acid or to change adjacent residues to reduce its reactivity.

4.3. Aggregation

Liquid formulations of protein therapeutics frequently exhibit a decrease in concentration due to adsorptivity to container walls at low concentration and can show aggregation events at high concentrations. Initial preparations of antibody drugs were administered in acute situations by IV infusion that could allow for medium protein concentration (1–10 mg/ml) formulations not dramatically affected by adherence or aggregation losses. As antibody-based drugs became more widely used in chronic care, high concentration formulations that would allow for clinically acceptable SC injection volumes became desirable and aggregation became an important concern for designing formulations. Along with aggregation events, protein solutions at concentrations >50 mg/ml may become sufficiently viscous as to no longer readily pass through the gauge of a needle that would be comfortable for patients' use. Some antibodies demonstrate high concentration viscosity issues while others do not. For example, the anti-respiratory syncytial viral antibody Synagis® is formulated at 100 mg/ml protein and is administered successfully by IM injection. Thus, addressing viscosity, adsorptive loss and aggregation in any specific antibody formulation will likely be performed on a case-by-case basis.

As protein–protein contact frequency increases at high concentrations, the opportunity for stable aggregate formation increases proportionally. Proteins are folded in such a way as to internalize hydrophobic domains and surface-expose more hydrophilic domains in a thermodynamically stable situation. As folded proteins undergo normal molecular motion transient surface exposure of hydrophobic domains can lead to protein–protein contacts that hide these

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surfaces in a more energetically favorable manner. The outcome of these events is the stabilization of these protein–protein contacts with the ultimate outcome of dimerization, trimerization and so on — leading to extended aggregation events [52]. As protein concentration and temperature goes up, so does the probability of energetically favorable contacts leading to aggregation with increased frequency and duration of reversible protein–protein contacts leading to a greater extent of stable (even irreversible) aggregation events. Aggregation (covalent, non-covalent, dissociable, non-dissociable) must be considered a likely degradation event for high concentration antibody formulations.

For some antibodies reduced temperatures can incite reversible self-associations; these molecules are known as cryoimmunoglobulins [53]. IgG3 cryoimmunoglobulins have been shown to self-associate through sugar structures of the Fc domain [54]. Self-association events involving IgG1 antibodies seem to occur primarily through weak ionic and hydrophobic interactions [55]. More recent studies have examined kinetic and thermodynamic aspects of antibody solution dimerization using a recombinant human monoclonal antibody that recognizes vascular endothelial growth factor (rhMab-VEGF) as a model [56]. It was found that for this IgG1 class antibody, aggregation rates were greater in slightly alkaline (pH 7.5–8.5) compared to slightly acidic (pH 6.5–7.5) conditions. A high salt environment (1 M NaCl) also enhanced dimerization. Not surprisingly, self-association events were accelerated as the storage temperature was increased. One of the most striking aspects of these dimerization studies on rhMab-VEGF is that trastuzumab, another genetically engineered antibody with the same IgG1 framework and a 92% sequence homology with rhMab-VEGF, fails to demonstrate aggregation events under the same conditions.

A number of additives have been identified that can reduce the rate of protein aggregation [52,57]. A rather wide spectrum of agents can reduce protein aggregation rates: urea, guanidinium chloride, amino acids (in particular glycine and arginine), various sugars, polyalcohols, polymers (including polyethylene glycol and dextrans), surfactants and even antibodies themselves. Studies have shown that different proteins benefit from different types of anti-aggregating factors, consistent with the notion that the interfacial surfaces that drive interactions leading to aggregation are protein-specific. Conceptually, anti-aggregating agents might fall into one of two categories: small agents that fit into channels or grooves and large agents that might interact with lower-curvature surfaces of the protein. In essence, the small agents would act to stabilize a protein from acquiring a conformation that might be more reactive toward aggregation while a large agent might act to reduce the frequency of surface contacts that lead to an aggregation event. In the case of antibody formulation, either or both may be important. As we will discuss, agents found to effectively protect an antibody from aggregation may differ for various stress conditions; antibodies in solution that are undergoing agitation and thermal stress may differ from those of the same antibody as it undergoes lyophilization and reconstitution.

Protection from aggregation can be limited in antibody formulations by the addition of surface-active agents such as detergents, both charged and uncharged. As an interesting aside, such surface-active agents can help reduce protein loss due to adherence to surfaces that can occur with low concentration protein formulations. Polysorbate 20 and 80 (now available from a vegetal source) are examples of detergents that are widely used to reduce the rate of protein aggregation in high concentration antibody drug formulations. A precautionary note must be sounded, however, as detergents perform this function optimally at low concentrations (0.01% to 0.05%) and can act detrimentally to participate in protein unfolding at higher concentrations, e.g., 1%.

That antibody aggregation can be accelerated by several physical conditions provides the potential for multiple methods to determine if a particular antibody drug is prone to aggregation and to rapidly test formulations for their ability to impede aggregation. Agitation studies are done, in addition to thermal stability studies, to determine if aggregation can occur in a given formulation. Different test methods are used, employing a shaker or a stirring apparatus. In one study done on an IgG1 antibody found sizes of aggregates produced by the two methods were comparable [58]. They concluded that a higher throughput test could be constructed using stirring analyzed by absorbance measurement. In their work,

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the stirred samples exhibited much higher absorbance and second particle species in DLS, indicating that stirring stress produces a higher amount of smaller protein aggregates. While Mahler found polysorbate 80 protects against aggregation, the stirred samples had an increase in turbidity and more aggregate products were detected by DLS as compared to a surfactant-free formulation. Polysorbate 80 stabilizes small aggregates and prevents further progress in the aggregation process.

4.4. Fragmentation

Intact, full-length antibodies are composed of several segments that have natural flexure sites. One site, in particular, exists at the flexure point between the Fc and Fv domain. Cleavage at this hinge region is a common concern when assessing antibody drug stability [25]. Molecular sizing methods such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion high performance liquid chromatography (SE-HPLC) can be used to rapidly identify fragmentation events in antibody drugs [59]. Fragmentation of full-length antibody drugs is a common occurrence and invariably affects their function. In general, cleaved forms are present in such low amounts that it is likely that little change in efficacy would be seen [60]. At the very least, the antibody drug may have some small reduction in efficacy following fragmentation or may be more prone to proteases, degradation and clearance events that would limit the duration of its efficacy. At the worst, a fragmented antibody preparation may result in a different biodistribution and thus a different safety profile. This can be of particular concern if the antibody is being used to delivery a cytotoxic chemotherapeutic agent. Fortunately, the extent of fragmentation in an antibody can be easily limited in the formulated product.

5. Strategies to stabilize antibody formulations

While liquid antibody formulations are less expensive, faster to develop and generally easier to prepare for administration than alternative formulation approaches, liquid antibody formulations are prone to oxidation, deamidation, aggregation and fragmentation as discussed above. In each of these events, water is the common culprit: water mediates electron transfer during oxidation and deamidation events and its required addition across a peptide bond can be a critical step in fragmentation. The thermodynamic stresses that lead to protein aggregation are caused by hydrophobic protein surfaces being exposed to water and trying to find a lower energy state by non-native protein–protein interactions. Thus, water is a big problem for stabilizing antibody-based drugs. Lyophilization or introduction into hydrophobic polymer systems can reduce the impact of water on antibody drug formulations.

5.1. Lyophilization

Although some antibodies have demonstrated a propensity to undergo precipitation as they are cooled from 37 °C [53], antibodies are typically robust to freeze/thaw cycles [61]. Early studies reported that antibodies were stable to the freeze-drying (lyophilization) process in the absence of excipients that function as cryoprotectants [62], however, with the technical advancement of certain analytical methodologies, became evident that antibodies are generally damaged in some way as a result of lyophilization as demonstrated by extensive formation of insoluble aggregates [61]. More recently, studies have shown that freeze-drying antibodies to a specific percent of residual water (usually between 1 and 8%) allows for optimal stabilization in the dry state and stability during reconstitution [63]. Higher moisture content in lyophilized antibody preparations correlates with increased aggregation (as seen by SEC) and asparagine deamidation and isomerization (as measured by HIC). Studies have also been performed to identify optimized lyophilization schedules where the secondary drying phase is performed at an elevated temperature [64]. This technique allows the lyophylized cake to achieve optimum moisture content, allowing the final product to be conformationally stable (as measured by DSC) during extended storage. Solid-state structural studies can be used to monitor conformational changes and aggregation events during the storage of a lyophilized monoclonal antibody preparation [65]. Improved native-like structure and a reduction in aggregation can be obtained by incorporation of a carbohydrate excipient in sufficient

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quantities to fulfill the hydrogen bonding requirements on the protein surface, suggesting a critical role for non-water molecules to act as placeholders for water molecules during drying and in the dry state. That the addition of excess carbohydrate failed to improve the stabilizing function of this excipient, reaffirms the conclusion that antibodies have a limited number of sites that must be protected in this fashion [65]. A variety of carbohydrates or polyol compounds such as sucrose, trehalose and mannitol have been shown to provide this stabilizing function [66,67].

The rate of rehydration when water is reintroduced during reconstitution of lyophilized formulations is a critical parameter. As water is removed during the different drying stages of the lyophilization cycle and replaced by a shell of non-water excipients, the antibody drug can undergo structural modifications that result in a non-native conformation as may be seen by differential scanning calorimetry [63]. If the rate of rehydration is sufficiently slow to allow recovery of native conformation as water replaces non-water excipient molecules, reconstitution will typically provide a satisfactory outcome for the antibody. If, however, the antibody does not have sufficient time or capacity to recover its native state during reconstitution, extensive aggregation can result. Efforts to protect monoclonal antibodies from the rigors of freeze-drying and reconstitution have been aided by a number of analytical methods that can examine protein modifications. In the solid state, circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy may be used to evaluate the protein; after reconstitution in the liquid phase, SEC or SDS-PAGE have been utilized to gather information from lyophilization studies [68,69]. A microscopic apparatus to visually monitor freeze-drying events has even been described [70]. Excipients that have been reported to slow the reconstitution rate and thereby preventing such changes in the rehydrated antibody are glycerol [71,72] and polymeric materials such as polyethylene glycol [73].

Pharmaceutical scientists frequently use increased temperatures for accelerated formulation stability studies. Although such studies can be used to assess formulation parameters such as pH and ionic strength, antibodies lyophilized in the presence of a cryoprotective sugar excipient provide a more complicated picture. Lyophilized amorphous solids composed of a monoclonal antibody and a sugar cryoprotectant material such as sucrose or trehalose form glassy structures, liquids that are too viscous to flow. That antibody-excipient complexes exhibit a glass transition temperature (Tg) provides the basis for observations showing that these materials have unique properties above and below this value. Thus, in predicting shelf life in accelerated degradation studies, it is important to use testing temperatures that do not exceed the Tg threshold of the complex [67]. In general, lyophilized formulations containing trehalose may likely show less tendency toward antibody aggregation than those with sucrose, since trehalose preparations have a higher Tg [67]. With this in mind, a promising approach to assess secondary structural parameters in evaluating antibody formulation stability is the use of amide I band Raman spectroscopy. This method demonstrates a good correlation between structural protein perturbations immediately after lyophilization and the rate of aggregation during long-term storage under accelerated conditions [74].

5.2. Polymer delivery systems

Removal of water from antibody preparations, either by lyophilization or spray freeze-drying, provides a provides a starting point for incorporation of this dried material into a hydrophobic polymer matrix such as polylactide-co-glycolide (PLGA) that can be made into microspheres generated through a solid-in-oil-in-water (S/O/W) encapsulation process [75]. Although both mannitol and trehalose can stabilize human IgG during spray freeze-drying, the double-emulsion solvent evaporation method used to load spray freeze-dried human IgG into PLGA microspheres has been shown to produce extensive antibody aggregation [75]. Such an outcome is likely the result of thermodynamic stress experienced at the water/organic solvent interface and a variety of process methods and excipients can be use to minimize this concern [76].

One feature of PLGA microparticle preparations is their recognition by immune elements and their capacity to stimulate immunization against incorporated proteins [77–79]. While this has clear advantages for vaccines, it poses a serious concern for therapeutic protein delivery. Hydrophobic microparticles have been reported to incite local inflammation

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at subcutaneous injection sites [80], events that may be partially mediated through macrophage recruitment [81]. Not only is the potential for antibody formation increased with local inflammation, the resulting injection site reaction may be clinically unacceptable.

In spite of some serious caveats, polymeric microspheres and delivery systems can be successful antibody formulations if administered to the appropriate sites, using certain types of polymers or preparing microspheres or polymeric formulations by methods that are more protective of the antibody. An example of a certain delivery site that may have less proclivity towards an inflammatory response is reported, where PLGA microspheres loaded with an anti-VEGF antibody were injected into the intravitreal humor of the eye, an immune-privileged site, for the treatment of age related macular degeneration (AMD) [82]. Resorbable polymers that show less potential to incite inflammation, e.g., have smoother surface characteristics, might be used to deliver antibodies in a sustained manner. Direct, local delivery of antibodies using carboxymethylcellulose aqueous gels can be an effective post-surgical anti-infective strategy [83]. Antibodies covalently coupled to a biodegradable hyaluronic acid hydrogel through a labile hydrazone linkage have been used for the sustained delivery of antibodies to sites within the central nervous system [84]. Introduction of dry powder IgG into ethylene–vinyl acetate copolymer (EVAc) has also been examined as a potential polymer-based antibody delivery system [85]. Similar to PLGA systems, EVAc microspheres can extend the time course of IgG release. This property makes polymers loaded with antibodies interesting opportunities for injectable sustained release delivery formats but also for topical application at mucosal surfaces. For example, long-term vaginal delivery of an antibody can be achieved in a mouse model using polymer vaginal rings [86,87]. Formulations of a biomedical grade polyurethane hydrogel with an antibody coating have also been prepared and shown to release bioactive protein [88]. It is important to note that one complicating aspect of many of these approaches is the requirement of a polymer solubilization step using a solvent, such as methylene chloride or isopropanol, which can compromise protein drug stability. Sustained release formulation approaches such as microspheres prepared using water, such as the PROMAXX microspheres (Epic Therapeutics) or crystallization of proteins [89] can bypass the use of solvents that are incompatible with proteins and antibodies.

6. Formulation issues for alternative forms of antibody delivery

To date, antibody development has focused primarily on injectable routes of administration: intravenous (IV), intramuscular (IM) and subcutaneous (SC). A long history of IV antibody infusions exists [90] and the advantages are clear for antibody-based drugs that are used in cancer treatments in order to reach tumor sites that may be diffuse or not otherwise accessible. In fact, the majority of the approved antibody-based drugs are administered by IV infusion (Table 1) with the total dose administered approaching one gram of protein per treatment in some cases. For many antibody-based therapies a critical safety concern involves the onset of acute or delayed systemic reactions following infusion [91,92]. Parameters and characteristics of the "infusion reaction" as well as treatment protocols have been well described with acute events commonly occurring within 10 min to 4 h and delayed events occurring 24 h to 4 days post dosing [93]. Although formation of antibodies to the administered antibody drug appear to be a risk factor for infusion reaction events [94], infusion reactions can occur following the initial exposure [93] and these reactions do not appear to be due to IgE responses or mast cell activation [95]. Most importantly for the patient, there appear to be treatment protocols to provide re-treatment of patients even after an infusion reaction event [93] with concomitant administration of immunomodulation molecules [94]. There are many unanswered questions about the mechanism and prevention of adverse infusion reaction events associated with IV infusion of antibody drugs. Certainly, one method is to explore IM and SC delivery when possible, although these delivery approaches may also have confounding aspects of tissue response and volume limitations.

Other routes are also amenable to antibody delivery. The increased number of antibody therapeutics on the market and the greater exploration of non-life threatening indications being treated by antibodies, drive efforts to deliver these agents by routes other than IV

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Table 1
Formulations of approved products

Antibody product; company	Generic name; description	Delivery route	Formulation concentration	Buffer components; pH[a]	*Excipients*
Othroclone OKT3®; J & J-Ortho Biotech	Muromonab-CD3; Murine IgG2a, anti-CD3	IV	1 mg/ml	Na,K phosphate; pH 7	NaCl, polysorbate 80
Reopro®; Centocor-Lilly	Abciximab; Chimeric IgG1, anti-GPIIb/IIIa, Fab	IV	2 mg/ml	Na phosphate; pH 7.2	NaCl, polysorbate 80
Rituxan®; Idec-Genentech	Rituximab; Chimeric IgG1, anti-CD20	IV	10 mg/ml	Na citrate; pH 6.5	NaCl, polysorbate 80
Zenapax®; Roche	Daclizimab; Humanized IgG1, anti-CD25	IV	5 mg/ml	Na phosphate; pH 6.9	NaCl, polysorbate 80
Simulect; Novartis[b]	Basilixamab; Chimeric IgG, anti-CD25	IV	4 mg/ml	Na,K phosphate, glycine	NaCl, sucrose, mannitol, polysorbate 80
Synagis®; MedImmune[b]	Palivizumab; Humanized IgG1, anti-RSV	IM	100 mg/ml	Histidine, glycine	Mannitol
Remicade®; Centocor[b]	Infliximab; Chimeric IgG1, anti-TNFα	IV	10 mg/ml	Na phosphate; pH 7.2	Sucrose, polysorbate 80
Herceptin®; Genectech[b]	Trastuzumab; Humanized IgG1, anti-HER2	IV	21 mg/ml	Histidine; pH 6	Trehalose, polysorbate 20, benzyl alcohol[c]
Mylotarg®; Wyeth[b]	Gemtuzumab ozogamicin; Humanized IgG4, anti-CD33, immunotoxin	IV	4 mg/ml	Na phosphate	NaCl, sucrose, dextran 40
Campath®-1H; Millennium-ILEX	Alemtuzumab; Humanized IgG1, anti-CD52	IV	Unclear — 30 mg /vial	Na,K-phosphate pH 7	NaCl, KCl, NaEDTA, polysorbate 80
Zevalin®; Biogen Idec	Ibritumomab tiuxetan; Murine IgG1, anti-CD20; radiolabeled (^{90}Y or ^{111}In)	IV[d]	1.6 mg/ml	Na Acetate, Na, K-phosphate pH 7	NaCl, human albumin
Humira®; Abbott	Adalimumab; Human IgG1, anti-TNFα	IV	50 mg/ml	Na phosphate, Na Citrate; pH 5.2	Mannitol, polysorbate 80
Xolair®; Genentech[b]	Omalizumab; Humanized IgG1, anti-IgE	SC	125 mg/ml	Histidine	Sucrose, polysorbate 20
Bexxar®; Corixam-GSK	Tositumomab-I131; Murine IgG2a, anti-CD20, radiolabeled (^{131}I)	IV	14 mg/ml	Na phosphate; pH 7.2	NaCl, maltose
Raptiva®; Genentech	Efalizumab; Humanized IgG1, anti-CD11a	SC	100 mg/ml	Histidine; pH 6.2	Sucrose, polysorbate 20
Erbitux®; Imclone Systems	Cetuximab; Chimeric IgG1, anti-EGFR	IV	2 mg/ml	Na phosphate; pH 7.2	NaCl
Avasatin®; Genentech	Bevacizumab; Humanized IgG1, anti-VEGF	IV	25 mg/ml	Na phosphate; pH 6.4	Trehalose, polysorbate 20
Tysabri; Biogen Idec	Natalizumab; Humanized IgG4, anti-4α-integrin	IV	20 g/ml	Na phosphate; pH 6.1	NaCl; polysorbate 80

Ordered by approval dates in the US.
Abbreviations: RSV=respiratory syncytial virus; TNFα=tumor necrosis factor alpha; EGFR=epidermal growth factor receptor; VEGF=vascular endothelial growth factor; NaEDTA=sodium edentate.
[a] Some lyophilized formulations do not stipulate a pH (or range) following reconstitution.
[b] Lyophlized product, concentration, pH, etc. values are obtained after reconstitution.
[c] Added as a preservative to generate a multi-dose vial configuration.
[d] Kit where components are mixed just prior to administation.

infusion. These routes may be more effective, less invasive, provide easier delivery, permit self-administration and thereby be more convenient and possibly have lower costs if less active agent can be used, as in the case where the alternate route of administration delivers the therapeutic agent to the intended site directly. Interestingly, some studies have suggested that antibodies administered by IV infusion are more

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susceptible to proteolysis than via IM injection [59] and sucrose-stabilized immunoglobulins have been associated with acute renal failure following IV infusion [96]. The antibody used to protect neonates from respiratory syncytial viral (RSV) infection (Synagis®) is one example of an antibody administered by IM injection. For reasons discussed above, the majority of antibody-based drugs have moved toward SC delivery with formulations efforts being focused to this end. Based upon the desired actions for a number of antibody-based drugs, however, routes of administration other than IV, IM or SC may be desirable. These alternative routes can provide unique formulation and stability issues.

6.1. Oral

Uptake of antibodies from the intestinal lumen can occur through fetal Fc receptors (FcRn) expressed at the apical surface of enterocytes [97]. This natural antibody uptake mechanism was initially identified in the gastrointestinal (GI) tract of neonatal rodents and its expression shown to vanish following "closure" of the intestine after weaning. It was only recently that the continued expression of this receptor in the adult intestine was demonstrated and this finding has prompted efforts to examine this receptor system as a means of delivery antibodies after oral administration. Although this pathway appears to be a viable route for the delivery of therapeutic antibodies, issues associated with antibody stability in the GI tract [98] and competition from endogenous antibodies can dramatically reduce the efficiency of uptake after oral delivery. Outside of this specific mechanism, no other transcellular mechanism of efficient antibody transport across epithelial cell of the GI tract has been demonstrated to date. Topical application of antibodies to produce a desired clinical outcome have been shown, however, with the oral delivery of a chicken-derived (IgY) antibodies for treating and preventing infectious diseases of the GI tract [98–100]. It should be noted that IgY antibodies have been shown to not bind to (or be transported by) FcRn [97].

6.2. Respiratory tract

Intranasal and pulmonary administration of protein therapeutics has shown more promising clinical outcomes than oral delivery. While the surfaces of both the respiratory tract and the gastrointestinal tracts are readily accessible, the respiratory tract is a far less hostile environment for an administered protein therapeutic. Nonetheless there are physical clearance mechanisms in place to remove foreign particles which act to reduce residence time dramatically following administration. In the case of nasal administration the use of a cream emulsion and a polymer delivery system has been shown to significantly decrease the clearance rate for a monoclonal antibody when applied to the nasal mucosa [101]. This study provides one approach for the topical application of a therapeutic antibody designed to provide clinical benefit at the mucosal surface. It does not, however, suggest that an appreciable amount of an antibody might be absorbed into the systemic circulation from this site. The nasal mucosa affords only a small absorptive surface area compared to the epithelial linings of the lung.

Aerosol delivery of antibodies to the lung has been explored as either a liquid or dry powder formulation, where in both cases the droplets or particles must be of sufficiently small size that they are respirable perhaps to the level of the deep lung but large enough that they are not readily exhaled or just impact the back of the throat [102]. The sizes of particles that provide this performance have a mass mean aerodynamic diameter and density such that they enter the alveoli and remain in the lung. This size window has been reported to be between 1 to 5 μm depending on the formulation used and in one case, far larger, 20 μm where the particles are dense (<0.4 g/cm^3) and highly porous [102].

Formulation efforts were made for producing fine dry powder particles of an anti-IgE monoclonal antibody (E25) in the size range desired for delivery to the lung [103]. These studies showed that sodium phosphate was capable of extending the usefulness of mannitol as a protective excipient in spray-dried antibody formulations and that mannitol can play a role both in maintaining protein stability as well as producing a suitable aerosol preparation. Further studies to assess carbohydrate excipients for spray-dried aerosol E25 formulations showed that excipient to protein ratios were critical. Degradation issues involved not only protein aggregation but also protein glycation or lactosylation, a protein modification

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event where a reducing sugar can add covalently at a free amine [104]. The ability to formulate and delivery an antibody-based drug to the lung provides the potential for systemic delivery of that protein via uptake through the FcRn expressed at this mucosal surface. Elegant studies showing the distribution and function as well as the application of this transport route for systemic deliver of an Fc-containing protein chimera has been demonstrated [105,106]. Thus, the potential or systemic delivery of antibody-based drugs via the respiratory tract looks promising.

6.3. Miscellaneous delivery sites of the body

Unique aspects of antibody–polymer formulations designed for intravitreal injection [82] or topical vaginal delivery [86,87] have been discussed above. Additionally, antibody formulations using hyaluronic acid hydrogels for sustained brain delivery have been noted [84]. These methods focus on strategies to administer large amounts of an antibody at a specific site in order to provide a sustained level to improve efficacy and/or to minimize injection frequency. Other strategies have been described to deliver antibodies to miscellaneous sites throughout the body, For example, an anti-transferrin antibody (OX-26) has been described as a carrier for delivery of covalently attached therapeutic molecules across the blood–brain barrier following IV injection [107]. An anti-tumor necrosis factor antibody has been adsorbed onto cardiovascular stent wires for local neutralization of this cytokine in order to limit restenosis events following stent placement [108]. A CMC gel containing antibodies to prevent post-surgical infections has been administered to the intraperitoneal cavity [83].

6.4. Intracellular targeting

Some promising antibody drug targets are intracellular. Although techniques exist where a cell can synthesize an antibody and secrete it into the cell cytoplasm, successful use of this technology requires gene therapy. Instead, it is possible to chemically couple an antibody to a pH-sensitive polymer that functioned to disrupt membranes at acidic pH. Many antibodies are internalized by cells through a receptor-mediated uptake process that leads to their delivery into early and then late endosomes. During endosomal maturation after receptor–antibody internalization the pH of these vesicles will drop from near neutral to below pH 6. This approach was demonstrated in a format where the pH-responsive polymer poly(propylacrylic acid) was covalently attached to a monoclonal antibody to facilitate its delivery to the cytoplasm of target cells [12].

7. Other delivery issues

A wide range of novel drugs and drug conjugates are being developed using monoclonal antibodies and engineered antibody fragments [20]. For some of these novel therapeutic approaches to be successful, they require additional or non-traditional delivery strategies. A cursory discussion of some of these approaches as they relate to intracellular targeting, site placement and engineered antibodies fragments follows.

7.1. Antibody fragments

An entire spectrum of antibody fragments have been identified through protein engineering studies with many of these showing remarkable potential for unique and novel clinical opportunities [109]. These smaller recombinant antibody fragments, the monovalent antibody fragments, Fab and single chain Fv and the engineered variants, diabodies, triabodies, minibodies and single-domain antibodies are an emerging form of antibody therapy that are less costly to manufacture while still retaining the targeting specificity of full length antibodies. In fact some may have greater efficacy and more applications than whole monoclonal antibodies. It may be anticipated that this new subset of antibodies are posing new formulation challenges, however, to date, publications detailing stability studies and formulation development for these molecules are rare in the scientific literature so formulation data on antibody fragments is scant. The single V domains have been found to be poorly soluble and have a tendency to aggregate [109], but these problems are being overcome by the identification of mutants that minimize the hydrophobic interface and selected by phage display [110]. Five Fab molecules have been approved as of this writing with the one humanized

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Fab (ranibizumab rhFab) awaiting approval in the very near future, but many are in early clinical trials and most in preclinical development. One study has been described that focused on Fab fragments. In general, the binding of an antibody to an effector protein does not necessarily inactivate that target's function [111]. When an antibody is effectively inactivating, however, the Fab fragments in the absence of an Fc domain can frequently function as highly potent binding agents capable of masking binding sites or enzymatic elements on macromolecules. For example, Fab fragments prepared from sheep anti-snake venom antibodies (Digibind, from GSK) can be an effective anti-venom. Storage of liquid formulations of such anti-venoms in sub-tropical countries where refrigeration may not be possible provides a significant formulation challenge. Although lyophilized products can perform well under these conditions, a pH 4.0 (acetate buffer) formulation was found to also be stable and potent for at least one year at room temperature [112], presumably room temperatures characteristic of the tropical and subtropical regions where the material would be used. Strikingly, this stable formulation did not require the presence of a protective carbohydrate excipient. To improve drug delivery, antibody fragments have been fused to many types of molecules, such as radionuclides, cytotoxic drugs, toxins, peptides and proteins, enzymes and liposomes [113]. An ımmunoliposome made with a Mab fragment is reported to deliver drugs to the brain as such a molecule is able to cross the formidable barrier of the blood brain barrier [114].

7.2. Immunoconjugates

Arming antibodies with materials useful for detection or cell killing has become a prominent approach to increase the utility of these proteins, reviewed by Wu and Senter, [20]. Antibodies provide a useful format for the production of radiopharmaceuticals for site- or tissue-specific localization using gamma scintigraphy [10,115] or for enhanced cell killing. There are currently three approved antibody–radionuclide drugs: two are murine radiolabeled Ab for B cell lymphomas ibritumomab tuixetan (^{90}Y, Zevalin) and tositumomab (^{131}I Bexxar) and the third is a humanized, gemtuzumab ozogamicin (calicheamicin, Mylotarg) for leukemia. The presence of a high energy emitting radionuclide, however, will compromise long-term stability of any antibody formulation; not only would the released energies act to damage the protein but there is strong preference to use short half-life isotopes for these procedures [116]. Thus, in these instances, a stable formulation based upon the principles discussed above can provide a product that can be radiolabeled and then used soon after this step. One example of this approach is a lyophilized antibody formulation that is reconstituted and prepared by a kit method prior to patient dosing [117].

Antibodies coupled with cytotoxins for targeted cell killing [118] are particularly useful in antibody-based cancer therapies [119]. Beyond the identification of a stable formulation for the antibody, such materials have several additional problems for the formulation scientist to solve: stability issues of the attached agent and stability of the linker used to join the attached agent to the antibody. In the case of a monoclonal antibody-vinca alkaloid conjugate, a pH range of 4.5–5.5 in a phosphate buffer showed improved solution stability compared to material stored at pH 6.5–7.4 as evidenced by antibody aggregation and hydrolysis of the chemical linker used to conjoin the alkaloid to the antibody [120]. In most cases, cytotoxic materials are coupled to antibodies using reversible chemistries to provide a release mechanism for maximal activity at the target site. Many of these antibody conjugates use a disulfide bond linkage that can be reduced by a novel redox enzyme present in endosomes and lysosomes of target cells [121]. Slightly oxidizing conditions that would provide added stability of this linker in the formulation could, over time, act to cause detrimental oxidation events in the antibody and/or the linked compound. Other conjugates use an acid-labile linker [122]. In this case, formulation pH must be restricted to either neutral or alkaline conditions. Enzymatic separation of antibody and a cytotoxic material could also be achieved by coupling the two through an amino acid linker that is recognized as a substrate by a specific protease or peptidase [123]. Formulation instability of this linker sequences can be corrected to some extent by selective changes of the amino acids used.

Thus, the challenges of immunoconjugates include maintaining both drug and antibody potency and stability. While some antibodies are efficacious by

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themselves, many are much more therapeutically active after being armed with a toxin, drug and radionuclide [124]. These changes make the resulting product more difficult to prepare, stabilize and deliver. Yet, there is tremendous enthusiasm for these approaches as they provide the promise for some companies to transform their antibody into a potentially curative drug. For this reason many antibodies are combined with a generic delivery system, such as a liposome, that can be used to delivery a variety or even multiple agents simultaneously through antibody-directed targeting [125]. Examples of such an approach include studies performed using an antibody directed against the Her2/neu antigen to delivery liposomes containing a chemotherapeutic have been described [126], antibodies used to deliver cationic liposomes for the administration of nucleic acid material for gene therapy [127] and antibodies used in the targeted delivery of modified gelatin nanoparticles [128] that can be coupled through a disulfide linkage [129].

8. Conclusions — challenges and opportunities

As antibody-based drugs come to the forefront of therapeutics derived from biotechnology, they provide a range of new and exciting opportunities to treat a number of currently unmet medical needs. Successful clinical application of these novel agents requires the development of stable formulations that can be used for specific delivery methods. Antibodies, because of their endogenous nature, have built-in features that may pose problems for stability as biotherapeutics, that function in the normal clearance of these proteins from the body [40]. Thus, despite our best efforts to prepare stable antibody-based drug formulations, the ultimate stability of these products may be limited to inherent mechanisms of degradation pre-designed for the rate of clearance optimal for their function and turnover in the body. Protein engineering has led to significant advances in improving some of these parameters and the trend would predict that additional advances will be made in the near future (reviewed in [109]) Protein modifications can be engineered such that stability and efficacy can be improved in ways that cannot be achieved solely, if at all, with formulation alterations. While it is clear that more research is needed in the relatively new area of engineered antibody fragments for clinical use [109], they have great potential for diagnostic and therapeutic application.

In general, successful antibody formulation designs are simply the best compromise of minimizing several competing degradation pathways. One approach is to find a pH where the minimal amount of degradation events such as deamidation and oxidation will occur. Another is to identify a buffer system that can stabilize the formulation at that pH without damaging the antibody. One common tactic taken to reduce the rate of many of these chemical degradation events is to prepare lyophilized or freeze-dried antibody preparation but even these can be compromised by aggregation issues due to thermodynamic stress that occurs during freezing, drying and reconstitution. A number of protective excipients, mostly carbohydrates, can reduce the propensity of antibodies to aggregate during these stressful events. Another issue is to produce a formulation that is compatible with delivery — neither hypotonic — nor hypertonic solutions are well-tolerated upon injection. Added to this is the potential use of a surface active agent to help with liquid stability such as a non-ionic detergent at a low concentration [58] as well as the possible addition of preservatives required for multi-use systems [130].

The future of antibody based drugs is likely to include more complex systems than those currently approved for use in man. Possibly the most critical aspect of generating a pharmaceutically successful formulation for antibody based drugs is an understanding the critical aspects of stability for the protein or the materials associated with the protein. It has been known for some time that even a single amino acid change in the Fv region of an antibody can result in dramatic differences in antigen-binding specificity [31]. Also, alterations at specific amino acids in the Fc domain can dramatically change functional aspects of an antibody. Thus, these kinds of changes must be considered more important in the search for a successful formulation than those changes that do not appear to affect function. In the case of antibody or antibody-like agents being used to delivery a payload such as a toxin or chemotherapeutic, the stability of the material to be delivered and its continued association with the delivery antibody is crucial.

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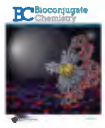
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Bioconjugate Chemistry

a wide variety of targeting small molecules and peptidomimatic compounds. Here, we exploited a keto-oxime method for conjugation of protease susceptible linkers to an antibody. This modified method involves two steps: (i) introduction of methyl ketone linkers (referred to as linker moiety) to the primary amines present in the antibody and (ii) conjugation of ketone linkers to aminoxy functional group present in the conjugated moiety (referred to as functional moiety). We have optimized this conjugation method and shown that ~10 functional moieties can be conjugated to antibody. Conjugation was verified by MALDI-TOF MS and Western blot analysis. The acidic pH conditions used in this method did not change the immune reactivity of the Ab. In addition, *in vitro* protease susceptibility assay was performed to validate this method for prodrug release assay as well as to remove excess radioimmune conjugates from circulation. This orthogonal method is compatible with peptides containing a thiol, amino, or carboxyl groups in the conjugation moiety.

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Subjects

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1 Introduction

Ketones selectively react with amino-oxy groups and form oxime bonds at slightly acidic pH. This type of chemoselective ligation is more preferable for making bioconjugates because it does not require anhydrous conditions, excessive heat, or any catalyst (1, 2). It is also tolerant of a wide range of functional groups that are found in proteins and oligonucleotides. This ligation has been successfully used in various applications such as making small molecule drug conjugates (3), peptide and oligonucleotide conjugates (4), protein conjugates (5), glycoconjugates (2), polymer conjugates (6, 7), and coating biomolecules on plannar microarray slides (8) and sialoglycoproteins present on cell wall (9, 10). Except for N-terminal labeling (11), this ligation method has not been used to make antibody drug conjugates especially in the area of cancer targeted therapy. Antibodies are important biomolecules developed by nature to protect hosts by eliminating disease-causing pathogens. Several conjugation methods have been developed to conjugate radionuclides, toxins, or drugs to antibody for cancer therapy (12-17). Here, we report conjugation of tissue plasminogen activator (t-PA) substrate rqYKYkf (18) to ChL6 antibody by the keto-oxime method. Chemical synthesis of methylketo linkers, aminoxy-functional peptides and optimization of conjugation

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Bioconjugate Chemistry

DMF (*N,N*-dimethylformamide) was purchased from VWR (Brisbane, CA). DCM (dichloromethane), MeOH (methanol), diethyl ether, and acetonitrile were purchased from Fisher (Houston, TX). All other chemical reagents were purchased from Aldrich (Milwaukee, WI). Analytical HPLC analyses (Vydac column, 4.6 mm × 250 mm, 5 μm, 300 Å, C_{18}, 1.0 mL/min, 25 min gradient from 100% aqueous media (0.1% TFA) to 100% CH_3CN (0.1% TFA), 214, 220, 254, and 280 nm) and preparative HPLC purification (Vydac column, 20 mm × 250 mm, 5 μm, 300 Å, C_{18}, 7.0 mL/min, 45 min gradient from 100% aqueous media (0.1% TFA) to 100% CH_3CN (0.1% TFA), 254 nm) were performed on a Beckman System Gold HPLC system (Fullerton, CA). MALDI-TOF MS analysis was performed on a Bruker BIFLEX III mass spectrometer (Billerica, MA). All of the experiments are carried out at room temperature unless otherwise noted. ChL6 (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) was a chimera consisting of human IgG1-constant regions and the murine L6 mAb antigen-binding site.

2.1 Synthesis of Biotin-rqYKYkf-spacer-K(Aoa)-NH_2 Peptide

Solid-phase synthesis of biotin-rqYKYkf was carried out using Rink amide MBHA resin after the treatment of resin with 20% piperidine solution in DMF (2 mL) for 15 min to remove Fmoc protecting group. Onto the Rink resin (200 mg, 0.09 mmol) with free amino groups, Fmoc-Lys(Alloc)-OH (122.2 mg, 0.27 mmol) was coupled following the standard Fmoc chemistry using HOBt (36.5 mg, 0.27 mmol) and DIC (42.3 μL, 0.27 mmol) as coupling agents. The coupling was monitored using ninhydrin test agents until a negative result was obtained. The resin was washed with DMF, MeOH, and DCM 3 times, respectively. The resin was treated twice with $Pd(PPh_3)_4$ (26.0 mg, 0.0225 mmol) in the presence of excessive $PhSiH_3$ (266.5 μL, 2.16 mmol) in DCM under argon for 30 min to remove the alloc protecting groups on the side chain of lysine. After thoroughly washing the resin with DCM, DMF, MeOH, and DMF, Boc-Aoa-OH (51.6 mg, 0.27 mmol) was coupled onto the resin using HOBt (36.5 mg, 0.27 mmol) and DIC (42.3 μL, 0.27 mmol) as coupling agents until the ninhydrin test was negative. After deprotection of Fmoc groups by piperidine treatment, a linear hydrophilic linker was coupled to the α-amino group of the lysine as a spacer following a published procedure (19). The peptide was constructed on the linker using standard Fmoc chemistry using DIC/HOBt as coupling agents (20). After the coupling of the last amino acid, the N-terminus Fmoc protection group was removed by piperidine treatment. The resin was treated with a solution of biotin (44.0 mg, 0.18 mmol), DIEA (diisopropylethylamine, 42.3 μL, 0.36 mmol), and HBTU (*O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate, 68.3 mg, 0.18 mmol) in DMF (2 mL) until the ninhydrin test was negative. The resin was thoroughly washed with DMF, MeOH, and DCM, and then dried *in vacuo*. To the dried resin was added 4 mL of cleavage mixture (TFA

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This keto-oxime method has two steps. First, ketone linkers will be conjugated to primary amines of the antibody followed by conjugation of functional derivatives such as DOTA chelators, immunotoxin, or chemotoxin. Before conjugation, the required amounts of antibody were purified by passing through a Sephadex-G100 molecular sieving column to remove impurities and are equilibrated with 40 mM of ketone linker conjugation buffer triethanolamine (pH 7.8). The linker conjugation can be done with phosphate buffer 50 mM (pH 7.8). In order to identify optimum ketone group introduction to Ab, conjugation was done with antibody at different molar ratios (10:1, 20:1, 30:1, and 300:1) for 1 h at room temperature. After conjugation, the excess free linkers were removed by passing through the Sephadex-G100 spin columns. In the second step, aminoxy functional conjugates were conjugated to the ketone linker of Ab with different molar ratios of linker to Ab (10:1, 20:1, and 30:1) at RT for 4 h in the present of TEA (40 mM, pH 5.5). We have selected a biotin-tagged rqYKYkf peptide, which is cleavable by tissue plasminogen activator (t-PA, recombinant version of t-PA, trade name TNKase) (18), as a functional derivative to verify this conjugation method for antibody studies. Conjugation was confirmed by determining the mass of the antibody and peptide conjugated antibody by using MALDI-TOF mass spectrometry.

2.3 Western Blot Analysis and Protease Cleavage Studies

Breast adenocarcinoma cancer cell targeting antibody (ChL6) (21) and biotin-rqYKYkf-conjugated ChL6 was separated at 10% SDS-PAGE gel and transferred onto nitro cellulose membrane. The transferred membrane was blocked with 1% BSA for 3 h at RT and incubated with HRPO-conjugated avidin for 30 min. Unbound avidin was removed by washing three times with TBS containing 0.2% Tween-20 and bands were developed by ECL method (Perkin-Elmer life Sciences). Biotinylated anti-insulin antibody was used as control. For protease susceptibility studies, biotin-rqYKYkf-conjugated ChL6 was incubated with TNKase at different concentrations (0, 10, and 25 μg/mL) in TBS buffer pH 8.0 for 1 h at 37 °C. Digested products are separated, transferred to membrane, and stained with avidin-HRPO as described above.

2.4 Immunohistochemistry

Breast adenocarcinoma cancer cells were seeded on a six-well plate one day before the experiments were performed. 1 μg of ChL6 and biotin-rqYKYkf-conjugated ChL6 were added directly into 1 mL of growth media in duplicate and kept for 1 h on ice. Unbound antibodies were removed by washing three times with PBS containing 1% BSA. FITC-conjugated streptavidin was added to each well and kept on ice for 30 min, followed by washing three times with PBS containing 1% BSA

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yield was obtained (8, 22). Synthetic scheme of biotin-rqYKYkf-spacer-K(Aoa)-NH_2 peptide was shown in Figure 1 A total of 25 mg of biotin-rqYKYkf-spacer-K(Aoa)-NH_2 was obtained from 200 mg of resin (yield: 16%). The synthetic product was verified by MALDI-TOF MS $(M+H)^+$ calculated: 1688.9; found: 1688.5.

Figure 1

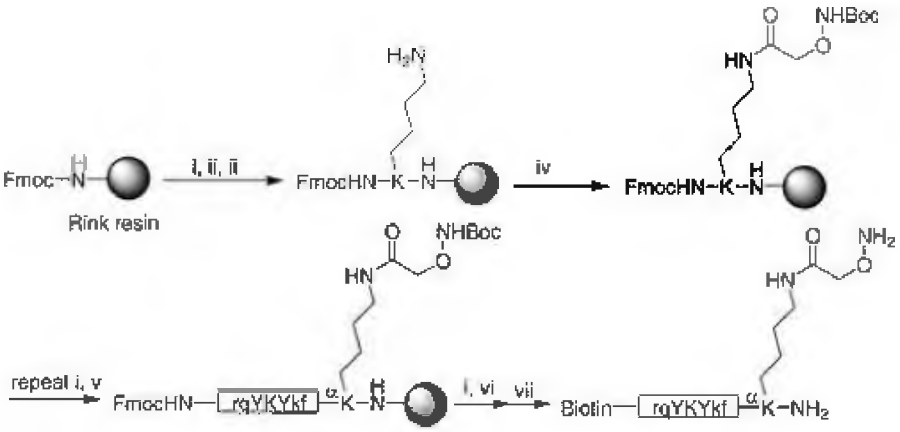


Figure 1. Schematic representation of the synthesis of aminoxy peptide. (i) 20% Piperidine in DMF; (ii) Fmoc-Lys(Alloc)-OH, DIC/HOBt; (iii) $Pd(PPh_3)_4/PhSiH_3$; (iv) Boc-Aoa-OH, DIC/HOBt; (v) Fmoc-AA_i, DIC/HOBt; (vi) Biotin, HBTU/DIEA; (vii) K Reagent (TFA/phenol/thioanisole/water/propylsilane, 82.5/7.5/5/5/2.5, v/w/v/v/v.

3.2 Optimization of Ketone-Oxime Conjugation

This modified ketone-oxime method has two steps. (i) introduction of the NHS-ketone linkers (referred as linker moiety) to the primary amines present in the antibody and (ii) conjugation of ketone group to aminoxy functional group present in the conjugated moiety (referred to as functional moiety). Steps involved in this conjugation are shown in Figure 2

Figure 2

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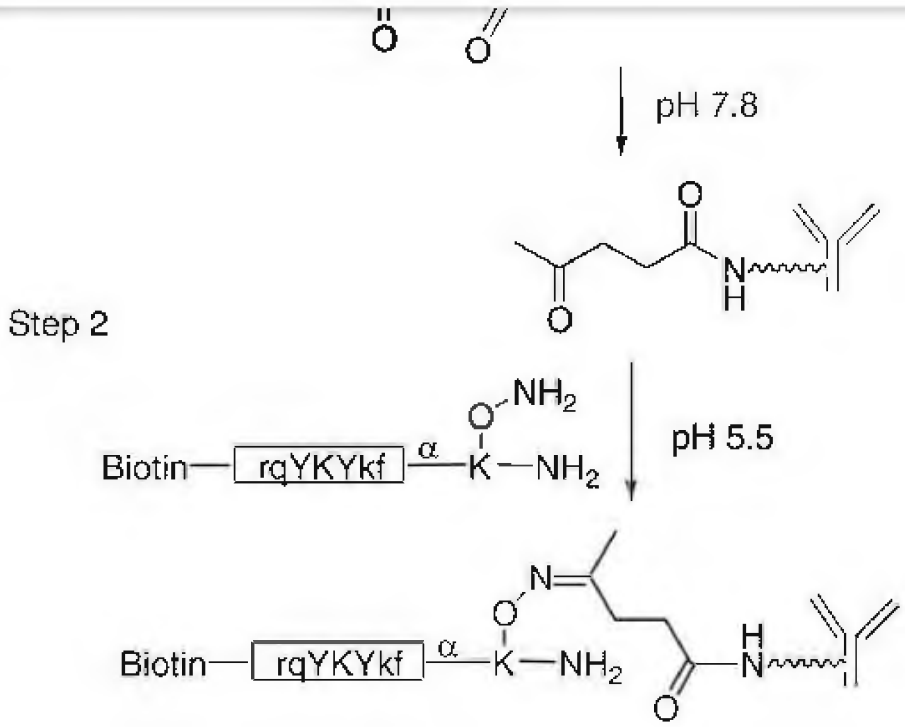


Figure 2. Schematic representation of ketone-oxime conjugation reaction.

First, ketone linkers were introduced by reacting the NHS group with primary amines of the antibody at various molar ratio (L/Ab 5:1, 10:1, 30:1, and 300:1). MALDI-MS results revealed that antibody derivatives occurred when a 20-fold excess or more of ketone linker was used in the initial antibody modification step. No conjugation was observed when 5:1 or 10:1 molar ratio of L/Ab was used. Table 1 summarizes the level of antibody derivatives under various L/Ab ratios. At 30:1 (L/Ab) ratio, ~5−6 linker moieties were conjugated to each Ab molecule (Figure 3A). The same preparation will yield 1−3 functional conjugates (Figure 3B). At (L/Ab) ratio of 300:1, mass spectrometry analysis of ChL6 Ab showed a distinct peak (Figure 3C) suggesting that most of the primary amines present in the Ab were conjugated. When functional moiety is conjugated at 30 molar ratio to an antibody, ~3 functional moieties were generated from this preparation. Previous reports have shown that, in order to retain the biological function of antibodies, conjugation of a functional moiety should not exceed 8. Therefore, we have selected 30:1 L/Ab ratio to perform our conjugation studies.

Table 1. Optimization of Ketone Linkers Per Ab Molecule

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30:1	5−6	1−3
300:1	~80	7−11

*Functional moiety conjugation was done at 30:1 ratio with antibody.

Figure 3

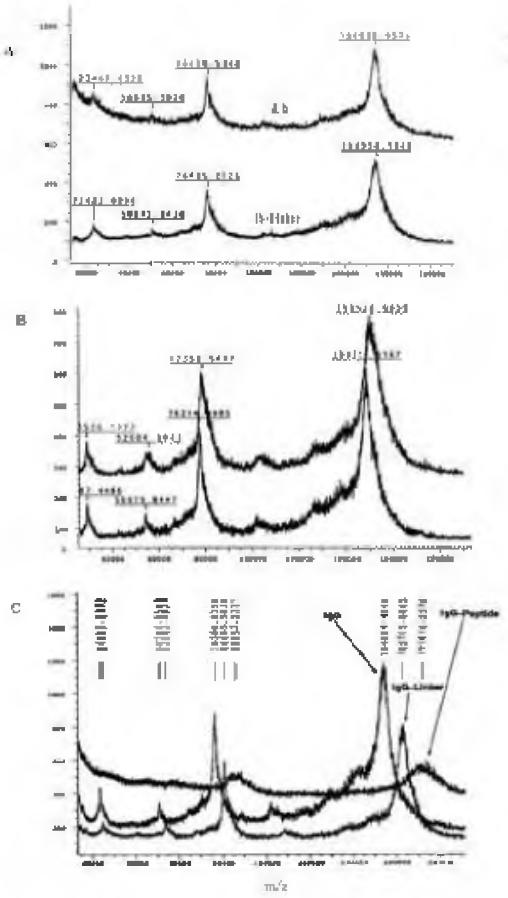


Figure 3. MALDI-MS analysis of ChL6 Ab, ChL6-linker moiety, and ChL6-functional moiety: (A) At 30:1 molar ratio of linker to Ab, ~5−6 linker moiety was conjugated to ChL6 Ab. (B) At 30:1 molar ratio, ~1−3 functional moiety (MW 1688) was conjugated to ChL6. (C) At 300:1 molar ratio, ~7−11

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unconjugated ChL6 antibody were binding HBT breast cancer cells with similar intensity, confirming that the conjugation method did not alter the antigen binding property of CHL6 (Figure 4)

Figure 4

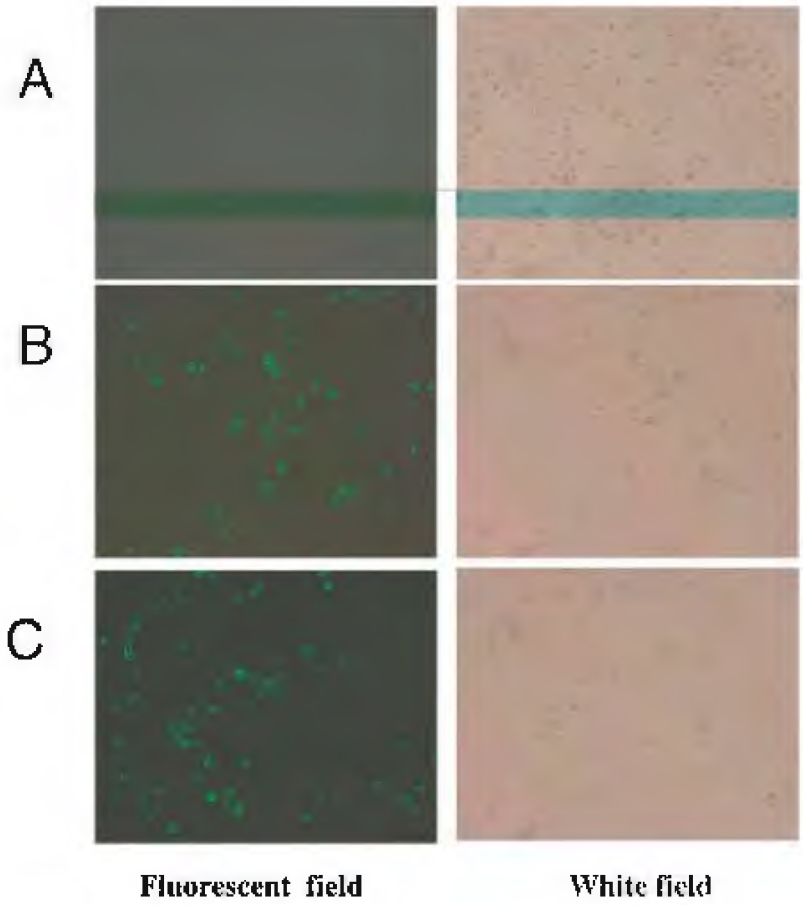


Figure 4. Immunoreactivity assay: Breast cancer cells (HBT) were incubated with ChL6 (B) and ChL6 conjugated to functional moiety (C) followed by FITC conjugated murine antihuman Ab. ChL6 conjugated with functional moiety (C) showed binding as good as native unaltered ChL6 (B). FITC conjugated murine antihuman IgG did not show any binding (A).

3.4 Protease Susceptibility Assay

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already in clinical use to dissolve fibrin clots (26, 27). Protease susceptibility was evaluated by incorporating t-PA substrate "rqYKYkf", identified by screening one-bead−one-compound combinatorial chemistry libraries in linker moiety with biotin tag (18). After conjugation with ChL6 at 30:1 molar ratio, 5 μg/mL samples were taken in triplicate and digested with 0, 10, and 25 μg/mL of recombinant t-PA (TNKase) for 2 h at RT. Digested samples were identified by Western blot analysis as described in the Materials and Methods section. ECL showed bands only in the conjugated ChL6 and not with ChL-6 alone or in TNKase-digested conjugated ChL6 samples, suggesting that biotin was removed by protease digestion. This confirms that this method can be used as an alternative method to conjugate therapeutic drugs, especially prodrugs (Figure 5)

Figure 5

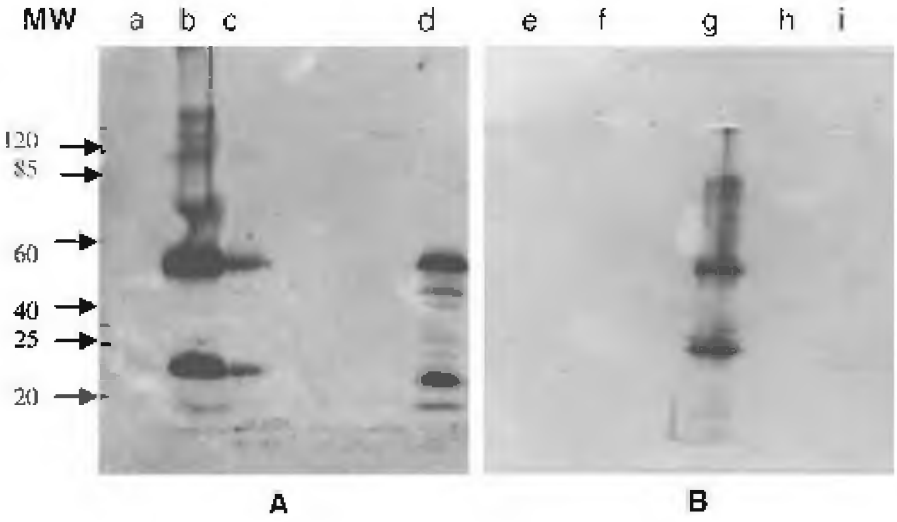


Figure 5. Western blot and protease susceptibility analysis: (A) ChL6−10μg (a), ChL6-conjugated with biotin-rqYKYkf−10μg (b), ChL6-conjugated with biotin-rqYKYkf−1μg (c), biotinylated anti-insulin Ab (d) was electrophoresed in 10% SDS-PAGE gel and developed using ECL after transferring to a nylon membrane. Positive bands appeared only on conjugated ChL6 (b,c,d) and not the native ChL6 (a). (B) To evaluate protease susceptibility of the functional moiety (t-PA substrate), conjugated ChL6 was incubated with various concentrations of TNKase, 0 μg/mL (g),10 μg/mL (h), and 25 μg/mL (i). The band has disappeared in TNKase-treated lanes (h,i) and not in untreated lanes (g). Native ChL6 and TNKase were run together as a negative control. Standard MW markers are shown on the left side of the figure.

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making therapeutic antibody conjugates. This method is compatible with peptides containing a thiol, amino, or carboxyl group in the conjugation moiety.

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Appendix KUMARESAN02

TECHNICAL NOTES

Evaluation of Ketone-Oxime Method for Developing Therapeutic On-Demand Cleavable Immunoconjugates

Pappanaicken R. Kumaresan,* Juntao Luo, Aimin Song, Jan Marik, and Kit S. Lam. Received January 17, 2008; Revised Manuscript Received March 31, 2008

The use of antibody molecules in immunoassay, molecular targeting, or detection techniques encompasses a broad variety of applications affecting nearly every field of medical science. In cancer therapy, monoclonal antibodies (mAb) have been used as vehicles to deliver radionuclides, toxins, or drugs to the target cancer cells. New conjugation methods are most needed to conjugate a wide variety of targeting small molecules and peptidomimatic compounds. Here, we exploited a keto-oxime method for conjugation of protease susceptible linkers to an antibody. This modified method involves two steps: (i) introduction of methyl ketone linkers (referred to as linker moiety) to the primary amines present in the antibody and (ii) conjugation of ketone linkers to aminoxy functional group present in the conjugated moiety (referred to as functional moiety). We have optimized this conjugation method and shown that ~10 functional moieties can be conjugated to antibody. Conjugation was verified by MALDI-TOF MS and Western blot analysis. The acidic pH conditions used in this method did not change the immune reactivity of the Ab. In addition, *in vitro* protease susceptibility assay was performed to validate this method for prodrug release assay as well as to remove excess radioimmune conjugates from circulation. This orthogonal method is compatible with peptides containing a thiol, amino, or carboxyl groups in the conjugation moiety.

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1. INTRODUCTION

Ketones selectively react with amino-oxy groups and form oxime bonds at slightly acidic pH. This type of chemoselective ligation is more preferable for making bioconjugates because it does not require anhydrous conditions, excessive heat, or any catalyst (*1, 2*). It is also tolerant of a wide range of functional groups that are found in proteins and oligonucleotides. This ligation has been successfully used in various applications such as making small molecule drug conjugates (*3*), peptide and oligonucleotide conjugates (*4*), protein conjugates (*5*), glycoconjugates (*2*), polymer conjugates (*6, 7*), and coating biomolecules on plannar microarray slides (*8*) and sialoglycoproteins present on cell wall (*9, 10*). Except for N-terminal labeling (*11*), this ligation method has not been used to make antibody drug conjugates especially in the area of cancer targeted therapy. Antibodies are important biomolecules developed by nature to protect hosts by eliminating disease-causing pathogens. Several conjugation methods have been developed to conjugate radionuclides, toxins, or drugs to antibody for cancer therapy (*12–17*). Here, we report conjugation of tissue plasminogen activator (t-PA) substrate rqYKYkf (*18*) to ChL6 antibody by the keto-oxime method. Chemical synthesis of methylketo linkers, aminoxy-functional peptides and optimization of conjugation method and its bioapplications are discussed.

2. MATERIALS AND METHODS

Rink amide MBHA resin (0.45 mmol/g) was purchased from Tianjin Nankai Hecheng (Tianjin, China). Levulinate *N*-succinimidyl ester was prepared using our previously published procedure (*8*). All the amino acid derivatives and HOBt (1-hydroxybenzotriazole) were purchased from CPC (San Jose, CA). DIC (diisopropylcarbodiimide) was purchased from Advanced ChemTech (Louisville, KY). DMF (*N,N*-dimethylformamide) was purchased from VWR (Brisbane, CA). DCM (dichloromethane), MeOH (methanol), diethyl ether, and acetonitrile were purchased from Fisher (Houston, TX). All other chemical reagents were purchased from Aldrich (Milwaukee, WI). Analytical HPLC analyses (Vydac column, 4.6 mm × 250 mm, 5 μm, 300 Å, C_{18}, 1.0 mL/min, 25 min gradient from 100% aqueous media (0.1% TFA) to 100% CH_3CN (0.1% TFA), 214, 220, 254, and 280 nm) and preparative HPLC purification (Vydac column, 20 mm × 250 mm, 5 μm, 300 Å, C_{18}, 7.0 mL/min, 45 min gradient from 100% aqueous media (0.1% TFA) to 100% CH_3CN (0.1% TFA), 254 nm) were performed on a Beckman System Gold HPLC system (Fullerton, CA). MALDI-TOF MS analysis was performed on a Bruker BIFLEX III mass spectrometer (Billerica, MA). All of the experiments are carried out at room temperature unless otherwise noted. ChL6 (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) was a chimera consisting of human IgG1-constant regions and the murine L6 mAb antigen-binding site.

2.1. Synthesis of Biotin-rqYKYkf-spacer-K(Aoa)-NH_2 Peptide. Solid-phase synthesis of biotin-rqYKYkf was carried out using Rink amide MBHA resin after the treatment of resin with 20% piperidine solution in DMF (2 mL) for 15 min to remove Fmoc protecting group. Onto the Rink resin (200 mg, 0.09 mmol) with free amino groups, Fmoc-Lys(Alloc)-OH (122.2 mg, 0.27 mmol) was coupled following the standard Fmoc chemistry using HOBt (36.5 mg, 0.27 mmol) and DIC (42.3 μL, 0.27 mmol) as coupling agents. The coupling was monitored using ninhydrin test agents until a negative result was obtained. The resin was washed with DMF, MeOH, and DCM 3 times,

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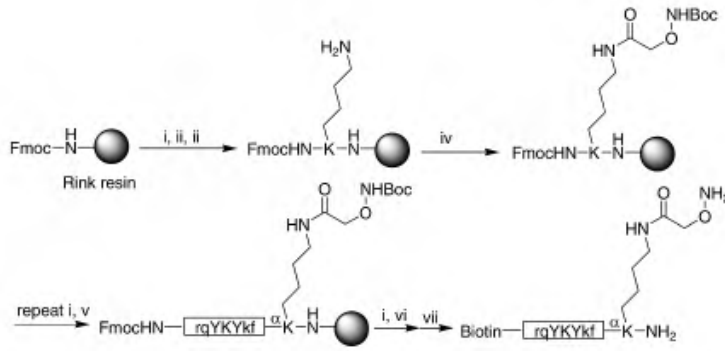


Figure 1. Schematic representation of the synthesis of aminoxy peptide. (i) 20% Piperidine in DMF; (ii) Fmoc-Lys(Alloc)-OH, DIC/HOBt; (iii) $Pd(PPh_3)_4/PhSiH_3$; (iv) Boc-Aoa-OH, DIC/HOBt; (v) Fmoc-AA_i, DIC/HOBt; (vi) Biotin, HBTU/DIEA; (vii) K Reagent (TFA/phenol/thioanisole/water/propylsilane, 82.5/7.5/5/5/2.5, v/w/v/v/v.

respectively. The resin was treated twice with $Pd(PPh_3)_4$ (26.0 mg, 0.0225 mmol) in the presence of excessive $PhSiH_3$ (266.5 μL, 2.16 mmol) in DCM under argon for 30 min to remove the alloc protecting groups on the side chain of lysine. After thoroughly washing the resin with DCM, DMF, MeOH, and DMF, Boc-Aoa-OH (51.6 mg, 0.27 mmol) was coupled onto the resin using HOBt (36.5 mg, 0.27 mmol) and DIC (42.3 μL, 0.27 mmol) as coupling agents until the ninhydrin test was negative. After deprotection of Fmoc groups by piperidine treatment, a linear hydrophilic linker was coupled to the α-amino group of the lysine as a spacer following a published procedure (*19*). The peptide was constructed on the linker using standard Fmoc chemistry using DIC/HOBt as coupling agents (*20*). After the coupling of the last amino acid, the N-terminus Fmoc protection group was removed by piperidine treatment. The resin was treated with a solution of biotin (44.0 mg, 0.18 mmol), DIEA (diisopropylethylamine, 42.3 μL, 0.36 mmol), and HBTU (*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, 68.3 mg, 0.18 mmol) in DMF (2 mL) until the ninhydrin test was negative. The resin was thoroughly washed with DMF, MeOH, and DCM, and then dried *in vacuo*. To the dried resin was added 4 mL of cleavage mixture (TFA (trifluoroacetic acid)/phenol/thioanisole/water/TIS (triisopropylsilane), 82.5:7.5:5:5:2.5, v/w/v/v/v) at ice-bath temperature. The mixture was slowly warmed to room temperature and allowed to agitate for 6 h. The supernatant was then collected and the resin was washed with TFA (3 × 1 mL). The combined supernatants were concentrated to 1 mL under a stream of nitrogen. The peptide was precipitated by dilution with cold ethyl ether (10 mL), collected by centrifuge, washed with ethyl ether, and dried *in vacuo*. The crude peptide was analyzed and purified by HPLC, and characterized by MALDI-TOF MS.

2.2. Antibody Conjugation. This keto-oxime method has two steps. First, ketone linkers will be conjugated to primary amines of the antibody followed by conjugation of functional derivatives such as DOTA chelators, immunotoxin, or chemotoxin. Before conjugation, the required amounts of antibody were purified by passing through a Sephadex-G100 molecular sieving column to remove impurities and are equilibrated with 40 mM of ketone linker conjugation buffer triethanolamine (pH 7.8). The linker conjugation can be done with phosphate buffer 50 mM (pH 7.8). In order to identify optimum ketone group introduction to Ab, conjugation was done with antibody at different molar ratios (10:1, 20:1, 30:1, and 300:1) for 1 h at room temperature. After conjugation, the excess free linkers were removed by passing through the Sephadex-G100 spin columns. In the second step, aminoxy functional conjugates were conjugated to the ketone linker of Ab with different molar ratios of linker to Ab (10:1, 20:1, and 30:1) at RT for 4 h in the present of TEA (40 mM, pH 5.5). We have selected a biotin-tagged

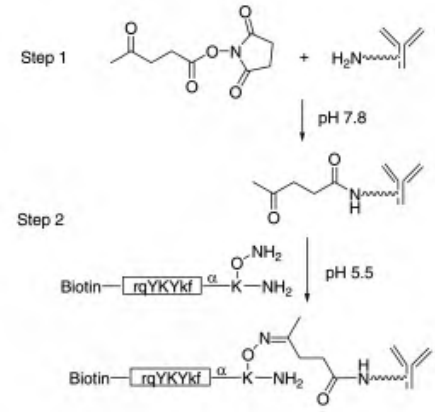


Figure 2. Schematic representation of ketone-oxime conjugation reaction.

Table 1. Optimization of Ketone Linkers Per Ab Molecule

linker moiety / Ab ratio	# linker moiety per Ab molecule	# functional moieties per Ab molecule*
5:1	none	none
10:1	none	none
30:1	5−6	1−3
300:1	~80	7−11

* Functional moiety conjugation was done at 30:1 ratio with antibody.

rqYKYkf peptide, which is cleavable by tissue plasminogen activator (t-PA, recombinant version of t-PA, trade name TNKase) (*18*), as a functional derivative to verify this conjugation method for antibody studies. Conjugation was confirmed by determining the mass of the antibody and peptide conjugated antibody by using MALDI-TOF mass spectrometry.

2.3. Western Blot Analysis and Protease Cleavage Studies. Breast adenocarcinoma cancer cell targeting antibody (ChL6) (*21*) and biotin-rqYKYkf-conjugated ChL6 was separated at 10% SDS-PAGE gel and transferred onto nitro cellulose membrane. The transferred membrane was blocked with 1% BSA for 3 h at RT and incubated with HRPO-conjugated avidin for 30 min. Unbound avidin was removed by washing three times with TBS containing 0.2% Tween-20 and bands were developed by ECL method (Perkin-Elmer life Sciences). Biotinylated anti-insulin antibody was used as control. For protease susceptibility studies, biotin-rqYKYkf-conjugated ChL6 was incubated with TNKase at different concentrations (0, 10, and 25 μg/mL) in TBS buffer pH 8.0 for 1 h at 37 °C. Digested

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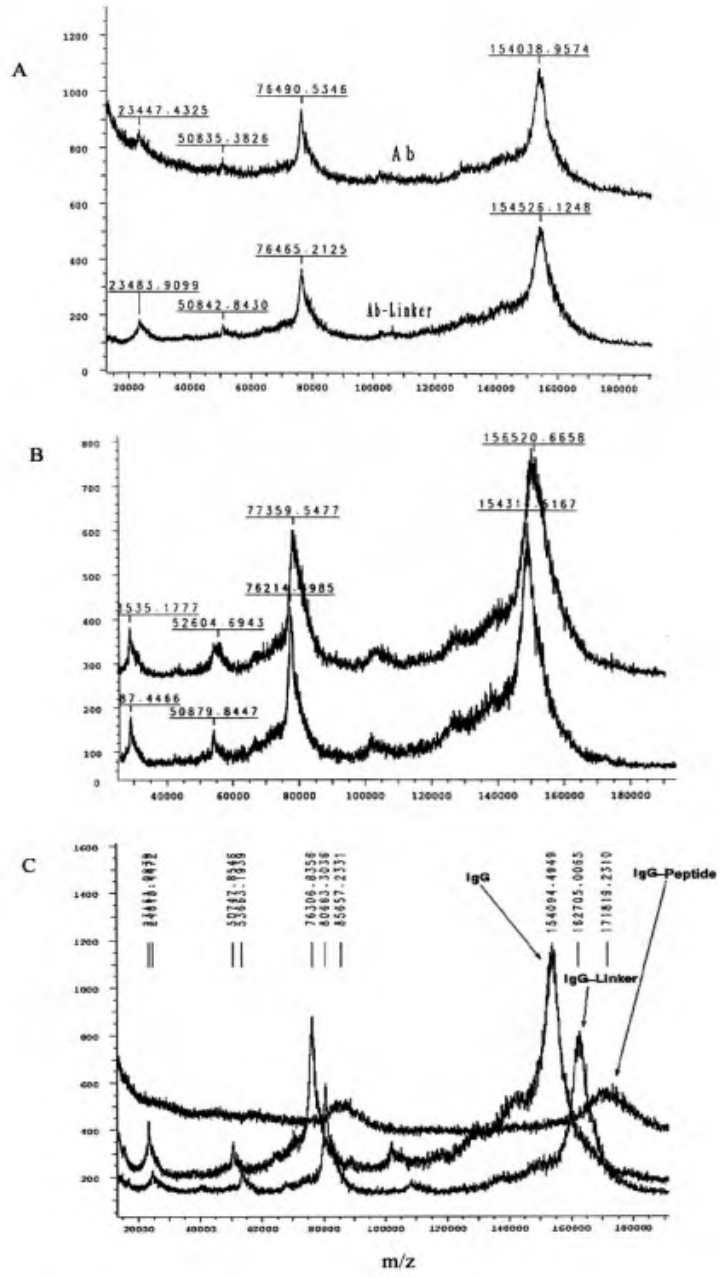


Figure 3. MALDI-MS analysis of ChL6 Ab, ChL6-linker moiety, and ChL6-functional moiety: (A) At 30:1 molar ratio of linker to Ab, ~5−6 linker moiety was conjugated to ChL6 Ab. (B) At 30:1 molar ratio, ~1−3 functional moiety (MW 1688) was conjugated to ChL6. (C) At 300:1 molar ratio, ~7−11 functional moieties were conjugated. The average incorporated values are shown. At this ratio, ChL6 with linker moiety showed a distinct peak suggesting that most of the primary amines in ChL6 are conjugated with linker moiety.

products are separated, transferred to membrane, and stained with avidin-HRPO as described above.

2.4. Immunohistochemistry. Breast adenocarcinoma cancer cells were seeded on a six-well plate one day before the experiments were performed. 1 μg of ChL6 and biotin-rqYKYkf-conjugated ChL6 were added directly into 1 mL of growth media in duplicate and kept for 1 h on ice. Unbound antibodies were removed by washing three times with PBS containing 1% BSA. FITC-conjugated streptavidin was added to each well and kept on ice for 30 min, followed by washing three times with PBS containing 1% BSA to remove the unbound streptavidin. Fluorescent staining was observed under the fluorescence microscope in green filter (U-M51004 F/R-V2; Olympus).

3. RESULTS AND DISCUSSION

3.1. Characterization of Biotin-rqYKYkf-spacer-K [Aminoxy (Aoa)]-NH_2 Peptide. The methyl ketone linker (Levulinate *N*-succinimidyl ester) was synthesized according to the reported procedure using levulinic acid and *N*-hydroxyl succinimide as starting materials and 85% yield was obtained (*8, 22*). Synthetic

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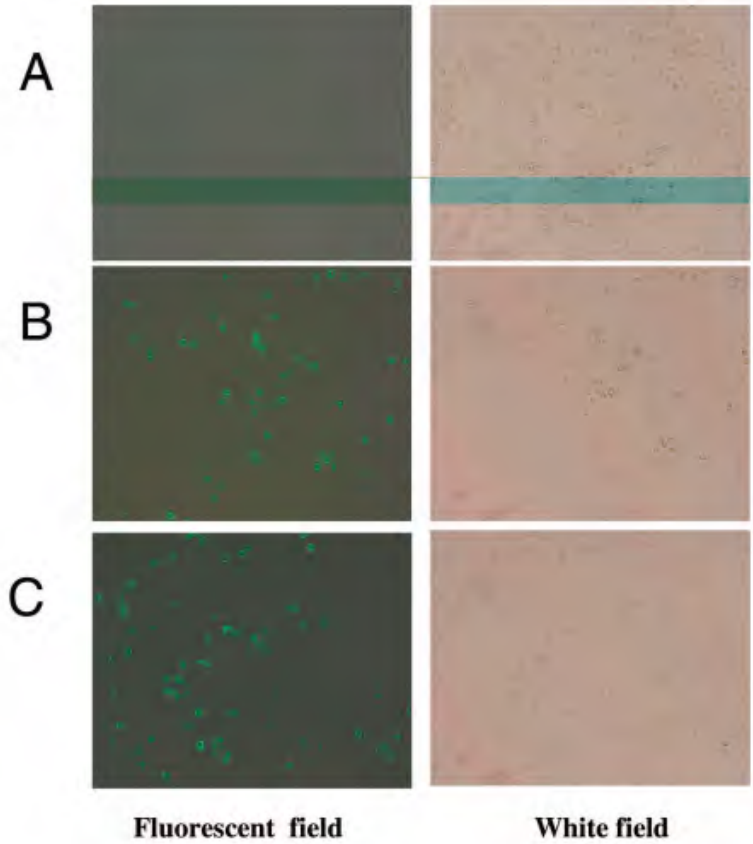


Figure 4. Immunoreactivity assay: Breast cancer cells (HBT) were incubated with ChL6 (B) and ChL6 conjugated to functional moiety (C) followed by FITC conjugated murine antihuman Ab. ChL6 conjugated with functional moiety (C) showed binding as good as native unaltered ChL6 (B). FITC conjugated murine antihuman IgG did not show any binding (A).

scheme of biotin-rqYKYkf-spacer-K(Aoa)-NH_2 peptide was shown in Figure 1. A total of 25 mg of biotin-rqYKYkf-spacer-K(Aoa)-NH_2 was obtained from 200 mg of resin (yield: 16%). The synthetic product was verified by MALDI-TOF MS $(M+H)^+$ calculated: 1688.9; found: 1688.5.

3.2. Optimization of Ketone-Oxime Conjugation. This modified ketone-oxime method has two steps. (i) introduction of the NHS-ketone linkers (referred as linker moiety) to the primary amines present in the antibody and (ii) conjugation of ketone group to aminoxy functional group present in the conjugated moiety (referred to as functional moiety). Steps involved in this conjugation are shown in Figure 2.

First, ketone linkers were introduced by reacting the NHS group with primary amines of the antibody at various molar ratio (L/Ab 5:1, 10:1, 30:1, and 300:1). MALDI-MS results revealed that antibody derivatives occurred when a 20-fold excess or more of ketone linker was used in the initial antibody modification step. No conjugation was observed when 5:1 or 10:1 molar ratio of L/Ab was used. Table 1 summarizes the level of antibody derivatives under various L/Ab ratios. At 30:1 (L/Ab) ratio, ~5−6 linker moieties were conjugated to each Ab molecule (Figure 3A). The same preparation will yield 1−3 functional conjugates (Figure 3B). At (L/Ab) ratio of 300:1, mass spectrometry analysis of ChL6 Ab showed a distinct peak (Figure 3C) suggesting that most of the primary amines present in the Ab were conjugated. When functional moiety is conjugated at 30 molar ratio to an antibody, ~3 functional moieties were generated from this preparation. Previous reports have shown that, in order to retain the biological function of antibodies, conjugation of a functional moiety should not exceed 8. Therefore, we have selected 30:1 L/Ab ratio to perform our conjugation studies.

3.3. Immunoreactivity Assay. CHL6, a chimeric antibody that targets a breast cancer antigen, was used for conjugation studies (*21*). To verify that this conjugation method does not alter antigen binding properties of CHL6 antibody, it was conjugated with biotin-tagged peptide at 30:1 L/Ab ratio, and immunoreactivity assay was performed on breast cancer cells (HBT). Figure 3 shows that both conjugated and unconjugated ChL6 antibody were binding HBT breast cancer cells with similar intensity, confirming that the conjugation method did not alter the antigen binding property of CHL6 (Figure 4).

3.4. Protease Susceptibility Assay. In clinics, antibodies have been used for targeting as well as delivering therapeutic drugs, especially prodrugs to eliminate infected and transformed cells. These nontoxic prodrugs get converted to a toxic metabolite when it is cleaved by a specific protease such as Cathepsin-B at the target site (*23*). A similar strategy has been used in radioimmunotherapy (RIT), to remove circulating radioimmunoconjugates after tumor targeting. This will boost the therapeutic potential of RIT which was greatly limited by exposure of radiation to vital organs (liver, bone marrow) during the treatment. These linkers can be cleaved either internally by proteases expressed in vital organs such as the liver (e.g., Cathepsin-D) (*24*) or externally by administering enzymes such as β-lactamase (*25*) and TNKase (*18*). We have selected TNKase as an on-demand cleavable enzyme because it is already in clinical use to dissolve fibrin clots (*26, 27*). Protease susceptibil-

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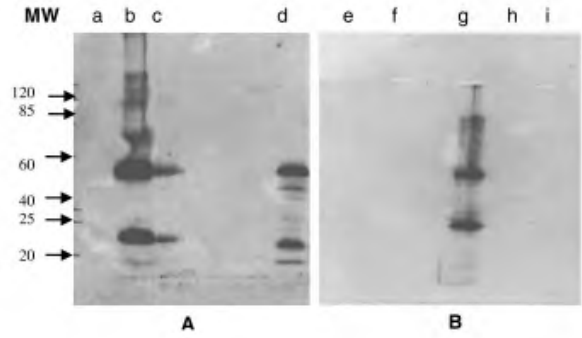


Figure 5. Western blot and protease susceptibility analysis: (A) ChL6−10μg (a), ChL6-conjugated with biotin-rqYKYkf−10μg (b), ChL6-conjugated with biotin-rqYKYkf−1μg (c), biotinylated anti-insulin Ab (d) was electrophoresed in 10% SDS-PAGE gel and developed using ECL after transferring to a nylon membrane. Positive bands appeared only on conjugated ChL6 (b,c,d) and not the native ChL6 (a). (B) To evaluate protease susceptibility of the functional moiety (t-PA substrate), conjugated ChL6 was incubated with various concentrations of TNKase, 0 μg/mL (g),10 μg/mL (h), and 25 μg/mL (i). The band has disappeared in TNKase-treated lanes (h,i) and not in untreated lanes (g). Native ChL6 and TNKase were run together as a negative control. Standard MW markers are shown on the left side of the figure.

ity was evaluated by incorporating t-PA substrate "rqYKYkf", identified by screening one-bead−one-compound combinatorial chemistry libraries in linker moiety with biotin tag (*18*). After conjugation with ChL6 at 30:1 molar ratio, 5 μg/mL samples were taken in triplicate and digested with 0, 10, and 25 μg/mL of recombinant t-PA (TNKase) for 2 h at RT. Digested samples were identified by Western blot analysis as described in the Materials and Methods section. ECL showed bands only in the conjugated ChL6 and not with ChL-6 alone or in TNKase-digested conjugated ChL6 samples, suggesting that biotin was removed by protease digestion. This confirms that this method can be used as an alternative method to conjugate therapeutic drugs, especially prodrugs (Figure 5).

CONCLUSION

Previously, we used the methods described here to print peptides and small molecules in microarrays over a planar surface (*8*). Previously, *in vivo* small molecule drug conjugate studies have demonstrated oxime bond stability at the physiological pH of body fluids (*28, 29*). Here, we have demonstrated this method to conjugate protease (TNKase) cleavable peptides to antibodies without affecting their antigen binding properties. We also showed that conjugated substrates were susceptible to TNKase at the clinical dosage. We conclude that the ketone-oxime method can be substituted in place of other methods like maleimide (*12*) and click chemistry conjugation (*30*) for making therapeutic antibody conjugates. This method is compatible with peptides containing a thiol, amino, or carboxyl group in the conjugation moiety.

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Expanded Utility of the β-Glucuronide Linker: ADCs That Deliver Phenolic Cytotoxic Agents

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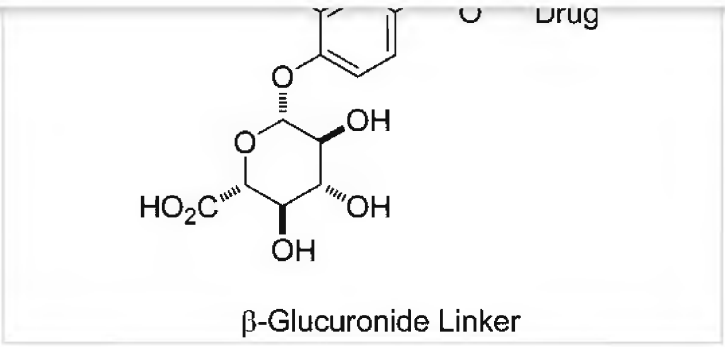
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β-Glucuronide Linker

The β-glucuronide linker has been used for antibody−drug conjugates (ADCs) to deliver amine-containing cytotoxic agents. The linker is stable in circulation, hydrophilic and provides ADCs that are highly active in vitro and in vivo. To extend the utility of the β-glucuronide linker toward phenol-containing drugs, an *N,N*-dimethylethylene diamine self-immolative spacer was incorporated with the linker for release of the potent cytotoxic phenol psymberin A. Exposure of the drug-linker to β-glucuronidase resulted in facile drug release. The corresponding ADCs were active and immunologically selective against CD30-positive L540cy and CD70-positive Caki-1 cell lines.

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Subjects

Aromatic Compounds Cells Drug Release Peptides And Proteins Toxicity

Keywords

B-Glucuronide Linker Antibody−Drug Conjugates Phenolic Cytotoxic Agents *N,N*-Dimethylethylene Diamine CD30-Positive L540cy Cell Line CD70-Positive Caki-1 Cell Line

Antibody−drug conjugates (ADCs) consist of three primary components: a monoclonal antibody that targets a tumor antigen with high tumor expression, a cytotoxic agent, and a linker system that releases drug from the antibody upon internalization into cancer cells. When a linker system with high circulation stability and tumor specificity is used, tolerability and efficacy can be significantly augmented. (1-3) In an effort to develop safe and effective ADCs, we have focused on linker systems meeting these criteria. We have reported on ADCs employing the β-glucuronide linker

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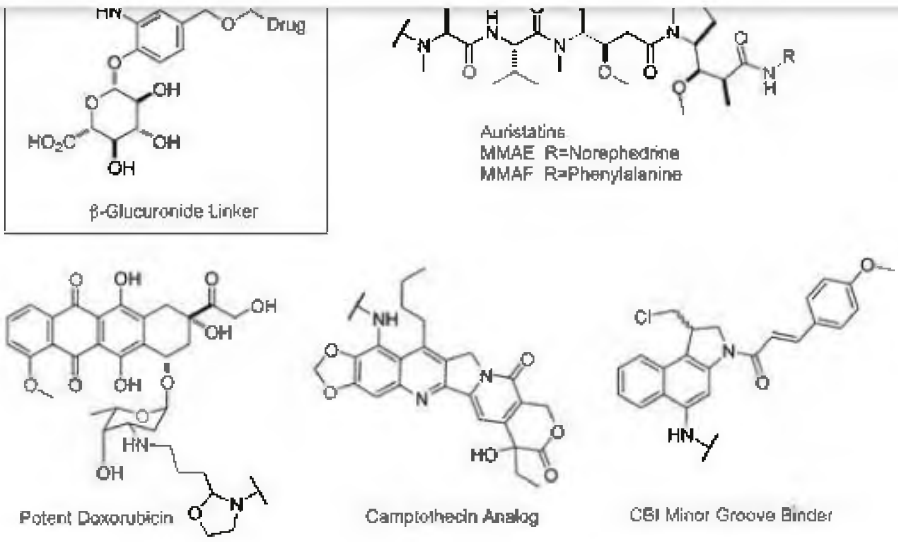


Figure 1. Examples of ADCs with the β-glucuronide linker.

Potent cytotoxic agents with basic amine residues are attractive molecules for targeted delivery using the β-glucuronide linker because the amine can be used for linker attachment. We were interested in extending the application of the β-glucuronide linker beyond amines to phenol-containing drugs, since the phenol functional group is a common element in many anticancer drugs. For example, the camptothecin derivative SN38 (**1**) and duocarmycins such as **2** possess phenol residues. Another phenol-containing drug, psymberin (aka irciniastatin A, **3**), is a potent anticancer molecule with subnanomolar cytotoxic activity (Table 1) (11). The treatment of cells with **3** leads to apoptosis through an undefined mechanism.

Table 1. IC_{50} Values of Psymberin (3) and β-Glucuronide Drug-Linker 8 Conjugates[a]

	Caki-1 (CD70+/CD30−)	L540cy (CD70−/CD30+)
psymberin (**3**)	0.3	0.7
cAC10−**8** (5.4 dr/mAb)	62	0.15
h1F6−**8** (5.4 dr/mAb)	0.8	58.6

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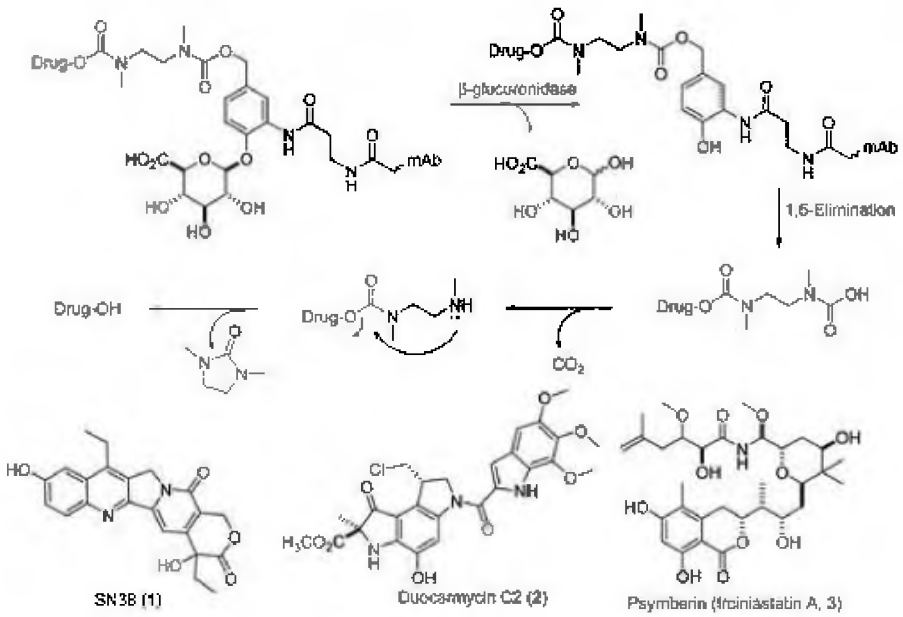
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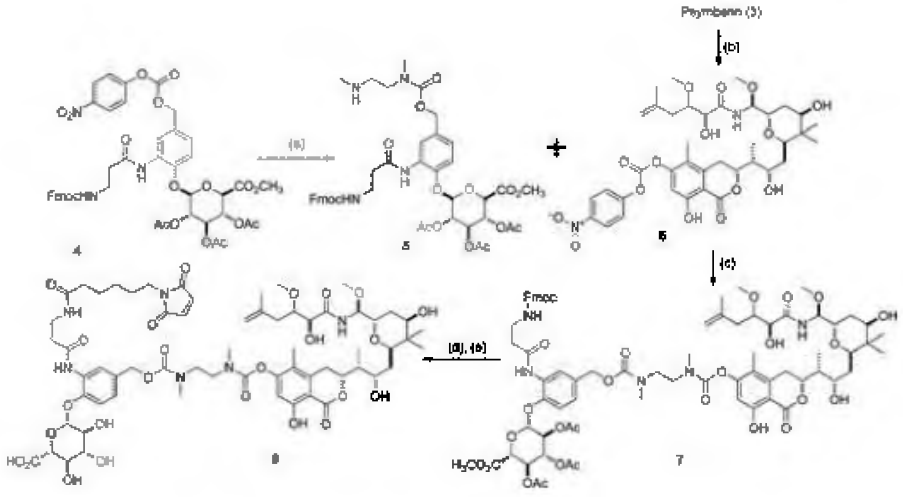
maleimidocaproyl-*N*-hydroxy succinimide ester provided the final drug-linker **8**.

Scheme 1



Scheme 1. Release Mechanism from β-Glucuronide Linker

Scheme 2



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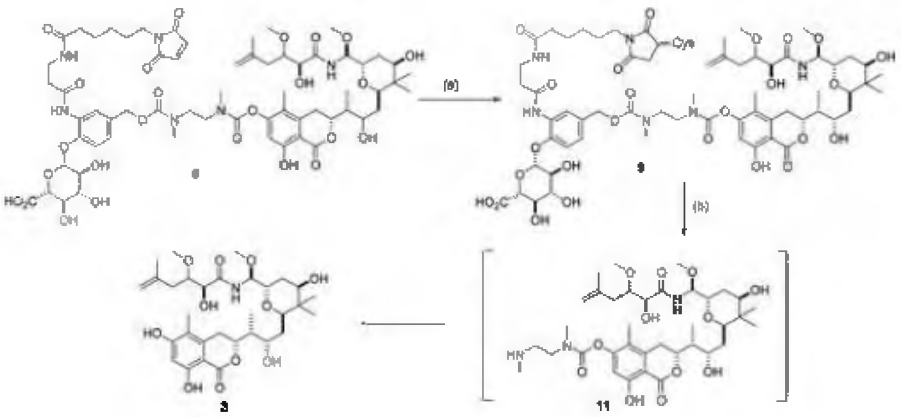
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completely consumed. The specific activity for enzymatic hydrolysis is 0.21 μmol/min/mg, which is consistent with a previous measurement on an auristatin-based drug-linker. (4) The products were **3** and the residual linker components, specifically the diastereomeric cysteine adduct **10**. The absence of any detectible intermediate carbamate **11** demonstrates that self-immolation of the DMED intermediate **11** to release **3** was rapid.

Scheme 3



Scheme 3. [a]

Scheme a Reagents and conditions: (a) Cysteine. (b) β-Glucuronidase (*E. coli*), 30 min.

Figure 2

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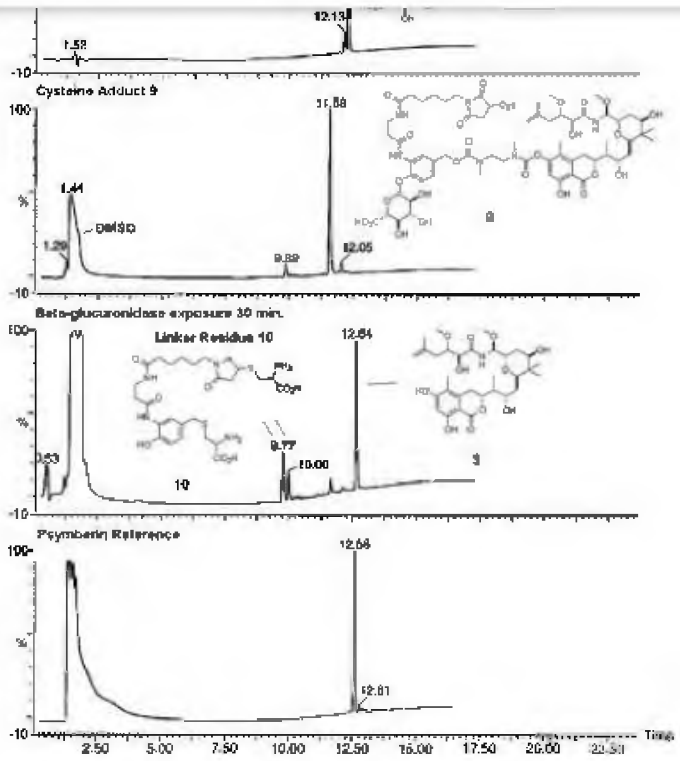


Figure 2. HPLC analysis of β-glucuronidase cleavage of psymberin drug linker **9**. Peaks were observed using a diode array detector.

Drug-linker **8** was conjugated to the monoclonal antibodies cAC10 (anti-CD30 mAb) and h1F6 (anti-CD70 mAb) via reduced interchain disulfides. An average loading of 5.4 drugs/mAb was achieved, and the conjugates were >95% monomeric. Exposure to the CD30-expressing cell line L540cy (Hodgkin's lymphoma) to cAC10–**8** over a 96 h period gave an IC_{50} value of 0.15 nM (Table 1) The cell killing was immunologically selective as exposure to the CD30-negative cell line Caki-1 (renal cell carcinoma) gave an IC_{50} value of 62 nM. Conversely, the h1F6–**8** ADC showed good activity on the CD70-expressing line Caki-1 but significantly reduced activity against the CD30-negative line L540cy. Unconjugated **3** was highly potent on these cell lines. The selectivity observed over the 96 h exposure assay suggests a high degree of stability, which is consistent with previous findings with the β-glucuronide linker. (7)

ADCs continue to demonstrate their value in the clinic, (3, 13-16) and as a consequence, investigations into new chemotypes and linkers for ADCs will continue. The favorable properties of

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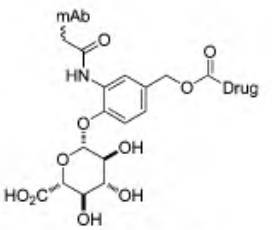
Expanded Utility of the β-Glucuronide Linker: ADCs That Deliver Phenolic Cytotoxic Agents

Scott C. Jeffrey,*,† Jef De Brabander,‡ Jamie Miyamoto,† and Peter D. Senter†

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ABSTRACT The β-glucuronide linker has been used for antibody−drug conjugates (ADCs) to deliver amine-containing cytotoxic agents. The linker is stable in circulation, hydrophilic and provides ADCs that are highly active in vitro and in vivo. To extend the utility of the β-glucuronide linker toward phenol-containing drugs, an *N*,*N*′-dimethylethylene diamine self-immolative spacer was incorporated with the linker for release of the potent cytotoxic phenol psymberin A. Exposure of the drug-linker to β-glucuronidase resulted in facile drug release. The corresponding ADCs were active and immunologically selective against CD30-positive L540cy and CD70-positive Caki-1 cell lines.

KEYWORDS β-Glucuronide linker, antibody−drug conjugates, phenolic cytotoxic agents, *N*,*N*′-dimethylethylene diamine, CD30-positive L540cy cell line, CD70-positive Caki-1 cell line



β-Glucuronide Linker

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Antibody−drug conjugates (ADCs) consist of three primary components: a monoclonal antibody that targets a tumor antigen with high tumor expression, a cytotoxic agent, and a linker system that releases drug from the antibody upon internalization into cancer cells. When a linker system with high circulation stability and tumor specificity is used, tolerability and efficacy can be significantly augmented.[1−3] In an effort to develop safe and effective ADCs, we have focused on linker systems meeting these criteria. We have reported on ADCs employing the β-glucuronide linker (Figure 1) with several drug classes including auristatins,[4] a highly potent doxorubicin derivative,[5] CBI minor groove binders,[6] and camptothecin analogues.[7] The β-glucuronide linker is readily cleaved by β-glucuronidase, an enzyme present in lysosomes and tumor interstitium.[8,9] The linker is highly stable in circulation and hydrophilic, which allows the use of hydrophobic drugs for ADCs that otherwise would lead to high degrees of aggregation.[6,10]

Potent cytotoxic agents with basic amine residues are attractive molecules for targeted delivery using the β-glucuronide linker because the amine can be used for linker attachment. We were interested in extending the application of the β-glucuronide linker beyond amines to phenol-containing drugs, since the phenol functional group is a common element in many anticancer drugs. For example, the camptothecin derivative SN38 (**1**) and duocarmycins such as **2** possess phenol residues. Another phenol-containing drug, psymberin (aka irciniastatin A, **3**), is a potent anticancer molecule with subnanomolar cytotoxic activity (Table 1).[11] The treatment of cells with **3** leads to apoptosis through an undefined mechanism.

Our goal was to modify the β-glucuronide linker to contain a dimethylethylene diamine (DMED) self-immolative spacer[1,2] for stable linkage and facile release of **3**. Drug release would involve enzymatic deglucuronidation, 1,6-elimination, decarboxylation, and cyclization of the DMED carbamate to liberate free phenol (Scheme 1). Toward this end, the glucuronide *para*-nitrophenyl (pNP) carbonate **4** was reacted with excess DMED to readily afford the stable coupling partner **5** (Scheme 2). Psymberin (**3**) was activated with pNP carbonate to give the phenolic carbonate **6**. The coupling of **5** and **6** gave a modest yield of **7**. Saponification of the glucuronide acetate and methyl ester groups was accomplished concomitantly with Fmoc removal, and coupling to maleimidocaproyl-*N*-hydroxy succinimide ester provided the final drug-linker **8**.

To confirm the reactivity of **8** toward enzymatic cleavage,[4] the linker was treated with cysteine (Scheme 3) to form the cysteine−succinimide adduct **9** (80 μM), which was then exposed to β-glucuronidase (*Escherichia coli*, 36 μg/mL) at 37 °C. After 10 min, inspection of the reaction mixture by liquid chromatography−mass spectrometry (Figure 2) demonstrated that **9** had been almost completely consumed. The specific activity for enzymatic hydrolysis is 0.21 μmol/min/mg, which is consistent with a previous measurement on an auristatin-based drug-linker.[4] The products were **3** and the residual linker components, specifically the diastereomeric cysteine adduct **10**. The absence of any detectible intermediate carbamate **11** demonstrates that self-immolation of the DMED intermediate **11** to release **3** was rapid.

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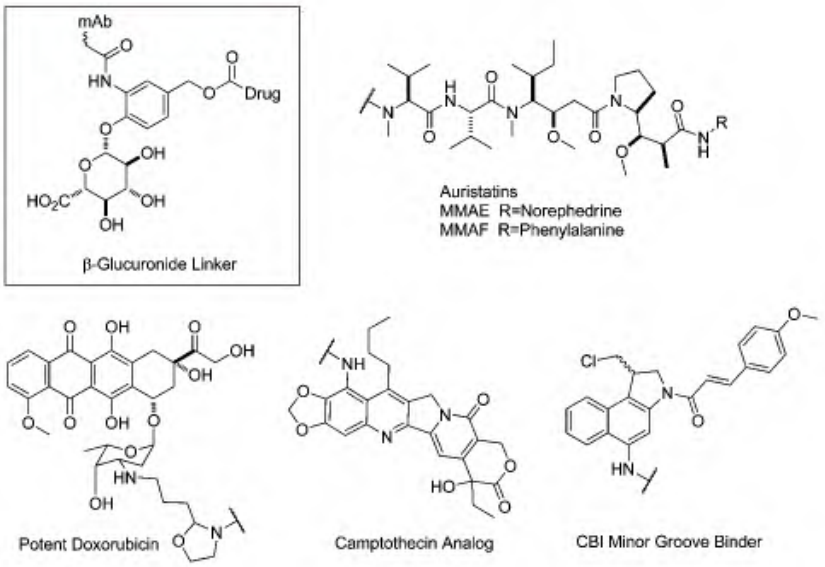


Figure 1. Examples of ADCs with the β-glucuronide linker.

Scheme 1. Release Mechanism from β-Glucuronide Linker

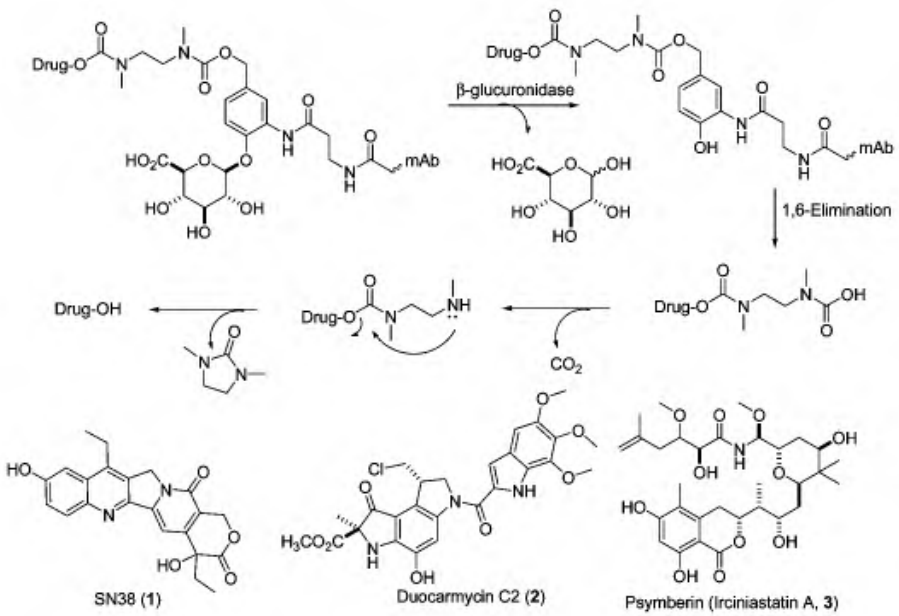


Table 1. IC_{50} Values of Psymberin (3) and β-Glucuronide Drug-Linker **8** Conjugates[a]

	Caki-1 (CD70+/CD30−)	L540cy (CD70−/CD30+)
psymberin (3)	0.3	0.7
cAC10−**8** (5.4 dr/mAb)	62	0.15
h1F6−**8** (5.4 dr/mAb)	0.8	58.6

[a] Values are listed in nM.

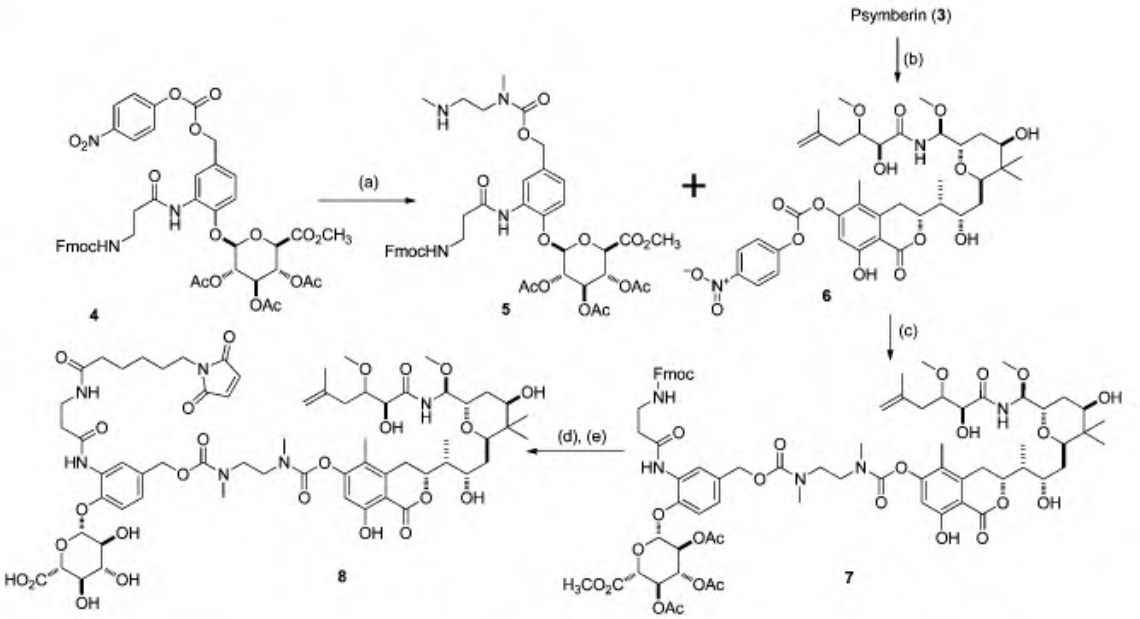
Drug-linker **8** was conjugated to the monoclonal antibodies cAC10 (anti-CD30 mAb) and h1F6 (anti-CD70 mAb) via reduced interchain disulfides. An average loading of 5.4 drugs/mAb was achieved, and the conjugates were >95% monomeric. Exposure to the CD30-expressing cell line L540cy (Hodgkin's lymphoma) to cAC10−**8** over a 96 h period gave an IC_{50} value of 0.15 nM (Table 1). The cell killing was immunologically selective as exposure to the CD30-negative cell line Caki-1 (renal cell carcinoma) gave an IC_{50}

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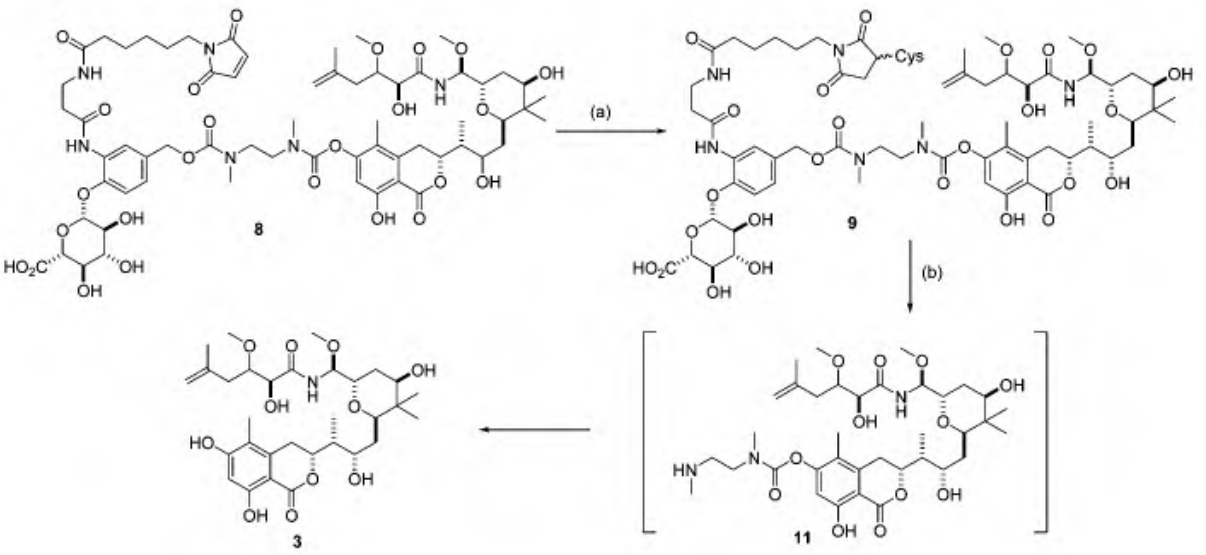
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Scheme 2[a]



[a] Reagents and conditions: (a) DMED (10 equiv), CH_2Cl_2, 61%. (b) pNP carbonate, DIPEA, THF, 49%. (c) Compound **5** and **6**, CH_2Cl_2, 42%. (d) LiOH (9 equiv), CH_3OH, THF, H_2O. (e) MC−OSu, DMF, DIPEA, 62% two steps.

Scheme 3[a]



[a] Reagents and conditions: (a) Cysteine. (b) β-Glucuronidase (*E. coli*), 30 min.

value of 62 nM. Conversely, the h1F6−**8** ADC showed good activity on the CD70-expressing line Caki-1 but significantly reduced activity against the CD30-negative line L540cy. Unconjugated **3** was highly potent on these cell lines. The selectivity observed over the 96 h exposure assay suggests a high degree of stability, which is consistent with previous findings with the β-glucuronide linker.[7]

ADCs continue to demonstrate their value in the clinic,[3,13−16] and as a consequence, investigations into new chemotypes and linkers for ADCs will continue. The favorable properties

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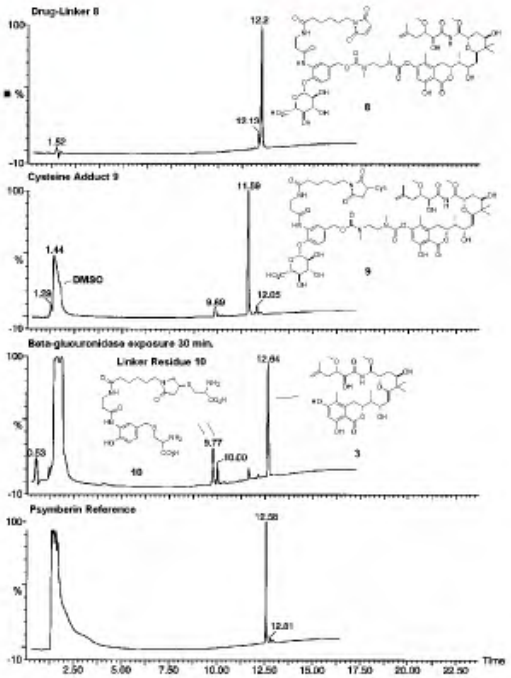


Figure 2. HPLC analysis of β-glucuronidase cleavage of psymberin drug linker **9**. Peaks were observed using a diode array detector.

of the β-glucuronide linker, including its hydrophilicity, stability in circulation, tumor selectivity, and efficient drug release inside tumor cells elevate this linker as an appealing component for new ADC development and chemotype screening. The expansion of its utility for the release of a phenol-containing cytotoxic such as psymberin (**3**) has been demonstrated in this communication and will be an avenue of continued investigation in our laboratory.

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Michael addition reactions in macromolecular design for emerging technologies

Brian D. Mather [a], Kalpana Viswanathan [a], Kevin M. Miller [b], Timothy E. Long [a]

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Abstract

The Michael addition reaction is a versatile synthetic methodology for the efficient coupling of electron poor olefins with a vast array of nucleophiles. This review outlines the role of the Michael addition reaction in polymer synthesis with attention to applications in emerging technologies including biomedical, pharmaceutical, optoelectronic, composites, adhesives, and coatings. Polymer architectures, which broadly range from linear thermoplastics to hyperbranched polymers and networks are achievable. The versatility of the Michael reaction in terms of monomer selection, solvent environment, and reaction temperature permits the synthesis of sophisticated macromolecular structures under conditions where other reaction processes will not operate. The utility of the Michael addition in many biological applications such as gene delivery, polymer drug conjugates, and tissue scaffolds is discussed in relation to macromolecular structure.

Previous Next

Keywords

Michael addition; Conjugate addition; Gene transfection; Drug delivery; Tissue scaffolds; Acetoacetates; Acrylates; Bioconjugates; Cysteine

1. Motivation and scientific rationale

The Michael addition reaction, which is also commonly termed conjugate addition, has recently gained increased attention as a polymer synthesis strategy for tailored macromolecular architectures. The Michael

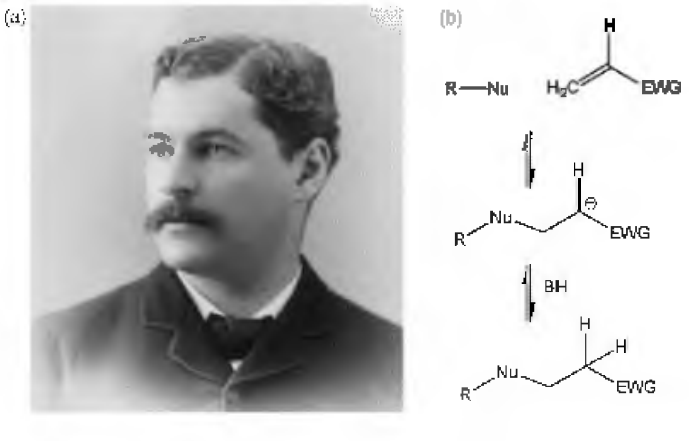
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addition, named for Arthur Michael, is a facile reaction between nucleophiles and activated olefins and alkynes in which the nucleophile adds across a carbon–carbon multiple bond [1]. The Michael addition benefits from mild reaction conditions, high functional group tolerance, a large host of polymerizable monomers and functional precursors as well as high conversions and favorable reaction rates [2]. The Michael reaction lends itself to both step growth [3] and chain growth polymerization [4] and has been employed in the synthesis of linear, graft, hyperbranched, dendritic and network polymers. Furthermore, post-polymerization modification [5] and coupling of biological and synthetic polymers are often facilitated by the Michael reaction [6]. These features make the Michael addition reaction well-suited to numerous emerging technologies including biomedical applications such as gene transfection [7], cell scaffolds [8] and tissue replacements [2].

The Michael addition reaction enables a wide range of polymers from diverse monomers, and corresponding polymers are prepared in environments in which other polymerization mechanisms will not operate. In biological applications such as protein derivitization the mild Michael addition reaction conditions are favorable since high temperatures, oxidizing radicals, and organic solvents are not feasible [9]. Furthermore, the Michael addition has recently found utility for the synthesis of crosslinked polymers such as hydrogels [10], thermoset resins [11], and coatings, where rapid cure and high conversions are necessary for performance. Few polymerizations offer sufficient rates to permit room temperature cure, and industrial coatings are often limited to toxic and environmentally hazardous isocyanate containing monomers. The Michael addition proceeds rapidly at room temperature, offers low cure times, and involves less toxic precursors. Non-linear optical materials were also realized using the Michael addition, which benefits from the absence volatile byproducts [12]. The Michael addition is also ubiquitous in classical polymer chemistry, such as the anionic polymerization of alkyl methacrylates and cyanoacrylates.

The Michael addition involves the addition of a nucleophile, also called a ‘Michael donor,’ to an activated electrophilic olefin, the ‘Michael acceptor’, resulting in a ‘Michael adduct’, as shown in Fig. 1b Although, the Michael addition is generally considered the addition of enolate nucleophiles to activated olefins, a wide range of functional groups possess sufficient nucleophilicity to perform as Michael donors. Reactions involving non-enolate nucleophiles such as amines, thiols, and phosphines are typically referred to as ‘Michael-type additions’. In this review, we will refer to Michael-type addition reactions with all nucleophilic donors as Michael additions. The Michael acceptor possesses an electron withdrawing and resonance stabilizing activating group, which stabilizes the anionic intermediate. Michael addition acceptors are far more numerous and varied than donors, due to the plethora of electron withdrawing activating groups that enable the Michael addition to olefins and alkynes. Acrylate esters, acrylonitrile, acrylamides, maleimides, alkyl methacrylates, cyanoacrylates and vinyl sulfones serve as Michael acceptors and are commercially available. Less common, but equally important, vinyl ketones, nitro ethylenes, α,β-unsaturated aldehydes, vinyl phosphonates, acrylonitrile, vinyl pyridines, azo compounds and even β-keto acetylenes and acetylene esters also serve as Michael acceptors [13].

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Fig. 1. (a) Arthur Michael (1855–1942), who discovered the Michael addition reaction. (b) Schematic depiction of the Michael addition reaction.

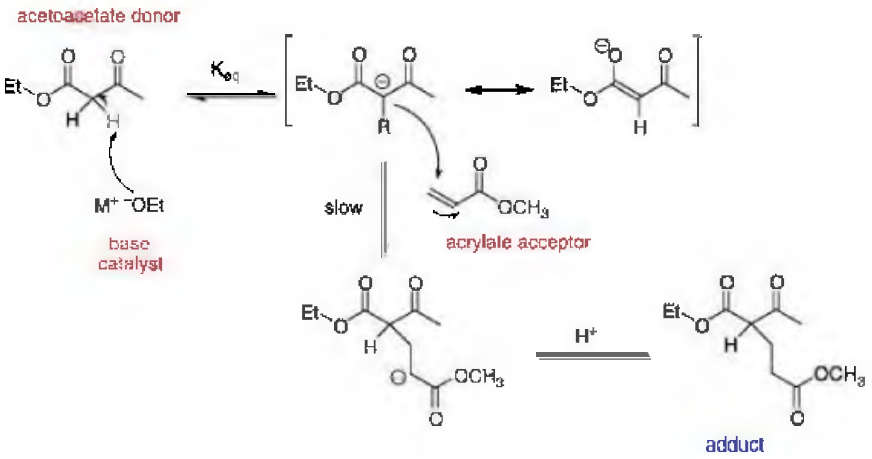
2. Introduction to the Michael addition reaction

The Michael reaction typically refers to the base-catalyzed addition of a nucleophile such as an enolate anion (Michael donor) to an activated α,β-unsaturated carbonyl-containing compound (Michael acceptor) as in Fig. 1b [14], [15], [16], [17], [18]. However, over the years, the scope of this reaction has increased dramatically to include a broad range of acceptors and the Michael-type additions of non-carbon donors. Due to the many types of Michael additions in the literature, we will focus here on investigating the mechanism and kinetics of only a few examples, namely the carbon–carbon bond forming Michael addition (referred to as the carbon-Michael addition), nitrogen (amine or aza) Michael additions, and the reaction of thiols with Michael acceptors.

2.1. The mechanism of the carbon Michael addition

One of the most well-known carbon-Michael transformations is the base-catalyzed addition of ethyl acetoacetate to methyl acrylate [19]. The mechanism of the reaction is fairly straightforward, with every step being in equilibrium and thermodynamically dependent on the relative strengths of the base and the type of acetoacetate. The acetoacetate is first deprotonated by the base, providing an enolate anion (Michael donor) in equilibrium (Fig. 2) The enolate anion then reacts in a 1,4-conjugate addition to the olefin of the acrylate (Michael acceptor). The carbonyl of the acrylate stabilizes the resulting anion until proton transfer occurs, regenerating the base. The overall driving force for the conjugate addition is the enthalpic change that accompanies replacement of a π-bond with a σ-bond. Thus, there is the preference for 1,4-addition over 1,2-addition. In some cases however, kinetically controlled reaction conditions can afford attack at the carbonyl carbon rather than at the β-carbon of the olefin [15], [20].

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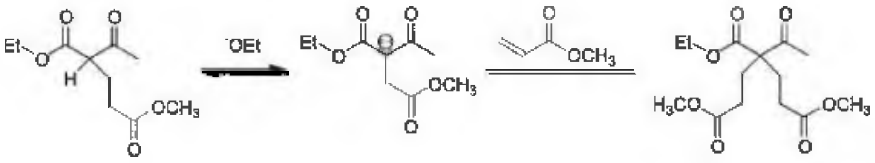


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Fig. 2. General carbon-Michael reaction mechanistic scheme.

From Fig. 2, one can observe that the rate determining step is the attack of the enolate anion on the activated olefin. The reaction rate is therefore second order overall and first order with respect to the enolate anion and the olefin acceptor. The concentration of the enolate is a function of the base strength and the K_{eq} of the deprotonation of the active methylene proton. It follows that the equilibrium constant is dependent on the relative strength of the base and the structure of the acetoacetate.

It is important to note that the product of the first Michael addition has a remaining active methylene hydrogen which can undergo a second addition to another acrylate (Fig. 3) Clemens et al. have documented that the second pK_a is expected to have a value of 13 (versus the pK_a of the initial active proton of 12) [21]. The second deprotonation therefore has a different equilibrium constant (K_{eq}) and it is assumed that the concentration of the first Michael adduct will be low. As a result, the concentration of the enolate at any rate (especially in the early stages of the reaction) is not strongly affected by this second reaction.

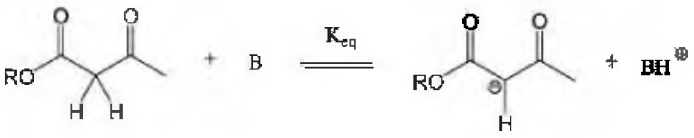


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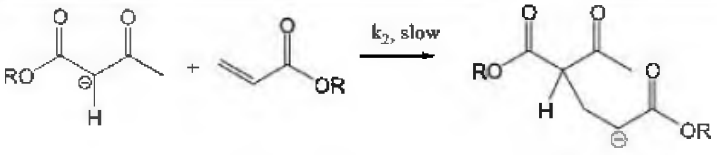
Fig. 3. Second Michael addition of acetoacetate group to methyl acrylate.

It follows that a rate law can be determined for the above reaction sequence. Writing the reactions in terms of the Michael addition, we arrive at the kinetic equations:

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And corresponding rate equations:

$$K_{eq} = [AcAc^-][BH^+]/[AcAc][B] \quad (3)$$

$$\text{Rate} = k_2[AcAc^-][\text{Acrylate}] = k_2 k_{eq}([B]/[BH^+])[AcAc][\text{Acrylate}] \quad (4)$$

2.1.1. Effect of base strength

The choice of base catalyst has a tremendous effect on the reaction kinetics. As previously noted, the concentration of the enolate and therefore the value of K_{eq} is highly dependent on the relative base strength. For strong bases, K_{eq} will lie far to the right and the concentration of the enolate anion is then approximately equal to the concentration of the base (achieving a steady state concentration). In the resulting rate expression Eq. (5), there is a pseudo first-order dependence on acrylate concentration, where k_{obs} is a function of base concentration.

$$\text{Rate} = k_{obs}[\text{Acrylate}] \quad (5)$$

In the case of weaker base catalysts, K_{eq} has a moderate value, but is not large. This introduces the equilibrium constant into the rate law and results in second order behavior Eq. (4). Clemens et al. completed an extensive study on the effect of base strength on reaction kinetics and molecular weight when the Michael addition is used to prepare crosslinked acetoacetate resins for thermoset coatings [21]. In a model study, Clemens analyzed the ability of bases such as TMG (tetramethylguanidine), triethylamine, DBU (1,8-diazabicyclo[5.4.0]undec-7-ene), and DBN (1,5-diazabicyclo[4.3.0]non-5-ene) and found that the rate of the reaction was dependent on catalyst concentration as indicated in Eq. (4). For strong bases such as hydroxide, a steady-state concentration of enolate anion is achieved and pseudo-first order kinetics were observed as in Eq. (5).

The pK_a of the Michael donor is important to the choice of base catalyst, which must have a pK_a for its conjugate acid in the same range as the Michael donor. Stronger bases such as tetramethylguanidine, with a pK_a of 13.6in its protonated form, are needed in the case of the carbon Michael addition of enolates due to the high pK_a of the acetoacetate groups (pK_{a1}=12, pK_{a2}=13.6). Base catalysts are often unnecessary in the case

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of amines, because of the strong nucleophilicity of the nitrogen atom, whereas weak bases aid in deprotonation of thiols.

2.1.2. Effect of solvent

Typical solvents for the carbon-Michael reaction include methanol, ethanol, diethyl ether, tetrahydrofuran, benzene, xylene, dioxane and mixtures of these solvents. Initially, protic solvents were desirable in the carbon-Michael reaction to promote rapid proton transfer and to stabilize charged intermediates, however, Schlessinger's group has shown that high yielding reactions were also achieved using aprotic solvents [22], [23], [24]. The choice of solvent strongly depends on the solubility of the catalyst, donor, and acceptor as well as sensitivity to side reactions. For example, if the reactants or products are susceptible to alcoholysis (ester exchange or hydrolysis), self-condensation of the substrate or the 'retro-Michael' reaction, a non-hydroxylic solvent is desirable [14], [25]. In some cases, reactions are carried out in the absence of solvent, especially for network or coating applications.

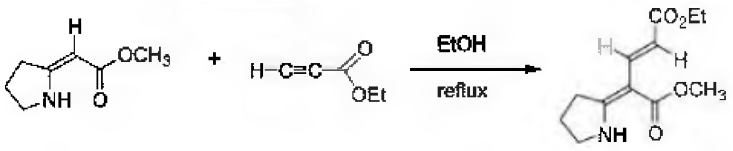
2.1.3. Effect of substrate

As mentioned before, the initial step of the carbon-Michael addition is deprotonation of an active methylene proton. Thus, donors must stabilize the resulting negative charge. Examples of functional groups, which stabilize enolate donors include ketones, aldehydes, nitriles, amides, nitrones, sulfones, malonates and acetoacetates. A great deal of thought must be directed to the selection of the proper donor and base, and knowledge of the pK_a of these various species is critical. Furthermore, strong bases such as hydroxide and methoxide may promote side reactions such as ester hydrolysis, so non-nucleophilic bases are often desirable.

Acceptors in the carbon-Michael addition are typically olefins that are activated for nucleophilic attack. The resulting anion from nucleophilic attack of the donor must also be stabilized for the reaction to occur. Thus, the use of α,β-unsaturated carbonyl compounds is prevalent in Michael addition chemistry. The reactivity of the acceptor will decrease if the substituent is electron rich, such as in the case of alkyl, aryl or carboethoxy substituted olefins such as styrene derivatives or vinyl ethers. Sterics are also an important factor. The larger the groups substituted at the α and β positions, the slower the reaction rate, although this can be offset by stronger polar effects [18], [26]. Differences in reactivity between acceptors require differences in nucleophilicity of the Michael donors. For instance, alkyl methacrylates are relatively poor Michael acceptors. Thus, stronger nucleophiles such as carbanions are necessary for successful Michael addition to alkyl methacrylates. Better acceptors such as alkyl acrylate or acrylamide groups readily accept weaker amine and thiol nucleophiles.

Alkynes have also been utilized as Michael acceptors. In Fig. 4, electron-rich enamines react readily with activated alkynes in refluxing ethanol [27]. Products of Michael reactions with alkynes have the additional variables of stereochemistry of the unsaturated product, and regiochemistry of addition for disubstituted alkynes acceptors.

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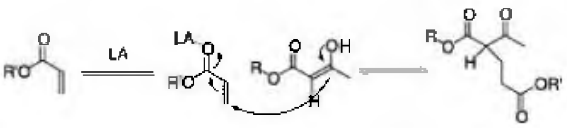


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Fig. 4. Michael Addition involving an alkyne acceptor.

2.1.4. Other carbon-Michael catalysts

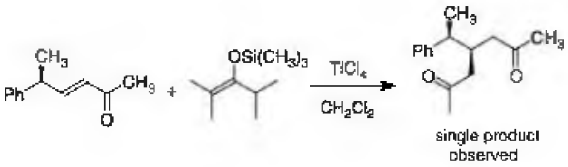
Although base-catalysis is most prominently used in the carbon-Michael addition, the reaction is also catalyzed with acids, particularly in the case of Lewis acids. Some of the earlier examples include the use of boron trifluoride, aluminum trichloride, and zinc chloride [28]. In these cases, the Lewis acid coordinates to the carbonyl of the acrylate to activate the olefin (Fig. 5) The coordinated complex will then react with the nucleophile to obtain the same adduct as in the base-catalyzed Michael addition. In the case where silyl enolates are donors, the reaction is often referred to as the Mukaiyama–Michael reaction.



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Fig. 5. Lewis-acid catalyzed Michael addition reaction.

Researchers have used Lewis acid complexes to their advantage over the years, especially in the area of enantioselective additions. For example, Heathcock's group has shown that silyl enolates will react enantioselectively with α,β-unsaturated ketones in the presence of $TiCl_4$ (Fig. 6) [29]. In the area of enantioselective carbon-Michael additions, the reader is directed to two recent reviews published on the subject [30], [31].

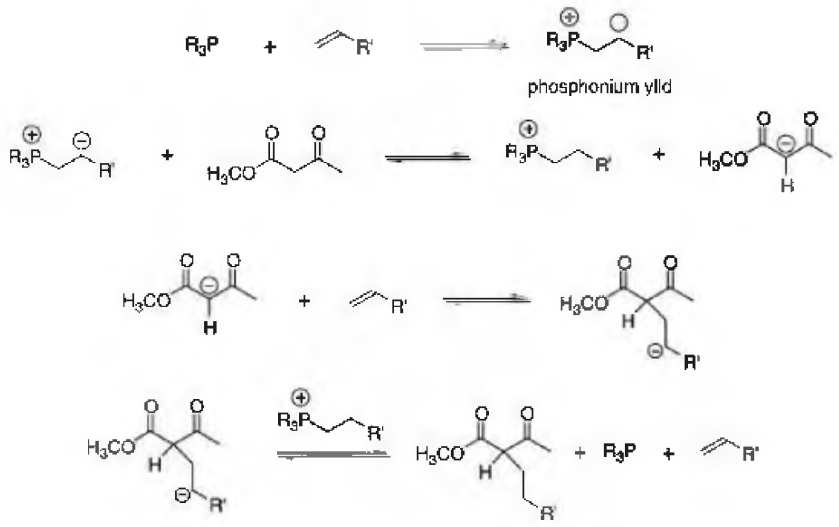


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Fig. 6. Stereoselective Michael addition between enone and silyl enolate in the presence of $TiCl_4$.

Phosphines also catalyze the carbon-Michael reaction [13]. The reaction sequence begins with nucleophilic attack of the phosphine on the β-position of the olefin, generating a reactive phosphonium ylid (Fig. 7) The resulting anion can then either react as a nucleophile or as a base. If an acetoacetate is present in the reaction, the ylid deprotonates the acetoacetate first, which adds to the β-position of another olefin in a Michael fashion. Proton transfer then results in regeneration of the initial olefin and the phosphine catalyst.

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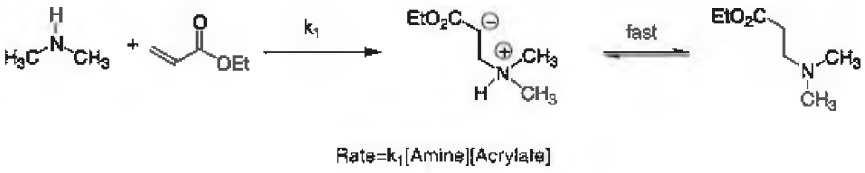
Fig. 7. Phosphine catalyzed Michael addition reaction.

2.2. Heteroatomic donors in the Michael reaction

Carbon-based nucleophiles represent only one class of donors used in Michael addition reactions. Heteroatomic nucleophiles involving nitrogen, sulfur, oxygen and phosphorus have also been used. In this section, two of these additions will be discussed: the aza-Michael reaction (amines) and the thiol Michael addition reaction. These are of primary importance due to the presence of sulfur and nitrogen nucleophiles in biological systems.

2.2.1. The aza-Michael reaction

The nitrogen-donor version of the Michael addition is often referred to as the aza-Michael reaction. Since only a few concepts will be touched upon here, the reader is encouraged to investigate several reviews for additional information [16], [32], [33], [34]. Since amines can act as both nucleophiles and bases, no additional base is typically needed in these reactions. The reaction tends to follow second-order kinetics based on the concentration of the olefin acceptor and the amine (Fig. 8)



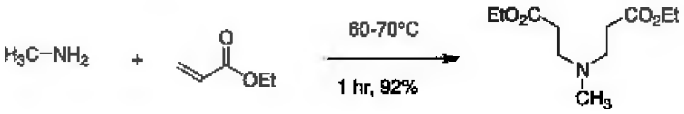
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Fig. 8. Aza-Michael addition reaction of dimethylamine with ethyl acrylate.

Primary amines can react with two equivalents of acceptor to form tertiary amines. In some cases, this second addition affects the observed kinetics, especially as the concentration of the secondary amine

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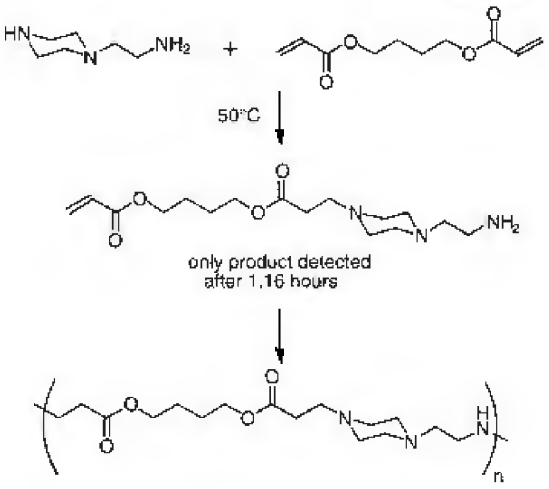
increases. An example is the reaction of methyl amine with ethyl acrylate, giving an excellent yield of the tertiary amine (Fig. 9) [35].



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Fig. 9. Aza-Michael addition of methyl amine to two equivalents of ethyl acrylate.

In the aza-Michael reaction, secondary amines are more nucleophilic than primary amines and are therefore more reactive. However, it is worth noting that this is highly dependent on the electronic and steric environment of the amine. For example, 1,4-butanediol diacrylate was allowed to react with 1-(2-aminoethyl)piperazine in an equimolar ratio (Fig. 10) [36]. During the initial part of the experiment, it was found that there was exclusive reaction with the secondary amine present in the piperazine ring. Only upon longer reaction times, did reaction occur with the primary amine, which led to polymerization.



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Fig. 10. Higher reactivity of secondary amines in aza-Michael addition reactions.

Acid catalyzed aza-Michael additions have also been studied extensively. Spencer and coworkers have determined a general and efficient way of reacting carbamates (NHCOOR) with α,β-unsaturated carbonyl compounds (Fig. 11), providing precursors to β-amino acids [32].



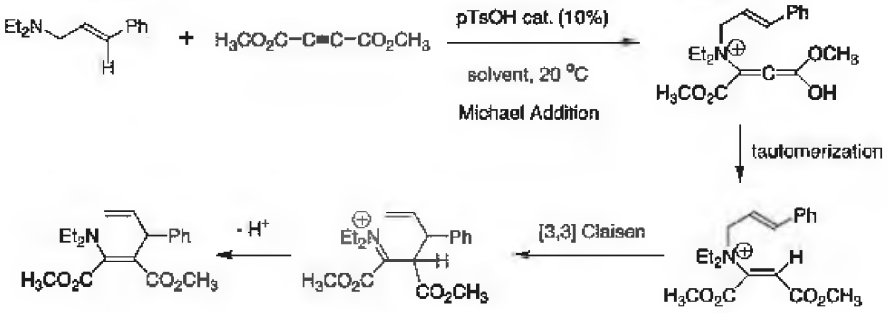
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Fig. 11. Acid catalyzed aza-Michael addition.

Vedejs and Gringas have also shown that acids catalyze the aza-Michael addition [37]. In Fig. 12, a tertiary amine, as the donor, adds to the alkyne in the presence of catalytic *p*-toluenesulfonic acid. The resulting

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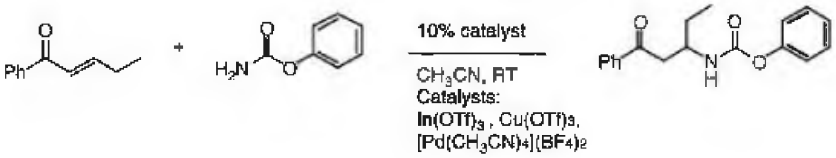
intermediate further undergoes a Claisen rearrangement and proton transfer to afford the final α,β-unsaturated amine.



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Fig. 12. Acid catalyzed aza-Michael addition to activated alkyne acceptors.

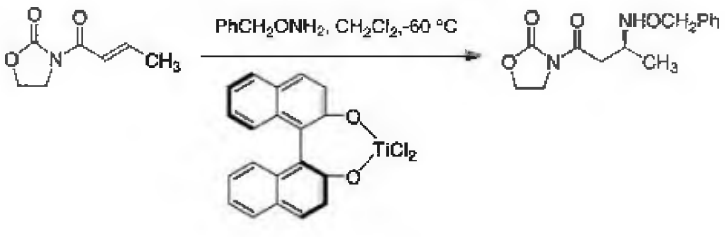
Lewis acids have also been used to catalyze the aza-Michael reaction, as in Fig. 13. The mechanism is thought to occur in an analogous fashion to the carbon-Michael addition where the Lewis acid coordinates to the carbonyl of the α,β-unsaturated olefin. However, Spencer has reported that, in the case of weakly basic amine nucleophiles, Brønstead acids that are formed during the reaction may catalyze the reaction [32].



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Fig. 13. Lewis acid catalyzed aza-Michael reaction.

The ability to generate β-amino carbonyl compounds has become increasingly important to the natural product and pharmaceutical areas. The aza-Michael reaction is a key transformation which enables the preparation of such compounds. Jørgensen was the first to report the enantioselective conjugate amine additions (Fig. 14) [38]. The $TiCl_2$-BINOL Lewis acid catalyst coordinates with the *N*-acyloxazolidinone, creating a chiral environment in which the primary amine will preferentially attack from one face of the complex, resulting in the formation of one major diastereomer. The oxazolidinone ring is then removed to afford the β-amino acid. Although, the enantioselectivity is low (maximum enantiomeric excess of 42%), the proof of concept was accomplished. The area has grown significantly and Sibi has published two reviews which discuss stereoselective aza-Michael additions to generate β-amino acids [30], [39].



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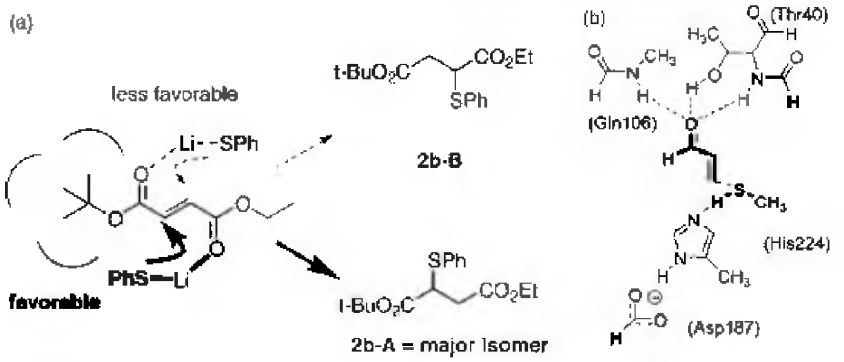
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Fig. 14. Stereoselective aza-Michael additions.

2.2.2. Thiols as Michael donors

Thiols can also be used as heteroatomic donors in the Michael addition. Many of the same reaction scenarios that were described above can be directly applied to the addition of thiols to activated olefins. Thiols are generally more nucleophilic than amines, although bases are often used to deprotonate them due to their comparatively higher acidity. The thiolate anion is often the active species in Michael additions involving thiols. The thiol Michael addition reaction rates increase with pH due to the increased concentration of the thiolate anion [10]. The thiol group is a useful Michael donor in biological systems where thiols are present on proteins in cysteine residues. One primary side reaction in thiol Michael additions is disulfide bond formation, which often prompts the use of protecting group chemistry.

Regioselective Michael additions of thiols to asymmetrically substituted fumarate esters or amides was achieved using lithium salts as Lewis acids or base catalysts to alter the charge environment around the double bond through coordination of carbonyls or deprotonation of the thiol (Fig. 15a) [40]. Enantioselective Michael additions of thiols to methacrylates and other 1-alkyl acrylates was accomplished with chiral lanthanide catalysts to enantiomeric excesses as high as 90% ee [41]. Enzyme catalysis of the thiol addition to 2-butenal was shown with *Candida antarctica* lipase B [42]. The active site of the enzyme in this reaction is described in Fig. 15b Suprisingly, aqueous sodium dodecyl sulfate solutions perform as powerful catalysts of the Michael additions of thiols or amines to enones [43]. Firouzabadi et al. proposed that the accelerating effect of the sodium dodecyl sulfate related to the concentrating of the hydrophobic species into the micellar core.



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Fig. 15. (a) Regioselective Michael addition through preferential coordination of substrate carbonyls with lithium cations, reprinted from Tetrahedron, 59, Kamimura et al. 'On the regioselectivity for the Michael addition of thiols to unsymmetrical fumaric derivatives,' 9537–9546, Copyright 2003, with permission from Elsevier [40] (b). Michael addition in the active site of wild *Candida antarctica* lipase B, reproduced from 'Exploring the active-site of a rationally redesigned lipase for catalysis of Michael-type additions,' Carlqvist et al. Copyright 2006 Society of Chemical Industry with permission from John Wiley and Sons, Ltd on behalf of the SCI [42].

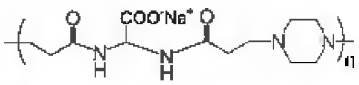
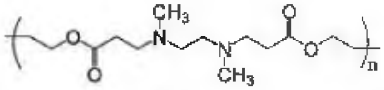
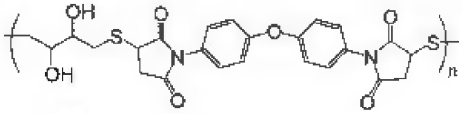
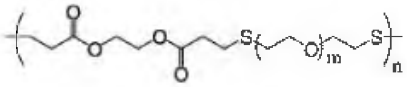
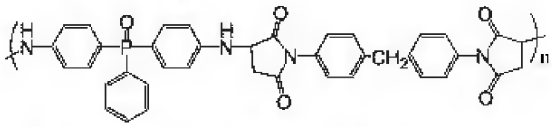
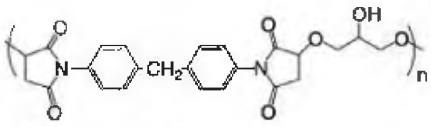
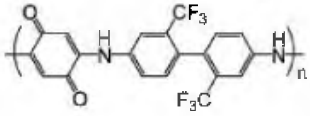
3. Linear step growth Michael addition polymerizations

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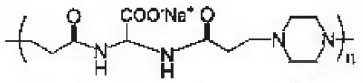
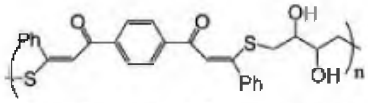
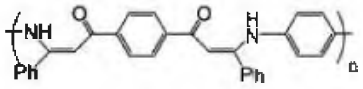
3.1. Overview

Significant earlier attention was devoted to linear step growth Michael addition polymerizations. Polymers that were produced via Michael addition polymerizations have ranged from segmented elastomers to high T_g engineering thermoplastics. Both rigid, amorphous, aromatic systems as well as semicrystalline aliphatic systems were pursued and a wide variety of monomers were employed. Numerous examples of step growth Michael addition polymerizations exist, however, a few main classifications exist, outlined in Table 1. Perhaps the earliest example is poly(amido amine)s, which were synthesized from bisacrylamides and diamines. A second class of step growth polymers are derived from bismaleimides and diamines, a type of poly(imido amine) referred to as poly(aspartamide)s in the literature. Thiol nucleophiles are involved in the synthesis of poly(imido sulfide)s, which are also based on bismaleimide monomers. Analogous poly(ester sulfide)s and poly(amino ester)s synthesized from diacrylates are potentially biodegradable due to the hydrolytic instability of the ester linkages. Poly(amino quinone)s are a unique class of polymers which possess redox activity [44] and serve as anticorrosion coatings on metals. α,β-unsaturated alkyne esters and ketones also serve as Michael acceptors in some cases and step growth polymerizations based on amine and thiol nucleophiles were pursued. Finally, hydrogen transfer polymerization allows the synthesis of polyamides and polyesters from AB-type Michael addition polymerizations. The high conversion of the Michael addition reaction and lack of side reactions are prerequisites for high molecular weight in step growth polymerizations, which is clearly obtained in numerous subsequent examples.

Table 1. Step growth polymers derived from Michael addition polymerisation

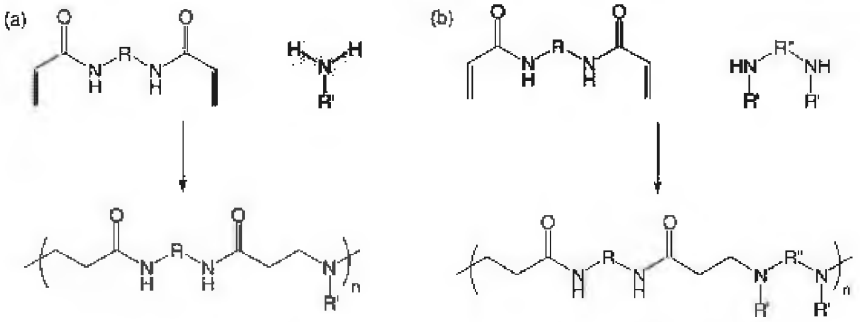
		References
Poly(amido amine)		**[53]**
Poly(amino ester)		[61]
Poly(imido sulfide)		[68]
Poly(ester sulfide)		[69]
Poly(aspartamide)		[70]
Poly(imido ether)		[76]
Poly(amino quinone)		[3]

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		References
Poly(amido amine)		[53]
Poly(enone sulfide)		[68]
Poly(enamine ketone)		[81]

3.2. Poly(amido amine)s

Poly(amido amines) are synthesized through the reaction of secondary diamines with bisacrylamides at 15–60 °C in water for durations of hours to days. Also, primary amines, which have difunctionality in the Michael addition are employed in poly(amido amine) synthesis (Fig. 16) Ferruti et al. synthesized linear poly(amido amine)s from a range of monomers including piperazine bisacrylamide and *N*,*N*′-dimethyl-1,6-hexanediamine [45], [46]. The poly(amido amine) polymerizations occurred rapidly at room temperature in protic media such as alcohols and water without catalyst. The rate of the Michael addition polymerization increased with protic solvents and was less dependent on solvent polarity or dielectric constant. Generally, these polymerizations do not proceed to high molecular weight in aprotic solvents. The rate of these polymerizations increased with the basicity of the secondary diamines, and with lower steric hindrance [47]. For instance, polymerization rates increased in the order: *N*,*N*′-diisopropylethylenediamine<*N*,*N*′-dimethylethylenediamine<2-methylpiperazine<piperazine.



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Fig. 16. Poly(amido amine) synthesis via (a) primary and (b) secondary amines.

Numerous variations of poly(amido amine) polymerizations exist. Amino acids may be copolymerized with bisacrylamides, as long as triethylamine is added to deprotonate the amino acid zwitterion and an amino acid with low steric bulk is employed. Successful polymerization occurred with glycine, taurine and β-alanine. Peptide oligomers may also be used as comonomers as long as the amine terminus is a simple amino acid such as glycine. Monomers such as divinylsulfones and diacrylates [48] can replace bisacrylamides as Michael acceptors. Segmented poly(amido amine ether)s were derived from PEG bis(piperazine) monomers [49]. Other routes to poly(amido amine)s yield different ordering of amido and amino functionalities including homopolymerization of protected aminoacrylamides with in situ

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deprotection. Yet another route is the reaction of activated acrylic acid derivatives with secondary diamines through amidation and the also Michael addition [50]. Hydrazine and alkylated hydrazines were also employed in the synthesis of poly(amido amine)s, however, if excess bisacrylamide monomer is used, then crosslinking occurs with residual NH groups along the polymer backbone at long reaction times [47], [51]. Thermal stability of aliphatic poly(amido amine)s is fairly low, and thermal decomposition occurs near 200°C. Poly(amido phosphine)s are synthesized in a similar manner to poly(amido amine)s, with the exception that free radical inhibitors are employed to prevent radical catalyzed addition of the phosphines to the bisacrylamides. Poly(amido phosphine)s exhibit greater thermal stability than poly(amido amines), with decomposition temperatures near 360°C.

Aliphatic poly(amido amines) often possess crystallinity and are water soluble due to the presence of tertiary amines in the backbone, which undergo partial protonation in water. Poly(amido amine)s exhibit a tendency toward amide hydrolysis. Ferruti et al. demonstrated increased solution viscosity versus time during polymerizations at high temperatures (100°C) in water, with a subsequent decrease in viscosity at later times. This attribute along with low toxicity of the amino acid degradation products enables poly(amido amine)s as degradable pro-drug conjugates [52]. Poly(amido amine)s based on hydrazine were found to decrease in molecular weight by a factor of four over 2d in water at physiological conditions, and were also found to possess low cytotoxicity [51].

Linear and crosslinked poly(amido amine)s were found to complex with heparin, an anticoagulant in the human body [45]. In medical practice, when heparin levels are too high in the body, protamine sulfate is administered, which is also an anticoagulant. A solution to this problem is the use of linear or crosslinked poly(amido amine)s to remove heparin from the blood stream. Poly(amido amine)s selectively remove heparin over other plasma components and do not cause hemolysis or affect other properties of blood such as prothrombin time. The poly(amido amine)s were also recycled through removal of heparin upon treatment with alkaline solutions of pH 11. Heparin, when coated on non-physiological objects, reduces the thrombus formation response of the human body. Thus, poly(amido amine)s also find utility in creating non-thrombogenic surfaces through covalent attachment to surfaces. Ferruti et al. developed synthetic methods for surface attachment of poly(amido amine)s to Dacron™ and poly(vinyl chloride) (PVC) using Michael addition of terminal acrylamide groups with surface tethered amine groups [46]. These surfaces readily adsorbed heparin without heparin release.

Ferruti et al. reacted ethylene sulfide with piperazine to yield dithiol monomers, which were polymerized with bisacrylamides. The resultant polymers were quaternized with methyl iodide, yielding water soluble antimicrobial polyelectrolytes [52]. These polymers exhibited antimicrobial activity even against resistant strains such as *Pseudomonas aeruginosa*. The polymers also possessed surprisingly low rates of hemolysis relative to commercial antimicrobial polymers.

Ferruti et al. investigated further biomedical applications of poly(amido amine)s in creating platinum (II) complexes with antitumor activity [53]. Polymer–drug conjugates have improved the effectiveness of drugs through a combination of increased solubility and slow drug release. The net result is called the enhanced permeability and retention (EPR) effect. Linear poly(amido amine)s based on 2-methylpiperazine, carboxylated bisacrylamides, and amino-β-cyclodextrin moieties were complexed directly with cisplatin. The degree of cisplatin complexation and release rate of platinum increased for polymers containing the cyclodextrin moiety. Release was suppressed however, with the carboxylated bisacrylamide, and non-

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carboxylated bisacrylamides were also studied. Both in vivo and in vitro toxicity of most complexes were lower than pure cisplatin, and similar antitumor activities were observed. Earlier work on poly(amido amine)s revealed effectiveness in reducing tumor metastasis in Lewis lung tumors and Sarcoma 180 carcinoma implanted in mice [54]. The reduction in cancer metastasis was attributed to specific interactions of these charged polymers with cell membranes. Neuse et al. also studied poly(amido amine) complexes of cisplatin, using 1,2-dihydroxylated, 1,2-dicarboxylated or 1-hydroxy-2-carboxyl functional amine monomers that chelate to the platinum center [55].

Recently, Ferruti et al. devoted considerable effort to studying the delivery of genes and proteins via complexation with poly(amido amine)s. One advantage of poly(amido amine)s is rapid protonation and expansion at low pH inside tumor cells which releases the genes or proteins and a compact geometry at plasma pH 7.4 to effectively hold the desired agent prior to delivery. Small angle neutron scattering (SANS) studies revealed that decreasing the pH- of aqueous poly(amido amine)s solutions increases the radius of gyration from ~2 to 8 nm [56]. Furthermore, poly(amido amine)s possess dramatically lower toxicity than conventional non-viral vectors such as poly(ethylene imine). In some cases, poly(amido amine)s were prepared that exhibited a 'stealth' behavior and maintained long circulatory lifetimes [57]. Duncan et al. recently investigated poly(amido amine)s that contained covalently bound endosomolytic protein melittin (a main component of bee venom) [58]. These conjugates were less toxic than pure melittin and were found to possess pH- selective hemolytic properties at pH 5.5 and not at pH 7.4 at low concentrations of the conjugates. Some of the conjugates were also effective in delivering the non-permeant toxin gelonin into cells. Segmented copolymers based on poly(amido amine)s containing carboxylic acid and hydroxyl functional blocks also showed promising endosomolytic and gelonin delivery activity [59]. Gene transfection studies of poly(amido amine)s have also demonstrated successful polyplex formation with λ Hind III DNA and transfection of β-galactosidase into HepG2 cells, with similar transfection efficiency as Lipofectin® and poly(ethylene imine) [7]. Ferruti et al. coupled bovine serum albumin and human serum albumin to poly(amido amine)s based on piperazine and piperazine bisacrylamide through Michael addition of protein amine groups to terminal acrylamide groups on the poly(amido amine)s [60]. The reaction was conducted under mild conditions (pH 8–8.5, 25 °C) to high conversion and the coupled product was verified by size exclusion chromatography (SEC).

Poly(amino ester)s are similar to poly(amido amine)s, however, they utilize diacrylates instead of bisacrylamides. Langer et al. studied the use of Michael addition step growth linear poly(amino ester)s as gene transfection agents (Fig. 17) [61]. Like poly(amido amine)s, poly(amino ester)s have benefits over conventional gene transfection agents (such as poly(ethylene imine) or poly(L-lysine)) due to their low cytotoxicity as well as biodegradability into low toxicity byproducts. Furthermore, the structure and degradability of the poly(amino ester)s are tailored through the incorporation of a wide range of diamines and diacrylates. Langer et al. obtained high molecular weight poly(amino ester)s (32,000 g/mol) and performed successful complexation with plasmid DNA. The absence of a retro-Michael addition in these systems eliminates the possibility of carcinogenic diacrylate production during degradation. The degradability of these transfection agents is expected to aid in the release of the DNA inside the cell.

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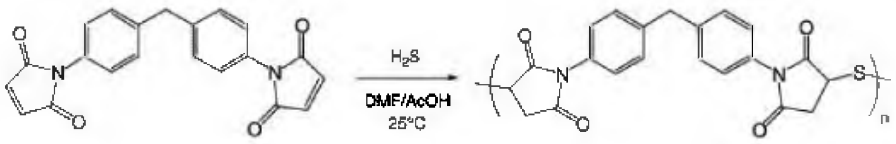
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Fig. 17. Poly(amino ester) synthesis from butanediol diacrylate and piperazine.

Non-biological applications of poly(amido amine)s also exist. Recently, Ferruti et al. examined the introduction of NLO chromophores into both linear and crosslinked poly(amido amine)s [12]. The Michael addition chemistry was well-suited to the primary amine containing chromophores and mild reaction conditions prevented chromophore degradation [62]. Initial studies showed that solution optical properties of the chromophores such as two photon pumped fluorescence were maintained in the polymer.

3.3. Poly(imido sulfide)s

Maleimides possess two carbonyl groups conjugated to the double bond, presenting a highly electron poor double bond susceptible to reaction with a range of nucleophiles. Poly(imido sulfide)s result from the step growth polymerization of dithiols with bismaleimide monomers. White and Scaia polymerized aromatic dithiols with aromatic bismaleimides in *m*-cresol with catalytic amounts of tri-*n*-butylamine at 80–100°C [63]. The acidity of the *m*-cresol prevented the anionic homopolymerization of the maleimide groups upon reaction with thiolate anions, which would have resulted in crosslinking. The poly(imido sulfide)s containing aromatic thiol units were susceptible to degradation from bases such as diethylamine in aprotic solvents which was attributed to retro-Michael addition regeneration of the maleimide group. Aliphatic dithiol-containing polymers were less susceptible to depolymerization. Poly(imido sulfide)s with molecular weights ranging from 70,000 to 450,000g/mol possessed glass transition temperatures from 185 to 212°C. Semicrystalline poly(imido sulfide)s were produced through the reaction of aliphatic dithiols with aliphatic bismaleimides [64]. Melting temperatures of approximately 77°C were observed. Semicrystalline poly(imido sulfide)s possessed stresses at break near 12MPa and elongations of 120%. Thermal decomposition of the poly(imido sulfide)s occurred between 336–380°C, and likely resulted from a retro-Michael addition depolymerization. Hydrogen sulfide serves as an effective dithiol in the synthesis of poly(imido sulfide)s and reacts very rapidly with bismaleimides in the presence of catalytic quantities of amines such as *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) or acids such as acetic acid (Fig. 18) [65]. Poly(imido sulfide)s were also obtained from biscitraconimides (methyl substituted maleimides), which resulted in geminal substitution of the sulfide group on the maleimide ring at the same position as the methyl group [66].



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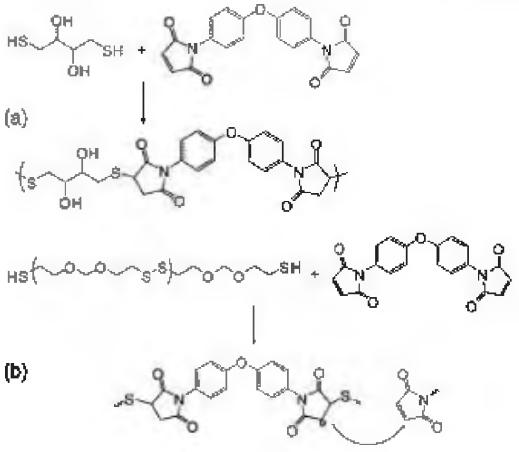
Fig. 18. Poly(imido sulfide) synthesis from a bismaleimide precursor and hydrogen sulfide gas.

Segmented polymers based on poly(imido sulfide) chemistry were developed by Crivello and Juliano [67]. These polymers were synthesized in an analogous fashion to polyurethanes, starting with an oligomeric ‘soft

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block' consisting of a thiol terminated polysulfide (Thiokol™ resin) and reacting with an excess of aromatic bismaleimide monomer in cresol with tri-*n*-butylamine catalyst at room temperature. The reactive oligomer is chain extended through reaction with hydrogen sulfide to produce the poly(imido sulfide) 'hard block'. The poor thermal stability of the polysulfide oligomers limited the processing temperature of the polymers to less than 150°C, but tensile strengths as high as 10MPa and 500% elongation were obtained. The thermal instability limited the choice of bismaleimide monomer to maintain a T_g that was less than 100°C. These segmented poly(imido sulfide)s exhibited two glass transition temperatures in dynamic mechanical analysis, indicating the presence of microphase separation. The rubbery plateau (service window) ranged from −30 to 130°C. Increasing the hard segment content above 30wt% led to higher tensile strengths with lower extensibilities and poor processability. The polysulfide containing segmented elastomers benefited from excellent solvent resistance.

Dix et al. showed that changing between protic and aprotic monomers in aprotic solvents influenced the topology of poly(imido sulfide)s (Fig. 19) [68]. Bismaleimides were reacted with oligomeric dithiols as well as low molar mass dithiols in the presence of catalytic quantities of triethylamine. Oligomeric bisthiols reacted with *N′*,*N′*-bismaleimido-4,4′-diphenylmethane to create crosslinked networks due to the reaction of the carbanion formed upon Michael addition with additional maleimide groups. The use of a protic small molecule dithiol monomer, dithiothreitol, resulted in linear polymers without crosslinking, due to protonation of the carbanions by hydroxyl groups on dithiothreitol. Triethylamine does not initiate maleimide polymerization, but deprotonates thiols, which are capable of polymerizing maleimides. For example, an oligomeric dithiol was reacted with excess monofunctional maleimide in the presence of triethylamine resulting in anionic polymerization of the maleimide from the chain ends of the oligomeric dithiol.



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Fig. 19. (a) A typical poly(imido sulfide)s synthesis. (b) Possible crosslinking side reaction through maleimide homopolymerization observed for aprotic oligomeric dithiols.

The acrylic analog to poly(imido sulfide)s, poly(ester sulfide)s, were synthesized using PEG dithiol and butanediol diacrylate or PEG diacrylate using triethylamine base catalyst [69]. Varying the length of the PEG spacer enabled hydrophilicity control. The reaction kinetics were studied in a number of aprotic solvents and increased in the order benzene<dioxane≪chloroform with increasing dielectric constant. Weight average

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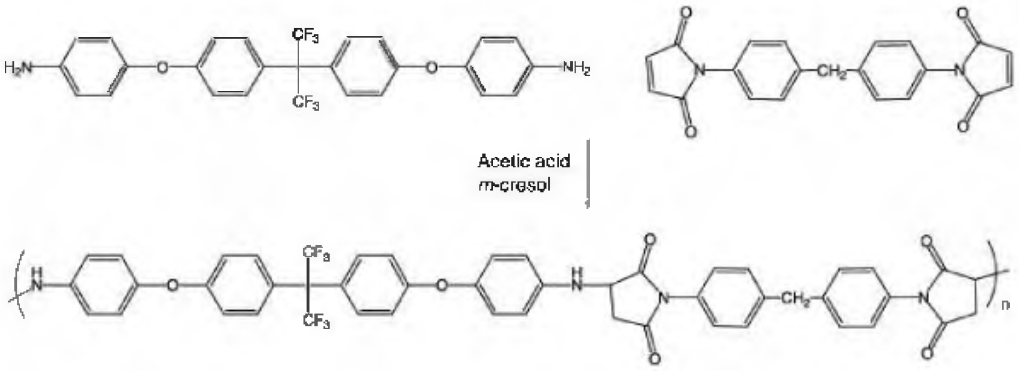
molecular weights ranged from 5000 to 28,000g/mol and glass transition temperatures from −50 to −60°C were observed. Crystallinity was observed in selected poly(ester sulfide)s.

3.4. Poly(aspartamide)s

Poly(aspartamide)s are a class of condensation polymers derived from bismaleimides and diamine monomers. Poly(aspartamide)s exhibit properties similar to poly(imide)s yet benefit from a facile, one-pot synthesis and increased solubility in organic solvents relative to aromatic poly(imide)s and poly(amide)s [70]. Poly(aspartamide) synthesis relies on a facile Michael addition of the amine functional group to the maleimide double bond and does not require a high temperature poly(amic acid) cyclization reaction as for poly(imide)s.

Crivello synthesized poly(aspartamide)s through the reaction of aromatic diamines with aromatic bismaleimides in cresol at 110°C for 3d with catalytic amounts of acetic acid [71]. The lower basicity of the aromatic amines allowed the use of acetic acid, which activated the maleimide group, through protonation [72]. Model reactions suggested that the polymerization mechanism involved charged intermediates due to increased rates in polar solvents. The poly(aspartamide)s exhibited glass transition temperatures near 210°C and thermal decomposition temperatures near 350°C. This decomposition occurred at the same temperature (TGA) in both air and nitrogen atmospheres, suggesting that depolymerization occurred rather than oxidation. The poly(aspartamide)s possessed tensile strengths near 95MPa and elongations of 5.5%. Flexural strengths and moduli as high as 140MPa and 3.1GPa, respectively, were reported.

Liaw studied poly(aspartamide)s based on arylene ether diamines with improved thermal stability and 10% weight loss temperatures near 400°C (Fig. 20) [73]. The molecular weight of the poly(aspartamide)s increased with increasing monomer concentration, from M_n=7900 at 0.38M to M_n=12,800 at 1.0M. Weight average molecular weights ranged from 24,000 to 68,000g/mol.



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Fig. 20. Thermally stable poly(aspartamide)s synthesized from arylene ether diamines.

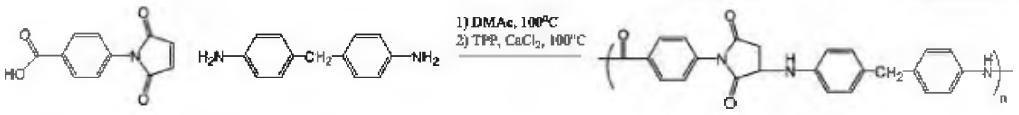
White and Scaia synthesized amorphous elastomeric poly(aspartamide)s via the polymerization of aliphatic secondary diamines and aliphatic bismaleimides [64]. Acetic acid was used in the polymerization of aromatic diamines and bismaleimides but not with aliphatic diamines due to the tendency to protonate the amine groups and prevent polymerization. The poly(aspartamide)s undergo slow crosslinking at ambient conditions likely due to Michael addition reaction of amine and maleimide end groups and subsequent

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homopolymerization of the maleimide group. Amorphous poly(aspartamide)s possessed elongations of 1700%, stresses at break near 9.3 MPa, and glass transition temperatures ranging from 0 to 86 °C.

Poly(aspartamide)s have potential application in second order non-linear optical (NLO) materials due to their high glass transition temperatures (~260 to 290 °C) which help maintain the critical orientation of the NLO chromophores. In addition, poly(aspartamide)s do not exhibit poling interferences due to water evolution as seen with polyimides during cyclization of the precursor poly(amic acid). Wu et al. used Michael addition to obtain an NLO-containing poly(aspartamide) pre-polymer with maleimide end groups that was thermally cured to obtain the final product [74]. At higher curing temperatures, the glass transition temperature of the cured poly(aspartamide)s appeared to increase, which demonstrated that curing continued to 250 °C.

Poly(amide aspartamide)s are similar to poly(aspartamide)s with the exception that polymerization occurs via both Michael addition of amines and maleimides and amidation reactions of amines and carboxylic acids [75]. Poly(amide aspartamide)s are synthesized through a step growth polymerization of an AA′ monomer containing a carboxylic acid and a maleimide functional group with a B_2 diamine monomer such as those shown in Fig. 21. The polymers are thus synthesized in a sequential process beginning with the Michael addition of the nucleophilic amine groups to the maleimide group followed by the amidation at the carboxylic acid. These polymers are controllably branched through the addition of AB_2 monomers. One drawback of the poly(amide aspartamide)s is their relatively low thermal stability, with typical thermal degradation temperatures of 300–360 °C at 10% weight loss. The char yields of the polymers increased on end-capping the carboxylic acid end groups with aniline.



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Fig. 21. Typical poly(amide aspartamide) polymerization. The first heating step produces the Michael adduct while the second step promotes amide bond formation.

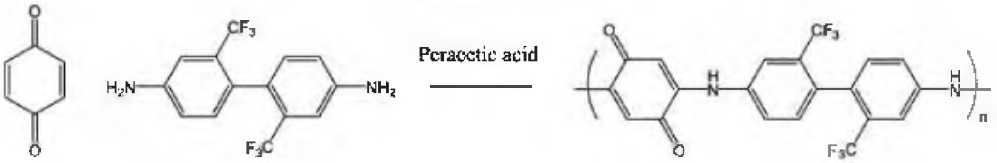
Oxygen nucleophiles are also effective in step growth polymerizations with bismaleimides. Hulubei and Rusu copolymerized glycerol and phenolphthalein with aliphatic bismaleimides resulting in poly(imido ethers) [76]. The reaction was performed in NMP using triethylamine or 2-mercaptobenzothiazole as catalysts at 85–115 °C for 2–3 d. In the case of glycerol, the difference in reactivity of the secondary and primary alcohols allowed synthesis of pendant hydroxyl polymers. Thermogravimetric analysis revealed initial weight losses ranging from 215 to 400 °C. Polymerization of bismaleimides with phenols was successful in solution (95–110 °C) and in the melt (180 °C), and fiber-glass reinforced composites were prepared [77].

3.5. Poly(amino quinone)s

Poly(amino quinone)s are synthesized from primary diamines and quinone, through Michael additions on both sides of the quinone (Fig. 22) [3]. The unsaturated nature of the quinone is maintained in the polymer, using either excess quinone or other oxidizing agents. Use of peracetic acid leads to improved yields and simplified purification. Poly(amino quinone)s have recently found application in anticorrosion coatings and

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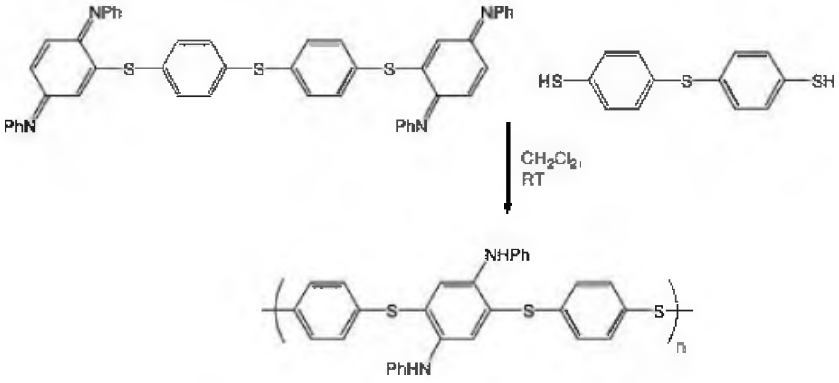
moisture resistant adhesives. Numerous traditional adhesives suffer from delamination from solid surfaces in the presence of water. This is due to a stronger interaction of water with the surface. Poly(amino quinone)s, however, displace water on metal surfaces and bind preferentially. The strong interaction of these polymers with metal surfaces is thought to arise from the electron donating capabilities of the quinone group. Poly(amino quinone)s inhibit corrosion on stainless steel surfaces even in the presence of sulfuric acid, through the formation of a protective coating that forms spontaneously on the metal surface. These corrosion inhibiting coatings are used for ship hull protection as well as protection of magnetic particles on data storage media. Diamino quinone containing polyimides are used to improve the corrosion resistance of interlayer dielectrics in microelectronics [78]. Poly(amino quinone)s have also been introduced into polyolefins and polyurethanes.



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Fig. 22. Poly(amino quinone) synthesis involving peracetic acid as an oxidizing agent.

Similar to poly(amino quinone)s are poly(thiophenylene)s formed from aromatic dithiols and bis(1,4-phenylenediimine)s, depicted in Fig. 23. The product of the Michael addition between the thiol groups and this nitrogen analog to quinone aromatizes through proton transfer to the imine nitrogens resulting in a poly(thiophenylene) with pendant amino groups [79]. The polymerization proceeds without a catalyst. These polymers exhibit electroresponsive behavior and are redox active under acidic conditions. The number average molecular weight of the polymer was 5400 g/mol and the glass transition temperature was 131 °C.



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Fig. 23. Polymerization of poly(thiophenylene)s via Michael addition and oxidation.

3.6. Step growth polymers derived from bisacetylene-containing monomers

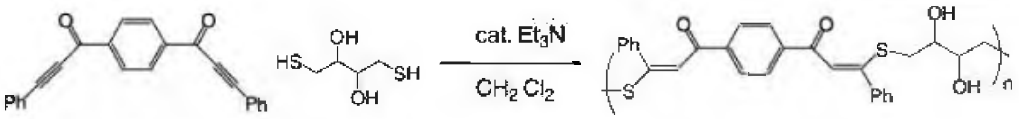
Numerous step growth Michael addition polymers are synthesized from bisacetylenic precursors. The acetylene bond reacts electrophilically when conjugated to electron withdrawing groups, in a similar fashion to an olefin as noted earlier. Typical functional groups that serve as Michael receptors are conjugated acetylene ketones and conjugated acetylene esters. These carbon–carbon triple bond Michael acceptors react

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with oxygen, sulfur and amine nucleophiles and adducts possess the additional feature of stereochemistry at the double bond [80]. Michael addition polymers based on acetylenic precursors possess surprising thermal stability and produce remarkably tough thermoplastics.

Sinsky et al. studied poly(enamine ketone)s synthesized through the Michael addition reaction of aromatic diamines with aromatic bis(alkynone)s [81]. These nucleophilic additions to acetylenic compounds result primarily in *Z* isomer formation. The reactions were performed in *m*-cresol at 100°C without catalyst. Glass transition temperatures between 198 and 235°C were obtained and thermogravimetric decomposition temperatures were nearly 300°C. The poly(enamine ketone)s were tough thermoplastics, with tensile strengths of 85MPa (63MPa at 93°C), tensile moduli of 2.27GPa, and elongations of 4%.

Bass et al. synthesized poly(enone sulfide)s, the sulfur analog to poly(enamine ketone)s through the reaction of aromatic dithiols (e.g. 1,3-benzenedithiol) with aromatic internal bis(alkynone)s in *m*-cresol at room temperature with *N*-methyl morpholine as a catalyst at temperatures from 25 to 40°C [82]. The polymers had moderately high glass transition temperatures (T_g~107–156°C) and thermal decomposition temperatures near 330°C. Tough, clear films were obtained from solution casting and exhibited tensile strengths of 78MPa and tensile moduli near 3.2GPa. Dix et al. also synthesized poly(enone sulfide)s from internal bis(alkynone)s and found a *Z:E* ratio of 60/40 (Fig. 24) [68]. Model reactions of thiols with phenylacetylene resulted in almost exclusive formation of the *Z* isomer [83].



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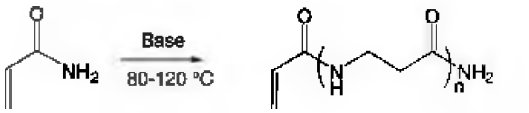
Fig. 24. Poly(enone sulfide) synthesis via Michael addition step growth.

3.7. Hydrogen transfer polymerization

Nylon-3 type polyamides are also synthesized via a Michael addition process. The base catalyzed polymerization of acrylamide to poly(β-alanine), as shown in Fig. 25, was originally proposed as an anionic chain growth process, which occurred with proton transfer of the amide NH_2 on the growing chain end to the enolate carbanion. This process was described as a 'hydrogen transfer polymerization'. However, it was later classified as a step growth polymerization due to proton transfer from the growing enolate carbanion to amide NH_2 groups on other monomers. Bush and Breslow demonstrated this via monitoring molecular weight as a function of monomer conversion [84]. At very short reaction times, high monomer conversion was achieved but high molecular weight product was not obtained suggesting initial dimerization and trimerization. Longer reaction times led to high molecular weight poly(β-alanine). These polymerizations were typically conducted at 80–120°C in aprotic solvents such as DMF in the presence of radical inhibitors. Further work from Lim et al. demonstrated that branching occurred in these polymerizations in aprotic solvents due to propagation of both enolate and amide anions, which are formed before and after hydrogen transfer, respectively [85]. Hydrogen transfer polymerization of acrylamide in the presence of carbon black and *n*-butyl lithium was effective in grafting of poly(β-alanine) onto the carbon black via polymerization from surface lithium alkoxides [86]. The grafted carbon black showed improved dispersability in water and organic solvents, and grafting ratios of 60–80% were achieved. Recently, the hydrogen transfer

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polymerization process was applied to *N*-acetylacrylamide and *N*-benzoylacrylamide and the proton transfer mechanism was preserved despite the steric hindrance and lower acidity of the amide NH groups [87]. Hydrogen transfer polymerization was also achieved with *N*-acryloyl-*N′*-*p*-tolylureas [88], *p*-styrenesulfonamides, heterocyclic base containing acrylamides [89], and maleimide.



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Fig. 25. Hydrogen transfer polymerization of acrylamide to poly(β-alanine).

In a similar nature to the hydrogen transfer polymerization of acrylamide, hydroxyalkyl acrylates are also polymerizable through Michael addition chemistry as AB monomers. The polymerization of hydroxyethyl acrylate was performed at temperatures from 85 to 150°C in the presence of free radical inhibitors using bases such as sodium hydride and potassium *tert*-butoxide as initiators [90]. Molecular weights as high as 9000g/mol were obtained and hydroxyethyl acrylate polymerized more effectively than hydroxyethyl methacrylate. The primary side reaction in these polymerizations is the disproportionation of hydroxyethyl acrylate to ethylene glycol diacrylate and ethylene glycol.

4. Linear chain growth Michael addition polymerizations

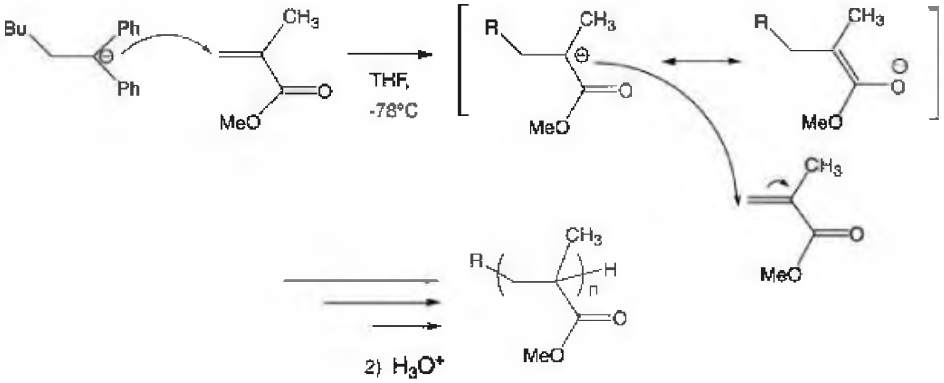
A classic example of Michael addition polymerization is the anionic chain-growth polymerization of methacrylate monomers. This review is not intended to comprehensively review anionic polymerization but rather to place the Michael addition in context for trends in polymer synthesis strategies. Anionic polymerization has been reviewed extensively in the literature [4], [91], [92]. The relatively low reactivity of the methacrylate, as noted earlier, towards Michael addition chemistry demands a stronger nucleophile, thus carbanionic species such as diphenylhexyllithium and nitrogen anions such as lithium diisopropyl amide(LDA) are suitable for the polymerization of alkyl methacrylates. One current limitation is the requirement for cryogenic temperatures to prevent potential nucleophilic attack of the enolate anion on ester groups. Group transfer polymerization, which was developed in the mid-1980s, enabled polymerization at ambient temperatures due to the use of a reversible silyl protecting group that was proposed to stabilize the propagating chain end. Anionic polymerization was demonstrated with acrylamides and acrylate monomers, later, and this may complicate step growth processes in some cases [93]. The polymerization of cyanoacrylates is another chain growth Michael addition process. Cyanoacrylates are initiated with very poor nucleophiles, such as water, due to the electron poor olefin and strong resonance contributions of the adjacent cyano and ester groups. Cyanoacrylate polymerizations are widely used in rapid cure adhesives, and adhesive products possess extremely high bond strengths.

4.1. Anionic polymerization of methacrylates

Anionic polymerization of alkyl methacrylates is an established chain growth polymerization technique, which relies upon Michael addition chemistry. Anionic polymerization is known for producing polymers of controlled molecular weights and narrow polydispersities. Anionic polymerization is particularly suited for well-defined block copolymers [94] and star-shaped polymers via chain-end coupling strategies. A further

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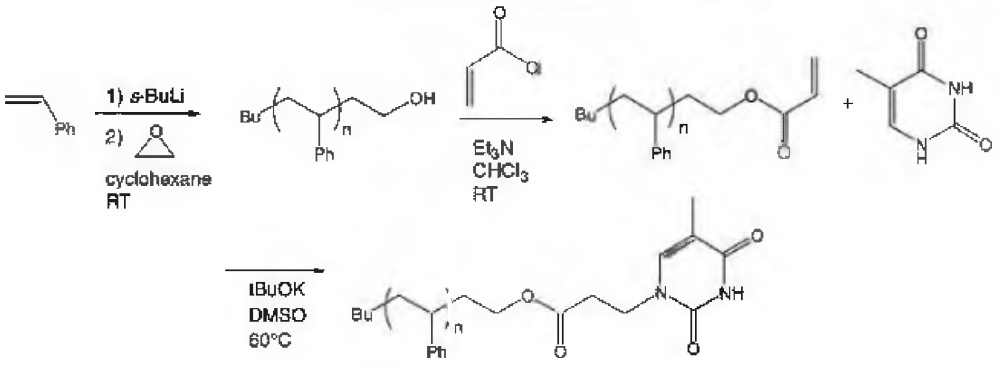
advantage of anionic polymerization is the potential for controlling tacticity, which was achieved for the sterically bulky *tert*-butyl methacrylate monomer in toluene by Long et al. [95]. Anionic polymerization is generally initiated using alkyllithium initiators with sufficient nucleophilicity to attack alkyl methacrylate monomers. However, due to the potential side reaction of the carbanionic chain end with ester carbonyls, the reaction must be conducted at low temperatures and with sterically hindered carbanions such as diphenylhexyllithium [94]. These bulky carbanions react selectively at the double bond, resulting in an enolate Michael adduct, as shown in Fig. 26.



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Fig. 26. Anionic polymerization of methyl methacrylate from diphenylhexyl lithium.

The strong nucleophilicity of the anionic PMMA growing chain end was used in chain end functionalization strategies in numerous cases [92]. For example, addition of di-*tert*-butyl maleate at the end of the polymerization results in succinate ester end groups [96]. These end groups were then pyrolyzed to produce reactive anhydrides capable of imide coupling with amine functional polymers. Long et al. acheived end functionalization with hydrogen bonding groups via Michael addition of heterocyclic base pairs to acrylated polystyrenes (Fig. 27) [97]. Termination of the anionic polymerization of styrene with ethylene oxide created hydroxyl functionality which allowed esterification with acryloyl chloride. Michael addition of nucleotide bases then allowed facile functionalization of the polystyrene chains, without detracting from hydrogen bonding properties. Adenine and thymine functionalized polystyrene demonstrated association in ^{1}H NMR spectroscopic investigations.



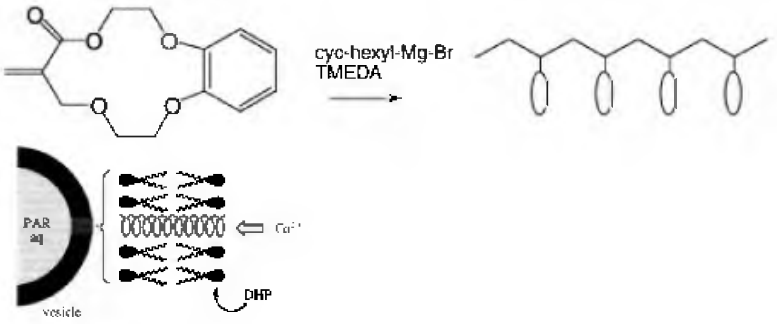
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Fig. 27. Michael addition of heterocyclic nucleotide bases to acrylated anionic polystyrene.

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Long et al. [94], [98] as well as Müller et al. [99] studied the synthesis of diene/acrylic, styrene/acrylic and all acrylic triblock copolymers containing *tert*-butyl methacrylate, which were later hydrolyzed and neutralized to obtain carboxylate block ionomers. The bulkier ester alkyl prohibits carbonyl ester attack at room temperature. Long et al. also established the utility of lithium diisopropylamide (LDA) as an initiator for methacrylate polymerizations in THF at −78 °C, despite the relatively low nucleophilicity of the hindered amide ion [100]. Anionic polymerization of acrylates, [93] maleimides, vinyl ketones [101] and acrylamides [102] are also known.

In some cases, anionically polymerized methacrylates have been designed for biologically related applications. For instance, Okamoto et al. synthesized macrocyclic ether containing acrylic monomers, which polymerized anionically to obtain highly isotactic polymers capable of functioning as synthetic ion channels in hexadecyl phosphate vesicles (Fig. 28) [103]. In contrast, radical polymerization of macrocyclic ether monomers yielded atactic polymer with dramatically lower ion transport.



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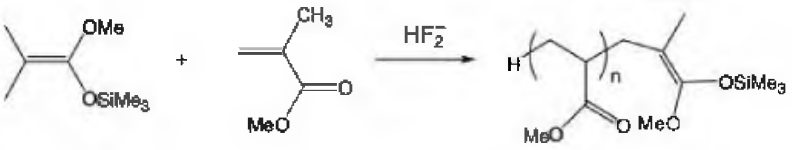
Fig. 28. Polymerization of macrocyclic ether acrylic monomers to form ion channels. Reprinted from Polymer, 43, Okamoto, et al. 'Stereospecific anionic polymerization of α-(hydroxymethyl)acrylate derivatives affording novel vinyl polymers with macrocyclic side chains', 3469–3474, Copyright 2002, with permission from Elsevier [103].

4.2. Group transfer polymerization

Commonly, anionic polymerization of alkyl methacrylates is conducted at cryogenic temperatures. Such temperatures are generally not industrially feasible, except in the cationic polymerization of isobutylene, and significant research effort was directed towards a methodology that was amenable to higher temperatures. Group transfer polymerization (GTP) is a polymerization methodology that was developed in the mid-1980s by DuPont as a method of producing narrow polydispersity methacrylate and acrylate polymers and block polymers above room temperature [104]. GTP is conveniently conducted at 80 °C using a silyl ketene acetal initiator in the presence of a nucleophilic anionic catalyst (Fig. 29) Initially, GTP was believed to proceed through a transfer of a silyl protecting group from the enolate oxygen on the chain end to monomers during the propagation step [105]. This initial mechanism has largely been disproven in favor of a dissociative anionic polymerization mechanism which involves a reversible dissociation of the silyl protecting group from the enolate oxygen, aided by anionic catalysts (HF_2^-, bibenzoate anion). The position of the equilibrium between the free enolate and the silyl protected enolate determines the rate of the polymerization. Similar to anionic polymerizations, the reaction is terminated through the addition of a

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protic species such as methanol. GTP was used by DuPont to produce acrylic block copolymers for pigment dispersing applications in automotive paints [104].

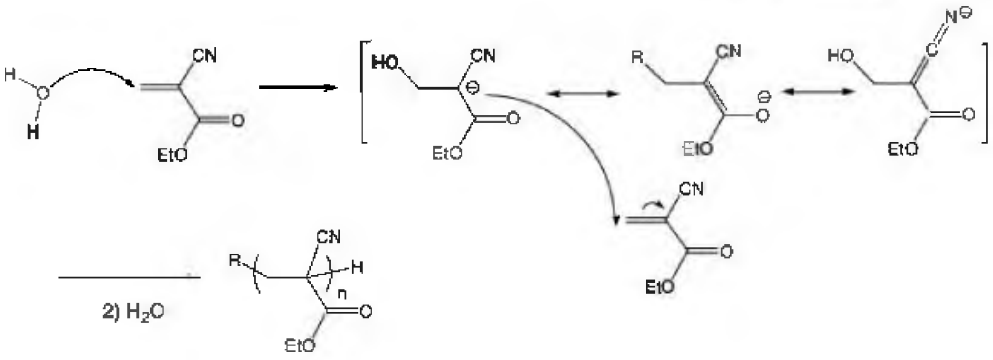


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Fig. 29. Group transfer polymerization of methyl methacrylate.

4.3. Anionic polymerization of α-cyanoacrylates

Cyanoacrylates are a class of monomers which undergo rapid anionic polymerization initiated by weak nucleophiles such as water, as shown in Fig. 30. These rapidly polymerizing monomers are used primarily in the adhesives industry as a high strength rapid setting adhesives [106]. The added electron withdrawing character of the nitrile group facilitates anionic polymerization of these monomers. Unlike conventional anionic polymerization, however, molecular weight control is absent. Klemarczyk studied the polymerization of ethyl cyanoacrylate using phosphine and amine initiators [107]. The initiated monomer species is zwitterionic, which makes it an effective nucleophile towards further monomer addition. Primary and secondary amines intiate more slowly than tertiary amines due to proton transfer after the first addition of monomer, which results in a neutral, less reactive adduct. In terms of current biomedical applications, octyl cyanoacrylate has recently found application as a tissue adhesive and gained FDA approval for surgical use [108]. This monomer polymerizes on contact with a wound and maintains flexibility due to the pendant octyl chain and furthermore possesses low water solubility.



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Fig. 30. Water-initiated polymerization of ethyl α-cyanoacrylate.

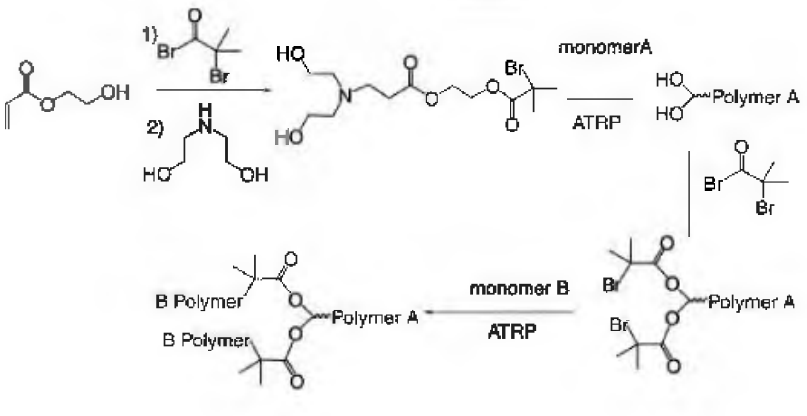
4.4. Living radical polymerization

Living radical polymerization encompasses several techniques for producing well-defined homopolymers, block copolymers, and star polymers. Due to the radical nature of this polymerization methodology, a wider range of monomers are accessible compared to traditional anionic polymerization and less stringent purification techniques are required. Numerous living radical polymerization methods are currently known, including atom transfer radical polymerization (ATRP), which involves activated alkyl halides and metal

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catalysts [109], stable free radical polymerization (SFRP), which utilizes nitroxides [110], and reversible addition fragmentation chain transfer polymerization (RAFT), which typically utilizes dithioester or dithiocarbamate chain transfer reagents [111]. Michael addition reactions have recently proven useful in many of these living radical polymerization strategies.

Functional initiation is of interest for the synthesis of well-defined polymers with the potential for block copolymer synthesis, coupling with proteins or reaction with surfaces. Recently, Michael addition was used to synthesize an ATRP initiator containing two hydroxyl groups [112]. This novel dihydroxyl initiator allowed the synthesis of Y-shaped polymers via a combination of ATRP on the initial alkyl halide site and post polymerization conversion of the hydroxyl groups to alkyl halides which allowed polymerization of a second monomer (Fig. 31) Matyjaszewksi et al. recently studied the synthesis of ATRP ligands through the Michael addition reaction of tris(aminoethyl)amine with several acrylates, resulting in ligands that were suitable for the polymerization of methacrylic, acrylic and styrenic monomers in nonpolar media [113]. Matyjaszewski et al. also synthesized ATRP ligands containing dimethoxymethylsilylpropyl acrylate, which aided in post-polymerization catalyst removal via passage through silica gel and coupling of the complexes to the silica gel via sol–gel reactions. Shen et al. synthesized novel diaminopyridine (DAP) containing ATRP ligands through acrylation of DAP followed by Michael addition using bis(diethylaminoethyl)amine [114]. Silica gel modified with thymine residues created hydrogen bonded associations with these DAP ATRP ligands, which dissociated at reaction temperatures, allowing polymerization to occur. After cooling the reaction, re-association allowed removal and recycling of the catalyst.



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Fig. 31. Synthesis of a dihydroxyl functional ATRP initiator.

Synthesis of functional polymers is also achieved through post-polymerization strategies based on the Michael addition. For example, hydroxyl functionalized RAFT polymerized PMMA were synthesized using hydrolysis of the dithioester end groups followed by Michael addition with hydroxyethyl acrylate to the thiol group [115].

5. Synthesis of branched polymers via the Michael addition reaction

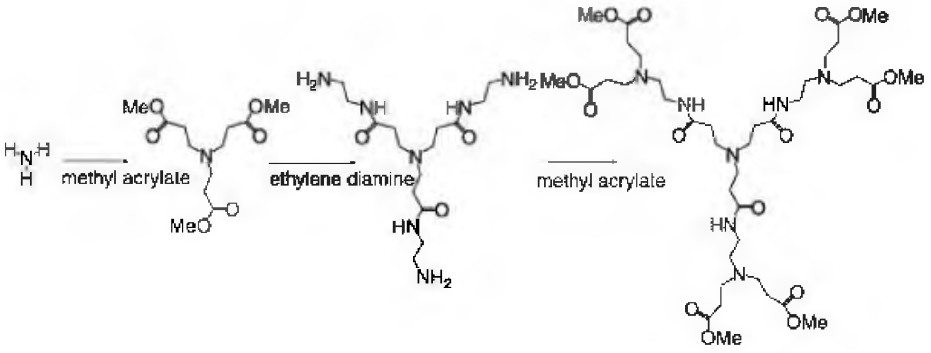
Branched polymers encompass a wide range of topologies, ranging from hyperbranched to graft and dendritic polymers. Michael addition polymerization mechanisms were broadly applied to each of these topological designs. Hyperbranched polymers have gained interest recently, due to a large number of

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functional termini and processing advantages in terms of lower melt viscosity relative to linear polymers of equivalent molecular weight. Michael addition provides the means to incorporate numerous types of terminal functionalities due to an inherent functional group tolerance and wide variety of monomers. Graft copolymers are also of increasing interest given the similarity to block copolymers and the ability to control the graft density and graft length. Graft copolymers often possess microphase separated morphologies and offer elastomeric properties. Dendrimers have traditionally been synthesized using Michael addition reactions, and in fact, Tomalia et al. synthesized the first dendrimer, poly(amido amine) (PAMAM) using Michael addition. Dendrimers and hyperbranched polymers, are interesting due to a plurality of functional groups. However, the absence of entanglements often limits the use of dendrimers to non-structural and mechanically non-demanding applications. Dendrimers based on Michael addition reactions are used in numerous applications, including biomedical applications and gene delivery [116], [117].

5.1. Dendrimers

Michael addition reactions are ideal for dendrimer synthesis due to the low probability of side reactions and mild reaction conditions. In the first dendrimer synthesis, dendritic poly(amido amine)s were synthesized via the alternating Michael addition of methyl acrylate to amine substrates (ammonia, f=3; ethylenediamine, f=4) and aminolysis of the resultant esters with excess ethylene diamine [118]. As depicted in Fig. 32, the PAMAM dendrimer synthesis is divergent, beginning from a small molecule core and enlarging as further generations are added. The high conversion and facile nature of the Michael addition led to nearly monodisperse individual dendrimer molecules as revealed using electron microscopy [119]. The Michael addition was most efficient in methanol, while aprotic solvents led to incomplete alkylation of amine groups. Retro-Michael additions fragmented the dendrimer molecules at elevated temperature (80°C) in solution [120].



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Fig. 32. Divergent PAMAM dendrimer synthesis via alternating Michael addition and amidation.

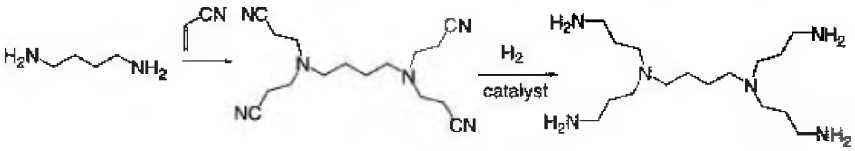
PAMAM dendrimers are effective gene transfection agents due to the plurality of terminal amine groups available to complex with the negatively charged DNA phosphate backbone. In aqueous solution, protonation of the terminal amine groups produces a cationic charge that favors complexation with DNA. A DNA-polymer complex must have a net positive charge in order to pass through a cell membrane. The PAMAM dendrimers effectively transfected genes for β-galactosidase expression, luciferase expression, and also antisense genes that selectively suppress luciferase genes [116]. PAMAM dendrimers received significant attention for effectiveness in gene transfection and numerous transfection studies were performed on

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diverse eukaryotic cell lines. Studies found increased transfection efficiency for higher generation dendrimers [117]. Higher generations were necessary to obtain efficient coverage of DNA, as higher generations possessed sizes close to the histone octamer. Heating the dendrimers in aqueous solution led to fragmentation, likely through retro-Michael additions and solvolysis, which significantly improved transfection. In fact, a commercial gene transfection agent, Superfectin™, is based on fragmented PAMAM dendrimers. PAMAM dendrimers of numerous sizes possess very low cytotoxicity and in vivo studies have also showed low toxicity.

Amine terminated PAMAM dendrimers were recently used to eliminate prion proteins from scrapie infected neuroblastoma cells [121]. Higher generation PAMAM dendrimers were more effective in removing the prion proteins and were most effective below pH 4, suggesting the importance of ammonium charge and activity in the lysosomal or endosomal regions. The importance of branching was demonstrated through the comparison of linear and branched poly(ethylene imine) controls. The ability to remove prions occurred at noncytotoxic dendrimer concentrations.

A second common dendrimer is poly(propylene imine) (PPI), marketed under the trade name Astramol™ [122]. PPI dendrimers are accessed through the Michael addition of primary diamines with acrylonitrile followed by hydrogenation of the nitrile groups to amines and repeated reaction with acrylonitrile (Fig. 33) First through fifth generation dendrimers are commercially produced in this manner. PPI dendrimers were recently synthesized with poly(propylene oxide) Jeffamine® cores, allowing greater flexibility and less steric congestion of the dendrimer molecules [123]. The terminal nitrile groups of poly(propylene imine) dendrimers are hydrolyzed under acidic conditions to obtain peripheral carboxylic acid functionality, but this often leads to degradation or discoloration of the polymer. Meijer et al. developed a route to carboxylic acid functional dendrimers via hydrogenation of the peripheral nitriles followed by the Michael addition of methyl acrylate and basic hydrolysis of the resultant ester functionalities [124]. In contrast to PAMAM dendrimers, PPI dendrimers have less potential for gene transfection and higher cytotoxicity [117]. Lower toxicity was achieved with PPI dendrimers containing ether and ester linkages, synthesized by the Michael addition of alcohols to acrylonitrile, reduction of the nitrile, Michael addition to methyl acrylate, and reduction back to alcohol terminal groups [125].

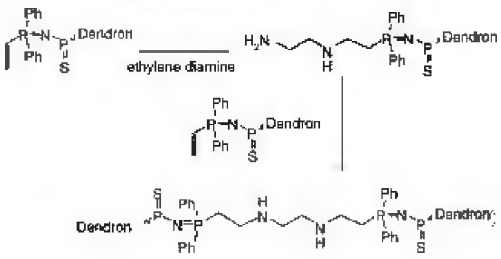


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Fig. 33. Synthesis of PPI dendrimers via alternating Michael addition and hydrogenation.

Majoral et al. developed novel dendrons containing Michael acceptors consisting of CH_2=CH–P=N–P=S–(O–R)$_2$ or CH_2=CH–P=N–P=O–(O–R)$_2$ groups at the dendrimer core. This allowed convergent synthesis of dendrimers via coupling of the dendrons through Michael addition with primary diamine donors (Fig. 34) [126], [127].

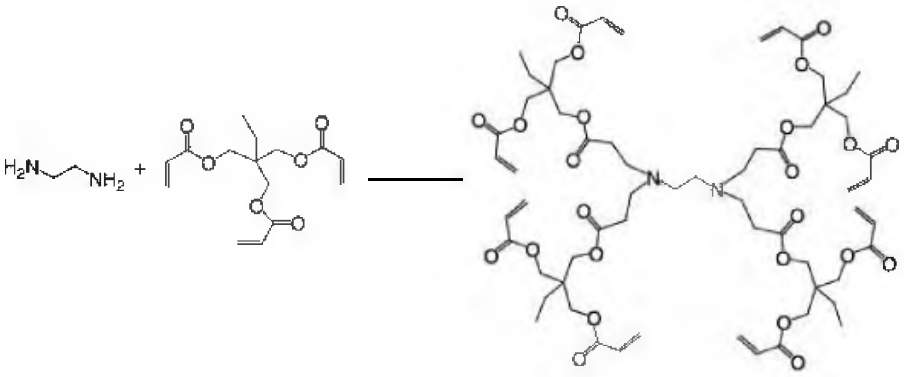
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Fig. 34. Convergent dendrimer synthesis via dendron core-linking Michael addition reactions.

Peripheral modification of dendrimers was also accomplished via Michael reactions with pendant Michael donors or acceptors. For instance, mesogenic cyanobiphenyl acrylates were introduced onto amine terminated poly(propylene imine) dendrimers resulting in liquid crystalline dendrimer molecules [128]. Michael addition of acryloxylethyl methacrylate to amine terminated dendrimers resulted in photocrosslinkable dendrimer methacrylates [129]. The Michael addition scheme was the only successful functionalization methodology as reaction with isocyanatoethyl methacrylate or acetoacetoxy methacrylate resulted in insoluble, crystalline products and reaction with methacrylic anhydride did not completely functionalize the terminal amines. The methacrylated dendrimers exhibited nearly quantitative cure reactions as determined through differential scanning calorimetry (DSC). In Fig. 35, dendritic acrylate oligomers were also synthesized through the Michael addition of trimethylolpropane triacrylate (TMPTA) with ethylene diamine in a 5:1 ratio [130], [131]. The desired product possessed a TPMTA:EDA ratio of 4:1, however, network formation occurred under these conditions. The branched oligoacrylates resembled dendrimers with low viscosities and facile cure conditions using UV-photocuring techniques. Purification of these dendrimers was achieved primarily through precipitation.



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Fig. 35. Crosslinkable dendrimer synthesis using Michael addition reactions.

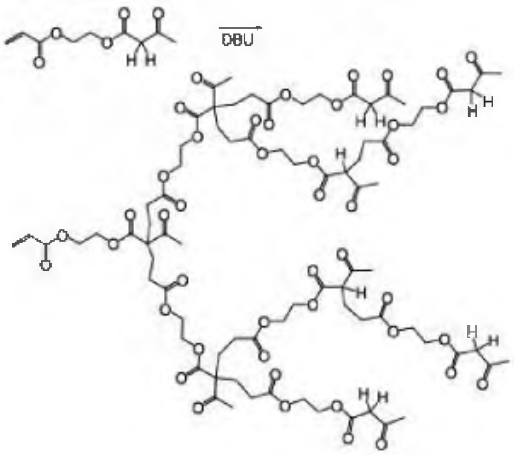
5.2. Hyperbranched and highly branched polymers

Hyperbranched polymers were initially developed as inexpensive, one-pot substitutes for dendrimers, but have developed a unique character over the last several years. Hyperbranched polymers were traditionally approached from asymmetric monomer synthesis (AB_n monomers). Difficult monomer synthesis is a major

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disadvantage of the AB_n method due to a lack of commercial AB_n monomer sources. Recently, A_n+B_m approaches were used to synthesize hyperbranched polymers, enabling a much wider range of monomers and topologies. A further development is the use of oligomeric A_n or B_m monomers, resulting in 'highly branched polymers', which allows control of the molecular weight between branch points, an important parameter that affects degree of entanglement and has performance implications [132]. Michael addition reactions were successfully applied to both the AB_2 and A_n+B_m strategies for hyperbranched polymer synthesis.

In a conventional AB_2 hyperbranching polymerization, Endo et al. conducted acetoacetylation of hydroxyethyl acrylate through a reaction with diketene [133]. This produced an AB_2 monomer that was capable of producing hyperbranched Michael addition polymers in the presence of base catalyst (Fig. 36) Polymerization of this novel monomer in the presence of DBU resulted in number average molecular weights between 2000 and 12,000g/mol with dispersities between 1.4 and 3.5 and degrees of branching between 43 and 83%. A high ratio of dendritic to linear units was observed, suggesting greater reactivity of the *mono*-Michael adduct towards the acrylate group compared to the reactivity of the original acetoacetate group.



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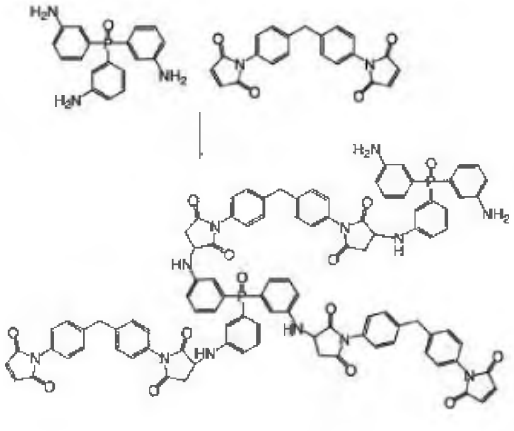
Fig. 36. Hyperbranched polymer synthesis via AB_2 Michael addition polymerization.

Trumbo studied the step growth polymerization of low molar mass diacrylates (tripropylene glycol diacrylate) with bisacetoacetates [134] and bisacetoacetamides [135]. These systems formed branched structures due to the difunctionality of the acetoacetate group. Gelation of the reaction mixture was avoided with an excess of bisacetoacetate monomer and through conducting the reaction in solution with a relatively mild base catalyst (DBU). Weight average molecular weights as high as 437,000g/mol were observed, however broad dispersities (M_w/M_n~10) were typical, which further suggested branching in this system. Decreases in molecular weight and dispersity with reaction time were observed during these polymerizations, suggesting the occurrence of a retro-Michael addition and subsequent equilibration.

Hyperbranched poly(aspartamide)s were synthesized from bismaleimides and aromatic triamines using the A_2+B_3 methodology [136]. The reaction stoichiometry was limiting in bismaleimide, thus controlling the extent of the reaction and favoring secondary amine terminated products. The hyperbranched polymers

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exhibited degrees of branching near 0.51 and 0.69, high glass transition temperatures (~210–250°C) as well as equivalent thermal stability to analogous linear polymers. In Fig. 37, a tris(4-aminophenyl)phosphine oxide triamine was used to create polymers with higher thermal stability and also to allow degree of branching determination through ^{31}P NMR.



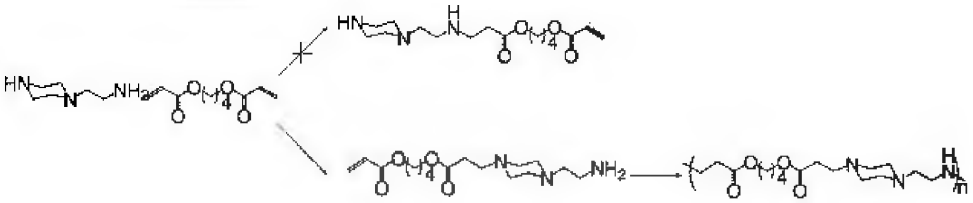
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Fig. 37. Hyperbranched poly(aspartamide)s via A_2+B_3 polymerization.

Feng et al. studied hyperbranched poly(amino ester)s that were synthesized from piperazine and trimethylolpropane triacrylate in molar ratios ranging from 1:1.08 to 1:2 [137]. These hyperbranched polymers formed aggregates in acetone/acidic water, which were proposed to contain a hydrophobic acrylate rich core and a hydrophilic, protonated amine periphery. The aggregates were crosslinked using UV light via reaction of residual acrylate groups. The size of the aggregates (DLS, TEM) decreased upon crosslinking and were smaller for higher molecular weight polymers, suggesting a packing efficiency limitation with the higher molecular weight, more disperse, polymers. Enhanced aggregation of similar poly(amino ester)s was achieved through reaction of peripheral amine groups with long chain acyl halides [138].

Wu et al. studied the effect of unequal functional group reactivity in a hyperbranching A_2+B_3 system with a butanediol diacrylate A_2 and a trifunctional amine B_3 containing both primary (f=2) and secondary (f=1) amines (Fig. 38) [36]. For an equimolar A_2:B_3 ratio, ^1H NMR studies indicated that secondary amines were consumed prior to primary amines. The secondary amine that was formed from reaction of the primary amine and the acrylate group was not consumed, and thus a predominantly linear product was obtained despite the functionality of the monomers. The reactivity of the amines was secondary > primary >> formed secondary. If the A_2:B_3 ratio was increased, participation of the formed secondary amine during the reaction of the primary amine occurred, thus resulting in a branched product. If the steric hindrance at the secondary amine position was increased, the primary amine was the most reactive and the reaction proceeded at the formed secondary amine during the reaction and again the product was branched. Thus, both the level of substitution of the amine groups and the stoichiometric ratio of the reactants controlled the degree of branching. Degrees of branching that ranged from 30 to 40% and weight average molecular weights as high as 23,500g/mol were obtained.

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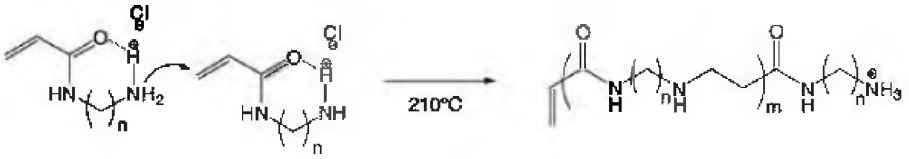
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Fig. 38. Hyperbranched poly(ester amine)s from A_2+B_3 polymerization with unequal reactivity in the B_3 monomer functional groups.

Wu et al. also studied hyperbranched poly(amino ester)s that were synthesized using two equivalents of butanediol acrylate and one equivalent of 1-(2-aminoethyl)piperazine [139]. Due to the higher reactivity of the secondary cyclic amine, the Michael addition with butanediol diacrylate occurred preferentially. The primary amino group reacted second, resulting in the in-situ generation of an AB_2 intermediate that undergoes a branching at the secondary amine formed from the primary amine. The ratio of R_h/R_g of 1.0 as measured by SAXS and DLS demonstrated the hyperbranched nature of the polymers. The hyperbranched polymers had number average molecular weights of 29,000–38,000g/mol and dispersities of 3.4–3.7.

In similar work, Gao et al. synthesized hyperbranched polymers from diethanolamine and methyl acrylate [140]. The reaction proceeded through an initial Michael addition reaction to form an ester diol AB_2 monomer in situ that self-condensed at elevated temperature (150°C) through transesterification in the presence of a zinc acetate catalyst. Number average molecular weights as high as 268,000g/mol were observed with a polydispersity of 2.4. Crosslinking occurred when the temperature in the second stage exceeded 160°C, whereas lack of polycondensation occurred if the temperature was less than 120°C. The degree of branching of these polymers was roughly 55%. These polymers degraded rapidly in water to form self-buffered pH 7 solutions, suggesting them as good candidates for drug delivery.

Hyperbranched poly(amido amine) hydrochloride polyelectrolytes were synthesized through the Michael addition polymerization of AB_2 monomers based on ammonium alkyl acrylamides, as shown in Fig. 39 [141]. The lack of nucleophilicity of the ammonium group toward the acrylamide functionality required the use of high temperatures (210°C) in order to drive the polymerization. It was postulated that a small equilibrium concentration of free amine that exists at these high temperatures undergoes the Michael addition. The shorter alkylene spacer (n=2) between the acrylamide group and the ammonium group resulted in faster reaction, and an intramolecular deprotonation through a six membered cyclic transition state was proposed. Model reactions suggested that the ammonium group reacts twice with acrylamide functionalities, resulting in a highly branched product. ^{15}N NMR was used to characterize the degree of branching in these materials. Signals from linear units were not observed, corroborating the highly branched nature of the polymer. This methodology is a more facile route to hyperbranched polymers resembling PAMAM dendrimers.

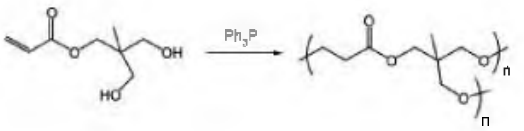


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Fig. 39. Homopolymerization of an AB_2 poly(amido amine) monomer.

Kadokawa et al. studied the triphenylphosphine initiated Michael addition polymerization of 2,2-bis(hydroxymethyl)propyl acrylate to yield phosphonium containing hyperbranched polymers of 1200–2700g/mol, as shown in Fig. 40 [142]. This is one of the few examples of oxygen based nucleophiles involved in a Michael addition polymerization. Degrees of branching near 50% were obtained. Similar polymers were produced through initiation using sodium hydride. The poor quality of the oxygen nucleophile for Michael addition is evident in the reaction temperatures required (80–100°C, with free radical inhibitors) [143].



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Fig. 40. Homopolymerization of an AB_2 poly(ester ether) monomer.

Highly branched gene transfection agents were synthesized through the reaction of low molecular weight linear PEI with PEG diacrylate [144]. The polymers clearly did not represent linear topologies due to branching in the PEI as well as reaction at multiple amine sites along the polymer backbone. The presence of ester groups in the polymers facilitated degradation. The half-lives of the polymers in PBS at 37°C were approximately 8d. The polymers demonstrated complex formation with plasmid DNA and transfection studies were performed. Branched gene transfection agents were also synthesized via reaction of trimethylolpropane triacrylate with primary amines such as *N*,*N*-dimethylethylenediamine [145]. Polyplex formation with plasmid DNA was verified with dynamic light scattering and transfection into various human and mouse cell lines exhibited transfection efficiencies similar to poly(ethylene imine). The branched nature of the poly(amino ester)s slowed polymer degradation and hence controlled the release of the plasmid DNA. The branched poly(amino ester)s showed little or no cytotoxicity.

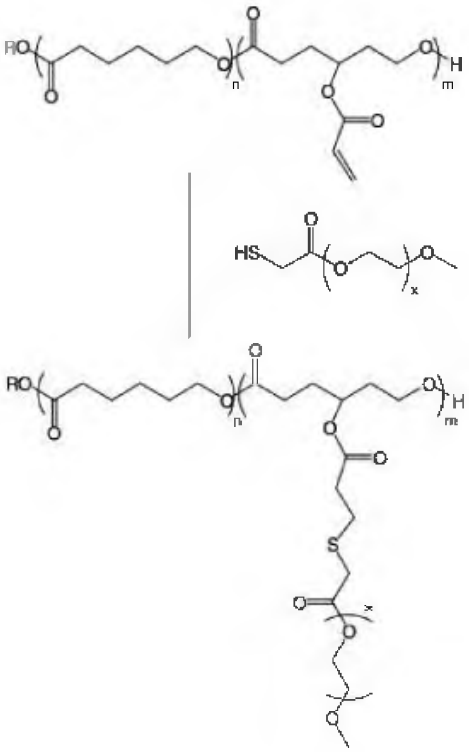
5.3. Graft copolymers

Graft copolymers are synthesized through numerous routes including chain or step growth polymerization of macromonomers and post-polymerization grafting. Ferruti et al. examined graft copolymer synthesis using poly(amido amine)s [146]. PEG chains with weight average molecular weight of 750g/mol were incorporated into poly(amido amine) backbones using monoamine terminated PEG as a comonomer. As a second route, PEG-NH_2 was first reacted with excess bisacrylamide monomer to obtain a PEG bisacrylamide, which was polymerized with piperazine. In both cases, vapor pressure osmometry indicated that the molecular weights obtained were quite low (~3000g/mol). Ferruti concluded that the reactivity of the PEG-NH_2 in the polymerization was lower than typical small molecule amines. Ferruti et al. also studied poly(amido amine)s grafted to polyethylene via a post-polymerization strategy, although Michael addition was not involved in the coupling [147].

Jerome et al. utilized γ-acryloxy-ε-caprolactone as a graftable comonomer in copolymerizations with ε-caprolactone [148]. In Fig. 41, pendant acrylate groups were introduced into poly(ε-caprolactone), permitting grafting of thiol terminated oligomeric PEG (M_n=900g/mol). The Michael addition reaction of various thiols (mercaptoacetic acid and triphenylmethane thiol) with PEG-acrylate model compounds proceeded to

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completion. However, conversions of only 65 and 70% were obtained with PEG-thiol and mercaptoacetic acid, respectively, in grafting studies with poly(γ-acryloxy-ε-caprolactone-*co*-ε-caprolactone).

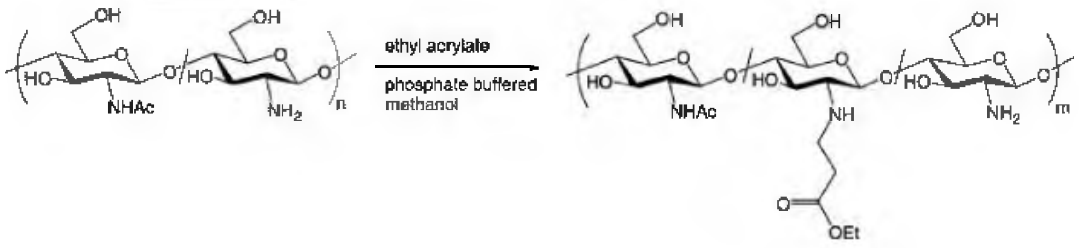


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Fig. 41. Graft copolymerization via grafting of thiol terminated PEG to pendant acryloxy side groups on a poly(ε-caprolactone) derivative.

Much work was done in the area of 'grafting to' syntheses utilizing the Michael addition, especially in the case of biopolymers such as chitin and chitosan. These materials typically possess poor solubility, which is improved through grafting techniques. Modification of chitosan with poly(amido amine) dendrimers was achieved by reacting chitosan surface amine groups with methyl acrylate followed by alternate reactions with ethylene diamine and further methyl acrylate [149]. The heterogeneity and steric hindrance of the backbone resulted in imperfect dendrimers. Grafting of living cationic poly(methyl oxazoline) and poly(isobutyl vinyl ether) onto the pendant amine groups of the grafted poly(amido amine) dendrimers was achieved successfully. Modification of chitin with small molecules was also achieved through Michael addition to pendant amine groups. Reaction with acrylonitrile yielded cyanoethylated chitin [5] and reaction with ethyl acrylate afforded ester-containing chitin [150] (Fig. 42)

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Fig. 42. Modification of chitin via Michael addition reaction with ethyl acrylate.

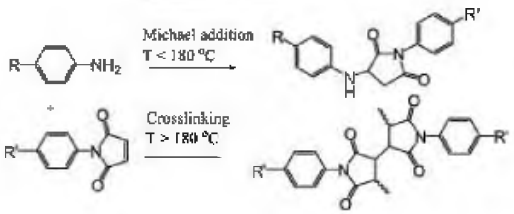
6. Novel networks via the Michael addition reaction

Network polymers that are synthesized via Michael addition reactions offer applications in diverse areas such as drug delivery systems, high performance composites, and coatings. Networks are often synthesized through a combination of Michael addition step growth chemistry combined with chain growth polymerization using photoinitiated radical or anionic processes. The Michael addition is suited to the formation of well-defined networks due to the commercial availability or facile synthesis of narrow molecular weight distribution functional oligomers. Commercially available reactive oligomers include PEG diacrylate and numerous oligomeric diamines such as Jeffamine®. Several classes of Michael addition networks are currently of interest.

6.1. Bismaleimide networks and composite materials

Currently, bismaleimide based networks are used in high performance fiber-reinforced composite materials [151] and also in thermoset resins. These materials have applications in the aerospace industry due to their high temperature strength, mechanical robustness, and low density [152]. In the absence of other components, bismaleimides crosslink through chain growth type reactions at high temperature to form highly crosslinked, high T_g networks. Vitrification in these systems leads to incomplete conversion of maleimide groups. Brittleness is the primary drawback of these homopolymerized networks. Efforts to reduce the crosslink density and hence the brittleness of these thermosets have involved Michael addition chain extension reactions with primary or secondary diamines to consume some of the maleimide functional groups [70]. The chain extension process results in greater toughness and improved mechanical properties for composites. The introduction of diamines such as methylene dianiline (MDA) causes a low temperature Michael addition polymerization (<180°C) during initial curing, followed on further heating (180–220°C) with a crosslinking homopolymerization of the maleimide groups (Fig. 43) [152], [153]. Under certain conditions, secondary amines formed during the initial Michael addition undergo further Michael addition crosslinking with maleimide groups. Hyperbranched amine terminated polyamides were also investigated as components of bismaleimide networks, successfully improving the toughness of these systems [154].

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Fig. 43. Crosslinking of bismaleimide networks via a combination of Michael addition and maleimide homopolymerization.

Kumar et al. incorporated bismaleimide crosslinking systems into silicon-containing epoxy resin networks [154]. The bismaleimides increased the flexural and tensile moduli, but decreased fracture toughness of the epoxy networks by introducing a more rigid, higher T_g component. This network reinforcement was due to the thermal cure and Michael addition reactions between the bismaleimide and the amines from the epoxy resin and the added diamine.

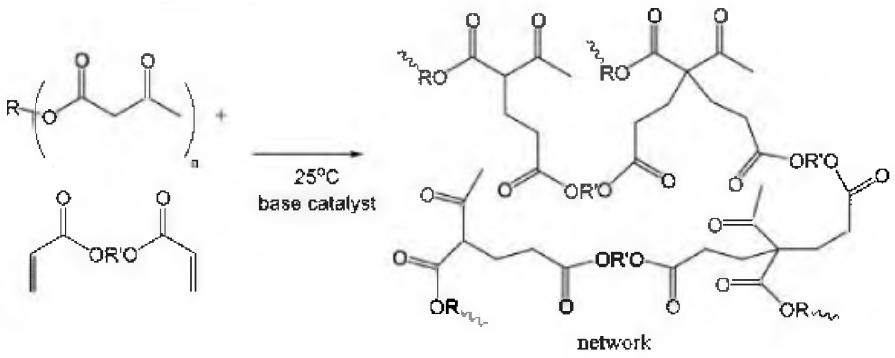
Bismaleimide networks are useful as NLO materials as well as composites due to their high glass transition temperatures and crosslink densities. These features are attractive for NLO materials for maintenance of the chromophore orientation. Furthermore, the absence of volatile by-products during cure allows poling during this process. Wu et al. synthesized a diamine NLO chromophore and incorporation into bismaleimide networks resulted in nonlinear optical coefficients as high as d_{33}=60pm/V at 1064nm [155]. Upon heating, the second harmonic generation (SHG) was stable to 120°C, although the cure process was continued 250°C in order to maximize crosslinking. Maintenance of the poling field during the cooling process maximized the NLO properties.

6.2. Carbon Michael addition networks utilizing acetoacetate chemistry

Considerable interest in Michael addition networks from acetoacetate and acrylate precursors has recently emerged. These networks are interesting due to the relatively low toxicity of the components and their room temperature processing in the absence of UV radiation. Furthermore, functionalization of common polyols with the acetoacetate group is achieved through transesterification with *tert*-butylacetoacetate [156] or reaction with diketene precursors [157] providing a facile route to crosslinkable precursors from a wide variety of hydroxyl containing feedstocks.

Room temperature thermosetting coatings is one of the primary applications of Michael addition networks. The Michael addition mechanism is advantageous as neither UV radiation nor heat are required, both of which could be deleterious to the substrate and require additional processing equipment. The base catalyzed carbon Michael addition of acetoacetylated resins to acrylate acceptors, as depicted in Fig. 44, is an ideal route to crosslinked networks due to the difunctionality of the acetoacetate group and the absence of undesirable toxic amine and malodorous thiol nucleophiles in industrial processes. Acrylic monomers and oligomers are useful candidates for the formation of coatings and adhesives with highly reduced volatile organic compounds (VOCs) and improved material performance properties. Although UV curing is not necessary for network formation, the presence of photocrosslinkable groups in these systems allows tandem Michael and radical curing mechanisms.

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Fig. 44. Carbon-Michael addition polymerization of acetoacetates with acrylates catalyzed by bases for network synthesis.

Clemens and Rector studied the carbon Michael addition reaction between model compounds, and concluded that the reaction mechanism involved an equilibrium deprotonation of the acetoacetate and subsequent rate limiting addition of the enolate to the acrylate group [21]. Thus, the rate of the reaction was unaffected by the acetoacetate concentration but was directly proportional to the base catalyst concentration. The effect of various catalysts was investigated and as expected, higher basicity led to faster reaction kinetics. Typical base catalysts included DBU and TMG as well as hydroxide and methoxide bases. A striking difference, however, was observed in the degree to which bisadducts of acrylates with acetoacetates formed as a function of catalyst. Amidine and guanidine bases resulted in higher formation of bisadducts while monoadducts were favored by methoxide and hydroxide bases. Weaker bases such as triethylamine were not strong enough to deprotonate the acetoacetate groups and did not catalyze the Michael addition reaction effectively.

Clemens and Rector prepared crosslinked coatings consisting of randomly functionalized acetoacetylated precursors such as methyl methacrylate/acetoacetyloxy-ethyl methacrylate random copolymers with low molar mass multiacrylates such as trimethylolpropane triacrylate [21]. The level of functionality of the methacrylate copolymers strongly influenced cure times, with a doubling of the acetoacetate functionality resulting in a 75% reduction in cure time. Acetoacetylated cellulose was also investigated, and the more rigid character of the polymer coupled with the lack of a spacer between the backbone and the acetoacetate groups resulted in slower cure reactions. One disadvantage of the Michael crosslinked coatings was hydrolytic instability that was attributed to hydrolysis of the ester linkages in the presence of the base catalyst. Heat treatments reduced the hydrolytic instability of the network.

Marsh described the use of high solids acetoacetate-containing resins for coatings applications [158]. High solids polyester resins containing hydroxyl groups were synthesized and acetoacetylated to various levels. It was found that increasing levels of acetoacetyl groups lowered the T_g of the polyester resins significantly to yield solvent-free low viscosity resins. The crosslinking of the pendant acetoacetyl groups using Michael type reactions with electron deficient olefins and DBU base catalyst (pH 12.6) afforded formaldehyde- and isocyanate-free room temperature curable coatings.

Tung published a review on the performance of coatings made from multifunctional acrylate resins containing reactive diluents and acid blocked catalysts and compared them to two-package polyurethanes

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[159]. The use of a formic acid neutralized DBU catalyst yielded better pot life and humidity resistance of the coatings when compared to the highly basic DBU catalyst. It was found that incorporation of 10–15 mol% of a reactive diluent such as 2,2,4-trimethyl-1,3-pentane-bis(acetoacetate) resulted in low VOC coatings with desirable ultimate properties. Various multifunctional acrylate crosslinkers were investigated to determine the influence of crosslinker functionality on the resulting resin properties. A mixture of tri- and tetraacrylates produced the best hardness, flexibility, solvent resistance, humidity resistance, adhesion, and weatherability compared to the triacrylates or hexaacrylates.

6.3. Curing mechanisms involving Michael addition with photocrosslinking or sol–gel chemistry

Cure mechanisms using a combination of Michael addition and photoinitiated radical curing are currently of interest due to higher crosslink densities and functional group conversions. The use of multifunctional acrylate monomers in the carbon Michael addition networks is ideal for photocrosslinking. Residual acrylate dangling ends must be limited to maximize chemical and physical durability. Moszner and Rheinberger synthesized networks from multiacrylates such as PEG diacrylates, trimethylolpropane triacrylate, and pentaerythritol tetraacrylate with bisacetoacetates such as PEG bis(acetoacetate) and hexanediol bis(acetoacetate) using DBN base catalyst [160]. Isothermal DSC studies revealed less than 25% of the double bonds were consumed via Michael addition during cure. The conversion of acrylate groups increased for longer, more flexible, spacers to approximately 71%. Subsequent photocuring of the Michael addition networks using camphorquinone/*N*-(2-cyanoethyl)-*N*-methylaniline resulted in dramatically improved hardness and conversion of the acrylate groups. Moy et al. developed photocrosslinked networks from crosslinkable oligomers that were synthesized using Michael addition with an excess of multiacrylates relative to acetoacetate precursors [161]. The Michael addition of acetoacetoxyethyl methacrylate with multifunctional acrylates is another route to photocrosslinkable methacrylate oligomers. The ketone-containing oligomers were capable of self-initiation in UV light due to Norrish type cleavage reactions. Klee et al. conducted Michael addition using diamines and acryloxylethyl methacrylate to create crosslinkable branched multimethacrylate oligomers for dental composites with lower shrinkage upon photocrosslinking [162]. Dammann and coworkers demonstrated the formation of liquid oligomeric Michael adducts by reacting di-, tri- and tetraacrylates with ethyl acetoacetate in a specific ratio in the presence of catalytic amounts of epoxide and quaternary ammonium salt. These compositions were further crosslinked to obtain coatings, laminates, and adhesives [163].

Pavlinec and Moszner further studied crosslinked networks from poly(propylene glycol) bis(acetoacetate) or pentaerythritol tetrakis(acetoacetate) and pentaerythritol tetraacrylate cured with DBN [11]. In these Michael addition networks, a 2:1 ratio of acrylate to acetoacetate was studied. The residual acrylate groups in the networks were studied using FTIR, following the acrylate vinyl absorbance at 810 cm^{-1}. The concentration of residual unreacted acrylate groups was lowest for networks that were prepared using poly(propylene glycol) bis(acetoacetate)s due to the molecular mobility and long distance between crosslink points. Higher conversion of reactive functional groups for longer precursors was also observed in photocrosslinking studies of diacrylate oligomers [164]. Ambient temperature aging and thermal postcure treatments to 200°C increased the conversion of acrylate groups from 88 to nearly 97%. The gel fractions that were obtained for the Michael addition networks ranged from 87 to 94%. The combination of Michael addition curing with free radical photocuring was also investigated with the addition of photoinitiators,

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excess pentaerythritol tetraacrylate, and PEG dimethacrylate monomers to selected compositions. The stability of the methacrylate against Michael addition prevented interference between the two cure mechanisms performed in a stepwise manner, beginning with a dark Michael cure and ending with the photocure. In the photocured systems, lower viscosity during the Michael addition stage was attributed to the additional PEG dimethacrylate monomer and excess acrylate monomer. This was postulated to improve the cure process by improving molecular mobility and homogeneity.

Michael reactions between multifunctional acrylates and acetoacetates have found widespread applications in the formation of radiation curable coatings, printing ink formulations, and adhesives. UV curable ink formulations have gained importance as a substitute to solvent-based ink systems. Currently available UV curable ink formulations exhibit rapid curing under commercial line speeds, however, large quantities of photoinitiator such as benzophenone are required to ensure complete curing throughout the thickness of the film for printing applications. These photoinitiators are known to be toxic, expensive and also cause odor and color in the material. In order to overcome this issue, current studies have involved the use of liquid oligomeric Michael adducts formed from multifunctional acrylates by reaction with Michael donors such as acetoacetate compounds, which are amenable to further crosslinking under UV irradiation [165]. Through the incorporation of suitable additives such as acrylic monomers and oligomers, vinyl monomers, vinyl ethers, and primary and secondary amines, ink formulations were modified to obtain rheological and adhesion characteristics that were desirable for printing applications on many different kinds of substrates.

Recently Liu et al. described the formation of renewable and environmentally benign wood adhesives based on derivatized soy protein using the Michael addition crosslinking approach [166]. Soy proteins used as renewable wood adhesives suffer from low shear strength and moisture resistance. In order to improve the properties of the adhesive, the authors first maleated the soy protein isolates (SPI) using maleic anhydride, which was then reacted with poly(ethylene imine) via Michael addition. Wood composites that were bonded with adhesive exhibited shear strength of 7.0MPa as compared to 2.0MPa of those bonded using SPI. In addition the joints also gained water resistance.

In addition to the application of the Michael addition in the formation of crosslinked coatings, the formation of reactive intermediates through Michael additions which are subsequently crosslinked using other mechanisms yields useful coatings and adhesives. This strategy has often involved sol–gel chemistry of the Michael adducts to form networks. For example, Wilkes et al. have reported the formation of bis- and tris(maleimide) functionalized trialkoxysilanes by Michael addition reaction between 3-aminopropyl triethoxysilanes and corresponding maleimides [167]. The functionalized silanes were crosslinked via sol–gel methods and used as protective coatings on polycarbonate substrates exhibiting enhanced abrasion resistance. Tilley et al. recently described the use of the Michael addition reaction for the preparation of acrylate-functionalized silane coupling agents to introduce acrylate functionality to colloidal silica particles [168]. Such acrylate functionalized silica particles were used as toughness enhancers for UV-curable acrylic coatings. Incorporation of these functionalized silica particles into a wide variety of organic matrices such as polyesters, epoxies, and polyurethanes was demonstrated. Such inorganic oxide modified coatings exhibited much greater micro-hardness and abrasion resistance compared to commercially prepared coatings. In a recent patent disclosure, Kobayashi et al. reported the synthesis of an acryloxy-functional silicone composition curable by high-energy radiation [169]. The silicones consisted of a mixture of multifunctional acrylates, amino-functional alkoxysilanes, organofunctional alkoxysilanes, and colloidal silica. The resultant coatings exhibited excellent storage stability, transparency, adhesiveness, water repellency, and abrasion

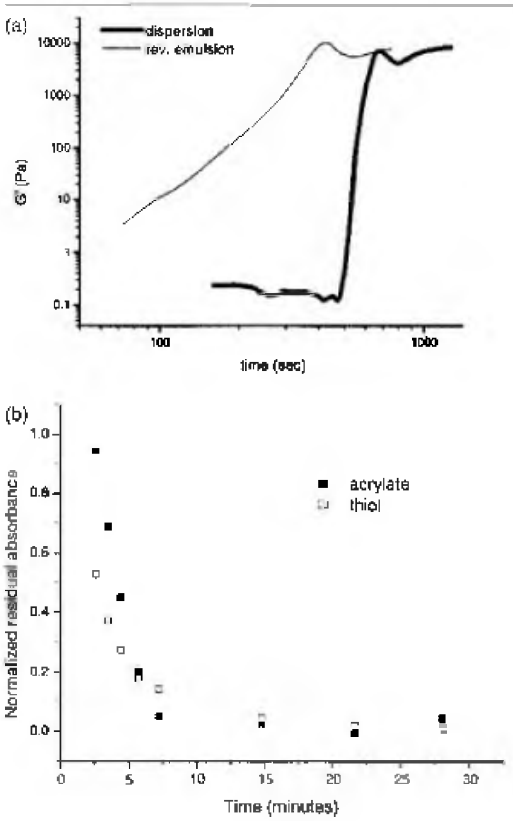
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resistance. Zhu et al. also demonstrated the formation of trimethoxysilane coupling agents containing *O*-butyrylchitosan via Michael addition of 3-acryloxypropyltrimethoxysilane with the amino groups of the butyrylchitosan [170]. Crosslinking of the modified silane derivatives resulted in colorless, flexible elastomeric coatings at 20 mol% incorporation of the modified silane. The resulting hydrogel films possessed good antithrombogenic properties as determined by blood-clotting and platelet adhesion.

6.4. Hydrogel networks for biomedical applications

Michael addition networks find application in areas beyond composites and coatings. Recently, numerous biomedical applications were investigated. Hubbell et al. studied in vivo gelling networks based on multifunctional thiols and multiacrylates as injectable tissue reinforcement [2]. Currently two mechanical ranges of in-vivo biomaterials exist: low modulus gels, which are used for drug delivery and high modulus bone cements. An intermediate strength material (0.5–50 MPa) is desirable for tissue reinforcement of invertebral disc annuli. Michael addition networks based on thiol and acrylate precursors are ideal due to the ability to control gelation with the addition of a basic catalyst, the absence of free thiols in the bloodstream, and the faster rate of the thiol-acrylate Michael addition in comparison to potential Michael reactions with amines in the bloodstream. Metters and Hubbell also recently demonstrated the biodegradability of these networks via slow hydrolysis of ester bonds adjacent to sulfide linkages, which leads to degradation on the order of days to weeks [171]. In a separate work, Hubbell et al. showed that the length of the alkyl spacer between the ester and the sulfide controlled the hydrolysis rate of these poly(ester sulfide)s, with higher degradation rates occurring for shorter alkyl spacers [172]. Both dispersion type and reverse emulsion type polymers were synthesized via mixing network components with basic PBS. Reverse emulsion type networks exhibited a slower change in shear storage modulus with time near the gel point and higher ultimate compressive strengths (6.7 vs. 1.8 MPa) due to greater continuity of the organic phase (Fig. 45) Dispersion networks exhibited rapid increases in shear storage modulus near the gel point, due to the percolation of dispersed crosslinking particles. Both materials exhibited maximum deformations near 37%. Modulus increased with increased amounts of tetraacrylate precursors versus diacrylate precursors.

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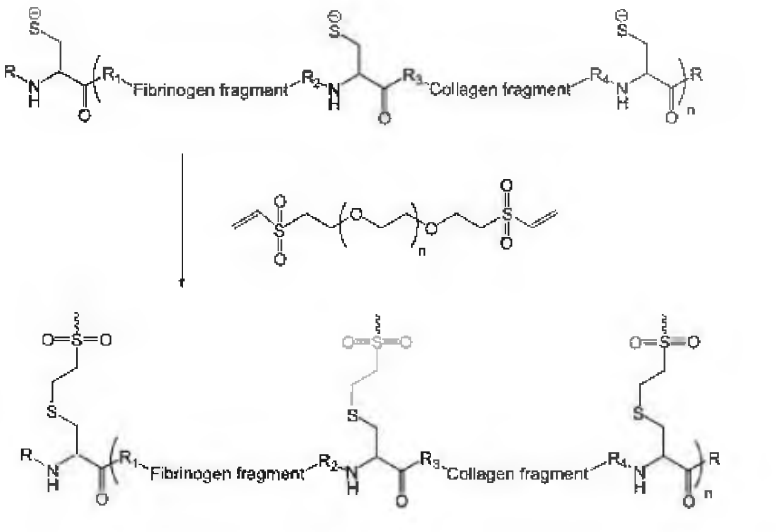
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Fig. 45. (a) Rheological monitoring of gel formation in networks synthesized from multifunctional PEG acrylates with PEG dithiol in both reverse emulsion and dispersion form. The steep rise in G′ with gel formation in the case of the dispersion network is an indication of a sudden percolation of crosslinked phases at later times relative to the continuous organic crosslinking phase in the reverse emulsion. (b) Depletion of functional groups with time during crosslinking. Reproduced from 'Water-borne, in situ crosslinked biomaterials from phase-segregated precursors,' Hubbell et al., Copyright 2003 Society of Chemical Industry with permission from John Wiley and Sons on behalf of SCI [2].

Rizzi and Hubbell [10] developed protein containing hydrogel networks for tissue repair applications. Cysteine-functionalized recombinant proteins containing sequences for integrin receptor ligation were synthesized and crosslinked with PEG bis(vinyl sulfone) (Fig. 46) Integrin receptors are cellular receptors, which play important roles in tissue development processes. These networks were stable to aqueous solution and did not exhibit hydrolytic degradation. Thus, protease cleavable units were introduced into the proteins to enable biodegradability. Glutamic acid units were placed strategically near cysteine residues to inhibit disulfide bond formation prior to crosslinking, while glycine residues were used to prevent hindrance of the Michael addition. Previous work on cysteine containing peptides revealed that positively charged arginine groups adjacent to cysteine lower the pK_a of the cysteine group, accelerating Michael reaction by a factor of 2 and decreasing gel time by a factor of 2.5 [173]. Numerous charged residues were also used to enhance solubility of the proteins. The crosslinking kinetics were studied using rheological measurements, which showed increasing storage modulus with time during the crosslinking reaction. The gel time decreased with increasing pH, due to the fact that the thiolate ion is the active species in the Michael

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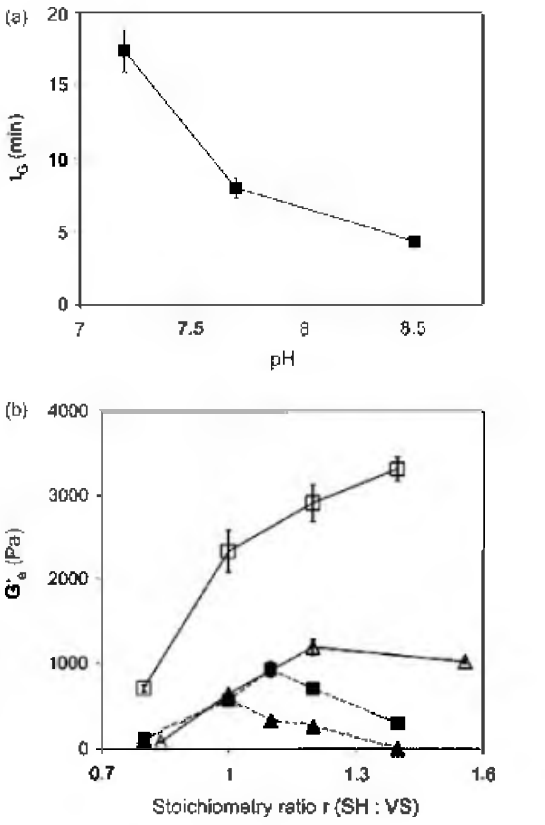
addition reaction with vinyl sulfone groups. A maxima in G′ and minimum in gel time was found for a ratio of thiol to vinyl sulfone (SH:VS) slightly greater than unity which differed with protein precursor. This non-stoichiometric maximum was likely due to disulfide bond formation during reaction. Higher storage modulus and lower swelling were generally observed with an increased molar ratio of thiol to vinyl sulfone (Fig. 47) A potential explanation for this phenomenon was decreased accessibility of reactive sites for crosslinking at lower SH:VS ratios. In similar work featuring non-degrading peptides with two cysteine residues, higher elastic modulus and lower swelling were observed for PEG bis(vinyl sulfone)s of higher functionality (3–8 arms) due to increased crosslink density [173]. Network elastic moduli in these systems reached a maximum at pH 8, likely because of slower kinetics at pH<8 and greater disulfide bond formation at pH>8. Furthermore, precursor concentration strongly influenced mechanical properties due to the propensity for cyclization reactions in dilute conditions.



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Fig. 46. Network formation of PEG bis(vinyl sulfone) and protein dithiols.

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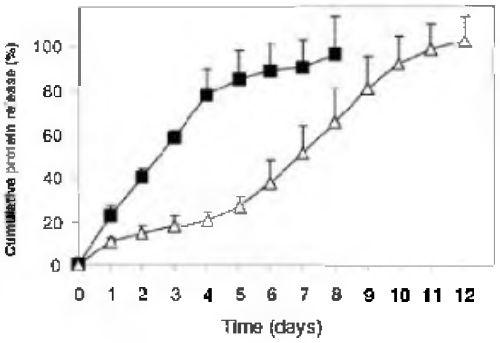
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Fig. 47. (a) Effect of pH on gel time for PEG bis(vinyl sulfone). Gel time decreases at higher pH due to the greater thiolate anion concentration. (b) Effect of thiol to vinyl sulfone ratio (SH:VS) on the storage modulus of PEG-protein networks. The G′ maxima occurs at ratios slightly greater than unity suggesting disulfide formation. Reproduced from 'Recombinant protein-*co*-PEG networks as cell-adhesive and proteolytically degradable hydrogel matrixes. Part I: development and physicochemical characteristics,' Rizzi and Hubbell, Copyright 2005 with permission from the American Chemical Society [10].

Michael addition networks were also developed for protein drug delivery applications. Protein drugs often suffer from rapid clearance from the bloodstream due to removal by the liver or kidneys. The use of drug delivery devices allows steady and long lasting delivery of protein drugs. Many current drug delivery materials such as poly(lactide)s can potentially denature the protein drug due to changes in pH associated with degradation byproducts. Another issue with current drug delivery materials is incorporating the protein into the material. Hubbell et al. studied Michael addition networks based on PEG multiacrylate and PEG dithiol that overcame many of these problems via incorporating bovine serum albumin protein during synthesis in aqueous media at physiological pH without denaturing the protein (Fig. 48) [9]. The crosslinking of these Michael addition networks required no basic catalyst or UV light that could potentially harm the protein as in photocuring of acrylate networks. Furthermore, the ester bonds in the PEG networks were degradable, allowing release of the protein as the network swells. The thiol-acrylate Michael addition reaction proceeded without significant side reactions with protein based amine nucleophiles, preserving the chemistry of the protein drugs. Furthermore, protein based free thiols are uncommon in extracellular proteins, lending greater selectivity to the approach. In cases where free thiols do exist, they are often

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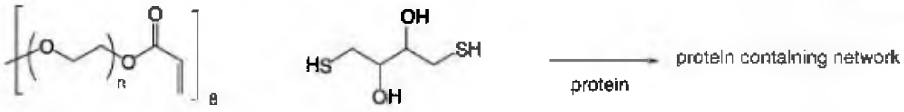
confined to sterically hindered locations in the tertiary structure of the protein. Hubbell et al. introduced the protein as well-defined ~100μm solid particles which were embedded into the Michael addition network during curing [9]. The release rate of the networks was tuned via the functionality of the PEG multiacrylate. PEG triacrylate networks resulted in rapid release of the protein. PEG tetraacrylate networks released protein over the course of 4d at a linear rate of 20% per day. PEG octaacrylate networks exhibited a slower release with a release rate of 10–15% per day. Hydrolytic degradation was observed in the swelling kinetics of these networks. A decrease in gel time with increasing solution pH (up to pH 8.2) indicated that the thiolate anion is the active species in the Michael addition with the acrylate.



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Fig. 48. Release of albumin from PEG networks based on PEG tetraacrylate (black squares) and PEG octaacrylate (white triangles). The lower functionality of the PEG tetraacrylate leads to lower crosslink density and more rapid release. Reprinted from J. Control Rel, 76, Hubbell et al. 'Protein delivery from materials formed by self-selective conjugate addition reactions,' 11–25, Copyright 2001, with permission from Elsevier [9].

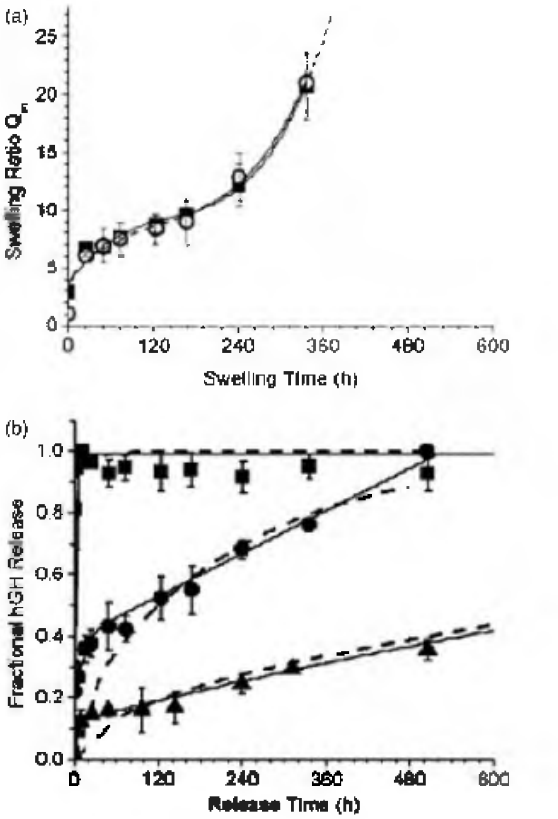
Hubbell et al. also studied protein drug delivery using Michael addition hydrogel networks from eight-arm PEG octaacrylate with dithiothreitol (Fig. 49) [174]. The protein drug human growth hormone (hGH), which suffers from rapid clearance was incorporated into these hydrogels without covalent attachment. Currently, daily administrations of this drug are required for treatment of the hormone deficiency as well as Turner's syndrome and renal failure. The crosslink density of the networks is controlled with the use of varying molecular weights of PEG octaacrylate, thereby influencing the drug diffusion. As shown in Fig. 50, the drug release rate was tuned to occur over periods from hours to months by changing the crosslink density of the networks. The ester sulfide linkages produced in the network degraded at a significantly faster rate than in free radically crosslinked acrylic networks, presumably due to neighboring group effects from the thioether group. Thus, the diffusion coefficient in these networks increases with time. The degradation rate of these networks varied from weeks to months and was directly related to the crosslink density.



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Fig. 49. Protein-containing PEG networks via Michael addition crosslinking of PEG octaacrylate with dithiothreitol. Human growth factor protein (hGH) was incorporated by mixing.

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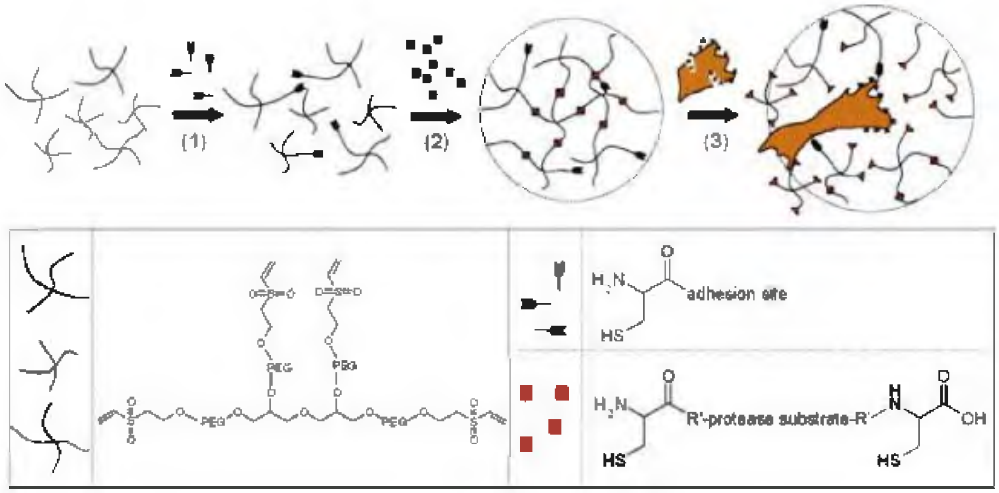
Fig. 50. (a) Swelling of PEG octaacrylate, dithiothreitol networks via initial solvent uptake followed by ester hydrolysis leading to degradation. (b) Release kinetics of hGH protein from networks as a function of PEG octaacrylate molecular weight (squares 10,000g/mol, circles 10,000g/mol mixed with 2000g/mol (50:50), triangles 2000g/mol). Reprinted from J Control Rel, 102, van de Wetering et al. 'The effect of the linker on the hydrolysis rate of drug-linked ester bonds,' 619–27, Copyright 2005, with permission from Elsevier [172].

Hubbell et al. also studied synthetic cell encapsulation networks [175]. These networks were synthesized from physically gelled four-arm Pluronics® (poly(ethylene oxide-*b*-propylene oxide-*b*-ethylene oxide)) functionalized with acrylate groups and thiols. These hydrogels have potential applications in cell encapsulation. The propensity of the thiol groups to undergo disulfide bond formation prompted the use of the thioacetic ester protecting group which was deprotected during crosslinking. The reversible physical gelation process for Pluronics occurred in aqueous solution when a cold, slightly acidic medium (5 °C, pH 6.8) was increased both in temperature and pH (37 °C, pH 7.4). Once the physical gel formed, the covalent crosslinking via the Michael addition reaction produced gels, which were biodegradable due to the presence of ester groups. Degradation in PBS at physiological conditions occurred over periods of 1–2 weeks.

In order to mimic the extracellular matrix and guide the growth of cells, synthetic matrices should be biocompatible, resistant to non-specific adsorption of proteins, degradable via cell- secreted matrix metalloproteinases (MMPs), and further promote cell-adhesion. Extending on an earlier study, Hubbell et al. synthesized hydrogels comprising multiarm vinyl sulfone-terminated PEG, a monocysteine containing adhesion protein, and a bis(cysteine) metalloprotein substrate protein. The adhesion peptide was introduced

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to provide adhesion sites on the surface of the hydrogel matrix. The unreacted vinyl sulfone groups were reacted with the bis(cysteine) terminated MMP substrate peptide. Fig. 51 depicts the stepwise formation of the hydrogel containing the adhesion proteins and the MMP substrate proteins as well as the attack on the substrate protein by a metalloproteinase [176].



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Fig. 51. Illustration of the stepwise synthesis of a PEG-based hydrogel by reacting multiarm vinyl sulfone-terminated PEG with small amounts of a monocysteine-containing adhesion peptide (1) followed by reaction of excess bis(cysteine) containing MMP substrate peptide (2). Attack of a cell-secreted MMP is also shown (3). Structures of the multiarm vinyl sulfone-terminated PEG (cross lines); monocysteine-containing adhesion peptide (green wedge shaped arrows), and bis(cysteine) containing MMP substrate peptide (orange divided squares) are shown in the legend. Reprinted from Proc Natl Acad Sci, 100, Lutolf et al., 'Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: Engineering cell-invasion characteristics.' 5413–5418, Copyright 2003, with permission from the National Academy of Sciences, USA [176].

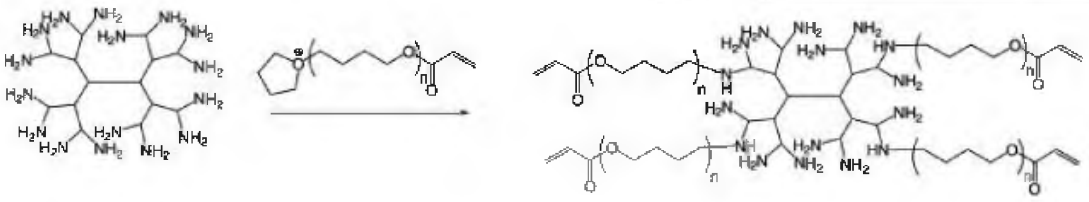
Analysis of the activity of an MMP to various substrates in the three-dimensional hydrogel indicated that the invasion of the cells was dependent on the enzymatic sensitivity of the substrate peptide and the rate of this process was dependent on the adhesion peptide concentration. Also the invasion of the cells decreased with an increase in crosslink density at lower PEG molecular weights. In order to demonstrate the capability of these hydrogels to promote tissue growth a bone protein containing hydrogel was placed in a rat cranial defect. Bone regeneration was observed after 4 weeks of hydrogel implantation and depended on the MMP sensitivity of the networks [176], [177]. Enhanced bone regeneration was observed due to the additional affinity sites for the bone protein in the matrix [178]. Similarly, the covalent incorporation of a vascular endothelial growth factor in the hydrogel matrix alongside adhesion peptides and MMP substrate peptides aided the regeneration of human endothelial cells and formation of new vascularized tissue in place of the biomaterial [179].

Ferruti et al. studied tissue scaffolds consisting of crosslinked poly(amido amine) hydrogels based on 2,2-bisacrylamidoacetic acid, which was crosslinked using primary diamines [8]. The networks degraded completely within 10d in Dulbecco's medium at physiological conditions, and cytotoxicity tests using direct

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contact with fibroblast cell lines demonstrated the non-cytotoxic nature of both networks as well as the degradation products. Bovine serum albumin was also incorporated into selected networks, through mixing with the network precursors prior to crosslinking. Ferruti et al. developed hydrogels containing agmatine, a guanidine functionalized amine precursor that resembles the RGD (arginine glycine aspartic acid) tripeptide, an agent for promoting cell adhesion [180]. The mechanical performance of these tissue scaffolds improved and degradation decreased through the use of a multi-amine functionalized crosslinker molecule, which was also synthesized through poly(amido amine) chemistry.

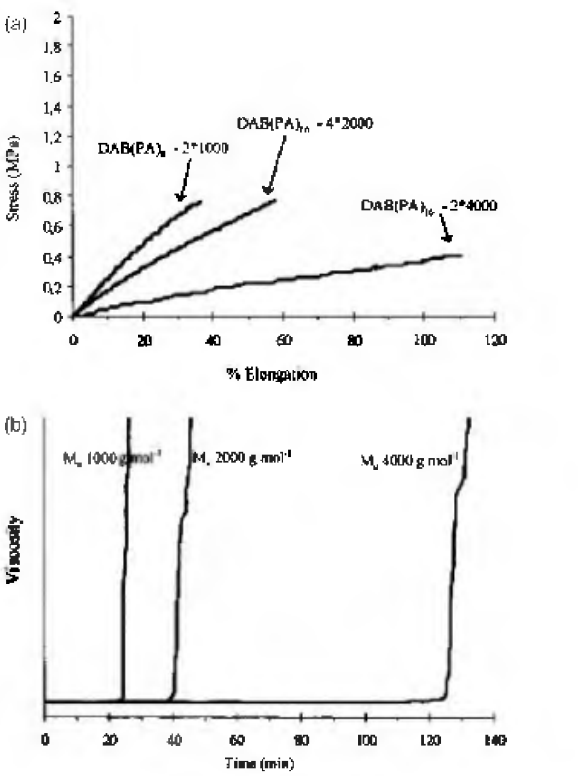
Goethals et al. reacted living cationic, acryloyloxybutyl triflate initiated poly(tetramethylene oxide) (PTMO) with Astramol™ dendrimers to produce acrylate functionalized dendrimers (Fig. 52) [181]. The presence of both amine and acrylate groups on the dendrimer molecules resulted in rapid gel formation with gel fractions of 88–99%. The pot life varied from 25 to 125 min with the molecular weight of the PTMO changing from 1000 to 4000 g/mol (Fig. 53) Higher degrees of swelling and higher tensile elongations were observed for higher molecular weight PTMO precursors. The pot life was also affected by the temperature of the reaction from 45 min at 25 °C to 4 min at 65 °C. The gel fraction increased with the incorporation of four PTMO arms as opposed to two arms.



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Fig. 52. Self-condensing dendrimer synthesized from PPI and acrylate initiated living PTMO.

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Fig. 53. (a) Effect of dendrimer generation and number of PTMO arms of various molecular weights on tensile strength. (b) Viscosity curves during crosslinking showed longer gel times at higher molecular weights. Reprinted from 'Segmented polymer networks containing amino-dendrimers,' Goethals et al., Copyright 2003 Society of Chemical Industry, with permission from John Wiley and Sons, Ltd on behalf of SCI [181].

7. Bioconjugates via the Michael addition

Strong interest in bioconjugates has developed in recent years. Bioconjugates consist of synthetic polymers covalently bound to biopolymers (proteins, polysaccharides, polynucleotides, antibodies) or bioactive species (pharmaceuticals). These materials possess special properties that make them useful in biomedical applications and fundamental studies. Coupling synthetic polymers to biomaterials is useful for numerous reasons. Polymer–protein bioconjugates may possess 'stealth' properties, resisting clearance from the bloodstream in terms of excretion from the liver or kidneys, thus increasing circulatory lifetimes of protein drugs. Furthermore, recognition properties may be imparted to synthetic polymers through the incorporation of receptor proteins. Recognition properties are important in artificial organs and implants as well as in biosensors. Polymer–protein and polymer–drug conjugates were used to improve solubility and circulation time within the body. In some cases, biomolecule linking using Michael addition chemistry was studied. Thus, Roy et al. used Michael addition to create sugar-protein conjugates from sialyloligosaccharides [182].

7.1. Polymer–protein and polymer-drug conjugates

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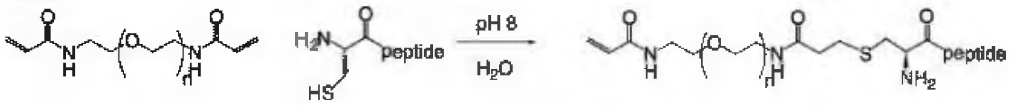
Poly(ethylene glycol) (PEG) is the most common synthetic polymer for coupling with proteins. PEG is a non-biodegradable polymer ideal for coupling to proteins due to its biocompatibility and nonabsorbent properties with respect to proteins. Currently, numerous functional PEGs are commercially available for applications in protein pegylation [183]. Michael addition of protein-bound thiol groups to maleimide functionalized PEG is one of the most common methods of polymer–protein conjugate synthesis. A second route is through Michael addition of thiol groups to vinyl sulfone functionalized PEG, resulting in greater hydrolytic stability. PEG containing two maleimide functional groups was used for mimicking the heavy chain end of antibodies and is also useful for binding two proteins in close proximity [183]. The coupling of thiol groups on cysteine residues with polymeric Michael acceptors benefits from several advantages. The Michael addition chemistry occurs readily at physiological conditions and is selective to thiol-containing proteins over amine nucleophiles. The reaction also produces no potentially toxic byproducts [184]. Hubbell et al. studied the reactivity of protein-bound cysteine residues as a function of charge environment [185]. Positively charged residues placed adjacent to the cysteine resulted in a lower pK_a of the cysteine group and increased Michael addition to PEG diacrylates. Negatively charged residues produced the opposite effect. The specific order of the residues adjacent to the cysteine groups had no apparent effect on reaction kinetics. Vinyl sulfone functionalized PEG molecules reacted selectively with cysteine residues over lysine residues at pH 8 and possessed greater hydrolytic stability. Harris et al. coupled reduced ribonuclease with PEG vinyl sulfone [6]. Reaction with lysine residues required a higher pH of 9.3 but was still dramatically slower than the reaction with cysteine at comparable pH.

Lysine residues of proteins also serve as functional group handles for the Michael reaction of acrylamides. This strategy benefits from the lack of dimerization of lysine residues in comparison to cysteine residues. A large number of protein-modifying PEG reagents are directed toward reaction with the amine groups contained in lysine residues. Such reagents include activated acylating agents such as *N*-hydroxysuccinimidyl ester-PEG (NHS-PEG). However, attachment to lysine residues is disadvantageous when the lysine residues are critical to protein binding or enzymatic active sites. Examples of proteins that show diminished activity upon pegylation using lysine targeting modified PEGs are asparaginase, α-galactosidase, and CD4 [186]. The reaction with lysine groups is also less controlled and often results in multiple PEG attachments due to the presence of numerous lysine residues. The use of cysteine via Michael addition to PEG reagents is advantageous in these cases and further benefits from the lack of synthetic byproducts. Thus, Kogan synthesized a maleimide terminated PEG capable of reaction with free cysteine residues in proteins [186].

Hubbell et al. have performed a great deal of work in the area of polymer–protein conjugates, often favoring the method of cysteine Michael addition [187]. PEG bisacrylamide and PEG diacrylate were studied as PEG substrates for coupling with proteins via the Michael addition. Residual acrylamide or acrylate groups permitted free radical crosslinking of the resulting pegylated proteins to create hydrogels with embedded proteins that serve as tissue scaffolds. The reaction of the acrylamide groups with cysteines was more than an order of magnitude slower than the corresponding acrylate reaction, but the acrylamide group possesses superior hydrolytic stability. The more hydrolytically stable and more Michael reactive vinyl sulfone linkage could not be used due to its inability to polymerize free radically. The tissue scaffold environment is critical to inducing particular phenotypes to embedded cells. PEG is well known to possess low adhesion to proteins, which allows the study of specific cell-adhesion agents such as RGD protein. Thus, Hubbell et al. introduced RGD protein to PEG bisacrylamide and formed networks [187] (Fig. 54) These networks were

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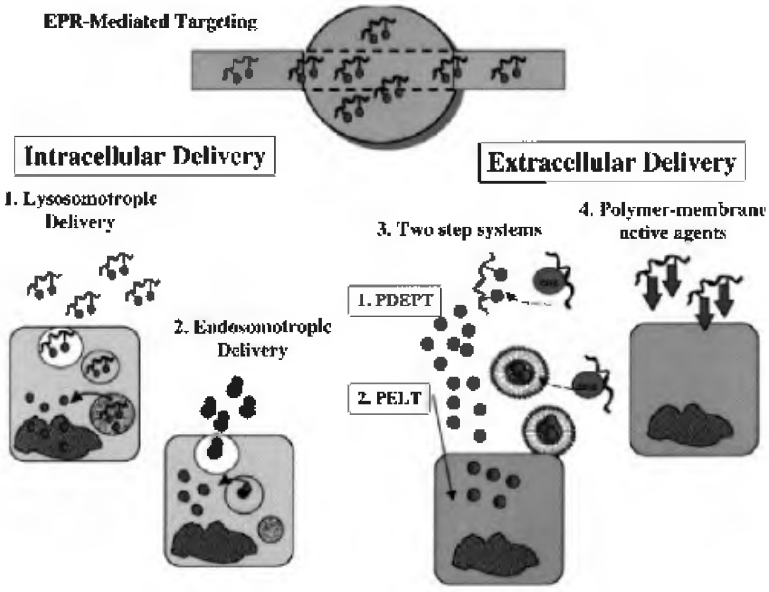
treated with cells that spread on the surfaces of the network, clearly well-adhered. Furthermore, the mechanical properties of the crosslinked tissue scaffolds were sufficient to produce durable materials.



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Fig. 54. Protein incorporation via Michael addition of thiol residues to PEG-acrylamide.

Polymer–drug conjugates are of special interest in anticancer therapy where numerous drugs have improved performance in comparison with the parent drug [188]. Conjugates of interest include poly(*N*-(2-hydroxypropyl)methacylamide) doxorubicin, poly(glutamic acid)-paclitaxel and PEG-camptothecin. Several reasons exist for the improved performance of these conjugates including improved solubility, lower toxicity, and longer retention in the bloodstream. Furthermore, some conjugates benefit from the enhanced vascular permeability and retention (EPR) effect, in which drugs are preferentially absorbed into tumor tissue and not released (Fig. 55) In fact, one reason for the lower toxicity of the polymer–drug conjugates is the absence of nonspecific uptake into nontargeted tissues and organs. Generally, polymer–drug conjugates must contain a hydrolysable or enzyme-cleavable linker to allow cellular uptake of the drug molecules at the tumor site. In some cases, linkers are introduced that cleave via lysosomic cysteine proteases. Generally, polymers used in these composites must either biodegrade or possess low enough molecular weight (<30,000g/mol) to allow removal through the kidneys.



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Fig. 55. Drug delivery via polymer–drug conjugates. Enhanced permeability and retention (EPR) mechanism of drug delivery relies on preferential permeation of the conjugate followed by drug release, without reverse permeation. Reprinted from J Control Rel, 74, Duncan et al., 'Polymer-drug conjugates, PDEPT and PELT: basic principles for design and transfer from the laboratory to clinic,' 135–146, Copyright 2001, with permission from Elsevier [188].

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Goodson and Katre accomplished pegylation of recombinant interleukin-2 protein using PEG-maleimide [189]. Interleukin-2 is a glycosylated protein that serves important functions in the immune system and is useful as an anticancer drug. The recombinant interleukin-2 was modified to include a cysteine site for Michael addition at the glycosidic linkage of the original protein, thereby preventing inhibition of the protein. The conjugation of recombinant interleukin-2 to PEG-maleimide resulted in an increase in hydrodynamic size and solubility, increasing systemic exposure four fold. An increase in hydrodynamic size results in less glomerular kidney filtration compared to the unmodified protein. Katre et al. showed a systematic decrease in clearance with increasing effective molecular weight up to 70,000 g/mol above which the rate of clearance was almost constant, possibly due to a different mechanism of clearance [190]. In previous work, modification of interleukin-2 via amine groups on lysine residues resulted in reduced bioactivity of the protein [191].

Jespers et al. studied recombinant staphlokinase pegylated at artificially introduced cysteine sites using PEG-maleimide [192]. Staphlokinase is an enzyme that is used to treat acute myocardial infarction, through thrombolysis of blood clots within blood vessels. Numerous sites on the enzyme were targeted for introduction of the cysteine residue. The circulatory lifetime of the staphlokinase was increased through pegylation while thrombolytic activity was maintained. The plasma clearance rates of the staphlokinase conjugate decreased with increasing PEG molecular weight. Clinical trials revealed successful restoration of circulation for patients suffering from acute myocardial infarction [193].

Currently, strong interest exists in functionalizing polymers with the RGD protein to promote cell adhesion for tissue scaffolds. The RGD protein selectively promotes cell adhesion, while avoiding nonspecific adsorption of proteins. Furthermore, cell spreading, a change in the cellular morphology, occurs in the presence of the RGD coating, indicating cell signaling has occurred. Hubbell et al. used a hetero-bifunctional PEG containing *N*-hydroxysuccinimide and vinyl sulfone linkages to couple the RGD containing peptides via cysteine linkages to the PEG and then to couple the PEG-RGD to poly(L-lysine) [194].

Surface grafting of biological molecules was achieved using the Michael addition reaction [184]. Model systems to achieve protein grafted surfaces were studied using PEG acrylated surfaces coupled to cysteine containing proteins such as portions of the Platelet factor 4 protein. A modification route beginning with thiol functionalized surfaces was also pursued, however, the surface grafted thiols were unreactive.

7.2. Coupling of biological molecules to Michael addition polymers

In addition to the Michael reaction as a strategy to couple synthetic polymers to biological molecules, it is also useful in the synthesis of polymers that will be coupled to biological molecules. Numerous Michael addition polymers are useful as precursors for bioconjugates due to their biocompatibility and biodegradability. Poly(amido amine)s are an excellent example of a biocompatible, biodegradable Michael addition polymer. Both linear poly(amido amine)s and dendritic poly(amido amine)s (PAMAM) proved useful in forming bioconjugates.

Ferruti et al. synthesized poly(amido amine) drug conjugates of the anticancer drug mitomycin C, which was bound to pendant hydroxyl groups on the poly(amido amine). In vivo studies showed similar antitumor activity for the conjugate compared to mitomycin C as well as lower toxicity values [45]. Poly(amido amine) dendrimers were also used to facilitate the release of the toxic anticancer drug 5-fluorouracil via covalent incorporation onto the terminal amines on the dendrimer structure [195]. Higher generation dendrimers

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released larger amounts of 5-fluorouracil, with complete release occurring over the period of about one week.

Glycodendrimers are dendrimers containing saccharide residues on the peripheral functional groups. Glycodendrimer interactions with proteins are often more pronounced than with individual saccharides due to increased 'valency'. However, the density of functional groups at the dendrimer surface may potentially lead to steric hindrance of the sugar groups. Meijer et al. introduced peripheral sugar groups at various distances from the surface of PPI dendrimers using alkyl spacers of various lengths, thus reducing steric congestion [196].

8. Conclusions and future directions

This review outlined the current applications of the Michael addition to polymer synthesis. The Michael addition reaction serves important roles in polymer synthesis, allowing the development of novel polymers from diverse monomer feedstocks. Polymers of numerous different topologies including linear, dendritic, hyperbranched and network polymers are enabled. Both step growth and chain growth techniques are utilized. The flexibility of the Michael reaction in terms of monomer functionality, solvent environment, and conversion at ambient or near ambient temperature allows the production of sophisticated macromolecular systems for technologically important applications. Other advantages include a general lack of sensitivity to oxygen and the absence of low molar mass byproducts, which require removal.

The applications of Michael addition polymers are as diverse as their compositions and topologies, ranging from networks for coatings and adhesives to linear, biodegradable polymers for gene transfection or drug delivery. Several Michael addition polymers such as poly(amido amine)s benefit from biocompatibility and non-toxic degradation products. Michael addition also serves as a synthetic tool for coupling polymers with biological systems such as proteins and enzymes. The ability to carry out the Michael addition under physiological conditions permits novel tissue replacements, cell scaffolds and direct application.

One aspect of the Michael addition largely unused in polymer science is the ability to carry out stereospecific Michael additions. The application of special catalysts to achieve stereospecific Michael addition may yield polymers with improved biological applications and potential crystallinity. Chirality is particularly important in biological systems including recognition processes with proteins. Another likely future direction for Michael addition polymerizations is the increased use of bio-based reactants. The number of bio-based precursors entering the market and the multitude of functionalities available for Michael addition chemistry are growing. Many of these materials are amenable to modification for introduction into Michael addition systems. Furthermore, the tolerance of the Michael reaction to protic impurities allows the use of less well-defined feed streams.

Acknowledgements

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Michael addition reactions in macromolecular design for emerging technologies

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Abstract

The Michael addition reaction is a versatile synthetic methodology for the efficient coupling of electron poor olefins with a vast array of nucleophiles. This review outlines the role of the Michael addition reaction in polymer synthesis with attention to applications in emerging technologies including biomedical, pharmaceutical, optoelectronic, composites, adhesives, and coatings. Polymer architectures, which broadly range from linear thermoplastics to hyperbranched polymers and networks are achievable. The versatility of the Michael reaction in terms of monomer selection, solvent environment, and reaction temperature permits the synthesis of sophisticated macromolecular structures under conditions where other reaction processes will not operate. The utility of the Michael addition in many biological applications such as gene delivery, polymer drug conjugates, and tissue scaffolds is discussed in relation to macromolecular structure.
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Keywords: Michael addition; Conjugate addition; Gene transfection; Drug delivery; Tissue scaffolds; Acetoacetates; Acrylates; Bioconjugates; Cysteine

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1. Motivation and scientific rationale

The Michael addition reaction, which is also commonly termed conjugate addition, has recently gained increased attention as a polymer synthesis strategy for tailored macromolecular architectures. The Michael addition, named for Arthur Michael, is a facile reaction between nucleophiles and activated olefins and alkynes in which the nucleophile adds across a carbon–carbon multiple bond [1]. The Michael addition benefits from mild reaction conditions, high functional group tolerance, a large host of polymerizable monomers and functional precursors as well as high conversions and favorable reaction rates [2]. The Michael reaction lends itself to both step growth [3] and chain growth polymerization [4] and has been employed in the synthesis of linear, graft, hyperbranched, dendritic and network polymers. Furthermore, post-polymerization modification [5] and coupling of biological and synthetic polymers are often facilitated by the Michael reaction [6]. These features make the Michael addition reaction well-suited to numerous emerging technologies including biomedical applications such as gene transfection [7], cell scaffolds [8] and tissue replacements [2].

The Michael addition reaction enables a wide range of polymers from diverse monomers, and corresponding polymers are prepared in environments in which other polymerization mechanisms will not operate. In biological applications such as protein derivitization the mild Michael addition reaction conditions are favorable since high temperatures, oxidizing radicals, and organic solvents are not feasible [9]. Furthermore, the Michael addition has recently found utility for the synthesis of crosslinked polymers such as hydrogels [10], thermoset resins [11], and coatings, where rapid cure and high conversions are necessary for performance. Few polymerizations offer sufficient rates to permit room temperature cure, and industrial coatings are often limited to toxic and environmentally hazardous isocyanate containing monomers. The Michael addition proceeds rapidly at room temperature, offers low cure times, and involves less toxic precursors. Non-linear optical materials were also realized using the Michael addition, which benefits from the absence volatile byproducts [12]. The Michael addition is also ubiquitous in classical polymer chemistry, such as the anionic polymerization of alkyl methacrylates and cyanoacrylates.

The Michael addition involves the addition of a nucleophile, also called a 'Michael donor,' to an activated electrophilic olefin, the 'Michael acceptor', resulting in a 'Michael adduct', as shown in Fig. 1b Although, the Michael addition is generally considered

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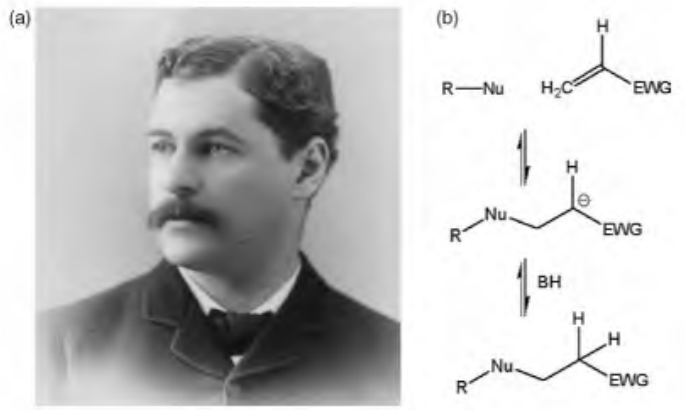


Fig. 1. (a) Arthur Michael (1855–1942), who discovered the Michael addition reaction. (b) Schematic depiction of the Michael addition reaction.

the addition of enolate nucleophiles to activated olefins, a wide range of functional groups possess sufficient nucleophilicity to perform as Michael donors. Reactions involving non-enolate nucleophiles such as amines, thiols, and phosphines are typically referred to as 'Michael-type additions'. In this review, we will refer to Michael-type addition reactions with all nucleophilic donors as Michael additions. The Michael acceptor possesses an electron withdrawing and resonance stabilizing activating group, which stabilizes the anionic intermediate. Michael addition acceptors are far more numerous and varied than donors, due to the plethora of electron withdrawing activating groups that enable the Michael addition to olefins and alkynes. Acrylate esters, acrylonitrile, acrylamides, maleimides, alkyl methacrylates, cyanoacrylates and vinyl sulfones serve as Michael acceptors and are commercially available. Less common, but equally important, vinyl ketones, nitro ethylenes, α,β-unsaturated aldehydes, vinyl phosphonates, acrylonitrile, vinyl pyridines, azo compounds and even β-keto acetylenes and acetylene esters also serve as Michael acceptors [13].

2. Introduction to the Michael addition reaction

The Michael reaction typically refers to the base-catalyzed addition of a nucleophile such as an enolate anion (Michael donor) to an activated α,β-unsaturated carbonyl-containing compound (Michael acceptor) as in Fig. 1b [14–18]. However, over the years, the scope of this reaction has increased dramatically to include a broad range of acceptors and the Michael-type additions of non-carbon donors. Due to the many types of Michael additions in the literature, we will focus here on investigating the mechanism and kinetics of only a few examples, namely the carbon–carbon bond forming Michael addition (referred to as the carbon-Michael addition), nitrogen (amine or aza) Michael additions, and the reaction of thiols with Michael acceptors.

2.1. The mechanism of the carbon Michael addition

One of the most well-known carbon-Michael transformations is the base-catalyzed addition of ethyl acetoacetate to methyl acrylate [19]. The mechanism of the reaction is fairly straightforward, with every step being in equilibrium and thermodynamically dependent on the relative strengths of the base and the type of acetoacetate. The acetoacetate is first deprotonated by the base, providing an enolate anion (Michael donor) in equilibrium (Fig. 2) The enolate anion then reacts in a 1,4-conjugate addition to the olefin of the acrylate (Michael acceptor). The carbonyl of the acrylate stabilizes the resulting anion until proton transfer occurs, regenerating the base. The overall driving force for the conjugate addition is the enthalpic change that accompanies replacement of a π-bond with a σ-bond. Thus, there is the preference for 1,4-addition over 1,2-addition. In some cases however, kinetically controlled reaction conditions can afford attack at the carbonyl carbon rather than at the β-carbon of the olefin [15,20].

From Fig. 2, one can observe that the rate determining step is the attack of the enolate anion on the activated olefin. The reaction rate is therefore second order overall and first order with respect to the enolate anion and the olefin acceptor. The concentration of the enolate is a function of the base strength and the K_{eq} of the deprotonation of the active methylene proton. It follows that the equilibrium constant is

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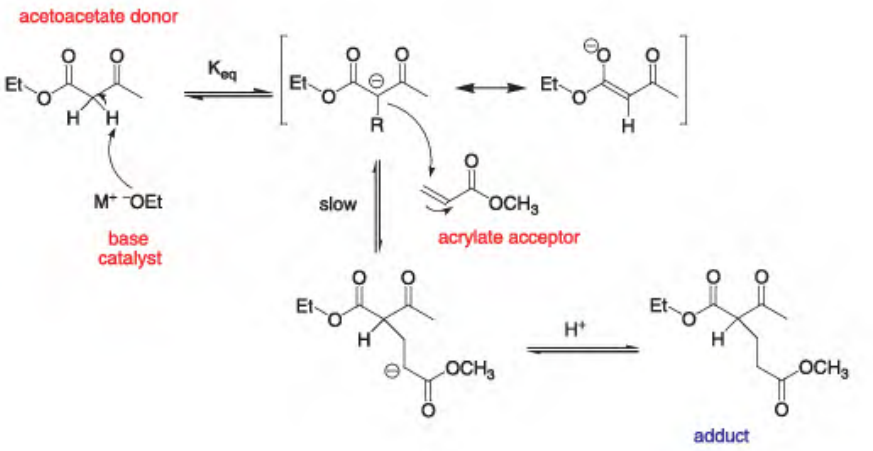


Fig. 2. General carbon-Michael reaction mechanistic scheme.

dependent on the relative strength of the base and the structure of the acetoacetate.

It is important to note that the product of the first Michael addition has a remaining active methylene hydrogen which can undergo a second addition to another acrylate (Fig. 3) Clemens et al. have documented that the second pK_a is expected to have a value of 13 (versus the pK_a of the initial active proton of 12) [21]. The second deprotonation therefore has a different equilibrium constant (K_{eq}) and it is assumed that the concentration of the first Michael adduct will be low. As a result, the concentration of the enolate at any rate (especially in the early stages of the reaction) is not strongly affected by this second reaction.

It follows that a rate law can be determined for the above reaction sequence. Writing the reactions in terms of the Michael addition, we arrive at the kinetic equations:

And corresponding rate equations:

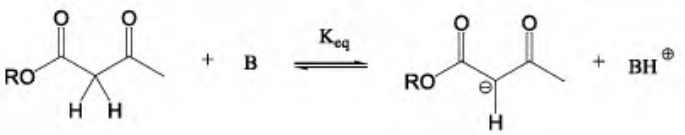
$$K_{eq} = [AcAc^-][BH^+]/[AcAc][B] \quad (3)$$

$$\text{Rate} = k_2[AcAc^-][\text{Acrylate}]$$

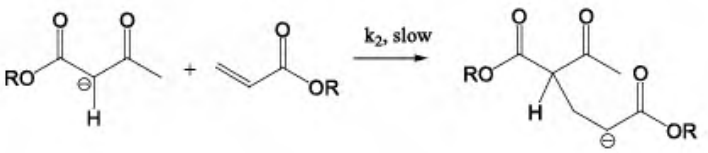
$$= k_2k_{eq}([B]/[BH^+])[AcAc][\text{Acrylate}] \quad (4)$$

2.1.1. Effect of base strength

The choice of base catalyst has a tremendous effect on the reaction kinetics. As previously noted, the concentration of the enolate and therefore the value of K_{eq} is highly dependent on the relative base strength. For strong bases, K_{eq} will lie far to the right and the concentration of the enolate anion is then approximately equal to the concentration of the base (achieving a steady state concentration). In the resulting rate expression



(1)



(2)

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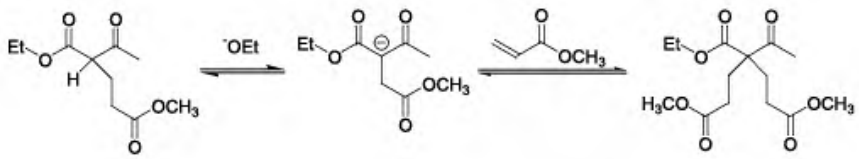


Fig. 3. Second Michael addition of acetoacetate group to methyl acrylate.

Eq. (5), there is a pseudo first-order dependence on acrylate concentration, where k_{obs} is a function of base concentration.

$$\text{Rate} = k_{obs}[\text{Acrylate}] \quad (5)$$

In the case of weaker base catalysts, K_{eq} has a moderate value, but is not large. This introduces the equilibrium constant into the rate law and results in second order behavior Eq. (4). Clemens et al. completed an extensive study on the effect of base strength on reaction kinetics and molecular weight when the Michael addition is used to prepare crosslinked acetoacetate resins for thermoset coatings [21]. In a model study, Clemens analyzed the ability of bases such as TMG (tetramethylguanidine), triethylamine, DBU (1,8-diazabicyclo[5.4.0]undec-7-ene), and DBN (1,5-diazabicyclo[4.3.0]non-5-ene) and found that the rate of the reaction was dependent on catalyst concentration as indicated in Eq. (4). For strong bases such as hydroxide, a steady-state concentration of enolate anion is achieved and pseudo-first order kinetics were observed as in Eq. (5).

The pK_a of the Michael donor is important to the choice of base catalyst, which must have a pK_a for its conjugate acid in the same range as the Michael donor. Stronger bases such as tetramethylguanidine, with a pK_a of 13.6 in its protonated form, are needed in the case of the carbon Michael addition of enolates due to the high pK_a of the acetoacetate groups (pK_{a1} = 12, pK_{a2} = 13.6). Base catalysts are often unnecessary in the case of amines, because of the strong nucleophilicity of the nitrogen atom, whereas weak bases aid in deprotonation of thiols.

2.1.2. Effect of solvent

Typical solvents for the carbon-Michael reaction include methanol, ethanol, diethyl ether, tetrahydrofuran, benzene, xylene, dioxane and mixtures of these solvents. Initially, protic solvents were desirable in the carbon-Michael reaction to promote rapid proton transfer and to stabilize charged intermediates, however, Schlessinger's group has shown that high yielding reactions were also achieved using aprotic solvents [22–24]. The choice of solvent strongly depends on the solubility of the catalyst, donor, and acceptor as well as sensitivity to side reactions. For example, if the reactants or products are susceptible to alcoholysis (ester exchange or hydrolysis), self-condensation of the substrate or the 'retro-Michael' reaction, a non-hydroxylic solvent is desirable [14,25]. In some cases, reactions are carried out in the absence of solvent, especially for network or coating applications.

2.1.3. Effect of substrate

As mentioned before, the initial step of the carbon-Michael addition is deprotonation of an active methylene proton. Thus, donors must stabilize the resulting negative charge. Examples of functional groups, which stabilize enolate donors include ketones, aldehydes, nitriles, amides, nitrones, sulfones, malonates and acetoacetates. A great deal of thought must be directed to the selection of the proper donor and base, and knowledge of the pK_a of these various species is critical. Furthermore, strong bases such as hydroxide and methoxide may promote side reactions such as ester hydrolysis, so non-nucleophilic bases are often desirable.

Acceptors in the carbon-Michael addition are typically olefins that are activated for nucleophilic attack. The resulting anion from nucleophilic attack of the donor must also be stabilized for the reaction to occur. Thus, the use of α,β-unsaturated carbonyl compounds is prevalent in Michael addition chemistry. The reactivity of the acceptor will decrease if the substituent is electron rich, such as in the case of alkyl, aryl or carboethoxy substituted olefins such as styrene derivatives or vinyl

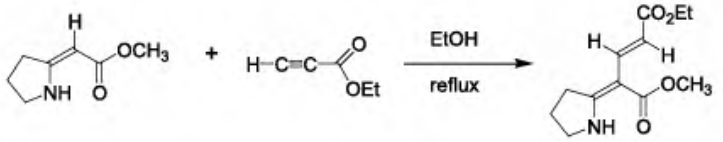


Fig. 4. Michael Addition involving an alkyne acceptor.

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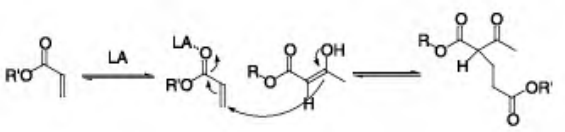


Fig. 5. Lewis-acid catalyzed Michael addition reaction.

ethers. Sterics are also an important factor. The larger the groups substituted at the α and β positions, the slower the reaction rate, although this can be offset by stronger polar effects [18,26]. Differences in reactivity between acceptors require differences in nucleophilicity of the Michael donors. For instance, alkyl methacrylates are relatively poor Michael acceptors. Thus, stronger nucleophiles such as carbanions are necessary for successful Michael addition to alkyl methacrylates. Better acceptors such as alkyl acrylate or acrylamide groups readily accept weaker amine and thiol nucleophiles.

Alkynes have also been utilized as Michael acceptors. In Fig. 4, electron-rich enamines react readily with activated alkynes in refluxing ethanol [27]. Products of Michael reactions with alkynes have the additional variables of stereochemistry of the unsaturated product, and regiochemistry of addition for disubstituted alkynes acceptors.

2.1.4. Other carbon-Michael catalysts

Although base-catalysis is most prominently used in the carbon-Michael addition, the reaction is also catalyzed with acids, particularly in the case of Lewis acids. Some of the earlier examples include the use of boron trifluoride, aluminum trichloride, and zinc chloride [28]. In these cases, the Lewis acid coordinates to the carbonyl of the acrylate to activate the olefin (Fig. 5) The coordinated complex will then react with the nucleophile to obtain the same adduct as in the base-catalyzed Michael addition. In the case where silyl enolates are donors, the reaction is often referred to as the Mukaiyama–Michael reaction.

Researchers have used Lewis acid complexes to their advantage over the years, especially in the area of enantioselective additions. For example, Heathcock's group has shown that silyl enolates will react enantioselectively with α,β-unsaturated ketones in the presence of $TiCl_4$ (Fig. 6) [29]. In the area of enantioselective carbon-Michael additions, the reader is directed to two recent reviews published on the subject [30,31].

Phosphines also catalyze the carbon-Michael reaction [13]. The reaction sequence begins with nucleophilic attack of the phosphine on the β-position of the olefin, generating a reactive phosphonium ylid (Fig. 7) The resulting anion can then either react as a nucleophile or as a base. If an acetoacetate is present in the reaction, the ylid deprotonates the acetoacetate first, which adds to the β-position of another olefin in a Michael fashion. Proton transfer then results in regeneration of the initial olefin and the phosphine catalyst.

2.2. Heteroatomic donors in the Michael reaction

Carbon-based nucleophiles represent only one class of donors used in Michael addition reactions. Heteroatomic nucleophiles involving nitrogen, sulfur, oxygen and phosphorus have also been used. In this section, two of these additions will be discussed: the aza-Michael reaction (amines) and the thiol Michael addition reaction. These are of primary importance due to the presence of sulfur and nitrogen nucleophiles in biological systems.

2.2.1. The aza-Michael reaction

The nitrogen-donor version of the Michael addition is often referred to as the aza-Michael reaction. Since only a few concepts will be touched upon here, the reader is encouraged to investigate several reviews for additional information [16,32–34]. Since amines can act as both nucleophiles and bases, no additional base is typically needed in these reactions. The reaction tends to follow second-order kinetics based on the concentration of the olefin acceptor and the amine (Fig. 8)

Primary amines can react with two equivalents of acceptor to form tertiary amines. In some cases, this

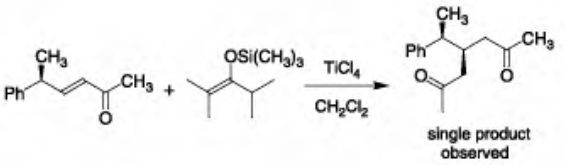


Fig. 6. Stereoselective Michael addition between enone and silyl enolate in the presence of $TiCl_4$.

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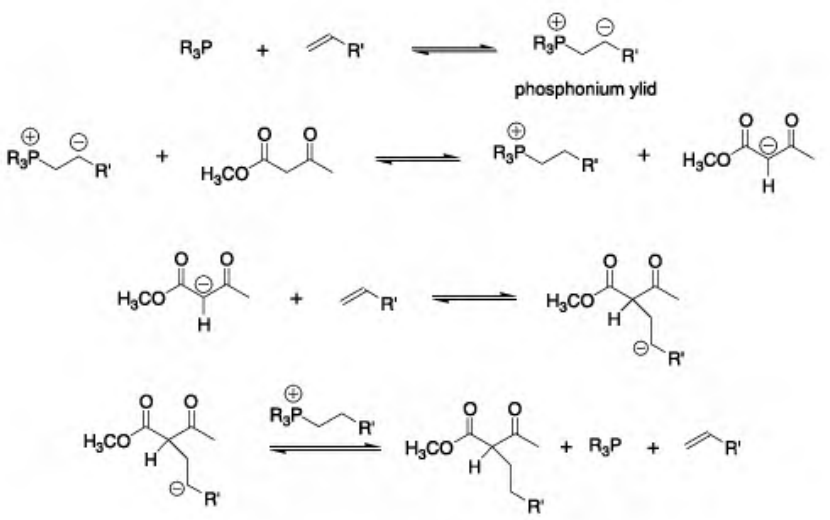


Fig. 7. Phosphine catalyzed Michael addition reaction.

second addition affects the observed kinetics, especially as the concentration of the secondary amine increases. An example is the reaction of methyl amine with ethyl acrylate, giving an excellent yield of the tertiary amine (Fig. 9) [35].

In the aza-Michael reaction, secondary amines are more nucleophilic than primary amines and are therefore more reactive. However, it is worth noting that this is highly dependent on the electronic and steric environment of the amine. For example, 1,4-butanediol diacrylate was allowed to react with 1-(2-aminoethyl)piperazine in an equimolar ratio (Fig. 10) [36]. During the initial part of the experiment, it was found that there was exclusive reaction with the secondary amine present in the piperazine ring. Only upon longer reaction times, did reaction occur with the primary amine, which led to polymerization.

Acid catalyzed aza-Michael additions have also been studied extensively. Spencer and coworkers have determined a general and efficient way of reacting carbamates (NHCOOR) with α,β-unsaturated carbonyl compounds (Fig. 11), providing precursors to β-amino acids [32].

Vedejs and Gringas have also shown that acids catalyze the aza-Michael addition [37]. In Fig. 12, a tertiary amine, as the donor, adds to the alkyne in the presence of catalytic *p*-toluenesulfonic acid. The resulting intermediate further undergoes a Claisen rearrangement and proton transfer to afford the final α,β-unsaturated amine.

Lewis acids have also been used to catalyze the aza-Michael reaction, as in Fig. 13. The mechanism is thought to occur in an analogous fashion to the

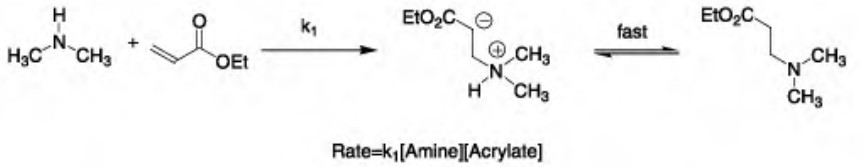


Fig. 8. Aza-Michael addition reaction of dimethylamine with ethyl acrylate.

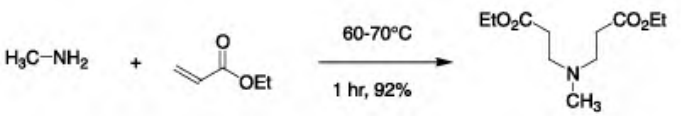


Fig. 9. Aza-Michael addition of methyl amine to two equivalents of ethyl acrylate.

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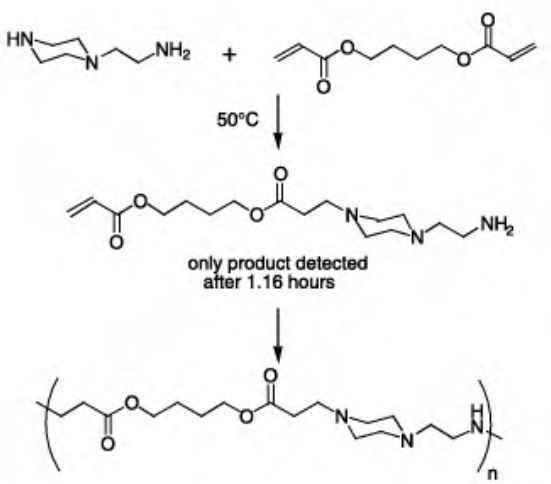


Fig. 10. Higher reactivity of secondary amines in aza-Michael addition reactions.

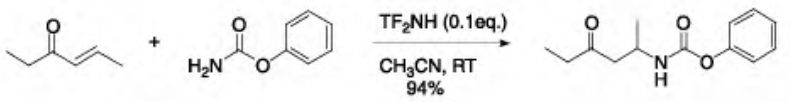


Fig. 11. Acid catalyzed aza-Michael addition.

carbon-Michael addition where the Lewis acid coordinates to the carbonyl of the α,β-unsaturated olefin. However, Spencer has reported that, in the case of weakly basic amine nucleophiles, Brønstead acids that are formed during the reaction may catalyze the reaction [32].

The ability to generate β-amino carbonyl compounds has become increasingly important to the natural product and pharmaceutical areas. The aza-Michael reaction is a key transformation which enables the preparation of such compounds. Jørgensen was the first to report the enantioselective conjugate amine additions (Fig. 14) [38]. The $TiCl_2$-BINOL Lewis acid catalyst coordinates with the *N*-acyloxazolidinone, creating a chiral environment in which the primary amine will preferentially attack from one face of the complex, resulting in the formation of one major diastereomer. The oxazolidinone ring is then removed to afford the β-amino acid. Although, the enantioselectivity is low (maximum enantiomeric excess of 42%), the proof of concept was accomplished. The area has grown significantly and Sibi has published two reviews which discuss stereoselective aza-Michael additions to generate β-amino acids [30,39].

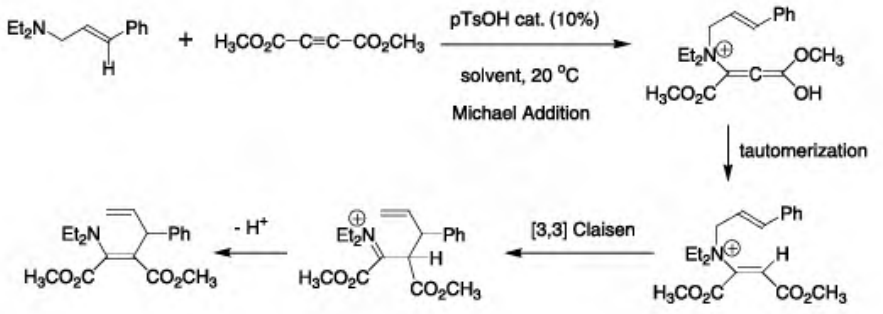


Fig. 12. Acid catalyzed aza-Michael addition to activated alkyne acceptors.

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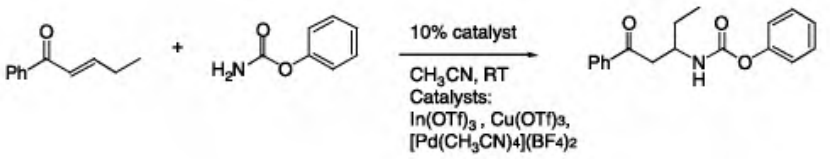


Fig. 13. Lewis acid catalyzed aza-Michael reaction.

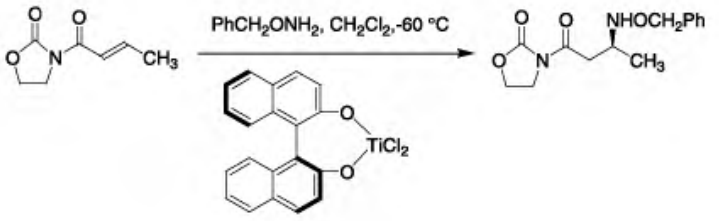


Fig. 14. Stereoselective aza-Michael additions.

2.2.2. *Thiols as Michael donors*

Thiols can also be used as heteroatomic donors in the Michael addition. Many of the same reaction scenarios that were described above can be directly applied to the addition of thiols to activated olefins. Thiols are generally more nucleophilic than amines, although bases are often used to deprotonate them due to their comparatively higher acidity. The thiolate anion is often the active species in Michael additions involving thiols. The thiol Michael addition reaction rates increase with pH due to the increased concentration of the thiolate anion [10]. The thiol group is a useful Michael donor in biological systems where thiols are present on proteins in cysteine residues. One primary side reaction in thiol Michael additions is disulfide bond formation, which often prompts the use of protecting group chemistry.

Regioselective Michael additions of thiols to asymmetrically substituted fumarate esters or amides was achieved using lithium salts as Lewis acids or base catalysts to alter the charge environment around the double bond through coordination of carbonyls or deprotonation of the thiol (Fig. 15a) [40]. Enantioselective Michael additions of thiols to methacrylates and other 1-alkyl acrylates was accomplished with chiral lanthanide catalysts to enantiomeric excesses as high as 90% ee [41]. Enzyme catalysis of the thiol addition to 2-butenal was shown with *Candida antarctica* lipase B [42]. The active site of the enzyme in this reaction is described in Fig. 15b Suprisingly,

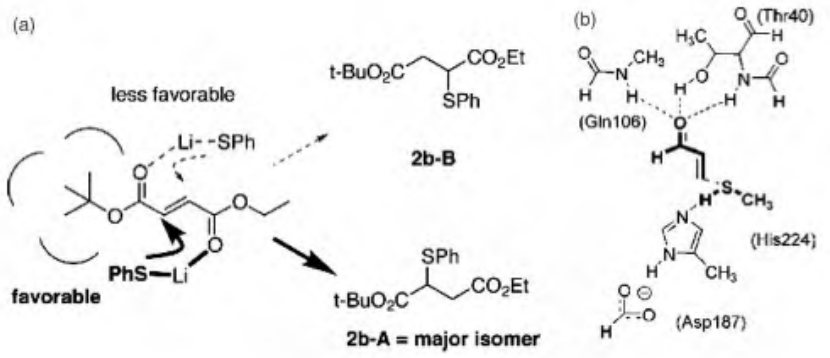


Fig. 15. (a) Regioselective Michael addition through preferential coordination of substrate carbonyls with lithium cations, reprinted from Tetrahedron, 59, Kamimura et al. 'On the regioselectivity for the Michael addition of thiols to unsymmetrical fumaric derivatives,' 9537–9546, Copyright 2003, with permission from Elsevier [40] (b). Michael addition in the active site of wild *Candida antarctica* lipase B, reproduced from 'Exploring the active-site of a rationally redesigned lipase for catalysis of Michael-type additions,' Carlqvist et al. Copyright 2006 Society of Chemical Industry with permission from John Wiley and Sons, Ltd on behalf of the SCI [42].

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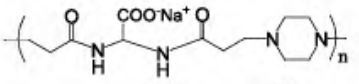
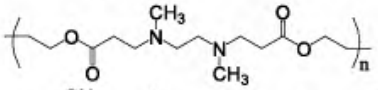
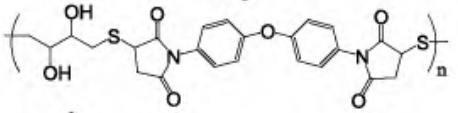
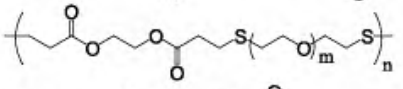
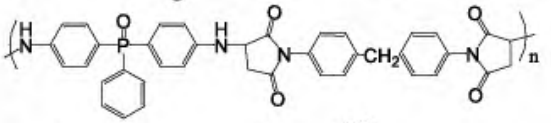
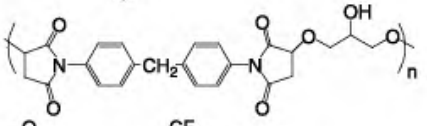
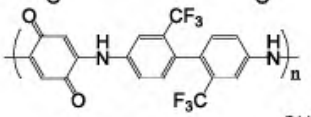
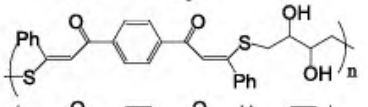
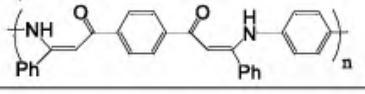
aqueous sodium dodecyl sulfate solutions perform as powerful catalysts of the Michael additions of thiols or amines to enones [43]. Firouzabadi et al. proposed that the accelerating effect of the sodium dodecyl sulfate related to the concentrating of the hydrophobic species into the micellar core.

3. Linear step growth Michael addition polymerizations

3.1. Overview

Significant earlier attention was devoted to linear step growth Michael addition polymerizations. Polymers that were produced via Michael addition polymerizations have ranged from segmented elastomers to high T_g engineering thermoplastics. Both rigid, amorphous, aromatic systems as well as semicrystalline aliphatic systems were pursued and a wide variety of monomers were employed. Numerous examples of step growth Michael addition polymerizations exist, however, a few main classifications exist, outlined in Table 1. Perhaps the earliest example is poly(amido amine)s, which were synthesized from bisacrylamides and diamines. A second class of step growth polymers are derived from bismaleimides and diamines, a type of poly(imido amine) referred to as poly(aspartamide)s in the literature. Thiol nucleophiles are involved in the synthesis of poly(imido sulfide)s, which are also based on bismaleimide monomers. Analogous poly(ester sulfide)s and poly(amino ester)s synthesized from diacrylates are potentially biodegradable due to the hydrolytic instability

Table 1
Step growth polymers derived from Michael addition polymerisation

		References
Poly(amido amine)		[53]
Poly(amino ester)		[61]
Poly(imido sulfide)		[68]
Poly(ester sulfide)		[69]
Poly(aspartamide)		[70]
Poly(imido ether)		[76]
Poly(amino quinone)		[3]
Poly(enone sulfide)		[68]
Poly(enamine ketone)		[81]

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of the ester linkages. Poly(amino quinone)s are a unique class of polymers which possess redox activity [44] and serve as anticorrosion coatings on metals. α,β-unsaturated alkyne esters and ketones also serve as Michael acceptors in some cases and step growth polymerizations based on amine and thiol nucleophiles were pursued. Finally, hydrogen transfer polymerization allows the synthesis of polyamides and polyesters from AB-type Michael addition polymerizations. The high conversion of the Michael addition reaction and lack of side reactions are prerequisites for high molecular weight in step growth polymerizations, which is clearly obtained in numerous subsequent examples.

3.2. Poly(amido amine)s

Poly(amido amines) are synthesized through the reaction of secondary diamines with bisacrylamides at 15–60 °C in water for durations of hours to days. Also, primary amines, which have difunctionality in the Michael addition are employed in poly(amido amine) synthesis (Fig. 16) Ferruti et al. synthesized linear poly(amido amine)s from a range of monomers including piperazine bisacrylamide and *N,N′*-dimethyl-1,6-hexanediamine [45,46]. The poly(amido amine) polymerizations occurred rapidly at room temperature in protic media such as alcohols and water without catalyst. The rate of the Michael addition polymerization increased with protic solvents and was less dependent on solvent polarity or dielectric constant. Generally, these polymerizations do not proceed to high molecular weight in aprotic solvents. The rate of these polymerizations increased with the basicity of the secondary diamines, and with lower steric hindrance [47]. For instance, polymerization rates increased in the order: *N,N′*-diisopropylethylenediamine < *N,N′*-dimethylethylenediamine < 2-methylpiperazine < piperazine.

Numerous variations of poly(amido amine) polymerizations exist. Amino acids may be copolymerized with bisacrylamides, as long as triethylamine is added to deprotonate the amino acid zwitterion and an amino acid with low steric bulk is employed. Successful polymerization occurred with glycine, taurine and β-alanine. Peptide oligomers may also be used as comonomers as long as the amine terminus is a simple amino acid such as glycine. Monomers such as divinylsulfones and diacrylates [48] can replace bisacrylamides as Michael acceptors. Segmented poly(amido amine ether)s were derived from PEG bis(piperazine) monomers [49]. Other routes to poly(amido amine)s yield different ordering of amido and amino functionalities including homopolymerization of protected aminoacrylamides with in situ deprotection. Yet another route is the reaction of activated acrylic acid derivatives with secondary diamines through amidation and the also Michael addition [50]. Hydrazine and alkylated hydrazines were also employed in the synthesis of poly(amido amine)s, however, if excess bisacrylamide monomer is used, then crosslinking occurs with residual NH groups along the polymer backbone at long reaction times [47,51]. Thermal stability of aliphatic poly(amido amine)s is fairly low, and thermal decomposition occurs near 200 °C. Poly(amido phosphine)s are synthesized in a similar manner to poly(amido amine)s, with the exception that free radical inhibitors are employed to prevent radical catalyzed addition of the phosphines to the bisacrylamides. Poly(amido phosphine)s exhibit greater thermal stability than poly(amido amines), with decomposition temperatures near 360 °C.

Aliphatic poly(amido amines) often possess crystallinity and are water soluble due to the presence of tertiary amines in the backbone, which undergo partial protonation in water. Poly(amido amine)s exhibit a tendency toward amide hydrolysis. Ferruti et al.

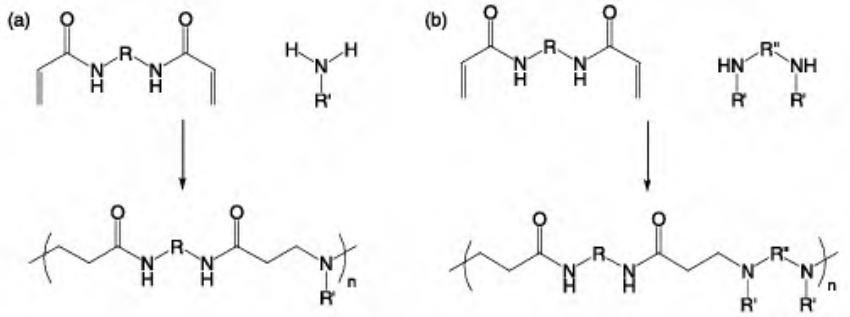


Fig. 16. Poly(amido amine) synthesis via (a) primary and (b) secondary amines.

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demonstrated increased solution viscosity versus time during polymerizations at high temperatures (100 °C) in water, with a subsequent decrease in viscosity at later times. This attribute along with low toxicity of the amino acid degradation products enables poly(amido amine)s as degradable pro-drug conjugates [52]. Poly(amido amine)s based on hydrazine were found to decrease in molecular weight by a factor of four over 2 d in water at physiological conditions, and were also found to possess low cytotoxicity [51].

Linear and crosslinked poly(amido amine)s were found to complex with heparin, an anticoagulant in the human body [45]. In medical practice, when heparin levels are too high in the body, protamine sulfate is administered, which is also an anticoagulant. A solution to this problem is the use of linear or crosslinked poly(amido amine)s to remove heparin from the blood stream. Poly(amido amine)s selectively remove heparin over other plasma components and do not cause hemolysis or affect other properties of blood such as prothrombin time. The poly(amido amine)s were also recycled through removal of heparin upon treatment with alkaline solutions of pH 11. Heparin, when coated on non-physiological objects, reduces the thrombus formation response of the human body. Thus, poly (amido amine)s also find utility in creating non-thrombogenic surfaces through covalent attachment to surfaces. Ferruti et al. developed synthetic methods for surface attachment of poly(amido amine)s to Dacron™ and poly(vinyl chloride) (PVC) using Michael addition of terminal acrylamide groups with surface tethered amine groups [46]. These surfaces readily adsorbed heparin without heparin release.

Ferruti et al. reacted ethylene sulfide with piperazine to yield dithiol monomers, which were polymerized with bisacrylamides. The resultant polymers were quaternized with methyl iodide, yielding water soluble antimicrobial polyelectrolytes [52]. These polymers exhibited antimicrobial activity even against resistant strains such as *Pseudomonas aeruginosa*. The polymers also possessed surprisingly low rates of hemolysis relative to commercial antimicrobial polymers.

Ferruti et al. investigated further biomedical applications of poly(amido amine)s in creating platinum (II) complexes with antitumor activity [53]. Polymer–drug conjugates have improved the effectiveness of drugs through a combination of increased solubility and slow drug release. The net result is called the enhanced permeability and retention (EPR) effect. Linear poly(amido amine)s based on 2-methylpiperazine, carboxylated bisacrylamides, and amino-β-cyclodextrin moieties were complexed directly with cisplatin. The degree of cisplatin complexation and release rate of platinum increased for polymers containing the cyclodextrin moiety. Release was suppressed however, with the carboxylated bisacrylamide, and non-carboxylated bisacrylamides were also studied. Both in vivo and in vitro toxicity of most complexes were lower than pure cisplatin, and similar antitumor activities were observed. Earlier work on poly(amido amine)s revealed effectiveness in reducing tumor metastasis in Lewis lung tumors and Sarcoma 180 carcinoma implanted in mice [54]. The reduction in cancer metastasis was attributed to specific interactions of these charged polymers with cell membranes. Neuse et al. also studied poly(amido amine) complexes of cisplatin, using 1,2-dihydroxylated, 1,2-dicarboxylated or 1-hydroxy-2-carboxyl functional amine monomers that chelate to the platinum center [55].

Recently, Ferruti et al. devoted considerable effort to studying the delivery of genes and proteins via complexation with poly(amido amine)s. One advantage of poly(amido amine)s is rapid protonation and expansion at low pH inside tumor cells which releases the genes or proteins and a compact geometry at plasma pH 7.4 to effectively hold the desired agent prior to delivery. Small angle neutron scattering (SANS) studies revealed that decreasing the pH- of aqueous poly(amido amine)s solutions increases the radius of gyration from ~2 to 8 nm [56]. Furthermore, poly(-amido amine)s possess dramatically lower toxicity than conventional non-viral vectors such as poly(ethylene imine). In some cases, poly(amido amine)s were prepared that exhibited a 'stealth' behavior and maintained long circulatory lifetimes [57]. Duncan et al. recently investigated poly(amido amine)s that contained covalently bound endosomolytic protein melittin (a main component of bee venom) [58]. These conjugates were less toxic than pure melittin and were found to possess pH- selective hemolytic properties at pH 5.5 and not at pH 7.4 at low concentrations of the conjugates. Some of the conjugates were also effective in delivering the non-permeant toxin gelonin into cells. Segmented copolymers based on poly(amido amine)s containing carboxylic acid and hydroxyl functional blocks also showed promising endosomolytic and gelonin delivery activity [59]. Gene transfection studies of poly(amido amine)s have also demonstrated successful polyplex formation with λ Hind III DNA and transfection of β-galactosidase into HepG2 cells, with similar transfection efficiency as Lipofectin® and poly(ethylene imine) [7]. Ferruti et al. coupled bovine serum albumin and human serum albumin to poly(amido amine)s based on

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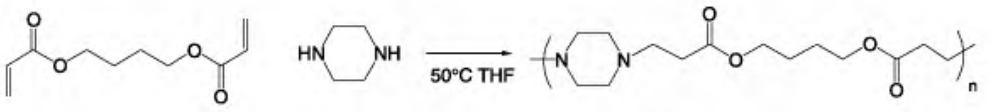


Fig. 17. Poly(amino ester) synthesis from butanediol diacrylate and piperazine.

piperazine and piperazine bisacrylamide through Michael addition of protein amine groups to terminal acrylamide groups on the poly(amido amine)s [60]. The reaction was conducted under mild conditions (pH 8–8.5, 25 °C) to high conversion and the coupled product was verified by size exclusion chromatography (SEC).

Poly(amino ester)s are similar to poly(amido amine)s, however, they utilize diacrylates instead of bisacrylamides. Langer et al. studied the use of Michael addition step growth linear poly(amino ester)s as gene transfection agents (Fig. 17) [61]. Like poly(amido amine)s, poly(amino ester)s have benefits over conventional gene transfection agents (such as poly(ethylene imine) or poly(L-lysine)) due to their low cytotoxicity as well as biodegradability into low toxicity byproducts. Furthermore, the structure and degradability of the poly(amino ester)s are tailored through the incorporation of a wide range of diamines and diacrylates. Langer et al. obtained high molecular weight poly(amino ester)s (32,000 g/mol) and performed successful complexation with plasmid DNA. The absence of a retro-Michael addition in these systems eliminates the possibility of carcinogenic diacrylate production during degradation. The degradability of these transfection agents is expected to aid in the release of the DNA inside the cell.

Non-biological applications of poly(amido amine)s also exist. Recently, Ferruti et al. examined the introduction of NLO chromophores into both linear and crosslinked poly(amido amine)s [12]. The Michael addition chemistry was well-suited to the primary amine containing chromophores and mild reaction conditions prevented chromophore degradation [62]. Initial studies showed that solution optical properties of the chromophores such as two photon pumped fluorescence were maintained in the polymer.

3.3. Poly(imido sulfide)s

Maleimides possess two carbonyl groups conjugated to the double bond, presenting a highly electron poor double bond susceptible to reaction with a range of nucleophiles. Poly(imido sulfide)s result from the step growth polymerization of dithiols with bismaleimide monomers. White and Scaia polymerized aromatic dithiols with aromatic bismaleimides in *m*-cresol with catalytic amounts of tri-*n*-butylamine at 80–100 °C [63]. The acidity of the *m*-cresol prevented the anionic homopolymerization of the maleimide groups upon reaction with thiolate anions, which would have resulted in crosslinking. The poly(imido sulfide)s containing aromatic thiol units were susceptible to degradation from bases such as diethylamine in aprotic solvents which was attributed to retro-Michael addition regeneration of the maleimide group. Aliphatic dithiol-containing polymers were less susceptible to depolymerization. Poly(imido sulfide)s with molecular weights ranging from 70,000 to 450,000 g/mol possessed glass transition temperatures from 185 to 212 °C. Semicrystalline poly(imido sulfide)s were produced through the reaction of aliphatic dithiols with aliphatic bismaleimides [64]. Melting temperatures of approximately 77 °C were observed. Semicrystalline poly(imido sulfide)s possessed stresses at break near 12 MPa and elongations of 120%. Thermal decomposition of the poly(imido sulfide)s occurred between 336–380 °C, and likely resulted from a retro-Michael addition depolymerization. Hydrogen sulfide serves as an effective dithiol in the synthesis of poly(imido sulfide)s and reacts very rapidly with bismaleimides in the presence of catalytic quantities of amines such as *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) or acids such as acetic acid (Fig. 18) [65]. Poly(imido sulfide)s were also obtained from biscitraconimides (methyl substituted maleimides),

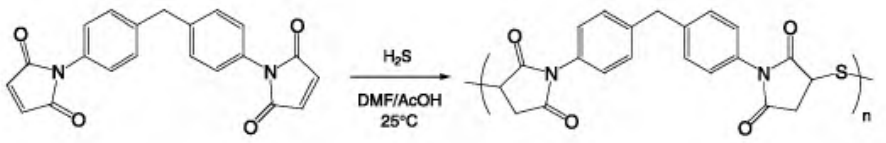


Fig. 18. Poly(imido sulfide) synthesis from a bismaleimide precursor and hydrogen sulfide gas.

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which resulted in geminal substitution of the sulfide group on the maleimide ring at the same position as the methyl group [66].

Segmented polymers based on poly(imido sulfide) chemistry were developed by Crivello and Juliano [67]. These polymers were synthesized in an analogous fashion to polyurethanes, starting with an oligomeric 'soft block' consisting of a thiol terminated polysulfide (Thiokol™ resin) and reacting with an excess of aromatic bismaleimide monomer in cresol with tri-*n*-butylamine catalyst at room temperature. The reactive oligomer is chain extended through reaction with hydrogen sulfide to produce the poly(imido sulfide) 'hard block'. The poor thermal stability of the polysulfide oligomers limited the processing temperature of the polymers to less than 150 °C, but tensile strengths as high as 10 MPa and 500% elongation were obtained. The thermal instability limited the choice of bismaleimide monomer to maintain a T_g that was less than 100 °C. These segmented poly(imido sulfide)s exhibited two glass transition temperatures in dynamic mechanical analysis, indicating the presence of microphase separation. The rubbery plateau (service window) ranged from −30 to 130 °C. Increasing the hard segment content above 30 wt% led to higher tensile strengths with lower extensibilities and poor processability. The polysulfide containing segmented elastomers benefited from excellent solvent resistance.

Dix et al. showed that changing between protic and aprotic monomers in aprotic solvents influenced the topology of poly(imido sulfide)s (Fig. 19) [68]. Bismaleimides were reacted with oligomeric dithiols

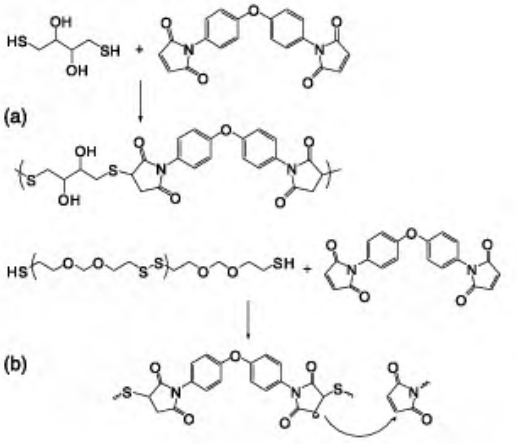


Fig. 19. (a) A typical poly(imido sulfide)s synthesis. (b) Possible crosslinking side reaction through maleimide homopolymerization observed for aprotic oligomeric dithiols.

as well as low molar mass dithiols in the presence of catalytic quantities of triethylamine. Oligomeric bisthiols reacted with *N′*,*N′*-bismaleimido-4,4′-diphenylmethane to create crosslinked networks due to the reaction of the carbanion formed upon Michael addition with additional maleimide groups. The use of a protic small molecule dithiol monomer, dithiothreitol, resulted in linear polymers without crosslinking, due to protonation of the carbanions by hydroxyl groups on dithiothreitol. Triethylamine does not initiate maleimide polymerization, but deprotonates thiols, which are capable of polymerizing maleimides. For example, an oligomeric dithiol was reacted with excess monofunctional maleimide in the presence of triethylamine resulting in anionic polymerization of the maleimide from the chain ends of the oligomeric dithiol.

The acrylic analog to poly(imido sulfide)s, poly (ester sulfide)s, were synthesized using PEG dithiol and butanediol diacrylate or PEG diacrylate using triethylamine base catalyst [69]. Varying the length of the PEG spacer enabled hydrophilicity control. The reaction kinetics were studied in a number of aprotic solvents and increased in the order benzene < dioxane ≪ chloroform with increasing dielectric constant. Weight average molecular weights ranged from 5000 to 28,000 g/mol and glass transition temperatures from −50 to −60 °C were observed. Crystallinity was observed in selected poly(ester sulfide)s.

3.4. Poly(aspartamide)s

Poly(aspartamide)s are a class of condensation polymers derived from bismaleimides and diamine monomers. Poly(aspartamide)s exhibit properties similar to poly(imide)s yet benefit from a facile, one-pot synthesis and increased solubility in organic solvents relative to aromatic poly(imide)s and poly(amide)s [70]. Poly(aspartamide) synthesis relies on a facile Michael addition of the amine functional group to the maleimide double bond and does not require a high temperature poly(amic acid) cyclization reaction as for poly(imide)s.

Crivello synthesized poly(aspartamide)s through the reaction of aromatic diamines with aromatic bismaleimides in cresol at 110 °C for 3 d with catalytic amounts of acetic acid [71]. The lower basicity of the aromatic amines allowed the use of acetic acid, which activated the maleimide group, through protonation [72]. Model reactions suggested that the polymerization mechanism involved charged intermediates due to increased rates in polar solvents. The poly(aspartamide)s exhibited glass transition temperatures near 210 °C and thermal

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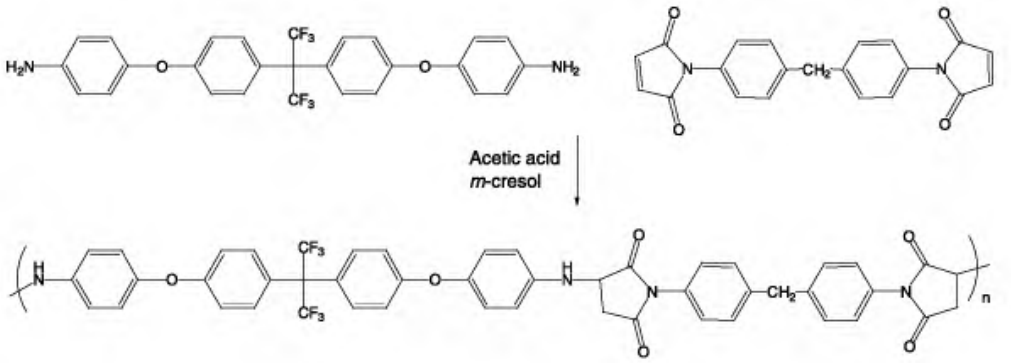


Fig. 20. Thermally stable poly(aspartamide)s synthesized from arylene ether diamines.

decomposition temperatures near 350 °C. This decomposition occurred at the same temperature (TGA) in both air and nitrogen atmospheres, suggesting that depolymerization occurred rather than oxidation. The poly(aspartamide)s possessed tensile strengths near 95 MPa and elongations of 5.5%. Flexural strengths and moduli as high as 140 MPa and 3.1 GPa, respectively, were reported.

Liaw studied poly(aspartamide)s based on arylene ether diamines with improved thermal stability and 10% weight loss temperatures near 400 °C (Fig. 20) [73]. The molecular weight of the poly(aspartamide)s increased with increasing monomer concentration, from $M_n = 7900$ at 0.38 M to $M_n = 12{,}800$ at 1.0 M. Weight average molecular weights ranged from 24,000 to 68,000 g/mol.

White and Scaia synthesized amorphous elastomeric poly(aspartamide)s via the polymerization of aliphatic secondary diamines and aliphatic bismaleimides [64]. Acetic acid was used in the polymerization of aromatic diamines and bismaleimides but not with aliphatic diamines due to the tendency to protonate the amine groups and prevent polymerization. The poly(aspartamide)s undergo slow crosslinking at ambient conditions likely due to Michael addition reaction of amine and maleimide end groups and subsequent homopolymerization of the maleimide group. Amorphous poly (aspartamide)s possessed elongations of 1700%, stresses at break near 9.3 MPa, and glass transition temperatures ranging from 0 to 86 °C.

Poly(aspartamide)s have potential application in second order non-linear optical (NLO) materials due to their high glass transition temperatures (~260 to 290 °C) which help maintain the critical orientation of the NLO chromophores. In addition, poly(aspartamide)s do not exhibit poling interferences due to water evolution as seen with polyimides during cyclization of the precursor poly(amic acid). Wu et al. used Michael addition to obtain an NLO-containing poly(aspartamide) pre-polymer with maleimide end groups that was thermally cured to obtain the final product [74]. At higher curing temperatures, the glass transition temperature of the cured poly(aspartamide)s appeared to increase, which demonstrated that curing continued to 250 °C.

Poly(amide aspartamide)s are similar to poly(aspartamide)s with the exception that polymerization occurs via both Michael addition of amines and maleimides and amidation reactions of amines and carboxylic acids [75]. Poly(amide aspartamide)s are synthesized through a step growth polymerization of an AA′ monomer containing a carboxylic acid and a maleimide functional group with a B_2 diamine monomer such as those shown in Fig. 21. The polymers are thus synthesized in a sequential process beginning with the Michael addition of the nucleophilic amine groups to

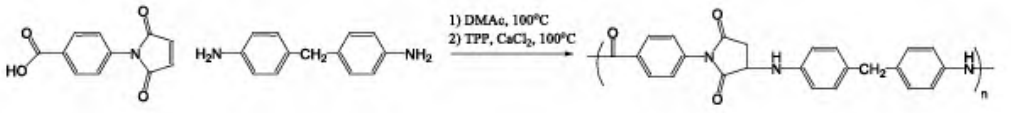


Fig. 21. Typical poly(amide aspartamide) polymerization. The first heating step produces the Michael adduct while the second step promotes amide bond formation.

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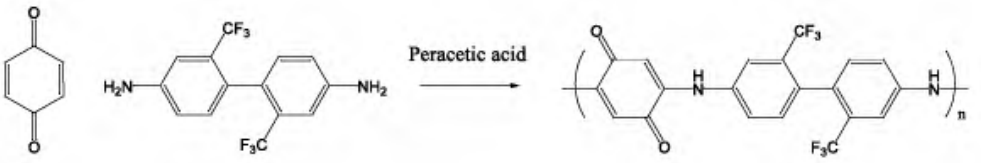


Fig. 22. Poly(amino quinone) synthesis involving peracetic acid as an oxidizing agent.

the maleimide group followed by the amidation at the carboxylic acid. These polymers are controllably branched through the addition of AB_2 monomers. One drawback of the poly(amide aspartamide)s is their relatively low thermal stability, with typical thermal degradation temperatures of 300–360 °C at 10% weight loss. The char yields of the polymers increased on end-capping the carboxylic acid end groups with aniline.

Oxygen nucleophiles are also effective in step growth polymerizations with bismaleimides. Hulubei and Rusu copolymerized glycerol and phenolphthalein with aliphatic bismaleimides resulting in poly(imido ethers) [76]. The reaction was performed in NMP using triethylamine or 2-mercaptobenzothiazole as catalysts at 85–115 °C for 2–3 d. In the case of glycerol, the difference in reactivity of the secondary and primary alcohols allowed synthesis of pendant hydroxyl polymers. Thermogravimetric analysis revealed initial weight losses ranging from 215 to 400 °C. Polymerization of bismaleimides with phenols was successful in solution (95–110 °C) and in the melt (180 °C), and fiber-glass reinforced composites were prepared [77].

3.5. Poly(amino quinone)s

Poly(amino quinone)s are synthesized from primary diamines and quinone, through Michael additions on both sides of the quinone (Fig. 22) [3]. The unsaturated nature of the quinone is maintained in the polymer, using either excess quinone or other oxidizing agents. Use of peracetic acid leads to improved yields and simplified purification. Poly(amino quinone)s have recently found application in anticorrosion coatings and moisture resistant adhesives. Numerous traditional adhesives suffer from delamination from solid surfaces in the presence of water. This is due to a stronger interaction of water with the surface. Poly(amino quinone)s, however, displace water on metal surfaces and bind preferentially. The strong interaction of these polymers with metal surfaces is thought to arise from the electron donating capabilities of the quinone group. Poly(amino quinone)s inhibit corrosion on stainless steel surfaces even in the presence of sulfuric acid, through the formation of a protective coating that forms spontaneously on the metal surface. These corrosion inhibiting coatings are used for ship hull protection as well as protection of magnetic particles on data storage media. Diamino quinone containing polyimides are used to improve the corrosion resistance of interlayer dielectrics in microelectronics [78]. Poly (amino quinone)s have also been introduced into polyolefins and polyurethanes.

Similar to poly(amino quinone)s are poly(thiophenylene)s formed from aromatic dithiols and

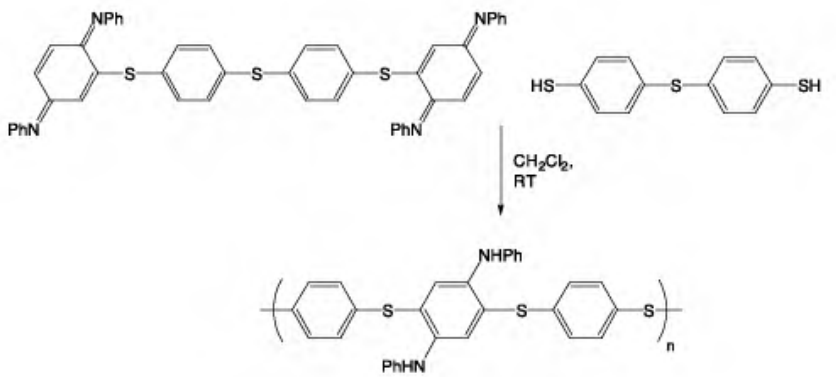


Fig. 23. Polymerization of poly(thiophenylene)s via Michael addition and oxidation.

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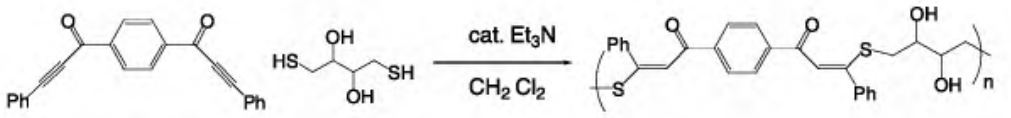


Fig. 24. Poly(enone sulfide) synthesis via Michael addition step growth.

bis(1,4-phenylenediimine)s, depicted in Fig. 23. The product of the Michael addition between the thiol groups and this nitrogen analog to quinone aromatizes through proton transfer to the imine nitrogens resulting in a poly(thiophenylene) with pendant amino groups [79]. The polymerization proceeds without a catalyst. These polymers exhibit electroresponsive behavior and are redox active under acidic conditions. The number average molecular weight of the polymer was 5400 g/mol and the glass transition temperature was 131 °C.

3.6. Step growth polymers derived from bisacetylene-containing monomers

Numerous step growth Michael addition polymers are synthesized from bisacetylenic precursors. The acetylene bond reacts electrophilically when conjugated to electron withdrawing groups, in a similar fashion to an olefin as noted earlier. Typical functional groups that serve as Michael receptors are conjugated acetylene ketones and conjugated acetylene esters. These carbon–carbon triple bond Michael acceptors react with oxygen, sulfur and amine nucleophiles and adducts possess the additional feature of stereochemistry at the double bond [80]. Michael addition polymers based on acetylenic precursors possess surprising thermal stability and produce remarkably tough thermoplastics.

Sinsky et al. studied poly(enamine ketone)s synthesized through the Michael addition reaction of aromatic diamines with aromatic bis(alkynone)s [81]. These nucleophilic additions to acetylenic compounds result primarily in *Z* isomer formation. The reactions were performed in *m*-cresol at 100 °C without catalyst. Glass transition temperatures between 198 and 235 °C were obtained and thermogravimetric decomposition temperatures were nearly 300 °C. The poly(enamine ketone)s were tough thermoplastics, with tensile strengths of 85 MPa (63 MPa at 93 °C), tensile moduli of 2.27 GPa, and elongations of 4%.

Bass et al. synthesized poly(enone sulfide)s, the sulfur analog to poly(enamine ketone)s through the reaction of aromatic dithiols (e.g. 1,3-benzenedithiol) with aromatic internal bis(alkynone)s in *m*-cresol at room temperature with *N*-methyl morpholine as a catalyst at temperatures from 25 to 40 °C [82]. The polymers had moderately high glass transition temperatures ($T_g \sim 107$–156 °C) and thermal decomposition temperatures near 330 °C. Tough, clear films were obtained from solution casting and exhibited tensile strengths of 78 MPa and tensile moduli near 3.2 GPa. Dix et al. also synthesized poly(enone sulfide)s from internal bis(alkynone)s and found a *Z:E* ratio of 60/40 (Fig. 24) [68]. Model reactions of thiols with phenylacetylene resulted in almost exclusive formation of the *Z* isomer [83].

3.7. Hydrogen transfer polymerization

Nylon-3 type polyamides are also synthesized via a Michael addition process. The base catalyzed polymerization of acrylamide to poly(β-alanine), as shown in Fig. 25, was originally proposed as an anionic chain growth process, which occurred with proton transfer of the amide NH_2 on the growing chain end to the enolate carbanion. This process was described as a 'hydrogen transfer polymerization'. However, it was later classified as a step growth polymerization due to proton transfer from the growing enolate carbanion to amide NH_2 groups on other monomers. Bush and Breslow demonstrated this via monitoring molecular weight as a function of monomer conversion [84]. At very short reaction times, high monomer conversion was achieved but high molecular weight product was not obtained suggesting initial dimerization and trimerization. Longer reaction times led to high molecular weight poly(β-alanine). These polymerizations were typically conducted at 80–120 °C in aprotic solvents such as DMF in the presence of radical inhibitors. Further work from Lim et al. demonstrated that branching occurred in these polymerizations in aprotic solvents due to propagation of both enolate and amide anions, which

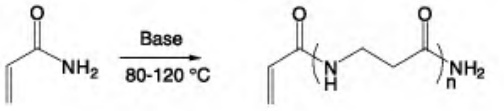


Fig. 25. Hydrogen transfer polymerization of acrylamide to poly(β-alanine).

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are formed before and after hydrogen transfer, respectively [85]. Hydrogen transfer polymerization of acrylamide in the presence of carbon black and *n*-butyl lithium was effective in grafting of poly (β-alanine) onto the carbon black via polymerization from surface lithium alkoxides [86]. The grafted carbon black showed improved dispersability in water and organic solvents, and grafting ratios of 60–80% were achieved. Recently, the hydrogen transfer polymerization process was applied to *N*-acetylacrylamide and *N*-benzoylacrylamide and the proton transfer mechanism was preserved despite the steric hindrance and lower acidity of the amide NH groups [87]. Hydrogen transfer polymerization was also achieved with *N*-acryloyl-*N'*-*p*-tolylureas [88], *p*-styrenesulfonamides, heterocyclic base containing acrylamides [89], and maleimide.

In a similar nature to the hydrogen transfer polymerization of acrylamide, hydroxyalkyl acrylates are also polymerizable through Michael addition chemistry as AB monomers. The polymerization of hydroxyethyl acrylate was performed at temperatures from 85 to 150 °C in the presence of free radical inhibitors using bases such as sodium hydride and potassium *tert*-butoxide as initiators [90]. Molecular weights as high as 9000 g/mol were obtained and hydroxyethyl acrylate polymerized more effectively than hydroxyethyl methacrylate. The primary side reaction in these polymerizations is the disproportionation of hydroxyethyl acrylate to ethylene glycol diacrylate and ethylene glycol.

4. Linear chain growth Michael addition polymerizations

A classic example of Michael addition polymerization is the anionic chain-growth polymerization of methacrylate monomers. This review is not intended to comprehensively review anionic polymerization but rather to place the Michael addition in context for trends in polymer synthesis strategies. Anionic polymerization has been reviewed extensively in the literature [4,91,92]. The relatively low reactivity of the methacrylate, as noted earlier, towards Michael addition chemistry demands a stronger nucleophile, thus carbanionic species such as diphenylhexyllithium and nitrogen anions such as lithium diisopropyl amide(LDA) are suitable for the polymerization of alkyl methacrylates. One current limitation is the requirement for cryogenic temperatures to prevent potential nucleophilic attack of the enolate anion on ester groups. Group transfer polymerization, which was developed in the mid-1980s, enabled polymerization at ambient temperatures due to the use of a reversible silyl protecting group that was proposed to stabilize the propagating chain end. Anionic polymerization was demonstrated with acrylamides and acrylate monomers, later, and this may complicate step growth processes in some cases [93]. The polymerization of cyanoacrylates is another chain growth Michael addition process. Cyanoacrylates are initiated with very poor nucleophiles, such as water, due to the electron poor olefin and strong resonance contributions of the adjacent cyano and ester groups. Cyanoacrylate polymerizations are widely used in rapid cure adhesives, and adhesive products possess extremely high bond strengths.

4.1. Anionic polymerization of methacrylates

Anionic polymerization of alkyl methacrylates is an established chain growth polymerization technique, which relies upon Michael addition chemistry. Anionic polymerization is known for producing polymers of controlled molecular weights and narrow polydispersities. Anionic polymerization is particularly suited for well-defined block copolymers [94] and star-shaped polymers via chain-end coupling strategies. A further advantage of anionic polymerization is the potential for controlling tacticity, which was achieved for the sterically bulky *tert*-butyl methacrylate monomer in toluene by Long et al. [95]. Anionic polymerization is generally initiated using alkyllithium initiators with sufficient nucleophilicity to attack alkyl methacrylate monomers. However, due to the potential side reaction of the carbanionic chain end with ester carbonyls, the reaction must be conducted at low temperatures and with sterically hindered carbanions such as diphenylhexyllithium [94]. These bulky carbanions react selectively at the double bond, resulting in an enolate Michael adduct, as shown in Fig. 26.

The strong nucleophilicity of the anionic PMMA growing chain end was used in chain end functionalization strategies in numerous cases [92]. For example, addition of di-*tert*-butyl maleate at the end of the polymerization results in succinate ester end groups [96]. These end groups were then pyrolyzed to produce reactive anhydrides capable of imide coupling with amine functional polymers. Long et al. acheived end functionalization with hydrogen bonding groups via Michael addition of heterocyclic base pairs to acrylated polystyrenes (Fig. 27) [97]. Termination of the anionic polymerization of styrene with ethylene oxide created hydroxyl functionality which allowed esterification with acryloyl chloride. Michael addition of nucleotide

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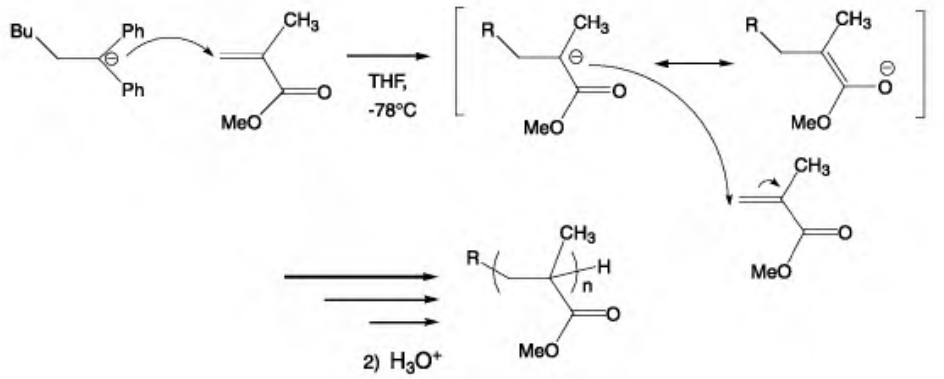


Fig. 26. Anionic polymerization of methyl methacrylate from diphenylhexyl lithium.

bases then allowed facile functionalization of the polystyrene chains, without detracting from hydrogen bonding properties. Adenine and thymine functionalized polystyrene demonstrated association in ^{1}H NMR spectroscopic investigations.

Long et al. [94,98] as well as Müller et al. [99] studied the synthesis of diene/acrylic, styrene/acrylic and all acrylic triblock copolymers containing *tert*-butyl methacrylate, which were later hydrolyzed and neutralized to obtain carboxylate block ionomers. The bulkier ester alkyl prohibits carbonyl ester attack at room temperature. Long et al. also established the utility of lithium diisopropylamide (LDA) as an initiator for methacrylate polymerizations in THF at −78 °C, despite the relatively low nucleophilicity of the hindered amide ion [100]. Anionic polymerization of acrylates, [93] maleimides, vinyl ketones [101] and acrylamides [102] are also known.

In some cases, anionically polymerized methacrylates have been designed for biologically related applications. For instance, Okamoto et al. synthesized macrocyclic ether containing acrylic monomers, which polymerized anionically to obtain highly isotactic polymers capable of functioning as synthetic ion channels in hexadecyl phosphate vesicles (Fig. 28) [103]. In contrast, radical polymerization of macrocyclic ether monomers yielded atactic polymer with dramatically lower ion transport.

4.2. Group transfer polymerization

Commonly, anionic polymerization of alkyl methacrylates is conducted at cryogenic temperatures. Such temperatures are generally not industrially feasible, except in the cationic polymerization of isobutylene, and significant research effort was directed towards a methodology that was amenable to higher temperatures. Group transfer polymerization (GTP) is a polymerization methodology that was developed in the mid-1980s by DuPont as a method of producing narrow polydispersity methacrylate and acrylate polymers and block polymers above room temperature

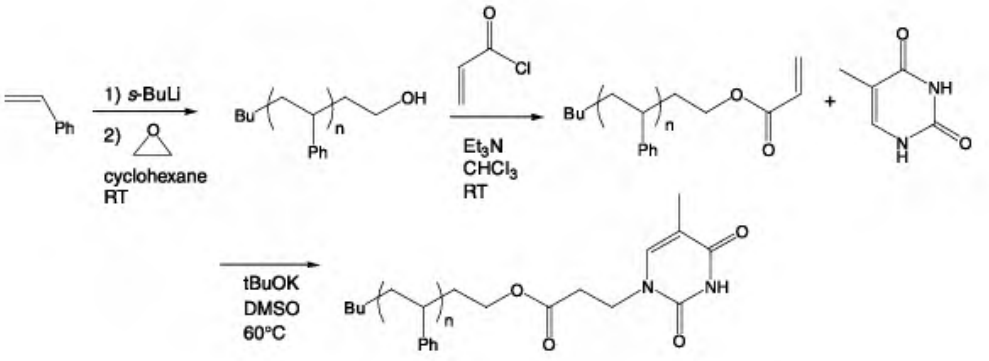


Fig. 27. Michael addition of heterocyclic nucleotide bases to acrylated anionic polystyrene.

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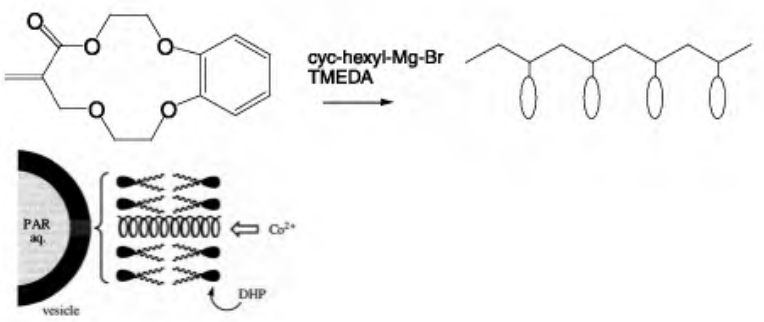


Fig. 28. Polymerization of macrocyclic ether acrylic monomers to form ion channels. Reprinted from Polymer, 43, Okamoto, et al. 'Stereospecific anionic polymerization of α-(hydroxymethyl)acrylate derivatives affording novel vinyl polymers with macrocyclic side chains', 3469–3474, Copyright 2002, with permission from Elsevier [103].

[104]. GTP is conveniently conducted at 80 °C using a silyl ketene acetal initiator in the presence of a nucleophilic anionic catalyst (Fig. 29) Initially, GTP was believed to proceed through a transfer of a silyl protecting group from the enolate oxygen on the chain end to monomers during the propagation step [105]. This initial mechanism has largely been disproven in favor of a dissociative anionic polymerization mechanism which involves a reversible dissociation of the silyl protecting group from the enolate oxygen, aided by anionic catalysts (HF_2^-, bibenzoate anion). The position of the equilibrium between the free enolate and the silyl protected enolate determines the rate of the polymerization. Similar to anionic polymerizations, the reaction is terminated through the addition of a protic species such as methanol. GTP was used by DuPont to produce acrylic block copolymers for pigment dispersing applications in automotive paints [104].

4.3. Anionic polymerization of α-cyanoacrylates

Cyanoacrylates are a class of monomers which undergo rapid anionic polymerization initiated by weak nucleophiles such as water, as shown in Fig. 30. These rapidly polymerizing monomers are used primarily in

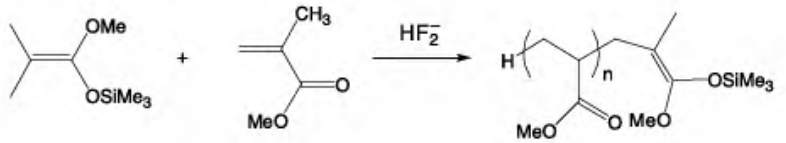


Fig. 29. Group transfer polymerization of methyl methacrylate.

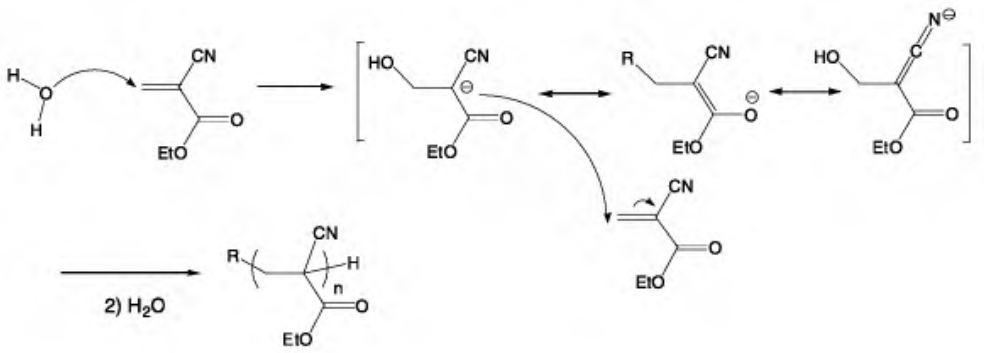


Fig. 30. Water-initiated polymerization of ethyl α-cyanoacrylate.

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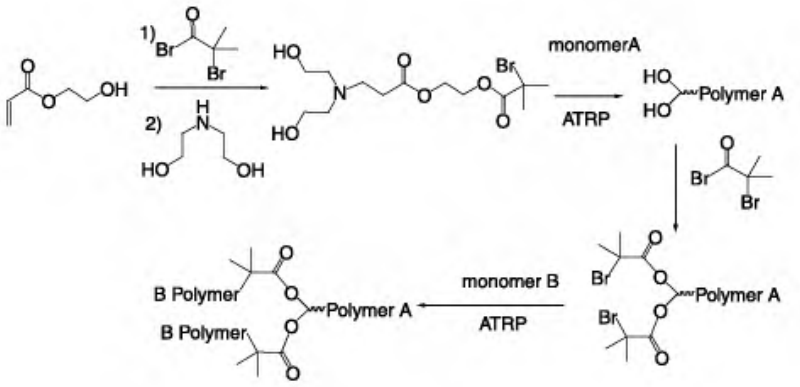


Fig. 31. Synthesis of a dihydroxyl functional ATRP initiator.

the adhesives industry as a high strength rapid setting adhesives [106]. The added electron withdrawing character of the nitrile group facilitates anionic polymerization of these monomers. Unlike conventional anionic polymerization, however, molecular weight control is absent. Klemarczyk studied the polymerization of ethyl cyanoacrylate using phosphine and amine initiators [107]. The initiated monomer species is zwitterionic, which makes it an effective nucleophile towards further monomer addition. Primary and secondary amines intiate more slowly than tertiary amines due to proton transfer after the first addition of monomer, which results in a neutral, less reactive adduct. In terms of current biomedical applications, octyl cyanoacrylate has recently found application as a tissue adhesive and gained FDA approval for surgical use [108]. This monomer polymerizes on contact with a wound and maintains flexibility due to the pendant octyl chain and furthermore possesses low water solubility.

4.4. Living radical polymerization

Living radical polymerization encompasses several techniques for producing well-defined homopolymers, block copolymers, and star polymers. Due to the radical nature of this polymerization methodology, a wider range of monomers are accessible compared to traditional anionic polymerization and less stringent purification techniques are required. Numerous living radical polymerization methods are currently known, including atom transfer radical polymerization (ATRP), which involves activated alkyl halides and metal catalysts [109], stable free radical polymerization (SFRP), which utilizes nitroxides [110], and reversible addition fragmentation chain transfer polymerization (RAFT), which typically utilizes dithioester or dithiocarbamate chain transfer reagents [111]. Michael addition reactions have recently proven useful in many of these living radical polymerization strategies.

Functional initiation is of interest for the synthesis of well-defined polymers with the potential for block copolymer synthesis, coupling with proteins or reaction with surfaces. Recently, Michael addition was used to synthesize an ATRP initiator containing two hydroxyl groups [112]. This novel dihydroxyl initiator allowed the synthesis of Y-shaped polymers via a combination of ATRP on the initial alkyl halide site and post polymerization conversion of the hydroxyl groups to alkyl halides which allowed polymerization of a second monomer (Fig. 31) Matyjaszewksi et al. recently studied the synthesis of ATRP ligands through the Michael addition reaction of tris(aminoethyl)amine with several acrylates, resulting in ligands that were suitable for the polymerization of methacrylic, acrylic and styrenic monomers in nonpolar media [113]. Matyjaszewski et al. also synthesized ATRP ligands containing dimethoxymethylsilylpropyl acrylate, which aided in post-polymerization catalyst removal via passage through silica gel and coupling of the complexes to the silica gel via sol–gel reactions. Shen et al. synthesized novel diaminopyridine (DAP) containing ATRP ligands through acrylation of DAP followed by Michael addition using bis(diethylaminoethyl)amine [114]. Silica gel modified with thymine residues created hydrogen bonded associations with these DAP ATRP ligands, which dissociated at reaction temperatures, allowing polymerization to occur. After cooling the reaction, re-association allowed removal and recycling of the catalyst.

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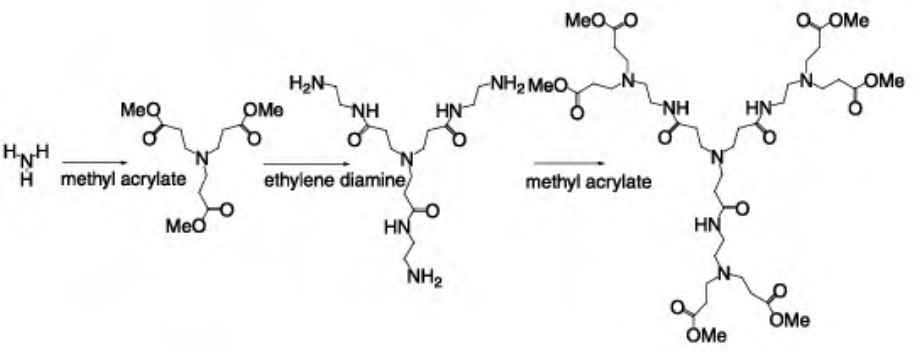


Fig. 32. Divergent PAMAM dendrimer synthesis via alternating Michael addition and amidation.

Synthesis of functional polymers is also achieved through post-polymerization strategies based on the Michael addition. For example, hydroxyl functionalized RAFT polymerized PMMA were synthesized using hydrolysis of the dithioester end groups followed by Michael addition with hydroxyethyl acrylate to the thiol group [115].

5. Synthesis of branched polymers via the Michael addition reaction

Branched polymers encompass a wide range of topologies, ranging from hyperbranched to graft and dendritic polymers. Michael addition polymerization mechanisms were broadly applied to each of these topological designs. Hyperbranched polymers have gained interest recently, due to a large number of functional termini and processing advantages in terms of lower melt viscosity relative to linear polymers of equivalent molecular weight. Michael addition provides the means to incorporate numerous types of terminal functionalities due to an inherent functional group tolerance and wide variety of monomers. Graft copolymers are also of increasing interest given the similarity to block copolymers and the ability to control the graft density and graft length. Graft copolymers often possess microphase separated morphologies and offer elastomeric properties. Dendrimers have traditionally been synthesized using Michael addition reactions, and in fact, Tomalia et al. synthesized the first dendrimer, poly(amido amine) (PAMAM) using Michael addition. Dendrimers and hyperbranched polymers, are interesting due to a plurality of functional groups. However, the absence of entanglements often limits the use of dendrimers to non-structural and mechanically non-demanding applications. Dendrimers based on Michael addition reactions are used in numerous applications, including biomedical applications and gene delivery [116,117].

5.1. Dendrimers

Michael addition reactions are ideal for dendrimer synthesis due to the low probability of side reactions and mild reaction conditions. In the first dendrimer synthesis, dendritic poly(amido amine)s were synthesized via the alternating Michael addition of methyl acrylate to amine substrates (ammonia, $f=3$; ethylenediamine, $f=4$) and aminolysis of the resultant esters with excess ethylene diamine [118]. As depicted in Fig. 32, the PAMAM dendrimer synthesis is divergent, beginning from a small molecule core and enlarging as further generations are added. The high conversion and facile nature of the Michael addition led to nearly monodisperse individual dendrimer molecules as revealed using electron microscopy [119]. The Michael addition was most efficient in methanol, while aprotic solvents led to incomplete alkylation of amine groups. Retro-Michael additions fragmented the dendrimer molecules at elevated temperature (80 °C) in solution [120].

PAMAM dendrimers are effective gene transfection agents due to the plurality of terminal amine groups available to complex with the negatively charged DNA phosphate backbone. In aqueous solution, protonation of the terminal amine groups produces a cationic charge that favors complexation with DNA. A DNA-polymer complex must have a net positive charge in order to pass through a cell membrane. The PAMAM dendrimers effectively transfected genes for β-galactosidase expression, luciferase expression, and also antisense genes that selectively suppress luciferase genes [116].

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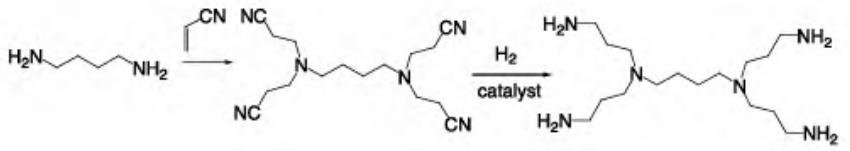


Fig. 33. Synthesis of PPI dendrimers via alternating Michael addition and hydrogenation.

PAMAM dendrimers received significant attention for effectiveness in gene transfection and numerous transfection studies were performed on diverse eukaryotic cell lines. Studies found increased transfection efficiency for higher generation dendrimers [117]. Higher generations were necessary to obtain efficient coverage of DNA, as higher generations possessed sizes close to the histone octamer. Heating the dendrimers in aqueous solution led to fragmentation, likely through retro-Michael additions and solvolysis, which significantly improved transfection. In fact, a commercial gene transfection agent, Superfectin™, is based on fragmented PAMAM dendrimers. PAMAM dendrimers of numerous sizes possess very low cytotoxicity and in vivo studies have also showed low toxicity.

Amine terminated PAMAM dendrimers were recently used to eliminate prion proteins from scrapie infected neuroblastoma cells [121]. Higher generation PAMAM dendrimers were more effective in removing the prion proteins and were most effective below pH 4, suggesting the importance of ammonium charge and activity in the lysosomal or endosomal regions. The importance of branching was demonstrated through the comparison of linear and branched poly(ethylene imine) controls. The ability to remove prions occurred at noncytotoxic dendrimer concentrations.

A second common dendrimer is poly(propylene imine) (PPI), marketed under the trade name Astramol™ [122]. PPI dendrimers are accessed through the Michael addition of primary diamines with acrylonitrile followed by hydrogenation of the nitrile groups to amines and repeated reaction with acrylonitrile (Fig. 33) First through fifth generation dendrimers are commercially produced in this manner. PPI dendrimers were recently synthesized with poly(propylene oxide) Jeffamine® cores, allowing greater flexibility and less steric congestion of the dendrimer molecules [123]. The terminal nitrile groups of poly(propylene imine) dendrimers are hydrolyzed under acidic conditions to obtain peripheral carboxylic acid functionality, but this often leads to degradation or discoloration of the polymer. Meijer et al. developed a route to carboxylic acid functional dendrimers via hydrogenation of the peripheral nitriles followed by the Michael addition of methyl acrylate and basic hydrolysis of the resultant ester functionalities [124]. In contrast to PAMAM dendrimers, PPI dendrimers have less potential for gene transfection and higher cytotoxicity [117]. Lower toxicity was achieved with PPI dendrimers containing ether and ester linkages, synthesized by the Michael addition of alcohols to acrylonitrile, reduction of the nitrile, Michael addition to methyl acrylate, and reduction back to alcohol terminal groups [125].

Majoral et al. developed novel dendrons containing Michael acceptors consisting of $CH_2{=}CH{-}P{=}N{-}P{=}S{-}(O{-}R)_2$ or $CH_2{=}CH{-}P{=}N{-}P{=}O{-}(O{-}R)_2$ groups at the dendrimer core. This allowed convergent synthesis of dendrimers via coupling of the dendrons through Michael addition with primary diamine donors (Fig. 34) [126,127].

Peripheral modification of dendrimers was also accomplished via Michael reactions with pendant Michael donors or acceptors. For instance, mesogenic cyanobiphenyl acrylates were introduced onto amine terminated poly(propylene imine) dendrimers resulting in liquid crystalline dendrimer molecules [128]. Michael addition of acryloxylethyl methacrylate to amine terminated dendrimers resulted in photocrosslinkable dendrimer methacrylates [129]. The Michael addition scheme was the only successful functionalization methodology as reaction with isocyanatoethyl methacrylate or acetoacetoxy methacrylate resulted in insoluble, crystalline products and reaction with methacrylic anhydride did not completely

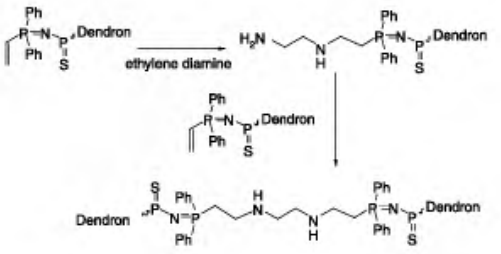


Fig. 34. Convergent dendrimer synthesis via dendron core-linking Michael addition reactions.

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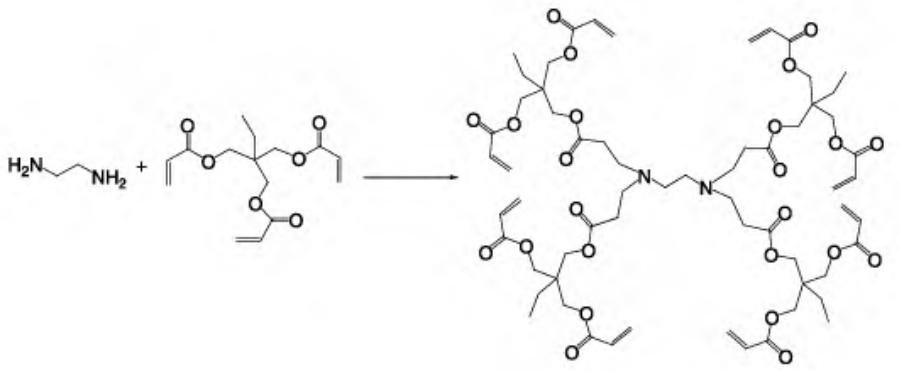


Fig. 35. Crosslinkable dendrimer synthesis using Michael addition reactions.

functionalize the terminal amines. The methacrylated dendrimers exhibited nearly quantitative cure reactions as determined through differential scanning calorimetry (DSC). In Fig. 35, dendritic acrylate oligomers were also synthesized through the Michael addition of trimethylolpropane triacrylate (TMPTA) with ethylene diamine in a 5:1 ratio [130,131]. The desired product possessed a TPMTA:EDA ratio of 4:1, however, network formation occurred under these conditions. The branched oligoacrylates resembled dendrimers with low viscosities and facile cure conditions using UV-photocuring techniques. Purification of these dendrimers was achieved primarily through precipitation.

5.2. Hyperbranched and highly branched polymers

Hyperbranched polymers were initially developed as inexpensive, one-pot substitutes for dendrimers, but have developed a unique character over the last several years. Hyperbranched polymers were traditionally approached from asymmetric monomer synthesis (AB_n monomers). Difficult monomer synthesis is a major disadvantage of the AB_n method due to a lack of commercial AB_n monomer sources. Recently, A_n+B_m approaches were used to synthesize hyperbranched polymers, enabling a much wider range of monomers and topologies. A further development is the use of oligomeric A_n or B_m monomers, resulting in 'highly branched polymers', which allows control of the molecular weight between branch points, an important parameter that affects degree of entanglement and has performance implications [132]. Michael addition reactions were successfully applied to both the AB_2 and A_n+B_m strategies for hyperbranched polymer synthesis.

In a conventional AB_2 hyperbranching polymerization, Endo et al. conducted acetoacetylation of hydroxyethyl acrylate through a reaction with diketene [133]. This produced an AB_2 monomer that was capable of producing hyperbranched Michael addition polymers in the presence of base catalyst (Fig. 36) Polymerization of this novel monomer in the presence of DBU resulted in number average molecular weights between 2000 and 12,000 g/mol with dispersities between 1.4 and 3.5 and degrees of branching between 43 and 83%. A high ratio of dendritic to linear units was observed, suggesting greater reactivity of the *mono*-Michael adduct towards the acrylate group compared to the reactivity of the original acetoacetate group.

Trumbo studied the step growth polymerization of low molar mass diacrylates (tripropylene glycol

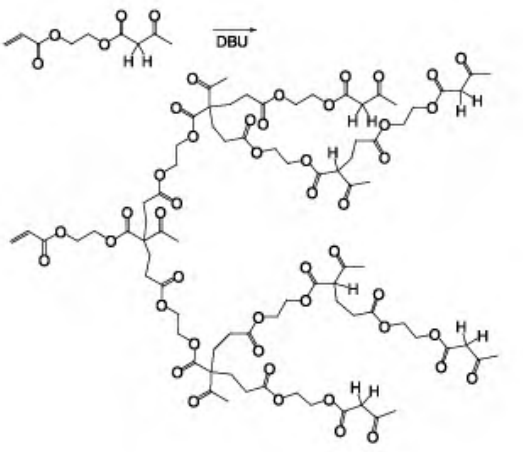


Fig. 36. Hyperbranched polymer synthesis via AB_2 Michael addition polymerization.

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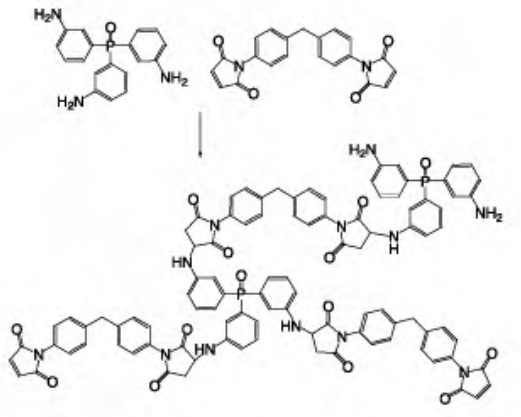


Fig. 37. Hyperbranched poly(aspartamide)s via A_2+B_3 polymerization.

diacrylate) with bisacetoacetates [134] and bisacetoacetamides [135]. These systems formed branched structures due to the difunctionality of the acetoacetate group. Gelation of the reaction mixture was avoided with an excess of bisacetoacetate monomer and through conducting the reaction in solution with a relatively mild base catalyst (DBU). Weight average molecular weights as high as 437,000 g/mol were observed, however broad dispersities ($M_w/M_n \sim 10$) were typical, which further suggested branching in this system. Decreases in molecular weight and dispersity with reaction time were observed during these polymerizations, suggesting the occurrence of a retro-Michael addition and subsequent equilibration.

Hyperbranched poly(aspartamide)s were synthesized from bismaleimides and aromatic triamines using the A_2+B_3 methodology [136]. The reaction stoichiometry was limiting in bismaleimide, thus controlling the extent of the reaction and favoring secondary amine terminated products. The hyperbranched polymers exhibited degrees of branching near 0.51 and 0.69, high glass transition temperatures (~210–250 °C) as well as equivalent thermal stability to analogous linear polymers. In Fig. 37, a tris (4-aminophenyl)phosphine oxide triamine was used to create polymers with higher thermal stability and also to allow degree of branching determination through ^{31}P NMR.

Feng et al. studied hyperbranched poly(amino ester)s that were synthesized from piperazine and trimethylolpropane triacrylate in molar ratios ranging from 1:1.08 to 1:2 [137]. These hyperbranched polymers formed aggregates in acetone/acidic water, which were proposed to contain a hydrophobic acrylate rich core and a hydrophilic, protonated amine periphery. The aggregates were crosslinked using UV light via reaction of residual acrylate groups. The size of the aggregates (DLS, TEM) decreased upon crosslinking and were smaller for higher molecular weight polymers, suggesting a packing efficiency limitation with the higher molecular weight, more disperse, polymers. Enhanced aggregation of similar poly(amino ester)s was achieved through reaction of peripheral amine groups with long chain acyl halides [138].

Wu et al. studied the effect of unequal functional group reactivity in a hyperbranching A_2+B_3 system with a butanediol diacrylate A_2 and a trifunctional amine B_3 containing both primary ($f=2$) and secondary ($f=1$) amines (Fig. 38) [36]. For an equimolar A_2:B_3 ratio, ^1H NMR studies indicated that secondary amines were consumed prior to primary amines. The secondary amine that was formed from reaction of the primary amine and the acrylate group was not consumed, and thus a predominantly linear product was obtained despite the functionality of the monomers. The reactivity of the amines was secondary > primary >> formed secondary. If the A_2:B_3 ratio was increased, participation of the formed secondary amine during the reaction of the primary amine occurred, thus resulting in a branched product. If the steric hindrance at the secondary amine position was increased, the primary amine was the most reactive and the reaction proceeded at the formed secondary amine during the reaction and again the product was branched. Thus, both the level of substitution of the amine groups and the stoichiometric ratio of the reactants controlled

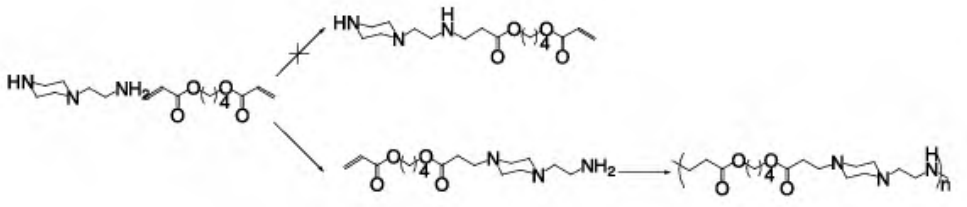


Fig. 38. Hyperbranched poly(ester amine)s from A_2+B_3 polymerization with unequal reactivity in the B_3 monomer functional groups.

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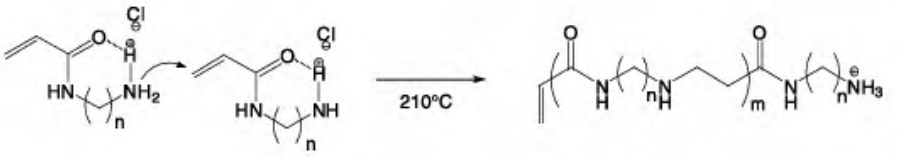


Fig. 39. Homopolymerization of an AB_2 poly(amido amine) monomer.

the degree of branching. Degrees of branching that ranged from 30 to 40% and weight average molecular weights as high as 23,500 g/mol were obtained.

Wu et al. also studied hyperbranched poly(amino ester)s that were synthesized using two equivalents of butanediol acrylate and one equivalent of 1-(2-aminoethyl)piperazine [139]. Due to the higher reactivity of the secondary cyclic amine, the Michael addition with butanediol diacrylate occurred preferentially. The primary amino group reacted second, resulting in the in-situ generation of an AB_2 intermediate that undergoes a branching at the secondary amine formed from the primary amine. The ratio of R_h/R_g of 1.0 as measured by SAXS and DLS demonstrated the hyperbranched nature of the polymers. The hyperbranched polymers had number average molecular weights of 29,000–38,000 g/mol and dispersities of 3.4–3.7.

In similar work, Gao et al. synthesized hyperbranched polymers from diethanolamine and methyl acrylate [140]. The reaction proceeded through an initial Michael addition reaction to form an ester diol AB_2 monomer in situ that self-condensed at elevated temperature (150 °C) through transesterification in the presence of a zinc acetate catalyst. Number average molecular weights as high as 268,000 g/mol were observed with a polydispersity of 2.4. Crosslinking occurred when the temperature in the second stage exceeded 160 °C, whereas lack of polycondensation occurred if the temperature was less than 120 °C. The degree of branching of these polymers was roughly 55%. These polymers degraded rapidly in water to form self-buffered pH 7 solutions, suggesting them as good candidates for drug delivery.

Hyperbranched poly(amido amine) hydrochloride polyelectrolytes were synthesized through the Michael addition polymerization of AB_2 monomers based on ammonium alkyl acrylamides, as shown in Fig. 39 [141]. The lack of nucleophilicity of the ammonium group toward the acrylamide functionality required the use of high temperatures (210 °C) in order to drive the polymerization. It was postulated that a small equilibrium concentration of free amine that exists at these high temperatures undergoes the Michael addition. The shorter alkylene spacer ($n=2$) between the acrylamide group and the ammonium group resulted in faster reaction, and an intramolecular deprotonation through a six membered cyclic transition state was proposed. Model reactions suggested that the ammonium group reacts twice with acrylamide functionalities, resulting in a highly branched product. ^{15}N NMR was used to characterize the degree of branching in these materials. Signals from linear units were not observed, corroborating the highly branched nature of the polymer. This methodology is a more facile route to hyperbranched polymers resembling PAMAM dendrimers.

Kadokawa et al. studied the triphenylphosphine initiated Michael addition polymerization of 2,2-bis(hydroxymethyl)propyl acrylate to yield phosphonium containing hyperbranched polymers of 1200–2700 g/mol, as shown in Fig. 40 [142]. This is one of the few examples of oxygen based nucleophiles involved in a Michael addition polymerization. Degrees of branching near 50% were obtained. Similar polymers were produced through initiation using sodium hydride. The poor quality of the oxygen nucleophile for Michael addition is evident in the reaction temperatures required (80–100 °C, with free radical inhibitors) [143].

Highly branched gene transfection agents were synthesized through the reaction of low molecular weight linear PEI with PEG diacrylate [144]. The polymers clearly did not represent linear topologies due to branching in the PEI as well as reaction at multiple amine sites along the polymer backbone. The presence of ester groups in the polymers facilitated degradation. The half-lives of the polymers in PBS at 37 °C were approximately 8 d. The polymers demonstrated complex formation with plasmid DNA and transfection studies were performed. Branched gene transfection

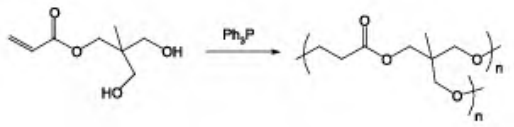


Fig. 40. Homopolymerization of an AB_2 poly(ester ether) monomer.

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agents were also synthesized via reaction of trimethylolpropane triacrylate with primary amines such as *N*,*N*-dimethylethylenediamine [145]. Polyplex formation with plasmid DNA was verified with dynamic light scattering and transfection into various human and mouse cell lines exhibited transfection efficiencies similar to poly(ethylene imine). The branched nature of the poly(amino ester)s slowed polymer degradation and hence controlled the release of the plasmid DNA. The branched poly(amino ester)s showed little or no cytotoxicity.

5.3. Graft copolymers

Graft copolymers are synthesized through numerous routes including chain or step growth polymerization of macromonomers and post-polymerization grafting. Ferruti et al. examined graft copolymer synthesis using poly(amido amine)s [146]. PEG chains with weight average molecular weight of 750 g/mol were incorporated into poly(amido amine) backbones using monoamine terminated PEG as a comonomer. As a second route, PEG-NH_2 was first reacted with excess bisacrylamide monomer to obtain a PEG bisacrylamide, which was polymerized with piperazine. In both cases, vapor pressure osmometry indicated that the molecular weights obtained were quite low (~3000 g/mol). Ferruti concluded that the reactivity of the PEG-NH_2 in the polymerization was lower than typical small molecule amines. Ferruti et al. also studied poly(amido amine)s grafted to polyethylene via a post-polymerization strategy, although Michael addition was not involved in the coupling [147].

Jerome et al. utilized γ-acryloxy-ε-caprolactone as a graftable comonomer in copolymerizations with ε-caprolactone [148]. In Fig. 41, pendant acrylate groups were introduced into poly(ε-caprolactone), permitting grafting of thiol terminated oligomeric PEG (M_n=900 g/mol). The Michael addition reaction of various thiols (mercaptoacetic acid and triphenylmethane thiol) with PEG-acrylate model compounds proceeded to completion. However, conversions of only 65 and 70% were obtained with PEG-thiol and mercaptoacetic acid, respectively, in grafting studies with poly(γ-acryloxy-ε-caprolactone-*co*-ε-caprolactone).

Much work was done in the area of 'grafting to' syntheses utilizing the Michael addition, especially in the case of biopolymers such as chitin and chitosan. These materials typically possess poor solubility, which is improved through grafting techniques. Modification of chitosan with poly(amido amine) dendrimers was

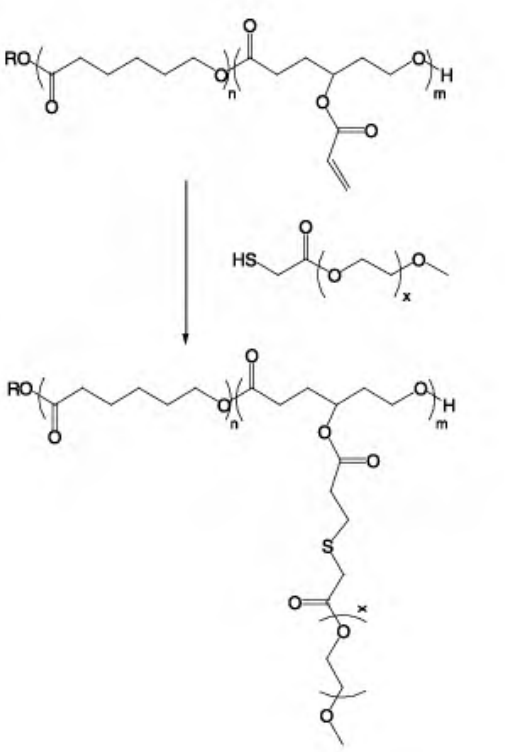


Fig. 41. Graft copolymerization via grafting of thiol terminated PEG to pendant acryloxy side groups on a poly(ε-caprolactone) derivative.

achieved by reacting chitosan surface amine groups with methyl acrylate followed by alternate reactions with ethylene diamine and further methyl acrylate [149]. The heterogeneity and steric hindrance of the backbone resulted in imperfect dendrimers. Grafting of living cationic poly(methyl oxazoline) and poly (isobutyl vinyl ether) onto the pendant amine groups of the grafted poly(amido amine) dendrimers was achieved successfully. Modification of chitin with small molecules was also achieved through Michael addition to pendant amine groups. Reaction with acrylonitrile yielded cyanoethylated chitin [5] and reaction with ethyl acrylate afforded ester-containing chitin [150] (Fig. 42)

6. Novel networks via the Michael addition reaction

Network polymers that are synthesized via Michael addition reactions offer applications in diverse areas such as drug delivery systems, high performance composites, and coatings. Networks are

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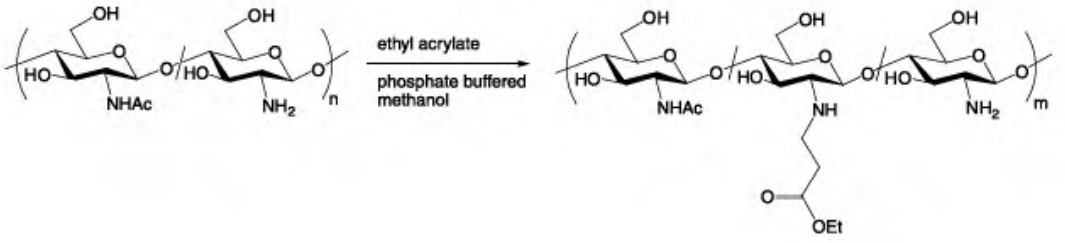


Fig. 42. Modification of chitin via Michael addition reaction with ethyl acrylate.

often synthesized through a combination of Michael addition step growth chemistry combined with chain growth polymerization using photoinitiated radical or anionic processes. The Michael addition is suited to the formation of well-defined networks due to the commercial availability or facile synthesis of narrow molecular weight distribution functional oligomers. Commercially available reactive oligomers include PEG diacrylate and numerous oligomeric diamines such as Jeffamine®. Several classes of Michael addition networks are currently of interest.

6.1. Bismaleimide networks and composite materials

Currently, bismaleimide based networks are used in high performance fiber-reinforced composite materials [151] and also in thermoset resins. These materials have applications in the aerospace industry due to their high temperature strength, mechanical robustness, and low density [152]. In the absence of other components, bismaleimides crosslink through chain growth type reactions at high temperature to form highly crosslinked, high T_g networks. Vitrification in these systems leads to incomplete conversion of maleimide groups. Brittleness is the primary drawback of these homopolymerized networks. Efforts to reduce the crosslink density and hence the brittleness of these thermosets have involved Michael addition chain extension reactions with primary or secondary diamines to consume some of the maleimide functional groups [70]. The chain extension process results in greater toughness and improved mechanical properties for composites. The introduction of diamines such as methylene dianiline (MDA) causes a low temperature Michael addition polymerization (<180 °C) during initial curing, followed on further heating (180–220 °C) with a crosslinking homopolymerization of the maleimide groups (Fig. 43) [152,153]. Under certain conditions, secondary amines formed during the initial Michael addition undergo further Michael addition crosslinking with maleimide groups. Hyperbranched amine terminated polyamides were also investigated as components of bismaleimide networks, successfully improving the toughness of these systems [154].

Kumar et al. incorporated bismaleimide crosslinking systems into silicon-containing epoxy resin networks [154]. The bismaleimides increased the flexural and tensile moduli, but decreased fracture toughness of the epoxy networks by introducing a more rigid, higher T_g component. This network reinforcement was due to the thermal cure and Michael addition reactions between the bismaleimide and the amines from the epoxy resin and the added diamine.

Bismaleimide networks are useful as NLO materials as well as composites due to their high glass transition temperatures and crosslink densities. These features are attractive for NLO materials for maintenance of the chromophore orientation. Furthermore, the absence of volatile by-products during cure allows poling during this process. Wu et al. synthesized a diamine NLO chromophore and incorporation into bismaleimide networks resulted in nonlinear optical coefficients as high as $d_{33}=60$ pm/V at 1064 nm [155]. Upon heating, the second harmonic generation (SHG) was stable to 120 °C, although the cure process was continued 250 °C in order to maximize crosslinking. Maintenance of the poling field during the cooling process maximized the NLO properties.

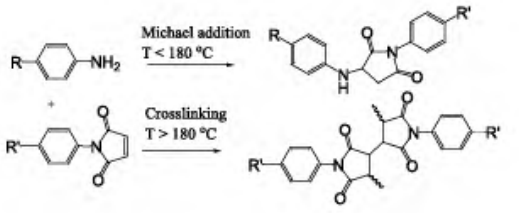


Fig. 43. Crosslinking of bismaleimide networks via a combination of Michael addition and maleimide homopolymerization.

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6.2. Carbon Michael addition networks utilizing acetoacetate chemistry

Considerable interest in Michael addition networks from acetoacetate and acrylate precursors has recently emerged. These networks are interesting due to the relatively low toxicity of the components and their room temperature processing in the absence of UV radiation. Furthermore, functionalization of common polyols with the acetoacetate group is achieved through transesterification with *tert*-butylacetoacetate [156] or reaction with diketene precursors [157] providing a facile route to crosslinkable precursors from a wide variety of hydroxyl containing feedstocks.

Room temperature thermosetting coatings is one of the primary applications of Michael addition networks. The Michael addition mechanism is advantageous as neither UV radiation nor heat are required, both of which could be deleterious to the substrate and require additional processing equipment. The base catalyzed carbon Michael addition of acetoacetylated resins to acrylate acceptors, as depicted in Fig. 44, is an ideal route to crosslinked networks due to the difunctionality of the acetoacetate group and the absence of undesirable toxic amine and malodorous thiol nucleophiles in industrial processes. Acrylic monomers and oligomers are useful candidates for the formation of coatings and adhesives with highly reduced volatile organic compounds (VOCs) and improved material performance properties. Although UV curing is not necessary for network formation, the presence of photocrosslinkable groups in these systems allows tandem Michael and radical curing mechanisms.

Clemens and Rector studied the carbon Michael addition reaction between model compounds, and concluded that the reaction mechanism involved an equilibrium deprotonation of the acetoacetate and subsequent rate limiting addition of the enolate to the acrylate group [21]. Thus, the rate of the reaction was unaffected by the acetoacetate concentration but was directly proportional to the base catalyst concentration. The effect of various catalysts was investigated and as expected, higher basicity led to faster reaction kinetics. Typical base catalysts included DBU and TMG as well as hydroxide and methoxide bases. A striking difference, however, was observed in the degree to which bisadducts of acrylates with acetoacetates formed as a function of catalyst. Amidine and guanidine bases resulted in higher formation of bisadducts while monoadducts were favored by methoxide and hydroxide bases. Weaker bases such as triethylamine were not strong enough to deprotonate the acetoacetate groups and did not catalyze the Michael addition reaction effectively.

Clemens and Rector prepared crosslinked coatings consisting of randomly functionalized acetoacetylated precursors such as methyl methacrylate/acetoacetyloxy- ethyl methacrylate random copolymers with low molar mass multiacrylates such as trimethylolpropane triacrylate [21]. The level of functionality of the methacrylate copolymers strongly influenced cure times, with a doubling of the acetoacetate functionality resulting in a 75% reduction in cure time. Acetoacetylated cellulose was also investigated, and the more rigid character of the polymer coupled with the lack of a spacer between the backbone and the acetoacetate groups resulted in slower cure reactions. One disadvantage of the Michael crosslinked coatings was hydrolytic instability that was attributed to hydrolysis of the ester linkages in the presence of the base catalyst. Heat treatments reduced the hydrolytic instability of the network.

Marsh described the use of high solids acetoacetate-containing resins for coatings applications [158]. High

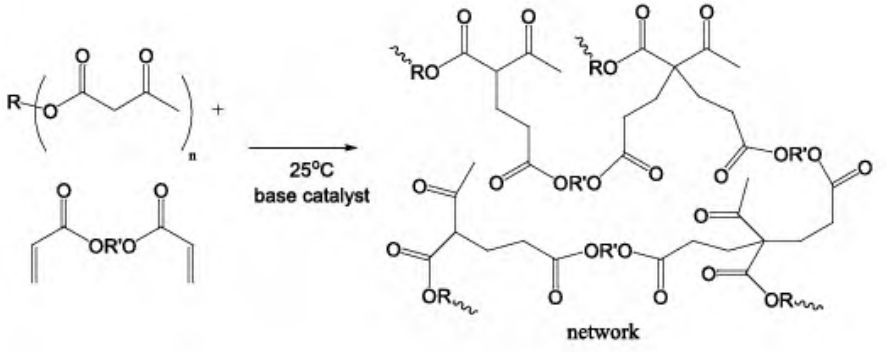


Fig. 44. Carbon-Michael addition polymerization of acetoacetates with acrylates catalyzed by bases for network synthesis.

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solids polyester resins containing hydroxyl groups were synthesized and acetoacetylated to various levels. It was found that increasing levels of acetoacetyl groups lowered the T_g of the polyester resins significantly to yield solvent-free low viscosity resins. The crosslinking of the pendant acetoacetyl groups using Michael type reactions with electron deficient olefins and DBU base catalyst (pH 12.6) afforded formaldehyde- and isocyanate-free room temperature curable coatings.

Tung published a review on the performance of coatings made from multifunctional acrylate resins containing reactive diluents and acid blocked catalysts and compared them to two-package polyurethanes [159]. The use of a formic acid neutralized DBU catalyst yielded better pot life and humidity resistance of the coatings when compared to the highly basic DBU catalyst. It was found that incorporation of 10–15 mol% of a reactive diluent such as 2,2,4-trimethyl-1,3-pentane-bis(acetoacetate) resulted in low VOC coatings with desirable ultimate properties. Various multifunctional acrylate crosslinkers were investigated to determine the influence of crosslinker functionality on the resulting resin properties. A mixture of tri- and tetraacrylates produced the best hardness, flexibility, solvent resistance, humidity resistance, adhesion, and weatherability compared to the triacrylates or hexaacrylates.

6.3. Curing mechanisms involving Michael addition with photocrosslinking or sol–gel chemistry

Cure mechanisms using a combination of Michael addition and photoinitiated radical curing are currently of interest due to higher crosslink densities and functional group conversions. The use of multifunctional acrylate monomers in the carbon Michael addition networks is ideal for photocrosslinking. Residual acrylate dangling ends must be limited to maximize chemical and physical durability. Moszner and Rheinberger synthesized networks from multiacrylates such as PEG diacrylates, trimethylolpropane triacrylate, and pentaerythritol tetraacrylate with bisacetoacetates such as PEG bis(acetoacetate) and hexanediol bis(acetoacetate) using DBN base catalyst [160]. Isothermal DSC studies revealed less than 25% of the double bonds were consumed via Michael addition during cure. The conversion of acrylate groups increased for longer, more flexible, spacers to approximately 71%. Subsequent photocuring of the Michael addition networks using camphorquinone/*N*-(2-cyanoethyl)-*N*-methylaniline resulted in dramatically improved hardness and conversion of the acrylate groups. Moy et al. developed photocrosslinked networks from crosslinkable oligomers that were synthesized using Michael addition with an excess of multiacrylates relative to acetoacetate precursors [161]. The Michael addition of acetoacetoxyethyl methacrylate with multifunctional acrylates is another route to photocrosslinkable methacrylate oligomers. The ketone-containing oligomers were capable of self-initiation in UV light due to Norrish type cleavage reactions. Klee et al. conducted Michael addition using diamines and acryloxylethyl methacrylate to create crosslinkable branched multimethacrylate oligomers for dental composites with lower shrinkage upon photocrosslinking [162]. Dammann and coworkers demonstrated the formation of liquid oligomeric Michael adducts by reacting di-, tri- and tetraacrylates with ethyl acetoacetate in a specific ratio in the presence of catalytic amounts of epoxide and quaternary ammonium salt. These compositions were further crosslinked to obtain coatings, laminates, and adhesives [163].

Pavlinec and Moszner further studied crosslinked networks from poly(propylene glycol) bis(acetoacetate) or pentaerythritol tetrakis(acetoacetate) and pentaerythritol tetraacrylate cured with DBN [11]. In these Michael addition networks, a 2:1 ratio of acrylate to acetoacetate was studied. The residual acrylate groups in the networks were studied using FTIR, following the acrylate vinyl absorbance at 810 cm^{-1}. The concentration of residual unreacted acrylate groups was lowest for networks that were prepared using poly(propylene glycol) bis(acetoacetate)s due to the molecular mobility and long distance between crosslink points. Higher conversion of reactive functional groups for longer precursors was also observed in photocrosslinking studies of diacrylate oligomers [164]. Ambient temperature aging and thermal postcure treatments to 200 °C increased the conversion of acrylate groups from 88 to nearly 97%. The gel fractions that were obtained for the Michael addition networks ranged from 87 to 94%. The combination of Michael addition curing with free radical photocuring was also investigated with the addition of photoinitiators, excess pentaerythritol tetraacrylate, and PEG dimethacrylate monomers to selected compositions. The stability of the methacrylate against Michael addition prevented interference between the two cure mechanisms performed in a stepwise manner, beginning with a dark Michael cure and ending with the photocure. In the photocured systems, lower viscosity during the Michael addition stage was attributed to the additional PEG dimethacrylate monomer and excess acrylate

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monomer. This was postulated to improve the cure process by improving molecular mobility and homogeneity.

Michael reactions between multifunctional acrylates and acetoacetates have found widespread applications in the formation of radiation curable coatings, printing ink formulations, and adhesives. UV curable ink formulations have gained importance as a substitute to solvent-based ink systems. Currently available UV curable ink formulations exhibit rapid curing under commercial line speeds, however, large quantities of photoinitiator such as benzophenone are required to ensure complete curing throughout the thickness of the film for printing applications. These photoinitiators are known to be toxic, expensive and also cause odor and color in the material. In order to overcome this issue, current studies have involved the use of liquid oligomeric Michael adducts formed from multifunctional acrylates by reaction with Michael donors such as acetoacetate compounds, which are amenable to further crosslinking under UV irradiation [165]. Through the incorporation of suitable additives such as acrylic monomers and oligomers, vinyl monomers, vinyl ethers, and primary and secondary amines, ink formulations were modified to obtain rheological and adhesion characteristics that were desirable for printing applications on many different kinds of substrates.

Recently Liu et al. described the formation of renewable and environmentally benign wood adhesives based on derivatized soy protein using the Michael addition crosslinking approach [166]. Soy proteins used as renewable wood adhesives suffer from low shear strength and moisture resistance. In order to improve the properties of the adhesive, the authors first maleated the soy protein isolates (SPI) using maleic anhydride, which was then reacted with poly(ethylene imine) via Michael addition. Wood composites that were bonded with adhesive exhibited shear strength of 7.0 MPa as compared to 2.0 MPa of those bonded using SPI. In addition the joints also gained water resistance.

In addition to the application of the Michael addition in the formation of crosslinked coatings, the formation of reactive intermediates through Michael additions which are subsequently crosslinked using other mechanisms yields useful coatings and adhesives. This strategy has often involved sol–gel chemistry of the Michael adducts to form networks. For example, Wilkes et al. have reported the formation of bis- and tris(maleimide) functionalized trialkoxysilanes by Michael addition reaction between 3-aminopropyl triethoxysilanes and corresponding maleimides [167]. The functionalized silanes were crosslinked via sol–gel methods and used as protective coatings on polycarbonate substrates exhibiting enhanced abrasion resistance. Tilley et al. recently described the use of the Michael addition reaction for the preparation of acrylate-functionalized silane coupling agents to introduce acrylate functionality to colloidal silica particles [168]. Such acrylate functionalized silica particles were used as toughness enhancers for UV-curable acrylic coatings. Incorporation of these functionalized silica particles into a wide variety of organic matrices such as polyesters, epoxies, and polyurethanes was demonstrated. Such inorganic oxide modified coatings exhibited much greater micro-hardness and abrasion

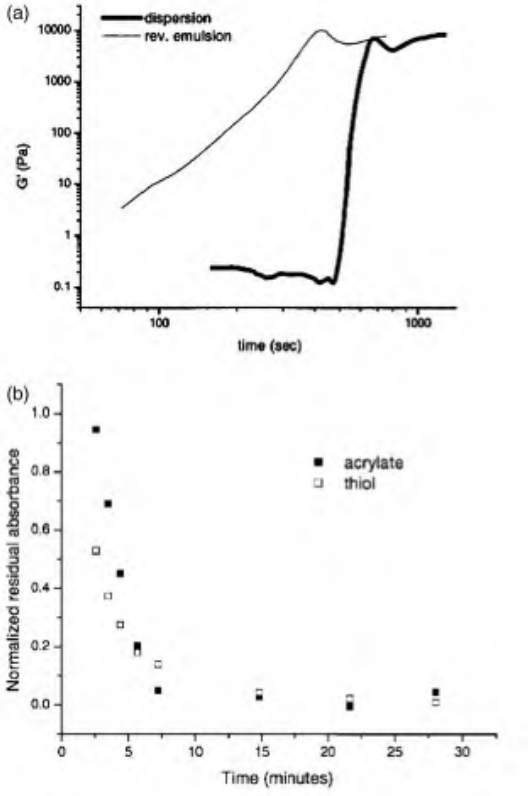


Fig. 45. (a) Rheological monitoring of gel formation in networks synthesized from multifunctional PEG acrylates with PEG dithiol in both reverse emulsion and dispersion form. The steep rise in G′ with gel formation in the case of the dispersion network is an indication of a sudden percolation of crosslinked phases at later times relative to the continuous organic crosslinking phase in the reverse emulsion. (b) Depletion of functional groups with time during crosslinking. Reproduced from 'Water-borne, in situ crosslinked biomaterials from phase-segregated precursors,' Hubbell et al., Copyright 2003 Society of Chemical Industry with permission from John Wiley and Sons on behalf of SCI [2].

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resistance compared to commercially prepared coatings. In a recent patent disclosure, Kobayashi et al. reported the synthesis of an acryloxy-functional silicone composition curable by high-energy radiation [169]. The silicones consisted of a mixture of multifunctional acrylates, amino-functional alkoxysilanes, organofunctional alkoxysilanes, and colloidal silica. The resultant coatings exhibited excellent storage stability, transparency, adhesiveness, water repellency, and abrasion resistance. Zhu et al. also demonstrated the formation of trimethoxysilane coupling agents containing *O*-butyrylchitosan via Michael addition of 3-acryloxypropyltrimethoxysilane with the amino groups of the butyrylchitosan [170]. Crosslinking of the modified silane derivatives resulted in colorless, flexible elastomeric coatings at 20 mol% incorporation of the modified silane. The resulting hydrogel films possessed good antithrombogenic properties as determined by blood-clotting and platelet adhesion.

6.4. Hydrogel networks for biomedical applications

Michael addition networks find application in areas beyond composites and coatings. Recently, numerous biomedical applications were investigated. Hubbell et al. studied in vivo gelling networks based on multifunctional thiols and multiacrylates as injectable tissue reinforcement [2]. Currently two mechanical ranges of in-vivo biomaterials exist: low modulus gels, which are used for drug delivery and high modulus bone cements. An intermediate strength material (0.5–50 MPa) is desirable for tissue reinforcement of invertebral disc annuli. Michael addition networks based on thiol and acrylate precursors are ideal due to the ability to control gelation with the addition of a basic catalyst, the absence of free thiols in the bloodstream, and the faster rate of the thiol-acrylate Michael addition in comparison to potential Michael reactions with amines in the bloodstream. Metters and Hubbell also recently demonstrated the biodegradability of these networks via slow hydrolysis of ester bonds adjacent to sulfide linkages, which leads to degradation on the order of days to weeks [171]. In a separate work, Hubbell et al. showed that the length of the alkyl spacer between the ester and the sulfide controlled the hydrolysis rate of these poly(ester sulfide)s, with higher degradation rates occurring for shorter alkyl spacers [172]. Both dispersion type and reverse emulsion type polymers were synthesized via mixing network components with basic PBS. Reverse emulsion type networks exhibited a slower change in shear storage modulus with time near the gel point and higher ultimate compressive strengths (6.7 vs. 1.8 MPa) due to greater continuity of the organic phase (Fig. 45) Dispersion networks exhibited rapid increases in shear storage modulus near the gel point, due to the percolation of dispersed crosslinking particles. Both materials exhibited maximum deformations near 37%. Modulus increased with increased amounts of tetraacrylate precursors versus diacrylate precursors.

Rizzi and Hubbell [10] developed protein containing hydrogel networks for tissue repair applications. Cysteine-functionalized recombinant proteins containing sequences for integrin receptor ligation were

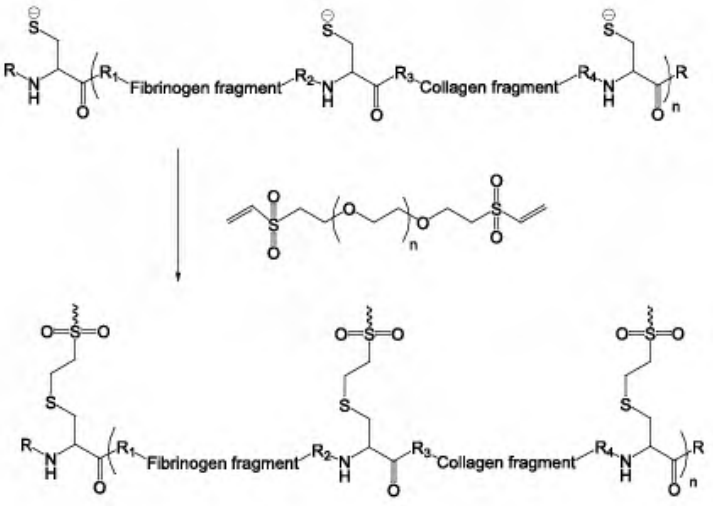


Fig. 46. Network formation of PEG bis(vinyl sulfone) and protein dithiols.

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synthesized and crosslinked with PEG bis(vinyl sulfone) (Fig. 46) Integrin receptors are cellular receptors, which play important roles in tissue development processes. These networks were stable to aqueous solution and did not exhibit hydrolytic degradation. Thus, protease cleavable units were introduced into the proteins to enable biodegradability. Glutamic acid units were placed strategically near cysteine residues to inhibit disulfide bond formation prior to crosslinking, while glycine residues were used to prevent hindrance of the Michael addition. Previous work on cysteine containing peptides revealed that positively charged arginine groups adjacent to cysteine lower the pK_a of the cysteine group, accelerating Michael reaction by a factor of 2 and decreasing gel time by a factor of 2.5 [173]. Numerous charged residues were also used to enhance solubility of the proteins. The crosslinking kinetics were studied using rheological measurements, which showed increasing storage modulus with time during the crosslinking reaction. The gel time decreased with increasing pH, due to the fact that the thiolate ion is the active species in the Michael addition reaction with vinyl sulfone groups. A maxima in G′ and minimum in gel time was found for a ratio of thiol to vinyl sulfone (SH:VS) slightly greater than unity which differed with protein precursor. This non-stoichiometric maximum was likely due to disulfide bond formation during reaction. Higher storage modulus and lower swelling were generally observed with an increased molar ratio of thiol to vinyl sulfone (Fig. 47) A potential explanation for this phenomenon was decreased accessibility of reactive sites for crosslinking at lower SH:VS ratios. In similar work featuring non-degrading peptides with two cysteine residues, higher elastic modulus and lower swelling were observed for PEG bis(vinyl sulfone)s of higher functionality (3–8 arms) due to increased crosslink density [173]. Network elastic moduli in these systems reached a maximum at pH 8, likely because of slower kinetics at pH < 8 and greater disulfide bond formation at pH > 8. Furthermore, precursor concentration strongly influenced mechanical properties due to the propensity for cyclization reactions in dilute conditions.

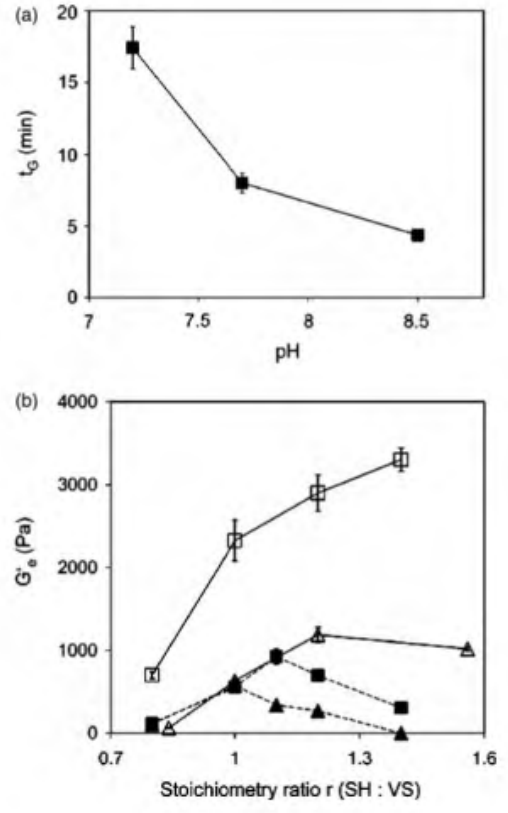


Fig. 47. (a) Effect of pH on gel time for PEG bis(vinyl sulfone). Gel time decreases at higher pH due to the greater thiolate anion concentration. (b) Effect of thiol to vinyl sulfone ratio (SH:VS) on the storage modulus of PEG-protein networks. The G′ maxima occurs at ratios slightly greater than unity suggesting disulfide formation. Reproduced from 'Recombinant protein-*co*-PEG networks as cell-adhesive and proteolytically degradable hydrogel matrixes. Part I: development and physicochemical characteristics,' Rizzi and Hubbell, Copyright 2005 with permission from the American Chemical Society [10].

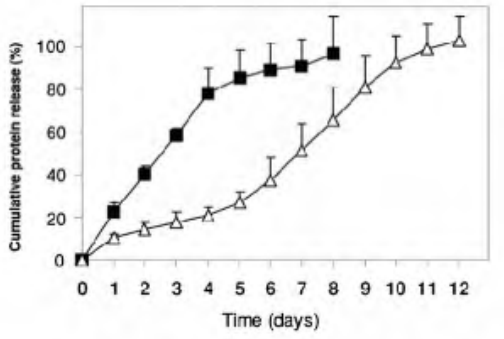


Fig. 48. Release of albumin from PEG networks based on PEG tetraacrylate (black squares) and PEG octaacrylate (white triangles). The lower functionality of the PEG tetraacrylate leads to lower crosslink density and more rapid release. Reprinted from J. Control Rel, 76, Hubbell et al. 'Protein delivery from materials formed by self-selective conjugate addition reactions,' 11–25, Copyright 2001, with permission from Elsevier [9].

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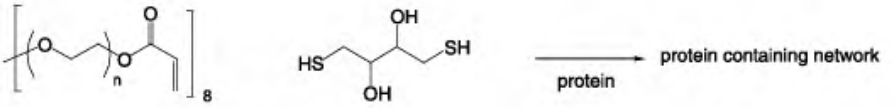


Fig. 49. Protein-containing PEG networks via Michael addition crosslinking of PEG octaacrylate with dithiothreitol. Human growth factor protein (hGH) was incorporated by mixing.

Michael addition networks were also developed for protein drug delivery applications. Protein drugs often suffer from rapid clearance from the bloodstream due to removal by the liver or kidneys. The use of drug delivery devices allows steady and long lasting delivery of protein drugs. Many current drug delivery materials such as poly(lactide)s can potentially denature the protein drug due to changes in pH associated with degradation byproducts. Another issue with current drug delivery materials is incorporating the protein into the material. Hubbell et al. studied Michael addition networks based on PEG multiacrylate and PEG dithiol that overcame many of these problems via incorporating bovine serum albumin protein during synthesis in aqueous media at physiological pH without denaturing the protein (Fig. 48) [9]. The crosslinking of these Michael addition networks required no basic catalyst or UV light that could potentially harm the protein as in photocuring of acrylate networks. Furthermore, the ester bonds in the PEG networks were degradable, allowing release of the protein as the network swells. The thiol-acrylate Michael addition reaction proceeded without significant side reactions with protein based amine nucleophiles, preserving the chemistry of the protein drugs. Furthermore, protein based free thiols are uncommon in extracellular proteins, lending greater selectivity to the approach. In cases where free thiols do exist, they are often confined to sterically hindered locations in the tertiary structure of the protein. Hubbell et al. introduced the protein as well-defined ~100 μm solid particles which were embedded into the Michael addition network during curing [9]. The release rate of the networks was tuned via the functionality of the PEG multiacrylate. PEG triacrylate networks resulted in rapid release of the protein. PEG tetraacrylate networks released protein over the course of 4 d at a linear rate of 20% per day. PEG octaacrylate networks exhibited a slower release with a release rate of 10–15% per day. Hydrolytic degradation was observed in the swelling kinetics of these networks. A decrease in gel time with increasing solution pH (up to pH 8.2) indicated that the thiolate anion is the active species in the Michael addition with the acrylate.

Hubbell et al. also studied protein drug delivery using Michael addition hydrogel networks from eight-arm PEG octaacrylate with dithiothreitol (Fig. 49) [174]. The protein drug human growth hormone (hGH), which suffers from rapid clearance was incorporated into these hydrogels without covalent attachment. Currently, daily administrations of this drug are required for treatment of the hormone deficiency as well as Turner's syndrome and renal failure. The

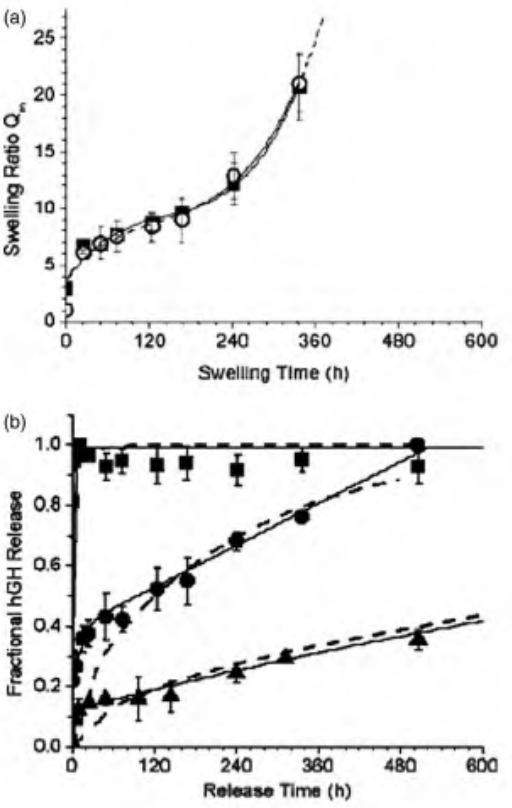


Fig. 50. (a) Swelling of PEG octaacrylate, dithiothreitol networks via initial solvent uptake followed by ester hydrolysis leading to degradation. (b) Release kinetics of hGH protein from networks as a function of PEG octaacrylate molecular weight (squares 10,000 g/mol, circles 10,000 g/mol mixed with 2000 g/mol (50:50), triangles 2000 g/mol). Reprinted from J Control Rel, 102, van de Wetering et al. 'The effect of the linker on the hydrolysis rate of drug-linked ester bonds,' 619–27, Copyright 2005, with permission from Elsevier [172].

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crosslink density of the networks is controlled with the use of varying molecular weights of PEG octaacrylate, thereby influencing the drug diffusion. As shown in Fig. 50, the drug release rate was tuned to occur over periods from hours to months by changing the crosslink density of the networks. The ester sulfide linkages produced in the network degraded at a significantly faster rate than in free radically crosslinked acrylic networks, presumably due to neighboring group effects from the thioether group. Thus, the diffusion coefficient in these networks increases with time. The degradation rate of these networks varied from weeks to months and was directly related to the crosslink density.

Hubbell et al. also studied synthetic cell encapsulation networks [175]. These networks were synthesized from physically gelled four-arm Pluronics® (poly (ethylene oxide-*b*-propylene oxide-*b*-ethylene oxide)) functionalized with acrylate groups and thiols. These hydrogels have potential applications in cell encapsulation. The propensity of the thiol groups to undergo disulfide bond formation prompted the use of the thioacetic ester protecting group which was deprotected during crosslinking. The reversible physical gelation process for Pluronics occurred in aqueous solution when a cold, slightly acidic medium (5 °C, pH 6.8) was increased both in temperature and pH (37 °C, pH 7.4). Once the physical gel formed, the covalent crosslinking via the Michael addition reaction produced gels, which were biodegradable due to the presence of ester groups. Degradation in PBS at physiological conditions occurred over periods of 1–2 weeks.

In order to mimic the extracellular matrix and guide the growth of cells, synthetic matrices should be biocompatible, resistant to non-specific adsorption of proteins, degradable via cell- secreted matrix metalloproteinases (MMPs), and further promote cell-adhesion. Extending on an earlier study, Hubbell et al. synthesized hydrogels comprising multiarm vinyl sulfone-terminated PEG, a monocysteine containing adhesion protein, and a bis(cysteine) metalloprotein substrate protein. The adhesion peptide was introduced to provide adhesion sites on the surface of the hydrogel matrix. The unreacted vinyl sulfone groups were reacted with the bis(cysteine) terminated MMP substrate peptide. Fig. 51 depicts the stepwise formation of the hydrogel containing the adhesion proteins and the MMP substrate proteins as well as the attack on the substrate protein by a metalloproteinase [176].

Analysis of the activity of an MMP to various substrates in the three-dimensional hydrogel indicated that the invasion of the cells was dependent on the enzymatic sensitivity of the substrate peptide and the rate of this process was dependent on the adhesion peptide concentration. Also the invasion of the cells decreased with an increase in crosslink density at lower PEG molecular weights. In order to demonstrate the

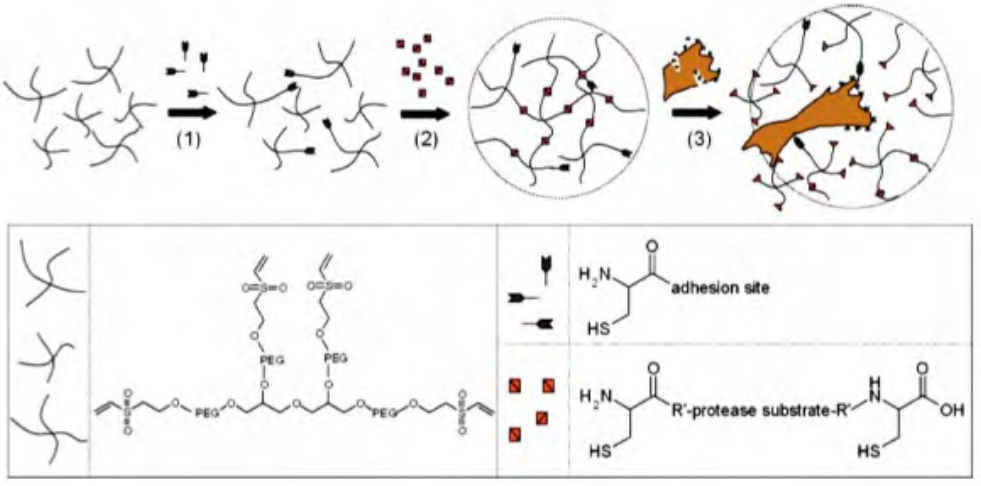


Fig. 51. Illustration of the stepwise synthesis of a PEG-based hydrogel by reacting multiarm vinyl sulfone-terminated PEG with small amounts of a monocysteine-containing adhesion peptide (1) followed by reaction of excess bis(cysteine) containing MMP substrate peptide (2). Attack of a cell-secreted MMP is also shown (3). Structures of the multiarm vinyl sulfone-terminated PEG (cross lines); monocysteine-containing adhesion peptide (green wedge shaped arrows), and bis(cysteine) containing MMP substrate peptide (orange divided squares) are shown in the legend. Reprinted from Proc Natl Acad Sci, 100, Lutolf et al., 'Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: Engineering cell-invasion characteristics.' 5413–5418, Copyright 2003, with permission from the National Academy of Sciences, USA [176].

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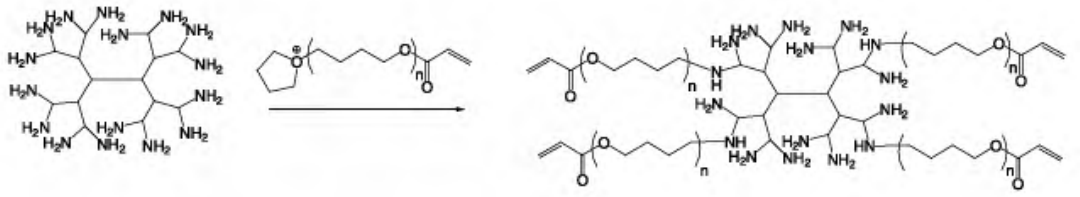


Fig. 52. Self-condensing dendrimer synthesized from PPI and acrylate initiated living PTMO.

capability of these hydrogels to promote tissue growth a bone protein containing hydrogel was placed in a rat cranial defect. Bone regeneration was observed after 4 weeks of hydrogel implantation and depended on the MMP sensitivity of the networks [176,177]. Enhanced bone regeneration was observed due to the additional affinity sites for the bone protein in the matrix [178]. Similarly, the covalent incorporation of a vascular endothelial growth factor in the hydrogel matrix alongside adhesion peptides and MMP substrate peptides aided the regeneration of human endothelial cells and formation of new vascularized tissue in place of the biomaterial [179].

Ferruti et al. studied tissue scaffolds consisting of crosslinked poly(amido amine) hydrogels based on 2,2-bisacrylamidoacetic acid, which was crosslinked using primary diamines [8]. The networks degraded completely within 10 d in Dulbecco's medium at physiological conditions, and cytotoxicity tests using direct contact with fibroblast cell lines demonstrated the non-cytotoxic nature of both networks as well as the degradation products. Bovine serum albumin was also incorporated into selected networks, through mixing with the network precursors prior to crosslinking. Ferruti et al. developed hydrogels containing agmatine, a guanidine functionalized amine precursor that resembles the RGD (arginine glycine aspartic acid) tripeptide, an agent for promoting cell adhesion [180]. The mechanical performance of these tissue scaffolds improved and degradation decreased through the use of a multi-amine functionalized crosslinker molecule, which was also synthesized through poly(amido amine) chemistry.

Goethals et al. reacted living cationic, acryloyloxybutyl triflate initiated poly(tetramethylene oxide) (PTMO) with Astramol™ dendrimers to produce acrylate functionalized dendrimers (Fig. 52) [181]. The presence of both amine and acrylate groups on the dendrimer molecules resulted in rapid gel formation with gel fractions of 88–99%. The pot life varied from 25 to 125 min with the molecular weight of the PTMO changing from 1000 to 4000 g/mol (Fig. 53) Higher degrees of swelling and higher tensile elongations were observed for higher molecular weight PTMO precursors. The pot life was also affected by the temperature of the reaction from 45 min at 25 °C to 4 min at 65 °C. The gel fraction increased with the incorporation of four PTMO arms as opposed to two arms.

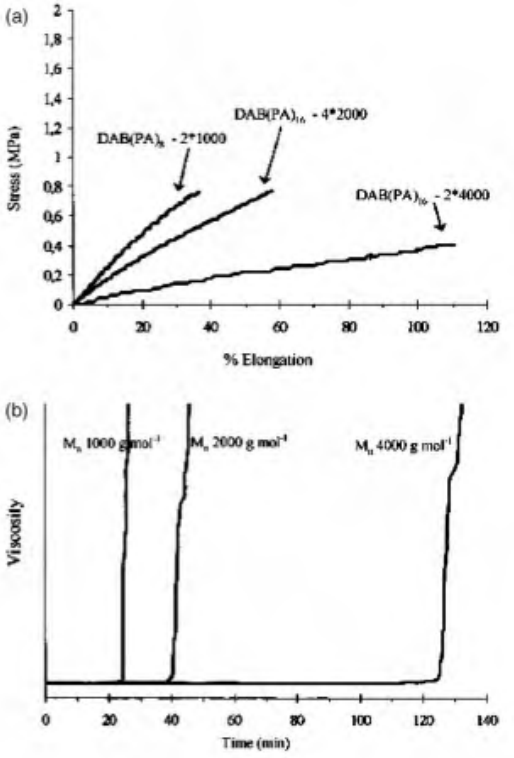


Fig. 53. (a) Effect of dendrimer generation and number of PTMO arms of various molecular weights on tensile strength. (b) Viscosity curves during crosslinking showed longer gel times at higher molecular weights. Reprinted from 'Segmented polymer networks containing amino-dendrimers,' Goethals et al., Copyright 2003 Society of Chemical Industry, with permission from John Wiley and Sons, Ltd on behalf of SCI [181].

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7. Bioconjugates via the Michael addition

Strong interest in bioconjugates has developed in recent years. Bioconjugates consist of synthetic polymers covalently bound to biopolymers (proteins, polysaccharides, polynucleotides, antibodies) or bioactive species (pharmaceuticals). These materials possess special properties that make them useful in biomedical applications and fundamental studies. Coupling synthetic polymers to biomaterials is useful for numerous reasons. Polymer–protein bioconjugates may possess 'stealth' properties, resisting clearance from the bloodstream in terms of excretion from the liver or kidneys, thus increasing circulatory lifetimes of protein drugs. Furthermore, recognition properties may be imparted to synthetic polymers through the incorporation of receptor proteins. Recognition properties are important in artificial organs and implants as well as in biosensors. Polymer–protein and polymer–drug conjugates were used to improve solubility and circulation time within the body. In some cases, biomolecule linking using Michael addition chemistry was studied. Thus, Roy et al. used Michael addition to create sugar-protein conjugates from sialyloligosaccharides [182].

7.1. Polymer–protein and polymer-drug conjugates

Poly(ethylene glycol) (PEG) is the most common synthetic polymer for coupling with proteins. PEG is a non-biodegradable polymer ideal for coupling to proteins due to its biocompatibility and nonabsorbent properties with respect to proteins. Currently, numerous functional PEGs are commercially available for applications in protein pegylation [183]. Michael addition of protein-bound thiol groups to maleimide functionalized PEG is one of the most common methods of polymer–protein conjugate synthesis. A second route is through Michael addition of thiol groups to vinyl sulfone functionalized PEG, resulting in greater hydrolytic stability. PEG containing two maleimide functional groups was used for mimicking the heavy chain end of antibodies and is also useful for binding two proteins in close proximity [183]. The coupling of thiol groups on cysteine residues with polymeric Michael acceptors benefits from several advantages. The Michael addition chemistry occurs readily at physiological conditions and is selective to thiol-containing proteins over amine nucleophiles. The reaction also produces no potentially toxic byproducts [184]. Hubbell et al. studied the reactivity of protein-bound cysteine residues as a function of charge environment [185]. Positively charged residues placed adjacent to the cysteine resulted in a lower pK_a of the cysteine group and increased Michael addition to PEG diacrylates. Negatively charged residues produced the opposite effect. The specific order of the residues adjacent to the cysteine groups had no apparent effect on reaction kinetics. Vinyl sulfone functionalized PEG molecules reacted selectively with cysteine residues over lysine residues at pH 8 and possessed greater hydrolytic stability. Harris et al. coupled reduced ribonuclease with PEG vinyl sulfone [6]. Reaction with lysine residues required a higher pH of 9.3 but was still dramatically slower than the reaction with cysteine at comparable pH.

Lysine residues of proteins also serve as functional group handles for the Michael reaction of acrylamides. This strategy benefits from the lack of dimerization of lysine residues in comparison to cysteine residues. A large number of protein-modifying PEG reagents are directed toward reaction with the amine groups contained in lysine residues. Such reagents include activated acylating agents such as *N*-hydroxysuccinimidyl ester-PEG (NHS-PEG). However, attachment to lysine residues is disadvantageous when the lysine residues are critical to protein binding or enzymatic active sites. Examples of proteins that show diminished activity upon pegylation using lysine targeting modified PEGs are asparaginase, α-galactosidase, and CD4 [186]. The reaction with lysine groups is also less controlled and often results in multiple PEG attachments due to the presence of numerous lysine residues. The use of cysteine via Michael addition to PEG reagents is advantageous in these cases and further benefits from the lack of synthetic byproducts. Thus, Kogan synthesized a maleimide terminated PEG capable of reaction with free cysteine residues in proteins [186].

Hubbell et al. have performed a great deal of work in the area of polymer–protein conjugates, often favoring the method of cysteine Michael addition [187]. PEG bisacrylamide and PEG diacrylate were studied as PEG substrates for coupling with proteins via the Michael addition. Residual acrylamide or acrylate groups permitted free radical crosslinking of the resulting pegylated proteins to create hydrogels with embedded proteins that serve as tissue scaffolds. The reaction of the acrylamide groups with cysteines was more than an order of magnitude slower than the corresponding acrylate reaction, but the acrylamide group possesses superior hydrolytic stability. The more hydrolytically stable and more Michael reactive vinyl sulfone linkage could not be used due to its inability to polymerize free

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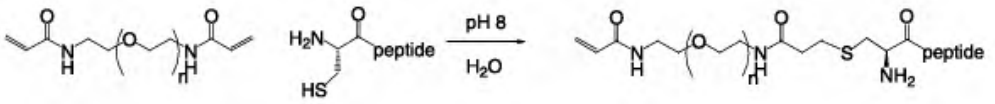


Fig. 54. Protein incorporation via Michael addition of thiol residues to PEG-acrylamide.

radically. The tissue scaffold environment is critical to inducing particular phenotypes to embedded cells. PEG is well known to possess low adhesion to proteins, which allows the study of specific cell-adhesion agents such as RGD protein. Thus, Hubbell et al. introduced RGD protein to PEG bisacrylamide and formed networks [187] (Fig. 54) These networks were treated with cells that spread on the surfaces of the network, clearly well-adhered. Furthermore, the mechanical properties of the crosslinked tissue scaffolds were sufficient to produce durable materials.

Polymer–drug conjugates are of special interest in anticancer therapy where numerous drugs have improved performance in comparison with the parent drug [188]. Conjugates of interest include poly(*N*-(2-hydroxypropyl)methacylamide) doxorubicin, poly(glutamic acid)-paclitaxel and PEG-camptothecin. Several reasons exist for the improved performance of these conjugates including improved solubility, lower toxicity, and longer retention in the bloodstream. Furthermore, some conjugates benefit from the enhanced vascular permeability and retention (EPR) effect, in which drugs are preferentially absorbed into tumor tissue and not released (Fig. 55) In fact, one reason for the lower toxicity of the polymer–drug conjugates is the absence of nonspecific uptake into nontargeted tissues and organs. Generally, polymer–drug conjugates must contain a hydrolysable or enzyme-cleavable linker to allow cellular uptake of the drug molecules at the tumor site. In some cases, linkers are introduced that cleave via lysosomic cysteine proteases. Generally, polymers used in these composites must either biodegrade or possess low enough molecular weight (<30,000 g/mol) to allow removal through the kidneys.

Goodson and Katre accomplished pegylation of recombinant interleukin-2 protein using PEG-maleimide [189]. Interleukin-2 is a glycosylated protein that serves important functions in the immune system and is useful as an anticancer drug. The recombinant

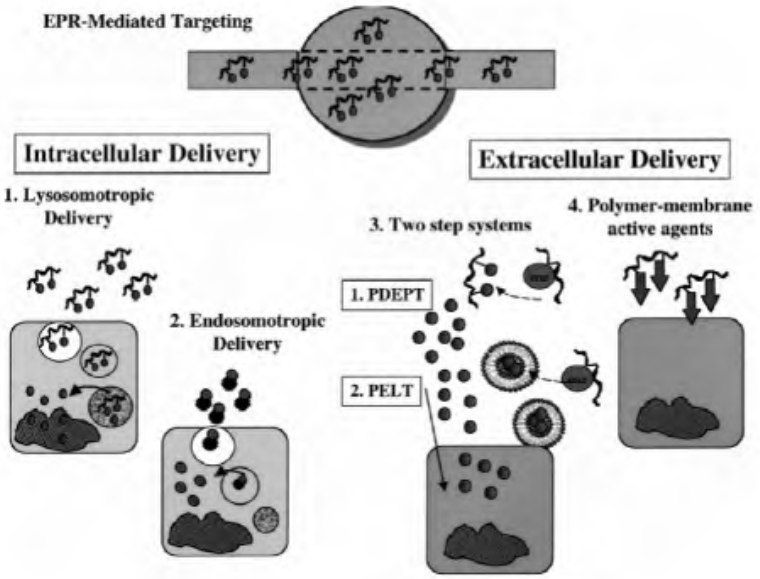


Fig. 55. Drug delivery via polymer–drug conjugates. Enhanced permeability and retention (EPR) mechanism of drug delivery relies on preferential permeation of the conjugate followed by drug release, without reverse permeation. Reprinted from J Control Rel, 74, Duncan et al., 'Polymer-drug conjugates, PDEPT and PELT: basic principles for design and transfer from the laboratory to clinic,' 135–146, Copyright 2001, with permission from Elsevier [188].

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interleukin-2 was modified to include a cysteine site for Michael addition at the glycosidic linkage of the original protein, thereby preventing inhibition of the protein. The conjugation of recombinant interleukin-2 to PEG-maleimide resulted in an increase in hydrodynamic size and solubility, increasing systemic exposure four fold. An increase in hydrodynamic size results in less glomerular kidney filtration compared to the unmodified protein. Katre et al. showed a systematic decrease in clearance with increasing effective molecular weight up to 70,000 g/mol above which the rate of clearance was almost constant, possibly due to a different mechanism of clearance [190]. In previous work, modification of interleukin-2 via amine groups on lysine residues resulted in reduced bioactivity of the protein [191].

Jespers et al. studied recombinant staphlokinase pegylated at artificially introduced cysteine sites using PEG-maleimide [192]. Staphlokinase is an enzyme that is used to treat acute myocardial infarction, through thrombolysis of blood clots within blood vessels. Numerous sites on the enzyme were targeted for introduction of the cysteine residue. The circulatory lifetime of the staphlokinase was increased through pegylation while thrombolytic activity was maintained. The plasma clearance rates of the staphlokinase conjugate decreased with increasing PEG molecular weight. Clinical trials revealed successful restoration of circulation for patients suffering from acute myocardial infarction [193].

Currently, strong interest exists in functionalizing polymers with the RGD protein to promote cell adhesion for tissue scaffolds. The RGD protein selectively promotes cell adhesion, while avoiding nonspecific adsorption of proteins. Furthermore, cell spreading, a change in the cellular morphology, occurs in the presence of the RGD coating, indicating cell signaling has occurred. Hubbell et al. used a heterobifunctional PEG containing *N*-hydroxysuccinimide and vinyl sulfone linkages to couple the RGD containing peptides via cysteine linkages to the PEG and then to couple the PEG-RGD to poly(L-lysine) [194].

Surface grafting of biological molecules was achieved using the Michael addition reaction [184]. Model systems to achieve protein grafted surfaces were studied using PEG acrylated surfaces coupled to cysteine containing proteins such as portions of the Platelet factor 4 protein. A modification route beginning with thiol functionalized surfaces was also pursued, however, the surface grafted thiols were unreactive.

7.2. Coupling of biological molecules to Michael addition polymers

In addition to the Michael reaction as a strategy to couple synthetic polymers to biological molecules, it is also useful in the synthesis of polymers that will be coupled to biological molecules. Numerous Michael addition polymers are useful as precursors for bioconjugates due to their biocompatibility and biodegradability. Poly(amido amine)s are an excellent example of a biocompatible, biodegradable Michael addition polymer. Both linear poly(amido amine)s and dendritic poly(amido amine)s (PAMAM) proved useful in forming bioconjugates.

Ferruti et al. synthesized poly(amido amine) drug conjugates of the anticancer drug mitomycin C, which was bound to pendant hydroxyl groups on the poly(amido amine). In vivo studies showed similar antitumor activity for the conjugate compared to mitomycin C as well as lower toxicity values [45]. Poly(amido amine) dendrimers were also used to facilitate the release of the toxic anticancer drug 5-fluorouracil via covalent incorporation onto the terminal amines on the dendrimer structure [195]. Higher generation dendrimers released larger amounts of 5-fluorouracil, with complete release occurring over the period of about one week.

Glycodendrimers are dendrimers containing saccharide residues on the peripheral functional groups. Glycodendrimer interactions with proteins are often more pronounced than with individual saccharides due to increased 'valency'. However, the density of functional groups at the dendrimer surface may potentially lead to steric hindrance of the sugar groups. Meijer et al. introduced peripheral sugar groups at various distances from the surface of PPI dendrimers using alkyl spacers of various lengths, thus reducing steric congestion [196].

8. Conclusions and future directions

This review outlined the current applications of the Michael addition to polymer synthesis. The Michael addition reaction serves important roles in polymer synthesis, allowing the development of novel polymers from diverse monomer feedstocks. Polymers of numerous different topologies including linear, dendritic, hyperbranched and network polymers are enabled. Both step growth and chain growth techniques are utilized. The flexibility of the Michael reaction in terms of monomer functionality, solvent environment, and conversion at ambient or near ambient temperature

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allows the production of sophisticated macromolecular systems for technologically important applications. Other advantages include a general lack of sensitivity to oxygen and the absence of low molar mass byproducts, which require removal.

The applications of Michael addition polymers are as diverse as their compositions and topologies, ranging from networks for coatings and adhesives to linear, biodegradable polymers for gene transfection or drug delivery. Several Michael addition polymers such as poly(amido amine)s benefit from biocompatibility and non-toxic degradation products. Michael addition also serves as a synthetic tool for coupling polymers with biological systems such as proteins and enzymes. The ability to carry out the Michael addition under physiological conditions permits novel tissue replacements, cell scaffolds and direct application.

One aspect of the Michael addition largely unused in polymer science is the ability to carry out stereospecific Michael additions. The application of special catalysts to achieve stereospecific Michael addition may yield polymers with improved biological applications and potential crystallinity. Chirality is particularly important in biological systems including recognition processes with proteins. Another likely future direction for Michael addition polymerizations is the increased use of bio-based reactants. The number of bio-based precursors entering the market and the multitude of functionalities available for Michael addition chemistry are growing. Many of these materials are amenable to modification for introduction into Michael addition systems. Furthermore, the tolerance of the Michael reaction to protic impurities allows the use of less well-defined feed streams.

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