

Engineering antibodies for clinical applications

Maneesh Jain, Neel Kamal and Surinder K. Batra

Department of Biochemistry and Molecular Biology, Department of Pathology and Microbiology, Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198-5870, USA

Molecular engineering has contributed immensely to the clinical success of antibodies in recent years. The modular structure of antibodies has permitted their modification in numerous ways, to meet various clinical requirements. With the help of antibody engineering, it has been possible to modify the molecular size, pharmacokinetics, immunogenicity, binding affinity, specificity and effector function of antibodies. In addition, fusion proteins of antibodies with various proteins and peptides have yielded targeted biological modifiers, toxins and imaging agents. This review focuses on the recent trends in antibody engineering for improving their clinical utility.

Introduction

Almost a century ago, antibodies were envisioned as ‘magic bullets’ for the specific targeting of a disease site. The recent success of antibodies in clinics has justified this reputation and has revolutionized treatment options for various diseases. Antibodies can be used as unarmed therapeutic agents that inhibit a target involved in disease progression or by causing the cytotoxic death of target cells, mediated by modulators of the immune response. Alternatively, antibodies can act as carriers to target cytotoxic and imaging agents, such as radioisotopes, toxins, and drugs, to the site of a disease [1,2]. The diverse clinical applications of antibodies have necessitated their modification in a variety of ways, thus laying the foundation of antibody engineering. Antibodies have been engineered to alter their size, pharmacokinetics, immunogenicity, specificity, valency and effector functions [3,4]. In addition, antibodies have been expressed as fusion proteins for the targeting of various cytokines, toxins and enzymes for therapeutic applications [5]. Out of ~20 antibody-based products approved by the Food and Drug Administration (FDA) for human use, 85% are the fruits of antibody engineering [6]. These include chimeric (see Glossary), humanized (see Glossary), and fully human antibodies; antibody fragments; phage-derived antibodies; and toxin-conjugated antibodies. Various other versions are under development, including bispecific antibodies, single-chain antibodies, single-domain antibodies and minibodies [1,3,6]. In this review, we have highlighted the recent developments in the field of antibody engineering that have helped in the clinical advancement of these magic bullets. We have placed more emphasis on the

application of antibodies in cancer because ~89% of the antibodies have been approved for oncological and immunological indications [6]. The focus is on the modification of antibodies using genetic engineering approaches, for clinical applications.

Antibody engineering for desired pharmacokinetics

Several antibodies have been approved for the treatment of various cancers, cardiovascular diseases, infectious diseases, rheumatoid arthritis and other autoimmune diseases. The pharmacokinetic requirements of a given antibody depend upon the nature of its application. In the case of therapeutic antibodies, which generally act either by inhibiting a signaling pathway or by inducing antibody-dependent cell-mediated cytotoxicity (ADCC; see Glossary) or complement-dependent cytotoxicity (CDC; see Glossary), a prolonged serum half-life is desirable, for longer bioavailability and to avoid repeated injections (making it cost effective). Alternatively, if an antibody is being used as a vehicle for the delivery of a cytotoxic agent, such as a radionuclide, drug or toxin, the rapid elimination of the untargeted conjugate is preferable, to avoid non-target-tissue toxicity. In general, intact antibodies and antibody fragments >60 kDa are above the renal threshold and exhibit prolonged circulation, whereas smaller antibody fragments (<60 kDa) exhibit a shorter serum half life.

Glossary

Antibody-dependent cellular cytotoxicity (ADCC): lysis of target cells (e.g. parasites or cancer cells) that have surface-bound specific antibody, by immune effector cells. The most common effector cells include Natural Killer (NK) cells or eosinophils that recognize the target-cell surface-bound antibody through their Fc receptors.

Antibody-directed enzyme prodrug therapy (ADEPT): use of tumor antigen-specific antibody linked to a drug-activating enzyme for the activation of prodrug (inactive drug) specifically at the tumor site.

Chimeric antibody: recombinant antibodies where the murine variable region is fused to the human constant region.

Complement-dependent cytotoxicity (CDC): a mechanism of killing cells in which an antibody bound to the target cell surface directs the recruitment of proteins of the complement system (complement fixation). This results in the assembly of the membrane attack complex that forms holes in the target cell membrane leading to cell lysis.

Complementarity-determining regions (CDR): short stretches of amino acid residues in the hypervariable regions of the variable domains involved in the specific recognition of the antigen.

Framework region: the amino acid stretches in the variable region that intervene and flank the three CDRs.

Humanized antibody: recombinant antibodies resulting from the grafting of murine hypervariable regions to the human antibody framework.

Radioimmunotherapy (RIT): use of tumor-antigen-specific antibodies or antibody fragments to deliver therapeutic radioisotopes specifically to tumor cells.

Corresponding author: Batra, S.K. (sbatra@unmc.edu).

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Small antibody fragments – from IgGs to scFvs

The driving force for the development of small antibody fragments was to make these molecules suitable for the radioimmunotherapy (RIT; see Glossary) of cancer. The large molecular size of intact IgG resulted in increased circulation times, which caused dose-limiting myelotoxicity in therapeutic applications and a high background in imaging. In addition, effector functions mediated by the Fc region are generally undesirable in some applications. The initial attempts to make smaller fragments were based on the digestion of intact IgGs by the proteolytic enzymes pepsin or papain, to generate F(ab')₂ or Fab' fragments. These fragments retained their specific antigen-binding capabilities and exhibited faster blood clearance and homogenous tumor penetration compared with intact IgGs. Advances in recombinant DNA technology facilitated the production of smaller recombinant antibody fragments.

Antibody engineering started with the development of antibody fragments encoded by a single gene, wherein only the regions involved in antigen binding – the variable heavy (V_H) and light (V_L) chains – were linked by a short peptide. The resulting protein was called a single-chain Fv (scFv) and was monovalent in binding [4]. In general, the affinity of a given scFv is comparable with the parental Fab fragment but owing to their monovalent binding such antibodies are of limited therapeutic significance. However, because of their small size (30 kDa), most scFvs have a short serum half life (<10 min) compared with the intact IgG, which can make them useful for diagnostic applications such as imaging [4]. Nanobodies, which are derived from the V_HH domains of heavy-chain antibodies, are the smallest antigen-binding domains and have recently generated enthusiasm (Box 1). Nanobodies have been recently generated against various tumor antigens, including MUC1 and EGFRvIII, and exhibited tumor targeting in animal models [7–9].

Multimerization of antibody fragments was undertaken to improve their serum half-life and also binding affinity and/or avidity [4,10–12]. Divalent scFvs were developed from the monovalent scFvs, either through the spontaneous non-covalent association of two scFv molecules (diabodies), or through the covalent disulfide bond [13–16]. Divalent and multivalent scFvs were also generated by linking two or more scFv molecules using a peptide linker [10,17]. The design of various antibody fragments is indicated in Figure 1. The initial strategies relied on the

use of linkers or the spontaneous formation of dimers or higher-order structures [4,10,13]. Other approaches involved the use of multimerization domains, including streptavidin, P53 and leucine zippers, fused to the antibody fragments [18–20]. Similar to scFvs, V_HHs have also been engineered into divalent fragments by connecting them with a peptide linker [8]. Zhang *et al.* described the use of the B-subunit of *E. coli* verotoxin (VTB), which self assembles into a homopentamer, for the pentamerization of V_HH fragments [21]. Furthermore, the same investigators were able to generate bispecific, decavalent molecules by the fusion of a single-domain antibody of one specificity to the N-terminus and a different specificity to the C-terminus of VTB [22].

Back to the intact antibody

The earlier, smaller antibody fragments were generated primarily because of the need for rapid blood clearance and homogenous target localization. With the advent of display libraries (Box 2), direct screening of the antibodies in a single-chain format, or even the Fab, is possible [23]. For clinical applications, these antibody fragments are often re-engineered back to the intact antibody [24]. An excellent example is adalimumab (Humira), an FDA-approved anti-TNF α antibody, which was selected using a human Fab library and later engineered into IgG1. Similarly, scFvs selected from the scFv-phage libraries are redesigned to incorporate the Fc region to form scFv–Fc fusion proteins [25,26]. The fusion of scFvs with Fc not only prolongs the serum half-life and stability of the scFvs but also improves their therapeutic efficacy owing to the ADCC and CDC mediated by the Fc portion [26,27]. An anti-ErbB2 compact antibody Erbicin (Figure 1) is an example of this approach, which is currently being evaluated in preclinical studies. It was shown to downregulate ErbB2, induce apoptosis and inhibit cell cycle progression of ErbB2 positive cells *in vitro*, and inhibit tumor growth *in vivo* [26,27].

Box 1. Unique *Camelidae* antibodies

The members of *Camelidae* (camels and llamas) produce a unique class of immunoglobulins called heavy-chain antibodies [104,105]. These antibodies are completely devoid of light chains and their variable domain, termed V_HH or nanobody, is the smallest domain (15 kDa) capable of independently binding an antigen [106]. In contrast to the conventional V_H domains, V_HHs have longer CDR loops that enable improved penetration in the cryptic epitopes on target antigens. The H3 loop forms a large convex paratope that interacts with the cryptic concave epitopes that are generally inaccessible to the conventional V_L–V_H pair [107]. V_HHs exhibit greater thermal stability, solubility and higher affinities (usually in nanomolar range) compared with the conventional variable domains. In addition, the V_HH domains exhibit greater intracellular stability and can be useful for targeting cytoplasmic proteins.

Box 2. Antibody phage display technology

Phage display is a system in which a combinatorial library encoding proteins or peptides fused to the gene encoding coat protein of a filamentous phage is generated. The resulting proteins or peptides are displayed on the surface of a phage as a fusion with the coat protein of the virus. The phage library can be screened by successive rounds of panning and amplification to isolate phages displaying proteins or peptides that bind to the target of interest. Because the DNA that encodes this protein is housed within the virion, the technology enables the isolation of proteins of interest and the genetic information that encodes it, in a single step.

Various naïve, immunized and synthetic libraries enabling the display of murine and human antibody fragments (both in Fab and scFv formats) have been created [23]. Immunized or naïve phage libraries are constructed by isolating the peripheral lymphocytes from immunized or non-immunized donors respectively. The mRNA is isolated and variable regions are amplified using degenerate primers and cloned in a suitable display vector. Although such libraries enabled the rapid selection of high-affinity antibodies against various antigens, their major limitation is their inability to produce antibodies against the self antigens. Synthetic libraries derived from a combinatorial arrangement of various germ line antibody gene segments (V_H, D_H, and J_H or V _{κ} and J _{κ} for heavy and light chain, respectively) have the potential to encode for self antigens. Immunized and naïve phage libraries have also been generated for isolation of camelid antibody fragments [108,109].

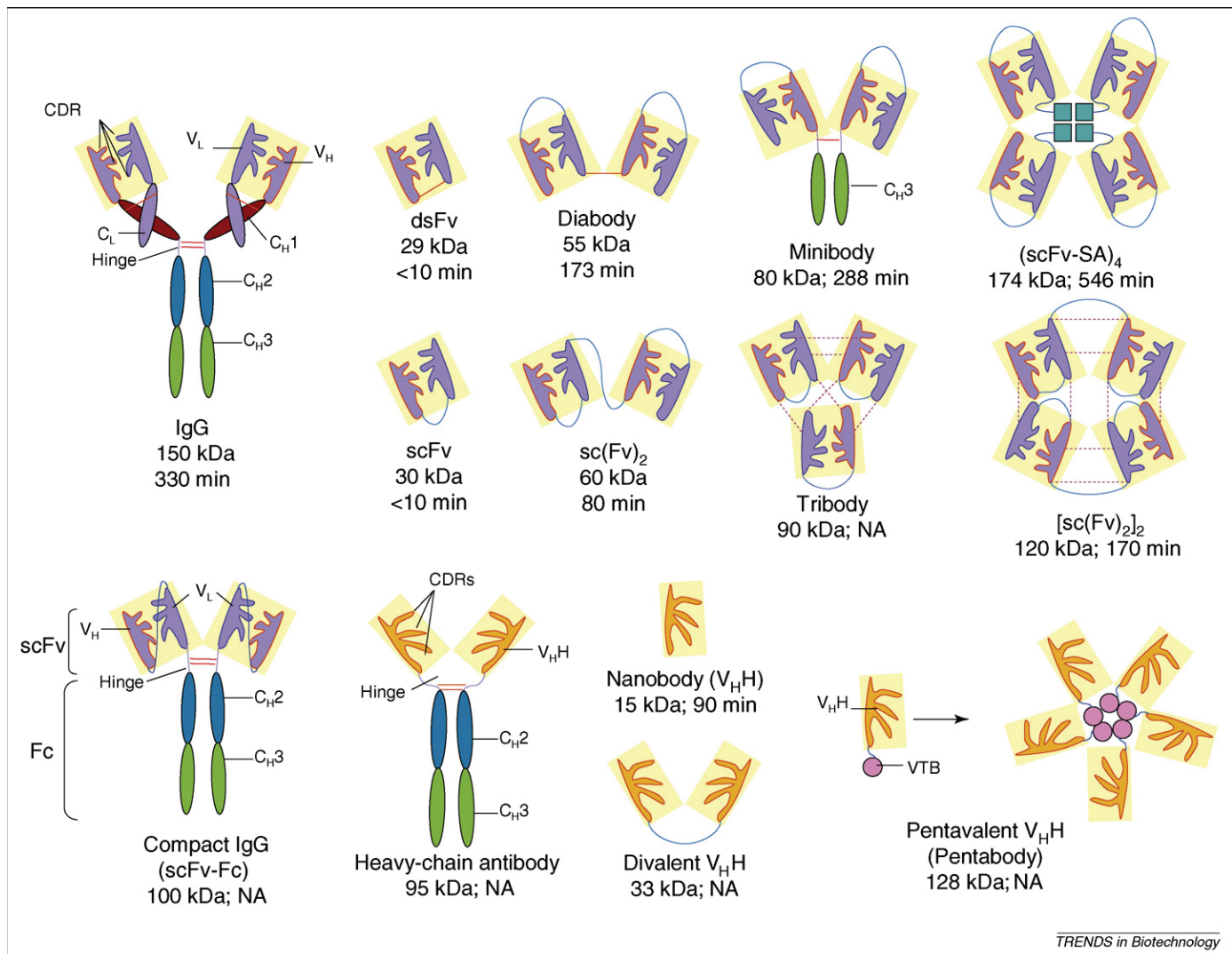


Figure 1. Schematic structure of intact antibody and various genetically engineered antibody fragments. The six CDR loops in the variable regions of heavy (V_H) and light chains (V_L) are involved in antigen binding. V_L and V_H together form a monovalent antigen binding site called Fv (shaded yellow) and can be stabilized either by a disulphide bond (dsFv) or connected through a peptide linker (scFv). Divalent and multivalent antibody fragments can either form by spontaneous association of monovalent scFvs (diabodies and tribodies) or can be generated by coupling monovalent scFvs by a peptide linker, such as divalent sc(Fv)₂. Tetravalent antibody fragment [(scFv)₂]₂ can form by spontaneous non-covalent association of covalent dimeric sc(Fv)₂ or can form by inclusion of multimerization domains of proteins, such as streptavidine [(scFv-SA)₄] or p53. Sometimes, constant region domains of the antibodies are included in scFvs for dimerization and improved serum stability and pharmacokinetics (minibody, which contains C_H3) or to incorporate effector domains (compact IgG, containing Fc region). Camelids express a unique class of antibodies called 'heavy chain antibodies' that are completely devoid of light chains (see Box 1 for details). The variable domain of heavy chain antibodies, V_{HH} (also called nanobody) is the smallest independent domain capable of antigen binding. Similar to scFvs, V_{HH} s have also been engineered into divalent (using peptide linker) and multivalent fragments (using pentamerization domain of bacterial toxin-see text for details). The molecular size and the serum half life of various fragments are indicated. The disulphide bonds are represented by solid red lines, the peptide linkers with blue lines, whereas the non-covalent protein-protein interactions are represented by dotted brown lines. (Abbreviations: NA, not available; SA, streptavidine; VTB, B-subunit of *E. coli* verotoxin.)

Altering FcRn-antibody affinity for improved pharmacokinetics

The neonatal Fc receptor (FcRn) has an important role in controlling the levels of serum IgGs by facilitating their recycling and preventing their catabolism in the lysosomes of the vascular endothelial cells [28]. The pharmacokinetics of IgG can be modulated by altering the binding affinities of the Fc region to the FcRn. Mutations resulting in an increased binding affinity of IgG to the FcRn translate into prolonged persistence [29], whereas those that decrease the binding affinity result in shorter half life of IgGs in the serum [30,31]. By mutating the crucial amino acid residues in the Fc region involved in the binding to FcRn receptors, Kenanova *et al.* recently generated a series of anti-CEA scFv-Fc fusion proteins that exhibit a

spectrum of pharmacokinetic properties in mice [32]. The terminal half-lives of the mutant scFv-Fc proteins ranged from 7.96 h to 83.4 h, whereas that of the wild-type protein was ~12 days [32]. By contrast, mutations that improved the binding affinity of human IgG to FcRn at pH 6.0 were used to generate antibodies that had prolonged serum half-life in primates [33]. In a recent report, Vaccaro *et al.* generated a mutant antibody that exhibits a higher binding affinity and reduced pH dependence to FcRn. The mutant antibody inhibited the FcRn-IgG interaction, enhanced IgG degradation and resulted in the rapid decrease in IgG levels when administered into mice [34]. Such an approach has therapeutic potential for treating autoimmune diseases where self-reactive antibodies are involved in the pathogenesis.

Antibody engineering for reducing immunogenicity

Mouse monoclonal antibodies, when administered in humans, elicit a human anti-mouse antibody (HAMA) response that can alter their biodistribution and accelerate clearance, thus reducing the efficacy of subsequent administrations [35]. To reduce their immunogenicity, antibodies are engineered in several ways. The first forms were mouse–human chimeric antibodies, in which the mouse heavy and light chain variable regions (involved in the antigen binding) were fused to the constant regions of the human heavy and light chains, respectively, resulting in a chimeric molecule. Currently, ~20 antibodies have been approved for use in humans to treat various diseases, of which approximately half are chimeric. When the chimeric antibodies were administered into the humans, a human anti-chimeric antibody response (HACA) was observed [35]. To further reduce the immunogenicity, the complement-determining regions (CDRs; see Glossary) of the mouse IgG were grafted onto the human framework region (see Glossary), resulting in the generation of humanized antibodies [36,37]. However, the affinity of the initial CDR-grafted antibodies was lower than that of the parental murine or rodent IgGs. To improve the binding affinity of the humanized antibodies, either amino acid residues in the framework region of the human heavy chain were altered or the CDR grafting was performed on the human framework regions with closest sequence similarity to the parental murine or rat antibody [38,39]. Nevertheless, CDR-grafted, humanized antibodies were shown to evoke an anti-variable region immune response. An analysis of the known antigen–antibody complexes suggested that not all the residues in the CDRs are crucial for antigen binding. Only ~20–33% of the residues, termed specificity-determining residues (SDRs), which are commonly located at positions exhibiting highest variability, are involved in antigen contact [40]. Minimally immunogenic antibodies have been generated by grafting the SDRs of murine antibodies onto either a human antibody framework [41] or on to multiple human germ line templates [42]. Another approach to reduce the immunogenicity of murine antibodies is based on the premise that only the surface residues contribute to the immunogenicity of the variable regions. Therefore, only the surface residues of the murine antibody are replaced with the human ones, while leaving the CDR and buried amino acids unaltered. Using homology-based molecular modeling, an anti-TNF α antibody was generated with a resurfaced variable domain [43]. Although the antibody was tested for its ability to neutralize TNF α , its immunogenicity is still to be evaluated.

De-immunization is another approach to reduce the immunogenicity of chimeric or mouse antibodies. It involves the identification of linear T-cell epitopes in the antibody of interest, using bioinformatics, and their subsequent replacement by site-directed mutagenesis to human or non-immunogenic sequences. Using this approach, an EGF-blocking, murine antibody, R3, was de-immunized by replacing six amino acid residues in the heavy chain variable region [44]. Similarly, two other antibodies, one directed against CD6 and another recognizing a carcinoma glycoprotein, were de-immunized by altering seven or eleven residues, respectively [45]. Although these de-immunized antibodies exhibited reduced immunogeni-

city in monkeys, compared with their chimeric counterparts, some loss of binding affinity was observed. Recently, pre-clinical studies and clinical trials have been performed with de-immunized antibodies directed against complement C3 and prostate-specific membrane antigen (PMSA) [46,47]. In a phase I trial, anti-PSA de-immunized MAb resulted in targeting of prostate cancer metastases in bone and soft tissues and did not elicit an immune response [46].

Although humanization reduced the immunogenicity of the antibodies, humanized antibodies elicited human anti-human antibody (HABA) response [35]. For the humanized antibodies approved for human use, the HABA response rate ranges between 0.1% (Herceptin) and 34% (Zenapax). Furthermore, chimerization and humanization of antibodies is a labor-intensive endeavor, involving sequence analysis, the development of engineering methodologies, analysis and optimization of binding affinity, and evaluation of immunogenicity for each antibody.

Recent technological advancements have facilitated the development of fully human antibodies. One approach involves the use of transgenic mice, in which the mouse immunoglobulin loci are inactivated and replaced with human immunoglobulin genes. Several groups have developed various mouse strains using different transgenic methodologies. Some of the popular strains of mouse include Xenomouse (Abgenix; <http://www.abgenix.com/>), HuMab mouse (GenPharm; <http://www.genpharm.ca/>), TransChromo (TC) mouse (Kirin Brewery Company; <http://www.kirin.com/>) and KM mouse (Kirin Brewery Company and Mederex; <http://www.medarex.com/>). The development of these mouse strains is described in recent review articles [1,48]. Panitumumab, directed against EGFR and generated using Xenomouse, was recently approved for the treatment of metastatic colorectal cancer [49]. Another fully human monoclonal antibody, Zanolimumab (<http://zanolimumab.com/>), directed against CD4, was developed using HuMab technology and is in a phase III clinical trial for cutaneous T-cell lymphoma [48]. In addition, as many as 14 other fully human monoclonal antibodies, developed using different strains and directed against various antigens, are in advanced clinical development for treating various infectious and inflammatory diseases, allergies and several types of cancers [48].

Advances in phage display technology (Box 2) have provided another route for the development of fully human antibodies [23]. Adalimumab (Humira; <http://www.humira.com/>), an anti-TNF α antibody, was developed using a human phage-display library and has been approved for treating rheumatoid arthritis. Approximately, 15 fully human antibodies derived from phage libraries and directed against as many antigens are in clinical trials for the treatment of asthma, autoimmune diseases and various malignancies [23].

Bispecific antibodies

Naturally occurring antibodies are directed against a single antigen: they are monospecific. Advances in antibody engineering and expression methodologies have made it possible to combine the specificities of two antibodies into a single molecule, called a bispecific antibody (BsAb), for various clinical applications (Box 3) [50]. There

Box 3. Application of bispecific antibodies in immunotherapy and pretargeted radioimmunotherapy

The earliest dual specificity antibodies were designed to activate and target immune effector cells to the tumor cells. In these antibodies, one arm is specific for the tumor cell surface antigen, whereas the other arm recognizes and activates the signaling receptor on the effector cells. The combination results in the tethering of immune effector cells to the target tumor cells and their activation, resulting in the killing of the target cells. A majority of BsAbs are designed to attract cytotoxic T-cells, with one arm specific to CD3, and the other arm is directed toward a tumor antigen such as EGFR, Her2/*neu*, CA-125 or CD20 [50]. Such BsAbs have been used in the immunotherapy of non-Hodgkin's lymphoma, breast, prostate and ovarian cancers.

BsAbs have also shown promise in an emerging therapeutic and diagnostic modality called pre-targeting [110]. In a pre-targeting approach, one arm of the antibody is directed toward the tumor antigen, whereas the second arm specifically recognizes a small molecular weight, radiolabeled hapten. Initially the tumor is targeted using the specificity of an anti-tumor antigen antibody, and the unbound antibody is cleared through the circulation. In the next step, radiolabeled hapten is administered and this is captured and retained specifically in the tumor through its interaction with an anti-hapten antibody, and cleared rapidly from the circulation owing to its smaller size. Several dual specificity antibodies directed against tumor antigens and small molecular weight radioconjugates have been described [110].

are several ways in which antibodies specific for two or more antigens can be generated (Figure 2), many of which are discussed in recent review articles [51,52]. Here, we have highlighted some of the recent approaches for the generation of BsAbs.

One of the earliest BsAb resulted from the fusion of two hybridomas producing antibodies of two desired specificities. However, the yields and purity of the desired BsAbs from these hybrid hybridomas (also termed as quadromas) was poor because the pairing between the heavy and light chains of the two specificities was a purely random event. This problem was partially addressed with the development of a mouse–rat quadroma, which resulted in preferential pairing of heavy and light chains from one species (Figure 2). The single-step binding to protein A followed by the differential elution of the BsAb at pH 5.8 further reduced contamination with parental antibodies [53]. Ertumaxomab (Trion Pharma; <http://www.trionpharma.de/>) is an intact trifunctional, BsAb developed using the quadroma approach and has recently entered phase I clinical trial for metastatic breast cancer [54]. It is directed against HER2 and CD3 and exhibits selective binding to Fcγ type I/III receptors, thereby resulting in the formation of a complex between tumor cells, T cells and accessory cells, such as macrophages or dendritic cells.

Using identical light chains on each arm and forced heterodimerization of the heavy chains, Merchant *et al.* generated bispecific IgG recognizing HER3 and human thrombopoietin receptor Mpl, using the 'holes and knob' methodology (Figure 2) [55]. The six conserved residues present at the interface of the pairing C_H3 domains were mutated such that the residues on one heavy chain were replaced with an amino acid with a shorter side chain (hole), whereas the corresponding residues on the other heavy chain were replaced with an amino acid with a larger side chain (knob). This approach yielded bispecific IgG with

>90% purity, and the mutations in the C_H3 domains did not have an effect on the effector function of IgG [55].

Recently, Rossi *et al.* described an elegant approach, called the 'dock and lock' method, to generate a trivalent BsAb and demonstrated its utility in a pretargeting approach (Box 2) [56]. Using the dimerization and docking domain (DDD) of the R subunit of cAMP-dependent protein kinase (PKA) and the anchoring domain (AD) of the A kinase anchor proteins, a stable, bivalent, anti-CEA–monovalent anti-HSG was generated for pretargeted radioimmunotherapy (Figure 2) [56]. The main advantage of this system is that an antibody fragment of any specificity can be produced, separately, as fusion proteins with either DDD or AD and, when needed, a simple mixing of the two purified products will give the desired BsAb.

Another recent approach to generate BsAbs involves the attachment of a single variable domain (sVD) antibody to the N terminus of the light chain of a functional IgG of a different specificity [57]. A BsAb was generated by genetically attaching a sVD directed against mouse platelet-derived growth factor receptor (mPDGFR)-α to the light chain of an IgG specific for mouse vascular endothelial growth factor receptor (mVEGFR) (Figure 2) [57]. Although the sVD fragments used in the study were selected from a naïve human Fab library, the availability of libraries derived from naturally occurring single domain antibodies (V_HH) from *Camelidae* (Box 1) and Shark V-NAR has facilitated the discovery of sVDs of various specificities [3]. The usefulness of V_HH libraries in the generation of bispecific antibodies is highlighted in a recent review article [58].

Optimization of effector functions by antibody glycoengineering

Many therapeutic antibodies act by killing the target cells through ADCC or CDC. These effects are mediated by the interaction of surface-bound immunoglobulins with Fcγ receptors or the components of the complement pathway. Recent clinical evidence shows that the efficacy of a therapeutic antibody depends on its affinity for the FcγRIII receptor. Polymorphisms in the FcγRIII receptor were shown to predict the response to Rituximab therapy in patients with follicular lymphoma [59,60]. The genotypes predictive of higher affinity of FcγRIII with Fc were associated with increased response rates. The N-linked glycans attached at the Asn297 residue in the Fc region of IgG are crucial for the optimal interaction of the Fc region with the Fcγ receptors. The lack of fucose sugar in the N-linked glycans has been shown to improve the binding of IgG1 to the FcγRIII receptor and enhance ADCC by as much as 50-fold [61,62].

The fucose content on the IgGs can be modulated in several ways. Most rat hybridomas produce IgG with low fucose content, whereas the fucose content of IgG produced by CHO cells is higher [63]. Alternatively, IgGs with low fucose content can be produced by expressing them in a cell line that is deficient in its ability to add fucose to the proteins [61] or in which the fucosyl transferase have been stably knocked-down using siRNA [64]. Another approach to generate antibodies with low fucose content is by the co-expression of acetyl-glycosaminyl transferase III. This enzyme catalyzes the addition of a bisecting GlcNAc residue, which

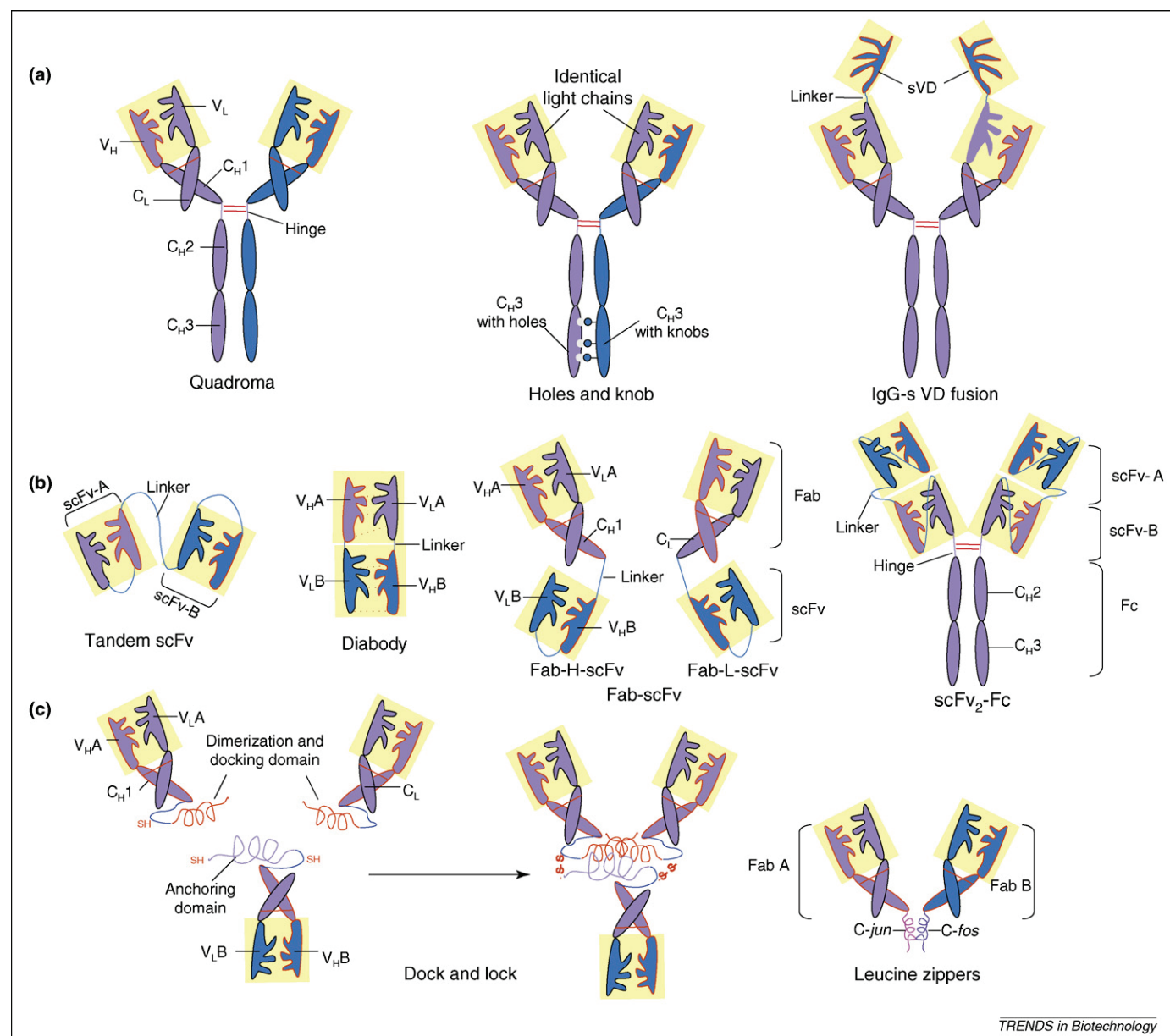


Figure 2. Various methodologies employed for the generation of bispecific antibodies. BsAbs have been generated in IgG formats (a), scFv formats (b) or Fab format (c). Initial approach to generate BsAbs relied on fusion of two hybridomas secreting antibodies of different specificities (quadroma). 'Holes and Knob' approach was employed to avoid chain mispairing and involved mutation of interacting amino acid residues in the C_H3 domain for forced pairing of heavy chains of different specificities. The residues on one heavy chain are replaced with an amino acid with shorter side chain which creates a 'hole'; on the other heavy chain the corresponding residues are replaced with the ones with bulkier side chains, thus creating a 'knob'. In another approach, a single variable domain (sVD), which can bind to antigen independent of light-chain, is fused to the N-terminal of V_L of different specificity, thus giving rise to IgG-sVD. Similarly, scFvs of two specificities are joined by a peptide linker to form tandem sc(Fv)₂ or associated non-covalently into a diabody. Bispecific tandem sc(Fv)₂ can be fused to Fc fragment to yield a bispecific-tetravalent protein [sc(Fv)₂-Fc]. Fab-scFv results from the fusion of scFv of one specificity with, either C_H1 (Fab-H-scFv) or C_L (Fab-L-scFv) domain of the Fab, of another specificity. In the 'Dock and Lock' method, Fab of one specificity is fused with a docking and dimerization domain (DDD) of protein kinase A (PKA), whereas the Fab of another specificity is fused to the anchoring domain (AD) of kinase anchor protein and expressed separately. When purified proteins are mixed, a bispecific, trivalent complex formed containing two Fabs containing DDD and one with AD. BsAbs can also be formed by using fusion proteins containing heterodimerization domains such as Leucine zippers.

affects the subsequent glycosylation steps involved in the addition of fucose [65]. Using this approach, the ADCC activity of an anti-Lewis Y antibody was increased nearly tenfold. Similarly, enhancement in ADCC was achieved for a bispecific, tetravalent scFv-Fc fusion protein (Figure 2) targeting MUC1 and TAG72. The fusion proteins expressed in *FUT8* knockout CHO cells exhibited a 100-fold higher ADCC compared with those expressed in wild-type cells [66]. Nowadays, several methods are used for the rapid production of antibodies for therapeutic applications. These

include yeast- and plant-based systems. The glycosylation machinery of these systems is different from that of humans and the resulting IgGs might have glycosylation patterns different from those of humans. The altered glycosylation will not only influence the effector function of the IgGs but also increase their immunogenicity. Recent studies have successfully demonstrated the feasibility of engineering metabolic pathways in yeast [67] and plants [68] for glycosylation of IgGs that is suitable for use in humans.

Antibody fusion proteins for biological activity

Fusion constructs comprising antibodies and proteins or peptides have been generated for various therapeutic applications (Table 1). Antibody molecules in the fusion constructs are generally used to direct therapeutic agents, such as toxins, cytokines or a drug-activating enzymes, to the tumor microenvironment. These and other agents (e.g. radioisotopes) have also been chemically conjugated to antibodies, and such immunoconjugates have been reviewed elsewhere [1,2]. We have, therefore, limited this discussion to fusion proteins only.

Owing to their immunomodulatory and anti-tumor effects, various cytokines (e.g. IL-2, TNF α , IFN γ and GM-CSF) have been explored as therapeutic agents, either alone or in adjuvant settings. Unfortunately, their clinical application has been hampered owing to their high degree of systemic toxicity, when administered in physiologically relevant doses, their relatively short serum half-lives and their inability to target tumors specifically. Antibody–cytokine fusion proteins were developed to circumvent these hurdles and to deliver the cytokines specifically to the disease site, with a relatively prolonged half-life and reduced systemic toxicity [69]. Among the cytokines used, fusion proteins of IL-2 with antibodies and antibody fragments have been most studied [70–74]. Antibody–IL-2 fusion proteins are in phase I clinical trials for the treatment of melanoma and neuroblastoma [70,72], and in preclinical studies for improving RIT [75,76]. Similarly, fusion proteins of anti-tumor antibodies with GM-CSF have been shown to exhibit anti-tumor activity [77]. Moreover, the utility of antibody–IL-2 and antibody–GM-CSF fusion proteins as adjuvants with anti-tumor vaccines, to induce anti-tumor immune response, has also been demonstrated [74,78–80]. Fusion proteins of antibodies, both in

IgG format and scFvs, have also been generated with TNF α , to direct the delivery of this pleiotropic cytokine to the tumor microenvironment [81–84]. Recently, a fusion protein between TNF α and an anti-HER2/*neu* scFv was described [85]. TNF α is active as a trimer and likewise the scFv–TNF α fusion protein formed trimers spontaneously. The fusion protein elicited Her2/*neu* signaling, prevented apoptosis and promoted wound healing of injured epithelium *in vitro* [85]. Epstein *et al.* recently generated fusion proteins for tumor immunotherapy, comprising anti-tumor antibodies and a co-stimulatory molecule (B7.1) or a minor histocompatibility antigen (H60), for activation of T cells and NK cells, respectively, [86,87].

Immunotoxins represent another class of fusion proteins, and various toxins have been expressed as fusion proteins with antibodies or their fragments. Several plant and bacterial toxins coupled with antibodies directed against various antigens, including CD22, CD25, Lewis Y antigen, EGFR and mesothelin, are being evaluated to treat both hematological malignancies and solid tumors [88–90]. Recently, a fusion protein containing an anti-CEA diabody fused to *Renilla* luciferase was developed and used as an optical probe to visualize CEA-positive xenografts in mice [91]. A fusion protein of anti-TAG 72 scFv with streptavidin has been used for pretargeted radioimmunotherapy [92], whereas an anti-TAG 72 scFv– β -lactamase fusion protein is being evaluated for antibody-directed, enzyme pro-drug therapy (ADEPT; see Glossary) [93]. A recombinant protein comprising anti-EGFR scFv fused to a pro-apoptotic serine protease granzyme B exhibited selective killing of EGFR-positive tumor cells [94]. Several fusion constructs have also been generated with nanobodies; nanobody– β -lactamase [95] and nanobody–GFP [96] constructs were recently designed for ADEPT and live-cell

Table 1. Antibody-based fusion products under development for therapy and diagnosis

Fusion partner	Antibody format	Intended function	Application	Refs
Immunomodulators				
IL-2	IgG/scFv	Targeted delivery of IL-2, enhancement of vascular permeability	Melanoma and neuroblastoma (phase I) Optimization of RIT of solid tumors (preclinical)	[70,72] [75,76]
TNF- α	IgG/scFv		Antitumor (preclinical)	[81–84]
GM-CSF	IgG	Improved antigen presentation, activation of humoral and cellular immune response	Antitumor (preclinical) Vaccination (preclinical)	[77] [78–80]
B 7.1	IgG	Co-stimulation of T cells	Immunotherapy (preclinical)	[87]
H60	IgG	Activation of NK cells	Immunotherapy (preclinical)	[86]
Toxins				
PE	scFv, dsFv	Cytocidal	Carcinoma: breast cancer, NHL, CLL, leukemia Mesothelioma (phase I)	[88,89]
Gelonin	Humanized IgG, scFv	Cytocidal	Melanoma (preclinical)	[88,89]
Miscellaneous				
Granzyme	scFv	Induction of apoptosis	Solid tumor therapy (preclinical)	[94]
Luciferase	Diabody	Optical imaging	Preclinical imaging	[91]
VEGF	scFv	Enhanced vascular permeability	RIT (Preclinical)	[103]
β -lactamase	scFv, nanobody, IgG	ADEPT	Tumor therapy (preclinical)	[93,95]
Tr-apo-1	nanobody	Lysis of trypanosomes	Anti-parasitic (preclinical)	[97]
Peptides				
Ang II	scFv	Increased tumor blood flow	RIT of tumors (preclinical)	[101]
TAT	scFv	Improved cellular penetration	Anti-HIV (<i>in vitro</i>); induction of mast cell apoptosis (<i>in vitro</i>); improved RIT (preclinical)	[98,99] [100]

Abbreviations: Ang II- angiotensin II; CLL- chronic lymphocytic leukemia; GM-CSF, granulocyte macrophage colony stimulating factor HIV- human immunodeficiency virus; IL-2, interleukin 2; NHL, non Hodgkin's lymphoma; PE, truncated *Pseudomonas* exotoxin A; RIT, radioimmunotherapy; TAT, transactivator of transcription; TNF- α , tumor necrosis factor α ; Tr-apo-L1, truncated apolipoprotein L1; VEGF, vascular endothelial growth factor.

imaging, respectively. A construct of a nanobody targeting cryptic epitopes of the variant surface glycoprotein (VSG) of trypanosomes fused with truncated apolipoprotein L-1 cured both chronic and acute infections with trypanosomes in mice [97].

Several peptides have also been fused to antibodies for various applications. Fusion proteins of scFvs with a cell-penetrating peptide have been used to inhibit intracellular targets. An anti-Bcl-2 scFv tethered to a cell-penetrating peptide TAT was shown to neutralize the activity of Bcl-2 in mast cells and induce apoptosis [98]. Similarly, a recombinant protein containing an anti-HIV TAT scFv fused to a cell-penetrating TAT peptide was able to neutralize TAT function in the cell nucleus [99]. In addition, combinations of antibody fragments directed against tumor cell antigens with cell-penetrating peptides have shown promise in improving the uptake and retention of radiolabeled antibodies [100]. Similarly, an scFv–angiotensin II fusion protein has been generated for the homogenous distribution of radiolabeled scFvs [101].

Conclusion and perspectives

The engineering of antibodies has contributed enormously towards their clinical advancement. The pharmacokinetics of antibodies has been prolonged or shortened by the generation of small antibody fragments and by modification of their interactions with Fc receptors, respectively. The immunogenicity in humans has been reduced by humanization, SDR grafting and de-immunization, and fully human antibodies, developed using transgenic animals and human antibody libraries, have been approved for clinical use. Several novel approaches have been recently described, using intact antibodies, antibody fragments and single domain antibodies, for the generation of BsAbs. The advances in display technologies have facilitated the rapid screening of antibodies in Fab, scFv or single-domain formats. The generation of fusion constructs of antibodies with various proteins (e.g. biological modifiers and toxins) and peptides have further diversified the clinical application of antibodies.

Of the 266 approved drug targets from the human genome, only 15 targets are approved for therapeutic antibodies for various diseases [102], and almost all of the targets of these antibody-based therapeutics are cell surface antigens. Recent research has facilitated rapid screening, production and engineering of antibodies. With the advances in the approaches to discover antibody frameworks that are stable and function intracellularly, and also the capabilities to transduce antibodies across biological membranes, it is now possible to target intracellular targets with the antibodies. Such advancements are expected to increase the number of possible antibody targets. Antibodies are, undeniably, set to have a more dominant role in disease management in years to come.

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