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REVIEW

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Evolution of phage display libraries for therapeutic antibody discovery

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ABSTRACT
Monoclonal antibodies (mAbs) and their derivatives have emerged as one of the most important classes of biotherapeutics in recent decades. The success of mAb is due to their high versatility, high target specificity, excellent clinical safety profile, and efficacy. Antibody discovery, the most upstream stage of the antibody development pipeline, plays a pivotal role in determination of the clinical outcome of an mAb product. Phage display technology, originally developed for peptide directed evolution, has been extensively applied to discovery of fully human antibodies due to its unprecedented advantages. The value of phage display technology has been proven by a number of approved mAbs, including several top-selling mAb drugs, derived from the technology. Since antibody phage display was first established over 30 years ago, phage display platforms have been developed to generate mAbs targeting difficult-to-target antigens and tackle the drawbacks present in in vivo antibody discovery approaches. More recently, the new generation of phage display libraries have been optimized for discovery of mAbs with "drug-like" properties. This review will summarize the principles of antibody phage display and design of three generations of antibody phage display libraries.

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Introduction

Monoclonal antibodies (mAbs) and their derivatives[1] represent a major class of therapeutics and have become the best-selling drugs in United States in recent years.[2,3] The global mAb market size reached ~$150 billion in 2020 and is expected to double in the next five years.[2] To date, approved fully human therapeutic antibodies were discovered from either in vivo animal immunization (including humanized mice and convalescent human donors) or in vitro phage display technology. For in vivo animal immunization, antibodies are generated through repeated immunization of target of interest. Antibodies with high affinity and developability are selected during the development of immune response. However, this process is time intensive and requires the antigen to be immunogenic and nontoxic. In addition, because the selection is entirely completed in vivo, there is limited control over properties, such as specificity and epitope. As the other approach, phage display technology has greatly advanced the therapeutic mAb discovery process by providing a highly versatile approach and overcoming many drawbacks present in in vivo antibody generation technologies. Due to the completely in vitro selection system, phage display surpasses in vivo discovery approaches by enabling the discovery of antibodies against virtually any targets or epitopes, including those that are either toxic or nonimmunogenic for animal immunization.[4–6] Moreover, due to fully controlled selection conditions, phage display can be tailored for selection for desired properties that may not be achievable by in vivo approaches, e.g., selection for a specific epitope recognition,[7,8] pH-dependent binding,[9,10] antibody internalization,[11,12] and even catalytic activity.[13] Furthermore, phage display libraries have successfully led to antibody discovery against the most challenging targets or epitopes, e.g., the stem region of influenza hemagglutinin,[14] G-protein-coupled receptors (GPCRs),[15] and specific conformations of ion channels.[16–18] A recently developed new generation of phage libraries with protein quality control steps in the library construction will further close the developability gap between antibodies derived in vivo and in vitro. Moreover, there is an increasing consideration that development of antibody-based affinity reagents and therapeutics should be moving from animal-based to in vitro approaches due to animal protection.[19,20]

Although the majority of approved therapeutic mAbs were discovered through animal immunization to date, the number of phage-derived antibodies has increased in the recent years, including some of the blockbuster drugs, Humira®, Lucentis®, and Tecentriq® (Table 1). As of November of 2022, 17 phage-derived mAbs have been granted approvals and a multitude of them are actively being evaluated in clinical trials (Table 1).[21–23] Due to the continuous evolution of the technology, as well as the expiration of related key patents,[23] more and more phage-derived mAbs are anticipated to enter clinical trial and the market in the future.

The success of phage display mainly relies on the quality of library and the selection (a process also known as "panning") strategy. The two most widely used panning strategies are solid phase and liquid phase panning. In solid phase panning, the target is immobilized on a microtiter plate where specific phage binders are captured. By elution and propagation in the *E.coli* host cells, specific phage hits are amplified. After iterative panning cycles, target-specific phages are enriched

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Table 1. Approved mAbs derived from phage display technology.

Generic Name	Product Name	Company	Format	Target	First Indication	Approved Year	Phage display technology
Adalimumab	Humira	Abbvie	IgG1	TNFα	RA	2002	Humanization
Ranibizumab	Lucentis	Novartis, Roche/Genentech	Fab-IgG1	VEGFA	nAMD	2006	Humanization, affinity maturation
Belimumab	Benlysta	Human genome Sciences (HGS), GlaxoSmithKline (GSK)	IgG1	BLyS	SLE	2011	Initial discovery
Raxibacumab	ABThrax	HGS, GSK	IgG1	*Bacillus anthracis* PA	Anthrax	2012	Initial discovery
Ramucirumab	Cyramza	Lilly/Imclone	IgG1	VEGFR2	GC, NSCLC	2014	Initial discovery
Necitumumab	Portrazza	Eli Lilly	IgG1	EGFR	NSLCC	2015	Initial discovery
Ixekizumab	Taltz	Eli Lilly	IgG4	IL-17a	Psoriasis	2016	Humanization
Atezolizumab	Tencentriq	Roche/Genentech	IgG1	PD-L1	UC	2016	Initial discovery
Avelumab	Bavencio	Merck Serono/Pfizer	IgG1	PD-L1	MCC	2017	Initial discovery
Guselkumab	Tremfya	Morphosys, Janssen,	IgG1	IL-23	Psoriasis	2017	Initial discovery
Lanadelumab	Takhzyro	Dyax, Shire	IgG1	pKal	HAE	2018	Initial discovery
Caplacizumab	Cablivi	Ablynx	VHH	vWF	aTTP	2018	Initial discovery
Moxetumomab pasudotox	Lumoxiti	AstraZeneca/Medimmune	dsFv-PE38	CD22	HCL	2018	Affinity maturation
Emapalumab	Gamifant	Novimmune	IgG1	INFγ	HLH	2018	Initial discovery
Inebilizumab	Uplizna	AstraZeneca/Medimmune, Viela Bio	IgG1	CD19	NMOSD	2020	Affinity maturation
Tralokinumab	Adbry	AstraZeneca/Medimmune, Leo Pharma	IgG4	IL-13	Asthma	2021	Initial discovery
Faricimab	Vabysmo	Roche	Bi-Fab	VEGFA, Ang2	nAMD, DME	2022	Initial discovery and affinity maturation

Abbreviations: Ang2: angiopoietin 2; aTTP: acquired thrombotic thrombocytopenic purpura; Bacillus anthracis PA: Bacillus anthracis protective antigen; Bi-Fab: bispecific Fab; BLyS: B-lymphocyte stimulator; DME: diabetic macular edema; EGFR: epidermal growth factor receptor; GC: gastric carcinoma; HAE: hereditary angioedema; HCL: hairy cell leukemia; HLH, hemophagocytic lymphohistiocytosis; IFN: interferon-gamma; IL-13: interleukin-13; IL-17A: interleukin-17A; IL-23: interleukin-23; MCC: Merkel cell carcinoma; nAMD: neovascular age-related macular degeneration; NMOSD: neuromyelitis optica spectrum disorder; NSCLC: non-small cell lung carcinoma; PD-L1: programmed death-1 ligand-1; PE38: Pseudomonas exotoxin A; pKal: plasma kallikrein; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; TNFα: tumor necrosis factor-alpha; UC: urothelial carcinoma; VEGFA: vascular endothelial growth factor A; VEGFR2: vascular endothelial growth factor receptor 2; vWF: von Willebrand factor.

(Figure 1).[24,25] In liquid phase panning, biotin-labeled targets are incubated with the antibody phage libraries and briefly pulled down by streptavidin-coated magnetic beads to capture bound phage.[26,27] Epitope-directed antibody selection can be achieved by alternative panning strategies or incorporating other technologies, e.g., competitive panning[7,28–30] and site-specific photocrosslinking.[8] Some other panning strategies, such as cell-based and nanodisc-based panning, have been developed for challenging targets, e.g., multi-spanning membrane proteins.[31–33] Moreover, functional selection can be directly integrated in the selection process.[34,35] Although various panning strategies have been developed to meet different antibody discovery goals, the selection procedures are generally well-established and standardized.[36] However, library design is highly variable and plays a central role in phage display-based antibody discovery. In this review, we will briefly summarize the principle of antibody phage display and phage engineering. This review will focus on the design principles of three generations of phage display libraries, as well as several specially designed libraries.

Phage display

Display of foreign peptide on the bacteriophage surface without affecting phage infection was first described by George P. Smith in 1985.[37] Since then, phage display technology has been extensively applied to various applications, e.g., protein evolution (also known as directed evolution), epitope determination, identification of enzyme substrates, and drug discovery.[38] Since phenotype and genotype are physically linked in a phage display system, protein variants with desired properties can be rapidly selected through panning and the genetic information can be readily extracted. In a phage display system, a library in which diversified peptide or protein variants are displayed on phage surface is constructed (typically 10^{10} – 10^{12} diversity). Variants with favored properties are enriched during iterative selection cycles. Unique sequences can then be screened for further applications. While the success of phage display has been demonstrated in a wide variety of areas, this review will focus on its application in therapeutic antibody discovery.

Antibody phage display was originally developed in three institutes: Institute of Cell and Tumor Biology at German Cancer Research Center (Germany),[39] MRC Laboratory of Molecular Biology (United Kingdom),[40,41] and Scripps Research Institute (United States).[42,43] Since then, various antibody fragment formats have been commonly used for phage display, such as single chain variable fragment (scFv),[44,45] antigen binding fragment (Fab),[24,46] single-domain antibody (VHH),[47,48] and bispecific antibody fragment.[49–51] Although display of full-length IgG on phage was also reported,[52,53] this platform was not widely used, likely due to unstable display, low display level, and bias on phage propagation, which resulted in limited functional diversity of the library. However, strategies for functional screening in full-length IgG format after phage panning have been reported, e.g., dual host vector,[54] donor-acceptor system,[55] high-throughput reformatting, and mammalian expression of phage-derived antibodies.[56,57] Nevertheless, the display of full-length antibody can be readily achieved by eukaryotic

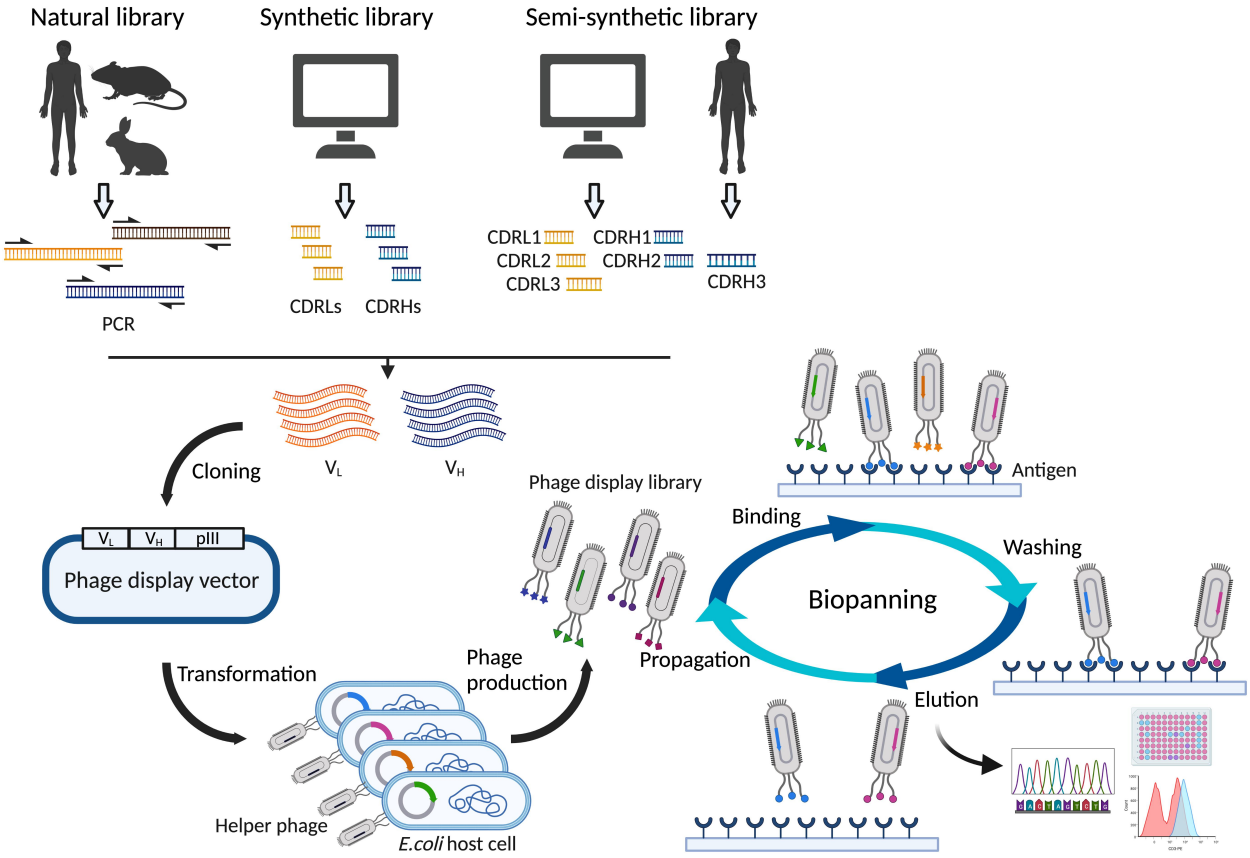

Figure 1. Types of antibody phage display libraries (upper left panel), library construction (lower left panel) and antibody selection (right panel). According to source of diversity, antibody phage display libraries can be categorized into three types: natural library, synthetic library, and semisynthetic library. In a typical phagemid system, variable regions of light and heavy chains, either cloned from a natural immunoglobulin repertoire or designed and synthesized in vitro, are cloned into a phage display vector (phagemid), with one of the chains genetically fused to pIII of phage for display. The library pool is then transformed to E.Coli host cells. By infection with helper phage, which provides all the components for phage production, a phage library is generated. Taking solid-phase panning as an example, the phage library is incubated with immobilized antigen. After washing, nonspecific phages are removed and antigen-specific phages stay with the antigen. The bound phages are then dissociated from the antigen by diverse methods, e.g., low-Ph elution, enzymatic cleavage. Lastly, eluted phages are subjected to propagation in E.Coli host cells. After iterative rounds of panning, antigen-specific clones are enriched. Typically, output phages from middle and late rounds of panning are subjected to sequencing and binding characterization for obtaining both sequence diversity and high affinity, respectively. Alt-text: Three types of antibody phage display libraries, including natural, synthetic, and semisynthetic libraries. Construction of an antibody phage library includes generation of antibody variable region fragments, cloning, transformation, and phage production. The antibody selection includes antigen immobilization, phage binding, washing, elution, and phage propagation.

display platforms, such as yeast and mammalian cell display.[58–60] Choice of display format depends on the final format that will be used in the application. Since reformatting sometimes causes substantial changes of antibody properties, e.g., affinity and stability, keeping format consistent from library to final product is considered a "rule of thumb." As such, phage display offers a highly versatile platform with regards to choice of antibody format.

Among several well-characterized filamentous bacteriophages (Ff), including f1, M13, and fd, M13 is the most widely used for phage display. Ff bacteriophages infect *E. coli* host cells through a specific interaction between host F pilus and phage minor coat protein pIII.[61,62] The Ff phage genome encodes 11 proteins, five of which are coat proteins (pIII, pVI, pVII, pVIII, and pIX). Although all five coat proteins have been demonstrated for protein display,[63,64] pIII is the most widely used as an antibody fusion partner in phage display platforms, because it can accommodate large proteins with minimal interference on phage function and is compatible with monovalent phage display.[65,66] Antibody-pIII fusion is generally achieved through genetic fusion, but can also be achieved through other conjugation approaches, such as leucine zipper dimerization,[67] synthetic Fc-binding ZZ domains,[52,68] etc.

In the early display system, the peptide of interest was fused to pIII in frame in the phage genome.[69,70] Thus, the peptide is displayed on all copies of pIII on the phage surface, which results in a potential decrease in phage infectivity. Also, the size of the peptide that can be efficiently displayed is limited to 12 amino acids.[71] In a phagemid-based system, antibody-pIII fusion is encoded in a separate plasmid (designated phagemid) and coinfection with a helper phage is required to provide all proteins for phage proliferation.[66,72,73] As a result, each phage particle contains both wild type and antibody-fusion pIII proteins. A key advantage of this system is that monoclonal display can be achieved, which facilitates selection of high affinity clones by avoiding the avidity effect during panning.[63,66] Meanwhile, phage infectivity is maximally retained due to the presence of wild type pIII. One drawback of the system is that wild type pIII is more efficiently assembled in the phage particle, resulting in only a small percentage (<10%) of antibody-displaying phage particles in the population.[74] As

a consequence, either panning efficiency or sensitivity of any phage-mediated binding assays can be greatly compromised due to excessive wild type phage background. One strategy is to increase multiplicity of display, which can be achieved by different methods, such as the pVIII/pVII display system,[75,76] the adapter-directed display,[77] and using an inducible promoter for the antibody-pIII fusion gene. To maximize the display valency, hyperphage was developed by generating a helper phage featuring a pIII gene-deficient genotype and wild type infectivity phenotype in a pIII-supplying *E. coli* host.[78,79] Thereby, application of the hyperphage in a regular phagemid system resulted in production of phage particles that exclusively display antibody-pIII fusion protein. This not only substantially increased the sensitivity of phage ELISA, but also significantly improved the panning efficiency.

The pIII protein is comprised of N1 and N2 domains required for phage infection and a C-terminal domain (CT) for pIII assembly. In another effort, a mutant helper phage, named CT helper phage, was generated by deletion of N1 and N2 domains in the genome. Similar to the hyperphage system, the infectivity of CT helper phage was restored by propagation in a *E. coli* host that heterogeneously expresses an intact pIII gene. Therefore, use of CT helper phage substantially improved the panning efficiency since only phages incorporated with antibody-pIII fusion are infective.[80] Some other pIII-deficient helper phage systems were also reported, e.g., ex-phage,[81] phaberge,[82] and a helper phage engineered with low N1 expression.[71] Of note, since these variants of helper phage adopt multivalent display, they result in either reduction in phage production or decrease in phage infectivity. Therefore, they may not be ideal for phage library construction. In order to balance display level and phage functionality, XP5 helper phage was developed to reduce WT pIII production by introducing multiple rare codons in the pIII gene and an altered ribosome binding site spacing.[83]

Three generations of antibody phage display libraries

Universal antibody phage display libraries, typically consisting of over ten billion unique sequences, have become a valuable source for discovery of mAbs against any types of targets. According to the source of diversity, antibody phage libraries can be divided into natural, fully synthetic, and semisynthetic libraries (Figure 1). In a natural library, diversity is derived entirely from natural repertoires, which can be either healthy, autoimmune, or immunized donors (named naïve, nonimmunized, and immunized libraries, respectively). In theory, a large naïve library can be used to isolate antibodies against any targets, but, if a library is constructed from immunized donors, diversity will be highly biased toward a specific target. For an immunized library constructed from human repertoires, it can be constructed from either vaccinated donors or donors who have recovered from infection or disease. In a fully synthetic library, the antibody frameworks are usually chosen from human antibody germlines that are well-represented and have shown superior developability. Complementarity-determining regions (CDRs) are designed based on antibody structure and application purpose. The quality of the synthetic DNA that are used to create CDR diversity plays a pivotal role in determining the functional size of a synthetic library. By using mono- or trinucleotide phosphoramidites (TRIM technology) as building blocks,[84,85] diversified CDRs can be synthesized in a high-throughput manner, such as array-based oligonucleotide synthesis and Slonomics.[86,87] In the TRIM technology, each amino acid is encoded by a defined codon. Therefore, amino acid distribution at the desired position can be precisely defined. Moreover, because the codon for each amino acid is selected for optimal antibody expression, the functional library size is increased. In the natural antibody repertoire, the diversity of CDRs, except CDRH3, is a collection of defined sequences that exhibit canonical structures. Compared with the traditional column-based DNA synthesis approaches in which one single gene is produced per column, the high-throughput DNA synthesis technologies allow parallel synthesis of a large number of predetermined sequences in the same footprint on a silicon-based chip.[88,89] This enables not only removal of liability motifs that impact developability, but also precise mimic of the natural antibody diversity. Semisynthetic libraries, therefore, combine synthetic CDRs (typically three light chain CDRs and heavy chain CDR1 and CDR2) and heavy chain CDR3 from natural repertoires. Phage libraries can be designed for either antibody discovery or engineering. The design of the latter is based on one parental antibody and tailored for one specific target. This review will focus on design of three generations of universal antibody phage display libraries for antibody discovery (Table 2).

First-generation library

Generation of a universal antibody phage library and its successful application for panning was first described in 1989.[42] The Fab library containing 2.5×10^7 plaque-forming units (PFUs) was constructed by amplification of variable regions of light and heavy chains from the mouse antibody repertoire. Soon after, an immunized scFv phage library was created using a similar procedure.[41] Despite the small library size (2×10^5), the hit rate was substantially improved (>90% after two rounds of panning). The success of natural libraries for in vitro antibody discovery has been proven at different institutions.[103–106] A common feature of these natural libraries is that light and heavy chains are randomly combined, thus these are designated combinatorial library. Due to the heavy and light chain rearrangement, the library diversity is dramatically increased. For example, if a natural antibody repertoire contains a diversity of 10^6 (i.e., 10^6 unique light and heavy chain pairs), the diversity would be 10^{12} after full light and heavy chain recombination. One significant advantage is that this approach generates specificities that do not exist in natural repertoire, which enables discovery of antibodies targeting any given antigen, including self-antigens.[107] In contrast, self-antigen targeting can be challenging for natively paired antibodies due to immune tolerance (a protection mechanism in which immune system does not respond to self-antigen).[108,109] In addition, a large naïve library comprises most of the germline genes, which further increases the structural complexity of the library due to diverse framework structures. Although the germline gene distribution in a naïve library is usually consistent with that in natural repertoire, it has been observed that germline gene usage post-selection is biased

Table 2. Selected examples of three generations of antibody phage display libraries.

	Library Name (year)	Organization	Format	Library Type	Framework	Library Size	Key Feature	Highest affinity (nM) (format)[a]	Reference
First generation library	N/A (1996)	Cambridge Antibody Technology	ScFv	Naïve	Naïve	1.4×10^{10}	* Repertoires from 43 healthy donors	4.2 (scFv)	90
	N/A (1994)	MRC Centre for Protein Engineering	Fab	Synthetic	49 V_H frameworks; 26 V_κ frameworks; 21 V_λ frameworks	6.5×10^{10}	• Fully randomized CDR3 • Heavy and light chains assembled using combinatorial infection	7 (Fab)	91
	HAL (2015)	Technical University of Braunschweig	ScFv	Naïve	Naïve	1.5×10^{10}	• Repertoires from 98 healthy donors • Optimized Myc/His tag • Improved antibody production by removal of C-terminal phenylalanine of V_κ light chain	0.9 (IgG)	92,110
	CAT2.0 (2009)	MedImmune	ScFv	Naïve	Naïve	1.29×10^{11}	* Repertoires from 160 healthy donors	N/A	93
	n-CoDeR (2000)	BioInvent	ScFv	Naïve	Single V_H framework; single V_L frameworks	2×10^9	* Rearranged 6 CDR segments from natural repertoire in single light and heavy chain frameworks	0.9 (scFv)[b]	94
Second generation library	ETH2-GOLD (2005)	ETH Zurich	ScFv	Synthetic	Single V_H framework; 2 V_L frameworks	3×10^9	• Fully randomized CDR3 • CDR diversity concentrated in the center of antigen binding region	23.6 (scFv)	95
	PHILODiam ond (2014)	ETH Zurich	ScFv	Synthetic	Single V_H framework; 2 V_L frameworks	4×10^{10}	* Introduction of S52N in heavy chain CDR2	27 (scFv)	96
	Dyax (2005)	Dyax	Fab	Semisynthetic	Single V_H framework; naïve V_L frameworks	3.5×10^{10} (FAB-310) 1.0×10^{10} (FAB-410)	• Randomization of selected antigen-contacting residues in heavy chain CDR1 and CDR2 • Diversity of light chain and CDRH3 derived from autoimmune donors	0.22 (Fab)[c]	97
	HuCAL- GOLD (2008)	MorphoSys	Fab	Synthetic	7 V_H framework; 4 V_κ frameworks; 3 V_λ frameworks	1.6×10^{10}	• Natural amino acid distribution of six CDRs by using trinucleotide mixtures • Elimination of frame-shifted clones by β-lactamase system • Antibody and pIII linked by disulfide bond	0.04 (Fab)	98
	HuCAL- PLATINUM (2011)	MorphoSys	Fab	Synthetic	7 V_H framework; 3 V_κ frameworks; 3 V_λ frameworks	4.5×10^{10}	• Removal of post-translational modification hot spots • Loop length dependent amino acid distribution in CDRH3 • Optimized sequence for high expression in both prokaryotic and eukaryotic cells	0.002 (Fab)[d]	99
	Ylanthia (2013)	MorphoSys	Fab	Synthetic	21 V_H/V_κ pairs; 15 V_H/V_λ pairs	1.3×10^{11}	• Selected V_H/V_L framework pairs with optimal biophysical properties • CDRH3 length and amino acid composition based on JH4 and JH6 sequences • CDR optimized to be devoid of PTM motifs • Optimized sequence for high expression in both prokaryotic and eukaryotic cells	0.7 (Fab)	100

(*Continued*)

Table 2. (Continued).

	Library Name (year)	Organization	Format	Library Type	Framework	Library Size	Key Feature	Highest affinity (nM) (format)[a]	Reference
Third generation library	ALTHEA Gold libraries (2019)	GlobalBio	ScFv	Semisynthetic	Single V_H framework; 2 V_L frameworks	2.5×10^{10}	• Natural CDRH3 from 200 donors • Removal of developability liability residues • Enhanced stability by heat shock and protein A recovery during library construction	3.3 (scFv)	101
	N/A (2021)	Specifica	ScFv	Semisynthetic	4 V_H framework; 3 V_κ frameworks; 1 V_λ frameworks	9×10^9	• Selection of clinically validated V_H/V_L pairs as library scaffolds • Synthetic CDRs except CDRH3 from replicated natural diversity. Natural CDRH3 sequences amplified from 10 donors • Removal of CDR sequence liabilities informatically • Comprehensively improved developability, including affinity, polyspecificity, stability, aggregation, expression and display level by yeast display filtration	0.034 (IgG)	102

Abbreviations: HC, heavy chain; LC, light chain; VH, heavy chain variable region; VL, light chain variable region; Vκ, kappa light chain variable region; Vλ, lambda light chain variable region.
a. Affinity was measured by SPR unless specified
b. Affinity was measured by ELISA.
c. Method was not specified.
d. Affinity was measured by solution equilibrium titration.
by combining donor-derived and synthetic

toward certain families,[93,110] e.g., V_H1–69, V_H1–46 (Kabat nomenclature) and/or IGHV3–30 (IMGT nomenclature) for heavy chain. For the light chain, V_λ1-c, V_λ2-2a (Kabat nomenclature) and/or IGLV1–47, IGKV1–12, IGKV1-D33 (IMGT nomenclature) were enriched after selection. Meanwhile, several synthetic and semisynthetic antibody libraries were generated in the early 1990s.[111–113] Although only antibodies with low affinity (micromolar range) were isolated due to the small library size (10^7-10^8), these libraries demonstrated that artificial antibodies can be generated in vitro. Later studies showed that, by generating larger naïve libraries with library size of 10^{10}-10^{12}, antibodies with low- to sub-nanomolar affinity can be directly isolated.[90,91,93,114–118] Of note, the large naïve library and related phage display technology developed by Cambridge Antibody Technology has led to approval of six mAbs: adalimumab,[119] belimumab,[120] moxetumomab pasudotox,[121,122] raxibacumab,[123] emapalumab,[124] and tralokinumab.[125] Another example is the n-CoDeR library in which heavy and light chain CDR segments were separately amplified by using primers specific to VH3–23 and VL1–47 germlines. The six CDRs were then reassembled in a single light and heavy chain framework that is well-presented in human repertoire and well-expressed in bacterial host cells.[94] This strategy overcomes the drawback with conventional naïve antibody phage libraries in which part of the diversity might be lost due to low expression and/or display level of certain germline genes in prokaryotic system. The n-CoDeR library was used to discover five mAbs and their derivatives that entered clinical trials.[126–130] In our experience, high hit rate (>90%) can be readily achieved and antibodies with high affinity (low nanomolar) can be routinely isolated from a large naïve library (library size = 10^{12}) constructed in-house. Depending on the target, 10–20% of total sequences screened are unique.

Taken together, first-generation phage libraries demonstrated that antibody discovery can be performed completely in vitro and antibodies with high affinity and sequence diversity can be isolated directly from phage libraries.

Second-generation library

In the design of the second-generation libraries, represented by several semisynthetic and fully synthetic libraries, amino acid distribution and positions to be diversified were carefully selected based on knowledge of natural antibody structure and antibody–antigen interaction. By leveraging TRIM technology which uses pre-synthesized trinucleotides as building blocks for oligonucleotides synthesis, the CDR amino acid composition can be precisely tailored to meet specific design criteria. Moreover, developability was taken into consideration in the library design.

In the design of several synthetic libraries, e.g., HelL-11, HelL-13, and Library F, amino acids that are frequently used in the antibody-antigen interaction dominate in CDRL3 and CDRH3.[131,132] More precisely, in the design of ETH2 and ETH-2-Gold libraries, selected residues predicted to be in direct contact with the antigen in silico were randomized, while structure-supporting CDR residues were kept constant.[133,134] Based on ETH-2-Gold library, PHILO and PHILODiamond libraries were designed to cover a broader epitope landscape by incorporation of hydrophilic residues at specific positions in the antigen contacting site.[96,135] ETH serial libraries led to two mAbs, Teleukin and Dekavil, being investigated in clinical trials.[136–140] Although CDRL3 and CDRH3 play crucial roles in germline diversity, it has been observed that CDR1 and CDR2 are preferentially selected for affinity maturation during the course of somatic hypermutation.[141–143] In the design of the Dyax library, a semisynthetic library, diversity of light chain and CDRH3 were derived from antibody repertoires of autoimmune donors to increase the likelihood of discovery of antibodies targeting self-antigens. CDRH1 and CDRH2, however, were fully synthetic, and residues that were predicted to be surface-exposed were randomized using all amino acids except cysteine.[97] Indeed, by panning against four selected antigens, the average antibody affinities indicated by K_D were below 20 nM for three of the four targets. Of note, the moderate average affinity (131 nM) of the other one target is likely due to usage of a single V_H/V_L framework pair in the library construction. The Dyax library was used in the discovery of four approved mAbs: ramucirumab,[144] necitumumab,[145] avelumab,[146] and lanadelumab.[147] In a systematic analysis of antibody-protein and antibody-peptide interaction propensity, machine learning was leveraged to predict CDR hot spot residue distributions.[148] By densely enriching the hot spot residues across all CDRs, GH libraries resulted in enhanced antibody–antigen recognition indicated by high affinity and specificity. In fact, by using minimalist libraries, it has been demonstrated that only a small subset of amino acid types (a four-amino-acid code (Tyr, Asp, Ser, and Ala) or a binary code (Tyr and Ser)) is sufficient to mediate interactions with proteins.[149,150]

In the HuCAL serial libraries, frameworks that cover major types of CDR canonical structures were included to maximize structural diversity. By leveraging TRIM technology, six CDRs were designed to precisely mimic the amino acid distribution that occurs in natural repertoire.[98,99,151] Thus, the HuCAL libraries are more likely to yield antibodies with nature-like properties. Since TRIM technology allows precisely defined nucleotide composition in the oligonucleotide synthesis, it also enables stable and high expression of antibody in *E.coli* host cells through optimized codon usage and avoidance of stop codons, which are the drawbacks of using degenerate codons. As a result, functional diversity of the synthetic library can be greatly improved. It is notable that the percentage of alanine at heavy chain H137 can be tuned to favor binding for either protein or hapten/peptide. Another feature of the HuCAL library is that restriction sites were introduced to flank all six CDR loops. Thus, antibody engineering can be facilitated by rapid CDR shuffling. In the design of the HuCAL-PLATINUM library, post-translational modification hot spots, e.g., N-glycosylation, were avoided in CDR design to further improve the antibody's developability. Moreover, amino acid distribution in CDRH3 was tuned based on the loop length to further mimic the natural repertoire.[99] In another effort to further improve the overall developability of antibodies selected from a phage library, 36 of 400 V_H/V_L pairs were identified for optimal biophysical properties and used for construction of the Ylanthia library.[100] As a result, antibodies directly selected from the library showed superior affinity,

protein expression level, thermal stability, and aggregation propensity. Of note, the HuCAL-GOLD library yielded guselkumab, which was approved in 2017.[152]

In summary, in the design of the second-generation libraries, antibody structure and developability properties were taken into consideration through the removal of sequence liability motifs, inclusion of natural amino acid distributions in CDR, and selection of heavy and light chain frameworks with superior biophysical properties.

Third-generation library

In general, the improvements of developability properties in the second-generation libraries were achieved by sequence-based optimization. While our knowledge regarding sequence-based prediction of protein liabilities is still limited, many biophysical properties, such as stability, solubility, and expression, are closely related to higher order structure of a protein. Although scaffolds with high stability and expression can be selected for library construction, the overall biophysical properties of antibodies in a phage library are also highly determined by CDR sequence. Therefore, these developability properties were experimentally improved during construction of the third-generation libraries.

In order to enhance the library solubility and thermostability, a heat shock step, followed by a protein A recovery, was incorporated in the construction of the semisynthetic ALTHEA Gold library.[101] This is based on the observation that heat denaturation selected stable and well-folded antibodies.[153] Validation of the library showed that the overall frequency of hydrophobic residues at diversified CDR positions decreased after heat shock and protein A selection. In contrast, charged residues were positively selected by the filtration process. By panning the library against a diverse panel of antigens, the scFvs selected exhibited high affinity (K_D ranges from single-digit nM to sub-nM), solubility (>50 mg/L), and thermal stability (Tm > 70°C), which agree with biophysical parameters of therapeutic antibodies.[101,154,155]

In another effort to create a semisynthetic phage library with "drug-like" properties, a yeast display filtration was applied to select sequences with optimal developability properties required for clinical development, including affinity, aggregation, thermostability, polyspecificity, and expression level.[102] This step leverages the eukaryotic protein quality control systems for selection of correctly behaving proteins for secretion.[156–158] In detail, five single-CDR yeast display libraries, in each of which one CDR (except CDRH3) was synthesized from replicated natural diversity, were individually created. The antibody sequences that were displayed correctly and in a high level were selected and subjected to next-generation sequencing (NGS) analysis. CDRH3 sequences, however, were sourced from human natural antibody repertoires. This was done for two reasons: 1) depending on length and amino acid usage, synthetic CDRH3 diversity typically far exceeds the actual library size; and 2) the natural CDRH3 source can provide enough high diversity and the sequences have also been filtered in vivo for optimal biophysical properties, e.g., high stability and expression, low immunogenicity. To validate the library, panning was conducted against four antigens. Remarkably, from a total number of 81 antibodies isolated, around 80% showed single-digit to sub-nM affinity. More strikingly, by determining developability parameters, including thermostability, polyspecificity, and self-interaction, 97% of the measurements of the antibodies behaved similarly or better than that of the corresponding approved parental antibodies. This study highlights the importance of the eukaryotic quality control system in the selection of high-quality antibodies.

In summary, the third-generation libraries leverage in vitro or *i*n vivo experimental approaches to further improve the overall library quality, which yield antibodies with properties comparable to therapeutic antibody drugs.

Library designed for specific applications

Although the aforementioned universal phage libraries provide valuable resources for antibody discovery, their performance may be compromised for challenging targets and epitopes (e.g., GPCR, concave-shaped epitope) and specific applications (e.g., pH-dependent antibodies). This is due to inherent characteristics of conventional antibody libraries, either natural libraries or synthetic libraries that mimic the human antibody repertoire. An advantage of phage display technology is that the library can be tailored to adapt to distinct applications. Indeed, several specialized phage libraries have been designed and created.

GPCR library

GPCR represents a class of seven transmembrane receptors. GPCRs have been recognized as successful drug targets as approximately one third of the US Food and Drug Administration (FDA)-approved drugs target GPCRs.[159,160] However, due to high hydrophobicity, conformational flexibility, and limited accessibility of epitopes on the extracellular portion, GPCRs are challenging targets for antibodies. To date, there are only two FDA-approved antibodies drugs targeting GPCRs: mogamulizumab and erenumab, which target CC chemokine receptor 4 and calcitonin gene-related peptide receptor, respectively.[161] Phage display offers a valuable antibody discovery platform for targeting challenging targets, including ion channels, transporters, and GPCRs.[162] For example, one synthetic antibody phage display library was designed by mining the sequences of all known GPCR ligand interactions and incorporating the identified binding motifs into CDRH3. As a result, this GPCR-focused library successfully led to discovery of a panel of antagonistic antibodies targeting glucagon-like peptide-1 receptor with high affinity.[15]

Library for selection of pH dependent antibodies

Elimination of soluble targets by conventional high affinity antibodies usually requires a large dose. This is because an antibody usually binds with an antigen with similar strength at both neutral and slightly acidic pH (pH 5.5–6.0); therefore the antigen bound with antibody in the extracellular environment does not dissociate from the complex in the endosome. As a result, the antigen can escape from lysosomal

degradation and return to the circulation mediated by the neonatal Fc receptor (FcRn).[163,164] Thus, antibodies that are capable of neutralizing the target at physiological pH and releasing it at acidic endosomal pH would be expected to enhance the therapeutic index. In the past, pH-dependent binding has been achieved through antibody engineering, e.g., histidine scanning.[165,166] Alternatively, sweeping antibody technology was developed to enhance binding with FcRn at neutral pH for rapid uptake of antibody–antigen complex for target clearance.[167] While these technologies proved successful, they are labor-intensive and all based on preexisting antibodies. In another attempt, pH-dependent antibodies were isolated de novo from a synthetic antibody phage display library.[9] In the library design, histidine residue was enriched in CDRH3 for two reasons. First, histidine is neutrally charged at physiological pH but becomes positively charged at pH 6.0. Therefore, histidine residues within the protein–protein interaction region can exert pH-dependent binding. Second, histidine is less frequently found in natural repertoires.[168] Combined with a modified selection strategy, several anti-CXCL10 antibodies with high binding affinity and strong neutralizing activity at pH 7.4, but weak binding at pH 6.0 were isolated.

Library with elongated CDRH3

One limitation of human or mouse antibodies is that targeting a concave epitope of a target, e.g., pore of ion channels and pocket of enzymes, can be challenging. This is largely due to the relatively short CDRH3 length, typically 7–12 and 8–20 amino acids for mouse and human respectively, which tend to form a cave or flat paratope.[169,170] Therefore, concave-shaped conformation of a target is usually inaccessible for conventional antibodies. Although antihuman immunodeficiency virus (HIV) broadly neutralizing antibodies with extended CDRH3 of around 30 amino acids have been isolated, they were only found in a minority of infected population and require years of development.[171,172] Interestingly, antibodies from some species, such as cow and camelid, are unusual in having a natural elongated CDRH3 (e.g., up to 70 amino acids for cow), which provides extra diversity and paratope complexity.[173,174] The cow ultralong CDRH3 generally adopts a ”stalk and knob” structure, in which a β strand ”stalk” supports a structurally complex ”knob” domain stabilized with multiple disulfide bonds.[175] The ”knob” domain protrudes out from the antibody surface, making it accessible to the concave epitope. In a proof-of-concept study, a synthetic Fab phage display library carrying elongated CDRH3 (23–27 amino acids) was constructed.[176] By panning against the library, a number of antibodies that potently inhibited matrix metalloproteinase-14 were identified. Of note, one of the antibodies was indicated to bind to the vicinity of the enzyme activity pocket. Libraries displaying atypical antibodies with elongated CDRH3 further extend the application of phage display technology.

Conclusions and prospects

Phage display has proven to be an unequivocal success for antibody discovery, evidenced by 17 approved mAbs and an increasing number of phage-derived antibodies under clinical investigation. As an entirely in vitro technology, phage display not only compensates for many limitations inherited by in vivo antibody discovery approaches, it also provides a highly versatile and customizable platform that continuously evolves to meet distinct development goals.

Affinity is a key factor used to evaluate the quality of a phage library. It has been observed that affinity that can be achieved is correlated with the library size. We investigated the correlation between highest affinity values obtained from published universal phage libraries and library size (data not shown). In agreement with previous observations, there is a positive correlation between the two parameters. This is because a universal library is designed for antibody discovery against any given target. Therefore, a larger library (higher diversity) offers a greater chance of identifying high affinity antibodies. In the case of a synthetic library, although a limited number of frameworks are used, the designed CDR diversity usually far exceeds the natural CDR diversity. Therefore, the chance to obtain high affinity antibodies mainly depends on CDR sequence diversity. In the case of a naïve library, the library diversity comes from not only the sequence diversity, but also from light and heavy chain rearrangement. Of note, the random heavy and light chain rearrangement is a process highly resembling chain shuffling, which is a routine strategy for in vitro antibody affinity maturation. This may explain why antibodies with very high affinity were isolated from naïve phage libraries, even when the libraries were constructed from germline sequences with no or very limited somatic hypermutations. While it was expected that identification of library size is a key factor that determines the affinity, a correlation with either library type or library generation was not observed (data not shown). One reason is that, except for a few early libraries, the library size difference among three generations of phage libraries is minimal. Also, the advancements of three generations of phage libraries mainly reflects improvements of overall developability properties.

It has been reported that the presence of library clones that do not display antibody fragments (bald phage) is partially due to stop codons or frameshifts present in the antibody gene. These clones often outgrow because of the decreased burden on production of antibody fusion proteins and higher infectivity due to all wild type pIII molecules on phage particle, resulting in loss of library diversity. In efforts to increase functional library size, several strategies have been developed. For example, anti-tag antibody was used for proofreading panning to select in-frame sequences due to the tag being in-frame with the antibody sequence.[177] In the design of the HuCAL GOLD library, the β-lactamase gene was used to eliminate frame-shifted sequences.[98]

Regarding framework usage in a phage display library, it is generally accepted that a library using multiple frameworks

will perform better than one using a single pair of frameworks, since multiple scaffolds provide more structural diversity.[100] For selection of framework, parameters, such as frequency in natural repertories, stability, expression, and display level, have been taken into consideration. Moreover, it should be noted that several heavy chain germline genes, e.g., certain IGVH4 family genes, have been found to be deselected during phage panning.[93,98] Also, VH4–34 has been found to be associated with B cell cytotoxicity.[178] Thus, these germlines should be excluded from the library design. For the majority of the phage display libraries, V_H and V_L are randomly rearranged, which agrees with the observation that there is no obvious preference of V_H/V_L pairing in natural repertories.[179] However, in terms of drug development, since different V_H/V_L pairings do exhibit very distinct biophysical characteristics,[100,180] attention should be given to the choice of framework pairing.

Compared to in vivo antibody discovery approaches, another advantage of phage display is that the sequence information can be retrieved rapidly and readily. However, this advantage is partially attenuated by the conventional screening approach of characterization of individual clones, in which only a small percentage of sequence information, i.e., the most abundant sequences, from panning output is assessed. This is partially due to intrinsic amplification bias of antibody-displaying phages in *E.coli* host cells, which leads to some of the sequences becoming rare over several cycles of panning.[181,182] To overcome the limitation, NGS, which allows deep mining of sequence space in a sample, has recently been applied to antibody phage display technology, especially for identification of those rare sequences with potential interesting features.[183,184] Most recently, machine learning combined with NGS has been applied to predict binding features (e.g., affinity, epitope, developability) of sequences from phage panning, and even generate new sequences with improved properties.[185,186]

Despite many advantages of phage display, developability has been a concern for mAbs derived in vitro due to lack of *in vivo* protein quality control process.[187–189] As aforementioned, either in vitro or eukaryotic quality control steps were integrated into the construction of the third-generation phage display libraries. Thus, only library members with favorable developability were selected. Indeed, it has been reported that mAbs selected from the third-generation libraries showed overall enhanced developability properties, including high affinity, improved stability and solubility, and less self-interaction. Alternatively, mammalian display can be used to further screen or optimize developability properties of mAbs, based on a strong correlation between optimal biophysical properties and display level.[190] It is worthwhile to mention that the emergence of in vivo delivery of nucleic acid-encoded biologics, i.e., DNA and mRNA technologies, enables direct production of therapeutic mAbs in vivo. These delivery technologies bypass the complex protein manufacturing, storage, and transport processes, which require proteins with excellent biophysical properties.[191,192] Therefore, it would be envisioned that, with the advancements of new drug delivery technologies, many requirements on developability, particularly manufacturability, can be mitigated in the future.

As phage display technology continues to evolve, and in concert with other state-of-the-art technologies, such as NGS and machine learning, phage display technology will continue to make great contributions to innovative drug discovery in the future.

Abbreviations

Ang2	angiopoietin 2
aTTP	acquired thrombotic thrombocytopenic purpura
Bacillus anthracis PA	Bacillus anthracis protective antigen
Bi-Fab	bispecific Fab
BLyS	B-lymphocyte stimulator
CDR	complementarity-determining region
DME	diabetic macular edema
EGFR	epidermal growth factor receptor
Fab	antigen binding fragment
FcRn	neonatal Fc receptor
FDA	US Food and Drug Administration
GC	gastric carcinoma
GPCR	G-protein-coupled receptor
HAE	hereditary angioedema
HC	heavy chain
HCL	hairy cell leukemia
HIV	human immunodeficiency virus
HLH	hemophagocytic lymphohistiocytosis
IFN	interferon-gamma
IL-13	interleukin-13
IL-17A	interleukin-17A
IL-23	interleukin-23
LC	light chain
mAbs	monoclonal antibodies
MCC	Merkel cell carcinoma
nAMD	neovascular age-related macular degeneration
NGS	next-generation sequencing
NMOSD	neuromyelitis optica spectrum disorder
NSCLC	non-small cell lung carcinoma
PD-L1	programmed death-1 ligand-1
PE38	Pseudomonas exotoxin A
PFU	plaque-forming unit
pKal	plasma kallikrein
RA	rheumatoid arthritis
scFv	single chain variable fragment
SLE	systemic lupus erythematosus
SPR	surface plasmon resonance
TNFα	tumor necrosis factor-alpha
TRIM	trinucleotide mutagenesis
UC	urothelial carcinoma
VEGFA	vascular endothelial growth factor A
VEGFR2	vascular endothelial growth factor receptor 2
V_H	heavy chain variable region
VHH	single-domain antibody
V_κ	kappa light chain variable region
V_λ	lambda light chain variable region
V_L	light chain variable region
vWF	von Willebrand factor

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