EXPERT OPINION

Advances in phage display technology for drug discovery

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Introduction: Over the past decade, several library-based methods have been developed to discover ligands with strong binding affinities for their targets. These methods mimic the natural evolution for screening and identifying ligand–target interactions with specific functional properties. Phage display technology is a well-established method that has been applied to many technological challenges including novel drug discovery.

Areas covered: This review describes the recent advances in the use of phage display technology for discovering novel bioactive compounds. Furthermore, it discusses the application of this technology to produce proteins and peptides as well as minimize the use of antibodies, such as antigen-binding fragment, single-chain fragment variable or single-domain antibody fragments like VHHs.

Expert opinion: Advances in screening, manufacturing and humanization technologies demonstrate that phage display derived products can play a significant role in the diagnosis and treatment of disease. The effects of this technology are inevitable in the development pipeline for bringing therapeutics into the market, and this number is expected to rise significantly in the future as new advances continue to take place in display methods. Furthermore, a widespread application of this methodology is predicted in different medical technological areas, including biosensing, monitoring, molecular imaging, gene therapy, vaccine development and nanotechnology.

Keywords: drug discovery, library, phage display, therapeutic applications

Expert Opin. Drug Discov. [Early Online]

1. Introduction

Drug discovery is a sophisticated procedure that incorporates scientific innovations with cutting-edge technologies. Developments of novel therapeutics or biological substances have gained considerable momentum in recent years. Discovery of new drugs has always required a broad search among many thousands of potential targets using well-defined *in vitro* screening analyses. Afterward, the hits are chosen to mimic, as closely as possible, the desired *in vivo* performance of the new drug [1,2].

Nowadays, new library methods provide a lot of alternatives and powerful ways to recognize the most interesting lead candidates, by combining the generation of billions of components with a fast screening or selection procedure [3-8]. One of the most widely used library methods is based on the use of filamentous phages, which seems to play an increasingly important role in the future of drug discovery [1,2,9,10]. Phage display was first described more than two decades ago and was defined as a simple and efficient functional genomics methodology for screening and identifying specific ligands for numerous types of molecular targets [11,12]. Phage display libraries not only allow the presentation of large peptide and protein libraries on the surface of filamentous phages but also permit the selection of novel biological components, with high affinity and specificity for almost any target [13-15].

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Article highlights

- Phage display technology enables the presentation of protein/peptide libraries on the surface of phage particles.
- Novel therapeutics or biological components, with high affinity and specificity, were developed by phage display.
- This technology has been applied to answer many technological problems, including the discovery of novel drugs.
- Use of phage display facilitates the production of protein and peptide and minimizes antibodies for therapeutic use.
- It is predicted that phage display will play an important role in different areas of medical technologies in the near future.

This box summarizes key points contained in the article.

Since its invention in 1985 [11], phage display has been successfully used in many different areas of bioscience, such as drug discovery, cancer research, immunology, neurobiology, nanostructured electronics, biosensing and agriculture. It has shown great potential in the discovery of new therapeutics in the 1990s. This review summarizes the extensive literature researches on application of this technology in drug development during the past two decades. It also highlights recent advances and lends a perspective for the future growth of phage display technology.

2. Phage display technology

2.1 Filamentous bacteriophage, structure and life cycle

Phage display technology is based on the presentation of a heterogeneous peptide or protein libraries on the surface of phage by fusion to bacteriophage coat proteins. Ligands fused to capsid proteins incorporate into the mature phage coat and are displayed on the phage surface, whereas the genetic material encoding each variant resides within the phage.

The most commonly used phage display methods are based on the use of M13 and related filamentous phages such as f1 and fd, as they do not lyse infected cells through their lifecycle. However, T4, T7 and λ phage have also been used for phage display [16-18]. Filamentous phages are a group of viruses that infect gram-negative bacteria using pili as receptors. These particles consist of a circular single-stranded DNA (ss-DNA) with 98% similarity across different strains. They also contain a flexible, rod-shaped cylinder ~ 6 nm in diameter and 1 μm long. The whole genome of the phage consists of 11 genes whose products were grouped according to their functions [19,20].

The body of a phage is covered by 2700 copies of tightly packed α-helical, rod-shaped protein named pVIII. One end of the particle is capped by 5 copies of pIII and pVI that bind to bacteria for injecting DNA into the host cell, whereas the opposite end has 3 – 5 copies of pVII and pIX. Although all the five capsid proteins have been used to display exogenous ligand on the surface of the phage, pIII is the most commonly applied anchor protein.

The size of the foreign ligand presented on each copy of selected coat protein limits the use of phage display, as larger inserts hamper the coat protein function in viral packaging and bacterial infectivity. Although the pVIII is mainly appropriate for display of peptides up to 8 amino acids in length, many short peptides and a variety of proteins (up to 300 amino acids) can usually be displayed at the N terminus of mature pIII, even if phage infectivity is thereby slightly reduced [10,13-15,21]. To overcome the size drawbacks, phagemid vectors that require helper phage superinfection were designed afterward. The helper phage provides all wild-type proteins necessary for phage assembly and infectivity. Therefore, the desired heterologous recombinant ligand is displayed on the phage surface as a mixture with wild-type proteins [19,20,22].

The pIII protein appears to have three distinct domains. The N1 domain begins translocation of the viral DNA into *Escherichia coli* during infection, whereas the N2 domain confers host cell recognition by the attachment of phage pIII to the F pilus of a male *E. coli*. The phage genome is then transferred to the cytoplasm of the bacterial cell where it is converted from single strand DNA to double-stranded plasmid replicative form by host DNA replication machinery. By rolling circle replication, the replicative form produces ss-DNA and also serves as template for phage protein expression. The C-terminal domain anchors the pIII in phage coat by interacting with other phage coat proteins. Therefore, it is responsible for the integration of pIII into the phage coat. The C-terminal part of pVIII is within the particle, close to the DNA, whereas its N-terminal is exposed to the environment (**Figure 1**).

Based on vector design, the phage display systems can be classified into three categories. Type 3, which represents an ss-DNA vector, is the first one according to the natural filamentous phage genome. Libraries are constructed by splicing foreign DNA inserts to the pIII gene, resulting in display of the fusion gene product on all of the coat proteins. The second is type 3 + 3, which represents the phagemid system. A phagemid vector is a plasmid that generally carries bacterial and phage origins of replication, a leader sequence, appropriate multiple cloning sites, an antibiotic-resistance gene and the fusion gene with a weak promoter.

The main advantage of phagemid systems is their smaller size and ease of cloning, which can help to construct large libraries. The major difference between these two systems is in the display level of the ligands. A phage vector displays 3 – 5 copies of the ligand on its surface, whereas a phagemid system is developed for monovalent display of binder on the surface of phages. The third system, named type 33, is a phage vector that contains both recombinant and wild-type gIII genes and expresses a hybrid pIII in addition to a wild-type pIII protein. Types 8, 8 + 8 and 88 are the same as 3, 3 + 3 and 33, respectively, which use pVIII for display (**Figure 2**).

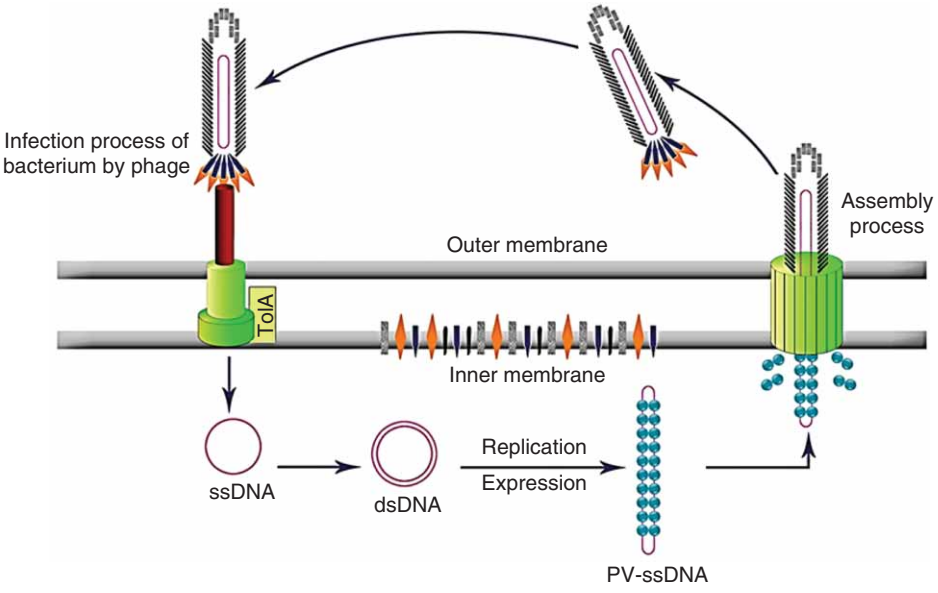

Figure 1. **Life cycle of filamentous phages.** Filamentous phage attaches to the tip of F-pilus on the bacterium through pIII N terminus. Next, the host TolA protein begins to depolymerize the phage coat proteins, which stay in the inner membrane for recycling. The ss-DNA of the phage is injected into the bacterial cell, which changes to double-stranded DNA (ds-DNA) and then replication and expression begins by host enzymes. The precursors of the phage is first produced by ss-DNA and then coated by pV protein dimers. pV protein dimers attach to the ss-DNA and avert or prevent conversion to replicative DNA. The C terminus of pI and pXI interact with pIV to form a channel to facilitate secretion of phage. PV, attached to ss-DNA, is replaced by pVIII in the channel and then mature phage particles are assembled and released.
ss-DNA: Single stranded DNA.

The monovalent display system is generally achieved by using pIII as the fusion partner in the 33 and 3 + 3 vector systems and permits the selection of higher affinity (nanomolar) ligands. In 88 and 8 + 8 vector systems, the valency of the displayed element is between 25 and 100 copies per viral particle. Therefore, the remarkable difference between pIII and pVIII display systems is the avidity effect caused by the high valency of component displayed on pVIII. In general, this can result in considerable differences in the affinity of the ligands that are subjected to selection either by pIII or pVIII display libraries against similar targets [23,24].

2.2 Construction of libraries

The starting point in phage display technique is library construction. A specific ligand for the target of interest is isolated through an affinity selection procedure. Like other selection methods, the size and quality of libraries are critical for a successful phage display process. Phage libraries usually contain billions of phage particles, some of which have as high as 10^{12} diverse particles. It is possible to display functional antibody fragments and peptides with an extensive variety of sizes and structures on the phage surface. However, all protein or peptide libraries cannot be chosen by phage display because some DNA sequences may be sensitive to bacterial enzymes or toxic to *E. coli*, which can cause interference with the assembly of phage. According to the specific goal of the studies, two types of libraries, including antibody fragments and peptides, are now extensively used for novel drug discovery [25-27].

2.2.1 *Peptide library*

Random phage peptide libraries are one of the most common types of phage display constructs. Using degenerate oligonucleotides introduced into the phage genome, the linear random peptide library can be obtained. The random oligonucleotides are cloned between the coding sequence of the signal peptide and the N-terminus of the protein pIII. The linear library permits screening of ligands against targets whose residues are involved in interaction sites with the ligand; those residues can be either continuous or widely separated in the primary sequence.

Random peptide libraries varying in length from 6 to 43 amino acids have been successfully cloned and expressed as peptide-capsid fusion proteins. One of the most common approaches to construct random peptides is to use $(NNK)_n$ codon degeneracy, where N indicates an equimolar mixture of all four nucleotides (A, G, C and T), and K indicates a 1:1 mixture of G and T. By adding an amber stop codon (TAG) at the beginning of gene III of phage genome, monovalent peptide libraries can be generated. In an amber-suppressor host strain, the number of stop codons is decreased from three (TAA, TGA and TAG) to one (TAG), by applying $(NNK)_n$ codons in place of $(NNN)_n$.

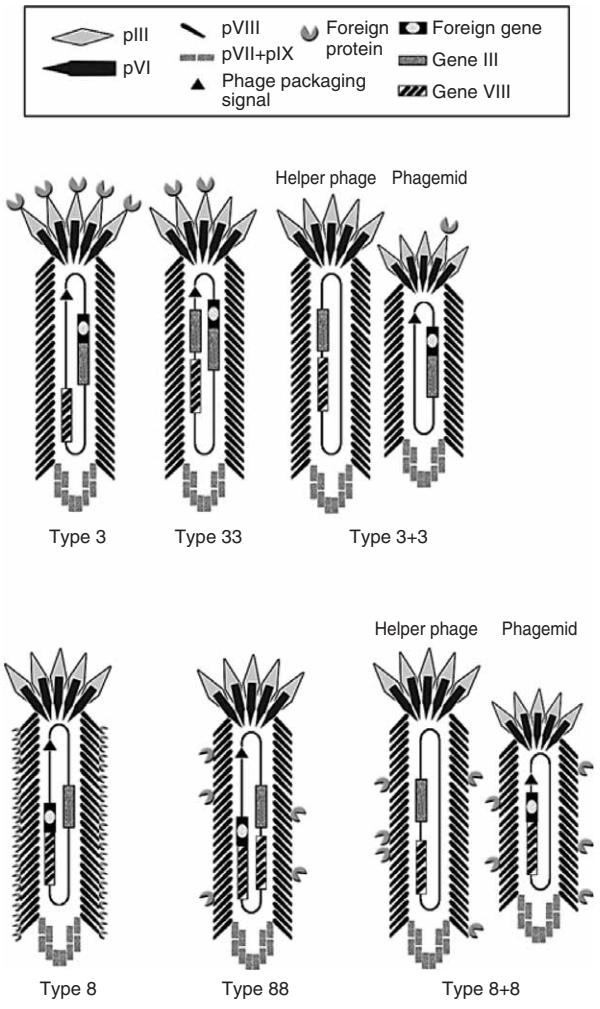

Figure 2. **Comparison of different types of phage display vector systems.** The names of the systems are listed at the bottom. The ss-DNA of M13 phage encodes all phage proteins and contains a phage packaging signal. Based on whether the foreign gene is fused to pIII or pVIII proteins, the vector system is classified as 3 or 8. See text for more information including the classification.
ss-DNA: Single stranded DNA.

Phage display libraries with a loop scaffold are constructed when the random peptide sequences are flanked by a pair of cysteine residues and form a disulfide cross-link. Such scaffolds have been successfully used to identify novel targets that did not bind to any ligands from the linear random peptide libraries. These libraries are also valuable in yielding high affinity ligands. Although hundreds of libraries have been established until now, the resources of commercial peptide libraries displayed on phages are very restricted. The most widely used commercial libraries are New England BioLabs, which consists of 10^9 independent peptides, and Creative Biolabs, which contains 10^{7-10} variants [26,28-30].

2.2.2 *Antibody library*

The conventional antibody is comprised of two identical heavy (H) and light (L) chains inter-connected by disulfide bridges. The chains composed of constant (C) and variable (V) domains, combine to form one interaction site for the antigen. The Fab consists of the L chain and two domains of the H chain located at its N terminus (VH and CH1). The single-chain fragment variable (scFv) is a small engineered fragment composed of two variable domains. A short flexible glycine-rich linker peptide of 10 – 25 amino acids serves as a linker between VH and VL domains (**Figure 3**) [31-34].

Historically, the first antibody gene libraries using phages were created and screened by means of lytic phage Lambda [35]. However, those libraries gained limited success. McCafferty *et al.* successfully expressed antibody variable domains on the surface of filamentous phages in 1990 [36]. Since then, two antibody formats including scFvs and Fabs have been used for generating an antibody phage display library by Winter's and Lerner's groups [37,38].

Owing to folding problems of heterologous proteins in *E. coli*, only antibody fragments can be generally used for antibody phage display. It is worth noting that the conformation of amino acids involved in complementarity determining regions (CDRs) of hypervariable domains on both H and L chains of an antibody plays the main role in determining antigen-binding activity [31,39].

However, production of full-length IgG in *E. coli* is rarely achievable. ScFvs are fairly stable on average and frequently have a high tendency to aggregate and form unstable multimers owing to the presence of an oligopeptide linker, which is susceptible to proteolytic cleavage and consequent unfolding of the antibody fragments [31,39,40]. Fab fragments were discovered to have comparably higher structural stability because of additional interface in their constant domains that result in overall diminished aggregation and consequently a larger proportion of monomers. Therefore, instead of the scFv, Fab fragments have been used for library construction in many of the more recent phage display systems. Besides the aforementioned advantages, which are significant aspects through selection and screening procedures, conversion of Fab fragments into full-length IgG is also possible without impairing their functionality [41].

The smallest fragment of an antibody with antigen-binding property is composed of just one variable domain (VH or VL) [31,41,42]. Because these isolated single domain antibodies (sdAb) often expose large hydrophobic regions to the solvent, such fragments are apparently unstable, with a tendency to form aggregates. The concept of sdAb derived from antibody variable domains was introduced > 20 years ago by Ward and his colleagues [43].

Camelids and nurse sharks have a substantial fraction of functional antibodies, known as heavy-chain only antibodies (HCAbs) (**Figure 3**). In an HCAb, the Ag-binding fragment of a classical antibody – the Fab – is diminished into a single

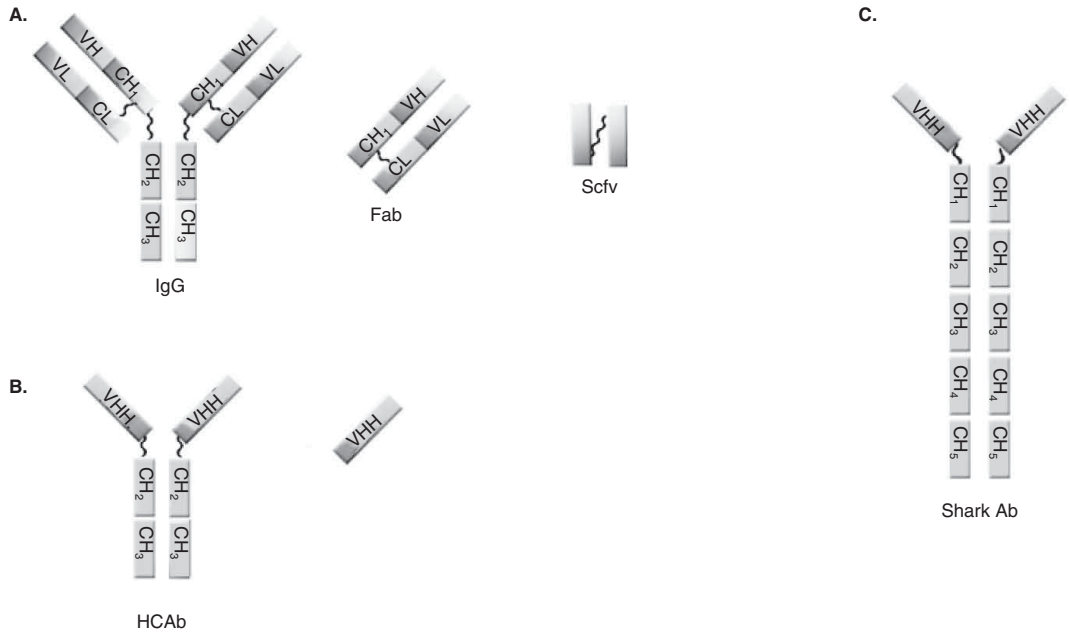

Figure 3. Fab and scFv fragments from conventional antibodies (**A**), VHH fragments from heavy-chain (**B**) and cartilaginous fish antibodies (**C**).

HCAb: Heavy-chain only antibody; scFv: Single-chain fragment variable.

variable domain, which is referred to as VHH or nanobody (**Figure 3**). Lacking VL, this domain is adapted to become functional in antigen binding. In contrast to human VH domains, the VHH fragments are well expressed in bacteria and yeasts and also demonstrate notable resistance to high temperatures and aggregation. VHH antibodies have longer CDR1 and CDR3 with different canonical structures that increase the structural repertoire of the antigen-binding sites and compensate for the lack of the VL CDRs. This permits a better contact with the antigens and a higher capability to identify them. The camelid heavy-chain and shark Ig new antigen receptor antibody fragments with smaller size, higher solubility and greater stability have become subjects of special interest for therapeutic and medical applications [44-46].

Regarding the source of library sequences, various types of antibody gene libraries can be constructed [47]. Immune libraries are generated by amplification of V genes isolated from IgG-secreting plasma cells from immunized donors. These types of libraries are typically created and used in medical researches and applications to obtain an antibody for a specific target [44,48,49]. Naive libraries are constructed from rearranged V gene pools of a non-immunized individual. These two libraries are completely based on naturally occurring sequence diversity. The major advantage of immune libraries over naive libraries is related to affinity maturation of the desired antibodies, which leads to selection of antibodies with a higher affinity [50,51].

Semi-synthetic libraries are derived from unrearranged V genes from pre-B cells (germline cells) or an antibody framework with genetically randomized CDR3. In fact, semi-synthetic libraries combine natural diversity for certain aspects of the library with *in silico* design. Fully synthetic libraries are made of human frameworks with randomly integrated CDR cassettes [52-55]. Semi-synthetic and synthetic libraries are developed based on Fab, scFv or even dAb fragments. Repertoires of camelized VH domains have been generated via randomization of CDR3 residues and simultaneous variation in the length of CDR3 [56-58]. Naive, semi-synthetic and synthetic libraries are listed as single-pot libraries as they are constructed to select antibody fragments that bind to each possible antigen. Lack of necessity for immunizing animals (especially humans) and rapid processing for producing antibodies are considered as the main advantages of these libraries compared to immune libraries [41,47].

Most of the antibody candidates are created from a few company-owned libraries: Cambridge Antibody Technology's scFv-fragment library, Dyax Corp's human Fab-fragment libraries, and MorphoSys's human combinatorial antibody scFv-fragment (HuCAL®) and Fab-fragment (HuCALGold®) libraries [9].

2.3 Biopanning

Screening of phage libraries, referred to as 'biopanning' is a characteristic aspect of phage display technology based on affinity selection, which isolates ligands against any target.

By carefully controlling selection and screening, display technologies enable the generation of antibodies/peptides against defined antigen conformations or epitopes. One of

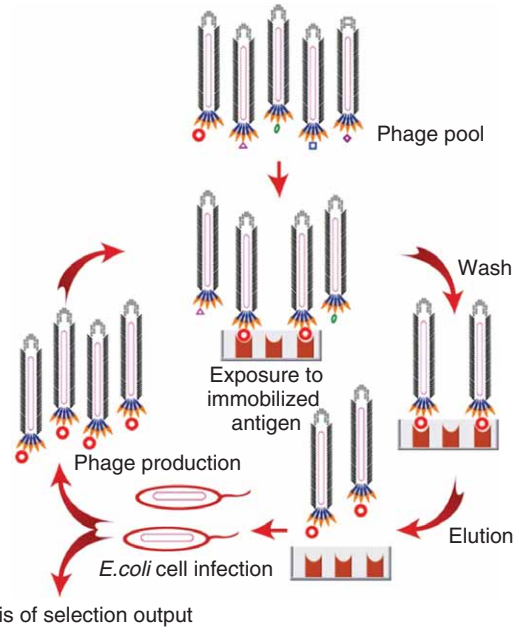

Figure 4. **Schematic presentation of a typical phage display selection cycle.** First, initial pools of functional foreign peptides, proteins or antibodies on the surface of phages are exposed to an antigen immobilized on a solid surface. Then, unbound phages are removed by washing and bound phages are eluted and permitted to infect *Escherichia coli* to produce phages for the next round of panning or to analyze the selected clones.

the advantages of using *in vitro* methods is its capability of overcoming limitations of immunological tolerance and permitting the isolation of affinity binders that identifies extremely conserved targets. Basically, selection process can be divided into four key steps, including incubation of phage display library with target molecules, washing the unbound phages, elution of specifically bound phages and amplification of the phage binders in *E. coli* (**Figure 4**). Most protein, peptide, nucleic acid, and carbohydrate targets can be used in screening of phage display libraries [39,47].

The target may be immobilized directly or indirectly (biotin-streptavidin interaction) on a solid surface such as nitrocellulose, magnetic beads, column matrices, plastic surfaces like polystyrene tubes and microtitreplates [37,44,59-62]. Direct selection of binders against markers on cell surfaces may be achieved on either monolayers of adherent cells or on cells in solution. Whole-cell panning is normally the best choice when antigen is unavailable. It is also useful in the discovery of unknown biomarkers [8,47,63]. Phage particles that bind weakly to the selector and the vast excess of non-binding phages are eliminated by harsh washing. To enhance the efficiency of biopanning, counter selections against carrier molecules or nonspecific targets can also be beneficial.

The captured phages, a 10^{-8} – 10^{-7} fraction of the initial library population, are usually eluted with mild acid, alkaline or detergent solutions without affecting phage infectivity and then reamplified by infecting with *E. coli*. Afterward, the phagemid-bearing cell is infected with a helper phage to create new antibody/peptide phage, which can be applied for further panning rounds until a significant enrichment of target-specific phage is obtained. In a biopanning process, three – four rounds of selections are usually performed [8,37,44]. *In vivo* panning is also possible to target specific cell types or even organs. This method was first described by Pasqualini and Ruoslahti in 1996 [64].

They isolated peptides that were home to renal and cerebral vascular endothelium *in vivo*. Such peptide ligands, or antibodies directed against specific vascular receptors, could be used to deliver imaging agents or therapeutic compounds across the endothelial cells *in vivo*. In addition, phage-derived ligands that are specific for an organ or a tissue could be valuable both for diagnostic and therapeutic applications by conjugating phage to a drug or assembling phage on drug-containing nanoparticles. Selection of tissue-specific or organ-specific ligand is obtained through injecting a phage library into the vasculature of a human volunteer or animals [14,65-70].

After panning, soluble antibody/peptide fragments are constructed, and their specific binding is characterized using ELISA or immunocytochemistry to identify individual binders. Subsequently, the selected binders can be sequenced, biochemically analyzed and further modified for specific applications [8,44].

3. Alternatives to phage display

Several alternative display systems, such as display on bacterial surfaces, yeast surfaces and eukaryotic viruses, have been introduced by a number of groups [1,71,72].

All of these systems still require transformation of a cellular host, and so they have not been successful in raising library sizes. Although these alternative methods have demonstrated advantages in special applications, M13 phage display platform remains the leading technology today [1,2].

4. Applications of phage display

Phage display technology offers potential tools for development of therapeutic agents, vaccines, diagnostic reagents, as well as gene and drug delivery systems [10,14,73-75].

Determination of interaction partners of organic (proteins, polysaccharides or DNAs) or inorganic compounds and also *in vitro* protein engineering are the major applications of phage display technology. More recently, a number of attempts have been made for using *in vitro* phage display technology in medical science by designing humanized antibodies or peptides and development of new pharmaceutics for various maladies such as cancer, autoimmune and inflammatory diseases, metabolic and allergic disorders. This methodology has usually been applied for the production and isolation of

the antibodies [31,76-79], identification of peptide agonists and antagonists for receptors [28,80-82], characterization of novel protein/peptide-protein/DNA interactions [13,14,81], demonstration and mapping of highly carbohydrate-dependent epitopes and protein functional epitopes [83-85], recognition of specific antigens in or on the surface of the tumor cells as the therapeutic targets [1,2], in addition to molecular imaging with the use of fluorescently labeled phage [86,87].

At present, > 20 phage display-derived antibody and peptides are in late-stage clinical trials or have been approved [2,9,39,88,89].

4.1 Antibody drugs derived from phage display technology

The first mAbs was produced by using the hybridoma technique in 1975 [90]. Over the past 40 years, a great number of mAbs have been prepared and characterized against various antigens. Nevertheless, only a few of these antibodies have presented clear clinical benefit in treatment of diseases [88,89,91-94]. These antibodies are widely used for development of diagnostic tests [42,95,96], such as enzyme immunoassays [97,98], immunochromatography [42,99,100] and immunosensors [101-103].

The generation of antibodies was revolutionized by progression of molecular biology techniques in recombinant DNA expression. Phage display technology, as a powerful tool for construction and isolation of recombinant antibodies, has provided a valuable alternative method for the production of antibodies of desired specificity. Study of antigen-antibody binding for the recognition of epitopes and mimotopes was one of the initial applications of antibody phage display technology. Later, it was found that large molecules, such as antibody fragments (scFV, Fab fragment and VHH domains), can successfully be presented on the phages. In comparison to polyclonal and monoclonal antibodies, recombinant antibodies based on phage display technology can be produced faster, in more automatic procedure and with decreased utilization of laboratory animals. Millions of different antibody fragments displayed on the phages are often used for selecting highly specific therapeutic antibodies [2,8,39,40,47,88,104].

Therapeutic mAbs have considerably advanced both in their molecular structure and clinical efficiency. Over the past two decades, the production of therapeutic antibodies via phage display technique has been found to be an effective alternative to the conventional immunization. Initial developmental efforts mainly focused on expanding of the target antigen repertoire and humanizing the antibody proteins to overcome problems caused by immunogenicity. In parallel, antibody-drug conjugates have been developed for targeted delivery and as imaging agents in screening, especially in cancer. At present, several phage display-derived mAbs are in clinical or preclinical stages of development, but only a few selected antibodies are reaching the market. Until now, phage display technology has presented ~ 30 – 35% of the total mAbs, which are currently in clinical trials [2,8,9,39,47,79]. The most successful phage display-derived antibodies that underwent clinical or preclinical studies are summarized later and in **Table 1** [2,8,9,39,47,79,89].

Adalimumab is a recombinant human IgG1 mAb that is used as a TNF-inhibiting anti-inflammatory drug. It was the first fully human mAb drug approved by the FDA for treatment of seven symptoms: rheumatoid arthritis, juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, ulcerative colitis and plaque psoriasis [39,73,105].

Belimumab is a human antibody that inhibits B-cell activating factor. It was generated by phage display for treatment of autoimmune diseases, mainly systemic lupus erythematosus. Belimumab is approved in the US, Canada and Europe for treatment of systemic lupus erythematosus [78,92].

Ranibizumab binds to VEGF-A and neutralizes its activity as an mAb Fab. It was affinity matured by phage display and is an interesting example of humanized antibody production by means of phage display technology. According to the crucial role of VEGF-A in angiogenesis, this antibody is approved for the treatment of wet age-related macular edema [106,107].

Raxibacumab is a human mAb intended for the prophylaxis and treatment of inhaled anthrax. This antibody binds to the protective antigen (PA83) of *Bacillus anthracis*. Its efficacy has been proven in rabbits and monkeys. In December 2012, raxibacumab was approved for the treatment of inhalational anthrax raised from *B. anthracis* in combination with appropriate antibacterial drugs. It can also be used for prophylaxis of inhalational anthrax when alternative therapies are not available or are not appropriate [89].

1D09C3 is a fully human IgG4 antibody directed against MHC class II (HLA-DR) cell surface antigens. IgG4 subclass antibodies offer the advantage of poor induction of the complement system and as a result have obtained great attention as therapeutic compounds. Currently, this antibody is under the Phase I clinical trial in patients with relapsed or refractory B-cell lymphomas, who have not shown positive response prior to standard therapy [47,108,109].

Lexatumumab (HGS-ETR2) is a fully human experimental agonistic mAb against TNF-related, apoptosis-inducing ligand receptor 2 (TRAIL-R2). It activates the extrinsic apoptosis pathway and is undergoing Phase I clinical trials for the treatment of cancer [110,111].

Moxetumomab pasudotox (CAT-8015) is an anti-cluster of differentiation-22 (CD22) immunotoxin fusion protein between a murine anti-CD22 disulfide-linked Fv (dsFv) antibody fragment and a *Pseudomonas* exotoxin PE38. CAT-8015 is a second-generation CD22-targeted immunotoxin. Phase I/II clinical trials using CAT-8015 for leukemia or lymphomas have been performed [112].

Fresolimumab (GC1008) is a human IgG4 mAb that binds and neutralizes all isoforms of TGF-β. It is developed for

Table 1. Selected approved or under clinical development phage display-derived therapeutic antibodies.

Product	Manufacturer	Type	Target	Phase
Adalimumab (Humira)	Abbott Laboratories	IgG1	TNFα	Approved
Belimumab (Benlysta)	GlaxoSmithKline	IgG1	BLyS	Approved
Raxibacumab (ABthrax)	GlaxoSmithKline	IgG1	PA83 of *Bacillus anthracis*	Approved
Ranibizumab (Lucentis)	Genentech	Fab fragment	VEGF-A	Approved
Ramucirumab (IMC-1121B, Cyramza)	Eli Lilly	IgG1	VEGFR2	Approved for treatment of stomach cancer/III
Necitumumab (IMC-11F8)	Eli Lilly	IgG1	EGFR	III
Moxetumomab Pasudotox (CAT-8015)	AstraZeneca	Antibody-fusion protein	CD22	III
Fresolimumab (GC1008)	Genzyme/Sanofi-Aventis	IgG4	TGF-β	II
Mapatumumab (HGS-ETR1)	Cambridge Antibody Technology/ Human Genome Sciences	IgG1	TRAIL-R1	II
ALX-0081 (Nanobody)	Ablynx	Camelid single-domain antibody	vWF	II
Anti-LINGO-1	Biogen Idec	IgG	LINGO-1	II
ART621	Arana Therapeutics	Human VH or VL single domain antibody	TNFα	II
Cixutumumab (IMC-A12)	ImClone Systems	IgG1	IGF-1R	I/II
1D09C3	GPC Biotech	IgG4	HLA-DR	I
DX-2930	Dyax	IgG1	Plasma kallikrein	I
Lexatumumab (HGS-ETR2)	Cambridge Antibody Technology/ Human Genome Sciences	IgG1	TRAIL-R2	I

BLyS: B lymphocyte stimulator; CD22: Cluster of differentiation-22; HLA-DR: Human IgG4 antibody directed against MHC class II; IGF-1R: IGF-1 receptor; LINGO-1: Leucine rich repeat and Ig domain containing 1; TRAIL-R1: Tumour necrosis factor apoptosis-inducing ligand receptor-1; vWF: von Willebrand factor.

treatment of idiopathic pulmonary fibrosis, focal segmental glomerulosclerosis and cancer [113,114].

Cixutumumab is a fully human IgG1 mAb directed against the human IGF-1 receptor (IGF-1R). It selectively binds to membrane-bound IGF-1R, thereby preventing the binding of the natural ligand IGF-1. Cixutumumab with an affinity of around 10^{-11} M can be used for blocking ligand-induced receptor activation [115,116].

Mapatumumab (HGS-ETR1) is a human mAb that specifically binds to TRAIL receptor-1 and induces apoptosis through activation of death receptors TRAIL-R1 and TRAIL-R2. TRAIL receptor 1 is expressed in all of the tumors in various degrees. The results of Phase I and II clinical studies demonstrated the safety and tolerability of mapatumumab in cancer patients with advanced solid tumors or non-Hodgkin lymphomas [117,118].

Necitumumab is a fully human IgG1 mAb against the EGFR. EGFRs are present in various tumors, including colorectal and NSCLC. Necitumumab binds to the EGFR with high affinity and blocks the binding of EGFR ligands and neutralizes ligand-induced EGFR phosphorylation. Phase III clinical trial of necitumumab in combination with gemcitabine and cisplatin in squamous NSCLC is ongoing [47,119-121].

Ramucirumab is a fully human IgG1mAb that was produced for the treatment of solid tumors. It directly binds to the VEGFR2 and works as a receptor antagonist. It blocks the binding of VEGF to VEGFR2. Ramucirumab is being tested in several Phase III clinical trials for the treatment of metastatic gastric adenocarcinoma and NSCLCs. It was approved by the FDA for the treatment of stomach cancer in 2014 [47,122,123].

DX-2930 is a fully human mAb developed by Dyax as an inhibitor of plasma kallikrein. Excessive activity of plasma kallikrein leads to increase in bradykinin production, a vasodilator for the localized swelling, inflammation and pain, characteristically associated with hereditary angioedema. DX-2930 is now under clinical study Phase Ib as a subcutaneous injection for the prevention of hereditary angioedema [124,125].

Anti-LINGO-1 targets leucine rich repeat and Ig domain containing 1 (LINGO-1), a protein expressed in the CNS that is known as a negative regulator of axonal myelination and axonal regeneration. Currently, this fully human mAb is in Phase II clinical trial for the treatment of multiple sclerosis [126,127].

Currently, most of FDA-approved therapeutic antibodies and the vast majority of those in clinical studies are full-size antibodies typically in IgG1 format. In spite of clinical success of fully human mAb, an important problem for such large molecules (150 kDa) is their poor penetration into solid

tissues and weak or absent binding to regions on the surface of some molecules such as the HIV envelope glycoprotein [2,39,79]. Therefore, during the past decade, a large number of studies were conducted particularly to develop new scaffolds of much smaller size and higher stability [31,104,128,129]. Such compounds are based on different human and non-human molecules of high stability and could be divided into two main groups including antibody [2,41,79] and protein-derived scaffolds [2]. In addition to domain-based protein scaffolds, short peptide sequences have also been used as therapeutic agents [2]. In this section, antibody-derived scaffolds, specifically those derived from antibody domains, are discussed. Protein-derived scaffolds and short peptides are discussed in separate specific sections.

SdAb fragments represent a new generation of therapeutic agents, and they could potentially act as a substitute for conventional therapeutic drugs in the treatment of human diseases, owing to their unique physicochemical and pharmacological properties [2,41,79]. SdAb can be obtained using phage display methods from HCAbs of camelids (camels, llama and alpacas) or sharks [31,44,45,104].

Camelized sdAb can also be made from VH or VL region of conventional murine or human IgG of immunized or naive libraries. A problem with this approach is that the binding domain of IgG consists of two variable L chain and H chain, which tend to dimerize or aggregate, owing to their lipophilicity. Monomerization is generally accomplished by substitution of lipophilic amino acids with hydrophilic ones [56-58].

Currently, some sdAb-based drugs are subjected to preclinical studies in animal models or *in vitro*. Some sdAb, in Phase I or II of clinical trials, are listed later.

ALX-0081 is a camelid single-domain antibody targeting von Willebrand factor. The neutralization of this factor can decrease the risk of thrombosis in patients with acute coronary syndrome and thrombotic thrombocytopenic purpura. Phase II clinical trial of ALX-0081 in high risk percutaneous coronary intervention was started in September 2009 [130,131].

ART621 is a human VH or VL sdAb targeting TNFα. The Phase I clinical trial was successfully completed in November 2007. The drug was well-tolerated in healthy volunteers. In March 2008, the Phase II clinical trial was conducted on psoriasis [79].

4.2 Peptide drugs derived from phage display technology

Through drug design, research and development efforts, peptides (10 – 50 amino acids) were introduced and immediately found their way into the market. Peptides offer some favorable advantages over biopharmaceutical polypeptides, including higher affinity and specificity, greater stability, easier manufacturing, lower cost of large-scale production and better tissue penetration [132]. These bioactive small molecules can interact selectively with proteins *in vivo* and act as ligand, inhibitor, substrate, antigen, epitope mimetic and so on, with therapeutic potential [133,134]. Hence, it is estimated that the expanding market of peptide-based drugs will be worth US $25.4 billion in 2018, according to the reports of Transparency Market Research [135]. In recent years, considerable numbers of peptide drugs have been approved or are under clinical studies for a wide variety of diseases such as AIDS and malignancies [20,136,137]. In **Table 2**, several selected successful therapeutic peptides isolated from phage display, which have been approved or are under clinical studies, are listed along with their functions, indications and manufacturers.

Peptides fused to the Fc region of an IgG are called peptibodies. They have a long circulating time and are considered as desirable alternatives to mAbs [138,139].

4.3 Protein drugs derived from phage display technology

Here, in a short overview, various applications of protein phage display in drug discovery are mentioned.

4.3.1 Protease inhibitors

Proteolytic enzymes have regulatory roles in cellular processes, including hormone release, coagulation and complement. This class of enzymes also has a function as the pathogenic factor in various diseases including inflammatory diseases, malignancies, cancers and so on. Because modification of the natural protease inhibitors seems to be laborious and the synthetic ones have been accompanied by unwanted or even dangerous effects owing to their complicated metabolism, phage display technology has been proposed as a practical approach that couples combinatorial chemistry and the synthetic power of biology to discover appropriate inhibitors. The role of phage display is mostly concentrated on isolation of ligands (< 100 amino acids), which contains special inhibitory scaffolds and provides information regarding functional groups and their relative positions required for efficient binding [140,141]. The identified candidates are then subjected to further modification to develop protease inhibitors in peptidomimetic form [142]. Nixon has comprehensively discussed phage display-derived inhibitors for tissue factor-factor VIIa complex, kallikrein and thrombin as major targets of inhibition [140].

4.3.2 Minimizing proteins

Mini-proteins are defined as polypeptides or peptides composed of < 100 amino acids, which have functional or structural roles. There are three approaches in using these kinds of proteins: i) natural occurring mini-proteins, including animal or bacterial toxins and cyclotides; ii) artificial mini-proteins, which are modeled after natural ones; and iii) minimized proteins obtained by random or directed mutagenesis [143-146]. Natural toxins (e.g., channels blocker) have shown great potential for therapeutic applications and serve as drugs directly in the treatment of various diseases [147].

Reduction in the size of a protein without affecting its biofunction has been an attractive field of study in drug design.

Table 2. Selected approved or under clinical development phage display-derived peptides or peptide-based therapeutics.

Product	Manufacturer	Function	Indication(s)	Phase
Romiplostim (Nplate®)	Amgen	Ligand for thrombopoietin receptor	Idiopathic (immune) thrombocytopenic purpura	Approved
Ecallantide (Kalbitor®)	Dyax	Plasma kallikrein inhibitor	Hereditary angioedema	Approved
Peginesatide (OMONTYS®)	Affymax, Takeda	Erythropoietin mimetic	Chronic kidney disease associated anemia	Approved*
AMG-386	Amgen	Inhibitor for binding of angiopoietin-1 and angiopoietin-2 to Tie2	Anti-angiogenic	III
CNTO530/CNTO 528	Ortho Biotech	Erythropoietin mimetic	Chronic kidney disease associated anemia	I

*In February 2013, Affymax and Takeda announced a nationwide voluntary recall of all lots of OMONTYS (peginesatide) injection to the user level as a result of new post-marketing reports regarding serious hypersensitivity reactions, including anaphylaxis, which can be life-threatening or fatal.

During such a process, the length of a protein is shortened using directed mutagenesis. Here, phage display methods can be applied to select the mutants with retained function. Arterial natriuretic peptide is a polypeptide hormone that reduces blood pressure and is considered as a successful example of minimized protein by means of phage display. Li *et al.* reduced its residues from 28 to 15 and, at the same time, maintained its high binding affinity and biopotency [148].

4.3.3 *New scaffolds*

These protein scaffolds with appropriate properties could have undeniable advantages in drug targeting. Although this approach is directly related to antibodies, there are also interesting applications of scaffold libraries in affinity ligand generation and chromatography [149]. Phage display helps developing affibody molecules, which are designed based on the Z domain of protein A and remarked as a viable and sometimes superior alternative to antibodies [150,151]. Specific binding of affibody molecules to a variety of different proteins (e.g., insulin, fibrinogen, transferrin, TNFα, IL-8, glycoprotein 120, CD28, human serum albumin, IgA, IgE, IgM, EGFR2 and EGFR) can result in gaining remarkable achievements in drug discovery in the next few years [150,152,153]. Currently, affibody has a broad pipeline with the most advanced projects in clinical studies such as ABY-025/GE226 (for tracing human EGFR2 in cancer diagnostics and imaging), which is in clinical trial Phase II [154].

It is noteworthy that successful application of these protein scaffolds relies on several crucial clinical aspects. Among various parameters such as appropriate affinity for the target in *in vivo* settings, the route of administration and plasma half-life, the *in vivo* immunogenicity profile of these protein scaffolds still remains an open question in designing process of novel protein scaffolds for therapeutic applications [155]. Like other proteins, entering these scaffolds into the human body can lead to generation of anti-drug antibodies. Especially in repeated administration, anti-drug antibodies interfere with or neutralize the effect of the biopharmaceutical and even result in serious and life-threatening adverse events such as allergic or autoimmune reactions [156,157]. Moreover, misfolded proteins, aggregated protein scaffolds or adjuvant substances also promote the risk of immunogenicity by breaking B-cell tolerance [158,159]. Because of poor understanding of main contributed factors in immunogenicity and the ambiguous mechanisms of specific tolerance induction, the prediction and evaluation of immunogenicity level is not accurately possible [155].

4.3.4 *DNA binding proteins*

The most ideal model for studying protein-DNA interaction is a famous protein class called zinc fingers (ZFPs). These proteins contain multiple finger-like motifs, which are different in sequence and structure, displaying a significant specificity in binding affinity even between members of the same class (DNA, RNA or proteins). Such capacity presents them as the stable scaffolds with specialized functions and therapeutic potentials [160].

Available protein engineering methods seem to be imperfect in redesigning ZFP domains particularly in drug development aspects. In contrast, phage display technology becomes one of the most successful rapid selecting methods that can be easily adapted to a high-throughput automated format in this area. Barbas groups have succeeded in construction of complete libraries of ZFP domains by means of phage display [37,161]. Sangamo Biosciences was also granted by FDA to enter clinical studies on ZFPs entitled Therapeutic® programs. Their platform has two main approaches: ZFP transcription factors that are used for gene regulation and Zinc-Finger nucleases that specifically modify gene sequences via different ways [160,162]. Detailed information on the products and ongoing studies are available on their website (http://www.sangamo.com) [163,164].

There is another interesting application for engineered ZFPs. Special artificial ZFPs are able to differentiate between cytosine, thymine and 5-methylcytosine. Considering the established role of DNA methylation in various diseases

such as cancer, designing such ZFPs could be very useful in diagnosis and therapy [165].

5. Phage display for target validation

Prediction, identification and validation of novel drug targets are an extremely difficult and resource-intensive procedure, which necessitates an integral use of numerous tools, methods and information. Validating potential drug targets is one of the most critical steps in drug discovery.

There are many tools and technologies that have been applied in different approaches for accelerating target discovery and validation. Some of the approaches to target validation might involve the use of small interfering RNAs, antisense oligonucleotides, microarray [166], phage display technologies [167,168], engineered transgenic or knockout mice [169].

Among these strategies, phage display technology can be used as a powerful tool for validating the most interesting targets. Identified targets could be examined for potential inhibition strategies with small molecules, antibodies and synthetic peptides. To validate a subset of targets, phage display was used to generate high-affinity inhibitors or antibodies against the predicted targets and their biological effects were examined by *in vitro* and *in vivo* assay. Of course, phage display technology is only preliminary validation strategy, and more tests prior to the trial are not only required to find the predictions as real new targets, but should provide crucial information regarding the validity of the chosen target. In particular, several human diseases, such as cancers and autoimmune disorders, can be emulated in mouse, making it an ideal tool for accelerating the validation procedure of new compounds in addition to the risk and toxicity assessment.

6. Publications and patents in phage display

The importance of phage display systems and their various applications brings more attention to this technology [170]. Currently, there are 114,752 publications about phage display and related subjects such as antibody, peptide, protein and nanobody phage display in the SCOPUS database (www.scopus.com), which includes 81,395 patents. Approximately 10% of these documents are in the fields of pharmacology, toxicology and pharmaceutics. There are also 146,068 phage display-related patents cited in Free Patent Online database (www.freepatentsonline.com). All databases were accessed in February 2015.

7. Current trends in phage display technology

Traditionally, filamentous phages are used for phage libraries construction. Thanks to their nanosized dimensions, they have been presented as an excellent alternative for synthetic nanoparticles, nanorodes and nanotubes. Parallel evolution of biotechnology and nanotechnology sciences and growing accessibility to genetic engineering toolboxes have recently made these phages expand their mere use in molecular screening to advanced applications in medical diagnosis and clinical management, including tissue targeting, imaging, gene/drug delivery and biosensing [171,172].

The accessibility of commercial libraries and ease of use have made this technology available for screening and identifying large number of ligands or receptor mimics for tumor targeting, such as integrin, somatostatin, gastrin-releasing peptide and so on [173]. Furthermore, conjugation of radiolabels, fluorescent probes and even MRI contrast agents to phage coat proteins, make phage particles the ideal non-invasive imaging compounds at molecular level. By assembling both selective ligands and imaging agent phage particles, intact phages can be used in monitoring patient's diseased condition and progression, especially in radiotherapy [173]. In contrast to anatomic imaging that measures tumor size, molecular imaging offers more information on tumor metabolism, response to treatments, apoptosis or proliferation. Thus, it is more applicable especially when the treatment affects tumor by preventing its progression instead of shrinking its size [87]. Recently, fabricating biocompatible nanoparticles conjugated with phage display-derived peptides has led to production of more favorable targeting and imaging agents, which can essentially reduce toxic effects of chemotherapeutics in anti-cancer therapy. In drug development, such interesting approach is used in assessing a drug's behavior *in vivo*, ensuring efficient drug delivery to the target site and evaluating drug metabolism pathway [86,173].

Phages have been also applied as drug and gene delivery vehicles. In addition to therapeutic peptide display, they can serve as targeted nanocarriers, which encapsulate and deliver drug (chemical or biological) molecules to specific tissues. Such ideas have recently been applied in phage display vaccination by presenting appropriate antigen peptides on phage surface. Because of their immunostimulatory nature, these phage particles additionally show adjuvant activity, which is extremely valuable in designing vaccines. As an advanced application in effective gene therapy, the phage coat can truly protect the DNA from degradation after injection and display probe molecules on the phage coat for targeting specific cell types [141,174].

In another remarkable aspect of application, filamentous phages have been applied in nanoscale biosensing. Their three-dimensional surface enables them to display various desirable peptides that result in providing the stronger multivalent bioreceptors for targeted molecules compared to conventional ones. There are numerous studies that have directly used whole engineered phage particles, reporter phages or phage-displayed peptides as the bioprobes in biosensors [172]. As a successful example, Biotech Laboratories have marketed FASTPlaque-Response™ and FASTPlaqueTB™ for rapid detection of *Mycobacterium tuberculosis* in human sputum samples [175]. Although most of phage biosensors have been designed for pathogen detection especially in food industry

until now [176], there are expanding efforts in this field with high expectation to obtain clinical biosensors in near future [177-179].

Natural ability of phages to target bacteria is used to develop fascinating area in phage therapy against bacterial infections. Modified lytic phages are directly administered to specifically kill pathogenic bacteria as an alternative to antibiotics. This application has been found more attractive because of sudden increase of incidence in antibiotic-resistant bacteria [180]. BiophagePharma has developed a large bank of isolated phages for use in phage therapy applications, PDS® Biosensor and Bactrapping® System. Moreover, Phico Therapeutics modifies a fully characterized bacteriophage for each type of target bacterium; their SASPject™ vectors target only bacterial cells. Ryan *et al.* have completely reviewed commercial phage-based products [175].

8. Conclusion

Phage display was first described in 1985 by presenting a peptide on the surface of filamentous phage M13. This technology was further developed and improved by Greg Winter and John McCafferty. Later, the first libraries of random peptide and also antibody fragments were made. During the past decade, various researches and commercial applications of phage display technology have shown its attractive potential for development of new drugs and vaccines, selective delivery of therapeutic agents and improved diagnosis tests. The ability of this system for designing drugs with desired characteristics using different strategies and also recent innovations in automation of the selection process suggests that this technology will continue to provide valuable therapeutic candidates for clinical development.

9. Expert opinion

A growing number of approved drugs and novel drug candidates established by phage display technology are currently being examined in clinical or preclinical trials, which demonstrate the value of this technology as a practical and reliable drug discovery platform. FDA-approved compounds including three antibodies (Adalimumab, Belimumab and Raxibacumab) and three peptides (Romiplostim, Ecallantide and Peginesatide) are the most advanced drugs developed using phage-displayed libraries. Beside, many others are also in clinical trials including ramucirumab, an antibody against VEGFR2, and AMG-386, an inhibitor of protein-protein interaction.

Most of the drug candidates explored via phage display libraries are mAb and antibody fragments that are frequently characterized by lower stability, inefficient tumor targeting and undesired uptake in healthy tissues compared with nanobodies and peptides.

The use of nanobodies as therapeutic agent in human diseases is a fast growing concept. There is a high homology between nanobodies and human VH domain. Until now, no immunogenicity in humans has been documented. New preclinical data are highlighting the important role of nanobodies as therapeutic compound, but their influence in the clinic is yet to be proved. It seems that additional preclinical experimental studies are necessary to confirm their clinically efficacy and safety.

Moreover, in spite of all the advantages of peptide therapeutics, there are still considerable challenges in using them *in vivo*. Common risks in this issue have been related to alteration of peptides while released from display format. This may decrease their binding affinity in soluble form. Sometimes peptides that have shown remarkable activity *in vitro* become completely dysfunctional *in vivo*, which could raise the possibility of aggregation followed by dangerous side-effects. Moreover, conjugation of displayed ligands to radiolabel or dye agents for imaging applications can change their pharmacokinetic profile and distort the required binding conformation of the ligands. There are additional problems of stability owing to proteolytic degradation of polypeptide drugs, their short circulation time and potential immunogenicity. Fortunately, advances in protein engineering methods can improve the pharmacodynamic properties of phage display-based compounds. A variety of techniques such as peptide cyclization, N- and C-termini blocking, use of fusion proteins, scaffold proteins and substitution of L-amino acids with D-amino acids, unnatural amino acids and chemically modified amino acids are used for enhancing peptide stability. To increase peptide half-life and reduce immunogenicity, conjugation to polyethylene glycol and polysialic acids would be useful. Furthermore, conducting secondary biased library screenings and peptide multimerization can reasonably improve the affinity of peptides. It is believed that using secondary synthetic peptide libraries provides further screening level through the ability to incorporate altered amino acids. Such combined screening approach could promisingly increase the success of experimental processes in identification of potential targets for developing novel drugs.

Even so, merging of phage display with nanotechnology not only seems to be helpful in overcoming some of the drawbacks, but also enriches the industrial aspects of this technology. Such intelligent fabrication is anticipated to facilitate the development of ligand-based chips and arrays that will lead to rapid determination of millions of target molecules simultaneously. Successful reports of phage-based biosensor models and efficient phage-mediated drug/gene delivery systems have brought hope to the research community that phage nanobiotechnology and phage nanomedicine will likely grow into a discipline with its own methods and strategies in the near future. Development of non-immunogenic phage with desired biological, physical and chemical properties that can be tailored using genetic engineering methods will be a new perplexing goal of phage nanobiotechnology, especially for theranostic applications and customizing patient-specific therapeutics.

In the authors' opinion, phage display technology will remain as a steady platform in future years providing more and more useful therapeutic candidates for clinical development as new disease markers are discovered and applications of phage display systems are extended. Because the peptide-based therapeutics mostly target cell surface receptors and proteins, recently, the cell-penetrating peptides and protein transduction domains have gained interests for screening and developing intracellular peptide therapeutics and even organelle-specific drugs by using phage display technology. In this regard, a new class of internalizing phage vectors has been developed for ligand-directed targeting of organelles and recognizing molecular pathways within mammalian cells. This unique technology is appropriate for applications ranging from fundamental basic science studies to novel drug development. In addition, advances in phage display biopanning and high-throughput screening of combinatorial chemical libraries on the surface of phage offer a powerful, rapid and economical tool to identify targeted molecules in different fields of science. The identification of appropriate disease targets and validating them are presently considered the main challenges in development of biopharmaceuticals, which will be attractive subjects in phage display researches.

We envision that in the years to come, phage-derived technologies will bring many more contributions to clinic and market. Widespread application of this methodology has great potential in different fields of therapeutics, diagnostics, industrial processes, nanotechnology and in other areas.

Declaration of interest

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