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Teaser
Available for near three decades, has the full potential of phage display been realized in peptide drug discovery?

Phage display as a technology delivering on the promise of peptide drug discovery

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Phage display represents an important approach in the development pipeline for producing peptides and peptidomimetics therapeutics. Using randomly generated DNA sequences and molecular biology techniques, large diverse peptide libraries can be displayed on the phage surface. The phage library can be incubated with a target of interest and the phage which bind can be isolated and sequenced to reveal the displayed peptides' primary structure. In this review, we focus on the 'mechanics' of the phage display process, whilst highlighting many diverse and subtle ways it has been used to further the drug-development process, including the potential for the phage particle itself to be used as a drug carrier targeted to a particular pathogen or cell type in the body.

Introduction

From a historical point of view, drug discovery can be divided into three periods; (i) before, (ii) during and (iii) after the twentieth century. Almost all of the drug discoveries before the twentieth century relied on serendipity. However, as the result of pronounced advances in the different disciplines involved, drug discovery quickly became a more rational process. Among the important technologies available to contribute to this by the end of the twentieth century were the determination of the molecular structures of drugs using a variety of instrumentation, molecular modeling, combinatorial chemistry, high-throughput screening and advanced molecular biology methods. The most recent era of drug discovery is marked by the increase in biopharmaceuticals, backed by the introduction and acceleration of omics technologies. In this context, phage display technology, a combinatorial biology technique introduced by G.P. Smith in 1985 [1–4], is likely

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1359-6446/06/$ - see front matter http://dx.doi.org/10.1016/j.drudis.2013.09.001

GLOSSARY

Capsid The protein coat that surrounds the phage genome in a phage particle.
Helper phage A phage that is introduced into a host cell in conjunction with a related cloning vector in order to provide enzymes required for replication of the cloning vector.
Library A population of clones with each clone containing one random piece of chromsomal DNA cloned into a vector.
Ligand Any molecule that binds to another; usually a soluble molecule such as a hormone or neurotransmitter that binds to a protein receptor.
Peptidomimetic A compound containing non-peptidic structural elements that is capable of mimicking or antagonizing the biological action(s) of a natural parent peptide.
Phage A virus that infects bacterial hosts and may be utilized to introduce genes. Phage are widely used as cloning and expression vectors.
Phage display A technique that fuses peptides or proteins to capsid proteins on the phage surface. Libraries of phage-displayed peptides may be screened for binding to specific ligands; determination of the gene sequence of the selected phage is used to identify the peptide/protein sequence.
Phagemid A type of plasmid which carries within its sequence a bacteriophage replication origin. When the host bacterium is infected with helper phage, the phagemid is replicated along with the phage DNA and packaged into phage capsids.

to play an increasingly more important role in the future of drug discovery. The concept is simple: a population of phage is engineered to express random-sequence peptides, proteins or antibodies on their surface. From this population, a selection is made of those phage that bind the desired target. In order to effect this presentation (or display), randomized cDNA sequences are inserted into the genome of the phage, such that they will be expressed as a fusion protein with one of the coat proteins of phage [2,4–6]. Proteins/peptides with a wide range of sizes and properties have been successfully displayed by filamentous phage such as alkaline phosphatase (60 kDa) [7], mustard trypsin inhibitor (7 kDa) [8], Src homology 3 (SH3) (6.5 kDa) [9] and cytochrome b562 (11 kDa) [10].

Phage display technology is a powerful tool in drug discovery, particularly for the identification of ligands with novel functions [11–25]. However, its application covers very diverse areas such as nanostructured electronics [26,27], agriculture [28], medical diagnosis [29] and neurobiology [30], just to mention a few. The success of phage display can be credited to the fact that highly diverse libraries can be constructed followed by rapid isolation and identification of specific proteinaceous ligands for numerous types of macromolecular targets [6,31]. In its relatively short existence, the phage display approach has been used to create libraries of random peptides and proteins for the purpose of identifying ligands for receptors, identifying enzyme blockers, studying protein/DNA–protein interactions, screening cDNA expression, epitope mapping of antibodies, engineering human antibodies, optimizing antibody specificities, identifying peptides that home to specific organs or tissues, generating immunogens for vaccine design, and for use in affinity chromatography [2,3,32–36]. Libraries have also been used to identify peptide/protein binders to small molecules such as explosive dinitrotoluene derivatives [37], prostaglandin E2 [38], 15-ketocholestane [39], and taxol [40].

Phage display has several advantages over traditional random screening methods used in drug discovery such as simplicity, cost effectiveness, and speed. But the major strength of this technique is in generating the enormously diverse exogenous peptides or proteins displayed on the surface of the phage using standard yet rapid molecular biology methods as opposed to using genetically engineered protein or peptide variants individually. Libraries can be screened rapidly for binding to a target and the 'selectants' eventually identified through DNA sequencing [2,31,41].

Once identified through phage display, selected ligands can be analyzed structurally to provide more detailed understanding of the ligand–target interaction [42]. This additional information is useful when proceeding to the next stages of drug discovery and pipeline development. For example, Feng et al. investigated the molecular basis for the affinity and selectivity of phage display-derived SH3 domain binding ligands using 2D-NMR spectroscopy [43]. The structural results were used to develop a general model for SH3–ligand interactions applicable to further drug design.

Filamentous bacteriophage biology

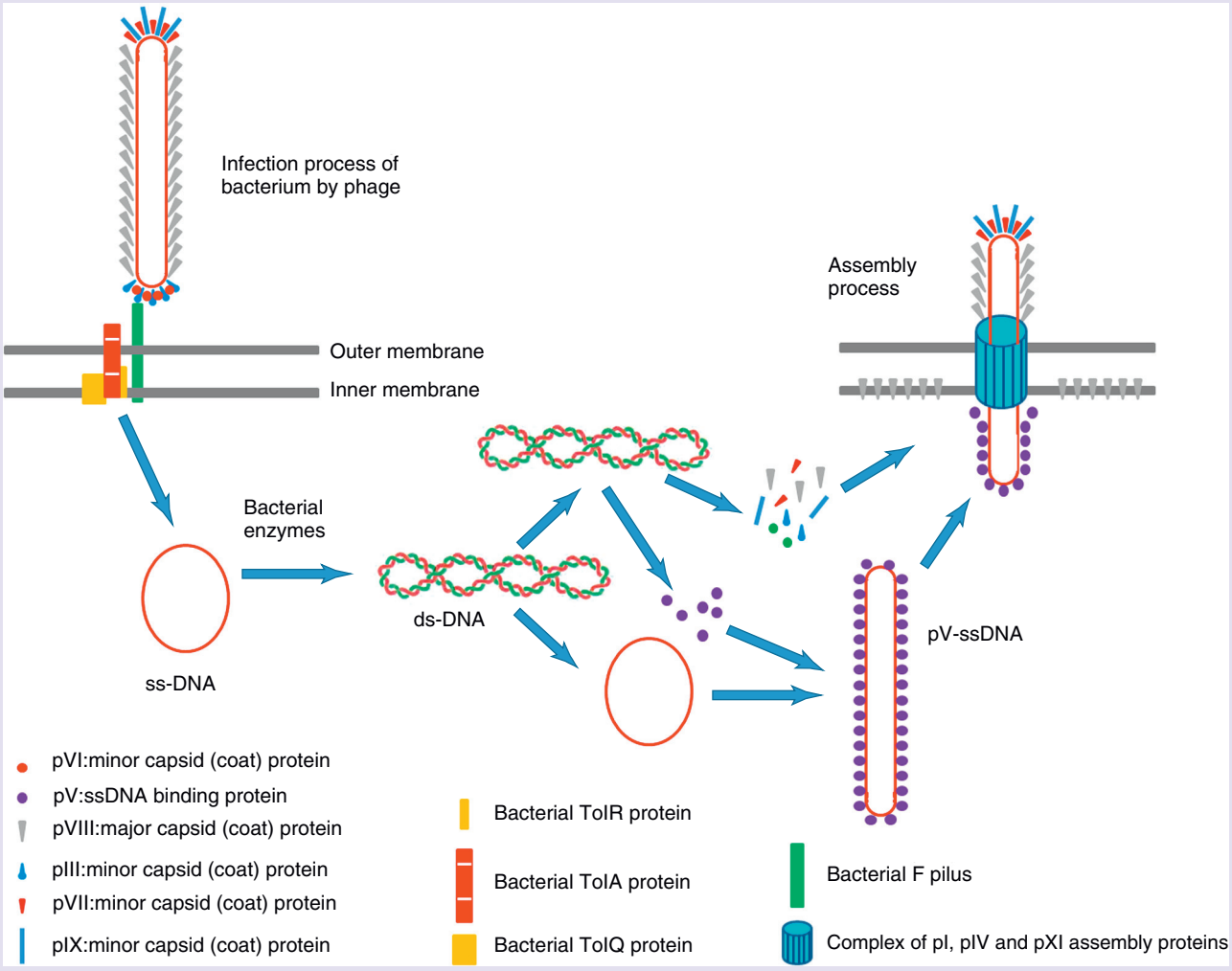
Filamentous bacteriophage (also commonly referred to as phage) is a group of viruses that infects F plasmid-containing gram-negative bacteria, such as *Escherichia coli* cells. They include members of the *Inoviridae* family, of the genus *Inovirus*, such as, phage M13, f1 and fd [44]. Generally, filamentous phage is not lytic and so strains of phage-infected *E. coli* can release new phage particles without bacterial lysis. Other types of virus-like systems used in phage display are the phagemid, which are plasmids containing an f1 origin of replication from a phage to enable their single-stranded replication and packaging into phage particles, as well as an origin of replication (ori) for double-stranded replication. Phagemid can be considered as cloning vectors but need helper phage for completing their infection process by providing the structural and functional proteins necessary for packaging phagemid into virion particles [45].

M13 and other strains of Ff phage contain circular single-stranded DNA (ss-DNA) with 98% identity across different strains. The ss-DNA is enclosed in a protein coat with the entire particle being ~6.5 nm (diameter) × 930 nm (length) (Box 1). The genome consists of 11 genes (Table 1) [3,33,36,46,47]. These genes are grouped according to function: (i) Capsid proteins comprise protein III (pIII), pVI, pVII, pVIII and pIX, (ii) DNA replication proteins consist of pII, pV and pX, and (iii) the assembly proteins pI, pIV and pXI.

The most important coat proteins for the display of exogenous proteins and peptides on the surface of the phage are pIII (406 residues) and pVIII (50 residues), known as the minor and major coat proteins, respectively, with the former being the most exploited coat protein for display. That the coat proteins are on the exterior of the phage literally does mean they are on display. Lack of surface accessibility of some of the other coat proteins in the context of intact phage particle may account for their reduced suitability for efficient display. For example, using sera directed against the minor proteins, Endemann et al. showed that the minor coat protein, pIX, is accessible in intact phage but at least some parts of pVI and pVII are not [48]. Nevertheless, there are

BOX 1

Life cycle



Structure of bacteriophage and its life cycle. Infection begins by attachment of pIII N-terminus to the tip of F-pilus on the bacterium. The binding leads to injection of ss-DNA, (+) strand, of the phage into the bacterial cell. Host polymerase then uses the (+) strand as template to produce the complementary (−) strand resulting in a double stranded- (ds-) or replicative form (RF) of phage. Phage proteins are synthesized from mRNA generated from the (−) strand of RF DNA. For replication of the genome to produce new phage, newly synthesized protein pII nicks the RF DNA to initiate replication of the (+) strand. As a result, a pool of RF DNA molecules can be produced by host enzymes. pII also ligates the molecular ends of newly synthesized (+) strands to form ss-DNA. pV protein dimers bind this new ss-DNA to prevent conversion to RF DNA. The amount of pV determines the ratio of RF to (+) strand DNA synthesis. pX is involved in the replication and is believed to regulate RF/(+) strand DNA synthesis as well as inhibition of pII function. Assembly occurs at the inner membrane of the cell and involves pI, pIV and pXI. The C-termini of pI and pXI interact with pIV to form a channel to facilitate secretion of phage. pVII and pIX are required for the secretion step, and they interact with the pV-ss-DNA complex. During extrusion pV which is bound to ss-DNA is replaced by pVIII followed by the addition of pVI and pIII to the end of the proximal end of the releasing particle [2,3,35,36,46,151]. About 1000 phage particles are produced during the first generation after infection and then bacterial cells produce approximately 100–200 phage per generation [152].

numerous reports of successful protein display using pVI, pVII and IX coat proteins [49,50]. Low efficiency of display using coat proteins other than pIII and pVIII may to some extent be related to the adverse effects of the fused peptides/proteins on the coat proteins during phage assembly.

An individual phage particle consists of 3–5 pIII proteins which form a knob-like structure at one end. These proteins are responsible not only for infection (via the F-pilus of the bacterium) but also for virion stabilization and assembly termination [35,36,46].

The displayed peptide or protein is presented at the N-terminus of pIII separated by a spacer from pIII's N-terminal residue (Fig. 1a). There is little restriction on the length of the insert so that peptides and proteins, though relatively large, can be accommodated.

About 2700 copies of pVIII are present on the coat of phage and are packed quite tightly [46]. pVIII has an α-helical architecture with some deviations from ideality, such as gentle kinking [51] or curvature [52]. The helical axis of pVIII is tilted about 20° relative

TABLE 1

Genes and gene products of fl bacteriophage

Gene	Function	No. of amino acids	Protein size (Da/mol)
I	Virion assembly	348	39 502
II	DNA replication	410	46 137
III	Minor capsid protein	406	45 522
IV	Virion assembly	405	43 476
V	Binding ssDNA	87	9682
VI	Minor capsid protein	112	12 342
VII	Minor capsid protein	33	3599
VIII	Major capsid protein	50	5235
IX	Minor capsid protein	32	3650
X	DNA replication	111	12 672
XI	Virion assembly	108	12 424

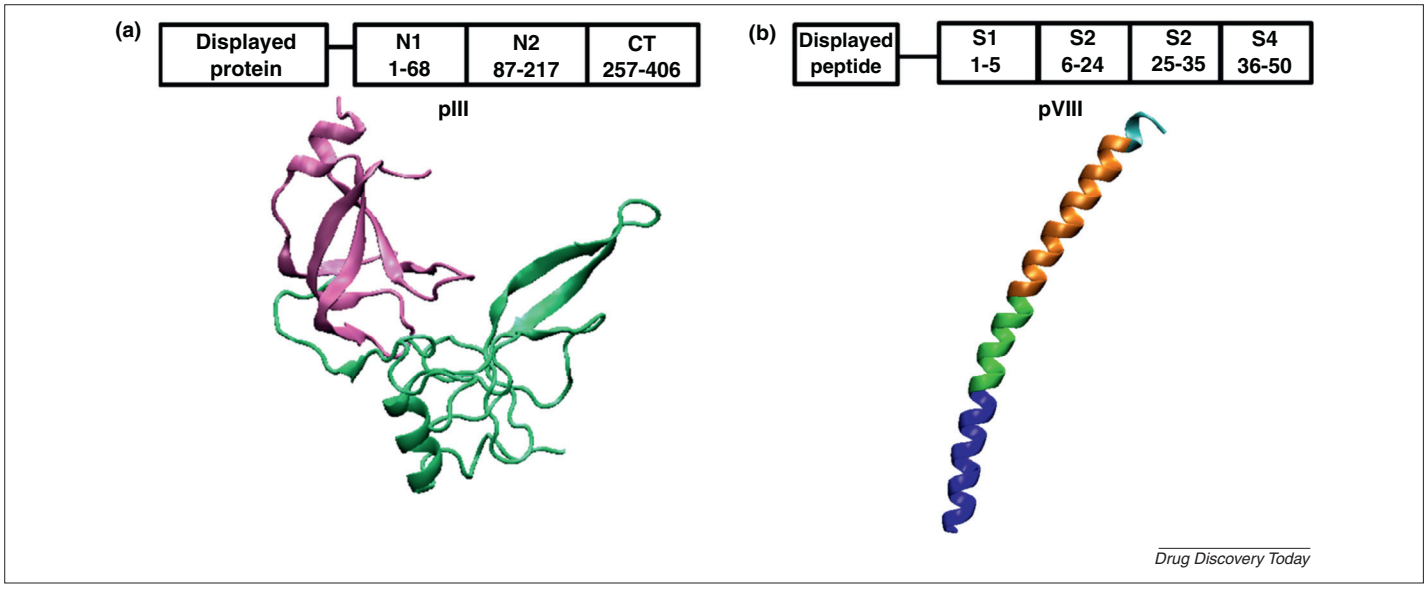


FIGURE 1

Structural and domain descriptions for pIII and pVIII proteins. Schematic diagrams at the top of panels a and b show the domains for pIII and pVIII, respectively. Each domain is represented by a rectangle with the domain name and residue numbers inside. In both pIII and pVIII the displayed protein/peptide is linked to the N-terminus via a spacer shown by a horizontal line. The 3D structure shown for pIII protein (PDB ID 1G3P) consists of only N1 and N2 domains colored in magenta and green, respectively [153]. The model of pVIII refined to fit X-ray fibre diffraction data (PDB ID 1IFI) shows distinct regions: surface segment (S1, cyan), amphipatic mainly α-helical region (S2, orange), hydrophobic helix (S3, green), and amphipatic helix extending to the C-terminal end (S4, blue) [154].

to the main axis of the phage particle [53]. The sequence of pVIII can be divided into four regions (S1–4) (Fig. 1b): The amino acid sequence at the C-terminus of pVIII contains four positively charged lysine residues which interact with the phosphate groups from the backbone of the ss-DNA inside the phage. The N-terminus of pVIII is present on the outside of the particle with only the first three residues accessible for digestion by proteases. Unlike pIII, only short peptides (<10 residues) can be tolerated at the N-terminus of pVIII [5,47,54] for the insert to be successfully displayed on every copy.

It is not clear why large inserts cannot be tolerated in pVIII, but it has been suggested they affect the assembly process of phage. Another suggestion for the lack of tolerance for large inserts is their physical dimensions prevent new virus particles passing through the pIV exit pore of the outer membrane of the bacterium [46]. However, to circumvent this problem, it has been shown that if the wild-type pVIII protein is supplied (i.e. pVIII without any fusion protein displayed) along with fused pVIII, large proteins can be displayed [33,55]. Kang et al. demonstrated the successful display of Fab fragments on the surface of phage particles by fusing them to the major coat protein of a phagemid/helper phage system [56].

Another striking difference between the use of pIII and pVIII in the display is the avidity effect caused by the display valency. Generally, this leads to significant differences in the affinity of the proteins or peptides that can be selected either by pIII or pVIII display libraries to the same target. There are only 3–5 copies of pIII per phage particle and this can be an advantage compared with pVIII libraries since avidity is reduced. As a result, relatively high

TABLE 2

Classification of most of the common phage display vectors

Display type	Coat protein used for display	Display on all or some copies of coat protein	# of coat protein genes	Fusion encoded in phage or phagemid genome	Examples of vectors
Type 3	pIII	All	1	Phage	M13KE
Type 8	pVIII	All	1	Phage	M13KE
Type 33	pIII	Some	2	Phage	M13KE
Type 88	pVIII	Some	2	Phage	f88-4
Type 3 + 3	pIII	Some	2	Phagemid	pComb3
Type 8 + 8	pVIII	Some	2	Phagemid	pComb3

affinity peptides and proteins can be isolated with dissociation constants (K_d) of 1–10 μM, whereas pVIII-fused peptides tend to have lower affinity (K_d of 10–100 μM) because of the higher number of the displayed peptides [31,54,57]. However, peptide and protein ligands with higher affinities, in the range of nanomolar and low picomolar, have also been reported using phage display of antibodies (such as those selected for binding the tumor antigen c-erbB-2 and murine vascular endothelial growth factor [58,59]).

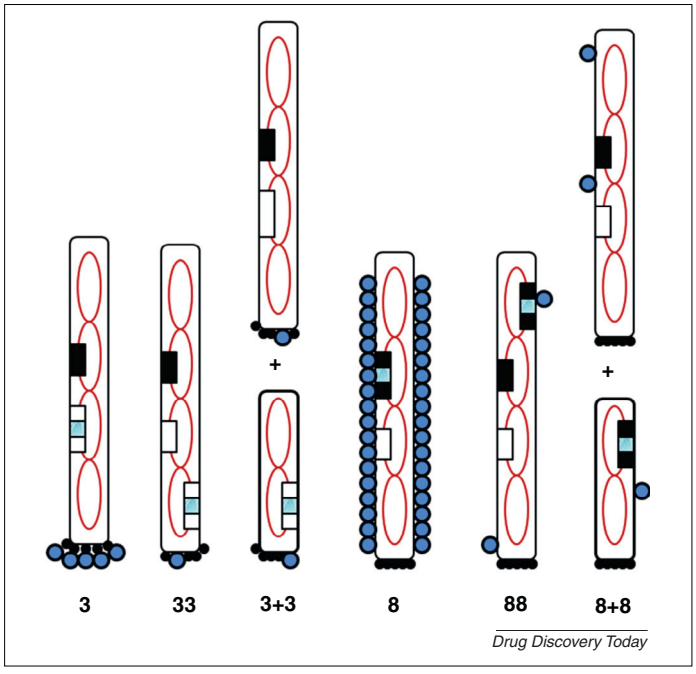


FIGURE 2

Schematic presentation of different types of phage display vector systems. The names for the systems are shown at the bottom. See text and Table 2 for more information including the nomenclature. Where two large rounded rectangles are shown the longer refers to helper phage and the shorter to phagemid. The twisted red line in each rectangle represents ss-DNA. The small black and white boxes show the location of the pVIII and pIII genes in the ss-DNA, respectively. The insert coding DNA for foreign peptide or protein is represented by the light blue box. The dark blue circles on the surface of particles represent the displayed peptide or protein. The pIII proteins are represented as black circles, while other coat proteins are not shown for the clarity.
Figure is adapted from [2].

Vectors and modes of display

The most common bacteriophage used in phage display are the filamentous phage including M13, f1 and fd; although T4, T7, and λ phage have also been used [34]. Vectors used in phage display can be classified according to the following parameters:

- The type of coat protein used for display (pIII or pVIII).
- The displayed protein or peptide fused to all copies of pIII or pVIII or a fraction of them.
- Whether the insert is encoded by the phage genome or another genome such as phagemid.

Table 2 shows a classification of vectors commonly employed in phage display and a schematic representation of the different types of display is shown in Fig. 2. For example, with the type 3 vector (Fig. 2), the insert encoded by the pIII gene results in the display of the foreign protein/peptide in all pIII copies. Similarly, the type 8 vector results in the display of peptide in each of the expressed pVIII molecules. In type 33, the phage genome bears two types of pIII molecule; one is recombinant and the other is wild type. As a result, only some of the expressed pIII proteins are fused with foreign peptide or protein. Type 3 + 3 differs from 33 in that two copies of the pIII gene are present but are on the separate systems: that is, the wild type version is on the phage (called helper phage), whereas the recombinant form is located on the phagemid genome (a plasmid carrying the filamentous phage intergenic region, a replication origin and antibiotic resistance gene). Types 88 and 8 + 8 are the same as 33 and 3 + 3, respectively, but with pVIII used for display [2,33,46,60].

Construction of libraries

Library construction is the starting point in the process of selecting and isolating the ligand(s) for the target of interest. Depending on the specific aims of the study to be undertaken, two types of libraries are extensively used – peptide libraries and antibody libraries.

Peptide libraries

Each of the 20 natural amino acids is encoded by codons. A random peptide library can be constructed using degenerate oligonucleotides introduced into the phage genome. One of the most common strategies to generate random peptides is to use $(NNK)_n$ codon degeneracy, where N is an equimolar mixture of all four nucleotides (adenine, guanine, cytosine and thymine) and K is a 1:1 mixture of guanine and thymine. By using $(NNK)_n$ codons instead of $(NNN)_n$ codons, the number of stop codons is reduced from three types (TAA, TGA and TAG) to one (TAG, Amber stop codon) [36,61,62].

Peptide libraries can be generated with lengths of the displayed peptides varying from 6 to 30 residues. Strategies can be used to present peptides in a more constrained conformation; for example, by including two cysteine residues in order to make a disulfide bond [31,36]. It is usually difficult to predict the optimum length required for the randomized displayed peptides as this depends on a number of factors including the folding properties of the displayed peptide, the characteristics of the target, and the purpose of investigation [31].

The construction of the library is a key step because the probability of being able to select ligands that bind the target is highly dependent on library diversity and sequence length. For a peptide library employing $(NNK)_n$ codons, each *NNK* is a mixture of the 32 different possible codon sequences that encode for all 20 amino acids (plus one stop codon). For such a library, the number of possible *n*-mer peptide sequences is given by 20^n, where 20 is the number of standard amino acids and *n* is the number of randomized positions. For example, for a peptide library with seven randomized positions, there are 20^7 (1.3×10^9) possible heptamers. However, these considerations represent an oversimplification of reality and lead to an overinflated estimate due to a number of factors such as degeneration in the amino acid code leading to random occurrences of the termination codons as well as transformation efficiency. The maximum concentration of phage particles is $\sim 10^{14}$ particles/mL (~170 nM), which sets the upper limit for diversity. Transformation efficiency allows only 10^8–10^{10} phage constructs to be transformed into *E. coli* by electroporation or other techniques [2,5,62,63]. Typically, the diversity of commercially available libraries is $\sim 10^9$ [64].

Antibody libraries

The antibody molecule comprises heavy (H) and light (L) chains, which both include variable (V) and constant (C) domains (Fig. 3). The antigen-binding fragment, Fab, and single-chain fragment variable (scFv) (Fig. 3) can be displayed on the surface of phage. This approach can be used to identify antibodies which recognize a specific target.

An scFv is an engineered component of an antibody which consists only of the variable regions of the heavy (V_H) and light chains (V_L) connected by a short flexible glycine-rich linker peptide of 10 to 25 amino acids; for example, in the form $(GGGGS/T)_3$ [65]. The linker can either connect the N-terminus of the V_H with the C-terminus of the V_L, or vice versa [66]. The purpose of serine or threonine in the linker is to increase solubility.

In the construction of antibody libraries, the initial decision is whether to construct a library based on scFv or Fab fragments as each has advantages and disadvantages. In the case of scFv libraries, construction can be achieved simply by overlap extension PCR, as explained in more detail by Andris-Widhopf and colleagues [67]. A similar method can be used for Fab fragment library construction [68,69].

The other advantage of such libraries is that the scFv molecules can be engineered in multivalent forms and as a consequence the avidity toward the target can be increased.

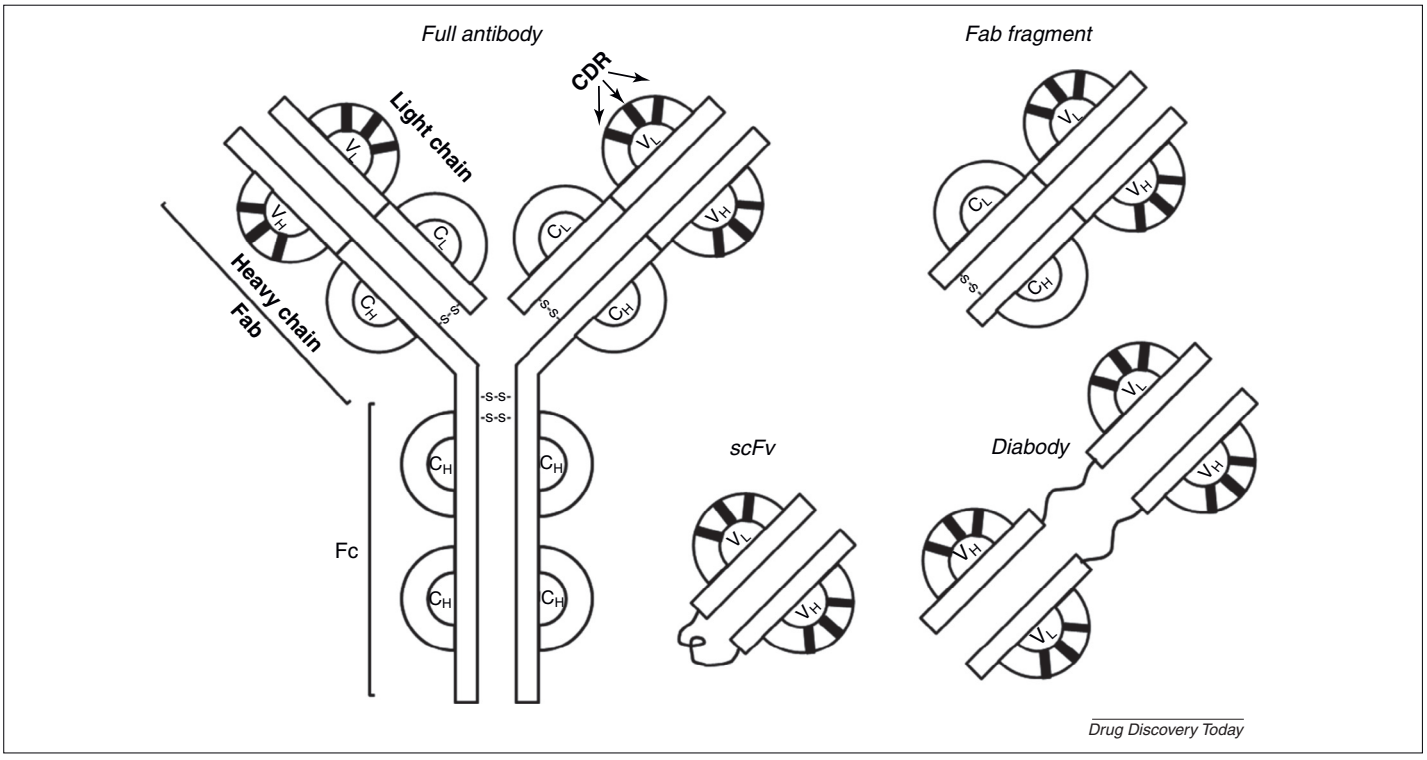


FIGURE 3

Schematic representation of full antibody (IgG), antibody fragments (Fab and scFv), diabody formed by dimerization of scFv. C_H segments are the constant domains and V_H and V_L denote the variable domains of the heavy (H) and light (L) chains, respectively. Hyper variable CDR regions (complementarity determining regions) are shown in black and are pointed to by arrows in the full antibody molecule. V_H and V_L are linked via linker sequence (shown by curly solid line) to generate scFv molecules, which can self associate to form diabody or triabody (not shown) polyvalent complexes. Fab fragment and scFv are monovalent, while full antibody and diabody are divalent species.

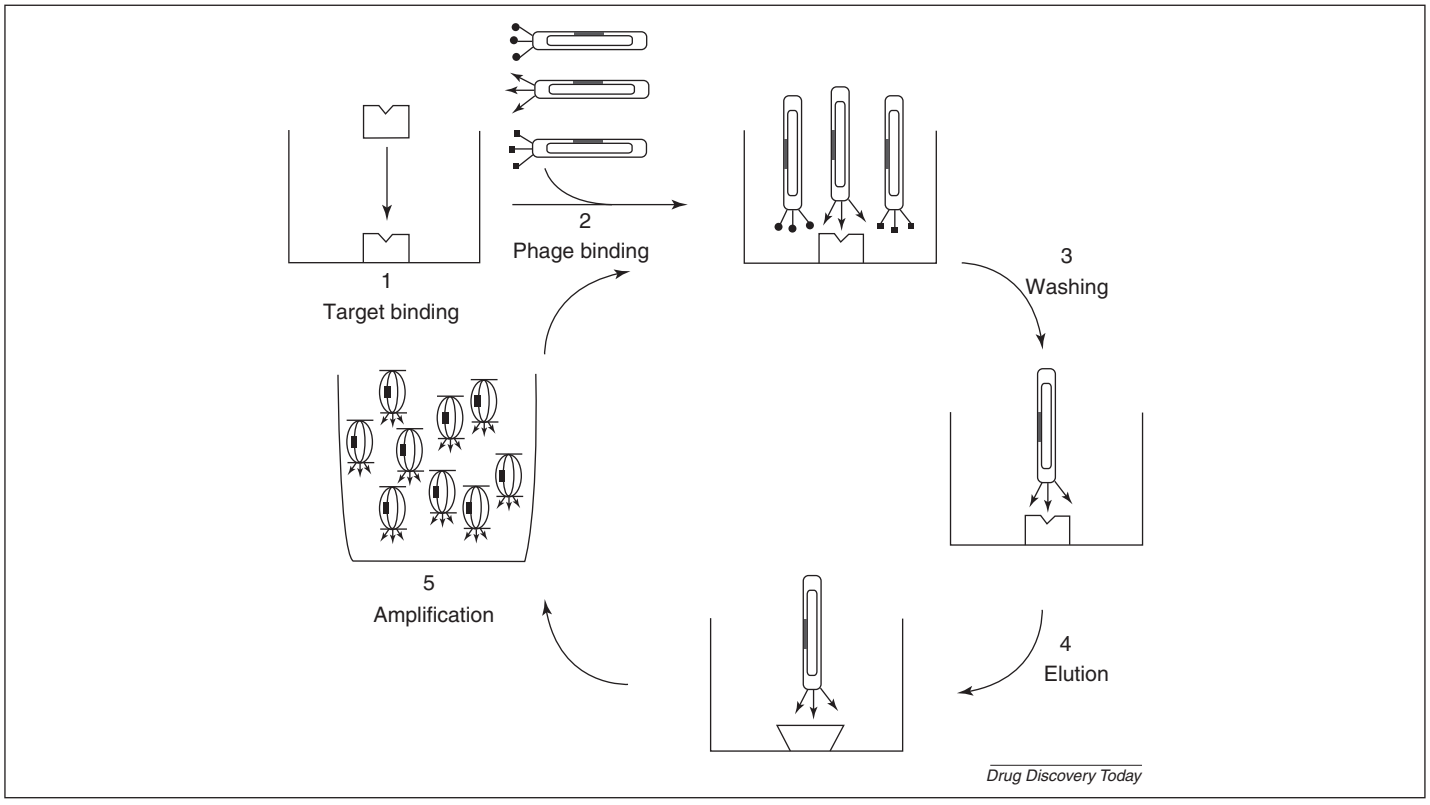


FIGURE 4

Biopanning. The process for affinity selection of phage-displayed peptide or protein: Step 1, target is immobilized. Step 2, phage library is added. Step 3, washing to remove unbound phage. Step 4, elution of bound phage as the result of conformational changes to the binding site caused by pH change or other means which disrupts the interaction between displayed ligand and the target. Step 5, amplification of eluted phage for next round of biopanning.
Figure is adapted from [32].

One of the advantages of using Fab libraries compared to scFv libraries is that the folded structure of Fab fragments tends to be more thermodynamically and kinetically stable [70]. In addition, Fab fragments generally do not multimerize nearly to the same extent as scFvs and so are more likely to provide information on affinity rather than avidity. The main disadvantage of Fab libraries is the generally lower expression levels in *E. coli* compared to the smaller scFv molecules [3,71].

Selection and screening methods

In vitro *screening*

Biopanning is the most common *in vitro* screening method for identifying and isolating ligands that bind the target of interest (Fig. 4) [2,4,32,72]. Biopanning involves the following steps: (i) target immobilization: the purified target of interest is immobilized on plates. Alternatively, selection can be performed on adherent cultured cells [73,74], or even cells in suspension culture, which contain the desired target such as a cell-surface receptor. (ii) Phage binding: the phage library is added and allowed to bind to the target in conditions suitable for binding. (iii) Washing: the unbound phage are removed. (iv) Phage elution: due to the high stability of filamentous phage, a wide variety of methods can be applied to elute the bound phage. Common methods for recovering bound phage are disruption of the interaction between the displayed ligand and the target by changing the pH or adding a competing ligand, denaturant or protease (e.g. because a protease-cleavage site has been engineered between the displayed protein/peptide at the N-terminus and the coat protein itself). (v) Increasing stringency: the eluted phage are then amplified in bacterial cells and biopanning repeated for several rounds (usually 3–5). This tends to select against phage with low affinity/non-specific binding to the target of interest. (vi) Identification of selectants using DNA sequencing.

In vivo *screening*

In vivo selection can be used to identify phage ligands capable of homing to a specific tissue or organ. For example, phage can be administered intravenously to an animal and allowed to circulate for a period of time. Phage are then recovered from the organ of choice, amplified, and the DNA sequenced. With this approach, 'nonspecific' phage tends to be distributed throughout the entire animal while phage with more 'selective' target ligands cluster in particular tissues. Phage-derived ligands specific for an organ or tissue potentially can be used as diagnostic tools or as a treatment for disease by conjugating phage to a drug or assembling phage on drug-containing nanoparticles [72,75–77].

Phage display provides leads for therapeutic drugs

Peptide phage display employing large libraries accompanied by high throughput screening has played an important role in the development of clinically useful peptides and peptidomimetics [78]. Once peptide ligands have been selected and identified from the phage library as outlined above, they generally need to be modified in order to be useful clinically. This is because, in part,

TABLE 3

Approved or under clinical development peptides or peptide-based therapeutics and diagnostic agents

Product	Manufacturer	Indication(s)	Phase
Humulin®	Lilly	Diabetes	Approved
Lupron®	Takeda Abbott Pharmaceuticals	Endometriosis, prostate cancer, precocious puberty	Approved
Zestril®, Prinivil® (lisinopril)	AstraZeneca, Merck	Hypertension, Congestive heart failure	Approved
Sarenin® (saralasin acetate)	Norwich-Eaton Pharms, Procter & Gamble	Hypertension	Approved
Stilamin® (somatostatin acetate)	Merck-Serono	Acute variceal bleeding	Approved
Zoladex® (goserelin)	AstraZeneca	Breast and prostate cancer, endometriosis	Approved
Sandostatin® (octreotide)	Novartis	Acromegaly, diarrhea	Approved
Miacalcin® (calcitonin)	Novartis	Hypercalcemia, osteoporosis, pagets disease	Approved
Integrillin® (eptifibatide)	Millenium Pharms, GSK	Angina, myocardial infarction	Approved
Natrecor® (nesiritide)	Scios	Congestive heart failure	Approved
Angiomax® (bivalirudin)	Medicines Company	Angina	Approved
Fuzeon® (enfuvirtide)	Roche, Trimeris	AIDS	Approved
Byetta® (exenatide)	Amylin Pharms, Eli Lilly	Type 2 diabetes	Approved
Kalbitor® (Ecallantide)*	Dyax	Acute hereditary angioderma	Approved
Vasotec® (enalapril maleate)	Merck Sharp & Dohme	Hypertension	Approved
Victoza® (liraglutide)	Novo Nordisk	Type 2 diabetes	Approved
Cibcalcin® (human calcitonin)	Novartis Pharma	postmenopausal osteoporosis, Paget's diseases, hypercalcaemia	Approved
Firazyr® (icatibant acetate)	Jerini AG	Hereditary angioedema	Approved
Prialt® (ziconotide acetate)	Elan Pharms	Severe chronic pain	Approved
Bigonist® (buserelin)	Sanofi-Aventis	Advanced prostate cancer	Approved
Synarel® (nafarelin acetate)	Pfizer	Central precocious puberty, endometriosis	Approved
Cetrotide® (cetrorelix acetate)	AEterna Zentaris, Merck-Serono	Inhibition of premature LH surges	Approved
Firmagon® (degarelix acetate)	Ferring Pharms	Advanced prostate cancer	Approved
Antocin® (atosiban acetate)	Ferring Pharms	Delaying the birth in case of threat of premature birth	Approved
Duratocin® (carbetocin acetate)	Ferring Pharms	Prevention of uterine atony	Approved
Syntocinon® (oxytocin)	Novartis Pharma	Initiation or improvement of uterine contractions	Approved
Somatuline Depot® (ianreotide acetate)	Beaufour Ipsen Pharma, Tercica, Globopharm	Acromegaly	Approved
Octastatin®, Sanvar® (vapreotide acetate)	Debiopharm, H3 Pharma	BOV	Approved
Rhinaaxia® (spaglumat magnesium)	Laboratoire Thea	Allergic rhinitis and conjunctivitis	Approved
Agifutol® (glutathione)	Prothera	Hepatic insufficiency, wound healing and asthenia	Approved
Velcade® (bortezomib)	Millennium Pharms, Janssen-Cilag	Multiple myeloma	Approved
Zadaxin® (thymalfasin)	SciClone Pharms International	Chronic hepatitis B and C	Approved
Diapid® (lypressin)	Sandoz-Novartis Pharma	Cushing's syndrome, central diabetes insipidus	Approved
Nplate® (Romiplostim)	Amgen	Idiopathic (immune) thrombocytopenic purpura	Approved
Hematide® (Peginesatide)*	Affymax, Takeda	Chronic kidney disease associated anemia	III
DX-890*	Dyax/Debiopharm	Cystic fibrosis, chronic obstructive pulmonary disease	II
Xerecept® (corticorelin acetate)	Celtic Pharma	Peritumoral brain edema	III
Onalta® (edotreotide)	Molecular Insight Pharms	Neuroendocrine tumors	II
Exubra®	Inhale, Pfizer, Aventis Pharmaceuticals	Diabetes type I and II	III
Gattex® (teduglutide)	NPS Pharms, Nycomed	Short bowel syndrome	III
AMG 386*	Amgen	Anti-angiogenic (oncology)	III
Thymogen® (oglufanide disodium)	Altika, Cytran, Implicit Bioscience	Immune system related diseases	II

TABLE 3 (*Continued*)

Product	Manufacturer	Indication(s)	Phase
Oralin® (insulin)	Generex	Diabetes type I and II	II/III
Apan®	Praecis	Alzheimer's disease	I
Exendin-4 (glucagon-like peptide)	Amylin	Diabetes type II	II
Iseganan	IntraBiotics	Pneumonia	II/III
Oral insulin	NOBEX, GlaxoSmithline	Diabetes type I and II	II
AMG 819*	Amgen	pain	Terminated-phase I
Oratonin®	NOBEX	Osteoporosis	I
HER-2/neu vaccine	Corixa	Breast and ovarian cancer	I
CNTO 530/CNTO 528*	Ortho Biotech	Chronic kidney disease associated anemia	I
Reptavlon® (pentagastrin)	Cambridge Labs, Wyeth-Ayerst Labs	Diagnosis of the gastric secretion	–
Thypinone® (protirelin)	Abbott	Diagnostic assessment of thyroid function	–
OctreoScan® (pentetreotide)	Mallinckrodt, Bristol-Myers Squibb	Diagnosis of primary and metastatic neuroendocrine tumors	–
Kinevac® (sincalide)	Bracco Diagnostics	Diagnosis of the functional state of the gallbladder and pancreas	–
Cortrosyn® (cosyntropin)	Amphastar Pharms, Sandoz-Novartis Pharma	Diagnosis of adrenocortical insufficiency	–
ChiRhoStim® (secretin)	ChiRhoClin	Diagnosis of pancreatic exocrine dysfunction	–
NeoTect™ (depreotide trifluoroacetate)	Amersham Health, Nycomed imaging	Diagnosis of lung tumors	–

For more comprehensive information see [86,88,90,92,95,96,101,149,150].

despite the advantage they may have over intact proteins, they still often suffer from poor pharmacokinetic properties, including a generally short half-life in the body (especially after oral administration), rapid enzymatic degradation, poor penetration through the intestinal membrane, and rapid excretion – all of which contribute to low bioavailability [79,80]. Nevertheless, peptides constitute a key class of biologically active molecules including hormones, neurotransmitters, cytokines, antigens and growth factors involved in peptide–protein non-covalent interactions and hence help to control a wide variety of biological processes and biochemical pathways [81,82]. Consequently, peptide-based therapeutics have attracted a significant level of interest in drug discovery and development projects [79,83].

Peptides are now used widely as therapeutic drugs and diagnostics (Table 3) in clinical applications such as endocrinology, oncology, urology, and obstetrics [84]. The current annual market of peptide drugs is $300–500 million with an annual growth rate of 25% [85]. Peptides often have numerous advantages over proteins, including antibodies, with respect to manufacturing costs, activity, stability, immunogenicity, efficiency of organ penetration, and patenting issues [86–88].

Several peptide drugs developed using phage display have been approved for use in the clinic or are in clinical trials (asterisks in Table 3). For example, ecallantide, a highly potent inhibitor of human plasma kallikrein, has been approved by the US Food and Drug Administration for the treatment of acute hereditary angioedema (HAE) [16–20]. DX-890, an inhibitor of human neutrophil elastase (HNE), with potential application in the treatment of pulmonary diseases such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), is another example of a therapeutic under clinical studies which had its origins in phage display [18,24].

The first marketed peptibody, Nplate® (Romiplostim, AMG 531), is an agonist of the thrombopoietin receptor used for the treatment of immune thrombocytopenic purpura. The peptide component of this peptibody has undergone substantial development, but owes its origin to a phage-displayed peptide library [89–92]. AMG 386, a peptibody neutralizing angiopoietins 1 and 2, is another example in which the active peptide was initially derived by screening of a peptide-displaying phage library [93,94]. It is utilized for prevention of endothelial cell proliferation and tumor growth and currently is in phase III clinical trail [92]. Nerve growth factor neutralizing peptibody known as AMG 819 is also in the aforementioned category in the area of analgesia and currently is under investigation in phase I clinical trials [92].

More recently, two novel peptides that bind epidermal growth factor receptor (EGFR) were identified [73], each capable of inhibiting the EGF-induced phosphorylation of EGFR in a concentration-dependent manner. Such ligands can be used for designing peptidomimetic anti-EGFR agents or targeting cancer drugs to EGFR over-expressing tissues in cancers with epithelial origin by making peptide–drug conjugates.

CNTO 530 and CNTO 528 are erythropoietin receptor agonists identified from peptide libraries which are in phase I clinical trial in management of chronic kidney disease associated anemia [95–97].

Peginesatide (Hematide)®, also a novel synthetic dimeric PEGylated erythropoietin-mimicking peptide, was designed to bind and activate the erythropoietin (EPO) receptor in order to stimulate erythropoiesis in the treatment of anemia associated with chronic kidney disease. The sequence of peginesatide was originally

obtained by phage display [98] followed by modification in sequence [99,100] and has now progressed to phase III trials [96,21–23]. PEGylation is often used in the generation of peptidomimetics because it generally improves bioavailability from the gut, increases plasma half life, decreases immunogenicity, reduces proteolysis and enhances solubility and stability [101,102]. Apart from its common use to improve the biomedical efficacy and physicochemical properties of therapeutic proteins, PEGylation has its own limitations and pitfalls, such as variations in the degree of PEGylation leading to polydispersity of the PEGylated product and separation and purification issues [103,104].

Other strategies are also frequently employed to improve the pharmacokinetics of peptides. These include: (i) Adding a group such as a phosphate ester to the N-terminus of the peptide to improve binding by serum albumin or other serum proteins to extend half-life [86]. (ii) Cyclization to constrain conformation and increase stability [105,106]. (iii) D-Amino acid substitution or incorporation of unnatural amino acids to improve resistance to proteolysis [101,107]. (iv) Producing a pro-drug to protect the peptide from premature proteolysis [79].

Peptide and protein delivery technologies

Recent advances in pharmaceutical technology have enabled the delivery of peptides and proteins in different pharmaceutical dosage forms via parenteral, buccal, transdermal, rectal, sublingual, vaginal, pulmonary and nasal routes. For example, injectable depot delivery systems have been developed to increase the effect of peptides and proteins by using micro- and nanoparticulate systems. *In situ* depot-forming (ISDF) systems, implant systems, and crystallization are other means of parenteral delivery techniques [104]. Schoenhammer et al. (2010) used ISFD containing poly(D,L-lactide-co-glycolide) (PLGA) to prepare a sustained release formulation of pasireotide, a cyclohexapeptide somatostatin analogue engineered to bind to multiple somatostatin receptor subtypes currently in phase III development for treatment of acromegaly, and then evaluated its functionality using *in vitro* and *in vivo* tests [108].

Microneedle delivery systems provide a means to overcome the stratum corneum barrier, gastrointestinal degradation, and liver first-pass metabolism by delivering macromolecules such as insulin, growth hormone and other proteins and peptides into the blood stream thereby increasing bioavailability [109]. However, oral delivery is still the patient-preferred route for delivering all types of drugs including peptide and protein drugs. New strategies are being used to enhance the oral absorption of peptides and proteins, evident in currently marketed peptide-based drugs. These strategies include use of absorption enhancers and enzyme inhibitors, preparation of encapsulated peptides and proteins in particulate delivery systems using polymeric and lipid particles, and mucoadhesive oral-drug delivery systems [110].

Phage as carriers in drug delivery

Phage particles themselves can also be used as the therapeutic agent. For example, the M13 bacteriophage was used successfully to treat a bacterial infection by delivering DNA encoding for bactericidal toxin proteins Gef (guanine nucleotide exchange factor) and ChpBK [111]. Phage delivery of the lethal agents reduced target bacterial numbers by several orders of magnitude *in vitro* and in a bacteremic mouse model of infection [111]. Antibacterial agents such as chloramphenicol can be targeted to pathogenic bacteria using phage which display a bacteria-specific binding peptide along with covalently bound chloramphenicol carried on the surface of the phage as a pro-drug [112–114]. Given that, for example, M13 phage has the capacity to cross the gastrointestinal mucosal barrier with [115] or without [116] a displayed peptide facilitating uptake, this provides the possibility of oral delivery of a drug payload targeted to a pathogen or specific cell type in the body (e.g. a cancer cell).

Filamentous phage has also been used as an immunogenic carrier useful in vaccine development [117–119]. The advantages include high immunogenicity, for example, because of the addition of foreign CD4 T-cell epitopes to pIII or pVIII, low production costs, and high stability due to the relatively low surface complexity of the phage [120]. For instance, f1 phage displaying the B2.1 peptide elicits stronger immune responses in mice compared to coupling the peptide to traditional carriers such as ovalbumin [118]. A phage clone displaying a 9-mer peptide which binds to the *zona pellucida* of the pre-implantation embryo has been shown to have contraceptive properties due to its ability to stimulate the production of anti-sperm antibodies. UV-inactivated (non-viable) phage can also be used to stimulate an immune response while eliminating problems associated with infectivity, therefore providing a safer alternative to live-phage vaccines [117].

Phage can also act as a gene-delivery vehicle. For example, phage can deliver functional genes (e.g. a eukaryotic reporter gene such as GFP) to mammalian cells through receptor-mediated endocytosis [121–123]. Furthermore, endocytic uptake can be targeted to particular cell types: phage displaying an scFv specific to the growth factor receptor ErbB2 results in receptor-mediated uptake only into mammalian cells expressing this receptor, and the infection can be followed by phage-encoded expression of GFP driven by the CMV promoter of a mammalian expression vector (e.g. pcDNA3) [124]. There has been some success in the isolation of phage with the ability to enter the bloodstream from the gastrointestinal tract and circulate in the blood for prolonged periods of time [125].

Related display methods

Finding high affinity peptides and proteins useful for diagnostic and therapeutic purposes can also be achieved by alternative display methods which have their roots in the original phage concept. These methods can be broadly categorized into either cell-surface or cell-free display systems. Bacteria [126,127], yeast [128,129], insect cells/baculovirus [130,131], and mammalian cells [132] are the common host organisms for cell-surface display platforms.

E. coli cells are the most common hosts used for bacterial surface display [133] and usually a membrane protein of *E. coli* is used as the anchor for the display. Due to the relatively high transformation efficiency of *E. coli*, the library size can be large (10^9–10^{11}). Other important properties are the large particle size of the bacterium and the multivalent surface display, which enable detection and subsequent analysis of binding to the target using flow cytometry. *E. coli* can be used to display peptides [134], antibodies and antibody fragments [135]. An important issue for bacterial display is that library sequences are inserted within the coding sequence of

the displaying protein (e.g. OmpX) rather than at the N-terminus, as is the case for phage. The use of circularly permuted OmpX, CPX, which allows library sequences to be displayed at a redefined N-terminus exposed at the surface of the bacterium has been shown to yield sequences with greater diversity, affinity and modularity for binding targets compared to OmpX itself [136]. Other bacteria such as Gram-positive *Staphylococcus* genus, including *S. aureus* and *S. carnosus* strains, have also been used for cell-surface display [137–139].

Compared to bacterial display [127,140], yeast surface display has fewer problems with regard to proper protein folding. ScFv (~25 kDa) and Fab (~50 kDa) antibody fragments, the extracellular domain of epidermal growth factor receptor (EGFR) (~69 kDa), human epidermal growth factor (hEGF) (~6 kDa) and cytokines (10–15 kDa) have all been successfully displayed on the surface of yeast cells. The most common strain for yeast surface display is *Saccharomyces cerevisiae* [129,141]. Although the yeast surface display system is a suitable tool for affinity maturation of antibody–antigen interactions, the high degree of glycosylation can be problematic through affecting the folding and function of the engineered proteins [140]. Insect cell/baculovirus display and mammalian cell display systems are relatively recently trialed display formats and have not been widely used for protein engineering so far. However, the use of these systems enables the display of proteins with complex folds and will more frequently allow the appropriate post-translational modifications to occur [140].

All of the display platforms mentioned above are cell-based methods and limited by the size of the library due to DNA transformation efficiency and any toxicity of the displayed molecules to the host cell. These problems can be overcome by using cell-free display systems which can support larger library sizes while eliminating the toxicity problem [128,142]. A range of cell-free methods have been developed including those using the ribosome, mRNA, and covalent DNA cell-free systems.

Ribosome display is an *in vitro* selection method used for displaying large libraries of proteins and peptides. In this system, proteins and their corresponding mRNA form a protein–ribosome–mRNA (PRM) complex [24,143]. To this end, a library of DNA constructs is generated. The library can be designed so that the constructs do not have a stop codon. The library is transcribed into mRNA *in vitro*, which in turn gets translated into the polypeptide. The newly synthesized polypeptide folds whilst part of the PRM. The PRM does not dissociate even with the addition of antibiotics such as chloramphenicol and cycloheximide to stop translation randomly. It can then be used to bind to immobilized target molecules [143,144]. The PRM complex thus provides the link between genotype and the phenotype of the displayed protein, which is selected using a panning procedure. The benefits of using ribosome display are (i) lack of a transformation step which makes it possible to generate a library with large diversity of 10^{12}–10^{14} members, (ii) elimination of the possibility of producing toxic proteins, and (iii) the possibility of displaying proteolytically sensitive and unstable proteins. Contamination with RNase and the intrinsic affinity of the ribosome or mRNA toward the target molecules compared to displayed peptides and proteins are two concerns for this method [145].

For the mRNA display method (as with ribosome display), the DNA library is constructed and then transcribed into mRNA *in vitro*. Protein–mRNA complexes are generated by *in vitro* translation of mRNA followed by linkage to displayed protein via a DNA–puromycin linker, which previously was added to the 3′ end of the transcribed mRNA. Because of the covalent link between protein and mRNA, the complex is more stable compared to the PRM and the library size is as large as that for the ribosome display [24,128,146]. The advances in cell-free display have lead to the covalent DNA display technique in which the displayed protein is linked to its cDNA through a covalent bond (see [145] and [24] for more information).

Concluding remarks

The discovery and development of a drug is a time-consuming, expensive and complex process and involves experts from a range of disciplines such as medicinal chemistry, biochemistry, molecular biology, medicine, and pharmacology. It has been estimated that from about 10 000 new chemical entities identified or synthesized as potential therapeutic agents, only one will reach the market in an average time of 16 years [147]. Prior to this century, drugs have been discovered either by identifying chemicals by trial-and-error or, not uncommonly, by serendipity. The advent of new technologies such as combinatorial chemistry and high-throughput screening has made it possible to prepare and assay rapidly large numbers of biologically active molecules [148]. Phage display, and particularly peptide phage display, has played a major role in the development pipeline for bringing peptide therapeutics into the clinic. The number of clinically useful peptides is expected to increase substantially as the new advances continue to take place in display methods.

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