REVIEW ARTICLE

Phage display and other peptide display technologies

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One sentence summary: Phage display and other peptide display technologies, which allow to expose peptides on various surfaces, have been successfully employed in numerous fields, from material sciences through diagnostics to the development of novel therapeutic approaches, while novel platforms are still being developed.

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ABSTRACT

Phage display technology, which is based on the presentation of peptide sequences on the surface of bacteriophage virions, was developed over 30 years ago. Improvements in phage display systems have allowed us to employ this method in numerous fields of biotechnology, as diverse as immunological and biomedical applications, the formation of novel materials and many others. The importance of phage display platforms was recognized by awarding the Nobel Prize in 2018 'for the phage display of peptides and antibodies'. In contrast to many review articles concerning specific applications of phage display systems published in recent years, we present an overview of this technology, including a comparison of various display systems, their advantages and disadvantages, and examples of applications in various fields of science, medicine and the broad sense of biotechnology. Other peptide display technologies, which employ bacterial, yeast and mammalian cells, as well as eukaryotic viruses and cell-free systems, are also discussed. These powerful methods are still being developed and improved; thus, novel sophisticated tools based on phage display and other peptide display systems are constantly emerging, and new opportunities to solve various scientific, medical and technological problems can be expected to become available in the near future.

Keywords: phage display; peptide display; bacteriophages; diagnostic methods; drug development; novel materials; display technologies

PHAGE DISPLAY TECHNOLOGY—HISTORY AND EVOLUTION

Since the discovery of bacteriophages over 100 years ago, they have been intensively exploited in basic and applied life sciences (Sulakvelidze, Alavidze and Morris 2001). Shortly after this discovery, a new trial was launched administering phages to patients with bacterial infections, but due to variable success rates and lack of knowledge about phage biology, this method has aroused much controversy (Hess and Jewell 2020). Furthermore, due to the spread of antibiotic therapy, most researchers

lost their interest in studying bacteriophages in the context of the phage therapy. With the arrival of genetic engineering during the late 1970s, phage-based vectors were among the early cloning vehicles (Maniatis *et al.* 1978; Hines and Ray 1980; Zacher *et al.* 1980).

In the late 1980s, George P. Smith, a biologist at the University of Missouri, presented a peptide junction to the outer protein of the phage coat, which enabled surface display. In 1985, he and his coworkers introduced the term 'phage display', which denotes the display/presentation of foreign molecules, usually peptides, on the surface of filamentous phages. The

original article (Smith 1985) described insertion of the DNA sequence encoding a 57-mer peptide into the filamentous phage gene III to develop a fusion protein with the external peptide sequence within coat protein III (pIII). The chimeric protein was presented 'in an immunologically accessible form' on the phage surface, which allowed preservation of the infectivity of this virus. In 1988, Parmley and Smith attained a noticeable enhancement of phage display technology by relocating the cloning site of external DNA sequences into another part of the gene coding for the pIII protein, which made it possible to obtain an entirely functional coat protein III (Parmley and Smith 1988). This upgrade was necessary for phage multiplication through 'the affinity purification of target phages' (termed 'biopanning') from a library of billions of clones, which significantly decreased the antibody demands in comparison to the primary procedure (Barderas and Benito-Peña 2019). In the same year, whole phage particles were used for the first time to induce an immune response against displayed external peptides (De La Cruz, Lal and McCutchan 1988). In the 1990s, Winter and coworkers achieved the display of human antibody fragments, such as single-chain variable fragments (scFv), on filamentous phages (McCafferty *et al.* 1990). Since then, phage display has become a molecular powerful tool for selection of peptide/antibody fragments with specific binding properties from a huge number of variants (libraries) by presenting such fragments on the phage surface, and therefore generating molecular probes against specific targets (Willats 2002). In acknowledgment of these advances, George P. Smith shared Nobel Prize with Sir Gregory P. Winter 'for the phage display of peptides and antibodies'. In fact, they revolutionized chemistry as well as biopharmaceutical development, and created the foundations for new antibody discoveries, epitope mapping and affinity selection (Barderas and Benito-Peña 2019; Hess and Jewell 2020). Since the establishment of phage display libraries, significantly more applications have been available and such libraries can include sequences encoding antibodies (Parhami-Seren, Viswanathan and Margolies 2002), containing cDNA (complementary DNA) (Crameri and Suter 1993), random oligonucleotides (Noren and Noren 2001) or even enzyme-coding DNA fragments (Brunet *et al.* 2002). Examples of proteins/peptides functionally displayed by filamentous phages include alkaline phosphatase (60 kDa), mustard trypsin inhibitor (7 kDa), Src homolog 3 (6.5 kDa) and cytochrome b562 (11 kDa) (McCafferty *et al.* 1990; Ku and Schultz 1995; Hiipakka, Poikonen and Saksela 1999; Volpicella *et al.* 2001). Functional display of cDNA libraries was achieved by C-terminal fusion to the proteins pIII, pVI or to the Fos leucine zipper (described later) (Crameri and Suter 1993; Sidhu 2001; Brunet *et al.* 2002; Fernandez-Gacio, Uguen and Fastrez 2003; Amery *et al.* 2012). In 2012, the initial phage-display-selected molecule adalimumab (tumor necrosis factor binder) was approved by the Food and Drug Administration for the treatment of human disease (Deutscher 2019). Phage display technology made it possible to produce the first six commercialized medicines derived from antibodies, and several others, based on human antibodies and antibody fragments, are under development (Barderas and Benito-Peña 2019).
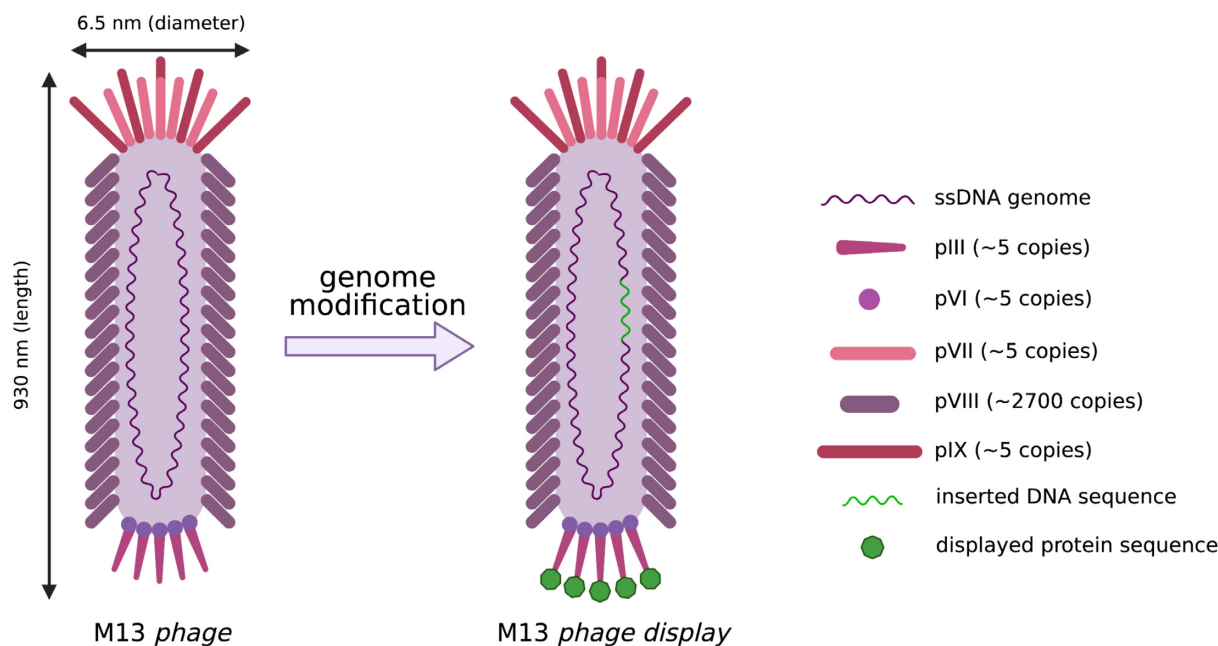
PRINCIPLE OF PHAGE DISPLAY

A successful use of any method requires good understanding of its principles, benefits and drawbacks. Thus, we aimed to start the description of the phage display technology from the presentation of its principles. Then, we will discuss various systems in more detail, presenting their advantages and disadvantages.

Phage display is a molecular technique based on a genetic modification of phage DNA in order to enable the expression of a peptide/protein/antibody fragment on the phage surface by combining them with one of the phage coat proteins (Sioud 2019). Exogenous DNA sequences of interest are introduced into a specific location in the phage genome nucleotide sequence, which encodes one of the phage coat proteins (Fig. 1). When phage infection occurs, phage gene expression begins inside the bacterial host, and the inserted peptide/antibody fragment is subsequently displayed on the surface of the phage as a combination product of the relevant genes encoding the coat protein and the cloned sequence (Arap 2005; Aghebati-Maleki *et al.* 2016). Therefore, if the cloned sequence is randomized, phage display libraries, providing $>10^{10}$ variants, can be constructed at the same time and stored for long term as DNA clones, rather than requiring the individual construction of various peptides or antibody fragments and the subsequent expression, purification and analysis of each particular construct (Arap 2005; Marintcheva 2017). The power of phage display technology also arises from its capability to form a physical connection between the displayed molecule (phenotype) and a DNA sequence that encodes the displayed molecule (genotype) (Aghebati-Maleki *et al.* 2016). Such phenotype–genotype linkage supports the selection of specific clones and allows the instant determination of the amino acid sequence of particular binders (peptides or proteins) by DNA sequencing of certain inserts in the phage genome (Azzazy and Highsmith 2002).

There are several key steps in phage display experiment, and the first one is library design, which can include several million or more DNA clones carrying target sequences that encode peptide/antibody fragments that can subsequently be replicated, transcribed, translated and displayed on the phage surface (Marintcheva 2017). Next, the library is cloned into the phage genome (either a classic vector or a phagemid system) and validated that it is functionally expressed, displaying peptides on the produced phage particles. The selection procedure, called biopanning, enables fairly easy identification of a concrete phage particle (Bábíčková *et al.* 2013; Marintcheva 2017). When the expression of a functional peptide/protein is proven, a library of different variants can be developed using mutagenesis methods (Marintcheva 2017). The capability to identify interactive regions of peptides, proteins or antibody fragments without prior knowledge about the type and nature of interaction is a great advantage of the phage display technology, as is the possibility of selecting from a vast number of phage particles in a small sample volume (the titer of phage lysate can be as high as 10^{14} virions per ml) due to the small size of phage virions (Arap 2005; Bábíčková *et al.* 2013).

BIOLOGY OF FILAMENTOUS PHAGES

Development of the phage display technology was possible only after detailed understanding of the biology of bacteriophages, and especially filamentous phages. Viruses infecting bacterial cells were used for years as models in microbiology and molecular biology, though their practical use remained rather illusory for a long time. Nevertheless, accumulating knowledge on the structure and functions of bacteriophages made it possible to employ them in developing sophisticated tools in genetic engineering and biological systems allowing for screening of huge numbers of molecules to select those of specific and desired purposes. In fact, creation of the phage display method is an excellent example for indispensability of advanced basic studies in

Figure 1. M13 bacteriophage virion architecture and principle of the phage display technology. M13 bacteriophage belongs to the family of filamentous phages. It has a single-stranded DNA genome, and its capsid is built of five different proteins. 'Classical' phage display is based on phage genome modification that consists of an in-frame insertion of a foreign sequence into one of the genes encoding capsid proteins. As a result, the phage virion particle presents heterogeneous peptide/protein on its surface (Bratkovič 2010; Hamzeh-Mivehroud *et al.* 2013; Aghebati-Maleki *et al.* 2016; Gagic *et al.* 2016).

further development of useful practical tools that can revolutionize various fields of (bio)technology and medicine.

Ff phages (constituting a part of the filamentous phage family) are a group of elongated viruses that are capable of infecting Gram-negative bacteria, such as *Escherichia coli*, bearing F plasmid. They belong to the *Inoviridae* family and the *Inovirus* genus. In terms of phage display, the Ff group, which includes phages M13, fd and f1, is the most important of the whole filamentous phage family (Aghebati-Maleki *et al.* 2016). These rod-shaped bacteriophages feature the simplest capsids in terms of the number of certain protein types (Aghebati-Maleki *et al.* 2016). The most popular filamentous phage used in phage display technology is bacteriophage M13, mainly due to its ability to adapt long fragments of foreign DNA into its genome (Falciani *et al.* 2005; Pande, Szewczyk and Grover 2010; Castel *et al.* 2011).

The Ff group has a single-stranded (ss) DNA genome with 98% similarity across different strains, and thus, it is perfect for genetic engineering purposes (Aghebati-Maleki *et al.* 2016). The M13 circular 6.4 kb ssDNA genome (Fig. 2) is sealed in a protein cylinder, which measures 6.5 nm in diameter and 930 nm in length (Fig. 1) (Hamzeh-Mivehroud *et al.* 2013; Hess and Jewell 2020). Interestingly, insertions of up to 12 kb of exogenous DNA (nearly twice the genome length of the wild-type phage) do not affect Ff phage functionality (Marintcheva 2017). The genome of an Ff phage is composed of 11 genes divided into groups based on the following functions of proteins encoded by corresponding genes: (i) capsid proteins including pIII, pVI, pVII, pVIII and pIX, (ii) DNA replication proteins called pII, pV and pX, and (iii) the assembly proteins including pI, pIV and pXI (Arap 2005; Hamzeh-Mivehroud *et al.* 2013) (Fig. 2). Two minor coat proteins, pIII and pVI, are situated at one end of the elongated phage virion, and at the other end, and there are two additional minor coat proteins, pVII and pIX (Webster 2001; Hess and Jewell 2020). The phage capsid is composed of the major capsid protein pVIII, which polymerizes from many thousands of copies around the genome (Marintcheva 2017) (Fig. 1).

Filamentous phage infection evokes a specific kind of lysogenic state in which the infected bacteria assemble and secrete phages into the growth medium (when cultured under laboratory conditions) (Arap 2005). At the initial stage of phage development, infection is triggered by the attachment of the phage protein pIII to the F pilus of a male *E. coli* cell (Arap 2005). During this phase, the circular ssDNA passes to the bacterial cell, where it is reshaped into a double-stranded plasmid replicative form by the replication machinery of the host cell (Arap 2005). In the course of rolling-circle replication, the replicative form produces single-stranded DNA and creates patterns for the expression of phage proteins (Arap 2005). When an appropriate concentration of phage pV protein is achieved, it attaches to the genome packaging sequence and triggers the assembly of the virion. The ssDNA is packaged into a capsid and excreted through the bacterial membrane (Russel 1991).

PRINCIPLES OF FILAMENTOUS PHAGE DISPLAY

As mentioned earlier, the first phage display system has been developed using filamentous phages (Smith 1985). Although many other display systems (based on different phages, various bacterial, yeast and mammalian cells, as well as cell-free ones) have been constructed subsequently, those based on Ff phages are still the most numerous and most used. This is due to the simplicity of the phage structure, ease of genetic manipulations with phage DNA and high efficiency of propagation of phages in bacterial cells giving a possibility to obtain huge number of virions in a short time.

The working principle of filamentous phage display relies on cloning DNA fragments that encode billions of versions of certain ligands into the phage genome, linked to the gene encoding one of the phage coat proteins (Benhar 2001). The key coat proteins for the display of foreign molecules on the phage
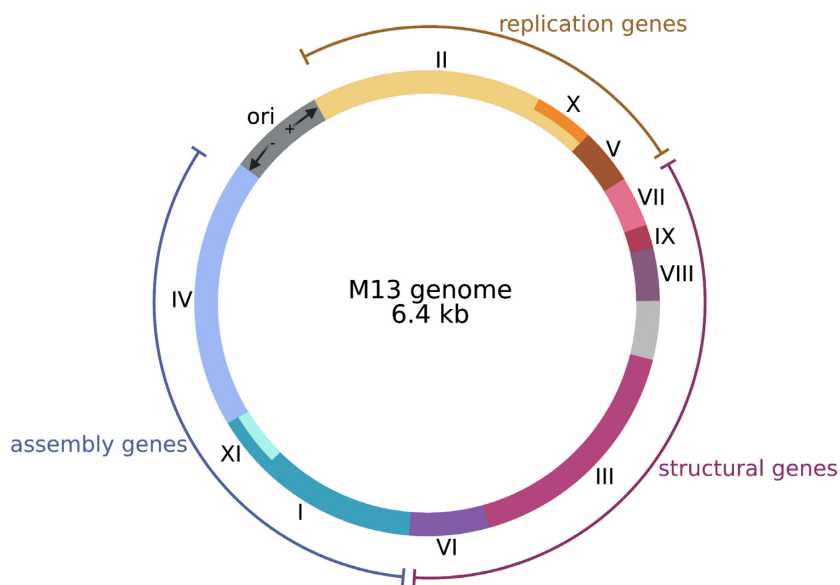
Figure 2. M13 genome organization. The genome of M13 bacteriophage contains 11 genes that can be classified into three distinct groups depending on encoded protein function: capsid structure (genes III, VI, VII, VIII, IX), DNA replication (genes II, V, X) and phage assembly (genes I, IV, XI) (Arap 2005; Hamzeh-Mivehroud *et al.* 2013).

surface are pIII (406 amino acid residues) and pVIII (50 amino acid residues) (Hamzeh-Mivehroud *et al.* 2013).

The pIII protein occurs at the tip of the phage in 3 to 5 copies and it is composed of three distinct domains (Omidfar and Daneshpour 2015) (Fig. 3). The N1 domain accounts for the start of the translocation of viral DNA into *E. coli* during infection, while the N2 domain provides host cell recognition by attaching the phage protein pIII to the F pilus of male *E. coli* (Omidfar and Daneshpour 2015). Presentation of the displayed protein or peptide occurs at the N-terminus of pIII, but the displayed molecules are isolated by a spacer (linker peptide) from pIII's N-terminal residue (Fernandez-Gacio, Uguen and Fastrez 2003; Hamzeh-Mivehroud *et al.* 2013). The C-terminal domain accounts for pIII integration into the phage coat and is an integral part of the capsid structure (Arap 2005; Omidfar and Daneshpour 2015). The display of the pIII protein results in a library of 3–5 copies of each particular peptide at the phage tip (Smith and Scott 1993). Relatively large molecules, up to 38 amino acids, may be incorporated into the N-terminus of the pIII protein without losing any phage infectivity (Arap 2005). In fact, among all filamentous phage coat proteins, only pIII is large enough to present several epitopes to B and T cells (Irving, Pan and Scott 2001; Aghebati-Maleki *et al.* 2016).

Approximately 2700 copies of the pVIII protein are tightly arranged on the phage coat, which means that by inserting the peptide coding sequence into the phage copy of the pVIII gene, up to 2700 copies of peptides with a maximum length of six amino acids can be presented along the phage capsid (Greenwood, Willis and Perham 1991; Webster 2001; Arap 2005). It is worth noting that higher copy number does not always translate into more efficient display, as factors like avidity and steric hindrance need to be taken into account. Protein pVIII has an alpha-helical structure with subtle deviations in curvature and kinking (Zeri *et al.* 2003; Woodburn *et al.* 2009; Hamzeh-Mivehroud *et al.* 2013). The pVIII helical axis is inclined ∼20 degrees to the main phage particle axis (Hamzeh-Mivehroud *et al.* 2013; Rodi, Mandava and Makowski 2015). The C-terminal part of pVIII is located

inside the phage particle near the genetic material, while the N-terminal end is exposed to the environment (Arap 2005).

In summary, two main coat proteins are suitable for the display of different types of molecules: pVIII is well suited for peptide and small protein display, but it is an ineffective platform for the display of large peptides; the pIII protein, despite its low copy number, can be utilized efficiently to display large peptides or proteins (Aghebati-Maleki *et al.* 2016). The key distinction in using one of the two major capsid proteins as a molecule presentation platform is display valency: peptide/antibody fragment attached to pIII can be displayed at a maximum of five copies per virion, while display on the pVIII protein allows the presentation of hundreds (type 88–described later on) or thousands of copies of peptides/antibody fragments (type 8–described later on) on a single phage particle (Bratkovič 2010). For experiments such as high-affinity antibody isolation, the use of pIII fusion is most suitable because a decreased valency is preferred. On the other hand, antibody-pVIII fusion results in high-avidity selection but probably low-affinity binding (Ebrahimizadeh and Rajabibazl 2014). In addition to the two main coat proteins, all other filamentous phage coat proteins were tested for the presentation of foreign peptides or small proteins, especially proteins pVI, pVII and pIX. However, the minor coat protein pIII and the major coat protein pVIII are by far the most widely used (Gao, Huang and Zhu 1999; Løset, Bogen and Sandlie 2011; Barderas and Benito-Peña 2019).

The most commonly used vectors in phage display systems are equipped with the P_{lac} promoter. This promoter is used to carry out the expression of fused genes coding for molecules attached to the pIII protein. The pIII display is obtained by removing or depleting the catabolic repressor (glucose) of the *lacZ* promoter, which results in a fusion product sufficient for production of the 'monovalent' phage display (described later on) (Hoogenboom *et al.* 1998; Fagerlund, Myrset and Kulseth 2008). Unfortunately, display systems relying on the P_{lac} promoter are very often leaky because of the absence of the O_2 operator situated in the *lac* operon structural genes (Fagerlund,
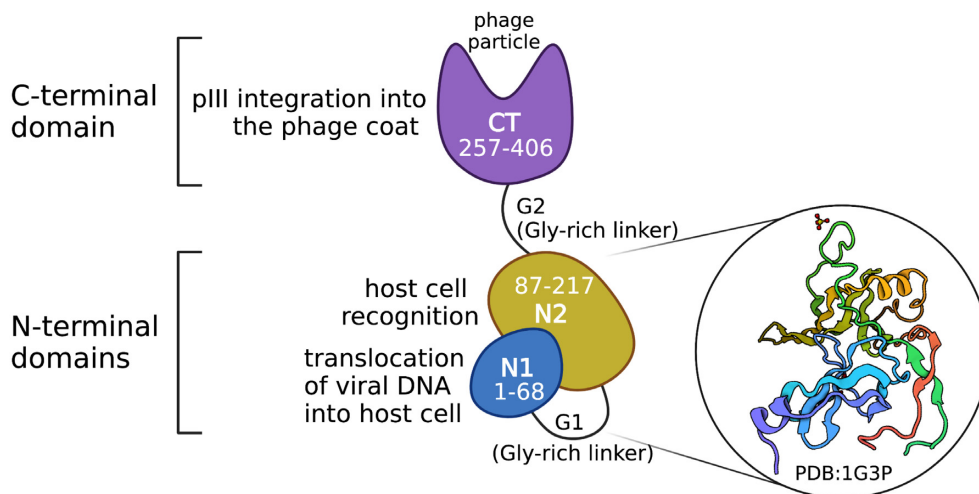
Figure 3. Structure of the pIII protein of M13 bacteriophage. The pIII protein contains two N-terminal domains, N1 and N2, joined with the G1 linker, and the C-terminal domain, CT. N- and C-terminal domains are connected with the G2 linker (Bratkovič 2010).

Myrset and Kulseth 2008). To overcome this problem, two different promoters, P_{BAD} and P_{tet} (also called tet$^{p/o}$), have been successfully used to modify the display of entire proteins connected to pIII (Fagerlund, Myrset and Kulseth 2008).

FILAMENTOUS PHAGE DISPLAY FORMATS

Depending on the specific requirements of desired constructs, and specific purposes of peptides displayed on virions, various systems of the filamentous phage display can be used. They were constructed to find optimal solutions for the efficiency of the system in the light of contradictions related to the number of displayed molecules and their size. Specific goals of experiments might require a display of either a few copies of longer peptides or maximal possible number of copies of short peptides, presentation of a desired peptide attached to either each molecule of the phage coat protein or to only some of such molecules, and location of the DNA fragment coding for a recombinant polypeptide in either a phage genome or a phagemid. All these possibilities can be achieved by using various formats of the filamentous phage display system.

All vectors used in filamentous phage display technology can be categorized according to the following aspects: (i) phage coat protein type used for display, (ii) connection of the displayed peptide/protein to all copies of pIII/pVII or to some of them and (iii) the type of matrix on which the insert for display is encoded—the phage genome or other genome, such as a phagemid (Hamzeh-Mivehroud *et al.* 2013). According to Smith's classification, there are six types of phage display formats (Fig. 4) (Smith and Petrenko 1997).

Type 3 is a display vector system referring to the natural filamentous phage genome, which is represented by the ssDNA vector type (Omidfar and Daneshpour 2015). In the type 3 vector, the insert encoded by the pIII gene leads to the display of the fusion protein/peptide in all of the five pIII protein copies (Smith and Petrenko 1997). Likewise, the type 8 display results in the presentation of foreign molecules in each of the produced pVIII proteins (Smith and Petrenko 1997). The major difference between types 3 and 8 is the number of copies of the displayed peptide, up to 5 in type 3 and ~2700 in type 8. Those formats are highly effective in displaying short peptides up to 12 amino acids, because longer peptides significantly decrease infectivity

and amplification of the phage that limits the construction of diverse peptide libraries (Sidhu 2000). Type 33 is characterized by the presence of two types of pIII protein, wild type and recombinant, as two different copies of the corresponding gene are present in the phage genome. Since wild type pIII carries out phage-related functions, and it is not linked to any peptide, only some of the expressed pIII molecules are fused with foreign peptides/proteins (Smith and Petrenko 1997; Chang *et al.* 2020). Type 33 vectors, in comparison with type 3, are able to display longer polypeptides (proteins or antibody fragments) but with significantly lower valency (Chang *et al.* 2020). The same concerns type 88 vectors. To summarize, types 3, 8, 33 and 88 are based on natural phage vectors and allow for polyvalent phage display in which a foreign molecule/peptide is connected to all copies of the phage coat protein (mainly pIII and pVIII, but also sometimes pVI, pVII, pIX are used, as described later), and the genome of a phage vector contains a single recombinant coat protein gene (Petrenko 2018). Phage vectors were the first used platforms in early phage display experiments, and generally they are easier to work with. However, some drawbacks are associated with this type of vectors, such as pIII infectivity loss due to foreign peptide fusion. Moreover, the resulting polyvalent display carries a higher risk of selecting less affine binders (Castel *et al.* 2011). To address those issues, phagemids (hybrids of phage and plasmid vector, representing types 3 + 3 and 8 + 8), which allow monovalent display, were developed (Bass, Greene and Wells 1990).

Types 3 + 3 and 8 + 8 describe the phagemid system, and similarly to type 33 or 88, they have two copies of the pIII or pVIII gene, though they are located on distinct replicons (Smith and Petrenko 1997). The recombinant form of the pIII or pVIII gene with foreign sequences for display is located in the phagemid genome, while the wild-type gene is present in the phage genome and called the helper (Hamzeh-Mivehroud *et al.* 2013). Specifically, phagemids are designed to contain the origin of replication for both the M13 phage and the *E. coli* plasmid in addition to gene III or VIII, an antibiotic-resistance gene and relevant multiple cloning sites (Mead and Kemper 1988). However, they do not contain any of the other structural or non-structural genes necessary for folding a complete, functional phage (Azzazy and Highsmith 2002). Phagemids can be used as plasmids, which allows the use of molecular biology tools for phagemid manipulation, or can be packaged as a recombinant
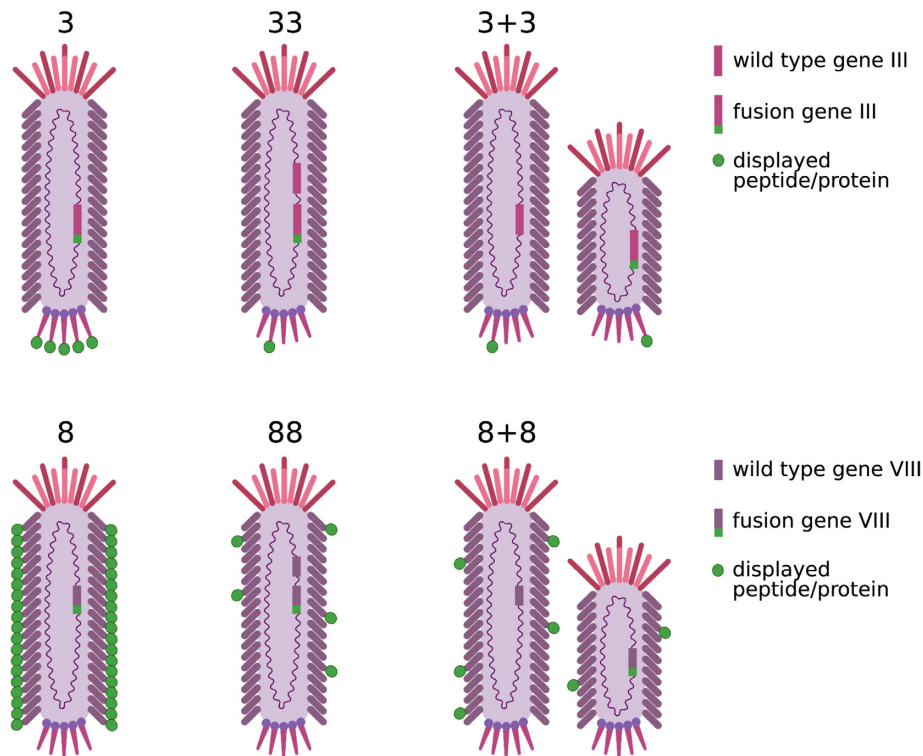
Figure 4. Major phage display formats. The initial classification of phage display formats, proposed by Smith, distinguished six types of phage display formats and took into account the phage coat protein type used for display, connection of the displayed peptide/protein to all copies of specific coat protein or to a part of them, and the type of replicon in which the insert for display is located (Bratkovič 2010; Huang, Bishop-Hurley and Cooper 2012; Hamzeh-Mivehroud *et al.* 2013).

M13 phage using the helper phage, which contains a somewhat defective origin of replication and provides, *in trans*, all the wild-type structural proteins essential for the production of a phage particle (Azzazy and Highsmith 2002; Marintcheva 2017). 'Wild-type helper phage' refers to a helper phage that encodes all virion proteins. This phage usually has an Ff origin of replication that is resistant to interference and/or a curtailed packaging signal and an additional (plasmid-derived) origin of replication, which allows selective replication and packaging of phagemid ssDNA, so that ~90% of secreted virions are 'phagemid particles' instead of helper phages (Barbas *et al.* 2001). A major benefit of a phagemid vector is the ability to produce one copy of each protein/peptide per phage particle (monovalent display), preventing avidity effects because of the expression of many copies of the displayed peptide, and therefore enhancing the selection stringency (O'connell *et al.* 2002; Jestin 2008; Castel *et al.* 2011). Phagemid vectors are also applicable in toxic protein display because their expression can be triggered at the same time as phage infection under inducible promoter control (Beekwilder *et al.* 1999). Nonetheless, an increased incidence of plasmid instability and gene deletion is the main drawback of the phagemid-helper phage system. On the other hand, this problem can be overcome by placing a gene coding for the fusion coat protein under the control of a strong transcriptional promoter, although this approach is likely to increase bacterial stress (Krebber, Burmester and Plückthun 1996).

The major difference between phagemid display and phage vector display lies in the valency (Fig. 5)—the viral vectors create only polyvalent display, while the phagemid/wild-type helper vectors could create a monovalent display, where valency is decreased, because displayed peptides/antibody fragments are diluted (Armstrong *et al.* 1996). Monovalent display is most commonly accomplished with the 3 + 3 vector system, and it allows to discriminate between low- and high-affinity binders due to decreased valency, and hence decreased avidity, whereas polyvalent display (especially on pVIII) is more suitable for low-affinity binders' selection, since the virion avidity offsets the low affinity of individual peptides (Armstrong *et al.* 1996; Shallom and Shoham 2003; Huang, Bishop-Hurley and Cooper 2012; Omidfar and Daneshpour 2015). Monovalent display technology is highly efficient during selection of strong binders from complex antibody libraries due to low valency of antibody fragments on the phage surface. Moreover, it decreases the risk for modifications in phage infection because of the low number of chimeric proteins on the phage surface (Sidhu 2001; Castel *et al.* 2011). On the other hand, polyvalent display is preferred for selection of weakly binding or rare clones, and it also might be required if the initial library does not include all possible sequences, and therefore, there may be no high-affinity binders included (Lowman 1997; Sidhu 2001; Castel *et al.* 2011).

BENEFITS AND OBSTACLES OF FILAMENTOUS PHAGE DISPLAY

Phage display technology offers a wide range of benefits, such as swiftness, simplicity and a low-cost methodology for binders' identification, compared with traditional screening methods including blue-white screening, restriction mapping or colony PCR (Hamzeh-Mivehroud *et al.* 2013). The major strength of filamentous phage display is its capacity to generate an immensely diverse library of peptides or proteins displayed on the phage surface. Furthermore, it is possible to adjust affinity selection conditions and formats for desired properties, and the selected clones can be easily identified through DNA sequencing
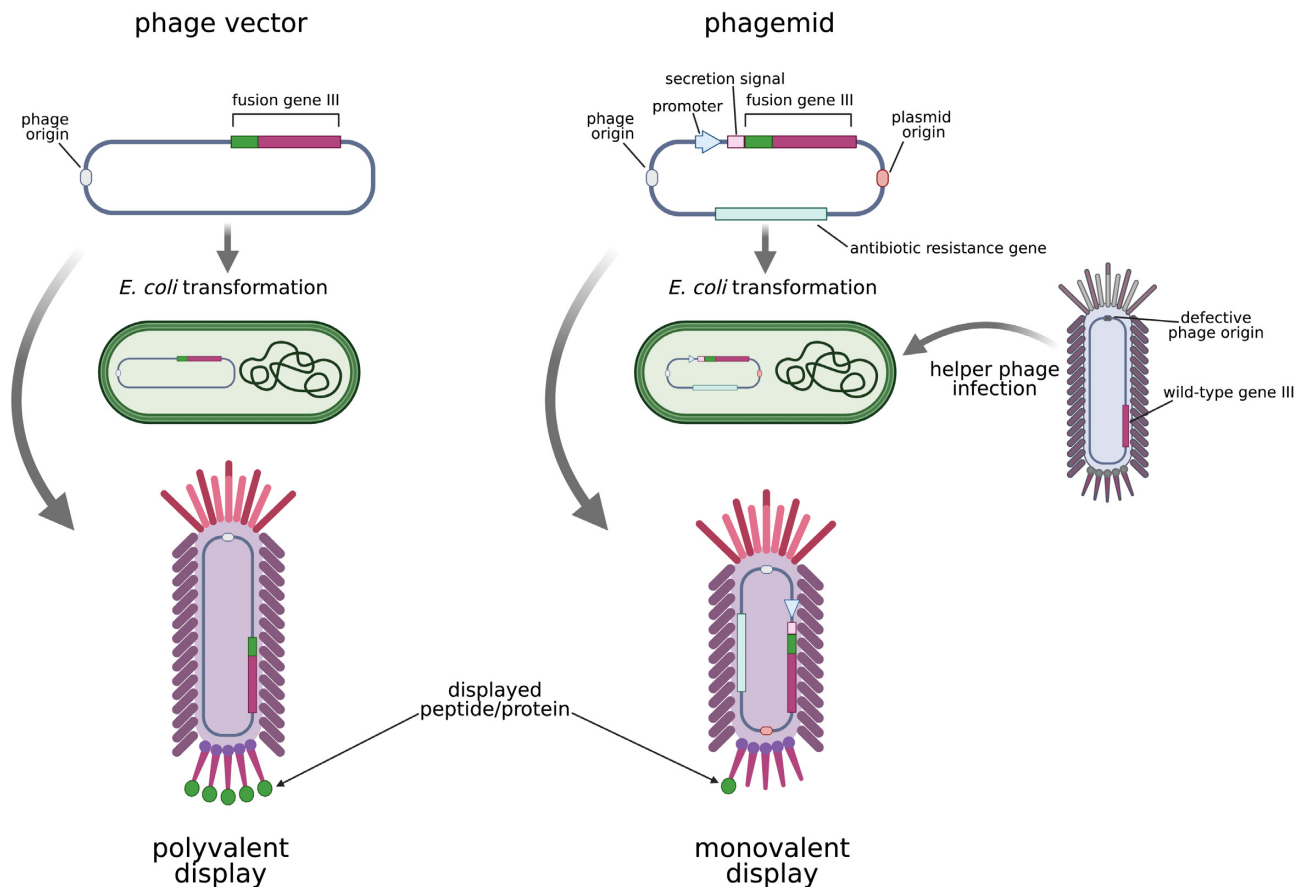
Figure 5. Comparison of a phage vector and a phagemid. The main difference between phage vector and phagemid is display valency. If the phage vector contains only one copy of a recombinant gene encoding displaying capsid protein, the resulting phage display is always polyvalent. The use of a phagemid, in turn, generates the display of lower valency (in the case of pIII mostly monovalent) as the helper phage provides the wild-type copy of the coat protein that is usually expressed more efficiently than the recombinant one (Hoess 2001; Willats 2002).

(Hamzeh-Mivehroud *et al.* 2013). Importantly, because filamentous phages are semi-lysogenic, they can replicate and shed constantly without killing the bacterial host (Hess and Jewell 2020). Another major advantage of filamentous phages is their ability to produce a very high titer (up to 10^{14} phages per ml). This ability to produce very large viral stocks comes from the lack of replication regulation and the capacity for episome transfer during cellular division into daughter cells (Marintcheva 2017). These properties allow phage production to be very fast and sustainable, facilitating rapid selection of protein-based hits for therapeutic purposes (Marintcheva 2017; Hess and Jewell 2020). Additionally, phage display provides certain benefits in the antibody discovery field in comparison to *in vivo* techniques that require animal immunization, including the full control of the experiment at every stage, which provides a high level of customization for direct and rational antibody discovery, thus, providing a significant increase in their therapeutic efficacy, as well as the reduction of animal usage (Laustsen *et al.* 2021).

On the other hand, traditional filamentous phage display, including production and display of peptides on the filamentous phage surface, followed by colony picking (biopanning), functional testing (e.g. ELISA test) and Sanger sequencing of positive clones, presents certain drawbacks (Bratkovič 2010). All filamentous phage capsid proteins are necessary for phage growth; therefore, there are large restrictions on protein arrangement changes (Marintcheva 2017). It is possible to insert long fragments of foreign DNA, but the subsequent phages are then

much larger and therefore are less stable and have a slower propagation rate (Marintcheva 2017). The traditional filamentous phage display approach is incompatible if the cDNA has an inherent stop codon at the 3′ portion of the gene transcript (Bratkovič 2010). Both the main capsid proteins pIII and pVIII require a leader peptide sequence at the N-terminus to reach the periplasm, and any fusion genes encoding those proteins must therefore be inserted in the exact reading frame between the leader sequence and the mature coat protein-coding region (Bratkovič 2010). Another drawback of the filamentous phage display arises from the fact that peptides or proteins fold in the *E. coli* periplasm only when the phagemid has periplasm leader sequences upstream of the gene of interest, otherwise cytoplasmic expression occurs. However, cytoplasm contains multiple pathways for the reduction of disulfide bonds, which results in the impaired conformation and biological function of the displayed peptide or protein, and therefore, display on Ff phages is suitable mainly for surface and secreted proteins folded in the periplasm of *E. coli* (Gagic *et al.* 2016). The filamentous phage-specific semi-lysogenic life cycle also presents some limitations for the successful display of peptides or proteins. Filamentous phages are released from the host cell without disrupting the cell membrane integrity, therefore, proteins forming the capsid must cross the lipid bilayer of the inner membrane. Thus, if a peptide or protein has properties that prevent it from crossing the membrane, the proper transfer and assembly of the hybrid capsid protein might be stopped (Castagnoli *et al.* 2012).

Despite these drawbacks of the filamentous phage display system, the elasticity of this technology coupled with the unique phage properties, including phage to phage conservation, packaging and sustainability, has definitely caused changes, and it is continually implemented in discoveries and innovations in molecular and chemical engineering (Petrenko 2008; Peltomaa *et al.* 2016; Barderas and Benito-Peña 2019).

ALTERNATIVE FORMATS OF FILAMENTOUS PHAGE DISPLAY

Drawbacks and limitations of the classical filamentous phage display systems, described in the previous section, inspired researchers to develop alternative formats. They employ modifications of the cloning systems using 'classical' genes coding for pIII and pVIII proteins with specific modifications, the use of other phage coat proteins (pVI, pVII, pIX) as carriers of the recombinant peptides, and combinations with other systems, like antigen-displaying bacteria or synthetic peptides. Details of such alternative formats are discussed later to indicate diversity of the already available systems.

Indirect display on pIII

Indirect display on the pIII coat protein, in the form of a specific 3 + 3 display system, was achieved by Crameri and Suter in 1993 in order to resolve the filamentous phage incapacity to display cDNA (Crameri and Suter 1993). This system relies on high-affinity interactions between the Jun and Fos proteins that form a super-secondary structural subject (the leucine zipper motif) (Crameri and Suter 1993) (Fig. 6). The Jun leucine zipper is attached to the N-terminus of the pIII coat protein that results in the display of the Jun protein on the phage surface, whereas the library of cDNA is cloned to code for a C-terminal fusion to the Fos leucine zipper protein (Crameri and Suter 1993). In periplasm, Jun and Fos proteins form a coiled complex—leucine zipper, and thus, the cDNA-encoded protein is indirectly bound to the pIII and displayed of the surface of the phage (Crameri and Suter 1993). This approach theoretically solves the problem of weak cDNA display because the library inserts are fused to the C-terminus of the Fos protein that eliminates the need for an in-frame connection downstream of the insert (since cDNA inserts require to be in frame with both upstream and downstream sequences) (Rakonjac *et al.* 2011). However, the required procedures are more laborious, and the obtained library involves only a small fraction of all the potential recombinant gene products (Bratkovič 2010). Moreover, a major limitation of indirect pIII display is that filamentous phage particles have to be secreted across the bacterial membrane that can act as a barrier to certain clones and possibly bias the selection (Piggott and Karuso 2016).

Fusion to the C-terminus of pVI

Successful display of proteins anchored to the C-terminus of pVI in a phagemid format 6 + 6 has been described by Jespers and coworkers (Jespers *et al.* 1995). In 1999, Hufton et. al. presented a functional cDNA display based on fusion to the pVI protein (Hufton *et al.* 1999). However, this format has lower efficiency than the similar 3 + 3 format (Bratkovič 2010). Perhaps due to those lower production capacities or to restrictions of the chimera size, the use of this pVI display system has not yet gained much popularity (Piggott and Karuso 2016).

pVII and pIX display

Ff phage proteins pVI and pIX are synthesized without N-terminal signal peptides (in contrast to proteins pIII and pVIII that rely on a N-terminal leader peptide for periplasmic targeting and virion integration), and thus, they do not undergo post-translational processing (Simons, Konings and Schoenmakers 1981). This feature of pVII and pIX proteins may allow to overcome some signal sequence-dependent events that can disrupt an infectious phage particle assembly and function or negatively affect the survival of individual phage particles during biopanning (Løset and Sandlie 2012). Loset and coworkers established three peptide tags displayed on pVII that were up to 17 aa long, and had varying charges and pI values (Løset, Bogen and Sandlie 2011). In 2020, the first phage-displayed protease substrate library, employing the pVII capsid protein, was reported (Kara *et al.* 2020). Signal sequence-independent pIX display indicated better performance in the antibody affinity selection than the pIII display, including stronger enrichment of particular clones and selection of higher affinity and stability clones, and therefore, it could be effectively used in antigen display (Høydahl *et al.* 2016).

Landscape phages

The origin of 'landscape phages' is related to the significant changes in surface architecture in phages with pVIII fusions in type 8 vectors (Petrenko 2008) (Fig. 7). These changes are caused by the arrangement of thousands of copies of the foreign peptide in an ultra-tight, repeating pattern around the tubular capsid (Petrenko 2008, 2018). The guest peptides presented on the phage capsid build well-defined organic surface structures called landscapes that differ from one phage clone to another, creating a variant of polyvalent display (Petrenko 2008, 2018). In other words, landscape phages are M13 phage particles with 2–4 amino acid molecules on every wild-type pVIII coat protein substituted with random octamers (Petrenko *et al.* 1996). This replacement results in a fixed peptide framework that enables phage particles to have features that rely on the introduced variable peptides (Petrenko *et al.* 1996). Moreover, if the guest peptide is displayed on every subunit of the pVIII protein, additional features may arise due to the global architecture 'landscape' of the entire phage surface (Petrenko *et al.* 1996; Petrenko and Smith 2000). Polyvalent display on landscape phages impairs selection for high affinity because individually strong and weak peptide ligands cannot be differentiated due to the significant increase in avidity (Petrenko 2018).

Selectively infective phage

In selectively infective phage (SIP) technology, contrary to traditional phage display, the desired specific protein–ligand interaction is directly responsible for returning infectivity in an otherwise noninfective display phage (Krebber *et al.* 1997) (Fig. 8A). The main advantage of SIP over the traditional phage display is the direct junction of the productive protein–ligand interaction with phage infectivity and amplification, without a need for the elution step (Jung *et al.* 1999). This technology is based on the modular structure of the gene encoding the pIII protein, which is composed of three domains, N1, N2 and C-terminal (Krebber *et al.* 1997). In SIP, the filamentous phage infectivity is destroyed by deletion of fragment(s) of the gene III coding for the N1 and/or N2 domain(s) of pIII (Jung *et al.* 1999). To replace the N1 or N1-N2 regions, a peptide or protein is genetically fused to
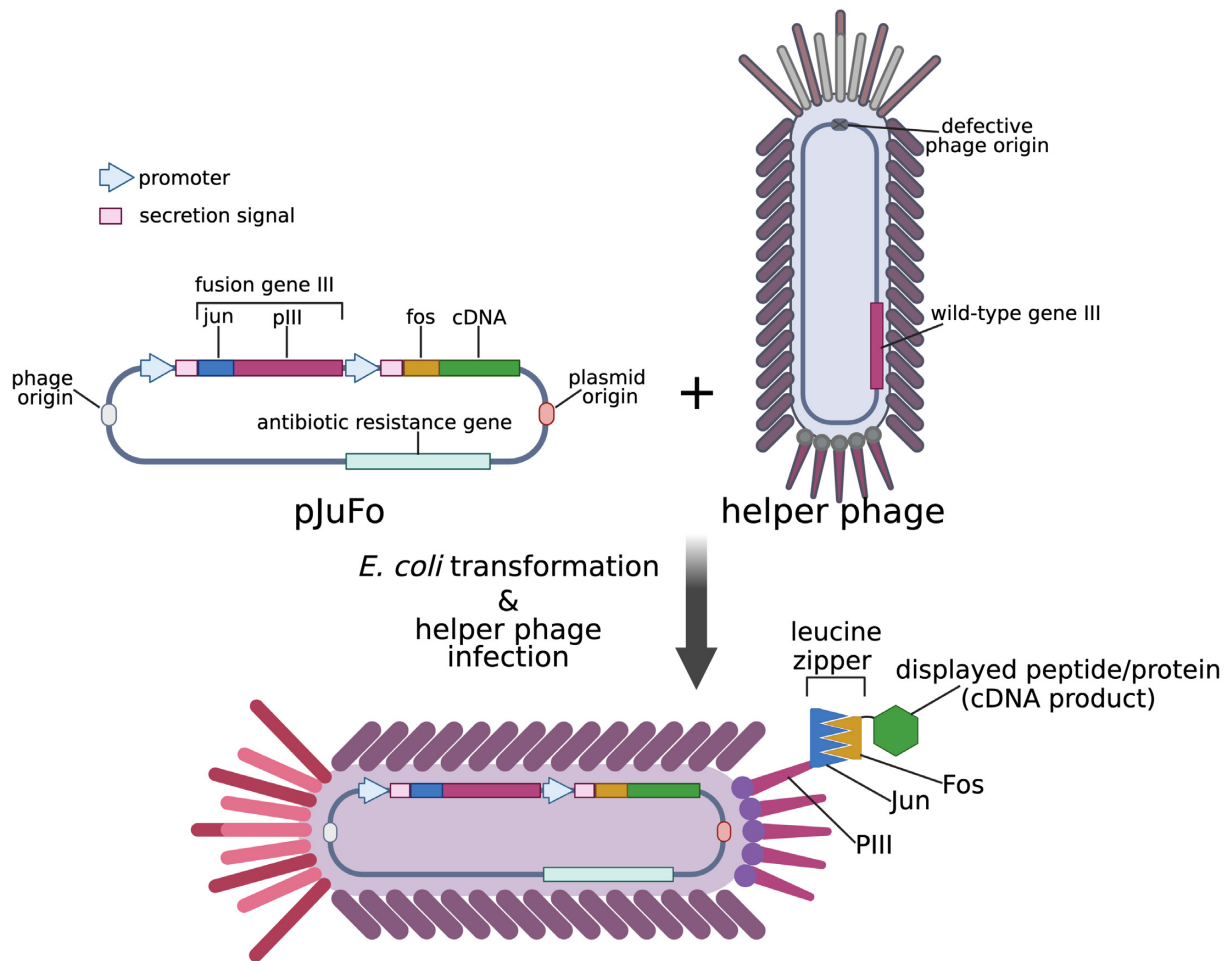
Figure 6. Indirect pIII display. An example for the system with the use of fragments of eukaryotic genes coding for Jun and Fos proteins is shown (Crameri and Suter 1993).
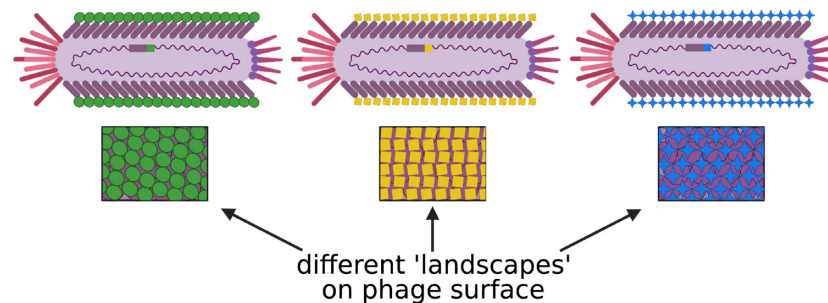
Figure 7. The idea of landscape phages. Different 'landscapes' of peptides displayed on phage surface are presented (Petrenko 2008, 2018).

the C-terminal domain or the N2-C-terminal domains of pIII that results in formation of a noninfective phage particles (Krebber *et al.* 1997). Since the N1 domain is necessary for infection, the N1 or N1-N2 domains must be added to restore the phage infectivity (Spada, Krebber and Plückthun 1997) (Fig. 8B). The infectivity restoring molecules, further referred to as 'adaptors', provide the missing N-terminal domains coupled to a ligand that binds to the peptide or protein displayed on the phage surface (Krebber *et al.* 1997). The adaptor molecule and the peptide-displaying phage particles (which are noninfective) are combined, and if the displayed peptide can bind the ligand from the adaptor,

peptide–ligand complexes are formed (Krebber *et al.* 1997). The N-terminal domains of pIII reconnect with the phage particles, and therefore, phages infectivity is restored (Krebber *et al.* 1997).

By linking protein–protein interactions to phage infectivity, the SIP technology enables for rapid identification of interacting proteins. Moreover, it is a low-background process that removes the need for the ineffective physical separation of specific and nonspecific binders, thus, it is proposed as an effective and rapid method for selecting peptide/antibody fragments with high affinity toward the antigen (Krebber *et al.* 1997; Hertveldt, Robben and Volckaert 2002).
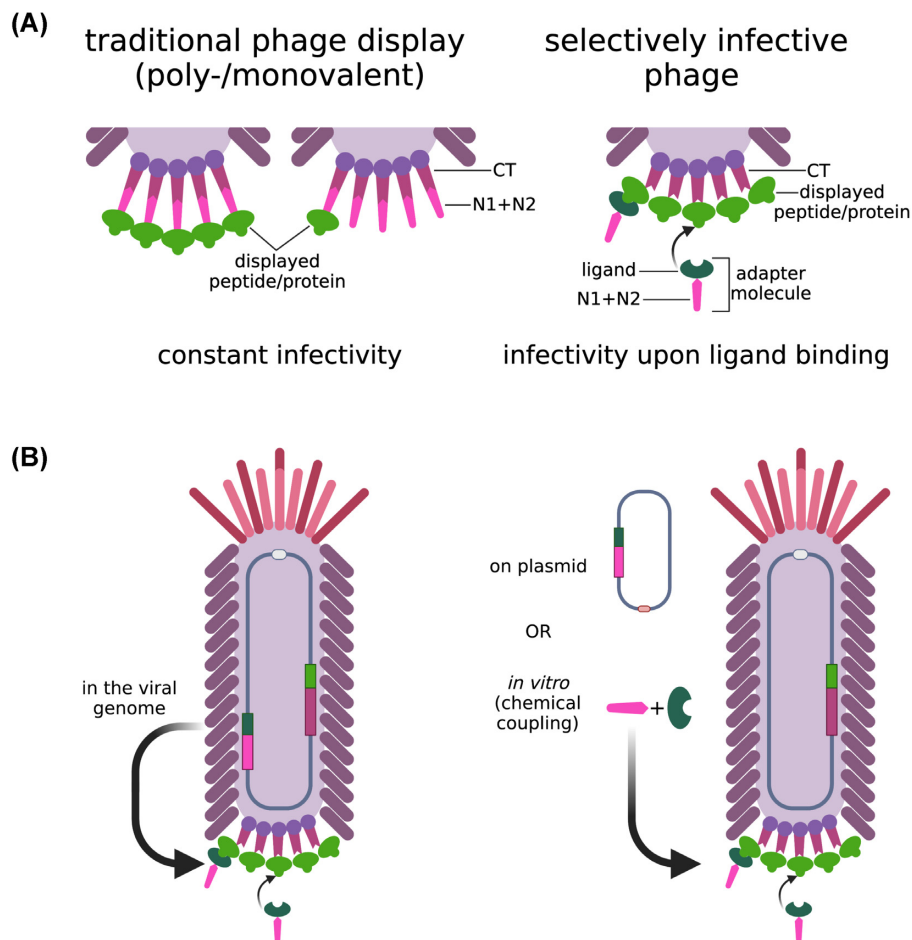
Figure 8. Selectively infective phage (SIP). **(A)** In the SIP technology, displayed peptide/protein is fused to the C-terminal domain of the pIII protein, whereas ligand is fused to its N-terminal domains that are responsible for phage infectivity. **(B)** Adapter molecule can be provided either as an phage genome-encoded molecule ('in cis') or as phagemid-encoded molecule or *in vitro* ('in trans') (Krebber *et al.* 1997).

Delayed infectivity panning

Delayed infectivity panning (DIP) technology combines conventional phage-displayed libraries with bacterial antigen display, where antigen-displaying bacteria are used to select antibody-displaying phage (Benhar *et al.* 2000) (Fig. 9). The principle of DIP is based on display of the protein sequences in multiple copies at the *E. coli* surface, obtained by constructing fusions with the hybrid Lpp-OmpA' sequence (Lpp, *E. coli* major outer membrane lipoprotein; OmpA, *E. coli* outer membrane protein) (Benhar *et al.* 2000). At low temperatures, bacteria do not form the F-pilus, which is necessary for phage infection, and under these conditions, target-displaying *E. coli* cells are used to capture phage (Benhar *et al.* 2000). Subsequently, after washing steps, bacteria with captured phages are transferred to 37°C, which facilitates F-pilus formation, and thus, enables infection by the captured phages (Benhar *et al.* 2000). The DIP process is very effective, therefore, it is relevant for the effective isolation of rare clones present in a large library, and should also be suitable for the isolation and characterization of not only antibodies but also any protein pairs that can be displayed on phage virions or on the surface of bacteria (Benhar 2001).

Biosynthetic phage display

Another display technology named 'biosynthetic phage display' was developed by Dwyer and coworkers in 2000 (Dwyer *et al.* 2000). It involves displaying one part of the protein as a fusion to the pIII or pVIII coat protein, while the other part is synthetically produced (Dwyer *et al.* 2000). Both chemically synthesized nonnatural amino acid parts of the displayed protein and pIII or pVIII peptide extensions are chemically connected before selection is carried out on the library (Dwyer *et al.* 2000). Biosynthetic display technology involves the fusion of phage display mutagenesis and peptide synthesis, thereby providing a new level of protein engineering (Bratkovič 2010).

OTHER PHAGE-BASED SYSTEMS

Filamentous phages are commonly used in the most often employed phage display systems. However, detailed understanding of structures, genetics and life cycles of some other bacteriophages provided a basis for construction of phage display systems with the use of other bacteriophages. Such systems have some important advantages over those based on filamentous phages, like possibility to display relatively large polypeptides or significantly more effective screening of cDNA libraries. Hence, the details of the use of other phages in developing display systems are described later, and features of such systems are discussed.

Tailed phages, which constitute the order named *Caudovirales*, have a common origin, and their virions consist of a head and a structured tail (Ackermann 1998). Lytic phages are capable of displaying larger proteins than filamentous phages due
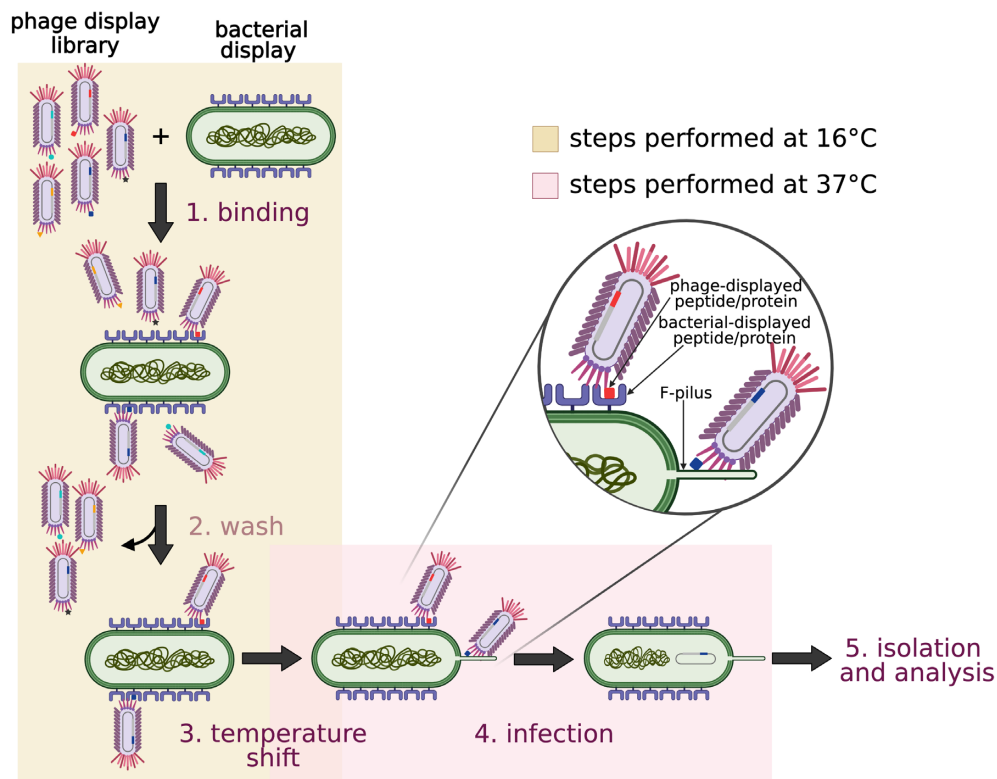
Figure 9. Delayed infectivity panning (DIP) procedure. Five steps of this procedure are distinguished, including binding of phages to bacterial cell surface, washing unadsorbed phages out, temperature shift, actual infection, and isolation and analysis of resultant phages (Benhar *et al.* 2000).

to their ability to carry larger fragments of foreign DNA (Marintcheva 2017). In lytic species, phage assembly occurs within the infected cell, and host cell lysis releases the progeny phages, creating new opportunities in display technologies (Bratkovič 2010). Peptides displayed on the surface of lytic phages do not have to be consistent with the host cell synthesis and secretion apparatuses; therefore, libraries may exhibit far more diversity than filamentous phage libraries (Krumpe and Mori 2006). Aside from filamentous and tailed phages, small icosahedral phages are also used in phage display systems, as they might be especially useful in presenting short peptides. Types of bacteriophages used in phage display systems (filamentous, tailed and icosahedral phages) are summarized in Fig. 10.

Bacteriophage T7

The T7 phage, a member of the *Podoviridae* family, has a 56-nm icosahedral capsid head, which is made up of 415 copies of capsid protein gp10 and has 40-kb lineal double stranded (ds) DNA genome (Bratkovič 2010). Other major proteins in the T7 phage virion are the fiver protein gp1, the connector protein gp8, the tail proteins gp11 and gp12, and proteins gp15 and gp16, which assist in phage DNA insertion (Deng *et al.* 2018). Both tail proteins, which form the 29-nm-long tail, enable binding of the phage to the host bacteria (Hess and Jewell 2020). The main capsid protein gp10 occurs in two different forms: the shorter 10A (344 aa), and the longer 10B (397 aa); the latter is produced due to a translational frameshift (Bratkovič 2010). A functional T7 phage capsid can be composed entirely of either 10A or 10B protein or from various ratios of the two. The assembly of T7 virions occurs in the *E. coli* cytoplasm, and progeny phages are released by cell lysis; thus, there is no need for secretion of the displayed

peptides via the periplasm and the cell membrane, as necessary for filamentous phages (Russel 1991). This knowledge prompted Rosenberg et. al. to implement restriction sites at the 3′ end of gene 10, which enabled the display of (poly)peptides as fusions to the C-terminus of the truncated protein 10B (Rosenberg *et al.* 1996). They developed a series of phage vectors for peptide and protein display on the T7 phage surface that were commercialized by Novagen, Merck Biosciences (Rosenberg *et al.* 1996). Larman and coworkers reported the establishment of a T7 phage-display polypeptide library derived from a synthetically obtained human proteome (Larman *et al.* 2011). The library was designed from 'tiles' with overlapping residues, and each library included 413 000 various peptides, enough to cover the human proteome (Larman *et al.* 2011). In this way, the entire human proteome can be screened with only one or two rounds of affinity selection (Larman *et al.* 2011).

A distinctive feature of the T7 phage particle is its stability in harsh environmental conditions that inactivate other phages used for display, including high temperature and low pH (Gao *et al.* 2010; Deng *et al.* 2018). Additionally, comparing to filamentous bacteriophages, T7 phages grow faster, their genome is more stable and have the capacity to display large proteins (>3000 bp DNA fragments can be successfully cloned) (Deutscher 2019). In terms of display technology, varied valency was achieved through upstream cloning of promoters of various strengths and the presence of modified translation initiation sites (Bratkovič 2010). T7 phage displaying only relatively short peptides (<50 amino acids) can be obtained at high valency. Large proteins (up to 1200 amino acid residues) are feasible but are restricted to a few copies per phage (Bratkovič 2010). The accessibility of an effective packaging system is certainly a major advantage of the T7 phage display system (Tan *et al.*
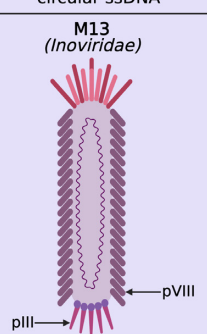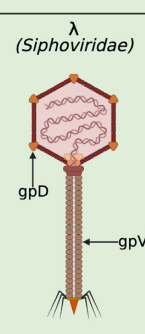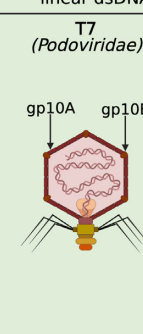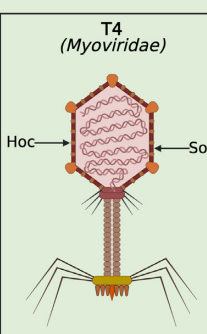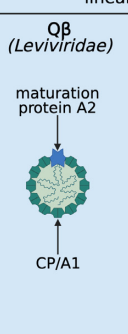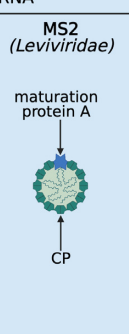
type	filamentous phages	icosahedral 'tailed' phages			small icosahedral phages	
genetic material	circular ssDNA	linear dsDNA			linear ssRNA	
phage (family)	M13 (*Inoviridae*)	λ (*Siphoviridae*)	T7 (*Podoviridae*)	T4 (*Myoviridae*)	Qβ (*Leviviridae*)	MS2 (*Leviviridae*)
capsid dimensions	930 nm x 6.5 nm	60 nm x 150 nm	56 nm x 29 nm	120 nm x 86 nm	28 nm	26 nm
genome length	6.4 kb	48.5 kb	40 kb	171 kb	4.2 kb	3.6 kb
protein(s) used for display	pIII, pVIII (rarely: pVI, pVII, pIX)	gpD, gpV	gp10B	Hoc, Soc	CP, A1 (coat proteins)	CP (coat protein)

Figure 10. Types of bacteriophages used in phage display. Apart from M13 bacteriophage that belongs to filamentous phages family, phage display can also be based on 'tailed' phages, such as T7, T4 and λ, and small, ssRNA icosahedral phages, Qβ and MS2. Main capsid proteins available for peptide/protein display are indicated by arrows (Tan *et al.* 2016).

2016). Furthermore, a more diverse library can be presented on the phage T7 surface than on the phage M13 capsid, and the display sequence shows lower charge and hydrophobicity bias (Krumpe and Mori 2006).

However, the T7 display system has some significant limitations (Piggott and Karuso 2016). Important posttranslational modifications of eukaryotic proteins, like phosphorylation, glycosylation, prenylation, and C- and N-terminal tailoring, cannot be displayed on T7 phage (Piggott and Karuso 2016). Very large, complicated, multidomain and membrane-associated proteins are unlikely to be displayed properly on the T7 phage surface (Piggott and Karuso 2016). In addition, two-thirds of the exogenous genes in T7 libraries are inserted with the phage coat protein gene out of frame, resulting in the display of random peptide fragments that are not encoded by the attached gene (Piggott and Karuso 2016). However, it is worth pointing out that many of those theoretical T7 phage display limitations are, in practice, irrelevant due to the very large number of phage particles that can be received in a small sample volume (Piggott and Karuso 2016).

Bacteriophage λ

Lambda (λ) phage is a member of the *Siphoviridae* family. This phage is characterized by an ~54 nm diameter icosahedral capsid head with ~4-nm shell thickness, a flexible tail ~150 nm long, and a 48.5-kb linear dsDNA genome (Nicastro 2014). Upon cell infection, λ phage can enter either a lytic life cycle and lyse the host cell or a lysogenic development in which the phage genome is integrated into the host cell genome (Hess and Jewell 2020). The λ phage genome encodes the major capsid proteins gpD (415 copies) and gpE (135–140 trimers of gpE connect the prohead to stabilize the λ capsid), the portal protein gpB, the major tail protein gpV (tubular tail is built of major tail protein V hexamer rings and is connected to the virion head (Hoess 2005), the viral protease gpC, and the scaffolding protein gpNu3 (Gao *et al.* 2010; Hess and Jewell 2020).

Referring to current research, polypeptides have been displayed on the λ phage surface linked to gpV and gpD proteins (Anand *et al.* 2021). The first λ display vector was constructed using the C-terminus of the gpV tail protein fused with foreign polypeptides (Maruyama, Maruyama and Brenner 1994).

Although constructs with the gpV protein have been used successfully for biopanning of antibody libraries, the gpV fusion system presents certain drawbacks, such as low display level and subsequently low phage recovery after affinity purification (Beghetto and Gargano 2011). To overcome these problems, the major capsid protein gpD has been explored as a display platform (Sternberg and Hoess 1995). The attachment of foreign molecules to the N- or C-terminus of gpD protein does not disturb λ phage assembly. Moreover, comparing to the filamentous phage coat proteins pIII or pVIII, higher capacity to display large domains of proteins of different sizes with less degradation of the fusion protein and in higher density, can be achieved using a two-gene system, in which both recombinant D-fusion protein and wild-type gpD are co-packaged into lambda head and subsequently co-exposed on the surface of the capsid (Sternberg and Hoess 1995; Gupta *et al.* 2003; Beghetto and Gargano 2011; Tan *et al.* 2016). Furthermore, λ vectors provide an easy way to assemble a chimeric phage because of the intracellular encapsidation of the fusion protein, similarly to phage T7 (Santi *et al.* 2000). This feature, combined with the protein gpD capacity to display large molecules, makes the λ display system particularly attractive to display cDNA libraries (Santi *et al.* 2000). Several cDNA libraries with a complexity of 10^7–10^8 independent clones displayed on λ was reported, such as two large cDNA repertoires from mammalian tissues (human brains and mouse embryo) (Santi *et al.* 2000), library of cDNA fragments from *Toxoplasma gondii* (Beghetto *et al.* 2001), and cDNA libraries from solid human tumors (Minenkova *et al.* 2003). In 2014, the λ display system was combined with the next-generation sequencing (NGS) to make a new platform, called PROLIFER (for 'phage-based representation of immunoligand epitope repertoire'), which enabled simultaneous analysis of dozens of phage-displayed antigen-specific libraries during selection, and provided increased resolution in epitope identification (Domina *et al.* 2014).

Bacteriophage T4

T4 phage belongs to the *Myoviridae* family, and it is characterized by an elongated icosahedral head measuring ~120 nm by 86 nm (Tao *et al.* 2019). After infection, the phage particles can enter only a lytic life cycle, which means that the bacterial host cell has to be destroyed to release replicated viral progenies

(Nicastro 2014). The genome consists of 171 kb of dsDNA that passes through the portal vertex formed by the portal protein gp20 to infect bacteria (Hess and Jewell 2020). The T4 phage capsid head has a more complicated architecture than the T7 phage, and it consists of the hexagonally stacked major capsid protein gp23 with pentamers of the corner protein gp24 at the peaks (Bratkovič 2010). Two redundant proteins symmetrically occur at the viral head: Soc (small outer capsid protein; 10 kDa, 870 copies per capsid) forms a constant entwined array, and Hoc (highly antigenic protein; 39 kDa, 155 copies per capsid), which is located in the middle of each gp23 (major capsid protein) hexagon (Bratkovič 2010). Under laboratory conditions, mutant T4 phage in which both *soc* and *hoc* genes are defective do not show a significant decrease in infectivity or replicative capacity (Ishii and Yanagida 1977). Moreover, Hoc⁻Soc⁻ capsids can be assembled *in vitro* from purified recombinant Soc and Hoc proteins with high specificity and nanomolar affinity (Qin *et al.* 2010). Therefore, phage T4 delivers a useful platform to display large proteins (as Soc or Hoc fusions), multi-protein complexes, or antigen fragments without negatively affecting the Soc and Hoc ability to bind to the capsid (Tao *et al.* 2017). The molecules displayed on the T4 phage surface can be connected to the N- and C-termini of the Soc and Hoc proteins, however, the N-terminus of Hoc is more suitable for display, because the C-terminal domain is required for the binding of the Hoc protein to the capsid (Ren, Baumann and Black 1997; Ren and Black 1998; Shivachandra *et al.* 2007; Fokine *et al.* 2011).

In 1996, Ren and coworkers designed two T4-Soc display system formats (Ren *et al.* 1996). In the first, peptides to be displayed were produced as C-terminal fusions of SOC and linked *in vitro* with the display platform (the phage capsid without the wildtype Soc) to create artificial T4 phage particles (Ren *et al.* 1996). The second display format relies on the *in vivo* incorporation of the recombinant *soc* gene into the T4 phage genome, transmitted by the positive selection plasmid pRH (Ren *et al.* 1996). The *soc* gene was flanked by specific T4 gene fragments in the pRH genome, which enabled homologous recombination with the T4 genome (Ren *et al.* 1996). Finally, bacterial cells with recombination plasmids were infected by a specifically designed phage with wild-type *soc* deletion to form fusion viral particles (Ren *et al.* 1996; Ren and Black 1998).

Bacteriophage T4 promotes protein display mainly as fusions of the nonessential capsid proteins Hoc and Soc, and hence, decreases the potentially destructive effects of fusions on phage stability (Marintcheva 2017). The main advantage of the T4 phage display system over other display formats is the use of T4 Hoc/Soc dual sites to present two different molecules simultaneously as fusions to Hoc and Soc on the T4 capsid surface (both the Hoc and Soc proteins have been used) (Wu *et al.* 2007; Tao *et al.* 2017). This system is appealing for cDNA expression and displays a high copy number of peptides and proteins on the surface of the T4 phage capsid (Gao *et al.* 2010).

Bacteriophage Q*β*

Q*β* phage is a 28-nm-diameter lytic virus of the *Leviviridae* family (Hess and Jewell 2020). A distinctive feature of this family is that it consists of the smallest and simplest phages (Hess and Jewell 2020). Their short 4.2-kb single-stranded (ss) RNA genome encodes only a few proteins: the major coat protein CP, the maturation protein A2, the coat protein A1 and the replicase (Kashiwagi and Yomo 2011). This phage binds to the F pilus of *E. coli* to insert its genome in the same way as filamentous phages, whereas the maturation protein A2 inhibits cell wall synthesis,

which triggers lysis and the release of new Q*β* phage particles (Reed *et al.* 2013). The A1 coat protein is only occasionally generated because of the leaky stop codon, and it is a target for display technology (Nchinda *et al.* 2021). This protein contains a 196-amino acid extension of the C-terminus of the major coat protein CP, and is necessary for infection of the host cell (Rumnieks and Tars 2011). Despite the Q*β* phage display is still under development, this novel display platform can be applicable in the directed evolutionary biotechnology and affinity maturation (Nchinda *et al.* 2021).

Bacteriophage MS2

MS2 phage, like QB phage, belongs to the *Leviviridae* family (Hess and Jewell 2020). The infection process of MS2 phage, like that of Q*β* phage, has a lytic character. The 26 nm icosahedral head contains the 3.6 kb ssRNA genome that encodes the major coat protein CP, the maturation protein A, the replicase and the lysis protein L (Fu and Li 2016). While both the N- and C-termini of the major coat protein CP are necessary for assembly, there is a prominent beta-hairpin that has been used for antigen display (Fu and Li 2016). The MS2 virus-like particle (VLP) has been developed for random peptide libraries (Spingola and Peabody 1997). Furthermore, Lino and coworkers presented a functional MS2 VLP platform that displayed four genetically fused fragments of scFv (Lino, Caldeira and Peabody 2017).

EUKARYOTIC VIRUS-BASED, CELL-BASED AND CELL-FREE SYSTEMS USED IN DISPLAY TECHNOLOGIES

Apart from bacteriophage-based systems for display peptides, other biological entities have also been applied for such purposes. Peptides can be displayed on surfaces of viruses infecting eukaryotic cells, as well as on those of bacterial, yeast and mammalian cells. Moreover, cell-free display systems were developed, including ribosome display, mRNA display, covalent DNA display and CIS display. These systems are depicted schematically in Fig. 11, and major eukaryotic virus-based, cell-based and cell-free peptide display systems are discussed later.

Bacterial display

Bacterial cell surface display is a commonly used technique for presenting heterologous proteins on the Gram-positive and Gram-negative bacterial cells' surfaces, and the first examples of heterologous bacterial surface display systems were reported approximately three decades ago (Agterberg *et al.* 1990; Janssen *et al.* 1994). Peptides or proteins to be displayed on the bacterial cell surface (named the target or passenger protein) are attached to an anchoring motif (named the carrier protein) by C-terminal fusion, N-terminal fusion or sandwich fusion, depending on the features of passenger and carrier proteins (Lee, Choi and Xu 2003). For anchoring motifs, bacterial cell-surface proteins or their fragments are usually applied (Lee, Choi and Xu 2003).

The most commonly used bacterium for the bacterial display is *E. coli* due to availability of various genetic tools and mutants, and the high transformation efficiency (Lee, Choi and Xu 2003). However, the major flaw in the case of all Gram-negative bacteria is that the display of proteins may cause the outer membrane fragility (Lee, Choi and Xu 2003). Bacterial display in Gram-positive bacteria was achieved in the *Staphylococcus* and
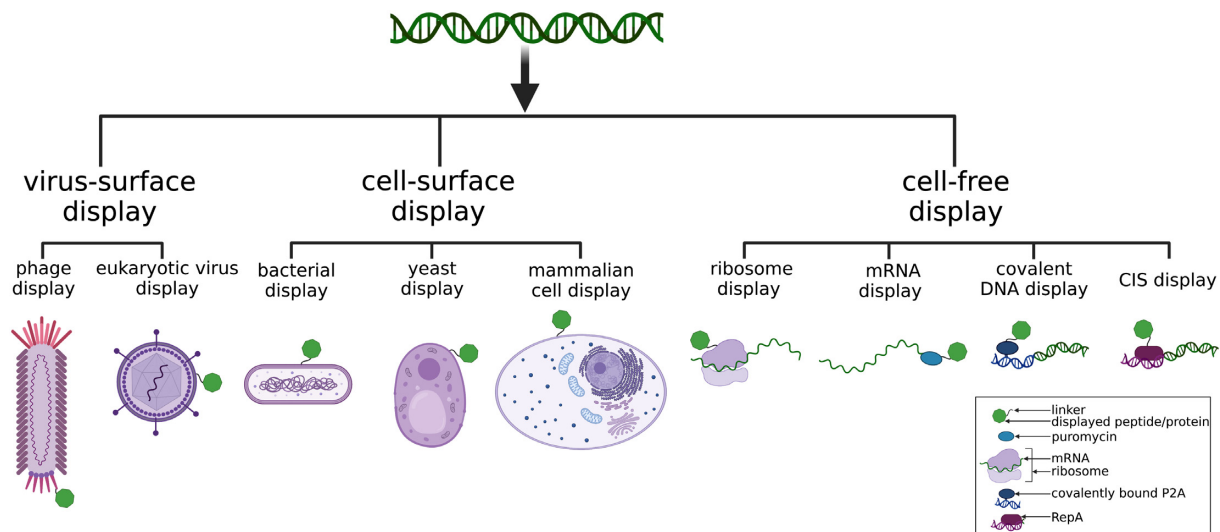
Figure 11. Types of surface display systems. The three main systems are based on virus-surface, cell-surface and cell-free displays (Lim, Choong and Lim 2017).

Bacillus genera (Lee, Choi and Xu 2003). *Bacillus subtilis* spore surface display (BSSD) technology, based on anchoring exogenous functional proteins on the spore surface (bacterial endospores are highly resistant, dormant structures formed in response to nutrient starvation and other stresses), was developed (in 2001) in order to increase the stability and functionality of these proteins, and since then it was intensively exploited in the fields of biodegradation, biocatalysts, pharmaceuticals and industrial enzymes (Isticato *et al*. 2001). In 2012, the first single-domain antibody library was displayed on Gram-positive bacteria, using the *Staphylococcus carnosus* system (Fleetwood *et al*. 2012).

Bacterial surface display technology has the capability to present antibody fragments, functional enzymes, foreign peptide epitopes and whole polypeptide libraries (Samuelson *et al*. 2002). Moreover, there are many potential applications of bacterial display, including the production of whole-cell biocatalysts, biosensors or bioadsorbents for heavy metals and harmful chemicals removal, and the development of live bacterial vaccine delivery systems (Benhar 2001; Lee, Choi and Xu 2003). However, the existing bacterial display systems still have some drawbacks, such as low display efficiency or passenger protein size restriction, furthermore, most applications are in the laboratory research stage (Li, Chavali and Babu 2018). Another limitation is the incapability to express complex eukaryotic proteins that require posttranslational modifications, including glycosylation and disulfide isomerization, to exhibit activity (Li, Chavali and Babu 2018).

Yeast display

Display on the yeast cell wall is possible due to incorporation a protein of interest into cell surface glycoproteins, ag-alpha-1 or aga2, which mediate interactions between yeast cells during mating of the yeast (Kondo and Ueda 2004). *Saccharomyces cerevisiae* is the most frequently used yeast species in construction of display platforms (Kondo and Ueda 2004; Lee *et al*. 2006; Hamzeh-Mivehroud *et al*. 2013). Several different molecules have been presented on the yeast surface, such as enzymes, cytokines, scFv and Fab (antigen-binding) antibody fragments, the extracellular domain of the epidermal growth factor receptor (EGFR) (Cochran *et al*. 2004), and peptides encoded by human testis cDNA fragments (Bidlingmaier and Liu 2006). In addition,

yeast display has been used to engineer and affinity mature several antibodies, including antibodies against cholera toxin (Tasumi *et al*. 2009), antibodies against antihuman immunodeficiency virus (HIV)-1 (Walker, Bowley and Burton 2009), murine monoclonal antibodies against the pandemic H1N1 virus (Shembekar *et al*. 2013), human antibodies against glioblastoma stem-like cells (Zorniak *et al*. 2017), and many more.

In recent years, there have been reported innovative studies that described posttranslationally modified peptides, displayed on yeast cells and effective incorporation of nonnatural amino acids with orthogonal reactivity in yeast-displayed polypeptides (Van Deventer *et al*. 2016; Hetrick, Walker and Donk 2018; Stieglitz *et al*. 2018). In 2018, first *in vitro* platform for nanobody discovery (nanobodies are small (12–15 kDa) and stable single-domain fragments of the naturally occurring heavy chain-only antibodies) were developed based on yeast surface display (McMahon *et al*. 2018). Yeast display technology is more suitable than bacteria-based technology when the displayed proteins require endoplasmic reticulum-specific posttranslational processing for efficient activity and folding; moreover, this type of display is compatible with fluorescence-activated cell sorting (FACS) that enables biophysical characterization and high-throughput screening of large combinatorial peptide and protein libraries (Kondo and Ueda 2004; Sergeeva *et al*. 2006). Even though yeast surface display technology is a relevant technique for the affinity maturation of antibody–antigen interactions, a higher glycosylation degree can impact the function and folding of engineered proteins (Cochran *et al*. 2009; Hamzeh-Mivehroud *et al*. 2013).

Eukaryotic virus display

Eukaryotic viruses, contrary to bacteriophages, can infect cells of protists, fungi, plants and animals, including humans. Three eukaryotic viral families have been adapted for use in display technology: *Adenoviridae*, *Retroviridae* and *Baculoviridae*. Among them, adenoviruses are the most potent group for their use as gene delivery vehicles, however, in terms of direct library display and selection, they lag behind other viral groups (Sergeeva *et al*. 2006). Nonetheless, numerous peptides and antigens have been displayed on adenoviral capsid-display vectors, such as ovalbumin (Johrden *et al*. 2013), influenza A antigens (Zhou *et al*. 2013) or

human papillomavirus (HPV) L2 protein (Wu *et al.* 2015; Vujadinovic *et al.* 2018).

Retroviral display, another eukaryotic virus-based display platform, can be applied to display proteins, antigens and enzymes (Urban and Merten 2011). Limited library displaying laminin-binding human synthetic scFv on retrovirus surface resulted in significant antigen binder enrichment in one selection cycle (Urban *et al.* 2005). In 2010, Granieri *et al.* developed a high-throughput technique for screening of structurally complex enzyme variants by encapsulation of retroviruses displaying complex mammalian enzymes into droplets of a water-in-oil emulsion, which could be manipulated using microfluidic devices, thus, each droplet acts as an independent reaction vessel allowing for active selection of enzyme variants under multiple turnover conditions (Granieri *et al.* 2010).

The key feature of baculovirus/insect cell display is the ability to tolerate large insertions of exogenous DNA and to display proteins that require posttranslational modifications for full functionality. Moreover, studies on the baculovirus display format have shown that both infected insect cells and the virus itself can present peptide libraries on their surfaces (Crawford *et al.* 2006; Marintcheva 2017). Baculovirus/insect cell display has been applied in such areas as recombinant protein production, tissue therapy and construction of biopesticides (Tsai *et al.* 2020). An important application of the baculovirus display technology is its use in studies on human-infecting viruses or pseudoviruses, especially their structural proteins, as baculoviruses are safe instruments to humans (Tsai *et al.* 2020). Some examples of molecules displayed on the baculovirus surface include Spike (S) protein from SARS coronavirus (SARS-CoV) (Chang *et al.* 2004), the γ-secretase complex implicated in Alzheimer's disease (Hayashi *et al.* 2004), a maize cDNA-based library of peptides displayed on insect cells (Meller Harel *et al.* 2008) and the envelope protein of the Japanese encephalitis virus, also displayed on the insect cell surface (Du *et al.* 2015). Therefore, the eukaryotic virus display systems provide a promising technology. They have already been used to study the complex eukaryotic transmembrane protein functions, to replace human-infecting viruses in diagnostic or antiviral assays, and to serve as vaccine antigens (Tsai *et al.* 2020).

Mammalian cell display

In principle, mammalian cell display depends on antibody-encoded DNA transient transfection, and initially this technology, adapted from yeast display, was used for isolation of high-affinity antibodies in human embryonic kidney 293T (HEK-293T) cells (Ho and Pastan 2009). This type of display has certain advantages over other display systems, such as the presence of the endogenous eukaryotic secretion machinery that enables the proper folding and biophysical characteristics of displayed antibodies, moreover, this system allows high-throughput and rapid screening of millions of antibody-displayed cells (Parthiban *et al.* 2019; Robertson *et al.* 2020).

Originally, only relatively small libraries (up to 10^7 clones) could have been achieved through mammalian cell display, however, recent advances mostly overcome this issue, for example by expanding the library size with the use of libraries derived from immunized animals, in which initial selection and maturation of antibodies occurred *in vivo*, or by using CRISPR/Cas9 integration methods, which further helped to reduce other developability issues, such as polyreactivity, aggregation propensity and immunogenicity of displayed antibodies (Parthiban *et al.*

2019; Dyson *et al.* 2020). In 2020, Robertson and coworkers developed a novel mammalian display system allowing for antibody selection against membrane proteins in their native configuration, which increased the chance of identifying functional antibodies because only physiologically relevant epitopes were displayed (Robertson *et al.* 2020). Other recently described mammalian cell display system, the tetraspanin anchored (tANCHOR) display, enables reliable presentation of heterologous peptides and proteins on human cell surface and provides a new alternative platform for antibody binding studies, and other applications (Ivanusic *et al.* 2020). In summary, a variety of antigens, peptides and proteins have been displayed on mammalian cell surfaces (Sergeeva *et al.* 2006), and further development of this display system should become important in biomedical applications.

Cell-free display

Cell-free *in vitro* display techniques are transcription/translation systems extracted from various ribosome-rich sources, such as wheat germs or *E. coli*, and used for the high-throughput screening, selection and evolution of biomolecules, and thereby provide genotype–phenotype connection by covalent linkage between proteins, DNA, RNA and ribosomes without the need to introduce DNA into a cellular host (Park, Lui and Cochran 2017; Contreras-Llano and Tan 2018). The major limitations of all cell-based display platforms are the size of the library, which affects the DNA transformation effectiveness, and the eventual toxicity of the displayed molecules to the host cell (Hamzeh-Mivehroud *et al.* 2013). Furthermore, most cell-based display experiments are associated with the presence of amplification bias, due to slight variations in growth rate, which can have significant influence on the overall diversity of the library, and hence, effective binders can be lost in the repeatable amplification process after each round of binding (Park, Lui and Cochran 2017). The implementation of cell-free display systems can overcome these problems because there is no transformation step, and therefore, the diversity and size of libraries are limited only by the amount of DNA that can be physically added to cell-free display systems (potentially up to 10^{14}–10^{15} molecules), and also the possibility of toxicity or amplification biases is eliminated (Hanes and Plückthun 1997; Fitzgerald 2000; Lipovsek and Plückthun 2004). A range of cell-free *in vitro* display platforms have been developed, including ribosome display, messenger RNA (mRNA) display, covalent and noncovalent DNA display, and *in vitro* compartmentalization (Rothe, Hosse and Power 2006; Hamzeh-Mivehroud *et al.* 2013).

Ribosome display
Transformation efficiency is a significant limiting phase of creating a large phage display library (Azzazy and Highsmith 2002). To address this issue, ribosome display of protein as a model of the first cell-free *in vitro* display was constructed (Mattheakis, Bhatt and Dower 1994). In ribosome display, DNA encoding a protein library is initially transcribed into mRNA (lacking a stop codon), which is subsequently purified and used for cell-free translation *in vitro* (Azzazy and Highsmith 2002; Marintcheva 2017). Then, mRNA–ribosome–peptide complexes are used for affinity selection, and afterward, mRNA is dissociated from the ribosomal complexes and undergoes reverse transcription into cDNA (Azzazy and Highsmith 2002). The produced cDNA is next amplified by PCR and employed for the next cycle of enrichment and PCR, and/or sequenced and analyzed (Azzazy and Highsmith 2002).

Cell-free *in vitro* ribosome display technology strives to concurrently select and evolve proteins from various libraries without any bacterial transformation; therefore, large libraries of peptides and folded proteins can be designed and used for selection (Hanes and Plückthun 1997; Hanes *et al*. 1998). The ribosome display technology has been applied to select picloram-specific variable fragments of heavy chain antibodies (Yau *et al*. 2003), or streptavidin (STA) binding peptides that confer higher affinity to bovine heart fatty acid-binding protein (FABP) (Lamla and Erdmann 2003). The advantages of ribosome display technology include the possibility of constructing a large and diverse library of up to 10^{12}–10^{14} members, as well as production of proteolytically sensitive and unstable proteins, generation of toxic proteins and incorporation of modified amino acids at specific positions. Two possible limitations of this technology are RNase contamination and the intrinsic affinity of the mRNA or ribosome toward the target molecules in comparison to displayed peptides and proteins (Rothe, Hosse and Power 2006; Hamzeh-Mivehroud *et al*. 2013).

mRNA display

The mRNA display technology is based on the construction of a DNA library followed by cell-free *in vitro* transcription into mRNA (Nemoto *et al*. 1997; Roberts and Szostak 1997; Takahashi, Austin and Roberts 2003). In this approach, first, protein–mRNA complexes are developed by the cell-free translation of mRNA *in vitro*. Second, the linkage to the displayed protein is created via a short DNA-puromycin linker, which was added previously to the 3' end of the transcribed mRNA (Nemoto *et al*. 1997; Roberts and Szostak 1997). The resulting complex is more stable than ribosome display due to the covalent link between mRNA and displayed protein (Nemoto *et al*. 1997; Roberts and Szostak 1997). Moreover, the size of the puromycin DNA linker is significantly smaller than ribosome, and thus the chance to interact with an immobilized selection target is significantly lower. Therefore, mRNA display results are less biased than those in ribosome display (Hamzeh-Mivehroud *et al*. 2013). Continuous attempts to use mRNA display to isolate high-affinity ligands resulted in the selection of various combinatorial or semicombinatorial libraries of peptides (Baggio *et al*. 2002). Other mRNA applications include the identification of drug-binding targets, protein–protein or DNA–protein interaction mapping, and the recent elucidation of protease–substrate interactions (Valencia *et al*. 2008).

Covalent DNA display

Bacteriophage P2 replicates by attaching replication initiator protein (P2A) to its own DNA (Lindahl 1970). This unprecedented attribute has been applied in covalent display technology (CDT) (Lindahl 1970). In covalent DNA display, linkage between the displayed polypeptide or protein and the gene is attained by forming a covalent bond between the DNA-binding protein P2A (produced as a conjunction with polypeptide) and the DNA encoding the fusion (Fitzgerald 2000). As a result, it is possible to connect the displayed protein to its own cDNA through a covalent bond (Rothe, Hosse and Power 2006). It is likely that CDT has the ability to overcome many limitations of other display systems, but it has not yet been widely used. Nevertheless, some libraries that display linear peptides or scFv attached to either the N- or the C-terminus of P2A have been described (Fitzgerald 2000; Reiersen *et al*. 2005).

CIS display

CIS display, a library selection system based on DNA, connects an expressed peptide/protein library to its own DNA sequence without the need for cloning (Mathonet *et al*. 2011). This technique is based on the bacterial initiator protein RepA, which has a specific feature called 'cis activity'. This means that the RepA protein can be bound only to the same DNA template from which it was generated (Mathonet *et al*. 2011). In the translation process, the cis-element stops the ribosome, the translated RepA interacts noncovalently with its *ori* and DNA-nascent protein complexes are created (Rothe, Hosse and Power 2006). As a result, by encoding a peptide/protein library linked to RepA, the expressed library is fused to its coding DNA, and can be easily sequenced to reveal the peptide sequence (Odegrip *et al*. 2004). Unlike phage display, CIS display can be carried out *ex cellulo*, and as a result, it can overcome the limitations resulting from the need to transfer the DNA into bacterial cells (Mathonet *et al*. 2011). CIS display technique has been applied to select a high-affinity binder toward the extracellular region of human vascular endothelial growth factor receptor isoform 2 (VEGFR-2) (Patel *et al*. 2013), and has been integrated with NGS and bioinformatics to facilitate the design of peptides with a potential therapeutic target (Mathonet *et al*. 2011).

PHAGE DISPLAY LIBRARIES

A crucial point in the practical use of peptide display is vast majority of experiments and procedures, with employment of any kind of display technology, is creation and analysis of a library of displayed molecules of peptides. In this section, we discuss types of libraries, their analysis and selection, considering phage display libraries as examples of the most commonly used display systems.

A phage display library is a diversified phage clone population in which every clone contains a random foreign DNA insert and hence presents a different molecule on its surface (Smith and Petrenko 1997). The key advantage of phage display libraries is the possibility to test a vast amount of phages in every round due to the high transformation efficiency that allows to obtain the most promising binders (Aghebati-Maleki *et al*. 2016). Other benefits of phage libraries include low propagation costs and ease of handling due to the large number of commercial kits available to perform ordinary molecular biology tasks (Aghebati-Maleki *et al*. 2016). In addition, a single phage display library is generally sufficient for multiple selection cycles (biopanning) once produced, and can be stored for a long time (Chan *et al*. 2014). Some possible disadvantages of phage display libraries can include potential mistakes in the selection procedure because of variable expression levels of certain sequences of peptides in *E. coli* (Castel *et al*. 2011). Moreover, consecutive library amplifications can quickly reduce phage population diversity (Castel *et al*. 2011). Despite these limitations, phage display libraries are widely used especially in discovering antibodies for therapy and diagnostic purposes, and several reports about the development and implementation of this technology have been released. Based on the purpose of a specific study, two library types are commonly employed: antibody libraries and peptide libraries (Hamzeh-Mivehroud *et al*. 2013). Library preparation and biopanning procedures are exemplified schematically in Fig. 12.

Peptide libraries

Peptide libraries are generated by using typical molecular cloning methods to insert fragments of oligonucleotides into the frame of the Ff phage coat protein genes (usually the N-terminus of pIII or pVIII) (Krumpe and Mori 2006). Subsequently, particular

LIBRARY PREPARATION
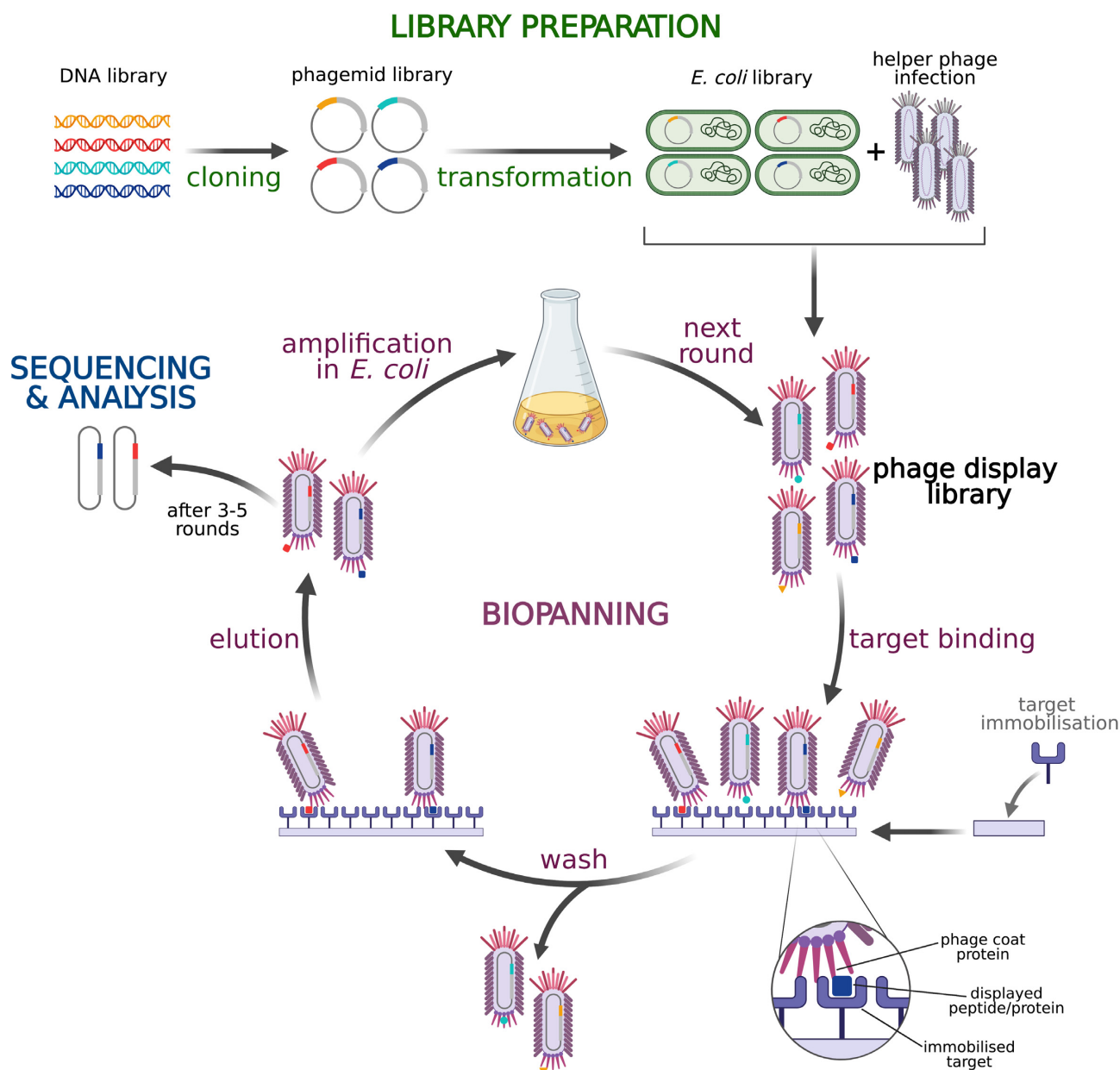
Figure 12. Library preparation and biopanning procedures based on phagemid and helper phage M13 pIII display. DNA sequences that constitute the basis of displayed peptides/proteins are primarily cloned to a phagemid vector. *Escherichia coli* cells are transformed with resulting phagemid library and, after helper phage infection, phage display library is produced. The library is affinity-screened against previously immobilized target of interest. The careful washing step is required to get rid of unbound phages. After washing, phages that interact with the target are eluted and amplified in *E. coli*. The whole cycle is repeated several (usually 3–5) times to select the most specific binders. After consecutive rounds of biopanning, the obtained sequences are functionally analyzed (Hamzeh-Mivehroud *et al.* 2013; Gagic *et al.* 2016; Barderas and Benito-Peña 2019).

peptides displayed by phages interact with a molecular target, and thus can be selected by affinity selection from a whole mixture of billions of unique displayed peptides (Krumpe and Mori 2006). Peptides are displayed on the phage surface in a linear form without well-defined 3D structure, which allows for high conformational elasticity and adaptability, however, such flexibility decreases their affinity to the target (Smith and Petrenko 1997). To overcome this problem, peptides can be structurally constrained in order to limit the number of possible conformers, and to increase independence of peptides on the phage environment (Castel *et al.* 2011). The most common strategy to obtain conformationally constrained peptides involves flanking

of random peptide sequences with two cysteine residues to create disulfide bridges that results in cyclization of peptides and reduction of available conformations range (Castel *et al.* 2011). In general, constrained peptides have a higher specificity and affinity, moreover, they exhibit a lower conformational entropy, which increases the probability of binding capacity preservation after removal from the phage context (Luzzago *et al.* 1993). Two different types of phage display libraries have been established: random peptide libraries (RPLs) and natural peptide libraries (NPLs) (Mullen *et al.* 2006).

Random peptide libraries are by far the most popular type of phage display library (Pande, Szewczyk and Grover 2010). In RPL,

the displayed variants of peptides (from 6 to 43 amino acids in length) are encoded by synthetic inserts of random degenerate oligonucleotides (Burritt *et al.* 1995; McConnell *et al.* 1996). These libraries are most often constructed with generic CX_nN structures flanked on either side by cyclized cysteines, resulting in the expression of random peptides (7–9 amino acids in length) (Sergeeva *et al.* 2006). The main advantage of random peptide libraries is their universality, which leads to numerous applications, such as the identification of linear antigenic epitopes or other molecules with various binding capacities, or application in directed *in vitro* evolution (Mullen *et al.* 2006; Sergeeva *et al.* 2006; Kubo 2017). However, one of the RPLs disadvantages is their universal nature, because their construction mode creates the possibility to display surface peptides that are not present inside the antigen or unchanged pathogen (Lundin *et al.* 1996). On the other hand, natural peptide libraries (NPLs) are constructed by random fragmentation of DNA of the selected species, such as pathogenic microbes (Mullen *et al.* 2006). Therefore, in these libraries, the phage particles display only natural peptide fragments (Mullen *et al.* 2006). NPLs provide an important alternative to RPLs in vaccine component identification and bacterial adhesin identification (Mullen *et al.* 2006). The reason for this is the higher efficiency of NPL-selected peptides in providing an antibody response that interacts with the native pathogen compared to that of RPLs (Matthews, Davis and Smith 2002). However, it should be noted that most clones obtained from natural peptide libraries are nonfunctional (Mullen *et al.* 2006).

Antibody libraries

The first antibody library was constructed in 1990, in which the display of functional antibody fragments on the surface of phage was obtained by the introduction of the heavy (V_H) and light (V_L) chain variable regions of the antilysozyme antibody (D1.3) into a fd-tet phage vector at the N-terminus of the minor coat protein pIII (McCafferty *et al.* 1990). Antibody phage display technology was also independently developed and improved by two other research groups, namely by Breitling and Dübel (Breitling *et al.* 1991), and by Lerner and Barbas (Barbas *et al.* 1991). In principle, antibody libraries involve cloning and shuffling the V_H and V_L chain-coding regions, and there are three types of antibody libraries that can be generated: naïve, immune and synthetic libraries.

Construction of immune libraries from immunized donors is the simplest method of obtaining antibody phage display libraries (Barbas *et al.* 1991). Immune library construction involves the isolation of immunized IgG-secreting plasma cells from immunized donors, followed by amplification of the coding DNA sequences and packaging them into phage library vectors (Omidfar and Daneshpour 2015). Immune libraries are usually used to produce antibodies for a certain target in medical research and applications, moreover, they provide selection of high-affinity antibodies as a result of the affinity maturation process (Clackson *et al.* 1991; Hust *et al.* 2011; Omidfar and Daneshpour 2015). However, they present certain drawbacks, such as time-consuming donor immunization process and a requirement for construction of a new antibody library for each antigen (Bazan, Całkosiński and Gamian 2012). Additionally, generation of human antibodies and prediction of the immune response are not always possible, and often there is no immune response to toxic antigens or auto-antigens (Bazan, Całkosiński and Gamian 2012). The naïve and synthetic antibody libraries are referred to as 'universal' or 'single-pot'. Unlike immune libraries, the generation and implementation of these

libraries make it possible to determine antibodies against every possible form of antigen, but far more effort is required (Frenzel *et al.* 2017). To provide suitable binders for all potential antigen configurations, these single-pot libraries must include a far more diverse range of antibody genes in comparison to immune libraries (Frenzel *et al.* 2017). Naïve libraries are generated using reconfigured V genes from IgM (B cells) obtained from nonimmunized donors (Hust and Dübel 2004). Semisynthetic libraries can be obtained from a single antibody with synthetic randomized complementarity-determining region 3 (CDR3) on the heavy chain or from rearranged V genes derived from germline (pre-B) cells (Pini *et al.* 1998; Hust and Dübel 2004; Frenzel *et al.* 2017). In universal fully synthetic libraries, random CDRs (complementarity-determining regions) are incorporated into completely synthetic framework sequences (Tiller *et al.* 2013; Frenzel *et al.* 2017).

In phage display antibody libraries, both Fab and scFv fragments have been effectively designed and used to identify antibodies that bind a specific molecular target, however, both present some benefits and obstacles (McCafferty *et al.* 1990; Winter *et al.* 1994; Hoogenboom *et al.* 1998; Hamzeh-Mivehroud *et al.* 2013; Barderas and Benito-Peña 2019). The construction of scFv antibody fragment libraries is significantly easier by using overlap extension PCR (SOE-PCR), which can be used also for construction of Fab antibody fragment libraries, but with more difficulty (Andris-Widhopf, Steinberger and Barbas 2001; Zhu and Dimitrov 2009). The other benefit of scFv libraries includes the possibility of engineering single-chain variable fragments in multivalent formats, and thus improving the avidity toward the target (Hamzeh-Mivehroud *et al.* 2013). One of the advantages of Fab antibody fragment libraries is that the Fab fragment folded framework seems to be kinetically and thermodynamically robust (Röthlisberger, Honegger and Plückthun 2005). Moreover, Fab fragments typically have a much lower tendency to multimerize than scFv fragments, and are therefore more prone to provide affinity information rather than avidity (Barbas *et al.* 2001). However, the key drawback of Fab libraries is the overall lower levels of expression in *E. coli* in comparison with the smaller scFv fragments (Azzazy and Highsmith 2002).

Selection of the phage display libraries

When a fusion protein or antibody library is built and displayed on the phage surface, a phage clone with a specific binding affinity can be separated from the mixture of all other phages, such as nonbinding phages by a method called affinity selection (Parmley and Smith 1988). Other generic names for this procedure include immunological panning or biopanning (this term was first mentioned in a scientific article where the phage selection/isolation was based on the strong biotin/streptavidin interaction) (Parmley and Smith 1988).

The step-by-step affinity selection process involves five stages. Step I is a preparation of a phage display primary library or amplification of the already existing library (Arap 2005). Step II relies on an exposure of phage particles to an immobilized target for which specific ligands are planned to be identified (Arap 2005). Target molecule can be presented on a solid surface (such as nitrocellulose, column matrices, microplates, magnetic beads or polystyrene tubes), or alternatively the biopanning can be carried out in a solution phase (but it must be followed by an affinity capture step to isolate the target-phage complexes) (Lou and Marks 2010; Aghebati-Maleki *et al.* 2016). Step III is a washing step in which unbound or weakly bound

phages are removed (Smith 2019). In Step IV, after the washing procedure, the bound phages are eluted by breaking target-displayed molecule bond without compromising phage infectivity, usually by applying an enzymatic cleavage with trypsin, or changes in pH, but sometimes also by adding a competing ligand or denaturant agents (Pande, Szewczyk and Grover 2010; Aghebati-Maleki *et al.* 2016). Step V is amplification of the collected phages by infecting *E. coli* to produce an enriched pool of phages, as the new library for the next round of affinity selection (Tan *et al.* 2016; Smith 2019). The repetition of binding–elution–amplification steps significantly increases the proportion of the target-specific phages in high-diversity libraries; therefore, usually 3 to 5 rounds of affinity selection are required to collect phages displaying high-affinity ligands (Aghebati-Maleki *et al.* 2016; Ledsgaard *et al.* 2018). A crucial affinity selection parameter is stringency (selection pressure), which reflects the degree to which phage particles displaying high-affinity molecules are favored over phages that display low-affinity molecules (Smith 2019). The stringency during biopanning rounds is crucial in order to retain the best binding phages and it can be intensified by a gradual increase in the number or duration of washing step (Vodnik *et al.* 2011; Aghebati-Maleki *et al.* 2016). Finally, after the last round of biopanning, phage clones or the whole phage pool are analyzed in terms of specificity, and a variety of methods can be used to validate target-specific phages, such as immunostaining with antiphage antibodies through enzyme-linked immunosorbent assay (ELISA) and immunocytochemistry techniques, or other competitive assays such as surface plasmon resonance (SPR) (Pande, Szewczyk and Grover 2010). After biopanning procedure, DNA sequencing by Sanger method is commonly used to analyze selected phage clones individually; however, in the last two decades high-throughput methodologies, denoted as NGS, have been developed and adapted to sequencing phage display libraries in a large scale (Dias-Neto *et al.* 2009). NGS technology enables identification of extremely rare clones and also provides the complete sequence spectrum through analysis of the entire phage pool after every round, or last round of biopanning. Moreover, it increases the possibility to select phages with binding affinity to certain targets (Vodnik *et al.* 2011; Liu *et al.* 2015).

A number of modifications in biopanning protocol or additional selection techniques have been developed in order to improve and expand the possibilities of the standard affinity selection procedure described earlier. By *in vivo* biopanning, phage ligands able to bind to a specific organ or tissue may be identified (Arap, Pasqualini and Ruoslahti 1998). In this technique, phage particles are provided intravenously to an animal, and unspecific phages have a tendency to spread across the whole animal organism, whereas phage particles with specific target ligands accumulate in certain tissues (Takagi *et al.* 2007). Phage-derived ligands unique to a tissue or organ may theoretically be used for medical diagnosis or treatment by combining phages with a medication, or by attaching phage particles to nanoparticles combined with a drug, especially since a number of tumor-homing peptides have been identified through an *in vivo* phage display (Thapa *et al.* 2008; Pleiko *et al.* 2021). Recently, *in vivo* panning have been connected with NGS, allowing the identification of target-selective homing peptides and is likely to facilitate progress toward the development of affinity-guided smart drugs (Pleiko *et al.* 2021).

Another selection technique, the SPR biosensor technology, depends on the SPR, which is an optical phenomenon that allows monitoring the interactions between biomolecules without labeling (Jonsson *et al.* 1991; Malmborg *et al.* 1996). The SPR

biosensor system consists of the sensor microchip on which one of the interacting biomolecules is immobilized, a microfluid channel system that provides the constant injection of the other interacting biomolecule over the sensor surface, and an optical detection system that measures an SPR response that reflects the changes in mass concentrations at the surface of the sensor chip as a result of association and dissociation between molecules (Hashimoto 2000). The SPR-based biosensor analysis of phage-displayed antibodies provides more specific kinetic information, and therefore, facilitates the selection of antibodies based on their kinetic binding properties (Malmborg *et al.* 1996). Many applications of the SPR biosensor selection technology, combined with phage display, have been described, including detection of pathogens such as *Salmonella* (Karoonuthaisiri *et al.* 2014) or *Staphylococcus aureus* (Liu *et al.* 2016), optical detection of cancer cells (Wang *et al.* 2016), or isolation of antibodies against dengue virus (Lebani *et al.* 2017).

Cell-based panning is an alternative for membrane protein binder selection in antibody libraries, since membrane proteins are often targets for therapeutic antibodies, but their purification is challenging due to the presence of the hydrophobic domain and easy denaturation during biochemical processes (Jones *et al.* 2016). Another selection alternative, the subtractive panning strategy, allows for selection of high-affinity and high-specificity antibodies, and it is based on changing the selection matrix and elution conditions during panning rounds (Eisenhardt *et al.* 2007). This selection technique allows for generation of conformation-specific antibodies that are highly demanded in biological research, therapeutics and medical diagnosis (Eisenhardt *et al.* 2007).

The next method, cell-based selection, employs the display of cell-surface antigens in their native form by using whole cells as the antigen source (Jones *et al.* 2016). Several other specific selection strategies have been developed such as antibody-guided selection using capture-sandwich ELISA (Itoh and Suzuki 2002), selection by epitope masking (Ditzel 2002), capture-lift screening (Watkins 2002), ultra-rapid selection of antibodies (Hogan, Rookey and Ladner 2018), rescue and *in situ* selection and evaluation (Vanhercke *et al.* 2005), selection by affinity isolation of antigen-specific B cells (Ditzel 2009), sequential antigen panning (Euler and Schuitemaker 2012), invert biopanning (Rahbarnia *et al.* 2016) or Yin-Yang biopanning (Lim, Woo and Lim 2019).

CONCLUDING REMARKS

The introduction of phage display technology over 30 years ago provided excellent tools to expose peptides on the surface of bacteriophages. Although phage display systems based on filamentous bacteriophages are the most commonly used platforms, there are many different, sophisticated alternative forms of this technique that allow the search for peptides or proteins with remarkably large variability in properties. Therefore, the applications of phage display are extremely broad, from material sciences through diagnostics to the development of novel therapeutic approaches. In fact, there are examples that the use of phage display technology made a clear advance or had an advantage over non-phage display systems. These include discovery of antibodies under nonphysiological conditions, to identify pH-dependent antibodies with special binding properties (Bonvin *et al.* 2015), development of recombinant antibodies against strong toxins (Laustsen *et al.* 2018; Føns *et al.* 2020) and introduction of a methodology based on phage display technology and a cross-panning strategy, enabling the selection of

cross-reactive monoclonal antibodies (Ahmadi *et al.* 2020). However, despite their usefulness, the phage display methods might still be improved. For example, problems with the production and presentation of hydrophobic peptides on phage surfaces or the formation of aggregates by recombinant proteins remain unsolved. It might be assumed that the construction of a phage display system in thermophilic bacteriophages could provide a solution. However, to date, no thermophilic phages or bacteria have been used for this purpose. This is perhaps due to the requirement for detailed knowledge on phages and their hosts, while the understanding of the structure and biology of thermophilic bacteriophages is still relatively poor. Although many phage display systems are available and there are thousands of publications describing specific applications of this technique, novel and improved phage display platforms are still desired, and further studies on the practical use of phages that expose peptides on their surfaces will bring many new fascinating discoveries in various fields of science and medicine.

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