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Bacteriophage Vehicles for Phage Display: Biology, Mechanism, and Application

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Abstract The phage display technique is a powerful tool for selection of various biological agents. This technique allows construction of large libraries from the antibody repertoire of different hosts and provides a fast and high-throughput selection method. Specific antibodies can be isolated based on distinctive characteristics from a library consisting of millions of members. These features made phage display technology preferred method for antibody selection and engineering. There are several phage display methods available and each has its unique merits and application. Selection of appropriate display technique requires basic knowledge of available methods and their mechanism. In this review, we describe different phage display techniques, available bacteriophage vehicles, and their mechanism.

Introduction

Phage display technology was first introduced by Smith in 1985 and since then, it has been employed in various research fields such as drug discovery [1, 2], vaccine development [3, 4], antibody isolation and engineering [5–7]), and epitope mapping [4, 8]. Phage display provides a fast and powerful technique for isolation of peptides or proteins that recognize a selected target with high affinity among millions or even billions of candidates.

Phage display is a reliable method for selection of high-quality antibodies from libraries consisting of numerous antibody members. Fast, simple, and high-throughput antibody isolation make this technology a preferred method for selection of antibodies against new antigens or cell markers [9–11]. Phage display technology has been used for selection and identification of human therapeutic antibodies [12–14], and it is estimated that near 30 % of human therapeutic antibodies that are under clinical trial have been developed by this method [15]. In addition to the therapeutic values, phage display technique is frequently used for identification and confirmation purposes in research fields [16, 17].

In the phage display method, foreign peptides are expressed on the surface of a bacteriophage. Several features make this technique a powerful tool for selection and identification of antibodies. It allows construction of large libraries consisting of numerous antibody genes. Mass cloning of antibody gene pool into the phagemid vectors, without need of individual identification of each gene, allows effortless construction of diverse and rich collection of antibodies. Direct linkage between phenotype and genotype allows isolation of antibody genes based on their products function. Presentation of an antibody or foreign peptide on the surface of the phage is usually achieved by genetic fusion of nucleic acid sequence of the desire peptide to the sequence of one of the bacteriophages coat proteins. In this way, isolation of a bacteriophage, which expresses the antibody, results in isolation of genetic information of that antibody as a single phage package. In phage display technique, the bacteriophage is used as an expression system that carries within the nucleotide sequence of a peptide expressed on its surface and in

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addition, it has the capability to replicate and release from the host bacteria [18].

Filamentous phages that infect the bacterium *Escherichia coli* are frequently used for this purpose [19]. One of the most popular applications of phage display is antibody isolation and engineering. This technique allows creation of large antibody libraries consisting up to 10^{12} unique members [20, 21].

Screening for antibodies from a phage display library is a multiple step process and after each step, antibodies that show highest affinity are chosen for amplification and the next round of panning. This can be achieved by increasing the washing solution stringency in sequential rounds, so while tight binders are retained, phages with lower affinity are removed. After these panning processes only antibodies with highest affinity toward a selected target are remained, thus characterization of all antibodies in the library is not necessary. After the last round of panning, few clones can be isolated and characterized. These selection steps allow fast and easy isolation of monoclonal antibodies.

The phage display technique has several advantages over conventional hybridoma techniques. Hybridoma technique is based on immortalization of lymphocytes [22] while phage display relies on isolation of lymphocyte and cloning of genetic material encoding immunoglobulins antigen binding region [23]. In phage display technology, researchers can choose a host for immunization based on the immunological traits of the organism and characteristics of their antibodies and even choose different classes of antibodies, while hybridoma technique is limited to the mouse antibodies [24]. Because some proteins are naturally tolerated by the mammalian immune systems and are not immunogenic in mice, the application of hybridoma technique could be restricted. Phage display allows cloning of immunological repertoire of the host of choice and screening of the library to select the antibodies with the most affinity and specificity to the target. The only obstacle in constructing a phage display library is that the desired immunoglobulin gene sequence must be known in sufficient details to allow design of primers and amplification of selected genes.

Another advantage of phage display libraries is the choice of using immune or non-immune (naïve) libraries [25–28]. The naïve libraries are driven from the host immunological repertoire without the immunization step and in this way, they are considered antigen independent [29]. These universal libraries can be panned against various antigens [30]. This allows selection of antibodies against haptens or antigens that are tolerated by the host immune system [31]. Furthermore, many antigens undergo metabolic changes such as dephosphorylation, reduction of cysteine bonds, or degradation in the biological environment and these could result in lack of specificity in the produced antibodies [15, 32]. Additionally, recent studies showed that naïve libraries are more reliable sources for selection of antibodies against cell surface antigens as the host tolerance mechanisms remove antibody clones that react to the cell surface proteins [31].

Different bacteriophages can be used in the phage display technique. Although T4, T7, and lambda phages have been used as a display vector, the most used bacteriophage is the M13 filamentous phage [33–37]. Based on each bacteriophage and even their coat proteins, many cloning vectors have been developed [38–40]. Here, we briefly review these bacteriophages biology and their application in phage display technology.

Filamentous Bacteriophage

The M13, f1, and fd are called Ff phages because of their filamentous appearance and dependence on the F pilus for infection [41]. Filamentous phages are rod shape long (900 nm long and 7 nm width) viruses that carry a single-stranded (ss) DNA genome [42]. Unlike lytic phages which assembly is completed in the cytoplasm and progeny phages are released by cell lysis, Ff phages are assembled in the periplasmic environment and then secreted out of the bacterial cell without killing the host. An infected bacterium with a Ff phage can continue living, but due to utilization of the bacterium machinery by the phage, the growth rate significantly decreases [42, 43].

Filamentous phages unique characteristics make them appropriate vectors for phage display applications. Because the Ff phages only infect strains of *E. coli* that express the F pilus, infection conditions can be controlled, and because the F pilus is depolymerized after the infection, each bacterium can only be infected by one phage [44]. Therefore, each bacterial clone represents one specific phage which codes a unique peptide or antibody. Additionally, the insertion of foreign sequence within the phage genome is not size dependent, and insertion of relatively larger sequence simply results in production of longer phage particles. More importantly, filamentous phages are resistant to extreme conditions such as acidic pH, high temperatures, and enzymatic cleavage [19]. Hence, they are adaptable to panning process and even in vivo applications. Among filamentous phages, the M13 bacteriophage has served as a reliable display platform in phage display technology [44].

M13 Bacteriophage Biology

The M13 DNA is coated by major coat protein pVIII which has 2,700 copies in the mature phage and has 50 amino

acid residues. The pVIII is coded by a single gene with the same name (gVIII). The minor coat proteins include pIII and pVI at one end and pVII and pIX at the other end of the phage. There are four or five copies of each minor coat protein on the phage [42]. The process of infection begins with the attachment of pIII to the F pilus and insertion of the ssDNA into the host cytoplasm. Consequently, coat proteins dissolve into the cell envelop. The phage genome (positive strand) uses the host machinery to synthesize the complementary DNA strand (also known as the negative strand) and form a double-stranded (ds) DNA known as replicative form (RF). The RF serves as a template for RNA and protein synthesis, production of progeny RFs and ssDNAs that are used in production of phage particles [45]. Phage genome consists of two coding regions. One with a powerful promoter that codes proteins that are required in high numbers like pVIII, and a second coding region that contains less efficient promoter and codes proteins that are needed in fewer amounts [42]. All five coat proteins are inserted into the cell envelop after synthesis. Signal peptides of pIII and pVIII are removed upon insertion into the inner membrane, but proteins remain attached to the membrane through hydrophobic interaction [42].

Phage genome replicates in a rolling circle form. The pII protein nicks the ori site in the positive strand, and the exposed 3′ end serves as a primer for DNA polymerase. After completion of replication, the pII ligates both ends to form a close circle. Production of RFs from ssDNA continues until sufficient numbers of pV protein are expressed. These ssDNA binding proteins accumulate on the positive strand and prevent the RF production. Phage secretion involves ATP hydrolysis and the virions are exported through the membrane channels created by pI and pIV complex. As the virions are exported, the pV is replaced by major coat protein VIII, and other minor coat proteins attach to each end and create a complete phage [46].

Choice of Coat Protein for the Fusion Partner

All five M13 coat proteins have been used as a fusion partner [47–49]. Depending on the size of the protein to be displayed and the number of copies that are needed to be expressed at the phage surface, the choice of coat fusion protein may differ. Given that there are four or five copies of pIII protein per phage, the maximum amount displayed pIII fusion proteins cannot exceed five copies per phage. If the displayed protein is needed in higher copies, pVIII fusion partner is recommended. Given that there are 2,700 copies of pVIII per phage, its fusions are suitable for tests such as avidity assessment, protein–protein interactions, or immunological assays. Although it is expected that all pIII coat proteins present the fusion partner on the phage surface, on average only one or two coat proteins display the fusion. Therefore, for experiments such as antibody isolation, use of pIII fusion is most appropriate and it results in selection of high-affinity binders in the panning processes. On the other hand, antibody fusion with pVIII results in selection of high avidity but possibly low-affinity binders [50].

As mentioned previously, M13 phage assembly is completed in the periplasmic environment and all coat proteins are inserted in the bacterial outer membrane prior to phage assembly, hence, fusions that disturb the export process of the coat protein are not included in the mature phage structure [51]. Unlike pVIII, pIII membrane insertion is sec (member of *E. coli* secretion system) dependent, thus it is more likely that large fusions to pIII are exported into the periplasm more easily than large pVIII fusions [52, 53]. However, this limitation could be resolved by using lytic phages such as T7 or Lambda as will be described later, these phages assemble entirely in the cytoplasm. Studies showed that Lambda phages are capable to express and display large fusion proteins in high densities [54]. Combinations of different phage types in one experiment could also be advantageous. In a research by Castillo et al. in 2001 [55] for ScFv selection, target peptides were displayed on the surface of T7 bacteriophages, and ScFv antibodies were displayed on the surface of M13 bacteriophages. This technique eliminates expression and purification processes and allows the target protein to have native folding and conformation in the panning steps.

The experiment objective has a significant influence on the selection of a coat protein as a fusion partner. However, size and characteristics of the fusion protein may interfere with the phages stability or viability [48, 49]. In the recent years, development of new variants of coat proteins and even entirely new artificial ones have opened new options and reduced limitation of M13 phage display [49].

Types of M13 Phage Display Systems

M13 phage display is categorized based on the coat protein which is used as a fusion, expression of coat protein as a fusion and/or wild form and vectors being used [43]. Examples of these systems are, but not restricted to, type 3, 33, 8, or 88. For example, in type 3 phage display, the gene of interest is inserted downstream of the pIII gene in the M13 genome. Therefore, all the pIII proteins are expressed in the recombinant form and carry the fusion protein, thus the display rate is high. However, this could be a problem in large proteins and causes delay in the phage assembly and disturbs the arrangement of the pIII on the phage particle which leads to reduction of phage infection rate. The absence of pIII at the time of phage assembly causes

production of polyphages. Polyphages are long phages that carry two or more genomes. Polyphages are naturally produced in the wild population of Ff phages and comprise 5 % of the produced phages [56, 57]. Similarly, in type 8 system, there is a single phage vector that carries a single pVIII gene which is fused with the inserted gene. In this case, all progeny phages carry the recombinant pVIII coat protein. This system is mainly used for display of small peptides no longer than eight amino acids. Displaying large peptides in fusion with pVIII without providing a wild-type version of the coat protein causes stereochemical interference. This disturbs the arrangement of the pVIII around the genome and prevents the phage assembly [16, 43].

Type 33 and 88 phage display use the same single phage vector but with two copies of pIII or pVIII gene, respectively, and only one of the copies is used as a fusion partner. In this way, progeny phages have two types of the coat protein, the wild type and the recombinant type. This approach resolves the drawbacks mentioned in type 3 and type 8 systems and enables display of larger peptides without disturbing the phage stability.

The difference between type 33 or 88 with type 3+3 or 8+8 is that the two copies of the coat proteins are separated into two genomes. The coat protein that serves as a carrier of the recombinant protein is coded by the phagemid; a plasmid that in addition to the bacterial origin of replication also carries filamentous phage origin of replication and an antibiotic resistance gene that facilitates selection. The wild-type version of the coat protein is coded by a defective phage called "Helper phage" [16]. In this method, production of recombinant phage particles is triggered by infecting the phagemid-bearing bacteria with the helper phage. Helper phage provides the necessary proteins that are required for phage assembly. These proteins also act on the phagemid ori and produce phage particles carrying phagemid genome. Two different coat proteins are presented by this approach, one coded by the phagemid and is fused to the recombinant protein, and a wild type coded by the helper phage. Also, two types of phage are secreted from the infected bacteria, one carrying the phagemid genome and another one carries the helper phage genome. Either phage carries both the wild-type and the recombinant coat protein. Since the helper phage and the phagemid are carrying different antibiotic resistance genes, selection of the phage particles carrying phagemid genome in the subsequent panning steps is archived by antibiotic selection. Therefore, only the bacteria infected by the phagemids are able to propagate [16, 58].

For reducing the background amount of helper phage and increasing the production phage particles carrying phagemid, usually the helper phages ori or packaging signal is defected by the mutation. Hence, replication and packaging of helper phage genome are far less efficient than the phagemids, thus contamination with helper phage can be reduced up to 1/1,000 [23, 39, 58]. In addition, to enable superinfection of the phagemid carrying bacteria in the 3+3 systems, the pIII gene of these vectors lacks the N-terminal domain [39, 58].

In hybrid systems, both the wild-type and the recombinant coat proteins are produced, as a consequence the display of larger proteins is possible. On the other hand, copy number of recombinant coat proteins displayed on the phage surface is decreased. By employing engineered helper phages, these issues can be resolved. Several helper phages have been developed that lack one of the coat proteins but code all the necessary proteins for phage replication and packing, for example helper phages with deletion or optionally untranslatable gene III [59–62].

Another way to reduce helper phage contamination after the selection rounds is to insert a protease cleavage site in the pIII protein of the helper phage. In this way after elution of the capture phages by use of proteases such as trypsin, the N-terminal domain of helper phage pIII will be removed. Since the N-terminal domain of pIII is necessary for phage infection, helper phages that lack this domain are no longer infectious [63, 64].

New methods of M13 phage display are now available, for example, HuCAL® GOLD uses a unique display method called CysDisplay. In this method, the fusion protein is not genetically fused to the coat protein; instead the protein is attached to the phage in post translation modification by formation of a disulfide bond to the specially engineered pIII coat protein. Thus, the elution step in panning process can be accomplished by addition of reducing agents. This could resolve the elution of ultrahigh affinity antibodies that cannot be eluted by conventional methods [15].

T4 Bacteriophage

Unlike M13, the T4 bacteriophage uses dsDNA genome. T4 genome is completely sequenced and contains 168895 bp, which is considerably larger compared to other phage display platforms such as T7 (39937 bp), Lambda (48502 bp), or M13 (6407 bp) [65–67]; Kutter). The larger genome size could be advantageous and allows insertion of longer sequences. T4 bacteriophage is structurally divided into two major parts, the head and the tail.

T4 Structure

The head has icosahedral structure and is mainly composed of three coat proteins, gp23, gp24, and gp20. The major capsid protein 23 or gp23 weighs 48.4 kDa and has 960

copies arranged in hexamer patterns [68]. The gp24 has 55 copies, weighs about 46 kDa and exists in pentamers while gp20 has 12 copies and exists in dodecamers. The round appearance of T4 head achieves by pentamers of gp24 that are inserted among gp23 hexamers to form angles [68]. There are two non-essential proteins coating the gp23 hexamers. SOC or small outer capsid protein is only 9 kDa and bonds the hexamer corners. Each SOC can bind to two gp23 subunits. This protein encircles the gp23 hexamers and occupies all six corners. However, when gp24 pentamers are neighboring the gp23, SOC proteins only cover the gp23 interface avoiding the gp24 protein. SOC proteins are unable to bind the gp24 or gp23 that neighbors the gp24 therefore, they only attach to gp23/gp23 interface [69]. Some studies even suggest that SOC–gp23 interaction is favored over SOC–SOC interaction [70]. Although SOC proteins are not necessary for the T4 viability, it is assumed that SOC–gp23 interactions could form a protective layer around the head and protect the phage in harsh environments such as alkaline pH, presence of detergent, or high temperatures [71].

Highly immunogenic outer capsid protein (HOC) is another non-essential T4 capsid protein. This 39.1 kDa protein is located at the center of gp23 hexamers and extends away from the surface causing protrudes in the phages head. The HOC consists of three domains, a round base, a thin stem, and a globular head [71].

Interaction of the T4 bacteriophage with the host and injection of its DNA to the bacteria cytoplasm are carried out by the tail section. The tail is composed of two concentric protein tubes. The inner tube includes 144 copies of gp19 and serves as a channel for transferring the virus DNA into the cell upon injection. The outer tube or tail sheath is constructed of 144 copies of gp18 and is contractible. When the outer sheath contracts, it shortens to a third of its original length, but the inner tube length remains the same and penetrates the bacterium membrane and provides a channel for DNA transfer [72].

Baseplate and fibers are at the end of the tail. The baseplate consists of several proteins and forms a hexagonal platform. The gp9 and gp12 tetramers keep the hub at the center of the baseplate. The baseplates hub is formed by gp5, gp27, gp29, and probably gp26 and gp28 [71]. The lysozyme activity of gp5 is necessary for membrane digestion and infection. There are two types of tail fibers on the T4 bacteriophage. The long tail fibers (LTFs) are used for recognition of certain receptors on the bacterial surface. After the LTF interaction, the baseplate structure changes and tail sheath contracts which results in the injection of DNA in the bacterial cytoplasm. The short tail fibers (STFs) are used for stabilizing and binding of the phage to the bacterial surface during the infection process [73].

T4 Phage Display

T4 phage display relies on employing two dispensable capsid proteins, HOC, and SOC. Given that there are about 155 copies of HOC and 810 copies of SOC on the capsid, bacteriophage T4 provides a rich-binding platform for displaying foreign peptides or proteins. Additionally, in filamentous phages such as M13, foreign peptides that are fused to the coat proteins must undergo membrane excretion, which is considered a drawback in large fusions and limits the insertions size and also brings toxicity problems to the host and could affect protein folding [39]. Assembly of lytic phages such as T4 occurs within the cytoplasm, thus avoid those problems and enables fusions of large complexes on the phages surface.

SOC's size (9 kDa), position, and characteristics make it a suitable vehicle for phage display. SOC is a non-essential protein and its absence does not change the phage morphology or infectability, and it binds to mature phage with high affinity [74]. SOC proteins are also able to attach to the polyheads, which are non-mature capsids [75]. Moreover, SOC is easily restored after chemical or thermal denaturation and regains full binding capability [76]. This suggests that SOC fusions could also be restored after purification from bacterial inclusion bodies and then be used for decorating the phage capsid. T4's HOC proteins share the same characteristics. Proteins have been fused to both C and N-terminal of SOC and HOC proteins [77].

As mentioned before, fusion to C or N-terminal of SOC and/or HOC proteins is used in T4 phage display. For this, first the foreign sequence is genetically fused to one or both proteins separately and expressed in the bacterial host. The recombinant proteins are then purified from the bacteria and used to decorate the soc^{-}/hoc^{-} T4 bacteriophages in a controlled environment. In the T4 phage display, fusion proteins are added in vitro to the T4 phages lack the HOC and SOC proteins, and unlike filamentous phage, binding occurs in more controlled conditions, thus it could be modified to reach optimum results. Since SOC/gp23 interaction is performed over SOC/SOC interaction, the attachment of recombinant SOC to the page capsid is usually achieved at high rates [77].

T4 phage display holds a unique advantage over other bacteriophage display methods. T4 phage display enables presenting sizeable and complex proteins in high-copy numbers at the surface of the bacteriophage. Several large proteins such as *E. coli* β-galactosidase enzyme with 116 kDa [78] or anthrax toxin weighing 710 kDa [79] have been successfully expressed on the T4 bacteriophages surface in high-copy numbers. The former has also been expressed in λ and T7 bacteriophages [78]. The expression of β-galactosidase fused to gpV coat protein of λ phage which has 192 copies per phage or fusion to gp 10 of T7

bacteriophage with 415 copies per phage resulted in display of only one copy of the fused protein per phage. Fusion to the λ gpD which has 420 copies per phage, resulted in display of 34 copies of fused protein. However, no other report has adapted this system for phage display applications [78]. Li et al. [78] have reported expression of anthrax toxin complex consisting of three components; protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa), and edema factor (EF, 89 kDa), with a total size of about 710 kDa on the T4 bacteriophage SOC and HOC proteins. The T4 system allowed them to display up to 229 toxin complexes, equivalent to a total of 2,400 protein molecules per capsid particle. The displayed protein mass expressed at the phage surface reached up to 133 mega dalton per phage particle. Expression of such complex molecules at the phage surface has a great advantage, as some immunological processes require complete proteins rather than epitopes or chosen domains which lack the native folding and could skip conformational epitopes.

T7 Bacteriophage

Similar to the bacteriophage T4, T7 has a head and tail structure. The head is icosahedral and consists of gp10 proteins [80]. T7 tail is short and not contractible, so in order to inject the genome into the bacterial cytoplasm, the virus builds a protein channel from its tail to the bacterial cytoplasm [81]. Tail fibers are used for attachment and in some strains of the bacteriophage, they possess enzymatic activity that cause the lysis the bacterial membrane [82]. The viruses 39937 bp dsDNA is trapped inside of 415 copies of T7 capsid proteins, encoded by gene 10. There are 60 hexamers of gp10 proteins on the bacteriophage capsid; the corners are shaped by 11 copies of gp10 pentamers [83]. The tail is attached to the capsid by gp8 or the head–tail connector protein. Short-conical tail is formed by gp10, gp11, and six tail fibers that are shaped by gp17 proteins. The DNA replication is similar to that of T4 bacteriophage [84].

T7 bacteriophage has unique features that are useful for a phage display vector. The lytic life cycle of the bacteriophage allows the assembly process to occur within the bacterial cytoplasm. The independency from membrane secretion expands the choice for fusions to be displayed and removes the limitations of the M13 in display of globular or hydrophobic fusions. Additionally, the T7 bacteriophage has a shorter life cycle than lambda or filamentous phages. The plaque forms within 3 h at 37° C and the culture lyses 1–2 h after infection [85]. This significantly decreases the time needed for performing multiple rounds of selection in a phage display screening process. Furthermore, the T7 bacteriophage is very resistant to various environmental conditions and can endure conditions that inactivate other bacteriophages.

T7 Phage Display

The T7Select® phage display system is introduced by Novagen and employs the capsid protein gp10 for display of foreign peptide and proteins. Natural translational frameshift in the gp10 gene results in two forms of gp10 protein, 10A with 344 amino acids and 10B with 397 amino acids. Although functional capsid could be made from each protein, both proteins are found in the wild-type T7 mature capsid. 10B protein is codes from the same mRNA that produces the 10A protein, however, it translates with a ribosomal shift at amino acid 341. 10B composes up to 10 % of the capsid proteins. Novagen T7Select® system uses a surface region of the 10B protein for phage display proposes. To achieve this, the natural translational frameshift site of the gp10 gene has been removed so the vectors express only one type of capsid proteins. The multiple cloning site has been inserted after the amino acid 348 of the 10B protein.

There are three types of T7Select® phage display vectors made by Novagen. The T7Select415 vectors have the ability to display proteins up to 50 amino acids in high-copy number of 415 copies per phage. The T7Select10 vector with mid-copy number can display 5 to 15 copies of proteins with up to 1,200 amino acids and the low-copy number vector T7Select1 can display 0.1 to 1 copies of protein with 900 amino acids (up to 1,200) in length [86].

Lambda Bacteriophage

The structure of Lambda phage is similar to those of T4 and T7 bacteriophages. The phage contains head, tail, and fibers. The icosahedral head is composed of 415 copies of major coat protein gpE and 405–420 copies of major coat protein gpD [87]. The tail is an assembly of 32 disks, and consists of hexamers of major tail protein or gpV [88].

Lambda has a double-stranded linear DNA which is 48490 bp long. Lambda genome has 12 nucleotides single-strand extensions at both 5′ ends, which are complementary to each other. These GC rich sticky ends are referred as cos sites or cohesive ends and after infection are ligated together by the host DNA ligase to form 48502 bp long circular DNA [33].

Lambda Life Cycle

The infection begins with attachment of the tail J protein to the *E. coli* lamb protein. After injection of the phage DNA

into the cytoplasm, cos sites are ligated together by the *E. coli* DNA ligase. Negative supercoils are formed by the DNA gyrase and force the AT-rich region of the phage circular DNA to unwind; this signals the start of transcription [89]. However, after infection, lambda can enter the lytic or lysogeny lifecycle. Continuous replication and packaging of the phage genome are followed by lysis of the host bacterium and release of progeny phage particles. This form of propagation results in cell lysis and called lytic cycle. Alternatively, in the lysogeny mode, phage genome can integrate into the bacterial chromosome and propagate along with the host. In this case, the host cell is termed lysogeny and the phage is called the prophage. Prophage does not kill the infected bacteria, but under certain conditions such as DNA damage, the lysogeny cycle can change into a lytic cycle and results in mass production of phage and cell lysis [90].

Replication and Genetic Structure

The lambda genome has three coding regions. Each region encodes groups of genes that are responsible for one of the phages functions. The left arm gene cluster codes proteins that pack the phage DNA into the bacteriophage head. The central domain contains genes that start the lysogenic life cycle and establish persistent lysogenic mode, and the right arm genes are mostly responsible for the phage replication and the lysis cycle of the bacteriophage [33].

Lambda DNA replication starts with a θ (theta) replication. After *E. coli* DNA gyrase forces negative supercoils on the phage circular genome, the AT-rich region in the *o* gene unwinds and serves as the origin of replication. The replication is carried out by the host machinery. Then, rolling circle replication replaces the theta replication. In the rolling circle replication, the ori is nicked and the 3′ end serves as a primer for DNA polymerase. Unlike other rolling circle replications in which after one round of replication, the nicked ori is rejoined to form a daughter circular DNA, in lambda replication the polymerization is continued to form a single DNA molecule containing multiple copies of phage genome called concatemer [91]. These concatemers are cleaved at the cos sites while packaging into the bacteriophage head. Packaging and cleavage of concatemers into the bacteriophage head is based on the length of DNA, and sequences less than 38000 bp and longer than 53000 bp are not properly packed. Genomes with length greater than 105 % or less than 78 % of the wild-type lambda DNA reduce the phages viability. Hence, cloning of large insertion in the wild lambda genome is not possible [92].

As mentioned before, the central domain of lambda genome contains genes that are not essential for lytic cycle and can be removed to enable insertion of longer sequences. Deletion of the central region does not affect phage viability or infection, and mutants that lack this region are infectious and viable. Since deletion of non-essential genes reduce the length of genome below the minimum packaging range, the difference must be replaced with the insertion sequence. This only allows packaging of the phages that contain the insertion sequence and genomes that lack the insertion are not assembled into the progeny phages.

Lambda Phage Display

Both the head major coat protein gpD and the tail major coat protein gpV have been used as a fusion partner in lambda phage display [23]. Earliest lambda display vectors used gpV protein with the truncated tail to display the foreign peptide. The foreign peptide replaced the last 70 amino acids on the C-terminal of the gpV protein [93]. Although gpV fusions successfully present the foreign peptides and have been used for isolation of specific antibodies in lambda libraries, they have some limitations. Poor display rate of the fusion proteins is one the limitations of this system. Fusion to gpV results in expression of only few recombinant proteins per phage and yields in low-recovery rate in panning process. On the other hand, in antibody libraries, low-copy number of displayed antibodies results in isolation of high-affinity binders [33].

For higher display rates, gpD protein can be used as a fusion partner. The gpD weighs 11.4 kDa and has up to 420 copies on the phage capsid [94]. Although gpD is an essential protein, fusion to C-terminal or N-terminal dose not interferes with the phage assembly, viability, or infection. Because the gpD has a small size, both C- and N-terminal fusions are accessible and can interact with ligands or receptors [95]. This display system has a very high-display rate that can reach up to 90 % of the gpD copies [96]. In this case due to morphological interference, large proteins are preferably displayed using a two-gene system. In a two-gene system, two copies of gpD protein are presented into the phage genome, while one is used as a fusion partner, and the other copy expresses the wild-type of the gpD. This reduces any morphological difficulties and enables expression of several hundred copies of fusion protein at the phage surface.

Applications of Phage Display Technology

Phage display is a high-throughput method that facilitates protein and antibody selection. Although selection of proteins is the main feature of this technology, it has been modified to function in other aspects of biotechnology such

as vaccine development, protein interactions, determination of enzyme specificity, and epitope mapping.

Vaccine Development

Phage display is a powerful technique for vaccine design and development. Phage display has been used to construct and design vaccines for many diseases [97]. Unlike, subunit vaccine that mainly induces humoral immune response, phage-based vaccine is able to induce both cellular and humoral immune response [97, 98]. Because bacteriophages are not infectious for humans, phage display vaccines are considered to be safer than other viral vaccines, therefore, more suitable for vaccine development [97]. Recently, phage display technology has been employed to produce vaccines for cancer [99–101]. Tumor associated antigens are expressed on the phage surface to induce tumor-specific immune response. Previous studies have suggested that co-expression of pIII protein of M13 phage with a scFv fragment can enhance T_{H1}-mediated response and induce specific humoral and cellular response [102].

Protein–Protein Interaction

The main application of phage display method is investigation of protein–protein interaction. Many of the cell proteins function as a complex multimer protein that is assembled from several subunits. One of the most effortless methods to investigate interaction of different proteins is the phage display technique. Different domains of each protein can be used to construct a phage library. These libraries can be panned against a specific protein or each other to discover domains involved in protein–protein interaction.

Selection of Substrates and Inhibitors

Phage display facilitates analysis of enzyme activity and specificity. Mutant stains of an enzyme can be expressed on a phage display vehicle and be used to investigate activity levels at a particular condition, such as pH or temperature [103]. Filamentous phages are resistance to wide ranges of pH, temperature, and protease cleavage and are appropriate for these studies [19]. Enzyme specificity can also be investigated using phage display method. Phage libraries can be prepared from different substrates and panned against an enzyme to isolate substrates. Same method can be applied to isolate enzyme-specific inhibitors [103].

Epitope Mapping

Phage display is widely used in epitope mapping [104–106]. Epitopes recognized by an antibody can easily be identified using random peptide libraries. Random peptide libraries are constructed from large numbers of peptides that are varied in length and sequence. The exact amino acid sequence recognized the antibody can be identified through few rounds of panning.

Selection of Appropriate Vehicle

Phage display is a fast, reliable, and easy method to construct large peptide libraries and provides a straightforward approach to screen those libraries for isolation and propagation of unique clone with desired characteristics. As described above, many bacteriophages have been used in this technique and each one has their advantages and weaknesses. Basically, M13 phage display is the most used among other systems. M13 various coat proteins provide comprehensive display choice that can be used to display variety of peptides with distinctive characteristics. The pIII or pVIII is the preferred choice for M13 phage display. pVIII with 2,700 copies per phage is usually used for high-density display of peptides, but as the phage's major coat protein that layers the M13 DNA, it cannot endure large fusions that cause functional limitation, and peptides with more than eight amino acids interrupt the phage assembly. To overcome this barrier, pVIII fusions are used in presence of the wild-type pVIII. This allows display of larger fusions without disturbing the phages integrity. pIII fusion is the choice of phage display antibody selection and enables display of larger proteins but with less copy numbers. Less copy number allows selection of antibodies with more affinity. Major shortage of the M13 phage display rests in the bacteriophage life cycle. Although a non-lytic life cycle of bacteriophage ensures production of large progeny and facilitates handling, it also requires secretion of the coat proteins though the bacterial envelop. Therefore, fusions that prevent such process due to their conformation or hydrophobicity are not suitable for M13 display. Alternatively, in lytic phage display, only proteins that have an impact on the phage assembly are not tolerated. Disadvantages of lytic phage display are their large genome sizes that intricate handling. Because disulfide bonds are only formed in the bacterial periplasm (as in M13 assembly), incapability of formation of disulfide bonds also impacts the fusions folding in lytic display. In lytic phage display, the copy numbers of fusion proteins are varied in each phage particle, which affects the sensitivity of the assay (Table 1).

Although many display systems have been developed, M13 phage display represents the first choice among different phage display systems. Other display systems, as powerful as they are, are only employed when M13 phage display fails, and as mentioned in a review by Zoltán

Table 1 Different phage display vehicles and their characteristics. Number of coat proteins that can be used as a fusion partner, and their display capacity varies in each bacteriophage. The life cycle of the bacteriophage also plays an important role in the selection of the appropriate vehicle

	M13	T4	T7	Lambda
Genome size	6407 bp	168895 bp	39937 bp	48502 bp
Highest displayed size	>110 kDa on pIII [107] and less than 10 kDa on pVIII [108]	710 kDa	132 kDa	116 kDa
Displayed on protein	All coat proteins, but usually; pIII or pVIII	SOC and HOC	gp10 (10A or 10B)	gpD or gpV
Display density	Up to 5 copies per phage on pIII and up to 2700 on pVIII	Up to 810 on SOC and 155 on HOC	Up to 415	Up to 420 for gpD and up to 32 on gpV
Lysogeny or lytic	Lysogeny	Lytic	Lytic	Lytic and lysogeny

Konthur and Reto Crameri (2003), "the different display concepts should be regarded as complementary."

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