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An Overview of Filamentous Bacteriophages for Phage display and its potential clinical applications

Dhawan Samriti*

Department of Biotechnology, Goswami Ganesha Dutta SD College, Chandigarh, India

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ABSTRACT

Viruses are a transition between living and non-living organisms. Their biological machinery is tailored in collaborating with cellular organisms to very effectively reproduce themselves. This inherent potential of hi jacking the transcription and translation machinery of the cell, and picking up and moving about DNA from the organisms with which they interact is exploited by the biologists. Bacteriophage is a kingdom of viruses that infect bacteria, and filamentous bacteriophages which are made up of long, thin filaments containing a circular single-stranded DNA genome which reproduce without killing the host, belong to genus *Inovirus*. This review article is an overview of filamentous bacteriophages, various vectors developed for use in the molecular biology and gene cloning. However, the major emphasis of the review is on their use in phage display technology. Although selection of proteins is the main feature of this technology, it has been modified to be utilized for diverse applications. A comprehensive detail of all the applications is beyond the scope of this review, so recent developments using phage display in cancer diagnosis and therapy are discussed in this article.

* Corresponding Author

Name: Dhawan Samriti
Phone: +91-9417095951
Email: samriti.dhawan@ggdsd.ac.in

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INTRODUCTION

Modern virology began about a century ago when two bacteriologists, Twort, and d'Hérelle independently in 1915 and 1917 respectively discovered the existence of bacteriophages as filterable bacteriolytic agents. Since then pioneering studies to elucidate the structure and physiology of host/phage interactions are done by various scientists (Adams, 1959; Cairns *et al.,* 1966). These studies proved to be the basis for development of various molecular biology and genetic engineering techniques/methods to utilize the cryptic potential of bacteriophages, but for welfare of mankind. Bacteriophages have been extensively exploited as vectors for gene manipulation, cloning and gene transfer. Some of the most widely used viruses include bacteriophaghe λ (used in molecular cloning, genomic library), P1 (as an alternative to cosmid and YAC for cloning large fragments) and Ff (Westwater, 2004). Filamentous bacteriophages are getting enormous amount of attention due to their potential to be used as antibacterial agents, phage display systems, and vehicles for vaccines delivery. Even phage therapy is coming up as one of the possible solution for increasing antibiotic resistance among bacteria (Kutateladze, 2010). Previously, bacteriophages have been reviewed for various clinical and therapeutic applications (Clark and March, 2006). Major developments in DNA engineering has led to the development of phage display technology (Bazan *et al.,* 2012; Rakonjac *et al.,* 2011). This update will provide a brief overview on role of filamentous bacteriophages in the use of phage display technology and its role for discovering novel anti cancer peptides reported in recent years for diagnostic and therapeutic potential.

Filamentous Phages

Filamentous bacteriophages (family Inoviridae) are long and thin filaments containing a single stranded DNA molecule. It is shaped like a long fibre about 6 nm in diameter by 900 to 1,900 nm in length; its length reflects the size of their genomes. Filamentous bacteriophage can infect a wide range of Gram-negative bacteria, including *Escherichia*, *Salmonella*, *Pseudomonas*, *Xanthomonas*, *Vibrio, Thermus* and *Neisseria* (Hyman and Abedon, 2010). Among *E. coli* filamentous phage, M13, f1 and fd, are most extensively studied. These bacteriophages use the tips of the F conjugative pilus as a receptor and thus are specific for *E. coli* containing the F plasmid, so generally called Ff- {F specific filamentous viruses}. Infected cells continue to grow and divide and extrude virions. Phage DNA is replicated via a double stranded intermediate form called replicative form (RF) and is replicated and transcribed to produce viral proteins. The build-up of viral protein II eventually forces asymmetric DNA replication of RF to produce single DNA strands. These are packaged into new viral particles, which are extruded from the bacteria up to 1000 particles per cell per generation without lysing the host cell. The switch between the double-stranded RF form and the single-stranded + form of the M13 viral genome made it an ideal candidate for exploitation as a vector. Many different filamentous phages have been described till date as per 10th report of (ICTV-International Committee on Taxonomy of Viruses, 2017).

As single stranded vectors, these have been developed as cloning vectors (Russel, 1991), because of advantages over other vectors. Single stranded DNA is required as starting material for many applications of cloned DNA like for sequencing by Sanger's method, for site directed mutagenesis and for certain methods of probe preparation. If purified in double stranded form (RF), it can be manipulated like a plasmid, can be transfected in *E.coli.* to yield either plaques or infected colonies. The major difficulty with M13 vectors is their instability when larger DNA fragments (few kB) are cloned into them (Russel, 1991). This short coming of M13 phage was overcome by building a hybrid vector i.e. Phagemid (Zinder and Boeke, 1982).

Phagemids themselves are small plasmids that has the ability to accept larger DNA inserts (10-20 kb) than M13-based vectors. It contain the pUC (Col E1) origin of replication as well as M13 phage origin of replication and a modified version of major intergenic region (this region encodes no protein but contains all the *cis*-acting sequences which are required for viral DNA synthesis). The M13 replication origin was not sufficient to direct single-stranded DNA production, but if a bacterium carrying a phagemid was superinfected with a helper phage, then the production of single-stranded phagemid DNA would occur. The helper phage (such as M13KO7 or VCSM13) has a slightly defective origin of replication (inserted origin of replication of plasmid p15A and a kanamycin resistance gene), and also has a mutation in the gene II, but can supply all the structural proteins required for generating a complete virion. When cells harbouring these phagemids are infected with a suitable helper phage, the mode of replication of the phagemid changes under the influence of the gene II product of the incoming helper virus. Interaction of the intergenic region of the phagemid with the gene II protein initiates the rolling-circle replication to generate copies of one strand of the phagemid DNA. The phagemid single-stranded DNA would be packaged into viral particles and secreted into the surrounding medium in the same way that M13 phage particles are produced (Zagursky and Brman, 1984).

Much of the acquired knowledge about filamentous phage replication and vectors derived from it has been exploited to derive many applications in the diverse areas of biotechnology (Abedon,2016; Atias *et al.,* 2008; Dalmasso et al). Due to their ease of manipulation filamentous phages can serve as reliable vehicles for combinatorial technologies, such as phage display (Smith, 1985).

Phage display

Filamentous phages are ideal for display because of their small genome, tolerance for insertions into intergenic regions, recovery of foreign DNA either as ssDNA or as ds DNA forms, choice of vectors as coat proteins can be modified with retention of infectivity and accumulation of phages to high titres as it does not lyse the host cell.

Phage display is a molecular technique used for display of foreign proteins and peptides on the surface of bacteriophages. M13 is the most commonly used phage for this work, though other filamentous phages f1 and fd are also used, as they share more than 97% sequence homology. The concept of phage display was first described by Smith, G. in 1985 (Smith, 1985). Since then it has become a powerful method for identifying proteins with novel properties and modifying the properties of existing ones.

It is used predominantly to study protein-protein interactions (Sambrook and Russel, 2001).

Novel ligands (e.g., antigenic determinant sites, antagonists, agonists, substrates) which have the

ability to bind to defined amino acid sequences were identified.

Antibody phage display libraries for affinity selection of peptides and monoclonal antibody were developed to identify human monoclonal antibodies with defined reactivity against specific targets.

Other examples of the uses of this technology include the use of recombinant bacteriophage displaying antigens from infectious disease agents as candidate vaccines (Clark and March, 2004), to confer immune responses against the encoded peptides or proteins. The ease of use, low cost, its safety and immunogenicity make this technique highly advantageous.

Phage display system is a result of powerful combination of affinity selection and biological amplification. Because filamentous bacteriophages can be exposed to ligand at concentrations as high as of 10^{13} particles/ml; a vast number of foreign peptides can be displayed on the surface of viral particles and surveyed for their ability to interact with a target ligand, library of phage particles expressing a wide diversity of peptides is used to select those that bind the desired target.

Although bacteriophages that bind to the target may be very rare in the original population they can be screened by repeated rounds of in-vitro affinity selection (biopanning) and phage propagation (Barbas, 2001).

Vector systems used for Phage Display*:*

M13 coat protein is made up of amazingly versatile proteins which pass through several distinct environments during its life cycle. The filament tube is formed of helically arranged (≈ 2700) copies of pVIII, a small α- helical protein of only 50 amino acids. The ends of the filament are built by two different pairs of proteins – The distal end, which is assembled first, consists of pVII-pIX and and the proximal end, which enters the host first consists of pIII-pVI. Peptides can be displayed on M13 phage in either a high- or low-valency format by fusing to either the gene-VIII major coat protein (protein-VIII, P-VIII) or the gene-III minor coat protein (protein-III, PIII), respectively. Minor coat protein (pIII) consists of 406 amino acid residues and occurs at the phage tip in 3 to 5 copies (Lopez and Webster, 1983) The peptides and folded proteins are displayed as fusions with pIII protein, as its functions in morpho-genesis are not affected by the presence of foreign peptide at its amino terminus, whereas to maintain the functionality of pVIII, it could be coupled only with short peptides. Six or more residues can disturb its conformation and prevent assembly of viral coat. The loss of coat protein functionality was the major limitation of the phage display technology; however this problem was overcome by hybrid phages.

In terms of the copy number of fusion proteins two types of phage display vectors have been developed:

Multivalent (or polyvalent) and paucivalent (or monovalent) (Lowman *et al.,* 1991; Lubkowski, 1998)

Multivalent display- Phage particles display peptides encoded by the foreign DNA on *all copies* of coat protein. It has been accomplished using bacteriophage display system vectors- a segment of foreign DNA is inserted either into gene III or gene VIII, a few nucleotides downstream from the cleavage site and recombinant DNA is transfected into *E.coli.,* secreting a population of virions. It is usually required for the initial screening of scaffold proteins, which have lower affinity. These vectors are designated as *type 3 or type 8.*

Paucivalent display- Phage particles display peptides encoded by the foreign DNA on only *some copies* of the coat protein. It is generated in two ways: bacteriophage vectors and phagemid systems with "helper" phages.

Phage vectors -If two copies of the coat protein genes (gIII or gVIII) i.e. native gene and fusion gene with foreign DNA insert reside within the phage genome, these vectors are designated as *type 33 or 88.*

Bacteriophage system: If two copies of the coat protein genes (gIII or gVIII) i.e. native gene on wild type phage genome and a copy of fusion gene with foreign DNA occur as an insert in a Phagemid, these vectors are designated as *type 3+3 or 8+8.* Moreover, the phagemid encoded polypeptide (gIII

Table 1: Types of Phage display Vector systems

Type of Vector	Coat protein for Display	No. of genes	Display on copies	Example
Type 3 (Phage)	pIII	1	All (Multi)	M13 KE
Type 8 (Phage)	pVIII	1	All (Multi)	F1
Type 33 (Phage)	pIII	2	Some (Pauci)	-
Type 88 (Phage)	pVIII	2	Some (Pauci)	F88-4
Type 3+3 (Phagemid)	pIII	2	Some (Pauci)	pCOMB3H
Type 8+8 (Phagemid)	pVIII	2	Some (Pauci)	pCOMB8

Adapted from Molecular cloning 3rd Ed. by Sambrook and Russell

or gVIII) fusion requires helper phage for packing into M13 particle.

Thus, both wild coat protein and polypeptide-fusion protein will be present on the phage surface.

Design and screening of peptide display libraries:

Each of the five Ff virion proteins have been used as platforms for phage display, but most commonly used are the minor protein pIII and the major protein pVIII. Both proteins in Ff phage have an essential N-terminally located signal sequence which is required for their targeting to the inner membrane. During translocation, the signal sequence is cleaved by signal peptidase, and the N terminus of the mature protein is localized in the periplasm (Rakonjac, 2005). Therefore, to be displayed on surface, proteins need to be inserted, in frame, between the signal sequence and the mature portion of these two proteins.

The design of random peptide phage display libraries involves the in-frame insertion of a short oligonucleotide into the M13 minor coat protein gene, gIIIp (gene III protein) or gVIIIp. Different sets of genes are inserted into the genome of multiple phages. As a result, the peptide is displayed on the surface of the bacteriophage and each phage will only display one protein, peptide or antibody. The phage remains infectious for *E. coli* (as the functional activity of the gIIIp remains intact). Whereas in the case of phagemid vectors, the vector is not infectious, since it requires the presence of a helper phage for replication and packaging. Typical peptide inserts range from 6 to 15 amino acids in length, and they may be either linear or cysteine-constrained. Linear inserts will assume a three-dimensional configuration (shape) that may be influenced by the coat protein residues immediately flanking the inserted peptide, while disulfide-constrained inserts will assume a more defined shape since they will be contained within an exposed, covalently closed loop on the surface of the phage particle. Inclusion of cysteine residues in displayed peptides reduces the infectivity of filamentous phages of constrained libraries, thus lowering the titer of display library (Makowski, 1993).

Advantages of using a phage display peptide library are numerous as they are inexpensive, commercially available, and easy to amplify and reuse, can be easily preserved at −80 °C for years. Importantly, phages can accommodate different peptide sequences. Peptide selection can occur under in vitro, ex vivo, or in vivo conditions. Phage libraries are tolerant of a variety of selection conditions and can endure harsh washing conditions.

Despite these many advantages, phage display is not without disadvantages.

The method is constrained for displaying complicated chemical structures, such as branched or bicyclic compounds, and is typically limited to naturally occurring amino acids; even d-Amino acids are traditionally difficult to incorporate. Moreover Small differences in growth rates during the amplification of the phage pool and amino acid and sequence biases existing in the library, further result in a decrease in library diversity (Russel *et al.,* 2004).

Phage Display applications

The premise behind the application of phage display technology is the process of *Biopanning*- A method for obtaining small number of phage clones specific for a ligand from initial large bacteriophage pool displaying peptides on its surface (each representing an individual peptide).

This method which was originally developed for affinity selection of proteins expressed from cDNA fragments later developed into an advancing technology. Phage display played instrumental role in improving our understanding of the protein-protein interactions in biological systems whether it's an enzyme substrate interaction, subunit domains of a multimeric protein involved in peptide interaction (Sidhu, 2001; Elena *et al.,* 2017), analysis of enzyme activity and specificity, novel enzyme inhibitors, protease cleavage sites, epitope mapping, peptide mimics of non-peptide antigens (Hamzeh *et al.,* 2013). It was also used for vaccine design and development (Gao *et al.,* 2010). The advantage of bacteriophages being unable to infect humans, is considered to be a safe option for humans than other viral vaccines.

Phage display based vaccine is reported to be able to induce both cell mediated and humoral immune response. The ability offered by phage display to correlate protein structure and function in a systematic way makes possible new methods of finding novel drugs and therapeutics.

In cancer therapy

Screening for many anticancer peptides or proteins is carried out by phage display in recent years (Table 2). Cancer cells exhibit certain distinct characteristics as compared to normal cells. Like enhanced expression of certain receptors, which allows for their uncontrolled proliferation and promotes metastatic processes (Carmeliet, 2005). This category includes growth factors and growth- factor receptors, as well as oncogene-encoded proteins.

Table 2: Summary of anticancer peptides discovered using phage Display

Phage Displayed Peptide (targeting ligands)	Target on Cancer cells/ Targeted Enzymes	Used in treatment	Inhibitory Effect	Refer ence
P12 FGF8b binding Heptapeptide	FGF8b Fibroblast growth factor 8b	prostate cancer	inhibition of FGF8b-induced cell proliferation and blockade of the activation of Erk1/2 and Akt cascades, prostate cancer cells and vascular endothelial cells	Wang *et al.,* 2013
DMPGTVLP	MCF-7	Breast Cancer	down-regulation of *PRDM14* gene expression and inhibit PRDM14 protein synthesis responsible for carcinogenesis in the target MCF-7 cells.	Bedi *et al.,* 2011
M204C4 and M205C4	Matrix metalloproteinase (MMP)-2	pancreati c cancer	Reduced MMP-2 mediated invasion of the pancreatic cancer cell lines PANC-1 and CFPAC-1, suppressed tumor growth in vivo	Lu *et al.,* 2012
peptide III (WHWLPNLRHYA S)	Vascular endothelial growth factor receptor 3 (VEGFR-3/Flt-4)	ovarian cancer	peptide III will be a potential inhibitor of VEGF signalling	Li *et al.,* 2015
Peptide SWQIGG N	ovarian cancer cells, cell line HO8910	ovarian cancer	Peptide was able to reduce tumor cell viability, migration, invasion, and adhesion capacity bind to ovarian cancer cells and control ovarian cancer *in vitro* and *in vivo*.	Zhou *et al.,* 2015
pHCT74	α-enolase Colorectal carcinoma cells	Colorectal cancer	Exhibited an enhanced antitumor effect and extended the survival of mice with human colorectal cancer in subcutaneous and orthotropic models.	Wu *et al.,* 2015
peptide LSLITRL	IL-6 receptor subunit alpha IL-6Rα	Glioblasto mas	inhibits IL-6-induced vascular endothelial growth factor-A expression and angiogenesis in different cancer cell lines	Wei *et al.,* 2017, Su *et al.,* 2005
F 56 peptide (WHSDMEWWYL LG)	VEGFR1 receptor	Mice xenograft with Human Gastric Tumor	inhibited tumor growth in vitro and in vivo	An *et al.,* 2004 Zhu *et al.,* 2017
MT1-MMP binding Bicycles within BT1718	MT1-MMP (MMP-14) Membrane type-1 Matrix metalloproteinase	Lung cancer TNBC, NSCLC, Sarcoma	Cell killing of lung tumor cells in vitro as well as efficacy across a panel of lung tumor xenograft mouse models.	Benn *et al.,* 2017.

Note: This list is a summary of selected recent publications and is not to be considered complete.
MCF-7: cell Line; NSCLC: Non small cell lung cancer; TNBC: Triple Negative Breast Cancer

Peptide display libraries can express random peptides and use of phage display in cancer therapy is to identify targeting ligand molecules that bind stably to unique receptors/cell surface markers expressed on targeted cancerous cells. A suitable receptor/ cell surface biomarker must have negligible expression on normal cells and it must not be shed from cell surface, as soluble form will act as a sink for targeting peptides.

In order to discover novel antagonistic molecules, a library of phage derived peptides was sorted against different growth factors. Growth factors

are also found to be responsible for angiogenesis. Few therapies are directed towards anti-angiogenesis approach. Peptides as antagonists that block receptor-mediated angiogenesis have been obtained by phage display (Yang *et al.,* 2017). Though monoclonal antibodies have been successfully used in cancer therapy. Peptide-based therapies have several advantages over monoclonal antibodies in that their smaller size, favours increased tissue penetration. When compared with antibodies, filamentous phage and their isolated coat proteins maintain their target binding functionality even after exposure to harsh environmental conditions, including high temperatures and low pH (Cui et al 2017), which makes them intriguing candidates for use as targeting ligands.

Besides, phage displayed peptides have been used to inhibit tumor growth and most attention is focused on identifying progressive disease markers and therapeutic agents. Furthermore, Such tumor-specific peptides are considered as targeting vehicles for the delivery of therapeutic genes, cytokines, agents for imaging, proapoptotic peptides, and cytotoxic drugs (Bedi *et al.,* 2011; Lang *et al.,* 2014). Selected peptides could also serve as vectors to transport therapeutic agents to tumors, which might increase the efficacy of therapy while decreasing probable side effects.

A number of phage-borne peptides (targeting ligands) specific to various tumors and enzymes responsible for metastasis and invasion have been identified in recent years. Affinity selection of phage displayed targeting ligand represents a potent mean of identifying peptide ligand on cancer cells. Targeting ligands can be used to identify cell surface markers and develop ligand-targeted therapy. These observations show the feasibility of phage displayed targeting peptide interaction using the small peptide and highlight its potential in the clinical applications. Through the application of these combinatorial methods (Bennet, 2017; Zhu *et al.,* 2017; Webster, 1996) the successful isolation of unique peptides acting as antagonists, as treatment options and vaccine development tools continues to be reported.

CONCLUSION

This article details the use of filamentous viruses for development and use of the phage display technology. Phage display technology is a powerful tool for studying protein-protein interactions, which can be used for multitude of applications. Phage display has been used to analyze the function of tumor vasculature and to select tumor specific antigens. Current review has described many novel peptides reported recently for playing role in understanding mechanisms of disease, to improve diagnostic potential and as possible therapeutic agents for cancer treatment. Many prominent cancer-associated molecules have been identified over the recent years which include FGF8b, EGFR, IL-6Rα, VEGFR1, MMP-2, HER2, MMP-14 etc. Though, phage display technology is only a preliminary validation strategy, it can also be used in conjunction with other methods to provide crucial information regarding the validity of the chosen target. The past innovations and those in pipeline promise a bright future for this field.

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