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Review article

Bacteriophage Fusion Peptides Based Targeted Delivery of Therapeutics to Cancer Cells

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ABSTRACT

A very paramount strategy for effective therapeutic management of tumors is the selective delivery of cancer therapeutic to the site of the malady employing the concept of targeted delivery. Although, a pleothora of ligands have been developed for targeting therapeutics to the site of the disease, only a few have been successfully used because of the need of their conjugation to therapeutic nanocarriers. A promising approach is harnessing bacteriophages displaying fusion peptides on all their major coat proteins (landscape phages) for targeting therapeutic-loaded nanocarriers to breast and prostate cancer cells. Screening of multibillion landscape phage has resulted in generation of peptide ligands targeting cancer-specific receptors. Also, spontaneous insertion of isolated phage proteins into therapeutic loaded nanocarriers such as liposomes can enhance their cancer-specific cytotoxicity and exclude the need for complex bioconjugations and derivitization procedures required for targeting. Various breast cancer, prostate cancer and metastatic prostate cancer cells specific landscape phage peptides have been generated by screening landscape phage libraries. These cancer specific-landscape phage clones and their peptides are promising platforms for various applications such targeted drug and gene delivery, cancer cell receptor identification and phage-like particles for gene therapy. As such, these applications are described in this review.

Keywords: *Bacteriophage. Fusion peptides. Targeted delivery. Cancer. Therapeutics*

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INTRODUCTION

Cancer is a principal cause of death and a public health burden worldwide. The incidence and fatality of this malady is expected to grow rapidly especially in low- and middle-income countries due to lifestyle changes (Torre *et al*., 2015). Accordingly, the development of an adequate treatment modality to constrain cancer mortality rate to its nadir remains a formidable challenge and a major goal of research in biomedical sciences and clinical oncology.

Traditional treatments of cancer inaccessible to surgery or as adjuvant to surgery are multi-modal comprising chemotherapy, immunotherapy, hormonal therapy and radiotherapy. Although, there have been improvements in cancer patient survival by employing cytotoxic chemotherapeutic regimens, this approach is encumbered by poor accessibility of medications to tumor site and non-specific cytotoxicity to normal cells. Selectivity of common cancer chemotherapeutics are often based on their preferential uptake to rapidly dividing cells, leading to side effects stemming from toxicities towards rapidly dividing normal tissues such as bone, gastrointestinal tract and hair follicles (Allen, 2002; Dasari and Tchounwou, 2014). Novel nanovehicular pharmaceutical carriers are available to achieve site-specific delivery thereby minimizing non-specific toxicity (Lammers *et al*., 2008). There is an array of nanovehicular pharmaceutical carriers comprising liposomes, micelles, dendrimers, nanotubes and nanoparticles which have been used to deliver cytotoxic cargos to cancer cells. Two of such drug loaded pharmaceutical nanocarriers, Doxil™ (Ortho Biotech Inc, Bedford, OH) encapsulating doxorubicin hydrochloride and Abraxane™ (Abraxis BioScience Inc., Los Angeles, CA) which is an albumin matrix embedded paclitaxel, have been approved by the United States Food and Drug Administration. The use of nanocarriers for drug delivery is based on their accumulation in solid tumors because of the "enhanced permeability and retention" (EPR) effect that promotes drug loaded nanocarriers to localize in the areas of increased vascular permeability in angiogenic tumors and is referred to as passive targeting (Seymour, 1992; Allen, 2002). Some of these clinically approved drug-loaded systems

have been made unrecognized (stealth) for the reticuloendothelial system and physiologically stable by their coating with polyethylene glycol to evade immune surveillance (Gullotti and Yeo, 2008; Wagner *et al*., 2017). Although, passive targeting can enhance accumulation of pharmaceutical nanocarriers in the tumor interstitium for a prolonged time, it cannot control their translocation into the cell. Consequently, the carrier allows only release of the drug into the vicinity of the tumor resulting in a bystander effect (Prokop and Davidson, 2008). Furthermore, drugs ultimately delivered into the cytoplasm are prone to being trapped and eliminated through the endo-lysosomal route. Besides, in multidrug resistant cells, for example with phenotype MCF-7/Adr, overexpression of multidrug resistance protein 1 (permeability glycoprotein) on plasma membranes, Golgi apparatus and nuclear membrane results in extrusion of drugs out of the intracellular compartment thereby leading to reduced drug efficacy (Vasir and Labhasetwar, 2005; Calcabrini *et al*., 2000).

As a means to improve selective interaction of the drug loaded nanocarriers with the target cell, active targeting has been explored. Active targeting is based on the principle of molecular recognition whereby a ligand-targeted nanocarrier, encapsulating a lethal cargo, is delivered to tumor cell's membrane, microenviroment, cytoplasm or nuclear membrane displaying the counterpart receptor (Haley and Frenkel, 2008). A plethora of ligands comprising small molecules, peptides, aptamers, proteins and antibodies are currently in use for targeting nanoparticles (Veiseh *et al*., 2010; Balestrieri and Napoli, 2007). In this set, the application of peptides offers an almost universal approach to targeting nanoparticles because of their intrinsic diversity of binding potentials. Two main types of combinatorial libraries have been a source of binding ligands: one-bead-one-compound (OBOC) combinatorial libraries and phage display libraries, the diversity of OBOC being much lower than the phage display libraries (Aina *et al*., 2007). Using phage display libraries, with a clone complexity greater than 1 x 109, phage clones can be selected and propagated in E. coli. The screening procedure can be iterated to select the highest affinity binding phage clones. Consequently, this review describes phage display library especially landscape phage library, their application as platforms for targeted drug and gene delivery to cancer cells, their capability for cancer cells receptor identification and phage-like particles generation for gene therapy.

Phage display library

Smith and colleagues invented the phage display by inserting foreign oligonucleotides into the pIII gene of filamentous bacteriophage which resulted into the display of fusion peptides on the minor coat protein pIII of the bacteriophage (Smith, 1985; Parmley and Smith, 1988). They also developed efficient affinity selection procedures for recognizing the displayed peptides' specific ligands. Il'ichev and other collaborators displayed foreign peptides at the N-terminal of the major coat protein pVIII (Il'ichev *et al* 1985; Minenkova *et al*. 1993). MacCafferty and co-workers displayed the antigen binding fragments of immunoglobulins on the surface of the fd phage (McCafferty *et al*., 1990). Thereafter, phage libraries generation emerged as a coaction of fusion phage and combinatorial chemistry. There are a variety of phage display libraries which include random peptides library, antibody fragment library, genomic library, cDNA library and protein fragment library. The random peptide libraries are the most common and are derived from degenerate oligonucleotides synthesized chemically by adding mixtures of nucleotides (instead of single nucleotides) to a growing nucleotide chain (Smith and Petrenko, 1997). Peptides specific for various tumor cells, tissues and organs in model animals have been identified using phage display random libraries with fusion peptides displayed on pIII protein in vitro, ex vivo and in vivo, respectively (Pasqualini *et al*., 1997; Rajotte *et al*., 1998; Rasmussen *et al*., 2002). In another group, random peptide phage display libraries were used for selection of tumor cell binding ligands in cancer patients (Krag *et al*., 2006). Phage display libraries were used to generate peptides specific for 59 NCI-60 cell (National Cancer Institute panel of cell lines from different histologic origins and grades) (Kolonin *et al*., 2006). The panel includes carcinomas of several origins (kidney, breast, colon, lung, prostate, and ovarian), tumors of the central nervous system, malignant melanomas, leukemias, and lymphomas. In addition, phages specific for the target tumor cell lines, tissues or mouse xenografts can be selected from the multi-billion landscape phage libraries, as described previously (Mount *et al*., 2004; Romanov *et al*., 2001; Samoylova *et al*., 2003; Fagbohun *et al*., 2012) and converted to targeted gene- and drug-delivery vehicles (Mount *et al*., 2004; Fagbohun *et al*., 2013; Wang *et al*., 2017).

The landscape phage library

Landscape phage libraries are collections of filamentous phages displaying random guest peptides on all 4,000 surface domains of the major coat protein type 8 system, whereas in type 88 and 8+8 systems, only a few of the pVIII copies display the random guest peptide. In the landscape phage library, each bacteriophage displays thousands of copies of the peptides in a repeating pattern subtending a major fraction of the viral surface which is different from the typical phage display library where guest peptides are fused to the pVIII coat proteins (Petrenko and Smith, 2000). The F-pilus-specific filamentous bacteriophages Ff (fd, f1 and M13) are part of a large family of bacterial viruses that infect Gram-negative bacteria. They are long, flexible thin with a diameter of 7 nm and length of 800-900 nm (determined by the size of the genome). Their single stranded DNA is enclosed in a cylindrical capsid composed of the major coat protein pVIII, and a few copies of the minor coat proteins capping the tips of the phage. The phage display library is an ensemble of up to 10 billion different phage clones, each harboring a unique foreign DNA, and therefore displaying a specified guest peptide on the virion surface. Foreign peptides were displayed on the pVIII protein soon after display on the minor coat protein pIII was pioneered (Il'ichev *et al*., 1989) (reviewed in (Petrenko and Smith, 2005). The foreign peptides replacing the three or four mobile amino acids close to the N-terminus of the wild-type protein pVIII do not greatly affect the general architecture of virions and do not change the conformation of the fusion proteins in membranes (Jelinek *et al*., 1997; Monette *et al*., 2001). Consequently, such fusion phages retain their ability to infect the host bacteria E. coli and form up to 1000 identical phage particles per bacterial cell during the doubling period. Such particles were eventually

termed "landscape phage" to emphasize the dramatic change in surface architecture caused by arraying thousands of copies of the inserted peptides in a dense, repeating pattern around the tubular capsid (Petrenko *et al*., 1996; Marvin *et al*., 1994). The foreign peptides decorating the phage body create defined organic surface structures (landscapes) varying from one phage clone to the next (Fig. 1). A library is a huge population of such phages, encompassing billion of clones with different surface structures and biophysical properties (Petrenko *et al*., 1996). Therefore, the landscape phage is a unique micro-fibrous material that can be selected in the affinity binding protocol. The binding peptide comprising up to 20% of the phage mass and up to 50% of the phage surface may be easily prepared by cultivation of the infected bacteria and isolation of the phage particles by precipitation.

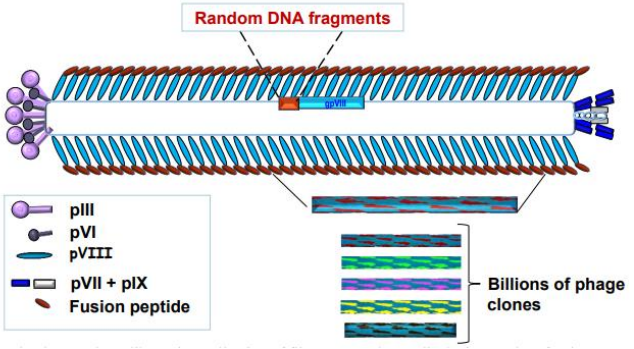

Figure 1
A landscape phage library is a collection of filamentous phages displaying random foreign peptides on the all 4,000 surface domains of the major coat protein

Targeted therapeutic delivery and phage coat protein biochemistry

Solid tumors are made up of two main cellular components, namely the tumor parenchyma and the stroma, with the stroma providing vasculature and other supporting cells (Munn, 2003). As tumor growth progresses, there is greater demand for more nutrients and structures. This results in the formation of new vasculature structures from endothelial cells from neighboring vessels vasculature and from bone marrow (Lyden *et al*., 2001). However, these vessels are deficient in their architectural integrity compared to newly form blood vessels in normal tissues such as those found at healing sites (Jain, 2001). In other words, neovasculature in tumors is typically highly "fenestrated," implying that it has many holes in the endothelial layer, allowing big particles such as liposomes, phage particles, or even whole cells to leak out into the tumor itself. This defect has been exploited in passive drug delivery of nanocarriers (nanomedicines) to various tumors.

There are plethora of drug delivery nanocarriers including liposomes, polymeric micelles, polymeric nanoparticles, dendrimers, viral-based nanoparticles and carbon nanotubes (Cho *et al*., 2008; Peer *et al*., 2007). Of all these nanocarriers, liposomes have been the most extensively studied. Liposomes are self-assembling phospholipids with a bilayer and a spherical shape. Doxil™, a liposomal formulation encapsulating the anthracycline doxorubicin hydrochloride, is currently used for systemic management of breast and ovarian carcinomas (Markman, 2006). As has been previously indicated, these systems exploit the defective tumor vasculature and lymphatics for the delivery of their toxic cargos. However, passive targeted delivery has intrinsic limitations such as inadequate permeation of tumor tissues and some therapeutics may require intracellular delivery into organs such as the nucleus or mitochondria for their action (Moghimi and Szebeni, 2003). Therefore, active targeting or targeting of nanomedicines with tumor specific ligands is currently being employed as an improvement to the passive delivery strategy. There are arrays of ligands available for targeting, including growth factors, antibodies and their fragments, carbohydrates, glycoproteins and receptors ligands. Most actively targeted nanomedicines require conjugation of ligands to the nanomedicines which often involves chemistry which may alter the structure and function of the ligands. Targeting of cancer nanomedicines with cancer selective-phage coat proteins provide a simple and economic approach for development of cancer targeted nanomedicines (Wang *et al*., 2010; Jayanna *et al*., 2009). Previously, Jayanna *et al*. (2009) have developed a new approach to preparation of targeted liposomes that relies on the use of the phage fusion coat proteins as targeting ligands. In this approach, a cancer cell-specific phage protein was inserted into the liposome exploring its intrinsic "membranophilic" properties (Jayanna *et al*., 2009). Fusion proteins carrying tumor-cell binding peptides inherit the major structural features of the "wild-type" major coat protein pVIII. They have a positively charged C-terminus (amino acids 45-55), which navigates the protein through the liposome membrane, probably using the mechanisms intrinsic for cationic cell-penetrating (Tseng *et al*., 2002). The highly hydrophobic "membranophilic" segment (amino acids 27-40) allows the protein to accommodate readily in the membrane (Tseng *et al*., 2002) while the amphiphilic N-terminus (amino acids 1-26), which are soluble in water, can interact with PEG residues on the surface of the "stealth" liposomes (Fig. 2) and display the N-terminal cancer cell-binding octamer or nonamer on the liposome shell.

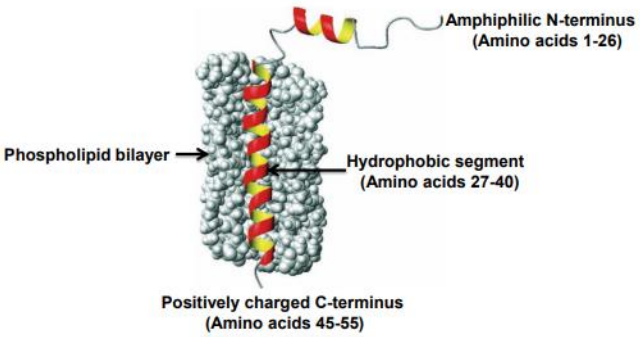

Figure 2
The model of the pVIII protein in the phospholipid bilayer environment. Adapted from (Stopar et al., 2003)

This concept was effectively proved when the cancer- selective phage with the coat protein structure:**ADMPGTVLPDPAKAAFDSLQASATEYIGYA WAMVVVIVGATIGIKLFKKFTSKAS** (the fusion peptide involved in cancer-selectivity is underlined) (Fagbohun *et al*., 2012) was grafted into drug encapsulated liposomes (Doxil™)

to enhance cytotoxicity to breast cancer cells MCF-7 (Wang *et al*., 2010a). The major coat proteins of the breast cancer cancer-specific phage particles were isolated with cholate solubilizing solution and chloroform and purified by separating the phage coat protein from coat proteins from phage DNA and cholate micelles using gel exclusion chromatographic separation. The purified protein was appended to Doxil™ (Ortho Biotech, Bedford, OH), stealth long circulating liposome encapsulated doxorubicin hydrochloride (Fig. 3). And, in a more recent study, this peptide was also demonstrated to translocate into the cell and exhibited endosomal escape (Wang *et al*., 2010b).

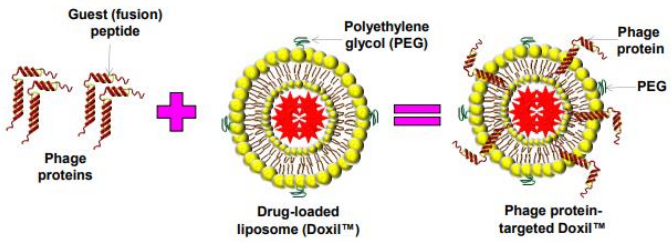

Figure 3
Doxil™ targeted by the pVIII protein created by exploiting the amphiphilic nature of the phage coat protein. The hydrophobic segment of the pVIII is anchored in the lipid bilayer, whereas the N-terminal tumor-specific peptide is displayed on the surface of the liposome. The doxorubicin hydrochloride molecules are pictured as particles inside the liposomes.

Another very promising approach to targeted therapeutic delivery is targeting the navigating peptides on nanocarriers to their cognate receptors on cancer cells. This is possible because the mammalian membrane is a dynamic scaffold displaying an array of macromolecules which are required for cell survival, proliferation and differentiation. These macromolecules, including receptors, acquire information from the extracellular milieu and transduce these signals into the intracellular environment through a variety of signal transduction pathways to maintain cellular homeostasis. This process is initiated by binding of monovalent or multivalent ligands to their cognate receptors. This cellular mechanism has been harnessed for active targeted delivery of nanomedicines. Targeting of anti-tumor drugs with monoclonal antibodies or their fragments toward tumor cell receptors is based on their specific monovalent binding (Carlson *et al*., 2007). However, this mode of drug delivery can be hindered by non-specific cytotoxicity of monovalent conjugates. Therefore, multivalent ligand-targeted drug delivery is currently being adopted to improve selectivity of targeted drugs toward cancer cells (Wang *et al*., 2010a; Jayanna *et al*., 2010). Wang and others have used multivalent ligands based on modifications of the landscape phage coat protein to target Doxil to MCF-7 cells. The study demonstrated the ease of insertion of the phage coat protein into Doxil and enhanced cytotoxicity of the modified Doxil toward MCF-7 cells (Wang *et al*., 2010a). To reveal the identity of receptors targeted by these nanomedicines and the basis of their selectivity towards MCF-7 cells, Fagbohun and others harnessed the microfibrous nature and multivalent feature of the landscape phage (Brigati *et al*., 2008; Petrenko, 2008; Fagbohun *et al*., 2012) in affinity chromatography to isolate phage-binding receptors on the surface of MCF-7 breast cancer cells. This was accomplished by immobilizing breast cancer-selective phage as an affinity ligand on a solid support in affinity chromatography.

The procedure entails immobilization of breast cancer-selective phages as affinity ligands on macroporous (dimethylacrylamide) monolithic columns, cryogel® (Protista, Sweden) as described (Noppe *et al*., 2006). Cryogel® is a hydrophilic, spongy, elastic, methacrylate containing macropores (1-2 µm) and activated epoxy groups (Mallik and Hage, 2006; Peskoller *et al*., 2009). The activated epoxy group was used for phage immobilization through reaction of epoxy groups on the resin with amine groups on the phage (Fig. 4). For effective immobilization of the landscape phage with multiple copies of the pVIII, a long circulation time (16 h) at 4ºC was used. The breast cancer cell MCF-7-selective phage previously selected by Fagbohun *et al*. (2012) designated DMPGTVLP was used for this study. The phages were immobilized on cryogel columns and allowed to react with the MCF-7 cell lysate. After washing the columns with phosphate buffered saline (PBS), bound proteins were eluted using mechanical force. Eluted proteins were separated on SDS-PAGE and identified by Nano-LC MS/MS. Nucleolins were the main proteins identified in the procedure. Identification of nucleolin as the receptor for the phages and their basis for selectivity toward MCF-7 cells was confirmed in phage competition assays with nucleolin polyclonal antibodies (Fagbohun *et al*., 2012).

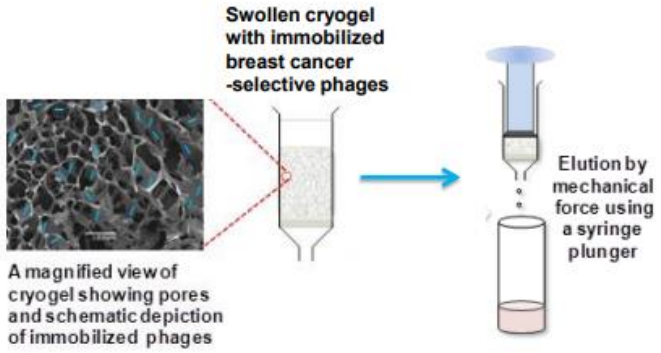

Figure 4
Affinity capture of breast cancer cell receptors using immobilized phages on macroporous monolithic columns. Breast cancer-selective phage particles were immobilized on macroporous monolithic gels (cryogels). Plasma membrane proteins were passed through the column and recirculated. Unbound proteins were washed off whereas bound proteins were eluted from the column using mechanical elution, which involves applying mechanical force to compress the cryogel gel and thereby eluting bound proteins.

siRNA as an attractive payload for tumour-targeted liposomes

Small (short) interfering fragments of RNA (siRNA) suppress specific protein synthesis by suppressing target gene expression at the mRNA level by RNA interference (RNAi). They are promising anticancer therapeutics due to their high specific gene silencing efficacy and low toxicity (Iorns *et al*., 2007). Nonetheless, systemic delivery of siRNAs into tumor cells is encumbered with the instability of siRNA in the blood stream and their ineffective penetration of the plasma membrane (Whitehead *et al*., 2009). As such, alternative siRNA delivery approaches to target cells have been devised. These include the

encapsulation of siRNA in liposomes or other nanocarriers, viral and bacterial delivery of siRNA precursors, or stabilization of siRNA molecules through their chemical modification (Kim *et al*., 2009). Bedi *et al*. (2011) developed the phage-based targeting strategy for siRNA delivery to breast cancer cells. This involved the integration of landscape phage proteins with liposomes for construction of siRNA-loaded nanocarrier specifically interacting with cancer cells. The basis of the targeting concept is that a cancer cell specific landscape phage peptide serves a dual function in liposome targeting: its surface-exposed N-terminus navigates the liposomes to the target cellular receptors while the hydrophobic C-terminus anchors the targeting peptides to the liposomal membrane. As a target for siRNA-mediated gene silencing, the PRDM14 gene - a member of the family of genes encoding proline rich domain proteins (PRDM) and that may play a crucial role in breast cancer carcinogenesis (Nishikawa *et al*., 2007) was employed. The studies revealed that gene-specific siRNA duplexes, encapsulated in phage protein-targeted PEGylated liposomes, specifically inhibited the expression of the target PRDM14 gene in breast cancer cells.

Phage-like particles for targeted gene delivery

Gene and vector-based molecular therapies as treatment modalities for cancer are usually intended to restore cancer cells to normal cells. However, efficacious gene therapy requires specific delivery of therapeutic genes to a target tissues and its sustained expression in the effected cancer cells (Urbanelli *et al*., 2001). Current gene delivery approaches are based on the use of viral and non-viral delivery systems. Non-viral systems comprise naked DNA, or DNA associated with polymers, dendrimers, liposomes, polyethylenimine, polylysine, cationic peptides and, other non-infective vehicles. Although, non-viral delivery systems assure safety and modularity (Urbanelli *et al*., 2001; Douglas, 2008), they rank in efficiency of cell transduction below mammalian viral therapeutic oligonucleotides delivery vesicles based on herpesviruses, retroviruses, adenoviruses, or adeno-associated viruses, which have been evolved to acquire natural tropism for mammalian cells. For example, phages with fusion peptides as targeting ligands on the pIII proteins have been shown to transduce mammalian cells with subsequent transgene delivery (Schaffer *et al*. 2008; Larocca *et al*., 1999; Poul and Marks, 1999; Larocca and Baird, 2001). Another type of display, with fusion peptides displayed on all multiple copies of the major coat protein pVIII can be even more advantageous for cell transduction since the multivalent display results in more strong binding of the phage to cellular receptors due to avidity effect, which provokes receptor dimerization and clustering (Urbanelli *et al*., 2001; Il'ichev *et al*., 1989; Minenkova *et al*., 1993). In the light of this, Fagbohun *et al* (2013) screened a 1 × 109 clone landscape phage library to isolate metastatic prostate cancer-specific phage clone EPTHSWAT (EPTHSWAT is the fusion peptide displayed on the phage coat protein). This phage clone was converted into phage-like particles by transforming K91 blue E. coli cells with a phagemid vector pcDNA™6.2-GW/EmGFP-miR capable of expressing a miRNA of interest and emerald green fluorescent protein (EmGFP). The phagemids were rescued with phage EPTHSWAT (helper phage) as illustrated (Fig. 5). The PC-3M cell-transforming activity was observed using emerald green fluorescent protein marker. This indicates that phage-like particles capable of expressing miRNA can be used in cancer cells miRNA replacement therapy.

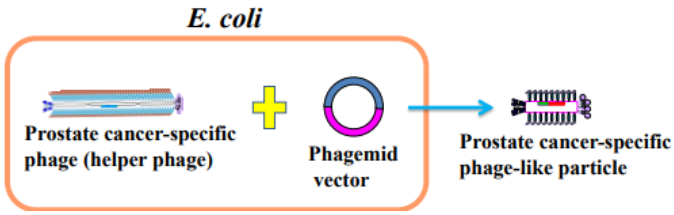

Figure 5
A simplified schematic representation of generation of a phage-like particle. The phage-like particle has the phagemid vector harbouring a therapeutic gene as its genome which is encapsidated by the cancer-specific protein from the helper phage.

Conclusion

Effective management of cancer is encumbered by non-specific cytotoxicity of therapeutics to normal tissues and organs. An explosion of knowledge regarding the molecular pathogenesis of disease and the emergence of new methods of nanotechnology has resulted in the development of the concept of active targeted therapeutic delivery. Applications of this new concept have the potential to improve cancer therapeutic efficacy and to reduce their side effects. Arrays of nanomaterials are currently being tested for use in cancer diagnosis and treatment. Among these, the landscape phage libraries which are collections of filamentous phages displaying random guest peptides on all 4,000 surface domains of the major coat protein are very promising. They are attractive nanotechnology platforms for application in biomedical research because of robustness of the phage protein membranophilic properties. The screening of multibillion landscape phage libraries against cancer cells offers an innovative approach to the generation of a repertoire of ligands for active cancer therapeutic targeted delivery. These ligands can be can be incorporated into nanocarriers such liposomes targeted the ligands' cognate receptors for precise targeted delivery of therapeutics. Furthermore, cancer selective landscape phage particles can be converted to phage-like particles harbouring therapeutics genes such as therapeutic oligonucleotides, siRNAs and miRNAs. Application of landscape phage is unlimited because of the high diversity of the library coupled with the multivalency effect of the displayed peptides

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