Chapter 4
Bacteriophages Functionalized for Gene Delivery and the Targeting of Gene Networks

Abstract Bacteriophages (phages) offer many potential and existing applications to biotechnology, including their modification and use as protein/gene carriers. Phages possess many intrinsic physicochemical attributes that make them excellent candidates for use in gene therapy. In this chapter we will explore how phages have been employed in gene delivery as well as their future utility in this exciting medical application.

1 Introduction to Phage Mediated Delivery of Genetic Material

Bacteriophages were among the first entities to be manipulated for modern gene transfer and gene targeting strategies. The small size, relative ease of production, capacity for genomic isolation and manipulation position phage attractively for this pursuit. The bacteriophage genome can be manipulated to incorporate heterologous sequences designed to be expressed in, or otherwise modify, a recipient cell. Gene expression in a recipient cell can be governed either by prokaryotic or eukaryotic genetic systems, making it possible to deliver genetic cargo that can be expressed in any host cell. The natural host specificity of a phage governs its tropism and when manipulated, provides an endless potential of biotechnological applications. Alternatively, phages can and have also been exploited for use as display instruments (Smith and Petrenko 1997). Known as phage display, this is a strategy of conjugating or translationally fusing peptide molecules onto the coat surface of the phage. Phage display of targeting ligands or antibodies can be generated against a range of mammalian cells for which the phage would naturally have no tropism nor capacity for propagation (Nicastro et al. 2014). Under such applications the phage would no longer function as a (bacterial) virus, but rather as an inert, nanoscale particle employed to deliver nucleic acid cargo and enact a specific activity to a targeted

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mammalian cell. In a seemingly endless assortment of activities and targets, the focus of this chapter will be on the specific characteristics of the engineered phage delivery vehicles and their delivery of encapsulated nucleic acids to their targeted cells.

Phage-delivered nucleic acid cargo to a recipient cell can be benign or toxic. Benign treatments, including gene therapy (Larocca and Baird 2001), immunomodulation (Willats 2002), and phage DNA vaccines (Clark et al. 2011) are intended to leave the cell metabolically active so that the host cell can express the DNA cargo provided by the phage. Phages may also be modified to encode toxins (Vilchez and Jacoby 2004) or other genes that are damaging to the cell (Abedon 2009), which can be used as a means of biocontrol in prokaryotic systems or as a means to kill or damage non-bacterial targets such as tumour cells. The expression of the cargo can be temporary, which is the goal in bacterial identification, biocontrol and phage mediated DNA vaccine applications. In other cases, such as in gene therapy in eukaryotic systems and gene cloning in prokaryotic systems, the expression of the phage encoded genetic cargo can and is aimed to be long lasting.

2 Bacteriophages as Gene Delivery Vehicles

Most commonly, eukaryotic viral vectors are employed to deliver DNA to eukaryotic cells to correct for a genetic defect or otherwise augment a desired cell phenotype. However, the use of such vectors poses important safety concerns, particularly with respect to the control of inherent virulence and immunogenicity (Clark et al. 2012; Seow and Wood 2009), which can and have previously resulted in mortality (Somia and Verma 2000). An additional challenge is the effective design of viral therapeutics to target the desired organ or tissue and to avoid prior immunity against viral vectors (Nayak and Herzog 2010). As such, there is a need for gene therapy systems that are benign and yet more precise, which has drawn attention to alternate approaches such as bacteriophage-mediated gene delivery.

Phages are stable (Jepson and March 2004), inexpensive to produce (Bakhshinejad et al. 2014), easy to manipulate genotypically and phenotypically (Clark et al. 2011), and can be targeted to their intended cellular targets (Nicastro et al. 2013). In their application to gene delivery, the outer capsid coat proteins of the phage vehicle can simultaneously protect the intended DNA cargo against degradation during delivery (Clark and March 2006; Dunn 1996) and tolerate capsid peptide/protein fusions, making it possible for the vehicle to target intended cells (Clark and March 2006); a cornerstone of successful gene therapeutic design. Phage can be safely administered to mammals as evidenced by the long history of phage therapy against bacterial infection (Abedon et al. 2011) and do, in fact, naturally penetrate mammalian tissue (Dabrowska et al. 2005) without intrinsically infecting mammalian cells. Despite the high penetrance of phage particles, they are also quickly cleared by the reticuloendothelial system (RES), which will negatively impact uptake by target cells (Molenaar et al. 2002). To circumvent clearance, long-circulating phages capable of evading the RES have been developed

(Merril et al. 1996). Alternatively, the addition of polyethylene glycol (PEG) can also improve phage circulation (Kim et al. 2008).

While the simplicity of phages positions them as attractive cloning vectors, they are generally limited by the size of the gene(s) of interest that can be cloned into the phage head, and in most cases lack natural nuclear honing and expression abilities in eukaryotic cells (Clark et al. 2011; Larocca and Baird 2001). While filamentous bacteriophage have a far more flexible packaging minimum and maximum (Specthrie et al. 1992), they are instead limited by the fact that they must carry circular single-stranded DNA, a detriment to expression in eukaryotic hosts where conversion to double stranded DNA is required for gene expression (Clark et al. 2012; Yacoby and Benhar 2008). Some of these limitations can be addressed by the modification of the phage particle including: the display of cell-penetrating peptides (Trabulo et al. 2012) and/or the use of chemical agents such as DEAE-dextran (Yokoyamakobayashi and Kato 1993), co-administration with cationic lipids (Eguchi et al. 2001; Yokoyamakobayashi and Kato 1994), and the inclusion of nuclear localization peptides and/or sequences (Lam and Dean 2010; Miller and Dean 2009). Despite carrying single-stranded DNA, filamentous phage gene delivery has resulted in successful uptake and expression in mammalian cells (Larocca et al. 1999; Poul and Marks 1999). Additionally, the introduction of self-complementary sequences in filamentous phage single-stranded DNA can lead to the formation of double-stranded DNA in eukaryotic cells (Prieto and Sánchez 2007).

Despite the above limitations, phages have validated their utility as functional gene delivery agents, with the first reported use for this application more than two decades ago by Hart et al. (1994). In this study, phage fd was employed to display a cyclic-binding peptide to the major coat protein pVIII. This amino (N)-terminal fusion, occurring at approximately 300 copies/phage particle, was bound to cells and was efficiently internalized. The same peptide sequence was then fused to the major tail protein (gpV) of bacteriophage λ. These modified phages proved to be more suitable gene delivery candidates—transfecting mammalian cells at a remarkable frequency in comparison to the undecorated controls (Dunn 1996; Hart et al. 1994).

Lankes et al. (2007) expanded the application of λ as a gene delivery vector by executing phage-mediated gene transfer in vivo. This group constructed recombinant λ particles encoding firefly luciferase (*luc*) in order to visualize gene delivery in real-time via the use of bioluminescence imaging (Lankes et al. 2007). They fused a $\alpha_v\beta_3$ (CD51/CD61) receptor integrin-binding peptide to gpD to increase its uptake by receptor-mediated endocytosis. This peptide, the tenth human fibronectin type III domain, was chosen because it is known to play a role in the binding/internalization in a number of mammalian viruses and it had been used to enhance the targeting of modified viral vectors to a variety of cells including professional antigen-presenting dendritic cells. The study showed preferential internalization of fused phage over their non-fused counterparts, where internalization decreased significantly in a dose-dependent fashion.

Integrin receptors are also overexpressed on cancer cells. To exploit this, Choi et al. (2014) modified filamentous phage M13 to controllably display the integrin binding motif RGD (Arg-Gly-Lys), a widely used cancer targeting peptide, on either the minor coat protein pIII or the major coat protein pVIII (Choi et al. 2014). The study examined the display of circular and linear RGD, resulting in the display of 5 or 2700 copies of linear RGD in comparison to 140 copies of circular RGD and found that the display of circular RGD facilitated more than threefold internalization of phage in HeLa cells when compared to the display of linear RGD. This highlights the importance of not only choosing a suitable targeting ligand, but also the necessary considerations of ligand display conformation.

Vaccaro et al. (2006) noted an increasing pattern of internalization following the addition of competitor proteins, indicative of a receptor-mediated process. The recombinant phage outperformed the control cells both in vitro and in vivo. In addition, they noted a 100-fold increase in phage internalization into integrin-positive versus control cells, but only a 3-fold increase in phage-mediated gene expression. This indicated that the level of internalization is not necessarily comparable to the successful delivery of genetic material (Vaccaro et al. 2006). Overall, this study provided a proof-of-concept for the use of recombinant phage to increase gene transfer in vivo, and a compelling argument for the use of phages in transgene delivery (Lankes et al. 2007; Vaccaro et al. 2006). However, it also underscores the importance of integrating mechanisms for overcoming intracellular barriers past cell entry in order to successfully deliver a genetic therapeutic.

Zanghi et al. (2007) further explored construct design, where they attempted to improve phage-mediated mammalian gene delivery of a luciferase gene through the simultaneous fusion of proteins to both the head and tail of phage λ (Zanghi et al. 2007). Multiple intracellular barriers such as cell attachment, cytoplasmic entry, endosomal escape, uncoating and nuclear import, must be overcome for successful gene transfer (Seow and Wood 2009). Multiple peptides could theoretically be displayed *in tandem*, where each peptide could function to circumvent a separate barrier. This research group reported, on average, the simultaneous display of two separate peptides: ~100 copies per phage particle to gpV of a CD40-binding peptide to facilitate endocytic uptake and ~400 fusions per phage particle to gpD of a ubiquitinylation motif to enhance intracellular trafficking of the internalized phage (Zanghi et al. 2007). Display of both the CD40-binding motif and the ubiquitinylation motif improved gene expression two-fold over display of either the CD40-binding motif or ubiquitinylation motif alone.

Although numerous methods have been developed for gene delivery, an efficient platform for protein delivery *in tandem* with gene delivery does not currently exist. Recently, Tao et al. (2013) developed a T4 DNA packaging machine using T4-based "progene" nanoparticles that were targeted to antigen-presenting cells and were expressed both in vitro and in vivo. The group fused DNA molecules onto the T4 major capsid proteins, Soc and Hoc, that would later be displayed on the phage heads. Foreign cell penetration peptides (CPPs) and proteins (β-galactosidase, dendritic cell specific receptor 205 monoclonal antibody, and CD40 ligand) were chosen for display onto Hoc. The encapsidated DNA included *gfp* (green fluorescent

protein) and *luc* (luciferase) genes to enable quantifiable expression within mammalian cells. Overall, the group demonstrated efficient in vitro and in vivo progene delivery and expression of self-replicating genes into mammalian cells. While promising, further investigation particularly with respect to in vivo studies is warranted, as the strongest luciferase signal in this study was unexpectedly generated in mice treated with nanoparticles that did not display targeting ligands. The authors have attributed this finding to the migration of the targeted cells to other parts of the body, an inference that will require further investigation (Tao et al. 2013).

3 Phages as Cytotoxic Agents in Eukaryotes

While the application of phage as gene delivery vehicles could be employed to restore a gene defect or alter the physiology of the host cell, phage could further prove therapeutic as cytotoxic agents. Tissues such as tumour cells or unwanted white fat cells can be deleterious to the mammalian host and beneficially targeted for reduction and removal. Toward such therapies, the physicochemical attributes of phages can be exploited to direct cytotoxic outcomes. In a targeted manner, phage can be manipulated to deliver toxins to targeted cells (Vilchez and Jacoby 2004), or alternatively employed to stimulate immune responses and clearance of unwanted cells (Ahmadvand et al. 2011; Clark et al. 2012). While the former strategy is greatly dependent upon precise targeting and uptake of the phage particle to kill or inactivate the host cell, the latter relies upon the natural adjuvant properties of the phage to stimulate and confer immunogenicity against targeted cells (Gamage et al. 2009).

The evolution of phages as therapeutics in these capacities requires focused targeting, typically facilitated through phage display technologies. Fibroblast growth factor, in particular, has enabled the specific targeting of cancer cells with the appropriate receptors (Haq et al. 2012; Hart et al. 1994; Sperinde et al. 2001). In a series of studies by Yacoby, Benhar and others, cytotoxic bacteriophages were designed from a template of their previous cytotoxic phage used for the treatment against bacterial pathogens (Yacoby et al. 2006, 2007). This group developed a filamentous phage that was engineered to display a eukaryotic cell-binding ligand conjugated to a the cytotoxic drug, either hygromycin or doxorubicin, to be released within the targeted tumor cells. These engineered phages were shown to be effectively endocytosed, resulting in the preferential release of the cytotoxin in targeted cells (Bar et al. 2008). In another study by Chung et al. (2008), tumour cells derived from Hodgkin's-derived cell lines were targeted for apoptosis by antibody-targeted phage particles (Chung et al. 2008). Their proof-of-principle study employed an in vitro GFP expression system as a measure of phage uptake, based on the premise that efficient expression of GFP could be replaced with the expression of a cytotoxic agent in the future (Chung et al. 2008). In a similar study, Eriksson et al. (2007, 2009) also used filamentous phage to target tumour cells. However, these studies differed where the delivered phage were designed to target

the host cells for removal by the host immune system without carrying a cytotoxic agent (Eriksson et al. 2007, 2009).

4 Phages for Delivery to the Central Nervous System

Delivery of therapeutics to the brain and the central nervous system (CNS) remains a challenging problem due to its complex structure, sensitivity to toxicity, and the impermeability of the blood-brain barrier (BBB). Gene delivery to the CNS has been achieved with some degree of success through direct injection into the eye and/or the cerebral spinal fluid, or direct implantation of transduced cells into brain parenchyma (Davidson and Breakefield 2003; Hampl et al. 2000); however, such methods are highly invasive, have limited penetration, and can be traumatic to the neural tissue. Overall, the capability to pass the blood brain barrier and penetrate heterogeneous neural tissue is highly desirable in a CNS-targeted therapeutic. Phage have been observed to exhibit this ability (Dabrowska et al. 2005; Frenkel and Solomon 2002) and may therefore be exploited for CNS drug and gene delivery.

Drug addiction is an important health and social problem world-wide, a prevailing culprit of which is the highly addictive recreational drug cocaine. It has been previously shown that protein-based therapeutics designed to bind to cocaine can reduce the drug load and attenuate its psychoactive effects. However, this strategy has not generally demonstrated significant therapeutic value due to the inability of these cocaine-binding proteins to cross the BBB and gain access to the CNS. To address this issue, Carrera et al. (2004) engineered a filamentous bacteriophage displaying cocaine sequestrating antibodies on its surface which blocks this drug in the brain. The modified phages were administered intranasally to rats twice a day for three consecutive days before the brains were examined, confirming the presence of the phage. The results of this study highlights the potential for phage to serve as a new system for treatment of cocaine addiction as well as serving as a platform for treatment of drug abuse (Clark and March 2006; Dickerson et al. 2005).

More recently, filamentous phages have also been demonstrated to accumulate in gliobastoma after intranasal delivery (Dor-On and Solomon 2015), potentiating their use in the treatment of brain tumours and other brain malignancies. Phage display has also been useful in the identification of several ligands capable of bypassing the BBB and targeting neural tissue (Li et al. 2011; Wan et al. 2009), which can functionalize other non-viral vectors.

5 Conclusions

Bacteriophages have evolved the natural ability to efficiently carry and deliver a genetic payload to their natural host cells—a function that continues to be exploited in the development of highly efficient, engineered phage delivery systems that can

specifically target and modify non-natural host targets. The ability of the phage to cross the BBB makes it an attractive vector against neural malignancies. One major area of improvement for phage gene delivery lies in enhancing its ability to traverse intracellular barriers: notably, transport across the plasma membrane and escape from the endosomal pathway. Viral peptides such as the adenoviral penton base have been shown to mediate entry, attachment and endosomal release (Haq et al. 2012; Piersanti et al. 2004) and can be conjugated to the phage through phage display. Similarly, the protein transduction domain of HIV (TAT protein) and the simian virus 40 (SV40) T antigen nuclear localization signal have also been used to enhance the uptake and nuclear targeting of phages (Haq et al. 2012; Nakanishi et al. 2003). Additional future improvements to phage delivery technologies may exploit the display of DNA reducing DNase II inhibitor to protect DNA (Haq et al. 2012; Sperinde et al. 2001).

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