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Targeted filamentous bacteriophages as therapeutic agents

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Bacteriophages (phages) have been used for therapy of bacterial infections, for genetic research, as tools for the discovery of specific target binding proteins and for vaccine development. The aim of this article is to present advances in genetic and chemical engineering of filamentous bacteriophages that facilitated their application for therapeutic purposes. We review studies where phages were applied for *in vivo* imaging, as gene delivery vehicles and as drug carriers. Target specificity is based on peptides or proteins displayed on the phage coat. The cargo may be a packaged gene incorporated into the phage genome for gene delivery applications, or imaging agents or cytotoxic drugs chemically conjugated at high density onto the phage coat. We believe that the combination of those separately developed methodologies would result in clinical applications of phage-based therapeutics.

Keywords: antibiotic resistance, antibody – drug conjugates, target-specific peptides, targeted therapy

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1. The biology of Ff filamentous phages

The Ff class of filamentous coliphages consists of M13, f1 and fd. These *Escherichia coli* (*E. coli*) male-specific bacteriophages (phages) form particles of nanometric dimensions (~ 1 μm in length but less than 10 nm in diameter); hence we refer to them as phage nanoparticles. The particle consists of a single-stranded DNA core surrounded by a proteinaceous coat [1]. The coat contains five different proteins (**Figure 1**). Several thousand copies of the major coat protein – g8p (also referred to in this review as p8) cover the length of the particle. The four minor coat proteins are present at about five copies per particle. The g7p and g9p proteins cap one end of the particle, while g3p and g6p cap the other end. All five coat proteins contribute to the structural stability of the phage particle, but g3p (also referred to in this review as p3) is also necessary for host cell recognition and infection. Consequently, p3 is the largest and most complex of the phage coat proteins and is composed of three distinct domains [2]. The N-terminal domain initiates translocation of the viral DNA into *E. coli* during infection, while the second domain confers host cell recognition by binding to the F pilus on the *E. coli* surface [2]. The C-terminal domain of p3 interacts with other phage coat proteins, and is thus responsible for the integration of p3 into the phage coat.

2. Phage display technology

The fact that polypeptides fused to the phage coat proteins are displayed on the surface of the phage particle enabled the development of phage display technology [3]. Phage display is a robust technique for obtaining libraries containing

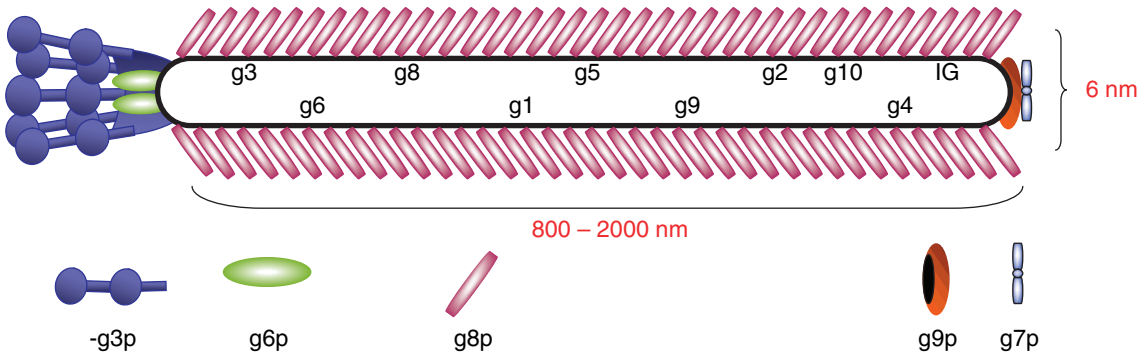

Figure 1. Structure of the filamentous bacteriophage. The diagram shows a single-stranded DNA core (thick black line) surrounded by a proteinaceous coat. The positions of the 10 phage genes and the intergenic region that contains the phage origin of replication are marked.

millions or even billions of different peptides or proteins. Phage display [3] has been used for affinity screening of combinatorial peptide libraries to identify ligands for peptide receptors, define epitopes for monoclonal antibodies, select enzyme substrates and screen cloned antibody repertoires [4]. One of the most successful applications of phage display has been the isolation of monoclonal antibodies, and fragments thereof, using large phage antibody libraries [4-6]. Screening by using phage display can be done in several ways. In type 3 or type 8 display systems, the open reading frame coding for the displayed polypeptide is fused to gene 3 or gene 8 in the viral genome and displayed on every copy of the structural phage coat protein. This polyvalent display system, which permits avidity, excels at selecting binders with low monovalent binding but with higher functional affinity when the antigen is multivalent [7]. With type 33 or 88- hybrid phage, the displayed fusion protein is included as an additional component of a phage coat that also contains wild type copies of all five coat proteins. That is, the fusion gene is added in addition to a complete phage genome [1]. Alternatively, hybrid display can be achieved with a phagemid-based system, as in the type 3 + 3 or 8 + 8 systems. A phagemid is a plasmid that contains DNA sequences that allow packaging into phage particles. The phagemid replicates in *E. coli* as a double-stranded plasmid, but co-infection with helper phage results in the production of single-stranded phagemid DNA, which is packaged into phage particles [1]. The resulting phage contains both wild-type coat proteins (provided by the helper phage) and the fusion protein (encoded from the phagemid). If the fusion is with p3, these phages will bear between none to five copies of fusion polypeptide per phage [7]. Such monovalent display has the potential advantage of allowing more efficient selection of higher affinity antibodies. The filamentous bacteriophages' natural capabilities to selectively infect host bacteria and the discovery of internalizing phages [8] has enabled its use for therapeutic purposes, such as imaging and drug carrying, which will be reviewed here. Phage display technology also paved the way to applying peptide or antibody-displaying phages directly as probes for biological threats and environmental contaminants, which have been recently reviewed elsewhere [9].

3. Imaging with phages

The revolutionary phage library-based screening methods recently expanded into the exciting field of *in vivo* targeting of various targets. The tailoring of peptides and proteins that bind various targets and imaging agents had opened the way to *in vivo* screening, as well as monitoring of specific targets using the whole phage body as a targeting as well as an imaging vector. Here we describe recent studies that described such applications.

The first report by the Arap and Pasqualini group [10] presented a bi-functional ligand display system for receptor targeting. This group pioneered the approach of *in vivo* panning of peptide phage display libraries in mice [11] and humans [12]. The major aim of these studies was to identify peptide ligands that home to tissue-specific endothelial cell receptors following intravenous administration. Such peptide ligands, or antibodies, directed against specific vascular receptors, can be used to target therapeutic compounds or imaging agents to endothelial cells *in vitro* and *in vivo*. The authors claimed that despite extensive advances in the understanding of the bacteriophage biology [13], as well as methods for simultaneous display of ligands on different proteins generating bi-functional phages, such constructs have been restricted to *in vitro* applications. To take the leap into an *in vivo* application, the authors expanded this concept by constructing and validating a bi-functional phage that displays both an integrin binding motif and a streptavidin binding motif. Imaging was demonstrated by luminescent quantum dots localization, surface plasmon resonance-based binding detection, and an *in vivo* tumor model. The target cells were KS1767 cells that express αv integrins in high levels and resemble the phenotype of activated endothelial cells [14]. These authors could show

that cell attachment by the phages could be inhibited by the corresponding synthetic peptide RGD-4C. These results have confirmed that cell adhesion in this system is specific and RGD dependent. Having shown the viability of using the bi-functional construct *in vitro*, the authors developed a targeting application *in vivo* as proof-of-principle for the chimera phage. Using a mouse mammary gland tumor model [15] they found preferential homing of the targeted phages to the tumor, with a fourfold higher accumulation of targeted phage in tumors in comparison to control, non-targeted phages. The authors' main achievement was the proof of *in vivo* application of targeted phages as imaging agents.

A recent study by Weissleder *et al.* [16] aimed to overcome existing bottlenecks in identifying phage clones with the highest likelihood of *in vivo* success and to determine whether phages could be used as a targeted imaging agent. They set out to develop comparative *in vivo* screening tools, reasoning that such comparative screens could be of value in eliminating *in vitro*-identified clones that would ultimately fail because of unfavourable *in vivo* pharmacokinetics, delivery barriers, opsonization, or insufficiently high target-to-background ratios [17,18]. To deal with these challenges, they directly labelled viable phage clones with far-red fluorochromes and comparatively imaged them *in vivo* by multichannel fluorescence ratio imaging in mouse tumor models. Using Secreted Protein Acidic and Rich in Cysteine (SPARC) as a model targeting moiety [19,20] and vascular cell adhesion molecule-1 (VCAM-1) as a target specific for inflammatory endothelium [21], they have shown how novel targeted peptide sequences presented on phages can be rapidly selected against this target. They also reported that a fluorescently labelled phage retained target specificity on labelling, and that *in vivo* distribution can be quantified (detection thresholds of 300 phage/mm^3 tissue) throughout the entire depth of the tumor using fluorescent tomographic imaging. Finally, they showed that the fluorescently labelled phage itself can serve as a replenishable molecular imaging agent. The ease of preparation of the phage imaging agent compared favourably with peptide-modified magnetofluorescent nanoparticles that were evaluated using the same model system.

In a study by Rusckowowski *et al.* [22], phages were used for the imaging of a bacterial infection *in vivo*. These authors were motivated by 'the need for techniques for anatomically delineating the primary and metastatic sites of infection; and the need for noninvasive imaging techniques that could be performed repetitively to assess the response to therapy' [23]. Although this statement is over 20 years old, even today the identity of the specific microbe remains undefined in approximately 30% of community-acquired pneumonia cases, which is one of the leading infections ending with hospitalization. This undefined cause of infection leads physicians to treat the patients with a very broad spectrum antibiotic therapy, as opposed to suspected pneumonia cases.

To that end, the authors radiolabelled M13 phage with ^{99m}Tc via mercaptoacetyltriglycine (MAG3). After radiolabelling, the phages were tested for binding ability 1, 5 and 10 min post-IV injection of *E. coli* strain 2537 (target bacteria), or *E. coli* strain 25922 and *Staphylococcus aureus* (*S. aureus*) strain 29213 (both are non-target control bacteria). In addition, the radiolabelled phages were also tested for specificity in mouse models that had received a subcutaneous injection of either live (infection/inflammation model) or heat-inactivated (inflammation model) bacteria in the thigh. The results showed that as early as 1 min, 84% ± 2% of the label was associated with *E. coli* 2537, and this percentage was unchanged over 10 min. By contrast, the negative controls *E. coli* 25922 and *S. aureus* bound only 41% ± 2% and 48% ± 3% of the label, respectively, at 1 min, followed by a slight decrease to 33% ± 6% at 5 and 10 min in both cases. Thus, even though the labelled M13 phage showed preferential binding to a single *E. coli* strain, it also bound to a second strain and to *S. aureus*, although at a lower level. Moreover, a thorough analysis of the biodistribution of the ^{99m}Tc-labelled phages in normal mice was carried out at various times. The main observation was that the lungs and liver were the organs of greatest phages accumulation at the earliest time, with about 31% in liver, 8% in lungs and 2% in spleen at 30 min. Activity in all organs gradually decreased over time so that at 6 h the kidneys, spleen and lungs contained only about 1% of the injected dose and by 24 h liver activity was reduced to 5% and lung activity to 0.39%. Values for blood were 2.5% at 30 min and decreased to 0.2% at 24 h post-phage injection.

To conclude, these authors have shown that the M13 phage may be labelled with ^{99m}Tc using MAG3 without *in vitro* evidence of label instability in saline or serum. They have also shown that the radiolabelled phage binds immediately and essentially equally to both live and heat-killed bacteria and that, once bound, the phage is surprisingly stable to dissociation. Furthermore, when they tested M13 binding to three different bacterial strains in culture, they observe a gradual specificity in which *E. coli* 2537 appeared to be preferred to *E. coli* 25922 and *S. aureus* 29213.

The application of targeted phages as new tools for MRI imaging was recently demonstrated by Segers *et al.* [24], who coined the term 'magnetophage' to described the particles they developed. In a previous study, they had isolated peptide-displaying phages that specifically bind phosphatidylserine (PS), a marker of apoptosis. These phages did not bind to phosphatidylcholine, and competition with annexin V confirmed their specific interaction with PS [25]. Magnetophages were prepared by reacting those phages with ultra-small particles of iron oxide (USPIO) previously reacted with epichlorhydrin, so that the USPIO reacted with amine groups on the phage coat. When these magnetophages were injected IV to BALB/c mice, they were mostly sequestered

by the liver (similar to the observation in the ^{99m}Tc-labelled phages described above). To overcome the non-specific liver uptake, 'stealthy magnetophages' were prepared by using polyethylene-glycol (PEG)-modified USPIO that were linked to the PS-specific phages. These stealthy magnetophages no longer accumulated in the liver of the mice, and could be used as MRI contrast agents as they induced a decrease in the relative enhancement of apoptotic, but not healthy, mouse livers. The authors suggested that USPIO-labelled phages adsorbed serum proteins onto the dextran coating of the particles, which opsonized them for uptake by the reticuloendothelial system (RES), which was prevented by PEGylation. The fact that phages are taken up by RES cells is well documented, and considered as an obstacle for their potential as therapeutics [26,27]. It was already shown that the biodistribution of phages in mice can be modulated by conjugating sugar groups to the phage coat [28]. By preparing 'stealthy magnetophages' that are coated with PEG, Segers *et al.* presented an additional useful solution to the unfavourable biodistribution of phages.

4. Phage-mediated gene delivery into mammalian cells

This technology was developed following discoveries by several groups that identified 'internalizing phages' while searching for cell surface molecules that could be used as targets in targeted immunotherapy. Antibodies that bind cell surface receptors in a manner whereby they are endocytosed are useful molecules for the delivery of drugs, toxins or DNA into the cytosol of mammalian cells for therapeutic applications. Traditionally, internalizing antibodies have been identified by screening hybridomas. Several groups turned to phage display as an alternative approach. In a pioneering work from the Marks group at UCSF, an anti ErbB2 antibody was used to determine the feasibility of directly selecting internalizing antibodies from phage libraries and identifying the most efficient display format [8]. They used the anti-human epidermal growth factor receptor 2 (ErbB2) antibody C6.5 (in a scFv format) displayed monovalently on phage as a model, and demonstrated that anti-ErbB2 phage antibodies can undergo receptor-mediated endocytosis. This group went on and isolated internalizing phages by selecting antibody phage libraries on cultured tumor cells [29]. Soon thereafter, David Larocca's group at Selective Genetics suggested using internalizing phages for gene delivery [30]. Although phage display has had until then limited use in the development of targeting agents for gene therapy vectors, by adapting a filamentous bacteriophage for gene delivery to mammalian cells, these authors have shown that it is possible to screen phage libraries for functional ligands capable of delivering DNA into cells. As an example, when targeted with epidermal growth factor (EGF), M13 bacteriophages were capable of delivering a green fluorescent protein (GFP) gene to EGF receptor-bearing cells in a ligand-, time- and phage concentration-dependent manner. They further demonstrated that EGF phages could be selected by their ability to transduce EGF receptor bearing cells from peptide display phage libraries. These data suggest the feasibility of applying molecular evolution to phage gene delivery to select novel cell-specific DNA-targeting ligands. The same approach could be used to select genetically altered phages that are specifically designed and evolved as gene therapy vectors [30]. Selective Genetics patented the technology of 'methods using genetic package display for selecting internalizing ligands for gene delivery' (US patent US 6,451527 B1). The Marks group soon published a work stating that prokaryotic viruses can be re-engineered to infect eukaryotic cells, resulting in expression of a reporter gene inserted into those bacteriophage genomes. Still working with the ErbB2 model system, phages capable of binding mammalian cells expressing ErbB2 and undergoing receptor mediated endocytosis were isolated by the selection of a phage antibody library on breast tumor cells and recovery of infectious phages from within the selecting cells. One of the isolated phages was engineered to package the GFP reporter gene driven by the cytomegalovirus promoter. When applied to cells, these phages underwent ErbB2-mediated endocytosis leading to GFP expression. These results further demonstrated the potential of phage antibodies as an *in vitro* or *in vivo* targeted gene delivery vehicle [31]. Two years later, Larocca and Baird reviewed the technology while listing the criteria for efficient selection of internalizing phages and comparing them to the better known viral and synthetic vectors used for gene therapy [32]. In a related study, the Selective Genetics group, while seeking to improve transduction efficiency so as to improve the usefulness of targeted phage vectors for gene therapy and ligand discovery, described the use of multivalent phagemid vectors that were specifically designed for ligand-targeted mammalian cell transduction. This phagemid system had certain advantages over phage vectors that were described in the earlier studies, such as larger insert size and vector stability, and they retained the multivalent display necessary for efficient cell binding and internalization. That system was most effective when the targeting ligand, EGF, was fused to the C-terminal domain of the p3 coat protein. By comparing phagemid particles displaying EGF at high or low valence by rescuing the vector with R408d3 (p3 deleted) or wild-type R408 helper phage, respectively, the more efficient display of EGF correlated with increased internalization, vector potency and transduction efficiency [33].

To prepare a modular system for gene delivery into tumor cells, Mount *et al.* developed a gene delivery system that uses a cell-binding helper phage pre-selected from a landscape phage display library, and a phagemid harboring a reporter gene and all regulatory elements (origins of replication and promoter – enhancer cassettes) necessary for replication of the phagemid and expression of the reporter gene in the targeted cell. In their system, all the proteins

that are required for encapsulation of the phagemid DNA and cell targeting are provided by the helper phage, while each different packaged phagemid can provide a different gene to be transduced. The resultant phagemids, which were named phagemid infective particles (PIPs), were able to bind and infect target cells and express the reporter gene from within the cell. Mount *et al.* tested their system using glioma cells. The authors suggested that because of its versatility, the PIPs system may be readily used for optimization of the gene delivery strategies applied to specific cell and tissue targets [34].

While the former studies used protein displaying phages for gene delivery, Paolo Monaci *et al.* soon followed suit with peptide displaying phages [35]. By screening a random peptide library displayed on phages as fusion to the major capsid protein, p8, they identified a ligand that specifically bound human ErbB2 (HER2). By mutating the sequence of this ligand, a 'secondary' library was generated, whose panning on HER2-positive cells isolated a phage-borne peptide with improved binding to HER2. The identified peptide recognized HER2 also when expressed as an N-terminal fusion to the minor coat protein, p3. When that phage was engineered to include a mammalian expression cassette for a reporter gene within its genome, it transduced HER2-expressing cells with very high specificity and with an efficiency comparable to that of chemical transfection protocols [35]. The criteria and steps of cell transduction by peptide displaying phages was reviewed by Uppala and Koivunen, who made a very thorough comparison of retroviral, lentiviral, adenoviral, adeno-associated virus (AAV) and naked/lipid-DNA vectors for gene therapy with the filamentous phage data available at the time [36]. Phages were referred to as the 'future vehicles for gene delivery' in a review in 2001 describing delivery systems intended for *in vivo* gene therapy of cancer, where the focus was on targeted viral vectors [37].

A recent work by the group of Pasqualini and Arap [38] used phages presenting a ligand-directed tumor targeting, combining gene delivery with molecular imaging. They developed a unique vector that was based on a chimera of filamentous phage and AAV. Their hypothesis was that combining the favourable biological attributes of eukaryotic viruses to those of prokaryotic viruses might yield chimeric particles with potential as targeted delivery tools for molecular imaging. The inclusion of the AAV sequences was designed to cause the conversion of the single-stranded DNA delivered by the phage to double-stranded DNA, resulting in improved transduction efficiency. For this goal they constructed a chimeric virus comprised of recombinant AAV and an fd – tet phage clone displaying the integrin-specific double-cyclic peptide CDCRGDCFC (termed RGD-4C) phage [11,39,40]. The RGD-4C peptide binds to αv integrins, a cell surface receptor overexpressed in both tumor and endothelial cells [11,41]. To obtain chimeric viruses (hereafter referred to as AAV/phage; AAVP), they inserted a eukaryotic gene cassette from AAV in an intergenomic region of RGD-4C phage (RGD-4C AAVP). Moreover they also demonstrated that RGD-4C AAVP carrying reporter genes can mediate ligand-directed internalization and transduction of mammalian cells relative to controls.

To further evaluate the tumor targeting *in vivo* they used an RGD-4C AAVP variant encoding the GFP gene as a reporter to determine, by using *in situ* immunofluorescence microscopic imaging, whether this vector (RGD-4C AAVP-GFP) could transducer KS1767-derived tumor xenografts in mice. Seven days post-administration, immunofluorescence revealed GFP expression largely in tumor blood vessels and surrounding tumor cells in mice that received RGD-4C AAVP-GFP. These results suggested that RGD-4C AAVP particles can specifically target tumor xenografts by a ligand directed mechanism and transduce them after systemic administration *in vivo*. Next, the authors introduced into the AAVP vector the HSV-tk gene, which can serve as both a 'suicide' gene (when combined with ganciclovir [GCV]) and a reporter transgene for clinically applicable PET imaging with HSV-tk-specific radiolabelled nucleoside analogues. After GCV therapy, the volume of tumors in mice that received RGD-4C AAVP-HSV-tk was significantly smaller than in mice that received non-targeted AAVP-HSV-tk. Finally, they assembled a panel of tumor cell lines from different species and histological origins and generated tumors in immunosuppressed or immunocompetent mice. Cohorts of Kaposi sarcoma (KS1767) derived, tumor bearing mice systemically received a single intravenous dose of the RGD-4C AAVP-HSV-tk, followed by GCV treatment in all groups. Marked tumor growth inhibition was observed in tumor-bearing mice receiving RGD-4C AAVPHSV-tk as compared to mice treated with vehicle or mice that received non-targeted AAVP. In conclusion of this work, the native tropism of AAV for mammalian cells was eliminated, since there is no AAV capsid formation and the displayed ligand peptides allow homing to tissue-specific receptors as well as specific killing of target cells.

5. Phage-mediated drug delivery

As a universal, modular solution for applying phages as targeted drug-carrying platforms, the author's group has been developing a novel application of filamentous phages as targeted drug carriers for the eradication of pathogenic bacteria and cancer cells. The phages (as illustrated in **Figure 2**) were used to deliver a large payload of a cytotoxic drug to the target cells. In the antibacterial application, the drug was linked to genetically modified phages by means of chemical conjugation through an esterase cleavable linker subject to controlled release by serum esterases. In parallel, the phages were genetically engineered to display a targeting moiety on their coat. This concept demonstrated the potential of using such multi-engineered filamentous phages as universal drug carriers. The main achievement of this

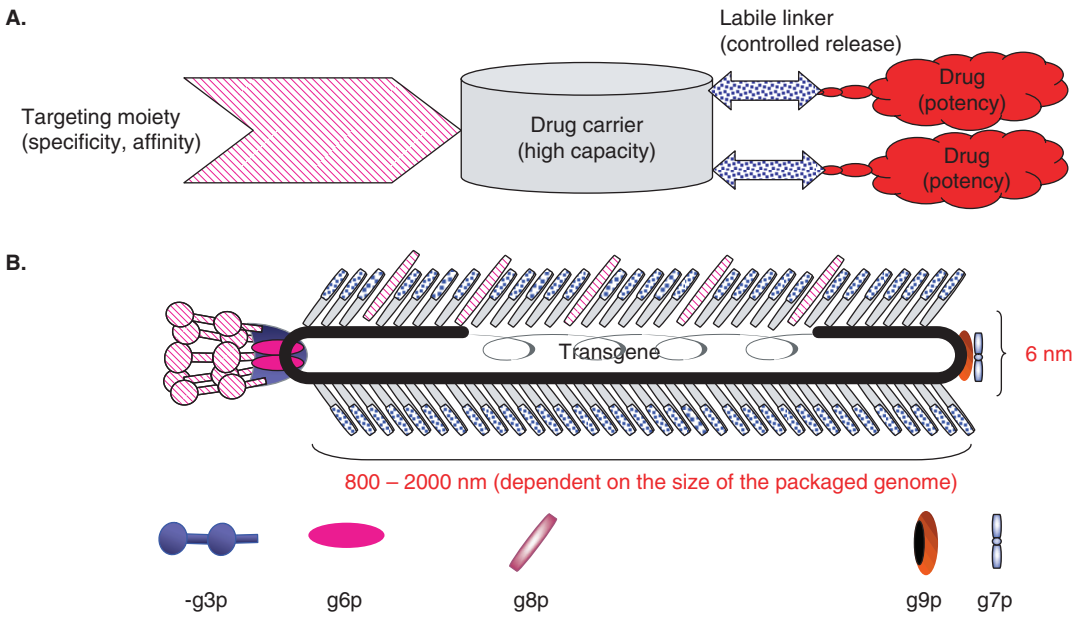

Figure 2. The filamentous phage as a targeted drug carrying platform. A. illustrates the basic components of a targeted drug carrying platform. In the phage example, **B.** a coat protein, either g3p or g8p, carries the targeting moiety while the drug (or imaging agent) and the engineered release mechanism are carried on g8p. In a gene delivery setting, the transgene is incorporated into the packaged, single-stranded DNA phage genome (depicted as a single-stranded helix).

approach was the transformation of the drug selectivity itself to a target selectivity borne by the targeting moiety, a feature that may shed new light on neglected non-specific drugs that, due to toxicity or low selectivity, have thus far been excluded from clinical use. The feasibly of this approach was demonstrated by using the bacteriostatic, non-potent antibiotic chloramphenicol (which is mostly excluded from systemic therapeutic application due to its haemolytic properties) [42] as a model drug. In our proof of concept study [43] targeting was accomplished by using two different targeting moieties: i) target-specific peptides, selected from a peptide phage display library, that were displayed on the major coat protein of the phage; or ii) antibodies linked to the phages via an IgG Fc-binding ZZ domain that was fused to the p3 minor coat protein of the phage. As a model target, we used the bacterial pathogen *Staphylococcus aureus*. The preliminary system [43] suffered from limited ability to inhibit bacterial growth due to a limited arming capacity of less than 3,000 drug molecules/phage, a limitation mainly due to the hydrophobicity of the drug. This led to a second generation of the platform by the development of a unique drug conjugation chemistry, based on the application of hydrophilic aminoglycoside antibiotics as branched, solubility enhancing linkers [44]. The replacement of the arming chemistry and a modification of the antibody – phage conjugation method improved our system into a viable and versatile tool for the targeting of a broad range of pathogenic bacteria such as *S. aureus, S. pyogenes and E. coli*, each targeted by microbe-specific antibodies. Experimentally the new drug conjugation approach led to an arming rate of over 40,000 chloramphenicol molecules/phage. These results had proven an impressive improvement factor in drug potency of about 20,000 in comparison to the free drug, as measured by bacterial growth inhibition in liquid culture. This large drug carrying capacity was made possible by using the exterior of the phage coat, which offers many docking sits on the 3000 coat protein monomers. Other phage-based drug delivery model systems pack the drug within the particle and hence have a limited drug carrying capacity [45].

While anti-bacterial targeted drug carrying phages release their cargo outside the target cells, we recently evaluated such phages for internalization-dependent retardation of tumor cell growth in cell culture. In these examples (Bar *et al.*, 2007, submitted) we used anti-ErbB2 and anti-ERGR antibodies as targeting moieties, the drug hygromycin conjugated to the phages by a covalent amide bond, or the drug doxorubicin conjugated to genetically engineered cathepsin-B sites on the phage coat. We could show that targeting of phage nanomedicines via specific antibodies to receptors on cancer cell membranes results in endocytosis, intracellular degradation and drug release, resulting in growth inhibition of the target cells with a potentiation factor of > 1000 over the corresponding free drugs. Our results revealed important features regarding the potential of filamentous phages to serve as an internalization-dependent drug delivery platform and define targeted drug carrying filamentous phage nanoparticles as a unique type of antibody – drug conjugates.

In conclusion, drug carrying phages represent a versatile therapeutic nanoparticle that, due to the tailoring of its coat, the simplicity by which it can be equipped with a targeting moiety and the massive drug carrying capacity, may become an important general targeting drug delivery platform. By comparison to particulate drug-carrying devices such as liposomes or virus-like particles, the arrangement of drug that is conjugated in high density on the exterior of the targeted particle is unique. A dense coating of the phage with aminoglycosides and drugs might produce advantages that are regarded as challenges in the application of phages as therapeutics; primary, of course, is the immunogenicity upon *in vivo* administration. Indeed, our unpublished results have shown that drug carrying phages are not recognized by anti-phage antibodies that were generated by vaccinating mice with 'naked' phages (unpublished data). Further, we found that drug carrying phages are non-toxic, as well as much less immunogenic to BALB/c mice up to a high dose of 10^{11} phage particles injected intravenously or intraperitoneally. The therapeutic capacities of targeted drug carrying phages in mouse models are currently being studied.

6. Conclusion

In this review we presented studies that demonstrated the potential of filamentous phages to be applied as targeted therapeutic agents. These studies were initiated when the investigators independently realised that time was ripe to combine the wealth of knowledge in the field of phage display technology in general, and *in vivo* phage panning in particular, with chemical modification of the phage coat to load it with imaging agents or with drugs. For gene delivery, the fact that phages can be used to identify internalizing antibodies and peptides was translated into using the entire internalizing phage as a gene delivery platform. For gene delivery purposes, a major hurdle for efficiency was overcome by the preparation of hybrid phage – virus vectors for conversion of the delivered single-stranded DNA to a double-stranded form for more efficient transduction of the target cells [38].

A wealth of targets and targeting moieties has been presented for using bacteriophages as targeted therapeutic agents. Phages were targeted by displaying antibodies, peptides, or by the natural affinity of the phage p3 coat protein for the F plus of the *E. coli* target. Targets included cell surface receptors, integrins, cell adhesion molecules, bacterial cell wall components and phosphatidylserine. Examples of cargo included imaging agents, radioisotopes, quantum dots, MRI contrast agents and cytotoxic drugs. Some of the studies dealt with obstacles to *in vivo* application of phages due to uptake by RES cells or immunogenicity, and provided solutions to overcome those hurdles.

To conclude, although other phages are being considered for therapeutic applications, as in phage therapy [46,47], filamentous phages, with their robust structural features, high drug (or imaging agent) carrying capacity and diversity of displayed targeting moieties, have the greatest potential of becoming a significant addition to the fast evolving arsenal of nanomedicines.

7. Expert opinion

This review had presented several *in vitro* and *in vivo* oriented applications of phage-based therapeutics. Such applications became possible when it was realised that in addition to facilitating the identification of target-specific peptides or proteins, or to interrogate vascular targets, that phage particle itself can serve as a platform allowing packaging on a genetic cargo (inside), or imaging agents or drugs (outside) the phage particle. The beauty of combining sophisticated genetic approaches such as foreign peptide and protein display on the phage coat with a chemical conjugation of compound from a battery of imaging as well as drugs to phages has been developed in recent years. In this review we covered *in vitro* and *in vivo* oriented applications of such tailored phages. In our view, the reports presented here generated two exciting new ways based solely on the unique properties of the phage platform. The first is the ability to screen *in vivo* for challenging unique targets such as tumor and vascular endothelial targets, as pioneered by the Arap and Pasqualini group. The second is the phage-based drug carrying, which opens up a new way in the ongoing battle against pathogenic bacteria. Since most antibiotics are considered non-potent drugs in terms of drug/bacterium ratio, and the lack of a general bacterial internalization process, none of the conventional drug delivery approaches fits this job. The phage coat drug carrying capacity of over ~ 10^4 molecules, as well as the extracellular drug release, resulting in local high drug concentration, nicely addresses those challenges.

The foreseeable challenges for a broader application of targeted phages in nanomedicine drug delivery approaches in general [48,49], will be those of immunogenicity and toxicity. These issues are of particular importance when long-term therapeutic applications are considered, and less so for imaging purposes. However, some of our unpublished evidence suggests that chemical modification of the phage coat, specifically with sugar-based compounds, limits the immune system reactivity. The possibility of altering the pharmacokinetic and pharmacodynamic properties of phages by chemical modification is particularly important since drug delivery platforms should behave differently from imaging agents. A more intensive and thorough research should address the many remaining questions about the efficiency and safety of using phage particles in *in vivo* therapeutic approaches.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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