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Bacteriophage T4 as a nanovehicle for delivery of genes and therapeutics into human cells

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The ability to deliver therapeutic genes and biomolecules into a human cell and restore a defective function has been the holy grail of medicine. Adeno-associated viruses and lentiviruses have been extensively used as delivery vehicles, but their capacity is limited to one (or two) gene(s). Bacteriophages are emerging as novel vehicles for gene therapy. The large 120 × 86-nm T4 capsid allows engineering of both its surface and its interior to incorporate combinations of DNAs, RNAs, proteins, and their complexes. *In vitro* assembly using purified components allows customization for various applications and for individualized therapies. Its large capacity, cell-targeting capability, safety, and inexpensive manufacturing could open unprecedented new possibilities for gene, cancer, and stem cell therapies. However, efficient entry into primary human cells and intracellular trafficking are significant barriers that must be overcome by gene engineering and evolution in order to translate phage-delivery technology from bench to bedside.

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Introduction

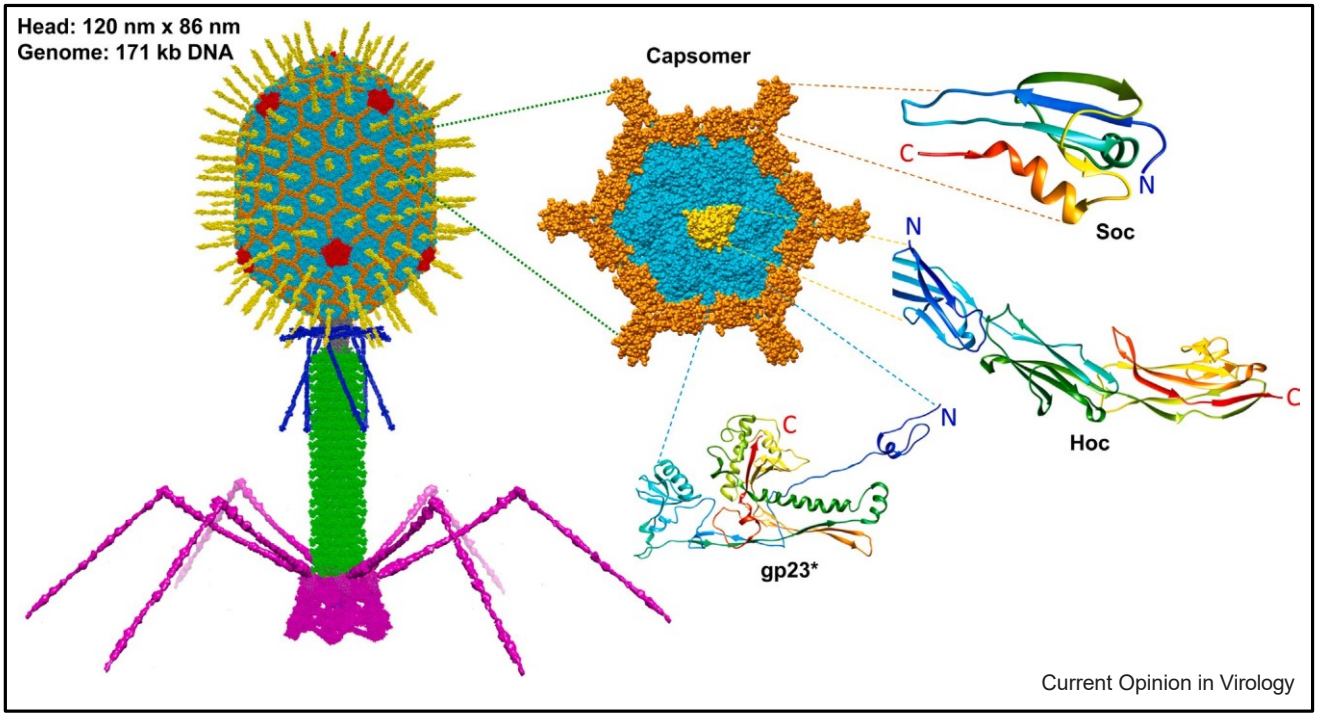
Bacteriophages (phages) probably constitute the largest biomass on Earth. They are widely distributed on lands and waters, in extreme environments, and in the guts of animals [1,2]. Of these, the tailed, double-stranded DNA (dsDNA) phages such as T4 (*Myoviridae*), lambda (*Siphoviridae*), and T7 (*Podoviridae*) are the most prevalent [3].

Historically, bacteriophages provided powerful tools for genetic manipulations that led the biotechnology revolution. Phage vectors have been extensively used to construct genome libraries, produce protein drug products, display peptides and antibodies, and evolve molecules with novel biological functions [4–6]. Their potential as antibiotics to kill multidrug-resistant bacterial pathogens (phage therapy) was demonstrated nearly a century ago and has re-emerged in recent years, and many clinical trials are underway [7]. However, the use of phages as therapeutics (drugs) to treat/cure genetic and metabolic diseases (phage medicine) is only beginning. Phages provide unlimited varieties of precisely assembled natural nanostructures that can be engineered to deliver therapeutic cargo molecules into human cells. While phage T4 due to its size and architectural features has emerged as a leading candidate, many other phages such as λ, T7, P22, M13, and Qβ have been considered for various biomedical applications [5,8–17].

Phage T4 is comprised of a prolate, 1200-Å long and 860-Å wide, head (capsid) with a 1400- Å long contractile tail to which six ~1600-Å long tail fibers are attached [18,19] (Figure 1). T4 infects *Escherichia coli*, and its infection efficiency is one of the highest, near the theoretical maximum of 1.0 [20]. It encapsidates ~171 kbp of linear dsDNA genome and its exterior is decorated with 1025 molecules of nonessential outer capsid proteins, 870 molecules of small outer capsid protein (Soc), and 155 molecules of highly antigenic outer capsid protein (Hoc) [18].

Phage T4 has a rich genetic history and well-understood structural framework. Its circularly permuted genome consisting of ~300 open-reading frames has been mapped and functions of most of the genes have been described in detail [21]. A CRISPR engineering strategy to manipulate the genome and generate recombinant phages has been developed [22–24]. The atomic structures of virtually every component of the T4 virion have been determined by X-ray crystallography and/or cryo-electron microscopy (EM) [25–31]. Decades of molecular analyses led to a wealth of mechanistic information on capsid assembly, genome packaging, and genome delivery. This review focuses on how this genetic and mechanistic knowledge combined with T4's architectural features and genome engineering strategies can be harnessed to develop novel human gene therapies. The review also identifies key barriers that must be overcome in order to navigate these from bench to bedside.

Figure 1



Structural components of bacteriophage T4 nanovehicle. The T4 head can encapsidate up to ~171 kb of DNA cargo, whereas the exterior outer capsid proteins Soc and Hoc serve as adapters to display foreign proteins. One of the capsomers is enlarged to depict the arrangement of major capsid protein gp23*(cyan), Soc (brown), and Hoc (yellow) subunits. The structures of gp23*(PDB ID 5VF3), Hoc (PDB ID 3SHS), and Soc (PDB ID 5VF3) are shown in rainbow colors, with their N- and C-termini labeled.

Phage T4 capsid architecture

The prolate icosahedral capsid of T4 is built with 930 copies of a single major capsid protein gp23* that form 155 hexameric capsomers ('*' represents the cleaved mature form) (Figure 1). Eleven of the icosahedral corners (vertices) are occupied by pentamers of the vertex protein gp24*, and the twelfth corner, the unique portal vertex, is occupied by 12 copies of the portal protein gp20 [18,19]. The latter is a dodecameric ring with a 3.5–4-nm-diameter central channel through which the dsDNA genome enters during packaging and exits during infection [26,32].

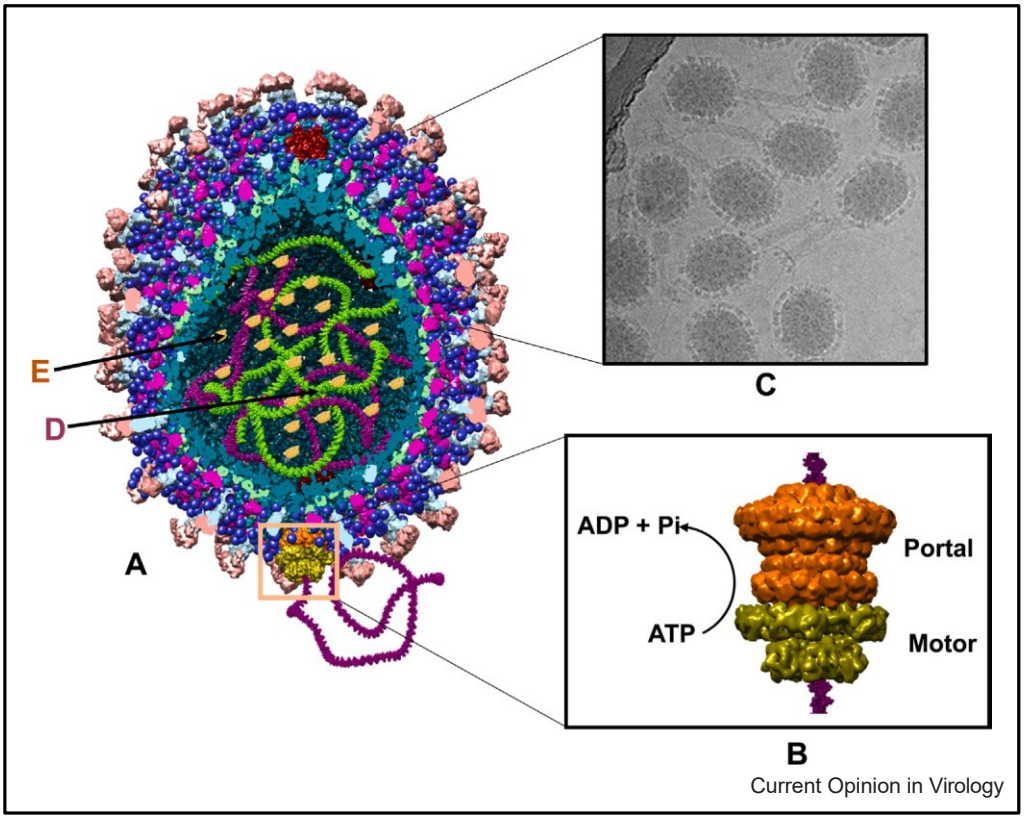
The major capsid protein gp23* has HK97 fold, a fold commonly found in all phage capsid protein structures examined thus far [28,33] (Figure 1). The atomic structure shows complex networks of intra- and inter-subunit interactions that stabilize the capsid. These are further reinforced by the binding of the triplex protein Soc, a 9.1-kDa tadpole-shaped molecule that exists as a monomer in solution but trimerizes when it assembles on the capsid at the quasi-threefold axes. Each Soc subunit, through interactions with two gp23* molecules, clamps adjacent capsomers [34] (Figure 1). Eight hundred and seventy such clamps form a molecular cage around the shell, making the T4 capsid one of the most stable viral structures [34]. These features not only allow the virus to withstand the high internal pressure of the tightly packed genome (~25 pN, 4–5 times the pressure in a champagne bottle) [35,36], but also maintain its integrity in hostile extracellular environments such as the human gut.

The second molecule that decorates T4 capsid is the 40.4-kDa Hoc, a linear fiber containing a string of four Ig-like domains connected by flexible linkers (Figure 1). It binds at the center of each capsomer as a monomer through the C-terminal capsid-binding domain, while its N-terminus is projected at ~170 Å away from the surface [37]. Consequently, 155 symmetrically arranged Hoc fibers emanate from the surface of the phage head. Hoc fibers at best provide marginal additional stability to the capsid. However [37], they might serve as environmental sensors and impart survival advantages, such as capturing host bacteria [38], or adhering to human cells through interactions with cell-surface molecules [39], though the mechanisms are yet unknown.

Capsid surface engineering

The above architecture provides unique features for engineering T4's surface lattice. First, it provides a high density of sites that can be used to decorate the virus

Figure 2



Compartmentalization of T4 nanoparticle cargos. (A) DNAs (purple and green) are packaged in the capsid interior by the packaging machine. Proteins (brown) are packaged as part of the scaffolding core. Proteins (magenta and blue) are displayed on the capsid surface as Soc-fusion proteins. Cell-targeting ligands (light pink) fused at the tip of Hoc fibers (cyan) decorate the capsid. (B) The T4 packaging machine is enlarged to show the dodecameric portal attached to a pentameric packaging motor. Utilizing energy from ATP hydrolysis, the packaging machine translocates DNA into the capsid. (C) Cryo-EM image showing T4 capsids decorated with oligomeric anthrax toxin complexes.

with foreign peptides and proteins [18]. Second, since both the N- and C-termini of Soc, and the N-terminus of Hoc are solvent-exposed (Figure 1), molecules can be fused to termini with minimal structural perturbation [34,37]. Third, since Soc and Hoc bind to capsid with exquisite specificity and nanomolar affinity, molecules fused to Hoc and Soc assemble efficiently on the capsid [40–47]. Fourth, since the tip of the Hoc fiber is ~17 nm away from the capsid, a cell-targeting ligand displayed here is well-positioned to capture surface receptor molecules present on targeted cells [37,48] (Figure 1).

The basic principles of assembling recombinant Soc and Hoc proteins on phage T4 nanoparticles have been extensively demonstrated [40–47,49,50]. Large full-length proteins (e.g. 83-89 kDa anthrax toxin proteins and their complexes) [51] (Figure 2) and oligomeric complexes as large as 433.5 kDa (e.g. trimeric SARS-CoV-2 spike ectodomain) [50], or even the 25-nm adeno-associated virion (AAV) particles [47,52] can be displayed on T4 capsid by *in vitro* assembly. Display could also be accomplished through *in vivo* assembly by expressing Soc- and Hoc-fusion proteins during phage infection [50,53,54]. However, expression of such fusions is inefficient *in vivo* and copy numbers of displayed molecules are relatively low. On the other hand, full occupancy can be achieved *in vitro*, though there are inherent limitations imposed by spatial hindrances, especially for the display of large proteins and complexes. A series of studies showed that the T4-displayed molecules (peptide/protein antigens) are highly immunogenic, stimulating robust cellular and humoral immune responses and complete protection against bacterial and viral infectious diseases, including anthrax, plague, flu, and COVID-19 in animal models [45–47,50,51,54–56].

Capsid interior engineering

The interior of T4 capsid can accommodate up to ~171-kb foreign DNA, making it one of the largest-capacity viral vectors available today. In comparison, the commonly used AAV vectors have a capacity of up to ~4.8 kb and the lentivirus (LV) vectors have a capacity of up to ~8 kb [57]. Another key feature of T4 is that the interior

space can be customized by filling it *in vitro* using a powerful packaging machine. Decades of research on the T4 packaging machine reduced the assembly system to a simple reaction involving two components, namely capsid and motor protein (gp17) [58–60]. The motor protein gp17 (69.8 kDa) assembles as a pentamer on the capsid portal vertex [19] (Figure 2). Powered by ATP, it translocates DNA into the shell at a rate of up to ~2000 bp/s and with a power density of ~5000 kW/m^3, making it the fastest and most powerful motor reported to date [35,61–64]. Furthermore, the T4 motor lacks sequence specificity and initiates packaging at any end of DNA [58,65]. Therefore, any foreign DNA can be packaged into T4 capsid, and there is no length requirement. It can package a single piece of genome-length DNA (up to ~171 kb), multiple plasmid DNAs, or even short oligonucleotides successively, until the head is full (headful packaging) [46,58]. Thus, the capsid would be filled with the same combination of DNAs as presented to the motor in the reaction mixture.

In addition to the DNA genome, the capsid also contains a cluster of ~1000 molecules of proteins that originate from a scaffolding core around which the capsid shell is assembled [66–68]. The core is then degraded by proteolytic cleavages creating 'empty' space for packaging the genome. However, some larger cleavage products remain in the shell, which include the 'internal proteins'; IPI, II and III, gpAlt, gp68, and others. Most of these can be replaced by foreign proteins by fusing a 10-amino acid capsid-targeting sequence (CTS) to the N-terminus, a homing signal to localize the protein in the scaffold structure [66,68] (Figure 2). By CRISPR engineering, a variety of foreign proteins could be packaged inside the capsid, including GFP, mCherry, Cre, and SARS-CoV-2 nucleocapsid protein [50,53,67]. This provides a novel feature to deliver combinations of DNAs, proteins, and their complexes such as transcription factors, recombinases, and genome editing enzymes for a variety of gene therapy operations.

Assembly of capsid nanoparticle payloads

The ability to engineer both the exterior and the interior of T4 capsid through self-assembly *in vitro* provides unusual flexibility to customize, and even personalize, the therapeutic payloads. We have optimized a sequential assembly line that merely involves a series of incubations with premade components, starting with an empty capsid shell [46] (Figure 3). The shells are purified from *E. coli* cells infected with packaging- and tail-deficient phage mutants. These mutants accumulate empty shells, up to ~10^{14} particles per liter, which can be stored at − 70°C for long periods of time [58]. Powered by the gp17 packaging motor assembled at the dodecameric portal vertex, these shells are first filled with DNA molecules. Then, the outer surface is decorated with proteins and complexes such as the genome editing molecules through fusion to Soc, as well as cell-targeting molecules through fusion to Hoc (Figure 3). In addition, proteins can also be incorporated into the capsid interior to form complexes with the packaged DNA(s). Thus, T4 nanoparticles carrying customized payloads of genes, proteins, and complexes can be created by mixing and matching appropriate combinations of biomolecules and their copy numbers can be quantified and tuned by varying the ratios of the components [46].

Transduction of human cells by T4 nanoparticles

The *in vitro*-assembled T4 nanoparticles can be transduced into cultured human cells. Nearly 100% transduction into HEK293 cells was observed, as determined by the expression of the reporter genes eGFP, mCherry, and luciferase. In a head-to-head comparison with AAV, the expression efficiency of the luciferase reporter gene is several-fold higher for T4 transduction probably because each T4 capsid can deliver multiple copies of the genetic payload in a single-transduction event, whereas AAV can transduce only one copy [46]. Furthermore, the T4 nanoparticle payloads can be targeted to specific human cells (e.g. CD4 + T cells) [48].

Since T4 nanoparticles have no tropism or ability to replicate in human cells, the mechanisms by which they enter a human cell and undergo intracellular processing are poorly understood. Current evidence indicates that the nanoparticles are internalized by endocytosis or macropinocytosis, escape the endosome, and disassemble in the cytosol [47] (Figure 4). While the released proteins and complexes exert their function in the cytosol or are transported to the nucleus when fused to a nuclear localization signal (NLS), the DNA molecules are translocated into the nucleus, apparently through a microtubule-dependent pathway [47].

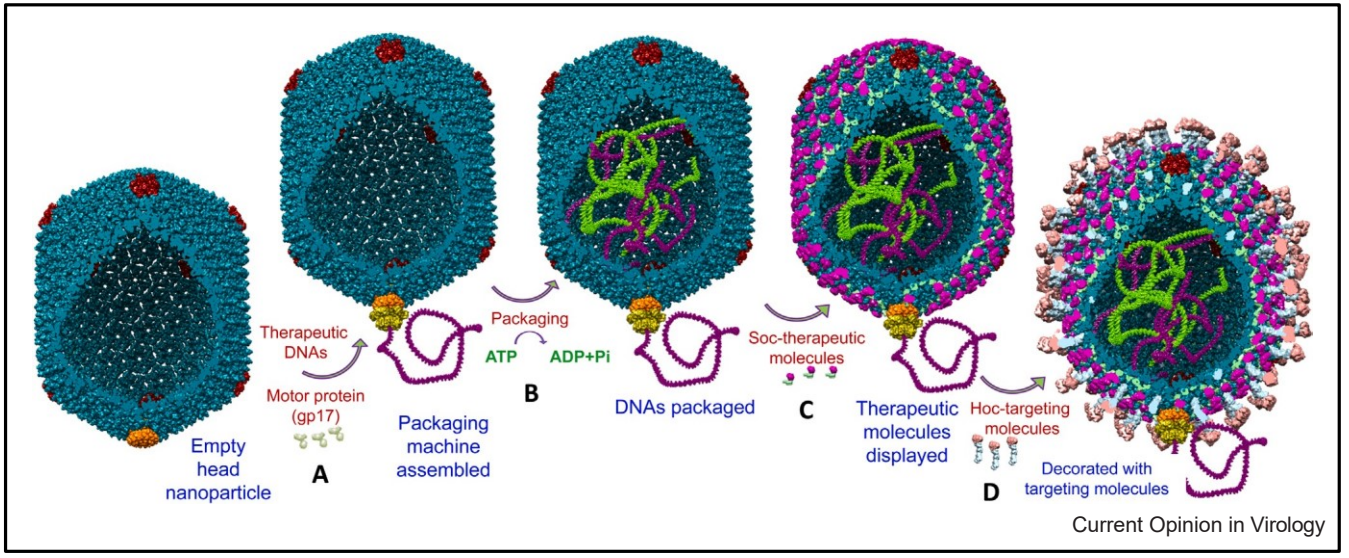
Distinct advantages and potential barriers

T4 and many phages have inherent size advantage and are amenable for deep engineering to create designer recombinant phages. These represent distinct advantages for creating gene therapy vehicles with considerable precision. However, phages possess no evolved mechanisms for entry into human cells and for intracellular trafficking (Figure 4). These barriers must be overcome to harness the potential for therapeutic applicability.

Payload capacity

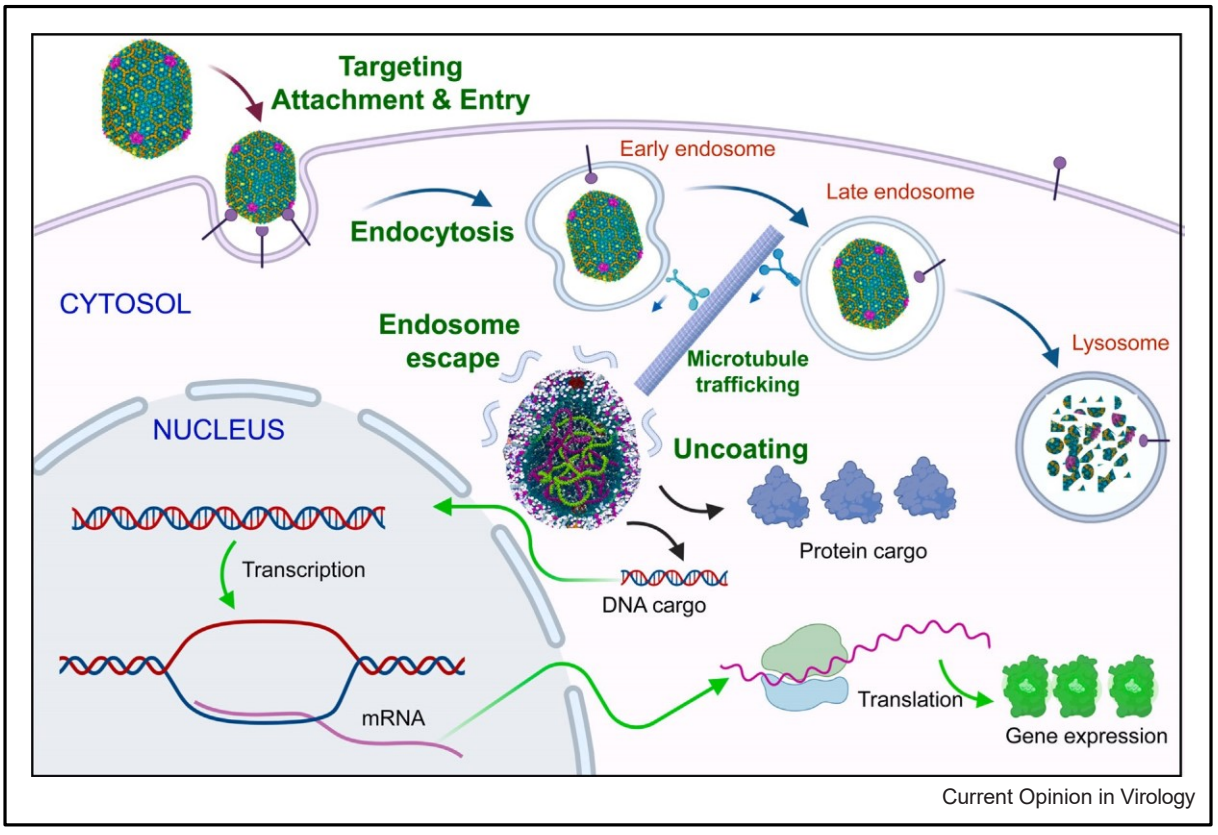
Until now, almost all gene therapy applications are directed toward monogenic diseases, mainly because of the limited capacity of the AAV and LV vectors. Though most single genes can be delivered by these viral vectors, large genes such as the muscular dystrophin gene

Figure 3



Sequential assembly of T4 nanoparticle cargos *in vitro*. (A) Assembly of the packaging motor protein gp17 at the unique portal vertex of the empty-head nanoparticle. (B) DNAs are packaged into capsid by the packaging machine fueled by ATP hydrolysis. (C) Soc-fused protein molecules are displayed on the surface by adding the purified protein to the reaction mixture. (D) The nanoparticles are decorated with cell-targeting molecules by adding the Hoc-fusion protein.

Figure 4



A proposed pathway for delivery of T4 nanoparticle cargos into human cells. T4 nanoparticles decorated with targeting ligands are captured by cell-surface receptors and internalized through an endocytic vesicle. Some of the nanoparticles escape the endosomes into the cytosol and are translocated to a perinuclear site through microtubule trafficking. Uncoating or disassembly follows releasing the cargos. The protein cargos exert their function in the cytosol, whereas the DNA cargos enter the nucleus and prime transcription under the control of a strong promoter. The mRNAs are transported to the cytosol and translated to proteins encoding a desired therapeutic function(s).

that has a size of ~11 kb [69], or the genome editing machinery that requires delivery of more than one gene [70], could not be accommodated. Multigenic and multifactorial diseases that include many cancers, diabetes, neurodegenerative, and cardiovascular diseases, which require correction of more than one gene, are beyond the capability of the current vectors. Furthermore, though LVs have been extensively used for CAR T-cell cancer therapies, the efficacy of such therapies can be greatly enhanced and tuned, if additional genes encoding targeting antigens, cytokines, and safety switches could be codelivered [71].

Phage T4, thus far, stands out as a high-capacity vector for its ability to incorporate varieties of biomolecules and complexes into a single nanoparticle. The ~1025 sites of Hoc and Soc can be used to build a therapeutic surface lattice consists of arrays of biomolecules and complexes through interactions between binding partners. Heptameric anthrax toxin complexes [43] and even the entire AAV particle [47] have been assembled on T4 capsid in this manner. In addition, the exposed loops of the major capsid protein gp23 (930 subunits per capsid) can be used to insert foreign peptides and domains [28]. In the capsid interior, multiple copies of multiple plasmids, up to ~170 kb, can be packaged, along with up to ~1000 molecules of proteins and protein–DNA complexes [46,68]. Coupled with CRISPR engineering and *in vitro* assembly [22,23,46,47], varieties of such 'artificial viruses' can be created for different disease indications and can be individualized for personalized medicine.

Cell targeting

T4 phage and phages in general have no specificity to interact with mammalian or human cells. In fact, its host-recognition molecules are located at the tips of tail fibers that would not even be present in the capsid-based nanoparticle vectors [31]. While this appears as a disadvantage, it is also a distinct advantage because the broad tropism exhibited by the AAV and LV vectors often requires cumbersome and expensive isolation of target cells (e.g. T cells and stem cells) to perform *ex vivo*

transfections. Otherwise, *in vivo* administration leads to rapid loss of therapeutic particles due to entry predominantly into nontarget cells [72]. This also poses a considerable safety risk. On the other hand, cell-targeting specificity can be engineered into T4 and phage nanoparticles by displaying targeting molecules through fusion to Hoc or Soc. The ~17-nm Hoc fiber with the targeting ligand at the tip is particularly suited to capture surface receptors present on human cells. Furthermore, the 155 symmetrically arranged Hoc fibers confer high specificity and avidity that can be exploited for a desired application [46]. Indeed, T4 capsid nanoparticles displaying Hoc-fused CD4Darpin (15 kDa), a CD4 receptor-binding ligand, titrated ~98% of CD4 + T cells from human Peripheral Blood Mononuclear Cells PBMCs, demonstrating extraordinary specificity [48]. Thus, *in vivo*-targeted therapies can be customized by simply changing the targeting ligand, a distinct advantage for T4 and phage-based delivery systems. This has also been amply demonstrated using filamentous phage M13 by fusing targeting ligands to the capsid proteins pIII and pVIII [73].

Internalization

Once bound to a human cell, nanoparticles can be internalized either by direct penetration through the plasma membrane, or by pinching off a small piece of the plasma membrane enclosing the nanoparticle within it, a process known as endocytosis. The former requires direct fusion with the membrane bilayer, a mechanism used by many enveloped viruses containing special trimeric spike proteins embedded in the viral envelope. With respect to endocytosis, there are three main pathways: clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), and macropinocytosis [74]. CME internalizes nanoparticles that are less than 200 nm in size, CvME does so smaller, ~80-nm-size nanoparticles, whereas macropinocytosis internalizes bulky particles that can be as large as 10 μm [75].

T4 and most other phages lack envelopes and fusion machinery, hence fusion with the plasma membrane is not possible. However, it might be possible to engineer a surface coating containing these components, which merits investigation. On the other hand, a number of studies have demonstrated that phages and synthetic nanoparticles once attached to human cells would be taken up by endocytic pathway(s) at reasonable efficiencies (Figure 4). For instance, phage PK1A2 bound to neuroblastoma cell-surface polysialic acid was internalized by endocytosis [76]. The targeting ligand and cell type also determine the endocytic pathway used. Folate–polyethylene glycol-coated, cationic tetra- decyl–cysteinyl–ornithine nanoparticles were inter- nalized by CvME, whereas the same nanoparticles displaying transferrin ligand were taken up by CME [77,78]. M13 phages were taken up by macropinocytosis by HeLa and MCF-7 cells, whereas CvME was used for entry into dermal microvascular endothelial cells [79,80].

Endosome escape

Endosomes are classified into three major types: early endosomes, late endosomes, and recycling endosomes [74]. After an endocytic vesicle pinches off the plasma membrane (early endosome), the pH inside the vesicle drops to ~6.0 due to acidification by the proton-translocating pumps in the inside-out vesicle. These would mature into late endosomes, leading to a further drop in pH to ~5.0–6.0. The late endosomes fuse with lysosomes (pH 4.5–5), exposing the nanoparticles to a variety of degradative enzymes that digest the payload molecules [74,81,82]. Inhibitors of lysosomal proteases cathepsin B and L substantially increased the expression of luciferase reporter gene delivered by the λ phage [83]. Therefore, it is essential that phage nanoparticles escape early endosomes before they enter lysosomes in order to preserve the functional integrity of the payload [84] (Figure 4).

The efficiency of early endosome escape by phage nanoparticles is low, hence they probably end up in lysosomes, although those entering via CvME and macropinocytosis might avoid lysosomes because of the endoplasmic reticulum-mediated transportation of CvME and the inherently leaky nature of macropinosomes [82,85]. Thus, designing phage nanoparticles that could be internalized by CvME and macropinocytosis will have a greater chance for efficient payload delivery. Indeed, the incorporation of H3-targeting peptides that shifted the endocytic pathway to CvME enhanced the efficacy of gene delivery [86].

Alternatively, phage nanoparticles can be equipped with motifs that allow efficient early endosomal escape, as evolved by natural viral pathogens. Mammalian viruses contain peptides that might be hidden in the virion structure, but get exposed through conformational changes triggered by the acidic endosomal milieu, and/or through interactions with the endosomal membrane. These lead to pore formation, membrane fusion, and rupture of the endosomal membrane allowing the escape of the viral nucleocapsid core into the cytosol. Fusogenic peptides such as diINF-7 and HA2 from influenza virus [87,88], Pb from adenovirus [89], lytic ɣ-peptides from Flock House, and Omega viruses [90] when displayed on a nanoparticle are reported to induce pore formation, fusion with endosomal membrane, or membrane lysis, resulting in effective endosome escape. Additionally, synthetic amphiphilic helix-forming peptides that contain acidic protonating residues (such as glutamate or aspartate) on one face of the helix and hydrophobic residues (such as leucine or alanine) on the opposite face have been reported to cause endosomal leakage and release of cargoes into the cytoplasm [91]. The 30-amino

acid pH-sensitive amphiphilic peptide GALA is a typical example of synthetic fusogenic peptides used for endosome escape of nanoparticles [92]. Histidine-rich peptides were also shown to promote endosomal escape via protonation of their imidazole groups in the acidic environment of the endosomes. This causes influx of water into endosome, resulting in osmotic swelling, rupture, and release of the endosomal contents ('proton-sponge effect') [93]. For example, a five-histidine peptide H5WYG displayed on filamentous phage particles substantially improved gene delivery into human cells [88].

Another approach is to conjugate phage to AAV to create a hybrid vector that combines the useful features of both vectors [47,52]. The AAV particle 'piggy-backed' on a phage capsid serves as a 'driver' to navigate the cargo through the intracellular traffic. An average of four ~25-nm AAV particles were conjugated to the 120 × 86-nm phage T4 capsid using this approach and both the nanoparticle compartments could be used to incorporate cargo molecules. A large payload consisting of multiple transgenes packaged inside T4 and AAV capsids and hundreds of protein molecules displayed on T4 capsid through Hoc and Soc were efficiently delivered into human cells *in vitro* and into mice *in vivo*. Evidence suggests that the T4-AAV hybrid vector co-opted the lipolytic pore-forming mechanism evolved by AAV for efficient endosome escape.

Cytosolic trafficking

Active trafficking of nanoparticle payloads to the perinuclear area is essential for efficient gene delivery into the nucleus (Figure 4). Otherwise, passive diffusion of large nanoparticles through the viscous and crowded cytosol would be limited [75]. Endosomes provide a fast shuttle for the endocytosed nanoparticles [82]. Microtubules provide a 'highway track' for moving endocytic vesicles and other cargoes toward the nucleus [94]. Typically, this movement is mediated by kinesin and dynein motors that propel the vesicles or cargoes toward the microtubule minus-end (nuclear periphery) [95]. Many eukaryotic viruses, including vaccinia virus, adenovirus, and AAV, take advantage of this intracellular transport system by having the dynein-binding domains on the surface of virions [96]. Such peptides and domains when conjugated to synthetic nanoparticles enhanced gene delivery. It is unclear to what extent phage nanoparticles can take advantage of these pathways and if the presentation of dynein-binding ligands on phage capsid would promote active trafficking and enhance delivery.

Disassembly

The next step in the delivery pathway is disassembly (uncoating) of the nanoparticle cargoes, which likely occurs before entry into the nucleus. However, very little is known about the mechanisms, even in well-studied eukaryotic viruses. There is evidence in the case of T4 that at least some of the nanoparticles are disassembled releasing the packaged DNA into the cytosol. Tubastatin A, a microtubule-binding agent that stabilizes microtubules and facilitates transport of DNA from the cytosol to the nucleus, significantly enhanced the T4-delivered reporter gene expression signal [47].

Nuclear entry

The nuclear membrane imposes another major barrier for phage-mediated cargo delivery into the nucleus probably due to the limited size of the nuclear pore channel (~5 nm) (Figure 4). However, the nanoparticles can passively enter the nucleus in dividing cells when the nuclear membrane disassembles. For nondividing or slow-dividing cells, eukaryotic viruses usually interact with the nuclear pore complex via the importin protein [97]. A nuclear localization signal (NLS) is identified as the importin-interacting ligand and facilitates active nuclear entry [98]. This was demonstrated for the NLS-containing phage cargoes. For example, the NLS peptide sequence from the simian virus 40 (SV40) antigen or the human immunodeficiency virus Tat protein when displayed on λ phage increased the localization of NLS-λ phage particles in the nucleoplasm and the expression of λ-encoded GFP reporter gene [99,100]. Intriguingly, NLSs are naturally present in the terminal proteins (TPs) of many bacteriophages, such as Φ29, Bam35, Cp-1, Nf, PRD1, and YS61, and these TPs can target the fused cargoes to the nucleus of mammalian cells [12,101]. In addition to NLS, DNA nuclear targeting sequence (DTS) derived from SV40 was also reported to improve nuclear import of DTS-containing DNA. Apparently, DTS binds to NLS-containing transcription factors allowing codelivery of DNA–protein complexes [102].

Perspectives and conclusions

Over the past three decades, tremendous amounts of research effort and funding have been infused into the development of eukaryotic AAV and LV gene therapy vectors. Having evolved as human viral pathogens, these vectors remain the most promising gene therapy vehicles because of their high infectivity and delivery efficiency. However, their inherent characteristics: one or two gene capacity, broad tropism, lack of targeting, inability to codeliver other biomolecules, safety concerns, and high cost of manufacture continue to remain as barriers for broad applicability, and the applications thus far have largely been for *ex vivo* gene corrections of monogenic diseases. Newer, safer, and large-capacity vectors with superior engineering flexibility and *in vivo* targeting capability are desperately needed to transform future human therapies. We believe that the prokaryotic bacteriophages can fill this critical gap.

As discussed above, the tailed bacteriophage T4 with its architectural, genetic, and engineering advantages is a strong candidate. Each of the phage T4 nanoparticle's spacious compartments can be custom-engineered, the payloads can be self-assembled, tuned, and programmed with functionally well-characterized components, and the particles can be precisely targeted to specific cells and tissues. These provide unprecedented opportunities for creation of targeted *in vivo* gene therapies for monogenic as well as complex diseases, and for personalized medicine. The principal challenge now is to break through the efficiency barrier for delivery into primary human cells that the phages are not equipped with. Powerful *in vitro* and *in vivo* evolution strategies using CRISPR engineering could endow phages with novel motifs and pathways for efficient endosomal escape and nuclear localization. The next decade is expected to see rapid progress in these areas and establish a new category of phage-based targeted *in vivo* gene therapy 'drugs' to treat/cure a variety of human genetic diseases.

Conflict of interest statement

The authors declare no conflict of interest.

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