Advanced Review

Plant viral and bacteriophage delivery of nucleic acid therapeutics

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Nucleic acid therapeutics have emerged as a powerful method for treatment of many diseases. However, the challenge lies in safe and efficient delivery of nucleic acids to their target site, as they need to cross various extracellular and intracellular barriers. Mammalian viruses have initially been favored for delivery of nucleic acid therapeutics, but safety concerns regarding their immunogenicity and potential of integration have fueled the search for alternative delivery strategies. For example, chemistry and bioengineering have led to advances in the use of nonviral vectors composed of lipids and other polymers; nevertheless, the synthetic systems often do not match the efficiency achieved using the biological systems. More recently, researchers have turned toward the development of plant viruses and bacteriophages and virus-like particles as an alternative or complementary approach. These systems unite the properties of both the viral and nonviral systems and as such are a new exciting avenue toward nucleic acid delivery. This review highlights the benefits of plant viral and bacteriophage delivery of nucleic acids and provides a summary of the current progress in research in this field.

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NUCLEIC ACID THERAPEUTICS: OPPORTUNITIES AND CHALLENGES

Nucleic acid-based therapeutics are comprised of wide-ranging forms of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Therapeutic use of nucleic acids has had a long history, beginning with gene delivery, and its initial conceptualization over 40 years ago.[1] Since then, there has been an extensive body of work dedicated to gene therapy for treatment of diseases, such as Parkinson's,[2] cystic fibrosis,[3] hemophilia,[4] and cancer.[5] The discovery of small noncoding RNAs that regulate gene expression,[6] has led to the use of microRNAs (miRNAs) and small interfering RNAs (siRNAs) as possible therapeutics.[7] Nucleic acid-based therapies are on the horizon to become a clinical reality: the first approved RNA-based therapy in the market, Macugen, targets vascular endothelial growth factor (VEGF) in the eye to treat neovascular age-related macular degeneration.[8] UniQure's Glybera was approved for clinical use in Europe in 2012.[9] The adeno-associated virus (AAV)-based therapy is used to treat patients with lipoprotein lipase deficiency, an orphan metabolic disorder associated with increased levels of fat in the blood. Other nucleic acid-based therapies are poised to push toward commercialization in the coming year.[10,11] The road to bring gene

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therapy to the market has not been an easy one, with tragedy striking in 1999 when a patient died during a clinical trial from adverse immune response instigated by the adenoviral vector meant to correct for ornithine transcarbamylase metabolic deficiency.[12] A few years later, despite great success for many children, retrovirus-based gene treatment for X-linked severe combined immunodeficiency resulted in leukemia due to gene insertion near oncogenes.[13] Despite these misfortunate events in the past, more research has led to a better understanding of nucleic acid delivery, its potential pitfalls, and how to overcome them, leading to enhanced specificity and control of nucleic acid delivery with less severe and fewer adverse effects.

APPLICATIONS OF NUCLEIC ACID THERAPIES

Nucleic acids provide many different applications in gene therapy including gene delivery, gene vaccines, gene inhibition/silencing, and gene editing. One approach to nucleic acid delivery that is gaining clinical attention is gene-directed enzyme prodrug therapy (GDEPT). GDEPT is the delivery of a gene encoding for an enzyme that is able to convert prodrugs into their active components.[14] Since the target cells control the expression of the delivered gene, activation of the prodrug can be limited to a specific site instead of all cells, allowing for controlled expression and stimulation of the activator and prodrug. GDEPT has been used for tissue-specific treatment of tumors postsurgery,[15] or the delivery of neural growth factors to protect Alzheimer's patients from neural degeneration.[16]

Since the discovery that messenger RNA (mRNA) injected into the skeletal muscles of mice results in protein expression,[17] mRNA vaccines encoding an antigen have become an attractive alternative to traditional vaccines, such as attenuated or inactivated pathogens. Research indicates that mRNA vaccines can trigger both specific cytotoxic T lymphocytes and/or antibodies against the target,[18] thus providing many-fold opportunities for mRNA vaccines. The main advantages of mRNA vaccines are that they can be produced quickly and can be tailored with ease for specific virus strains. Much work has been focused on developing delivery mechanisms for mRNA vaccines including lipid-based delivery systems,[19,20] RNA-dendrimer nanoparticles,[21] and naturally derived nanoparticles.[22,23] mRNA vaccines have been developed for influenza[24] and cancer.[25]

Other strategies focus on the delivery of regulatory nucleic acids, for example, siRNAs and miRNAs to regulate gene expression. siRNAs and miRNAs are short RNAs (21–24 nucleotides (nt) in length) that can bind to complementary mRNA. This binding results in cleavage of the mRNA, and its degradation by exonucleases, thereby controlling gene expression through transcriptional silencing. This strategy has been shown to be powerful, for example, to knockdown Hepatitis B virus replication,[26] or in treatment of various types of cancer.[27]

Furthermore, the delivery of immune-stimulatory nucleic acids is applied in immunotherapy: for example, nonmethylated CG (CpG) motifs are delivered to potentiate antitumor immune responses,[28,29] and the oncolytic virus therapy T-VEC (Amgen, Thousand Oaks, CA) delivers the cytokines for immune stimulation.[30] T-VEC was recently approved for treatment of melanoma patients.

Beyond the clinical applications, nucleic acid delivery as a research tool has advanced our fundamental understanding of cell biology and physiology across kingdoms. Gene knock-in and knock-out models as well as transient expression or silencing approaches provide foundational techniques to the molecular biologist and advanced our understanding of structure–function relationships of proteins and signaling cascades guiding the fundamental processes of life. The recent discovery of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system (CRISPR/Cas), a prokaryotic machinery for genome editing, has changed the way molecular biologists and engineers approach genetic engineering and nucleic acid delivery, both in the setting of basic science and medical applications.[31–36] In particular, the type II CRISPR/Cas9 system derived from *Streptococcus pyogenes* that has been adapted for gene editing. The system was engineered into a useable two-component system making use of the Cas9 nuclease, and a guide RNA (gRNA) that directs sequence-specific digestion of DNA by Cas9. Through homologous base pairing, the gRNA will bind to a target region within the genome, guiding Cas9 to introduce double-stranded DNA breaks. Co-transfection with donor DNA allows for insertion of a new target sequence at the cut site, making use of the cell's DNA repair machinery and homologous recombination.[37,38]

From these examples, it is clear that nucleic acid delivery is a powerful technology to advance the progress of science, bioengineering, and medicine. Gene editing is highly sophisticated at the molecular level, however, the need for a safe and efficient delivery platform has fueled the research to develop and investigate a library of viral and nonviral systems.

DELIVERY OF NUCLEIC ACID THERAPEUTICS: VIRAL VERSUS NONVIRAL VECTORS

The delivery of nucleic acids is a highly bifurcated field; the division exists as a result of the unmatched inherent strengths and weaknesses of viral versus nonviral systems (Table 1). Recombinant viruses such as retrovirus, lentivirus, adenovirus, adeno-associated virus (AAV) and herpes simplex virus (HSV) have been widely utilized as vectors for gene delivery.[39–41] Viral-based vectors evolved mechanisms for cell entry and trafficking that enable integration into mammalian cells, and clinical trials have shown that viral systems often outperform nonviral vectors as they achieve higher levels of expression and genomic incorporation.[42] On the other hand, because of their efficient integration machinery, mammalian vectors bear risks of insertional mutagenesis leading to oncogene activation and onset of malignancies.[13] Another drawback and potentially the biggest challenge for viral gene therapy is the immune response from the body.[12] Prevalence or development of carrier-specific antibodies may neutralize the therapeutic after repeat administration. Addressing this challenge, several approaches have been developed to overcome immune surveillance, including the use of stealth and camouflage coatings to shield and hide the carrier from immune recognition.

Nonviral delivery systems include liposomes and polymer complexes as well as physical methods, such as gene gun delivery, electroporation, and intercellular microinjection.[39–41] Physical methods are useful for laboratory investigations or *ex vivo* manipulation of cells, but invasive for medical applications, highlighting the potential for nanotechnology. For nonviral gene delivery, engineers and chemists have developed highly sophisticated chemistries to mimic cell entry and trafficking machineries[43–51]; nevertheless, nonviral systems do not (yet) match the beauty and effectiveness of nature's gene delivery vehicles: the viruses. Therefore, nonviral methods are generally less efficacious than the viral methods, and in many cases, the gene expression is short-lived. On the other hand, nonviral systems offer safety; as a result of inefficient integration, nonviral systems do not bear a risk of insertional mutagenesis and the materials developed are generally less immunogenic. Nevertheless, the positively charged polyplexed nonviral systems introduce problems, such as aggregation and instability in biological media through interaction with serum components.[52,53]

In summary, gene therapies from mammalian viral vectors effectively deliver genes but they involve safety issues. Nonviral systems offer safety but often lack efficacy. In recent years, plant viral and bacteriophage systems have been developed and explored for delivery of nucleic acid therapeutics. The plant viral and bacteriophage system unite advantages of both the viral and nonviral systems, and may be regarded as a hybrid platform, the next generation of viral delivery systems (Table 1).

PLANT VIRAL AND BACTERIOPHAGE DELIVERY OF NUCLEIC ACID THERAPEUTICS

Plant viruses and bacteriophages can be considered safer for use in humans compared to their mammalian counterparts,[54] because plant viruses or bacteriophages do not replicate in or infect mammals. The system is nonintegrating and therefore does not bear

TABLE 1 | Pros (Blue) and Cons (Red) of Viral versus Nonviral versus Plant Virus-Based Vectors and Bacteriophages for Nucleic Acid Delivery

	Viral Vector	Nonviral Vector	Plant Virus Vector and Bacteriophage
Efficiency	High efficiency: trafficking machineries enable effective integration	Less efficient	Feasibility has been demonstrated; efficiency remains to be demonstrated
Safety	Genome integration bears risk of insertional mutagenesis	No risk of insertional mutagenesis	No risk of insertional mutagenesis
	Immunogenic	Less immunogenic	Shape-engineering and tailor-made surface coatings render them less immunogenic
	Stability in biological media	Aggregation and instability in biological media	Stability in biological media
Manufacture	Milligrams per liter tissue culture	Large-scale chemical synthesis of components	1–2 g/kg leaf tissue or per liter culture
QA/C	Monodisperse nanoparticles with long-term stability	Polydisperse assemblies exhibiting colloidal instability	Monodisperse nanoparticles with long-term stability

the risk insertional mutagenesis, as has been reported for adenoviral vectors.[13] Bacteriophage therapies have already advanced as standard therapy in Eastern European countries to treat antibiotic resistant bacterial infections.[55,56] Several clinical trials were performed to determine the safety and efficacy of phage.[57–59] No adverse effects were found and there was a reduction in bacterial load in one trial.[57]

For the plant viruses, we and others have shown that plant virus-based delivery systems can be administered at doses of up to 100 mg (10^{16} particles) per kilogram body weight without clinical toxicity.[60,61] The platform demonstrates excellent blood and tissue compatibility.[62,63] We also recently demonstrated that plant viruses show excellent stability in biological media and are less prone to interact with serum proteins compared to synthetic nanoparticles.[64] While 'naked' plant viruses are moderately immunogenic, we have shown that stealth[63,65] or camouflage[66] coatings can overcome the immunogenic properties and most importantly allow evasion from carrier-specific antibodies. Another design parameter to impart immune evasion is shape-engineering: we demonstrate that high aspect-ratio plant viruses have reduced interaction with cells of the mononuclear phagocyte system compared to their low aspect ratio or spherical counterparts,[67] therefore rendering these particles less immunogenic.

Another considerable advantage of plant virus-based biologics is the availability of scalable manufacturing processes and quality control and assurance. Bacteriophages, plant viruses, their virus-like particles (VLPs, genome-free versions), and chimeras (genetically engineered capsids encoding for epitopes or peptide ligands facilitating cell entry) can be produced through fermentation, in plants, or through expression in heterologous systems in high yields: for example, large-scale fermentation of the filamentous M13 bacteriophage in *Escherichia coli (E. coli)* results in 3.49×10^{11} transducing units (TU)/mL culture.[68]

Similarly, the manufacture of plant viruses in plants produces up to 1–2 g of plant virus-based particles per kilogram leaf tissue. Production of large quantities of leaf material is highly feasible in a research greenhouse or indoors using reach-in or walk-in growth chambers; a standard $11'' \times 22''$ plant growth tray can produce around 100 g of leaf tissue. Productions in insect cells, bacteria, or yeast are also feasible strategies for the production of plant viruses and VLPs thereof.[69] This is in stark contrast to production of mammalian viral vectors, which are produced in tissue culture. While mammalian viruses grow to high titers in tissue culture, typical yields obtained are only a few milligrams per liter cell culture.[70] Therefore, compared to other systems, yields are only moderate. Heterologous expression of plant viruses or bacteriophages gives rise to yields that are 10–100 times higher.

The plant virus/bacteriophage-based nanomanufacturing approach also provides advantages compared to synthetic systems; while the building blocks used for the assembly of nonviral delivery systems are generally easy to manufacture through scaled-up chemical synthesis, one of the major barriers to the widespread use of these novel pharmaceutical agents is their high instability in aqueous suspensions.[71–73] While several advances have been made to counter the storage instability of nonviral systems,[74] the synthetic platforms do not reach the level of stability that can be achieved using the viral counterparts, which are stable in biological media and can be stored as purified solutions or in infected leaf tissue. Furthermore, it remains challenging to synthetically produce nanomaterials with truly narrow size distributions. Plant virus and bacteriophage-based vectors are genetically encoded and the materials are monodisperse offering a level of quality assurance not yet achievable with synthetic systems.

The development and study of plant virus and phage vectors in drug delivery and imaging is an active area of research with several promising examples, such as the recent development of a plant virus therapeutic for *in situ* vaccination of tumors,[75] plant virus and bacteriophage contrast agents enabling magnetic resonance imaging (MRI) and positron emission tomography (PET) imaging of cardiovascular disease and cancer,[76,77] or plant virus-based drug delivery vehicles targeting human health application as well as agricultural applications.[78–80] Recognizing the potential of plant viral and bacteriophage delivery systems, several platforms have been investigated for delivery of nucleic acid therapeutics. In the following sections, we review the state-of-the-art of frequently used plant viruses and bacteriophages (Figure 1).

The Cowpea Chlorotic Mottle Virus Platform

The plant virus cowpea chlorotic mottle virus (CCMV) belongs to the family *Bromoviridae*. CCMV infects mostly legumes and causes disease in black-eyed peas as well as in soybeans. The virus is found exclusively in the USA where it is transmitted by beetles.[81] CCMV forms particles with a diameter of about 30 nm. CCMV has a tripartite, positive-sense, single-stranded RNA (ssRNA) genome which is contained in capsids composed of 180 identical copies of a single coat protein arranged in a $T = 3$ symmetry.[82]

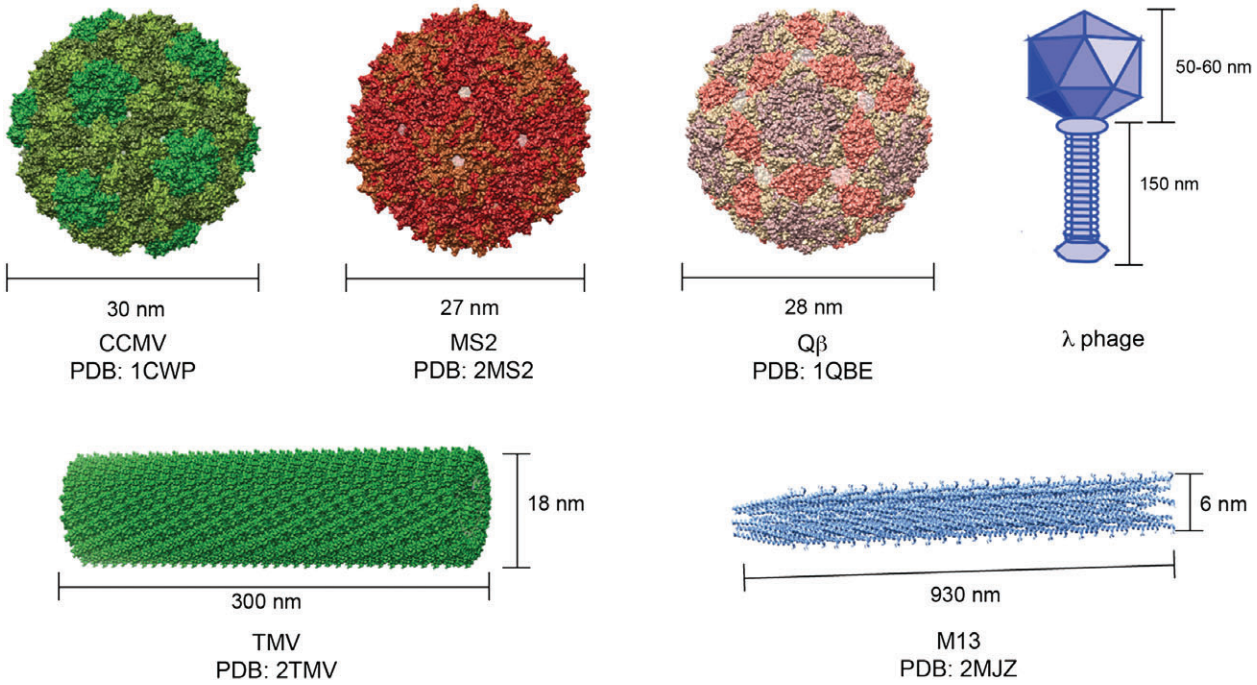

FIGURE 1 | Structures of the plant viruses and bacteriophages discussed in this review. Plant viruses: cowpea chlorotic mottle virus (CCMV), tobacco mosaic virus (TMV); bacteriophages: MS2, Qβ, λ, and M13. Virus structures were created using the UCSF Chimera software package (University of California, San Francisco, CA) and the respective PDB files, except bacteriophage λ: here a cartoon was drawn using ChemDraw (PerkinElmer Informatics, Cambridge, MA). Viruses are not drawn to scale.

CCMV manufacture can be achieved through infection of natural hosts, for example, black-eyed peas (*Vigna unguiculata*), or by expression in heterologous culture. In the natural host, high titers of CCMV accumulate and 1–2 g can be isolated from 1 kg of infected leaf material. Heterologous expression systems give rise to comparably high yields; for example, expression of CCMV VLPs in yeast (*Pichia pastoris*) yields up to 0.5 g VLPs per kilogram wet cell mass.[83] Furthermore, coat protein monomers can be expressed in *Pseudomonas fluorescens* and in *E. coli* and then be self-assembled *in vitro* into intact empty CCMV protein cages.[84] Typical yields of coat proteins made in *E. coli* range from 10 to 100 mg/L of culture, depending on the purification method whereas expression in *P. fluorescens* in a bioreactor yields up to 2.6 g/L of culture.[85–87]

CCMV has a remarkable capacity to dis- and reassemble in appropriate bathing conditions *in vitro* (i.e., in the test tube). Not only does it facilitate assembly around its genome, RNA-free VLPs can also be obtained.[88] Furthermore, the assemblies of CCMV can accommodate a variety of cargos: CCMV has been shown to encapsulate heterologous ssRNAs,[89,90] organic anionic polymers,[91–93] metal oxide and gold nanoparticles,[94] and nanoemulsions.[95] A close relative of CCMV, brome mosaic virus (BMV) is also capable of disassembling into protein dimers, and reassembling into empty capsids and around various cargoes such as gold particles,[96,97] iron oxide cores,[98] and quantum dots.[99] The empty capsids can also be engineered for biomedical applications.[100] Though BMV was shown to be able to interact with and package tobacco mosaic virus (TMV) subgenomic RNA when BMV coat proteins were expressed from a genetically engineered TMV,[101] to date development of this platform for nucleic acid therapy has not been described.

Cadena-Nava and Comas-Garcia in the laboratories of Gelbart and Knobler at UCLA have dissected the assembly of CCMV, specifically addressing the design rules for packing RNA into the viral capsids.[89,90] For example, they demonstrated that ssRNAs between 140 and 12,000 nucleotides can be packaged into CCMV capsids and that each of these RNAs is completely packaged at a 'Goldilocks' protein:RNA mass ratio of 6:1 (independent of the length of the RNA). At this critical value, the charge of the RNA is equal to the *N*-terminal coat protein charges, therefore favoring electrostatic assembly. Using less protein leads to reduced RNA packaging efficiency, whereas more protein leads to protein aggregation and polydispersity in the assembled particles. It was also shown that multiple RNAs are packaged when the RNA is shorter than 3000 nt whereas RNAs longer than 4500 nt yield multiplet

assemblies.[89] More recent studies detailed the degree of cooperativity in the assembly process as a function of RNA length.[102] At the same time, building on fundamental understanding of RNA-triggered assembly of CCMV, the group developed a 'cherry bomb' assembly with symmetry-broken features enabling higher-order assembly of CCMV onto surfaces or co-assembly with metal nanoparticles.[103]

Turning toward delivery of nucleic acid therapeutics, Azizgolshani et al. recently demonstrated heterologous RNA delivery to mammalian cells using CCMV.[104] CCMV coat proteins were assembled to encapsulate a heterologous RNA cassette derived from Sindbis virus (SINV). While the 5′ untranslated region (UTR) and 3′UTR were retained as *cis*-acting elements enabling replication, the nonstructural coding region of SINV was replaced to encode the reporter protein enhanced yellow fluorescent protein (eYFP), yielding a defective-interfering RNA template (DI-eYFP). Cell delivery of the CCMV-based gene delivery vector carrying DI-eYFP was achieved through the use of Lipofectamine-2000. The machinery for transcription and replication of the DI-eYFP cargo was then supplied through an SINV-like particle. Successful expression of eYFP in human embryonic kidney 293 (HEK293) cells indicates cytoplasmic delivery of the cargo[104] (Figure 2). This study thus demonstrates a first proof-of-concept for the use of CCMV as a delivery agent for nucleic acid therapeutics. To be useful for *in vivo* medical applications, further optimization of the platform would be desired to avoid the use of transfection agents and the need for co-delivery of SINV-like particles.

In a different approach, Mikkilä et al. from the Kostiainen lab at Aalto University in Finland recently united DNA origami with viral assembly.[105] The concept of DNA origami allows programming any genetic code to assemble into defined nanoscale architectures. While DNA origami is attractive for the programming of complex soft matter, barriers to application as nucleic acid therapeutics are the instability of DNA origamis in biological media as they are prone to degradation by nucleases, and the negative charge of the complexes prevent efficient cell interactions and uptake. To overcome these challenges, the coat proteins from CCMV were utilized to encage the DNA origami assembly and therefore provide stability and a platform for delivery. Stoichiometric mixing of CCMV coat proteins and DNA origami sheets yielded protein-coated sheets or wrapped tubes. The assembly is driven through electrostatic interactions: the negatively charged DNA origami structures mimic CCMV's natural cargo, its RNA genome. The morphology of the CCMV coat protein–DNA origami assembly could be controlled by the ratio of protein: DNA base pairs with an excess of 0.64 coat proteins per DNA base pair yielding sheets and 0.08 coat proteins per DNA base pair yielding tubes. Cell delivery studies using fluorescent-labeled DNA origami cargos indicated that the protein coating improves cellular attachment and delivery of DNA origamis into the cells by 13-fold compared to bare DNA origamis.[105] Nevertheless, the intracellular fate of the nucleic acid cargo as well as a demonstration of function is yet to be determined.

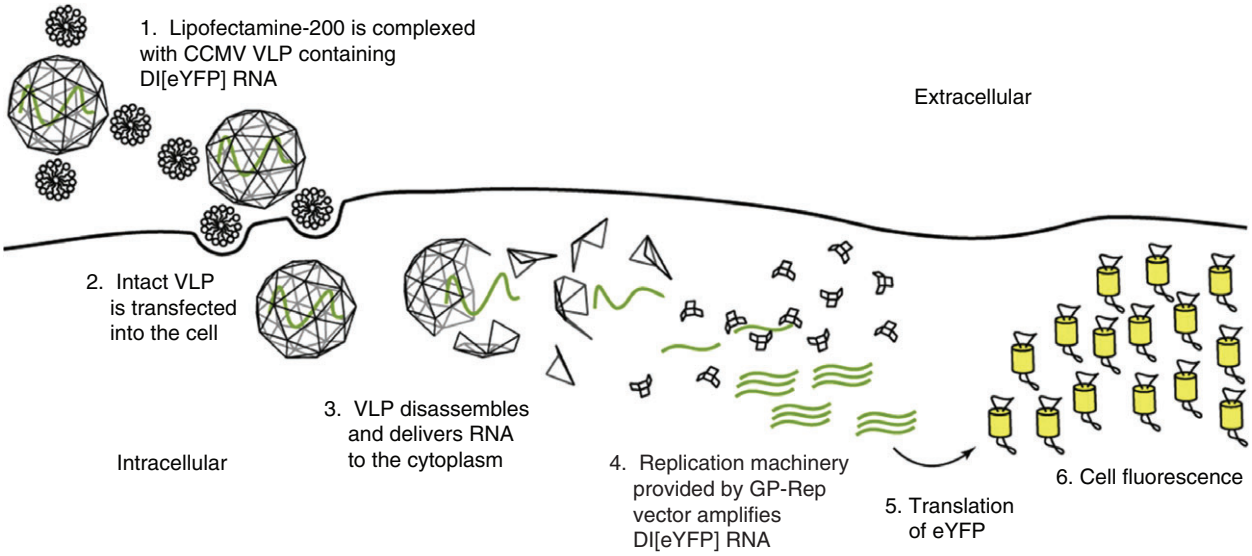

FIGURE 2 | Delivery of cowpea chlorotic mottle virus (CCMV) virus-like particles (VLPs) into cells using lipofectamine. Once in the cell, the VLP disassembles allowing for release and expression of enhanced yellow fluorescent protein (eYFP). (Reprinted with permission from Ref 104. Copyright 2013 Elsevier)

The TMV Platform

The plant virus, TMV is a rod-shaped tobamovirus. TMV is distributed worldwide and has a broad host-range. TMV is transmitted by mechanical contact between plants and man. The particles can be produced in high titers in tobacco plants (*Nicotiana tabacum*, *benthamiana* and related species); yields up to 2 g/kg infected leaf material can be obtained.

TMV particles contain a positive-sense ssRNA genome. The nucleoprotein components assemble into a rigid soft matter, hollow nanotube with dimensions of 18 × 300 nm with a 4-nm wide interior channel. Each TMV particle is composed of 2130 identical protein subunits closely packed in a helix with a pitch of 2.3 nm and 16 1/3 subunits per turn.[106,107] In addition to the rod structure, TMV can also take other forms such as stars and stripes, tripods, and boomerang structures.[108] The self-assembly of the TMV platform technology is well understood[109] and has led to wide-ranging applications in nanotechnology, including MRI imaging,[110] vaccine[111,112] and drug delivery,[78,113] Ebola diagnostics,[114,115] tissue engineering,[116,117] trinitrotoluene (TNT) sensing,[118] batteries and data storage,[119] and light harvesting.[120] Further applications are reviewed by Koch et al.[121]

Experiments to assess the potential for gene transfer using the TMV platform technology date back 30 years.[122] TMV coat proteins were programmed to package synthetic RNA transcripts containing the reporter gene chloramphenicol acetyltransferase (CAT) as well as TMV's origin of assembly sequence (OAS) to promote the assembly. Transgene expression was demonstrated in a number of cell systems, including epidermal cells of tobacco, the natural host, and pea, a nonhost plant, as well as animal cells. More specifically, transgene expression was confirmed after microinjection of TMV-encapsidated mRNA into oocytes from *Xenopus laevis*. Expression of the reporter gene CAT provides supporting evidence that animal cells have the machinery to both uncoat TMV and translate delivered mRNA.[122]

To overcome the aforementioned challenges involved in using viral vectors for therapy (high immunogenicity and risk of transgene integration), TMV has been investigated for packaging and delivery. In work done by Smith et al. in the McCormick Lab at the Large Scale Biology Corporation in California, a recombinant viral vector containing RNA from the Semliki Forest virus (SFV) fused to the *lacZ* reporter gene was attached to a portion of the TMV OAS.[123] This RNA was then packaged *in vitro* by incubation with TMV coat proteins to generate a pseudovirus. To test the *in vitro* expression of this pseudovirus, the protein delivery agent, BioTrek (Stratagene, San Diego, CA), was used to transfect baby hamster kidney (BHK21) cells. After 24 h, expression of β-galactosidase was observed, indicating successful disassembly of the pseudovirus, and that the TMV OAS does not interfere with the replication of SFV. When tested *in vivo* in C57/B6 mice, immunization and immune reactivity to β-galactosidase was detected, further demonstrating successful delivery of the mRNA vaccine. However, SFV has some limitations: (1) its rather large genome (the RNA used was 13.6 kilobases (kb)) makes it technologically challenging to add additional transgenes and (2) SFV can induce apoptosis in cells, therefore bearing a safety concern. To address this problem, Maharaj et al. from the McCormick Lab has also encapsidated the self-replicating RNA elements from Flock House virus (FHV) into TMV.[124] FHV has high levels of replication but has a smaller genome than SFV, and does not induce apoptosis, making it an attractive alternative to SFV. A viral construct containing the FHV RNA1 gene, which encodes a 3.1 kb RNA-dependent RNA polymerase, was attached the enhanced green fluorescent protein (eGFP) reporter and the TMV OAS. The replication efficiency of FHV-eGFP-TMV OAS RNA was tested in transfected BHK21 cells by assessing the level of eGFP expression. The expression of eGFP was maintained even after 120 h posttransfection, indicating the robustness of the system. The next step was then to encapsidate the FHV-eGFP-TMV OAS RNA into TMV particles: this was accomplished first using an *in vitro* assembly protocol and second by synthesizing the recombinant particles *in planta*. For the latter strategy, the RNA template was capped at the 5′ end prior to insertion into TMV particles to yield more immunogenic transgenes. To achieve encapsulation *in planta*, the template RNA (FHV-eGFP-TMV OAS) was expressed in agrobacterium-inoculated *Nicotiana benthamiana* plants with excess TMV coat proteins supplemented *in trans*. After confirmation of successful *in planta* assembly of FHV-TMV particles, the anti-GFP immune response in mice was tested from *in vitro* and *in planta* assembled particles. It was found that *in planta* particles conferred a greater immune response than *in vitro* assembled particles, suggesting its potential use as a vaccine[125] (Figure 3).

The MS2 and Qβ Platforms

The MS2 and Qβ bacteriophages (viruses that infect and replicate in bacteria) are members of the *Leviviridae* family. They are small, icosahedral viruses (27 and 28 nm in diameter, respectively) containing a positive-sense ssRNA genome. Like CCMV, MS2

and Qβ are composed of 180 identical copies of a single coat protein arranged in a $T = 3$ symmetry.[126,127] The outer capsid of MS2 and Qβ can easily be genetically and chemically modified, and they can self-assemble into monodisperse VLPs.

MS2 bacteriophages can be produced in many different homologous and heterologous systems, including *E. coli*[128] and *Saccharomyces cerevisiae*.[129] The *in vitro* self-assembly of MS2 phages was initially described by Stockley et al. at the University of Leeds, UK. The wild-type virion can disassemble at low pH, such as through the addition of acetic acid. With an increase in pH, the particle will reassemble through interaction of the coat proteins and a 19-nt-specific region called the *pac* site located on the RNA genome.[130] MS2 coat proteins have been shown to reassemble around heterologous RNAs attached to the *pac* site, thereby making them attractive nanocarriers for delivery of nucleic acids.[131]

To enable targeted delivery of MS2 VLPs, Ashley et al. from the Peabody group at the University of New Mexico, Albuquerque showed that chemical conjugation of the SP94 peptide, a novel peptide found through phage display that is specific for human hepatocellular carcinoma (HCC) cells, to the surface of MS2 VLPs enabled delivery of various cargoes specifically to HCC cells with decreased uptake in nontarget cells such as hepatocytes, endothelial cells, monocytes, and lymphocytes.[132] Additionally, the Galaway and Stockley at the University of Leeds, UK, were also able to target HeLa cells through attachment of human transferrin to MS2 VLPs.[133]

Another attractive strategy to achieve efficient cellular uptake of nanoparticles and their cargoes is the use of cell penetrating peptides; the Tat peptide derived from human immunodeficiency virus-1 is a particularly attractive candidate. The Tat peptide has been used for delivery of MS2 VLPs to cells. The Li and Wang Labs at the National Center for Clinical Laboratories, China, showed that fusion of the Tat peptide to MS2 VLPs harboring antisense RNA targeting hepatitis C virus (HCV) was able to inhibit the translation of HCV in human hepatocyte derived cellular carcinoma (Huh-7) cells expressing HCV.[134] Additionally, the same group showed that Tat-labeled MS2 VLPs have been shown to also deliver miRNAs.[135,136] In these studies, miRNA146a delivered in Tat-labeled MS2 VLPs were able to decrease disease progression in lupus-prone mice by inhibiting autoantibody production and suppress osteoclast differentiation in human peripheral blood mononuclear cells, providing a potential therapy for osteoporosis.

Work on the Qβ bacteriophage as a delivery vehicle for nucleic acids has resulted in promising results in the delivery of DNA rich in CpGs. CpGs are of particular interest as an adjuvant (agents that enhance the induction of immune responses). In a study by Storni et al. from the Bachmann group at Cytos Biotechnology AG in Switzerland, when CpGs were packaged in Qβ particles and administered in mice, they saw an activation of CD8+ T cells, suggesting an immune-stimulatory effect.[137] The potent T cell response that was seen in this study matched that of live vaccines, highlighting the potential of this approach in vaccines. As such, clinical trials have been performed using a Qβ VLP encapsulating a CpG oligonucleotide that triggers toll-like receptor (TLR)-9 for treatment of melanoma.[138] Combination

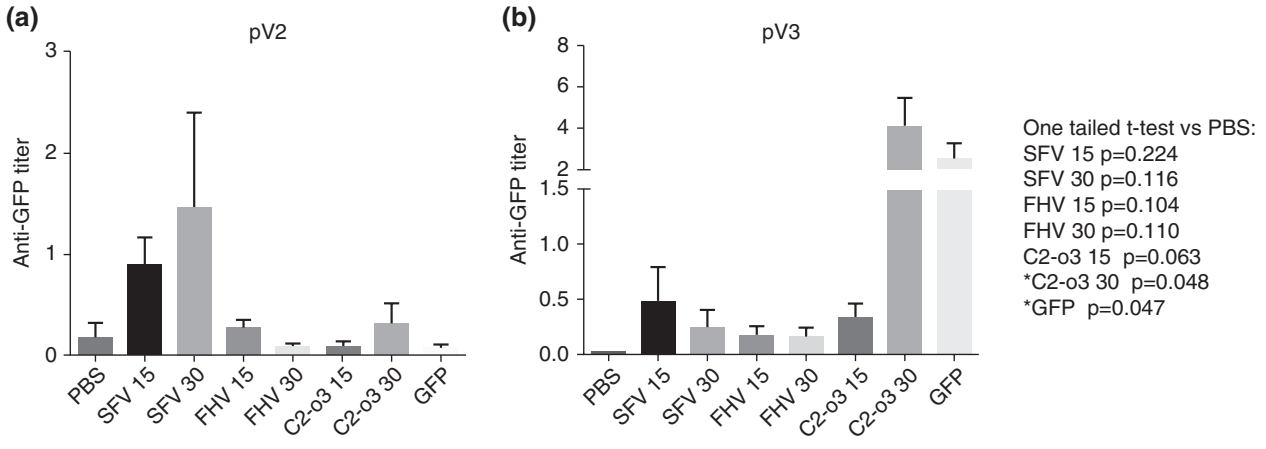

FIGURE 3 | Immune response after vaccination with transencapsidated Flock House virus (FHV) ribonucleic acid (RNA). After a second vaccination (pV2), weak immune responses were detected. However, after a third vaccination (pV3), all groups showed an augmented immunity against enhanced green fluorescent protein (eGFP), but most significant was the response from the *in planta* encapsidated FHV (treatment C2-o3 30). (Reprinted with permission from Ref 125. Copyright 2014 Springer Science + Business Media)

treatment with the nanoparticle formulation and the drug Imiquimod resulted in memory- and effector-phenotype T-cell responses.

Bacteriophage λ, T4, M13, and AAV/Phage Hybrid Systems

Other bacteriophages, including bacteriophage λ, T4, M13, and AAV/M13 phage hybrid (AAVP) system, also have been explored as vehicles for delivering nucleic acids. These bacteriophages are well-studied, provide stable nanoparticles, economic to produce in large scales through fermentation and just like the other virus-based platform encapsulate and protect nucleic acids.

The particles of bacteriophage λ are comprised of a capsid head measuring around 50–60 nm that contains the phage's dsDNA genome, and a tail structure measuring around 150 nm in length that is used to interact with host proteins for phage entry. The capacity for the capsid head to encapsulate large amounts of heterologous DNA (~20 kbp) makes bacteriophage λ an attractive medium for delivery of genes and DNA vaccines. The first instance of gene therapy using bacteriophage λ dates back almost 50 years, and was in the delivery of a gene for galactose transferase in human fibroblast cells that were deficient in the galactose transferase.[139] Preliminary work done by Clark and March at the Moredun Research Institute, UK, showed that when λ-phage carrying the eGFP gene under control of the cytomegalovirus (CMV) promoter was injected into mice, GFP antigens were detected 8 h later.[140] This antibody response was greater than that of naked DNA treatment in mice, suggesting λ particles can target and recruit antigen-presenting cells (APCs), leading to greater immune response.[140] Subsequent work from the same group has also used bacteriophage λ as a potential vaccine for hepatitis B.[141] λ-phage carrying a plasmid containing the hepatitis B surface antigen cloned under the CMV promoter (λ-HBsAg) was administered to rabbits intramuscularly. Following three vaccinations with λ-HBsAg, all rabbits treated showed a high level of anti-HBsAg response, suggesting that the previous doses aided in 'priming' the immune system against the DNA vaccine. Similarly, Ghaemi et al. from the Roohvand group at Tarbiat Modares University, Iran, used bacteriophage λ to generate a DNA vaccine against human papilloma virus (HPV)-derived cervical cancer.[142] Using the Lambda ZAP-CMV XR vector (Stratagene, San Diego, CA), they cloned in the human HPV-16 E7 gene. HPV-16 is associated with most cervical cancers, and the E7 gene encodes an oncoprotein that is highly expressed in HPV-containing cervical cancer.[143] C57BL/6 mice injected with TC-1 cells (a HPV-16 E+ tumor cell line) were immunized with phages packaged with the plasmid (λ-HPV-16 E7). Tumor volumes were monitored for 30 days, and those mice treated with λ-HPV-16 E7 showed significantly reduced tumor volumes compared to mice treated with just the λ-phage or PBS. These results suggest a potential antitumor effect for λ-phage-based vaccines.

Like bacteriophage λ, bacteriophage T4 is composed of an icosahedral capsid head 90 nm in diameter that packages the genome, and a tail measuring 200 nm in length, with tail fibers that are used in recognizing host cell surface receptors. Tao et al. from the Rao group at the Catholic University of America in Washington DC engineered the molecular motor assembly of the T4 bacteriophage to insert heterologous DNA into the T4 capsid head.[144] These particles, when decorated with the cell penetrating peptides Tat or ANTP, were able to deliver the luciferase gene into HEK293T cells at the same efficiency as lipofectamine-transfected cells.

The bacteriophage M13 is a high aspect ratio, rod-shaped, filamentous virus (930 × 6 nm) composed of five coat proteins: four minor proteins (p3, p6, p7, and p9) and one major (p8), which surround and encapsulate the ssDNA genome.[145] M13 is the workhorse platform for phage display and identification of novel peptide ligands to tailor cell and molecular specificity of nanoparticles and biologics used in imaging and therapy. To allow for targeted delivery in mammalian cells, peptides and targeting ligands can be chemically or genetically incorporated to the outer coat proteins.[146–148] Initial attempts to use the filamentous M13 phage as a gene delivery vehicle dates back to 1998 by Larocca et al. at Selective Genetics Inc., San Diego. They attached fibroblast growth factor 2 (FGF2) to the outer surface of the bacteriophage and assessed reporter gene expression (GFP or β-galactosidase under control of the CMV promoter) in target cells.[149] It was found that bacteriophages carrying FGF2 were more effective in gene delivery in COS-1 cells (cells with affinity for FGF2), than those cell lines that lack FGF receptors. COS-1 cells expressing the GFP reporter gene from the phage DNA were able to maintain expression of GFP under selective pressure, indicating a potential role for filamentous phage-mediated delivery of transgenes.

The combination of M13 phage with AAV has led to the creation of the AAV/M13 phage hybrid delivery system for gene delivery. AAVs are small icosahedral ssDNA viruses in the parvovirus family. The genome of AAV includes inverted terminal

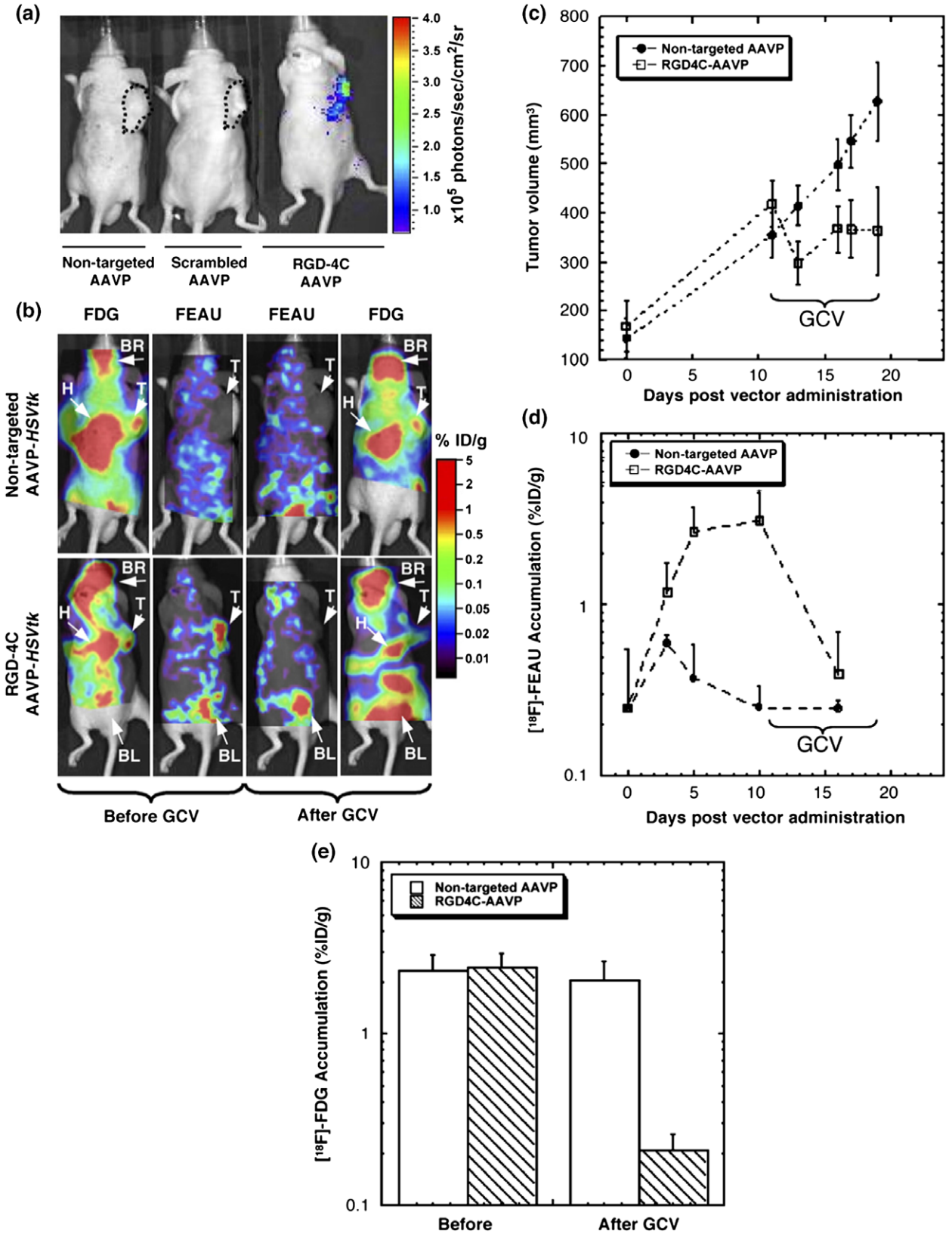

FIGURE 4 | Legend on next page.

repeat (ITR) sequences that help the virus integrate its genome into the host. Recombinant AAVs engineered to deliver genes are cloned with the gene of interest in between the ITRs. Currently, there are 11 serotypes of AAV, all of which differ in the types of cells they infect. One of the challenges of AAV as a gene delivery vehicle is unspecific delivery, due to the limited range in host cell type infection. To overcome this challenge, several groups have engineered various AAV/M13 hybrid phages. One construct, made by Hajitou et al. at the University of Texas, Houston, was developed for tumor targeting and treatment.[150] The phage coat proteins displayed the RGD peptide for cell uptake. The AAV ITR was inserted into the phage genome under the control of the CMV promoter. Initial tests attempted to deliver GFP to mammalian cells (human 293) as a proof of concept. After successful delivery of GFP, they sought to evaluate the efficacy and specificity of delivery of herpes simplex virus thymidine kinase (HSVtk) gene in a xenographic mouse model expressing human Kaposi sarcoma KS1767 tumor cells. Tumor cells that express HSCtk are rendered sensitive to prodrugs such as ganciclovir. When mice were treated with ganciclovir, and the phage hybrid, they showed reduced tumor size, indicating successful expression of HSVtk[150] (Figure 4). The Lee group from the UC Berkeley created a system where the hybrid phages could be made to form nanofibrous phage films.[151] The M13 phage expressed the RGD peptide on its surface to facilitate cell uptake. Inside the phage were M13 phage genes and the AAV ITR flanking recombinant GFP. When HeLa and MC3T3 cells were grown on top the phage films, after 24 h the cells were able to express the GFP protein, demonstrating successful gene delivery.

SUMMARY AND OUTLOOK

The delivery of DNA and RNA for treatment of diseases, such as cancer and other chronic diseases, is very promising. The development of plant virus and bacteriophage delivery methods for nucleic acid therapeutics is aimed at creating safer and more versatile platforms than conventional mammalian viral vectors. The design considerations for plant virus and bacteriophage delivery methods involve the synthesis of nonimmunogenic, biocompatible nanoscale materials able to protect the nucleic acid cargo from degradation that are able to successfully traffic to and enter the target cell, and once in the cell, release their cargo in the cytoplasm or nucleus. Inspired by nature and with bioengineering design, artificial viruses have also been developed and proposed as safer than mammalian viral vectors and more efficient than contemporary nonviral systems.[152,153]

While mammalian and nonviral vectors have been studied extensively with many preclinical and clinical studies available, the development of plant virus and bacteriophage vectors for nucleic acid therapy is still an emerging field. Plant viruses and bacteriophages offer intriguing attributes: they offer increased safety as they are nonintegrating or replicating in mammalian cells; furthermore, high production yields in homo- or heterologous expression systems and stability in biological media provide advantages over the mammalian and synthetic vectors systems (Table 1). Nevertheless, while research to date has demonstrated the feasibility of using plant viruses or bacteriophages for nucleic acid delivery, more research is required to determine efficiency of these systems. With a translational vision in mind, immunogenicity is an important consideration for any material whether biologic or synthetic origin; while some materials have been deemed nonimmunogenic, it should be noted that when assembled into or onto a particulate, any material may interact with and get processed by the mononuclear phagocyte system leading to innate and adaptive immune responses. Viruses are known to be immunogenic, based on their particulate and repetitive proteinaceous nature, viruses trigger innate and adaptive humoral and cellular immune responses. However, several engineering concepts have been developed to

FIGURE 4 Targeted AAV/phage (AAVP)-mediated molecular imaging of tumor-bearing mice. (a) In vivo bioluminescent imaging (BLI) of luciferase (Luc) expression after systemic AAVP delivery. Nude mice bearing DU145-derived tumor xenografts received an intravenous single dose of either RGD-4C AAVP-Luc (5×10^{11} TU) or controls (nontargeted AAVP-Luc or scrambled RGD-4C AAVP-Luc). Ten days later, BLI of tumor-bearing mice was performed. (b) Multitracer positron emission tomography (PET) imaging in tumor-bearing mice after systemic delivery of RGD-4C AAVP-HSVtk. Nude mice bearing DU145-derived tumor xenografts (n = 9 tumor-bearing mice per cohort) received an intravenous single dose (5×10^{11} TU) of RGD-4C AAVP-HSVtk or nontargeted AAVP-HSVtk. PET images with fludeoxyglucose ([^{18}F]FDG) and [^{18}F]FEAU (1-(29-deoxy-29-fluoro-b-D-arabinofuranosyl)-5-ethyluridine) obtained before and after ganciclovir (GCV) treatment are presented. T, tumor; H, heart; BR, brain; BL, bladder. Calibration scales are provided in (a) and (b). Superimposition of PET on photographic images of representative tumor-bearing mice was performed to simplify the interpretation of [^{18}F]FDG and [^{18}F]FEAU biodistribution. (c) Growth curves of individual tumor xenografts after AAVP administration. (d) Temporal dynamics of HSVtk gene expression as assessed by repetitive PET imaging with [^{18}F]FEAU at different days post-AAVP administration. (e) Changes in tumor viability before and after GCV therapy as assessed with [^{18}F]FDG PET. Error bars in (c)–(e) represent standard deviations (SD). (Reprinted with permission from Ref 150. Copyright 2006 Elsevier)

overcome or reduce the immunogenic properties of viruses; these include the coating of viruses with polymers,[63,154] the addition of self-recognition elements[155] or proteins[66,156], as well as their encapsulation into artificial lipid envelopes.[157] Another design parameter is shape engineering: we have shown that high aspect ratio materials have lower rates of phagocytosis thus rendering materials less immunogenic.[67] Going forward, comparative studies are required to test mammalian vectors, plant viruses/bacteriophages, and synthetic systems side-by-side and delineate their efficacy and safety.

In addition to discovery and development of improved delivery vehicles, the recently discovered CRISPR/Cas system offers new dimensions for genome editing with potential therapeutic applications. Packaging the components of the CRISPR/Cas9 system into a delivery vehicle to chaperone the gene editing machinery to a target cell thus has received much attention in recent years. For example, Qazi et al. from Indiana University and Montana State University, were able to create VLPs derived from the P22 bacteriophage to package Cas9 and a gRNA that can cleave a specific sequence DNA target *in vitro*.[158] Additionally, Li et al. from Sichuan University, Chengdu, China, successfully created an artificial virus that packages the entire CRISPR/Cas9 system.[159] Their artificial virus was able to enter cells, escape the endosome and enter the nucleus, thereby enabling gene disruption.

There have been many advances in the development of viral and nonviral delivery agents through collaboration bridging the disciplines of biology and nanotechnology, the next generation of nucleic acid delivery systems is on the horizon.

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