Plant Viruses and Bacteriophage-Based Reagents for Diagnosis and Therapy

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Abstract

Viral nanotechnology exploits the prefabricated nanostructures of viruses, which are already abundant in nature. With well-defined molecular architectures, viral nanocarriers offer unprecedented opportunities for precise structural and functional manipulation using genetic engineering and/or bio-orthogonal chemistries. In this manner, they can be loaded with diverse molecular payloads for targeted delivery. Mammalian viruses are already established in the clinic for gene therapy and immunotherapy, and inactivated viruses or virus-like particles have long been used as vaccines. More recently, plant viruses and bacteriophages have been developed as nanocarriers for diagnostic imaging, vaccine and drug delivery, and combined diagnosis/therapy (theranostics). The first wave of these novel virus-based tools has completed clinical development and is poised to make an impact on clinical practice.

Keywords

viral nanotechnology; plant viruses; bacteriophages; virus-like particles; drug delivery; imaging; vaccines; immunotherapy; nanomedicine

1. INTRODUCTION TO VIRAL NANOTECHNOLOGY

Nanomedicine is the use of nanoscale materials for medical applications. Some nanomedical formulations have already progressed through clinical development and are now approved in humans or companion animals (1); however, development and testing of novel nanomaterials are critical for further advancement of the field. These nanocarrier-based formulations are designed to optimize the delivery of diagnostic and/or therapeutic agents. This is achieved

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by improving the solubility, stability, and/or bioavailability of the cargo or by targeting disease sites and thus reducing off-target systemic toxicity. However, the nanocarrier may also play an active role by interacting with the immune system and enhancing its response to a vaccine. In the case of therapeutic vaccines and immunotherapies, the nanocarrier may even play a direct role in the eradication of an established disease. The development pipeline features several broad categories of nanocarriers, including liposomes, protein scaffolds, polymer micelles, dendrimers, and inorganic/metallic nanoparticles, with an even more diverse payload repertoire encompassing small-molecule drugs, nucleic acids, biologics, and contrast agents (2).

One promising category of nanocarriers is based on viruses. These ubiquitous nucleoprotein particles are naturally occurring nanomaterials that have perfected the art of cargo delivery to specific host cells during evolution, making them ideal for the development of nanocarriers. The size and shape of viruses vary according to the species, although most fall within the range of 20–500 nm with icosahedral or helical structural configurations. Helical viruses can be flexible filaments or rigid rods, whereas other viruses have more complex head-and-tail structures or outer envelopes (Figure 1).

Viruses have been conventionally used as vaccines, occasionally in their native form but more often as inactivated (e.g., heat-killed or chemically inactivated) or otherwise attenuated preparations and more recently as subunit vaccines. The development of viruses as delivery vehicles has broadened their applications in medicine and beyond, including in materials science (3, 4). In addition to their natural ability to deliver cargo to target cells, the replication of viruses in their natural host(s) or heterologous expression systems offers a low-cost production platform. Furthermore, the structure of the viral nucleocapsid is genetically encoded, which means that viral preparations are clonally identical. This provides an inbuilt quality control step that cannot yet be matched by synthetic materials (5). Finally, the nucleoprotein architecture of viruses is amenable to structural alterations introduced by genetic engineering (6). The endogenous structure can be modified through addition, deletion, or replacement of amino acids to alter the surface charge, or to introduce reactive side chains for chemical conjugation or for introduction of specific ligands or epitopes. Once optimized, such genetically engineered viruses can be replicated to make identical copies without batch-to-batch variations, just like the native virus. The modification of amino acid side chains can be achieved using various bio-orthogonal chemistries, allowing the conjugation of diverse ligands and small-molecule reagents to the external or internal surfaces, in some cases simultaneously (7, 8). The structural homogeneity and robustness of viruses ensures that chemical modifications can be introduced with atomic precision and the modified particles have a long shelf life, in some cases remaining stable at room temperature for years (this is particularly evident for plant viruses and bacteriophages) (9–12).

The medical applications of mammalian viruses are based on host-specific infection, replication, and propagation. Therefore, they cater to a highly specific niche of gene therapy and virotherapy (13). However, mammalian viruses pose the risk of undesirable events such as reversion of attenuated versions to virulent forms (e.g., by mutation and/or recombination) or the integration of partial or complete virus sequences into the host

genome. These drawbacks can be overcome by the use of plant viruses and bacteriophages, which cannot infect or replicate within mammalian cells and therefore provide nanocarriers with inbuilt safety. However, plant viruses and bacteriophages are still perceived as foreign antigens by the mammalian immune system and are therefore immunogenic. This can be advantageous in certain immunotherapy scenarios, but undesirable immunogenicity can be avoided by shielding the particles with molecules such as polyethylene glycol (PEG), a strategy also used with synthetic nanocarriers (14).

In this review, we highlight recent developments in the use of viral nanocarriers for the delivery of vaccines, drugs, and imaging reagents and in next-generation immunotherapy. We focus on plant viruses and bacteriophages but also draw on informative examples involving mammalian viruses and nonviral protein nanoparticles.

2. VIRAL NANOCARRIERS FOR THE DELIVERY OF SMALL MOLECULES, PROTEINS, AND NUCLEIC ACID THERAPEUTICS

Viruses that have been repurposed to carry a medically relevant payload are defined as viral nanoparticles (VNPs) if they retain the virus genome or virus-like particles (VLPs) if the genome is absent (15). As depicted in Figure 2, the molecular cargo can be tethered to the external or internal surface of the capsid by covalent or noncovalent interactions with the coat proteins (8, 16, 17) or can be packaged inside the capsid by programmed self-assembly (18, 19) or infusion (20, 21).

2.1. Viral Nanocarriers for Drug Delivery

Viral nanocarriers have been developed for the delivery of diverse therapeutic payloads including small-molecule drugs, protein drugs, enzymes (to convert prodrugs into their active ingredient), photosensitizers, and combinations thereof. In concert with targeting ligands, such as peptides, antibodies, and aptamers, these formulations improve the specificity and efficacy of treatment while minimizing systemic toxicity. Some prominent examples are discussed below.

The use of bacteriophages carrying small-molecule cancer drugs dates back more than 10 years. In one of the earliest studies, filamentous bacteriophage fUSE5 was genetically engineered to express the immunoglobulin G (IgG)-binding domain of staphylococcal protein A (the ZZ domain), and the resulting fUSE5-ZZ bacteriophage particles were loaded with hygromycin or doxorubicin by conjugation to peptide DFK (which is sensitive to the protease cathepsin B), allowing controlled drug release in target cells (22). The resulting particles could be targeted by mixing them with specific antibodies due to the high-affinity binding between the ZZ domain and IgG fragment crystallizable (Fc) domain. The particles were initially targeted using antibodies specific for two members of the epidermal growth factor receptor (EGFR) family, EGFR/ErbB-1 and HER2/neu/ErbB-2, resulting in specific interactions with cells displaying those receptors followed by receptor-mediated endocytosis, intracellular degradation of the virus carrier, and drug release. This increased the cytotoxicity of the payload by ~1,000-fold compared with the free drug (22). Similarly, bacteriophage M13 was loaded with doxorubicin via the DFK peptide tethered to the P8 coat

protein and also achieved targeted drug delivery and enhanced efficacy (22). In an alternative approach, a core-shell nano-assembly comprising bacteriophage M13 conjugated to folic acid (thus targeting the folic acid receptor) and block copolymer PCL-P2VP for the entrapment of doxorubicin molecules was taken up by tumor cells and degraded in the acidic environment of the endosome to release the drug (23). In this case, the bacteriophage was not a drug carrier per se but a targeting reagent that associated with the hydrogel-like nano-assembly via multivalent interactions. Other bacteriophages, including MS2, lambda, and HK97, have also been used for targeted drug delivery, increasing the cytotoxicity of their payload at lower drug concentrations and reducing off-target effects (24, 25).

Drugs such as doxorubicin and cisplatin have also been delivered by plant viruses. One of the simplest formulations was based on potato virus X (PVX), which was able to deliver doxorubicin by exploiting hydrophobic interactions involving $\pi-\pi$ stacking of the planar drug molecules and polar amino acids in the PVX coat protein (17). Targeted drug delivery systems have also been developed using VLPs derived from Johnson grass chlorotic stripe mosaic virus (JgCSMV) covalently modified on the external surface with folic acid as a targeting ligand and with doxorubicin infused into the central cavity (26). These concepts can be applied broadly to other drug targets and viral platforms. For example, the external surface of tobacco mosaic virus (TMV) has been modified with mannose or lactose as targeting ligands, and the central channel was loaded with cisplatin, exploiting the interaction between platinum and glutamic acid residues, 4,200 of which line the TMV central channel (27). Similarly, ~2,000 molecules of the platinum-based drug candidate phenanthriplatin were loaded into TMV using noncovalent interactions to achieve efficient delivery and antitumor activity against breast cancer xenografts in mice (20). The negative charge of the TMV channel has also been exploited to achieve the electrostatic coupling of nonplatinum drugs such as mitoxantrone, a type II topoisomerase inhibitor (28).

An additional beneficial aspect of viruses is their ability to adopt new conformations depending on the environment, meaning that the structure of VNPs and VLPs can be influenced by the salt concentration, pH, and temperature of the medium during assembly. The design space of TMV particles is particularly intriguing because self-assembly under different conditions can yield rods of a defined length, icosahedral capsids, and complex assemblies such as stars and boomerangs (29). Nanoparticle shape and size govern differential cellular interactions and pharmacokinetics in vivo (30, 31); therefore, such shape transitions can be used to meet customized delivery needs (32). For example, doxorubicin-loaded TMV nano-disks outperformed spherical and filamentous counterparts for the delivery of drugs to intracranial glioma in mice (33).

In addition to small-molecule drugs, high-aspect-ratio (aspect ratio of nanoparticles is defined as the ratio of length to width) VNPs and VLPs are particularly efficient for delivery of biologics (proteins and nucleic acids), which are challenging to deliver as free reagents because they are often targeted by the immune system. The rigid rods formed by TMV (300 × 18 nm) have the tendency to marginate when traveling through blood vessels, making them ideal for the treatment of vascular disease, such as targeting thrombi even without integrated molecular recognition chemistry (34). TMV has therefore been engineered to carry protein-based drugs such as streptokinase and tissue plasminogen activator,

encouraging thrombus resolution and reducing the risk of bleeding (35, 36). Similarly, the flexible nanofilaments formed by PVX tend to concentrate in tumors and have been used to deliver tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which binds to death receptors DR4 and DR5 overexpressed on cancer cells and induces caspase-dependent apoptosis (37). PVX-TRAIL was assembled by interactions between nickel-coordinated nitrilotriacetic acid modules displayed on the PVX surface and TRAIL with an N-terminal His$_6$ tag, resulting in the accommodation of ~500 TRAIL molecules per PVX particle. The multivalent PVX-TRAIL formulation was significantly more efficacious than soluble TRAIL against human triple-negative breast cancer in an athymic mouse model (37). In this case, multivalent display (rather than delivery) enhanced drug efficacy by promoting the crosslinking of DR4/DR5 and strongly triggering the proapoptotic signaling pathway in cancer cells.

Proteins can also be encapsulated in viral cages, and this can be achieved with high precision. For example, bacteriophage P22 VLPs enable protein loading with controlled cargo stoichiometry and packaging density. In a recent example, streptavidin was linked to the P22 scaffold protein to program the assembly of a hybrid capsid consisting of the capsid protein and chimeric scaffolding protein, thus allowing the internal presentation of streptavidin. The assembly of a mixture of native and streptavidin-linked scaffold proteins allows the number of streptavidin molecules loaded per particle to be controlled precisely. This in turn provides control over the density of biotinylated molecular cargo that can be loaded in or tethered to the viral capsid (38).

Cancer drugs can be administered as inactive prodrugs to prevent off-target toxicity, relying on endogenous enzymes to convert them into active drugs. However, the efficacy of this approach is dependent on endogenous enzyme expression and activity, which vary between individuals and between cells in the same individual, especially in the heterogeneous tumor environment. To achieve consistent drug doses, viral nanocarriers containing enzymes have been developed to perform specific biochemical reactions in situ, and these can be targeted to tumor cells to avoid off-target effects caused by systemic prodrug conversion. For example, the programmed disassembly and reassembly of cowpea chlorotic mottle virus (CCMV) in the presence of cytochrome P450 (CYP) enzyme (39) and the differential expression of bacteriophage P22 coat proteins and CYP enzyme in *Escherichia coli* each resulted in the encapsidation of ~100 CYP molecules in the virus (40). When the P22-CYP particles were conjugated to folic acid, they achieved the targeted conversion of prodrug tamoxifen in tumor cells (40). More recently, complementary single-stranded nucleic acid tags were used to achieve the co-encapsidation of multiple enzymes in a single CCMV particle, resulting in the confinement of a functional enzymatic cascade (41).

Virus particles have also been proven useful for the delivery of photosensitizers used in photodynamic therapy (PDT) and photothermal therapy (PTT). In the former approach, a photosensitizer can be activated by light to produce reactive oxygen species that kill cells by inducing oxidative stress, whereas the latter achieves the same aim by triggering an increase in temperature. Photosensitizers must be incorporated into nanocarriers to improve their solubility and biocompatibility and to allow cell-specific targeting. For example, bacteriophage MS2 was modified internally with ~180 porphyrin molecules and externally

with aptamers targeting Jurkat cells, resulting in the photo-dependent elimination of this cell population (42). CCMV-based PDT nanocarriers have been developed by assembling the viral coat proteins around phthalocyanine macrocycle dendrimers (43). Furthermore, the internal channel of TMV has been loaded with the photosensitizer 5-(4-ethynylphenyl)-10,15,20-tris(4-methylpyridin-4-ium-1-yl)porphyrin-zinc(II)triiodide (Zn-EpPor), allowing the uptake of the photosensitizer by endocytosis followed by endolysosomal drug release, thus enhancing the efficacy of PDT in melanoma cells compared with the free drug (44). PTT has been implemented via the programmed assembly of gold nanoparticles (AuNP) on the capsids of bacteriophage T7 carrying a ligand that targets prostate cancer cells. The T7-AuNP clusters were taken up by the target cells and localized within the endosomal compartments, resulting in targeted temperature increase that killed the cells efficiently in response to light (45). New developments in wirelessly activated implantable photonic devices (46, 47) can overcome the current limitations of delivering lights to deeper tissues for in vivo PDT/PTT using surgical or endoscopic insertion of optics fibers (48).

2.2. Phage Nanocarriers and Phage Therapy

Bacteriophages play a special role when targeting infectious diseases because they can act both as nanocarriers and as antibacterial agents in their own right. Filamentous phages were developed for the targeted delivery of antibiotics ~15 years ago, with early examples including particles loaded with chloramphenicol and neomycin to prevent the growth of *E. coli*, *Streptococcus pyogenes*, and *Staphylococcus aureus* (49, 50). Along these lines, a recent study demonstrated that conjugating azithromycine to bacteriophage Qβ enabled the trafficking and rapid accumulation of the antibiotic-laden VLPs in mouse lungs, and this could be developed as a strategy for pulmonary drug delivery (51). However, the prevalence of multidrug-resistant bacteria has increased the demand for new antimicrobial strategies because the development of new antibiotics can take more than 10 years from discovery to approval (52, 53). Phage therapy, which has long been applied in the ex-Soviet Bloc (54, 55), is now re-emerging in the West. The resurgence of phage therapy is attributed to improved knowledge of phage biology, phage interactions with the host immune system (56, 57), development of standard protocols (58), and technological advancements in production, purification, characterization, and handling (59). Recent developments include use of bacteriophage or phage cocktails in animal models against several clinically important pathogens including *Pseudomonas aeruginosa* (60), *Clostridium difficile* (61), multidrug-resistant *E. coli* O25:H4ST 131 strain (62), and *Vibrio parahaemolyticus* (63). Phage therapy has been also evaluated in human patients; for example, at the Eliava Institute of Bacteriophages in Georgia and the Institute of Immunology and Experimental Therapy in Poland, patients were treated with phages for antibiotic unresponsive diabetic foot ulcers (64), and at the University College London Ear Institute and the Royal National Throat, Nose and Ear Hospital in London (UK), patients received phage therapy to treat chronic otitis (65). More recently, at the University of California, San Diego, phage therapy was successfully used for the treatment of a faculty member infected with multidrug-resistant *Acinetobacter baumannii* who had failed to respond to 4 months of conventional antibiotic therapy (66). This was followed by the treatment of further patients (67, 68).

In addition to the use of wild-type bacteriophages, next-generation phage therapies use genetically modified bacteriophage. For example, removal of the immunity repressor gene *45* of bacteriophage ZoeJ, which is required for lysogeny, leads to improved lytic activity of the phage in *Mycobacterium abscessus* (69). Another new direction is the incorporation of bacteriophages into hydrogels to achieve sustained delivery, as recently demonstrated using bacteriophage PA5 to kill *P. aeruginosa* PA01 over prolonged treatment periods (70). Besides tackling antibody-resistant pathogens and overcoming adverse reactions, phage therapy offers several advantages. In contrast to antibiotics, phages can penetrate bacterial biofilms via enzymatic degradation (e.g., by extracellular polymeric substance depolymerase) of the biofilm (71, 72). Phages also show both species and strain specificity and are therefore less likely to affect the gut microbiome (73, 74). However, this strain specificity can also limit the general applicability of phage for infective wounds colonized by several strains of bacteria. Phage cocktails can overcome these shortcomings but present challenging issues that are beyond the scope of this review.

2.3.   Viruses and Virus-Like Particles for Gene Therapy

Gene therapy involves the delivery of nucleic acids to disrupt, replace, or repair nonfunctional or dysfunctional genes, thus restoring the functionality of native proteins. Nearly 3,000 gene therapy clinical trials have been conducted thus far (75), several of which have led to approved products (Table 1). Because viruses evolved naturally to protect their nucleic acid cargo and shuttle it between cells, they remain the most efficient gene delivery vectors, accounting for nearly 70% of all gene therapy trials. The operational complexity and therapeutic efficacy of viral vectors remain unmatched (76–78). The major gene therapy vectors are based on adenoviruses, adeno-associated viruses, lentiviruses/retroviruses, and herpesviruses. Plant viruses and bacteriophage have also been developed as safer alternatives to mammalian viral vectors that carry the inherent risk of insertional mutagenesis and genotoxicity (79). Plant viruses and bacteriophages can also be produced inexpensively on a large scale in their native hosts or in heterologous production systems, avoiding the time-consuming and expensive cultivation of mammalian cells.

A recent example of gene therapy involving a plant virus was the use of CCMV to deliver *gfp* messenger RNA (mRNA) [encoding green fluorescent protein (GFP)] to mammalian cells. The RNA cargo was stabilized by encapsulation and was released into the cytoplasm when co-delivered with the reagent Lipofectamine 2000 and then expressed, resulting in intracellular green fluorescence (88). CCMV has also been used to deliver mammalian replicons (89, 90), enabling the amplification and expression of constructs encoding a range of model antigens (91). Similarly, TMV was loaded with *gfp* mRNA as proof of concept for a vaccine delivery platform, resulting in an immune response to GFP in the immunized mice (92).

Nucleic acid therapeutics are not restricted to gene therapy and also extend to the delivery of small regulatory RNAs such as short interfering RNAs (siRNAs), microRNAs (miRNAs), and anti-miRNA oligonucleotides. Early proof-of-concept work showed that the cell-penetrating peptide M-lycotoxin L17E facilitated the uptake of CCMV VLPs loaded with siRNAs to knock down the expression of GFP and the forkhead box transcription factor

FOXA1 (93). Similarly, TMV vectors displaying the human immunodeficiency virus (HIV) transacting activator of transduction (TAT) peptide (YGRKKRRQRRR) were able to deliver GFP-specific siRNA to mouse epidermal stem cells in vitro and metastatic hepatocellular carcinoma in vivo. The TAT peptide facilitated the lysosomal/endosomal escape of the vector, releasing the siRNA in the cytoplasm (94). In a recent study, a VLP/RNA interference nanocomplex based on bacteriophage Qβ was designed to downregulate the hepatocyte growth factor receptor gene (*c-MET*). In malignant brain tumor cells, hepatocyte growth factor binds to c-MET to protect cells from the damage caused by alkylating agents such as temozolomide (TMZ). The nanocarrier was modified to display a cell-penetrating peptide and an apolipoprotein E (ApoE) peptide, allowing passage across the blood-brain barrier. The suppression of *c-MET* expression worked synergistically with TMZ in mouse models of intracranial glioblastoma (95). The encapsulation of MS2 phage RNA is mediated by a 19-nucleotide stem-loop structure known as the *pac* site, which interacts with the coat protein. Therefore, nucleotides, drugs, and proteins conjugated to RNA molecules containing a *pac* site are efficiently packaged into the self-assembling capsid. Using this strategy, antisense RNA complementary to the p120 mRNA of myelogenous leukemia cells was packed into MS2 capsids displaying transferrin and delivered to leukemia cells expressing the transferrin receptor, increasing the cytotoxicity of the formulation compared with free antisense RNA (96).

Finally, mammalian viruses (97, 98) and bacteriophage P22 (99) have been used to facilitate gene editing with the powerful CRISPR/Cas9 system by shuttling the components into target cells. For example, bacteriophage P22 VLPs have been produced by expressing the coat protein and a truncated scaffold protein genetically fused to Cas9 in *E. coli*, followed by spontaneous self-assembly with the synthetic guide RNA (sgRNA). This co-expression system allowed the temporal decoupling of the cargo and capsid and facilitated the assembly of the scaffold-Cas9 fusion protein with the sgRNA cargo prior to coat protein expression, resulting in the encapsulation of ~20 Cas9 enzymes per capsid. The encapsulated Cas9 achieved target-specific double-stranded DNA cleavage and was protected from proteolysis (99).

2.4. Viruses and Virus-Like Particles for Molecular Imaging

Molecular imaging involves the use of markers or labels to highlight cellular or tissue-level structures, providing information about biological processes and also enabling early diagnoses, accurate prognoses, and personalized therapy (100). Ideal molecular imaging techniques achieve the optimal signal-to-noise ratio at the target site with minimal toxicity. Viruses carrying imaging reagents are advantageous because their short circulation and retention time make them easier to eliminate than synthetic nanoparticles. Viruses can be tailored to carry a wide range of contrast agents and/or fluorescent labels, and the addition of aptamers, peptides, or antibodies to the external surface of the virus allows the targeting of particular cells and tissues. Viral nanocarriers have therefore been developed for multiple imaging modalities, including fluorescence imaging, magnetic resonance imaging (MRI), positron emission tomography (PET), and computed tomography (CT) (101) (Figure 3).

The use of VNPs/VLPs for fluorescence imaging initially required the conjugation or encapsidation of many fluorescent dye molecules per particle. However, genetic engineering has also been used to produce viral particles carrying intrinsically fluorescent proteins, such as GFP and mCherry (102). Furthermore, a combination of genetic engineering and programmed self-assembly has been used to prepare polyomavirus particles simultaneously loaded with GFP and m-Ruby3, which are suitable for fluorescence resonance energy transfer experiments (103). Preclinical imaging in live animals has been achieved using VLPs by incorporating near-infrared (NIR) fluorescent dyes and/or NIR2 contrast agents such as single-walled carbon nanotubes and quantum dots (104–106).

In one example of virus-based fluorescence imaging, fluorescent cowpea mosaic virus (CPMV) particles have been used to image the tumor neovasculature by intravital two-photon laser scanning microscopy, exploiting the natural interaction between CPMV and the endothelial protein vimentin (107). However, other targets can be selected by adding appropriate ligands to the CPMV surface. For example, the use of peptide ligands specific for epidermal growth factor-like domain 7 was sufficient to retarget CPMV, allowing its use as a diagnostic reagent for the stratification of prostate cancer (108).

Several groups have recently developed fluorescent VNPs/VLPs for cardiovascular imaging in order to stratify atherosclerotic plaques, thus improving the accuracy of prognoses and reducing the frequency of strokes and heart attacks. TMV particles loaded with the NIR dye Cy5 and conjugated to peptides targeting S100A9 (myeloid-related protein 14) have been used to detect the presence of macrophage-rich atherosclerotic lesions in ApoE$^{-/-}$ mice (109). Similarly, MS2 VLPs carrying the NIR fluorescent dye AlexaFluor 680 and displaying antibodies specific for vascular cell adhesion molecule were used to detect atherosclerotic plaques in the descending aorta and aortic arch (110). Although many VLPs are designed to bind membrane proteins on target cells, others target components of the extracellular matrix. For example, collagen and secreted protein acidic and rich in cysteine (SPARC) were targeted with fluorescent M13 VLPs for deep tissue imaging in lung cancer, and SPARC was also used for the fluorescence-guided resection of ovarian cancer using M13 particles carrying single-walled carbon nanotubes (111).

Viruses modified with MRI, PET, and CT contrast agents are promising formulations for clinical development. MRI is the most frequently used noninvasive diagnostic technique. Given the rapid recent progress in cryogen-free superconducting magnet technology, the current trend in clinical MRI involves the use of ultrahigh magnetic field strengths (7.0 T) to achieve a higher signal-to-noise ratio and greater spatial and temporal resolution (112). The signal-to-noise ratio in MRI is improved by contrast agents that selectively shorten the T1 or T2 relaxation times in the region of interest. VNPs/VLPs carrying such contrast agents can therefore improve the quality of MRI by concentrating these molecules at the target site. Simultaneously, the toxicity of the contrast agents is reduced because the VNPs/VLPs are cleared more rapidly than the free molecules. Accordingly, viruses and their VLPs have been loaded with contrast agents for both T1 and T2 imaging. For example, bacteriophage P22 has been used to encapsulate gadolinium-tetraazacyclododecane (Gd-DOTA) and Mn^{3+} chelators, leading to higher T1 relaxivities (113, 114). Similarly, TMV was loaded with Gd-DOTA and conjugated to vascular cell adhesion molecule 1 in order to target the

corresponding receptors, allowing the detection and imaging of atherosclerotic plaques at submicromolar doses. The high aspect ratio of the TMV nanoparticles promoted ligand-receptor interactions by enhanced margination to vessel walls, while the slower tumbling and enhanced relaxivity of the Gd-DOTA increased the signal-to-noise ratio and imaging sensitivity (115). For T2 imaging, brome mosaic virus (BMV) and bacteriophage M13 have been loaded with iron oxide nanoparticles. The BMV VLPs formed a shell around an iron oxide core, increasing the T2 relaxivity by 4–6.5-fold compared with commercially available T2 contrast agents (116). The iron oxide–loaded M13 VLPs were also conjugated to SPARC peptides for the targeted imaging of prostate cancer (117).

Bimodal contrast agents allow the use of multiple imaging techniques. In a key example, the internal cavity of TMV particles was loaded with a dysprosium (Dy^{3+}) complex to increase T2 relaxivity during MRI but also with the NIR fluorescent dye Cy7.5 (118). The external surface was conjugated to the peptide DGEA via a PEG linker to target integrin $\alpha_2\beta_1$ on prostate cancer cells and achieved high relaxivity during ultrahigh-field MRI (119). Interestingly, physalis mottle virus particles loaded with MRI contrast agents to target prostate cancer cells were cleared more slowly than TMV and therefore allowed tumor imaging for several days (106). Such longitudinal imaging modalities may be useful to follow disease progression and/or therapeutic responses.

Chemical exchange saturation transfer (CEST) and HyperCEST imaging are newer MRI modalities using reagents based on xenon in which exogenous nuclei are selectively saturated and enhanced water signals are observed due to saturation transfer to the surrounding bulk water (120, 121). Bacteriophage MS2, M13, and fd have been used to develop VNP/VLP formulations to exploit these new techniques. For example, cyptophane cages bound to filamentous M13 achieved HyperCEST contrast enhancement with sensitivities as low as 230 fM, 50-fold more sensitive than free cyptophane (122). Similarly, icosahedral MS2 particles containing 125 cyptophane molecules improved contrast, with sensitivity as low as 0.7 pM (123). In a more recent development, cyptophane-loaded MS2 particles were targeted to Burkitt's lymphoma cells using an aptamer specific for the membrane protein mIgM (124). Furthermore, cyptophane-loaded filamentous fd particles targeting EGFR/ErbB-1 have been used for the selective imaging of liver cancer cells (125). The unprecedented sensitivity of these new MRI modalities could significantly increase the accuracy of preclinical and clinical imaging.

In another new development, TMV was loaded with a metal-free paramagnetic nitroxide organic radical contrast agent (ORCA) to develop MRI and electron paramagnetic resonance probes for superoxide detection in vitro with enhanced r1 and r2 relaxivities. The probes therefore functioned as both T1 and T2 contrast agents, making them suitable for preclinical and clinical MRI scanners. Furthermore, bimodal TMV probes with fluorescent dyes conjugated to the internal surface and ORCA to the outer surface allowed the fluorophore to be used as a concentration marker regardless of the ORCA oxidation state (126).

PET imaging is based on the detection of radiotracers and has been used to map the biodistribution of bacteriophage MS2 in vivo by covalently attaching [18F] fluorobenzaldehyde or by loading the particle with DOTA-[64Cu] (127). Similarly, the

biodistribution and tumor homing ability of MS2 particles targeting EGFR/ErbB-1 in a breast cancer model (128) and T7 particles displaying RGD peptides (129) were also investigated by PET imaging after labeling the particles with ^{64}Cu. PET imaging has been also used to confirm successful gene delivery using viral vectors. For example, vectors expressing the HSV-1 thymidine kinase reporter system can be traced by PET imaging by monitoring the phosphorylation of radiolabeled thymidine analogs because the accumulation of radioactive tracer is proportional to the quantity and activity of the enzyme (130). Finally, gold-coated CPMV particles were recently developed for CT imaging, which uses scattered X-rays for three-dimensional visual reconstruction and tissue segmentation. The gold-coated CPMV particles improved the sensitivity of the technique, reducing the scan time to less than 2 min and achieving a resolution of nearly 150 HU (131).

Viruses loaded simultaneously with imaging reagents and drugs can be used for both diagnosis and therapy, a concept known as theranostics. Some theranostic modalities are constitutive, meaning that the imaging reagents and drugs are active simultaneously. Examples include a multifunctional bacteriophage M13 conjugated with chemotherapy, fluorophores, and targeting ligands designed for simultaneous imaging and drug delivery to prostate cancer cells (132) as well as tri-functional simian virus 40 VNPs displaying the peptide CGNKRTRGC to target atherosclerotic plaques and encapsulating NIR quantum dots (QD800) for imaging and the anticoagulant drug Hirulog for atherosclerotic imaging and therapy (133). A more refined approach is conditional or induced therapy, in which the drug is activated after imaging by applying an external trigger. This is beneficial because it allows for therapeutic decisions based on the imaging results (e.g., therapy can be withheld if the imaging results reveal that treatment is unnecessary). For example, TMV particles were loaded with MRI contrast agents and coated with polydopamine for photoacoustic imaging and PTT, the latter based on strong NIR absorption with high photothermal conversion efficiency (134). Similarly, hepatitis B virus core (HBc) particles were loaded with indocyanine green for image-guided cancer phototherapy and also conjugated to the RGD peptide for tumor targeting. The VLPs accumulated in U87MG tumors and facilitated sensitive NIR fluorescence/photoacoustic imaging followed by PDT/PTT for tumor ablation upon irradiation with an NIR laser (135). HBc VLPs have also been used to develop multifunctional theranostic agents carrying iron oxide and the cancer drug methotrexate (MTX) (136). The MTX was chemically conjugated to the iron oxide shell-core nanoparticles and embedded in the capsid. The iron oxide component enabled T2-weighted MRI as well as PTT for tumor ablation in response to an NIR laser, whereas the MTX killed the tumor cells with or without laser exposure. The particles were therefore suitable for image-guided therapy. When injected into 4T1 tumors in mice and irradiated with the NIR laser, the particles achieved synergistic tumor ablation by PTT and chemotherapy.

3. VIRUSES AS IMMUNOMODULATORS

Viral nucleocapsids act as pathogen-associated molecular patterns (PAMPs), which are danger signals that activate the innate immune system by triggering multiple signaling pathways (137). Cell surface receptors Toll-like receptor (TLR) 2 and TLR4 have been implicated in recognition of viral proteins (138–140), whereas intracellular receptors TLR3, TLR7, TLR8, and TLR9 can recognize viral nucleic acids (141). Although plant viruses and

bacteriophages are noninfectious in mammals, their nucleocapsid imparts similar immunological potency via TLRs (142–144). Furthermore, the size range of most viruses (20–500 nm) allows VLPs to drain into the lymph nodes and to be captured by innate immune cells to prime the immune system, making them ideal for the development of vaccines and immunotherapy platforms.

3.1.   Virus-Like Particles as Platforms for Vaccine Development

The prevention of infectious diseases by vaccination is arguably the greatest medical achievement in history. While viral diseases smallpox and rinderpest have been eradicated by vaccination, others such as poliomyelitis, rubella, yellow fever, and measles have been brought under control to a great extent (145). However, efficacious vaccines remain unavailable for many prevalent diseases, such as HIV/acquired immunodeficiency syndrome and malaria, and have not yet been developed for many emerging and resurgent diseases, such as coronaviruses including SARS-CoV, MERS-CoV, and the more recent SARS-CoV-2 (146–148). Therapeutic and prophylactic vaccines are also being developed for cancer, chronic diseases, and substance abuse.

Most vaccines are based on live, inactivated, or attenuated whole pathogens. The disadvantage of this approach is that the antigens required to prime immune memory are inextricably linked to the original pathogen, which incurs a risk of reversion to virulence and also means that the properties of the delivery platform cannot be changed. The introduction of subunit vaccines was the first step toward separating the antigen from the delivery platform, but this also removed the advantages of the virus as a self-contained adjuvant. The more recent development of recombinant VLPs has expanded the versatility of vaccines by combining antigens with the most potent immunostimulatory virus-based nanocarriers (149–151). Several VLP-based vaccines have been approved or are undergoing clinical evaluation (Table 2). While VLP vaccines allow for surface display of antigens and induce neutralizing antibody response, in the absence of de novo synthesis of viral antigens, they often induce a weak cytotoxic T cell response as compared with live vaccines.

3.1.1.   Virus-like particle–based vaccines against infectious diseases.—VLP-based vaccines have been approved for the prevention of hepatitis B virus, hepatitis E virus, and human papillomavirus (HPV) infections, the latter also preventing HPV-derived cervical cancer. Other VLPs are undergoing clinical trials to prevent infections with influenza A virus (https://www.clinicaltrials.gov/ identifiers NCT02768805, NCT02233816), chikungunya virus (NCT02562482), human cytomegalovirus (NCT02826798), and Norwalk virus (NCT02669121, NCT03039790, NCT02661490). VLPs based on plant viruses and bacteriophage have also been developed to present the HPV L2 epitope, including grapevine fanleaf virus and bacteriophage MS2, leading to the production of HPV-neutralizing antibodies in preclinical studies (152, 153). Similarly, a VLP displaying the Ebola virus glycoprotein and matrix protein (VP40) was used in conjugation with the TLR4 agonist glucopyranosyl lipid adjuvant to enhance immunogenicity and promote durable protection against a mouse-adapted Ebola virus strain (154). Plant and bacteriophage VLPs have also been used to develop vaccines against Zika virus, influenza A virus, Norwalk virus, and the bacterial diseases brucellosis, anthrax, and bubonic plague (155). In a recent study,

bacteriophage Qβ displaying the cell-transversal protein from the human malaria parasite *Plasmodium vivax* (PvCeITOS) was evaluated as a preclinical malaria vaccine and produced PvCeITOS-specific antibodies and CD8^{+} T cells in mice (156).

3.1.2. Virus-like particle–derived vaccines against cancer.—Cancer vaccines are designed to prime the immune system to recognize tumor-associated antigens and launch an endogenous adaptive immune response that can eliminate primary tumors as well as any residual or recurring disease by inducing immune memory. Tumor antigens are often overexpressed self-antigens, and cancer vaccines must therefore overcome self-tolerance by providing potent adjuvants, which can be achieved by presenting the vaccines as VLPs (157, 158).

Several VLP-based vaccines have been developed targeting HER2/neu/ErbB-2, some of which accomplished a true virus-like display of the correctly folded multi-epitope antigen to induce a strong polyclonal antibody response. For example, using the Spy-tag/Spy-catcher ligation method, *Acinetobacter* bacteriophage AP205 VLPs were engineered to display multivalent human HER2 on the particle surface, overcoming B cell tolerance and inducing a strong polyclonal anti-HER2 autoantibody response (159). Prophylactic vaccination reduced the spontaneous development of mammary carcinomas by 50–100% in human HER2 transgenic mice and inhibited the growth of HER2^{+} tumors implanted in wild-type mice. The comparison of vaccine platforms for HER2 epitope display based on VLPs differing in shape and structure revealed that icosahedral CPMV nanoparticles outperformed PVX filaments in terms of lymphatic draining and uptake by antigen presenting cells (APCs) (160). Efficacy studies showed that the CPMV-HER2 vaccine candidate delayed the progression of primary tumors and metastasis in mice, thereby prolonging survival (161).

Antibodies and T cell responses generated by VLPs target not only the epitope but also the VLP carrier. To overcome this drawback and focus the immune system solely on the epitope, the Steinmetz lab recently deployed a heterologous prime-boost strategy in which the different antigen delivery systems are used sequentially to boost immunity against a shared antigen (162, 163). Thus, we used distinct VLP carriers presenting the same epitope for primary immunization and subsequent boosts. We used three icosahedral plant viruses—CPMV, CCMV, and sesbania mosaic virus—displaying an HER2 epitope. The heterologous prime-boost significantly enhanced the HER2-specific immune response and induced an efficacious Th1-predominant response, as indicated by delayed tumor progression and improved survival (164).

Bacteriophage Qβ has been engineered to present tumor-associated carbohydrate antigens including the poorly immunogenic monomeric Tn antigen (165) and the ganglioside antigen GM2 (166). Recently, a Qβ-MUC1 glycopeptide vaccine targeting the epithelial cell marker mucin 1 was shown to induce high titers of anti-MUC1 IgG antibodies in MUC1 transgenic mice, conferring protection against primary and metastatic breast cancers (167, 168).

Current strategies for the development of cancer vaccines rely on the identification and targeting of neoantigens, thus offering the potential for patient-specific treatments (169). A multi-target vaccine approach based on bacteriophage Qβ VLPs was recently evaluated

against B16F10 murine melanoma, comparing vaccines containing germline epitopes identified by immunopeptidomics with mutated epitopes predicted by whole exome sequencing and a third set consisting of a mix of these antigens. This study indicated that the mixture of germline and mutated epitopes achieved the greatest therapeutic efficacy (170).

3.1.3.   The diverse applications of virus-like particle–based vaccine platforms.—VLP-based vaccines have been developed to treat a range of disorders, including cardiovascular, neurological, and autoimmune diseases. In the context of cardiovascular diseases, proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secreted protein that controls cholesterol homeostasis by enhancing the degradation of the low-density lipoprotein receptor. Mutations that increase PCSK9 activity can lead to hypercholesterolemia, atherosclerosis, and early-onset cardiovascular diseases. Monoclonal antibodies targeting PCSK9 reduce its activity. Accordingly, the vaccination of mice and macaques with bacteriophage Qβ VLPs displaying PCSK9-derived peptides resulted in high titers of PCSK9-specific antibodies, inhibiting the enzyme and lowering the levels of cholesterol, phospholipids, and triglycerides (171). In the context of neurological disorders, a VLP based on HPV displaying the β-amyloid 11–28 epitope (Aβ 11–28) was recently used as a multivalent scaffold to develop a candidate vaccine against a mouse model of Alzheimer's disease. The chimeric VLP elicited Aβ-specific antibodies that bound to β-amyloid plaques in a mouse model (172). In other examples, a cucumber mosaic virus (CuMV)-based vaccine displaying recombinant nerve growth factor (NGF) generated antibodies against NGF, a key factor underlying the chronic pain associated with osteoarthritis. Antibodies against NGF are potent analgesics, and a recent study showed that the vaccine induced high titers of anti-NGF antibodies in mice that had undergone partial meniscectomy to induce osteoarthritis, leading to the reversal of pain behavior (173). In the context of autoimmune diseases, interleukin 17 (IL-17) mediates the release of proinflammatory cytokines in a wide range of cells, and aberrant T helper cells producing this molecule (Th17) are implicated in several autoimmune disorders, including arthritis and multiple sclerosis. To mimic the blockade of IL-17 by monoclonal antibodies, mice were immunized with bacteriophage Qβ VLPs displaying IL-17. The vaccine overcame self-tolerance against IL-17 and generated high titers of anti-IL-17 antibodies, which slowed the progression of the disease (174). Vaccination strategies have also been explored for contraception. Previously, several mammalian viral vectors including recombinant myxoma virus, ectromelia virus, and cytomegalovirus-based contraception vaccines have been evaluated (175–177). Using similar approaches, contraception vaccines based on plant virus–derived VNPs were used to induce antibodies that blocked the ability of sperm to interact with the zona pellucida of the egg. JgCSMV particles were engineered to display the sperm peptide YLP12 and zona pellucida epitope ZP3, leading to a significant loss of fertility in immunized mice (178).

VLPs have also been developed into veterinary vaccines. For example, VLPs based on CuMV have been used as a vaccine scaffold for the major cat allergen Fel d1. Cats vaccinated with the VLPs produced high-affinity antibodies against Fel d1 with strong neutralizing effects in vitro and in vivo, which could potentially protect pet owners with

allergies (179). CuMV VLPs have also been developed into a vaccine against insect-bite hypersensitivity in horses using equine IL-5 as the antigen (180).

3.1.4.   Virus-like particle–based in situ vaccines for cancer therapy.—The immunostimulatory properties of VLPs are ideal for immunotherapy, either relying on the intrinsic properties of the virus or by exploiting them as nanocarriers for an immunostimulatory cargo. VLPs can also be used for in situ vaccination or oncolytic virotherapy. The latter exploits the tendency of some viruses to replicate selectively within cancer cells and trigger their lysis, thereby exerting anticancer effects by directly debulking the tumor and by instigating antitumor immune responses (181). For example, talimogene laherparepvec (T-VEC) is a live oncolytic herpesvirus that displays granulocyte stimulating factor, which is approved for the treatment of inoperable metastatic melanoma (182). T-VEC is injected directly into the tumor, where it preferentially replicates in dividing cells and triggers apoptosis, as well as promotes local and systemic immune responses. The lysis of cancer cells releases tumor-associated antigens that are sampled by infiltrating APCs, thus customizing the vaccination to the individual tumor (183, 184). These innate responses also prime the adaptive immune system, leading to systemic antitumor immunity at local and remote disease sites. Several other oncolytic viruses are undergoing clinical trials, including poliovirus, Newcastle disease virus, vaccinia virus, and measles virus (181).

To be effective, cancer immunotherapies must overcome the immunosuppressive tumor microenvironment (TME) (185). In situ vaccination refers to the administration of immunotherapy directly to a tumor or metastatic site to relieve immunosuppression in the TME and induce a systemic antitumor immune response (Figure 4). Recently, CPMV and papaya mosaic virus (PapMV) were shown to modulate the TME when applied as in situ vaccines. The efficacy of in situ CPMV was established in syngeneic mouse tumor models of melanoma; ovarian, colon, and breast cancer (186–188); and glioma (189) as well as in companion dogs with oral melanoma (190). The administration of CPMV to tumors leads to a cascade of events including the infiltration and activation of innate immune cells, the increased secretion of proinflammatory cytokines, and the recruitment and repolarization of immune cells to antitumor phenotypes leading to tumor cell death. Tumor antigens are then released and taken up by tumor-infiltrating APCs as discussed above for oncolytic viruses, leading to the augmentation of local and systemic antitumor responses mediated by antitumor CD8$^+$ T cells. However, unlike oncolytic viruses, the potency of in situ vaccines based on plant viruses is driven by their immunostimulatory multivalent capsids, which are recognized as PAMPs. Although PapMV immunotherapy is attributed primarily to the encapsidated nucleic acid, which activates TLR7 signaling, both the capsid and RNA of CPMV are required for a potent effect. In subsequent studies, a combination of the CPMV in situ vaccine and cyclophosphamide (191) or radiation treatment (192) was shown to elicit a synergistic effect, leading to long-term antitumor responses and immune memory in mouse models of melanoma and ovarian and breast cancer. In another study, filamentous PVX particles were used as an in situ vaccine combined with doxorubicin in a mouse melanoma model. The direct injection of nonconjugated PVX and doxorubicin into the tumor achieved a synergistic effect, slowing tumor progression and improving survival compared with the PVX or doxorubicin treatments alone (193).

Bacteriophage M13 targeting carcinoembryonic antigen has been used as an in situ vaccine in a mouse model of colorectal cancer (194). The cytotoxic response and immune memory were attributed to TLR9 signaling based on the single-stranded DNA genome of the virus. Bacteriophage Qβ VLPs and VLPs loaded with immunostimulatory CpG oligonucleotides have also shown promise as in situ vaccines (158). The encapsulation and delivery of immunostimulatory molecules such as CpG oligonucleotides has several advantages, including the protection of the cargo from premature degradation and the effective targeting of innate immune cells. Not only is this more efficient, but also it avoids the off-target effects of systemic immunomodulators, including systemic inflammatory responses, autoimmune disease–like symptoms, and organ dysfunction (195, 196). Indeed, several nanocarrier-based drug delivery strategies have been repurposed for immunotherapy (197, 198). Mammalian viruses or their components have been used to deliver immunotherapeutic agents, including HBc particles displaying tumor antigens and carrying CpG oligonucleotides that showed efficacy against fibrosarcoma in mouse models (199). Lentiviral vectors displaying breast tumor antigens have been designed to target dendritic cells using a sindbis virus glycoprotein that selectively binds the surface protein DC-SIGN (200, 201). Similarly, bacteriophages have been used in preclinical and clinical studies to present tumor antigens while encapsulating CpG oligonucleotides, resulting in the preferential uptake of VLPs by APCs and strong effector T cell responses in mice (202).

4. CHALLENGES AND NEW DIRECTIONS

VNPs and VLPs offer a remarkable combination of beneficial properties. The size and shape of the particles facilitate their vascular transport, cellular uptake, and interactions. Their robust structure can withstand harsh chemical and physical conditions, yet they remain biocompatible and biodegradable, and to a great extent they conform to the structure-function rules that apply to synthetic nanomaterials. Accordingly, large doses of viruses are well tolerated, but proteolytic degradation ensures their rapid and complete clearance. The genetically predetermined architecture minimizes structural variations and allows precision engineering to generate new structures that interact predictably with biological systems.

As discussed in this review, plant viruses and bacteriophages provide an added layer of safety over mammalian viruses, but they remain immunogenic. Therefore, they are susceptible to accelerated clearance and reduced therapeutic efficacy upon repeat administrations. For example, the immunostimulatory effects of PapMV were completely abolished after multiple treatments, attributed in part to the appearance of PapMV-specific antibodies (203). Such effects are not unique to viral platforms and have been observed for other protein-based therapeutics (204, 205). Several surface passivation strategies have been developed to shield the viral surface from non-specific adsorption of serum proteins or to prevent recognition by antibodies including coating viral surfaces with polymers such as PEG (14) or camouflaging with albumin (206). Other methods can be adapted from synthetic virology, where the selective alteration of immunodominant protein domains has been shown to modulate viral tropism and immunogenicity (207, 208).

Multiple administrations of viral nanocarriers can also be abolished via slow release implants, scaffolds, and patches. Recently, a solvent free melt-encapsulation strategy was

developed to load VNPs based on bacteriophage Qβ into poly(lactic-coglycolic) acid implants. As subcutaneous implants, the biodegradable polymers released VLPs over time without affecting the structural integrity of the particles and generated an anti-VLP immune response comparable to that achieved by multiple injections of the soluble particles (209). Similarly, the slow release of a CPMV-based in situ vaccine in a mouse model of ovarian cancer was as effective as multiple injections of soluble CPMV (210). In another example, bacteriophage M13 was embedded in responsive polymers by grafting NIPAM-cophenylboronic acid onto the M13 surface. The temperature-responsive gelation behavior of the polymer was exploited to incorporate insulin into the M13-polymer conjugate at 4°C, followed by conversion to a hydrogel at 37°C. This insulin-hydrogel formulation showed glucose-dependent release behavior, and hydrophilic conversion of the hydrogel matrix in the presence of glucose was used to tailor the insulin release rate (211). The development of VLP-based oral vaccines is another significant area for future exploration. For example, VLPs based on influenza A virus displaying an intestinal protozoan antigen showed remarkable stability to temperature and pH changes, enabling vaccination by oral delivery (212).

Scalable production platforms based on plants and microbes offer an inexpensive and reliable approach to produce large quantities of VNPs and VLPs for research as well as preclinical and clinical development. This will allow the development of ever more innovative and versatile virus-based diagnostic and therapeutic reagents in the future.

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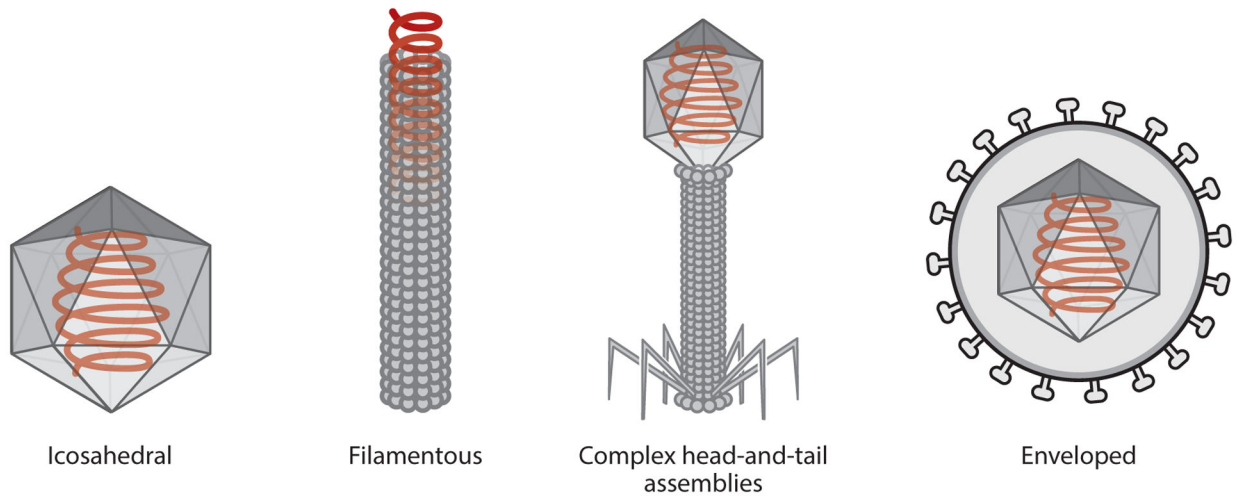
| Icosahedral | Filamentous | Complex head-and-tail assemblies | Enveloped |

Figure 1.
The structure of a virus depends on the species, and there is great diversity in nature, but the four basic configurations are icosahedral, filamentous (rigid or flexible), head-and-tail assemblies, and enveloped viruses.
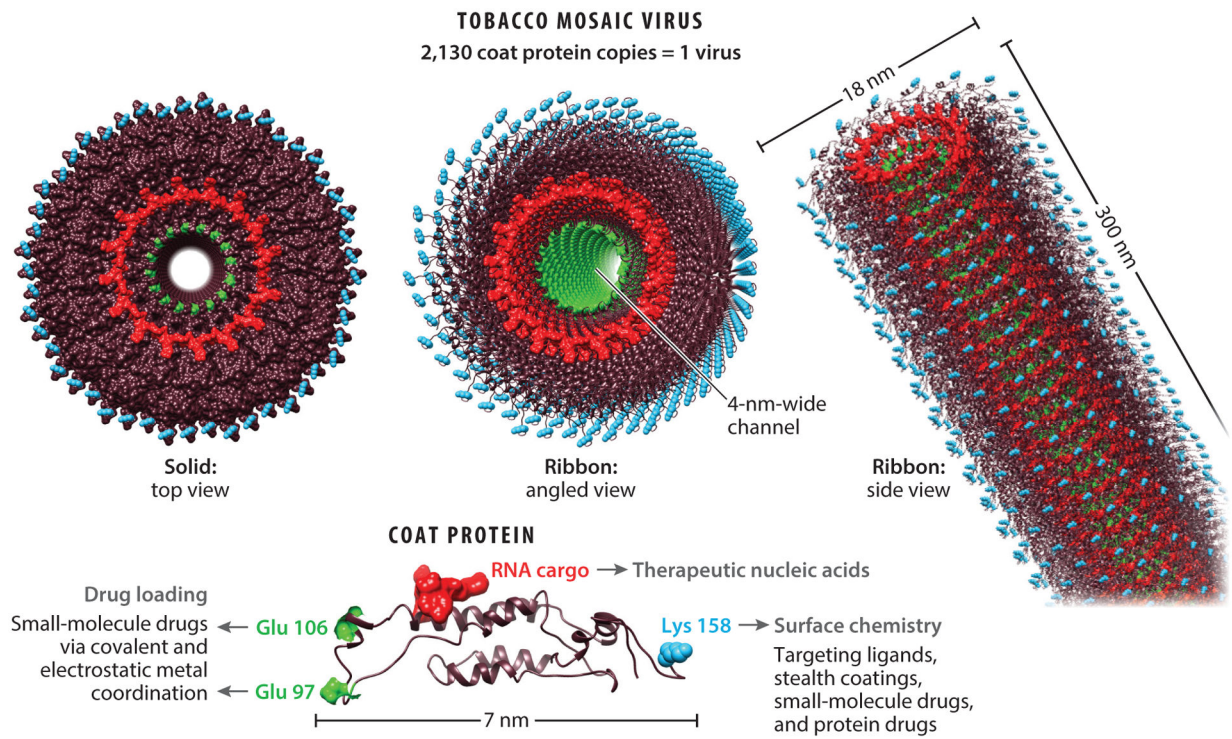
TOBACCO MOSAIC VIRUS
2,130 coat protein copies = 1 virus

18 nm

300 nm

4-nm-wide channel

Solid: top view

Ribbon: angled view

Ribbon: side view

COAT PROTEIN

RNA cargo → Therapeutic nucleic acids

Drug loading

Small-molecule drugs ← Glu 106
via covalent and
electrostatic metal
coordination ← Glu 97

Lys 158 → Surface chemistry

Targeting ligands,
stealth coatings,
small-molecule drugs,
and protein drugs

7 nm

Figure 2.
Gene and drug delivery by tobacco mosaic virus (TMV). Similar design and engineering concepts have been established for various filamentous and icosahedral plant viruses and bacteriophages.
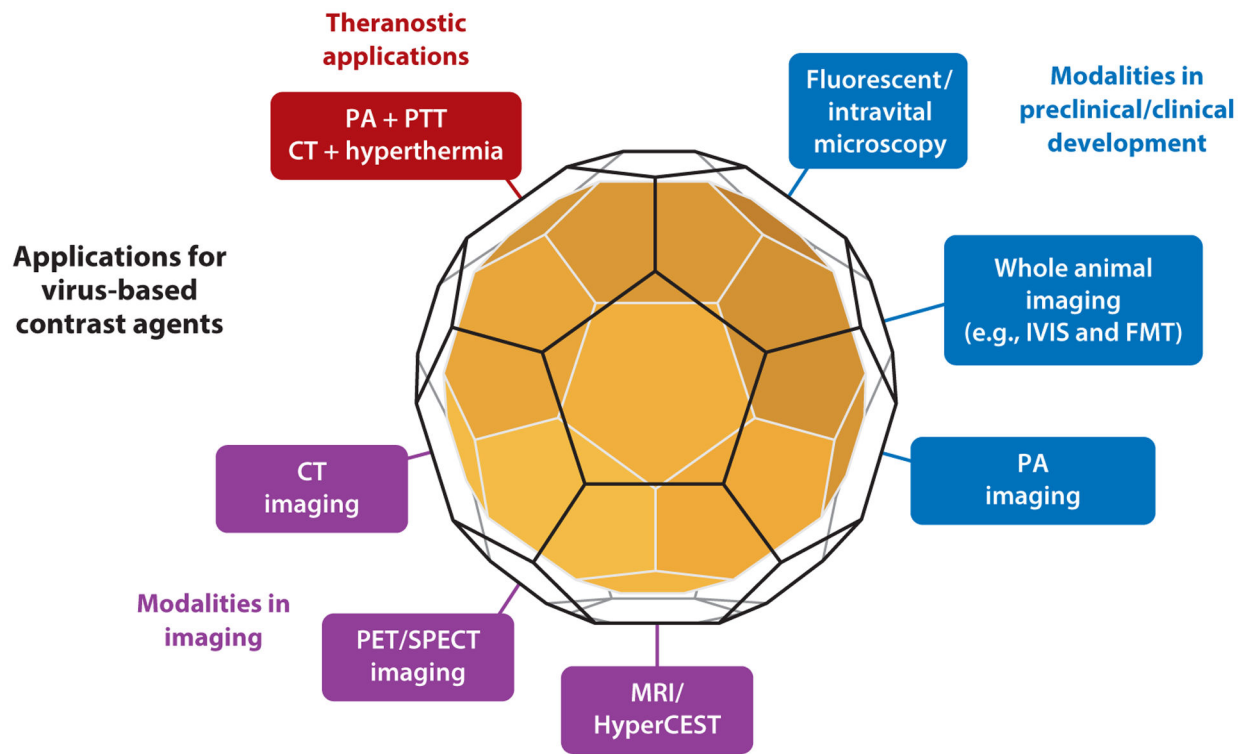
Figure 3.
Viral nanocarriers have been developed as a versatile platform for multiple diagnostic imaging modalities and theranostic applications. Abbreviations: CT, computed tomography; FMT, fluorescence molecular tomography; HyperCEST, hyperpolarized xenon chemical exchange saturation transfer; IVIS, in vivo imaging system; MRI, magnetic resonance imaging; PA, photoacoustic imaging; PET, position emission tomography; PTT, photothermal therapy; SPECT, single photon emission computed tomography.
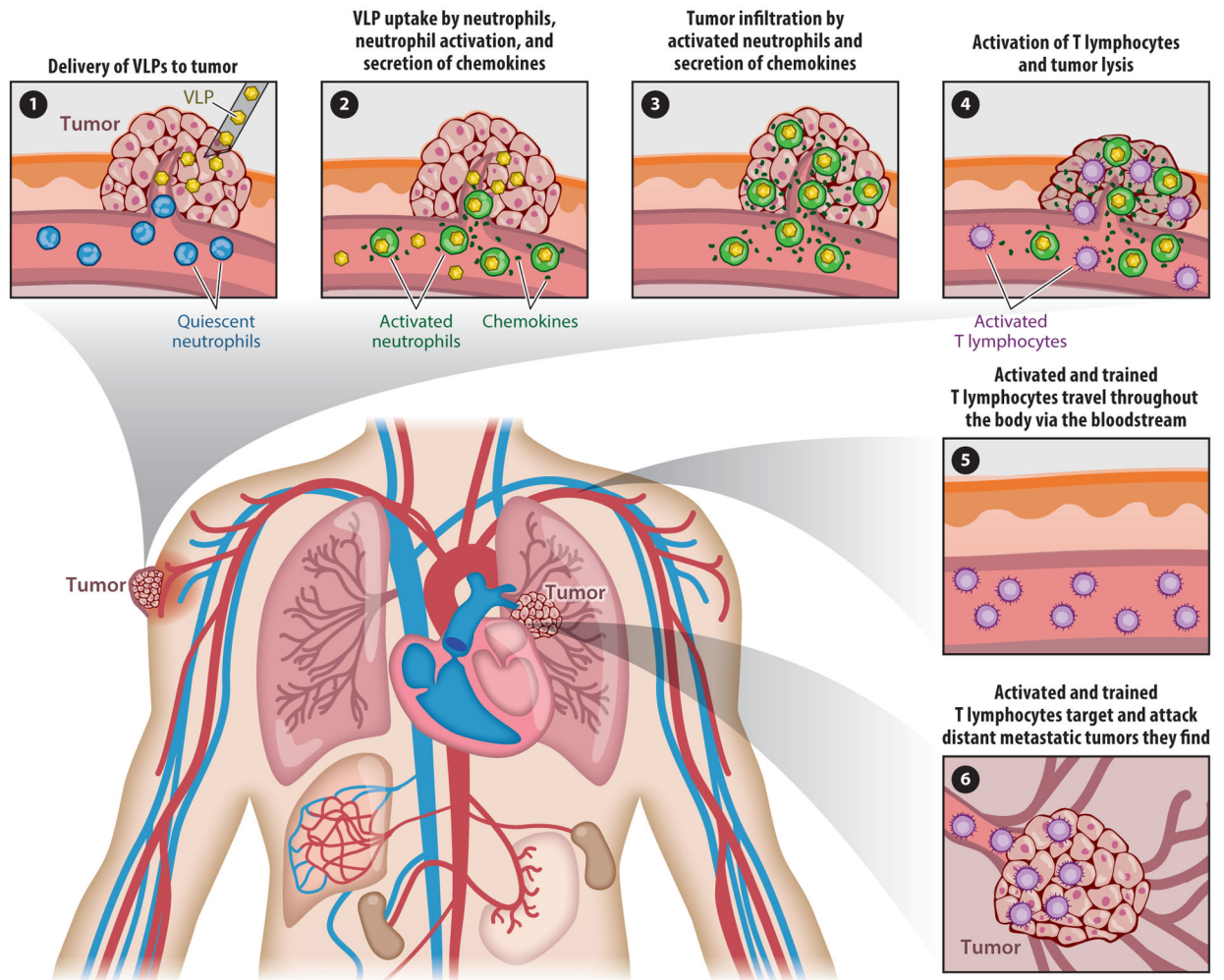
Figure 4.
Virus-like particle (VLP)-based in situ vaccination strategy. (❶) Direct administration of
VLPs into a tumor. (❷) Infiltration and activation of neutrophils, followed cytokine/
chemokine secretion, leading to (❸) the activation of T cells and tumor lysis (both
neutrophils and T cells can lyse tumor cells). (❹) Local priming of tumor antigen-specific T
cells results in the eradication of tumor cells at distant metastatic sites (❺, ❻).

Table 1

Approved gene therapies

Trade name (generic name)	Viral vector	Delivered gene/modification	Condition	Reference
ZOLGENSMA (onasemnogene abeparvovec-xioi)	AAV9	*Survival motor neuron 1*	spinal muscular atrophy	80
LUXTURNA (voretigene neparvovec-rzyl)	AAV2	*RPE 65*	retinal dystrophy	81
Gendicine	adenovirus	*TP53*	head and neck cancer	82
Strimveli	gamma-retrovirus	*Adenosine deaminase*	severe combined immunodeficiency	83
KYMRIAH (tisagenlecleucel)	lentivirus	genetically modified autologous T cells encoding an anti-CD19 chimeric antigen receptor	acute lymphoblastic leukemia	84
YESCARTA (axicabtagene ciloleucel)	retrovirus		large B cell lymphoma	85
Zalmoxis	retrovirus	genetically modified allogeneic T cells encoding a truncated form of the human low affinity nerve growth factor receptor and the herpes simplex virus type 1 thymidine kinase	hematopoietic stem cell transplant	85
Zynteglo	lentivirus	genetically modified autologous $CD34^+$ cells encoding β-globin gene (T87Q mutant)	transfusion-dependent β-thalassemia	86
Imlygic (talimogene laherparepvec/T-VEC)	herpes simplex virus type 1	*Granulocyte-macrophage colony-stimulating factor*	advanced melanoma	87

Abbreviation: AAV, adeno-associated virus.

Table 2

Clinical trials of VLP-based vaccines

Company	Trial number(s)	Phase(s)	Route	Formulation	Disease	Antigen	VLP vector	Status
Merck Sharp & Dohme	NCT00943722/ NCT01984697	III	Intramuscular	Nonavalent HPV VLP (V503), no adjuvant specified	HPV	L1 capsid protein	HPV6, 11, 16, 18, 31, 33, 45, 52 and 58	Completed (2017)
Fraunhofer Center for Molecular Biotechnology	NCT02013687	I	Intramuscular	Pfs25-CP VLP with allhydrigel	Malaria	Pfs25	Alfalfa mosaic virus	Completed (2017)
Fred Hutchinson Cancer Research Center	NCT03051516	IV	Intramuscular	Nonavalent HPV VLP (Gardasil 9), NaCl, aluminum hydroxyphosphate sulfate, L-histidine, sodium borate, pol, polysorbate 80	High-grade Squamous intraepithelial lesions	L1 capsid protein	HPV6, 11, 16, 18, 31, 33, 45, 52 and 58	Active
Merck Sharp & Dohme	NCT03158220	III	Intramuscular	Aluminum hydroxyphosphate sulfate, yeast protein, NaCl, sodium borate, pol, polysorbate 80	HPV	L1 capsid protein	HPV6, 11, 16, 18, 31, 33, 45, 52 and 58	Completed (2019)
Shanghai Zerun Biotechnology Co., Ltd.	NCT02740777, NCT02733068	II/III	Intramuscular	Bivalent VLP combination with aluminum phosphate, in a histidine buffer with sodium chloride and Tween–80	HPV	L1 capsid protein	HPV 16 and 18	Active
Takeda	NCT02669121, NCT03039790	II	Intramuscular	Bivalent VLP combination with aluminum hydroxide	Norovirus	VP1 capsid protein	GI.1 norovirus and GII.4 norovirus	Completed (2019)
Medicago	NCT03301051	III	Intramuscular	Quadrivalent VLP combination in phosphate buffer with sodium chloride and Tween–80	Influenza A virus	H1, H3, B hemagglutinin	Influenza A virus	Completed (2019)
Medicago	NCT02768805, NCT02233816, NCT02236052	II	Intramuscular	Quadrivalent VLP combination in phosphate buffer with sodium chloride and Tween–80	Influenza A virus	Unspecified	Influenza A virus	Completed (2019)
National Institute of Allergy and Infectious Disease	NCT02562482	II	Intramuscular	VRC-CHKVLP059– 00-VP VLP in phosphate buffered saline	CHIKV	E1, E2, and CHIKV, capsid protein	Chikungunya virus	Completed (2019)
Sentinext Therapeutics Sdn Bhd	ACTRN12617001027303	I	Intramuscular	Alhydrogel and aluminum hydroxide in Bis-Tris buffer with NaCl	Hand, foot, and mouth disease	EV-A71	Enterovirus 71	Active

Abbreviations: CHKV, chikungunya virus; HPV, human papillomavirus; VLP, virus-like particle.