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Virus-Based Nanoparticles as Versatile Nanomachines

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Abstract

Nanoscale engineering is revolutionizing the way we prevent, detect, and treat diseases. Viruses have played a special role in these developments because they can function as prefabricated nanoscaffolds that have unique properties and are easily modified. The interiors of virus particles can encapsulate and protect sensitive compounds, while the exteriors can be altered to display large and small molecules in precisely defined arrays. These properties of viruses, along with their innate biocompatibility, have led to their development as actively targeted drug delivery systems that expand on and improve current pharmaceutical options. Viruses are naturally immunogenic, and antigens displayed on their surface have been used to create vaccines against pathogens and to break self-tolerance to initiate an immune response to dysfunctional proteins. Densely and specifically aligned imaging agents on viruses have allowed for high-resolution and noninvasive visualization tools to detect and treat diseases earlier than previously possible. These and future applications of viruses have created an exciting new field within the disciplines of both nanotechnology and medicine.

Keywords

viruses; nanotechnology; drug delivery; vaccines; imaging

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INTRODUCTION: VIRUSES AS BIOMATERIALS

Advances in synthetic biology and chemistry have influenced many areas of research, industry, and medicine by allowing for the fabrication of nanoscale devices with increasingly controllable structures. Even so, the large-scale manufacturing of such materials remains challenging, and it is difficult to prepare structurally homogeneous populations of particles (1, 2). In contrast, bionanomaterials based on viruses allow for the templated assembly of millions of identical nanoparticles and their production in living cells. Viruses are ubiquitous in the environment, and those that infect bacteria, mammals, or plants have all been used to manufacture virus-based nanoparticles (VNPs). Viruses are an ideal starting point because they have evolved naturally to deliver nucleic acids and can therefore be subverted for the delivery of other molecules, such as drugs and imaging reagents. Finally, viruses replicate prodigiously, allowing the inexpensive manufacture of VNPs on an industrial scale.

VNPs comprise regular arrays of virus coat proteins and have highly defined three-dimensional structure, providing an engineering scaffold that is superior to synthetic particles (Figure 1). The structure of VNPs can be altered by modifying the nucleic acid template that codes for viral proteins prior to synthesis, and by chemically decorating the particles by adding conjugates to specific amino acid side chains. VNPs are composed primarily of protein and are therefore known for their biocompatibility, biodegradability, ability to cross biological barriers, and efficient delivery of cargo to target cells. Viruses have evolved to interact with specific cellular proteins, deliver their nucleic acid cargo, and hijack the intracellular machinery to produce the components of progeny viruses. These properties have led to the development of VNPs based on mammalian viruses for use in gene therapy, but it is difficult to rule out pathogenic effects resulting from natural virus-host interactions (3–5). In contrast, VNPs based on bacteriophages and plant viruses are regarded as safe because even the fully functional viruses cannot infect humans. The majority of this article therefore focuses on the medical applications of VNPs based on bacteriophages and plant viruses.

Bacteriophages and plant viruses are nucleoprotein assemblies in which the nucleic acids are tightly enclosed in a capsid comprising multiple copies of identical coat proteins. The capsids are generally icosahedral (roughly spherical in appearance), stiff tubes, or flexible filaments, the latter groups characterized by a high aspect ratio (Figure 1). Unlike many mammalian viruses, plant viruses and bacteriophages usually are not enveloped by a fragile lipid membrane because they must withstand more harsh environmental conditions in order to successfully infect their hosts.

The natural function of the virus capsid is to protect the viral genome from nucleases and physical hazards. Virus coat proteins are therefore stable and resistant to chemical and physical degradation, which is an advantage for the development of VNPs because it means they have a long shelf life and can withstand the chemical treatments necessary for conjugation with targeting ligands or loading with payloads such as drugs, fluorophores, or contrast agents (7, 8).

MODIFICATION STRATEGIES: FROM SCAFFOLDS TO FUNCTIONAL ENTITIES

A battery of techniques can be used to tailor and modify virus-based materials, including genetic engineering, encapsulation, biomineralization, infusion, and bioconjugation (Figure 2). Genetic engineering allows the basic structure of the coat protein to be changed by inserting, removing, or substituting particular amino acid residues (9–12). Such changes include terminal extensions (adding sequences to the N terminus or C terminus of each coat protein), the insertion of sequences that form surface loops, or the insertion or exchange of individual amino acids to introduce side chains that allow functionalization (13–15) or to alter the overall physicochemical properties of the VNP (16). Prominent examples of such modifications include the introduction of purification/immunodetection tags, the introduction of epitope sequences so that the VNP functions as a vaccine (17), and the introduction of targeting sequences that allow the VNP to target specific receptors (18). The incorporation of unnatural amino acids as unique handles for subsequent chemical reactions is also possible using similar recombinant expression strategies (19).

Virus coat proteins have evolved to self-assemble around nucleic acids under physiological conditions, and this property (which is shared by VNPs) can be exploited to disassemble VNPs and reassemble them into more desirable structures around other cargo molecules. Two basic principles can be used to trigger cargo encapsulation (20–23): (*a*) surface charge and electrostatic interactions or (*b*) unique binding interactions that occur during self-assembly. For example, bacteriophage MS2 contains a translational repression (TR) operator protein that binds to a TR RNA stem loop. TR operator proteins can be chemically engineered to carry small drug molecules. When intact MS2 particles are mixed with the modified TR operators, the latter diffuse inside the VNPs and bind stably to the capsid. Therapeutic molecules such as the ricin A chain and 5-fluorouridine have been successfully incorporated into MS2 particles using these design principles. In vitro cell studies using this approach have confirmed cargo delivery and the successful destruction of target cells (24, 25).

Biomineralization is the accumulation of minerals in and around the cells and tissues of living organisms, but in the context of VNPs it refers to the ability of virus coat proteins to assemble around a mineral core or nucleate mineralization. There are many applications of VNP biomineralization in the field of energy research (26), but there are also examples in medicine, particularly where mineral cargos are used as contrast agents. For example, an electrostatically engineered cowpea chlorotic mottle virus (CCMV) was used to facilitate the nucleation and oxidation of an Fe(II) cargo, leading to the formation of spatially constrained iron oxide nanocrystals suitable for magnetic resonance imaging (MRI) or hyperthermia treatment applications (16).

Whereas some materials must be encapsulated by encouraging the formation of capsids around a cargo, others can diffuse through the capsid and into the interior cavity, where they can be persuaded to remain inside by noncovalent interactions with nucleic acids or internally projecting amino acid side chains or can be permanently linked to handles by

bioconjugation (27). Fluorescent dyes for optical imaging, Gd^{3+} ions for MRI, and small drug molecules have all been loaded using this approach (28, 29).

One of the most powerful approaches for the modification of VNPs is the use of classical chemistry to functionalize particular amino acid side chains, such as the carboxylate groups on glutamic and aspartic acid residues, reactive amines on lysine residues, sulfhydryl groups on cysteine residues, and phenol groups on tyrosine residues (Figure 3). These groups can be directly conjugated to particular molecules or modified to display functional groups necessary for more sophisticated conjugation strategies. For example, carbodiimide coupling agents can be used to link any molecule containing a primary amine to the carboxylate groups of glutamic and aspartic acid, Michael addition can be used to link maleimides to the sulfhydryl groups of cysteine residues, and *N*-hydroxysuccinimide (NHS)-activated esters can be added to lysine side chains to link any molecule compatible with NHS chemistry (8, 30). The solvent-exposed phenol ring of tyrosine side chains can be modified by reacting it with the diazonium salts of a particular conjugate. Alkyne-based diazonium salts are more versatile because they provide a handle for further functionalization using click chemistry (31, 32). A popular click chemistry strategy used with VNPs is copper-catalyzed azide-alkyne cycloaddition (CuAAC) between azides and alkynes in the presence of Cu(I), irreversibly yielding the biocompatible 1,4-substituted triazole derivative (19, 28, 33–37). In addition, azo-coupling can be used to introduce payloads into VNPs (32, 38–40). Typically, an aldehyde is first introduced onto the VNP surface by azo-coupling to a bifunctional linker. The aldehyde can then be used in oxime or hydrazone condensation reactions (38, 39, 41). Another method is to selectively oxidize the primary N-terminal amine using pyridoxal 5′-phosphate. The reaction is specific to the N-terminal amines because it has a lower pK_a than the equivalent reaction with lysine side chains, and the resulting ketone (or aldehyde in the case of oxidizing terminal glycine residues) condenses with hydroxylamine-modified payloads to form oxime linkages. This approach has been used to conjugate imaging agents to the surface of bacteriophage M13 (42).

VIRUS-BASED NANOPARTICLES IN THERAPEUTIC INTERVENTIONS

The ability of bacteriophages and plant viruses to enter mammalian cells without further replication makes them suitable as tools for therapeutic interventions. Virus-based nanomaterials can be tailored to target particular cells, including cancer cells and specific cells of the immune system. They can present antigens to the immune system, meaning they can also be used as vaccines. VNP interactions with the immune system are advantageous for immunotherapy and immuno/chemo combination therapies, but often not for imaging applications or drug delivery. Therefore, several strategies have been developed to shield VNPs from the immune system while directing them to specific target cells. The clearance of VNPs by the mononuclear phagocyte system (43–45) can be overcome by tailoring the surface chemistry or shape of the particles. For example, surface PEGylation can minimize nonspecific interactions between VNPs and macrophages, thus prolonging their circulation time (46–48). Targeting can be achieved by the genetic or chemical addition of ligands that bind to receptors overexpressed on particular cell types, such as cancer cells. The ligands are either displayed on the capsid surface or added to the ends of the PEG chains; for example,

VNPs have been targeted using folic acid (49), transferrin (50, 51), epidermal growth factor (EGF) (52, 53), and RGD peptides (54) (Figure 4).

Tissue specificity can also be influenced by the shape, size, and aspect ratio of the VNP, so these are other properties that can be considered at the design stage. Notably, tubular or filamentous VNPs can have in vivo properties preferable to those of spherical VNPs—that is, enhanced flow and margination toward the vessel wall and reduced clearance by the mononuclear phagocytic system, thus leading to increased tumor homing (55) and thrombus targeting (56). Because VNP structures are monodisperse and can be tailored with precise and reproducible spatial control, they can be used to investigate the impact of VNP size and shape on the efficiency of drug delivery and imaging. For example, a bottom-up assembly strategy based on nucleic acids of different lengths was used to control the aspect ratio of VNPs based on tobacco mosaic virus (TMV). This allowed the comparison of particles that were identical in all properties except the aspect ratio, and showed that the aspect ratio had a significant impact on VNP biodistribution, longevity, and tumor penetration (57).

DRUG DELIVERY WITH VIRUS-BASED NANOPARTICLES

The development of VNPs that target specific cell types has allowed for the addition of toxic payloads by conjugation, infusion, and/or encapsulation so that the target cells are killed, thus allowing the selective elimination of cancer cells, or other diseased cells, without off-target effects. As discussed briefly above, conjugation involves the selective covalent addition of payload molecules to particular amino acid residues of the coat protein. Infusion is achieved by incubating the intact VNP in a solution containing the cargo (29, 58), and encapsulation requires the carrier to be assembled around the payload (24) (Figure 2). Many different types of therapeutic cargo have been delivered, including genes and short interfering RNAs (50, 59), conventional small-molecule drugs, photoactive molecules that support photodynamic therapy, and even heterologous viral genomes for gene therapy, such as an alphavirus genome encapsulated in a VNP based on CCMV (60).

Toxic cargos can be preferentially loaded into the VNP cavity, rather than coated onto the external surface, to protect them from enzymatic and chemical degradation in vivo and avoid interactions with nontarget cells. The capacity of VNPs and the efficiency of loading are generally improved by removing the native viral genome, which can be achieved by expressing the coat proteins from a plasmid (for the production of bacteriophage VNPs) or from a transgene (for the production of plant VNPs) so that the viral nucleic acid is never present; the resulting empty particle is described as a virus-like particle (VLP). Alternatively, the viral genome can be removed by selective chemical or enzymatic degradation.

The covalent attachment of toxic cargo molecules to internally exposed side chains ensures that there is no early release, but noncovalent methods generally allow for higher loading efficiency because there is space within the VNP for more cargo if the entire cavity is used rather than only the internal surface. For example, up to 300 doxorubicin molecules can be conjugated to the capsid surface of cowpea mosaic virus (CPMV) (61), but up to ~950 doxorubicin molecules could be loaded into the internal cavity of hibiscus chlorotic ringspot

virus (HCRSV) by encapsulation (62). Both viruses have icosahedral capsids 30 nm in diameter. Polymerization can achieve the best of both worlds by providing a branching network of functionalized groups for payload attachment that extends from the external surface of the VNP (63) or pervades the interior (64). Although most studies thus far have focused on the design aspects of VNPs and their in vitro toxicity, the preclinical testing of a VNP-based drug delivery vehicle has demonstrated in vivo efficacy and reduced cardiotoxicity of a doxorubicin-loaded VNP, specifically cucumber mosaic virus (CMV) modified with folic acid to target ovarian cancer (49).

As well as standard chemotherapy, VNPs have also been loaded with photosensitizers for photodynamic therapy applications. For example, a VLP based on bacteriophage Qβ was loaded with a metalloporphyrin derivative for photodynamic therapy and a glycan ligand targeting cells bearing the CD22 receptor (65). Furthermore, a multifunctional MRI contrast and photodynamic therapy agent (chelated Gd^{3+} and Zn^{2+} phthalocyanine) was successfully encapsulated in CCMV as a first demonstration of theranostic VNPs (66). Hybrid VNP-based materials carrying metal nanoparticles for photothermal therapy have also been investigated (67).

IMMUNIZATION AND IMMUNOTHERAPY USING VIRUS-BASED MATERIALS

The development of vaccines to prevent infectious diseases is one of the most significant medical advances in the past 300 years (68, 69) and has had an immense socioeconomic impact worldwide (70–72). Nevertheless, several key vaccines remain elusive, including those for HIV, respiratory syncytial virus, hepatitis C virus, and the hemorrhagic fever viruses. A current public health crisis, for example, is the Ebola outbreak affecting West African countries and visiting health care workers. Without effective antivirals and vaccines, detection and monitoring are currently the best options to prevent the spread of this life-threatening disease. The rapid development of a vaccine using a safe and effective platform is urgently needed.

Vaccination and other forms of immunotherapy also hold great promise as prophylactic and therapeutic interventions for the treatment of cancer and chronic diseases (73–76). Several monoclonal antibodies, antibody-based fusion proteins, and antibody-drug conjugates are already used for clinical immunotherapy, and many others are undergoing clinical and preclinical development (77–80).

Virus-based materials have repetitive, protein-based structures and therefore elicit immune responses that make them useful for the development of vaccines and immunomodulators. Particle-based vaccines fall into several classes, including (*a*) chemically inactivated virus vaccines, (*b*) attenuated virus vaccines with minimal virulence, (*c*) genome-free and noninfectious VLPs, and (*d*) chimeric and nanoparticle vaccines, in which pathogen-derived epitopes are displayed using a noninfectious carrier such as a plant virus, bacteriophage, or synthetic platform (81–83). Particulate vaccines, including VLPs and other nanoparticle vaccines, provide several distinct advantages over DNA vaccines (84–87) and subunit vaccines (88–90). The virus-based carrier confers antigen stability, carries multiple copies of the antigen (multivalent presentation), and has the potential to present two or more different

antigens. The formulation promotes passive or active uptake by antigen-presenting cells (91, 92) followed by their activation and the subsequent priming of the appropriate T and B cell responses (93–95) (Figure 5). The different categories of virus-based materials that have been developed as vaccines have been comprehensively reviewed (17, 96). Here we focus on important examples of VLPs and chimeric VNPs based on bacteriophages and plant viruses.

Vaccines for Infectious Diseases

VLP vaccines have enjoyed great success against viral diseases, particularly using strategies in which the structure of the noninfectious vaccine formulation closely mimics that of the natural virus [these have been described as native VLPs (96)]. The first successful example was the vaccine against hepatitis B virus (HBV), which has dramatically reduced HBV infections in immunized populations (97). Vaccines against human papillomavirus (HPV) elicit immunity against the virus that in turn protects against HPV-induced cervical carcinoma, and potentially other HPV-induced cancers (98).

Chimeric VLPs display heterologous antigens and can generate antipathogen and neutralizing antibodies such that immunization may also result in protection against pathogen challenge (99, 100). Many studies have been carried out with chimeric VLPs based on plant viruses (101–107), bacteriophages (108, 109), insect viruses (110), and animal polyomaviruses and papillomaviruses (97, 98). Chimeras have also been prepared from native vaccine platforms (e.g., HBV and HPV) and build on these platforms by displaying additional heterologous epitopes (111–113). These native-chimeric VLPs have the advantage of using an established and FDA-approved vaccine backbone.

Chimeric VLPs displaying complex antigen structures have been developed based on Flock House virus (FHV), which infects insects. This multivalent display system has been modified to include portions of the anthrax toxin receptor (ANTXR2), which in turn acts as a scaffold to display the *Bacillus anthracis* protective antigen. The virus-antigen complex induced protective immune responses after a single dose in the absence of adjuvant (110). Additional mechanisms for chemical attachment of multivalent antigens achieve a similarly efficient induction of immune responses (41). The FHV system has the advantage of accepting protein and peptide insertions in a variety of locations on the capsid surface and benefits from the availability of detailed structural and genetic information that allows the precise placement and arrangement of antigenic domains. For example, the influenza hemagglutinin (HA) protein is a major antigen for all strains of influenza, but it is difficult to develop broadly neutralizing immune responses due to antigenic variation. There are small regions of the protein that are highly conserved, but they are difficult to display in a structural context, which would allow the induction of specific and neutralizing antibody responses. Displaying the conserved regions of HA in a trimeric arrangement on FHV allows for the induction of these antibodies (114). The utility and scope of native and chimeric VLPs for vaccine applications continue to grow. For example, the combination of bioengineering VLP vaccines and administering them into the lungs was recently demonstrated as a powerful strategy for future vaccine development and immunotherapy (115).

Vaccines for Cancer

Cancer vaccines are based on our increasing knowledge of the molecular identities of tumor-associated antigens (116–118), combined with the ability to elicit efficient immune responses against these self-antigens despite active immunosuppression by the tumor. Anti-tumor vaccination has several advantages over chemotherapy, including a simplified outpatient procedure, fewer side effects, the avoidance of drug resistance, training the immune system to eliminate residual drug-resistant cells, and the induction of long-term immunological memory to protect against metastases and relapse.

Because tumor antigens are derived from the host, it is necessary to break immunological tolerance to induce immune responses against nonimmunogenic or weakly immunogenic targets. Several VNP-based cancer vaccine approaches that involve the patterned display of tumor-associated carbohydrate or peptide antigens have been evaluated. For example, CPMV particles were used as scaffolds to chemically display the Tn antigen, a glycoprotein that is overexpressed on numerous cancer cells including breast, colon, and prostate cancer cells. Conjugation to the virus-based scaffold and multivalent display resulted in production of high titers of Tn-specific antibodies (75). Similarly, Tn antigen conjugated to TMV can elicit antigen-specific IgG and IgM responses (76). The presentation of cancer epitopes on virus-based scaffolds enables the presentation of these self-epitopes in a nonnative molecular environment, which is a promising strategy to overcome self-tolerance, as shown for human epidermal growth factor receptor 2 (HER2)-positive breast cancer patients (119).

Vaccines for Neurological Diseases and Addiction

VLPs have been used as scaffolds to display the amyloid beta (Aβ) protein implicated in the progression of Alzheimer’s disease. Papillomavirus and Qβ VLPs displaying Aβ antigens elicited anti-Aβ antibodies in the absence of adjuvant, and with limited T cell responses. The antibody subclasses varied according to the use of the whole antigen or peptide antigens (73, 120). An HBV core antigen (HBcAg) carrying N-terminal epitopes of Aβ has also been developed as a vaccine and demonstrated efficacy in a transgenic mouse model of Alzheimer’s disease (121, 122).

A potential vaccine against nicotine addiction has been recently developed using the 30-nm icosahedral capsid of bacteriophage Qβ chemically modified to display nicotine in a multivalent fashion. The multivalent and particulate nature of the Qβ-based vaccine boosts the production of antinicotine neutralizing antibodies, thereby reducing blood nicotine levels and limiting transport across the blood-brain barrier. The nicotine vaccine is currently under investigation in clinical trials with the goal to reduce smoking addiction (123, 124).

VIRUS-BASED MATERIALS AS IMAGING PROBES

Imaging agents with an optimal signal-to-noise ratio, tailored circulation time, reduced toxicity, and targeted delivery can achieve higher-resolution imaging and earlier disease detection to facilitate better patient outcomes. VNPs are particularly suitable for this application, as demonstrated by recent advances in oncology and cardiovascular medicine (see Figure 4). As described above for drugs, imaging molecules can be added to the

external surface or internal cavity of VNPs by genetic modification (bioluminescent proteins), infusion, encapsulation, and/or bioconjugation. The precise spacing and controlled loading of imaging molecules can be used to tweak the signal-to-noise ratio depending on the dye or contrast agent used.

The attachment of fluorescent dyes to VNPs can facilitate flow cytometry, confocal microscopy, and in vivo imaging experiments because a large number of dye molecules can be incorporated per particle and the sensitivity of detection can be modulated by controlling the spacing of the molecules to optimize individual particle detection (125). Dye molecules are usually attached by conjugation to the surface of the VNP (126, 127), but some investigators have used genetic engineering to incorporate polypeptides such as green fluorescent protein (GFP) into the coat protein, thus creating stable virus chimeras that are fluorescent. In order to complete these genetic manipulations, viral genomes often must be refactored to eliminate overlaps in the protein-coding sequences that are to be modified (128). The genetic addition of GFP or the red fluorescent protein mCherry to the potato virus X (PVX) coat protein allowed for in vivo imaging of human tumor xenographs in mice (129). Similarly, bacteriophage λ has been coated with fluorescent molecules via bioconjugation to addressable lysine side chains (130) and also by the genetic engineering of the coat protein gpD to decorate the particle with GFP (131). Most VNPs for imaging are based on nonenveloped viruses, but the E2 spike protein of Sindbis virus (an enveloped alphavirus) has been used to decorate enveloped particles with the fluorescent proteins mApple and Venus, while maintaining gross particle morphology (132). Sindbis virus capsid proteins can also be manipulated to self-assemble around different imaging cargos (133). Filamentous bacteriophage M13 has been developed that incorporates 2,700 copies of an HPQ biotin-like sequence in the capsid, which was subsequently used as a handle to attach streptavidin-modified fluorophores (134). Infusion with imaging agents can be achieved by soaking VNPs in a solution of imaging agents that bind nucleic acids or proteins. For example, CPMV was shown to deliver noncovalently retained DAPI, propidium iodide, and acridine orange to mammalian cancer cells (29). In addition, these VNPs were stable for several weeks at 4°C without significant dye leakage (29). Such highly fluorescently modified VNPs can be used with new high-resolution technologies, such as stochastic optical reconstruction microscopy (135), to promote our understanding of viral delivery systems and their clinical deployment.

Imaging in vivo often requires light to penetrate deep into a tissue. Near-infrared (NIR) imaging can accomplish this goal because proteins and water absorb few photons in the NIR range (650–950 nm), allowing access to deeper layers of living tissue. There is also little tissue autofluorescence in the NIR window. Bacteriophage MS2 has therefore been loaded internally with NIR dyes and decorated on the outside with fibrin-targeting ligands, allowing for the detection of blood clots (136). Similarly, VLPs based on brome mosaic virus (BMV) were used to encapsulate the FDA-approved NIR imaging agent indocyanine green (137). Further studies showed that the presence of serum proteins enhanced the absorption and fluorescence emission properties of this VNP formulation (138). Second near-infrared light (NIR2) imaging (950–1,400 nm) has an even greater penetration depth than NIR. Single-wall carbon nanotubes (SWNT) can fluoresce in the NIR2 window and have previously been used alone for in vivo imaging (139). The coat protein of filamentous bacteriophage M13

was genetically modified to align a hydrophobic SWNT along the long axis of the VNP, and the resulting M13/SWNT complexes were also modified with targeting ligands on the distal end, allowing for the successful NIR2 imaging of deep tumors in vivo (140). A similar M13 formulation was successfully used for surgical guidance during tumor resection (141).

MRI is widely used for the in vivo noninvasive characterization of both healthy and diseased tissues. Tailored MRI contrast agents can further increase signal differences between types of tissues and help resolve anatomical differences. VNPs are advantageous in this field because they can concentrate contrast probes to increase the sensitivity of detection, they reduce tumbling rates and increase relaxivity to optimize the signal, and by encapsulating the contrast reagents they reduce their toxicity. Paramagnetic gadolinium (Gd^{3+}) is often used as a positive MRI contrast agent, but it must be presented in a chelated form to reduce toxicity, usually with tetraazacyclododecane tetraacetic acid (Gd-DOTA) or diethylenetriamine pentaacetic acid (Gd-DTPA). In order to make a better MRI contrast agent, the internal cavity of spherical bacteriophage P22 capsids were decorated with multiple branched oligomers that each held several Gd-DTPA complexes (142). This formulation allowed researchers to achieve high contrast agent density with high relaxivity (142). Additional studies using bacteriophage P22 capsids showed that the internal or external attachment of chelated Gd^{3+} did not significantly change the relaxivity (143), but that larger particles caused slower tumbling and faster T_1 relaxation (143). Less toxic Mn^{3+} chelators have also been attached successfully to bacteriophage P22 (144). As stated earlier, a multifunctional MRI contrast and photodynamic therapy agent was created by the coencapsulation of chelated Gd^{3+} and Zn^{2+} phthalocyanine in a VNP based on CCMV particles (66). The amphipathic nature of Zn^{2+} phthalocyanine allowed the chelated Gd^{3+} to be loaded at a higher density, thus achieving a greater overall contrast (66).

The potential of virus-based MRI probes was validated in a recent study that achieved sensitive delineation of atherosclerotic plaques using a Gd-DOTA-loaded TMV nanoparticle followed by detection and imaging at submicromolar doses (400× lower than the typical clinical dose) (145, 146). The high contrast-to-noise ratio, and resulting sensitivity of the agent, was attributed to slower tumbling and enhanced relaxivity, multivalency, recognition chemistry (the probes were targeted to VCAM-1 receptors), and carrier shape. The elongated shape of the VNP enhanced its flow properties and promoted margination toward the vessel wall.

Virus scaffolds have also been developed for T_2 imaging. Whereas T_1 shortening agents create a bright signal, T_2 shortening agents create a dark signal. Iron oxide–based nanoparticles are used as T_2 contrast agents and have been combined with VNPs. For example, iron oxide nanoparticles have been aligned on bacteriophage M13 (147) or have been encapsulated into VLPs derived from BMV (148) to achieve both biomedical and agricultural imaging.

Newer approaches include the use of virus-based materials in chemical exchange saturation transfer (CEST) and hyperCEST imaging. These are MRI techniques in which exogenous nuclei are selectively saturated using radio frequency signals after the transfer of this saturation to the surrounding water protons, and are detected indirectly through the water

signal with enhanced sensitivity. Hyperpolarizable and chemically inert xenon-based MRI sensors, which can increase the signal sensitivity 10,000-fold, have therefore been added to both icosahedral bacteriophage MS2 (149) and filamentous bacteriophage fd (150).

VNPs have also been investigated as potential tools for radioimaging. DOTA groups covalently attached to the interior surface of PEGylated VNPs based on bacteriophage MS2 have been used to chelate ^{64}Cu radioisotopes for positron emission tomography (PET). This allowed PET imaging for up to several hours in mice, and small concentrations of these ^{64}Cu-carrying VNPs stayed in the bloodstream 24 h postinjection, much longer than similar-sized VNPs (151).

OPPORTUNITIES AND IMPLICATIONS

VNPs are high-precision materials that self-assemble into symmetrical and polyvalent structures that can be tailored at the atomic level. Virus-based materials come in a variety of shapes and sizes, but most are monodisperse with geometries that can be custom modified. For example, gold-core BMV-shell hybrid structures can be self-assembled in vitro from coat proteins into hierarchical structures, in which the overall size of the assembly is governed by the size of the core nanoparticle (23). Not only can different-sized particles be generated, but the geometries can also be switched. For example, isolated coat proteins from icosahedral CCMV particles form nanotubes when assembled in the presence of DNA templates (152). Going in the other direction, TMV rods can be converted into spherical nanoparticles by thermally controlled shape-switching (153).

Synthetic biology and orthogonal chemistry allow the addition of reproducible functionalities to VNPs. In contrast to synthetic nanoparticles (in which modifications follow a statistically randomized distribution), the proteinaceous scaffolds of VNPs allow the deterministic display of drugs, imaging reagents, targeting peptides, or epitopes, thus enabling structure-based engineering. Not only can small chemical modifiers such as contrast agents, drugs, and peptides be displayed, but there is also an opportunity to present entire proteins as genetic fusions. Examples include the display of full-length fluorescent proteins for optical imaging (129); the genetic fusion of functional enzymes to the internal (154) or external (155) surfaces of VNPs, allowing them to be used as nanoreactors; and the presentation of complex protein structures such as the I-domain of the anthrax toxin cellular receptor in a complex with the anthrax protective antigen as a vaccination platform (110). Another interesting avenue is the use of subviral structures such as the tail, which functions as a molecular motor (156).

As well as providing a three-dimensional scaffold to facilitate VNP design, plant viruses and bacteriophages can also be manufactured on a large scale by molecular farming in plants or fermentation in bacteria or yeast, providing a realistic avenue for commercialization (157). The commercialization of VNPs must also take into account any potential risks to health and the environment. As stated above, mammalian viruses pose a risk of infection, so bacteriophages and plant viruses are preferred for medical use, but even these pose a risk to the environment and in particular to agriculture. Procedures have therefore been established to render them noninfectious, including the heterologous expression of genome-free VLPs

(157) and in vitro disassembly and reassembly to yield VLPs from self-assembled purified coat proteins (11, 158). Alternatively, nucleic acids can be removed from intact particles by alkaline hydrolysis (159, 160) either during extraction or following the recovery of pure virus particles (161). Shortwave (254 nm) UV irradiation can be used to cross-link and inactivate the genomes of intact particles (162).

As is the case for any platform technology, preclinical safety and efficacy must be established before any promising strategies can be considered for clinical development and translational medicine. Bacteriophages and plant viruses have been shown to be well tolerated by mammalian cells in vitro and by animal models. For example, no evidence of toxicity was observed in mice injected with up to 10^{16} CPMV particles per kilogram of body weight (100 mg/kg) (44). Following intravenous administration, bacteriophage- and plant virus–based materials are cleared from the circulation by the mononuclear phagocytic system and deposited in liver and spleen, followed by hepatobiliary extraction (44, 55, 163, 164). High-aspect-ratio, anisotropic, and filamentous plant viruses, much like nanotubes (165, 166), are also cleared by renal filtration (167). Overall, the clearance of VNPs from the circulation follows the same rules observed with synthetic materials, but there are significant differences when it comes to the clearance of VNPs from tissues. Whereas some synthetic nanoparticles persist in the body for weeks or even longer (168–171), virus-based materials are subject to proteolytic degradation and thus are removed safely from the body within days (172, 173).

Like many other exogenous materials, protein carriers derived from bacteria and plants are immunogenic. Such immunostimulatory properties can be exploited in the development of VNP-based vaccines, for example, in which the capsid carrier can be exploited to overcome self-tolerance or boost the immunogenicity of otherwise unstable or weakly immunogenic molecules such as peptides and carbohydrates (17). The elicitation of an immune response is also desirable in certain therapeutic approaches (e.g., immuno/chemo combination therapies) but not for the straightforward delivery of drugs or imaging reagents, particularly when repeat administration is required and an immune response (particularly carrier-specific antibodies) would dampen the efficacy of the VNP by accelerating its clearance. Immunogenicity is not a challenge unique to VNPs, because antibodies are also elicited against many inorganic and synthetic carriers (164, 174–178). Several strategies have been developed to address this challenge. For example, polymers such as PEG grafted to and grafted from the capsid surface can prevent antibody recognition (179–181). Another elegant strategy to reduce interactions with the immune system (which ultimately lead to the production of antibodies and potential inflammatory responses) is to camouflage the particles as endogenous proteins. This approach was recently demonstrated using synthetic nanoparticles tagged with peptides derived from the active domain of CD47, which prevented clearance by the mononuclear phagocyte system (182).

A detailed understanding of the biological fate of VNPs will provide opportunities to control their fate in vivo and increase their efficacy. Natural molecular recognition between VNPs and target cells can be harnessed; for example, the discovery of CPMV-vimentin interactions has been exploited for molecular imaging and drug targeting applications in cancer, neurological disease, and atherosclerosis (183–189). Molecular functionality can also be

incorporated by shape engineering and molecular recognition chemistry. The internal and external surfaces, coat protein interfaces, and interior cavity are all amenable to functionalization with medically relevant cargos. Mammalian viruses have been investigated in detail for gene delivery applications (3), with numerous VLP and VNP systems undergoing clinical development and several already on the market, including the HPV vaccine Gardasil (17). Novel approaches in the development pipeline include VNPs used for theranostics and tissue engineering (190). Within the nanomedicine sector, at least one start-up company has been established focusing on the development of plant virus–based chemotherapy (Nanovector Inc.). Bacteriophages and plant viruses have therefore emerged as versatile research tools and as platforms for nanomedical research, and there is now a clear path for their adoption in the clinic.

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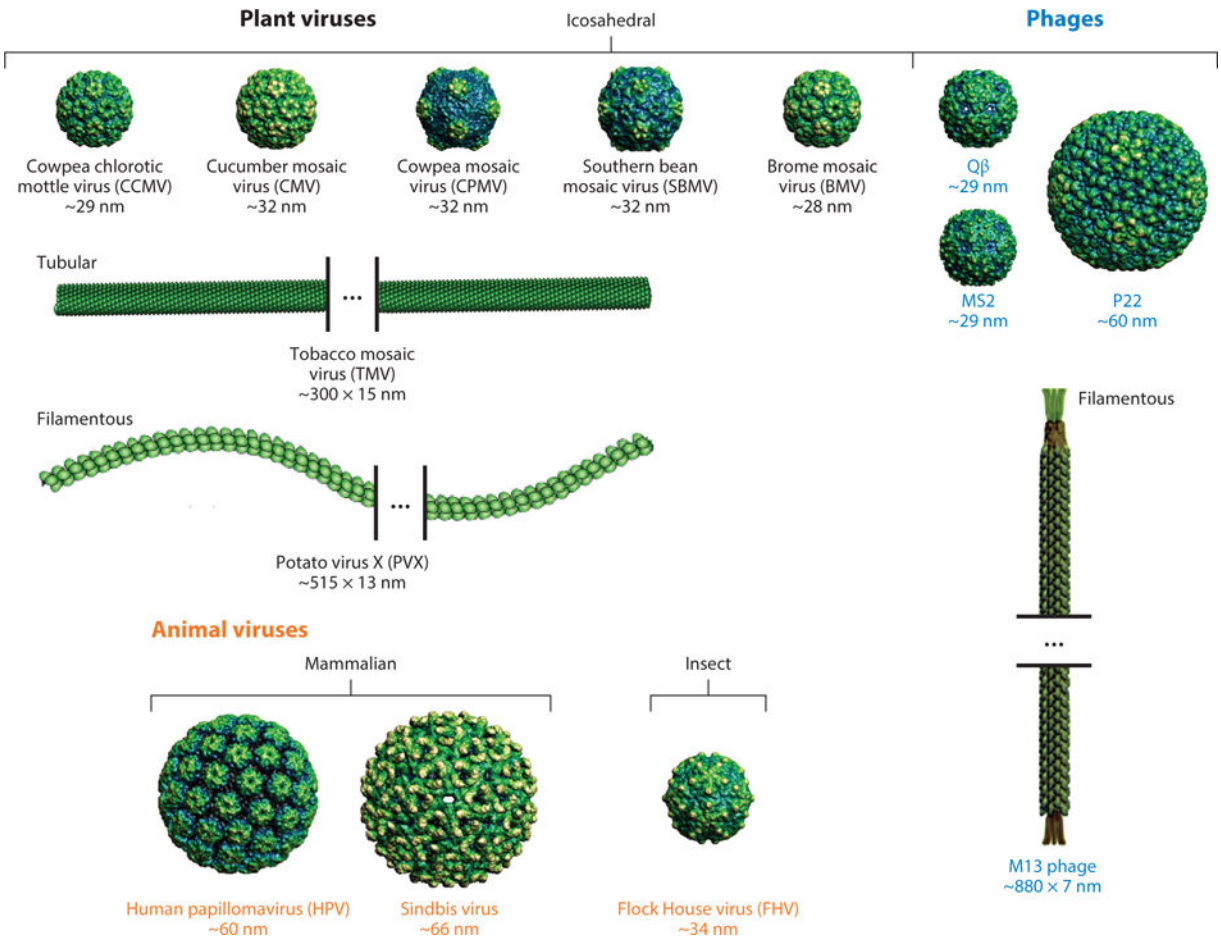


Figure 1.

Structures of a selection of plant, bacteriophage, and animal viruses that have been used as virus-based nanoparticles. Structural data for icosahedral viruses were obtained from http://viperdb.scripps.edu/, and structural data for TMV were obtained from http://www.rcsb.org; images were produced using Chimera software (6). For PVX and M13, schematic representations of the virus particles are shown.

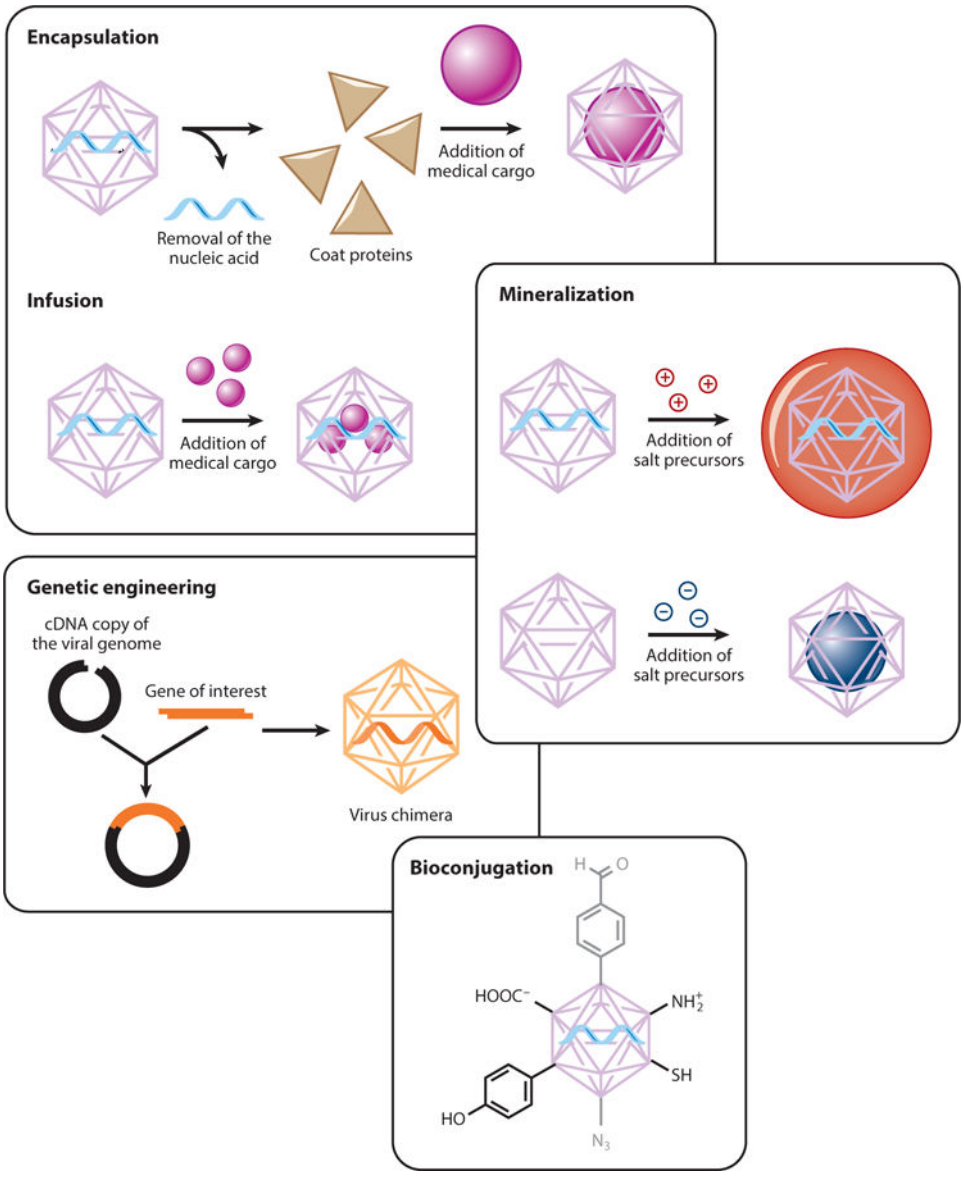
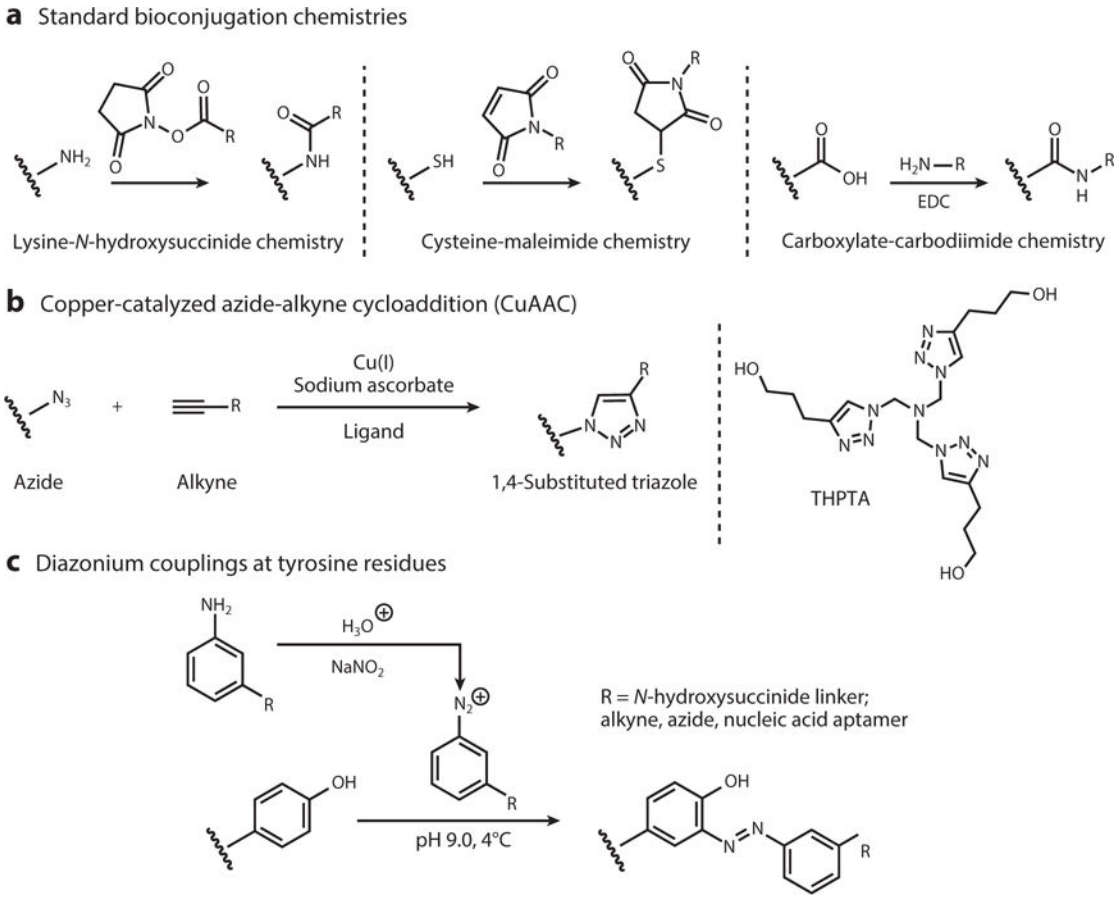


Figure 2.
Generalized modification strategies to develop functionalized virus-based nanoparticles.



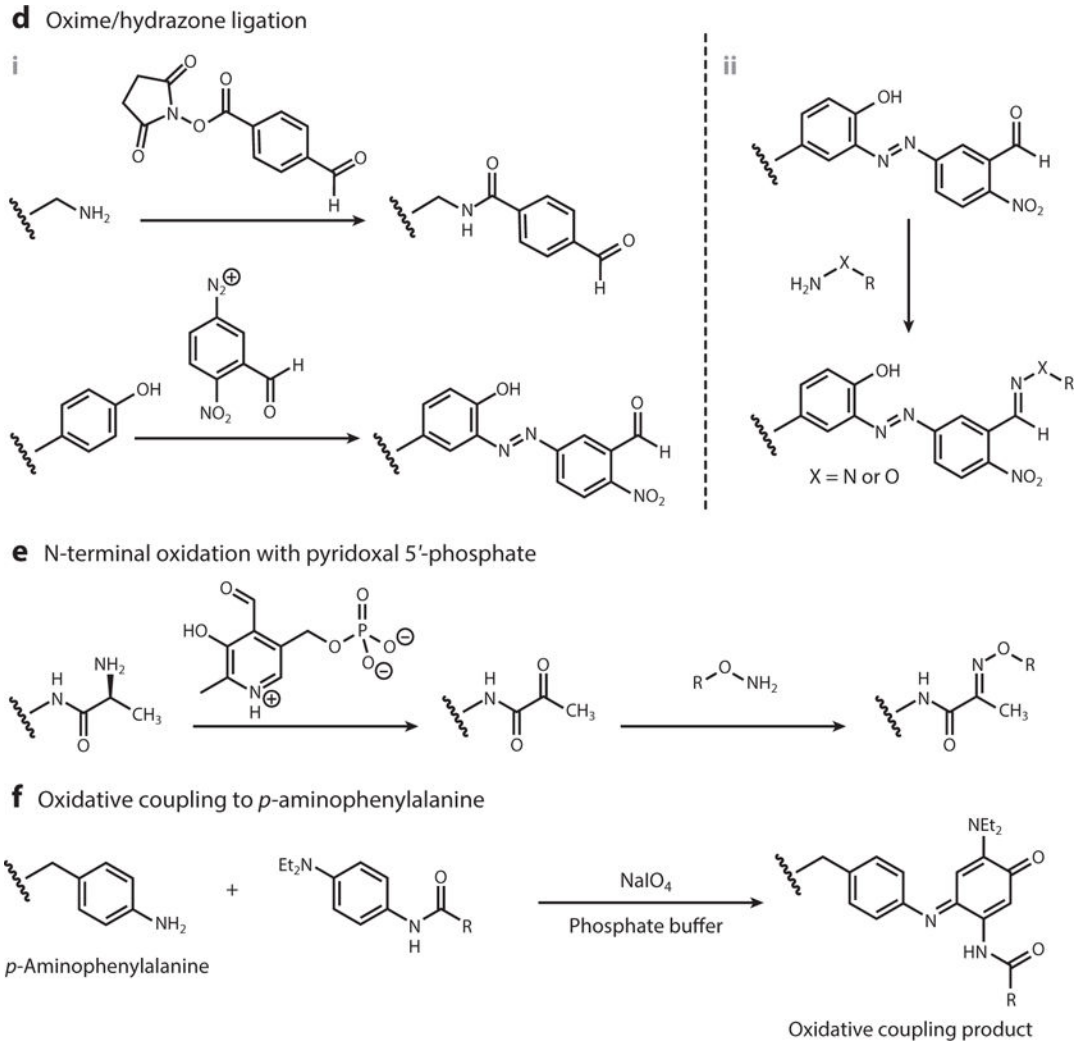


Figure 3.

Frequently used virus-based nanoparticle amino acid bioconjugation schemes. Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; THPTA, tris(3-hydroxypropyltriazolylmethyl)amine.

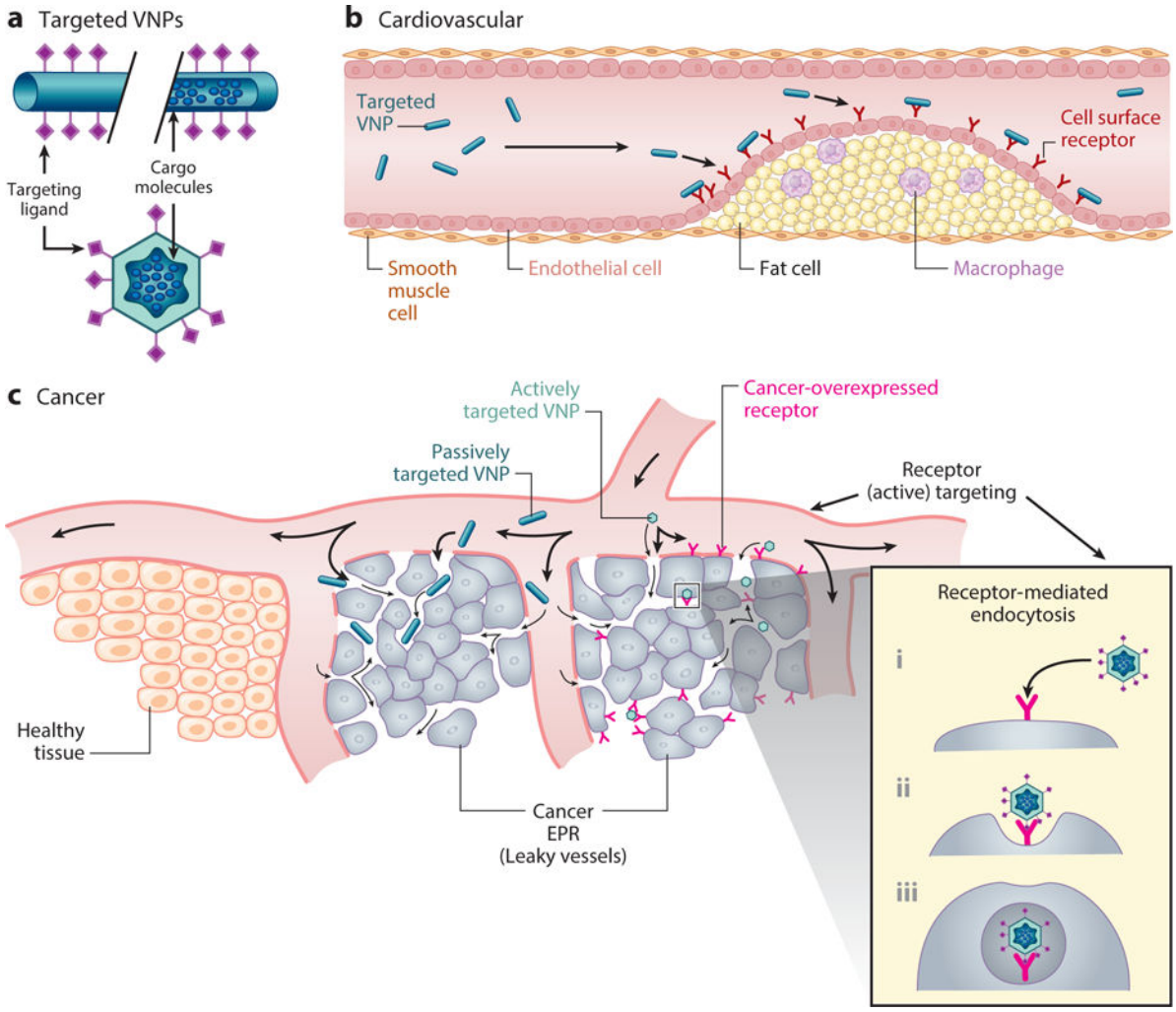


Figure 4.

Fundamentals of tissue- or disease-targeted virus-based nanoparticles (VNPs). Targeting ligands are presented on the exterior of the VNP to facilitate active cell-specific surface binding and/or receptor-mediated endocytosis. Additional unique factors, such as the enhanced permeability and retention (EPR) effect in tumor masses or VNP shape, can further aid in passive localization.

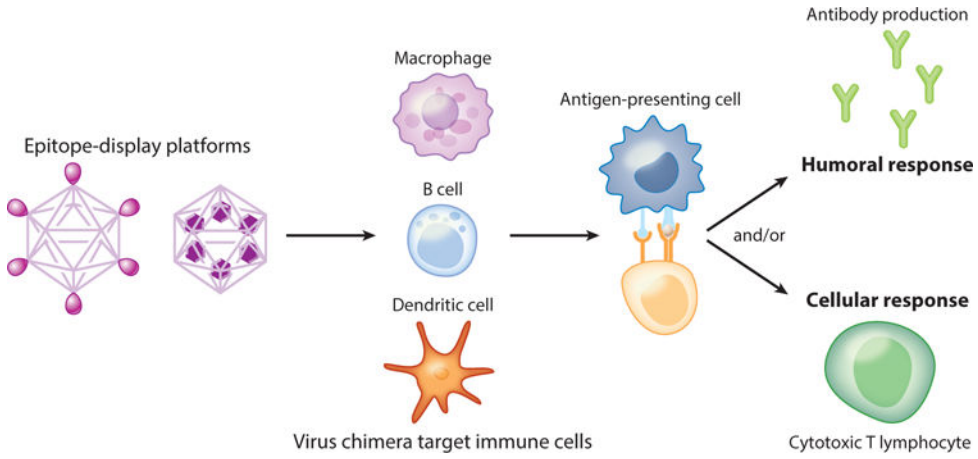


Figure 5.

Immune response activation by virus-based nanoparticles. Homogeneous or heterogeneous epitopes displayed on the surface of virus-based nanoparticles are taken up by antigen-presenting cell types and initiate activation of the humoral immune response, the cellular immune response, or both.