Synthetic plant virology for nanobiotechnology and nanomedicine

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Nanotechnology is a rapidly expanding field seeking to utilize nano-scale structures for a wide range of applications. Biologically derived nanostructures, such as viruses and virus-like particles (VLPs), provide excellent platforms for functionalization due to their physical and chemical properties. Plant viruses, and VLPs derived from them, have been used extensively in biotechnology. They have been characterized in detail over several decades and have desirable properties including high yields, robustness, and ease of purification. Through modifications to viral surfaces, either interior or exterior, plant-virus-derived nanoparticles have been shown to support a range of functions of potential interest to medicine and nano-technology. In this review we highlight recent and influential achievements in the use of plant virus particles as vehicles for diverse functions: from delivery of anticancer compounds, to targeted bioimaging, vaccine production to nanowire formation. © 2017 John Innes Centre. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology Published by Wiley Periodicals, Inc.

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INTRODUCTION

Nanobiotechnology focusses on the use of biologically derived structures with at least one dimension smaller than 100 nm, which can be adapted to perform specific functions. In this review, we will highlight the key advancements in the use of synthetic plant virology as a basis for a number of nanobiotechnology and medical applications.

There are certain characteristics that determine the usefulness of a nanobiotechnology system. Ideally it should be possible to produce species (particles) of consistent size, structure and biophysical properties. Such particles should be amenable to the introduction of additional functional groups such as dyes, enzymes, peptides, or inorganic compounds. Also such technologies should have minimal toxicity and low impact to the environment, particularly in the case of medical applications. Additional factors that will ultimately affect the viability of a nanobiotechnology platform include ease and cost of production, ease of containment and the low risk of cross-contamination with mammalian pathogens.

Viruses, and noninfective virus-like particles (VLPs) in particular, possess these desirable features. They are capable of self-assembling into defined structures of known dimensions, show a degree of genetic flexibility to allow functionalization with proteinaceous species, and possess reactive amino acid side-chains, that can be used for conjugation to inorganic or less amenable species. Using plant viruses reduces many of the risks associated with biological materials as a basis for medical nanotechnologies, and the use of noninfective VLPs results in low risk to the environment. These lower risks allow greater ease in handling, transportation and processing of viral nanoparticles (VNPs), making plant virus-based particles particularly attractive.

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Platforms for a range of nanobiotechnological applications. Throughout this review, we will use VNP as a generic term to denote any particle derived primarily from viral proteins, and VLP to distinguish VNPs that do not contain viral genetic material required to be infectious.

Plant viruses have played a prominent role in biochemical and structural research, despite the chemical nature of viruses only being revealed in the mid-1930s. Plant viruses were an ideal system for early biochemical experiments as high viral titres, and simple purification protocols, meant high yields of reasonably pure material were easily achievable in an era preceding sophisticated recombinant technologies. These properties also led to plant viruses being in the vanguard of studies on viral architecture and how viral replication protocols, meant high yields of reasonably pure material were easily achievable in an era preceding sophisticated recombinant technologies. This review seeks to introduce readers to some of the key concepts surrounding the field. This review seeks to introduce readers to some of the key concepts surrounding the ability to engineer plant viruses, either by genetic or chemical means, to generate synthetic nanoparticles for a wide range of applications.

**Plant Virus Structures**

The vast majority of plant virus genera are nonenveloped and have genomes consisting of one or more strands of positive-sense RNA. In common with all viruses, the particles are composed of highly-repetitive motifs of protein subunits that assemble around the genome to form large, well-ordered macromolecular structures. Though the detailed nature of these repeating motifs differs between viruses, there are essentially two classes of symmetry—helical and icosahedral (Figure 1). The classic example of a helical virus is tobacco mosaic virus (TMV, *Virgaviridae*) in which 2130 identical protein subunits surround the genome to form rigid rods of 300 nm. Other helical viruses include potato virus X (PVX, *Alphaflexiviridae*) and potato virus Y (PVY, *Pospiviroidae*), both of which form flexuous rods. In terms of icosahedral symmetry, some viral capsids, such as that of Cowpea chlorotic mottle virus (CCMV, *Bromoviridae*), are composed of multiple copies of a single-coat protein that form icosahedral cages around its genome. Other Icosahedral viruses, such as cowpea mosaic virus (CPMV, *Secoviridae*) are composed of more than one type of capsomeric protein, though typically in this case these are produced by proteolytic processing of a precursor, meaning that the mature capsid proteins are present in equal numbers in the assembled particle. Assembly of mature viruses typically involves formation of smaller assembly intermediates before these associate to form the large multimeric viral structures; however, the precise details of how capsid proteins mature, how assembly intermediates are initiated, and how complete structures form varies between viruses. Regardless, the periodicity of capsid proteins in mature virions allows accurate predictions and regulation of the degree of modification that can be introduced into particles.

The precise size and highly symmetric nature of plant viruses makes them a powerful tool for structural biology, and has aided the development of structural techniques such as (cryo)electron microscopy. Improvements in electron microscopic techniques can be easily traced through studies into TMV structure, from development of negative stain transmission electron microscopy (TEM) methods in the late 1950s to the advent of cryo-EM structures defining helical arrangements 30 years later, and the rapid progression from near-atomic resolution to atomic resolution structures that can now be produced without the need to crystallize proteins of interest. Such advances in structural biology are important for development of future biotechnological applications. Knowledge of the detailed three-dimensional structure of virus particles resulted in identification of exposed loop regions of the coat proteins that permit genetic modification of the coat protein without interfering with the capsomeric interactions essential for assembly.

**Production of Virus and VLPs**

VNPs must be produced in plants in a different way, depending on whether they are infectious viruses or noninfectious VLPs. Indeed, infectious virus can be produced by infecting plants with the relevant virus; whether it is wild-type or genetically modified, while VLPs must be produced by the expression of just those proteins necessary for particle formation. The latter approach has been carried out in a variety of standard heterologous expression systems including *Escherichia coli*, *yeast*, *insect cells*, and the infection approach results in the production of particles containing the viral genome which may require downstream processing to remove or inactivate the viral nucleic acid for reasons of safety or containment. However, using infectious virus usually has the advantage that it is possible to produce large quantities of material as the virus is able to spread within plants. The capsid expression approach often
results in the production of assembled particles \textit{in vivo}, but unlike infectious virus, these VLPs are either devoid of nucleic acid or encapsidate host RNA. Whichever method is chosen, the assembled particles can be further processed by, for example, \textit{in vitro} disassembly and reassembly. The capsid expression approach has the advantage that it does not require the handling of infectious material and can be used to produce mutant coat proteins that are incompatible with a ‘live’ virus infection.

Recent work in the field of synthetic plant virology has led to the development of ‘deconstructed’ viral vectors, which can be used to produce high yields of plant virus-derived VLPs in plants rather than in a heterologous system.\textsuperscript{31,32} The term deconstructed here refers to the removal of viral genes not required for high levels of transcription and translation (such as viral coat proteins), resulting in vectors which contain only those viral elements that lead to enhanced protein yields, such as promoters and UTRs. The first example of deconstructed viral vector use was the production of VLPs based on CPMV.\textsuperscript{33} Though CPMV particles produced via infection have been extensively used in bio- and nanotechnology (see sections below), 90\% of the particles contain the viral RNA and no \textit{in vitro} disassembly/reassembly system has yet been devised for this virus; this has limited its potential as a system for encapsidation of cargo molecules. By transiently coexpressing the precursor of the two (L and S) viral coat proteins, VP60, and the protease necessary for its processing, Saunders et al.\textsuperscript{33} were able to produce particles which were morphologically similar to wild-type CPMV but which were devoid of RNA and hence were termed empty VLPs or eVLPs. Structural studies by both cryo-EM and crystallography\textsuperscript{34,35} revealed the particles produced in this way to be identical to wild-type CPMV apart from the absence of the genomic RNA. Moreover, the cryo-EM structure allowed the visualization of a 24-amino acid chain at the C-terminus of the S coat protein.\textsuperscript{34} This 24-amino acid region plays an important role in particle assembly and controls the permeability of the particles to small molecules,\textsuperscript{36,37} with implications for loading desired

\textbf{FIGURE 1} Structure and dimensions of plant viruses commonly used in nanobiotechnology. (a) Negative stain TEM of TMV showing rods of \textasciitilde300 nm length. (b) Negative stain TEM of CPMV particles. (c) Cryo-EM structure of a cross-section of TMV (pdb 4udv). (d) Cryo-EM structure of the external (left) and internal (right) surfaces of CPMV. Repeating motifs for cryo-EM structures are shown as ribbons (monomer for TEM and pentamer for CPMV) [Correction added on 23 February 2017 after first online publication: labels (b) and (c) have been switched to match with the images.].
In Vitro Assembly of Structures

The coat proteins of a number of plant viruses have been shown to be able to self-assemble to form VLPs in vitro, including the well-studied TMV. In this case, the TMV origin of assembly sequence (OAS) from the genomic RNA alone can direct particle formation by the coat protein in vitro under appropriate buffer conditions. By this means the assembly of rod-shaped particles with modified coat protein conferring desirable properties to the resulting rod-like particles has been attempted. Here, Eiben et al. note that mutant rod formation was possible only by mixing mutant coat protein synthetized in E. coli with wild-type coat protein derived from a plant TMV infection in the assembly reaction. Similar in vitro assembly has been demonstrated for flexuous rod and icosahedral plant viruses allowing the encapsidation of a range of foreign species, discussed further in Section Plant Viruses as Nanocarriers of Useful Cargo.

In addition to in vitro assembly to produce particles with wild-type morphologies, filamentous viruses can be modified to produce novel structures, altering the biophysical properties of the resulting nanoparticles. Most dramatic is the production of nanospheres of various dimensions from the rod virus TMV, which maintain high thermal stability.

Of potential interest to nanobiotechnological applications, nonnative TMV structures such as ‘kinked nanoboomerangs’ can be made by introducing RNA molecules with multiple OASs, with ‘multipods’ produced in a similar manner. Such structure may allow the development of biocatalysis nanostructures with high surface area for efficient enzyme display. Metal-nucleic acid conjugates with multiple TMV OASs results in the formation of ‘nanostars,’ and similarly large networks of Turnip mosaic virus ‘Nanons’ bound to Candida antarctica Lipase B resulted in catalytically active supramacromolecular complexes (Figure 2).

Such hybrid architectures may find favor in future nano-technical applications such as nanowires and nanobiocatalysis assemblies. Not only can changes in nanoparticles structure alter physical properties such as surface area and thus for example reaction efficacy, but different architectures also affects in vivo behavior, raising the possibility of choosing specific architectures with different pharomokinetic profiles. Although heterologous expression of TMV may be appealing for biotechnology, wild-type TMV coat protein expressed in yeast and bacteria led to the formation of rod-like particles of indeterminate length without the necessity of the OAS nucleotide region in the template RNA. Thus compared to in vitro rod formation, the heterogeneous nature of the particles formed, and lack of control over assembly normally mediated by RNA means that heterologous expression systems appears to be of limited use in this instance.

Once a particle of desired physical properties has been selected, both icosahedral and filamentous viruses structures can be functionalized via both surface modifications and use of their internal cavities.

Functionalizing Viral Surfaces

The advent of modern recombinant technologies has allowed greater functionalization of the outer surface of viruses either by the direct genetic insertion of functional enzymes or peptides into predetermined loci, or the addition of nonnative amino acids to facilitate chemical conjugation using reactive side chains, such as those of cysteine, lysine and glutamate.
Genetic insertion of a peptide or protein may appeal to researchers with knowledge of the target virus’ structure as the position of such inserts should be predictable, and once purified the VNPs may not require additional processing for functionalization. One of the problems encountered with this approach, however, is that the presence of the inserted sequence often adversely affects the yield of virus that can be obtained, particularly if the insert is large or positively charged.52,53 For CPMV, structural studies have been useful in rationalizing such limitations, and improving insertion sites to maximize VNP activity22,52,54; however, such data are not available for every prospective VNP. A potential solution to this problem is to use specific antibodies or antibody fragments to mediate interactions with antigens or other proteins of interest. For example, mosaic PVX VNPs have been produced in planta, which display engineered antibody fragments capable of antigen-binding, despite such insertions typically preventing VNP formation.55,56 This was achieved by including a short peptide that resulted in a mixed population of wild-type and modified proteins via a ribosomal stutter mechanism. By choosing different antibodies for fusion to PVX coat protein in this system, it should be possible to produce VNPs functionalized with a wider range of species than would otherwise be possible.

Alternatively, researchers may choose to modify the outside of wild-type particles by chemical conjugation in order to avoid the potentially destabilizing effect of genetic insertion. This process is not always straightforward however. For example TMV lacks exposed reactive cysteine and lysine amino acids, and so modification of wild-type particles relies on the less commonly used azo-coupling through existing tyrosine residues.57 Nevertheless, mutant TMV particles have been synthesized that include nonnative amino acids to allow thiol- or amine-selective chemistry.58,59 Similarly, modified TMV possessing a surface-exposed cysteine residue has been coupled with sensor enzymes such as glucose oxidase and horseradish peroxidase, resulting in multivalent nanoscale platform for the ordered presentation of bioactive proteins.60 The scope for genetic fusions to the termini of TMV capsid proteins is limited, as this can inhibit virus formation, with wild-type morphologies requiring mosaics of wild-type and mutant protein.41

Chemical modifications to the solvent-exposed external surface of icosahedral particle can carried out using either virus particles or VLPs, while modification of the internal surface is usually confined to empty VLPs as the presence of genomic RNA tends to occlude access to the reactive amino acid side chains.37 Multiple examples of surface modifications of icosahedral particles exist, using the carboxyl groups of aspartic and glutamic acid,61 the ε-amino group of lysine,62–65 the thiol group of cysteine65,66 and the phenol side chain of tyrosine.67 It is also possible to genetically introduce or remove amino acids with reactive side changes in order to better control the levels of modification, or facilitate alternative chemistries for conjugation. For example, Wang et al.66 and Gillitzer et al.68 introduced cysteine residues to the surface of CPMV and CCMV, respectively while Chatterji et al.69 successively reduced the number of lysine residues on the surface of CPMV. Once a desirable chemistry has been identified (using either wild-type or mutant VNPs), conjugation can facilitate functionalization with a wide range of species including fluorescent dyes, electroactive compounds, drug molecules, quantum dots, specific metals, and even active enzymes.63,70–72

The interior surface of certain VNPs is also amenable to modification: the interior surface of CPMV VLPs can be chemically modified with fluorescent dyes via naturally occurring cysteines.37 Modification of the interior surface of VLPs derived from CCMV has also been achieved: the wild-type coat protein is highly positively charged due to the presence of the basic amino acids lysine and arginine.
This positively charged interior surface was used to promote mineralization within the preformed capsid to produce defined inorganic nanoparticles.\(^7^3\) By producing CCMV VLPs with an altered interior charge, it was possible to alter the range of materials that could be encapsulated.\(^7^4\)

Modifications to both the solvent-exposed surfaces of VLPs and to the internal cavity of many plant viruses allow synthetic viruses to interact with a variety of organic and inorganic substrates for a range of biotechnology applications, and to act as nanocarriers for both medical and bioimaging use.

Plant Viruses as Nanocarriers of Useful Cargo

Plant viruses have a strong track record of being used as carriers of useful cargo for a plethora of nanotechnological and biomedical applications,\(^7^5,7^6\) which we will divide here into three broad categories: delivery of therapeutics, bioimaging, and metallization. Cargo carrying utilizes the interior viral cavity as a vehicle for specific molecules; however, the introduction of defined cargoes into viruses is nontrivial. There are two main strategies for loading plant viral particles with foreign cargo: the infusion technique seeks to allow diffusion of a cargo of interest into the preformed viral particle, whereas the caging strategy aims to trigger particle formation around the cargo of interest (Figure 3). Each strategy has been used successfully with different viruses for different types of cargo, described below.

**Infusion**

The infusion strategy relies on causing viral particles to swell in such a way that pores open in the capsid, allowing diffusion of small cargo. Reversing the swelling then causes these pores to close, thus trapping the cargo inside. The method required to achieve such reversible swelling in tomato bushy stunt virus (TBSV, *Tombusviridae*) was described by Perez et al.,\(^7^7\) and relies on the chelation then addition of divalent cations to cause opening then closing of pores. This method has been used to load TBSV virions with ethidium bromide,\(^7^8\) and a similar strategy was used by Loo et al.\(^7^9\) as well as Lockney et al.\(^8^0\) to load the chemotherapy drug doxorubicin into particles of red clover necrotic mosaic virus (RCNMV, *Tombusviridae*) thanks to the interaction between the drug and viral nucleic acid. The same technique was used by Zeng et al. to load doxorubicin into particles of the distantly related cucumber mosaic virus (CMV, *Bromoviridae*).\(^8^1\) This technique has also found a use in agronomy, where the soil mobility of RCNMV led...
Cao et al.\textsuperscript{82} to load the nematicide Abamectin into the viral particles through infusion. This encapsulated form of Abamectin had similar bioavailability to nematodes as the free form of the pesticide, but with improved soil mobility, making it more effective at protecting crop roots from nematode infection.

In some cases, infusion does not even require swelling of the viral particles to allow ingress of cargo: it is possible to use the native nucleic acid content of the virions as an electrostatic sponge to attract and retain positively charged cargo. This is the strategy that was used by Yildiz et al.\textsuperscript{83} as well as Wen et al.\textsuperscript{84} to load particles of CPMV with imaging agents and therapeutic molecules. This relies on the natural affinity of these molecules with nucleic acid, and these experiments demonstrate that potential to use infused CPMV particles as imaging and therapy vehicles. A similar method was employed to load the platinum-containing anticancer drug candidate Phenanthriplatin into the channel of the rod-shaped TMV through a one-step loading protocol that also exploited the electrostatic interaction between the positively charged cargo and the negatively charged interior of the viral particle.\textsuperscript{85}

**Caging**

The caging strategy relies on assembling viral particles around the cargo of interest, either \textit{in vivo} or after disassembling virions \textit{in vitro}. The plant virus that has been most often used for caging of foreign cargo is almost certainly CCMV. The conditions required to disassemble the virus particles were described by Adolph,\textsuperscript{86} and had the advantage of being very simple: shifts in pH and ionic strength allowed for easily controlled swelling, and eventually complete disassembly of the viral particles. This mechanism has been extensively studied,\textsuperscript{87} and the reversibility of this process allowed later groups to use the disassembly–reassembly mechanism to remove native nucleic acid from the inside of the particles and replace it with cargo of interest. In one example, CCMV particles have been used as gene therapy candidates: the native viral RNA is removed after disassembly of the virions, and the capsid dimers are reassembled around heterologous RNA originating from the mammalian Sindbis virus.\textsuperscript{88} The authors even demonstrated that the heterologous RNA was released and expressed in mammalian cells upon transfection with the chimeric viral particles. Proteins can also be specifically packaged inside CCMV particles by using the caging strategy in concert with some targeted genetic engineering of the CCMV capsid protein. Indeed, the N-terminus of the CCMV capsid protein can be fused to the K-coil of a leucine zipper, while the C-terminus of a protein cargo is fused to the \textit{E. coli}, allowing noncovalent binding of the cargo to the capsid dimers, and self-assembly of VLPs around the protein cargo.\textsuperscript{89}

More stable covalent binding of cargo has also been induced through the use of bacterial Sortase A: The C-terminus of the CCMV capsid protein is modified to end in a glycine residue, and covalent binding can take place with any cargo (small molecule of protein) fused to a leucine proline glutamate threonine glycine (LPETG) amino acid motif via the action of trans-acting Sortase A.\textsuperscript{90} Encapsulation of whole proteins into CCMV VLPs allows the creation of nanorreactors; nanoscale cages with enzymatic activity. This was demonstrated by Comellas-Aragones et al.,\textsuperscript{91} who disassembled CCMV virions and reassembled them around individual molecules of horseradish peroxidase, with the enzymatic substrate and product capable of diffusing in and out of the nanoreactors. This has huge implications for enzyme-based therapeutics, as demonstrated by Sanchez-Sanchez et al.,\textsuperscript{92} who used a similar method for the encapsulation of a bacterial cytochrome p450 inside CCMV VLPs. These VLPs were enzymatically functional and could process prodrugs into cytotoxic active forms, which has important implications for targeted drug delivery. The closely related virus CMV can also be used for caging of cargo by functionalization of a nucleic acid intermediate. Disassembled CMV capsids can be made to reassemble around heterologous DNA, which can be used directly as cargo of interest, but if the DNA is functionalized with dyes or protein (through biotin–streptavidin interactions), the capsid–DNA interaction allows encapsulation of a wide range of cargoes.\textsuperscript{93}

Two areas which are attracting a considerable amount of attention with regards to loading cargo inside plant viral particles are the fields of biodelivery and mineralization of nano-scale structures. To achieve their goals, researchers in both of these fields have made use of both the infusion as well as the caging strategies, each with great success.

**VNP \textit{s} for Biomedical Delivery and Bioimaging**

Efficient drug delivery is greatly affected by the biochemical properties of the target compound and its interactions with the host. For compounds that are quickly eliminated from the body, are poorly soluble, or may not be able to efficiently pass cell membranes, the use of carrier molecules can improve drug delivery and thus efficacy. Such carriers must be small enough to move through the bloodstream, nontoxic, biocompatible and able to enter cells. Plant viruses have all of these properties, and have been the
subject of much research as potential biomedical vehicles. With distinct surface and interior residues, VNP.s are good candidates for manipulation to protect poorly soluble drugs while maintaining good biocompatibility.

In addition to drug biocompatibility, efficient transport to target tissues is a major challenge in pharmacology. This is a particular issue in the case of anticancer drugs which usually discriminate between cancerous and normal cells by the fact that the cancer cells are dividing more rapidly and differentially certain cell-surface receptors. However, anticancer drugs are toxic to all cells and thus often have severe side effects.

Nanotechnology-based drug delivery is aided by physiological differences between tumorous and healthy tissue. Tumors have been shown to retain more nanoparticles than healthy tissue and display increased permeability to larger nanomaterials on the basis of disrupted tight junctions. This results in preferential penetration of nanoparticles into tumorous tissue, known as the enhanced permeability and retention effect. Viruses specifically are useful for biomedical delivery due to their bioavailability, biodistribution, and persistence in a mammalian organism. Studies to ascertain these characteristics have been undertaken with CPMV, CCMV, TMV, and PVX. Taken together, these studies reveal that plant viral particles are generally safe, well tolerated, with high bioavailability, broad biodistribution, and relatively short persistence in animal models.

Many wild-type plant viruses display nonspecific cell entry, or at least entry into a wide range of cells, which may be deleterious for delivery of cytotoxic drugs. Untargeted uptake can be reduced by conjugation of surface residues to polyethylene glycol (PEGylation), and more targeted delivery facilitated by specific surface modifications. CPMV has been shown to interact with the intermediate filament protein vimentin, and this interaction facilitates CPMV uptake into a range of cells (including macrophages and certain cancerous cells). Using this natural uptake, Wen et al. were able to decorate CPMV with a photosensitizing agent to target macrophages and tumorous cells for destruction. Similarly, the loading of TMV with Zn-EpPor has been shown to enhance photosensitizer uptake into melanoma cells.

Integrins are another example of cell-surface receptors that are upregulated in many cancers. Specific peptide motifs, such as the RGD motif seen in Adenovirus are recognized by subtypes of integrins, and when this peptide is introduced to the surface of CPMV, either by genetic fusion to exposed loops or via chemical conjugation, uptake of CPMV into multiple cancer cell lines can be increased. Similar approaches have been used to target ovarian cancer cells. Folic acid receptors (FR) are overexpressed in ovarian cancer cells, and so FR-mediated endocytosis is a potential strategy for cell-specific drug delivery. This strategy was successfully employed by Ren et al. and Zeng et al.: modification of the surfaces of two unrelated viruses with folic acid for cell targeting, and infusion of the internal cavity of the VNP.s with doxorubicin resulted in a significant increase in drug delivery into cancer cells.

The ability to incorporate custom cargoes into protective protein shells, and targeting of these to specific tissue types allows the use of VNP.s as biologic agents. Using NHS ester chemistry both flexible rod virus PVX and the icosahedral virus CPMV can be functionalized with a number of commercial fluorescent dyes for imaging in cell cultures and to mark embryo vasculature and tumor tissue in chick and mouse systems. The use of more complex two-step bioconjugation methods allows further functionalization of PVX to target fluorescent particle uptake into cancer cells. In addition to commonly used single-photon fluorescent markers, two-photon dyes can be preferable for bioimaging as such dyes give lower background fluorescence and can reduce radiation damage to surrounding tissue. Recently the coupling of TMV particles to the two-photon dye BF3-NCS has been demonstrated to allow visualization of diseased brain vasculature in mice. Imaging using nonoptical methods such as MRI is also achievable by loading metals such as gadolinium into VNP.s. Such metal-VNP-specific interactions, referred to as mineralization, are further discussed in Section Mineralization of Viral Scaffolds.

VNP.s provide a good basis for biomedical technology, not only as chassis capable of transporting specific cargo, but also as scaffolds for antigen presentation and vaccination. With the development of plant-virus based technologies capable of producing a wide range of non-infectious VLPs, plant-derived VLPs is an area of considerable interest.

**Synthetic Plant Virology for Vaccine Production**

Vaccines provide acquired immunity to the infection of a particular micro-organism by stimulating the immune system against it. Vaccines against viral diseases are commonly produced from lab-cultured
Deconstructed viral vectors to produce high yields of some of the coat proteins is fused to epitopes from maeric TMV particles in which the C-terminus of have also been used to display heterologous epitopes: get epitope in test animals. 125 could stimulate an immune response against the target disease virus epitope, human rhinovirus epitope, or HIV epitope fused to the S coat protein have been produced in cowpea plants, and these particles to mouth disease virus epitope, human rhinovirus epitope, or HIV epitope fused to the S coat protein have been produced in cowpea plants, and these particles could stimulate an immune response against the target epitope in test animals.125–127 Moreover, this technique also permitted the development of a vaccine against mink enteritis virus that provides protective immunity in target animals.128 TMV particles have also been used to display heterologous epitopes: a leaky stop codon strategy was used to produce chimaeric TMV particles in which the C-terminus of some of the coat proteins is fused to epitopes from the malaria parasite, influenza virus, or HIV.130 It has subsequently shown that TMV particles presenting a short epitope from murine hepatitis virus was able to stimulate protective immunity in mice.131

Chemical conjugation of antigens provides a further alternative to genetic fusion, with tyrosine-mediated linkage of the weakly immunogenic hapten estriol to the surface of TMV leading to a significant immune response in mice.132

Although VLP-mediated antigen display can increase immune protection, this is not always the case.133–135 Furthermore, the use of a single VLP scaffold for antigen delivery may not be sustainable as exposure to the scaffold may lead to an immune response against the VLP moiety. Instead, recent work in the field of synthetic plant virology has used deconstructed viral vectors to produce high yields of pharmaceutically interesting proteins (for a recent review, see Peyret & Lomonossoff122).

Although beyond the scope of this review to cover in detail, the use of such vectors and heterologous plant systems such as Nicotiana benthamiana to produce nonplant VLPs for vaccines is an exciting area of research that cannot go without mention. In this approach the structural proteins for a target virus are cloned into a high-expression vector and transiently expressed in hosts such as N. benthamiana. Protein expression results in particles that morphologically resemble their infectious counterparts, and thus can be expected to act as an effective vaccine. Similar methods can be used in traditional mammalian and insect cell-culture; however, the use of plants has a number of significant benefits. This approach has been used successfully to produce a number of different VLPs as vaccine candidates (Table 1). It is possible to produce VLPs composed of a small number of capsid proteins, such as Hepatitis B core-like particles and Human Papillomavirus VLPs,142,149 and more complex nonenveloped proteins such as Bluetongue virus.148 The transient expression in plants of VLPs from a variety of animal viruses is further reviewed in Marsian and Lomonossoff, 2016.150

Mineralization of Viral Scaffolds

Many VNP technological and medical applications, such MRI, are dependent on the ability to form complexes between VNPs and specific metals and metal-based compounds. In the case of mineralization, inorganic nanoparticles and microstructures can be deposited and assembled on virus structures that act as biological templates or scaffolds.151,152 Mineralization has been reported for both the interior and exterior surfaces of icosahedral and rod-shaped virus particles (Figure 4). VNPs present several advantages like their nanoscale size, symmetry, polyvalence and monodispersity.75,152 Hybrid organic–inorganic species such as metallized VNPs can be useful for a broad range of applications such as catalysis, semiconductors, drugs and contrast agents.73,75

Icosahedral viruses such as CCMV and CPMV have been demonstrated to be able to interact with a variety of metals, with the positively charged internal cavity of CCMV allowing inorganic crystal nucleation.153 pH-dependent swelling, as described above, results in the formation of 60 pores in the capsid, which can then be used to load paratungstate and vanadate.73 CPMV shows similarities to CCMV in its ability be loaded with metals cobalt or iron-oxide154; however, in order to load CCMV with...
iron, nine basic amino acids at the N-terminus of the coat protein must be substituted with glutamate residues to alter the charge of the inner cavity to allow oxidative mineralization. The surface of CPMV can be decorated directly with ferrocene derivatives. Alternatively, custom decoration with cobalt–platinum, iron–platinum or zinc sulfide can be achieved by conjugating mineralizing peptides to the viral surface, or genetic fusion of hexa-histidine motifs to the C-terminus of the small coat protein subunit.

Filamentous viruses such as TMV can also be modified to facilitate mineralization, either externally or internally. Immobilization of both silica and a range of metals on the surface of TMV has been demonstrated, and the inner cavity can likewise be utilized for mineralization of nickel and cobalt. With a range of available VNP s with different physical characteristics (icosahedral, rigid, or flexuous rod), and by identifying the appropriate modifications required for specific metal interactions, VNPs appear to be a viable platform for the generation of a number of organic–inorganic hybrids.

CONCLUSION

Plant viruses are an integral tool for nanobiotechnology. Much of this is due to the extensive foundational work regarding plant virus structure, genetics, and biochemistry. Viruses have many properties making them ideal chassis for biotechnological functions, with plant viruses having additional benefits with regards to ease of production and purification. Production is one of the main limiting factors for widespread use of plant-derived VNPs, as current research can be relatively labor-intensive; activities such as prick ing out seedlings for growth are typically done manually. This issue is by no means insurmountable, as automation is feasible for most aspects of plant growth, and such issues must be weighed against the benefits of using simple media and growth conditions. The success of companies such as North America-based Medicago using plants as an expression system demonstrate the viability of industrial-scale use of plants for protein expression.

As both wild-type viruses and noninfectious VLPs pose little threat to human health, they are a good choice for nanomedical uses. As with any protein-based system there are risks of inducing an immune response to VNPs which may limit their long-term usefulness in both antigen display and live-animal bioimaging; however, to what extent this will prevent their clinical usefulness is uncertain. With very promising preclinical data regarding VNP toxicity, retention, and biodistribution, a key area of research will be the demonstration of clinical efficacy. If VNPs can be demonstrated to be viable clinically, improved methods for cell targeting will be crucial for widespread use of VNPs for drug delivery and gene therapy. The development of resources such as TumorHOPE, a database for tumor homing peptides, will play a vital role in developing novel

| Virus                      | Summary                                                                 | Reference          |
|----------------------------|------------------------------------------------------------------------|--------------------|
| Hepatitis B                | Tabletized transgenic lettuce containing HBsAg VLPs is orally immunogenic in mice | Pniewski 2011      |
| Hepatitis C                | Cucumber mosaic virus nanoparticles carrying a Hepatitis C virus-derived epitope, orally immunogenic in rabbits | Nuzzaci 2010       |
| Hepatitis C                | Papaya mosaic virus-like particles fused to a hepatitis C virus epitope: evidence for the critical function of multimerization, mixed response in mice | Denis 2007         |
| Influenza                  | Influenza virus-like particles induce a protective immune response against a lethal viral challenge in mice, produced for H7N9 outbreak virus | D’Aoust 2008       |
| Papillomavirus             | HPV-16 L1 VLPs via agroinfiltration-mediated transient expression or via transplastomic expression | Maclean 2007, Fernandez-San 2008 |
| Papillomavirus             | Expression of HPV-8 L1 VLPs                                            | Matic 2012         |
| Papillomavirus             | transient expression of chimaeric L1::L2 VLPs and proof of increased breadth of immune response | Pineo 2013         |
| Bovine papillomavirus      | Transient expression of BPV L1 VLPs                                     | Love 2012          |
| HIV                        | Expression of Gag VLPs in transgenic tobacco chloroplasts               | Scotti 2009        |
| Human norovirus            | NaVCP VLPs in which generate a mucosal and serum antibody response      | Mathew 2014        |
| Rotavirus                  | Immunogenic rotavirus-like particles in transgenic plants               | Yang 2011          |
| Bluetongue virus           | Protective bluetongue virus-like particles                              | Thuenemann 2013    |

TABLE 1 | Key Vaccine Candidates Produced Using Deconstructed Viral Vectors. Further Details Can Be Found in Marsian and Lomonossoff 2016.
targeting methods that may be ultimately beneficial where existing drug treatments show little discrimination between diseased and healthy tissue.

Functionalization of external surfaces combined with manipulation of viral morphologies opens a large number of potential applications in nanotechnology, from nanowires to nanoreactors. Increasing our understanding of virus tolerance to manipulation, generation of more novel structures, and methods of functionalizing with active enzymes without affecting activity is going to be key in expanding the range of VNPs with custom properties.

Currently there are no plant-based nanobio-technology products on the market. We believe that as researchers are able to demonstrate greater success in producing VNPs with desirable properties, increased interest from both private and public bodies will drive investment in both fundamental research and infrastructure required to produce economically viable technologies. Part of this is likely to be due to economies of scale: despite low running costs for plant production, high upfront costs for industrial-scale expression means that very few sites are capable of producing industrial levels of plant-derived nanotech materials. Specialized medium-scale production facilities that will allow researchers to test the feasibility of novel VNPs will be an important step in demonstrating viability of plants as an expression platform, thus hopefully stimulating wider interest in the field.

Furthermore, new technologies are likely to be a driving force in plant-based VNP production, and plant-based heterologous expression in general. Cell-based methods such as the BY2 cell-pack method for transient expression\textsuperscript{162} that allow medium- to high-throughput screening will be essential to allow candidate screening analogous to existing \textit{E. coli} based methods. Although the production timescales are unlikely to match those of \textit{E. coli} (expression typically requiring several days as opposed to several hours), transient expression screens are still a viable means to facilitate construct design and optimization for enhanced yield, and improved particle characteristics.

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REFERENCES

1. Stanley WM. Isolation of a crystalline protein possessing the properties of tobacco-mosaic virus. *Science* 1935, 81:644–645.

2. Bawden FC, Pirie NW, Bernal JD, Fankuchen I. Liquid crystalline substances from virus-infected plants. *Nature* 1936, 138:1051–1052.

3. Caspar DL, Klug A. Physical principles in the construction of regular viruses. *Cold Spring Harb Symp Quant Biol* 1962, 27:1–24.

4. Stubbs G, Warren S, Holmes K. Structure of RNA and RNA binding site in tobacco mosaic virus from 4-A map calculated from X-ray fibre diagrams. *Nature* 1977, 267:216–221.

5. Bloomer AC, Champness JN, Bricogne G, Staden R. Protein disk of tobacco mosaic virus at 2.8 A resolution showing the interactions within and between subunits. *Nature* 1978, 276:362–368.

6. Harrison SC, Olson AJ, Schutt CE, Winkler FK, Bricogne G. Tomato bushy stunt virus at 2.9 A resolution. *Nature* 1978, 276:368–373.

7. Abad-Zapatero C, Abdel-Meguid SS, Johnson JE, Leslie AG, Rayment I, Rossmann MG, Suck D, Tsukihara T. Structure of southern bean mosaic virus at 2.8 A resolution. *Nature* 1980, 286:33–39.

8. Porta C, Lomonossoff GP. Use of viral replicas for the expression of genes in plants. *Mol Biotechnol* 1996, 5:209–221.

9. Varma A, Gibbs AJ, Woods RD, Finch JT. Some observations on structure of filamentous particles of several plant viruses. *J Gen Virol* 1968, 2:107–114.

10. Rossmann MG, Johnson JE. Icosahedral RNA virus structure. *Annu Rev Biochem* 1989, 58:533–573.

11. Lomonossoff GP, Johnson JE. The synthesis and structure of comovirus capsids. *Prog Biophys Mol Biol* 1991, 55:107–137.

12. Bancroft JB. The self-assembly of spherical plant viruses. *Adv Virus Res* 1970, 16:99–134.

13. Casjens S, King J. Virus assembly. *Annu Rev Biochem* 1975, 44:555–611.

14. Klug A. The tobacco mosaic virus particle: structure and assembly. *Philos Trans R Soc Lond B Biol Sci* 1999, 354:531–535.

15. Atabekov J, Dobrov E, Karpova O, Rodionova N. Potato virus X: structure, disassembly and reconstitution. *Mol Plant Pathol* 2007, 8:667–675.

16. Zhang W, Olson NH, Baker TS, Faulkner L, Aghandj-McKenna M, Boulton ML, Davies JW, McKenna R. Structure of the Maize streak virus geminate particle. *Virolology* 2001, 279:471–477.

17. Brenner S, Horne RW. A negative staining method for high resolution electron microscopy of viruses. *Biochim Biophys Acta* 1959, 34:103–110.

18. Jeng TW, Crowdther RA, Stubbs G, Chiu W. Visualization of alpha-helices in tobacco mosaic virus by cryo-electron microscopy. *J Mol Biol* 1989, 205:251–257.

19. Sachse C, Chen JZ, Coureux PD, Stoupe ME, Fandrich M, Grigorieff N. High-resolution electron microscopy of helical specimens: a fresh look at tobacco mosaic virus. *J Mol Biol* 2007, 371:812–835.

20. Fromm SA, Bharat TA, Jakobi AJ, Hagen WJ, Sachse C. Seeing tobacco mosaic virus through direct electron detectors. *J Struct Biol* 2015, 189:87–97.

21. Lomonossoff GP, Johnson JE. Use of macromolecular assemblies as expression systems for peptides and synthetic vaccines. *Curr Opin Struct Biol* 1996, 6:176–182.

22. Lin T, Porta C, Lomonossoff G, Johnson JE. Structure-based design of peptide presentation on a viral surface: the crystal structure of a plant/animal virus chimera at 2.8 A resolution. *Fold Des* 1996, 1:179–187.

23. Bragard C, Duncan GH, Wesley SV, Naidu RA, Mayo MA. Virus-like particles assemble in plants and bacteria expressing the coat protein gene of Indian peanut clump virus. *J Gen Virol* 2000, 81:267–272.

24. Zhao X, Fox JM, Olson NH, Baker TS, Young MJ. In vitro assembly of cowpea chlorotic mottle virus from coat protein expressed in Escherichia coli and in vitro-transcribed viral cDNA. *Virology* 1995, 207:486–494.

25. Brumfield S, Willits D, Tang L, Johnson JE, Douglas T, Young M. Heterologous expression of the modified coat protein of Cowpea chlorotic mottle bromovirus results in the assembly of protein cages with altered architectures and function. *J Gen Virol* 2004, 85:1049–1053.

26. Kadri A, Wege C, Jeske H. In vivo self-assembly of TMV-like particles in yeast and bacteria for nanotechnological applications. *J Virol Methods* 2013, 189:328–340.

27. Mueller A, Kadri A, Jeske H, Wege C. In vitro assembly of tobacco mosaic virus coat protein variants derived from fission yeast expression clones or plants. *J Virol Methods* 2010, 166:77–85.

28. Young M, Willits D, Uchida M, Douglas T. Plant viruses as biotemplates for materials and their use in nanotechnology. *Annu Rev Phytopathol* 2008, 46:361–384.

29. Langeveld JP, Brennan FR, Martinez-Torrecuadrada JL, Jones TD, Bosshuizen RS, Vela C, Casal JJ, Kamstrup S, Dalsgaard K, Meloen RH, et al. Inactivated recombinant plant virus protects dogs from a lethal challenge with canine parvovirus. *Vaccine* 2001, 19:3661–3670.
30. Ochoa WF, Chatterji A, Lin T, Johnson JE. Generation and structural analysis of reactive empty particles derived from anicosahedral virus. Chem Biol 2006, 13:771–778.

31. Sainsbury F, Lomonossoff GP. Transient expressions of synthetic biology in plants. Curr Opin Plant Biol 2014, 19:1–7.

32. Peyret H, Lomonossoff GP. When plant virology met Agrobacterium: the rise of the deconstructed clones. Plant Biotechnol J 2015, 13:1121–1135.

33. Saunders K, Sainsbury F, Lomonossoff GP. Efficient generation of cowpea mosaic virus empty virus-like particles by the proteolytic processing of precursors in insect cells and plants. Virology 2009, 393:329–337.

34. Hesketh EL, Meshcheriakova Y, Dent KC, Saxena P, Thompson RF, Cockburn JJ, Lomonossoff GP, Ranson NA. Mechanisms of assembly and genome packaging in an RNA virus revealed by high-resolution cryo-EM. Nat Commun 2015, 6:10113.

35. Huynh NT, Hesketh EL, Saxena P, Meshcheriakova Y, Ku YC, Hoang LT, Johnson JE, Ranson NA, Lomonossoff GP, Reddy VS. Crystal structure and proteomics analysis of empty virus-like particles of cowpea mosaic virus. Structure 2016, 24:567–575.

36. Sainsbury F, Saunders K, Aljabali AA, Evans DJ, Lomonossoff GP. Peptide-controlled access to the interior surface of empty virus nanoparticles. Chembiochem 2011, 12:2435–2440.

37. Wen AM, Shukla S, Saxena P, Aljabali AA, Yildiz I, Dey S, Mealy JE, Yang AC, Evans DJ, Lomonossoff GP, et al. Interior engineering of a viral nanoparticle and its tumor homing properties. Biomacromolecules 2012, 13:3990–4001.

38. Belval L, Hemmer C, Sauter C, Reinbold C, Fauny JD, Berthold F, Ackerer L, Schmitt-Kechinger C, Lemaire O, Demangeat G, et al. Display of whole proteins on inner and outer surfaces of grapevine fanleaf virus-like particles. Plant Biotechnol J 2016, 14:2288–2299.

39. Saunders K, Lomonossoff GP. The generation of turnip crinkle virus-like particles in plants by the transient expression of wild-type and modified forms of its coat protein. Front Plant Sci 2015, 6:1138.

40. Butler PJ. Self-assembly of tobacco mosaic virus: the role of an intermediate aggregate in generating both specificity and speed. Philos Trans R Soc Lond B Biol Sci 1999, 354:537–550.

41. Eiben S, Stitz N, Eber F, Wagner J, Atanasova P, Bill J, Wege C, Jeske H. Tailoring the surface properties of tobacco mosaic virions by the integration of bacterially expressed mutant coat protein. Virus Res 2014, 180:92–96.

42. Arkhipenko MV, Petrova EK, Nikitin NA, Protopopova AD, Dubrovin EV, Yaminskii IV, Rodionova NP, Karpova OV, Atabekov JG. Characteristics of artificial virus-like particles assembled in vitro from potato virus X coat protein and foreign viral RNAs. Acta Naturre 2011, 3:40–46.

43. Chen C, Daniel MC, Quinkert ZT, De M, Stein B, Bowman VD, Chipman PR, Rotello VM, Kao CC, Dragnea B. Nanoparticle-templated assembly of viral protein cages. Nano Lett 2006, 6:611–615.

44. Hu Y, Zandi R, Anavitarte A, Knobler CM, Gelbart WM. Packaging of a polymer by a viral capsid: the interplay between polymer length and capsid size. Biophys J 2008, 94:1428–1436.

45. Sun J, DuFort C, Daniel MC, Murali A, Chen C, Gopinath K, Stein B, De M, Rotello VM, Holzenburg A, et al. Core-controlled polymorphism in virus-like particles. Proc Natl Acad Sci U S A 2007, 104:1354–1359.

46. Hart RG. Morphological changes accompanying thermal denaturation of tobacco mosaic virus. Biochim Biophys Acta 1956, 20:388–389.

47. Atabekov J, Nikitin N, Arkhipenko M, Chirkov S, Karpova O. Thermal transition of native tobacco mosaic virus and RNA-free viral proteins into spherical nanoparticles. J Gen Virol 2011, 92:453–456.

48. Eber FJ, Eiben S, Jeske H, Wege C. RNA-controlled assembly of tobacco mosaic virus-derived complex structures: from nanoboomerangs to tetrapods. Nanoscale 2015, 7:344–355.

49. Cuenca S, Mansilla C, Aguado M, Yuste-Calvo C, Sanchez F, Sanchez-Montero JM, Ponz F. Nanonets derived from turnip mosaic virus as scaffolds for increased enzymatic activity of immobilized candida antarctica lipase B. Front Plant Sci 2016, 7:464.

50. Shukla S, Eber FJ, Nagarajan AS, DiFranco NA, Schmidt N, Wen AM, Eiben S, Twyman RM, Wege C, Steinmetz NF. The impact of aspect ratio on the biodistribution and tumor homing of rigid soft-matter nanorods. Adv Healthc Mater 2015, 4:874–882.

51. Shukla S, Ablack AL, Wen AM, Lee KL, Lewis JD, Steinmetz NF. Increased tumor homing and tissue penetration of the filamentous plant viral nanoparticle Potato virus X. Mol Pharm 2013, 10:33–42.

52. Porta C, Spall VE, Findlay KC, Gergerich RC, Farrance CE, Lomonossoff GP. Cowpea mosaic virus-based chimaeras. Effects of inserted peptides on the phenotype, host range, and transmissibility of the modified viruses. Virology 2003, 310:50–63.

53. Lico C, Capuano F, Renzone G, Donini M, Marusic C, Scaloni A, Benvenuto E, Baschieri S. Peptide display on Potato virus X: molecular features of the coat protein-fused peptide affecting cell-to-cell and phloem movement of chimeric virus particles. J Gen Virol 2006, 87:3103–3112.

54. Taylor KM, Lin T, Porta C, Mosser AG, Giesing HA, Lomonossoff GP, Johnson JE. Influence
of three-dimensional structure on the immunogenicity of a peptide expressed on the surface of a plant virus. J Mol Recognit 2000, 13:71–82.

55. Smolenska L, Roberts IM, Learmonth D, Porter AJ, Harris WJ, Wilson TM, Santa CS. Production of a functional single chain antibody attached to the surface of a plant virus. FEBS Lett 1998, 441:379–382.

56. Cruz SS, Chapman S, Roberts AG, Roberts IM, Prior DA, Oparka KJ. Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. Proc Natl Acad Sci U S A 1996, 93:6286–6290.

57. Schlick TI, Ding Z, Kovacs EW, Francis MB. Dual-surface modification of the tobacco mosaic virus. J Am Chem Soc 2005, 127:3718–3723.

58. Demir MaS MH. A chemoselective biomolecular template for assembling diverse nanotubular materials. Nanotechnology 2002, 13:541.

59. Yi H, Nisar S, Lee SY, Powers MA, Bentley WE, Payne GF, Ghodssi R, Rubloff GW, Harris MT, Culver JN. Patterned assembly of genetically modified viral nanotemplates via nucleic acid hybridization. Nano Lett 2005, 5:1931–1936.

60. Koch C, Wabbel K, Eber FJ, Krolla-Sidenstein P, Azucena C, Gliemann H, Eiben S, Geiger F, Wege C. Modified TMV particles as beneficial scaffolds to present sensor enzymes. Front Plant Sci 2015, 6:1137.

61. Steinmetz NF, Lomonossoff GP, Evans DJ. Cowpea mosaic virus for material fabrication: addressable carboxylate groups on a programmable nanoscaffold. Langmuir 2006, 22:3488–3490.

62. Wang Q, Kaltsgrad E, Lin T, Johnson JE, Finn MG. Natural supramolecular building blocks. Wild-type cowpea mosaic virus. Chem Biol 2002, 9:805–811.

63. Aljabali AA, Barclay JE, Steinmetz NF, Lomonossoff GP, Evans DJ. Controlled immobilization of active enzymes on the cowpea mosaic virus capsid. Nanoscale 2012, 4:5640–5645.

64. Sen Gupta S, Kuzelka J, Singh P, Lewis WG, Manchester M, Finn MG. Accelerated bioorthogonal conjugation: A practical method for the Ligation of diverse functional molecules to a polyvalent virus scaffold. Bioconjug Chem 2005, 16:1572–1579.

65. Gonzalez MJ, Plummer EM, Rae CS, Manchester M. Interaction of Cowpea mosaic virus (CPMV) nanoparticles with antigen presenting cells in vitro and in vivo. PLoS One 2009, 4:e7981.

66. Wang Q, Lin T, Johnson JE, Finn MG. Natural supramolecular building blocks. Cysteine-added mutants of cowpea mosaic virus. Chem Biol 2002, 9:813–819.

67. Meunier S, Strable E, Finn MG. Crosslinking of and coupling to viral capsid proteins by tyrosine oxidation. Chem Biol 2004, 11:319–326.

68. Gillitzer E, Willits D, Young M, Douglas T. Chemical modification of a viral cage for multivalent presentation. Chem Commun (Camb) 2002, 20:2390–2391.

69. Chatterji A, Ochoa WF, Paine M, Ratna BR, Johnson JE, Lin T. New addresses on an addressable virus nanoblock: uniquely reactive Lys residues on cowpea mosaic virus. Chem Biol 2004, 11:855–863.

70. Steinmetz NF, Lin T, Lomonossoff GP, Johnson JE. Structure-based engineering of an icosahedral virus for nanomedicine and nanotechnology. Curr Top Microbiol Immunol 2009, 327:23–58.

71. Lomonossoff GP, Evans DJ. Applications of plant viruses in bionanotechnology. Curr Top Microbiol Immunol 2014, 375:61–87.

72. Shah SN, Steinmetz NF, Aljabali AA, Lomonossoff GP, Evans DJ. Environmentally benign synthesis of virus-templated, monodisperse, iron-platinum nanoparticles. Dalton Trans 2009, 40:8479–8480.

73. Douglas TY. M Host-guest encapsulation of materials by assembled virus protein cages. Nature 1998, 393:152–155.

74. Douglas T, Strable E, Willits D, Aitouchen A, Libera M, Young M. Protein engineering of a viral cage for constrained nanomaterials synthesis. Advanced Materials 2002, 14:415–418.

75. Men AM, Steinmetz NF. Design of virus-based nanomaterials for medicine, biotechnology, and energy. Chem Soc Rev 2016, 45:4074–4126.

76. van Kan-Davelaar HE, van Hest JC, Cornelissen JJ, Koay MS. Using viruses as nanomedicines. Br J Pharmacol 2014, 171:4001–4009.

77. Perez J, Defrenne S, Witz J, Vachette P. Detection and characterization of an intermediate conformation during the divalent ion-dependent swelling of tomato bushy stunt virus. Cell Mol Biol (Noisy-le-Grand) 2000, 46:937–948.

78. Grasso S, Lico C, Imperatori F, Santi L. A plant derived multifunctional tool for nanobiotechnology based on Tomato bushy stunt virus. Transgenic Res 2013, 22:519–535.

79. Loo L, Guenther RH, Lommel SA, Franzen S. The Red clover necrotic mosaic virus capsid as a multifunctional cell targeting plant viral nanovirus. Transgenic Res 2013, 22:519–535.

80. Loo L, Guenther RH, Lommel SA, Franzen S. Infusion of dye molecules into Red clover necrotic mosaic virus. Chem Commun (Camb) 2008, 88:90. DOI: 10.1039/B714748A.

81. Lockney DM, Guenther RN, Loom L, Overton W, Antonelli R, Clark J, Hu M, Luft C, Lommel SA, Franzen S. The Red clover necrotic mosaic virus cap- sid as a multifunctional cell targeting plant viral nanoparticle. Bioconjug Chem 2011, 22:67–73.

82. Wang Q, Wen H, Wen Q, Chen X, Wang Y, Xuan W, Liang J, Wan S. Cucumber mosaic virus as drug delivery vehicle for doxorubicin. Biomaterials 2013, 34:4632–4642.

83. Cao J, Guenther RH, Sit TL, Lommel SA, Opperman CH, Willoughby JA. Development of
94. Koudelka KJ, Pitek AS, Manchester M, Steinmetz NF. Virus-Based Nanoparticles as versatile nanomachines. *Annu Rev Virol* 2015, 2:379–401.

95. Rusch V, Baselga J, Cordon-Cardo C, Orazem J, Zaman M, Hoda S, McIntosh J, Kurie J, Dmitrovsky E. Differential expression of the epidermal growth factor receptor and its ligands in primary non-small cell lung cancers and adjacent benign lung. *Cancer Res* 1993, 53:2379–2385.

96. Ross JF, Chaudhuri PK, Ratnam M. Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. *Cancer* 1994, 73:2432–2443.

97. Rae CS, Khor IW, Wang Q, Destito G, Gonzalez MJ, Singh P, Thomas DM, Estrada MN, Powell E, Finn MG, et al. Systemic trafficking of plant virus nanoparticles in mice via the oral route. *Virology* 2005, 343:224–235.

98. Singh P, Prasuhn D, Yeh RM, Destito G, Rae CS, Osborn K, Finn MG, Manchester M. Bio-distribution, toxicity and pathology of cowpea mosaic virus nanoparticles in vivo. *J Control Release* 2007, 120:41–50.

99. Kaiser CR, Flenniken ML, Gillitzer E, Harmsen AL, Harmsen AG, Jutila MA, Douglas T, Young MJ. Biodistribution studies of protein cage nanoparticles demonstrate broad tissue distribution and rapid clearance in vivo. *Int J Nanomedicine* 2007, 2:715–733.

100. Bruckman MA, Randolph LN, VanMeter A, Hern S, Schoffast AJ, Taurog RE, Steinmetz NF. Biodistribution, pharmacokinetics, and blood compatibility of native and PEGylated tobacco mosaic virus nanorods and -spheres in mice. *Virology* 2014, 449:163–173.

101. Shukla S, Wen AM, Ayat NR, Commandeur U, Gopalkrishnan R, Broome AM, Lozada KW, Keri RA, Steinmetz NF. Biodistribution and clearance of a filamentous plant virus in healthy and tumor-bearing mice. *Nanomedicine (Lond)* 2014, 9:221–235.

102. Lee KL, Shukla S, Wu M, Ayat NR, El Sanadi CE, Wen AM, Edelbrock JF, Pokorski JK, Commandeur U, Dubyak GR, et al. Stealth filaments: polymer chain length and conformation affect the in vivo fate of PEGylated potato virus X. *Acta Biomater* 2015, 19:166–179.

103. Steinmetz NF, Manchester M. PEGylated viral nanoparticles for biomedicine: the impact of PEG chain length on VNP cell interactions in vitro and ex vivo. *Biomacromolecules* 2009, 10:784–792.

104. Koudelka KJ, Destito G, Plummer EM, Trauger SA, Siuzdak G, Manchester M. Endothelial targeting of cowpea mosaic virus (CPMV) via surface vimentin. *PLoS Pathog* 2009, 5:e1000417.

105. Lee KL, Carpenter BL, Wen AM, Ghiladi RA, Steinmetz NF. High aspect ratio nanotubes formed by tobacco mosaic virus for delivery of photodynamic
agents targeting melanoma. ACS Biomater Sci Eng 2016, 2:838–844.

106. Koistinen P, Heino J. Integrins in Cancer Cell Invasion. Madam Curie Bioscience. Austin, TX: Landes Bioscience; 2000-2013.

107. Hovlid ML, Steinmetz NF, Laufer B, Lau JL, Kuzelka J, Wang Q, Hyppia T, Nemerow GR, Kessler H, Manchester M, et al. Guiding plant virus particles to integrin-displaying cells. Nanoscale 2012, 4:3698–3705.

108. Bareford LM, Swaan PW. Endocytic mechanisms for targeted drug delivery. Adv Drug Deliv Rev 2007, 59:748–758.

109. Ren Y, Wong SM, Lim LY. Folic acid-conjugated protein cages of a plant virus: a novel delivery platform for doxorubicin. Bioconjug Chem 2007, 18:836–843.

110. Steinmetz NF, Mertens ME, Taurog RE, Johnson JE, Commandeur U, Fischer R, Manchester M. Potato virus X as a novel platform for potential biomedical applications. Nano Lett 2010, 10:305–312.

111. Lewis JD, Destito G, Zijlstra A, Gonzalez MJ, Quigley JP, Manchester M, Stuhlmann H. Viral nanoparticles as tools for intravital vascular imaging. Nat Med 2006, 12:354–360.

112. Cho CF, Ablack A, Leong HS, Zijlstra A, Lewis J. Evaluation of nanoparticle uptake in tumors in real time using intravital imaging. J Vis Exp 2011, 21:2808.

113. Leong HS, Steinmetz NF, Ablack A, Destito G, Zijlstra A, Stuhlmann H, Manchester M, Lewis JD. Intravitral imaging of embryonic and tumor neovascu-lature using viral nanoparticles. Nat Protoc 2010, 5:1406–1417.

114. Chariou PL, Lee KL, Wen AM, Gulati NM, Stewart PL, Steinmetz NF. Detection and imaging of aggressive cancer cells using an epidermal growth factor receptor (EGFR)-targeted filamentous plant virus-based nanoparticle. Bioconjug Chem 2015, 26:262–269.

115. Kim D, Moon H, Baik SH, Singha S, Jun YW, Wang T, Kim KH, Park BS, Jung J, Mook-Jung I, et al. Two-photon absorbing dyes with minimal autofluorescence in tissue imaging: application to in vivo imaging of amyloid-beta plaques with a negligible background signal. J Am Chem Soc 2015, 137:6781–6789.

116. Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. Science 1990, 248:73–76.

117. Squirrell JM, Wokosin DL, White JG, Bavister BD. Long-term two-photon fluorescence imaging of mammalian embryos without compromising viability. Nat Biotechnol 1999, 17:763–767.

118. Nieh A, Appaix F, Bosca S, van der Sanden B, Nicoud JF, Bolze F, Heinlein M. Fluorescent tobacco mosaic virus-derived bio-nanoparticles for intravitral two-photon imaging. Front Plant Sci 2015, 6:1244.

119. Shriver LP, Plummer EM, Thomas DM, Ho S, Manchester M. Localization of gadolinium-loaded CPMV to sites of inflammation during central nervous system autoimmunity. J Mater Chem B 2013, 1:5256–5263.

120. Bruckman MA, Yu X, Steinmetz NF. Engineering Gd-loaded nanoparticles to enhance MRI sensitivity via T(1) shortening. Nanotechnology 2013, 24:462001.

121. Rezapkin GV, Chumakov KM, Lu Z, Ran Y, Dragunsy EM, Levenbook IS. Microevolution of Sabin 1 strain in vitro and genetic stability of oral poliovirus vaccine. Virology 1994, 202:370–378.

122. Clarke BE, Newton SE, Carroll AR, Francis MJ, Appleyard G, Syred AD, Highfield PE, Rowlands DJ, Brown F. Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. Nature 1987, 330:381–384.

123. Francis MJ, Hastings GZ, Brown AL, Grace KG, Rowlands DJ, Brown F, Clarke BE. Immunological properties of hepatitis B core antigen fusion proteins. Proc Natl Acad Sci U S A 1990, 87:2545–2549.

124. Plummer EM, Manchester M. Viral nanoparticles and virus-like particles: platforms for contemporary vaccine design. WIREs Nanomed Nanobiotechnol 2011, 3:174–196.

125. Usha R, Rohll JB, Spall VE, Shanks M, Maule AJ, Johnson JE, Lomonossoff GP. Expression of an animal virus antigenic site on the surface of a plant virus particle. Virology 1993, 197:366–374.

126. Porta C, Spall VE, Loveland J, Johnson JE, Barker PJ, Lomonossoff GP. Development of cowpea mosaic virus as a high-yielding system for the presentation of foreign peptides. Virology 1994, 202:949–955.

127. McLain L, Porta C, Lomonossoff GP, Durrani Z, Dimmock NJ. Human immunodeficiency virus type 1-neutralizing antibodies raised to a glycoprotein 41 peptide expressed on the surface of a plant virus particle. AIDS Res Hum Retroviruses 1995, 11:327–334.

128. Dalsgaard K, Utenthal A, Jones TD, Xu F, Merryweather A, Hamilton WD, Langeveld JP, Boshuizen RS, Kamstrup S, Lomonossoff GP, et al. Plant-derived vaccine protects target animals against a viral disease. Nat Biotechnol 1997, 15:248–252.

129. Turpen TH, Reiln SJ, Charoenvit Y, Hoffman SL, Ballarre V, Grill NK. Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. Biotechnology (N Y) 1995, 13:53–57.

130. Sugiyama Y, Hamamoto H, Takemoto S, Watanabe Y, Okada Y. Systemic production of
foreign peptides on the particle surface of tobacco mosaic virus. *FEBS Lett* 1995, 359:247–250.

131. Koo M, Bendahmane M, Lettieri GA, Paoletti AD, Lane TE, Fitchen JH, Buchmeier MJ, Beachy RN. Protective immunity against murine hepatitis virus (MHV) induced by intranasal or subcutaneous administration of hybrids of tobacco mosaic virus that carries an MHV epitope. *Proc Natl Acad Sci U S A* 1999, 96:7774–7779.

132. Zhao X, Chen L, Luckanagul JA, Zhang X, Lin Y, Wang Q. Enhancing antibody response against small molecular hapten with tobacco mosaic virus as a polyanivalent carrier. *Chembiochem* 2015, 16:1279–1283.

133. Arora U, Tyagi P, Swaminathan S, Khanna N. Virus-like particles displaying envelopelike protein III of dengue virus type 2 induce virus-specific antibody response in mice. *Vaccine* 2013, 31:873–878.

134. Middelberg AP, Rivera-Hernandez T, Wibowo N, Lua LH, Fan Y, Magor G, Chang C, Chuan YP, Good MF, Batzloff MR. A microbial platform for rapid and low-cost virus-like particle and capsomere production. *Vaccine* 2011, 29:7154–7162.

135. Mazei K, Gedvilaite A, Blohm U. Induction of insert-specific immune response in mice by hamster polyomavirus VP1 derived virus-like particles carrying LCMV GP33 CTL epitope. *Virus Res* 2012, 163:2–136.

136. Pniewski T, Kapusta J, Bociag P, Wojciechowicz J, Kostrzak A, Gdula M, Fedorowicz-Stronska O, Wojcik P, Otta H, Samardakiewicz S, et al. Low-dose oral immunization with lyophilized tissue of transgenic plants. *Adv Mater* 2010, 22:8140–8143.

137. Nuzzaci M, Vitti A, Condelli V, Lanorte MT, Tortorella C, Boscia D, Piazzolla P, Piazzolla G. In vitro stability of Cucumber mosaic virus nanoparticles carrying a Hepatitis C virus-derived epitope under simulated gastrointestinal conditions and in vivo efficacy of an edible vaccine. *J Virol Methods* 2010, 165:211–215.

138. Denis J, Majeau N, Acosta-Ramirez E, Savard C, Bedard MC, Simard S, Lecours K, Bolduc M, Pare C, Willems B, et al. Immunogenicity of papaya mosaic virus-like particles fused to a hepatitis C virus epitope: evidence for the critical function of multimerization. *Virology* 2007, 363:59–68.

139. D’Aoust MA, Lavoie PO, Couture MM, Trepanier S, Guay JM, Dargins M, Mongrand S, Landry N, Ward BJ, Vezina LP. Influenza virus-like particles produced by transient expression in Nicotiana benthamiana induce a protective immune response against a lethal viral challenge in mice. *Plant Biotechnol J* 2008, 6:930–940.

140. Maclean J, Koekemoer M, Olivier AJ, Stewart D, Hitzeroth II, Rademacher T, Fischer R, Williamson AL, Rybicki EP. Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. *J Gen Virol* 2007, 88:1460–1469.

141. Millan AFS, Ortigos SM, Hervas-Stubbbs S, Corral-Martinez P, Segui-Simarro JM, Gaeta J, Coursaget P, Veramendi J. Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic. *Plant Biotechnol J* 2008, 6:427–441.

142. Matic S, Masenga V, Poli A, Rinaldi R, Milne RG, Vecchiati M, Noris E. Comparative analysis of recombinant Human Papillomavirus 8 L1 production in plants by a variety of expression systems and purification methods. *Plant Biotechnol J* 2012, 10:410–421.

143. Pinoe CB, Hitzeroth II, Rybicki EP. Immunogenic assessment of plant-produced human papillomavirus type 16 L1/L2 chimaeras. *Plant Biotechnol J* 2013, 11:964–975.

144. Love AJ, Chapman SN, Matic S, Noris E, Lomonossoff GP, Taliansky M. In planta production of a candidate vaccine against bovine papillomavirus type 1. *Planta* 2012, 236:1305–1313.

145. Scotti N, Alagna F, Ferraiole E, Formigoni S, Sannino L, Buonaguro L, De Stradis A, Vitale A, Monti L, Grillo S, et al. High-level expression of the HIV-1 Pr55gag polypeptide in transgenic tobacco chloroplasts. *Planta* 2009, 229:1109–1122.

146. Mathew LG, Herbst-Kralovetz MM, Mason HS. Norovirus Narita 104 virus-like particles expressed in Nicotiana benthamiana induce serum and mucosal immune responses. *Biomed Res Int* 2014, 2014:807539.

147. Yang Y, Li X, Yang H, Qian Y, Zhang Y, Fang R, Chen X. Immunogenicity and virus-like particle formation of rotavirus capsid proteins produced in transgenic plants. *Sci China Life Sci* 2011, 54:82–89.

148. Thuenemann EC, Meyers AE, Verwey J, Rybicki EP, Lomonossoff GP. A method for rapid production of heteromultimeric protein complexes in plants: assembly of protective bluetongue virus-like particles. *Plant Biotechnol J* 2013, 11:839–846.

149. Mechtcheriakova IA, Eldarov MA, Nicholson L, Shanks M, Skryabin KG, Lomonossoff GP. The use of viral vectors to produce hepatitis B virus core particles in plants. *J Virol Methods* 2006, 131:10–15.

150. Marsian J, Lomonossoff GP. Molecular pharming – VLPs made in plants. *Curr Opin Biotechnol* 2016, 37:201–206.

151. Shenton W, Douglas T, Young M, Stubbbs G, Mann S. Inorganic-organic nanotube composites from template mineralization of tobacco mosaic virus. *Adv Mater* 1999, 11:253–256.
152. Pokorski JK, Steinmetz NF. The art of engineering viral nanoparticles. *Mol Pharm* 2011, 8:29–43.

153. Douglas T, Young M. Virus particles as templates for materials synthesis. *Adv Mater* 1999, 11:679–681.

154. Aljabali AA, Sainsbury F, Lomonossoff GP, Evans DJ. Cowpea mosaic virus unmodified empty viruslike particles loaded with metal and metal oxide. *Small* 2010, 6:818–821.

155. Heddle JG. Protein cages, rings and tubes: useful components of future nanodevices? *Nanotechnol Sci Appl* 2008, 1:67–78.

156. Aljabali AA, Lomonossoff GP, Evans DJ. CPMV-polyelectrolyte-template gold nanoparticles. *Biomacromolecules* 2011, 12:2723–2728.

157. Tseng RJ, Tsai CL, Ma LP, Ouyang JY. Digital memory device based on tobacco mosaic virus conjugated with nanoparticles. *Nat Nanotechnol* 2006, 1:72–77.

158. Gorzny ML, Walton AS, Wnek M, Stockley PG, Evans SD. Four-probe electrical characterization of Pt-coated TMV-based nanostructures. *Nanotechnology* 2008, 19:165704.

159. Knez M, Bittner AM, Boes F, Wege C, Jeske H, Maiss E, Kern K. Biotemplate synthesis of 3-nm nickel and cobalt nanowires. *Nano Lett* 2003, 3:1079–1082.

160. Tsukamoto R, Muraoka M, Seki M, Tabata H, Yamashita I. Synthesis of CoPt and FePt3 nanowires using the central channel of tobacco mosaic virus as a biotemplate. *Chem Mater* 2007, 19:2389–2391.

161. Kapoor P, Singh H, Gautam A, Chaudhary K, Kumar R, Raghava GP. TumorHoPe: a database of tumor homing peptides. *PLoS One* 2012, 7:e35187.

162. Rademacher T. Method for the generation and cultivation of a plant cell pack. EP 2012-2623603 A1: 20130807, Fraunhofer Institute, 2013.