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Virus-based nanomaterials as PET and MR contrast agents: from technology development to translational medicine

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Abstract

Viruses have recently emerged as ideal protein scaffolds for a new class of contrast agents that can be used in medical imaging procedures such as positron emission tomography (PET) and magnetic resonance imaging (MRI). Whereas synthetic nanoparticles are difficult to produce as homogeneous formulations due to the inherently stochastic nature of the synthesis process, virus-based nanoparticles are genetically encoded and are therefore produced as homogeneous and monodisperse preparations with a high degree of quality control. Because the virus capsids have a defined chemical structure that has evolved to carry cargoes of nucleic acids, they can be modified to carry precisely defined cargoes of contrast agents and can be decorated with spatially defined contrast reagents on the internal or external surfaces. Viral nanoparticles can also be genetically programed or conjugated with targeting ligands to deliver contrast agents to specific cells, and the natural biocompatibility of viruses means they are cleared rapidly from the body. Nanoparticles based on bacteriophages and plant viruses are safe for use in humans and can be produced inexpensively in large quantities as self-assembling recombinant proteins. Based on these considerations, a new generation of contrast agents has been developed using bacteriophages and plant viruses as scaffolds to carry positron-emitting radioisotopes such as [¹⁸F] fluorodeoxyglucose for PET imaging and iron oxide or Gd³⁺ for MRI. Although challenges such as immunogenicity, loading efficiency and regulatory compliance remain to be address, virus-based nanoparticles represent a promising new enabling technology for a new generation of highly biocompatible and biodegradable targeted imaging reagents.

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Introduction

Viruses are natural nanomaterials that have evolved to deliver cargos to specific cells and tissues. In the simplest form, a virus consists of nucleic acids (its genome) and a protein capsid, the latter protecting the genome cargo during delivery. Viruses have been studied for more than 100 years but a new discipline has emerged over the last decade, i.e. chemical or physical virology, in which chemists, engineers and physicists have turned toward the study of viruses and developed them as platforms that provide a three-dimensional scaffold for the controlled arrangement and display of functional molecules. Viruses have thus become building blocks for the design, development and application of novel nanoscale materials with potential applications in materials science and medicine. The study and application of viruses for potential medical applications is a rapidly evolving discipline in which virus-based and virus-inspired materials have been developed and tested as contrast agents for molecular imaging, gene and drug delivery vehicles, vaccines and scaffolding materials for tissue engineering. Several excellent review articles have recently been published in covering virus-like particle (VLP) and viral nanoparticle-based vaccines [1], virus-based tissue engineering scaffolds [98], viruses as carriers for drug delivery [2] and gene transfer [99], and the engineering of virus-based materials as optical probes for cell labeling and imaging [3]. However, the field has advanced beyond optical imaging towards the development of virus-based contrast agents for techniques such as positron emission tomography (PET) and magnetic resonance imaging (MRI). This focus article investigates the development of virus-based PET and MRI contrast agents and their testing in preclinical animal models. We discuss recent advances in the field and highlight current challenges and opportunities.

1. Virus-based nanomaterials for molecular imaging

Nanomaterials have diverse applications in molecular imaging, e.g. quantum dots are fluorescence imaging reagents and iron oxide nanoparticles are used as contrast agents for MRI. Nanoparticles can also be used as carriers enclosing large payloads of concentrated imaging reagents. By providing such nanoparticles with specific ligands, the payloads can be delivered to particular sites in the body to achieve tissue-specific contrast enhancement. Several classes of nanomaterials, each with its own advantages and disadvantages, are currently undergoing preclinical and clinical testing trials. For example, dendrimers are inexpensive and easily synthesized but may suffer from low biocompatibility [4, 5], whereas metallic nanoparticles and quantum dots have useful physicochemical imaging properties but some formulations are toxic or slow to clear from tissues [6]. Polymers and liposomes are promising because they are biodegradable and non-toxic. Some formulations are already approved for clinical use, including liposome-formulated doxorubicin (Doxil, Roche) for the treatment of cancer. For imaging applications, polymer-based contrast agents are well suited for ultrasound imaging [7].

Protein cages and viruses are a relatively new class of contrast agents in the early stages of technological development. They are easily produced as recombinant proteins in whole plants or cultivated plant cells, and because the structure is genetically encoded the material is generally homogeneous and monodisperse thus offering a high degree of quality control.

The capsids provide a scaffold that can carry internally enclosed cargos (such as contrast agents) and that can also be decorated selectively on the internal and/or external surfaces by targeting ligands with three-dimensional spatial control [8]. The combination of synthetic biology and genetic engineering allows new conjugation sites to be introduced at specific positions to provide additional functionality, e.g. additional targeting ligands [8] or reporter molecules, such as *Potato virus X* (PVX) particles displaying the fluorescent markers mCherry and green fluorescent protein (GFP) for imaging applications in plants, human cells and preclinical animal models [9]. This level of control cannot yet be achieved with synthetic nanoparticles.

Plant virus-based nanoparticles have the potential to make a broad and global impact on medicine because this technology can be deployed 'in the region for the region' in developing countries lacking a conventional refrigeration system and health infrastructure simply by providing the plants that acts as production hosts. While the molecular farming of MR and PET contrast agents may be difficult to translate with current tracers and modification strategies involving chemical conjugation, future technologies may enable such development. For example, targeted molecular probes can be produced in plants without post-harvest modification [9]. The proteinaceous materials are generally non-toxic and naturally biodegradable. Nanoparticles based on plant viruses and bacteriophages offer additional advantages over mammalian viruses because they do not infect or replicate in humans and are therefore unlikely to cause disease or interact with endogenous signaling pathways. One potential drawback is that nanoparticles based on viruses can be immunogenic. It is therefore important to study their ability to provoke an immune response and to develop strategies that avoid such adverse effects. For example, the immunogenicity of protein based nanoparticles [10, 11] including plant viruses [12] can be attenuated by stealth polymer coating, e.g. using polyethylene glycol (PEG) [11, 13–16]. The hydrophilic PEG shield reduces serum protein adsorption, which in turn prevents clearance by macrophages and the deposition of nanoparticles in non-target cells and organs, such as components of the immune system. The development of more efficient stealth and camouflage techniques for nanomaterials will improve their biocompatibility and promote their translation to the clinic.

Viruses are highly dynamic structures that can self-assemble with cargoes to generate materials with diverse geometries and sizes, and some of these materials can switch between alternative conformations (e.g. sphere to rod and rod to sphere) [17–20]. Viruses are responsive to their environment and can undergo maturation and swelling, providing unique opportunities for sensing applications, drug delivery and controlled release. For example, the 30-nm nanoparticles formed by the plant virus *Brome mosaic virus* (BMV) can be disassembled into coat proteins and re-assembled around artificial cargos yielding hybrid core-shell nanoparticles of different sizes depending on the encapsulated molecules [21]. Similarly, *Cowpea chlorotic mottle virus* (CCMV) coat proteins can self-assemble into tubular structures in the presence of artificial DNA molecules whereas they form icosahedral structures in plants [17]. *Tobacco mosaic virus* (TMV), which normally exists as stiff and hollow rods, can be thermally re-shaped into spherical nanoparticles [22].

In our laboratory we have made use of different viral nanoparticle (VNP) shapes (Figure 1) to gain insight into the role of shape as a design feature to tailor *in vivo* properties such as pharmacokinetics, biodistribution, and tumor or vessel wall targeting [23]. We found that elongated, high-aspect-ratio VNPs (aspect ratio is defined as lengths over width, for example potato virus X measures 515×13 nm, and therefore has a aspect ratio of 40) show enhanced pharmacokinetic behavior [24], more efficient tissue penetration [25], enhanced cell targeting and uptake [26], enhanced tumor homing [27] and favorable vessel wall targeting compared to their spherical counterparts [28]. These findings agree with reports describing the structure–function relationships of synthetic nanomaterials [29–35].

These advances in the design and development of VLPs have encouraged research into the development of virus-based materials as imaging probes loaded with PET or MRI contrast agents, and as discussed below these materials are now making their way through preclinical and in some cases clinical development.

2. Virus-based PET and MRI contrast agents

2.1 Virus-based PET tracers

PET is a highly sensitive and noninvasive clinical imaging modality that uses positron-emitting radioisotopes such as [^{18}F] fluorodeoxyglucose (FDG) as imaging reagents. It is widely used for cancer diagnosis and longitudinal follow-up. Bacteriophages and mammalian viruses have been developed as PET imaging platforms. The proteinaceous capsid functions as a scaffold for the conjugation of large payloads of PET tracers such as FDG.

PET imaging has been used to gain a better understanding of the *in vivo* properties of bacteriophage MS2. In one study the interior of genome-free MS2 was labeled with [^{18}F] fluorobenzaldehyde by conjugation to tyrosine side chains. A two-step reaction was used in which the hydroxyl groups were first converted into alkoxyamine functional groups by diazonium coupling, followed by the oxime ligation of [^{18}F] fluorobenzaldehyde. Sprague-Dawley rats were used to evaluate the biodistribution and pharmacokinetics of MS2 particles. Free [^{18}F] was cleared rapidly from circulation, with no signal in the heart blood pool 15 s after administration, whereas the [^{18}F]-labeled MS2 nanoparticles were cleared in two phases, with an initial circulation half-life of 4.5 min. PET imaging revealed that the [^{18}F]-labeled MS2 particles were cleared from the circulation by the liver and spleen followed by excretion through the bladder and bowel [36].

More recently, these studies were extended to include the microPET-CT imaging of native and PEGylated MS2 capsids loaded with the radiotracer [^{64}Cu], which was non-covalently attached to the MS2 interior surface by chelation with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). The use of [^{64}Cu] as a tracer is advantageous because its half-life is 12.7 h compared to 1.8 h for [^{18}F]. The biodistribution and pharmacokinetic properties of the tracer were evaluated using Nu/Nu mice with orthotopic MCF7cl18 xenografts. Imaging data confirmed the expected biodistribution and clearance profiles for nanoparticles in this size range. Both [^{64}Cu]-labeled MS2 and MS2-PEG were deposited in the liver in spleen, although MS2-PEG was much less abundant in

the spleen indicating its reduced interaction with macrophages. Although free [^{64}Cu]-labeled reagents are cleared rapidly from the plasma, the [^{64}Cu]-labeled MS2 tracers remained in circulation for extended periods with > 20% ID/g detectable in blood 24 h post-administration. Although microPET-CT imaging was not sensitive enough to confirm tumor homing, passive tumor accumulation caused by the enhanced permeability and retention effect was confirmed by carrying out quantitative biodistribution studies in collected organs using a gamma well counter (Figure 2) [37].

More efficient tumor targeting can be achieved by adding targeting ligands that recognize molecular cancer signatures. For example, a microPET imaging study demonstrated that > 10% ID/g of RGD-modified T7 bacteriophages labeled with [^{64}Cu] tracers accumulated in U87MG tumor xenografts in mice [38]. RGD is a tripeptide ligand (arginylglycylaspartic acid) which was introduced into the T7 capsids by genetic modification. RGD has been shown to bind tumor-associated integrins expressed on tumor cells and neovasculature with high affinity and specificity. The [^{64}Cu] radionuclides were introduced using covalently attached chelators including DOTA and 4-((8-amino-3,6,10,13,16,19-hexaazabicyclo [6.6.6] icosane-1-ylamino) methyl) benzoic acid (AmBaSar) [38].

In another study, *Hemagglutinating virus of Japan* envelopes (HVJ-E) vectors, which are often used for gene delivery to the central nervous system, were synthesized with encapsulated iron oxide nanoparticle cores and [^{18}F] radionuclides. Biodistribution was investigated by PET imaging and although the larger HVJ-E formulations, measuring 300 nm in diameter, showed similar biodistribution profiles to MS2, the iron oxide cargos (ferumoxides) allowed magnetic targeting to the brain of Sprague Dawley rats [39]. The HVJ-E core-shell formulations containing superparamagnetic iron oxide nanoparticles would also be suitable tools for MRI [40].

In addition to monitoring the biodistribution of nanoparticles, PET imaging can also be used to confirm successful gene delivery if viral vectors are engineered to express the *Herpes simplex virus-1* thymidine kinase (HSV1-tk) reporter system (Figure 3). HSV1-tk phosphorylates thymidine to produce thymidine monophosphate, but unlike mammalian TK1, the HSV1 enzyme also phosphorylates thymidine analogs, including the radionuclides 2'-deoxy-2'-fluoro-5-iodo-1- β -D-arabinofuranosyluracil (FIAU) and 2'-fluoro-5-ethyl-1- β -D-arabinofuranosyluracil (FEAU). Such vectors can be used for tissue-specific PET imaging. The radioactive tracer is phosphorylated by HSV1-tk and thus accumulates in proportion to the level of enzyme produced, which can be imaged by PET. Based on similar principle, PET imaging has been combined with replication competent HSV-1 reporter vector for non-invasive detection of melanoma micrometastases in regional lymph nodes [97]. This topic is covered in detail in the review article by Brader and colleagues [41].

2.2 Virus-based MRI contrast agents

Although nuclear medicine is widely used for disease diagnosis and longitudinal imaging, MRI is an attractive alternative because it provides high spatial resolution and soft tissue contrast without using ionizing radiation. MRI is therefore safer, especially for repeated administration during longitudinal imaging to monitor disease progression or therapeutic success. However, diagnosis can be difficult in areas where disease tissue and healthy tissue

produce similar signal intensities. To overcome this challenge, contrast-enhancement agents have been developed including some using virus-based scaffolds as discussed below.

MR scanners use non-ionizing radiation to measure the aligned nuclear magnetization of hydrogen atoms (protons). In brief, after protons are excited with a radiofrequency (RF) pulse applied perpendicular to the magnetic field, the protons will realign with the magnetic field. This process is referred to as relaxation. MR images rely on the differences in tissue relaxation times, both longitudinal (T_1) and transverse (T_2), to generate image contrast [42]. Disease detection with MRI can be difficult in areas where healthy and diseased tissues have similar signal intensities. Contrast enhancement agents can be used to overcome this: Contrast agents interact with water molecules, leading to altered T_1 or T_2 proton relaxation [43]. Paramagnetic lanthanide ions interact with water protons, leading to decreased longitudinal relaxation (T_1). Gadolinium (Gd) is the most popular paramagnetic imaging contrast agent used to produce a *positive* MR contrast [44]. Alternatively, superparamagnetic iron oxide effectively shortens the transverse relaxation time (T_2) and produces a negative intensity effect in MRI [45]. Virus-based scaffold have been utilized for both approaches and are discussed in the following sections.

2.2.1 Iron oxide-loaded VNPs as T_2 contrast agents—Iron oxide-modified nanoparticles have been used for contrast enhancement by promoting T_2 shortening, and several materials have already been clinically approved for this purpose [45–48]. The first example of virus-based materials combined with iron oxide nanoparticles for MRI used the icosahedral capsids of BMV to produce core-shell nanoparticles comprising an iron oxide nanoparticle core and a virus-like protein shell. Specifically, cubic iron oxide cores were functionalized with carboxylate-terminated PEG-phospholipids. The negative head group mimics the negative charge of the natural cargo (the viral genome) and is necessary to trigger the templated self-assembly of coat proteins via electrostatic interactions. The resulting core-shell nanoparticles exhibited T_2 relaxivities of $376 \text{ mM}^{-1} \text{ s}^{-1}$, which is 4-fold higher than Feridex and 6.5-fold higher than Supravist, two commonly-used commercial contrast reagents. Although these BMV-based core-shell materials have not yet been used in medical research, their application as tools for high-contrast functional imaging in plants confirms the broad applicability of virus-based materials in medicine as well as agricultural biotechnology [49].

Similar approaches can also be applied to VLPs derived from mammalian viruses, as shown recently using the *Rotavirus* VP4 structural protein and the VP1 protein from *Simian virus 40* (SV40) [50, 51]. VP4 was produced by heterologous expression in *Escherichia coli*, and its assembly around iron oxide cores was chemically driven using bioconjugate chemistry. Cell imaging was demonstrated and it was shown that MRI signals increased using the VLP-based MRI probes compared to commercially-available Feridex dextran-coated iron oxide nanoparticles. The enhanced imaging reflected the more efficient cellular targeting, exploiting natural mechanisms for the uptake of viral coat proteins that interact with certain integrin receptors on the mammalian cell surface. The core-shell nanostructures were also combined with drugs by chemical conjugation to the external capsid surface, allowing the combined imaging and killing of cancer cells (theranostics) [50].

In a different approach, the filamentous bacteriophage M13 was used for the templated assembly of iron oxide nanoparticles and SPARC peptides specific for receptors that are upregulated in certain prostate tumors with poor prognosis. The M13 filament is 800 nm in length and the iron oxide nanoparticles were distributed evenly by promoting electrostatic binding to the tri-glutamic acid motif on each coat protein subunit. In contrast, the SPARC peptides were added by genetic engineering and were confined to one end of the filament, thus conferring tissue-specificity. These MRI contrast agents exhibited a relaxivity of $\sim 60 \text{ mM}^{-1} \text{ s}^{-1}$ (comparable with the commercial agents discussed above) but delivered a larger payload specifically to the tumor cells. Tumor-specific T_2 -weighted MRI was demonstrated in mouse models of prostate cancer. C4-2B tumors, which express the glycoprotein recognized by SPARC at high levels, were effectively targeted by the virus as demonstrated by the 6-fold reduction in pixel intensity per cm^2 (T_2 -weighted positive signals appear dark in the MRI scan), whereas DU145 tumors, which express SPARC sparsely, remained bright [52].

2.2.2 Gd-loaded VNPs as T_1 contrast agents— T_1 -shortening agents are preferred because they generate more contrast than T_2 -shortening agents, and a variety of small molecules chelated with Gd^{3+} have been approved for clinical use including Magnevist (Bayer-Schering) and Gadovist (Bayer Healthcare). Nevertheless, these reagents are untargeted and do not deliver a sufficient payload for sensitive disease detection. The formation of complexes in which these contrast agents are enclosed within virus-based nanoparticles allows larger payloads to be carried, and the decoration of the same nanoparticles with receptor-specific ligands achieves targeted delivery [53].

A set of virus-based nanomaterials have been used as scaffolds for the conjugation of Gd^{3+} ions, including the 30-nm icosahedral particles of *Cowpea mosaic virus* (CPMV) and CCMV. In the CPMV platform, the RNA genome was used to chelate Gd^{3+} ions [54] whereas the CCMV platform took advantage of the intrinsic metal binding sites at the three-fold axis [55, 56]. The CCMV structure was therefore able to yield nanoparticles with an ionic relaxivity of $T_1 = 202 \text{ mM}^{-1} \text{ s}^{-1}$ and a particle relaxivity of up to $T_1 = 36,120 \text{ mM}^{-1} \text{ s}^{-1}$ [57]. The ionic relaxivity of the CCMV-based nanoparticle was significantly higher than that of commercial agents, e.g. the T_1 relaxivity was 40 times higher than that of Gadovist. Nevertheless, the potential *in vivo* toxicity and lability of these structures has prevented their further development. These concerns have been overcome by covalently attaching chelated Gd^{3+} ions using reagents such as Gd(DOTA). CCMV-based constructs with 60 Gd(DOTA) conjugates per particle have achieved ionic and particle relaxivities of 46 and $2806 \text{ mM}^{-1} \text{ s}^{-1}$ respectively [57].

Several other viral nanoparticles have been used as scaffolds for chelated Gd^{3+} ions including CPMV [54], bacteriophage Q β [54], MS2 [58–60], P22 [61], and TMV [20, 28]. Strategies based on polymerization chemistry have been developed to increase the number of Gd^{3+} ions per particle. For example, PEG-based polymers were grafted from the external surface of 30-nm Q β particles using atom transfer radical polymerization, yielding nanoparticles containing 610 Gd^{3+} ions with ionic and particle relaxivities of 11.6 and $7094 \text{ mM}^{-1} \text{ s}^{-1}$ [62]. In another approach, a dendron network was polymerized by successive click chemistry reactions on the interior surface of 64-nm bacteriophage P22 particles,

yielding nanoparticles containing 1900 Gd^{3+} ions conjugated to diethylene triamine pentaacetic acid (DTPA) with ionic and particle relaxivities of 14.0 and $26,600 \text{ mM}^{-1}\text{s}^{-1}$ [61].

We recently developed TMV-based nanoparticles loaded Gd(DOTA) with a proton relaxivity of $36,562 \text{ mM}^{-1}\text{s}^{-1}$, which is four times that of the commercial agent Gadovist (Figure 4) [20, 28]. These TMV probes were also modified with fluorescent dyes and peptide ligands targeting the vascular cell adhesion molecule VCAM-1, which is upregulated at sites of inflammation. The dual-mode TMV probes were studied using the ApoE^{-/-} mouse model of atherosclerosis. Targeting and sensitive delineation of plaques was achieved using sub-micromolar concentrations of the contrast agent, three orders of magnitude lower than the typical clinical dose. The increased signal sensitivity reflected the combined impact of multivalency and molecular targeting, high payload delivery and reduced tumbling rates, and the elongated shape with high aspect ratio leading to improved vascular targeting [63–65].

The aforementioned examples highlight that various VNP platforms and coordination chemistries are being developed. Because free Gd ions are toxic, chelation is important. Beyond the safety aspect, the Gd-chelator impacts the relaxivity and hence contrast enhancement. The water exchange rate and number of coordinated water molecules can be tuned making use of different Gd-chelators: the most popular chelators used are based on diethylenetriaminepentaacetate (DTPA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) [42], both of which have been used for VNP-based MR contrast agents. Newer chelators include HOPO (4-carboxyamido-3,2-hydroxypyridinone) and AAZTA (6-amino-6-methylperhydro-1,4-diazepinetetraacetic acid) ligands that efficiently coordinate two water molecules (thereby increasing the contrast enhancement), while maintaining stable chelation with Gd ions suitable for *in vivo* application [66]. Novel chelation chemistries and bi- or multivalent chelators in combination with multivalent (virus-based) nanoparticle platform technologies will help to further increase the MR contrast agent concentration to pinpoint diseases at earlier stages.

2.2.3 VNPs as chemical exchange saturation transfer (CEST) and hyperCEST contrast agents—Chemical exchange saturation transfer (CEST) and hyperCEST imaging are relatively new MRI contrast approaches in which exogenous nuclei (that resonate at a frequency different than water protons) are selectively saturated via 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. For further background information on this technology, the reader is referred to the following review article [67].

In this context, the filamentous phage M13 was modified with cryptophane-A molecular cages as a hyperpolarized xenon MRI contrast agent. Saturation of xenon bound in the cryptophane cages was transferred to the pool of aqueous-solvated xenon via chemical exchange. The complimentary sensitivity enhancements of both hyperpolarized [^{129}Xe] and chemical exchange saturation transfer (CEST) detection caused the hyperCEST contrast enhancement mechanism, as previously described for cryptophane-A molecular cages.

However, the M13-based xenon sensor had a low detection limit of 230 fM (a 50-fold increase compared to free cryptophane-A) reflecting multivalent display on the M13 scaffold [68].

These strategies are also applicable to other virus-based nanomaterials, including 27-nm icosahedral MS2 and filamentous bacteriophage fd. The cryptophane-A cages were conjugated to the interior of MS2 resulting in a detection limit as low as 0.7 pM, using the HyperCEST imaging protocol. The increased contrast enhancement compared to free xenon host molecules was again explained by the multivalent nature of the viral capsids, in this case hosting 125 copies of cryptophane-A cages per nanoparticle [69].

Targeting and imaging cancer cells was achieved using a dual-modified filamentous bacteriophage fd carrying the xenon-binder cryptophane-A as well as a single chain variable fragment (scFv) targeted to the epidermal growth factor receptor (EGFR) [70]. The scFv targeting ligands were genetically engineered, whereas the cryptophane-A cages were chemically introduced using a combination of transamination/oxime ligation chemistry [71]. Target-specific live cancer cell imaging using MDA-MB-231 cells was demonstrated using ^{129}Xe nuclear magnetic resonance with the hyperCEST detection protocol [70].

3. Technology development to clinical translation: advances, challenges and opportunities

For any nanoparticle platform and contrast agent to make a clinical impact, its safety profiles must be evaluated carefully including *in vivo* testing. Several mammalian viruses, including the *Adenovirus*, *Adeno-associated virus* (AAV) and *Lentivirus* platforms, have been widely used as vectors for therapeutic gene delivery, so the potential medical applications of virus-based materials has clearly been recognized. VNPs from plants and bacteriophages do not infect or replicate in mammalian cells, and are therefore considered inert and safe for human use. Many plant viruses are part of the natural food chain and are consumed with plant-derived foods. A number of plant viruses have proven non-toxic when delivered in high doses to animals, e.g. 10^{16} CPMV particles per kg body weight (or 100 mg CPMV/kg body weight) [72]. Recent studies have also demonstrated the biocompatibility of viruses, e.g. the repeated administration of PEGylated PVX particles did not induce chronic inflammation in mice [Lee et al., in review], and TMV hydrogels implanted *in vivo* also showed no apparent toxicity in mice and were readily degradable [73]. Another study showed that TMV particles do not induce hemolysis or coagulation, which is important for the development of contrast agents [24]. These studies have completed essential steps on the path toward the application of TMV and other virus-based materials in medicine.

Virus-based materials are cleared mostly by macrophages via the liver and spleen after intravenous administration. Compared to synthetic nanoparticles, several of which persist within the body for weeks or months, virus-based materials are readily biodegradable and are therefore removed from the body within days [74, 75]. Certain synthetic nanoparticles (e.g. gold, silica and carbon nanostructures) are cleared slowly by the macrophage system, potentially causing adverse effects in the liver and spleen [6, 76–78]. Rapid tissue clearance is particularly important for MRI contrast agents containing Gd^{3+} because release of Gd^{3+}

ions from their chelators can induce toxic side effects and may lead to disorders such as nephrogenic systemic fibrosis (NSF) [79]. Protein-based scaffolds therefore provide an opportunity for the development of MRI contrast agents carrying a large payload with high T1 relaxivity which may not be possible with synthetic structures.

Like many other exogenous materials, protein carriers derived from bacteria and plants are immunogenic and this is a translational challenge that must be addressed for many systems, including those based on mammalian viruses and synthetic nanoparticles. The production of carrier-specific antibodies has been reported for many inorganic and synthetic systems [80–85]. The key questions to be addressed are whether the immune responses are part of the natural clearance mechanism or whether they represent potential adverse effects. Recent studies have shown that TMV induces the production of antibodies in mice [86] and human patients [87]. Whether or not this immune response represents an adverse effect, it is also necessary to consider the possibility that neutralizing antibodies may interfere with the medical application. For example, for imaging applications (especially longitudinal imaging to follow disease progression or treatment success) antibody neutralization of the contrast agent may affect the pharmacokinetic behavior and biodistribution, thereby preventing the particles accumulating at the target site. Several strategies have been developed to address this challenge. For example, polymers such as PEG grafted-*to* and grafted-*from* the capsid surface can overcome antibody recognition [reviewed in 13, 15, 16]. Alternative strategies include polysaccharide coating or immunoediting, where immunogenic epitopes on the surface of the virus are genetically deleted or altered, rendering the particles non-immunogenic. Another elegant strategy to reduce interactions with the immune system (which ultimately leads to the production of antibodies and potential inflammatory responses) is to camouflage the particles as endogenous proteins. This was recently demonstrated using synthetic nanoparticles tagged with peptides derived from the active domain of CD47, which prevented clearance by the mononuclear phagocyte system [88].

The potential restoration of virulence is a significant safety concern when mammalian viruses are used as carriers, and safety can only be ensured by removing all traces of genetic material using highly reproducible procedures that reliably produce a uniform distribution of nanoparticles completely devoid of DNA and/or RNA. Several *in vitro* techniques have been developed to release nucleic acids from capsids thus generating particles devoid of genetic material. Such VLPs can be self-assembled *in vitro* from TMV or CCMV coat proteins [89, 90]. Alternatively, nucleic acids can be released by making use of the pH-dependent swelling mechanism of particles such as CCMV and *Hibiscus chlorotic ringspot virus* (HCRSV) [91, 92]. Alkaline hydrolysis is an effective method for particles such as CPMV that do not undergo extensive swelling. Alkaline hydrolysis can be applied either to purified CPMV particles [93] or during the initial steps of the extraction protocol [94]. Another method for production of non-infectious particles is short wave (254 nm) UV irradiation to crosslink and inactivate the genome within intact particles [95]. Although it is feasible to remove the genomes from viruses after propagation using sequential disassembly and reassembly protocols, it may be better to produce the protein subunits in a heterologous expression system allowing them to assemble into VLPs in the absence of the genome [96].

In addition to safety and biocompatibility considerations, the manufacture of nanoparticle formulations is subject to regulatory scrutiny and they cannot be approved for clinical use without the complete and reproducible characterization of the material to confirm that it is homogeneous, i.e. each particle is identical. Particle size and dispersity have a significant impact on *in vivo* distribution, so the synthesis of uniform nanoparticle formulations is necessary to predict their behavior *in vivo* accurately. This is one of the strengths of the VNP and VLP platforms because the production of viruses and their subunits is genetically controlled. Genetic engineering provides a means of controlling the formulation with atomic precision. Most chemical modifications are stochastic, but it is relatively straightforward to determine VNP/VLP concentrations and/or how many sites are conjugated per nanoparticle through the use of colorimetric assays, gel electrophoresis, UV/vis spectroscopy, static light scattering (SLS) and other methods. The same level of quantitation is difficult to achieve with liposomes or other nanoparticle formulations because it is challenging to determine the number of nanoparticles in solution.

For imaging applications, the quantity of contrast agent per nanoparticle is a critical determinant: For TMV (MW = 40 MDa), approximately 4000 contrast agent molecules can be conjugated to the internal and external surfaces, resulting in a 0.05% imaging reagent load as a proportion of molecular weight. The relatively low loading efficiency of viruses is a setback that must be overcome with new loading strategies. Recent chemical methods that were explored using bacteriophage P22 can achieve much higher loading densities: dendron networks were polymerized inside the P22 cage structure yielding a contrast agent with a Gd³⁺ load of nearly 1% as a proportion of molecular weight [61].

Conclusion

Viruses can be used as protein scaffolds to carry contrast reagents for positron emission tomography (PET) and magnetic resonance imaging (MRI) and offer many advantages in terms of synthesis, quality control, cost and contrast density, as well as offering the ability to target specific disease tissues such as tumors. Synthetic nanoparticles are difficult to produce as homogeneous formulations because the synthesis process is stochastic, but the synthesis of virus-based nanoparticles is templated and each particle therefore has an identical structure which interacts with the contrast reagent in a predictable and controllable manner (e.g. by encapsulation or conjugation/non-covalent binding to specific amino acid side chains on the internal or external surface. Particles can be assembled and disassembled around cargoes by changing the environmental conditions and can also be functionalized with targeting ligands. Unlike inorganic contrast agents, which can persist in the body and cause adverse effects, viruses are cleared rapidly from the body by macrophages. Those based on bacteriophages and plant viruses cannot revert to virulence because they do not replicate in humans even in their natural state. Certain challenges remain such as immunogenicity, loading efficiency and regulatory compliance, but these are being addressed by strategies such as immunogenic cloaking, and the use of dendron networks to increase the loading density. Whereas each class of nanomaterial currently undergoing preclinical/clinical development and testing offers advantages and disadvantages, virus-based materials offer many favorable properties for use as nanocarrier systems. As the field continues to advance, more detailed understanding of the *in vivo* fates of functionalized

VNP materials, along with advanced biosynthesis methods will pave the way for many more translational applications.

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Fig. 1. Viral nanoparticles provide structural diversity

A) Icosahedral CPMV capsid present residues on both exterior (a) and interior surfaces (b) for chemical conjugation of contrast agents (ref: *Biomacromolecules*. 2012 Dec 10;13(12): 3990–4001); B) Genetic engineering enables expression of proteins of interest fused to the coat proteins (ref 9); C) Shape transformability can be achieved through the top-down approach using heat (ref 24) or D) using the bottom-up approach such as self-assembly (scale 50nm) (ref 26).

Fig. 2. MicroPT-CT imaging using bacteriophage MS2

A) Radiotracer [^{64}Cu] is placed at the interior through chelation by DOTA, while PEG forms the outer protective coating; B) PET-CT images of mice injected with free ^{64}Cu (a) and ^{64}Cu labeled DOTA-MS2 (b) shows distinct accumulation sites suggesting; C) Comparative biodistribution of free ^{64}Cu , ^{64}Cu labeled DOTA-MS2, and PEGylated ^{64}Cu labeled DOTA-MS2. (Ref 37).

Fig. 3. Combining PET imaging with replicating viral vectors

A) HSV-1 vector infection, replication and transgene expression leading to *in situ* uptake and generation of PET contrast agent; B) PET Imaging of melanoma micrometastases in regional lymph nodes with NV1023 HSV-1 vector *in vivo* (Ref: [PLoS One](#), 2009;4(3):e4789); C) [18F]-FEAU-PET imaging with NV1066 allows detection of cancerous neural invasion *in vivo* (ref 41).

Fig. 4. MRI of atherosclerotic plaques using targeted Tobacco mosaic virus

A) TMV structure and addressable amino acid residues on interior and exterior surfaces; B) Confocal images of aorta cryo-sections highlights enhanced localization of VCAM-targeted TMV (green) in atherosclerotic plaques (scale 250 μm); C) Pre and post injection MRI scans of VCAM-TMV (a) and Gd(DOTA) (b) in ApoE^{-/-} mice (figures adapted from ref 28).