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REVIEW

Using viruses as nanomedicines

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The field of nanomedicine involves the design and fabrication of novel nanocarriers for the intracellular delivery of therapeutic cargo or for use in molecular diagnostics. Although traditionally recognized for their ability to invade and infect host cells, viruses and bacteriophages have been engineered over the past decade as highly promising molecular platforms for the targeted delivery and treatment of many human diseases. Inherently biodegradable, the outer capsids of viruses are composed entirely of protein building blocks, which can be genetically or chemically engineered with molecular imaging reagents, targeting ligands and therapeutic molecules. While there are several examples of viruses as *in vitro* molecular cargo carriers, their potential for applications in nanomedicine has only recently emerged. Here we highlight recent developments towards the design and engineering of viruses for the treatment of cancer, bacterial infections and immune system-related diseases.

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Abbreviations
CCMV, cowpea chlorotic mottle virus; CMV, cucumber mosaic virus; CPMV, cowpea mosaic virus; CPV, canine parvovirus; HCRSV, hibiscus chlorotic ringspot virus; PEG, polyethylene glycol; RCNMV, red clover necrotic mosaic virus; SpA, *S. aureus* protein A; TfR, transferrin receptors

Introduction

The ability to control and target drug delivery is crucial for effective and efficient medical treatment; however, the lack of control over site-specific localization and, hence, bioavailability at the desired site still remains one of the major challenges in modern-day medicine. In particular, drug solubility is a major issue, with nearly half of newly developed and currently available pharmaceutical products being poorly soluble in water. This lack of solubility significantly reduces their overall performance, as only a fraction of the drug accumulates in the desired region, and importantly, it is the loss upon administration that is often the cause of undesirable side effects (Allen and Cullis, 2004). Some conventional ways to address poor solubility are to use the salt form or to use excipients such as organic solvents, oils or surfactants. More recently, the rational design of efficient, targetable carrier systems such as nanoparticles, nanocontainers and biomaterials has received considerable attention. With regard to the design of efficient carrier systems, the physical properties, such as size, morphology and charge, of the nanocarrier exterior have direct effects on cellular uptake, intracellular distribution and accumulation, retention and excretion times (see 'Toxicity and biodistribution' below; Figure 1) (Moghimi *et al.*, 2005; Doane and Burda, 2012). While semisynthetic carriers such as quantum dots, dendrimers, vesicles and liposomes all offer certain advantages (and disadvantages) (Steinmetz, 2010; Wen *et al.*, 2013), in terms of biocompatibility, pharmacokinetics, toxicity and immunogenicity, viruses consistently stand out as ideal molecular carrier systems (Wen *et al.*, 2013). Although evolved in nature to invade and infect host cells for the efficient delivery of genomic cargo, recent developments in nanotechnology have revolutionized viruses as safe delivery vehicles. Plant- and bacteria-derived viruses are particularly useful as they are ubiquitous in nature, are considered non-infectious and

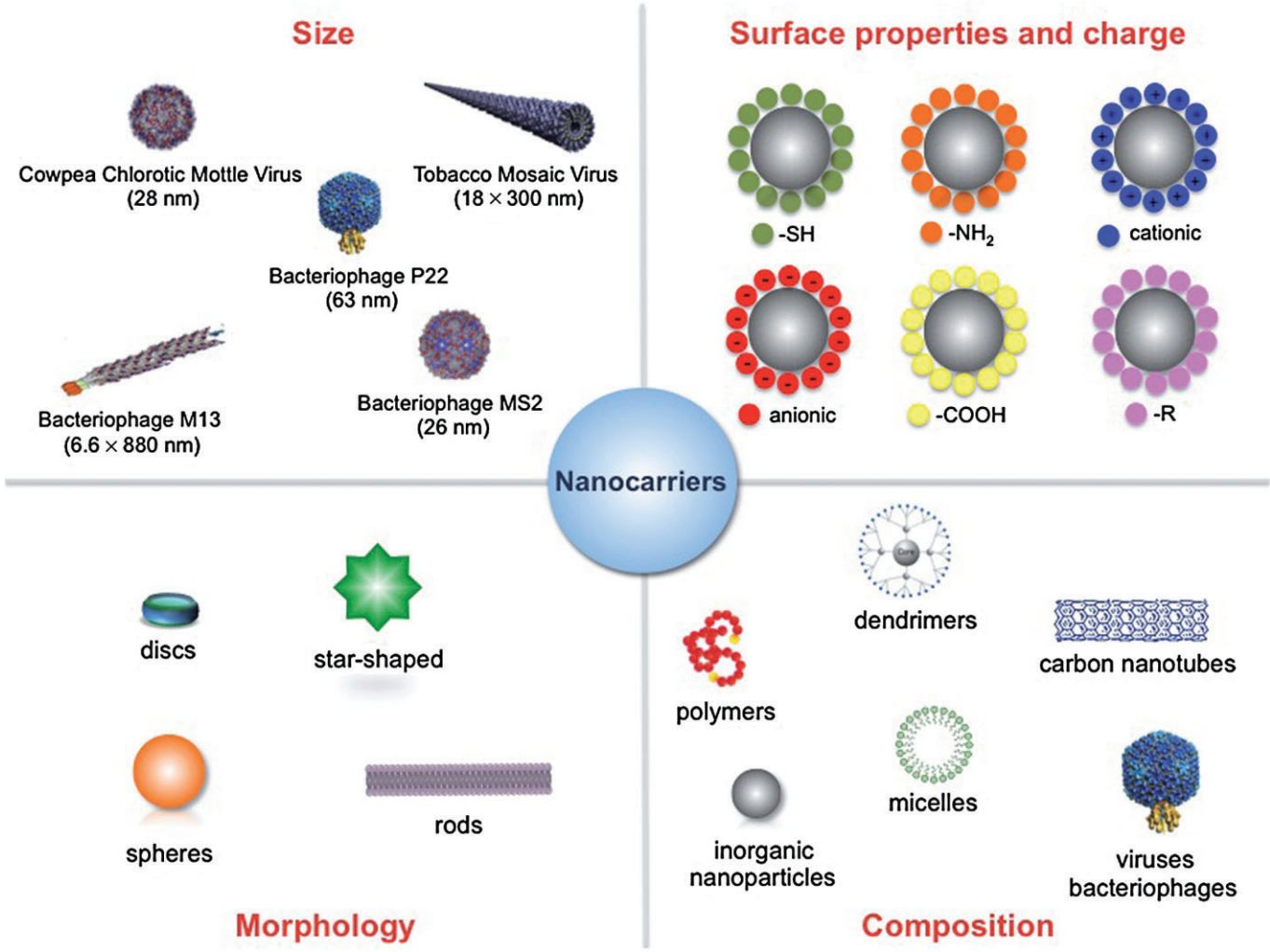

Figure 1

Overview of the main physical properties that play important roles in the cellular uptake, intracellular distribution and accumulation, retention and excretion times of nanocarriers.

non-hazardous in humans and other mammals, and are inherently biodegradable and highly biocompatible (Kaiser *et al.*, 2007; Singh *et al.*, 2007). Furthermore, they can be isolated from native sources in gram quantities or expressed recombinantly in bacterial hosts (Santi *et al.*, 2006; Saunders *et al.*, 2009). Although there are a variety of available sizes and morphologies, the majority of virus-based nanocarriers to date have been based on spherical or rod-shaped assemblies (Figure 2A).

Viruses as nanocarriers

Virus nanocarriers are highly promising scaffolds for the design of 'smart' delivery systems that are triggered to release their cargo in response to changes in pH, chemical stimuli, redox status or temperature (Moghimi *et al.*, 2005). Plant-based viruses and bacteriophages are typically considered safer delivery vehicles than mammalian viruses because they cannot proliferate in humans and hence are less likely to trigger negative downstream effects (Costa *et al.*, 2012; Yildiz *et al.*, 2012; Wen *et al.*, 2013). Non-enveloped plant and bacterial viruses are two-component systems composed of an outer protein shell (capsid) surrounding the genomic material. For some viruses, the capsids can be disassembled, the genomic RNA/DNA cargo can be removed and the virus capsid proteins can be reassembled to form empty virus-like structures that resemble native viruses in morphology (so-called virus-like particles) (Figure 2B) (Ochoa *et al.*, 2006). This ability to form a well-defined closed structure that can subsequently disassemble offers a convenient and powerful strategy for the controlled encapsulation and release of various functional (therapeutic) materials, such as proteins, enzymes, polymers, small inorganic molecules and micelles (Lockney *et al.*, 2010; Brasch *et al.*, 2011; 2013). As the exteriors of virus nanoparticles are composed entirely of proteins, by functionalizing their surfaces with appropriate ligands, viral nanocarriers can be targeted to specific cells and locations within the body. While viruses and virus-like particles have a long history in the field of vaccines and gene therapy

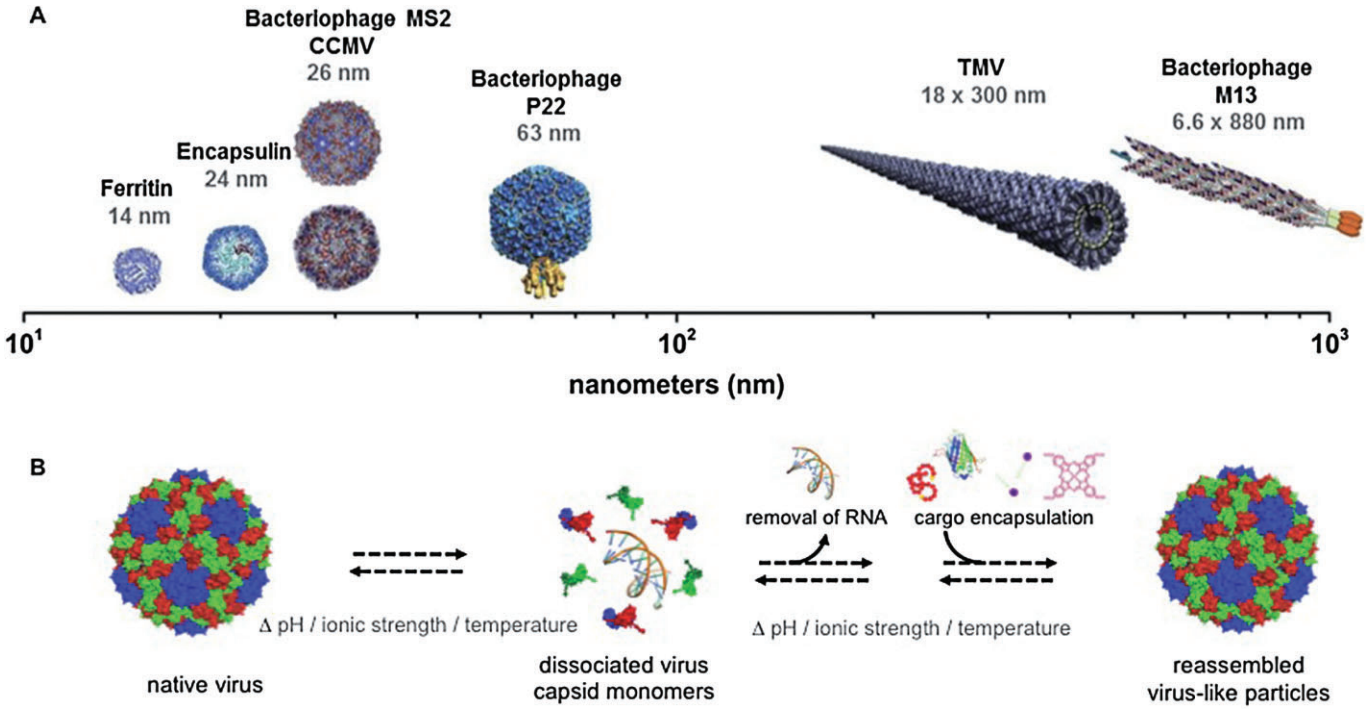

Figure 2

(A) An overview of the most common viral and non-viral assemblies used in nanomedicine; (B) Upon changes in pH, ionic strength and/or temperature, some viruses undergo reversible disassembly and reassembly. Once disassembled, the genomic RNA can be removed and the virus capsid can be used for the encapsulation of functional materials.

(Lee and Wang, 2006; Saini *et al.*, 2006; Ren *et al.*, 2007; Destito *et al.*, 2009; Grasso and Santi, 2010; Li *et al.*, 2010; Steinmetz, 2010; Yildiz *et al.*, 2011), here we highlight recent developments towards the use of viruses for the treatment of cancer, bacterial infections and immune-system-related diseases.

Viruses for the treatment of cancer

Metastasis, the uncontrollable proliferation of cancer cells from a primary tumour to other parts of the body, still remains one of the main challenges in cancer treatment (Chambers *et al.*, 2002; Schroeder *et al.*, 2012). To date, surgery has proven to be rather ineffective in treating metastatic cancer, and instead, current treatment often involves the use of highly potent cytotoxic compounds, which often lead to severe side effects (Schroeder *et al.*, 2012). In the push for new approaches, recent developments have turned towards nanotechnology, and several promising examples of plant viruses, such as cowpea mosaic virus (CPMV), hibiscus chlorotic ringspot virus (HCRSV), red clover necrotic mosaic virus (RCNMV), and the bacteriophages Hong Kong 97 (HK97) and M13 have emerged for applications for cancer treatment including molecular detection, targeting and trafficking (Steinmetz, 2010; Yildiz *et al.*, 2011). Although some viruses, such as CPMV and canine parvovirus (CPV), exhibit a natural affinity for a particular type of cell (Singh *et al.*, 2006; Shriver *et al.*, 2009; Leong *et al.*, 2010), cellular uptake is often inefficient. By making use of the reactive lysine, cysteine, aspartic acid and glutamic acid groups, the outer surface of the virus capsid can be made accessible to both genetic engineering and chemical modification strategies such as *N*-hydroxysuccinimide coupling, Michael addition to maleimides and carbodiimide activation (Steinmetz, 2010). This allows molecular cargo or prosthetic groups such as aptamers, proteins, antibodies, carbohydrates, fluorescent dyes and drugs to be functionalized and has been widely employed for applications in cell imaging and targeting (Douglas and Young, 2006; Singh *et al.*, 2006; Caruthers *et al.*, 2007; Grasso and Santi, 2010; Koudelka and Manchester, 2010; Steinmetz, 2010; Yildiz *et al.*, 2011; Ma *et al.*, 2012). Although different strategies for controlled functionalization and encapsulation have been explored extensively, significant challenges still remain, as virus cages are highly sensitive to structural and genetic mutations, which can easily interfere with the small interactions responsible for cage assembly. Therefore, many current examples are based on (or perhaps limited to) chemical modifications on the external surface of viruses.

Molecular detection

Using standard chemical conjugation strategies, wild-type virus particles can be labelled on the external surface with fluorescent moieties. For example, using *N*-hydroxysuccinimide ester chemistry, wild-type CPMV can be fluorescently labelled with Alexa Fluor 555, Alexa Fluor 488 or fluorescein, yielding exceptionally bright particles that could be used for long-term *in vivo* imaging of the vascular endothelium as well as the vasculature (blood vessels) and

blood flow in living mouse and chick embryos (Lewis *et al.*, 2006; Leong *et al.*, 2010). In an extensive study, Steinmetz and co-workers used CPMV fluorescently labelled with Oregon Green 488 to demonstrate that, upon injection, CPMV interacts specifically with the intermediate filament protein vimentin. Vimentin is important for the stabilization of cellular structure and architecture in mesenchymal cells and is crucial for cell adhesion, migration and cell signalling. Vimentin is also often overexpressed on the surface of tumour cells and has also been correlated with the malignant progression and invasion of cancer cells. While vimentin-associated uptake of CPMV particles was not (and could not be) predicted in advance, this work demonstrated the potential of fluorescent CPMV particles for the *in vitro* and *in vivo* detection of metastatic tumours (Steinmetz *et al.*, 2011). Although vimentin is not a specific target for tumour cells, its overexpression in cancer cells could still serve as a valuable factor in the development of a new treatment or method of detection.

The ability to specifically target particular cells or cellular regions is an important factor to be considered for applications in nanomedicine. Two examples of highly sought targets are transferrin and folate. Transferrin is an iron carrier protein that is overexpressed in rapidly dividing cells (including cancer cells) and has been the centre of much attention for targeted cancer therapy. Interestingly, some viruses, such as CPV, utilize transferrin receptors (TfRs) as a mechanism for cell uptake and entry. Taking advantage of this natural affinity, CPV particles fluorescently labelled with Oregon Green 488 were investigated for their cell uptake efficiency using several human tumour cell lines (Singh *et al.*, 2006). Surface-modified CPV Oregon Green 488 particles retained their natural TfR targeting ability, and confocal microscopy showed that the CPV particles localized specifically to endosomes. In subsequent independent work, TfRs have been successfully engineered on the surface of other viral nanoparticles. For example, cysteine-modified bacteriophage HK97 nanoparticles were dual-labelled with transferrin and fluorescent molecules. A distinct advantage of bacteriophage HK97 is that, due to the different maturation states of the bacteriophage capsid head, a range of HK97 particles with different sizes, internal capacity, stability and dynamics could be explored for cell uptake studies. Confocal imaging confirmed that the functionalized HK97 particles were internalized in cancer cells via endocytosis in clathrin-coated pits, which were subsequently targeted and localized to the endolysosomal compartment (Huang *et al.*, 2011).

Receptor-targeted imaging

Because folate receptors are up-regulated and over-expressed on the cell surface of tumour cells, folate receptor targeting has emerged as a powerful approach for the detection, imaging and treatment of many cancers and diseases. In particular, folic acid (nanoparticle) conjugates have been widely explored as a means to target and deliver molecular cargo to pathologic cells while minimizing delivery to normal cells and tissues. For example, Destito *et al.* were the first to develop CPMV particles that selectively bound *in vitro* to tumour cells expressing folate receptors (Destito *et al.*, 2007). Using copper(I)-catalysed azide–alkyne cycloaddition, folic acid–polyethylene glycol (PEG) conjugates were covalently attached to the CPMV surface, and their effective uptake was studied in KB and HeLa cells. Fluorescence microscopy studies showed folic acid–PEG conjugates were evenly distributed in the cytoplasm, and uptake was presumed to involve clathrin- and caveolin-mediated endocytic pathways. Interestingly, the presence of PEG on the surface of CPMV served two purposes, firstly to suppress and inhibit the natural targeting and uptake of wild-type CPMV and secondly to simultaneously act as a spacer to display folic acid on the ends. Surprisingly, HeLa and KB cells were not able to recognize folic acid coupled directly to the surface of CPMV particles, but only folic acid presented at the end of the PEG spacer. More recently, targeted CPMV particles have been used for the *in vivo* detection of prostate tumours in a chick embryo chorioallantoic tumour membrane model (Steinmetz *et al.*, 2011). In this particular example, CPMV particles were designed to target gastrin-releasing peptide receptors, which are overexpressed on prostate tumour cells, by functionalizing Alexa Fluor 647-bound CPMV nanoparticles with bombesin peptides. Rather than using copper-based chemistry, Brunel *et al.* developed a convenient strategy based on hydrazine ligation to generate CPMV nanoparticles bearing a fluorescent dye for imaging, a PEG polymer for improved plasma circulation time, and the peptide ligand F56 (Brunel *et al.*, 2010). Peptide F56 binds to vascular endothelial growth factor receptor 1 (VEGFR-1), which is often up-regulated in breast, gastric and nerve sheath tumours, allowing the functionalized CPMV nanoparticles to be targeted to endothelial cells.

Receptor-targeted delivery

Conventional chemotherapeutic agents, which are often non-specifically administered, typically affect both pathological and normal cells, inducing unwanted side effects. Various strategies have been investigated to improve the targeting capability of virus-based nanoparticles by engineering specific interaction to cellular receptors (Grasso and Santi, 2010). With the advances in using viruses for receptor-based imaging, the field has progressed towards the use of virus-based nanoparticles as a vehicle for cargo delivery. To date, the controlled release of the well-studied anticancer agent doxorubicin from virus-like particles has proven to be highly promising. In two independent examples, HCRSV and cucumber mosaic virus (CMV) loaded with doxorubicin were functionalized with folic acid for cell uptake studies in ovarian cancer cells (OVCAR-3) (Ren *et al.*, 2007; Zeng *et al.*, 2013). In an extensive study by Zeng *et al.*, accumulation of doxorubicin in the nuclei of mouse myocardial cells was shown to be significantly decreased and the doxorubicin uptake in the ovarian cancer cells increased, leading to diminished cardiotoxicity and improved antitumour effects. In another example, Lockney *et al.* used a different approach to release doxorubicin from modified RCNMV particles. The sensitivity of RCNMV to divalent cations such as Mg^{2+} and Ca^{2+} offers a distinct advantage for drug delivery applications, as RCNMV particles should stay intact in the blood, where the Ca^{2+} and Mg^{2+} concentrations are high, as well as in the endosome, where the pH is low (<6.0), and release should only occur in the cytosol, where divalent cation concentrations are sufficiently low. In the past, many approaches focused on the use of RGD peptides for cell targeting;

however, the lack of specificity and efficiency in cancer cell uptake has since led to a search for more specific targets. Here, the outer surface of RCNMV was functionalized with N-cadherin-targeting peptides. The E- and N-cadherins are Type I transmembrane proteins that play important roles in cell adhesion (Takeichi, 1990). The transition from E-cadherin expression to N-cadherin expression is common to many cancers and hence represents an interesting alternative for selective, targeted delivery. In this work, doxorubicin-loaded RCNMV particles were dual-functionalized with ADH304 and CD46 peptides, targeting N-cadherin and the group B adenovirus receptor (Lockney *et al.*, 2010). Although an increase in cytotoxicity was observed, the response behaviour was time-dependent and was not consistent with a typical dose–response curve. Such behaviour may be due to the inherent tendency of RCNMV particles to aggregate at neutral pH and may suggest that gaining control over dose, distribution and cargo release may be difficult.

In an alternative approach to cell-targeting peptides, Francis and co-workers reported the use of DNA aptamers for targeted cell uptake. In this example, the MS2 bacteriophage was functionalized with a specific DNA aptamer that targets the tyrosine kinase receptors of Jurkat leukaemia T cells (Tong *et al.*, 2009). In subsequent work, MS2 particles functionalized internally with porphyrins were used for applications in photodynamic therapy. Porphyrins were used as photosensitizers, whereby light irradiation initiated the formation of reactive oxygen species, inducing cell apoptosis and cell death. The *in vitro* application of MS2 particles dual-functionalized with porphyrins and DNA aptamers led to the highly selective death of Jurkat cells in a Jurkat/erythrocyte mixture, demonstrating the efficiency of the DNA aptamers in cell selectivity (Stephanopoulos *et al.*, 2010).

Viruses are a highly promising platform for receptor-targeted delivery. Their highly symmetrical structure allows for the multivalent presentation of surface molecules for enhanced molecular targeting and simultaneously enables exceptionally high payload capacities (for drug delivery). Although still in its early stages, with the diverse range of viruses that are readily available combined with the library of targeting peptides that are of interest, this field and the field of the use of viruses in general for the detection and treatment of cancers will continue to grow, making many exciting discoveries in the future.

Bacterial infections

Bacterial biofilms are dense communities or agglomerations of a specific bacterial cell type, and their formation continues to be one of the main causes of hospital-based bacterial infections. Embedded in a matrix of exopolysaccharides, biofilms are extremely difficult to treat owing to their high resistance to mechanical interference and their acquired resistance to antibiotics (Wainwright and Crossley, 2004; Suci *et al.*, 2007). In many cases, the use of antimicrobials alone is insufficient as they are often either inactivated prior to reaching their target site or are simply unable to penetrate the biofilm, thus lowering antimicrobial uptake and efficiency (Mah and O'Toole, 2001). Instead, new strategies have been explored to inhibit bacterial growth, disrupt biofilm formation or otherwise improve the uptake efficiency. The use of liposomes and virus-based nanoparticles to deliver molecular cargo offers a potentially attractive solution, as the naturally high surface area (and hence surface coverage) allows for both superior cargo loading and payload delivery efficiency. Furthermore, the size and morphology of nanoparticles could improve depth of penetration into biofilms. Early studies on *Staphylococcus aureus*, a common biofilm-forming pathogen, showed that biotinylated cowpea chlorotic mottle virus (CCMV) conjugates loaded with fluorescein dye molecules or Gd(III)-DOTA contrast agents were able to penetrate the exopolysaccharide matrix to a depth of an impressive 20 μm over a time period of 80 min in order to reach and target the cells (Suci *et al.*, 2007). *S. aureus* expresses a variety of surface proteins, such as protein A (SpA), that are involved in cell adhesion to host tissues and immune avoidance. In subsequent work, biotinylated anti-SpA monoclonal antibody was initially bound to *S. aureus* (Figure 3A). In the second step, streptavidin was then bound to these cells (Figure 3B) before they were finally exposed to biotinylated CCMV, functionalized with Ru(bpy2)phen-iodoacetamide photosensitizers

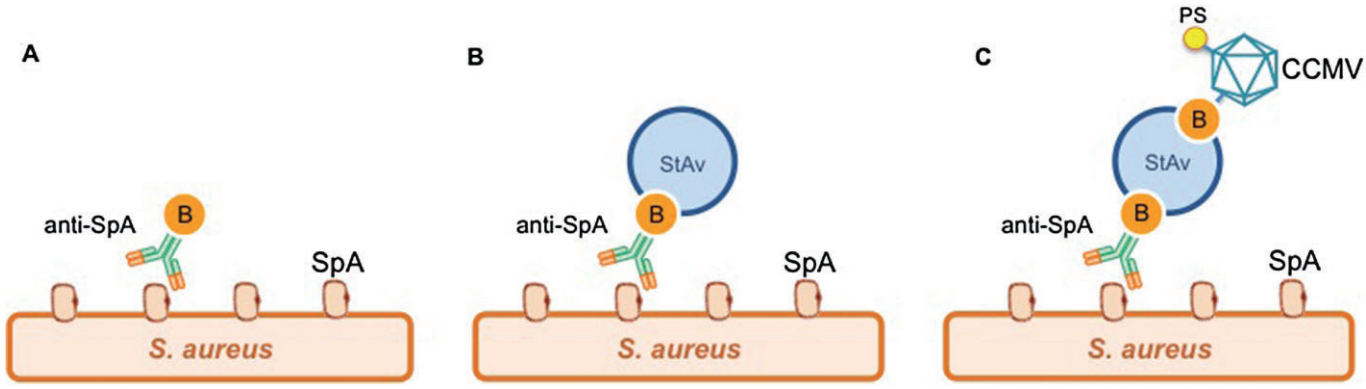

Figure 3

Schematic representation of virus-based nanoparticles engineered for the targeted treatment of bacterial infections. (A) Biotinylated anti-SpA monoclonal antibody (anti-SpA-B) binds to SpA expressed on the surface of *Staphylococcus aureus*, followed by (B) Streptavidin (StAv) binding before (C) binding of biotinylated CCMV, functionalized with a photosensitizer (PS).

(Figure 3C). These target-functionalized CCMV particles were used to penetrate and effectively kill *S. aureus*, demonstrating the potential of such nanoplatforms in the treatment of biofilm-associated infections (Suci *et al.*, 2007).

Although the use of icosahedral viruses remains highly promising, attention has more recently shifted towards the use of bacteriophages for the detection and treatment of bacterial infections (Carson *et al.*, 2010; Chibeu *et al.*, 2012; Hodgson, 2013; Mendes *et al.*, 2013; Seth *et al.*, 2013; Takemura-Uchiyama *et al.*, 2013; Vandenheuvel *et al.*, 2013; Yilmaz *et al.*, 2013). Bacteriophages have been used in the past in Eastern Europe and the former Soviet Union to treat bacterial infections; however, it is only over the past two decades, with the rising problem of antibiotic-resistant infections, that their potential use has re-emerged worldwide (Kaur *et al.*, 2012). Bacteriophages are considered ideal therapeutic systems owing to their natural specificity for bacteria. In addition, bacteriophages are effective and significantly cheaper to produce (and hence offer low scale-up costs) compared with antibiotic therapy (Kaur *et al.*, 2012), making them attractive platforms for applications as antibacterial nanomedicines. There are two types of bacteriophage infections, lytic and lysogenic. However, it is mostly lytic bacteriophages, which destroy bacteria rapidly, that are used for therapeutic applications. In one of the first reported examples, Yacoby *et al*. demonstrated the potential use of bacteriophages as drug carrier systems for the targeted treatment of bacterial infections. In this example, the antibiotic chloramphenicol (as a prodrug) was tethered to the exterior of the bacteriophage M13 subtype A12C. Although normally specific for *Escherichia coli,* the M13 bacteriophage displayed the highest affinity to *S. aureus* in a phage ELISA assay. The bacteriophage was targeted to *S. aureus*, where cleavage of the prodrug linker by esterase induced drug release, effectively retarding the growth of this bacterium in serum (Yacoby *et al.*, 2006). Despite the relatively slow kinetics, this allowed the targeted system to localize to the site prior to efficient release of the drug (see 'Toxicity and biodistribution' below). By optimizing the targeting with antibodies, the researchers further achieved complete growth inhibition of *S. aureus, S. pyogenes* and *E. coli* in serum (Yacoby *et al.*, 2007).

As an alternative to their use as drug carriers, an *in vivo* detection method has been reported for the optical imaging of bacterial infections using bacteriophage M13 (Bardhan *et al.*, 2013). Bacteriophage M13 naturally targets *F*-pili-expressing and *F*-negative strains of *E. coli*. In this work, fluorescent dyes were conjugated to the surface of M13. Biotin acceptor peptides were expressed on the surface of M13, which were enzymatically biotinylated using a biotin–protein ligase and reacted with streptavidin-complexed antibodies against a certain bacterium. Specifically, the authors report the development of an anti-*S. aureus* M13 fluorescent probe that was able to selectively localize and target *S. aureus* infections. Future coupling with the abovementioned drug carriers could lead to the development of bacteriophages with dual functionality.

Immune stimulation

Viruses and virus-like particles can be engineered or developed to stimulate an immune response against their cognate virus, or alternatively, as molecular platforms for the presentation of foreign epitopes (Grasso and Santi, 2010). To date, virus-based nanoparticles have been successfully used to target and neutralize self-proteins (i.e. immune stimulation) for the treatment of non-communicable chronic ailments such as inflammatory autoimmune diseases, allergy, Alzheimer's disease, hypertension, cardiovascular disease, cancer, chronic respiratory disease and diabetes (Chackerian *et al.*, 2006; Jennings and Bachmann, 2008; 2009; Do *et al.*, 2010; Röhn and Bachmann, 2010; Bachmann and Jennings, 2011; Klimek *et al.*, 2011).

For example, the pro-inflammatory cytokine IL-1β is thought to play an important role in the development of type II diabetes. Cytokines are very important factors in the pathogenesis of autoimmune and chronic inflammatory diseases. Treatment with high affinity anti-cytokine monoclonal antibodies is subject to limitations, as the treatment of chronic diseases requires frequent administration, which carries the risk of progressive antibody resistance over time (Aarden *et al.*, 2008). In a recent example, IL-1β coupled with bacteriophage Qβ was shown to successfully reduce type II diabetes in animal studies (Bachmann and Jennings, 2011). Efficacy studies with these modified Qβ conjugates in mouse and primate models were highly promising, and this vaccine has since entered phase I clinical trials for the treatment of mild type II diabetes to determine its toxicity, safety and immunogenicity.

Toxicity and biodistribution

For potential applications in biomedicine, the toxicity, biodistribution and pharmacokinetics of virus-based nanoparticles are all essential factors that determine their overall viability. One of the major challenges is finding an appropriate balance between tissue penetration, accumulation and systemic clearance (pharmacokinetics) (Steinmetz, 2010). On one hand, longer circulation times allow drugs to accumulate in target tissues (Yacoby *et al.*, 2007); however, on the other hand, prolonged circulation also increases the risk of exposure and potential toxicity. Recent studies suggest that pharmacokinetic properties are dependent not only on size and morphology, but on the composition, surface charge and surface modifications. In designing virus-based nanocarriers, Steinmetz and co-workers recently described some key physical parameters that influence the pharmacokinetic properties (Wen *et al.*, 2013).

Morphology. There is still an ongoing debate over whether spherical or elongated and rod-like assemblies are better cargo carriers for nanomedicine. On the one hand, spherical particles provide a higher surface-area-to-mass ratio, which is advantageous for improving cell targetability. On the other hand, model studies by Champion *et al*. showed that elongated filaments or particles are significantly more effective in avoiding clearance by phagocytosis compared with spherical nanoparticles (Champion and Mitragotri, 2006). By minimizing phagocytosis, the efficiency of delivery (as well as circulation times) would be improved; hence, a lower dose would be required, and potential side effects would also be minimized.

Targeting and multivalency. One common approach to promoting or enhancing cell uptake and internalization is to incorporate receptor-specific ligands that can be used to target specific cell types and enhance cellular uptake via endocytosis. However, it should be noted that while multivalency and increased ligand density can enhance cellular uptake, there is a threshold for optimal ligand density and spacing on the outer surface.

Size. Virus based nanoparticles come in a variety of different shapes and sizes. While it has been reported that larger nanoparticles have a larger surface area and are more likely to accumulate in the tumour, smaller nanoparticles induce significantly weaker hydrodynamic and shear forces upon entry, allowing them to penetrate more deeply than large nanoparticles. Interestingly, it has been reported that size is of less importance than shape or morphology during initial cell internalization.

Charge. Here, it should be noted that the pharmacokinetic properties of synthetic and biological carriers differ significantly. For example, it has been reported that positively charged synthetic carriers such as polymers, dendrimers and DNA-based delivery systems can induce toxic effects by disrupting the membrane structure (Hong *et al.*, 2004; Garnett and Kallinteri, 2006), whereas positively charged virus-based assemblies interact with mammalian cells more efficiently, show enhanced tumour transport properties and exhibit longer retention times (Wen *et al.*, 2013). In addition, it has been shown that positively charged virus nanoparticles such as bacteriophage Qβ have long circulatory half-lives of more than 3 h, which is significantly longer than those of negatively charged virus particles such as CPMV and CCMV, which have reported half-lives <15 min (Kaiser *et al.*, 2007; Singh *et al.*, 2007). Detailed investigations on the plasma clearance kinetics of CPMV functionalized with Gd-DOTA were performed by means of intravenous injection in mouse models, and the biodistribution showed rapid clearance rates from plasma (<20 min). After 30 min, the majority of injected virus particles accumulated in the liver, and some in the spleen (Singh *et al.*, 2007).

While most studies involve intravenous injection as a means of administration of virus-based nanoparticles, the biodistribution of CPMV by means of oral administration has also been studied (Rae *et al.*, 2005). While there are many potential routes of administration for nanoparticle drug delivery, such as intravenous, inhalation, transcutaneous, ocular and oral, from a patient perspective, oral administration is often highly preferred. However, until recently, the fate of the (virus) nanoparticles was unknown. As CPMV particles are highly stable, have a slightly negative surface charge at acidic pH and are sourced from an edible host plant, Manchester and co-workers investigated the suitability of CPMV for uptake in the intestinal epithelium as a potential oral delivery agent for vaccine or therapeutic applications. Here, the authors investigated not only the distribution of CPMV in the gastrointestinal tract but also its stability (i.e. whether the particles stay intact or are degraded and excreted), which has been reported to be problematic for synthetic nanoparticles (Wells *et al.*, 1988; Damgé *et al.*, 1990; Rae *et al.*, 2005). Initial studies on the stability of CPMV particles under simulated gastric conditions confirmed that the particles were stable and remained intact. Subsequent studies in mouse models were performed by oral administration or intravenous inoculation, and the stability, localization and persistence time were reported. In the days following oral administration, CPMV was found to accumulate in the spleen, kidney, liver, lung, stomach, small intestine, lymph nodes, brain and bone marrow, suggesting a similar biodistribution pattern as by intravenous injection. Interestingly, in this work, CPMV was also found to be bioavailable when administered in an edible form in cowpea leaves, further demonstrating the feasibility of oral ingestion as a means to administer virus-like nanoparticles.

Conclusion and future directions

While there is a certain aspect of (chemistry) design in terms of the choice of a particular targeting sequence and the mode of surface functionalization, the design and implementation of virus and bacteriophage assemblies as nanomedicines is still in its early stages, with only very few examples currently in preclinical trials. One of the major challenges and limitations is that until virus assemblies are tested *in vivo*, their immunological effects cannot be predicted in advance (as observed in the vimentin-associated uptake of CPMV), and hence there is still a significant lack of knowledge (more importantly, *pre*knowledge) that currently prevents their rapid implementation as nanomedicines.

Unlike adenovirus, viruses derived from bacteriophages and plants are generally considered to be safe for human treatment. Animal studies have been performed for a wide range of plant-based viruses such as CPMV and CCMV, as well as for bacteriophages Qβ and M13, and despite the broad biodistribution, no toxicity was observed (Rae *et al.*, 2005; Kaiser *et al.*, 2007; Steinmetz, 2010). However, to date, most cell studies have focused on either *in vitro* biochemical assays, cultured cells or model studies. Although it is important to identify essential physical properties and to establish a general set of rules for the design of viruses as nanomedicines, a certain caution needs to be taken, as such design rules can only be considered as rules of thumb, with many exceptions already having been found that contradict established design principles. For example, despite their similarity in size and morphology, CPMV, Qβ, and M13 particles were found to accumulate primarily in the liver and spleen, while CCMV was also found in the thyroid gland, bladder and salivary glands (Singh *et al.*, 2007; Steinmetz, 2010), demonstrating that not all spherical nanocarriers are rapidly cleared from the body.

Finally, it should be mentioned that in the recent Human Microbiome Project it has become increasingly apparent that certain bacteria and phages live symbiotically in the human gut and skin and are essential to human health. Therefore, while we continue to design viruses and nanocarriers as nanomedicines, it is essential to consider their immunogenicity and their influence on the human microbiome (Kaur *et al.*, 2012). Only then will the future of virus-like particles in nanomedicine truly be known.

Conflict of interest

None.

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