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Viruses, Artificial Viruses and Virus-based Structures for Biomedical Applications

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Abstract

Nanobiomaterials such as virus particles and artificial virus particles offer tremendous opportunities to develop new biomedical applications such as drug/gene-delivery, imaging and sensing but also allow to understand more about biological mechanisms. Recent advances within the field of virus-based systems give insights in how to mimic viral structures and virus assembly processes as well as understanding biodistribution, cell/tissue targeting, controlled and triggered disassembly or release and circulation times. All these factors are of high importance for virus-based functional systems and therefore mimicking and enhancing or controlling these aspects to a high degree as is illustrated in this review for delivery and imaging.

1. Introduction

Viruses and virus-like particles often introduce fear into the heart of the general public but also still in fellow scientists. The uneasiness is mainly due to the role viruses play in some of the major global incidences such as Ebola, AIDS and Influenza. Although viruses are generally feared, they also offer tremendous possibilities to be used in (bio)nanotechnology applications.^[1–8] Virus particles (VP) and virus-like particles (VLP) have several distinct features which can be utilized for (bio)medical applications such as drug and gene delivery, imaging, biosensors, anti-microbial agents and of course they are already used as a vaccine.^[9–12] Additionally, also in electronics, scaffold particle materials are utilized for defined structure formation and other functional surfaces and materials.^[13]

The reason for using VPs and to mimic these particle is based on their defined shapes and surface properties, their infectious nature, the availability from biomass (infected species, or bacterial expression) and the possibility to modify them genetically and chemically. These attributes make VPs multi-functional and broadly applicable as well as an inspiration for new materials. Furthermore, they are a powerful tool to better understand biological mechanisms.^[14–16]

Here we show how VPs are being mimicked in their structure, assembly processes and specific interactions with genetic material as well as some recent developments in changing their properties via genetic and/or chemical modifications (**Figure 1**). Additionally, we discuss examples of biomedical applications as imaging, delivery or viral scaffolding and provide still existing bottle-necks which hamper the use of VPs and VLPs in biomedical applications. The use of artificial virus particles as vaccines which is one of the most important applications, has recently been reviewed in several excellent articles we would like to refer to and is thus not discussed here^[17–20] Recent developments show that VPs can be targeted completely synthetically from structural features, to assembly processes and infecting

capabilities.^[21] Such new routes will open up the possibilities to standardized methods and providing large-scale production allowing VLPs to be used commercially to a greater extent. This limitation so far hampers true major breakthroughs in utilizing VPs and VLPs.

Figure 1. Different strategies to create artificial virus structures ordered by increasing artificial proportions discussed in this review: (a) functional molecules are adsorbed or attached chemically (b) the capsid contains a genetic modification (c) either a hollow capsid is filled by a substance or a capsid is assembled around a template (d) the entire coat protein is artificially engineered (e) the natural virus is only used to generate a negative copy of itself.

2. Mimicking Virus Structures and Viral Assembly Processes

VPs are hierarchically built, meaning that there is one or a few types of small coat proteins which assemble around the genetic material or in some cases also without.^[4,5,22] The build-up of virus particles from small components is associated with several features, which inspires us to use them and mimic their behavior. The defined self-assembly properties, the cooperative assembly process and the ability to assemble around genetic material (DNA, RNA) and thereby encapsulating it. Studying such properties will provide more insights in how viruses function, deliver their cargo and use these properties to construct new nano-, micro-, meso- and macromaterials. The different aspects have been studied by many in great detail. This efforts have led to new synthetic systems which are able to assemble around genetic material such as DNA. This process resembles coat proteins assembling around DNA/RNA.^[23,24] Self-

assembling peptides are frequently used to form novel self-assembling structures. The attractive feature is that peptide-based materials are not only capable of utilizing the same driving forces as found in natural peptide-based systems, they can also be genetically altered to introduce other specific features such as positive charges capable of interacting with negative polyelectrolytes e.g. RNA or DNA but also synthetic polymers. Two such works have been recently published by Ruff *et al.* and Garcia *et al.* who used peptide-polymer conjugates and fully peptide-based structures, respectively.

In the work of Ruff and co-workers, a triblock structure was used containing a cationic spermine (Sp) unit, a coiled-coil (CC) domain and a polyethyleneglycol (PEG) block.^[23] The coiled-coil allowed for discrete self-assembly producing small coiled-coil structures. The PEO ensures solubility and the three blocks combined, Sp-CC-PEG initially self-assembles into a small coiled-coil aggregate. This aggregate possesses a domain of multiple positive charges due to the clustered Sp-units which then coordinates via charge interactions with negatively charged double stranded DNA (dsDNA), forming elongated assemblies. The approach displays a very interesting hierarchical assembly, which also often occurs in natural systems where few protein units assemble initially and target a particular area of the RNA or DNA strand and then further assemble. While in this case a peptide-polymer conjugate was used, Garcia and co-workers achieved a similar system based on only peptide sequences which could be synthesized in a single step using yeast as an expression system.^[24] This offers a convenient approach since the synthesis using yeast can be scaled in principle to semi-industrial or even industrial scales. For the peptide-based system a diblock was used. A relatively small block of twelve amino acids (12 lysine residues or 6 histidine-lysine dyads), which has affinity towards DNA was connected to a collagen-like block of 400 amino acids long and this readily assembled around supercoiled plasmid DNA.

The length of the assembled structure as presented by Ruff *et. al* could be tuned simply by adding longer polynucleotide sequences. The assembled structure in both studies discussed provide elongated assemblies which are highly flexible indicated by low persistence length. In nature, flexibility is very diverse and although virus structures such as Tobacco Mosaic Virus (TMV), Potato Virus X and M13 Bacteriophage are all considered rod-like virus particles, the rigidity is very different. In order to obtain rigid assemblies of controllable length, the natural system is combined with genetic material of varying length as was shown by Ma *et al.* They used TMV which is prone to self-assemble also in the absence of RNA and then produces structures of varying length (**Figure 2**).^[25] When starting from the disassembled system and offering the solubilized coat proteins RNA strands of varying length, the length of the assembled rods was controlled allowing to work with a more homogeneous particle system.

Figure 2. Using the virus protein subunits, shown here for TMV, and offering RNA-templates enables the control over the length of the virus structure which can then be shorter as well as longer as compared to the wild-type structure.

To obtain also more rigid systems utilizing completely synthetic coat protein mimics would be interesting since then creating VLPs will be more in line with natural systems since in nature the flexibility is varied as well. In 2014, a true virus mimic including infection shown as transfection of the plasmid DNA in HeLa cells was developed by de Vries and co-workers.^[21] The only aspect missing is the self-replicating nature but all other aspects also observed for TMV were mimicked using completely synthetic triblock polypeptides expressed in yeasts. The triblock is composed of an oligolysine which is coordinating to the dsDNA template. A silk-like block drives the self-assembly process in a cooperative way in the presence of DNA. VPs assemble generally in a cooperative fashion to maximize the formation of active and infectious particles, which ensures the “survival” of the virus since partly assembled structures will not function properly. De Vries and co-workers managed to even mimic this behavior by introducing a silk-like motif between their initial DNA recognition and collagen-like block. The silk-like motifs will interact and stack on top of each other, choosing the block size carefully introduced the cooperative effect. They found that only a specific combination would function optimally. The cooperativity causes that only fully assembled particles were formed even in the presence of excess genetic material. This biotechnology approach for synthesis along with a careful design of the coat protein mimic with the precise relative ratio between the three blocks is a highly innovative incentive to the field of artificial virus particles.

At this point complex VP and VLP can be constructed which greatly resemble the natural structures. These are formed either using natural protein structures combined with non-native genetic material, natural-synthetic hybrids or completely natural based de novo designed virus structures.^[11,26–31] Although it is interesting to mimic the complete virus structure in great detail, in many cases taking only parts of the virus structures can be enough to induce new effects and is often a simpler approach. Hence,

on many occasions coat proteins are simply used with available artificial synthetic templates either organic or inorganic.

The use of templates for the assembly of virus capsid subunits often include inorganic nanoparticles onto which the subunits adhere, usually via electrostatic interactions.^{[32–}

^{34]} For the assembly around nanoparticles globular virus particles are used because they can accommodate the double curvature more easily than rod-like ones since those only have one curvature. Using a predefined template which dictates the size and shape of the artificial virus structure is very pragmatic and renders templates biocompatible. In principle possible interactions that are associated to the virus surface structure are transferred to the template. It can be debated whether or not such complexes should be regarded as artificial VPs or VLPs since it is simply a coating, which is also possible with other non-viral proteins. However, when it is the intention to use viral surface interactions, then the approach offers an easy and generally applicable approach to biofunctionalize nanostructures which would otherwise be toxic and not biocompatible e.g. quantum dots.^[13,35]

It is possible to assemble globular virus particles using an undefined template such as polyelectrolyte. Similar as the rod-like virus particles, negatively charged polyelectrolyte polymers can be used instead of the genetic material.^[36–38] For globular virus particles, the assembly is somewhat more critical. Rod-like VPs such as TMV will shorten or lengthen their structure based on the length of the polymer without altering their packing.^[25] However, globular VPs rely more on their symmetry to arrange the protein subunits, which is also reflected by the change in stability of subunit coatings around differently sized nanoparticles. The larger the size mismatch, the less stable the coating associated with a change in overall symmetry. The same applies to increasing the length of the polyelectrolyte since all polymer needs to be encapsulated by the virus capsid. The longer the polyelectrolyte polymer, the more

subunits are needed to encapsulate the polymer completely which alters the symmetry.^[38,39]

Only taking the polymer length as a determining factor would be over-simplifying the complex process of virus assembly. In addition to the polymer length also charge density, charge distribution, charge density on the protein subunits and medium ionic strength play crucial role in the assembly process.

In the structures mentioned so far, VPs are mimicked, used or modified. Another approach to make use of VPs is to extend the hierarchy found in the native structures beyond the natural sizes and shapes. One can utilize the assembly process, surface properties and polyanion/genetic material recognition capabilities of VPs to construct micro- and mesostructures with a high degree of morphology control. Achieving control over the VP assemblies is important for tuning the properties of the final structures. An interesting feature for such endeavors is that similar adhesion and coordination approaches can be used for mimicking the assembly behavior around the genetic material as discussed earlier.

Eber *et al.* used a nanoparticle template which was coated with different densities of RNA which is recognized by the TMV capsid protein subunits. These are able to self-assemble around the individual RNA-strands producing close to native TMV structures.^[40] Both density and length of the assembled virus structure can be controlled making it a very elegant approach for complex nanohybrid structures (**Figure 3A**). The same approach is also possible on planar surfaces allowing for a virus coating to be formed.^[41]

Belcher and co-workers used phage display methods to modify M13 bacteriophage at one end having a different composition than the rest of the virus (Figure 3B). Specific binding peptides can be expressed at this end such as streptavidin binding peptides.

This means that any structure displaying streptavidin moieties on the surface, the M13

will bind to it only with one end.^[42] The remainder of the surface is then still available for other modifications having other binding motifs displayed. This system offers the opportunity to bind complete structures selectively in a highly controlled fashion forming linear single substituted, linear double substituted and triangular triple substituted structures around a single inorganic core.

Figure 3. TEM images of complex inorganic-virus hybrid structures with novel morphologies. **A)** core coated with different density RNA-strands which increases the density of assembled TMV particles on the surface (Adapted from ref. [40] with permission from Wiley). **B)** TEM analysis of M13 bacteriophage which has adhesion peptides towards streptavidin on one end of the virus particle and thereby controlling the attachment of the particles onto a central streptavidin labelled inorganic core

(Adapted from ref. [42] with permission from American Chemical Society). C) TEM images of genetically modified potato virus X (PVX) displaying biomineralisation peptides on the surface which induces SiO₂-core formation and thereby incorporating PVX particles while the PVX surface is still accessible for immuno-gold labelling (Adapted from ref. [43] with permission from Wiley).

The system where TMV assembled around RNA-coated nanoparticles has similar morphology as the approach developed by van Rijn *et al.* but larger. Genetically modified PVX particles displaying silicification peptides on the surface were used which were able to convert TEOS into SiO₂.^[43] While the combination with TEOS gave isolated nanoparticles on the surface of the PVX, mixing TEOS and 3-aminopropyltrimethoxysilane in a 1:1 ratio induced a novel star-shaped mesostructure. The structure was organized around a mesoporous silicon dioxide core with PVX particles protruding from it which were attached at one end (Figure 3C). Virus particles may display different reactivity at either end however, via immuno-gold end-labelling it was verified that there was not a specific particle-end connected to the mesoporous core. The 3-D nature of the star-shaped particles was determined using cryo-TEM tomography. It has to be noted that PVX-wt (PVX wild type, the naturally occurring form) did not display any structure formation indicating that the biomineralization process is crucial for structure formation. It was shown that after the mineralization process, the PVX surface was still accessible for immuno-gold labelling and thereby adding additional features to the already complex architecture.

Different approaches may lead to similar structures. The approach by Eber *et al* and Belcher and coworkers is more controlled and hierarchically build. The last approach was based on simple mixing without any special designs other than the genetic modifications of the PVX. Morphology control and system complexity often are associated and the preferred approach

depends on the final application. VPs and VLPs are currently extensively investigated, too numerous to discuss them all, therefore the focus for the remainder will be on biomedical applications such as imaging, scaffolding and delivery.

3. Altering Chemical Properties of Virus Structures

One of the major hurdles and still a continuous endeavor is to modify the surface properties by changing the surface chemistry in order to include properties such as binding affinity, non-binding affinity (stealth), alter surface charge or even increase virus particle stability towards organic solvents and otherwise dis-assembling conditions. There are two approaches which are generally used: chemical or genetic modifications or combinations thereof. Genetic modifications enable the inclusion of non-native amino acids into the coat protein sequence and thereby exchanging or introducing single amino acids as well as polypeptide sequences when incorporating chain-end extensions either at the C- or N-terminus. The single amino acid addition is very convenient in combination with chemical modifications. This way several functional chemical groups can be introduced reactive towards specific types of modifications for which excellent reviews are available.^[44,45] Many virus particle surfaces are relatively unreactive and cannot be targeted by conventional peptide chemistry. Thus they need more sophisticated approaches or rely on the introduction of lysine (-NH₂), aspartic or glutamic acid (-COOH) or cysteine (-SH) which are able to participate in more conventional peptide chemical approaches (**Figure 4**).

Figure 4. Frequently used reactions for chemical modification of proteins with synthetic components. Most frequently used approaches for the modification of **A)** Lysine: 1) amide formation following standard peptide coupling approaches; 2) urea or thiourea formation using isocyanates or isothiocyanates, respectively; 3) Schiff base formation with subsequent reduction (3') to a secondary amine; **B)** Cysteine: 1) thioether formation; 2) Michael addition; 3) disulfide formation; 4) allylsulfide formation followed by olefin metathesis (4'); **C)** Tyrosine: 1) Tsuji-Trost reaction a palladium catalysed coupling of allylic acetates; 2) diazonium salt addition forming azobenzene derivatives used as handles (depending on "X") for further modifications such as Schiff base addition, Diels-Alder reaction or a copper catalyzed 1,3-dipolar cycloaddition reaction.

The addition of peptide sequences at the chain ends allow for the incorporation of attributes such as signaling, recognition, binding but also has great chemical relevance

for catalysis or biomineralization.^[4,46,47] These are valuable assets for biomedical applications as biological interactions and cell signaling are dominated by peptide sequences and fragments but also for applications in regenerative medicine.^[47] It is not the intention to fully review procedures for genetic and chemical modification of protein structures as there are many excellent reviews on this topic.^[48–51] Alternatively, a few chemical approaches are highlighted which are non-standard approaches developed for coping with relatively unreactive virus particles.

TMV is one of the lesser reactive virus particles basically lacking any surface exposed amino acid readily available for conventional peptide chemistry. Francis and coworkers developed a mild strategy to chemically modify the surface exposed tyrosine via the formation of a diazonium salt of an electron deficient aniline derivative. This was used in an electrophilic aromatic substitution reaction forming an azobenzene on the ortho-position of the tyrosine.^[52] The easy and mild approach opened up new possibilities changing the surface chemistry with genetically incorporating more reactive amino acids. However, the reaction is limited to electron deficient anilines. A very elegant use of the approach using a 3-ethynylaniline derivative as the diazonium precursor allowed for subsequent modification via the so-called “click-reaction” where an alkyne is coupled to an azide via a copper catalyzed 1,3-dipolar cycloaddition reaction forming a triazole derivative.^[53] The click-reaction is currently a well-established method to modify virus particles and proteins as it conveniently adds chemical functionality for further modifications such as polymerization initiators (e.g. Atom Transfer Radical Polymerization), fluorescent labels and binding motifs.^[54–57]

Tailoring the properties of virus particle enable the fabrication of more complex systems or allow for convenient post-modifications. Introducing surface charges via genetic modifications facilitates charge interactions. A frequently used and easily

performed approach is layer-by-layer deposition of charged polymers. Introducing a strongly negative surface charge will allow cationic polyelectrolytes to adhere. When a strongly positive surface charge is used, a negatively charged polyelectrolyte is attached.^[58] This process can be repeated thereby building a layered structure of alternately positively charged and negatively charged polyelectrolytes. That this approach proved to be successful for achieving enhanced transfection in vivo using baculoviral vectors coated with polyethylenimine which was electrostatically bound.^[59] The coating was effective in reducing serum-mediated inactivation which was considered to be associated with complement-mediated inactivation thereby enhancing treatment.

Altering the surface properties facilitates various biomedical applications. Below we will address virus structures for imaging, scaffolding and delivery which are among the most prominent applications of virus particles. The review finalizes with still remaining issues which are thought to be potentially solved by means of chemistry and genetic alterations.

4. Virus and Virus-based structures for Imaging

In the last years virus and virus-based structures have been increasingly used for imaging and detection purposes.^[60,61] Virus and virus-based structures entail both chemical and genetic modified particles as well as using the virus as carrier or the virus coat protein subunit as a coating material. Advantageous is that their structure can be controlled quite well. Both the protein shell and their core can be modified genetically and/or chemically (**Table 1**). Using the hollow capsids is also possible. Size and shape of virus particles have unprecedented reproducibility in nanoscience. They also often have the ability to self-assemble from their components under the right conditions. Additionally, virus raw materials can be produced in host organisms and thus the synthesis does not require toxic solvents or high temperature.^[40]

Table 1. Strategies for Artificial Virus Approaches

Modification	Virus	Application	Ref.
Genetically modified coat protein	PVX, TMV	Optical imaging	[63]
Chemical modification on coat protein	CPMV	Optical imaging and targetting	[42]
Hollow capsid	P22	Magnetic imaging	[64]
A combination	CPMV	Tumour targeting and optical imaging	[41]
Entirely artificial virion	Entirely artificial	Delivery and protecting DNA	[21,46,47]
Negative copy	TMV, HRV, TYMV (negatives)	Virus sensors, separation, artificial antibodies	[48–51]

Overview of different strategies to generate artificial viruses (Abbreviations: CPMV Cow pea mosaic virus, HRV human rhino virus, PVX potato virus X, TMV tobacco mosaic virus, TYMV turnip yellow mosaic virus)

4.1. Different choices of virus particles

All kinds of different virus particles have been used already for imaging purposes.

However, plant viruses and bacteriophages are particularly prominent since they are less likely to be pathogenic to humans and relatively easy to obtain.

Other factors to be considered are the size and shape of the virus particles. Mostly small labels are preferred since they are less likely to influence the properties (as biological function or surface chemistry) of the material, which should be labelled.

However, especially large viral capsids can offer the advantage that one can load larger amounts of labels. Some authors also chose large viruses for lower diffusion or tumbling rates.^[50] Shape can be an important factor if a certain natural property of the virus should be maintained. Elongated shapes for instance have favorable flow and tumor penetration properties.^[66,67] Other points, which are often considered, are the availability, yield and how well the structure and their properties are known. In addition to these general points there are a few virus particles, which have especially desirable properties. The cowpea chlorotic mottle virus for instance swells reversibly into a larger structure with defined holes⁵³. This stage can be utilized to load the

particle with different molecules. Defined holes can also form in red clover necrotic mosaic virus (RCNMV) when magnesium and calcium ions that are associated with it are removed.^[9,69] This effect can be used to load a cargo. On the other hand, the RCNMV has the disadvantage that its capsid is very unreactive. This obstacle can be circumvented, however, by introducing a certain assembling motif to the cargo molecules. Cowpea mosaic viruses (CPMV) on the other hand are valued for their high stability over a wide pH range or even if exposed to organic solvents.^[54] An attractive alternative is using entirely artificial virus like structures. To achieve this goal several proteins or peptides have been successfully assembled into complex symmetrical molecules.^[71,72] Matsuura has recently reviewed the design of such protein assemblies.^[30]

Table 2: Examples of particles based on different virus structures and their size as well as advantages and drawbacks (TMV: Tobacco mosaic virus, CCMV: Cowpea chlorotic mottle virus, RCNMV: Red clover necrotic mosaic virus, CPMV: Cowpea mosaic virus, HBV: hepatitis B virus)

Particle	Size	Pros/Cons	Ref.
TMV	Rod 18x300 nm	+Rod shaped structure allows penetration +well characterized, relatively simple +Non-pathogenic to humans -/+ relatively large	[52]
CCMV	30 nm	+reversible swelling into structures with defined holes +relatively easy to load +Non-pathogenic to humans	[73][74]
RCNMV	36 nm	+reversible swelling into structures with defined holes +Non-pathogenic to humans +relatively easy to load -unreactive capsid	[29,69,75]
CPMV	28 nm	+stable over a wide pH range (even in organic solvents) +/- relatively small +well characterized	[76–78]
HBV	42 nm	-human pathogen +very efficient self-assembly in different expression systems	[79,80]
Entirely artificial	different	-reproducibility +flexible (properties can be tuned)	[21]

4.2. Strategies to attach labels for imaging applications

There are several different approaches to generate artificial viruses for imaging. The options can be divided into chemical attachment/loading of labels and genetic modifications as was

described in more general terms above. The different approaches are summarized in Figure 1 illustrated above.

4.2.1 Chemical attachment/loading with labels

The first approach is based on labels, which are attached to the virus particle or loaded inside. The advantage of this approach is that the labels can be applied after assembling virus particle and thus the assembling process is not altered.

There are two different synthesis strategies for loading virus particles. The first one is using a nanoparticle core and self-assemble the capsid around it.^[57] This has been achieved for a variety of particles also used for imaging (**Figure 5A**).^[34,82–84] However, negative charges on the core particle surface are crucial.^[60] Interestingly, artificial virus particles can in some cases also form around nanoparticles which exceed the size of the inner cavity.^[59] However, as one would intuitively expect these structures are more prone to errors and defects in the capsid structure.^[61]

The second strategy starts with a preassembled (hollow) virus particle and the label is self-assembled inside.^[68,87]

Alternatively, labels can also be attached on the outside of the virus capsid.

Finally, also a combination of different strategies can be applied to attach different functional units. Steinmetz *et al.* demonstrated the attachment of multiple functional units onto a virus particle.^[43] They used CPMV with a targeting ligand to accumulate in prostate cancer.

Additionally, they attached polyethylene glycol (PEG) to increase circulation time in the blood flow and an IR dye to the capsid.

4.2.2 Genetic modifications

The second approach requires a genetic modification of the virus particles. To this end fluorescent proteins are genetically incorporated directly in the virus particles.^[84,89] This

approach allowed Oparka *et al.* to identify virus infections in plants. Infected cells expressed virus particles including green fluorescent protein (GFP) and thus the infection dynamics could be followed. Compared to the first approach this method has the advantage that no further processing steps are needed after virus harvesting. However, a potential problem is that the additional protein parts might hinder self-assembly of virus particles. To account for this fluorescent proteins have to be placed at a position that does not hinder the assembling. place the. If the viruses should still be infectious this is even more crucial.

4.3. Optical Imaging

In order to use artificial virus particles for optical imaging one needs to attach a fluorescent dye. In principle all the above described techniques can be utilized to achieve this goal. Loading the virus particles with dye has the advantage that the dye can be protected from quenching.^[84,90] Some examples of optical imaging with artificial virus particles are shown in (Figure 5A-C). This approach was used by Lewis *et al.* for vascular imaging of the tumor microenvironment (Figure 5B/C).^[90] For their study they used cowpea mosaic virus, which was loaded with different dye molecules. Using IR dyes is particularly favorable due to the low background fluorescence of biological samples in this range of the spectrum and the advantageous tissue penetration.^[43]

An interesting alternative for dyes is using a quantum dot. This approach was used by Dixit *et al.*^[31] and Li *et al.*^[66] who loaded brome mosaic virus (BMV) and the simian virus 40 (a mammalian virus) with quantum dots. These quantum dots have the advantage that they are very photo-stable and do not bleach. Thus, they allow for long term monitoring.

Attaching dyes on the outside has been realized also with organic dyes as well as quantum dots.^[67]

Figure 5. VLPs for optical imaging: (a) Fluorescence image showing the uptake of PVX (green) into HT-29 cells (cell membrane, red, nucleus, blue, Scale bar = 20 μm) (b) Tumour induced vasculature (model in chicken embryos) stained with fluorescent CPMV particles (red, cell nuclei: blue, Scale bar = 16 μm) (c) Zoom in on image of (b) (scale bar = 5.5 μm) Reprinted with permission from ref. [84,90].

4.4. Magnetic imaging

Another imaging method where artificial viruses have been utilized is magnetic imaging (for examples see **Figure 6**). VLPs with a “natural” protein coating containing a contrast bearing core have proven to be useful contrast agents.^[68] Using this approach has several advantages in addition to the ones mentioned in the general introduction of imaging techniques. Relatively large magnetic particles with a reproducible and narrow size distribution can be stabilized. Furthermore, since the particle is enclosed by the viral capsid, the metal is protected from biodegradation. Additionally, potentially toxic effects from the metal can be reduced this way.^[69]

To achieve this goal several different contrast agent cores have been utilized. In principle one can differentiate between longitudinal contrast agents (or T1 contrast agents) or transverse contrast agents (or T2 contrast agents). These can be used for different imaging modalities in MRI. Among the T1 contrast agents, Gd(III) is one of the most prominent examples. Qazi et al. successfully incorporated Gd(III) into P22 viral capsids^[50]. They chose this type of bacteriophage for its large size and the

resulting low tumbling rate. Another interesting property of this phage is that the cargo can be released by heating to 75°C. This causes the capsid to transform into the so-called wiffleball conformation, which has a larger size and 10 nm pores. Bruckman *et al.* used Gd loaded or coated TMV particles instead.^[68] They report that the elongated shape is beneficial for entering a tumor. Furthermore, they also synthesized Gd(III) loaded TMV particles with an artificial few hundred nm round shaped coat protein shell. Since they can tailor the size of the particle, this approach might be interesting for applications where non-naturally available shapes are needed. Smith *et al.* provide theoretical simulation about the self-assembly of such Gd(III) loaded virions.^[70]

Different iron oxide cores are suitable as T2 contrast agents. Malyutin *et al.* for instance used gold coated maghemite (γ -Fe₂O₃) particles which were incorporated into brome mosaic virus or hepatitis B virus capsids.^[78] FeO/Fe₃O₄ provides an alternative, which also has been utilized. The general approach is to functionalize these nanoparticles to allow self-assembly of viral protein around^[79]. Shen *et al.* for instance encapsulated Fe₃O₄ into engineered virus like particles based on hepatitis B virus (HBV)^[80]. Although HBV is a human virus, it is relatively convenient to use and available in a safe form due to its use as a vaccine.^[96] Furthermore, the fact that HBV proteins readily self-assemble in different expression systems is convenient.

Figure 6. Artificial virus particles utilized for magnetic imaging applications: (a) Magnetic image (T2) *N. benthamiana* leaf after infiltration with virus like particles with a magnetic core (b) transmission electron micrograph from HBV based particles with 6.1 nm iron cores. (c) T2 weighted magnetic resonance images of the above

mentioned HBV particles at different concentrations and different core sizes ((1) 3.4 nm, (2) 6.1 nm, (3) 11.7 nm) Reprinted with permission from [83] and [80].

4.5. Other imaging techniques

While magnetic imaging and fluorescence imaging of artificial virus particles has been utilized relatively frequently virus like particles could also be used for other imaging techniques. Although this is much less common, this might be interesting for future application. An interesting approach is to attach gold nanoparticles which can be used as labels for electron microscopy but are also visible optically.^[97] As other labels these can also be either used as a core^[34,98,99] with the VLP assembled around it or attached to the outside^[100,101]. Latham et al. for instance attached gold particles to artificial influenza viruses.^[71] These were used to visualize the particles under electron microscopy. In their study they investigated structural proteins and molecular interactions required for formation and release of influenza VLPs. Other imaging techniques where VLPs could be used as contrast agents in similar fashion as described here is PET scanning or radio-labelling.

4.6. Imaging specific structures

Additionally, to attaching certain labels it is often desirable to image the VLP at to a specific location as e.g. a tumor.^[72] For some viruses such targeting is to some extent already achieved by the native structure of the viruses which naturally accumulate in certain cell types.^[51] However, this process can also be improved by attachment of different molecules.^[43] Feasible examples for such molecules are: antibodies, peptides that interact with tumor cells or tumor vasculature or tissue specific ligands.

4.7. Drawbacks of using artificial viruses in imaging

A potential problem in using artificial viruses arises when the virus particles, which are used, are still infectious or if the infectious particles have to be used during the synthesis of the label. In this case necessary precautions have to be taken to avoid spreading the virus or harming people handling the material. This is especially problematic when working with viruses, which infect humans.

Another problem can arise during in vivo imaging from immune reactions towards the virus particles. Since the immune system has evolved several defense mechanisms to counter virus attacks these can limit their in vivo applications.^[73] Also care has to be taken since toxicology, immunology as well as clearance from the body it is expected to differ depending on surface chemistry, shape or physical properties of the particle.⁷⁴ Thus this has to be tested for every new artificial particle. For most VLPs this has not been tested yet but might seriously complicate their usability. For the particles that have been tested fast clearance and no toxic effects have been found. However, immune responses were observed in some cases (for instance to CCMV or CPMV based particles in mice, also see 6.2.).^[77,103]

Furthermore, a virus particle is usually larger than the simple dye/contrast agent molecule. This can pose a problem for some applications. Although viruses are relatively stable biomolecules, they can still be degraded by microorganisms or when subjected to harsh conditions. This can limit their lifetime or shelf life.

5. Scaffolding

An interesting approach uses the structure of the virus to generate a negative copy of a virus. The field of molecular imprinting has utilized this with the aim to generate artificial alternatives for natural anti-virus antibodies in different applications. The negative copies of viruses outperform natural antibodies in shelf life and reusability

and can also be used in organic solvents or other non-biocompatible conditions. The principle is shown in **Figure 7**.

Figure 7. Creating negative virus copies using the molecular imprinting principle. First virus particles are pressed into a prepolymer. While curing, functional groups in the polymer assemble around the virus. Simultaneously, the polymer is cross-linked and thus the orientation of these groups is preserved. When the virus particle is removed, cavities remain which reproduce the size and shape of the virus.

To synthesize such negative virus copies the VP is pressed into a prepolymer with high cross linker concentration. While the polymer is cured two reactions occur simultaneously: First, the functional units in the prepolymer self-assemble around the virus particle. This means e.g. that a hydrogen bond donor in the polymer will assemble next to a hydrogen acceptor on the virus surface. It has to be noted that hydrogen bonding is only an example and in principle all kinds of chemical interactions as charges, hydrophobic interaction, or pi-pi interactions can play a role. Second, the polymer is cross-linked. As a result the orientation of the functional groups is maintained even when the VP is removed. The remaining cavities resemble the virion in size shape, and surface chemistry. As a result these cavities can preferentially reincorporate the VPs that were used for the imprinting process over other particles. It is also believed that it is possible that the polymer remains to some extent flexible and that the recognition takes place via a so-called induced fit. Besides for virus particles

this approach has been successful to reproduce structures of small molecules,^[77] proteins^[106,107] or even entire cells.^[80] For general information about molecular imprinting the reader is referred to recent reviews.^[109–111] Molecularly imprinted polymers (MIPs) for viruses have mostly been utilized for chemical sensors for their respective virus template. This was for instance realized by Dickert *et al.* who synthesized an imprinted polymer for TMV or parapox virus ovis (PVO) on the gold surface of a quartz crystal microbalance.^[112,113] To generate the imprints they simply adhered virus particles to a glass plate and pressed them into a prepolymer (polyurethanes or polyacrylates). After removing the virions from the cured polymer they used the sensor to detect the virus in plant sap of infected tobacco leaves. Jenik *et al.* used a very similar approach to create molecularly imprinted polymers for HRV and foot and mouth disease virus (FMDV).^[47] The resulting sensor was capable of differentiating between different viruses. Remarkably, they were not only able to distinguish HRV from FMDV but even different serotypes of HRV (HRV1A, HRV2, HRV14 and HRV16). The work of Dultsev *et al.*, who also followed a similar approach, is remarkable due to the high sensitivity of 140-150 viruses which could be detected by their sensor.^[115] Another application for virus MIPs is affinity-based separation. Bolisay *et al.* demonstrated this by imprinting a flexible polymer hydrogel with TMV.^[48] These hydrogels can be used as stationary phases in chromatography. Since the polymer hydrogel has a higher affinity to it, it takes the template virus significantly longer to pass the column than it would take similar VPs.^[84] A slightly different approach was used to generate a sensor for human immunodeficiency virus (HIV) by Lu *et al.*^[85] They fabricated a sensor which was able to detect HIV in spiked human urine samples. The authors used a so-called epitope imprinting approach. This means instead of imprinting with the entire virus structure they imprinted with only a protein part

that is on the surface. The epitope approach has two advantages over conventional imprinting. First, one does not need to handle the entire (infectious) virus when making the negative copy. Second, the epitope approach is more suitable for virus particles with heterogeneous or varying surface structures.^[82]

5.1. Drawbacks of scaffolding systems

Since the functional groups in the polymer have to be complementary to the groups on the virus surface the method has to be optimized separately for different viruses.^[119]

Although seemingly endless choices of monomers are commercially available, the choice is restricted by the biological nature of the virus. This means that the polymer has to be chosen in a way that the native conformation of the virus is at least conserved in the prepolymer until the imprint is formed.

It might also pose a problem, that the virus particles are required for generating the imprinted polymers. This is especially problematic when handling human pathogenic viruses. Due to the possibility of incomplete removal of the template viruses, which might be released at a later step (e.g. when the polymer is swelling in a solvent, at elevated temperatures or polymer degrading conditions) also the final devices have to be handled with care. There are also a few issues specific for the use for artificial antibodies: The polymer particle itself might trigger an immune reaction during in vivo experiments.^[120] Furthermore, the resulting polymeric antibodies are relatively large and thus might pose a problem for circulation or uptake into tissues or cells. If a biodegradable polymer is used (which is usually preferred in clinical applications due to better clearance) the imprint structure might be destroyed or deteriorated or previously trapped virus particles might be released. Most of the safety issues, however, can be circumvented when using deactivated viruses or empty capsids.^[121] As mentioned above this safety concern is also circumvented when using the epitope approach.^[122] However, for the epitope approach to work one needs prior knowledge on which proteins or protein

parts are on the surface. Additionally, one might lose selectivity if the imprints are made in the wrong orientation (so that they would for instance recognize the inside part of a virus protein instead of the surface).

6. Virus Particles and Virus-like Particles for Delivery

Viruses are replicating by infecting a host, which means that genetic material needs to be delivered into the host cell in order to infect. This occurs with high efficiency which immediately stimulates the imagination providing ideas how to control this and use such infecting capabilities to deliver other genetic material than that of the virus.^[123,124]

Gene-delivery is therefore one of the applications which is often targeted but also to deliver other payloads.^[9] Delivery comprises of various important parameters including **1)** inclusion/encapsulation of bio-active components; **2)** release of encapsulated materials; **3)** location specific accumulation where delivery is intended; **4)** excretion from the host; **5)** circulation time and **6)** immune-response of the body towards the delivery agents. In the section below these parameters will be discussed.

6.1. Gene-delivery

When discussing artificial virus particles for delivery in the broadest sense ranges from genetically or chemically modified virus particles, non-viral protein systems for delivery of genetic materials, self-assembled small molecular systems and even polymer systems able to complex DNA combined with intracellular delivery. Since the important aspect for delivery is the ability of the virus to enter the cell and release its genetic material. Many approaches involving cell internalization combined with release of a payload is often regarded as a virus mimicking system and hence many variations are known composed of the systems mentioned above. Whether or not “artificial virus” is a proper term for something, which enters the cell and delivers

cargo is debatable however, since it is the general term used for all such systems, the same applies here. However, the focus will be on the use of actual virus particles displaying the actual protein capsid subunits or mimics thereof.

Gene delivery is still in its infancy however a cure for *severe combined immunodeficiency disease* (SCID) using gene transfer, be it ex vivo is currently available. Two other treatments are even approved. Firstly, Gendicine® treating head and neck squamous cell carcinoma (HNSCC) and the more recent Glybera® (alipogene tiparvovec) for the treatment of lipoprotein lipase deficiency (LPLD).^[125,126] These still ongoing developments and findings uphold the future promise to be able to cure many other diseases such as cancer, AIDS, cystic fibrosis, Parkinson's and Alzheimer's diseases, amyotrophic lateral sclerosis (Lou Gehrig's disease), cardiovascular disease and arthritis.^[127] Because of the still limited applications, many endeavors target new delivery systems. In vitro as well as in vivo, gene delivery is performed very frequently using adenovirus or adeno associated virus.^[128–131] The virus, unlike complexes of DNA with molecular or polymer structures such as N-[1-(2,3,-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), polyethyleneimine (PEI), poly (l-lysine) (PLL) or poly(2-(dimethylamino)ethyl methacrylate (pDMAEMA), has millions of years of evolution behind it.^[31,132] Therefore, it is already tailored to meet the requirements for entering/infecting/transfecting cells efficiently. However, the human body defends itself against invaders via the immune system and hence pure viral treatments only are effective for limited periods of time which is a critical drawback when prolonged and repetitive use is necessary.^[130]

6.2. Immune-response

Many investigations therefore target the outer surface of the VPs and VLPs to influence the response of the immune system but also to add species to target specific cells, follow and image the VPs and VLPs and deliver non-genetic therapeutics.

Similar approaches using nanoparticles have been nicely summarized by Medintz and coworkers.^[133] As mentioned earlier, there are various ways to target the surface.

Options are chemical modifications, genetically altering the protein capsid subunit expression, using physicochemical interactions to add charged species such as polymers or lipids to the surface.^[9,11,134–136]

One of the most frequently used approaches to circumvent the immune system is by the addition of polymers, particularly polyethyleneglycol (PEG), which are considered “stealth” polymers but also alternatives are being investigated since PEG is not unambiguously “stealth” and non-toxic with respect to the degradation products.^[137–139] Synthetic alternatives include poly-oxazoline derivatives which display similar behavior as PEG. These have also been used in combination with VPs which displayed reduced particle aggregation and increased stability towards high temperature.^[138,140] However, to the best of our knowledge, biomedical applications have not been investigated at present time using oxazoline-coated VPs. As alternatives, “wrapping” in biopolymers such as alginate is considered a potential approach to prevent a triggered immune response. Sailaja *et al.* encapsulated adenovirus containing the LacZ gene inside alginate microspheres and upon injecting either the encapsulated or non-encapsulated virus, a difference in vector-specific immune-response was detected in favor of the encapsulated system.^[141] Not only was the immune-response reduced, the expression of LacZ was of similar efficiency indicating that “smart wrapping” would aid in circumventing the immune-response drawback. Similar attributes were also found when the adenovirus was entrapped inside PLLA microspheres.^[142] So far many other biomaterials have been used to prevent triggering the immune response including PEI (polyethylene imine), HPMA (poly-N-(2-hydroxypropyl) methacrylamide), Chitosan and lipids.^[143] Although, the approach facilitates prolonged circulation and lowers immune-response, still the addition alone will not be enough to reach efficacy levels high enough to be considered therapeutically relevant.

6.3. Tissue targeting

Fooling the immune-system is an important attribute when using VPs and VLPs however; also there is the matter of getting the particles on the right location. When infecting isolated cells in vitro and placing these back after treatment is the most reliable way of knowing which cells are infected and for which purpose. When administering VPs and VLPs via systemic delivery then there is a significant chance that the majority of the particles will not reach their proper destination. In addition to the triggering of the immune-response, tissue targeting is another major drawback. There is no intrinsic directionality on the VPs and VLPs used which makes it difficult to determine where the particles go and how much one would need when the biodistribution is known. Although very recently, it was shown that “naked” TMV particles injected in mice with and without tumors displayed an altered biodistribution.^[144] This difference indicates that specificity is required to guide the particles under deviating circumstances to the same location/tissue/cell type. The general idea about targeting is that via the addition of recognition vector/peptides, cells and tissues interact/bind to certain structures (e.g. tumor tissue). It has to be noted that the particles are not actively brought to the area of interest. Instead they remain longer at these areas when applied systemically and thus accumulate. The delivery of particles to tumors is facilitated by increased porosity (“leaky”) of the vasculature and thereby collecting more particles. However, cell targeting and cell penetration can be enhanced using homing peptides such as RGD (Arg-Gly-Asp) which binds with high affinity to overexpressed α_v -integrins on angiogenic blood vessels. Although, frequently used, RGD is not restricted to tumor cells, overexpression does not mean that the relevant receptors are absent in on other cell surfaces. Therefore, there is a continuous search for new and alternative/more specific homing and cell penetrating peptides.^[26,143,145]

Using peptides as functional moieties for accumulation combined with VPs is an important combination since “empty” capsids devoid of genetic material can be expressed in bacteria and yeast to facilitate large-scale production. Genetically altering the protein structures by adding surface exposed peptides, which enable tissue/cell targeting, enhances the potential use of VPs in clinical settings. Also chemical addition of these peptides belongs to the possibility and can therefore be attached to the end of polymers, which in turn are tethered to the surface of the VP.

The group of Steinmetz is highly successful in establishing modification approaches and is able to combine several interesting properties and structures on a single particle including visualization, therapeutic, cell targeting and shielding using an engineered Brome Mosaic Virus (BMV).^[136] Although, the genetic engineering entails addition of a cysteine residue to enable easy modification chemistry, the approach is versatile. Similar approaches also have been demonstrated by others in combination with different types of virus particles such as Bacteriophage λ ,^[146] PVX,^[147] TMV,^[148] and many other.^[9,15] Similar strategies are also used for VLPs where the protein capsid subunits are sometimes combinations of protein capsid subunits from different but compatible viruses, called chimeric virus particles. This then allows for two different combinations of functionality. In the system designed by Deo *et al.*, a vaccination and drug delivery approach was targeted and together with surface exposed rscFv ligands, accumulating on LS174T cells (Human Caucasian colon adenocarcinoma), provides a good example of the versatility of such approaches.^[149]

6.4. Delivery of non-genetic pharmaceuticals

Not all VPs and VLPs are used for delivery of genetic material as also becomes clear from some of the examples above. Also as a drug delivery system (DDS), VPs and VLPs are recognized to be of great importance.^[11] Most often these are used to deliver anti-cancer drugs such as doxorubicin.^[9,75,136,150] This target is of course sensible as the

porosity of the blood vessels in cancerous tumors allows particles to pass more efficiently. Since most globular VPs and VLPs are hollow and can readily assemble/dis-assemble, they are just like many other protein cage structures, ideal to host small molecular, polymers, particles and even other proteins.^[11,75,80,135,151–158] It has to be noted that besides bioconjugation approaches, the covalent linking of bio-active moieties to the interior or the exterior of the virus particle is also a common way to deliver payloads.^[75,88,136,150,159]

Main advantages of using virus particles to incorporate bio-active components, which also hold for other non-viral cage structures, is that relatively toxic or delicate components are shielded and thereby their toxicity is decreased or their structure maintained *e.g.* prevention of degradation of proteins due to shielding from digestion enzymes.

In some cases, immobilization is enough to decrease toxicity of bio-active components. Confinement via electrostatic interactions or via supramolecular approaches can also help. Components such as doxorubicin and similar type of anti-cancer drugs can be loaded onto virus particle templates.^[75,160] This allows for the inner compartment to be used for additional components, which directly shows the broad applicability and tremendous number of possibilities using virus and virus-like particles. There are many viruses available in various shapes and sizes but the particles inspire as also shown earlier, to design new virus-like systems for complement and improve their use in biomedical applications. Rome and coworkers designed a nanovault based on an amphipathic α -helix unit derived from the non-structural protein 5A of Hepatitis C.^[161] The design allowed the subunits to self-assemble into a specific oval shaped structure of about 75nm in length and 40nm in width. The interesting aspect, in addition to the self-assembly process into defined structures and the hollow nature of the structure, is that the created interior was lipophilic allowing various hydrophobic drugs to be stored

inside such as doxorubicin, amphotericin B, retinoic acid and bryostatin 1.^[161] Such developments are highly important since many therapeutics are hydrophobic in nature and therefore poorly soluble in water. A simple disassembly/reassembly using virus particles will therefore not work since only few molecules will be entrapped. The delivery using virus particles can sometimes be triggered simply by a small pH change when being taken-up by lysosomes and thereby disassembly occurs and release as a result. Since most species initially end up in lysosomes, this is an often used strategy using polymer structures.^[162] Relying on such a release mechanism could potentially limit the application and therefore more sophisticated approaches are being developed. Fang et. al combined proteins such as lactoferrin with Fe₃O₄ magnetic nanoparticles and drugs (doxorubicin as well as paclitaxel) via a water (W) and chloroform (O) W/O/W emulsion.^[163] Subsequent modification with folic acid provides for targeting capabilities and drug release is initiated by high frequency magnetic fields inducing hyperthermia and drug release.

6.5. Circulation and Excretion

With in vivo systemic delivery, using vessels containing the active agents, body circulation times and excretion of the vessels is important. Longer circulation will provide more chance of delivery of the payload and proper excretion prevents accumulation of particles, which could then pose a problem due to toxicity. Therefore, together with circulation time, biodistribution and clearance from the body are important factors which only now are being identified. Steinmetz and coworkers are pioneering the field and comparing native filamentous, rod-like and globular virus particles together with PEGylated particles and determine the route through the body.^[164–166] Stealth like properties are indeed obtained using PEG-polymers on the surface of the particles but only in sufficient density and polymer length.^[165] Not only the polymer will enhance circulation half life, also the shape of the

particle influences this and rod-like structure had a longer circulation half life than the globular structure.^[164] Concerning the clearance, it was found that e.g. PVX accumulates in the liver and spleen while it is cleared via the hepatobiliary route (liver, gall bladder, bile ducts) and to a much lesser amount via renal pathway.^[166] Unfortunately, only PVX without PEGylation was examined for this and it would also be interesting to know whether this process becomes significantly different when coated with “stealth” polymers.

7. Conclusion

Refining and manipulating virus particles contribute not only to understanding biological mechanisms but also allows us to develop more sensitive and specific systems for applications such as biomedical imaging and drug/gene-delivery. Working systems are available in the form of cultivatable wild-type virus particles which can be modified chemically. However, biotechnological approaches for introducing peptide-based functionalities via genetic manipulations broadens the scope significantly.

Although virus particles and genetically altered variations of these are readily available, there is a tendency to go more into the direction where virus systems are mimicked both in assembly process as well as in infectious nature using non-viral components. By completely artificially mimicking every process provides fundamental understanding but also introduces the highest control possible.

For natural viruses or particles assembled from them, the bottleneck often is immunotoxicity or limited chemical functional groups. Using virus based systems in biomedical applications is still limited as the body will respond to foreign structures, trying to eliminate them which significantly reduce circulation times as well as biodistribution and clearance from the body. Only now these aspects are investigated using labelled wild-type particles but genetically altered particles as well as the addition of surface-bound polymers already demonstrated that in vivo events can be greatly affected by such alterations. Initial work shows that circulation

times are enhanced by the addition of “stealth” polymers which will greatly enhance biomedical applications where particles are injected systemically and need time to reach the site for either delivery, imaging or both. New molecular, polymeric, inorganic or peptide features are today still limited since structural organization and the control of shape/size uniformity is still extremely difficult. However, continuous improvement of complex particle design and synthesis still hold strong future promise despite the limited applications reached so far.

Another critical aspect is clearance from the body. As shown in section 4, virus capsid coat proteins subunits are often used to coat inorganic nanostructures or the virus cavity is able to encapsulate the inorganic structure. While proteins can be digested, the inorganic nanostructures be it gold or iron oxide will not be digested. Hence bioaccumulation in the liver poses then as a significant problem. This will not be the case when chemical or genetic modifications are being used which could be chelating agents for lanthanides such as gadolinium or europium.

Overall it is clear that virus-based therapies have not yet reached their full potential and is not yet as advanced with respect to commercial use as was expected many years ago. The recently developed systems discussed here along with many others not shown here offer a tremendous and highly sophisticated field of science. However, most virus-based systems are very well prepared on a laboratory scale by experienced scientists but the often complex multi-synthetic steps, purification methods hampers efficient upscaling. Therefore easier and more straightforward particle development with the potential of easy upscaling will be of great importance. Also in vivo testing is a complex and tedious endeavor and only pursued when enough corporate incentives have been raised to support the process.

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The table of contents entry

Virus particles have unique properties including a reproducible and controllable nanostructure, as well as their ability to deliver their genetic cargo. Thus they have inspired scientists to engineer them artificially. Our review covers the latest advances in this field and the use of artificial virus structures for biomedical applications.

Viruses

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Viruses, Artificial Viruses and Virus-based Structures for Biomedical Applications