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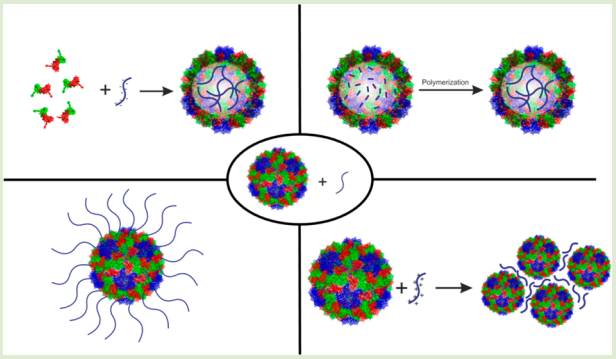
Combining Protein Cages and Polymers: from Understanding Self-Assembly to Functional Materials

Stan J. Maassen,[†] Anne M. van der Ham,[†] and Jeroen J. L. M. Cornelissen*

Laboratory for Biomolecular Nanotechnology, MESA+ Institute, University of Twente, P.O. Box 207, 7500 AE Enschede, The Netherlands

ABSTRACT: Protein cages, such as viruses, are well-defined biological nanostructures which are highly symmetrical and monodisperse. They are found in various shapes and sizes and can encapsulate or template non-native materials. Furthermore, the proteins can be chemically or genetically modified giving them new properties. For these reasons, these protein structures have received increasing attention in the field of polymer−protein hybrid materials over the past years, however, advances are still to be made. This Viewpoint highlights the different ways polymers and protein cages or their subunits have been combined to understand self-assembly and create functional materials.



In recent years proteins and polymers have been combined in a variety of hybrid materials that have interesting properties, often incorporating characteristics of both building blocks in the same material. While much research in this field focuses on the use of single proteins, protein cages like viruses and virus-like particles (VLPs) offer extra possibilities. Viruses are well-defined structures that occur in different shapes and sizes depending on the virus species (Figure 1). They are monodisperse and highly symmetrical, often corresponding to Casper-Klug icosahedral symmetry in which the number of protein subunits per capsid is given by 60 times the triangulation number (T) of the virus.[1] Furthermore, many viruses possess a natural self-assembly behavior which allows for the encapsulation a variety of materials. Finally, the proteins of these particles can be chemically and genetically modified giving them new, unique properties. This Viewpoint aims to give an overview of the interaction of synthetic macromolecules with virus (-based) proteins on different length scales, for example, in the self-assembly of virus proteins on polymer templates, using VLPs as templates for polymer growth, creating polymer-VLP hybrids, and finally the assembly of VLPs and polymers in larger micrometer sized aggregates. Other materials such as nanoparticles have been used in similar, hierarchical assemblies, but these examples are outside the scope of this review.

Packaging of (bio)polymers: The simplest viruses consist of a protein shell, called the capsid, and the viral genetic material. One of the interactions stabilizing viruses are the electrostatic interactions between the negatively charged DNA or RNA and the positively charged capsid interior. In this regard, the poly(nucleic acid) can be considered as a (bio)polymer template for virus particles. The first experiments focused on capsid assembly addressed the question whether the capsid can also form with different RNA templates such as homologous RNAs and RNAs from different viruses.[2−4]

It was shown that the capsids of Bromo Mosaic Virus (BMV) and Cowpea Chlorotic Mottle Virus (CCMV), viruses showing $T = 3$ icosahedral symmetry, which have a size of 30 and 28 nm, respectively, formed particles similar in size to the native viruses upon interaction with non-native RNA and did not have preferences for particular sequences.

RNA's ability to adopt different topologies by base pairing, however, strongly influences its templating behavior during capsid formation. An increasing number of branch points on the RNA leads increasing packaging efficiency. This phenomenon was observed experimentally when comparing packaging efficiencies of BMV RNA and CCMV RNA in CCMV CP[5] and was explained by modeling of free energies for varying RNA.[6,7] Similarly, molecular dynamic simulations by Zhang et al. showed that hyper-branched polyanions are more efficient at VLP formation.[8,9]

More studies focus on understanding the packaging of the RNA templates into viral capsids.[10−14] Upon increasing the RNA length it was observed that larger particles were formed and also the packaging of one RNA template in multiple capsids was observed. This is likely due to multiple nucleating centers forming on the same template. This partial encapsulation of long RNA strands has been applied by Garmann et al. to develop a method for monofunctionalization of CCMV.[15] By partially encapsulating a sufficiently long RNA strand one end extended out of the capsid which was available for functionalization. They suggest that this technique may be

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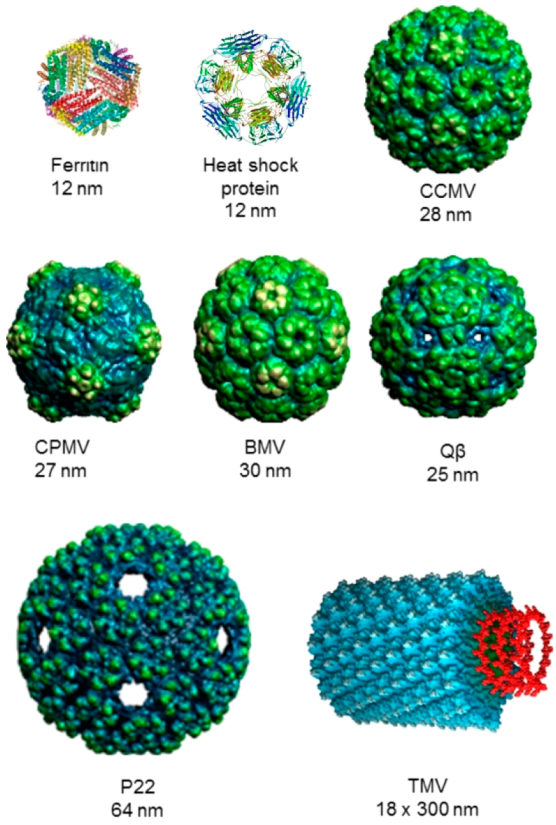

Figure 1. Structures of different protein cages (pictures taken from VIPER database (www.viperdb.scripss.edu) and Protein Data Bank (www.pdb.org).

used for monofunctionalization of icosahedral viruses in general.

Garman et al. also investigated the formation of VLPs around single-stranded (ss) RNA to elucidate the roles of subunit interactions during VLP formation.[12,13] In their work they vary the CP−RNA interaction by changing the ionic strength of the solution, while the CP−CP interaction was tuned by varying the pH. They suggest a two-step approach, in which first the ionic strength is lowered to induce CP−RNA interactions, and second the pH is lowered to induce CP−CP interactions, which gives the best yield of spherical VLPs.[12,16]

The use of biopolymers as templates for CCMV capsid assembly has also been extended to include DNA,[17,18] DNA-containing materials,[19] and DNA-origami.[20] Because of comparable charge distribution of DNA and RNA, these molecules will interact in a similar way with the positively charged protein interior. Double-stranded (ds)DNA, ssDNA,[17,18] and DNA micelles[19] have been studied as templates. Depending on the rigidity of the template, viral coat proteins were assembled into tubes, in the case of dsDNA, and into spherical particles, comparable to native capsids, for ssDNA and DNA micelles. It was shown by de la Escosura et al. that, by combining ssDNA with appropriate guest molecules, such as naphthalene and stilbene derivatives, the rigidity of the template could be altered, allowing for a transition of the capsid assembly from spheres to tubes.[17] Mikkilä et al. have shown that viral coat protein can also self-assemble onto DNA origami structures.[20] It was shown that the protein coating enhanced transfection of DNA origami structures into human cells.

In the cases discussed so far, the biopolymer acts as a template for the assembly of protein cages, however, the opposite case also occur when the capsids template the structure of the genetic cargo. The packaging of genetic material of several viruses and bacteriophages occurs by translocation of the genetic material into a preformed capsid using a molecular motor. Both theoretical and experimental studies have shown that large forces are involved in the packaging of the DNA.[21−24] The confinement forces the DNA into an out-of-equilibrium, glassy state and relaxation of the DNA is slowed significantly.[24,25] The conformational changes of the DNA are suggested to enhance DNA release during infection.[22,26,27]

Using a conceptually different approach neutral biopolymers have also been used as a template for capsid assembly.[28] Elastin-like proteins (ELPs) were fused to the CCMV coat protein. This fusion product retained the pH responsive capsid formation of the CCMV coat protein, but capsid formation could also be triggered by a salt- and temperature-response of the ELP part (Figure 2). Well-defined spherical particles of

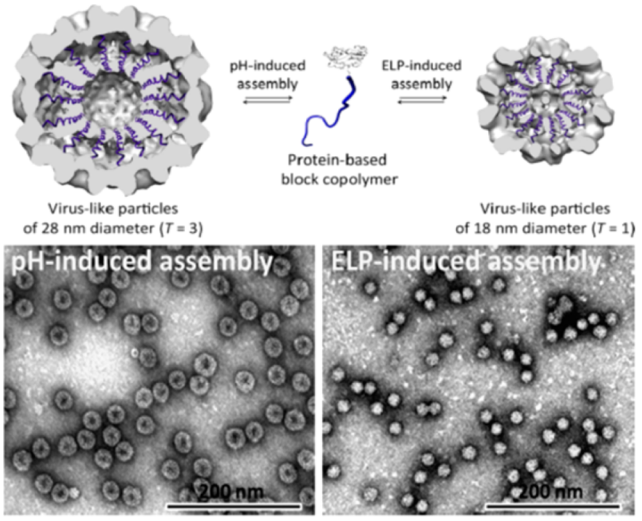

Figure 2. Schematic representation of the self-assembly of the CCMV CP-ELP product (upper) and TEM images of the two different assemblies (lower). Adapted with permission from Van Eldijk et al.[28] Copyright 2012 American Chemical Society.

different sizes were observed for the two assembly pathways. This opens up a new approach for the use of noncharged (bio)polymers as templates for capsid formation and allows for the formation of new responsive materials.

Using the same principle as with polynucleotides, synthetic polymers such as poly(styrenesulfonate) (PSS)[2,29−33] and poly(acrylic acid) (PAA)[31] have been shown to form virus-like particles. In fact, the self-assembly of CCMV coat protein with PSS is a widely studied model for capsid assembly.[29−32] It has been shown that depending on the molecular weight of the PSS template spherical particles with varying sizes are formed. Sikkema et al. showed the formation of 16 nm (T = 1) icosahedral particles when using low molecular weight PSS (average 9900 Da),[32] while experiments by Hu et al. utilizing high molecular weight polymers (400 kDa to 3.4 MDa) demonstrated the formation of 22 nm (T = 2) and 27 nm (T = 3) particles.[29] It has been observed that larger polymer templates induce larger assemblies, indicating that the size of

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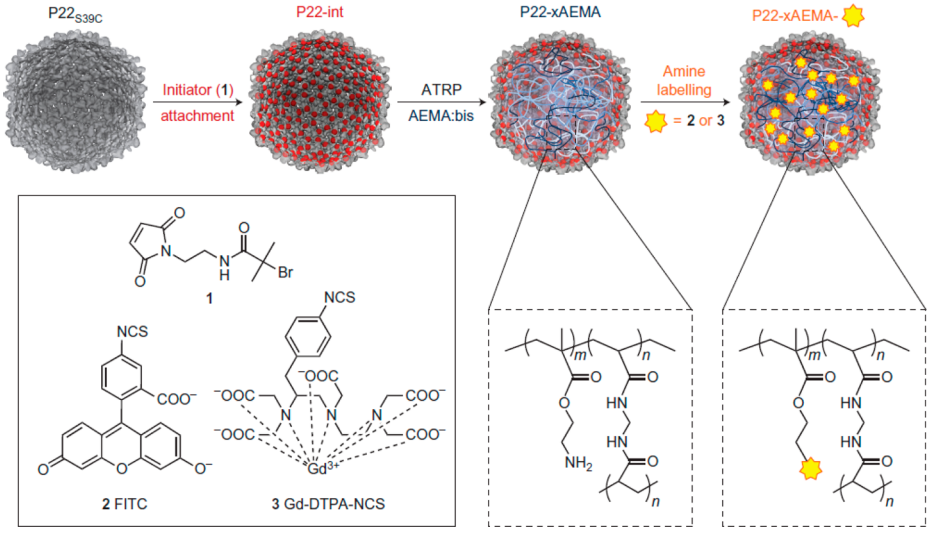

Figure 3. Representation of the confined ATRP polymerization inside the P22 capsid and subsequent labeling with a dye or Gd-DTPA complex. Reprinted with permission from Lucon et al.[38] Copyright 2012 Macmillan Publishers Ltd.

the polymer cargo is an important factor in directing the capsid size of the formed assemblies.

By fluorescent labeling of a PSS template, Cadena-Nava et al. were able to address the question how many polymer chains are packaged inside different sized capsids.[30] Their experiments demonstrated that larger capsids can accommodate more polymer chains of the same molecular weight, indicating that not only the charge ratio but also the molar ratio between template and coat proteins plays an important role in the formation of different sizes of capsids.

Theoretical studies into the assembly of coat proteins with a polymeric template have highlighted the influence of polymer length on capsid assembly.[34,35] Encapsulation is most efficient at polymer lengths that scale with the inner surface area of the capsid. Increased polymer length can cause the formation of malformed capsids where the polymer sticks out or may induce the encapsulation of one template by multiple capsids. Additionally, these studies have elucidated the contributions of the polymer template in the assembly mechanism.[34−37] The template lowers the nucleation barrier due to stabilization of assembly intermediates by the polymer and by increasing the local concentration of capsid protein due to absorption onto the template. Also, the electrostatic attraction between template and coat proteins enhances the growth rate of the capsid.

The possibilities that controlled polymerization techniques like ATRP and RAFT offer, such as control over polymer length and polydispersity, and different ways of modifying polymers, enables the design of specific polymeric structures. This can be used to create templates to address questions about capsid assembly that remain. For instance the influence of polymer topology on the assembly, as for example observed by Setaro et al. when encapsulating various dendrimers in CCMV VLPs,[39] could be investigated. However, no examples that exploit these possibilities in literature are known to our knowledge, except for the fluorescent labeling of the template.[30]

By using functional polymers as template, large materials can be loaded inside virus-like particles. Polymers that have been encapsulated as functional cargo include the fluorescent poly(5-methoxy-2-(3-sulfopropoxy)-1,4-phenylenevinylene) (MPS-PPV),[40−42] the redox-active polyferrocenylsilane (PFS),[43] and supramolecular polymers of zinc phtalocyanine (ZnPc), a photosensitizer.[39,44] Of great interest is the effect of encapsulation on the properties of the functional material. As with the native virus, the protein shell often provides protection to its cargo, that is, the encapsulated polymer. For example, Brasch et al. showed that MPS-PPV inside spherical particles could not be quenched by methyl viologen present in solution.[40] Interaction with the protein shell can interfere with the original properties as in the case of encapsulated PFS. Minten et al. observed that encapsulated PFS could only be oxidized and not reversibly reduced.[43] Finally, the shape of the formed structures and the conformations the polymer is able to adopt inside has consequences for the properties of the new material when these properties are conformation-dependent. Besides spherical particles, MPS-PPV can induce the formation of tubes when in its stretched form. Ng et al. showed that both the spherical particles and the tubes, both based on the same protein and polymer, possessed different optical properties.[42]

As polymers can act as templates for viral coat proteins, likewise the empty virus capsid can be envisioned as a scaffold for polymer growth. By functionalization of amino acids, either naturally occurring or recombinantly introduced, with a suitable initiating group polymerization can be induced. Using this approach, Abedin et al. constructed a branched polymeric networks inside the small heat shock protein, a protein cage of approximately 12 nm, via stepwise growth employing the Cu(I)-catalyzed azide−alkyne cycloaddition.[45] Also, the P22 capsid, a 60 nm $T = 7$ protein cage, was changed in to a macroinitiator for ATRP in this manner and linear polymers and networks of cross-linked polymer of 2-aminoethyl acrylate (AEMA) were polymerized in its interior (Figure 3). Lucon et al. showed that the polymers can be modified with functional molecules, yielding a MRI contrast agent (using Gd-diethylenetriaminepentacetate) or a photocatalytic active particle (using $[Ru(5\text{-methacrylamido-phenanthroline})_3]^{2+}$).[38,46] Hovlid et al. performed a similar experiment in which 2-dimethylaminoethyl methacrylate (DMAEMA) was polymerized inside the 25 nm $T = 3$ bacteriophage Qβ VLPs.[47] Furthermore, cellular uptake of these VLPs was studied, with and without modification of the outer surface, and showed greater internalization for cationic polymer-filled VLPs compared to similar VLPs lacking this polymer cargo. These

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results show the potential of the polymer−protein hybrids for biomaterial and biomedical applications.

Some capsids contain natural occurring motifs to anchor the necessary moieties for polymerization to the interior without the need for chemical functionalization. For example, apoferritin, a protein cage of approximately 12 nm, possesses metal-binding sites which can be used for other metals than iron. Abe et al. introduced rhodium(II)-catalysts for the polymerization of phenyl acetylene at the interior of apoferritin and subsequently used the inorganic-virus hybrids for formation of poly(phenyl acetylene).[48] Another example of a polymerization with the catalyst inside a protein cage was presented by Rengli et al., who performed ATRP inside the cavities of a 16 nm chaperonin by confining a copper catalyst.[49] This system was shown to yield polymer chains with a very low polydispersity.

So far, in all examples of confined polymerization using protein cages, it has been observed that the cage limits polymer growth, for both linear chains and branched networks.[38,45,47−49] The confinement of the polymer growth in some cases also creates products with narrower polydispersities compared to the same molecules created in solution.[48,49] However, details of the exact mechanism for polymerization and the influence of the confinement remain unknown. Theoretical simulations of, for example, catalytic reaction sites, provide more details,[50] yet experimental data investigating these mechanisms further are currently not available.

The capsid shell itself allows for certain selectivity in monomers for the confined polymerization. Monomers must pass through the pores of the protein shell, restricting the size of the molecules. When a high concentration of charged groups is present at the pore interior, selection may occur based on charge. Indeed, Abe et al. demonstrated positively charged phenyl acetylene derivatives could not be polymerized inside rhodium-containing apo-ferritin.[48]

From a materials perspective, the confined polymers inside capsids offer possible advantages for the introduction of functionality. This was demonstrated by Lucon et al. by the insertion of metal complexes to branched networks inside the small heat shock protein.[51] When the confined polymers possess free moieties, these are amenable for postpolymerization modification. It has been shown that in this manner a variety of small molecules, such as fluorescent dyes and imaging agents, can be incorporated with a dramatically increased loading compared to functionalization of interior amino acids only.[38,46,47]

Exterior modification: The surface functionalization of viruses and VLPs with polymers has mainly been focused on the development of hybrid materials for biomedical applications. To this end, poly(ethylene glycol) (PEG) and oligo (ethylene glycol) methacrylate (OEGMA) functionalized with carbohydrates has been attached to different viruses via the grafting to approach employing standard bioligation techniques, such as oxime ligation,[52,53] activated esters,[33,54−56] thiol-maleimide couplings,[57] and the Cu(I)-catalyzed azide−alkyne cycloaddition.[58−60] PEG is biocompatible, soluble in aqueous solutions and, most importantly, it reduces the immunogenic response. Indeed, reduced immunogenic response has been observed for PEG-covered virus-like particles compared to normal viruses.[54−57,61] For biomedical applications, these particles also need to be combined with other surface functionalities such as cell-targeting moieties for cell specific uptake. Functional groups can be introduced at the end of polymer chains attached to the capsid surface or to functional monomer side groups prior to attachment to the capsid. In this manner fluorescent dyes[54] and carbohydrates for tumor cell targeting[58] have been introduced. Additionally, the number of attached polymer chains can be decreased, leaving non-functionalized amino acids for modification with other molecules. However, it should be noted that in this approach the effective shielding of the PEG chains will be lowered, altering the immunogenic response to these particles, depending on polymer length and conformation.[55,61]

To a lesser extend the grafting from approach has been explored for the creation of virus-like particles with biomedical applications. Hu et al. coupled an ATRP initiator to a horse spleen ferritin protein cage, and polymerized both 2-methacryloyloxyethyl phosphorylcholine (MPC) and PEG methacrylate onto the surface.[62] Pokorski et al. modified the surface of the Qβ capsid with initiating groups for ATRP and used this macroinitiator for the polymerization of OEGMAs with and without pendant azide-moieties.[63] The great advantage of these virus-like particles is that they can act as a scaffold for many different functionalities by simply changing the molecules that can be attached to the monomer units.

Attachment of polymer chains on the surface of a protein cage can induce the dissociation of the protein shell as was observed by Comellas-Aragones et al. in the case of the CCMV virus.[33] However, the PEG-functionalized protein subunits could be reassembled using PSS as a template, resulting in VLPs with polymers on the interior and the exterior (Figure 4).

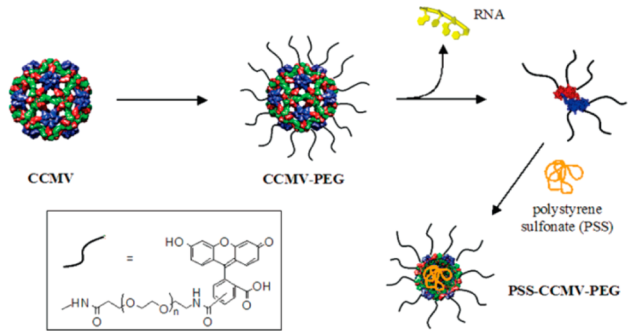

Figure 4. Controlled incorporation of polymers at the surface and the interior of the CCMV capsid. Reprinted with permission from Comellas-Aragonès et al.[33] Copyright 2009 American Chemical Society.

One of the greatest obstacles in the development of virus-based materials is the limited compatibility of many viruses with organic solvents. However, polymer-virus hybrids are a potential solution to this problem. For both PEG-functionalized Tabaco Mosaic Virus (TMV, 18 × 300 nm)[53] and Cowpea Mosaic Virus (CPMV, 28 nm, T = 3)[64] their solubility in organic solvents have been studied. PEG-TMV could be transferred into chloroform and even less polar solvents or solid polystrene.[53] PEG-CPMV was freeze-dried before successful introduction into organic solvents.[64] Interestingly, thermal annealing of the freeze-dried PEG-CPMV yielded a solvent-free liquid state of the polymer-virus hybrid. In all cases, the viruses remained intact. Polar organic solvents remain a problem because the viruses fall apart, likely due to hydrogen bonding between solvent and the proteins subunits, disrupting their structure. However, the compatibility of polymer-virus hybrids

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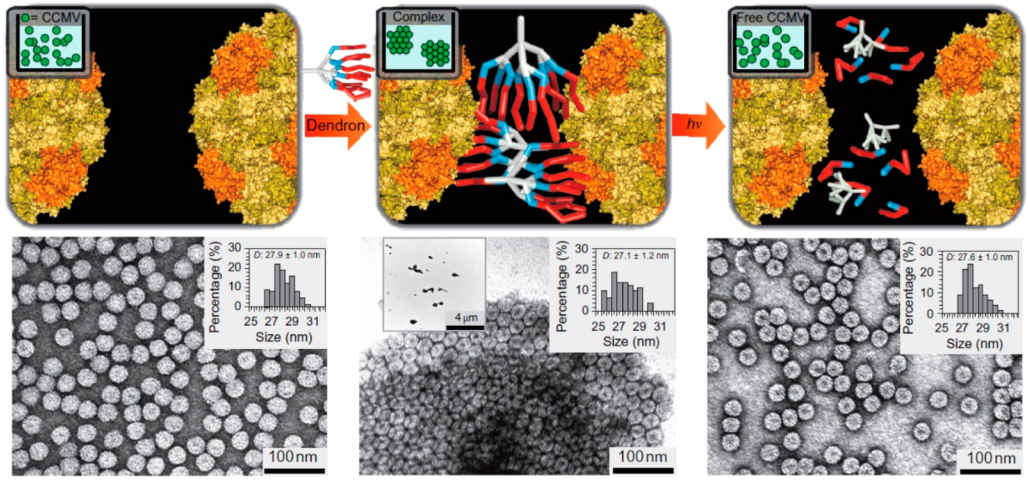

Figure 5. Assembly of CCMV with photocleavable dendrons and its optically triggered disassembly (above) and TEM images of the different stages of assembly and disassembly (below). Reprinted with permission from Kostiainen et al.[77] Copyright 2010 Macmillan Publishers Ltd.

with organic solvents opens up new possibilities for other virus-based materials in nonaqueous conditions.

The way a polymer is attached to the virus capsid can increase the stability of the particles. Manzenrieder et al. showed that multipoint attachment of poly(oxazolines) to the Qβ capsid effectively cross-linked the particle.[65] This yielded particles that were thermally stable upon heating to 100 °C. In contrast, when the polymer was attached monovalently the capsids were disassembled at these temperatures, even though the protein subunits retained their secondary structure. Control over the size of these polymer−virus hybrids was obtained by changing polymer length and attachment density.

As described above, polymer−virus hybrids offer a facile way to introduce different properties into virus-like particles by changing the polymer type attached to the surface or by adding functional groups to an attached polymer. For example, stimuli-responsive behavior could be inferred by a responsive polymer.

Higher order assemblies: Assembly of individual virus(-like) particles into larger, multiparticle, assemblies opens the way to more complex materials. For example, studies have shown that virus-polymer complexes can be used to improve gene delivery and allow for easier large-scale processing of viruses.[66]

Anisotropic particles, such as the TMV, can crystallize into ordered structures through depletion interactions.[67−69] It was even shown that filamentous bacteriophages M13 and fd, both having a diameter of 6,6 nm and a length of 800−900 nm, 3D structures can be formed using 3D guided extrusion.[70]

Virus particles that possess a negatively charged surface can complex with positively charged macromolecules, which will induce clustering. Kostiainen et al. investigated the assembly of CCMV with cationic linear polymers, dendrons, and dendrimers and found that the branched cationic templates were more efficient in the assembly of virus-like particles, indicating the need for multivalency.[71] This method can be extended to empty and loaded VLPs and several other protein cages, such as ferritin.[72] The size, and the corresponding icosahedral symmetry, of CCMV-based VLPs seems to affect the organization of the formed structures when it is clustered with linear poly-λ-lysine or dendritic poly(aminoamine).[73] Even more control over the assembly product can be obtained by using amphiphilic polymer structures with viral capsids.[74]

Stimuli-responsive assemblies between virus-like particles and polymers can be made introducing responsive groups in the employed polymers. Temperature-switchable assemblies have been made by using a thermoresponsive block-*co*-polymer. Such systems can reversibly be assembled and disassembled several times simply by increasing or lowering the temperature.[75,76] Furthermore, it is possible to create assemblies with optically triggered disassembly by using dendrons with a photocleavable group[77] (Figure 5).

The properties of free particles and assembled particles can differ as was shown by Kostiainen et al., who investigated the difference in magnetic properties of free and assembled magnetoferritin.[78] Therefore, it may be interesting to study assemblies formed by coaggregation of virus-like particles and polymers in order to form new functional materials. Co-assembly of VLPs with different cargos may yield materials with interesting optical or magnetic properties.

In the examples above, both the virus-like particles and the polymeric template are hydrophilic and therefore form homogeneous assemblies. Li et al. developed a method to assemble both spherical and rod-shaped viruses and polymers in large core−shell assemblies using an amphiphilic template, poly(4-vinylpyridine) (P4VP).[79−82] Investigation of the formed particles revealed a virus shell and polymer core. Varying the mass ratio virus/polymer allows control over the size of these colloidal assemblies. Furthermore, Suthiwangcharoen et al. reported on virus-polymer hybrid materials that could be used as nanosized drug delivery vehicles by loading the core with a small drug molecule and placing a cell-targeting group on the virus shell.[83] Inclusion of a pH-sensitive block in the polymer allows for the assemblies, which are stable at neutral pH, to disassemble at acidic pH.

Assembly of virus-like particles into multilayer films has been achieved by using layer-by-layer (LbL) assembly with polyelectrolytes.[84−87] Due to the overall negative surface charge, the virus-like particles can be used as negatively charged component instead of the polyanion. Steinmetz et al. showed that the spherical viruses are readily incorporated into a multilayer system, while rod-shaped viruses assemble in an ordered manner on top.[85] The virus-based films have mainly been developed as scaffolds for cell adhesion because of the biocompatibility due to the presence of viruses.[86−88] By surface functionalization of viruses with, for example, cell-targeting peptides, additional properties can easily be introduced into the thin films.[86,87] However, virus-based LbL assemblies are not

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restricted to biological purposes, also virus-based battery anodes[89,90] and porous imide films[91] are presented in literature. Furthermore, Li et al. showed that the incorporation of CPMV into oligo(9,9′-dioctylfluorene-*co*-bithiophene) (OF8T2) substrates enhanced the amplified spontaneous emission (ASE) performance of these thin films.[92] Another method for creating virus-covered surfaces was shown by Azucena et al., who showed the growth of protein nanotubes at various surfaces using the self-assembly of TMV-derived coat proteins on immobilized RNA.[93] This technique also allows for patterning of the surface with these nanotubes using lithography techniques.

Rod-shaped viruses like TMV and bacteriophage M13 have been able to template polymeric wires of poly(aniline) of several micrometers in length.[94−97] The rod-shape viruses assemble in a head-to-tail fashion and provide a scaffold for the aniline monomer. Upon addition of an initiator, the monomer is polymerized around the virus template.[94] Depending on the pH conditions, single wires (near neutral pH) and bundles of wires (acidic pH) could be made.[95,96] Addition of PSS in the wires increased their conductivity, which makes these materials interesting for electronic materials.[97] Rong et al. have explored the conductive properties of the virus-polymer wires combined with titanium oxide in LPG gas sensors films.[98] TMV was also included into poly(vinyl alcohol) (PVA) nanofibers as a universal method for including functionalities into such fibers.[99]

In summary, in this Viewpoint we have provided an overview showing that polymers and viruses or virus-like particles can be combined in different ways to influence the assembly of the constituent capsid proteins, to form new biohybrid architectures or to obtain large mesoscale structures, eventually leading to functional materials. The unique definition that virus-like particles have with respect to their size, shape, and constitution plays a key role in this. New developments, such as enhancing compatibility with organic solvents, give access to new possibilities with these materials. We think these developments will lead to various applications of polymer-virus constructs, for example, in vaccination or medical imaging. Still, a lot of aspects are still to be investigated, requiring both fundamental studies as well as studies focusing on applications.

AUTHOR INFORMATION

Corresponding Author

*E-mail: j.j.l.m.cornelissen@utwente.nl.

Author Contributions

†These authors contributed equally to this work (S.J.M. and A.M.v.d.H.).

Notes

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ABBREVIATIONS

AEMA, 2-aminoethyl acrylate; ATRP, atom transfer radical polymerization; BMV, Bromo Mosaic Virus; CCMV, Cowpea Chlorotic Mottle Virus; CPMV, Cowpea Mosaic Virus; CP, coat protein; DMAEMA, 2-dimethylaminoethyl methacrylate; ELP, elastin-like protein; LbL, Layer-by-layer; MPC, 2-methacryloyloxyethyl phosphorylcholine; MPS-PPV, poly(5-methoxy-2-(3-sulfopropoxy)-1,4-phenylenevinylene); OEGMA, oligo(ethylene glycol) methacrylate; P4VP, poly(4-vinylpyridine); PAA, poly(acrylic acid); PEG, poly(ethylene glycol); PFS, polyferrocenylsilane; PSS, polystyrenesulfonate; RAFT, reversible addition−fragmentation chain-transfer; TMV, Tobacco Mosaic Virus; VLP, virus-like particle; ZnPc, zinc phtalocyanine

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