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Designing Two Self-Assembly Mechanisms into One Viral Capsid Protein

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Abstract

A structural fusion protein of the thermally-responsive elastin-like polypeptide and a viral capsid protein (ELP-CP) was designed and the assembly properties were investigated. Interestingly, this protein-based block copolymer could be self-assembled via two mechanisms into two different, well-defined nanocapsules: (1) pH-induced assembly yielded 28 nm virus-like particles and (2) ELP-induced assembly yielded 18 nm virus-like particles. The latter were a result of the emergent properties of the fusion protein. This work shows the feasibility to create a self-assembly system with new properties by combining two structural protein elements.

Self-assembled nanocapsules have been studied intensively because of their controlled assembly and disassembly, and storage capacity. Nanocapsules have a range of applications, varying from drug delivery vehicles to nanoreactors. Different materials have been used to generate these capsules including lipids, synthetic polymers[1], proteins[2–4] or combinations thereof[2,5]. Protein-based building blocks have gained much interest, as these structures permit the formation of perfectly defined capsules due to the intricate three-dimensional folding of the protein constituents.[6,7] In nature many examples of protein cages are known, including nanovaults [8], viruses[9] and ferritin[10]. Spherical viruses generally consist of several hundreds of subunits, which self-assemble to encapsulate their genetic material for storage and transport. However, many virus capsid proteins can be induced to assemble even without their natural cargo.[11–13]

Cowpea chlorotic mottle virus (CCMV) is an excellent example of a virus whose assembly can be exquisitely controlled in the absence of its viral RNA. It can be disassembled and reassembled by adjusting the pH. At high pH (7.5) empty CCMV capsids dissociate into capsid protein (CP) dimers and at low pH (5.0) they reassemble.[14,15] Virus-like capsids are comprised of 90 CP dimers arranged with Caspar and Klug triangulation number[16] (T) =3

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Notes

The authors declare no competing financial interests.

Supporting Information

Methods for preparing plasmids and proteins, protein characterization, protein assembly, analysis of assembly and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

symmetry, though T=1 and pseudo T=2 forms have also been observed.[17] Virus-like particles (VLPs) of CCMV have been used as nanoreactors or as template for constrained synthesis of nanomaterials.[18–21] However, these VLPs are only stable at lower pH, which limits their applications.

Templated assembly has been described as an approach to generate VLPs at different conditions.[22,23] However, nanoparticle-templated capsids are already filled and for that reason it is more challenging to accommodate additional material. Therefore we need to develop additional methods to control assembly. In a recent study, a capsid protein with an N-terminal histidine-tag (H_6CP) was produced in *E. coli*. This histidine-tag is capable of binding nickel ions and is positioned on the inside of the VLPs in proximity of the N-termini of other CPs. It was shown that the H_6CP could be assembled and stabilized by the addition of nickel ions.[24] We realized that if the assembly of the CPs could be controlled by an external trigger, for example in a thermally-responsive manner, this would allow another level of control over assembly of CPs in addition to changing pH or the addition of other substances.

A well-defined protein-based class of stimulus-responsive polymers are the elastin-like polypeptides (ELPs). ELPs consist of repeating pentapeptides of Val-Pro-Gly-Xaa-Gly (Xaa= any natural amino acid except proline), which can be switched from an extended water soluble to a collapsed hydrophobic state in response to increasing temperature. This completely reversible phenomenon is also known as lower critical solution temperature (LCST) behavior and the transition temperature (T_t) can be influenced by changing the fourth residue of the pentapeptide repeat, the number of repeats, protein concentration and salt concentration.[25,26]

Herein we report the construction and assembly properties of a block copolymer in the form of a fusion protein consisting of the CP of CCMV with an N-terminal short ELP block (ELP-CP). In this design, the properties of both blocks are combined: the CP ability to form well-defined VLP morphologies and the stimulus-responsive character of the ELP fragment. Amazingly, two types of highly homogeneous self-assembled structures can be formed using a single capsid protein: (1) pH-induced assembly into 28 nm VLPs and (2) ELP-induced assembly into 18 nm VLPs (Scheme 1). The latter can only be accessed via the emergent properties of the ELP-CPs.

We expressed CCMV CPs with an N-terminal stimulus-responsive ELP domain (ELP-CPs) in *E. coli*. The ELP was placed at the N-terminus of the CP as it was expected that this would position the ELP-tags on the interior of the VLPs. The ELP design was based on our estimate that ELP[$V_4L_4G_1$-9] would give a transition temperature within the temperature and salt concentration range where CP is stable.[26] ELP constructs are described using the notation ELP[$X_iY_jZ_k$-n], where the capitals between the brackets indicate the single letter amino acid code for the Xaa replacing residue in the pentapeptide Val-Pro-Gly-Xaa-Gly. The subscript stands for the ratio of the guest residues and the n represents the total number of pentapeptide repeats. The ELP block replaced the RNA-binding domain of the wild type CP to ensure that the length of the new N-terminal domain would not interfere with the assembly of the CPs into VLPs.[17,27] The ELP-CP constructs were also equipped with an N-terminal histidine-tag to facilitate purification via affinity chromatography. The resulting plasmid encoded H_6-ELP[$V_4L_4G_1$-9]-CP(ΔN26) (for protein sequence Supporting Information, Table S2). The expressed protein, purified via affinity chromatography, was dialyzed against a pH 7.5 buffer (50 mM Tris, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA) (Supporting Information, Figure S1). The expected molecular weight of the product of 22253.4 Da was verified by electrospray ionization time-of-flight (ESI-TOF) mass

spectrometry (Figure S2). The capsid proteins were produced with a yield of 60-80 mg per liter of culture.

First it was shown that the modified capsid proteins still assembled into virus-like particles in response to pH. These are conditions where the ELP domain is not expected to self-assemble. To induce assembly, the CPs were dialyzed against an acetate buffer of pH 5.0 and visualized by transmission electron microscopy (TEM). ELP-modified capsid proteins formed virus-like particles (VLPs) with a diameter of 28 nm (Figure 1A and 1C). Dynamic light scattering (DLS) indicated that this assembly condition yielded a uniform assembly product with a diameter estimated to be 31 nm (Figure S3) with minimal aggregation. Size exclusion chromatography (SEC) inline with multi angle laser light scattering (MALLS) indicated that the VLPs, which eluted at 10.2 mL on a Superose 6 10/300 GL column (Figure 2), have a molecular weight of 4.0 MDa which is in line with 180 capsid proteins of 22253.4 Da (theoretically 4.0 MDa). These results are consistent with VLPs similar to native-like T=3 particles. Assembly at pH 5.0 was of high affinity as no peak was observed that corresponded to dimers (expected at 17.0 mL, Figure 2) and with high fidelity as no other aggregates or assembly products were observed. In comparison, assembly of wildtype CP (WT-CP) under similar conditions occurs with lower affinity and always leaves some residual dimer.[28]

To ascertain particle geometry and understand the disposition of the ELP blocks we performed cryo-electron microscopy (cryo-EM) and three-dimensional reconstruction of the pH-induced H_6-ELP[$V_4L_4G_1$-9]-CP(ΔN26) VLPs (Figure S4). It was observed that the VLPs consist of two shells (Figure 3A and 3C). As the morphology of the outer shell was identical to the empty T=3 CCMV capsid [17,29], the electron density of the inner shell can only be explained by the ELP, indicating that the ELP domain indeed was on the interior of the VLPs. Firstly, this indicates that the ELP domain was inside the VLPs. Tentatively, it appears that in this environment, the ELPs have self-assembled due to their high local concentration.

After having established that the ELP-CPs can be assembled into T=3 VLPs under pH-induced conditions, the ELP-induced assembly was assayed. The T_t of ELPs can be tuned by several factors. Both polymer length and the amino acid at the fourth position in the pentapeptide repeat are determined by the design of the polypeptide. Therefore, the salt concentration was used to adjust the T_t into a range suitable for CCMV CP. By increasing the NaCl concentration to 2.5 M the T_t of the ELP of the fusion protein could be reduced to below room temperature. Therefore, to induce assembly, ELP-CP dimers were dialysed against a buffer of pH 7.5 with 2.5 M NaCl and then the samples were analyzed by TEM. Similar to pH-induced assembly, we again observed assembly of the ELP-modified capsid proteins into monodisperse VLPs, but now with a diameter of 18 nm (Figure 1). These assemblies were then analyzed by DLS and a diameter of 25 nm was estimated (Figure S3). These small VLPs were also analyzed by SEC-MALLS (Figure 2). Triggering the stimulus-responsiveness of the ELPs led to assembly of the dimers into 1.3 MDa VLPs with no residual dimer, indicating high CP-CP affinity. This mass corresponds to 60 capsid proteins of 22253.4 Da, which suggests T=1 architecture (theoretically 1.3 MDa).

Cryo-EM and image reconstruction was employed to verify that the ELP-induced assembly yielded T=1 icosahedra (Figure 3B and 3D). Again, two shells were observed surrounding a small unoccupied inner volume. The outer shell shows the pentameric turrets expected for CCMV.[17] The electron density of the inner shell was again attributable to ELP. In our construct, the ELP-domain was designed to interact with neighbouring ELP-domains. This could explain the tighter packing and the resulting smaller VLPs after ELP-induced assembly. This hypothesis was corroborated by previous reports in literature in which it was

shown that CPs lacking both the positively-charged RNA-binding domain and the β-hexamer (residues 3–36) could be assembled into mainly pseudo T=2 and T=1 and a small fraction of T=3 icosahedrons in a pH-dependent manner.[17] Formation of T=1 particles was also observed for truncated CPs, lacking the N-terminal region, and for full length WT-CPs via kinetic trapping, by addition of short oligonucleotides or by addition of anionic polymers.[28,30–32] The quantitative formation of T=1 particles as reported in this paper is however unprecedented.

2.5 M of NaCl was used to ensure that all ELP-CPs were assembled; now a series of NaCl concentrations was used to investigate the minimum concentration required to obtain assembly at room temperature and at pH 7.5. It was found that a concentration of 1.8M NaCl in the pH 7.5 buffer was enough to get full assembly of the ELP-CPs (Figures S5 and S6).

To demonstrate that ELP was responsible for assembly at high salt concentrations, it was investigated whether capsid proteins lacking the stimulus-responsive peptide were capable of self-assembly under high salt conditions. WT-CP and the histidine-tagged capsid proteins (H_6CP)[19] were dialyzed into pH 7.5 buffer with 1.8 M NaCl. However, under these conditions, the control proteins were only detectable as dimers (Figure S6).

At NaCl concentrations below 1.8 M, only a fraction of the ELP-CP assembled resulting in a bimodal distribution of capsid and dimer consistent with virus assembly theory (Figure S5).[33] This is notable because when free ELP undergoes a phase transition it leads to complete aggregation. This difference is probably because the ELPs are sequestered on the inside of the VLPs, thus limiting the number of ELPs that can participate in a given nucleation event. To verify that this is the reason for incomplete assembly, a sample was diluted twice and measured again by SEC after overnight incubation. As expected, the ratio of VLP:dimer changed from 0.39:1 to 0.17:1 (based on area under the peak, Figure S8). This showed that relatively more ELP-CP is present as dimers at lower protein concentration and that the assembly is reversible.

Finally, the temperature-responsive assembly of ELP-CPs was examined. As concluded from the previous experiment, only a minor fraction of ELP-CPs was assembled at room temperature in the pH 7.5 1.3 M NaCl buffer. Therefore, a sample of ELP-CPs at these conditions was incubated at 35 °C for 15 minutes and then again analyzed by SEC at this temperature. This resulted in efficient assembly of the dimers into T=1 particles (Figure 4). From this experiment it can also be concluded that the assembly is a rather fast process as it was completed within 15 minutes. The temperature-responsive assembly was also confirmed by TEM (Figure S7).

In conclusion, this work shows the feasibility of combining two structural protein elements to generate a system with new properties. We applied this principle to create a viral capsid protein which can be self-assembled via two mechanisms into two different, well-defined structures. To construct such a system, thermally-responsive ELPs were genetically combined with CCMV CPs. Under the pH-induced assembly conditions these modified capsid proteins assembled into virus-like particles of 28 nm with T=3 icosahedral architecture. Thermally-responsive ELP-induced assembly resulted in the efficient production of T=1 icosahedra of 18 nm. This architecture is only accessible because of the combined properties of ELPs and CPs in one system, in which the assembly was induced by triggering the phase transition of ELP and in which the architecture was controlled by the capsid proteins.

The present system could find application in switchable encapsulation of enzymes to control their activity. The VLPs could also be used as a capture and release system for therapeutics

to protect them from the environment. The confined internal volume with relative high ELP-concentration can also lead to a better understanding of the ELP structural characteristics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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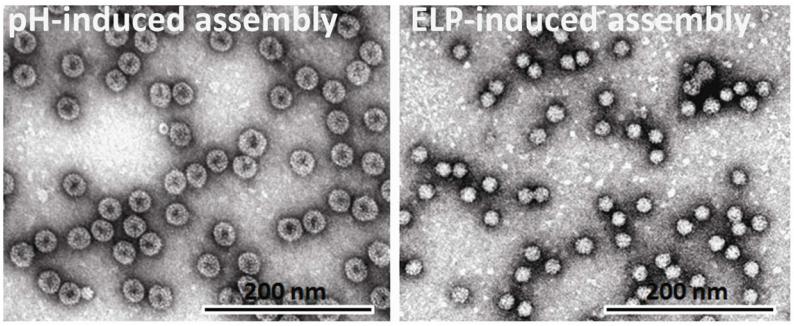


Figure 1.
Uranyl acetate stained TEM micrographs of CPs assembled into VLPs under pH-induced and ELP-induced assembly conditions.

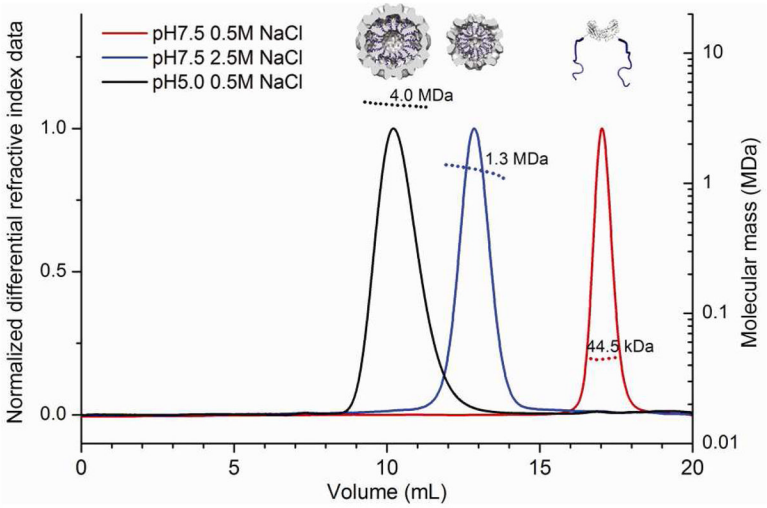


Figure 2.

SEC-MALLS chromatogram of H_6-ELP[$V_4L_4G_1$-9]-CP(ΔN26). The solid lines show the normalized differential refractive index and the dotted lines show the molecular mass of the complexes.

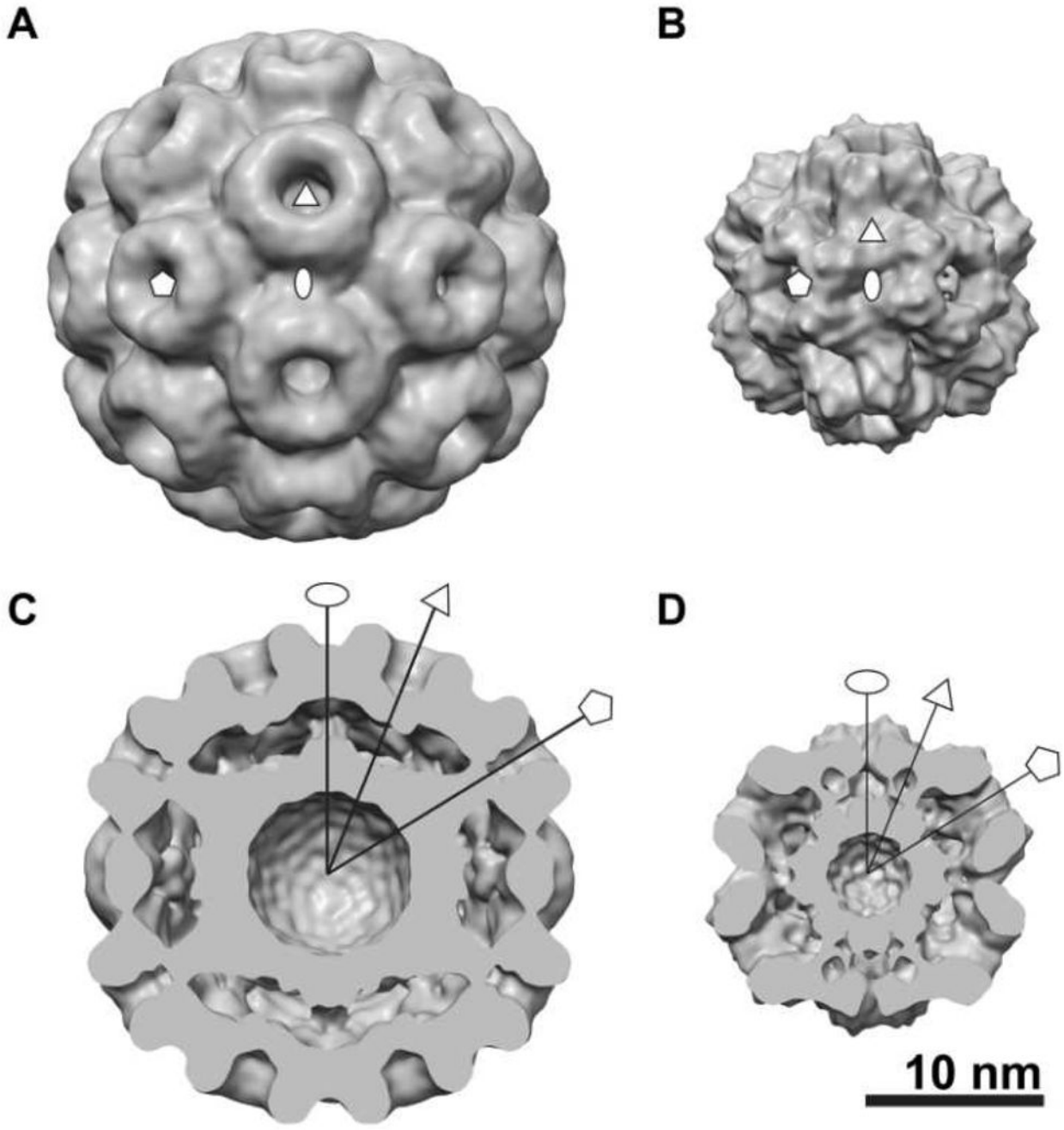


Figure 3.
Cryo-EM reconstruction of VLPs viewed along icosahedral twofold axis. pH-induced assembly (A and C) yields 90-dimer *T*=3 particles. ELP-induced assembly (B and D) yields 30-dimer *T*=1 particles. The top panels depict the surface views and the bottom panels present the equatorial views to show the interior. Oval, triangle, and pentagon indicate locations of twofold, threefold and fivefold axes, respectively.

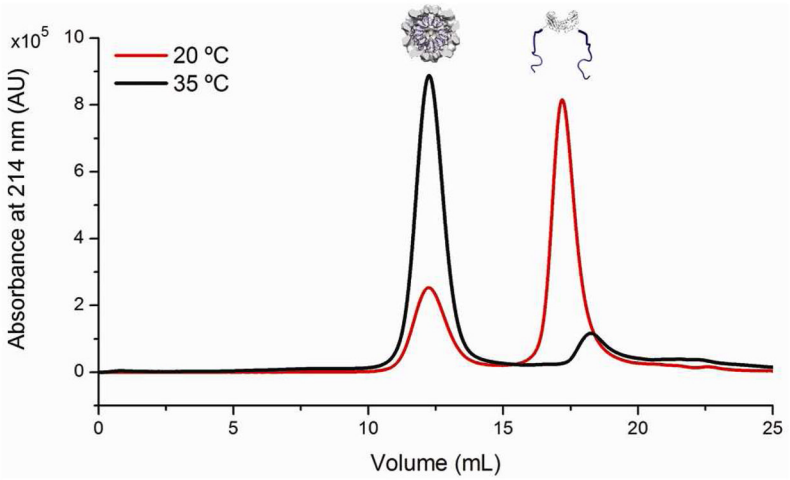
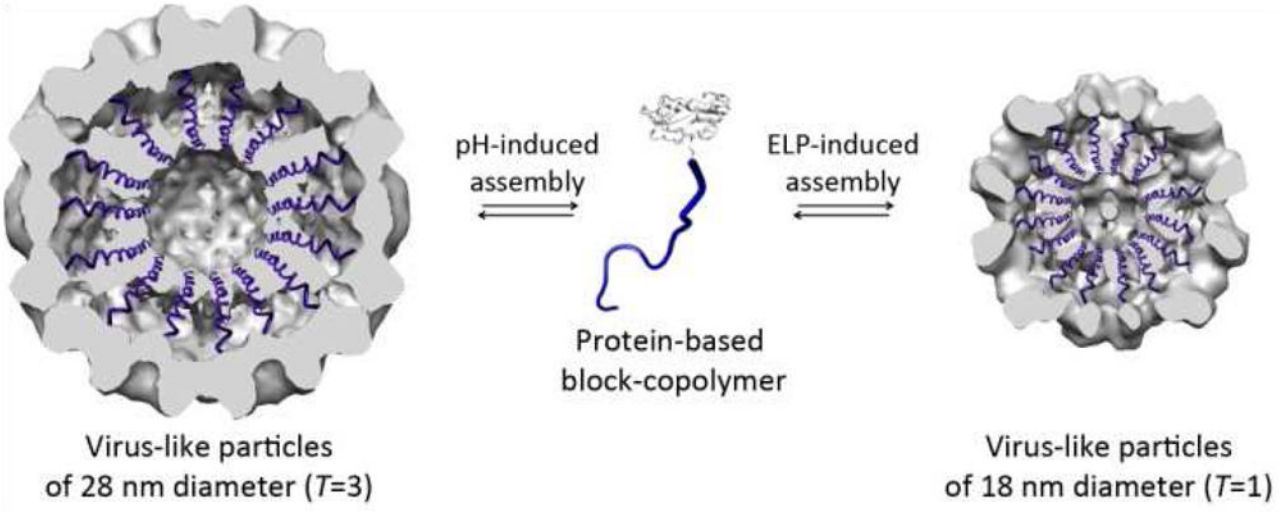


Figure 4.
SEC chromatogram of H_6-ELP[$V_4L_4G_1$-9]-CP(ΔN26) at pH 7.5 and 1.3 M NaCl at different temperatures.



Scheme 1.
Representation of ELP-functionalized capsid protein and its assembly products.