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Designing Two Self-Assembly Mechanisms into One Viral Capsid Protein

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Supporting Information

ABSTRACT: ELP-CP, a structural fusion protein of the thermally responsive elastin-like polypeptide (ELP) and a viral capsid protein (CP), was designed, and its 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 of creating 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] and 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] Many examples of protein cages in nature are known, including nanovaults,[8] viruses,[9] and ferritin.[10] Spherical viruses generally consist of several hundreds of subunits that self-assemble to encapsulate their genetic material for storage and transport. However, many virus capsid proteins (CPs) 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 CP dimers, and at low pH (5.0) they reassemble.[14,15] Virus-like capsids are composed of 90 CP dimers arranged with Caspar and Klug triangulation number[16] (T) = 3 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 templates for constrained syntheses of nanomaterials.[18−21] However, these VLPs are stable only at lower pH, which limits their applications.

Templated assembly has been described as an approach to generate VLPs under different conditions.[22,23] However, nanoparticle-templated capsids are already filled, and thus, it is more challenging to accommodate additional material. Therefore, we need to develop additional methods to control the assembly. In a recent study, a CP with an N-terminal histidine tag (H_6CP) was produced in *Escherichia coli*. This histidine tag was capable of binding nickel ions and was positioned on the inside of the VLPs in proximity to 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 (e.g., in a thermally responsive manner), this would allow another level of control over the assembly of CPs in addition to changing the pH or the adding other substances.

A well-defined protein-based class of stimulus-responsive polymers are the elastin-like polypeptides (ELPs). ELPs consist of repeating Val-Pro-Gly-Xaa-Gly pentapeptides, where Xaa is any natural amino acid except proline. ELP constructs are described using the notation ELP[$X_iY_jZ_k$-n], where the capital letters in the brackets are the single-letter codes for the amino acids replacing Xaa in the pentapeptides, the subscripts give the ratio of the guest residues, and n represents the total number of pentapeptide repeats. ELPs can be switched from an extended water-soluble state to a collapsed hydrophobic state in response to an increase in temperature. This completely reversible phenomenon is also known as lower critical solution temperature (LCST) behavior, and the transition temperature (T_t) can be varied by changing the fourth residue of the pentapeptide repeat, the number of repeats, and the protein and salt concentrations.[25,26]

Herein we report the construction and assembly properties of a block copolymer in the form of a fusion protein, ELP-CP, consisting of the CP of CCMV and an N-terminal short ELP block. This design combines the properties of the two blocks: the ability of CP to form well-defined VLP morphologies and

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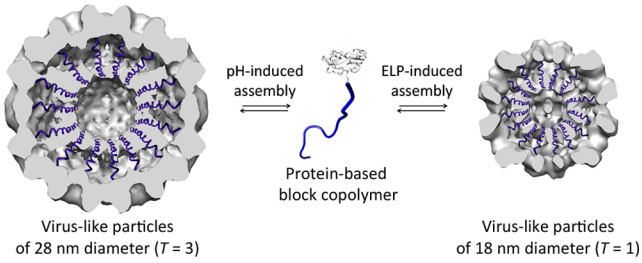
the stimulus-responsive character of the ELP fragment. Amazingly, two types of highly homogeneous self-assembled structures can be formed using a single CP: (1) pH-induced assembly into 28 nm VLPs and (2) ELP-induced assembly into 18 nm VLPs (Scheme 1). The latter can only be accessed via the emergent properties of the ELP-CPs.

Scheme 1. Representation of the ELP-CP and Its Self-Assembly Products



We expressed CCMV CPs with an N-terminal stimulus-responsive ELP domain 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 have a transition temperature within the temperature and salt concentration ranges where the CP is stable.[26] The ELP block replaced the RNA-binding domain of the wild-type CP (WT-CP), which ensured 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 the protein sequence, see Table S2 in the Supporting Information (SI)]. The expressed protein was purified via affinity chromatography and dialyzed against a pH 7.5 buffer (50 mM Tris, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA) (Figure S1 in the SI). The expected product molecular weight of 22253.4 Da was verified by electrospray ionization time-of-flight (ESI-TOF) mass spectrometry (Figure S2). The CPs were produced with a yield of 60−80 mg/L of culture.

First it was shown that the modified CPs would still assemble into VLPs in response to pH changes under conditions where the ELP domain would not be expected to self-assemble. To induce assembly, the CPs were dialyzed against a pH 5.0 acetate buffer and visualized by transmission electron microscopy (TEM). The ELP-CPs formed VLPs with a diameter of 28 nm (Figure 1 left). Dynamic light scattering (DLS) indicated that

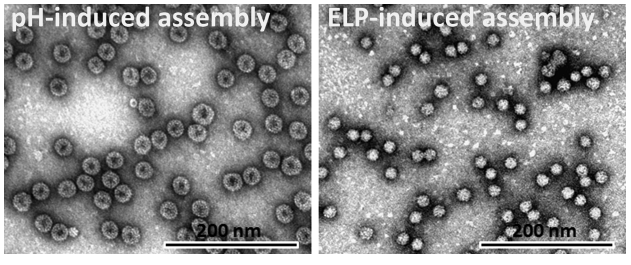


Figure 1. Uranyl acetate-stained TEM micrographs of CPs assembled into VLPs under (left) pH-induced and (right) ELP-induced assembly conditions.

these assembly conditions yielded a uniform assembly product having an estimated diameter of 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), had a molecular weight of 4.0 MDa, in

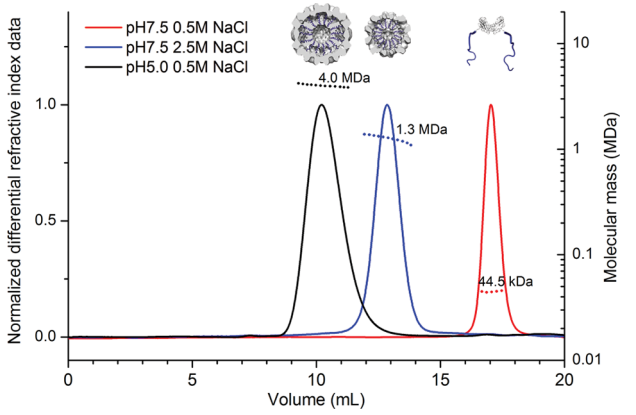


Figure 2. SEC−MALLS chromatograms of H_6-ELP[$V_4L_4G_1$-9]-CP(ΔN26). The solid lines show the normalized differential refractive index signals, and the dotted lines show the molecular masses of the complexes.

line with 180 CPs of 22253.4 Da (theoretically 4.0 MDa). These results are consistent with VLPs similar to native-like T = 3 particles. The assembly at pH 5.0 was of high affinity, as no peak corresponding to dimers (expected at 17.0 mL; Figure 2) was observed, and had high fidelity, as no other aggregates or assembly products were observed. In comparison, assembly of WT-CP under similar conditions occurred with lower affinity and always left some residual dimer.[28]

To ascertain the particle geometry and understand the disposition of the ELP blocks, we performed cryogenic 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 consisted of two shells (Figure 3A,C). As the morphology of the outer shell was identical to that of the empty T = 3 CCMV capsid,[17,29] the electron density of the inner shell could only be explained by the presence of the ELPs, indicating that the ELP domain indeed was in the interior of the VLPs. Moreover, it tentatively appeared that in this environment the ELPs self-assembled because of their high local concentration.

After it was established that the ELP-CPs could assembled into T = 3 VLPs under pH-induced conditions, ELP-induced assembly was assayed. The ELP T_t can be tuned by several factors. Both the polymer length and the amino acids at the fourth positions in the pentapeptide repeat were determined by the design of the polypeptide. Therefore, the salt concentration was used to adjust T_t into a range suitable for the CCMV CP. Increasing the NaCl concentration to 2.5 M lowered the T_t of the ELP of the fusion protein to below room temperature. Therefore, to induce assembly, ELP-CP dimers were dialyzed against a pH 7.5 buffer containing 2.5 M NaCl, and then the samples were analyzed by TEM. Similar to the pH-induced assembly, we again observed assembly of the ELP-modified capsid proteins into monodisperse VLPs, but these had a diameter of 18 nm (Figure 1 right). These assemblies were then analyzed by DLS, and a diameter of 25 nm was estimated

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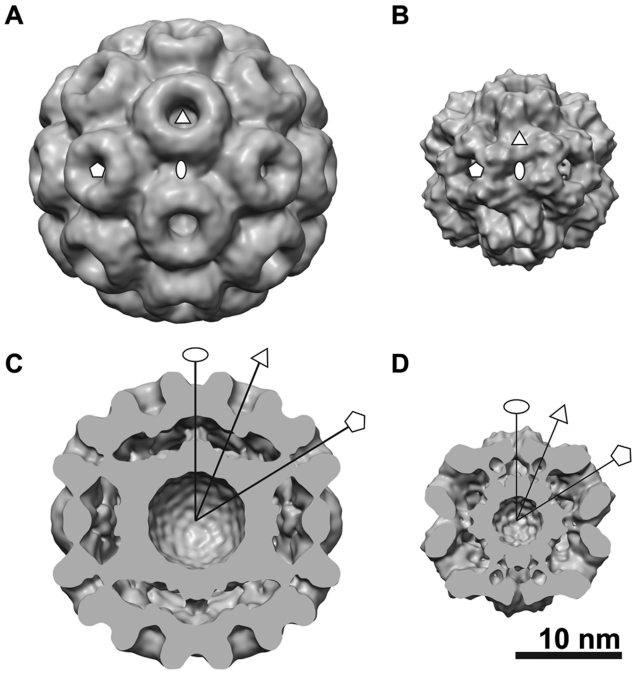

Figure 3. Cryo-EM reconstructions of VLPs viewed along the icosahedral twofold axis: (A, C) pH-induced assembly yielded 90-dimer $T = 3$ particles; (B, D) ELP-induced assembly yielded 30-dimer $T = 1$ particles. The top panels depict surface views, and the bottom panels present equatorial views to show the interior. Ovals, triangles, and pentagons indicate locations of twofold, threefold, and fivefold axes, respectively.

(Figure S3). These small VLPs were also analyzed by SEC–MALLS (Figure 2). Triggering the stimulus response of the ELPs led to assembly of the dimers into 1.3 MDa VLPs with no residual dimers, indicating high CP–CP affinity. This mass corresponds to 60 CPs of 22253.4 Da, suggesting a $T = 1$ architecture (theoretically 1.3 MDa).

Cryo-EM and image reconstruction were employed to verify that the ELP-induced assembly yielded $T = 1$ icosahedra (Figure 3B,D). Again, two shells surrounding a small unoccupied inner volume were observed. The outer shell showed the pentameric turrets expected for CCMV,[17] and the electron density of the inner shell was again attributable to ELP. In our construct, the ELP domain was designed to interact with neighboring ELP domains. This could explain the tighter packing that resulted in smaller VLPs after ELP-induced assembly. This hypothesis was corroborated by previous reports in the literature showing 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 upon addition of short oligonucleotides or anionic polymers.[28,30−32] However, the quantitative formation of $T = 1$ particles reported here is unprecedented.

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

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

At NaCl concentrations below 1.8 M, only a fraction of the ELP-CPs 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 a result of the fact that 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 was the reason for incomplete assembly, a sample was diluted twice and measured again by SEC after overnight incubation. As expected, the VLP:dimer ratio changed from 0.39:1 to 0.17:1 (based on the areas under the peaks; Figure S8). This showed that relatively more ELP-CP is present as dimers at lower protein concentrations 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 1.3 M NaCl buffer at pH 7.5. Therefore, a sample of ELP-CPs under these conditions was incubated at 35 °C for 15 min and then again analyzed by SEC at this temperature. The higher temperature resulted in efficient assembly of the dimers into $T = 1$ particles (Figure 4). From

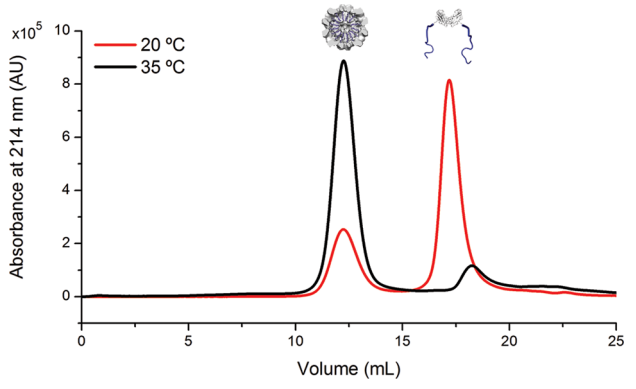

Figure 4. SEC chromatogram of H_6-ELP[$V_4L_4G_1$-9]-CP(ΔN26) in 1.3 M NaCl at pH 7.5 at different temperatures.

this experiment it can also be concluded that the assembly is a rather fast process, as it was completed within 15 min. 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 that 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 pH-induced assembly conditions, these modified CPs assembled into 28 nm diameter VLPs with a $T = 3$ icosahedral architecture. Thermally responsive ELP-induced

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assembly resulted in the efficient production of 18 nm diameter $T = 1$ icosahedra. This architecture is accessible only because of the combined properties of ELPs and CPs in one system, as the assembly was induced by triggering the phase transition of ELP but the architecture was controlled by the CPs. 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 a relatively high ELP concentration could also lead to a better understanding of ELP structural characteristics.

■ ASSOCIATED CONTENT

Supporting Information

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

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Notes

The authors declare no competing financial interest.

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