Self-assembly and stabilization of hybrid cowpea chlorotic mottle virus particles under nearly physiological conditions

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Abstract: Capsids of the cowpea chlorotic mottle virus (CCMV) hold great promise for use as nanocarriers *in vivo*. A major drawback, however, is the lack of stability of the empty wild-type virus particles under physiological conditions. Here, we report on the assembly behavior and stability under nearly-physiological conditions of protein-based block copolymers composed of the CCMV capsid protein and two hydrophobic elastin-like polypeptides. UV-vis spectrometric studies, dynamic light scattering analysis, and transmission electron microscopy measurements demonstrate that both hybrid variants form stable capsids at pH 7.5, physiological NaCl concentration and 37°C. The more hydrophobic variant also remains stable in a cell culture medium. These engineered hybrid CCMV capsid particles can therefore be regarded as suitable candidates for *in vivo* applications.

Introduction

Hollow particles with sizes in the nanometer range (nanocages) have found widespread application in the medical field. For instance, the encapsulation of pharmaceutical drugs inside nanocages has tremendously increased the control over drug delivery on the molecular level.[1,2] By modifying the exterior of the particles, targeting towards a desired tissue can be achieved, reducing the side effects of the drug. At the same time, the nanocage enhances the stability of the drug and increases its half-life *in vivo* by shielding it from interfering external influences. The success of these types of designs is illustrated by liposomal drug delivery systems that are now on the market or in clinical trials.[3] The same can be said for polymeric nanoformulations, of which many are now being evaluated clinically, showing great promise for future applications.[4] Potential *in vivo* applications of nanocarriers are not limited to the drug delivery field. There are also opportunities in the field of artificial organelles, where nanocarriers can be used to replace dysfunctional enzymes in diseased cells or to extend the endogenous biosynthetical pathways of the cell.[5]

Although liposomal and polymeric nanocages evidently show great potential as *in vivo* nanocarriers, they have some disadvantageous properties. For instance, the lipid- and polymer-based subunits intrinsically form assemblies with a relatively large size distribution, which can only be controlled to a certain extent. In contrast, protein-based nanocages are very well-defined in both size and shape, as they are formed from a specific number of proteins. In addition, these nanoparticles are biocompatible, biodegradable, and robust.[2,6] It is therefore not surprising that recent developments enabling (future) *in vivo* use have been made with both natural protein cages, such as ferritin[7–9], bacterial encapsulins[10,11] and the bacteriophage P22[12–14], as well as with designed protein cages[15–17]. In particular, virus-like particles (VLPs), i.e. viruses devoid of their endogenous genetic material, are promising vehicles for nanocarrier purposes, as their natural role is to safely transport the viral genome to the right place in the host organism.[18,19] *In vivo* studies have already indicated the great potential of these systems as nanocarriers for biological purposes.[20–24]

Of particular interest is the cowpea chlorotic mottle virus (CCMV) capsid. This VLP distinguishes itself from other viral nanocages through its reversible assembly behavior. At neutral pH, capsid protein dimers exist in solution, which spontaneously assemble into 28 nm sized capsids with a triangulation number $T = 3$ upon lowering the pH to 5.0.[25,26] This process is completely reversible, and, remarkably, can take place without the viral RNA being present. This allows facile loading of a cargo into the capsids, which is essential for the application of CCMV capsids as nanocarriers.[27] Cargo encapsulation can be achieved in two ways: (*i*) in a statistical fashion, i.e. the cargo is added to the capsid protein dimers and the pH of the solution is lowered to 5.0 to induce capsid formation and simultaneous cargo encapsulation;[28,29] or (*ii*) through the attachment of a cargo to the capsid protein N-termini. In this way the cargo ends up on the inside of the capsid after assembly.[30,31] A major disadvantage of the wild-type CCMV VLP is that it is not stable under physiological conditions: at neutral pH the capsids disassemble again into capsid protein dimers. Nevertheless, successful in vitro and *in vivo* studies with CCMV have been reported.[20,32,33] These studies, however, involved stabilized capsids, i.e. capsids encapsulating the viral RNA or a negatively charged synthetic polymer mimicking the nucleic acid. Although these polymers offer the required stabilization at physiological conditions, their presence makes the encapsulation of an additional cargo challenging. Therefore, we are interested in tuning the stability of the capsid proteins such, that no additional compound, e.g. the negatively-charged polymer, is required to keep the capsids stable at physiological conditions.

Previously, a variant of the CCMV capsid protein was developed in our laboratory, which involved the substitution of the cationic nucleic acid-binding domain at the N-terminus by a short elastin-like polypeptide (ELP).[34] ELPs are stimulus-responsive peptides, consisting of repeating VPGXG pentapeptides (where the guest residue X can be any amino acid except proline). These peptides can be reversibly switched from a water-soluble state to an insoluble, hydrophobic state, upon changes in the environmental conditions, such as the temperature or salt concentration.[35] In addition, ELP phase transitions are

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dependent on the hydrophobicity of the guest residues, number of repeats and ELP concentration. The introduction of this stimulus-responsive peptide to the CCMV capsid proteins resulted in an expansion of their assembly properties: upon an increase of the temperature or salt concentration, spontaneous assembly into smaller T = 1 particles of roughly 18 nm was achieved at pH 7.5.[34] Although 2 M NaCl was still needed to achieve assembly, this assembly could at least be realized at a physiologically relevant pH. Further studies were aimed at introducing more hydrophobic guest residues in the ELP domain, with the objective to lower the amount of NaCl needed to assemble the capsids at physiological concentrations.[36] It was shown that changing only one or two out of the nine guest residues in the ELP fragment was enough to observe a significant decrease in the so-called 'transition salt concentration'. In particular, the two most hydrophobic variants caught our attention, as these remarkably assembled at 150 mM NaCl, which is close to the physiological salt concentration, indicating the potential of these ELP modified CCMV capsids as nanocarriers for *in vivo* studies.

Here, we present studies aimed at further investigating the potential of these two ELP-CCMV variants as carrier vehicles at physiological conditions. To this end, we thoroughly studied the assembly behavior of these capsid proteins, taking into account the possibility to encapsulate a cargo in the capsids. We aimed at developing a working protocol that involves disassembly of the capsids for potential cargo encapsulation, followed by reassembly to stable particles, such that experiments at physiological conditions can be performed. Particular attention will therefore be given to the aspects of capsid stability and the reversibility of the assembly process for cargo loading purposes.

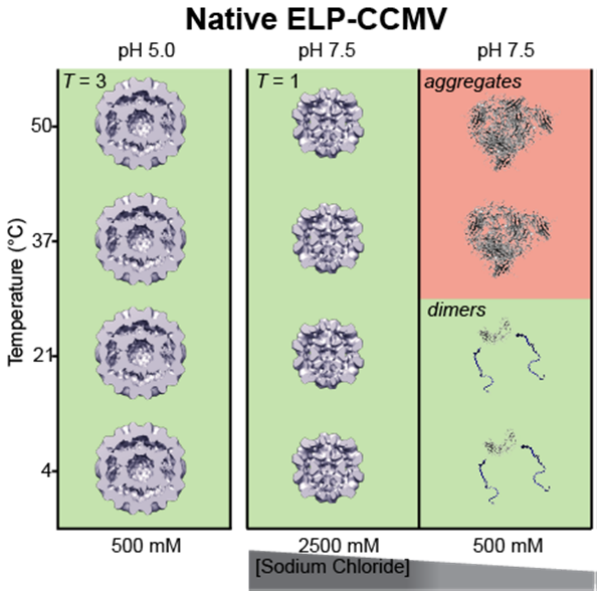

Figure 1: Overview of the assembly states of native ELP-CCMV in various buffers after incubation for 1 hour at the indicated temperature. The remaining soluble protein fraction (in either dimer or particle form) was determined by UV-vis spectroscopy and is highlighted with colours (red = 0%-24% and green = 75%-100%). The particle size was measured by DLS and is shown in the panels as pH-induced particles (pH 5.0, left), ELP-induced particles (pH 7.5 middle), dimers and aggregates (pH 7.5, right). The corresponding DLS intensity distributions and UV-vis absorbance spectra can be found in the SI (Figures S1-S6).

Results and Discussion

Design of hydrophobic ELP-CCMV variants

In order to create ELP-CCMV variants with higher stability at lower salt concentrations, hydrophobic residues were introduced in the ELP-domain of the ELP-CCMV capsid protein. Hereto, the native ELP-CCMV construct containing a H_6 tag (H_6-ELP-CCMV(ΔN26)) was used as the initial variant. This ELP sequence, VPGVG-VPGLG-VPGVG-VPGLG-VPGVG-VPGLG-VPGGG-VPGVG-VPGLG, contains as guest residues (the fourth amino acid of each pentapeptide repeat) four times a valine, four times a leucine, and once a glycine. This species can also be described by the general notation ELP[$V_4L_4G_1$-9], where the ratio of the guest residues is mentioned, followed by the number of pentapeptide repeats.

The two most promising hydrophobic ELP-CCMV variants, VY1-VY8 ELP-CCMV (ELP[$Y_2V_2L_4G_1$-9]) and VW1-VW8 ELP-CCMV (ELP[$W_2V_2L_4G_1$-9]) were developed by introducing valine to tyrosine and valine to tryptophan mutations, respectively, at the guest-residue of the first and eighth ELP pentapeptide. The introduction of the ELP fragment allows us to induce both pH-induced (T = 3 particles) and ELP-induced assembly (T = 1 particles), in which the ELP assembly behavior is dependent on salt and capsid protein concentration, pH, temperature, and time.[35]

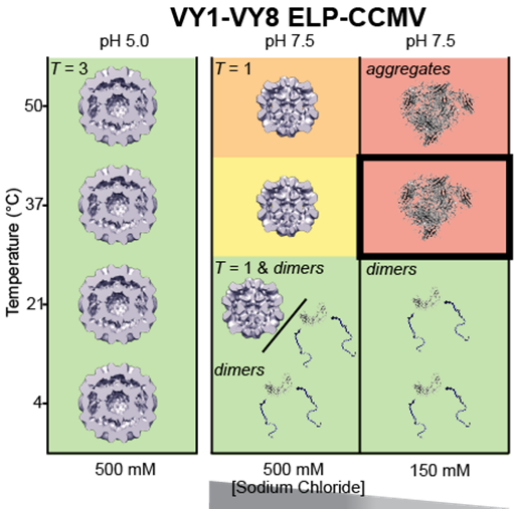

Figure 2: Overview of the assembly states of VY1-VY8 ELP-CCMV in various buffers after incubation for 1 hour at the indicated temperature. The remaining soluble protein fraction was determined by UV-vis spectroscopy and is highlighted with colours (red = 0%-24%, orange = 25%-49%, yellow = 50%-74% and green = 75%-100%). The particle size was measured by DLS and is shown in the panels as pH-induced particles (pH 5.0, left), ELP-induced particles and dimers (pH 7.5, middle), dimers and aggregates (pH 7.5, right). The assembly state at physiological conditions is indicated with a box. The corresponding DLS intensity distributions and UV-vis absorbance spectra can be found in the SI (Figures S9-S14).

Assembly behavior of the ELP-CCMV variants

In order to investigate the assembly behavior and stability of these ELP-CCMV variants, the pH-induced, hollow *T* = 3 particles were either diluted in a pH 5.0 buffer or spin-filtrated to a pH 7.5 buffer with NaCl concentrations ranging from 150 to 2500 mM and a final protein concentration of 20 μM. The stability of the ELP-CCMV variants in the pH 7.5 buffer was of interest for *in vivo* applications, while the experiments in the pH 5.0 buffer allowed a comparison of the stability of the ELP-induced particles with that of the pH-induced particles. The assembly behavior and stability was followed over time by dynamic light scattering (DLS) and UV-vis spectroscopy. When protein aggregation occurred, the samples became considerably turbid. The soluble protein concentration was measured at several time points by determining the absorbance at 280 nm after removal of the precipitated protein.

In a first series of experiments the assembly behavior and stability of the native ELP-CCMV were determined in several buffers (Figure 1). Interestingly, this native ELP-CCMV showed surprisingly high stability up to 50°C in pH 5.0 buffer (*T* = 3 particles by pH-induced assembly), however over an extended incubation period in these conditions a large fraction of the protein aggregated. In pH 7.5 buffer containing 2.5 M NaCl (*T* = 1 particles by ELP-induced assembly) the protein also demonstrated high stability, however at more physiological relevant salt concentrations (500 mM NaCl) capsid assembly did not occur, as we reported previously, indicating the need for more hydrophobic variants.[36]

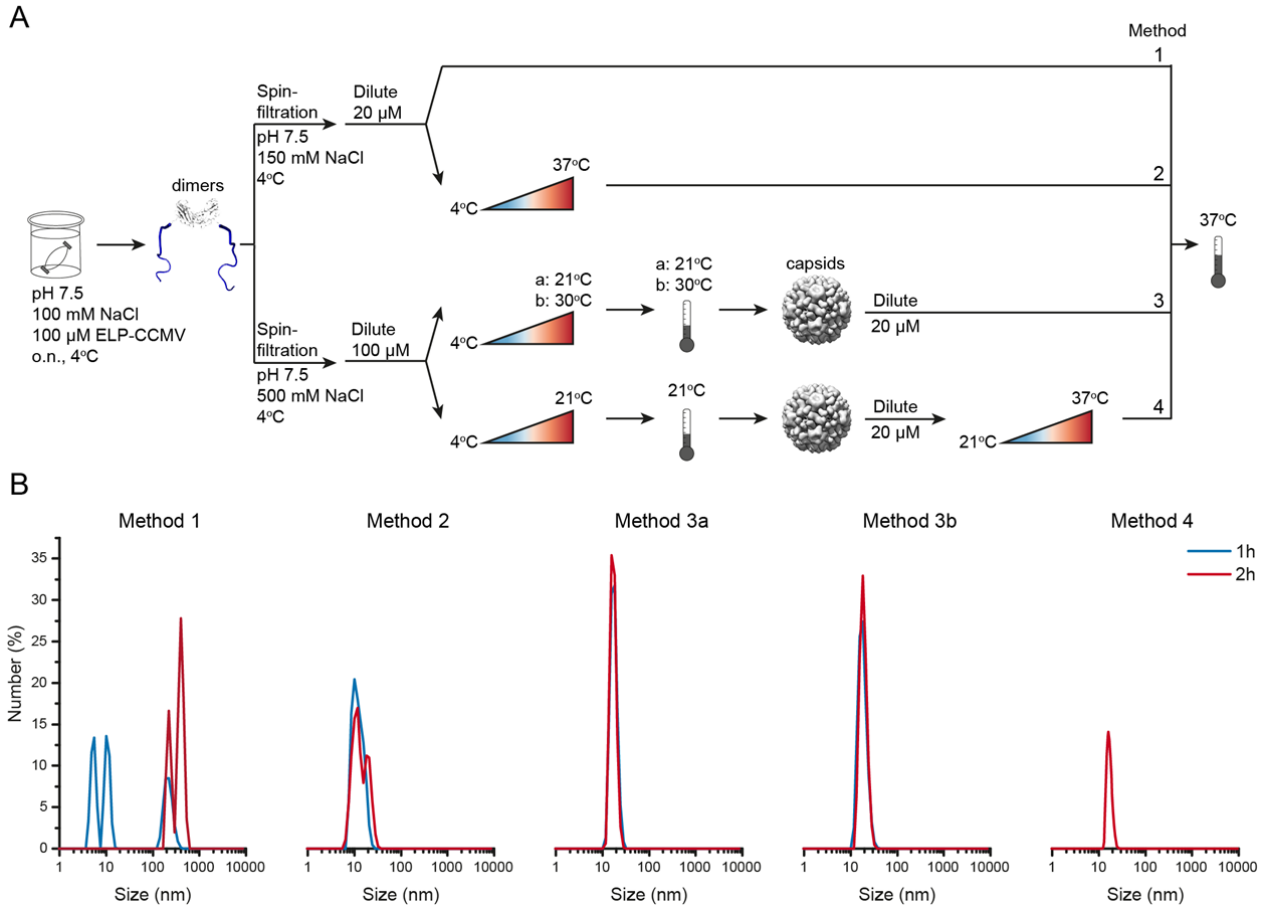

Figure 3: Optimization of capsid assembly at pH 7.5. A) Schematic representation of all steps of the different methods. Method 1 comprises overnight dialysis of a 100 μM VY1-VY8 ELP-CCMV solution to pH 7.5 buffer supplemented with 100 mM NaCl to render coat protein dimers, followed by spin-filtration to pH 7.5 buffer with 150 mM NaCl, dilution to 20 μM VY1-VY8 ELP-CCMV and subsequent incubation at 37°C. In method 2 the temperature is increased with 1°C per min. after dilution of the protein solution and before incubation at 37°C. In method 3 the solution of VY1-VY8 ELP-CCMV coat protein dimers is spin-filtered to pH 7.5 buffer supplemented with 500 mM NaCl, subsequently diluted to 100 μM protein, after which the temperature is increased to a) 21°C or b) 30°C. The protein solution is then incubated at these temperatures for 30 minutes to allow capsid self-assembly. Finally, the solution is diluted to 20 μM VY1-VY8 ELP-CCMV and 150 mM NaCl and incubated at 37°C. In method 4 the temperature is slowly increased with 1°C per min. after dilution of the solution to 20 μM protein and 150 mM NaCl, and before incubation at 37°C. B) Number distributions after 1 h and 2 h of incubation at 37°C and at a final protein concentration of 20 μM, as determined by DLS. The corresponding intensity distributions are given in the SI (Figure S15).

Next, the assembly behavior of both hydrophobic variants was determined (see SI Figure 7). DLS experiments showed that the capsids of these variants remained assembled in pH 5.0 buffer. Interestingly, in pH 7.5 buffer containing 500 mM NaCl the capsids did not completely disassemble, indicating the higher stability of these hydrophobic variants compared to the native ELP-CCMV. Since ELP-CCMV assembly behavior and stability are dependent on many variables, i.e. salt, capsid protein concentration, pH, temperature, and time,[35] a more extensive dialysis was performed overnight at 4°C to achieve full disassembly of these more hydrophobic variants. Although the native and VY1-VY8 ELP-CCMV showed complete disassembly towards dimeric capsid proteins after this extended protocol, the VW1-VW8 ELP-CCMV capsid proteins remained fully assembled. Additional experiments showed that disassembly of the VW1-VW8 ELP-CCMV variant even was not achieved after further reducing the NaCl concentration and protein concentration to 0 μM and 5 μM, respectively (see SI Figure 8).
Since the hydrophobic variant, VY1-VY8 ELP-CCMV, displayed reversible assembly behavior, we decided to continue the study of the assembly behavior and stability of this ELP-capsid protein variant in the same way as was done for the native ELP-CCMV, with the exception that an extensive dialysis overnight at 4°C was performed in order to achieve complete disassembly (Figure 2). Compared to the native ELP-CCMV, the VY1-VY8 ELP-CCMV variant showed comparable stability up to 50°C in pH 5.0 buffer. Interestingly, the stability in pH 7.5 buffer containing 500 mM NaCl was considerably increased, resulting in fairly stable T = 1 particles up to 37°C. However, self-assembly into stable capsids did not occur in a buffer with a nearly-physiological NaCl concentration (150 mM).

Optimization of capsid assembly in nearly-physiological buffer

As the VY1-VY8 ELP-CCMV coat protein was unstable in pH 7.5 buffer containing 150 mM NaCl, manifested by severe protein aggregation, we attempted to improve the protein stability in this buffer. Our initial data suggested (Figure 2, Figure 3**Error! Reference source not found.**) that aggregation of the VY1-VY8 ELP-CCMV coat protein occurred quicker than capsid assembly at a 20 μM concentration in pH 7.5 buffer with 150 mM NaCl. Therefore, we opted to find a procedure that reduced aggregation and at the same time accelerated capsid formation. At physiological pH, the assembly of VY1-VY8 ELP-CCMV capsids is driven by the N-terminal ELP domain[34] and is thus a kinetic process dependent on e.g. protein and NaCl concentration, and temperature[35], as opposed to thermodynamically driven self-assembly known for other peptide domains.[37] Accordingly, we initially adapted the existing protocol by adding a gradual temperature increase to 37°C before incubating the protein solution at this temperature (Method 2 in Figure 3 A). The rationale behind this approach was to allow the capsid proteins to slowly undergo temperature-induced changes at temperatures that are suitable for capsid assembly, but do not induce aggregation. Although this approach indeed reduced the degree of aggregation, capsid assembly was not improved sufficiently. Therefore, we focused on the other factors that can influence ELP self-assembly and increased the initial protein and NaCl concentrations to 100 μM and 500 mM, respectively, in order to further promote capsid formation. In addition, we allowed the VY1-VY8 ELP-CCMV coat

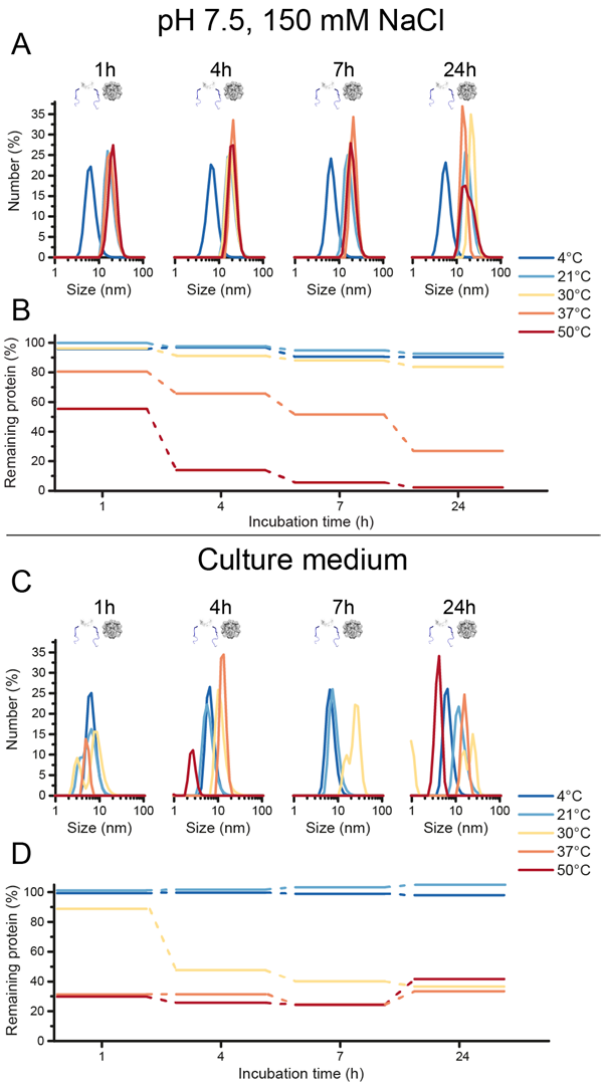

Figure 4: Capsid assembly state and protein stability of 20 μM VY1-VY8 ELP-CCMV in pH 7.5 buffer with 150 mM NaCl after initial capsid self-assembly at 100 μM VY1-VY8 ELP-CCMV, 500 μM NaCl and 21°C (a, b) and in DMEM after initial capsid self-assembly at 200 μM VY1-VY8 ELP-CCMV and 25°C in DMEM (c, d). Number distributions (a, c) were determined by DLS after 1 h, 4 h, 7 h, and 24 h of incubation at the indicated temperatures. The corresponding intensity distributions can be found in SI Figure S19 and S30. The remaining soluble protein percentage (b, d) was determined by UV-vis spectroscopy and is plotted against the incubation time. The corresponding spectra can be found in SI Figure S20 and S31.

protein to self-assemble at 21°C or 30°C, temperatures that did not induce protein aggregation, before dilution to 20 μM VY1-VY8 ELP-CCMV and 150 mM NaCl and subsequent incubation at 37°C (Method 3 in Figure 3 A). This approach resulted in the formation of capsids that remained assembled at 37°C for at least 2 hours, while protein aggregation was minimized. A further change in the procedure, i.e. by introducing a gradual temperature increase after capsid formation, right before incubation at 37°C, did not improve capsid self-assembly or stability further (Method 4 in Figure 3 A), indicating that VY1-VY8 ELP-CCMV capsids are less sensitive to temperature changes than coat protein dimers or intermediate assemblies.

Table 1. Particle sizes as determined by TEM analysis. The ELP-CCMV variants were incubated in either pH 7.5 buffer with 150 mM NaCl (called Buffer) or DMEM at 37 °C for the indicated time period prior to TEM sample preparation.

ELP-CCMV variant	Buffer, 1 h	Buffer, 24 h	DMEM, 1 h	DMEM, 24 h
Native ELP-CCMV	No particles	No particles	Not determined	Not determined
VY1-VY8 ELP-CCMV	22.8 ± 2.4 nm	23.1 ± 2.1 nm	21.7 ± 1.8 nm[a]	No particles[a]
VW1-VW8 ELP-CCMV	26.3 ± 2.0 nm	25.5 ± 2.0 nm	24.0 ± 2.2 nm	No particles

[a] Incubated at 30°C, as no particles were found to be present at 37°C

Stability of VY1-VY8 ELP-CCMV capsids in nearly-physiological buffer

After having established that we can stabilize VY1-VY8 ELP-CCMV capsids under nearly-physiological conditions, we were interested in determining the fate of these capsids during prolonged incubation. Hereto, we first assembled capsids via method 3a (Figure 3 A) and then incubated these particles at temperatures ranging from 4°C to 50°C for up to 24 hours. Capsid assembly state and protein stability were evaluated with the help of DLS and UV-vis spectroscopy, respectively. In order to get a better understanding of what factors are important in determining the protein stability and assembly state, we compared the fate of VY1-VY8 ELP-CCMV capsids with the nanocages formed by a less hydrophobic variant, i.e. native ELP-CCMV. We discovered that the capsids formed by VY1-VY8 ELP-CCMV coat proteins remained assembled at temperatures above 21°C in the buffer that mimics physiological conditions, while at 4°C no capsids
could be observed by DLS (Figure 4 A). In addition, the protein demonstrated remarkable stability at 30°C for at least 24 h. More importantly, even at 37°C it was quite stable in the considered time period, with the protein concentration gradually decreasing over time (Figure 4 B). Transmission electron microscopy (TEM) analysis (Table 1, Figure S34) confirmed that capsids were present at 37°C during the complete 24 hour incubation period. Interestingly, the native ELP-CCMV coat protein, lacking the two hydrophobic tyrosine residues in its ELP domain, did not remain assembled in the same buffer conditions (see SI Figure S16, S17, and S18), indicating the importance of capsid stabilization by ELP assembly. More importantly, while the protein sequences of the VY1-VY8 ELP-CCMV and native ELP-CCMV coat proteins only differ by the two valine to tyrosine substitutions in the ELP domain, the native ELP-CCMV protein is much less stable at 37°C and 50°C than the VY1-VY8 ELP-CCMV variant (SI). This suggests that the formation of capsids increases the stability of the ELP-CCMV coat proteins even at elevated temperatures.

Assembly and stability of VY1-VY8 ELP-CCMV capsids in cell culture medium

After having established that capsids of the VY1-VY8 ELP-CCMV variant were fairly stable in a buffer that mimicked physiological conditions, it was evaluated whether capsids would also remain intact in a cell culture medium (Dulbecco's Modified Eagle Medium, DMEM), which resembles *in vivo* conditions even closer. Hereto, it was first attempted to assemble VY1-VY8 ELP-CCMV capsids in DMEM by incubating a 100 μM protein solution in this buffer for 30 minutes at 21°C, in a procedure very similar to the optimized capsid assembly protocol described above. However, no capsid assembly was observed in this way (see Figure S23). Therefore, the assembly protocol was further optimized by increasing the VY1-VY8 ELP-CCMV concentration to 200 μM, the assembly temperature to 25°C, and the induction time to 1 hour (see Figure S25 and S26). These conditions provided a balance between fast capsid formation, which was accelerated by the increased coat protein concentration and elevated temperature (see Figure S23 and S25), and reduction of temperature-induced protein aggregation (see Figure S24 and S26). In addition, the prolonged incubation time ensured that capsids remained stable upon dilution to 20 μM (Figure S25).
We employed the optimized protocol for the evaluation of the stability of VY1-VY8 ELP-CCMV in DMEM at temperatures ranging from 4°C to 50°C. DLS analysis was performed in order to determine the assembly state, while the protein concentration in solution was monitored by UV-vis spectroscopy. Initially UV-vis measurements in DMEM were troublesome, as light-induced degradation of components in the DMEM solution interfered with our measurements, as also observed previously in the literature.[38] We could minimize the occurrence of these artefacts by protecting the DMEM and protein solutions therein from light irradiation during all steps of the procedure (Figure S27).
When we compare the results in DMEM with those in the nearly-physiological buffer we see similar trends, although the overall stability of the hydrophobic VY1-VY8 ELP-CCMV variant was reduced (Figure 4). The VY1-VY8 ELP-CCMV variant only remained assembled into capsids at temperatures from 30°C and above, while the coat protein itself was very stable at 4°C and 21°C (Figure 4 C, D). Most of the protein was lost within 1 hour at 37°C and 50°C. TEM analysis confirmed these findings (Table 1, Figure S35). It is most likely that some of the components in the DMEM solution have a destabilizing effect on ELP-CCMV capsid assembly, since we ruled out the possibility that the NaCl concentration is the limiting factor (Figure S28 and S29) and the ionic strengths of the nearly-physiological buffers and DMEM formulation are comparable. Nonetheless, the VY1-VY8 ELP-CCMV is promising for *in vivo* studies, especially since the stability can be further improved in the future by e.g. cross-linking of the capsid proteins.

Stability of VW1-VW8 ELP-CCMV capsids under nearly physiological conditions

Since the VY1-VY8 ELP-CCMV variant was found to have promising stability under nearly-physiological conditions, we decided to also evaluate the more hydrophobic and perhaps more stable VW1-VW8 ELP-CCMV variant. The fact that we could not disassemble its capsids, even at reduced protein and NaCl concentrations or in the absence of the stabilizing $MgCl_2$, (see SI Figure S8 A, B), was a first indication of improved stability. This was further supported by DLS data that suggested that the coat proteins already assembled into capsids during bacterial expression (see SI Figure S8 C, D). Therefore, we investigated the fate of VW1-VW8 ELP-CCMV capsids during prolonged incubation at nearly-physiological conditions. When we incubated capsids of this hydrophobic variant in the nearly-physiological buffer in the same way as for the VY1-VY8 ELP-CCMV variant, we found that the more hydrophobic VW1-VW8 ELP-CCMV coat protein remained assembled into capsids even at 4°C in the same buffer conditions (Figure 5 A, B). More importantly, the VW1-VW8 ELP-CCMV protein is much more stable at 37°C and 50°C than the VY1-VY8 ELP-CCMV variant. This suggests that the increased hydrophobicity of the ELP domain further enhances capsid stability at elevated temperatures. When we incubated capsids formed by the VW1-VW8 ELP-CCMV variant in DMEM, we again found the capsids of this variant to be more stable than the ones formed by the less hydrophobic VY1-VY8 ELP-CCMV variant. The VW1-VW8 ELP-CCMV protein remained assembled into capsids at all temperatures that were tested and was very stable at temperatures up to 30°C (Figure 5 C, D). However, at 50°C it quickly degraded, while at 37°C it degraded gradually over a time course of 7 hours, which allows sufficient time for *in vivo* uptake studies. Thus, with its increased stability compared to VY1-VY8 ELP-CCMV, the VW1-VW8 variant is very suitable for *in vivo* applications where especially a stable protein cage is required.

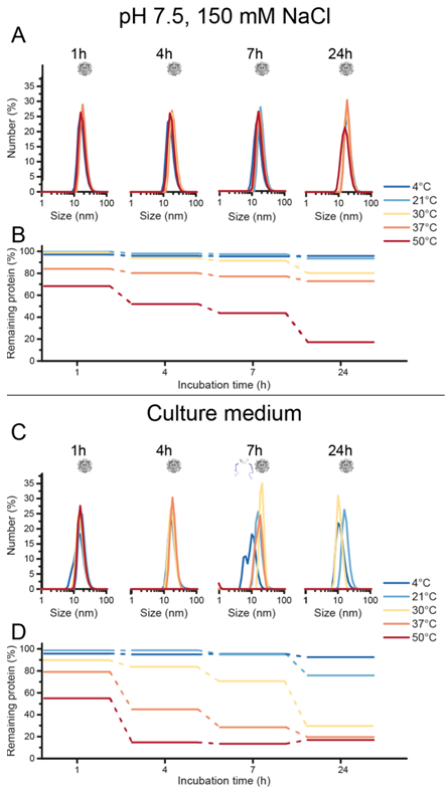

Figure 5: Capsid assembly state and protein stability of 20 μM VW1-VW8 ELP-CCMV in pH 7.5 buffer with 150 mM NaCl after initial capsid self-assembly at 100 μM VW1-VW8 ELP-CCMV, 500 μM NaCl and 21°C (a, b) and in DMEM after initial capsid self-assembly at 200 μM VW1-VW8 ELP-CCMV and 25°C in DMEM (c, d).Number distributions (a, c) were determined by DLS after 1 h, 4 h, 7 h, and 24 h of incubation at the indicated temperatures. The corresponding intensity distributions can be found in SI Figure S21 and S32. The remaining soluble protein percentage (b, d) was determined by UV-vis spectroscopy and is plotted against the incubation time. The corresponding spectra can be found in SI Figure S22 and S33.

Combined, our findings in nearly-physiological buffer and DMEM demonstrate the importance of stabilization of the ELP-CCMV capsids in order to increase the resistance of the protein nanocage to elevated temperatures. Between 21°C and 30°C the coat protein is sufficiently stable by itself, but above this temperature range capsid assembly is required to prevent uncontrolled protein aggregation. Furthermore, we demonstrated that by making subtle adjustments in the assembly conditions we could induce striking changes in capsid stability. In general the introduction of hydrophobic residues in the ELP domain greatly increases capsid stability and this stability may be further enhanced in the future by making modifications in the capsid exterior or encapsulating stabilizing cargoes. In addition, it is likely that a crowded environment such as the cell interior will further stabilize ELP-CCMV capsids by physically hampering disassembly.

Conclusions

We have extensively studied the assembly behavior and stability of two ELP-CCMV variants, which are promising for *in vivo* studies. As we demonstrated, the VY1-VY8 ELP-CCMV protein can be reversibly assembled and disassembled, which is advantageous for future cargo encapsulation prior to *in vivo* studies. After optimization of the assembly protocol, capsids of this variant were stable in pH 7.5, 150 mM NaCl buffer at 37°C for 7 hours and in DMEM for around 1 h, which is approximately the time scale for cellular uptake. Capsids assembled from the VW1-VW8 ELP-CCMV protein were even more stable, as they remained assembled for at least 24 h in pH 7.5, 150 mM NaCl buffer at 37 °C and for 4-7 hours in DMEM. So far, we did not succeed in fully disassembling capsids formed by this more hydrophobic variant. This may be problematic for cargo loading, although a solution could be to employ *in vivo* cargo loading during capsid protein expression, which is common practice for several other VLPs.[39,40] Remaining challenges for future research are the further improvement of the stability of the VY1-VY8 ELP-CCMV variant and to find other ways for encapsulating cargo inside capsids formed by the VW1-VW8 ELP-CCMV variant.

Experimental Section

Buffers. pH 5.0 capsid buffer: 50 mM NaOAc, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 5.0. pH 7.5 62.5 mM NaCl buffer: 50 mM Tris·HCl, 62.5 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.5. pH 7.5 100 mM NaCl buffer: 50 mM Tris·HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM

EDTA, pH 7.5. pH 7.5 150 mM NaCl buffer: 50 mM Tris·HCl, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.5. pH 7.5 500 mM NaCl buffer: 50 mM Tris·HCl, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.5. pH 7.5 2500 mM NaCl buffer: 50 mM Tris·HCl, 2500 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.5.

General expression and purification protocol. The pET-15b-H_6-ELP-CCMV(ΔN26), pET-15b-H_6-VY1-VY8-ELP-CCMV(ΔN26) and pET-15b-H_6-VW1-VW8-ELP-CCMV(ΔN26) vectors encoding for the three ELP-CCMV variants used in this paper have previously been constructed[34,36]. *E.coli* BLR(DE3)pLysS containing any of these vectors were cultured overnight at 37°C in 50 mL LB medium containing ampicillin (100 mg/L) and chloramphenicol (50 mg/L). The overnight culture was used to inoculate 2xTY medium (1 L), containing ampicillin (100 mg/L). Protein expression was done by inducing exponentially growing bacteria (OD_{600} between 0.4 – 0.6) with 1 mM IPTG at 30°C for 5-6 hours. Bacteria were lysed by ultrasonic disruption. Purification was done by immobilized metal affinity chromatography. Proteins were dialyzed against pH 5.0 capsid buffer (2 times 30-60 minutes), followed by overnight dialysis using a 12-14 kDa tubing) for stable storage at 4°C. The purity and characteristics of the protein were verified and determined by SDS-PAGE, SEC, Q-TOF, DLS and TEM.

General protocol for assembly and stability assays: For a typical assembly and stability assay, an ELP-CCMV variant (100 μM) was dialyzed overnight to pH 7.5, 100 mM NaCl buffer (12-14 kDa MWCO, 2 x 30 min + o.n., 4°C). Subsequently the protein was spin-filtered to either pH 7.5 buffer 500mM NaCl (buffer experiments) or DMEM (DMEM experiments) (10 kDa MWCO, 3 x 10 min, 4°C). For buffer experiments, a pre-incubation solution of 100 μM ELP-CCMV variant was prepared, heated to 21°C with 1°C/min and incubated for 30 minutes at this temperature to allow capsid assembly. For DMEM experiments, the pre-incubation solution contained 200 μM of the ELP-CCMV variant and was heated to 25°C with 1°C/min before incubation at the final temperature for 1 hour to induce self-assembly into capsids. The protein solutions were then diluted with pH 7.5, 62.5 mM NaCl buffer (for buffer experiments) or DMEM (for DMEM experiments) to a final concentration of 20 μM ELP-CCMV variant and incubated at temperatures ranging from 4°C to 50°C for up to 24 h. After 0, 1, 4, 7, and 24h samples were taken for DLS and UV-vis analysis to assess assembly state and stability of the ELP-CCMV variant respectively. All samples were centrifuged twice prior to analysis.

For further detailed procedures see the Supporting Information.

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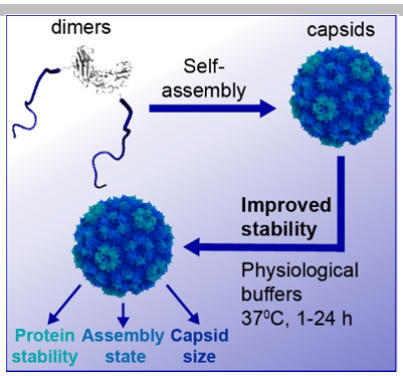
Layout 1:

FULL PAPER

Virus-like particles ready for *in vivo* applications: Protein-based block copolymers composed of the cowpea chlorotic mottle virus (CCMV) capsid protein and two hydrophobic elastin-like polypeptides are studied under nearly physiological conditions. Both hybrid variants form stable capsids under these conditions. These engineered hybrid CCMV capsid particles can therefore be regarded as suitable candidates for *in vivo* applications.



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Self-assembly and stabilization of hybrid cowpea chlorotic mottle virus particles under nearly physiological conditions