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Supporting Information

Engineered Protein Cages for Selective Heparin Encapsulation

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1. Materials and Methods

1.1 Materials

Ampicillin was purchased from MP Biomedicals. Chloramphenicol was obtained from Sigma-Aldrich. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Acros. Ni-NTA agarose beads were obtained from Qiagen. Heparin binding peptide KMEKKLHAVPAAKTVKFKGGLPETG-NH_2 was purchased from Pepscan. According to the heparin supplier (Sigma-Aldrich), heparin molecular weight ranges between 6 and 30 kDa and most chains are 17-19 kDa. Based on this we have used an average molecular weight of 18 kDa for heparin.

1.2 Buffers

Table S1. Name and composition of the buffer used in the study.

Name	Composition
encapsulation buffer	50 mM Tris-HCl, pH 7.4
dimer buffer	50 mM Tris·HCl, 500 mM NaCl, 10 mM $MgCl_2$, pH 7.5
capsid buffer	50 mM NaOAc, 500 mM NaCl, 10 mM $MgCl_2$, pH 5.0
Sortase buffer	50 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5

1.3 Protein concentration

Protein concentrations were measured on a Varian Cary 50 Conc UV-vis spectrometer using a quartz cuvette with a path length of 3 mm. Protein concentrations were calculated using the theoretical extinction coefficients. Samples were centrifuged prior to the measurements. After dialysis the protein concentration was measured with a Thermo Fisher Scientific NanoDrop Lite Spectrophotometer.

1.4 Dialysis

CCMV capsids were disassembled by dialyzing the CCMV (0.94 mg/mL) in the capsid buffer against 500 times in volume of dimer buffer for 18 hours. Dialyzed solutions were used directly for measurements.

1.5 Mass spectrometry

Protein mass characterization was performed by electrospray ionization time-of-flight (ESI-TOF) on a JEOL AccuTOF CS or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) on a Bruker UltrafleXtreme. Deconvoluted mass spectra were obtained using MagTran 1.03 b2 or FlexAnalysis 3.4 software. Isotopically averaged molecular weights were calculated using the 'Protein Calculator v3.4' at http://protcalc.sourceforge.net. Protein samples were desalted by spin filtration with MQ.

1.6 Dynamic light scattering (DLS)

Size measurements were conducted with a Zetasizer Nano ZS device (Malvern Instruments) with a 4 mW He-Ne ion laser at the wavelength of 633 nm and an Avalanche photodiode detector at an angle of 173°. Samples were measured in micro-cuvettes (ZEN0040). Each sample was measured at 25 °C at least two times and the results presented are the averages of these measurements. Zetasizer software (Malvern Instruments) was used to obtain the data. Cumulant analysis was used to give the volume mean value, the width parameter, i.e., polydispersity index, and the volume size distribution graphs together with the correlation function graphs.

Samples with heparin were prepared by mixing 70 µL of freshly dialyzed CCMV CP solution (0.65 mg/mL, 50 mM Tris-HCl, pH 7.4) with 20 µL of 0.05, 0.50 or 1.0 mg/ml fluorescein-heparin solution to yield heparin to CP mass ratios of 0.02, 0.22 and 0.44. Samples were brought to final volume of 200 µL and were measured within 30 s after mixing. For the selectivity samples, 75 µL of CCMV CP solution (0.38 mg/ml, 50 mM Tris-HCl, pH 7.4) was mixed with 0.50 mg/mL hyaluronic acid, 0.44 chondroitin sulfate or 1.45 mg/mL bovine serum albumin in 50 mM Tris-HCl, pH 7.4. Samples were brought to final volume of 100 µL. Disassembled CPs were measured in the beginning of each measurement before adding the other components. CCMV capsids were measured in 70 µL volume and 0.94 mg/mL concentration.

1.7 Transmission electron microscopy (TEM)

For the samples after expression and Sortase-A coupling, TEM grids (FCF-200-Cu, EMS) were glow-discharged using a Cressington carbon coater and power unit. Protein samples (0.2 mg/mL, 5 µL) were applied on the glow-discharged grids and incubated for 1 min. The samples were carefully removed using a filter paper and the grid was allowed to dry for at least 15 minutes. Then the grid was negatively stained by applying 2% uranyl acetate in water (5 µL). The staining solution was removed after 15 seconds and the grid was allowed to dry for at least 15 minutes. The samples were analyzed on a JEOL JEM-1010 TEM.

Tecnai 12 Bio Twin instrument was utilized for the TEM imaging after reassembling the capsids. Samples were prepared on Formvar carbon coated copper grids (FCF-400-Cu) by adding a 2 µL drop of the DLS sample solution or the CCMV capsid solution (0.094 mg/mL) or the CP solution (0.031 mg/mL). The sample drop was left on the grid for 1 min following removal of the excess solution with filter paper and the grid was allowed to dry for 20 minutes. Samples were stained with 1 % uranyl acetate water solution by adding 2 µL on to grid for 30 seconds after which the grid was dried with filter paper. Finally, the samples were dried under ambient conditions for minimum of 30 minutes. TEM images were further analyzed with the ImageJ program to define the diameters of the assemblies. In total 60 individual spherical particles were used from each sample to define the average diameters and standard deviations.

1.8 Cryogenic transmission electron microscopy (cryo-TEM)

The cryo-TEM images were collected using JEM 3200FSC field emission microscope (JEOL) operated at 300 kV in bright field mode with Omega-type Zero-loss energy filter. Prior to sample preparation, 200 mesh copper grids with lacey carbon support film (Electron Microscopy Sciences) were plasma cleaned using Gatan Solarus (Model 950) plasma cleaner for 30 seconds. The samples for Cryo-TEM imaging were prepared by placing 3 µL of a freshly prepared aqueous dispersion of the sample (protein concentration 0.59 mg/mL) on a plasma treated TEM grids and plunge-freezed into -170 °C ethane/propane mixture using Vitrobot™ 2 s blotting time under 100 % humidity. The vitrified specimen were cryo-transferred to the microscope. The images were acquired with Gatan Digital Micrograph®software while the specimen temperature was maintained at -187 °C.

1.9 Fast protein liquid chromatography (FPLC)

FPLC measurements were performed on a Superose 6 increase 10/300 column (GE Healthcare). Analytical and preparative FPLC measurements were executed on a Shimadzu LC-2010AHT HPLC and Agilent 1260 bio-inert HPLC, respectively. Samples were separated on the column with a flow rate of 0.5 mL/min.

FPLC measurements of the reassembled capsids were conducted with GE Healthcare Life Sciences ÄKTA Pure 25 M1 system together with Superose 6 10/300 column. Samples prepared for the DLS and TEM measurements were studied and a sample for the selectivity study was prepared by mixing 75 µL CCMV CP (0.38 mg/mL) with 15 µL bovine serum albumin (1.45 mg/mL). Samples were brought to final volume of 200 µL and 50 mM Tris-HCl (pH 7.4) buffer was used throughout the measurements. UV-Vis spectra were recorded using a U9-M multiwavelength detector and fractions were collected with Flexible fraction

collector F9-C in 1 mL aliquots. Fractions were concentrated with spin-filtering with 3 kDa filters (Amicon Ultra-0.5 Centrifugal Filter Unit).

1.10 Fluorescence spectroscopy

For the fluorescence measurements, a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) was used. Concentrated fractions collected from the FPLC runs were measured in a glass cuvette with excitation wavelength of 485 nm and the emission spectra were recorded between 500 and 700 nm.

1.11 Nuclear magnetic resonance (NMR) spectroscopy

NMR sample of the fluorescein labeled heparin was prepared in deuterium oxide. ^{1}H spectra were recorded with a Bruker AVANCE 400 MHz instrument at 298 K, and the solvent peak was used as reference.

1.12 Hemolysis assay

The detailed procedure for the hemolysis assay has been previously reported.[1] Generally, freshly-donated human red blood cells were purchased (Cambridge Bioscience Ltd, the United Kingdom) and stored at 4 °C. Before samples were added, 1 mL of blood was centrifuged at 500× g for 5 minutes and the plasma was removed gently. The remaining red blood cells were washed with 1× PBS for 3 times and redispersed to the initial volume in 1× PBS. The red blood cells were diluted 50× and split into 96-well culture plates (190 μL/well). 10 μL of concentrated sample solutions in 1× PBS were added to each well, resulting in desired final compound concentrations (50 ~ 500 μg/mL). 10 μL of 20% Triton X-100 in 1× PBS and 10 μL of 1× PBS were added as positive and negative controls, respectively. After incubation at 37 °C for 1 h, the plates were centrifuged for 5 min at 500× g to pellet intact erythrocytes, and 100 μL of supernatant from each well was delicately transferred into a clear 96-well plate. The resulting hemoglobin in supernatant was measured at 540 nm with a microplate reader (Cytation 3, Biotek). The percentage of hemolysis was calculated as follows:

$$\% \text{ Hemolysis} = [(A_{sample} - A_{negative\ control}) / (A_{positive\ control} - A_{negative\ control})] \times 100$$

The measurements were performed using triplicate samples.

2. Experimental results

2.1 SrtA-mediated CCMV CP-HBP synthesis and characterization

2.1.1 Expression of Sortase A

The expression was performed according to a literature procedure.[2] The pQE30-H_6-SrtA vector encoding for the hexahistidine-tagged SrtA protein was previously constructed by the group of Geerten W. Vuister (Department of Biochemistry, University of Leicester) and kindly donated to our group. *E. coli* BL21 AI cells were transformed with a pQE30 plasmid carrying the Sortase gene, followed by incubation in LB medium (1 mL) for 1h at 37 °C. After this short incubation phase, the cells were transferred into fresh LB medium (4 mL) with ampicillin (100 mg/L) and were incubated at 37 °C for 4h. This preculture was then transferred into TB medium (500 mL) with ampicillin (100 mg/L) and cells were incubated for 24h at 37 °C. Cells were pelleted and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole and supplemented with 1 mM phenylmethanesulfonyl fluoride, pH 8.0) and lysed by sonication. The lysate was centrifuged (14.000 g, 30 min, 4 °C) and the supernatant was incubated with Ni-NTA beads for 2 h at 4 °C. Ni-NTA beads were washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and the purified protein was eluted from the beads with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0). For storage the protein was dialyzed against Sortase buffer. The pure protein was obtained with a yield of 10-13 mg/L of culture. The purity was verified by SDS-PAGE. ESI-TOF: calculated 21947.5 Da, found 21948.7 Da.

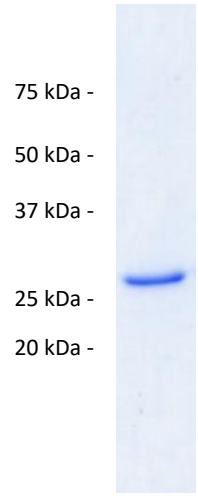

Figure S1. SDS-PAGE of purified Sortase A.

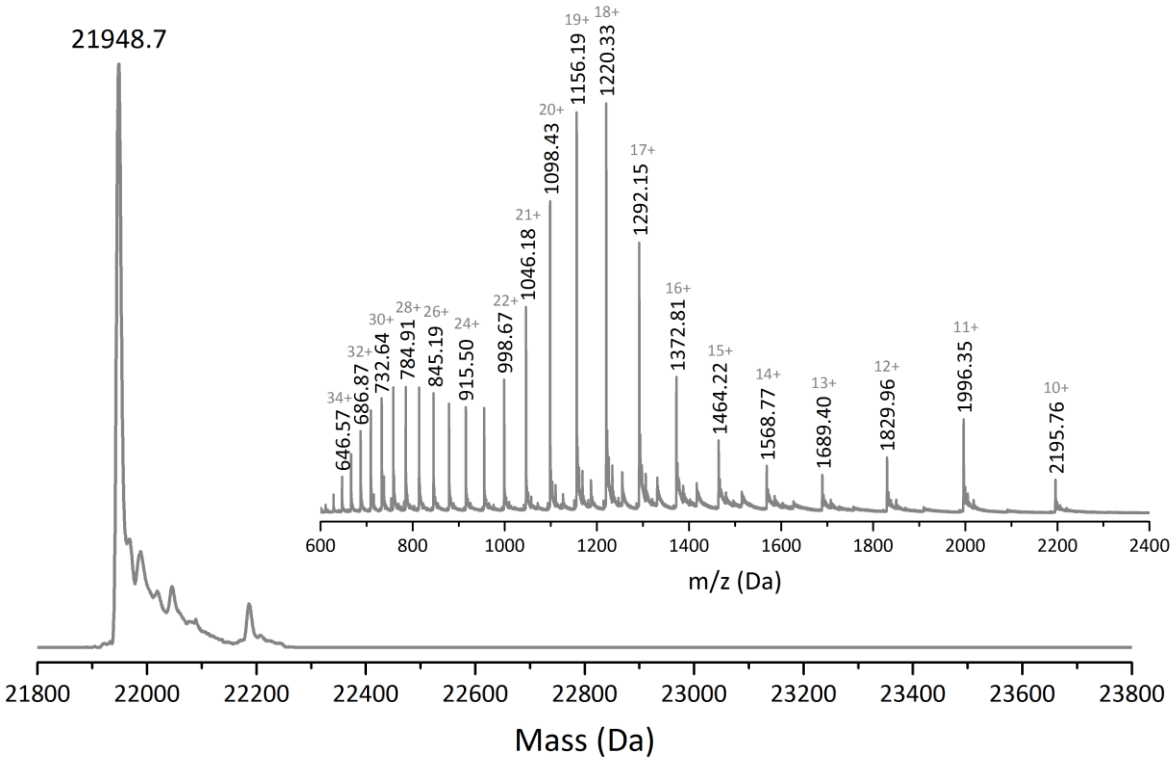

Figure S2. ESI-TOF mass spectrometry of purified Sortase A. Deconvoluted total mass spectrum and multiply charged ion series (inset). The expected molecular weight is 21947.5 Da.

2.1.2 Expression of G-ELP-CCMV

The pET-15b-G-H_6-[$V_4L_4G_1$-9]-CCMV(ΔN26) vector encoding for the hexahistidine-tagged ELP-CCMV protein was previously constructed as described by van Eldijk *et al.*[3] The expression was performed according to a literature procedure.[2] For a typical expression, LB medium (50 mL), containing ampicillin (100 mg/L) and chloramphenicol (50 mg/L), was inoculated with a single colony of *E. coli* BLR(DE3)pLysS containing the pET-15b vector encoding for the desired CCMV capsid protein, and was incubated overnight at 37 °C. This overnight culture was used to inoculate 2x TY medium (1 L), supplemented with ampicillin (100 mg/L). The culture was grown at 37 °C and protein expression was induced during logarithmic growth (OD_{600} = 0.4–0.6) by addition of IPTG (1 mM). After 6 h of expression at 30 °C, the cells were harvested by centrifugation (2700 g, 15 min, 4 °C) and the pellets were stored overnight at -20 °C.

After thawing, the cell pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 1.3 M NaCl, 10 mM imidazole, pH 8.0; 25 mL). The cells were lysed by ultrasonic disruption (3 times 30 s, 100% duty cycle,

output control 3, Branson Sonifier 250, Marius Instruments). Then the lysate was centrifuged (16.400 g, 15 min, 4 °C) to remove the cellular debris. The supernatant was incubated with Ni-NTA agarose beads (3 mL) for 1 h at 4 °C. The suspension was loaded onto a column, the flow-through was collected and the beads were washed twice with wash buffer (50 mM NaH_2PO_4, 1.3 M NaCl, 20 mM imidazole, pH 8.0; 20 mL). Then, the protein of interest was eluted from the column with elution buffer (50 mM NaH_2PO_4, 1.3 M NaCl, 250 mM imidazole, pH 8.0; 1 time 0.5 mL, 7 times 1.5 mL). The purification was analyzed by SDS-PAGE. The fractions containing the desired protein were combined and dialyzed against pH 7.5 dimer buffer to obtain the capsid protein dimers. For storage, the proteins were assembled by dialysis against pH 5.0 capsid buffer. The pure protein was obtained with a yield of 100 mg/L of culture. The purity of the proteins was verified by SDS-PAGE. The assembly properties of the capsid proteins and the geometry of the resulting capsids were analyzed by FPLC using a Superose 6 GL 10/300 column with pH 5.0 capsid buffer as the eluent and TEM. ESI-TOF: calculated 22253.4 Da, found 22253.5 Da.

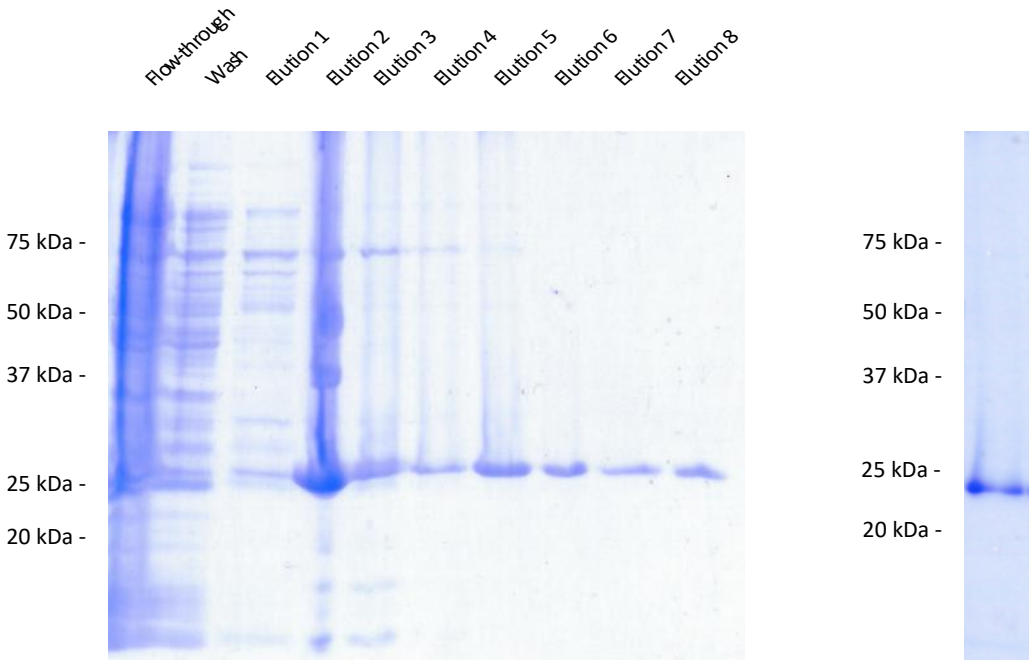

Figure S3. SDS-PAGE analysis of affinity purification of His6-ELP-CCMV (left) and purified His6-ELP-CCMV (right).

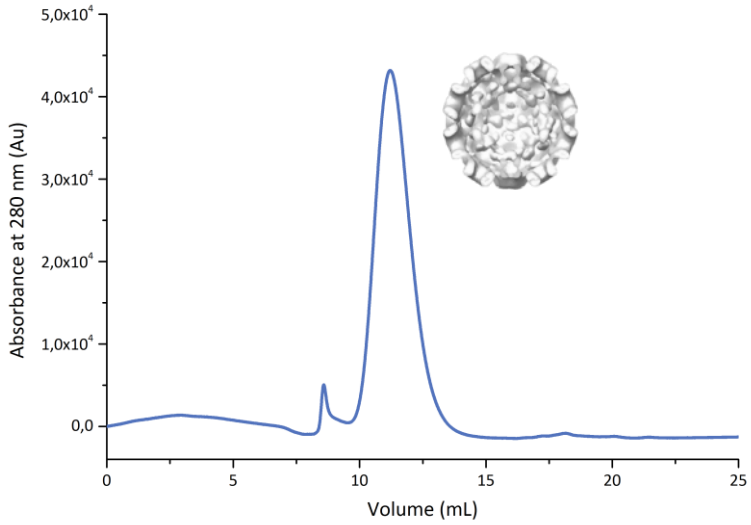

Figure S4. Size exclusion chromatogram of purified His6-ELP-CCMV in pH 5.0 capsid buffer.

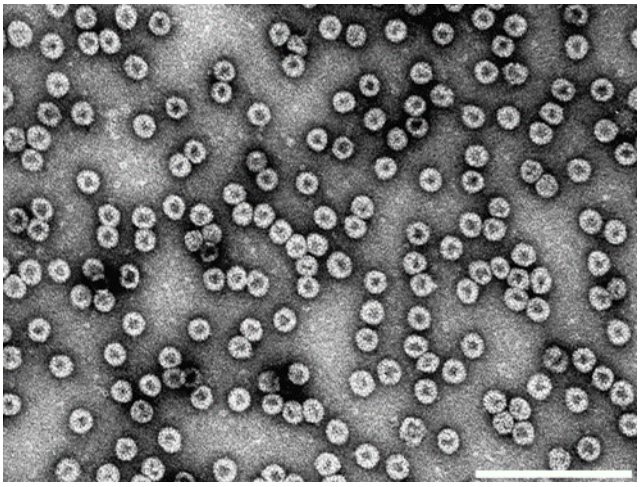

Figure S5. Uranyl acetate stained TEM micrograph of His6-ELP-CCMV. Average particle size = 29.2 ± 1.5 nm. Scale bar corresponds to 200 nm.

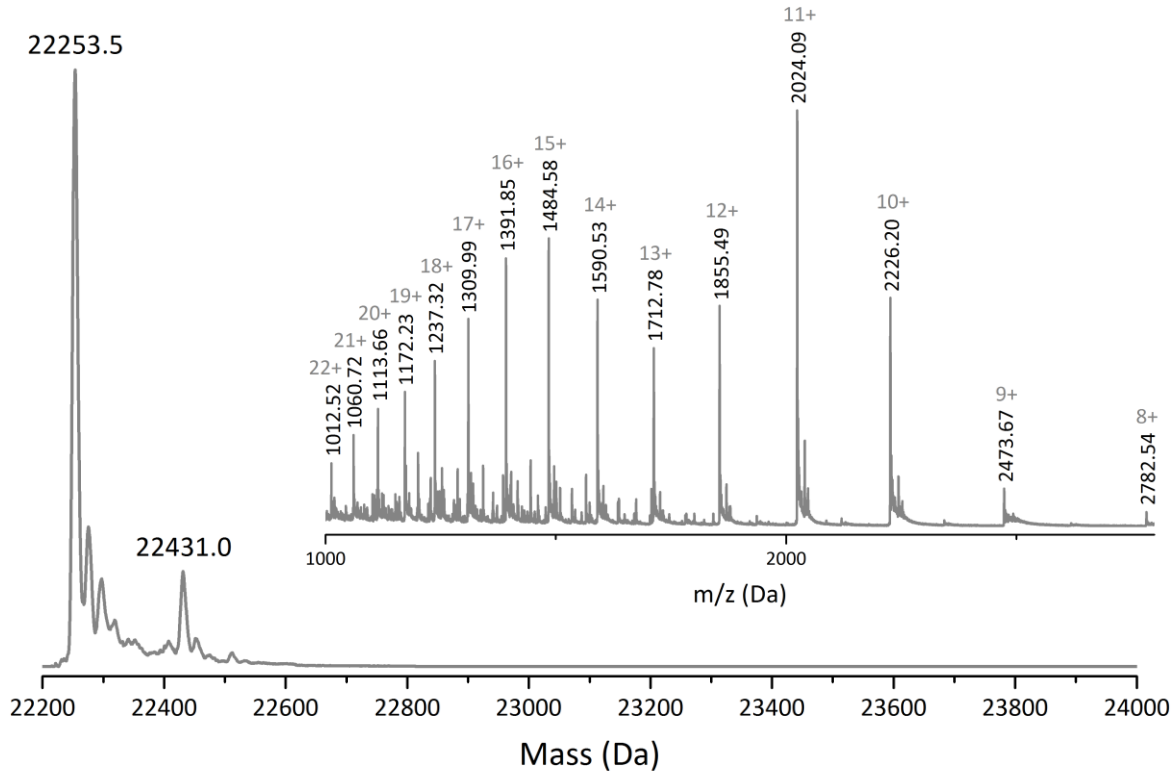

Figure S6. ESI-TOF mass spectrometry of purified His_6-ELP-CCMV. Deconvoluted total mass spectrum and multiply charged ion series (inset). The expected molecular weight is 22253.4 Da.

Table S2. Amino acid sequences of the proteins and peptides used in this study.

Name	Sequence
Sortase A	TGSHHHHHHGSKPHIDNYLHDKDKDEKIEQYDKNVKEQASKDKKQQAKPQIPK DKSKVAGYIEIPDADIKEPVYPGPATPEQLNRGVSFAEENESLDDQNISIAGHTFI DRPNYQFTNLKAAKKGSMVYFKVGNETRKYKMTSIRDVKPTDVGVLDEQKGK DKQLTLITCDDYNEKTGVWEKRKIFVATEVK
wild type CCMV	MSTVGTGKLTRAQRRAAARKNKRNTRVVQPVIVEPIASGQGKAIKAWTGYSVS KWTASCAAAEAKVTSAITISLPNELSSERNKQLKVGRVLLWLGLLPSVSGTVKS CVTETQTTAAASFQVALAVADNSKDVVAAMYPEAFKGITLEQLTADLTIYLYSS AALTEGDVIVHLEVEHVRPTFDDSFTPVY
G-ELP-CCMV	GHHHHHHVPGVGVPGLGVPGVGVPGLGVPGVGVPGLGVPGGGVPGVGVPGLG LEVVQPVIVEPIASGQGKAIKAWTGYSVSKWTASCAAAEAKVTSAITISLPNELS SERNKQLKVGRVLLWLGLLPSVSGTVKSCVTETQTTAAASFQVALAVADNSKD VVAAMYPEAFKGITLEQLTADLTIYLYSSAALTEGDVIVHLEVEHVRPTFDDSFT PVY
HBP-CCMV	KMEKKLHAVPAAKTVKFKGGLPETGHHHHHHVPGVGVPGLGVPGVGVPGLGV PGVGVPGLGVPGGGVPGVGVPGLGLEVVQPVIVEPIASGQGKAIKAWTGYSVSK WTASCAAAEAKVTSAITISLPNELSSERNKQLKVGRVLLWLGLLPSVSGTVKSC VTETQTTAAASFQVALAVADNSKDVVAAMYPEAFKGITLEQLTADLTIYLYSSA ALTEGDVIVHLEVEHVRPTFDDSFTPVY
HBP	H_2N-KMEKKLHAVPAAKTVKFKGGLPETG-NH_2

2.1.3 SrtA-mediated coupling, followed by capsid purification

For a SrtA-mediated coupling, stock solutions of SrtA, G-ELP-CCMV and the heparin binding peptide were prepared in Sortase buffer. If a component had been dissolved in another buffer, it was spin filtrated to Sortase buffer (10 kDa MWCO, 3 x 10 min). The components were added together to final concentrations of 50 μM SrtA, 50 μM G-ELP-CCMV and 50 μM heparin binding peptide. The solutions were incubated at 21 °C for 5 hours (400 rpm). The capsids were assembled and concentrated by spin filtration (10 kDa MWCO, 4 x 10 min) to pH 5.0 capsid buffer. Subsequently, the capsids were isolated using preparative

FPLC. The combined capsid fractions were concentrated by spin filtration, analyzed and used for further experiments.

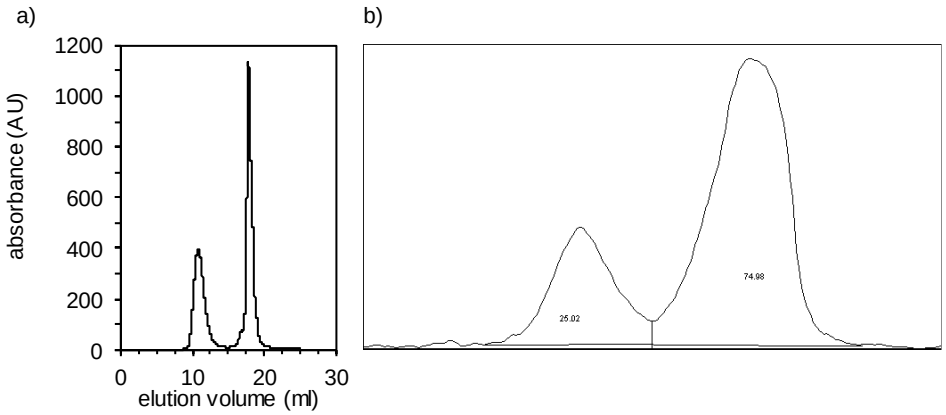

Figure S7. a) Size exclusion chromatography and b) ImageJ area analysis of the SDS-PAGE gel of the final product, HBP-CCMV.

2.2 FITC-heparin synthesis and characterization

FITC-heparin was synthesized following a previously published protocol.[4] In short, heparin (100 mg, 0.006 mmol) was solubilized into 25 mL of Na_2CO_3-$NaHCO_3$ buffer (pH 7.4). Fluorescein-5(6)-isothiocyanate (8.65 mg, 0.022 mmol) was solubilized into 2 mL pyridine. These two solutions were mixed and let to react under stirring in room temperature and in the dark overnight. The crude product was purified by dialysis (3.5 kDa MWCO dialysis tubing) against multiple changes of ultrapure water for 60 hours. The product was collected by lyophilization and characterized with FPLC and NMR.

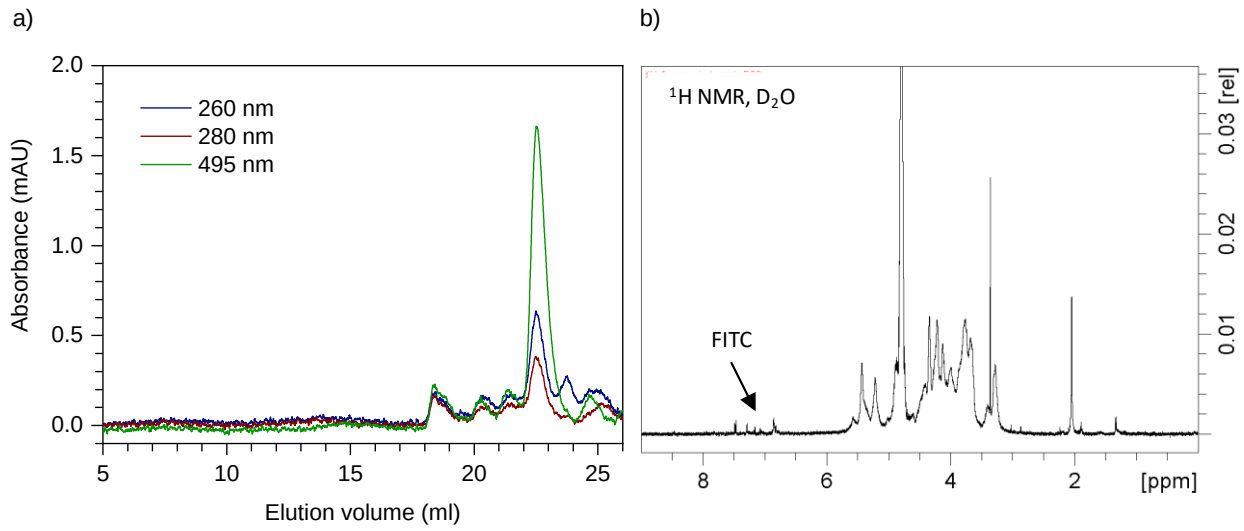

Figure S8. Characterization data of the FITC-heparin. a) Size exclusion chromatography and b) NMR spectrum.

2.2 Characterization of CCMV-HBP capsids and CPs

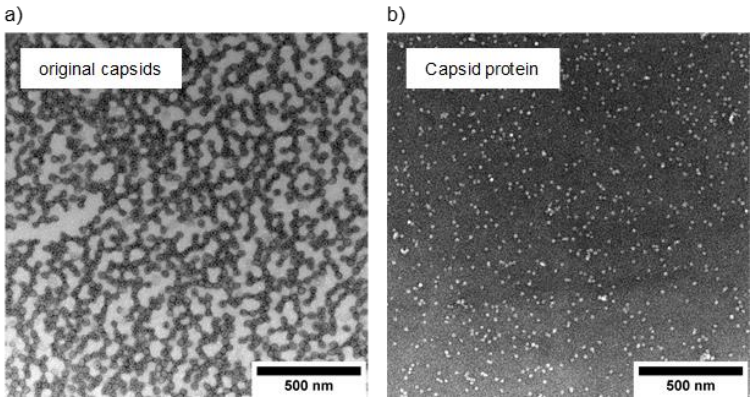

Figure S9. TEM image of a) the original CCMV-HBP capsids and b) the disassembled CCMV capsid.

2.3 Characterization of heparin-induced CCMV assembly

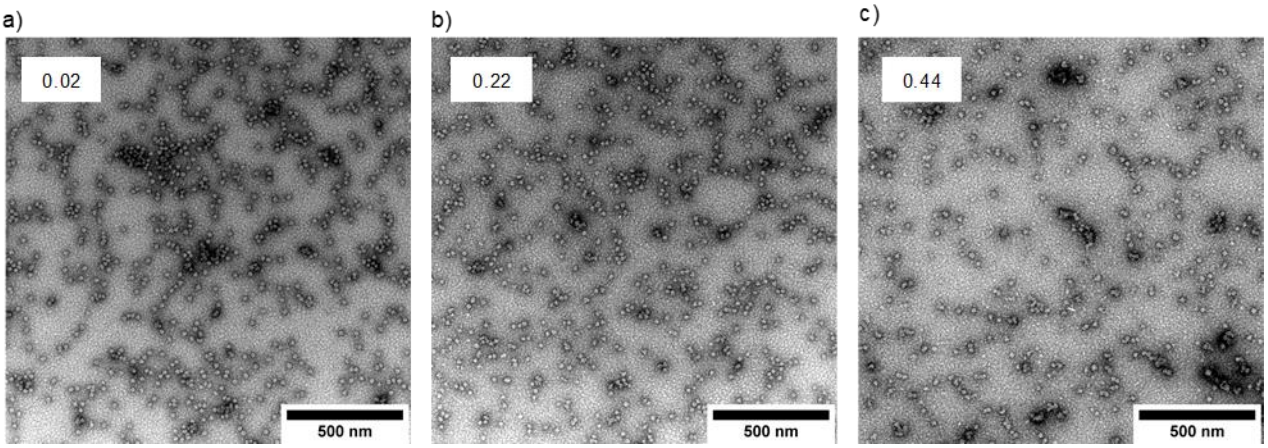

Figure S10. TEM images of the reassembled T = 1 CCMV with varying heparin: protein ratios: a) 0.02, b) 0.22, c) 0.44.

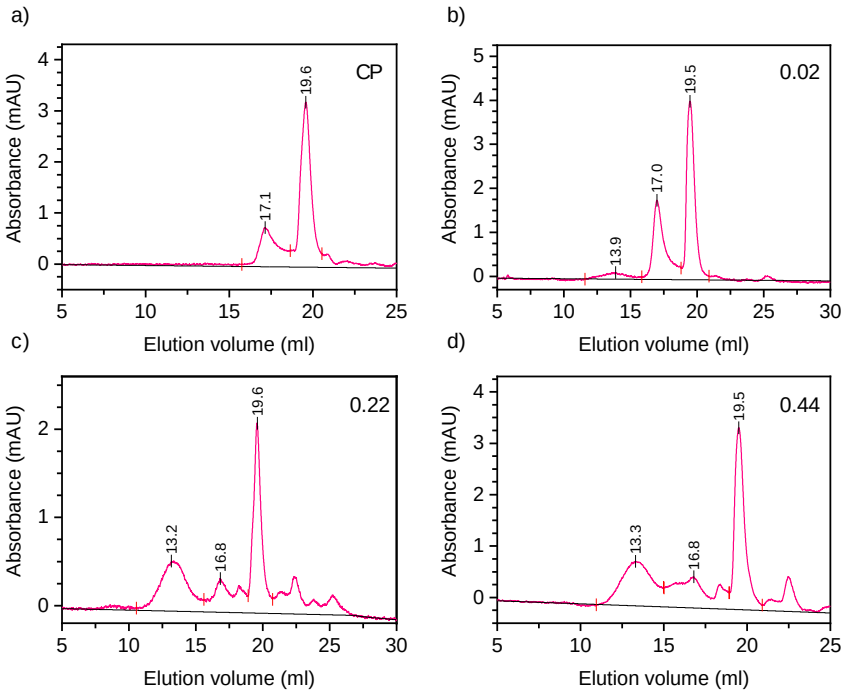

Figure S21. FPLC chromatograms (280 nm) used for determining the peak areas. Baseline marked with black line, peak values and markers for the integrated areas (red bars).

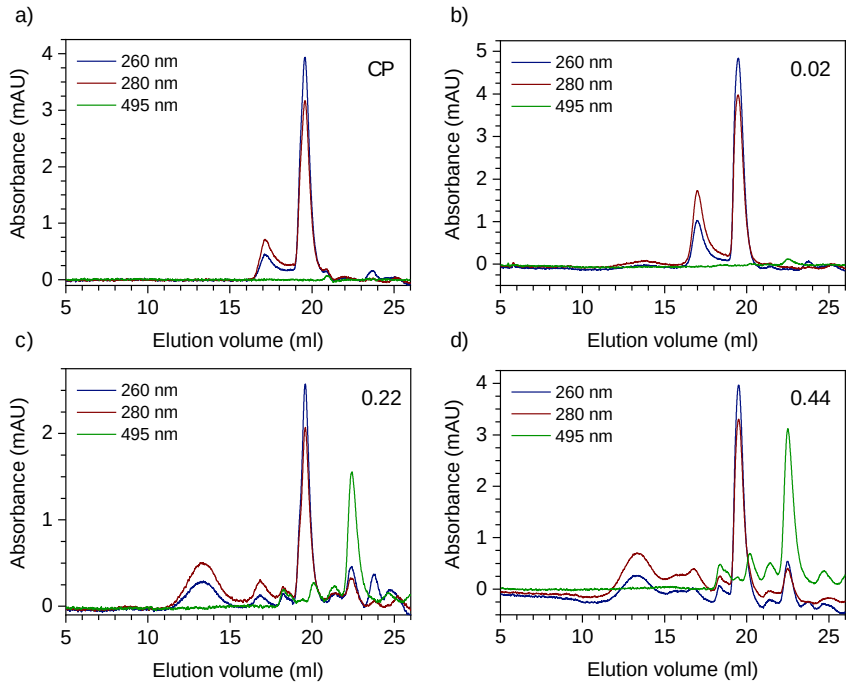

Figure S32. FPLC chromatograms for a) the disassembled CCMV and the reassembled CCMV with varying heparin to protein ratios b) 0.02, c) 0.22, d) 0.44.

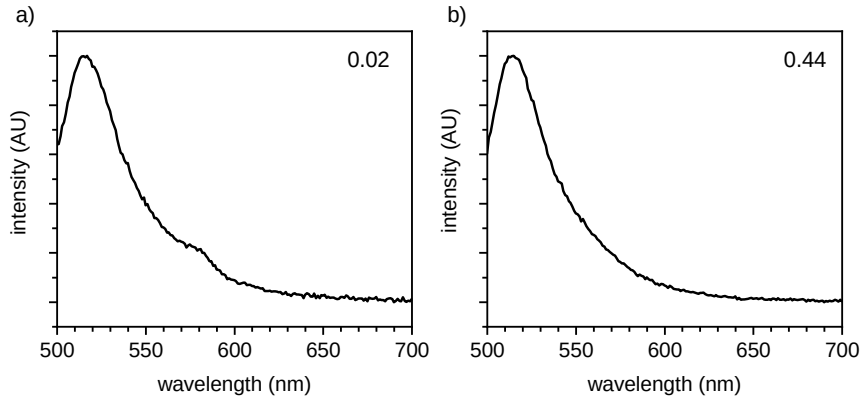

Figure S43. Fluorescence emission spectra for the T = 1 fractions collected from FPLC samples with heparin to protein mass ratio of a) 0.02 and b) 0.44.

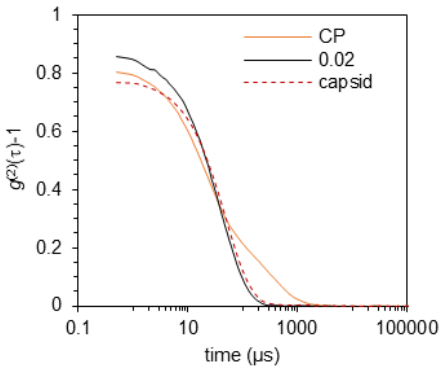

Figure S14. Second-order autocorrelation functions for the DLS samples including the capsids before disassembly and capsids after reassembly with heparin: protein mass ratio of 0.02.

3. References

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