Supporting Information for:

Self-Assembly and Characterization of Small and Monodisperse Dye Nanospheres in a Protein Cage

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Materials and methods

Materials

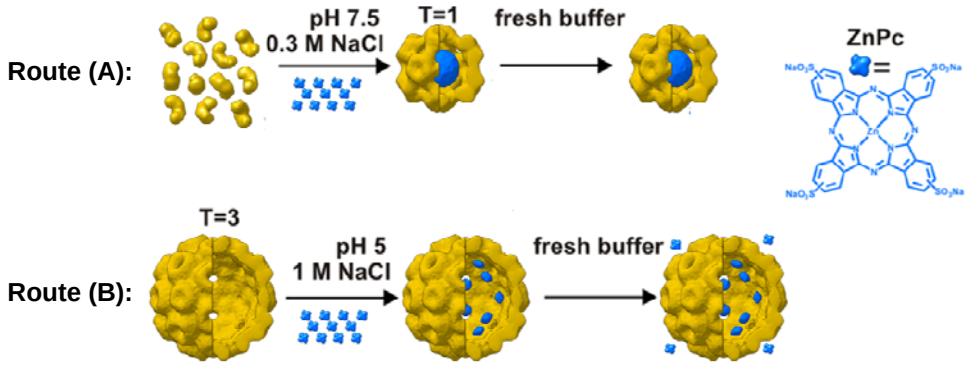
Purification of CCMV, removal of its RNA and isolation of the coat protein (CP) were carried out according to standard procedures.[1] The resulting viral CP was characterized by size exclusion chromatography (SEC; V_{CP} = 18.2 mL) and UV-Vis spectroscopy. The ZnPc tetrasulfonic acid sodium salt was prepared as described.[2]

General methods

SEC chromatograms were obtained in a FLPC system, using Tris-HCl (50 mM Tris-HCl, 300 mM NaCl and 1 mM DTT, pH 7.5) or sodium acetate buffer (50 mM NaOAc, 1 M NaCl, 1 mM NaN_3, pH 5). For preparative purposes, we used a superose 6 10/100 GL column (GE Healthcare) with a 100 μL injection volume. All compounds used to prepare buffers were of analytical quality, in ultrapure (Milli-Q) water. UV-Vis spectra were recorded using a Perkin Elmer Lambda 850 UV spectrophotometer.

Preparation of ZnPc-loaded VLP

ZnPc-loaded T=1 and T=3 VLP were prepared according to the following scheme, which is an extended version of Fig. 1 in the main text:



Scheme S1 Synthetic routes for the preparation of (A) T=1 and (B) T=3 ZnPc-loaded VLP.

*Preparation of ZnPc-loaded T=1 VLP (sample **1**; route A in Scheme S1)*

A stock solution of 100 μL ZnPc (3 mM) was added to 100 μL dimeric CP (0.35 mM), both prepared in Tris-HCl buffer. The sample was incubated (4ºC, overnight on a rollerbank), then SEC purified on a superose column and eluted with Tris-HCl buffer. Samples that eluted at 12.5 mL were further analyzed and dialyzed against the encapsulation buffer.

*Preparation of ZnPc-loaded T=3 VLP (sample **2**; route B in Scheme S1)*

A stock solution of 100 μL ZnPc (3 mM) was added to 100 μL CCMV capsid (0.35 mM), both prepared in sodium acetate buffer. The sample was incubated as above, then SEC purified on a superose column and eluted with sodium acetate buffer. Samples that eluted at 11.5 mL were further analyzed and dialyzed against the encapsulation buffer.

*Preparation of samples **3** and **4***

To obtain sample **3**, full-length CP (freshly isolated from CCMV as in ref. 1) was used as starting material. For sample **4**, truncated CP (generated by incubating disassembled CP in Tris-HCl buffer for several weeks) was used as the starting material. A stock solution of 100 μL full-length (for sample **3**) or truncated (for sample **4**) CCMV CP (0.35 mM) was dialyzed against sodium acetate buffer (24 h). Capsids were SEC purified on superose and eluted with sodium acetate buffer. Samples that eluted at 11.5 mL were collected for further study.

Mass spectrometry (MS) studies

Samples were buffer-exchanged to ammonium acetate buffer (50 mM NaOAc, 1 M NaCl, 1 mM NaN_3, pH 5) using Vivaspin 500K 10K MWCO centrifugal filter units. A 1-2 μL aliquot was loaded into gold-coated capillaries for nanoelectrospray ionization (nESI), using home-made capillaries (borosilicate glass tubes; 1.2 mm OD, 0.68 mm ID; World Precision Instruments) using a P-97 micropipette puller (Sutter Instruments) and gold-coated using a Scancoat Six Pirani 501 sputter coater (Edwards Laboratories). MS was performed on a modified Q-TOF II instrument (Waters and MS Vision).[3] Spectra were mass-calibrated with cesium iodide. Capillary voltage was in the 1300-1500V range; the sample cone, 120-200V. To optimize transmission of larger ions, backing pressure was raised to ~10 mbar.[4] Xenon was used as collision gas at $1\text{-}2*10^{-2}$ mbar.[5]

Cryo-electron microscopy

For cryo-EM, samples (5 μL) were applied to one side of Quantifoil R 2/2 holey grids, blotted, and plunged into liquid ethane using standard procedures[6] in a Leica EM CPC cryo-fixation unit.

CryoEM images were recorded on a FEI Eagle 4k CCD under low-dose conditions (~10 $e^-/Å^2$), in a Tecnai G2 electron microscope operating at 200 kV and equipped with a field emission gun. CCD frames were recorded at a detector magnification of 67,873 X (2.21 Å/pixel sampling rate).

Image processing

General image processing operations were performed using BSoft[7] and Xmipp[8] software. Graphics representations were produced with UCSF Chimera.[9] The Xmipp automatic particle picking routine was used to select individual images for T=3 (21,962), T=2 (6,362), ZnPc-loaded T=3 (5,406) and ZnPc-loaded T=1 particles (1,817). Defocus was determined with CTFfind,[10] with a 1.3 to 3.9 μm defocus range. CTF phase oscillations were corrected in images by flipping them in the required lobes, and individual particle images were extracted and normalized.

The published structures of CCMV T=3 capsid,[11] Brome mosaic virus (BMV) T=2 capsid,[12] and BMV T=1 capsid[13] were filtered to 30 Å, size-scaled and used as initial models for CCMV T=3, T=2 and T=1 capsid, respectively. We used Xmipp iterative projection matching to determine particle origin and orientation. After each refinement iteration, two independent reconstructions were computed using interpolation in Fourier space, and resolution was assessed by FSC between independent half-dataset maps, applying a correlation limit of 0.5. After the independent refinements, 19,766 (for T=3), 5,726 (T=2), 4,866 (ZnPc-loaded T=3), and 1,635 particles (ZnPc-loaded T=1) were included in the 3DR. Due to sample heterogeneity, T=3 capsids were further classified using the Xmipp ML3D routine[14] to select 9,429 A-particles and 9,925 B-particles; these particles were used for iterative projection matching and new 3DR were calculated including 8,486 (for A-) and 8,932 particles (B-T=3 capsids).

For A-, B- and ZnPc-loaded T=3 capsids, Uro fitting [http://mem.ibs.fr/JORGE/index.html] was used on the entire map with the X-ray structure of CCMV[11] (PDB entry 1CWP). A, B, and C conformers were initially treated as a single rigid body and the initial docking coordinates were refined, treating each subunit as an independent rigid body. For ZnPc-loaded T=1 capsid molecular modeling, the pseudo-atomic model of the CCMV swollen form[15] (Viper data base entry 203)[16] was fitted using Chimera to optimize the local correlation.

AFM studies

AFM substrate was prepared by cleaning glass slides overnight in an ethanol/KOH bath, thorough rinsing with Milli-Q water, dried in air and rendered hydrophobic by overnight incubation in hexamethyldisilazane vapor. Samples were prepared for AFM nanoindentation by incubating a 100 μL droplet of capsid solution in 50 mM sodium acetate, 1 M NaCl pH 5.5 (T=3) or 50 mM Tris, 0.3 M NaCl pH 7.5 (T=1) for ~20 min on silanised glass slides.[17,18] Buffer (100 μL) was added and the cantilever was wetted before placing the AFM head on the substrate. We used Olympus OMCL-RC800PSA rectangular, silicon-nitride cantilevers with a nominal spring constant of 0.05 Nm^{-1}. Cantilevers were calibrated by the method of Sader et al.[19], to give 0.0524 ± 0.002 SD N/m mean spring constant.

Capsids were imaged with ~50 pN maximum imaging force. To assess particle spring constant and breaking force, particles were indented by five consecutive approach-retraction series of 100 nm, with a 50 nm offset from the substrate. Probe velocity was set at 30 nm/s. Data were analyzed using Labview.[20]

Supporting information for ZnPc-loaded VLP synthesis

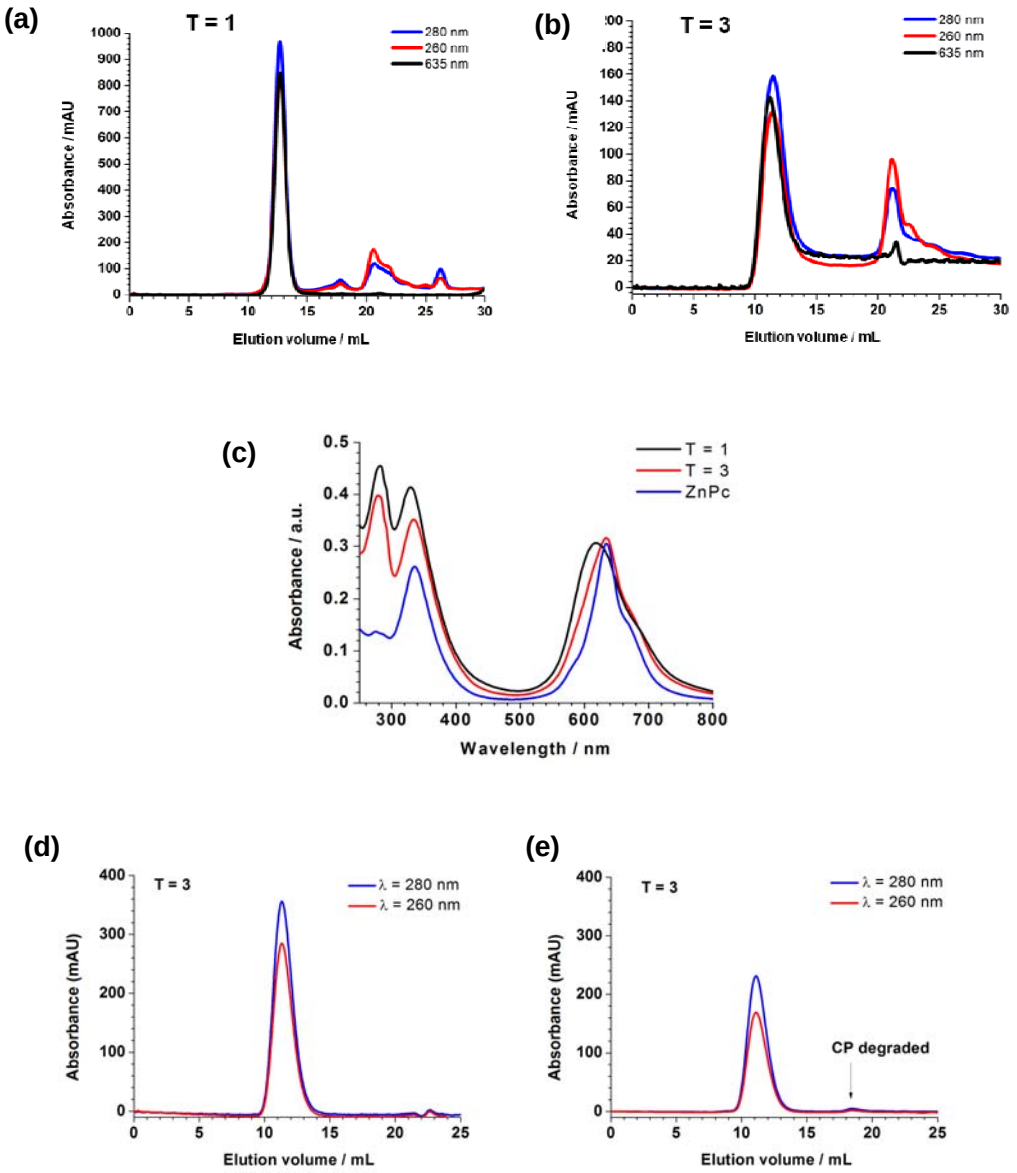


Fig. S1 Characterization of ZnPc-loaded VLP by size-exclusion and UV-Vis spectroscopy. (a, b) SEC chromatograms of samples **1** (a) and **2** (b), composed of ZnPc-loaded T=1 and T=3 VLP, respectively. (c) Normalized UV-Vis spectra of samples **1** (black line) and **2** (red line),

composed of ZnPc-loaded T=1 and T=3 VLP, respectively, and of ZnPc dimers in aqueous buffer (blue line). In its monomeric form, ZnPc absorbs at 680 nm; when it forms supramolecular H-type dimers in aqueous solution, at 635 nm. The hypsochromic shift for sample **1** indicates formation of longer H-type ZnPc stacks inside ZnPc-loaded T=1 VLP. (d, e) Characterization of reference samples. SEC chromatograms of samples **3** (d) and **4** (e), at pH 5 composed of capsids formed by non-degraded and degraded CP, respectively.

Supporting information for MS experiments

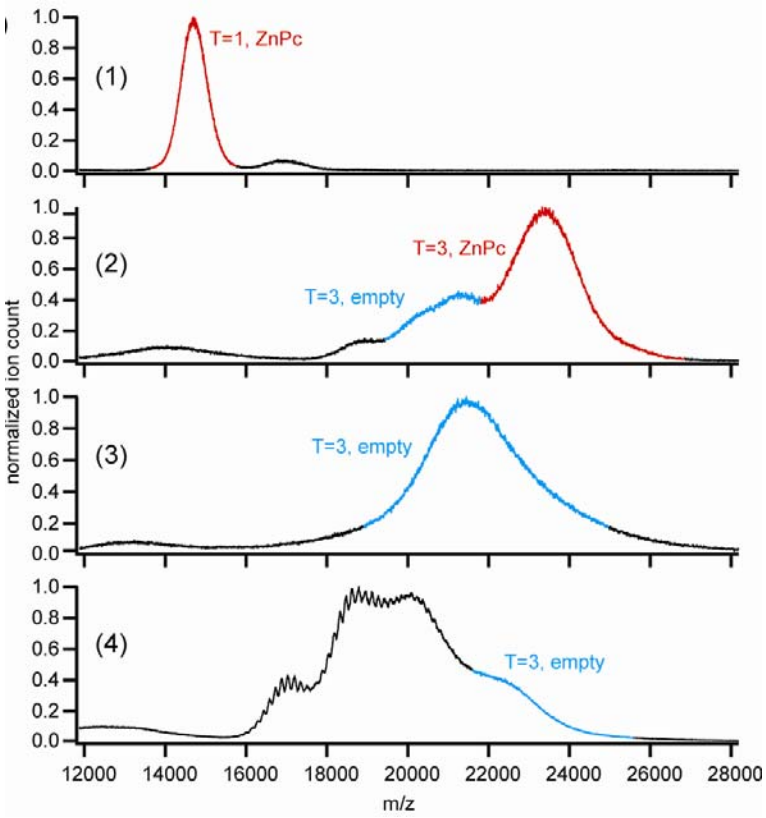


Fig. S2 Native MS spectra of samples (1) ZnPc-loaded T=1 VLP, (2) ZnPc-loaded T=3 VLP, (3) empty T=3 capsids, and (4) empty T=3 capsids with truncated CP. Signals assigned to ZnPc-loaded VLP are colored red, empty VLP is colored blue. The additional signal in (**1**) is attributed to 'T=2' particles, and to aberrant structures in (**2**) and (**4**).

Table S1 Experimental masses and estimated cargo encapsulation from Native MS analysis.

Coat protein				
type	**residues**	**average (kDa)**	**standard deviation (kDa)**	**deviation from empty VLP (kDa)[a]**
full length	2-190	20.251	0.0011	-0.0031
truncated	28-190	17.431	0.0010	-0.0010
truncated	33-190	16.910	0.0008	0.0002

[a] Compared to theoretical masses calculated from sequence, accession code AEI54615.1.

Particles					
type		**average (kDa)**	**standard deviation (kDa)**	**deviation from empty VLP (kDa)[b]**	**# of ZnPc[c]**
T=1, ZnPc (14,400 m/z)	precursor	1340	1.2	231	259
T=1, ZnPc (14,400 m/z)	product	1330	1.2	222	249
T=3, ZnPc (23,400 m/z)	precursor	3476	0.7	372	417
T=3, ZnPc (23,400 m/z)	product	3444	1.3	357	400
T=3, empty (20,500 m/z)	product	3113	1.3	9	-

[b] Compared to theoretical empty capsids. The CP mass was taken as intensity weighted average of the three forms observed. Copy number was considered 60 for T=1, 180 for T=3.

[c] based on stated values for deviation from empty VLP. The mass of ZnPc was considered 892 Da.

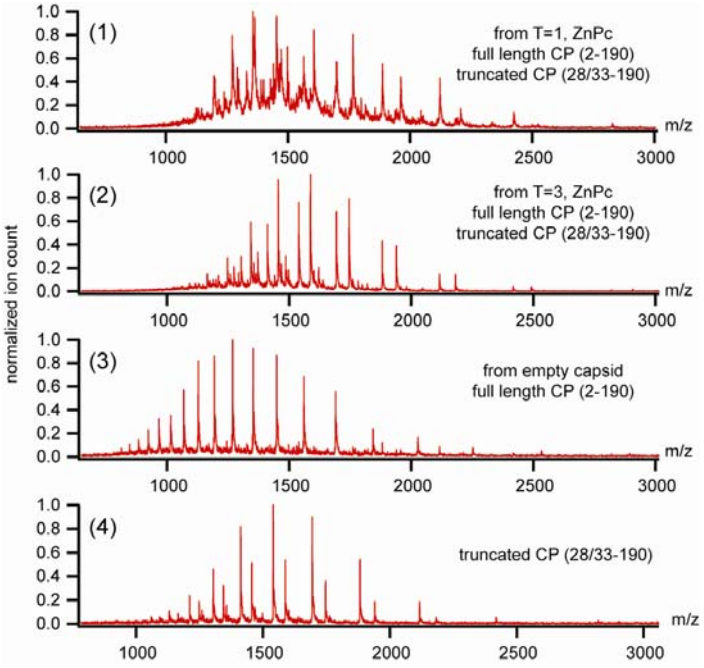


Fig. S3 CP analysis in empty and ZnPc-loaded particles from tandem MS. Signals corresponding to CP monomer were generated by collision induced dissociation of intact particles. In ZnPc-loaded VLP, the majority of the CP is truncated at the N-terminal residue 27 or 32. See Table S1 for masses.

Supporting information for cryo-EM analysis

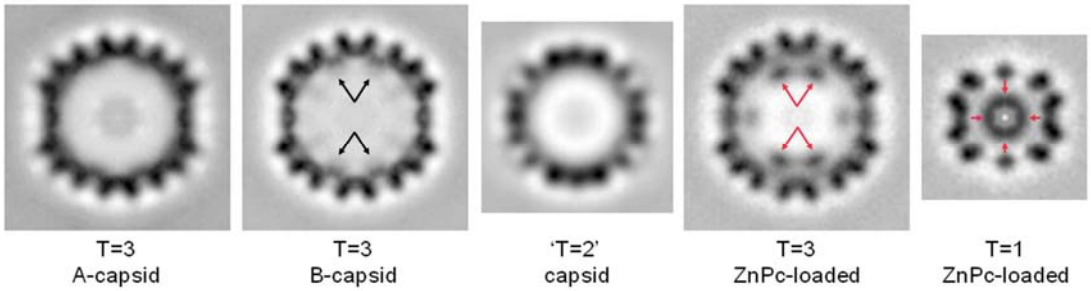


Fig. S4 Three-dimensional cryo-EM of empty and ZnPc-loaded CCMV capsids. Central sections from the 3D reconstructions viewed along a two-fold axis of T=3 A-capsid, T=3 B-capsid, 'T=2' capsid (all empty capsids), and ZnPc-loaded T=3 VLP and ZnPc-loaded T=1 VLP. Darker shading indicates higher density. Arrows indicate density due to the capsid protein β-annulus in B-capsid (black)and to ZnPc in T=3 and T=1 capsids (red).

(a) (b)

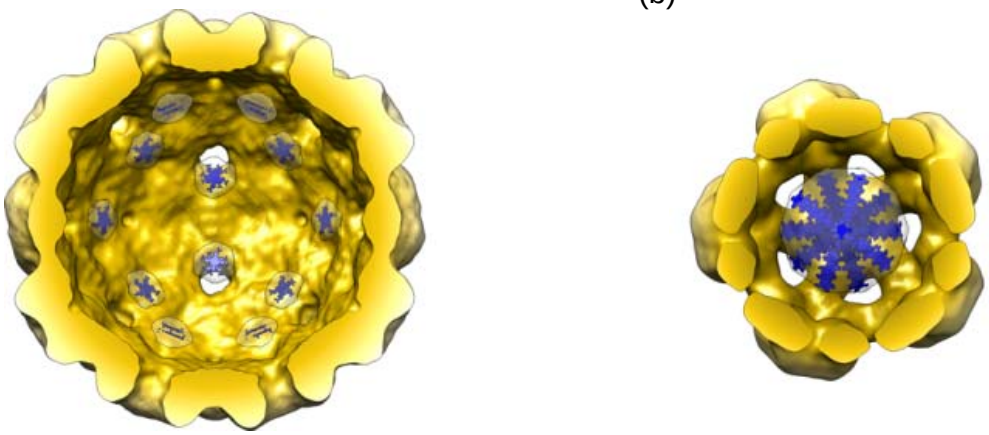


Fig. S5 Models for ZnPc-loaded T=3 and T=1 VLP. (a) ZnPc-loaded T=3. Fitting of 20 ZnPc dimers into the internal density located beneath the hexameric capsomers at the threefold axes in the ZnPc-loaded T=3 VLP (<9 nm radius). This model accounts for the fraction of ZnPc dimers that follow icosahedral symmetry; most ZnPc dimers do not follow icosahedral symmetry and remain invisible in cryo-EM 3DR. The ZnPc structure was modeled by molecular mechanics using Spartan'10 software. (b) Alternative model for ZnPc-loaded T=1 VLP. Fitting of 12 ZnPc 10-mer H-type stacks into the internal density of the ZnPc-loaded T=1 VLP (<5.5 nm radius). This model is considered less plausible, as it would lead to strong electrostatic repulsion between ZnPc stacks near the nanosphere core. The ZnPc structure was modeled using Spartan'10.

Supporting information for AFM experiments

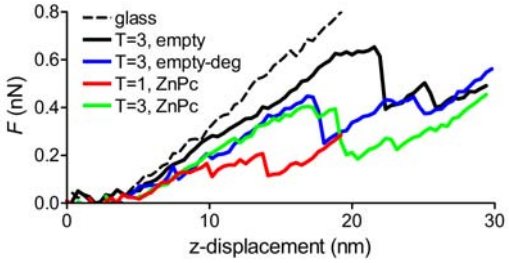


Fig. S6 Force-distance curves for CCMV-based VLP. The curve measured on the glass substrate is shown for reference.

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