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# Relative size selection of a conjugated polyelectrolyte in virus-like protein structures†‡

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**A conjugated polyelectrolyte poly[(2-methoxy-5-propyloxy sulfonate)-phenyl-ene vinylene] (MPS-PPV) drives the assembly of virus capsid proteins to form single virus-like particles (VLPs) and aggregates with more than two VLPs, with a relative selection of high molecular weight polymer in the latter.**

Biomolecules that self-assemble to form precisely shaped containers with a defined size are of growing interest in nanotechnology. In particular viruses represent some of the most diverse, robust yet adaptable nanoparticles found in nature. The self-assembly of the virus capsid protein protects its contents from extreme surroundings (pH and temperature) and ensures highly efficient delivery of its cargo. Virus-like particles (VLPs) are made up of coat proteins that form empty non-infectious capsids.[1]

VLPs have been used to encapsulate biomaterials, such as proteins[2] or enzymes[3] as well as synthetic material, *e.g.* micelles[4] or anionic polymers.[5] Here we report on the capsid protein assembly directed into different forms by using the templating properties of conjugated polymers. Since the encapsulation of coiled sulfonated polystyrene (PSS) with a highly variable radius of gyration ($R_g$)[6] leads to $T = 1$ or pseudo '$T = 2$' capsids,[5] we postulated that a conjugated, rod-like polyelectrolyte such as poly[(2-methoxy-5-propyloxysulfonate)-phenylene vinylene] (MPS-PPV) ($R_g$ = 26 nm)[7] would direct the protein assembly into a rod-like structure. Such structures have been reported for encapsulation of DNA[8,9] and DNA dye hybrids with tuneable rigidity.[10] In the case of MPS-PPV we were interested to see if tubular protein architectures could be formed in a similar way, by incubating the polymer with cowpea chlorotic mottle virus (CCMV) coat protein (CP). This could potentially lead to different polymer properties, *e.g.* an increased conjugation length.

The CCMV consists of 180 identical CP subunits, which self-assemble around the RNA to form a $T = 3$ icosahedral capsid with an external diameter of 28 nm. The native CCMV capsid can be disassembled into CP dimers and reassembled into empty capsids. CP dimer formation is favoured at pH 7.5 and an ionic strength higher than 0.3 M, and can be used for the controlled encapsulation of negatively charged materials into $T = 1$ VLPs with an average external diameter of 18 nm.[1–5]

Interestingly, the encapsulation of MPS-PPV into VLPs by incubating the polymer with CP at pH 7.5 not only led to single $T = 1$ VLPs (herein referred to as free particles, FP) but also to clusters of more than two $T = 1$ VLPs (herein referred to as aggregate particles, AP), see below. To understand what directs the assembly (whether the content dictates particle size) and if this process can be controlled, two different experimental sets were obtained.

First, a sample set containing MPS-PPV (100 μg ml$^{-1}$) and various CP concentrations (100–5000 μg ml$^{-1}$) was purified by size exclusion chromatography (SEC) (Fig. 1A). The elution of MPS-PPV was monitored at $\lambda = 451$ nm and that of capsid at $\lambda = 260$ nm and $\lambda = 280$ nm. Absorbance at all three wavelengths indicated successful encapsulation of the polymer in protein complexes. Three peaks were detected, eluting at $V = 8.5$ ml, 12.5 ml and 18.5 ml (Fig. 1A). The SEC fractions at $V = 8.5$ ml and 12.5 ml were studied by transmission electron microscopy (TEM) in order to visualize the formed

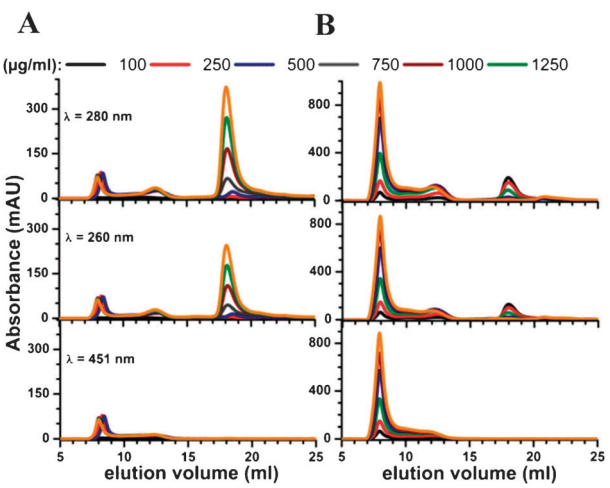


**Fig. 1** SEC chromatograms: CP ($\lambda = 260$ nm; $\lambda = 280$ nm) and MPS-PPV ($\lambda = 451$ nm). The FPLC was equipped with a superpose 6 size exclusion column, with a bed volume of 24 ml. AP elute at 8.5 ml, FP at 12.5 ml and CP at 18.5 ml. (A) Samples containing MPS-PPV (100 μg ml$^{-1}$) and various [CP]. (B) Samples containing CP (4000 μg ml$^{-1}$) and various [MPS-PPV].

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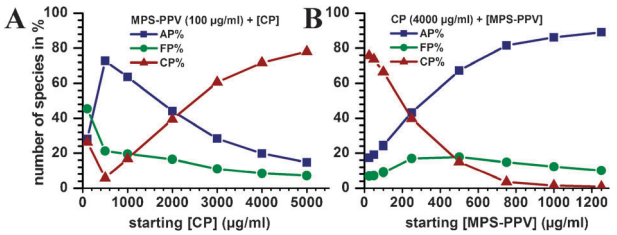


**Fig. 2** % of AP, FP, CP calculated from the SEC data ($\lambda$ = 280 nm). (A) Samples containing MPS-PPV (100 µg ml$^{-1}$) and various [CP]. (B) Samples containing CP (4000 µg ml$^{-1}$) and various [MPS-PPV].

structures (Fig. S1A and B, ESI†). TEM showed FP eluting at $V$ = 12.5 ml and AP at $V$ = 8.5 ml all within the expected size range of $T$ = 1 particles *i.e.* 18 nm in diameter. The third peak at $V$ = 18.5 ml corresponds to unassembled, dimeric CP. Interestingly, increasing the concentration of CP did not result in the exclusive formation of FP. In all samples both species, AP and FP, were found (Fig. 1A).

The ratios of AP, FP and CP were determined from the SEC data and plotted as a function of concentration (Fig. 2A). At very low CP concentration more FP than AP are observed. At higher CP concentrations more AP than FP are observed and the ratio between AP and FP approaches constant and is independent of CP concentration. The constant amount of FP and AP indicates that all polymer material is encapsulated, leaving the excess of CP free.

The relative high yield of FP formation at low CP concentration (Fig. S2A, ESI†) suggests that when there is insufficient CP to encapsulate all the polymer material, the formation of FP is more favorable compared to AP. The above data can be explained by assuming that low molecular mass polymers will be preferentially encapsulated in the FP, whereas the AP include more high molecular mass polymers (see below). To prove the stability of FP and AP, both species were isolated and analyzed again after 30 days by SEC. No clear shift in the equilibrium between the amount of FP and AP was observed (Fig. S3, ESI†).

To determine the optimal ratio to encapsulate the maximum amount of MPS-PPV into either FP or AP, the concentration of MPS-PPV was varied from 25 µg ml$^{-1}$ to 1250 µg ml$^{-1}$ whilst the concentration of CP was kept at 4000 µg ml$^{-1}$. We chose this high CP concentration to ensure full encapsulation of the polymer material. SEC analysis of these samples showed the same structures; AP and FP (Fig. 1B and 2B).

Interestingly, the formation of AP continuously increases with the polymer concentration whereas the formation of FP increases until a polymer concentration of 750 µg ml$^{-1}$ (Fig. 2B) is reached. Apparently, the encapsulation of FP is favored when CP is limiting. The results from both experimental sets indicate that the most efficient ratio, in which all CP is used for the encapsulation of the entire MPS-PPV into FP and AP, is around 1 µg ml$^{-1}$ MPS-PPV to 5 µg ml$^{-1}$ CP. The exact mechanism of this complex assembly behavior is still unclear. Given that both species are stable over a period of at least 30 days, a thermodynamic mechanism of formation is suggested. It is, however, at this point too premature to draw any conclusions on this and more experiments are needed.

The purified fractions of AP and FP containing MPS-PPV were characterized using UV-Vis spectroscopy. MPS-PPV exhibits characteristic absorbance peaks at $\lambda$ = 440 nm, 370 nm and

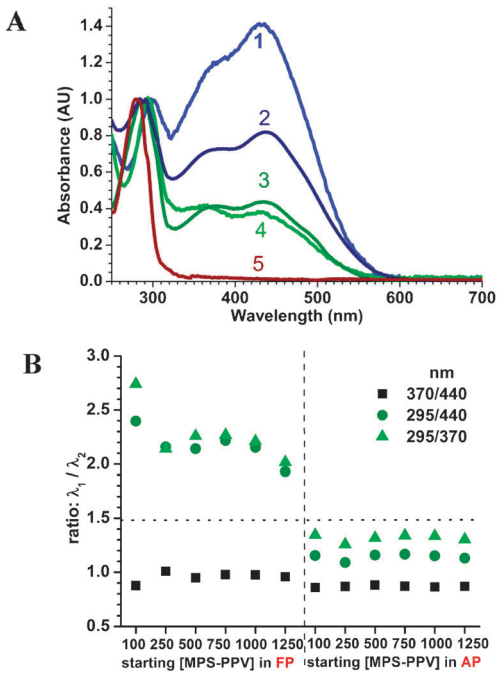


**Fig. 3** (A) UV-Vis spectra showing high (1) and low (2) molecular mass MPS-PPV, AP (3), FP (4) and coat protein (5) after SEC. (B) The ratio $\lambda$ = 295/440 nm or 370 nm calculated from the data in (A). On the *x*-axis the different starting concentrations of MPS-PPV are given. The ratio 370/440 nm is given as a reference. The estimated error in the data is <5%.

295 nm (Fig. 3A). These peaks originate from π-electron transitions in the conjugated polymer backbone.[11–13] Remarkably, the peak ratios of 295/440 nm and 295/370 nm are different for AP and FP. In the case of AP, the ratio of 295/440 nm is around 1.2–1.4, whereas for FP this ratio is between 2.0–2.4 (Fig. 3B).

To investigate if these different optical properties are related to the molecular weight of the polymer, UV-Vis spectra of SEC fractions of presumably mass separated MPS-PPV were measured (Fig. S4–S6, ESI†). From these data it is clear that the different ratios in absorbance are related to the relative molecular weight and that the FP indeed contain a substantially larger fraction of low molecular mass material compared to the AP.

The conformation of MPS-PPV chains has been reported to vary from extended (*i.e.* more conjugated) to coiled structures, depending on the environment.[14–17] The two conformations can be distinguished by their emission spectra, which shows a blue-shift for the coiled structure compared to that of extended structures.

To study whether the chains of MPS-PPV are extended or coiled inside AP and FP, the emission spectra were measured (Fig. 4) showing signals centered at $\lambda$ = 560 nm and 535 nm, respectively. The increase in absolute intensity in the emission spectra is due to the different concentration of AP and FP in these measurements (see above).

The observed blue-shift confirms that in FP the polymer has a more coiled conformation and is likely fully included in the protein capsule (average diameter of FP 10–30 nm, see above), whereas the AP particles do accommodate more stretched polymer chains. This suggests that polymer chains extend through the protein shell, leading to exposure of the polymer to the external environment (average diameter of AP 20–120 nm, see above).

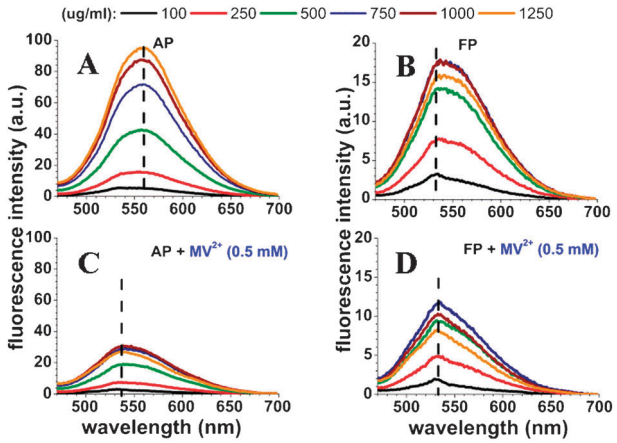


**Fig. 4** Fluorescence emission spectra of different concentrations AP (A), FP (B) and after adding $MV^{2+}$ to AP (C) or FP (D).

If the MPS-PPV is partly outside the VLP it might be better accessible for small molecules. Therefore, the fluorescence spectra of AP and FP were measured in the presence of the quencher methyl viologen ($MV^{2+}$) (0.5 mM, Fig. S10, ESI†).[18,19] After adding $MV^{2+}$ to a solution of AP, the emission intensity decreased by ~70% and a blue shift, from $\lambda_{max}$ = 560 nm to 535 nm, was observed (Fig. 4C). However, adding $MV^{2+}$ to a solution of FP showed a smaller decrease in intensity (~25%) and no blue-shift (Fig. 4D). When the particles are dialyzed to pH 5 the observed structures of FP and AP remained largely intact (Fig. S12 and S13, ESI†). Fluorescence quenching was still observed, however, it appears that the pH not only influences the protein assembly but also the polymer properties. These results indicate that at pH 7.5 MPS-PPV is accessible in the case of AP but interactions in the case of FP are substantially less. This can be quantified by the Stern–Volmer fluorescence quenching constant ($K_{sv}$),[20] which we determined to be $K_{sv} = 2 \times 10^6\ M^{-1}$ and $1 \times 10^6\ M^{-1}$ for AP and FP, respectively. The $K_{sv}$ for free MPS-PPV is approximately $2.7 \times 10^7\ M^{-1}$ (Fig. S11, ESI†). In both cases an amount of polymer is still protected against quenching by $MV^{2+}$, since both values ($K_{sv}$ of AP and FP) are 10 times lesser than the $K_{sv}$ value for free MPS-PPV.[5a]

Based on the obtained data, it can be concluded that MPS-PPV, in a coiled or more extended form, can be included in VLPs. Based on the optical data it is suggested that encapsulation of coiled up MPS-PPV by FP leads to a more efficient shielding of the polymer to external quenchers. AP accommodates more extended polymers with a relatively higher molecular mass that are furthermore better accessible to external fluorescence quenching (Scheme S12, ESI†). The delicate interplay between polymer conformation and capsid protein assembly results in an unexpected selection of molecular weight distributions, where longer conjugated MPS-PPV chains cannot sufficiently coil to be accommodated in a single VLP. In the present case this does not result in a morphology change of the protein assembly, *e.g.* from spheres to rods,[8–10] but instead VLPs cluster together. This minimizes the amount of non-encased polymer as is indicated by the fluorescence quenching studies. Although there are some differences in the conjugation length of the polymers in the two different protein structures, as can be concluded from the absorption spectra, the protein assembly does not influence the polymer structure substantially as is the case with DNA–chromophore hybrid materials.[10] Control over the uptake of guest materials in VLPs is of great importance for their application in biomedicine as, *e.g.*, cellular uptake is dependent on the particle size[21] and its surface chemistry.[1]

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