Monodisperse polymer–virus hybrid nanoparticles†

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Self-assembly of polystyrene sulfonate and modified cowpea chlorotic mottle virus protein yields monodisperse icosahedral nanoparticles of 16 nm size.

Organic and inorganic nanoparticles are currently receiving great interest because of their interesting physical and chemical properties, which are often different from those of microscopic systems.[1] These have been synthesized by a wide variety of methods, one being the self-assembly of amphiphilic block-copolymers, which yields vesicles, micelles and other structures. The use of biological materials to create nanoparticles is increasing.[2] Many efforts have been directed toward the encapsulation of drugs and other molecules in these particles.[3] Major disadvantages, however, are their low biocompatibility, their low solubility in water and in general their broad polydispersity, although small polydispersity indices have been attained.[4]

The well-characterized virus cowpea chlorotic mottle virus (CCMV) consists of 90 identical dimeric coat proteins (CP) enveloping a central RNA strand, yielding a highly defined 28 nm virion.[5] The icosahedral shape of the virus can be described according to the Caspar–Klug T (triangulation) number.[6] A larger T number describes a larger particle, with a set number of protein subunits. In CCMV, the 90 dimeric coat protein subunits give rise to a $T = 3$ particle, consisting of 12 pentameric arrangements and 20 hexameric faces. The monomeric 20 kDa CP consists of 189 amino acids with nine basic residues at the N-terminus. In its free form, the CP is dimeric. Ionic interactions between the RNA and these residues enhance the structural integrity of the virus, so that capsid assembly at *neutral* pH is only possible in the presence of RNA.[6] Removal of the RNA by precipitation yields pure CP which can be assembled by lowering the pH to $\sim$5, yielding empty $T = 3$ capsids. Very little or no empty capsid will form in neutral conditions (see below). It is believed that subtle electrostatic interactions between the RNA and the protein subunits assist in particle formation. Experiments to determine if a synthetic polymer gives rise to similar effects have been undertaken: It has been reported in literature that negatively charged sodium–polyanethole sulfonate and CP mixed at high pH (7.5) and dialyzed to pH 4.5 give rise to particles corresponding to the dimensions of the virus ($T = 3$).[7] No well-defined particles are observed at high pH. Incubation of the CP at pH 7.5 with ds-DNA of different sizes has been shown to yield 16–17 nm diameter tubular structures of various lengths.[8] This implies that at low pH (*i.e.* 5), interactions between protein subunits predominate capsid formation, while at higher pH ($\geq$7) other interactions (such as between CP and polyelectrolyte) become increasingly important.

We have investigated the interaction of polystyrene sulfonate (average mass: 9900 Da) with CP at neutral pH in different stoichiometric conditions and report here the formation of monodisperse 16 nm icosahedral $T = 1$ nanoparticles, characterized using fast performance liquid chromatography (FPLC), transmission electron microscopy (TEM) and dynamic light scattering (DLS).

CP was prepared according to a literature procedure.[9] Polystyrene sulfonate was synthesised using the atom transfer radical polymerization (ATRP) technique.[10] In order to facilitate detection by UV, the polymer was equipped with a dansyl group. To achieve this, dansyl chloride (Dns-Cl) was first reacted with 1,3-diaminopropane (Scheme 1) to yield **1**, which was coupled to 1-bromoethyl benzoic acid to provide the ATRP initiator **2**. Monomer **3** was prepared using a modified literature procedure.[11] Subsequent polymerization of **3** in the presence of **2**, CuBr and 2,2′-bipyridine yielded the desired polymer, DNs-PSS (**4**) after deprotection of the ethyl ester functions.

A fixed amount of CP (4.5 mg ml^{-1}) in a pH 7.5 Tris buffer (50 mM, 0.3 M NaCl) was incubated with varying amounts of DNs-PSS and the assembly of the particles was monitored by FPLC and TEM.[12] Fig. 1 shows the FPLC elution curves for CP, incubated with different amounts of DNs-PSS.

The incubation time appeared not to be a crucial parameter since no difference was detected between a sample injected 1 hour after incubation and one that had stood at 4 °C for several days. The presence of polymer was verified by UV (adsorption at 312 nm, see the ESI†). A very small ratio of DNs-PSS : CP (0.4)[13] yielded very little particle assembly, with small peaks at elution volumes of 1.0 and 1.35 ml, corresponding to $T = 3$ icosahedral particles (the naturally occurring capsid of 180 subunits) and non-natural, somewhat smaller particles. Increasing the ratio of DNs-PSS : CP to 4 and then to 40 caused the peak at 1.0 ml to decrease and the one at 1.35 ml to increase. At the ratio of 40 *only* the assembly eluting at 1.3 ml and free dimeric CP was detected, implying that this ratio is close to the ideal value for formation of this particular assembly. Increasing the ratio DNs-PSS : CP even more (400) yielded these particles as well, but the FPLC trace clearly showed a lower intensity of the 1.35 mL peak, and a large amount of material eluting at 1.6 ml (DNs-PSS). This inhibition of particle formation is probably caused by a polyelectrolyte effect, preventing capsid formation at high polyelectrolyte concentrations. It is, for

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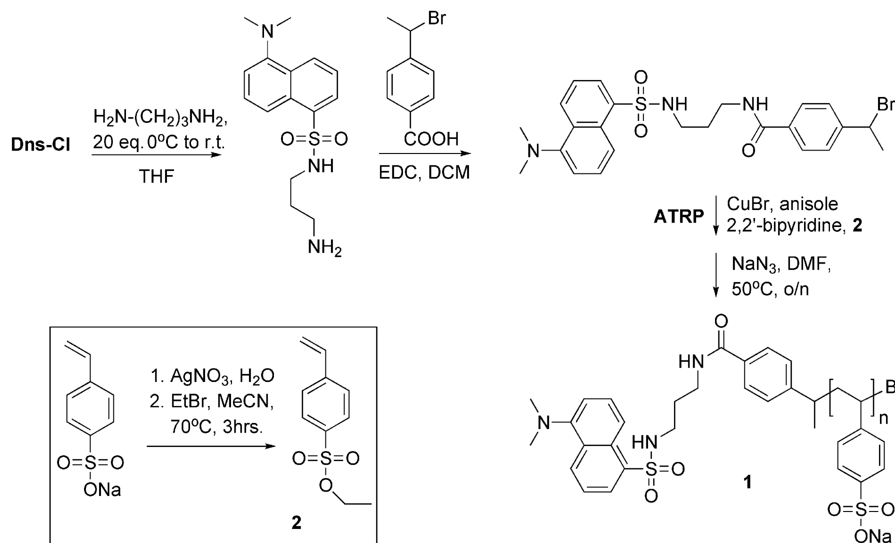

Scheme 1 Synthesis of labelled polystyrene sulfonate (DNs-PSS). For synthetic details see the ESI.†

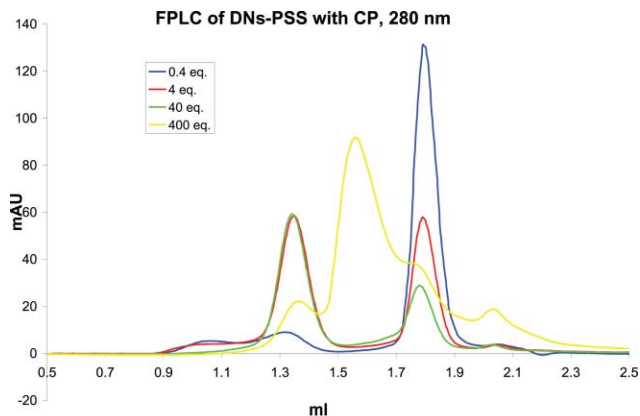

Fig. 1 FPLC traces of mixtures containing different ratios of DNs-PSS polymer and CP subunits The resolution of the FPLC systems does not allow discrimination between native and degraded CP (see below). Small ratios of DNs-PSS : CP (0.4 and 4)[13] induce formation of two types of particles, with elution volumes of 1.0 and 1.35 ml, indicating the presence of $T = 3$ and $T = 1$ protein aggregates. At a ratio of 40 only $T = 1$ particles and free CP appear. Higher ratios of DNs-PSS/CP (400) inhibit particle formation.

example, possible that at higher concentrations multiple polymer chains engage in an interaction with CP so that particle formation is inhibited. Since no peak appears for the polymer, not even at high magnification of the traces, we may conclude that, at lower stoichometries, all polymer is encapsulated within the particles. Blank FPLC runs with only polymer (not shown) did not show a significant change in elution volume (~1.55 ml) as a function of concentration, implying that free DNs-PSS is always detected at the same elution volume.

The peak intensity of a given elution volume, relative to total protein and polymer peak intensity was calculated (Table 1). For the $T = 1$ particle, indeed the highest relative peak intensity was found at the ratio DNs-PSS : CP = 40.

TEM images (Fig. 2) obtained from the fraction collected at 1.35 ml revealed the presence of well-defined monodisperse particles which were around 16 nm in diameter and remarkably

Table 1 Relative peak intensities in the FPLC chromatograms

Ratio DNs-PSS : CP	Relative peak intensity[a]		
	$T = 3$[b]	$T = 1$[c]	CP[d]
0.4	4	6	90
4	3	48	48
40	0	68	32
400	0	37	63

[a] In% based on the peak heights in the FPLC chromatogram detected at $\lambda = 208$ nm. [b] Eluting at $v = 1.0$ ml. [c] Eluting at $v = 1.35$ ml. [d] Eluting at $v = 1.8$ ml.

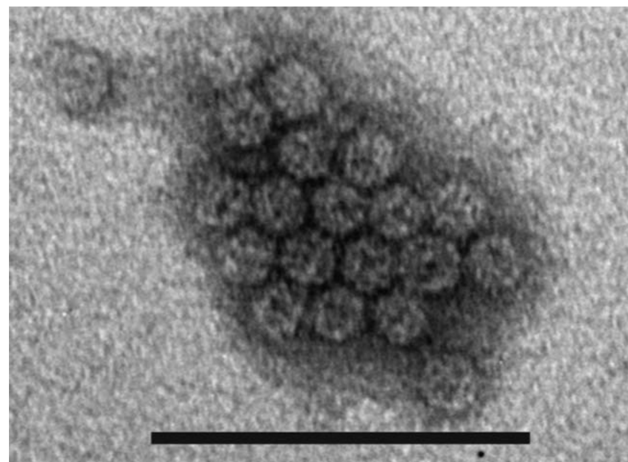

Fig. 2 Transmission electron micrograph of $T = 1$ assemblies of CP assembled around DnS-PSS. The scale bar represents 100 nm. Size analysis of the particles revealed that they have a diameter of 16.1 to 16.2 nm.

stable; storage at 4 °C for many weeks had no detectable effect on the particles.

Fig. 3 shows the results of dynamic light scattering experiments of the isolated aggregate, which clearly indicates that the new $T = 1$ particle is smaller ($R \sim 9.5$ nm) than unmodified CCMV. The discrepancy between the observed diameter in the TEM micrograph

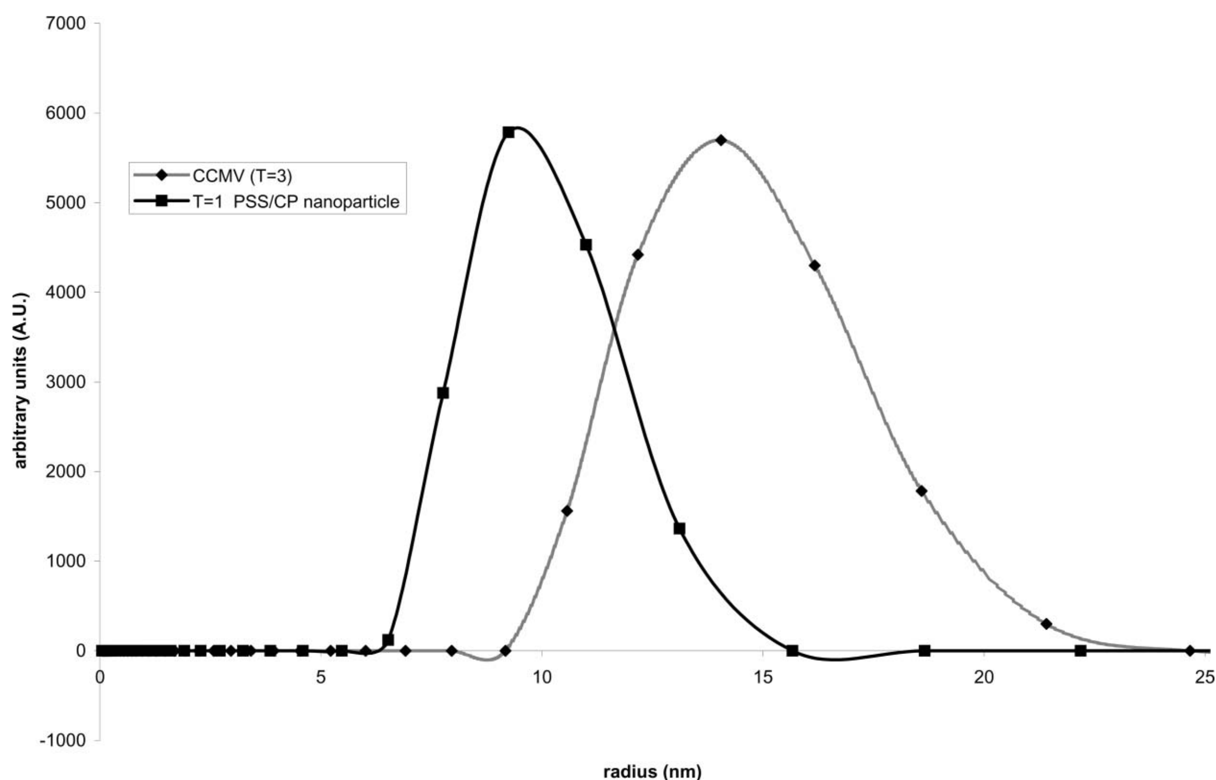

Fig. 3 Normalized CONTIN fits of dynamic light scattering data for the CCMV $T = 3$ particle and the DnS-PSS : CP $T = 1$ aggregate.

and the radius obtained from DLS measurements is likely caused by the fact that DLS measures the hydrodynamic radius and TEM does not. The FPLC fractions containing the DnS-PSS : CP aggregate (pH 7.5) were reinjected on the FPLC column, and also dialysed to pH 5.0 to verify the influence of pH change. Both at pH 7.5 and at pH 5.0, the same particles were detected, and no free CP was observed, implying high particle stability.

The spontaneous formation of the much smaller $T = 1$ particle, compared to the native-like $T = 3$ capsid, was initially quite surprising as it is well-established that native CP is unable to form the $T = 1$ shell.[14] Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the FPLC fraction corresponding to the $T = 1$ particle indicated that the polymer loaded particles contained modified capsid proteins lacking the (1–25) and (1–41–44) amino acid regions (Fig. 4). SDS-PAGE of the CP used for these experiments showed identical degradation products (not shown). Recent results from Johnson and coworkers[14] show that purified partially degraded protein building blocks (NΔ34) will form a mixture of $T = 1,2,3$ particles at low pH. They conclude that the N-terminal amino acid residues 1–36 are not essential for capsid formation but bias the $T = 3$ capsid arrangement. Despite the elimination of the N-terminal RNA binding sequence, there are still sufficient RNA binding sites left to account for the favorable interaction between the modified CP and the polyanion.[5] We speculate that the presence of a small quantity of non-degraded protein would not exert a large influence, since our work shows that particle formation is primarily driven by the presence of polyanion. Nevertheless, we are currently investigating the relation between protein degradation and particle formation.

The experiments presented above show that at very low polymer concentrations (DNs-PSS : CP = 0.4 and 4) larger $T = 3$ particles are formed, suggesting that in this case, the interactions between the CP itself determine the particle size.[14] We conclude that at pH 7.5 an equilibrium exists between free native and degraded CP and the $T = 3$ capsid, although strongly in favor of the free CP. Apparently, the very small amount of polyanion present has

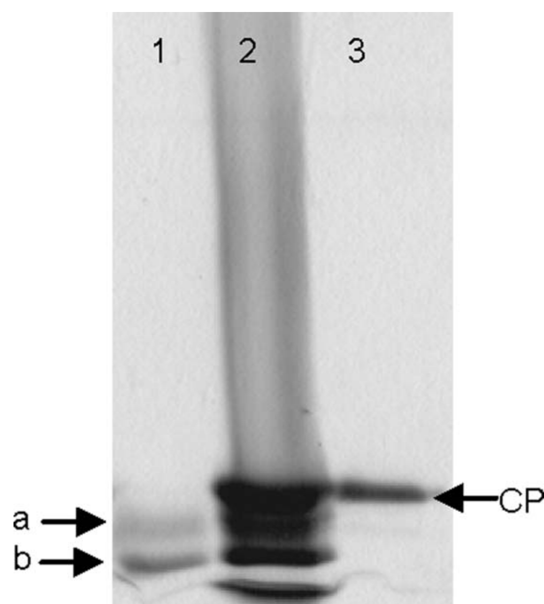

Fig. 4 SDS PAGE analysis of the isolated $T = 1$ fraction (lane 1), crude CP (lane 2) (the CP used for the inclusion experiments showed similar degradation products as seen in lane 1) and the purified virus (lane 3). The arrows indicate the degraded proteins lacking the (1–25) (arrow a) and the (1–41–44) (arrow b) regions.

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only a slight ionic interaction with the degraded CP, which is not strong enough to completely change the particle dimension, therefore, both the $T = 1$ and the $T = 3$ particles can be obtained. In this case, the equilibrium between free CP and the $T = 3$ particle still plays a role (Fig. 5). The absence of the pseudo $T =$ '2' particle in our experiments is in line with the dynamic character of this process, since this $D = 25$ nm particle is a kinetic product only observed upon swift lowering of the pH.[14]

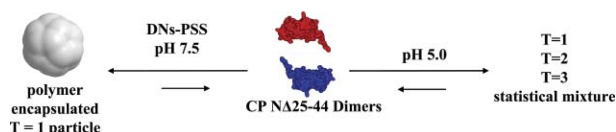

Fig. 5 The presence of DNs-PSS induces the formation of the $T = 1$ particle from modified CP (left) at pH = 7.5, whereas $T = 1,2,3$ particles are formed at pH = 5.0 in the absence of polymer.

At higher stoichiometric ratios, the presence of more attractive interactions force the modified CP to aggregate in a $T = 1$ architecture, which was found to be remarkably stable; equilibria existing between this $T = 1$ particle and the free CP mixture or other particles are strongly in favor of the former species. Since no free DNs-PSS was detected at the ratio 40,[13] we may conclude that all polymer is assembled into the particle, indicating a high affinity for the polymer. The results obtained at DNs-PSS : CP = 400 support this; although the yield of the smaller particle is lower, presumably due to a polyelectrolyte effect, no larger particles are observed. Therefore, in the presence of an excess of polyanion, only the $T = 1$ particle is generated. Calculations show that 60 subunits make up the shell of this $T = 1$ particle, yielding an icosahedron with 12 pentameric faces (Fig. 5).[14]

In conclusion, we have demonstrated that the self-assembling properties of CCMV CP lacking part of its N-terminus can be employed to create a monodisperse 16 nm $T = 1$ icosahedral nanoparticle upon encapsulation of a disperse polymer at pH 7.5. Whether or not non-degraded, or a mixture of partially- and non-degraded protein exhibits the same behaviour is still under investigation. In the experiments presented above, no free DNs-PSS was detected; we may therefore conclude that the equilibrium of the self-assembly process strongly favours the $T = 1$ particle. Since inclusion of this relatively short PSS only yields the $T = 1$ particles it is feasible that higher molecular weight polymers induce the formation of larger than $T = 1$ assemblies, opening the way to specifically tune the particle sizes. This is currently under investigation. Potential applications of these materials can be found in pharmaceutics (*e.g.* targeting and transport), nano-arrays or electronics.[15]

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