# CCMV capsid formation induced by a functional negatively charged polymer†

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A functional negatively charged polyelectrolyte, polyferrocenylsilane (PFS) was encapsulated in cowpea chlorotic mottle virus (CCMV) capsid proteins, yielding monodisperse particles of 18 nm in size with altered redox properties compared to the parent materials.

## Introduction

Nanometer sized materials have gained much interest over the past decades with a growing number of practical utilizations.[1] These structures are more and more often constructed from biomaterials like proteins and DNA.[2-9] Their major advantage with respect to non-biological nanomaterials is their exceptional ability to self-assemble into precisely defined structures. The Cowpea Chlorotic Mottle Virus (CCMV) is an example of such a defined biological system.[10] It consists of 180 identical capsid proteins, which self-assemble around the central RNA yielding highly defined icosahedral virions of 28 nm in size. The icosahedral shape can be described according to the Caspar and Klug T (triangulation) number as a T = 3 particle.[11,12] The capsid has a positively charged interior with a diameter of 18 nm, likely interacting with the RNA to stabilize the capsid structure. At high pH (7.5) the virus dissociates into protein and RNA, of which the latter can be removed by precipitation, yielding capsid dimers which can be reassembled at low pH (pH 5.0). This indicates that at low pH the interactions between the capsid proteins dominate the assembly. The pH dependent assembly and disassembly process is reversible, making it possible to use CCMV as an encapsulation vesicle.[13,14]

Recently, we have shown that a negatively charged polymer, polystyrene sulfonate (PSS), together with capsid dimers at pH 7.5 forms virus like particles of 19 nm.[15] These capsids likely consist of 60 protein subunits instead of 180 and can be described as T=1 particles.

In order to investigate whether the same principle would apply to other negatively charged polymers, and to add functionality to the capsid, we describe here the encapsulation of a functional negatively charged polyelectrolyte, polyferrocenylsilane (PFS) polyanions.[16] PFS contains redox active ferrocene units in the main chain and can therefore be oxidized and reduced reversibly,[17,18] either by chemical or electrochemical means.[19] In the reduced state, the polyion has a net negative charge due to the negatively charged polymer side groups. Complete oxidation of the polymer will introduce additional positive charges on the polymer main chain, which results in a net neutral charge. Since the formation of the capsid at neutral pH depends on the interaction of the protein dimers with a negatively charged species, switching the polymer towards its neutral state could have an effect on the stability of the capsid. Conversely, the capsid proteins might also have an effect on the redox chemistry of the polymer, since it is known from *e.g.* the cytochrome family, that proteins can have a significant impact on the redox potential and behavior of a metal center.[20] Here, we report on the investigation of the interaction of the PFS polyanions with the capsid proteins at neutral pH, the redox chemistry of the PFS capsid complex, and the stability of the PFS capsid complex under these circumstances.

## Results and discussion

CCMV capsid proteins and PFS polyanions were obtained according to literature procedures.[18,21,22] The PFS polyanions (Fig. 1) were dissolved in a small volume of milli-Q water (MQ) and added to the capsid protein at pH 7.5 at a ratio of 40 monomeric units per capsid protein, since previous experience with the encapsulation of PSS polymer indicated that 40 negative charges per capsid monomer is the optimal ratio.[15] The particles were allowed to form for at least 15 minutes at 4 °C and were subsequently analyzed using Fast Performance Liquid Chromatography (FPLC) and transmission electron microscopy (TEM). The formed particles eluted at V = 1.20 mL as detected at both λ = 280 and λ = 450 nm (Fig. 2). The PFS polyanions have a specific absorption at λ = 450 nm[23] when they are in the reduced state, whereas proteins do not absorb in this region. This

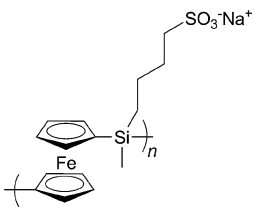


**Fig. 1** Structure of the PFS anionic polymer.

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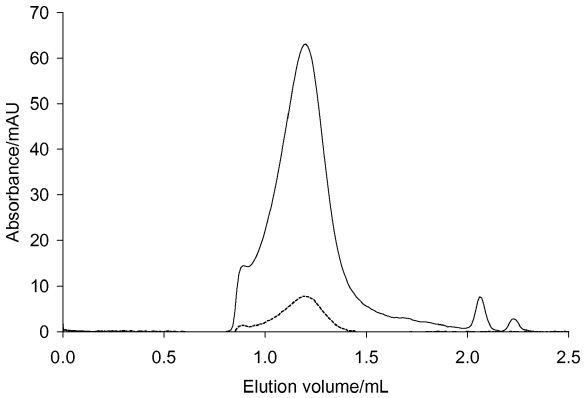


**Fig. 2** FPLC trace of capsid proteins mixed with PFS polyanions The FPLC was equipped with a superose 6 size exclusion column. The bed volume of this column is 2.4 mL. The solid (upper) line represents the UV absorption at $\lambda = 280$ nm, at which both the protein and the polymer absorb. The dashed (lower) line represents the UV absorption at $\lambda = 450$ nm, which is specific for the PFS polyanions. The elution volume of the peak is 1.20 mL. CCMV capsids of 28 nm in size usually elute at V = 1.1 mL, and the capsid dimers usually elute at V = 1.8 mL.

showed that the PFS polyanions co-elute with the protein (Fig. 2), indicating that it is indeed encapsulated. Non-encapsulated PFS polyanions did not seem to elute from the column, possibly because the PFS polyanions stick to the column, as is suggested by injecting a pure polymer solution into the FPLC system.

In order to visualize capsids with TEM, they are stained with uranyl acetate, a negative staining agent, revealing capsids with d = 18 nm (Fig. 3a), which was confirmed by additional measurements using Dynamic Light Scattering (DLS). This negative staining procedure visualizes the protein material, because the staining agent (uranyl acetate) does not adhere to proteins. In this way the particle is visible as a bright spot, often with a sharp rim, in a darker background. Since the PFS contains iron atoms in the main chain, the PFS-containing particles should also be visible without this staining technique. Indeed, particles of a slightly smaller size than the stained PFS containing capsids are visible without staining (Fig. 3b), exhibiting dark PFS loaded capsules. This is further support for the encapsulation of PFS.

The percentage of PFS that was encapsulated in the T = 1 particles purified by FPLC was determined using the UV absorbance ratio of $\lambda = 280$ and 450 nm. The capsid protein absorbs at $\lambda = 280$ nm, while the PFS absorbs at both these wavelengths and has a 280/450 nm absorbance ratio of 11.67. Since the 280/450 nm ratio of the T = 1 particles was consistently 20, the percentage of PFS loading can be calculated, indicating that about 12% of the added PFS is encapsulated. From this data, the concentration of PFS inside the capsids can be determined, yielding a value of $1.9\ 10^{-2}$ M PFS, whereas the concentration of the same amount of PFS free in solution would be $5.8\ 10^{-5}$ M (see ESI† for calculation details). This means that the concentration of PFS inside the capsids is about 330 times higher than one would expect from statistical encapsulation. This strongly indicates that the encapsulation of PFS is favored, probably because it is needed to stabilize the capsid structure in the absence of the natural RNA.

The PFS containing particles appear to be quite stable; they survive at least two months of storage at 4 °C, and even dialyses against MQ (Fig. 3c). This is in contrast to the empty T = 3 capsids

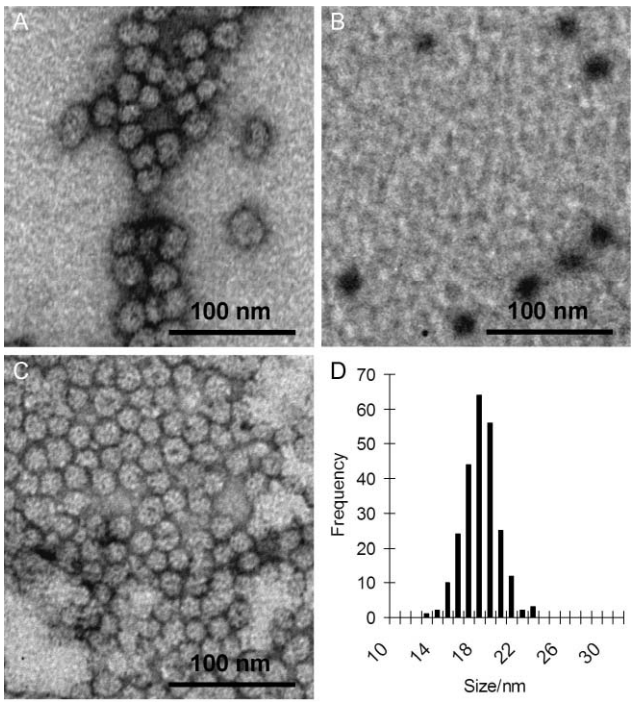


**Fig. 3** Size of the PFS-protein nanoparticles obtained from TEM imaging. (A) PFS loaded capsids stained with uranyl acetate. (B) Non-stained PFS loaded capsids, (the iron atoms of the PFS polyanions provide contrast in this case). (C) PFS loaded capsids stained with uranyl acetate which were dissolved in MQ. (D) Size distribution of PFS loaded capsids.

resembling the natural virus particle, which need large amounts of salt to stabilize their structure. This is a strong additional indicator that the PFS polyanions stabilize the T = 1 particle, presumably through ionic interactions of the negatively charged PFS polyanions with the positively charged interior of the capsid proteins. Taking these properties into account, it is of interest to study whether the PFS containing particles are still stable when these ionic interactions are altered. Since the PFS polyanion is redox responsive,[18] the polymer can be switched from a negative to a net neutral state, which could potentially disrupt the ionic interactions.

PFS can be oxidized chemically by $FeCl_3$. Unfortunately, this oxidant appeared to harm the capsid proteins. That is, when $FeCl_3$ was added to the capsid protein dimers, the dimer peak in the FPLC disappeared. A new peak at V = 2.06 mL appeared, but this is probably due to the oxidized polymer, since a Western blot of the material eluting at this volume showed that it did not contain any capsid protein. The addition of other oxidizing agents (KI, $KMnO_4$, $Cl_{2(g)}$) gave the same result. $(NH_4)_3$ $[Fe(CN)_6]$ did not appear to have such a detrimental effect on the protein, since the capsid protein dimer was still clearly visible on the FPLC graph. However, unfortunately, this compound did not oxidize PFS completely, as no change in optical appearance was observed. This is in contrast to the oxidation with $FeCl_3$, where the optical appearance changed immediately from yellow to dark green/blue, after addition of the $FeCl_3$. This finding was confirmed by UV–vis analysis of the samples (Fig. 1 of ESI†). Therefore it seemed that the balance between an oxidizing agent that was sufficiently strong to oxidize the PFS, but not strong enough

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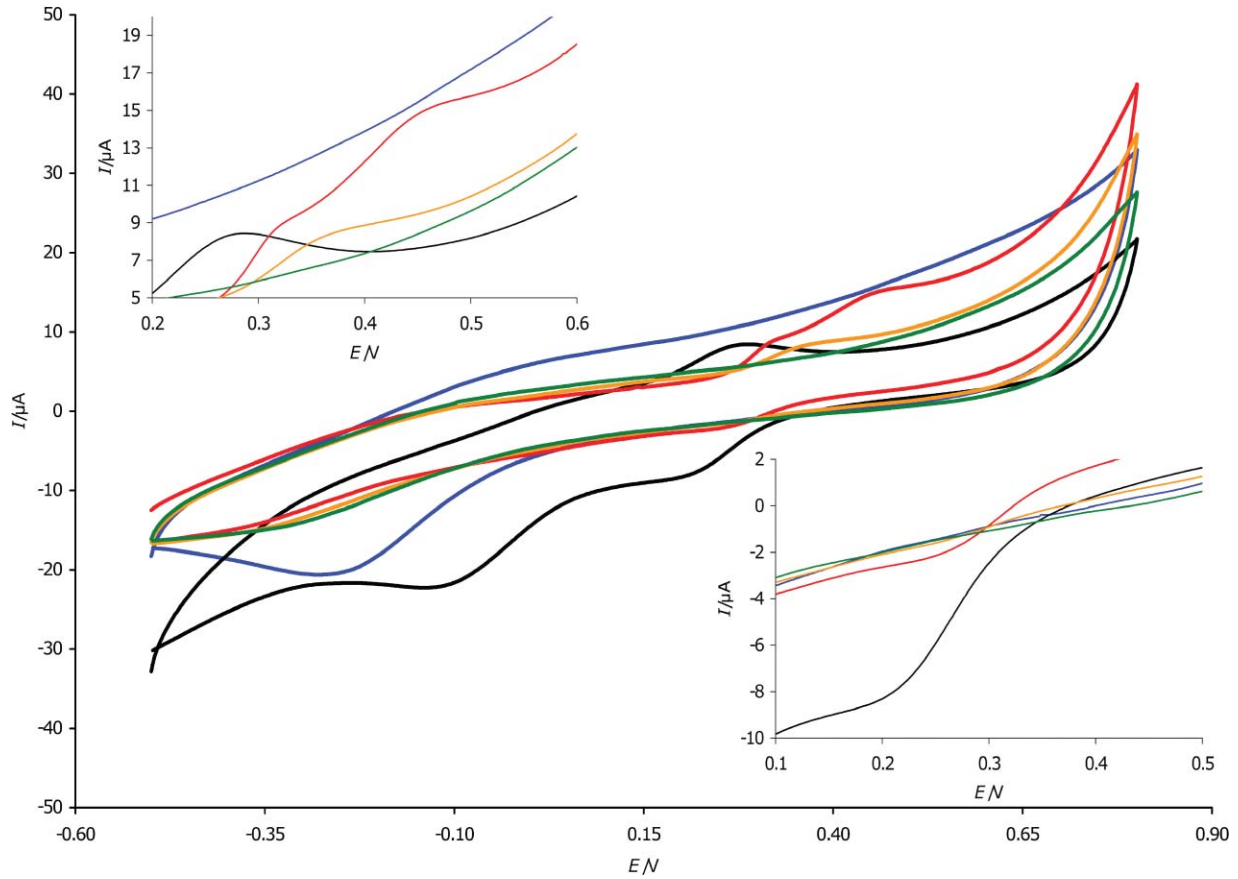


**Fig. 4** CV diagrams of PFS loaded capsids. Blue line: buffer, black line: free PFS in solution, red line: the first oxidation cycle of the PFS loaded capsids, orange line: the second oxidation cycle of the PFS loaded capsids, green line: the third oxidation cycle of the PFS loaded capsids. Inset top left corner: enlargement of oxidation peaks in CV diagram. Inset bottom right corner: enlargement of reduction peaks in CV diagram.

to oxidize the protein, would be very delicate or even non-existent. For this reason electrochemical oxidation was carried out to oxidize the PFS polyanions. Experimentally, 3 platinum wires acting as the working, reference and counter electrodes respectively were submerged into the T = 1 particle containing solution and attached to a potentiostat with a cyclic voltametry (CV) setup. The cyclic voltammograms recorded showed that the electrochemical behavior of the PFS inside the capsid deviated significantly from the behavior of PFS polyanions that are free in solution (Fig. 4). Normally, PFS can be oxidized and reduced reversibly, but once encapsulated this does not seem to be the case. In the first cycle, the oxidation peak is about as strong as the oxidation peak of the PFS free in solution, but the reduction peak is much weaker. In the subsequent cycle, the oxidation peak is also weaker, and the reduction peak decreases even further, until in the third cycle the peaks have disappeared completely. This could indicate an incomplete reduction of the PFS polyanion. With each successive cycle more PFS polyanions remain oxidized, until after only three cycles, no electrochemical response can be detected anymore. Also the position of the redox peaks in the voltammograms is shifted upon encapsulation with regard to normal PFS. This could be related to an effect of the protein environment, as proteins are also known to have a profound influence on the redox potentials of, for example, iron in the cytochrome protein family.[20] In order to check whether the redox response is really caused by the PFS polyanions and not by the protein shell surrounding it, a redox unresponsive polyanion, PSS, was encapsulated and CV was measured in the same way. Indeed, no redox response was observed (Fig. 2 of ESI†), confirming that the redox response of the PFS loaded capsids was caused by the ferrocene units, and not by the protein. To investigate whether the capsid stability was affected by the oxidation and reduction of the polymer, the particles were studied by TEM at three different time points: before oxidation, immediately after oxidation, and following reduction (Fig. 3 of ESI†). In order to obtain a representative view of the electrochemical process the TEM samples were prepared by dipping the specimen grids in the solution at designated time points. Since the concentration of particles is much higher in these experiments, as compared to the concentration normally used to prepare TEM grids, the images are somewhat blurred. At each time point capsids are still visible, although directly after oxidation the capsids seem less well-defined. In this case, however, the contours of the PFS containing particles are still clearly visible; therefore it is unlikely that the capsids are completely disassembled.

## Conclusions

The experiments described above have shown that negatively charged PFS is also able to assemble capsid proteins to form

T = 1 particles, similar to *e.g.* PSS.[15] As mentioned, this is probably due to the interaction of the negatively charged polymer with the positively charged capsid interior. The redox properties of the encapsulated PFS were quite different from those of the 'free' PFS. The initial shift of the oxidation and reduction potentials might be explained by the interaction of the polymer with the protein shell. In subsequent electrochemical cycles the peaks shift and their intensity is decreased. Apparently, the complex formed between PFS and the capsid protein can be oxidized but not reversibly reduced. The reduction therefore proceeds incompletely, so that the polymer/protein complex is likely still largely hydrophobic and inaccessible for the subsequent oxidation, also because a substantial part of the polymer is still oxidized. The oxidation peak is thus decreased in height and shifted to a higher voltage because compared to the blank specimen, PFS, it is getting more difficult to oxidize the polymer. This cycle repeats itself until all polymer is oxidized and becomes inaccessible for further oxidation. This might explain the CV results, but it still does not explain why the capsids appear to stay intact during this process. It might be that although the PFS is crucial for the initial formation of the T = 1 particle, it is no longer needed when the capsid is formed. Possibly the interactions between the capsid proteins in the spherical structure formed are sufficiently strong to maintain the capsid structure.

## Experimental

For all experiments a buffer solution containing 0.4 M NaCl, 0.05 M Tris-HCl, 0.01 M $MgCl_2$ and 0.001 M EDTA at pH 7.5 was used.

Protein concentrations were determined using a Cary 50 Conc (Varian, Middelburg) UV-VIS spectrophotometer.

FPLC measurements were performed using a superose 6 PC 3.2/30 analytical column from GE lifesciences, on an Amersham Ettan LC system, fitted with a fractionating device. Buffers for FPLC were filtered with a Millipore 0.2 μM filter before use. TEM grids (Formvar-Carbon) were exposed to an electron discharge treatment using a Cressington Carbon coater and power unit. Unless stated otherwise, the sample was applied to the grids by adding a drop of 5 μL solution (~0.2 mg/mL) to the grid and carefully removing it following 1 minute immersion using a filter paper. The grid was allowed to dry for at least 15 minutes before applying 5 μL of a 2% (w/v) uranyl acetate aqueous solution, which was removed after 15 seconds. The grid was again allowed to dry for at least 15 minutes. Samples were studied on a JEOL JEM-1010 TEM (Jeol, Japan). CV measurements were performed on a Autolab PGSTAT10 potentiostat from Echochemie. PFS loaded capsids were purified on FPLC and concentrated to a concentration of ~1 mg/mL protein with Millipore centrifugal devices (MWCO 100 kDa).


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