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The use of a viral template has allowed the synthesis of monodisperse Prussian blue nanoparticles with a diameter of $18 \pm 1.7$ nm and their organization into hexagonal patterns on mica and hydrophilic carbon surfaces.

The variety of structures and magnetic properties of Prussian blue (PB; $[\text{MFe}^\text{III} \text{II}(\text{CN})_6] (\text{M} = \text{NH}_4, \text{Li}, \text{Na}, \text{K})$) and its related metal hexacyanates have attracted intense interest because of their potential application as molecular magnets.1 Much attention has been focused on understanding the relationship between the unit cell structure and the magnetic properties of PB, as well as the preparation of Prussian blue analogues with high Curie temperatures ($T_c$)’s. Surprisingly few examples of PB nanoparticles (NPs) have been reported thus far,2 and controlling the growth and size of these particles remains an important issue.

It has been shown in recent years that biomolecular architectures can serve as nanoreactors or nanotemplates for crystallizations and other reactions. Among the repertoire of biological scaffolds for nanochemistry, protein cages such as apo-ferritin or a number of icosahedral viral capsids have been used as a nanoreaction vessel for the fabrication of NPs.3 In this paper, we describe the use of the cowpea chlorotic mottle virus (CCMV) as a template for the synthesis of monodisperse Prussian blue nanoparticles. Furthermore, the ability of CCMV to form hexagonal patterns on surfaces provides a new strategy for the self-organization of these nanoparticles.

The CCMV icosahedral virus4 is 28 nm in diameter and the protein shell defines an inner cavity with a diameter of approximately 18 nm. CCMV consists of 180 identical coat protein (CP) subunits self-assembled around a central RNA strand. The 20 KDa CP is formed from 189 amino acids with nine basic residues at the N-terminus. An interesting feature of this virus is its sensitivity to pH and ionic strength.5 Depending on these factors, the CCMV capsid can be rapidly disassembled in vitro into CP dimers and, after RNA removal, re-assembled. This reversible pH-dependent assembly/disassembly process provides a unique molecular gating mechanism for the entrapment of organic and inorganic materials.6

The wild-type CCMV capsid has been shown to be a suitable system for the crystallization and growth of polyoxometallate species (e.g., vanadate, molybdate and tungstate) based on its positively charged interior surface.6a However, the preparation of magnetic materials, such as maghemite ($\gamma$-Fe$_2$O$_3$), within the viral capsid has only been achieved with a genetic mutant of CCMV having the electrostatic character of its interior surface dramatically altered.7 The mutant was not stable under the conditions necessary to prepare maghemite, therefore the viral capsid had to be stabilized by cross-linking the protein subunits with a bifunctional cross-linker such as glutaraldehyde.3b The wild-type bromo mosaic virus (BMV) has also been used to host iron oxide superparamagnetic NPs by self-assembly of the CP around them, but the nanoparticles had to be previously synthesized and modified with phospholipids.5 Only one example has been reported concerning the direct growth of a magnetic material, i.e., cobalt, inside a wild-type viral capsid, i.e., the T7 bacteriophage.9 This DNA virus, however, does not possess the ability to reversibly assemble and disassemble, and its higher diameter (55 nm) led to relatively big cobalt nanoparticles (diameter ~40 nm). The method described herein (schematically depicted in Fig. 1) represents a fast and efficient route to prepare, under mild conditions, monodisperse Prussian blue nanoparticles with a diameter of $18 \pm 1.7$ nm. Considering that the application of magnetic nanoparticles in information storage devices and other magneto-optic applications requires their organization on a surface, preliminary results show that these PB-CCMV biohybrids can self-organize on mica and hydrophilized graphite surfaces to form hexagonally packed monolayers.

The purification of the CCMV virus and the removal of its RNA were carried out according to literature procedures.10

![Fig. 1 Schematic representation of the method employed to prepare PB nanoparticles inside the CCMV capsid.](image-url)
The resulting viral capsids were characterized by fast protein liquid chromatography (FPLC; \( V_{\text{capsid}} = 1.12 \text{ mL} \)), UV–Vis spectroscopy and transmission electron microscopy (TEM).

For the synthesis of Prussian blue, a photoinitiated stepwise reaction was chosen in which the photoreduction of \([\text{Fe(C}_2\text{O}_4)\text{]}^{3-}\) produces Fe\(^{II}\) ions that further react with \([\text{Fe(CN)}_6]^{3-}\) to form the aforementioned PB clusters.\(^{2a}\) Since the Prussian blue precursors (PBPs) employed in this method are negatively charged iron complexes, the inclusion within the CCMV capsid is not only statistical but likely also driven by the positively charged amino acids of the N-terminus of the coat protein that are pointing into the interior of the capsid (see below). Moreover, both reagents are not reactive towards each other and therefore it is possible to encapsulate them before the reaction is photoinitiated.

In order to carry out the encapsulation, a solution of capsid at pH 5.0 was incubated for 2 h with a solution of PBPs in the same buffer, attaining a final 0.1 M concentration of each of the precursor salts.\(^{f}\) The diffusion of small substrates through the capsid pores was previously proven and provides a mechanism for the inclusion.\(^{6}\) The solution containing the encapsulated PBPs was then irradiated for 6 h using a 405 nm laser beam. Very rapidly, a distinct blue colour was observed. The mixture was centrifuged every hour during the irradiation to remove solid particles that were formed outside the protein shell. The FPLC chromatogram obtained from the resulting sample shows an intense peak at \( V = 1.15 \text{ mL} \) that corresponds to the viral capsid (Fig. 2). The intense blue colour of the fractions corresponding to this peak together with their broad absorption at around 720 nm clearly indicate the presence of Prussian blue inside the capsid. The remaining PBPs elute at \( V = 2.1 \text{ mL} \) and show a colour ranging from green to yellow depending on the concentration.

Further purification by size exclusion chromatography on Sephadex G-100 allowed the complete removal of the non-reacted PBPs (Fig. S1),\(^{f}\) after which spectroscopic characterization of the pure PB–CCMV biohybrid material was carried out. The UV–Vis spectrum shows a broad band at 720 nm, which is consistent with an intermetal charge transfer between Fe\(^{II}\) and Fe\(^{III}\) in PB,\(^{11}\) together with an absorption below 300 nm that arises from both the coat protein and the Prussian blue. Furthermore, a linear combination of the spectra of CP and PB gives rise to a spectrum almost identical to the one of the capsid containing Prussian blue (Fig. S2).\(^{f}\) The FT-IR spectrum contained a peak at 2077 cm\(^{-1}\) that corresponds to the Fe–CN stretching mode in the cyanometallate lattice.

TEM images of the same sample confirmed the formation of PB inside the CCMV capsid (Fig. 3). CCMV capsids with the typical dimensions are observed over the whole grid when stained with uranyl acetate (Fig. 3A). These capsids appear as black dots surrounded by a bright shell when looking at the sample without any staining (Fig. 3B and 3C), which can be attributed to the high electron density cores of Prussian blue. The particle size distribution shows the dots to be monodisperse, with a diameter of 18 ± 1.7 nm. This corresponds accurately with the dimensions of the inner cavity of the CCMV capsid.

The above results are in clear contrast to the control experiments. The synthesis of Prussian blue in the absence of capsid gave a blue solid that was separated from the PBPs by centrifugation (Fig. S3).\(^{f}\) After resuspension of the blue solid in water and sonication, cubic structures greatly varying in size were observed by TEM (Fig. S4).\(^{f}\) In addition, a second control involving the irradiation of samples containing only capsids showed no changes in the FPLC, UV–Vis and IR curves, and TEM images revealed the absence of an electron dense core inside the viral particles.

In order to determine the concentration of coat protein and PB building units (\((\text{NH}_4)\text{Fe}^{III}[\text{Fe}^{II}\text{(CN)}_6]_{3}\)) in the solution of PB–CCMV biohybrid nanoparticles, analysis by ICP-OES (inductively coupled plasma-optical emission spectroscopy) was carried out.\(^{f}\) It was determined from this experiment that the molar ratio of Fe from the PB to S from the coat protein was 5.4. Given that there are 3 sulfur atoms per CP subunit, an average number of 1460 PB building units per viral capsid can be calculated. The concentration of PB building units within the capsid can then be estimated to be 0.8 M, a value substantially higher than the initial concentration of precursors. This result suggests that, while the crystallization of PB is occurring in the interior of the capsid, and the concentration of PBPs is therefore decreasing, diffusion of more precursors through the capsid pores is taking place, probably directed by the gradient of concentration and the electrostatic interactions between the positive N-terminus of the coat protein and the negative iron complexes. This diffusion effect provides a mechanism to enrich the amount of material within the capsid and allows it to be completely filled with Prussian blue, as indicated by the TEM data.

The organization of magnetic nanoparticles on surfaces is considered an important goal because it may allow their
application in, e.g., magneto-optic devices. Previous studies have shown that the CCMV virus self-organizes into hexagonal patterns and forms stretched monolayers. This ability can be exploited to prepare self-assembled arrays of the PB-CCMV biohybrid nanoparticles described above. Two different methods have been attempted for this purpose. First, the organization of the NPs was studied by TEM on carbon hydrophilic grids, using ammonium molybdate as a staining agent. Ammonium molybdate is known to intercalate between the viral particles and the surface, helping them to pack closely to each other. As a consequence, hexagonal patterns of the PB-CCMV nanoparticles were nicely observed in the TEM images (Fig. 4A and 4B). In order to get the same type of organization over large areas, mica was tested as the surface because of its natural hydrophilic character, and the samples were studied by atomic force microscopy (AFM). The AFM micrograph in Fig. 4C shows a 2 × 2 µm area completely covered with round-shaped particles of the same size as the CCMV capsid. In the image, different domains with hexagonal ordering can be clearly distinguished, showing the tendency of the PB-CCMV nanoparticles to self-organize on the surface.

In conclusion, the synthesis of Prussian blue inside the CCMV capsid has yielded highly monodisperse nanoparticles with a diameter of 18 ± 1.7 nm. The preparation method is fast and efficient, and the purification is easy; the whole process can be carried out in less than 12 hours. The organization of these PB nanoparticles on mica and on hydrophilic grids, using ammonium molybdate as a staining agent, can be carried out in less than 12 hours. The preparation process can be carried out in less than 12 hours. The organization of the NPs was studied by TEM on carbon hydrophilic grids, using ammonium molybdate as a staining agent. Ammonium molybdate is known to intercalate between the viral particles and the surface, helping them to pack closely to each other. As a consequence, hexagonal patterns of the PB-CCMV nanoparticles were nicely observed in the TEM images (Fig. 4A and 4B). In order to get the same type of organization over large areas, mica was tested as the surface because of its natural hydrophilic character, and the samples were studied by atomic force microscopy (AFM). The AFM micrograph in Fig. 4C shows a 2 × 2 µm area completely covered with round-shaped particles of the same size as the CCMV capsid. In the image, different domains with hexagonal ordering can be clearly distinguished, showing the tendency of the PB-CCMV nanoparticles to self-organize on the surface.

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