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Summary

The templated mineralization of metal or metal oxide on the external surface of wild-type Cowpea mosaic virus (CPMV), a plant virus, is facilitated by increasing the external surface negative charge. This is achieved by the chemical modification of surface lysine groups by succinamic anhydride. Hence, for example, treatment of charge modified CPMV-succinamate with a 2:1mixture of iron(II) and iron(III) salts, followed by raising the pH to 10.2, led to the formation of narrowly dispersed, CPMV-templated, magnetite (Fe_3O_4) nanoparticles.

Key words: Nanoparticles, Iron oxide, Magnetite, Cowpea mosaic virus, Templated mineralization

1. Introduction

The Cowpea mosaic virus (CPMV) capsid is comprised of 60 asymmetric units that assemble into an icosahedral structure of approximately 30 nm diameter ***(1-3)***. CPMV provides a useful platform for the attachment of molecules of interest either by genetic ***(4-6)*** or chemical modification ***(7-15)***. Previously, we have shown that CPMV can template on its external surface the mineralization of silica or metals either: by the use of CPMV chimaeras, in which peptides specific for particular minerals/metals are genetically inserted into loops on the surface of the virus (silica ***(5)*** and iron-platinum ***(6)***); by chemically-coupled-peptide-promoted mineralization (iron-platinum, cobalt-platinum and zinc sulfide ***(16)***); by an electroless deposition process (cobalt, nickel, iron, platinum, cobalt-platinum and nickel-iron ***(17)***); by polyelectrolyte surface modification (gold ***(18)***, see Chapter X). However, our observations ***(5, 6, 16)***, and those of others ***(19, 20)***, implied to us that to initiate mineralization on the external surface of CPMV it was necessary only to increase the surface negative charge by chemical modification. This is a much simpler process than those that we had previously developed.

To create suitable conditions for templated surface mineralization by increasing the virus capsid surface negative charge, CPMV particles were chemically modified with succinic anhydride (**Fig. 1**) ***(21)***. On reaction, the anhydride ring opens forming a covalent bond to lysine amine-nitrogen and a free carboxylate group at the other end of the ring-opened molecule ***(22)***. Coverage with succinamate of at least the 240 readily addressable surface lysines was achieved. CPMV-succinamate particles have a zeta potential (-28.3 ± 2.34mV) almost two-and-a-half fold more negative than

CPMV (-12.3 ± 1.40 mV), consistent with successful surface modification and consequent increase in surface negative charge.

"[Fig 1 near here]"

For mineralization with iron oxide (magnetite, Fe_3O_4), CPMV-succinamate particles in buffer were incubated under alkaline hydrolysis conditions with a 2:1 mixture of iron(II) chloride and iron(III) chloride followed by raising the pH to approximately 10.2 for 1 hour (**Equation**). The presence of succinamate, and hence the extra surface negative charge, was essential for templated-mineralization.

$2FeCl_3 + FeCl_2 + 8NaOH \rightarrow Fe_3O_4 + 8NaCl + 4H_2O$ **Equation**

The unstained transmission electron microscopy (TEM) image for iron oxide-CPMV (**Fig. 2**) showed narrowly dispersed, mineralized nanoparticles. The mineralized CPMV particles appeared similar to those that we had previously observed for templated mineralized-CPMV particles obtained by alternative methods ***(6, 16, 17)***. A range of other physical techniques also were used to characterise the iron oxide-CPMV particles and from dynamic light scattering the thickness of the iron oxide layer was estimated to be 2 nm. ***(21)***.

This method provides a simple route to narrowly disperse metal or metal oxide virus-templated nanoparticles that may have a range of potential applications in nanotechnology as diverse as, for example, materials science, catalysis and biomedicine.

2. Materials

Dimethyl sulfoxide ≥ 99.9% (DMSO), iron (III) chloride hexahydrate and iron(II) chloride tetrahydrate were purchased from Sigma-Aldrich; Sephadex G-25 PD-10 columns from GE Healthcare; 100 and 300 kDa molecular weight cut-off membranes from Spectrum Labs.

All reagents were used without further purification. All experiments were performed using Milli-Q water with resistivity of 18.2 MΩ cm (Millipore). The propagation and purification of wild-type CPMV particles were performed by standard procedures (***23***). Purified virions were stored at 4 ºC in 10 mM sodium phosphate buffer pH 7.0. (1 litre of 0.2 M Na_2HPO_4 (27.0 g) and 1 litre of 0.2 M NaH_2PO_4 (28.39 g) were prepared in Milli–Q water and autoclaved before mixing. To prepare 0.1 M buffer solution, 305 mL of Na_2HPO_4 solution and 195 mL of NaH_2PO_4 solution was added to 500 mL Milli–Q water that gave a pH of 7.0).

3. Methods

3.1 $^{\text{Succinamate}}$**CPMV** (superscript preceding the virus initialism refers to external surface modification).

1. CPMV particles (10 mg mL^{-1}, 1 mL) suspended in 10 mM sodium phosphate buffer pH 7.0 were reacted with a 2000 molar excess of succinic anhydride dissolved in DMSO and the final DMSO concentration was adjusted to 20% (v/v) by adding buffer as required.

2. Succinic anhydride was added in two additions (at the beginning of the reaction and after 1 hour).
3. The reaction pH was monitored and maintained, if needed, at pH 7.0 by the addition of a few drops of 1 mM NaOH (*see* **Note 1**).
4. The reaction was left to proceed overnight at 4 °C with gentle stirring at 1000 rpm to ensure complete conversion of all amine groups.
5. The modified particles were purified on PD–10 desalting columns pre-equilibrated with 10 mM sodium phosphate buffer pH 7.0.
6. The eluted sample containing ^{Succinamate}CPMV particles were further dialysed overnight (10-14 hours) using 100 kDa molecular weight cut–off membranes against 10 mM sodium phosphate buffer pH 7.0.
7. The yield of ^{Succinamate}CPMV conjugate, as determined by UV–visible absorption at 260 nm, was between 80–90% based on the initial virus concentration (*see* **Note 2**).
8. The particle integrity was established by native agarose gel electrophoresis (**Fig. 3**), TEM (**Fig. 2**), dynamic light scattering (DLS) and zeta potential measurements.

"[Fig 2 near here]"

3.2 ^{Succinamate}CPMV quantification

1. The number of succinamate moieties per particle was determined for ^{Succinamate}CPMV as follows.

2. ^{Succinamate}CPMV suspended in 10 mM sodium phosphate buffer pH 7.0 was reacted with a 2000 molar excess of *N*-hydroxysuccinamide (NHS) ester–activated amine specific dye, DyLight594, in DMSO.
3. The DMSO level was adjusted to 20% (v/v) if needed and the reaction left to proceed at 4 °C overnight while gently stirring.
4. The doubly-functionalised particles were purified on a PD–10 column pre-equilibrated with 10 mM sodium phosphate buffer pH 7.0.
5. The eluted samples (fractions) were concentrated on 100 kDa cut–off columns before being layered onto 5 ml 10% – 50% sucrose gradients (*see* **Note 3**).
6. The fractions containing the virus particles were collected and dialysed against 10 mM sodium phosphate buffer pH 7.0 for 2 days with change of buffer approximately every 12 hours.
7. After further concentration on 100 kDa cut–off columns, fluorescence was examined by either UV–visible spectroscopy or on agarose electrophoresis gels; a lack of fluorescence indicates complete modification of the virus by succinic anhydride.

"[Fig 3 near here]"

3.3 Iron oxide–CPMV mineralisation

1. ^{Succinamate}CPMV pre–activated particles (1–1.2 mg mL^{-1}, 2 mL) suspended in 10 mM sodium phosphate buffer pH 7.4 were incubated with freshly prepared aqueous solutions of 2 mM iron(II) chloride

tetrahydrate and 4 mM iron(III) chloride hexahydrate at ambient temperature, while gently shaking at 50 rpm for 1–2 hours. The reaction pH was measured as 3.8.

2. The pH was raised slowly to 10.2 by the dropwise addition of 1 mM sodium hydroxide.
3. The reaction was left to proceed for another hour at ambient temperature while gentle stirring at 500 rpm (*see* **Note 4**).
4. The fractions containing mineralized particles were collected and dialysed against 10 mM sodium phosphate buffer pH 7.0 for 15 hours.
5. Mineralized particles were collected and characterised by TEM, DLS, zeta potential, antibody detection, and agarose gel electrophoresis. (*see* **Note 5** and **Note 6**)

4. Notes

1. Monitoring pH after addition of succinic anhydride is crucial, as very high acidic conditions resulted in the disassembly of the virus particles.

2. UV-visible spectroscopy of CPMV particles: CPMV particles have an absorption maximum at a wavelength of $\lambda = 260$ nm (derived from the encapsidated RNA molecules) with molar extinction coefficient of $\varepsilon = 8.1$ mL mg^{-1} cm^{-1}. The law of Beer Lambert can be used to calculate the concentration:

$$A = c\ d\ \varepsilon$$

Where A is the absorbance, c is the concentration of the particles in mg mL^{-1}, d is the length of the light path in cm and ε the molar extinction coefficient in mL mg^{-1} cm^{-1}.

3. Sucrose gradients: Sucrose solutions of 50%, 40%, 30%, 20%, and 10% (w/v) in 10 mM sodium phosphate buffer pH 7.4 were prepared and used within 2 weeks.

Gradients were prepared by underlying sucrose solutions of decreasing density (175 or 500 µL) in a centrifuge tube of 2.1 mL or 5 mL, respectively, and carefully overlaying the sample to fill the tube. Gradients were ultracentrifuged in a swing–out rotor (AH–650) and centrifuged at 137000 g for 1.5–2.5 hours at 4 ºC; 175–300 µL fractions containing modified particles were collected and buffer exchanged for 10 mM sodium phosphate buffer pH 7.4.

4. Shorter incubation times resulted in incomplete reaction. Mössbauer spectroscopy (***21***) indicated mainly $Fe(OH)_2$; the alkaline hydrolysis and hence formation of iron oxide was not complete.

5. As a control experiment, unmodified CPMV particles (CPMVwt) were incubated with the same concentration of iron salts for 1 hour, prior to raising the pH to 10.2 and mixing for another hour, followed by purification by gel filtration columns, dialysis and sucrose gradients. No templated mineralization was observed.

6. A similar method can be used to form CPMV-templated cobalt nanoparticles by incubation of ^{Succinamate}CPMV with cobalt(II) ions followed by sodium borohydride reduction.

Acknowledgement

This work was supported by the Biotechnology and Biological Sciences Research Council, UK (Core Strategic Grant to the John Innes Centre, D.J.E., and JIC DTG, A. A. A. A.).

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Figure Legends:

Figure 1. A schematic representation of the covalent modification of CPMV with succinamate (at least 240 per virus) followed by mineralization (Adapted with permission from *Adv. Funct. Mater.*, 2011, **21**, 4137-4142. Copyright 2011 Wiley-VCH Verlag GmbH & Co.).

Figure 2. Transmission electron microscope images of (A) uranyl acetate stained $^{\text{Succinamate}}$CPMV particles and (B) unstained iron oxide-CPMV; inset showing particles at higher magnification (Adapted with permission from *Adv. Funct. Mater.*, 2011, **21**, 4137-4142. Copyright 2011 Wiley-VCH Verlag GmbH & Co.).

Figure 3. Agarose gel electrophoresis (1.2%) of CPMV intact particles visualized by (A) ethidium bromide staining and (B) Coomassie staining. Lane 1, CPMVwt; 2, $^{\text{Succinamate}}$CPMV; 3, iron oxide-CPMV. The two bands observed for CPMVwt arise from two different electrophoretic forms of the virus that each contain RNA. The third band observed on staining with Coomassie blue arises from the so-called "top" component of CPMVwt that contains no encapsidated RNA and hence is not visualized with ethidium bromide (Reprinted with permission from *Adv. Funct. Mater.*, 2011, **21**, 4137-4142. Copyright 2011 Wiley-VCH Verlag GmbH & Co.).

Figures

Figure 1.

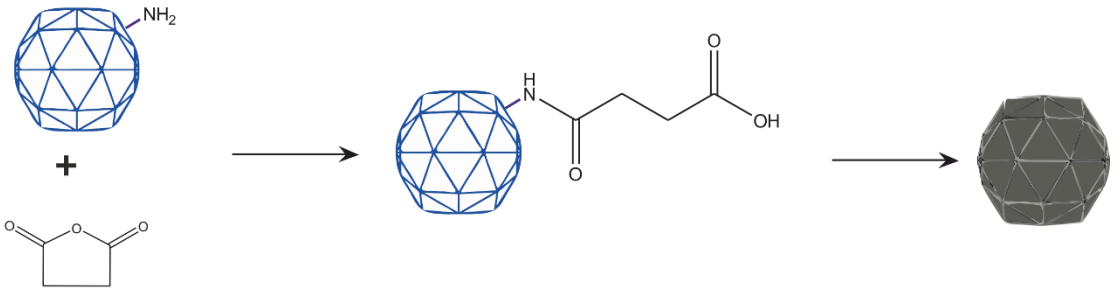

Figure 2.

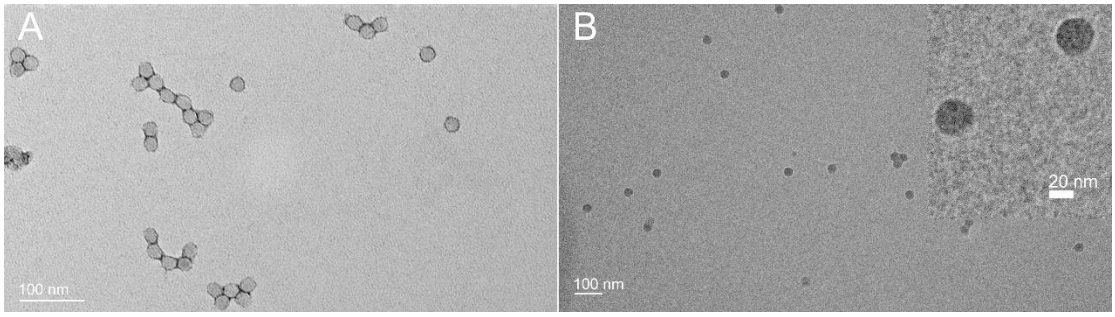

Figure 3.