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Review

The use of tobacco mosaic virus and cowpea mosaic virus for the production of novel metal nanomaterials

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ABSTRACT

Due to the nanoscale size and the strictly controlled and consistent morphologies of viruses, there has been a recent interest in utilizing them in nanotechnology. The structure, surface chemistries and physical properties of many viruses have been well elucidated, which have allowed identification of regions of their capsids which can be modified either chemically or genetically for nanotechnological uses. In this review we focus on the use of such modifications for the functionalization and production of viruses and empty viral capsids that can be readily decorated with metals in a highly tuned manner. In particular, we discuss the use of two plant viruses (Cowpea mosaic virus and Tobacco mosaic virus) which have been extensively used for production of novel metal nanoparticles (< 100 nm), composites and building blocks for 2D and 3D materials, and illustrate their applications.

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Introduction

In the 21st century, nanotechnology has become one of the most rapidly developing fields of science and technology (Mangematin and Walsh, 2006). Numerous nanomaterials have been manufactured to have characteristic electrical, mechanical, magnetic, thermal, dielectric, optical and catalytic properties (Chaturvedi and Dave, 2013). The broad range of functionalities associated with these materials has led to them being used in electronics, computing, communications, materials science, transport and energy-based sectors of the economy, as well as by the pharmaceutical and cosmetic industries (Chaturvedi and Dave, 2013). Generally, nanotechnology uses materials with at least one dimension in the range of 1–100 nm. In recent years, a great deal of attention has been drawn to the fabrication of biomimetic or bioinspired materials. A variety of highly organized nano-scale biological structures have evolved which have inspired researchers to design new systems for producing novel nanomaterials, and viruses are perfect examples of such materials.

Viruses have traditionally been studied as human, animal and plant pathogens and as tools for understanding molecular and cell biology. Through these studies a great body of information is available on the

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biological, chemical and physical properties of viruses, which have allowed precise chemical and genetic modifications to be made to their capsids (Steinmetz et al., 2009a). Viruses have very simple structures, consisting of multiple copies (up to thousands) of one or a few capsid protein subunits arranged in either icosahedral (spherical viruses) or helical (rod-shaped viruses) symmetry. Such repetitive elements can serve as a platform for the attachment or integration of various functional groups to multiple sites, offering a great variety of choice for fabrication of novel nanomaterials. For example, viral capsids can be modified to produce nanosized templates for material deposition (such as metals; Aljabali et al., 2010a, 2011a; Kobayashi et al., 2012) or engineered as containers for targeted delivery of drugs and other therapeutic agents (Zeng et al., 2013; Aljabali et al., 2013; Steinmetz et al., 2009a). Due to the highly controlled self-assembly of these viral capsid derived constructs, their batch-to-batch variability is often much lower than that observed in the traditional physically and chemically produced nanoparticles. Although viruses in general have great utility for exploitation in nanotechnology, plant viruses in particular have certain advantages. Plant viruses are not pathogenic to animals, and they and also their empty non-infectious capsids (empty virus-like particle; eVLP) can be easily and rapidly produced to high yields using plants (>1 g/kg fresh plant weight), in a manner which is conducive to up-scaling (Saunders et al., 2009; Fischer et al., 2004). In this case this approach for producing viruses and virus-like structures is generally regarded as being safer, more time and cost effective than other expression systems such as yeast, insect cells and bacteria (Ma et al., 2005; Rybicki, 2010; Penney et al., 2011). This review article will predominantly focus on how plant viruses have been exploited to produce nanoscale virus-metal biocomposites and metal nanoparticles, and where appropriate shall indicate the applications in which they can be used.

Plant viruses as scaffolds and templates for production of novel metal nanoparticles

Cowpea mosaic virus (CPMV) – an icosahedral framework for metal functionalization

The natural properties of CPMV make it an attractive nanoscale building block for biomedical and material science applications. CPMV is a 28 nm diameter icosahedral virus containing 5.9 and 3.5 kb (RNA-1 and RNA-2 respectively) positive sense genomic ssRNAs. These RNAs each contain a single open reading frame and are expressed through the synthesis and subsequent processing of precursor polyproteins. RNA-1 contains the 24 K proteinase, a proteinase co-factor, helicase and an RNA dependent RNA polymerase. RNA-2 encodes proteins responsible for cell–cell and long distance movement in plants and also the large (L) and small (S) coat proteins. The two domains of the L coat protein associate with the S coat protein, forming an asymmetric unit, of which 60 of these unite to form the CPMV capsid (for more information about the structural components of CPMV please see Sainsbury et al., 2010). This structure has been elucidated to near atomic resolution. The surface of CPMV particles have exposed and addressable amines (lysine), carboxylates (aspartic acid and glutamic acid) and hydroxyl (tyrosines) groups which have previously been used to selectively attach moieties such as redox-active molecules, fluorescent dyes, metallic and semi-conducting nanoparticles, carbohydrates, DNA, proteins and antibodies (Steinmetz et al., 2009a). In addition, these functional groups have facilitated the electroless deposition (ELD) of cobalt, nickel, iron, platinum, cobalt–platinum and nickel–iron onto the surface of CPMV (Aljabali et al., 2010a). ELD is a process utilizing chemicals for reduction of metals onto a surface which is not conductive enough for electric current deposition methods. The ELD method used in this study involved interaction of the CPMV surface amine groups with a palladium "activator", which then formed clusters after treatment with a reducing agent (dimethylamine borane); these clusters then acted as nucleation points for the subsequent absorbance and coalescence of ions of the metals listed above. This allowed production of metallized CPMV nanoparticles with a size of 35 nm in diameter, although deposition steps could be extended to produce larger metallized spheres (Aljabali et al., 2010a). In another report Aljabali et al. (2011a) utilized NHS/EDC carbodiimide chemical linkage techniques in order to conjugate surface exposed amine groups with peptides which can direct specific mineralization processes. In this case the peptides promoted coating of CPMV with metals such as cobalt–platinum, iron–platinum and zinc sulfide after exposure to a reducing agent; effectively producing 32 nm diameter metallized spherical particles. These particular types of CPMV metallic nanoparticles will likely have applications in catalysis and in ultra-high density magnetic data storage devices (FePt and CoPt; Hyeon, 2003), with semiconducting zinc sulfide nanoparticles having applications in optoelectronic devices such as solar cells (Chen and Lockwood, 2002; Gao et al., 2004). As an alternative strategy for CPMV metallization, Aljabali et al. (2011b) increased the negative surface charge of CPMV via the attack of at least 240 surface exposed amine groups by application of succinic anhydride. The increased negative charge enabled binding of cobalt or iron ions, which were then reduced to cobalt metal or iron oxide using mild ELD conditions, thus leading to 32 nm diameter metallized CPMV particles. Aljabali et al. (2011b) also demonstrated that the iron oxide coated CPMV could be further functionalized with a thiolated oligosaccharide, likely through interaction with iron or remaining hydroxyl groups, thus broadening the range of modifications which may be carried out. It was indicated that such particles have magnetic properties, which may enable them to be used as Magnetic Resonance Imaging (MRI) contrast agents or as particles which can bind cancer cells (after functionalizing with appropriate tumor recognition motifs) and kill them by generating heat upon application of an oscillating magnetic field (hyperthermia therapy; Chatterjee et al., 2011).

With regard to MRI, CPMV has also been decorated with Gd^{3+} ions and clinically approved Gd(DOTA) paramagnetic complexes. It has previously been shown that Gd^{3+} ions can coordinatively bind CPMV nucleoprotein, or alternatively Gd(DOTA) complexes can be attached via Cu-mediated azide–alkyne cycloaddition to linkers which have been NHS/EDC conjugated to the CPMV surface exposed amine groups (Prasuhn et al., 2007). It was found that gadolinium attached to CPMV had a three-fold enhancement in relaxivity than compared to free Gd(DOTA), thus indicating that the modified CPMV has potential as an improved contrast agent. The authors also indicated that the utility could be further refined by targeting the CPMV-Gd complexes to particular tissue types of interest by attachment of recognition motifs, which would serve to enhance the local concentration of Gd.

While substantial progress has been made in the exploitation of surface groups for mineralization of the CPMV surface, genetic modification has also been performed to enhance the surface chemistry for improved decoration with metals or metalloids. For example, work by Steinmetz et al. (2009b) demonstrated that CPMV can be genetically modified to surface display a dodecapeptide which favours silica binding and mineralization. This peptide promoted deposition of a 2 nm layer of silica on the modified CPMV surface after incubation with the tetraethylorthosilicate and aminopropyltriethoxysilane precursors. In another study, a chimeric CPMV was engineered to contain a surface exposed metal binding peptide, which facilitated metallization by iron and platinum after incubation with salts of these metals and subsequent exposure to a reducing agent (Shah et al., 2009). In this case the chimeric CPMV was coated

with iron and platinum to a thickness of 1 nm. Genetic modification of CPMV has also been carried out to introduce capsid surface exposed cysteine residues (which are not normally present on the surface of wildtype CPMV) at defined sites (Blum et al., 2004, 2005), which can anchor preformed gold nanoparticles via their thiol groups (Fig. 1a and b). The bound gold nanoparticles were then linked to each other by di-thiolated linkers containing metal atoms, in effect producing CPMV covered in a metal network (Fig. 1c; Blum et al., 2011). Subsequently, the surface lysines of the CPMV-based structure were chemically linked to biotin by EDC/NHS carbodiimide conjugation. Due to the metal networks, the modified particles were found to be highly conductive, and moreover this conductance significantly changed upon interaction and binding of avidin to biotin; thus forming an effective biosensor (Blum et al., 2011).

In addition to the surface modulation of CPMV, there has also been an interest in loading the interior with metals and other functional chemicals. It has previously been reported that icosahedral plant viruses such as Cowpea chlorotic mottle virus (CCMV) are able to encapsulate preformed nanoparticles such as those composed of vanadate, tungstate, titanium and Prussian blue; a process facilitated by the ease with which nucleic acid-free empty particles can be obtained by using *in vitro* assembly (Douglas and Young, 1998; Klem et al., 2008; Liepold et al., 2007). In contrast, with CPMV it has been very difficult to obtain RNA free capsids which can be loaded, since these exist in very small numbers in infected plant material. Using cutting edge plant-expression technology empty CPMV particles were obtained in abundance by co-expressing the coat protein precursor with the 24 K proteinase in plants; in this case the proteinase cleaved the coat protein precursors and permitted their self-assembly into empty capsids (Saunders et al., 2009; see also Montague et al., 2011 for improvements to this technology). The purified empty CPMV particles (eCPMV) could be loaded with any manner of cargo, such as drugs, fluorescent dyes or metals. For example, Aljabali et al. (2010b) successfully loaded the interior of eCPMV with cobalt or iron ions prior to reduction with sodium borohydride, which induced the formation of a cobalt metal or iron oxide core. Interestingly, this approach did not change the diameter of the particles or affect their capacity to be functionalized on their external surface. The ability to encapsulate materials within eCPMV and modify the external surface emphasizes the extent to which CPMV can be tailored for a desired nanotechnological purpose.

One such purpose is to utilize these diverse functionalities in the production of new devices. For this to occur, CPMV has to be appropriately arrayed or patterned into 2D and 3D architectures at the nanoscale and macroscopic scales. Early work by Fang et al. (2002) demonstrated that CPMV could form parallel lines or cross-like patterns when dried on freshly cleaved mica or acid-treated mica respectively. These patterns were found to be 9 capsid layers deep (250 nm), 600 nm wide and of 7 μm in length. CPMV can also be arranged into highly ordered monolayer membranes which can form at the liquid interphase of oil droplets in aqueous solutions, prior to being stabilized by cross-linking (Russell et al., 2005). In another approach chemoselective protein-surface linkers were patterned onto a gold substrate using scanning probe nanolithography into 30–50 nm width lines, and subsequently genetically modified CPMV which contains unique cysteine residues at defined locations was covalently attached onto these linker patterns (Cheung et al., 2003). Genetically modified CPMV containing surface exposed cysteine residues has also been used directly to produce monolayers on gold surfaces, via a gold–thiol interaction (Fig. 2a; Steinmetz et al., 2006). Alternatively, monolayers can be produced using biotinylated-linker-functionalized CPMV that can interact with gold surfaces decorated with streptavidin (Fig. 2b). The application of a layer of streptavidin onto the upper surface of the biotinylated CPMV monolayer permits the subsequent binding of a second biotinylated CPMV monolayer (Steinmetz et al., 2006). This process can be continued to produce multilayered arrays of CPMV (Fig. 3). Interestingly the length of the linker between the biotin molecule and the CPMV and the number of biotin molecules per CPMV can be altered in order to change the density and porosity of the CPMV array (Steinmetz et al., 2008). Short linkers and high amounts of biotin functionalization would lead to dense CPMV arrays, whereas less biotin and longer linkers leads to channels in the array; thus conferring flexibility in the types of applications in which they can be used. For example, dense packing of internally metallized or electroactive CPMV in the array would favor electron-transfer events in the case of electronic applications. Whereas the CPMV array with channels may allow fluid movement into the network, which could have applications in catalysis and biosensors (Evans, 2010). In another approach, Falkner et al. (2005) produced macroscale 3D structures from CPMV crystals. These crystals are body-centred cubic crystals

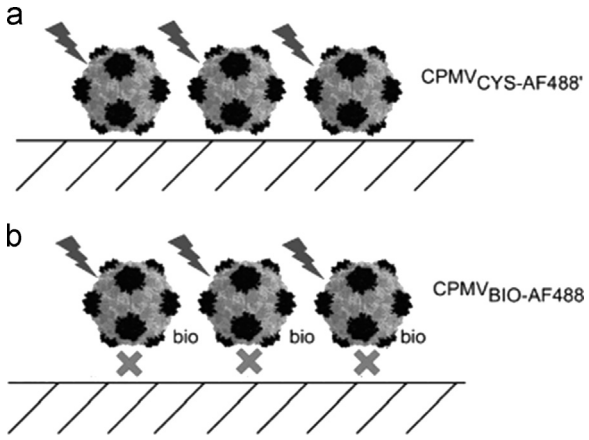

Fig. 2. (a) Schematic showing attachment of Cys modified and AlexFluor-AF488 labeled CPMV to a gold surface. (b) Attachment of biotin (bio) modified fluorescently-labeled CPMV to a gold substrate via thiolated streptavidin. The gray cross represents thiol-modified streptavidin and the gray flags represent the AlexFluor-AF488 fluorescent label. Adapted and reproduced in part from Steinmetz et al. (2006); with permission from the American Chemical Society.

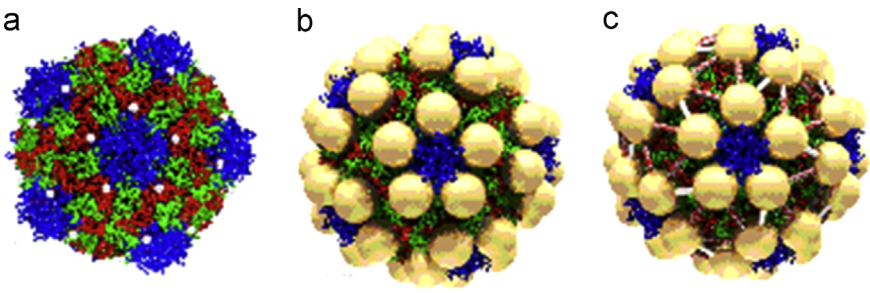

Fig. 1. CPMV genetically modified to surface display cysteine residues (shown in white) at defined sites (a). (b) Binding of gold nanoparticles to cysteine residues. (c) Networking of gold nanoparticles with di-thiolated Pt linkers. Adapted and reproduced in part from Blum et al. (2011); with permission from Elsevier.

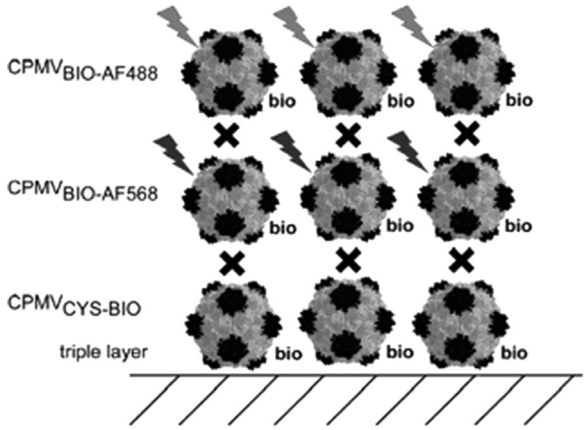

Fig. 3. Representation of a triple layer of biotinylated (bio) and/or fluorescent – labeled CPMV particles on a gold substrate. The dark cross represents streptavidin, while the gray and dark flags represent the AlexFluor-AF488 and AF568 fluorescent labels respectively. Adapted and reproduced in part from Steinmetz et al. (2006); with permission from the American Chemical Society.

that contain cavities, which the authors demonstrated are solvent accessible and could be used for the restricted growth of palladium and platinum. In summary, CPMV not only has immense possibilities for production of novel discrete monodisperse metallized nanoparticles with many applications, it can also be utilized as a highly tailorable building block for production of functional 2D and 3D macro- and nanoscale devices.

Tobacco mosaic virus (TMV)-rod shaped structures for metal nanomaterials

TMV rod-shaped virions encapsidate a plus-sense single stranded RNA genome of 6395 nucleotides in 2130 identical coat protein subunits of 17.5 kDa each, and forms a rigid helical tube with a length of 300 nm, a diameter of 18 nm, and a central channel 4 nm in width (Clare and Orlova, 2010). As this structure exposes several distinct and repeated functional groups on the inner and outer surfaces (Knez et al., 2004) it is suitable for differential modifications such as metallization or specific biochemical conjugation reactions. For example the exterior surface contains the COOH termini of the coat proteins and serine and threonine residues (OH groups), whereas the central channel contains OH groups in addition to primary amines. Similar to CPMV, the TMV surface chemistry permits binding of a variety of metal ions, which can then be reduced into a metal coating via techniques such as ELD. For example, one of the earliest reports on metallization of TMV involved exploiting the exterior surface glutamate and aspartate residues for nucleating the co-precipitation of CdS or PbS from H_2S gas and metal salt precursors, or for the deposition of iron oxide facilitated by oxidative hydrolysis (Shenton et al., 1999). While these authors reported metallization of the exterior surface of TMV, they also indicated that surface chemistry of the central channel may be exploited for metal deposition. Using ELD reducing baths consisting of dimethylamine borane or ascorbate, Knez et al. (2004) found that metal could be preferentially deposited in the central channel or alternatively on the exterior surface by varying the pH, metal ion variety and altering the phosphate concentration of the reactions. Such techniques have permitted the production of TMV based nanowires composed of different metals and alloys of copper, iron, nickel, cobalt and palladium (Balci et al., 2012, 2006). Balci et al. (2012) stated that the small TMV alloy nanowires (>200 nm length) may have applications in high density data storage, imaging, sensing, catalysis and drug delivery. For example, palladium coated TMV has been utilized in chemical catalysis for the reduction of dichromate, a system that has recently been improved by incorporation of the palladium-TMV into easy-to-handle hydrogel microparticles (Yang et al., 2013). In addition, TMV onto which TiO_2 has been deposited has shown utility as sensors for the detection of liquefied petroleum gases (Rong et al., 2009), or if ZnO is coated on the viral surface it may be used as semi-conducting transistor components (Atanasova et al., 2011). Moreover, work by Gorzny et al., (2010) showed that platinized TMV rods produced using methanol ELD were more stable and were 65% more efficient as anodes in direct methanol fuel cells (DMFCs) than conventional nanoparticles of a similar size. While DMFCs offer ten-fold more energy density storage than traditional lithium-ion batteries, the authors suggest that this might be further improved by using TMV-Pt particles as the anode.

In addition to directly depositing metals on to the surface of unmodified TMV using the ELD approaches discussed above, some research groups have tried to bind preformed nanoparticles to the surface of TMV. For example Khan et al. (2013) took citrate-coated negatively charged gold and iron oxide nanoparticles in an acidic solution and applied this to wildtype TMV (WT TMV). In this case binding of the nanoparticles occurred since the natural negative charge of TMV was abolished by the acidic protonation; thus allowing electrostatic attraction between the nanoparticles and the surface of TMV. Other studies (Wu et al., 2010) have found that TMV may associate with iron nanoparticles in ferrofluids to form a complex of quasi-linear TMV/nanoparticle scaffolds. Interestingly this arrangement is thought to account for the greatly enhanced magnetoviscosity upon the application of a magnetic field, when compared to the ferrofluids lacking the TMV (Wu et al., 2010). The authors indicated that the improved magnetoviscosity of the TMV "doped" ferrofluids is likely to have applications in the engineering and transport sectors (for example, production of better vehicular shock absorbers).

In other reports, purified TMV coat proteins which can self-assemble into ring-like disk structures under particular salt and pH conditions have been used as a scaffold for the attachment of preformed gold nanoparticles (Zahr and Blum, 2012). In this study, gold nanoparticles were pretreated with bis(p-sulfonatophenyl) phenylphosphine in order to enhance the electrostatic interaction of the nanoparticles with arginine residues on the top edge of the TMV coat protein disk. This led to the formation of a ring of gold nanoparticles on the outer circumference of the TMV disk. The authors also found that the inner channel of the TMV-nanoparticle assemblage could also bind a gold nanoparticle in a pH modulatable manner, whereby the pH dependent protonation of the negatively charged arginine and carboxyl groups in this location permits binding. The TMV-nanoparticle rings had very interesting local surface plasmon resonance spectra which could be easily tuned via inclusion of a gold nanoparticle in the central core. The authors indicated that such optically tunable structures could have broad applications in the areas of nonlinear optics, sensors and laser technology.

To further enhance the functionality of TMV, it has been genetically modified such that metal binding motifs have been included in the surface exposed C-terminal region of the coat protein. For example, a titanium binding motif was added to the C-terminal end of the CP, and this was found to increase the number but reduce the size of the associated TMV-bound gold nanoparticles formed from gold salts and $NaBH_4$ baths, when compared to WT TMV (Kobayashi et al., 2012). This study also elucidated that these TMV-gold nanoparticle hybrid materials had interesting spectral characteristics in the UV and visible light regions, with likely applications in the optics field. In another study, a hexahistidine tag was incorporated into the coat protein, and this improved the growth density of gold deposition in comparison to WT TMV (Nan et al., 2012). The hexahistidine tagged TMV CP has also been exploited in order to bind pre-

formed gold nanoparticles, which if desired can act as nucleation points for the subsequent deposition of metals (Wnek et al., 2013). These approaches produced gold TMV nanowires, which the authors found to have very reduced electrical resistivity when compared to conventionally produced gold nanowires; indicating that gold TMV wires may have utility for the electronics industry.

In other work, TMV was modified to contain a novel CP cysteine residue (TMV1cys) that enabled binding to gold surfaces (via a gold–thiol interaction) in a densely packed and vertically oriented manner (Royston et al., 2008), as a basis for production of battery components of 3D architecture. The vertically arrayed TMV1cys was subjected to dimethylamino-borane mediated ELD of cobalt and nickel after pre-activation of the TMV1cys with a palladium catalyst (Fig. 4); this led to formation of a metal coating ~40 nm thick (Fig. 5). The structure was found to be highly durable under a variety of vacuum, aqueous and electrical conditions, and moreover it

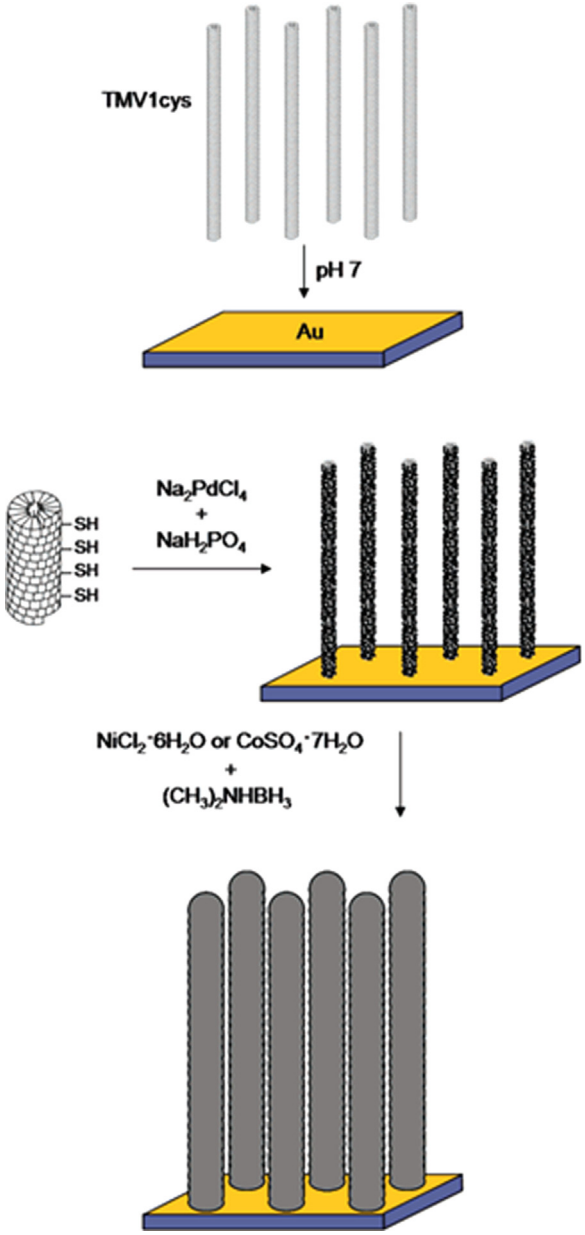

Fig. 4. Diagram showing the assembly of nickel- and cobalt-coated TMV1cys templates attached to a gold surface. Adapted and reproduced in part from Royston et al. (2008); with permission from the American Chemical Society.

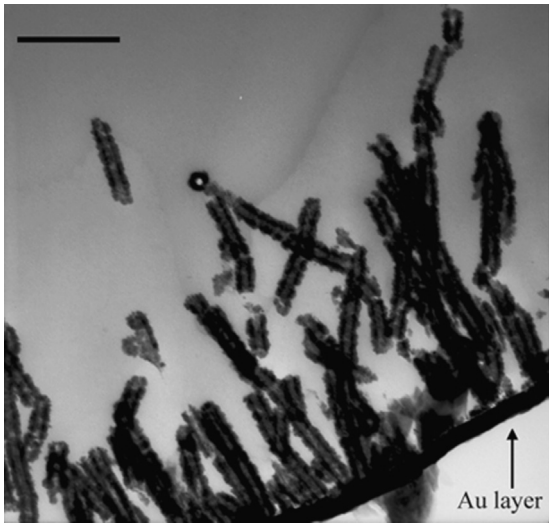

Fig. 5. TEM image showing a 70 nm thick cross section of nickel-coated TMV1cys attached perpendicular to a gold-coated mica surface. Scale bar is equal to 300 nm. Adapted and reproduced in part from Royston et al. (2008); with permission from the American Chemical Society.

displayed a two-fold enhancement in electrode capacity when compared to controls not containing virus in a nickel-zinc battery (Royston et al., 2008). The authors also indicated that this process is easily scalable such that as little as 450mg of virus would be required to coat a square meter of electrode surface.

Interestingly the cysteine modified TMV has been used in a different approach for production of battery components (Gerasopoulos et al., 2010). In this study the modified TMV was arrayed on a gold coated substrate and activated via palladium prior to dimethylaminoborane facilitated deposition of a 20 nm thick nickel layer on the virus surface. The nickel layer was subsequently coated with 20 nm of TiO_2 through an Atomic Layer Deposition (ALD) process. ALD is a method for controlled thin film deposition on surfaces whereby two or more chemical vapors or gaseous precursors are sequentially injected in short pulses into a gaseous medium containing the target substrate. These interact with the surface to produce the coating. It was found that the TMV based anode had a two-fold higher discharge capacity and can outperform the control planar films even at much higher currents and cycle number; remarkably the TMV based structures were intact in spite of such rigorous electrical testing. The authors of both these papers Gerasopoulos et al. (2010) and Royston et al. (2008) have suggested that factors such as the much higher surface areas imparted by the TMV templates may account for the improved electrical properties.

Given that that assembly of TMV is driven by the RNA Origin of Assembly (OA) motif's high affinity for 20S disk coat protein aggregates (comprising 34 CP subunits; Butler and Lomonossoff, 1980; Atabekova et al., 1975), this can be exploited in several ways to influence arraying and structural aspects of the assembled TMV rods. For example, purified WT or engineered TMV CP subunits (produced using yeast or bacterial expression systems or derived from TMV extracted from plants) can be mixed with synthetic RNA that contain multiple or modified OAs (Taliansky et al., 1982; Kaplan et al., 1982), leading to the assembly of branched TMV structures comprising, for example, star, tree and boomerang morphologies, which may be free (Gallie et al., 1987) or grown on RNA anchored to wafers (Mueller et al., 2011) or metal nanoparticle cores (Eber et al., 2013). These constitute a new class

of metal-TMV hybrids with a high surface-area protein corona which is amenable to further functionalization.

Conclusions

Plant viruses and empty plant virus capsids are easily customizable for particular functionalities that can be exploited for broad nanotechnological areas such as novel metal nanomaterials. Moreover, plant viruses and their modified derivatives, which can be safe and non-infectious, may be obtained to very high yields in plants; factors which make them suitable for upscaled production of custom made metal nanoparticles. Through simple downstream metal ELD processes, the binding of preformed metal nanoparticles and 2D and 3D arraying techniques, a diverse range of functional plant viral nanocomposites have been produced. Many of these have outperformed their conventional counterparts in a variety of applications, and moreover in several cases some of the plant virus derived metal nanoparticles have no functional equivalent, which likely broadens their usage in new nanotechnological areas. Given the utility of viruses in nanotechnology demonstrated thus far, and their great potential for customization, we believe that in future that they will be more rigorously exploited by the nanotechnology industry.

Acknowledgments

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