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A chemoselective biomolecular template for assembling diverse nanotubular materials

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Abstract
We describe the design and production of a tobacco mosiac virus mutant that provides for specific and stoichiometric attachment of a wide variety of ligand-linker groups. As a result, specific ligands could be chemoselectively linked to the virion to produce highly diverse nanomolecular materials. These included semi-crystalline protein arrays and metallic 'nanopipes', as well as nanomolecular 'light sticks'. The method described is facile and inexpensive with potential uses in such diverse areas as nanofabrication and biomolecular structure determination.

1. Introduction

Nanomolecular patterning with definable size and organization is of integral importance in the drive for further miniaturization in materials science and electronics. Biomaterials and biopolymers are an important source of potential templates for the production of such nanoscale structures. Template mineralization or photochemical polymerization on lipids [1, 2] and bacterial fibres [3] and methods for DNA driven nanocrystal organization [4] have been reported, as well as two-dimensional array fabrication using ferritin [5]. However, supramolecular protein self-assemblies such as viruses, actin filaments, and bacterial flagella have mostly been unexplored. Tobacco mosaic virus (TMV) is the prototypical tobamovirus which encapsidates a positive sense RNA genome of 6.5 kb. The virion is a rigid rod of approximately 18 nm diameter and 300 nm length with a central hole of 4 nm across. In addition, the virion is extraordinarily stable and can be manipulated under a variety of conditions. The 2130 identical coat protein subunits of 17.5 kD are arranged in a right-handed helix with 16 1/3 subunits per turn. The structure of the protomeric coat protein has been determined [6] and reveals that there are three potential regions for chemical modification on the exterior of the virion. These are the N and C termini as well as the 63–66 loop region. Both the C-terminus and the 63–66 loop region have previously been engineered for immunoreactive epitope expression [7]. We chose the C-terminus as the point of modification for our studies and a single site mutation (T158K) was engineered into native TMV. The rationale was that this single accessible lysine could be selectively coupled using facile N-hydroxy succinamide chemistry to afford a template with selectable chemospecificity (figure 1(a)). Here we describe the use of the T158K mutant virion for the production of a variety of chemospecific nanotubular materials.

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2. Mutant virion production, labelling and quantification

The *Nicotiana benthamiana* tobacco seeds and the T7 TMV cDNA plasmid were obtained from Cecille J Robinson in the University of Florida Citrus Research Center. The *in vitro* transcription kit was purchased from Ambion. All NHS labelling reagents were purchased from Pierce. The protocol of Picard *et al* [8] was used for the mutation of T7 TMV cDNA plasmid. We experienced difficulties using T7 TMV plasmid in PCR reactions due to the size (9064 bps). To overcome this problem the T7 TMV plasmid was cut with Acc 651/Bam H1 and the resulting 3 kb fragment which contained the coding region of the coat protein was subcloned into pUC 19 to be used in PCR mutagenesis reaction. After successful PCR mutagenesis the 3 kb fragment was cloned back into the original T7 TMV plasmid.

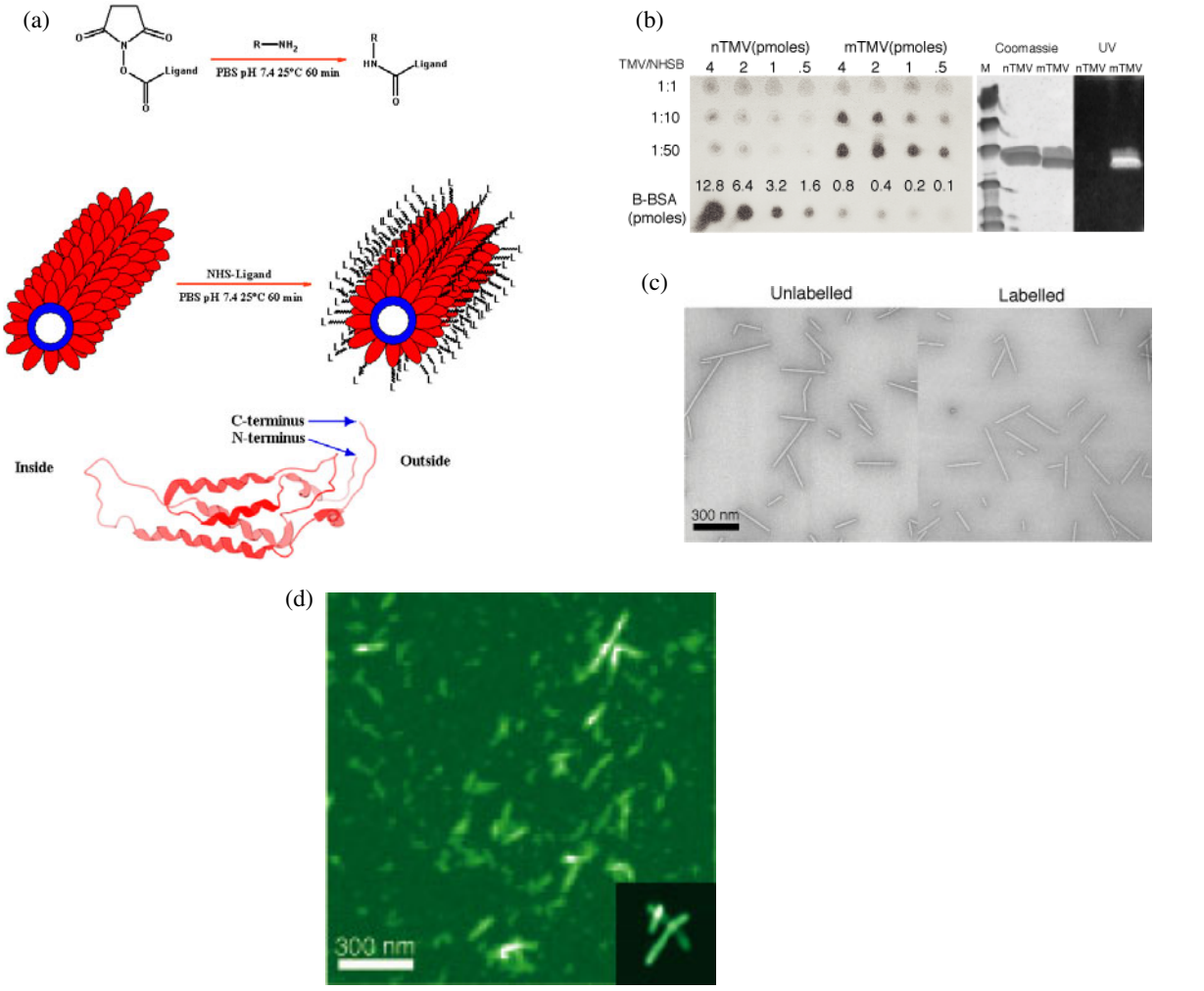

Figure 1. (a) Schematic of the overall method along with the secondary structure of the TMV coat protein and its arrangement in the virion; the upper panel shows the facile NHS chemical reaction; the middle panel shows a schematic of the intact virion coat protein assembly (red oblates) prior to and following labelling using an NHS–ligand compound; the lower panel shows the overall four-helix-bundle fold of the coat protein monomer: the N- and C-termini are labelled as well as the relative position of the outer and inner surfaces of the intact virion. (b) Selective and quantitative labelling of the T158K mutant as demonstrated by immunoblotting (left panel, biotin labelled TMV as detected by ECL dot blot) and SDS-PAGE (right panel, FITC labelled coat protein). The left panel is an ECL dot blot showing the relative amounts of biotin labelling observed using various ratios of TMV coat protein to NHS–biotin (NHBS). Right panel: 12% SDS-PAGE of FITC labelled TMV coat protein. Molecular weight markers *M* (kD) 3.8, 7.1, 14.3, 21.5, 31.0, 45.0; expected mass of TMV coat protein is 17.5 kD. Note the small shift to lower molecular weight of the FITC labelled mTMV and the evident contrast in the fluorescence (UV excitation) visualization. (c) EM field view of labeled (FITC) and unlabelled mTMV. Note that the number and length of the virions are similar, demonstrating the mild nature of the labelling reaction. (d) Similar field as (c) (right), observed using a confocal microscope in green fluorescence mode and collecting only a single plane, hence the wide variation in the apparent length of the virions. The inset shows a volume image of a small area showing three labelled virions with similar lengths and uniform labelling.

The plasmid was prepared for *in vitro* RNA transcription by linearization with Nar I and the reaction was carried out for 2 h at 37 °C. *Nicotiana benthamiana* grown from seeds and having five to eight leaves were used for inoculation. The middle two or three leaves of plants were dusted with carborundum and were inoculated. The plants were then transferred to 60–70% humidity, 30 °C incubators for 2–3 weeks. The method developed by Gooding and Herbert [9] was used to purify intact virions from systemically infected plants.

Labelling was performed by using approximately 0.3 nmol of TMV and incubating with NHS–ligand in PBS for 60 min at room temperature. Ratios of 1:1, 1:5 and 1:10 (TMV to NHS–ligand) typically were used. The samples were quenched by the addition of 5 μl 1.0 M Tris pH 7.4 for 5 min and then dialysed against PBS. Samples were used immediately or snap frozen in LN2 for storage and latter analysis.

To quantitate the labelling reactions, samples labelled with biotin were blotted on nitrocellulose, blocked with 5% milk in PBS and then incubated with 1/500 dilution of Avidin-HRP (1 mg ml^{-1}) for 30 min and visualized using the ECL method. Similar concentrations of biotinylated BSA were used as a quantitative control.

For TEM, 5 μl samples of labelled TMV solution was placed on an electron microscopy grid for 2 min, and then washed with six droplets of water. The grid was blocked with 1 mg ml^{-1} Cyt c by reversing the grid on a drop and leaving it for 1 min; the grid was transferred to a drop of Cyt c for 2 min

and then washed six times with water. The grid was placed on a drop of 1/10 dilution of avidin and left for 20 min. Finally, the grids were washed in DW and stained with uranyl acetate. The grids were examined in a Philips EM-208 operating at 80 kV.

Biotinylated TMV was produced using NHS–biotin following the procedure above. The sample was then dialysed using 20 kD molecular weight cut-off membranes against PBS. The sample was then treated with equimolar avidin and allowed to incubate at room temperature for one hour and then analysed by TEM, following negative staining with uranyl acetate.

Photolysis experiments were performed in ADA buffer at pH 6.5. Fluroescence labelled virion in PBS with 1 mM CuCl was bubbled with nitrogen for 10 min and then sealed in a quartz cuvette. The sample was then irradiated using a Hg lamp and a UV filter for 10 min. The sample was then analysed by TEM directly. Control samples without CuCl or UV irradiation showed no apparent deposition on the virions (figure 3).

3. Results and discussion

In vitro transcription of the mutant DNA and subsequent infection of *N. benthamiana* plants resulted in systemically infected plants that could be harvested for isolation of intact mutant virions. Labelling experiments using NHS–biotin or NHS–fluorescein demonstrated the specific and quantitative ($106 \pm 14\%$ for a 1:10 ratio of mTMV to NHS–biotin) labelling of the mutant virus with no apparent effect on the overall appearence of the virion (figures 1(b) and (c)). Furthermore no labelling of the native TMV was observed. This result was of particular importance because the native TMV contains two lysines which potentially could be labelled. Based upon the known structure we expected these two lysines to be inaccessible to chemical modification because they are located within the interior of the virion and interact directly with the RNA. Thus we were able to produce a mutant TMV that could be chemoselectively functionalized in a quantitative manner.

Further experiments using a number of fluorescent labelling reagents allowed us to produced a series of nanomolecular 'light sticks', with differing excitation and emission wavelengths commensurate with the fluorescent labelling reagent. Figure 1(d) is a confocal image of fluorescein labelled TMV and the inset is a close-up volume figure of several labelled virions showing the uniform labelling achieved. Further elaborations of such 'light sticks' could have interesting optical properties due to the well defined spatial location and arrangements of the fluorophores.

Having demonstrated the production of mutant virus that could be selectively functionalized, several template assembled microstructures were targeted for investigation. The first such system utilized the biotinylated virion as a template for assembly and crystallization of avidin. The addition of a slight excess of neutravidin to biotinylated virions resulted in the formation of densely coated viral particles when examined by electron microscopy (figure 2(b)). In some instances, short semicrystalline arrays of avidin could be formed (figures 2(c) and (d)). The extension of this method to a wide range of ligand and ligand binding proteins is readily foreseeable and would allow for the production of a number of fascinating biomolecular nanomaterials. This result further demonstrates the overall applicability of the method to template assembly and, importantly, the potential to form crystalline arrays that may be utilized for structural analysis, in particular the use of such a system for the template assisted crystallization of membrane proteins for structural analysis using helical reconstruction methods.

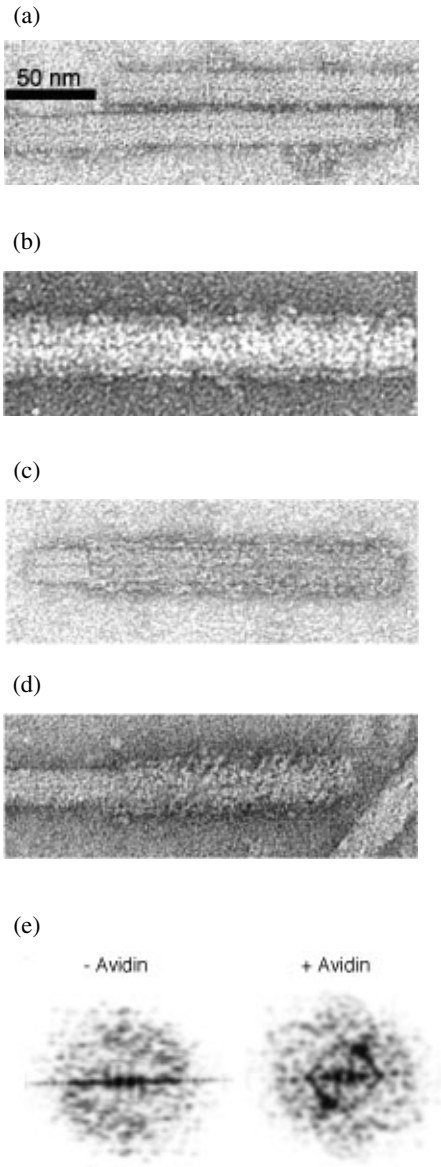

Figure 2. (a) Biotin labelled mTMV in the absence of avidin. Virions appear similar to unlabelled as expected due to the small size of biotin, F.W. 244. (b) Typical appearance (>90%) of the sample in (a) when treated with protein neutravidin which binds biotin with high affinity. Note the increase in diameter of the virions. (c), (d) Occasionally observed (<5%) semicrystalline arrays of avidin formed by the treatment of the sample in (a) with neutravidin. Particularly evident in (d), where distinct ribbons of bound avidin can be seen. (e) Computed diffraction pattern from the image of biotinylated mTMV in the absence of neutravidin (left panel) and following incubation with neutravidin (right panel). There is a clear higher order layer line observed for the neutravidin labelled sample.

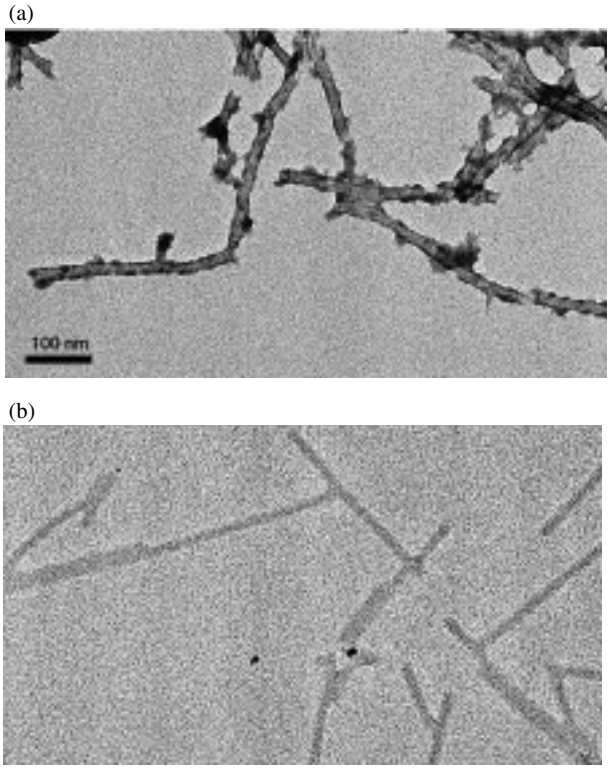

Figure 3. (a) Photoreduction of metals by fluorophor labelled mTMV. (b) As in (a) but in the absence of photoexcitation. Samples were maintained aerobic and directly applied to the grid following photoreduction, blotted without washing or staining and observed by TEM. Solutions contained 1 mM CuCl, 1 mM ADA pH 6.5 and were made anaerobic by bubbling with nitrogen prior to irradiation.

A final model system which was studied utilized the fluorescently labelled material as a starting point. It was expected that such material could serve as a site specific template for photo-reduction of metals at the surface of the virion. When 'cascade blue' labelled virion was placed in a solution of Cu(I)Cl and subjected to photoexcitation with UVB radiation, an electron dense coating of the virions was observed by EM (figure 3(a)). Control samples without photoexcitation or in the absence of Cu(I)Cl did not show such electron dense coating of the virions (figure 3(b)). While we have no direct evidence that the electron dense coating observed on the TMV particles is in fact copper the lack of such material in either control sample leads us to conclude that copper nanopipes have been produced by this method. In subsequent experiments, silver nanopipes could be produced, by utilizing silver chloride in place of copper chloride.

4. Conclusion

Previously TMV been shown to function as a non-specific template for inorganic–organic mineralization [10]. In contrast, the mutant TMV reported here offers a facile route to a number of intriguing nanomaterials beyond such a simple mineralization process. Furthermore the production of large quantities of mutant virion is straightforward and the NHS coupling chemistry is inexpensive, selective and quantitative. In the future, we hope to extend this method to a number of areas, in particular the formation of ordered crystalline arrays of proteins for structural determination using helical reconstruction methods, and to exploit the potential to form nanomolecular wires which can be self-assembled into nanoscale electronic devices.

Acknowledgments

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