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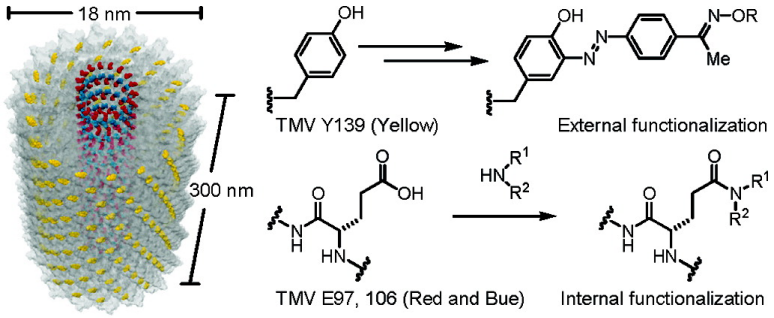

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Dual-Surface Modification of the Tobacco Mosaic Virus

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Abstract: The protein shell of the tobacco mosaic virus (TMV) provides a robust and practical tubelike scaffold for the preparation of nanoscale materials. To expand the range of applications for which the capsid can be used, two synthetic strategies have been developed for the attachment of new functionality to either the exterior or the interior surface of the virus. The first of these is accomplished using a highly efficient diazonium coupling/oxime formation sequence, which installs >2000 copies of a material component on the capsid exterior. Alternatively, the inner cavity of the tube can be modified by attaching amines to glutamic acid side chains through a carbodiimide coupling reaction. Both of these reactions have been demonstrated for a series of substrates, including biotin, chromophores, and crown ethers. Through the attachment of PEG polymers to the capsid exterior, organic-soluble TMV rods have been prepared. Finally, the orthogonality of these reactions has been demonstrated by installing different functional groups on the exterior and interior surfaces of the same capsid assemblies.

Introduction

The protein shells of viruses provide highly promising scaffolds for the construction of new materials.[1] In addition to possessing nanoscale dimensions overall, the subunits that comprise the capsid shell present regularly spaced attachment sites that could order material components. Recent reports have demonstrated this concept through the addition of new functionality to the exterior[1f,2] and interior[3] surfaces of icosahedral viruses, yielding efficient routes to spherical core/shell materials. The tubelike capsid of the tobacco mosaic virus (TMV) provides a useful complement to these structures, as each 300 nm particle could orient optical and electronic components into linear assemblies resembling wires, Figure 1.[4,5] Electrostatic differences between the capsid surfaces have already been exploited in this regard, leading to the selective deposition of inorganic materials on either the interior or exterior surface of the tube.[6] However, the covalent attachment of small molecules to the capsid has been more difficult to achieve, in part because the amino acid side chains commonly targeted by bioconjugation reactions are not found on the surface after assembly.[7] As future applications will depend critically on the introduction of new functional groups, we have developed two orthogonal synthetic

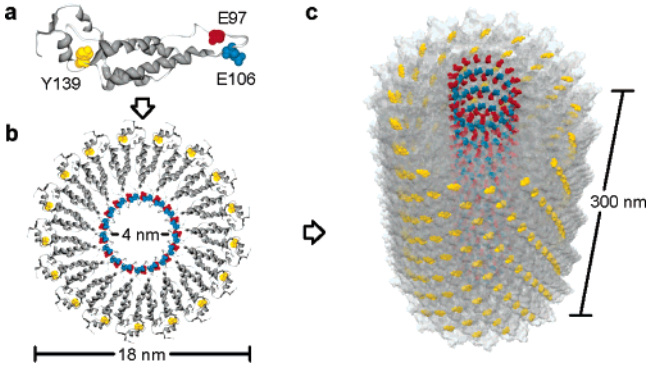

Figure 1. Reactive sites for the covalent modification of TMV. (a) The positions of tyrosine 139 (yellow), glutamate 97 (red), and glutamate 106 (blue) are indicated in a single TMV capsid monomer. In the assembled capsid, these residues provide reactive handles on both the exterior and interior surfaces. These residues are mapped in (b) a planar disk aggregate[5a] and (c) a fully assembled capsid.[5b]

(1) For lead references, see (a) Douglas, T.; Young, M. *Nature* **1998**, *393*, 152. (b) Douglas, T.; Young, M. *Adv. Mater.* **1999**, *11*, 679. (c) Douglas, T.; Strable, E.; Willits, D.; Aitouchen, A.; Libera, M.; Young, M. *Adv. Mater.* **2002**, *14*, 415. (d) Whaley, S. R.; English, D. S.; Hu, E. L.; Barbara, P. F.; Belcher, A. M. *Nature* **2000**, *405*, 665. (e) Lee, S.-W.; Mao, C.; Flynn, C. E.; Belcher, A. M. *Science* **2002**, *296*, 892. (f) Wang, Q.; Lin, T.; Tang, L.; Johnson, J. E.; Finn, M. G. *Angew. Chem, Int. Ed.* **2002**, *41*, 459.

(2) (a) Wang, Q.; Kaltgrad, E.; Lin, T.; Johnson, J. E.; Finn, M. G. *Chem. Biol.* **2002**, *9*, 805. (b) Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. *J. Am. Chem. Soc.* **2003**, *125*, 3192−3193. (c) Raja, K. S.; Wang, Q.; Gonzalez, M. J.; Manchester, M.; Johnson, J. E.; Finn, M. G. *Biomacromolecules* **2003**, *4*, 472. (d) Smith, J. C.; Lee, K. B.; Wang, Q.; Finn, M. G.; Johnson, J. E.; Mrksich, M.; Mirkin, C. A. *Nano Lett.* **2003**, *3*, 883.

(3) (a) Hooker, J. M.; Kovacs, E. W.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 3718. (b) Meunier, S.; Strable, E.; Finn, M. G. *Chem. Biol.* **2004**, *11*, 319. (c) For the interior modification of a heat-shock protein assembly, see: Flenniken, M. L.; Willits, D. A.; Brumfield, S.; Young, M. J.; Douglas, T. *Nano Lett.* **2003**, *3*, 1573.

(4) For a general review of TMV structure and self-assembly, see: Klug, A. *Philos. Trans. R. Soc. London B* **1999**, *354*, 531.

(5) Crystal structures from: (a) Bhyravbhatla, B.; Watowich, S. J.; Caspar, D. L. D. *Biophys. J.* **1998**, *74*, 604. (b) Pattanayek, R.; Stubbs, G. *J. Mol. Biol.* **1992**, *228*, 516.

(6) (a) Shenton, W.; Douglas, T.; Young, M.; Stubbs, G.; Mann, S. *Adv. Mater.* **1999**, *11*, 253. (b) Dujardin, E.; Peet, C.; Stubbs, G.; Culver, J. N.; Mann, S. *Nano Lett.* **2003**, *3*, 413. (c) Knez, M.; Bittner, A. M.; Boes, F.; Wege, C.; Jeske, H.; Maiss, E.; Kern, K. *Nano Lett.* **2003**, *3*, 1079. (d) Knez, M.; Sumser, M.; Bittner, A. M.; Wege, C.; Jeske, H.; Martin, P.; Kern, K. *Adv. Funct. Mater.* **2004**, *14*, 116−124. See also: (e) Mao, C. B.; Flynn, C. E.; Hayhurst, A.; Sweeney, R.; Qi, J. F.; Georgiou, G.; Iverson, B.; Belcher, A. M. *P. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 6946.

(7) The modification of recombinant TMV mutants bearing additional lysine residues has been reported: Demir M.; Stowell, M. H. B. *Nanotechnology* **2002**, *13*, 541.

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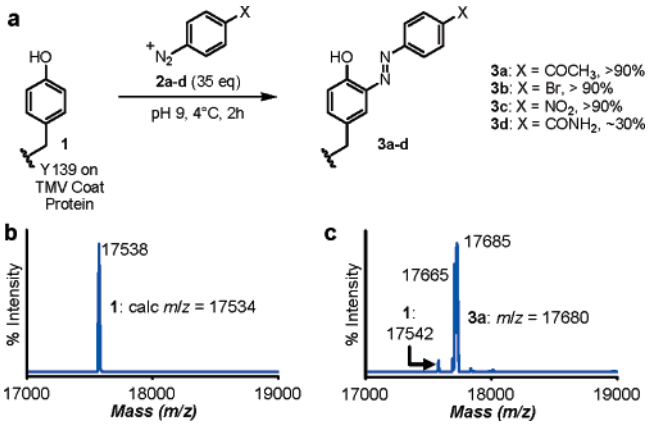


Figure 2. Exterior modification of TMV. (a) Tyrosine 139 can be modified selectively using diazonium salts bearing electron-withdrawing substituents. (b) After isolation of assembled capsids using gel filtration, comparison of ESI-MS spectra of unmodified TMV capsid protein and (c) acetophenone-functionalized conjugate **3a** indicated that >90% of the protein monomers were modified. The additional peak at $m/z = 17\,665$ arises from an elimination commonly observed for diazonium coupling reactions and does not interfere with the subsequent modification chemistry.

methods that can install small molecules on (1) the exterior surface of the capsid by targeting tyrosine 139 or (2) the interior surface by targeting glutamic acid residues 97 and 106, Figure 1. Both of these reactions can install a wide range of functionality without disrupting the capsid assembly. Herein we detail these strategies and demonstrate the use of surface modifications to prepare viral capsids that are soluble in organic solvents.

Results and Discussion

Exterior Modification of TMV. A significant practical advantage of TMV is the ease with which large quantities of the virus can be obtained. For these studies, TMV was propagated in Turkish tobacco plant hosts, *Nicotiana rustica*, and purified according to modified literature protocols.[8] Protein quantification using UV/vis spectrophotometry indicated a yield of 2.5 g of virus from 1.6 kg of infected leaf material. SDS-PAGE and ESI-MS analysis (Figure 2b) of the capsid monomers after disassembly confirmed that highly pure protein was obtained.

Recently we reported the efficient modification of tyrosine residues on the interior surface of bacteriophage MS2 using diazonium salts.[3a] It was found that this reaction proceeds with equally high levels of conversion for the functionalization of TMV, Figure 2a. Although diazonium salts bearing *p*-nitro groups were uniquely effective for the modification of MS2, the robust nature of the TMV capsid allowed the use of a wider range of reaction conditions and, thus, an expansion of the substrate scope. Specifically, modification of TMV capsids was accomplished through exposure of intact viral capsids (**1**) to 35 equiv of diazonium salts **2a**−**d** (per monomer) in pH 9 buffer for 2 h, Figure 2a.

After the coupling reaction, the remaining small molecules were removed from the samples using gel filtration. Sephadex S300 resin was found to be particularly efficient in this regard, as previous studies in our labs have confirmed that small molecules and dissociated monomers are retained by this resin, while intact capsids elute in the void volume. For all of the samples, UV−vis spectroscopy and SDS-PAGE analysis of the eluted capsids indicated little if any protein loss, and TEM analysis of the resulting material revealed no changes in the capsid structures. The level of modification was determined using ESI-MS analysis, which indicated that >90% of the capsid monomers were converted to azo adducts using diazonium salts derived from *p*-aminoacetophenone (**2a**), *p*-bromoaniline (**2b**), and *p*-nitroaniline (**2c**), Figure 2c.[9] Anilines bearing electron-donating substituents were generally found to be less effective in these reactions. In addition to the expected azo adduct, the mass spectra indicated that some of the monomers lost 17 mass units through an unidentified side reaction. The full characterization of this species is currently underway; nonetheless, its presence has had no effect on subsequent bioconjugation reactions.

Although there are four tyrosine residues on each monomer, ESI-MS data consistently indicated that only one of these sites participated in the reaction. The specific location of the modification was determined by digesting the modified capsids with endoproteinase Glu-C and characterizing the peptide fragments using ESI-MS and MS/MS.[10] These data confirmed that only tyrosine 139 was functionalized in this reaction. As shown in Figure 1, this residue is located in a shallow groove on the capsid exterior. Presumably the other tyrosine residues are buried within the capsid structure and are not accessible to the reaction solvent.

The ketones introduced on the protein surface using substrate **2a** provide convenient sites for further conjugation via oxime formation. Upon exposure of modified capsid **3a** to a series of alkoxyamines (Figure 3a), adducts **5a**−**c** were obtained with virtually complete conversion, as exemplified by the ESI-MS spectrum of biotin conjugate **5c** in Figure 3b. No coupling of the alkoxyamines was observed for wild-type TMV, indicating that this reaction is completely specific for the introduced ketone group. A combination of gel filtration and TEM imaging again indicated that the capsids remained intact after both modification steps.

This two step strategy can be carried out in as little as 6 h and affords 300 nm rods bearing >2000 copies of the desired functionality periodically spaced at 3.3 nm intervals on the capsid exterior. For initial applications, it was anticipated that these sites could be used to alter the solubility properties of the capsids through the installation of synthetic polymer chains. Starting with poly(ethylene glycol) monomethyl ethers with molecular weights of 2000 and 5000 (**6d** and **6e**, respectively), alkoxyamine derivatives **4d** and **4e** were prepared via displacement of the alcohol with *N*-hydroxyphthalimide, followed by hydrazinolysis. The polymers were then exposed to TMV conjugate **3a** over a period of 12 h. Gel filtration of the resulting samples confirmed that the capsids did not disassemble during this procedure (Figure 3d).

The attachment of the polymer chains was detected using SDS-PAGE after disassembly of the capsids, Figure 3e. In

(8) Chapman, S. *Methods in Molecular Biology, Vol 81: Plant Virology Protocols: From Virus Isolation to Transgenic Resistance*; Humana Press: Totowa, NJ; pp 123−129.

(9) The azo linkage could also be detected as a broad absorption in the UV−vis spectrum with a maximum at 330 nm. Upon comparison with model compounds prepared with *p*-cresol, the amount of azo absorption (relative to that of the TMV capsid) was approximately what would be expected for the reported level of conversion. However, this peak was not sufficiently resolved from the protein spectrum to allow quantitative measurement of the conversion by this method.

(10) See Supporting Information for spectra.

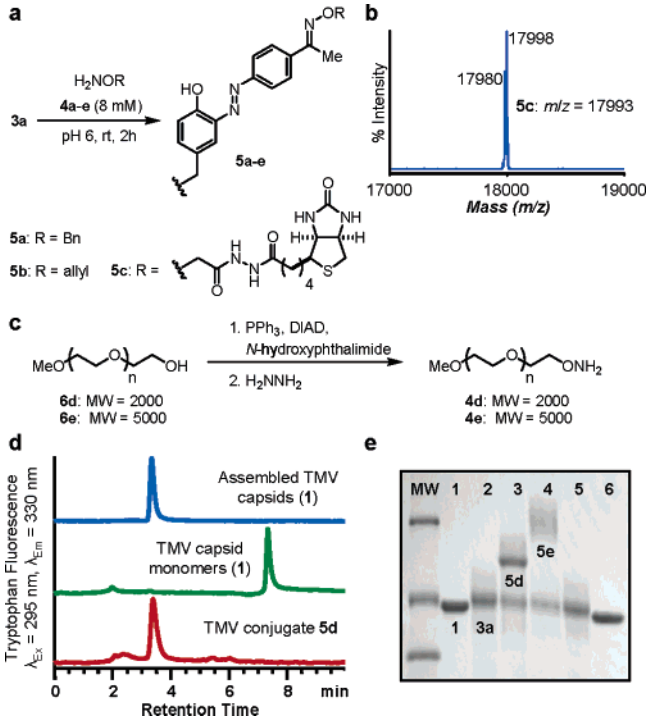


Figure 3. Secondary bioconjugation on the exterior surface of TMV. (a) Upon exposure to a variety of alkoxyamines, ketone-labeled **3a** can be functionalized through oxime formation. (b) ESI-MS analysis of biotinlabeled conjugate **5c** after isolation of assembled capsids using gel filtration. The species at $m/z = M - 17$, resulting from the diazonium coupling reaction, also formed the oxime linkage. (c) Synthesis of poly(ethylene glycol) alkoxyamines. (d) Size exclusion chromatographic (SEC) analysis of intact TMV capsids (blue), TMV capsid monomers prepared upon treatment with 66% HOAc (green), and PEG-conjugate **5d** (red). Conjugate **5e** produced an identical chromatogram. (e) SDS-PAGE analysis of TMV−PEG conjugates. MW markers are 14.4, 21.5, and 31 kD. Lane 1: wild-type TMV (**1**). Lane 2: conjugate **3a**. Lane 3: **3a** + **4d**. Lane 4: **3a** + **4e**. Lane 5: **3a** + **6d**. Lane 6: **1** + **4d**.

addition to unmodified TMV monomers, a single new band was observed at a higher molecular weight (lane 3), which was assigned as conjugate **5d**.[11] Similar results were obtained for conjugate **5e** (lane 4), although the increased polydispersity of the polymer resulted in a more diffuse band. Based on Coomassie blue staining, the coupling efficiency was estimated at 60% in both cases, corresponding to a total polymer mass of 2.6×10^6 Da per capsid for **5d** and 6.6×10^6 Da for **5e**. No reaction was observed when **3a** was incubated with PEG alcohol **6d** (lane 5) or when wild-type TMV was incubated with alkoxyamine **4d** (lane 6), confirming that the modification occurs through oxime formation. Both gel electrophoresis and gel filtration analyses indicated that little protein loss occurred during these procedures.

After attachment of the polymer coating, the solubility properties of the viral capsids were explored. Although **3a** and **5d** are fully soluble in aqueous solution (Figure 4a), they exhibit vastly different behavior after the addition of a precipitation salt (1.9 M $(NH_4)_2SO_4$), followed immediately by chloroform. PEG conjugate **5d** readily dissolves in the lower chloroform layer and remains in solution, while **3a** is confined to the upper

(11) For additional examples of PEG attachment to viral capsids, see: (a) O'Riordan, C. R.; LaChapelle, A.; DelGrado, C.; Parkes, V.; Wadsworth, S. C.; Smith, A. E.; Francis, G. E. *Human Gene Therapy* **1999**, *10*, 1349. (b) Wang, Q.; Raja, K. S.; Janda, K. D.; Lin, T.; Finn, M. G. *Bioconj. Chem.* **2003**, *14*, 38. Also see ref 2c.

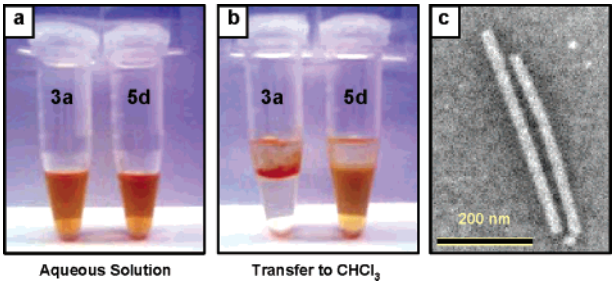
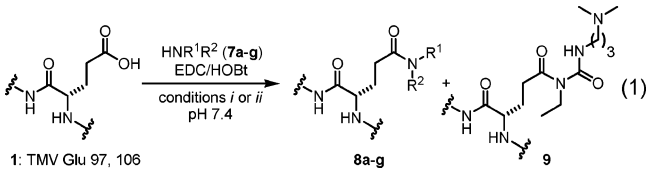


Figure 4. Solubilization of PEG-modified TMV capsids in chloroform. (a) Samples of **3a** and **5d** in aqueous solution. The orange color is from the azo linkage. (b) Upon the addition of 1.9 M $(NH_4)_2SO_4$ and $CHCl_3$ (bottom layer), the PEG-labeled sample is readily transferred to the organic solvent. The unconjugated sample remains insoluble in both layers. (c) TEM analysis of the particles in the $CHCl_3$ layer indicates that the capsids remain assembled.

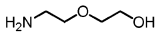
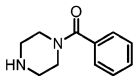
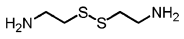
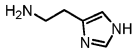
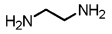
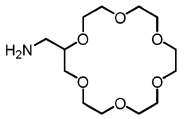
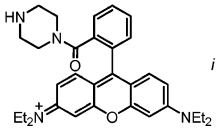
saturated aqueous layer as an insoluble precipitate (Figure 4b). After isolation of the chloroform layer, TMV conjugates were recovered through evaporation of the solvent. The TMV rods could be resuspended upon the addition of water to the dehydrated pellet, although this step was accompanied by a significant amount of protein loss (presumably due to the denaturation of some of the monomers). Subsequent TEM analysis revealed the presence of 300 nm rods that were virtually indistinguishable from wild-type capsids, Figure 4c, indicating that some of the capsids remain assembled despite the solvent transfer and rehydration steps. In addition, identical results were obtained for both PEG conjugates (**5d** and **5e**) when dichloromethane or THF was used in place of chloroform. In contrast, conjugate **3a** shows no detectable solubility in any of the organic solvents tested. A more detailed study of the stability of the modified capsids in organic solvents is underway, as are experiments to explore the ability of the polymer coating to reduce the immunogenicity of the capsids for drug and gene delivery applications. It is anticipated that the ability to transfer the assembly to organic solvents will greatly expand the conditions that can be used for subsequent bioconjugation reactions as well as future device fabrication.

Interior Modification of TMV. The interior modification strategy focused on glutamate residues as targets for amide bond formation, eq 1. Although these residues could be activated for



coupling using *N*-ethyl-3-(*N*′,*N*′-dimethylaminopropyl) carbodiimide (EDC) alone, substantial amounts of stable EDC adducts (assumed to be *N*-acyl urea **9**) were observed as byproducts in the ESI-MS spectra. It was found, however, that this undesired pathway could be suppressed through the addition of excess HOBT, which presumably functions to turn over the *O*-acylisourea intermediate before rearrangement can occur. Other additives, such as HABT, NHS, sulfo-NHS, and DMAP, were less effective in suppressing byproduct formation. Using a variety of amine substrates, 21−86% of the capsid monomers could be converted to singly and doubly modified products,

Table 1. Interior Modification of TMV Capsids[a]

	Amine **7**	Conditions	Unmod (%)	+1 Mod (%)	+2 Mod (%)	**9** (%)
a		*i*	32	48	11	9
b		*i*	42	46	8	4
c		*ii*	71	21	0	8
d		*ii*	8	60	26	6
e		*ii*	16	54	25	5
f		*ii*	58	26	0	16
g		*i*	52	26	12	10

[a] Conditions: 6.5 mg/mL capsid, 60 equiv of EDC, 250 equiv of HOBT, 250 equiv of amine in (i) 0.1 M phosphate buffer, pH 7.4, rt, 24 h or (ii) 0.1 M HEPES buffer, pH 7.4, rt, 24 h. Product distributions were determined from ESI-MS spectra.

Table 1.[12] Both primary and secondary amines were competent in the reaction, with only trace amounts of **9** observed in most cases. It is notable that even chromophores and crown ethers can be attached to the capsid despite the steric demands they introduce. No appreciable disassembly of the capsid was detected in any of these reactions.

The site-specificity of this reaction was determined through analysis of trypsin digest fragments by ESI-MS and MS/MS.[10] Glu 97 was identified as the primary site of modification, with Glu 106 also affording appreciable reactivity. Although both of these residues are exposed on the core surface of the capsid, it is interesting to note that modification of exterior aspartic acids and the carboxy terminus have not been observed. As both of the modification sites extend into the core of the TMV capsid helix (Figure 1), this method positions the new functional groups as closely as 1 nm apart on the inside of the tube.

The pathway by which TMV capsids self-assemble has been elucidated in detail.[4] One outcome of these studies is a predictable set of pH and ionic strength conditions that can be used to shift the aggregate states of purified TMV coat protein from double disks to micron-length rods. We have adapted a series of these literature protocols[13] to disassemble internally modified TMV conjugates and then reassemble the monomers into equivalent structures bearing the new functional groups. As an example, a sample of conjugate **8a** (in which >75% of the monomers had been modified using extended coupling reaction times) was disassembled and precipitated using the procedure described in the Experimental Section. The denatured protein was then redissolved in high pH solution and dialyzed against 100 mM Tris buffer at pH 8.0 to effect refolding of the capsid monomers. Following this, the monomers were dialyzed against 47 mM phosphate buffer (pH 7.0) for 12 h to yield 18 nm double disk structures (Figure 5a). Alternatively, dialysis of the refolded monomers against acetate buffer (pH 5.5) afforded long tubes that were similar to the native capsids but were not length templated by the genomic RNA. After isolation of the assembled structures from any remaining monomers using gel filtration, ESI-MS analysis of the samples indicated that >75% of the protein monomers were conjugate **8a**. This confirms that, at least in this case, the modified protein monomers retain their assembly capabilities. Although this procedure is bound to encounter limits as the steric demands of the internal substituents increase, it nonetheless provides a convenient way to access different scaffold structures using a single protein building block.

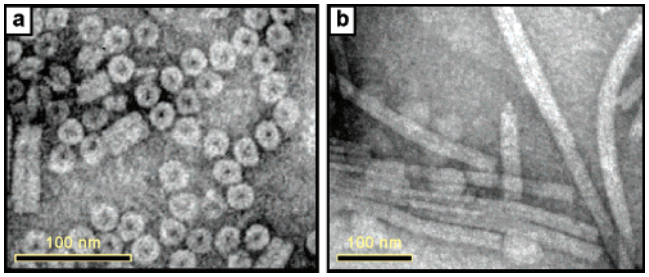


Figure 5. Reassembly of modified TMV conjugate **8a**. (a) After capsid disassembly, refolding of the protein, and dialysis into pH 7.0 phosphate buffer (47 mM), TEM analysis indicated the formation of 18 nm disk aggregates. (b) Alternatively, dialysis against pH 5.5 acetate buffer (100 mM) yielded extended tubelike structures that were microns in length. After isolation of the assembled structures using gel filtration, ESI-MS analysis indicated that in both cases >75% of the protein monomers were conjugate **8a**.

Importantly, the interior surface modification can also be carried out on azo-adduct **3a** to yield differentially functionalized viral capsids, albeit with substantial protein loss. As an example, rhodamine derivative **7g**[14] has been coupled to the inside surface of azo-modified capsids, and the presence of doubly modified capsid monomers has been confirmed using MALDI-TOF MS.[10] After isolation via gel filtration, TEM analysis of the dual-surface modified capsids revealed tubelike structures that were virtually identical to the unmodified virus, with the exception that some capsid fragmentation had occurred as a result of sample handling. These studies illustrate the durability of the TMV capsid for materials applications, as very high levels of modification (in the case of rhodamine **7g**, 2100 external and ~650 internal sites per 300 nm rod) can still be tolerated in assembled capsids.

Conclusion

As a result of these studies, the utility of TMV for the construction of nanoscale materials has been expanded dramatically. Through the development of efficient modification reactions, virtually any functional group can now be installed on either the outside or the inside surface, and preliminary studies have shown that these modifications can alter the solubility properties of the virus. Current experiments are exploring the modified assemblies as multivalent scaffolds for the display of biological ligands and for the preparation of linear arrays of chromophores and inorganic nanoparticles. Parallel

(12) In the case of rhodamine derivative **7g**, UV−vis absorption spectra agreed with the modification ratios reported in the table. Nonetheless, we consider the ESI-MS based ratios to be more accurate. See Supporting Information for details.

(13) (a) Fraenkel-Conrat, H. *Virology* **1957**, *4*, 1. (b) Scheele, R. B.; Lauffer, M. A. *Biochemistry* **1967**, *6*, 3076. (c) Durham, A. C. H.; Finch, J. T.; Klug, A. *Nature New Biol.* **1971**, *229*, 37.

experiments are also exploring the use of the modified capsids as carriers for drug and gene cargo.

Experimental Section

Propagation and Isolation of Tobacco Mosaic Virus (TMV). The general procedure of Chapman was used:[8] Turkish tobacco plants were infected by rubbing each leaf with a thin slurry of Celite mixed with a 5 mg/mL solution of TMV in 100 mM phosphate buffer (pH 7.4). Three weeks after infection, the leaves and stalks of the plants were cut into small pieces with a total mass of 1.6 kg. In a 4 °C cold room, the material was homogenized in 100 mM phosphate buffer (pH 7.4) containing 0.1% (v/v) mercaptoethanol. The resulting mixture was filtered through cheesecloth, and the plant debris was discarded. *n*-Butanol (8 mL per 100 mL of sap) was added to the thick green filtrate. After stirring, the mixture was clarified by centrifugation at 8000 rpm for 10 min. Sodium chloride and poly(ethylene glycol) (average MW 6000) were added to the supernatant (4 g of each per 100 mL of solution), which resulted in the formation of a white precipitate. The mixture was centrifuged at 8000 rpm for 10 min, after which the supernatant was discarded.

The pellet was resuspended in 10 mM potassium phosphate buffer at pH 7 with 1% Triton X-100, and the resulting solution was layered onto an 8 mL pad of sucrose solution (250 mg/mL) containing 1% Triton X-100 in a 70 mL polycarbonate ultracentrifuge tube. Ultracentrifugation at 40 000 rpm for 1 h in a Ti-45 rotor afforded a gelatinous pellet, which was resuspended overnight in 10 mM pH 7.0 phosphate buffer at 4 °C. The remaining debris was removed via centrifugation at 5000 rpm, and the supernatant was again layered onto an 8 mL sucrose pad (250 mg/mL) containing 1% Triton X-100 in an ultracentrifuge tube. After a final round of ultracentrifugation at 40 000 rpm for 1 h, the resulting pellet was resuspended overnight in 10 mM KH_2PO_4, pH 6.8, at 4 °C.

A final round of low-speed centrifugation (5000 rpm, 5 min) was carried out to remove remaining debris, and the resulting TMV stock solution was flash frozen in aliquots using liquid nitrogen. The TMV stock solutions were stored at −80 °C until use. The absorbance of a sample diluted 1000-fold was measured as 0.090 absorbance units at 260 nm. Assuming an extinction coefficient of 3.0 for a 1 mg/mL solution of TMV at this wavelength,[8] the original stock solution was calculated to be 30 mg/mL in virus. The overall yield for this process was approximately 2.5 g of virus from 1.6 kg of plant material. The purity of the virus was confirmed by LC/ESI-MS and by SDS-PAGE, both of which showed a single protein species with a mass corresponding to the TMV coat protein.

Exterior Surface Modification of TMV. Diazonium salts **2a**−**d** were synthesized by mixing the following solutions at 0 °C: 200 μL of 0.84 M (160 mg/mL) aqueous *p*-toluenesulfonic acid monohydrate; 200 μL of 0.46 M (32 mg/mL) aqueous sodium nitrite; and 400 μL of 0.15 M aniline in acetonitrile. The resulting solution was placed in an ice water bath for 1 h. Subsequently, 250 μL of a 15 mg/mL stock solution of TMV in 10 mM phosphate buffer (pH 7.4) was diluted with 650 μL of 150 mM aqueous borate buffer, pH 9, also containing 100 mM sodium chloride. To this solution was added 100 μL of the diazonium salt solution prepared above. The reaction mixture was placed in a water bath in a 4 °C cold room for 2 h, after which the solution was a deep brown color. To remove the small molecules, the reaction solution was passed through a disposable Sephadex G-25 size exclusion column, pre-equilibrated with 250 mM aqueous phosphate buffer (pH 6.0). The azo-functionalized TMV was characterized by LC/ESI-MS, MALDI-TOF MS, and size exclusion chromatography. For conjugates **3a**−**c**, ESI-MS and UV/vis analysis of intact capsids isolated via gel filtration confirmed that >90% of the monomers had been functionalized (see Figure S.1 in the Supporting Information). The reaction mixture was stored at 4 °C for up to 1 month.

(A) Oxime Formation with 4a−**c**. To 200 μL of a 2 mg/mL solution of TMV conjugate **3a** was added 50 μL of a 40 mM solution of the desired hydroxylamine compound in 250 mM aqueous phosphate buffer (pH 6.0). The reaction solution was vortexed briefly and left at room temperature for 2 h to allow oxime formation to occur. The resulting solution was then passed through a disposable Sephadex G-25 column to remove the small molecules. The modified viral capsids were analyzed by LC/ESI-MS, MALDI-TOF MS, and TEM.

***O*-(Methoxypoly(ethylene glycol))-hydroxylamine, Average Molecular Weight 2000 (4d).** Under an atmosphere of N_2, diisopropyl azodicarboxylate (0.212 mL, 1.09 mmol) was added dropwise to a mixture of poly(ethylene glycol) monomethyl ether, average molecular weight 2000 (**6d**) (2.00 g, 0.994 mmol), *N*-hydroxyphthalimide (194 mg, 1.19 mmol), and PPh_3 (312 mg, 1.19 mmol) in CH_2Cl_2 (10 mL). The resulting mixture was stirred for 18 h at room temperature. The solution was then transferred to a large flask, to which an excess (~400 mL) of diethyl ether was added to precipitate the polymer as a white solid. After vigorous stirring for 20 min, the suspension was filtered, and the obtained pellet was washed with three 70 mL portions of diethyl ether. After drying under reduced pressure, ^{1}H NMR analysis indicated a mixture of the *N*-hydroxyphthalimide (NHP) adduct (80%) and unreacted **6d** (20%). Resubjecting this material to the same initial reaction conditions yielded the pure NHP product (2.1 g, 99%) as a white powder that was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.37 (s, 3H), 3.5−3.7 (poly(ethylene glycol) signals), 7.75 (m, 2H), 7.82 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.6, 133.9, 128.2, 122.7, 76.3, 71.2, 70.0−69.5, 68.7, 58.3.

Cleavage of the phthalimide moiety was achieved by adding hydrazine hydrate (0.076 mL, 2.47 mmol) to a solution of the PEG-NHP product (1.00 g, 0.494 mmol) dissolved in CH_2Cl_2 (10 mL). The solution was stirred rapidly for 0.5 h, during which a white precipitate was observed. The mixture was then filtered over glass wool and concentrated under reduced pressure to afford **4d** as a white powder (983 mg, 99%) that was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.17 (s, 3H), 3.3−3.5 (poly(ethylene glycol) signals). ^{13}C NMR (100 MHz, $CDCl_3$): δ 74.0, 71.3, 69.9, 68.9, 58.4.

***O*-(Methoxypoly(ethylene glycol))-hydroxylamine, Average Molecular Weight 5000 (4e).** This compound was prepared using an identical two-step procedure to that used for **4d**. Compound **4e** was isolated in 93% overall yield (2.45 g scale) as a white powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.05 (s, 3H), 3.2−3.5 (poly(ethylene glycol) signals). ^{13}C NMR (100 MHz, $CDCl_3$): δ 73.6, 70.9, 69.6, 68.5, 58.0.

General Procedure for Oxime Conjugation of Ketone-Modified TMV with *O*-(Methoxypoly(ethylene glycol))-hydroxylamine (5d,e). To a vial containing 1.5 mL of a solution of TMV conjugate **3a** (2.0 mg/mL, 0.17 μmol modified Y139 in 50 mM NaH_2PO_4 buffer, pH 6.5) was added *O*-(methoxypoly(ethylene glycol))-hydroxylamine **4d** or **4e** (85 μmol). The reaction mixture was further diluted with buffer (50 mM NaH_2PO_4, pH 6.5) to reach a total volume of 3.0 mL. The resulting solution was then rotated on a LabQuake shaker at room temperature for 24 h. The reaction mixture was then diluted with buffer and subjected to at least three rounds of centrifugal ultrafiltration with 100 kDa molecular weight cutoff spin columns (Millipore) to remove the unreacted polymer. The relative extent of polymer conjugation was monitored via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the general protocol of Laemmli.[15]

General Procedure for the Solubilization of PEGylated TMV Capsids in Organic Solvents. To a 1.7 mL microcentrifuge tube was added 200 μL of PEGylated TMV capsid **5d** or **5e** (2.0 mg/mL in 0.05M NaH_2PO_4 buffer, pH 6.5) and 50 mg of $(NH_4)_2SO_4$. After gentle vortexing to dissolve the salt, the resulting mixture was briefly centrifuged (11 000 × *g*, 1 min) after which 150 μL of $CHCl_3$, CH_2Cl_2, or THF were added. The resulting biphasic mixture was again briefly centrifuged (11 000 × *g*, 1 min). Subsequent analysis of viral particles was performed by careful isolation of the organic layer,

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(15) Laemmli, U. K. *Nature* **1970**, *227*, 680.

concentration under reduced pressure, and rehydration of the solid residue with the appropriate buffered aqueous solution.

General Procedures for Interior Surface Modification of TMV. To 0.2 mL of TMV stock solution (15 mg/mL, 171 μmol) in 10 mM phosphate buffer, pH 6.8, were added successively 5.8 mg of 1-hydroxybenzotriazole (HOBT, 43 μmol), 20 μL of an aqueous buffered solution of 1-ethyl-3-*N*,*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 32.8 mg/mL, 3.4 μmol) in 100 mM phosphate buffer, pH 7.4, 40 μL of an aqueous buffered solution of the amine (42.8 μmol) in 100 mM phosphate buffer, pH 7.4, and 200 μL of additional phosphate buffer (100 mM, pH 7.4). The reaction mixture was vortexed briefly and rotated on a LabQuake shaker at room temperature for 6 h, at which time an additional 20 μL aliquot of a freshly prepared EDC solution in 100 mM phosphate buffer (pH 7.4) were added. A third and final 20 μL aliquot of the EDC solution (again, freshly prepared) was added 18 h after initial mixing. After 24 h, the reaction solution was passed through a gel filtration column (NAP-5) pre-equilibrated with 5 column volumes of elution buffer (100 mM, pH 7.4). EDC coupling reactions for some primary amines were carried out in HEPES buffer (100 mM, pH 7.4) in place of the phosphate buffer.

Dual-Surface Modification of TMV. Ketone-labeled TMV (2.5 mg/mL) prepared as described above was passed through a S-300 size exclusion chromatography column pre-equilibrated with 250 mM phosphate, pH 7.0, to effect buffer exchange. To 200 μL of this solution were added the following: 1.93 mg (14 μmol) of solid hydroxybenzotriazole (HOBT); 10 μL of a solution of 1-ethyl-3-*N*,*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 21.7 mg/mL, 140 mM; 1.4 μmol added) in 250 mM phosphate buffer (pH 7.0), and 30 μL of a solution of rhodamine derivative **7g**[13] (240 mg/mL, 439 mM; 13 μmol added) in 250 mM phosphate buffer (pH 7.0). The reaction mixture was vortexed briefly and left at room temperature for 6 h, at which time an additional 20 μL aliquot of a freshly prepared EDC solution in 100 mM phosphate buffer (pH 7.4) was added. A third and final 20 μL aliquot of the EDC solution (again, freshly prepared) was added 12 h after initial mixing. The mixture was allowed to react in Eppendorf tubes at room temperature for 48 h, after which small molecule reagents and any dissociated coat protein monomers were removed via two rounds of gel filtration through Sephadex S-300 resin. The sample was characterized by MALDI-TOF MS as described above and by TEM (see Figure S2 in the Supporting Information).

Refolding Procedure for Internally Modified TMV Capsids. The general prodecures of Fraenkel−Conrat, Scheele, and Durham were used:[13] To a solution of modified TMV capsids were added two additional volumes of acetic acid. The resulting suspension was centrifuged at 10 000 rpm for 10 min to pellet the precipitated RNA, and the supernatant was passed through a Sephadex G-25 column pre-equilibrated with three column volumes of 1% aqueous HOAc. The protein-containing eluent was added dropwise to 10 mL of 100 mM phosphate buffer (pH 7.4), which resulted in precipitation of the coat protein. After a period of 2 h, the precipitate was pelleted via centrifugation at 5,000 rpm for 10 min and the supernatant was discarded. The pellet was then dissolved in 100 mM KOH solution and added to a dialysis cartridge (MW cutoff = 10 kD). The sample was dialyzed against 100 mM Tris buffer (pH 8.0) at 4 °C overnight. For the formation of disk aggregates, the resulting solution was further dialyzed against 47 mM phosphate buffer (pH 7.0) at 4 °C overnight; alternatively, rodlike structures were prepared by dialyzing against 100 mM NaOAc buffer (pH 5.5) at 4 °C overnight. The resulting assemblies were applied to TEM grids and analyzed as described in the Supporting Information.

Proteolytic Digests of TMV Conjugates. Protein solutions at concentrations of 1−6 mg/mL were isolated from small molecules via gel filtration using Sephadex S-300 resin. Solid urea was added to a final concentration of 6 M, and the solution was heated to 100 °C for 30 min. Ammonium bicarbonate buffer, 150 mM at pH 7.8, was added to reduce the total urea concentration to 1 M. Trypsin (Promega) or endoproteinase Glu-C (Sigma) was dissolved and added to the solution of TMV protein at a ratio of roughly 1 μg of enzyme per 100 μg of protein. The digest solution was incubated with shaking at 37 °C for 3−12 h. The solution was then purified and concentrated using a Phenomenex C-18 cartridge (50 mg size) with aqueous 60% acetonitrile, 0.1% formic acid, as the elution buffer. This fraction was subsequently analyzed by MALDI-TOF MS and by ESI-Q-TOF MS (see Figures S3 and S4 in the Supporting Information).

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Supporting Information Available: General experimental procedures, instrumentation details, and MS/MS characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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