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COMMUNICATIONS

Published on Web 03/09/2004

Interior Surface Modification of Bacteriophage MS2

Jacob M. Hooker, Ernest W. Kovacs, and Matthew B. Francis*

Department of Chemistry, University of California, Berkeley, California 94720-1460, and Material Science Division, Lawrence Berkeley National Labs, Berkeley, California 94720-1460

Received December 17, 2003; E-mail: francis@cchem.berkeley.edu

There has been growing interest in the use of protein assemblies as scaffolds for nanoscale materials.[1] Viral capsids have received particular attention in this regard, as they offer robust and monodisperse structures in a range of sizes and shapes. For inorganic materials, the inside surface of these structures has been used to template the growth of metal oxide particles,[2] and phage libraries have been used to identify capsids that can recognize and order nanocrystals.[3] In terms of covalent modification, cysteine residues introduced using site-directed mutagenesis have proven useful for the positioning of dye molecules,[4] gold nanoparticles,[5] and carbohydrates[6] on capsid exteriors. Engineered cysteines have also been used to attach viruses to inorganic substrates.[7] These studies highlight the versatility of capsids as building blocks for material construction.

We are particularly interested in the covalent modification of the interior surface of viral capsids[8] for the attachment of drug cargo, site-isolated catalysts, and nucleation sites for crystal growth. To achieve this, we have developed an efficient four-step strategy for the interior functionalization of hollow capsid shells. This method features a new hetero-Diels−Alder bioconjugation reaction for the attachment of olefin substrates to selectively modified tyrosine residues.

Bacteriophage MS2 was chosen as the target for these studies because of its facile propagation and isolation from Hfr$^+$ *Escherichia coli* cultures. By following established protocols,[9] 30 mg of highly pure virus can be obtained from each liter of infected broth. The capsid shell comprises 180 sequence-identical protein monomers assembled into an icosahedral arrangement that is 27 nm in diameter. Of particular interest for internal modification is the presence of 32 pores that are 1.8 nm in diameter, providing access to the interior volume of the capsid, Figure 1a.[10]

Before interior functionalization, the RNA genome was removed by exposing native MS2 to pH 11.8 conditions for 4 h. It is assumed that the alkaline conditions degrade the RNA through phosphate hydrolysis and reduce its affinity for the capsid by deprotonating the associated lysine side chains, although the detailed process by which the RNA fragments escape the capsid shell is currently under investigation. Following alkaline hydrolysis, the empty capsids were isolated via precipitation with over 90% protein recovery. The RNA core found in native MS2 normally prevents the TEM staining agent ($UO_2(OAc)_2$) from entering the capsid interior; however, after removal of the genome, the center of the particles appears dark due to penetration by the stain, Figure 2a,b. The absence of RNA absorption at 260 nm confirms genome removal, Figure 2c. Stability studies have indicated that the genome-free capsids do not disassemble in the pH range of 3−10 over a 12 h period, Figure 2d.

As the exterior surface already possesses cysteine residues that have been modified in our lab,[11] we were reluctant to use site-directed mutagenesis to introduce new reactive sites. Instead, a native residue, tyrosine 85, was targeted to provide 180 modification sites on the interior surface, Figure 1b,c. Analysis of the crystal structure[10] suggested that the three other tyrosines in each monomer were significantly less surface accessible and therefore were not expected to participate in bioconjugation reactions.

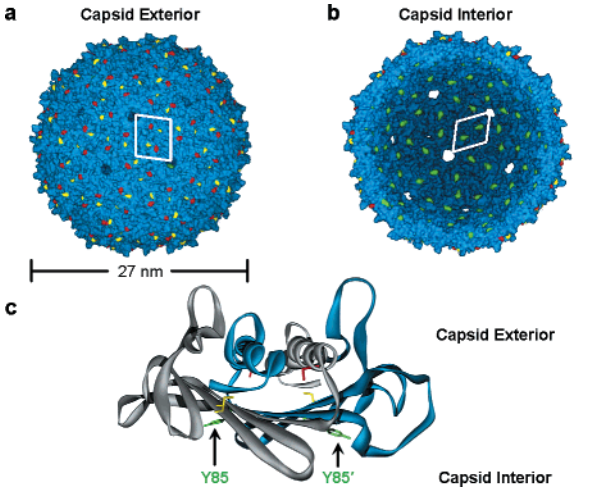


Figure 1. Bacteriophage MS2. (a) Access to the capsid interior is provided by 32 pores that are 1.8 nm in diameter. The positions of two modifiable cys residues are indicated in red and yellow. (b) A cut-away view of the capsid reveals tyr 85 (green) as an accessible residue on the interior surface of each capsid monomer. (c) The location of tyr 85 is indicated in a "side-on view" of two coat protein monomers. The position of these monomers is indicated by the white outline in a and b.

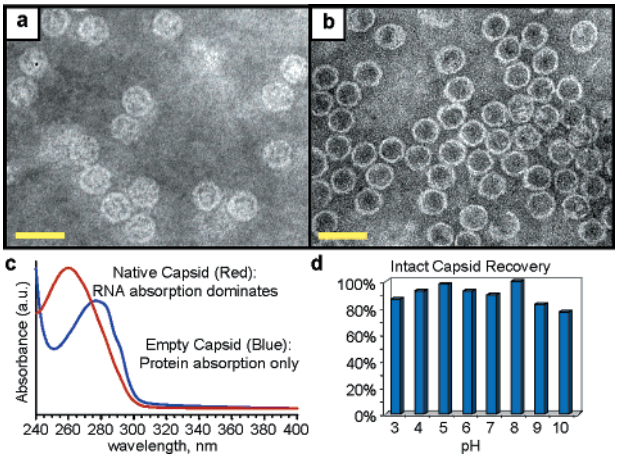
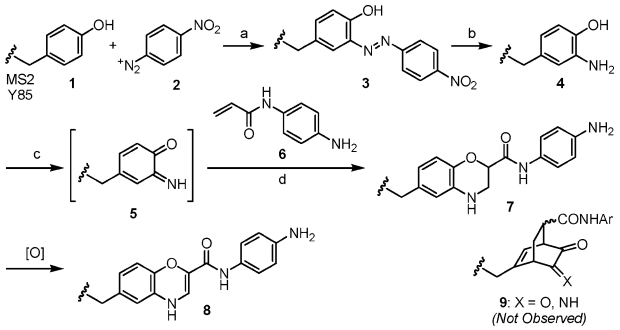


Figure 2. Preparation of genome-free capsid shells. (a) TEM image of RNA-containing MS2 capsids stained with $UO_2(OAc)_2$. (b) After exposure to pH 11.8 for 4 h the RNA genome is removed, allowing the stain to enter the capsid (for a and b, scale bar = 50 nm). (c) The loss of RNA is confirmed by the lack of absorption at 260 nm in samples isolated using SEC. (d) The "empty" capsids are stable for 12 h over a wide pH range.

Using diazonium-coupling reactions, tyrosine 85 was modified with high efficiency and selectivity. In particular, virtually complete

10.1021/ja031790q CCC: $27.50 © 2004 American Chemical Society

Scheme 1. Functionalization of Tyrosine 85[a]



[a] (a) 2 (5 equiv), pH 9, 4 °C, 15 min; (b) $Na_2S_2O_4$ (100 mM), pH 7.2, rt, 2 h, 80−85% protein recovery, 2 steps; (c) $NaIO_4$ (100 μM), pH 6.5, followed by (d) **6** (10 mM), 2 h, 75% protein recovery.

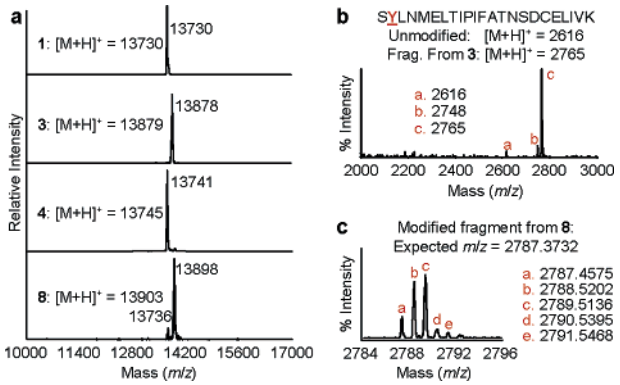


Figure 3. Analyses of protein modification reactions. (a) Intact capsid samples isolated using SEC were disassembled and characterized by MALDI-TOF MS. All *m*/*z* ratios are within 0.04% (5 amu) of the expected values. (b) After subjecting azo-adduct **3** to trypsin, the digest fragments were identified by MALDI-TOF MS. The expected mass for the modified fragment was observed, confirming the site selectivity for Y85. No other modified fragments could be identified. (c) High-resolution MALDI-TOF MS analysis of the analogous trypsin digest fragment for **8**.

coupling was achieved in 15 min through exposure to 5 equiv of diazonium salt **2**, Scheme 1. Analysis of modified capsids isolated using size exclusion chromatography indicated the presence of a new absorption band at 355 nm, and MALDI-TOF MS analysis of the protein monomers confirmed single modification, Figure 3a. Both methods verify that >95% conversion was achieved with excellent protein recovery.[12] The selectivity of this reaction for tyrosine 85 was confirmed through trypsin digest analysis, Figure 3b.

Unfortunately, diazonium salts lacking the nitro substituent attained lower levels of conversion, thus limiting the range of functionality that can be installed using this method. To overcome this problem, the azo bond of **3** was reduced with sodium dithionite[13] to afford *o*-amino tyrosine derivative **4**. This reaction reaches full conversion in 2 h at room temperature and can be followed by the absorbance loss at 355 nm. This functional group can then be oxidized to *o*-imino-quinone **5** with $NaIO_4$, affording a highly reactive functional group for further elaboration.

The reactivity of this functional handle was tested in the context of a Diels−Alder reaction. By screening a series of dienophiles under a variety of reaction conditions, it was found that acrylamide **6** gave particularly efficient conjugation, with conversion levels exceeding 90% in 2 h at room temperature, as determined by MALDI-TOF MS analysis, Figure 3a. No other modification sites were identified after trypsin digest analysis, and exposure of "empty" capsid **1** to **6** and $NaIO_4$ yielded no reaction. Although two Diels−Alder products (**7** and **9**) can be expected, current data are more consistent with initial adduct **7**.[14] High-resolution MS analysis of the modified fragment after trypsin digestion indicates that the nitrogen atom remains in the product and that a subsequent oxidation[15] occurs to afford benzoxazine **8** as the final reaction product. Although $BnONH_2$ reacts with **5** to form oximes, no such adducts are formed for the Diels−Alder product, further suggesting that **9** is not observed. Assignment of the regiochemistry of the reaction product is underway. As indicated by SEC and TEM analysis, no morphological changes were observed for the capsids after this modification procedure.

Starting with "empty" MS2 capsids, this efficient four-step procedure can be carried out in less than 4 h and offers an orthogonal modification strategy to more commonly used lysine- and cysteine-targeted reactions. Under mild reaction conditions, this reaction can reach high levels of conversion with exceptional protein recovery. A more thorough exploration of this new hetero-Diels−Alder bioconjugation reaction is in progress, as are efforts to build targeted drug delivery systems and other core/shell materials from dual-surface modified MS2 capsids.

Acknowledgment. We gratefully acknowledge the Lawrence Berkeley National Laboratory, Materials Science Division (U.S. Department of Energy Contract No. DE-AC03-76SF00098), and the Center for New Directions in Organic Synthesis for financial support. CNDOS is supported by Bristol-Myers Squibb as a Sponsoring Member and Novartis Pharma as a Supporting Member. J.M.H. was supported by a predoctoral fellowship from the NSF.

Supporting Information Available: Experimental procedures and characterization data for all intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA031790Q