Implementation of P22 Viral Capsids as Nanoplatforms

Sebyung Kang,†,‡ Masaki Uchida,† Alison O’Neil,† Rui Li,§ Peter E. Prevelige,*,§ and Trevor Douglas*,†

Department of Chemistry and Biochemistry and Center for Bio-Inspired Nanomaterials, Montana State University, Bozeman, Montana, 59717, Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama, 35294, and School of Nano-Biotechnology and Chemical Engineering, Ulsan National Institute of Science and Technology (UNIST), Ulsan, 689-798, Korea

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Viral capsids are dynamic macromolecular machines which self-assemble and undergo concerted conformational changes during their life cycle. We have taken advantage of the inherent structural flexibility of viral capsids and generated two morphologically different types of viral nanoplatforms from the bacteriophage P22 capsids. Their interior surfaces were genetically manipulated for site-specific attachment of a biotin linker. The extent of internal modifications in each capsid form was characterized by high-resolution mass spectrometry and the analyses revealed that the reactivity of the genetically introduced residues located on the internal surface changes according to the structural transformation of the capsid. Internally modified capsids having 10 nm diameter pores at the 12 icosahedral vertices, so-called wiffle-balls (WB), exhibited the capability to entrap the large tetrameric protein complex streptavidin via the biotin linker anchored onto the interior surface of the WB.

Introduction

Viral capsids self-assemble into precise and symmetric supramolecular architectures.1-4 Many undergo concerted conformational changes during maturation or infection and can be considered as dynamic macromolecular machines.5 Their remarkable plasticity allows viruses to respond to environmental changes as functional materials. We, and others, have taken advantage of the inherent structural flexibility of viral capsids and have generated a series of viral capsid based nanoplatforms and investigated their potential as nanoscale cargo delivery vehicles.6-11 The desired functionality, such as drug delivery or cell targeting, can be introduced to viral capsids via either chemical or genetic modification. Viral capsids up to \( T = 3 \) such as Cowpea chlorotic mottle virus (CCMV), Cowpea mosaic virus (CPMV) and bacteriophage MS2, whose diameter is around 30 nm, have been widely used.7-11 However, a viral capsid with higher loading capacity would be desirable for some applications such as entrapment of large molecules. In the current work we describe the use of the \( T = 7 \) bacteriophage P22 capsid for internal site-specific modification and the entrapment of protein complexes. To the best of our knowledge, this is currently the largest icosahedral viral capsid manipulated to attempt encapsulation of nongenomic materials.

The Salmonella typhimurium bacteriophage P22 assembles from 415 copies of the 46.6 kDa coat protein with the aid of approximately 300 copies of the 33.6 kDa scaffolding protein to form an icosahedral P22 procapsid.1 In the infectious phage, 1 of 12 pentameric vertices is occupied by a portal complex in the WB via the pentameric pores was investigated. The P22 procapsid has a diameter of 58 nm (Figure 1A and 2B) and undergoes a structural transformation initiated by DNA packaging to form the infectious 64 nm diameter mature capsid (Figure 1B).2-12

The P22 capsid transformation, from procapsid to mature capsid, can be mimicked in vitro by gentle heating (65 °C for 10 min).13,14 Extended heating (75 °C, ≥20 min) induces the selective release of subunits from the 12 5-fold icosahedral vertices (pentons) to produce another capsid form, affectionately known as the “wiffle-ball”, which has a 10 nm hole at each of the 12 5-fold vertices (Figures 1C and 2C).14,15 Wiffle-ball capsids (WB) are identical in structure to the mature capsids except for the absence of the subunits at the 5-fold vertices.14,15 The P22 capsid is, thus, a remarkable dynamic nanoplatform whose structural transformations lend themselves to synthetic utilization. As the transformation from procapsid to WB is associated with a conformational change of the tertiary structure of each subunit, the chemical reactivity of amino acid residues in the capsid could be altered by the transformation. Also, the large 10 nm diameter pores of WB at the 12 vertices ensure free molecular exchange between the interior and exterior environments of the capsid and could potentially be used as portals for entry of large molecular species to modify the interior of the capsid.

As a first step to exploit the P22 capsid as a platform for a nanoscale container and a cargo delivery vehicle, we genetically introduced cysteine residues at sites presumptively lining the interior surface and examined their reactivity in different capsid forms. Furthermore, the feasibility of entrapment of a protein complex in the WB via the pentameric pores was investigated.

Experimental Section

Mutagenesis and Capsid Purification. All of the mutants were generated by using established polymerase chain reaction protocols using pET-3a based plasmids encoding genes for scaffolding and coat proteins as templates. The amplified DNAs were transformed into a CaCl2-treated competent E. coli strain BL21 (DE) and selected for ampicillin resistance. Mutant procapsids were overexpressed in E. coli and purified by sucrose cushion centrifugation. The empty procapsid

* To whom correspondence should be addressed. Phone (406) 994-6566 (T.D.). Fax (406) 994-5117 (T.D.). E-mail: tdouglas@chemistry.montana.edu (T.D.); prevelig@uab.edu (P.E.P.).
† Montana State University.
‡ UNIST.
§ University of Alabama at Birmingham.

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Mass measurements using ESI-TOF mass spectrometry. The quaternary structure of MPB-treated ES and WB was examined by native agarose gel electrophoresis. The extent of the labeling was estimated by subunit chromatography (SEC: Amersham-Pharmacia, Piscataway, NJ) with a Superose 6 column. The extent of the labeling was estimated by subunit mass measurements using ESI-TOF mass spectrometry. The quaternary structure of MPB-treated ES and WB was examined by native agarose gel electrophoresis.

Maleimide-PEO2-Biotin or Maleimide-C2-Biotin Labeling of the Wt and Mutant Capsids. Wt and mutant P22 ES and WB were incubated with 3 mol equiv per subunit of maleimide-PEO2-biotin (MPB: Thermo Scientific, U.S.A.) or maleimide-C2-biotin (MC2B: Thermo Scientific, U.S.A.) or maleimide-C2-biotin (MC2B: Thermo Scientific, U.S.A.) or maleimide-C2-biotin (MC2B: Thermo Scientific, U.S.A.) or maleimide-C2-biotin (MC2B: Thermo Scientific, U.S.A.).

Mass Spectrometry. Subunit masses of MPB-treated, MC2-treated, and control P22 capsids were analyzed by ESI-Q-TOF mass spectrometry (Q-TOF Premier, Waters) interfaced to a Waters UPLC and autosampler. Samples were loaded onto the BioBasic SEC-300 (5 µm, 250 × 1.0 mm I.D, Thermo Scientific) and eluted with the buffer containing 40% isopropanol, 59.9% water, and 0.1% formic acid isocratically with a rate of 25 µL/min.

Binding of Fluorescein Conjugated Streptavidin (F-StAv) to MPB-Labeled K118C WB. F-StAv was purchased from Molecular Probes (Eugene, Oregon) and used without further purification. F-StAv (0.1 mg) was mixed with either MPB-labeled, MC2B-labeled, or unlabeled K118C WB and mixtures were incubated at room temperature overnight with vigorous shaking. The reactions were loaded onto a 10 × 300 mm Superose 6 (Amersham Bioscience) size exclusion column which as pre-equilibrated with 50 mM phosphate, 100 mM NaCl buffer (pH 7.0), and eluted with the same buffer at a rate of 0.5 mL/min, monitoring at 280 and 490 nm. Fractions were collected for further analysis.

Quantification of F-StAv Conjugated with K118C WB. The average number of F-StAv bound to MPB-labeled K118C WB cage was calculated from absorbance at 280 nm of the subtracted spectrum (ε = 2.7 × 10³ M⁻¹ cm⁻¹) (see Supporting Information), therefore, none of the absorbance at 280 nm was due to F-StAv. The number of F-StAv linked with MC2B-labeled K118C WB cage was estimated by the same way.

Results and Discussion

Although there is no atomic resolution structure of any form of the P22 capsid, there are cryo electron microscopy based image reconstructions of the procapsid and mature capsid at subnanometer resolution. Analysis of the reconstructions revealed that the coat protein was folded in the HK-97 fold typical of dsDNA containing bacteriophage and made it possible to identify the helical elements within the subunit and their positions within the capsids. This analysis suggested that long helix 1 is oriented on the internal surface and is therefore an excellent candidate site for internal modification of the viral capsid nanoplatform.

The residues constituting the helical elements were identified by secondary structure prediction and compared with recent 3-D models. Cysteine residues were introduced throughout the putative long helix 1 based on a helical wheel prediction scheme (Supporting Information (SI) Figure F1) and visual inspection of a recently obtained 3-D model. We individually substituted three residues with cysteine; Val 119 (V119) in the middle of the hydrophilic face, Lys 110 (K110) in the middle of the hydrophilic face, and Lys 118 at the helical border (K1118; SI F1). All the mutations were verified by DNA sequencing and mass spectroscopic measurements of purified capsids. All the mutants were overexpressed and purified using the same methods as previously described for wt P22 (SI F2A). Empty procapsid shells (ES) of the mutants were prepared by extracting scaffolding proteins as previously described.

The size and morphology of the mutant ES were identical to wt (SI F2B) and we routinely obtained 50 mg or more mutant ES from 1 L E. coli culture. Empty WB of the P22 mutants were also prepared in the same manner as wild type (wt). Native agarose gels are quite sensitive to changes in the diameter of the capsids and the larger WB form migrates more slowly than the smaller ES form. The size and morphology of the prepared mutants were identical to wt (Figure 2 and SI F3). Because the heterologously expressed wt and mutant capsids do not contain the tail assembly, the ES form is composed of 420 of the coat protein subunit, whereas WB forms are comprised of 360 copies of the subunit.

To investigate whether the introduced cysteine residues could be selectively and covalently modified with thiol reactive reagents in the different capsid forms, ES and WB forms of all
mutants (K110C, K118C, V119C) were treated with maleimide-PEO₂-biotin (MPB). MPB has a thiol reactive maleimide on one end and a biotin which can be used as an affinity tag to recruit reacting partners, streptavidin (StAv), on the other end. As controls, wt ES and WB were treated in parallel with the mutant WB capsids. Maleimide-PEO₂-biotin (MPB)-treated ES and WB migrated to the same position as untreated wt ES and WB on an agarose gel, respectively (SI F4), suggesting MPB conjugation does not alter capsid integrity or overall morphology.

The extent of MPB labeling per subunit of both mutant and wt samples was determined by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS; Figure 3). Even though wt capsid protein has one endogenous cysteine (404), neither wt ES nor WB showed any mass change upon MPB
treatment (Figure 3A), suggesting that this cysteine is buried and inaccessible to labeling with MPB. The 3-D structure supports this, as the cysteine is not fully exposed either to the exterior or the interior surface. Nonspecific labeling of MPB was also not observed (Figure 3A). Similarly, the mass of V119C was unchanged upon MPB treatment (SI F5) in either form of the capsid, suggesting that residue C119 is not reactive to maleimide reagents in either form. However, residues C110 and C118, which lie on the other face of the helix wheel (SI F1), showed different degrees of reactivity to the maleimide reagent, depending upon the capsid forms. In both cases, a maximum of one modification per subunit was observed, suggesting that only the introduced cysteine is reactive to MPB (Figure 3B, C). However, the microenvironment of these two residues changes according to the structural transformation of capsids. While almost all subunits of K110C were labeled with MPB when in the ES form, reactivity was completely blocked for MPB labeling in the WB form (Figure 3B), suggesting that the interior surface exposed C110 becomes buried upon structural transformation from ES to WB. In contrast, only 35% of subunits of K118C ES were labeled with MPB, whereas all the subunits of K118C WB were labeled with MPB (Figure 3C). These data suggest that residue C118 is partially exposed to the interior surface in the ES and becomes fully exposed to the interior surface as the capsid lattice transformation occurs. The alterations in reactivity are consistent with the changes in helix 1 during expansion seen in the cryo-EM reconstructions. The transition from ES to WB appears to induce a bending in helix 1 centered approximately at residue 118, which could render the side chain more accessible, as shown in Figure 4. Even though the structure of ES (Figure 1A) looks solid, it is not surprising that the MPB molecule can diffuse through the ES shell into the interior cavity, because in the native maturation process of the P22 virus, the 33.6 kDa scaffolding proteins get out of the interior cavity while the capsid transform from the procapsid to mature capsid.

The WB structure has 12 10 nm holes, which might be expected to allow free passage of macromolecules through the capsid. To investigate the potential use of WB as a high capacity nano delivery vehicle, MPB-labeled K118C WB was treated with streptavidin conjugated with fluorescein (F-StAv). StAv is an approximately 60 kDa homotetrameric protein of approximately 4 nm diameter, which has a strong affinity for biotin. As a control, unlabeled K118C WB was treated in parallel. While F-StAv coeluted with MPB-labeled K118C WB on size exclusion chromatography (SEC; Figure 5A, top), it did not elute in association with unlabeled K118C WB (Figure 5A, bottom). Only the SEC fraction of F-StAv-treated MPB-labeled...

**Figure 3.** Deconvoluted mass analyses of the maleimide-PEO₂-biotin (MPB)-treated ES (bottoms) and the MPB-treated WB (tops) of wt and mutant P22 capsids: (A) wt, (B) K110C, and (C) K118C. Theoretical masses of unmodified are marked at the bottom panels. K118C dominantly labels in the WB structure and K110C only labels in the ES form.

**Figure 4.** Reconstituted image of P22 WB subunit (cyan) and ES subunit (green). Amino acid residues, positions 97–130, are shown in a ribbon structure and position 118 is shown in magenta. The structural change in the helix occurs near position 118 and could account for the differences in reactivity between the two forms of P22.
K118C WB exhibited distinct absorption at 493 nm where fluorescein absorbs (Figure 5B, red line). To confirm the coelution of MPB-labeled K118C WB and F-StAv, SEC fractions of F-StAv-treated MPB-labeled K118C WB and unlabeled K118C WB were run on SDS-PAGE. Bright fluorescein-labeled StAv was detected only in the lane corresponding to the MPB-labeled K118C WB (Figure 6). These data suggest that F-StAv can interact, and stably associate with the biotin molecules anchored onto the interior surface of K118C WB. The amount of F-StAv bound to the biotin-modified K118C WB structure was determined spectroscopically to be about 13 F-StAv/cage, which corresponds closely to the number of holes and is substantially less than to the maximum capacity calculated based on the internal volume. The result suggests that on average only one F-StAv molecule is bound per pentameric hole. This implies that the F-StAv molecules are bound at the 5-fold hole but might not be able to fully enter the WB capsid, thereby preventing the binding of other StAv molecules. Indeed the distance from C118 to the nearest edge of the hole is about 2 nm, which is shorter than the length of MPB linker (2.9 nm). Therefore the MPB anchored on the K118C WB could bind with StAv even if StAv does not completely enter into the capsid. To address this, K118C WB was labeled with MC2B, which has a shorter linker (1.8 nm) than MPB. All subunits of K118C were labeled with MC2B (SI F6). MC2B-labeled K118C was incubated with F-StAv and the number of StAv bound to the cage was determined spectroscopically to be roughly 3/cage, which is significantly less than the case of the MPB-labeled capsid (SI F7). This result also suggests that it is difficult if not impossible for StAv to pass thorough the pentameric hole and get into the capsid. It is possible that the hole is functionally not really 10 nm in diameter, as suggested by the cryo image reconstruction, perhaps because it is occluded by the amino acid side chains, which cannot be visualized. Another possible explanation is that residue 118C is partially buried and the linker of MC2B is not long enough for F-StAv to access. There are a few potential approaches to overcome this limitation and establish a methodology to entrap macromolecules inside of the P22 capsid. First, proteins and other macromolecules smaller than StAv might be able to pass through the pentameric holes and access the interior cavity. This approach would require a strategy different than using biotin-StAv interaction to anchor the macromolecules. Second, new cysteine residues can be introduced at different sites lining the interior surface but away from 10 nm holes. Finally, encapsulation of cargo macromolecules could be achieved by controlled coassembly of capsids from subunits in the presence of the cargo. These investigations are currently underway. In addition, we have previously reported that the external loop region of the P22 procapsid can be utilized to present functional groups and affinity tags. Thus, the ability to modify the interior and exterior surface in separate steps will facilitate the development of well-defined binary nanoplatforms for biomedical applications and nanoscale devices.
Conclusion

In this study, we have generated two structurally and morphologically different types of viral capsid based nanoplatforms from chemically identical bacteriophage P22 procapsids and demonstrated that the interior surface of these two forms can be genetically manipulated and utilized for further chemical modifications. Mutants designed in this study show differential labeling depending on the particle morphology, that is, K118C preferentially labels in the WB structure and K110C only labels in the ES form, whereas V119C labeled neither the ES nor the WB form. Streptavidin could be bound to a biotin linker, anchored onto the interior surface of K118C WB. However, the accessibility of the StAv macromolecule to interior of the capsid appears to be less than expected based on the cryostructure, suggesting that the pores at the 5-fold axes might be functionally smaller than 10 nm.

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Supporting Information Available. Details on experimental procedures and data of TEM, DLS, agarose gel electrophoresis, and mass spectrometry analyses of chemically modified mutants are available. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes


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