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Using the Interior Cavity of the P22 Capsid for Site Specific Initiation of Atom Transfer Radical Polymerization with Tremendously Increased Cargo Loading

Janice Lucon^{1,2}, Shefah Qazi^{1,2}, Masaki Uchida^{1,2}, Gregory J. Bedwel³, Ben LaFrance^{1,2}, Peter E. Prevelige Jr.³, and Trevor Douglas^{1,2,*}

¹Chemistry and Biochemistry Department, Montana State University, Bozeman, Montana, 59717

²Center for Bio-Inspired Nanomaterials, Montana State University, Bozeman, Montana, 59717

³Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama, 35294

Abstract

Virus-like particles (VLPs) have emerged as important and versatile architectures for chemical manipulation in the development of functional hybrid nanostructures. Here we have successfully demonstrated the site selective initiation of atom transfer radical polymerization (ATRP) reactions to form an addressable polymer constrained within the interior cavity of a VLP. This protein-polymer hybrid, of P22 and crosslinked poly(2-aminoethyl methacrylate), is potentially useful as a new high-density delivery vehicle for encapsulation and delivery of small molecule cargos. In particular, the encapsulated polymer can act as a scaffold for the attachment of primary amine reactive molecules of interest, such as a fluorescein dye or a Gd-DTPA MRI contrast agent. Using this approach, a significant increase in labeling density of the VLP, compared to previous modifications of VLPs, can be achieved. These results highlight the use of multimeric protein-polymer conjugates for their potential utility in the development of VLP-based MRI contrast agents with the possibility of loading other cargos.

Introduction

The use of protein-polymer composite materials for medical and materials applications is a growing field, which aims to take advantage of the exquisite monodispersity and bioactivity of biomolecules while imparting new materials properties via polymer conjugation. When a responsive polymer is selected, new thermo, light, and pH sensitive macromolecular materials can be produced to more fully control the activity and phase solubility of the biomolecule.^{1,2} By adding a specific polymer to the biomolecule, the composite material may exhibit improved retention, lowered immunogenicity, and increased bioavailability.³⁻⁵ To attain the desired final material properties, the careful selection of both the protein and polymer components is essential. Much of this work has been focused on site-specific conjugation of polymers to monomeric proteins, but when more complex multimeric biomolecules are employed not only is the polymer location on the primary sequence of

*Corresponding Author: To whom correspondence should be addressed. Phone: (406) 994-6566. Fax: (406) 994-5117. tdouglas@chemistry.montana.edu.

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interest, but also the spatial relationship between the polymer and the overall protein architecture becomes increasingly important.

In particular, the use of virus-like particle (VLP) proteins, which are a special class of multimeric proteins that form symmetric protein shells surrounding an empty interior space, relies on two distinct environments that can be modified - either the exposed exterior or the confined interior. Utilizing the exterior surface, polymer formation or attachment has been employed as a means of appending molecules of interest designed to alter VLP solubility, increase stability, or introduce new functionalities.^{6,7} Electrostatic interactions have been used in several systems to package existing polymers or guide capsid assembly around polymers and polymer-nanoparticle composites providing a charge dependent occupation of the interior space.⁸⁻¹³ Synthesis of polymers in the interior space has previously been limited to small protein cages, which have been employed as a synthesis chamber for an untethered oligomer or for the development of an anchored addressable network.^{14,15}

We previously reported using azide-alkyne 'click' chemistry to construct an anchored polymer network inside a small protein cage architecture.¹⁵⁻¹⁷ In this step-wise synthesis approach, polymer growth was directed to the protein cage interior, resulting in a protein-confined hyperbranched polymer. The protein shell acted as a barrier, limiting polymer size and leaving only the protein exterior exposed to the bulk solution. By labeling the resulting protein-polymer construct with a Gd-based MRI contrast agent, an enhanced magnetic resonance contrast agent was obtained, highlighting the utility of using the interior space to maximize cargo loading.¹⁶ While this method is effective, the step-wise nature of the polymerization reaction makes this process onerous for larger constrained polymer synthesis.

An alternative route to achieving an anchored, addressable polymer would preferably proceed via a continuous polymerization of simple monomers from an easily modified initiator. Of the several suitable continuous biomolecule-anchored polymerization methods, we chose to use atom transfer radical polymerization (ATRP) as it is particularly suited for improved formation of polymer inside a protein cage. This method is not only rapid, but also results in products with relatively low polydispersity in bulk solutions and is promiscuous with respect to the range of monomers that can be used. Also, the simplicity of the ATRP initiator means that it can be readily attached to the protein cage in a site-specific manner, thereby controlling the site of polymer initiation. Thus, by combining ATRP with a container-like protein, the formation of a polymer scaffold constrained to the interior of a VLP architecture can be afforded in a single short reaction.

Here we report the use of ATRP to make addressable polymer networks within the confines of the bacteriophage P22 based VLP (Figure 1). The 2-aminoethyl methacrylate (AEMA) monomer was selected because the primary amine-rich polymer synthesized within the P22 capsid could be subsequently modified with small molecules of interest resulting in very high density loading of the capsid. The use of the AEMA network as a scaffold was demonstrated through the attachment of either fluorescein isothiocyanate (FITC) or Gd-DTPA-NCS. Using this method, we can achieve a substantial increase in the degree of labeling per VLP compared to previous reports, demonstrating the potential capacity of the capsid interior for directed cargo loading.

Results and Discussion

In this work we have utilized a VLP, derived from the bacteriophage P22, which consists of 420 subunits arranged on an icosahedral lattice with a resulting exterior diameter of 64 nm and an unoccupied internal cavity 54 nm in diameter.¹⁸ Recombinant expression in *E. coli* requires co-expression of the coat protein and scaffold protein for self-assembly. This VLP

is capable of transformation into a series of distinct morphologies including the procapsid (PC) form, which contains scaffold protein, an empty shell (ES) form, where the scaffold protein has been removed, an expanded form (EX), and a wiffleball structure (WB) where all 12 pentamers have been removed (Supplementary Figure S1).^{19,20} The EX form most closely mimics the morphology found in the DNA-containing infectious virion and is the form used in this study.²⁰ To attain the EX form, the scaffold protein is removed from the PC using successive guanidine-HCl extractions followed by heating at 65 °C which generates the capsid in its EX morphology.

A new P22 mutant was designed, for use with ATRP, containing a single point mutation in the wild-type coat protein, P22_{S39C}, introducing an addressable thiol suitable for attachment of an initiator (**1**). This site was specifically selected such that the introduced thiol is exposed exclusively to the interior according to the currently available P22 structural models (Figure 2).^{19,21} Although the wild-type protein contains an intrinsic cysteine (C405) it was not removed, as previous studies have demonstrated that this site is not addressable.^{22,23} This new mutant has been characterized, and behaves in the same manner as the wild-type P22 capsid, going through the same series of morphological transformations. To obtain the EX morphology, the scaffold protein was removed from P22_{S39C} to generate the empty shell (ES), heated to 65°C, and subsequently analyzed to ensure formation of the EX form (Figure 3). The characteristic shift of the particles to lower electrophoretic mobility was observed upon heating, consistent with expansion of the capsid. Precipitation of the protein was only observed at temperatures greater than 80 °C, indicating that the protein architecture is relatively thermostable (Supplementary Figure S2). The size of the VLP, by dynamic light scattering (DLS), was found to increase as expected from 60 ± 4 nm (PC morphology) to 71 ± 5 nm, consistent with the known range of values for the P22 particle in either the EX or WB morphologies (Figure 3D). By transmission electron microscopy (TEM) the overall structure of the VLP was retained and after heating to 75 °C and large pores in the structure became apparent (Figure 3C), which is a characteristic of the WB morphology.

To make the P22 macroinitiator, the P22_{S39C} mutant was labeled with initiator **1**, an amide derivative of a previously reported ATRP initiator. This cysteine reactive ATRP initiator (Figure 1) was selected because of its efficient labeling, satisfactory initiation, and demonstrated compatibility with biomolecules. Initiator **1**, unlike the ester containing form previously report, is expected to be less susceptible to bond cleavage.^{24,25} It was synthesized through modification of established protocols and selectively reacted with P22_{S39C} to make, P22_{S39C}-int with near quantitative single labeling of the introduced cysteine as observed by subunit mass spectrometry (Supplementary Figure S3).

Using the P22_{S39C}-int macroinitiator construct, crosslinked AEMA polymer strands were synthesized inside this protein cage under standard ATRP biomolecule conditions using a Cu(I)/bpy catalyst.²⁶ To explore the range of reaction conditions available to the P22_{S39C} system for internally directed polymerization reactions, a selection of AEMA:bisacrylamide monomer to subunit ratios (3,000 to 26,000) and temperatures (23, 40, 60 °C) as well as catalyst loading ratios and were initially investigated. Samples were monitored by gel electrophoresis and dynamic light scattering to determine reaction completion and suitable conditions (an example comparison is shown in Supplementary Figure S4). The purified protein polymer-hybrid constructs were stable after polymerization and only at the highest temperature and loading conditions did the diameter of the cage increase significantly, indicating that under most of the tested conditions the VLP effectively constrains the polymer growth to the interior of the capsid. From these test reactions it was apparent that the monomer loading had a greater impact on the extent of polymerization than the temperature of the reaction, and that the reactions were effectively complete after less than 3 hrs.

To further verify that our selected P22 mutant was confining the polymer we compared the behavior of P22_{S39C} to a second P22 coat protein mutant (P22_{K118C}), which has been described previously and which has a reactive cysteine site predicted to be partially exposed to the exterior (Supplementary Figure S5).²² Both mutants were labeled with an ATRP initiator (Supplementary Figure S6, S7) and analyzed under simple polymerization conditions. Using uncrosslinked AEMA at a loading of 26,000 monomers/subunit the partially exterior exposed P22_{K118C}, resulted in dramatically different material properties compared to the P22_{S39C} construct under the same conditions emphasizing the importance of site specificity for polymer initiation and growth in these protein architectures. When the initiator labeled P22_{K118C} was treated to make P22_{K118C}-AEMA, the construct exhibited an increase in diameter of about 20 nm, from 60 ± 3 nm to 81 ± 4 nm, as measured by DLS suggesting growth of polymer on the exterior of the cage (Supplementary Figure S8), while the P22_{S39C} based sample diameter remained constant before (67 ± 4 nm) and after (67 ± 4 nm) polymerization. In addition, unlike the P22_{S39C}-AEMA, the P22_{K118C}-AEMA hybrid was not very stable. While no precipitation was observed under the reaction conditions, mass precipitation of the P22_{K118C}-AEMA occurred upon purification. This loss could be partially alleviated by increasing the ionic strength of the buffer (> 250 mM NaCl). Taken together these data support a model where the initiation site determines the overall access of the AEMA polymer to the exterior environment and when polymer growth is exposed to the exterior of the cage, the protein-polymer composite is significantly destabilized.

We directed our efforts to the interior facing P22_{S39C}-int macroinitiator construct at a midrange monomer loading (6,000 monomers/subunit) and moderate temperature conditions (23°C) for further investigation with 4 experimental replicates. After 3 hr reaction time, the synthesis was halted by exposing each sample to air and the protein-polymer conjugate (P22_{S39C}-xAEMA) was purified away from remaining AEMA and bis-acrylamide monomers and the copper catalyst by pelleting the protein construct using ultracentrifugation, which easily separates large macromolecular complexes from small molecular species. The resulting construct exhibited a dramatic shift in electrophoretic mobility by native agarose gel indicating that the P22-int had become P22-xAEMA (Figure S9). On a subunit basis an increased subunit mass by denaturing gel analysis, as indicated by a shift to higher molecular mass was observed (Figure 4A and Supplementary Figure S9). By this analysis method it appears that not all of the initiator labeled subunits produce sufficiently long polymer chains for a mass shift to be apparent. Others have also observed this incomplete initiation in both monomeric and multimeric systems when making grafted-from protein-polymer composites.^{6,24-26}

To confirm that the polymer was confined to the interior of the P22, the size of the P22_{S39C}-xAEMA construct was compared to the initial macroinitiator P22 complex. When the particles were visualized using transmission electron microscopy (TEM) the morphology of the P22 was unchanged before and after the reaction (Figure 4B). The average particle diameter was 51 ± 3 nm in the unpolymerized P22_{S39C}-int and remained the same (53 ± 3 nm) after the reaction. In addition, the hydrodynamic diameter, as measured by dynamic light scattering (DLS), remained unchanged upon polymer formation (Figure 4C). The particle diameters were 70 ± 10 nm and 71 ± 3 nm respectively for the P22_{S39C}-int and P22_{S39C}-xAEMA further confirming that the polymer is confined to the interior of the protein cage.

Multi-angle light scattering (MALS) was used to further analyze the P22_{S39C}-xAEMA construct for radius and molecular weight (Figure 5). According to this method the radius of the particles remains nearly constant, measuring at 29.3 nm (P22_{S39C}-int) and 28.2 nm (P22_{S39C}-xAEMA), while the molecular weight increases upon polymerization. Using the standard dn/dc for protein (0.185) and the published value for AEMA (0.153)²⁷ the multi-angle light scattering data from the P22_{S39C}-int and P22_{S39C}-xAEMA samples were fit to

obtain molecular weights. The measured molecular weight for P22_{S39C}-int was 18.9 ± 0.2 MDa, consistent with the predicted value for the EX morphology of P22, while the P22_{S39C}-xAEMA had a combined molecular weight of 20.2 ± 0.6 MDa, with 18.7 ± 0.5 MDa contributed from protein and 1.5 ± 0.4 MDa contributed from polymer. This mass increase corresponds to the addition of $12,000 \pm 3000$ AEMA monomers/VLP or 28 ± 7 AEMA monomers/subunit on average.

The internally directed P22_{S39C}-xAEMA polymerization resulted in the introduction of a large number of addressable amines sequestered within the protein cage. To demonstrate that the introduced amines on the poly-AEMA inside P22_{S39C}-xAEMA construct were addressable, FITC (**2**) was used as an amine specific labeling agent. Both the P22_{S39C}-xAEMA and P22_{S39C}-int control were incubated with 100 fold excess of FITC per subunit to give P22_{S39C}-xAEMA-FITC and P22_{S39C}-FITC, respectively. Excess FITC was removed by pelleting the protein twice using ultracentrifugation followed by resuspension before analysis. The difference in the degree of labeling between the P22_{S39C}-FITC and P22_{S39C}-xAEMA-FITC was significant enough to be readily discernable (Supplementary Figure S10).

When the fluorescently labeled P22_{S39C}-xAEMA construct was analyzed using gel electrophoresis the fluorescein signal was observed to co-migrate with the protein. Under denaturing SDS-PAGE conditions, the fluorescein migrated with the subunit and the polymer-modified subunit, indicating that the fluorescent dye was covalently bound to the construct and not just sorbed onto the protein-polymer composite (Supplementary Figure S11). When analyzed by native agarose gel electrophoresis, a net shift in the electrophoretic mobility of the P22_{S39C}-xAEMA and P22_{S39C}-xAEMA-FITC was observed due to the polymer and FITC altering the charge of the construct (Figure 6A). The observed shift indicates that the polymer and dye were tightly associated with the protein cage. The P22_{S39C}-xAEMA-FITC was labeled to the extent that the migration of a distribution of species was visible prior to staining under ambient light, while P22_{S39C}-FITC was only weakly observed (Figure 6B). In contrast, the emission signal of the P22_{S39C}-xAEMA-FITC distribution was depressed in intensity compared to the much less heavily labeled P22_{S39C}-FITC (Figure 6C), which is likely due to self quenching of the fluorophore due to the abundance of polymer bound fluorescein in close proximity. This apparent loss of fluorescence intensity was confirmed by solution phase analysis of the constructs where a greater than 95% reduction in fluorescence was observed in the P22_{S39C}-xAEMA-FITC compared to P22_{S39C}-FITC (Supplementary Figure S12). Similar quenching has been shown in systems with high fluorophore concentrations, such as in micelles, where derivatives of fluorescein can lose greater than 98% of their fluorescence signal due to proximity and dimerization effects.^{28,29}

Having demonstrated polymerization within the P22 capsid and the post-synthetic modification of the functional groups, we have explored the P22_{S39C}-xAEMA composite system as a potential T₁ contrast agent through the attachment of Gd-DTPA-NCS (**3**) to the encapsulated polymer. Gd-DTPA-NCS was added, in 100 fold excess per subunit, to the P22_{S39C}-AEMA or P22_{S39C}-int and allowed to react overnight followed by pelleting by ultracentrifugation and resuspension of the protein 2 times in each of 4 experimental replicates. The resulting material (P22_{S39C}-xAEMA-Gd or P22_{S39C}-int-Gd) was analyzed for both sulfur and gadolinium content by ICP to determine both the Gd and protein concentrations. To rule out the possibility of simple electrostatic interaction between the polymer and Gd-DTPA, controls with both P22_{S39C}-xAEMA and P22_{S39C}-int were incubated and isolated, under the same conditions as above, with Magnevist® (Gd-DTPA), which lacks the amine reactive isothiocyanate. When these constructs were analyzed by native agarose gel electrophoresis (Supplementary Figure S13), a net shift in the

electrophoretic mobility of the P22_{S39C}-xAEMA-Gd was observed due to the polymer and Gd-DTPA-NCS altering the net charge of the construct, while the P22_{S39C}-xAEMA-Magnevist sample retained the same low electrophoretic mobility of the P22_{S39C}-xAEMA providing evidence of no significant electrostatic interactions between P22_{S39C}-xAEMA and Gd-DTPA.

The Gd-DTPA loading per P22 was determined quantitatively for each of the constructs from the ICP data. The P22_{S39C}-xAEMA-Gd contained 28 times more Gd/VLP than the P22_{S39C}-int-Gd control (320 Gd/VLP, <1 Gd/subunit), where endogenous lysines were modified, indicating that it is largely the polymer that is being labeled rather than the protein shell. The low reactivity of the endogenous P22 lysines has been observed previously where only a few of the 20 lysines per subunit were observed to be reactive.³⁰ This minimal background reactivity is advantageous because it means that in the P22_{S39C}-AEMA sample the vast majority (>95%) of the addressable sites are located on the encapsulated polymer. If necessary, endogenous lysines could be chemically blocked prior to polymerization.¹⁵ The P22_{S39C}-xAEMA-Gd loading per cage was found to be $9,100 \pm 800$ Gd/VLP (22 ± 2 Gd/subunit), corresponding to an internal concentration of 150 mM Gd within the VLP. This is significantly more Gd, both on a per-cage and per-subunit basis, than previous reports using VLPs, whose values range from less than 1.0/subunit to 6.6/subunit (60/VLP to 650/VLP) and highlights the advantage of using the full capacity of the interior volume.^{6,16,31-37} In addition, both the P22_{S39C}-xAEMA and P22_{S39C}-int incubated with Magnevist® (Gd-DTPA) contained Gd levels below the lower limit of quantification demonstrating that the Gd detected is covalently attached to the protein-polymer construct rather than associating via electrostatic interactions under the labeling reaction conditions.

To verify that the high loading observed was reasonable and occurred homogeneously across the population of P22 capsids, the particles were analyzed by analytical ultracentrifugation to investigate differences in sedimentation velocity (Figure 7). The measured sedimentation value of the P22_{S39C}-xAEMA (167 S) falls within the range observed for P22_{S39C}-int (142 S) and the scaffold protein containing PC morphology (191 S), indicating that the polymer content does not exceed the packing observed in the naturally occurring self-assembled system. Covalent modification of polymer with Gd-DTPA-NCS to make P22_{S39C}-xAEMA-Gd results in a shift to higher S value (227 S), which is consistent with the efficient incorporation of additional mass via Gd-DTPA-NCS to the interior of the VLP.

The ¹H relaxivity of water in the presence of these highly loaded P22_{S39C}-xAEMA-Gd particles and P22_{S39C}-int-Gd control was measured at 60 MHz (1.4 T), using an inversion recovery pulse sequence. The P22_{S39C}-xAEMA-Gd and P22_{S39C}-int-Gd were found to have ionic relaxivities (r_1) of $22.0 \text{ mM}^{-1}\text{s}^{-1}$ and $23.5 \text{ mM}^{-1}\text{s}^{-1}$, respectively. The observed improvement in r_1 for both constructs is consistent with enhancement over free Gd-DTPA ($4.0 \text{ mM}^{-1}\text{s}^{-1}$) corresponding to an increase in rotational correlation time arising from tethering the chelate to a large supramolecular particle.^{16,35} In addition, because the ionic relaxivity is similarly enhanced in both constructs it can be concluded that the polymer does not significantly restrict water exchange between bulk and the interior of the P22_{S39C}-xAEMA-Gd composite as water restriction would lessen the observed enhancement such as is observed in some micelles and liposomes.^{38,39}

In addition to the ionic relaxivity enhancement, each P22_{S39C}-xAEMA-Gd carries $9,100 \pm 800$ Gd/VLP leading to a per particle relaxivity of $200,000 \text{ mM}^{-1}\text{s}^{-1}$. The particle relaxivity of P22_{S39C}-xAEMA-Gd dramatically exceeds both the P22_{S39C}-int-Gd control ($7,500 \text{ mM}^{-1}\text{s}^{-1}$) and previous reports of VLP nanoparticle based MRI contrast agents, whose values fall in the range of 10^3 - $10^4 \text{ mM}^{-1}\text{s}^{-1}$.^{6,16,31-37} The particle relaxivity is also higher

than observed for many other macromolecular assemblies such as micelles, liposomes, and polymers, placing this construct comfortably in the upper end of observed macromolecular relaxivities for its size.³⁸⁻⁴²

Conclusions

In summary, the application of ATRP for site-directed polymer formation inside a VLP results in an anchored network that is unparalleled for labeling purposes and utilizes the previously largely untapped interior volume of the VLP. The P22_{S39C}-based macroinitiator effectively directs polymer growth to the VLP interior, resulting in confined polymer growth as the protein shell acts as a barrier to unconstrained polymer growth, leaving only the protein shell exposed to the bulk solution. By selecting an appropriate macroinitiator and monomer this new multimeric protein-polymer composite acts as a scaffold for the attachment of small molecules of interest such as the fluorophore (FITC) or paramagnetic MRI contrast agents (Gd-DTPA-NCS). The introduced polymer scaffold results in a significantly increased number of labels per cage compared to other VLP based systems. The improvement in labeling is important for the delivery of contrast agent on a per-particle basis allowing for high concentration delivery of contrast agent or other cargo molecules of interest. This material exhibits an order of magnitude improvement, in relaxivity per particle, over existing VLP systems. The use of this material as a targeted MRI contrast agent is particularly interesting because of the high relaxivity and is an application we are exploring further. Due to the simplicity, modular nature, and loading level of the ATRP based approach taken to make these P22-polymer internal composites, this same method is currently being employed to make a range of novel VLP-polymer composites with biomedical and catalytic applications.

Methods

All materials were analytical grade and purchased from either Sigma-Aldrich or Fisher Scientific and used as received unless otherwise noted. Dichloromethane was distilled over calcium hydride prior to use. All water was deionized using a NANOpure water purification system. Dynamic light scattering measurements were taken on a 90Plus particle size analyzer (Brookhaven) and the data was processed by volume distribution.

Synthesis of 2-bromoisobutyryl aminoethyl maleimide (1)

1 was synthesized by a modification of the procedures previously reported.^{24,25} *N*-2-aminoethyl-maleimide (250 mg, 0.98 mmol) was mixed on ice with triethylamine (300 μ l, 2.2 mmol) in 5 ml dry dichloromethane. 2-bromo-2-methylpropionylbromide (200 μ l, 1.6 mmol) was added dropwise. The reaction was allowed to warm to room temperature and was subsequently extracted 3 times from dichloromethane and water followed by drying over anhydrous sodium sulfate. The crude product was cleaned via column chromatography (silica gel, 10% ethyl acetate in dichloromethane) with a yield of 80%. ¹H NMR (500 MHz, CDCl₃) δ = 1.89 (s, 6H, CH₃); 3.46 (dd, *J* = 5.5, 5.5, 2H, NCH₂); 3.72 (dd, *J* = 5.0, 1.5, 2H, NCH₂); 6.71 (s, 2H, CH_{vinyl}), 6.97 (s, NH). ¹³C δ = 32.54 (CH₃), 37.34 (NCH₂), 39.84 (NCH₂), 62.51 (C), 134.59 (CH_{vinyl}), 170.96 (CO), 172.74 (CO). ESI-MS: *m/z* calculated 289.0188, 291.0167 (MH⁺), found *m/z* 289.0192, 291.0179.

P22-int Macroinitiator Formation Conditions

P22_{S39C} in PBS, pH 7.6 (4 ml, 7.2 mg/ml) was infused with 156 μ l of **1** (80 mM in DMSO, 10 fold excess per a subunit). The mixture was allowed to react for 3 hrs at room temperature. After 3 hrs the reaction was quenched with DTT (156 μ l, 80 mM in water). To remove excess DTT and **1**, the protein was pelleted at 48,000 rpm for 50 minutes in an ultra

centrifuge (Sorvall) followed by resuspension into PBS, pH 7.6. By subunit mass spectrometry >95% of the subunits were labeled. Yield: quantitative.

P22-AEMA Polymer Formation Conditions

Each experimental replicate was made in a large crimp-top vial with the addition of 20 mL monomer solution (4 wt% 16:4 AEMA:bis-acrylamide in PBS, pH 8.0) and 11 mL buffer (PBS, pH 8.0) followed by pH adjustment with concentrated sodium hydroxide solution as needed back to pH 8.0. To this mixture, 6 mL of P22-int (8.0 mg/mL, 1.0 μ moles subunit, pH 8.0) was added followed by pump and back filling with Ar 4 times to deaerate the mixture. The metal catalyst solution was made in a second crimp vial where 19.2 mg CuBr (0.13 mmoles), 29.9 mg CuBr₂ (0.13 mmoles), and 83.5 mg 2,2'-bipyridine (0.53 mmoles) followed by the addition of 10 mL deionized water which had been degassed by bubbling Ar through the liquid for 20 minutes. The vial was subsequently sonicated for 5 min to obtain a dark brown solution. To the monomer-protein vial, 3 mL of the metal catalyst solution was added and the vial was maintained at 23 °C for the remaining duration of the experiment. After 3 hr the reaction was quenched by exposure to air and the protein-polymer composite was purified away from unreacted monomer and the copper catalyst by pelleting and resuspending the protein 2 times into 100 mM sodium carbonate with 50 mM NaCl buffer, pH 9.0. Yield: 38.7 mg (81%).

FITC (2) Labeling Conditions

To 2.0 mL P22_{S39C}-xAEMA (0.086 μ moles subunit, carbonate buffer, pH 9.0) at 2 mg/mL, 343 μ L FITC (8.6 mmoles, 25 mM in DMSO) was added dropwise while vortexing the protein solution. The mixture was allowed to sit overnight at 4 °C followed by purification away from excess FITC by pelleting and resuspending (carbonate buffer, pH 9.0) the protein twice over.

Gd-DTPA-NCS (3) Labeling Conditions

Gd-DTPA-NCS was made according to established procedures.¹⁶ Briefly, 2.80 mg (4.31 μ moles) DTPA-NCS was dissolved in 40 μ L of 1M sodium bicarbonate and 200 μ L water. Once the DTPA-NCS was completely dissolved, 5.27 μ L GdCl₃ (900 mM in water, 4.74 μ moles) was added to the solution and stirred for 3 hours at room temperature. The solution was subsequently diluted to 10 mM with DMSO (186 μ L) and added to the protein-polymer conjugate as follows. To 4.0 mL (0.17 μ moles subunit, 2 mg/mL carbonate buffer, pH 9.0) P22_{S39C}-AEMA, 1700 μ L (17 μ moles) Gd-DTPA (10 mM in DMSO/H₂O) was added dropwise while vortexing the protein solution. The mixture was allowed to sit overnight at 4 °C followed by purification away from excess Gd-DTPA by pelleting and resuspending the protein twice. Yield: 5.2 mg (65%).

Multi-Angle Light Scattering

P22-int and P22-xAEMA samples were separated over a WTC-0100S (Wyatt Technologies) size exclusion column at flow rate of 0.7 mL/min (Agilent 1200 HPLC) in 50 mM phosphate, 500 mM sodium chloride, pH 8.0. Each sample injection was 25 μ L and the samples were run in triplicate. Elution profiles were for mass analysis were detected using a UV-Vis detector (Agilent), a Wyatt HELEOS multi angle light scattering detector, and a Wyatt Optilab rEX differential refractometer. Using the elution profile data the number average molecular weight, M_n , was calculated with Astra 5.3.14 software (Wyatt Technologies) using a dn/dc for protein of 0.185 and a dn/dc for the polymer component of 0.153.²⁷

Protein and Gd Concentration

The protein concentration was determined by analyzing samples for sulfur content and Gd concentration by inductively couple plasma mass spectrometry (ICP-MS) at Energy Labs (Billings, MT). Samples were submitted in triplicate and average concentrations were reported. The protein concentration was obtained by subtracting from the detected sulfur concentration the Gd contribution due to sulfur in the DTPA-NCS chelator. The remaining sulfur content was converted to subunit concentration using a conversion factor of 13 thiols per a subunit (the initial Met in the P22 coat sequence is never present in the final protein according to mass spectrometry).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Grover GN, Maynard HD. Protein-polymer conjugates: Synthetic approaches by controlled radical polymerizations and interesting applications. *Curr Opin Chem Biol.* 2010; 14:818–827. [PubMed: 21071260]
2. Krishna OD, Kiick KL. Protein- and peptide-modified synthetic polymeric biomaterials. *Biopolymers.* 2010; 94:32–48. [PubMed: 20091878]
3. Thordarson P, Le Droumaguet B, Velonia K. Well-defined protein-polymer conjugates-synthesis and potential applications. *Appl Microbiol Biotechnol.* 2006; 73:243–254. [PubMed: 17061132]
4. Depp V, Alikhani A, Grammer V, Lele BS. Native protein-initiated ATRP: A viable and potentially superior alternative to PEGylation for stabilizing biologics. *Acta Biomater.* 2009; 5:560–569. [PubMed: 18804423]
5. Klok HA. Peptide/protein-synthetic polymer conjugates: Quo vadis. *Macromolecules.* 2009; 42:7990–8000.
6. Pokorski JK, Breitenkamp K, Liepold LO, Qazi S, Finn MG. Functional virus-based polymer–protein nanoparticles by atom transfer radical polymerization. *J Am Chem Soc.* 2011; 133:9242–9245. [PubMed: 21627118]
7. Schlick TL, Ding ZB, Kovacs EW, Francis MB. Dual-surface modification of the tobacco mosaic virus. *J Am Chem Soc.* 2005; 127:3718–3723. [PubMed: 15771505]
8. de la Escosura A, Nolte RJM, Cornelissen J. Viruses and protein cages as nanocontainers and nanoreactors. *J Mater Chem.* 2009; 19:2274–2278.
9. Aniagyei SE, DuFort C, Kao CC, Dragnea B. Self-assembly approaches to nanomaterial encapsulation in viral protein cages. *J Mater Chem.* 2008; 18:3763–3774. [PubMed: 19809586]
10. Dixit SK, et al. Quantum dot encapsulation in viral capsids. *Nano Lett.* 2006; 6:1993–1999. [PubMed: 16968014]
11. Comellas-Aragones M, et al. Controlled integration of polymers into viral capsids. *Biomacromolecules.* 2009; 10:3141–3147. [PubMed: 19839603]
12. Douglas T, Young M. Host-guest encapsulation of materials by assembled virus protein cages. *Nature.* 1998; 393:152–155.
13. Hu YF, Zandi R, Anavitarte A, Knobler CM, Gelbart WM. Packaging of a polymer by a viral capsid: The interplay between polymer length and capsid size. *Biophys J.* 2008; 94:1428–1436. [PubMed: 17981893]

14. Abe S, et al. Polymerization of phenylacetylene by rhodium complexes within a discrete space of apo-ferritin. *J Am Chem Soc.* 2009; 131:6958–6960. [PubMed: 19453195]
15. Abedin MJ, Liepold L, Suci P, Young M, Douglas T. Synthesis of a cross-linked branched polymer network in the interior of a protein cage. *J Am Chem Soc.* 2009; 131:4346–4354. [PubMed: 19317506]
16. Liepold LO, et al. Supramolecular protein cage composite MR contrast agents with extremely efficient relaxivity properties. *Nano Lett.* 2009; 9:4520–4526. [PubMed: 19888720]
17. Lucon J, et al. A click chemistry based coordination polymer inside small heat shock protein. *Chem Commun.* 2010; 46:264–266.
18. Earnshaw W, Casjens S, Harrison SC. Assembly of head of bacteriophage P22: x-ray diffraction from heads, proheads and related structures. *J Mol Biol.* 1976; 104:387–410. [PubMed: 781287]
19. Parent KN, et al. P22 coat protein structures reveal a novel mechanism for capsid maturation: Stability without auxiliary proteins or chemical crosslinks. *Structure.* 2010; 18:390–401. [PubMed: 20223221]
20. Teschke CM, McGough A, Thuman-Commike PA. Penton release from P22 heat-expanded capsids suggests importance of stabilizing penton-hexon interactions during capsid maturation. *Biophys J.* 2003; 84:2585–2592. [PubMed: 12668466]
21. Chen DH, et al. Structural basis for scaffolding-mediated assembly and maturation of a dsDNA virus. *Proc Natl Acad Sci U S A.* 2011; 108:1355–1360. [PubMed: 21220301]
22. Kang S, et al. Implementation of P22 viral capsids as nanoplatfoms. *Biomacromolecules.* 2010; 11:2804–2809. [PubMed: 20839852]
23. Tuma R, Prevelige PE, Thomas GJ. Mechanism of capsid maturation in a double-stranded DNA virus. *Proc Natl Acad Sci U S A.* 1998; 95:9885–9890. [PubMed: 9707570]
24. Mantovani G, et al. Design and synthesis of N-maleimido-functionalized hydrophilic polymers via copper-mediated living radical polymerization: A suitable alternative to PEGylation chemistry. *J Am Chem Soc.* 2005; 127:2966–2973. [PubMed: 15740133]
25. Heredia KL, et al. In situ preparation of protein: “Smart” polymer conjugates with retention of bioactivity. *J Am Chem Soc.* 2005; 127:16955–16960. [PubMed: 16316241]
26. Peeler JC, et al. Genetically encoded initiator for polymer growth from proteins. *J Am Chem Soc.* 2010; 132:13575–13577. [PubMed: 20839808]
27. Alidedeoglu AH, York AW, Rosado DA, McCormick CL, Morgan SE. eBioconjugation of D-Glucuronic Acid Sodium Salt to Well-Defined Primary Amine-Containing Homopolymers and Block Copolymers. *J Polym Sci, Part A: Polym Chem.* 2010; 48:3052–3061.10.1002/pola.24083
28. Weinstein JN, Yoshikami S, Henkart P, Blumenthal R, Hagins WA. Liposome-cell interaction: Transfer and intracellular release of a trapped fluorescent marker. *Science.* 1977; 195:489–492. [PubMed: 835007]
29. Chen RF, Knutson JR. Mechanism of fluorescence concentration quenching of carboxyfluorescein in liposomes: Energy transfer to nonfluorescent dimers. *Anal Biochem.* 1988; 172:61–77. [PubMed: 3189776]
30. Kang S, Hawkrige AM, Johnson KL, Muddiman DC, Prevelige PE. Identification of subunit-subunit interactions in bacteriophage P22 procapsids by chemical cross-linking and mass spectrometry. *J Proteome Res.* 2006; 5:370–377. [PubMed: 16457603]
31. Allen M, et al. Paramagnetic viral nanoparticles as potential high-relaxivity magnetic resonance contrast agents. *Magn Reson Med.* 2005; 54:807–812. [PubMed: 16155869]
32. Liepold L, et al. Viral capsids as MRI contrast agents. *Magn Reson Med.* 2007; 58:871–879. [PubMed: 17969126]
33. Anderson EA, et al. Viral nanoparticles donning a paramagnetic coat: Conjugation of MRI contrast agents to the MS2 capsid. *Nano Lett.* 2006; 6:1160–1164. [PubMed: 16771573]
34. Prasuhn DE, Yeh RM, Obenaus A, Manchester M, Finn MG. Viral MRI contrast agents: coordination of Gd by native virions and attachment of Gd complexes by azide-alkyne cycloaddition. *Chem Commun.* 2007:1269–1271.
35. Datta A, et al. High relaxivity gadolinium hydroxypyridonate-viral capsid conjugates: Nanosized MRI contrast agents. *J Am Chem Soc.* 2008; 130:2546–2552. [PubMed: 18247608]

36. Hooker JM, Datta A, Botta M, Raymond KN, Francis MB. Magnetic resonance contrast agents from viral capsid shells: A comparison of exterior and interior cargo strategies. *Nano Lett.* 2007; 7:2207–2210. [PubMed: 17630809]
37. Dunand FA, Borel A, Helm L. Gd(III) based MRI contrast agents: improved physical meaning in a combined analysis of EPR and NMR data? *Inorg Chem Commun.* 2002; 5:811–815.
38. Mulder WJM, Strijkers GJ, van Tilborg GAF, Griffioen AW, Nicolay K. Lipid-based nanoparticles for contrast-enhanced MRI and molecular imaging. *NMR Biomed.* 2006; 19:142–164. [PubMed: 16450332]
39. Ghaghada KB, et al. New dual mode gadolinium nanoparticle contrast agent for magnetic resonance imaging. *PLoS One.* 2009; 4:1–7.
40. Karfeld-Sulzer LS, Waters EA, Davis NE, Meade TJ, Barron AE. Multivalent protein polymer MRI contrast agents: Controlling relaxivity via modulation of amino acid sequence. *Biomacromolecules.* 2010; 11:1429–1436. [PubMed: 20420441]
41. Schuhmanngiampieri G, Schmittwillich H, Frenzel T, Press WR, Weinmann HJ. In vivo and in vitro evaluation of Gd-DTPA-polylysine as a macromolecular contrast agent for magnetic resonance imaging. *Invest Radiol.* 1991; 26:969–974. [PubMed: 1743920]
42. Ananta JS, et al. Geometrical confinement of gadolinium-based contrast agents in nanoporous particles enhances T(1) contrast. *Nature Nanotech.* 2010; 5:815–821.

Figure 1. Schematic of the internally initiated ATRP polymerization within the P22 VLP
P22_{S39C} modified with a cysteine reactive ATRP initiator (**1**) was used as macroinitiator and a size constrained reaction vessel for ATRP growth of poly(AEMA) strands, crosslinked with bis-acrylamide, inside the P22 VLP architecture. This internal polymer scaffold was subsequently modified with primary amine reactive labeling agents, either (**2**) or (**3**), to introduce a high density of new functionality to the construct.

Figure 2. Structural model of the P22 capsid expanded morphology showing the location of the S39C mutation

The location of the modified residue, S39C (in red), has been derived from a structural model of P22 using coordinate data²¹ deposited as PDB file 2XYZ. Both a view of the exterior of the capsid (top) and a half shell cut-away view revealing the interior (bottom) of the capsid are included illustrating that according to this model this mutation site is interior exposed.

Figure 3. Characterization of the P22_{S39C} mutant to verify morphological transformation
 A) SDS-PAGE of the purified P22 before (PC) and after (ES) extraction of the scaffold protein. The lower band corresponds to scaffold (33.56 kDa), while the upper band is coat protein (46.64 kDa). L is the molecular weight standard. B) Native agarose gel of the empty P22 (ES) the expanded capsid (EX), and the wiffleball structure (WB). The observed shift in mobility, indicates the temperature induced transformation from ES to EX and WB morphologies. C) Negatively stained TEM images of the ES, EX, and WB morphologies showing the degree of particle size homogeneity. The inset shows the appearance of small voids (yellow arrows) in the shell of the WB morphology. D) Dynamic light scattering of the three morphological forms (ES, EX, and WB). The measured hydrodynamic diameter, expected for the expansion from ES (60 ± 4 nm) to EX (71 ± 5 nm) and WB (68 ± 4 nm) is observed.

Figure 4. Size and morphological characterization of the P22_{S39C}-xAEMA composite and P22_{S39C}-int

A) Representative SDS-PAGE gel of P22_{S39C}-int and P22_{S39C}-AEMA. The polymerized sample shows some streaking to higher Mw, indicating that the range of polymer chain lengths have been appended to the subunits as compared to the starting P22. In addition, highly crosslinked material can be observed in the well at the top of the gel. B) TEM images of P22_{S39C}-int (top) and P22_{S39C}-xAEMA (bottom) illustrating that the P22_{S39C}-xAEMA retains the size and shape homogeneity of the P22 capsid after the polymerization reaction. C) Dynamic light scattering of P22_{S39C}-int (top) and P22_{S39C}-xAEMA (bottom). The modified sample is monodisperse and has the same average diameter as the starting P22_{S39C}-int.

Figure 5. Molecular weight increase due to polymerization monitored by multiangle light scattering

Using size exclusion chromatography, both P22_{S39C}-xAEMA and P22_{S39C}-int exhibit the same elution time when monitored by absorbance (280 nm) indicating retention of particle size. Fitting the multiangle scattering data across the elution profile reveals the molecular weight of the material, which increases substantially for the P22_{S39C}-xAEMA (top) compared to the P22_{S39C}-int (bottom).

Figure 6. Verification of polymer formation and covalent modification with FITC by native agarose gel electrophoresis

Three different views of the same native agarose gel with P22_{S39C}-int (lane 1), P22_{S39C}-int-FITC (lane 2), P22_{S39C}-xAEMA (lane 3), and P22_{S39C}-xAEMA-FITC (lane 4). A) The gel was stained with Coomassie to detect the protein component. The P22_{S39C}-AEMA runs slightly out of the well in the opposite direction to the other proteins due to the net positive charge on the poly(AEMA) at the running buffer pH. Labeling this construct with the negatively charged FITC reverses the effective net charge on the construct resulting in shift in migration direction. B) The unstained gel under ambient light. The P22_{S39C}-FITC sample is faintly visible, while the P22_{S39C}-xAEMA-FITC sample is clearly visible signifying the relative degree of FITC labeling in each sample. C) The unstained gel illuminated with a laser at 488 nm and detected at 520 nm to highlight FITC. The P22_{S39C}-FITC is considerably brighter than the P22_{S39C}-xAEMA-FITC indicating the relative degree of fluorescence quenching in each sample.

Figure 7. Analysis of sample population homogeneity by analytical ultracentrifugation
Samples were centrifuged at 5,000-7,000 rpm and absorbance data were acquired at 280nm to attain sedimentation values for each sample. The measured sedimentation value of the P22_{S39C}-xAEMA (167 S) shows an increase from P22_{S39C}-int (142 S) and covalent modification of polymer with Gd-DTPA-NCS to make P22_{S39C}-xAEMA-Gd results in a shift to a higher S value (227 S), consistent with the incorporation of additional mass via Gd-DTPA-NCS labeling of the polymer. This data indicates that there is a homogeneous shift in the entire population to higher S values with each modification.