

THE DEVELOPMENT OF HYBRID BIOMATERIALS USING THE VIRUS-LIKE
PARTICLE (VLP) FROM BACTERIOPHAGE P22

by

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ABSTRACT

A broad range of bio-composite materials have been developed through inspiration from biology. In particular, natural systems that confine, co-localize and protect their contents has inspired the design and synthesis of the P22 virus-like particle (VLP) to effect a suite of biomaterials. These materials were realized by taking advantage of the native protein architecture of P22 as an initiation point and platform for material synthesis.

Introducing a reactive cysteine on the P22 coat protein provided an initiation point for polymer synthesis. Atom transfer radical polymerization (ATRP) was initiated creating a polymer framework on the interior of the P22 VLP. Using this polymerization technique (ATRP) a photocatalytic crosslinker was successfully incorporated for reduction of methyl viologen. Next, a manganese porphyrin imaging agent was loaded creating a T_1 -enhanced MRI contrast agent, as an alternative to the highly toxic Gadolinium currently used. Inspired by photosynthetic machinery, the P22-xAEMA system was labeled with a co-catalyst system, creating a co-localized photocatalytic nanoparticle capable of photochemically producing NADH/hydrogen. The production was controlled by labeling density of catalysts resulting in a tunable biomaterial.

The design of a complex bio-hybrid material was developed by combining both synthetic and genetic approaches. Coupling the enzyme Alcohol Dehydrogenase D from *Pyrococcus furiosus* with a small molecule catalyst led to a coupled catalytic system between a synthetic catalyst and biologically derived enzyme.

The P22 VLP system was studied by atomic force microscopy (AFM) and cryo-electron microscopy (cryo-EM) unraveling its biophysical properties and providing insights for further material design. 2D-crystal arrays were formed from a variety of P22-protein biomaterials, for the development of functional P22 arrays. Lastly, the P22 VLP was monitored by charge detection mass spectrometry, giving insight into the stability of the scaffolding protein. These studies show the versatility of this system for both material synthesis and fundamental biochemical understandings.

Overall, the work here continues to progress and push the boundaries of protein cage nanoparticles as platforms for material synthesis. The development of hybrid biomaterials from VLPs serve to improve our basic understandings of the natural systems they are derived from and provide additional design principles for improved complex bio-hybrid materials.

CHAPTER ONE

INTRODUCTION

Bio-inspiration – Overview

Biological organisms have evolved to possess sophisticated biomaterials and biocomposites. Biomaterials are synthesized for a wide range of physical properties giving them specific biological roles including, structural, adhesive, mechanical, and protective.^{1,2}

For instance, biocomposites like nacre, the inner shell of some molluscs is composed of organic/inorganic components, which acts as an extremely tough and protective material. Nacre are formed from layers of aragonite (type of calcium carbonate, inorganic component, hard) that are connected by elastic biopolymers (chitin and lustrin, organic component, soft). The combination of hard and soft components creates a new biocomposite that contains properties from both.^{3,4} Bones are another example of an exquisite biomaterial, which were designed to be light and strong for supportive, protective purposes as well as blood cell production.⁵ Like nacre, this biomaterial is made up of two main parts an inorganic (calcium phosphate) and organic component (collagen), making a new composite that contains properties of both in rigidity and fracture resistance, respectively.⁵

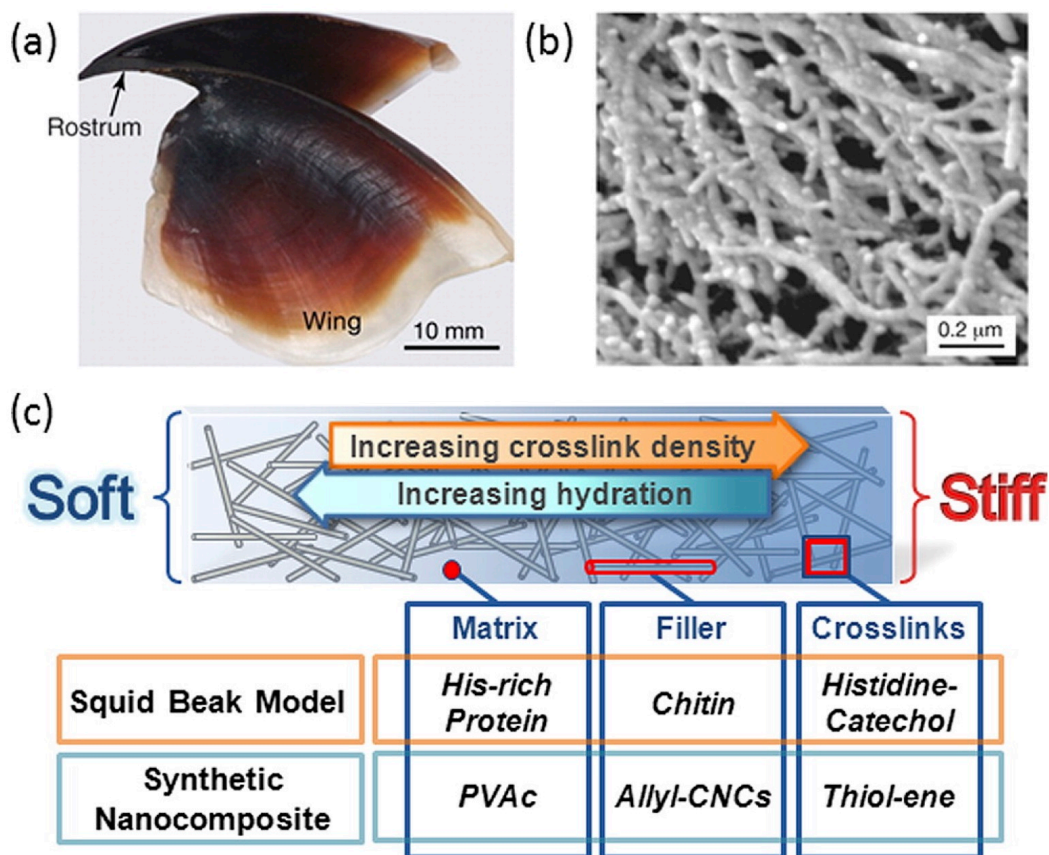


Figure 1.1 The Physical and Chemical Makeup of the Humboldt Squid Beak (from Fox *et al. JACS* (2013)¹) (a) An image of a split Humboldt Squid *Dosidicus gigas*. (b) A SEM image after chemical treatment with an alkaline peroxidation showing the chitin fiber network in the rostrum of the beak. (c) A diagram of the complex network of this nanocomposite material and proposed biomimetic synthetic design.

The beak of the Humboldt squid is another example of nature's ability to design and synthesize complex biomaterials.¹ The Humboldt squid has evolved to couple its extremely sharp and hard beak to the soft connective muscle tissues of the jaw (Figure 1.1). This extreme mechanical mismatch between the tip (elastic modulus ca. 5 GPa) and the wing edge (elastic modulus ca 50 MPa) is connected by a gradient of stiffness (controlled by crosslinking) that helps regulate the vast difference in modulus (Figure

1.1c).¹ This is another example of nature's remarkable ability of combining multiple components to design biomaterials that have numerous applications. These are just a few examples of biocomposites/biomaterials that organisms have created over millions of years of evolution for their overall survival. Therefore utilizing the design principles derived from nature is an extremely advantageous strategy known as biomimicry or bio-inspiration.

Taking inspiration from nature in the design of new biomaterials is at the forefront of material science today. This interface between biology and material science has been studied since the early parts of the twentieth century by D'Arcy W. Thompson (father of this field) and has influences dating back to Greek mythology, when Icarus' father Daedalus helped construct wings from feathers and wax.^{1,2} In addition to biocomposites nature has also evolved to form complex biological compartments to create confined and co-localized environments for chemical reactions.

Biological Compartments

The basic building block of all organisms, the cell, is an example of a complex biological compartment.⁶ Compartmentalization is needed to separate cellular components from the surrounding environment, the membrane confined space in a cell has a high density of macromolecules taking up about 30-40 % of space.⁷ The separation and subcellular organization in cells allows for the formation of multiple different local environments for a variety of roles, all contained in one functional package. By forming compartments out of lipid membranes unique catalytic centers can be sequestered and

confined from the external environment and have independent functions. Mitochondria are excellent examples of complex subcellular compartments.^{8,9} They are primarily used for the production of cellular energy in the form of ATP, by cellular respiration and metabolism. Mitochondria are confined reaction centers which have many functions and components that are all co-localized and confined inside two membranes made up of phospholipid bilayers and proteins. Each compartment has a function and local environment specialized for a specific biochemical process.⁸⁻¹⁰ All organisms take advantage of compartmentalization by either forming lipid membranes or protein cage structures that create specialized constrained environments. Organisms have evolved to contain these structures for diverse purposes ranging from protein folding to constraining metabolic pathways.^{9,10}

Bacterial microcompartments (BMCs), reminiscent of subcellular compartments, are examples of protein cages that sequester specific biochemical processes. These virus-like structures were first discovered by electron microscopy studies in the 60s from cyanobacteria, which allowed for biochemical confirmation that they were bacterial compartments rather than viruses.¹¹ This new protein species was called a carboxysome, which uses the interior of a ~80 nm protein container to confine enzymes essential for CO₂ fixation, significantly enhancing the overall enzymatic process. The carboxysome co-localizes two enzymes; carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco), allowing CO₂ to be concentrated around the slow/inefficient rubisco, increasing the pathway efficiency.^{12,13}

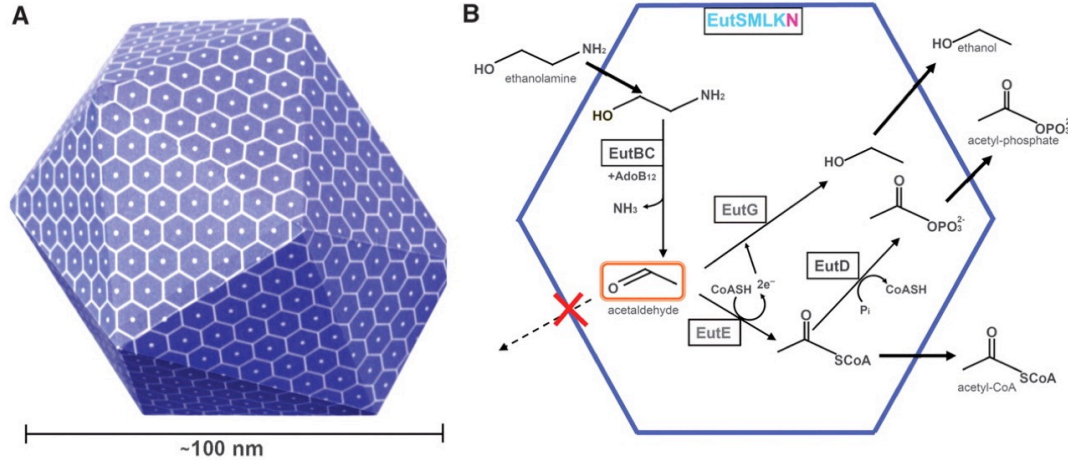


Figure 1.2 The Structure and Chemical Reactions of the Ethanolamine Utilization Microcompartment (from *Tanaka et al Science* (2010)¹⁴) (a) A structural model (b) A schematic representation of the complex chemistry that is sequestered on the inside by coupling multiple enzymatic reactions.

An example of a BMC from *Escherichia coli* and *Salmonella enterica* is the ethanolamine utilization microcompartment (EUT).¹⁴ These hollow protein cages are built from four essential protein components (EutS, EutL, EutK, EutM) and promote metabolism of ethanolamine while preventing diffusion of harmful intermediates out of the protein cage (Figure 1.2). Briefly, this is achieved by conversion of ethanolamine to acetaldehyde, a toxic intermediate that can't diffuse out of the cage, which in turn is degraded by the enzymes aldehyde and alcohol dehydrogenase. The structural protein genes for EUT and carboxysomes have strong sequence homology, but the EUT does not form a regular icosahedral cage like the carboxysome.¹³⁻¹⁵ Similar to the BMCs previously discussed, a protein cage produced by almost all living organisms, called ferritin is used for maintaining iron homeostasis.¹⁶ These small 24 subunit protein cages have specific catalytic sites for nucleation of iron minerals, which allow for storage and subsequent delivery. This type of structural compartmentalization exhibited by

organelles, and BMCs is a largely untapped design principle for biomimetic material design.

Viruses

Viruses are also amazing examples of complex protein compartments. Viruses are model systems for macromolecular and supramolecular assembly, assembling from a precise set of structural protein subunits that form a pre-defined protein cage architecture (similar to the BMCs described earlier). They are the most abundant biological agents on the planet, with $\sim 4 \times 10^{30}$ viruses in the oceans alone, and make up the second largest portion of the biomass. If all the viruses were stretched end to end it is estimated that they would span ~ 10 million light years (estimated $\sim 10^{31}$ viruses on the planet).¹⁷⁻¹⁹ Viruses are extremely small infectious particles that can only replicate inside a living cell of another organism and infect all types of life forms.^{20,21} Infectious virus particles are referred to as virions, which consist of at least genetic material (DNA or RNA) and a protective (protein/lipid) capsid. The sole purpose of the capsid is to protect the genetic material and ensure it reaches the desired destination (a cell).^{17,18}

There is a staggering variety of viral genome structures, with more genomic diversity than all other species on earth combined.¹⁹ But this diversity can be broken down to two main classes of viruses based on the type of genetic material they contain, RNA or DNA. Typically RNA and DNA viral genomes are either single or double stranded. Most RNA viruses contain a single stranded genome, which can have two different types of polarity, either negative- or positive-sense. Positive-sense viral RNA is

immediately ready for translation by the host, while negative-sense RNA viruses need to convert their genetic material to positive-sense by an RNA-dependent polymerase. The choice of genetic material is determined by many different factors, but most plant viruses contain single-stranded RNA while most bacteriophages contain double-stranded DNA. Typically RNA viruses have a smaller genome therefore the mechanisms for encapsulation of genetic material is different between the two types.^{19,22,23} Understanding the encapsulation of genetic material by viruses allows material scientists to mimic this in a synthetic system. Viruses have evolved to be efficient containers for protection and delivery of genetic material making them highly advantageous targets for the design and development of biomaterials.

Cowpea Chlorotic Mottle Virus (CCMV)

The cowpea chlorotic mottle virus (CCMV) is a single-stranded positive-sense RNA plant virus in the family of *Bromoviridae* that infects the cowpea plant.^{24,25} This family of viruses is unique because they encapsulate a tripartite genome (3 unique ssRNA) into three different viral capsids. This virus assembles from 180 identical protein subunits forming a T=3 icosahedral virus capsid and a near atomic structure has been solved to a 3.2 Å resolution. CCMV's genome size is ~8.2 kilobases (kb), made up of RNA 1 (3.2 kb), RNA 2 (2.9 kb), RNA 3 (2.1 kb) and a small fourth RNA strand (RNA-4) is encapsulated with RNA 3.^{25,26}

By amino acid deletion studies, it was found that the positively charged N-termini of the viral capsid subunit acts as an electrostatic encapsulation signal for RNA internalization.²⁵ If the positively charged amino acids (primarily lysine and arginine)

where deleted in the first 25 amino acids, the viral capsid could not encapsulate RNA, but if only the first seven amino acids were deleted infectious virions were still produced. From these results they determined that the positively charged amino acids responsible for RNA encapsulation are located on the interior of the capsid.^{24,25} Understanding the mechanism of the encapsulation of its genetic material was an important scientific advancement for the use of CCMV as a biomaterial.

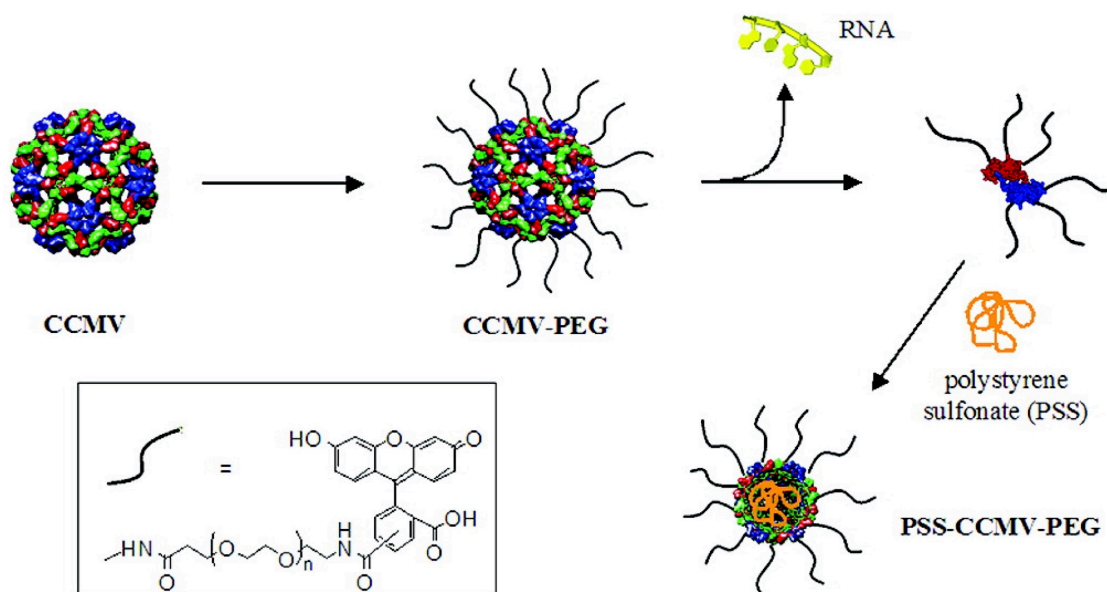


Figure 1.3 Schematic representation of the CCMV coat protein reassembling around a negatively charged polystyrene sulfonate (PSS) polymer. (from Comellas *et al.* Biomacro. (2009)²⁷) This process occurs by first labeling the lysines with a fluorescein-PEG, removing RNA, VLP disassembly, PSS addition, and reassembling into T=1 capsids.

From a biomaterial scientist's point of view, understanding the mechanism of the RNA encapsulation opens doors for its use as a biomaterial. With this knowledge and the added benefit of an atomic resolution structure of CCMV, precise engineering of amino acid sites could be installed for site-specific chemistry. An initial study using the CCMV

template for material synthesis focused on the mineralization of polyoxometalate species (decavanadate and paratungstate) on the interior cavity.²⁸ This approach took advantage of the positively charged interior, using it as a distinct chemical environment and nucleation site. Because CCMV's morphology changes with pH, lowering the pH trapped the mineral inside. CCMV was also shown to entrap an organic polyanion called polyanetholesulphonic acid (PAS).²⁸ Because of these advances, CCMV has been studied for its ability to package foreign genetic material and synthetic polymers. One example of this approach was performed by labeling the lysines with a fluorescein-PEG, which disassembled the capsid by destabilization, forming individual subunits as dimers. With the addition of the small polymer polystyrene sulfonate (PSS), the capsids reassembled into smaller T=1 particles with a diameter of ~18 nm (Figure 1.3).²⁷

This technique was taken a step further using inspiration from the iron storage protein cage, ferritin, where eight positively charged amino acids were replaced by glutamic acids. This mutant was called SubE, which now contained a negatively charged interior surface, which was able to sequester cationic Fe(II) ions and lead to the formation of ~24 nm lepidocrocite (γ -FeOOH) particle confined to the interior of the virus-like particle. This biomimetic approach shows the versatility of using this protein cage to sequester and confine cargo by electrostatic interactions.^{29,30}

Bacteriophage P22

The native bacteriophage P22 from *salmonella typhimurium*, a model short-tailed phage, is a double stranded DNA virus. The infectious virion consists of various gene products (gp) that are arranged to form a T=7 icosahedral particle by two linear,

independent pathways: capsid formation and tail assembly (Figure 1.4). These gene products are: a coat protein (CP; gp5), scaffold protein (SP; gp8), portal protein (gp1), and 12 copies of the three minor ejection proteins (gp7, gp16, gp20).^{31,32} The infectious bacteriophage P22 passes through a procapsid intermediate (~56 nm) that is made up of 415 copies of the coat protein and a range (100-300 copies) of scaffolding proteins (Figure 1.4). The scaffolding proteins role is to direct and template the assembly of the coat proteins.³²

In the infectious form of the bacteriophage, the 88 kDa dodecameric portal (gp1) protein replaces one of the pentamers at the five fold vertices, creating a symmetry mismatch. The 12 copies of the three minor ejector proteins (gp7, gp16, gp20) also are assembled into the procapsid, but their exact function and position are unknown, except that they are essential for genome delivery and infection. The portal protein along with two additional proteins (gp2 & gp3) help drive the packaging of the 43.5 kilobases (kb) of DNA by an ATP driven mechanism into the capsid with extremely high fidelity and pressure (Figure 1.4).³³ The bacteriophage packages about 103.5% of the genome known as “headful” DNA packaging. The genome of P22 is actually only 41.7 kb. The mismatch in symmetry is hypothesized to help in the packaging of DNA by providing rotational motion.³²

Once the DNA starts to be inserted the scaffold protein leaves the capsid and initiates the transition into the mature virion, which is larger and more angular. During this structural transition the thick procapsid walls become thinner, this structural rearrangement has been visualized by cryo-electron microscopy (cryo-EM) structural

studies creating structural models.³⁴⁻³⁶ To finish the DNA packaging process the capsid packages a specific amount of DNA by using a pressure sensor that initiates the terminase complex (gp2 & gp3) for DNA cleavage. These packaging proteins have a recognition site of a specific 22 base pair span of the viral DNA. Once this pressure sensor stops DNA packaging, the terminase complex is released from the capsid and the initiation of the tail assembly occurs. The tail is assembled first by closing the portal complex by the addition of multiple copies of three gene products that form of the base of the tail assembly (gp4, gp10 & gp26), and then the trimer-tail spike is attached (gp9) (Figure 1.4).³²

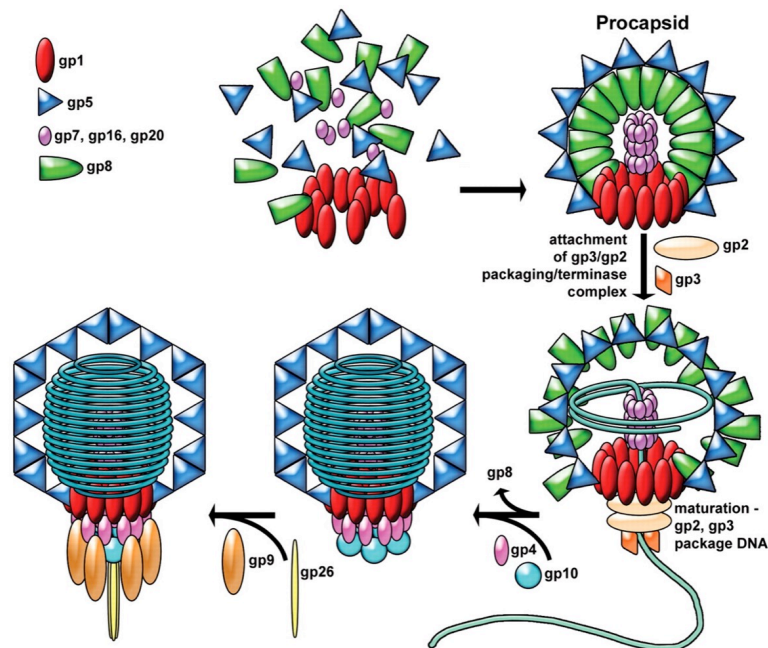


Figure 1.4 The Assembly of the Bacteriophage P22 (from *Lander et al Science* (2006)³²) The procapsid form of bacteriophage P22 is formed from 415 copies of the coat protein (gp5) and ~100-300 copies of the scaffolding protein (gp8). These proteins form the icosahedral particle, which contains a portal complex at a single five-fold symmetry axis, which assists in the packaging of DNA by a headful mechanism to form the infectious bacteriophage. Only gp5 and gp8 (coat and scaffold proteins) are for the formation of the P22 VLP.

Synthetic Compartments

Inspired by bacterial microcompartments, synthetic compartments such as protein cage nanoparticles (PCNs) have been sought as platforms for material synthesis. The use of supramolecular complexes allows for large amounts of cargo to be amended together, confining, protecting and co-localizing them. PCNs are protein cages often derived from viruses as well from nonviral sources, like ferritin. They are highly symmetric materials made up of a small amount of structural protein subunits. PCN's have advantages that other supramolecular complexes do not. First, they are gene products, which enable them to be synthesized by an organism at high yields. Second, their symmetry allows the size and structure to be precisely controlled. Lastly, these PCN's are robust protein structures that are highly resistant to chemical and thermal degradation. Taking advantage of the symmetry of these structures allows us to study the structural information and engineer specific sites on the protein cages. PCNs have three specific sites for genetic or synthetic modification: interior, interface, and the exterior (Figure 1.5).³⁷ Modifications to PCNs is preformed by either chemical and/or genetic, allowing complex materials to be synthesized with multiple functionalities. Combined, these various advantages make PCN's great candidates for uses in material design and synthesis.

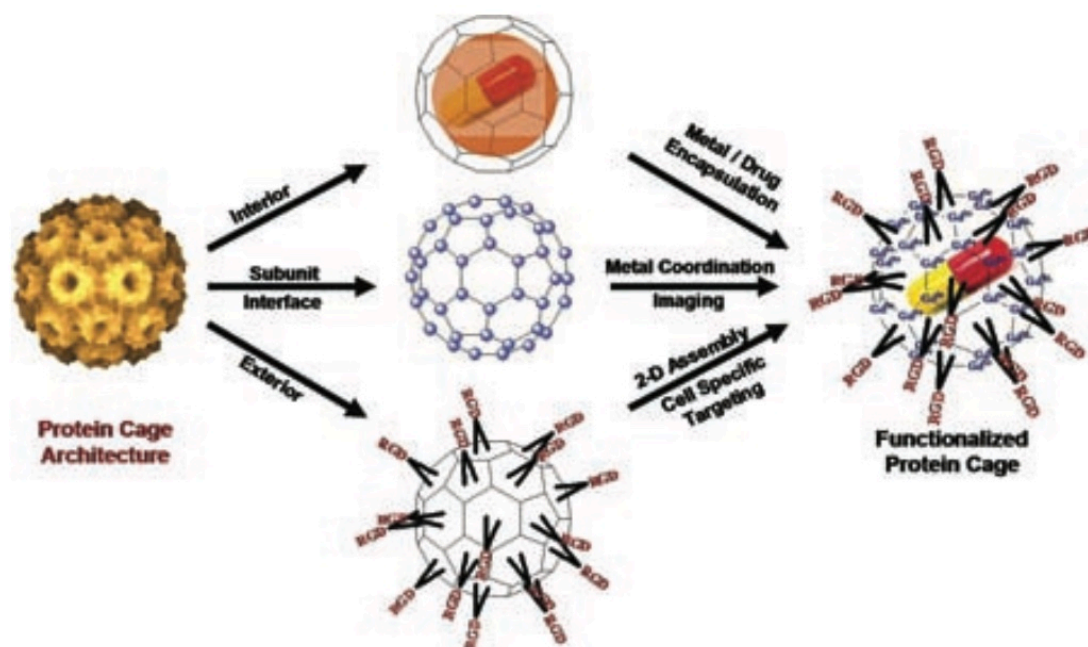


Figure 1.5 Cartoon scheme of the different surfaces available for functionalization. (from Flenniken et al. *Viruses and nanotechnology* (2009)³⁷) There exist three different surfaces that are available for labeling (interior, interface, exterior) on a protein cage nanoparticle.

Inspired by the structures and arrangement of BMCs a variety of PCNs have been used to confine and sequester cargo in a bio-inspired material approach. The interior of small heat shock protein (Hsp) was used for molecular confinement of mineralized platinum nanoparticle for the design and development of a material that produces hydrogen as a biomimetic hydrogenase particle.³⁸ This approach allowed for the synthesis of a HSP platinum particle that had similar rates of hydrogen production compared to most active hydrogenases.

Another example of using a PCN in a similar biomimetic approach was the use of *listeria innocua* DNA binding protein from starved cells (LiDps).³⁹ Instead of mineralization, platinum nanoclusters were formed by site specific labeling of an

engineered cysteine on the interior of the PCN. By labeling the cysteine with a small molecule chelator (5-iodoacetamido-1,10-phenanthroline), Pt^{2+} could be added forming Pt nanocluster that were monitored by noncovalent mass spectrometry. This was the first time that mineralization inside a protein cage was monitored by noncovalent mass spectrometry.³⁹ Using the same PCN, LiDps, chimeric nanoparticles were formed with distinct internal and external functionalities. This was achieved by creating two different nanoparticles with different sites for chemical modification, disassembling these PCNs and reassembling with specific ratios led to a random distribution of PCNs. This was monitored by noncovalent mass spectrometry and allowed them to determine the relative contributions of each chimeric cage (Figure 1.6).³⁹ This chimeric protein cage assembly approach allows for the production of complex and controlled multifunctional protein cage nanoparticles that could have a plethora of biomaterial synthesis applications.

As described earlier Ferritin (present in almost all living organisms), a small PCN that stores and maintains iron homeostasis, has been extensively used in material synthesis because of its unique ability to efficiently mineralize metals. Ferritin has been used to mineralize Fe, Pd, Ag, and CoPt cores with various applications including structural, catalytic, and improvements in electron transmission images.⁴⁰⁻⁴³ Despite the successes of these protein structures in biomaterial design, we set out to explore

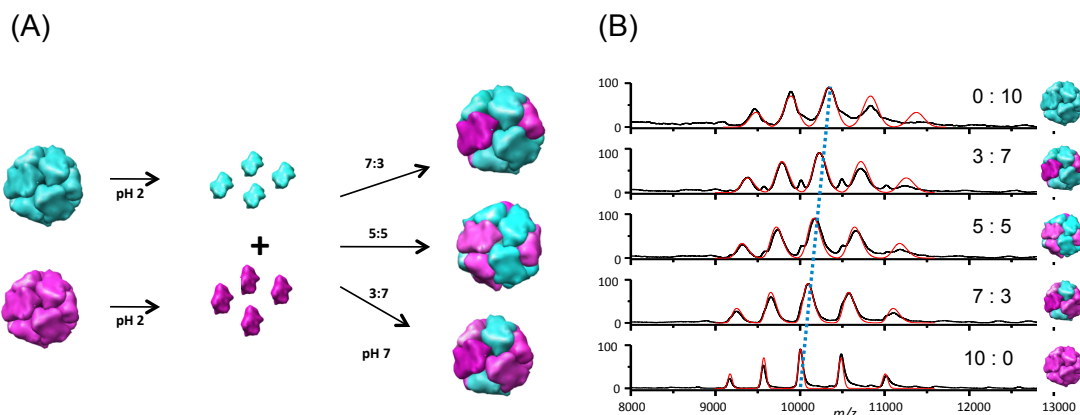


Figure 1.6. *Listeria innocua* DNA binding protein from starved cells (LiDps) used to make chimeric nanoparticles monitored by noncovalent mass spectrometry (from Kang *et al*, JACS (2008)³⁹) (A) Schematic representation of chimeric cage construction scheme by mixing two types of differentially modified S13C LiDps and reassembling into new cages. (B) Mass spectra of reassembled whole cages.

larger PCNs. One specific PCN that is interesting from a material science point of view is the virus-like particle (VLP) from P22 because of its ability to withstand both genetic and synthetic modifications while also being larger in size when compared to previously described examples.

P22 Virus-Like Particle (VLP)

The virus-like particle (VLP) from bacteriophage P22 has recently been used as a PCN for biomaterial design. The VLP from P22 used for biomaterial applications only contains gene products for the coat protein (gp5) and the scaffolding protein (gp8), as mentioned previously (Figure 1.4).^{39,44} These two gene products are heterologously expressed in *E.coli* as the non-infectious phage, and self-assemble from 420 subunits of coat protein (46.6 kDa) and 100-300 copies of the scaffold protein (33.6 kDa). The scaffold protein is needed to guide proper assembly of the P22 procapsid. It has been

shown that only ~65 amino acids of the C-terminus are needed for proper assembly, allowing for cargo to be attached via genetic fusion.^{45,46} There are four morphologies that the non-infectious P22 can obtain: procapsid (PC, 58 nm), empty shell (ES, 58 nm), expanded shell (EX, 64 nm), and wiffleball (WB, 64 nm) (Figure 1.7). Treatment with guanidine hydrochloride disrupts the scaffold and coat protein interactions, therefore converting the procapsid (PC) into empty shell (ES). Heating the ES morphology at 65 degrees Celsius creates the more angular expanded form (EX), and repeating the heating at 75 degree Celsius allows for the twelve pentamers at the vertices to be removed and form the wiffleball (WB) morphology.^{44,47} These large 10 nm diameter pores that are formed in the WB allow for free diffusion of molecules between the interior and exterior environments of the capsid. The P22 capsid has been genetically modified at amino acid residue 39 from a serine to a cysteine, S39C, to provide a site for chemical conjugation on the interior of the capsid. The EX morphology used in most of this work is the morphology that closely resembles the mature phage and has the largest interior volume. The interior volume doubles in size once it is in the more angular expanded form (EX), therefore allowing for large amounts of cargo to be placed on the interior, advantageous from a material synthesis perspective.^{47,48}

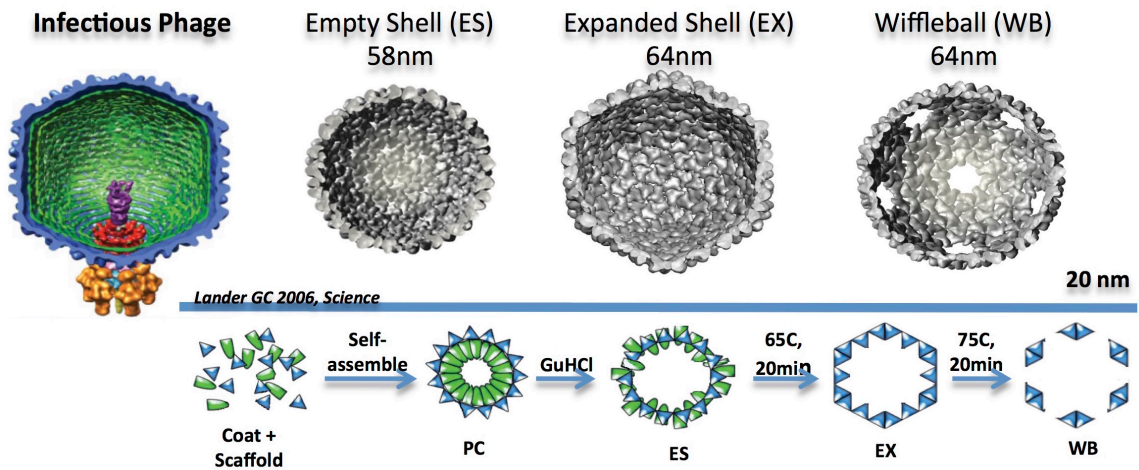


Figure 1.7 Bacteriophage P22: The different morphologies that this virus can obtain. From infectious phage on the left to non-infectious virus-like particles on the right. Below is a scheme of how each morphology is obtained.

The use of P22 VLP as a biomaterial is relatively new, but within the last few years it has been used extensively for the development of an array of biomaterials.^{46,47,49-}

⁵² Prior to its use as a biomaterial, our lab focused on a slew of other smaller PCNs.^{37,39,40,53,54} The scaffold protein (SP) from P22 can be truncated down to the essential amino acids in the C-terminus (first 65 amino acids), proving that the first 237 amino acids are not essential for proper assembly of the P22 VLP.⁴⁵ With this knowledge, the SP was truncated and genetically modified to have a small poly-acid peptide (ELEAE) on the N-terminus. The addition of this small peptide facilitated the mineralization of ferrihydrite on the interior of the P22 VLP.⁴⁶

The next advancement for the P22 biomaterial was the encapsulation of small fluorescent proteins. This was achieved by genetic fusion of the cargo (GFP & mCherry) to the N-terminus of the truncated scaffold protein (SP₁₄₁) and expressed on a plasmid containing the coat protein. Analysis of this material by multi-angle light scattering

revealed ~281 GFP and ~233 mCherry were encapsulated inside P22, while still maintaining the morphology of wild-type P22. By heating the capsids and inducing the morphological change to wiffleball a controlled release of cargo was obtained.⁴⁶ This study was followed by making concatenated fusions of GFP and mCherry to co-encapsulate this FRET pair, which lead to a significantly increased FRET response.⁵⁵ With these breakthroughs, the Douglas Lab wanted to test the limits of this scaffold mediate encapsulation method.

The next step was to use this *in vivo* expression method for the encapsulation of more complex cargos such as enzymes. A monomeric enzyme, alcohol dehydrogenase D (AdhD), from the hyperthermophile *Pyrococcus furiosus*, was first encapsulated.⁴⁹ AdhD, an enzyme that catalyzes the reduction of ketones to alcohols, was chosen because it hydrolyzes NADH, making detection easy. This enzyme can also withstand high temperatures allowing for multiple morphologies of P22 to be formed without destroying the cargo, which allowed them to test enzyme kinetics under different conditions. AdhD remained active after encapsulation and achieved the highest packing density of any active enzyme (7mM or 350 mg/mL) that had been reported.⁴⁹

This genetic scaffold mediated encapsulation was taken a step further by the encapsulation of the tetrameric β -glycosidase, CelB, from the hyperthermophile

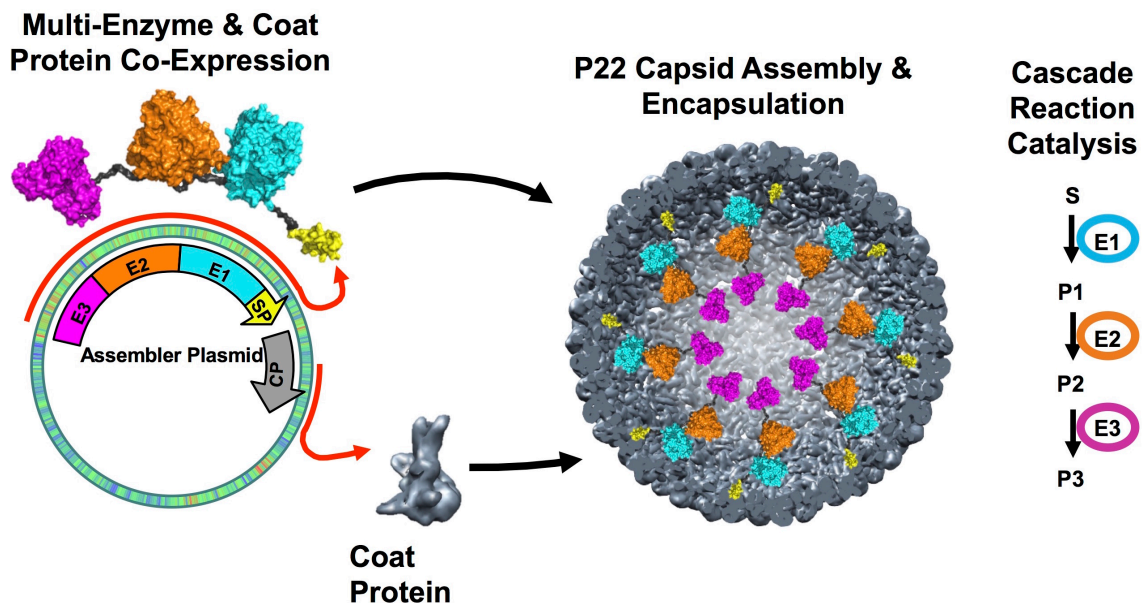


Figure 1.8 P22 used to create a synthetic metabolon inspired by bacterial microcompartments (BMCs). (from Patterson *et al.* ACS Chem. Biol. (2014)⁵¹) Creating a fusion protein multiple cargo can be encapsulated inside the P22 VLP creating an enzyme cascade.

Pyrococcus furiosus.⁴⁹ CelB was chosen because this enzyme has a strong quaternary structure needed for enzymatic activity and has a thermal dependence. CelB catalyzes the conversion of lactose to glucose and galactose by hydrolysis, but in P22-CelB studies 4-nitrophenyl- β -D-glucopyranoside was chosen because once it is cleaved the byproduct is 4-nitrophenol, which can be assayed by UV-Vis spectroscopy. Using this encapsulation scheme around 87 copies of the tetrameric enzyme were packaged and the efficiency of the enzyme was unchanged. This is most likely due to the result of using such a robust enzyme, but the over arching goal was to improve the catalytic efficiency.⁴⁹

Inspired by the BMC's discussed earlier, the next step was to encapsulate an enzyme pathway, creating a synthetic P22 metabolon or synthetic P22 micro-compartment (Figure 1.8).⁵¹ To achieve this, three enzymes were chosen to be fused to

the scaffold protein as one gene product, including: an ATP-dependent galactokinase (GALK, monomer), a ADP-dependent glucokinase (GLUK, dimer), and the tetramer CelB discussed previously. The enzyme metabolon was shown to be active, resulting in the most complex active cargo encapsulated in the P22 system to this point.⁵¹ These advancements with this versatile encapsulation scheme will allow for complex biomaterials to be synthesized creating synthetic BMCs with a multitude of potential to advance the field of bio-inspired materials.

The P22 VLP can encapsulate cargo by the genetic scaffold-mediated encapsulation scheme as described above. The P22 VLP can additionally be modified through alternative pathways that rely on synthetic and bioconjugation approaches. These synthetic approaches have been employed on a variety of protein cage systems and studied extensively,^{39,56,57} but now they were used with the P22 VLP a much larger protein cage nanoparticle. Uchida *et al.* were able to show that the P22 VLP could be used as a constrained vessel for site-directed coordination polymer chemistry on the interior of the nanoparticle.⁴⁷ To achieve this the 39th serine residue was mutated to a cysteine and labeled with 5-iodoacetamido-1,10-phenanthroline creating a site for various metals to bind. Once the bioconjugation was proven by subunit mass spectrometry, various metals such as Ni^{2+} , Co^{2+} , Fe^{2+} were added and analyzed by noncovalent mass spectrometry. Ni^{2+} was found to bind the strongest and was chosen as the metal of choice for the coordination polymer growth. This was achieved by introducing a ditopic metal coordinating ligand (1,3-di-1,10-phenanthroline-5-ylthiourea) diphen and Ni^{2+} in an iterative manner, until they grew a generation 4 (G4) coordination polymer.⁴⁷ This study

proved that the P22 VLP could constrain a site directed coordination polymer, and is an effective alternative method for the high loading of small molecules that otherwise could not be encapsulated. This versatile strategy could be employed for a wide range of small molecules ranging from cell-targeting ligands, small molecule drugs, and catalytic molecules in a “plug and play” manner.

The synthetic approach described above is effective for the introduction of small molecules, but multiple rounds of coordination polymer growth are needed to achieve high loadings of cargo, making this an inefficient process. Each round of coordination growth requires time to purify the material before another round can be initiated resulting in loss of material. Therefore, new synthetic approaches have been adopted and used in the P22 VLP system that allow for a one time polymerization event which generates a particle with a highly loaded polymer cargo on the interior of the capsid.^{47,48,58} This and other advances of bio-polymerization are discussed in the next section. A brief selection of the use of P22 VLP as a biomaterial has been discussed in this section. The expression and packaging of more complex cargos both genetically and synthetically for various applications are still being developed, explored and refined.

Atom Transfer Radical Polymerization (ATRP)/Bio-polymerization

Atom Transfer Radical Polymerization (ATRP) is a type of controlled radical polymerization (CRP) that has been extensively researched in the past few years for its biocompatibility.^{59,60} ATRP is a living radical metal catalyzed polymerization method developed by the Matyjaszewski group,^{24,59,61,62} which allows for the synthesis of

polymers that have a narrow molecular weight distribution in addition to predefined compositions and architectures. This method is extremely advantageous because a wide range of monomers have been successfully polymerized including acrylates, acrylamides, acrylonitriles, and styrenes.⁶¹ One important note is that for each monomer chosen, the reaction conditions (metal ligand, halide initiator, reaction solution) need to be optimized.

This polymerization technique is a catalytic process that uses an alkyl halide initiator that is activated by a transition metal catalyst (Cu(I)/Cu(II)). The interaction of the catalysts and the initiators forms an alkyl radical and the catalyst at a higher oxidation state. Propagation proceeds by adding monomers for a period of time before deactivation occurs. The repetition of this process allows for polymer chains to grow at similar rates, making well-controlled polymers (Figure 1.9).^{24,61} This type of controlled radical polymerization is advantageous for its robustness and is promiscuous towards a wide range of monomers.

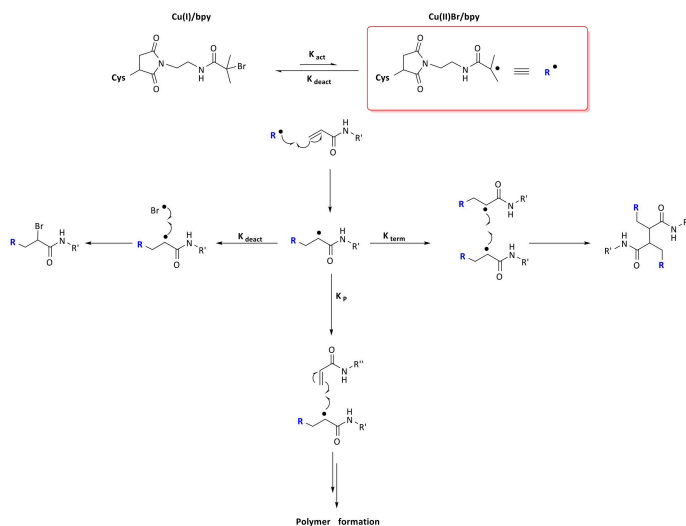


Figure 1.9 Atom transfer radical polymerization (ATRP) mechanism.

ATRP has been successfully used to synthesize protein-polymer hybrids by the attachment of the alkyl halide initiator to protein subunits. This breakthrough has allowed for significant advances of this polymerization technique in designing and synthesizing complex bio-hybrid materials.^{59,60,63,64} ATRP with biomolecules can be approached in two different manners, either by attaching a separately synthesized polymer to a biomolecule (grafted-to), or by growing the polymer directly off of the biomolecule (grafted-from). This latter approach was first employed by modifying streptavidin with a derivative of biotin (initiator) and this complex was used to grow a N-isopropylacrylamide (NIPAM) polymer from the protein framework.⁶⁵ Other early attempts using this polymerization method were used to grow NIPAM polymers from a cysteine initiation site on a BSA or lysozyme platform.⁶³ Some initial studies used ATRP to focus on functionalizing proteins with PEG-like polymers to increase pharmacokinetic properties. An example of this was grafting poly-(OEGMA) from the exterior of the Q β VLP, but this technique had limited success.⁶⁶ Each of these studies has helped understand more about the polymerization process, but was limited to a small set of monomers and focused mainly on monomeric proteins.

More recently ATRP has been employed on the interior of large macromolecular protein complexes from the Q β and P22 VLP. ATRP polymerization on the interior of the Q β VLP was achieved by removing the endogenous packaged RNA, attaching an initiator molecule to an unnatural amino acid on the interior, and then performing ATRP with a tertiary amine-bearing methacrylate.⁶⁷ The authors were able to show that the biotin-derivatized polymer strands were partially accessible indicating polymer formation

constrained to the interior. This Q β -polymer VLP brought about new physical and chemical characteristics including making it a positively charged particle allowing for easier entry into mammalian cells for delivery of functional small interfering RNA.⁶⁷ In the instance of the P22 VLP, a point mutation was made so that the P22 coat protein had a cysteine residue on the interior of the VLP. The mutation, S39C, was labeled with a cysteine reactive alkyl halide initiator, the initiation point for ATRP polymerization. Two monomers were chosen to be incorporated, 2-aminoethylmethacrylate (AEMA) and bis-acrylamide, for post-polymerization modification and crosslinking to constrain the polymer to the interior, respectively. Using this approach Lucon *et al* was able to load a MRI contrast agent, Gd-DTPA, with high labeling density improving the overall relaxivity by an order-of-magnitude compared to existing VLP systems (Figure 1.10).⁴⁷

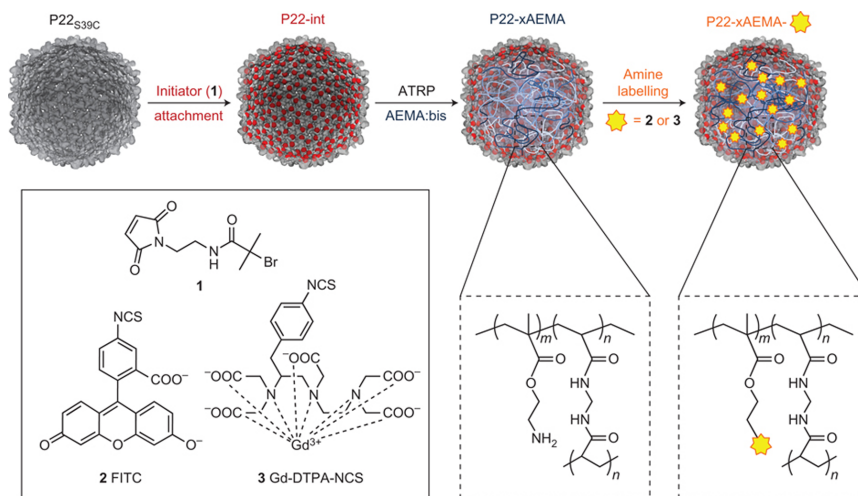


Figure 1.10 Schematic of atom transfer radical polymerization (ATRP) on the interior of the P22 VLP, for the synthesis of a high loaded P22-Gd-DTPA (from Lucon *et al.* Nat. Chem. (2012)⁴⁷). The P22 VLP's cysteine is labeled with 2-bromoisobutyryl aminoethyl maleimide (1). Poly-2-aminoethyl methacrylate (AEMA) was formed via atom transfer radical polymerization (ATRP). Post-polymerization modification with FITC (2) or Gd-DTPA-NCS (3) loads nearly 10,000 of the cargo molecules.

This breakthrough of high labeling density has set the stage for the use of the P22 ATRP bio-polymerization technique. The diverse protein-polymer biocomposites described in Chapters 2, 3, 4, and 5 are an extension to the previous work done with ATRP based protein-polymer composites.

Conclusions and Research Directions

The use of biology to synthesize uniformly sized nanoparticles as a template to make biomaterials is highly desirable. Using recombinant protein expression we can synthesize homogenous mixtures of protein-based nanoparticles. The bacteriophage P22 VLP has emerged as a model system for both assembly and DNA packaging, as well as a model system for biomaterial synthesis. The simplicity, size and versatility of this system allows for material design and synthesis for numerous applications. For these reasons the P22 VLP is a remarkable system that has a ton of potential for material design and synthesis.

Inspired by the bacteriophage P22 and its protein architecture, facilitated the design of new nanomaterials. In the current work, the P22 VLP was our template for material design and synthesis. In chapters 2, 3, 4, and 5 polymerization on the interior of the P22 VLP is further explored, pushing the boundaries with new photocatalytic crosslinkers, unique MRI contrast agents, and stoichiometric sequential labeling of a co-catalyst system to test effects of co-localization. In Chapter 6, the P22 VLP was used as a bio-hybrid nanoreactor, demonstrating that both genetic and synthetic modifications can

produce complex biomaterials. Lastly, in chapters 7, 8 and 9 the P22 VLP was used to study the biophysical and biochemical properties as well as its use in material assembly.

CHAPTER TWO

ATOM TRANSFER RADICAL POLYMERIZATION ON THE INTERIOR OF THE
P22 CAPSID AND INCORPORATION OF PHOTOCATALYTIC MONOMER
CROSSLINKSContribution of Authors and Co-Authors

Manuscript in Chapter 2

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Contributions: Designed studies, developed synthetic platform for all experiments, carried out preliminary experiments, wrote and edited the manuscript.

Co-Author: Ethan Edwards

Contributions: Synthesized photocatalytic P22, characterized and helped design experiments, help write and edit the manuscript.

Co-Author: Shefah Qazi

Contributions: Critically read and edited the manuscript.

Co-Author: Masaki Uchida

Contributions: Critically read and edited the manuscript.

Co-Author: Trevor Douglas

Contributions: Obtained funding for the research, designed studies and experiments, assisted with writing, critically reading and editing the manuscript.

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CHAPTER TWO

ATOM TRANSFER RADICAL POLYMERIZATION ON THE INTERIOR OF THE P22 CAPSID AND INCORPORATION OF PHOTOCATALYTIC MONOMER CROSSLINKS

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Abstract

We have successfully used atom transfer radical polymerization (ATRP) to form linear and crosslinked polyacrylamide and polyacrylate polymers, constrained within the virus like particle (VLP) derived from the bacteriophage P22. Polymerization of Tris(hydroxymethyl)methylacrylamide was initiated, in a spatially controlled manner, using macroinitiators derived from two different mutants of P22, S39C and K118C. Initiation from the S39C mutant results in spatially confined polymer growth on the interior of P22 while initiation from the K118C site results in a polymerized VLP in which some of the polymer is partially exposed on the outside of the capsid. Using the S39C macroinitiator we have demonstrated polymerization of aminoethyl methacrylate (AEMA) monomers, crosslinked by co-polymerization with the multifunctional monomer $[\text{Ru}(\text{5-methacrylamido-phenanthroline})_3]^{2+}$ resulting in an active photocatalytic P22 capsid particle.

Introduction

The use of protein-polymer composite materials is a growing field that takes advantage of the monodispersity and biosynthesis of biomolecules while imparting new materials properties via polymer conjugation.^{59,60,62,64,66,68,69} While much of the work in this area has been focused on conjugation of polymers to monomeric proteins there is a large, relatively untapped, landscape of complexity when these approaches are applied to more elaborate protein architectures. When multimeric biomolecules are employed for polymer conjugation the spatial relationship between initiation/attachment sites of the polymer and the symmetry of the overall protein architecture is important in directing the desired properties and can be utilized for controlling the morphology of the composite.^{47,59,70}

Virus-like particles (VLP) are protein cage architectures, derived from viral capsids, which form symmetric protein shells surrounding an interior cavity. These architectures define two distinct environments that can potentially be chemically modified by polymer attachment - either the exposed exterior or the confined interior.^{27,39,42,44,54,71-73} 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.^{66,74} We have previously demonstrated that the interior cavity of protein cages can be used as a confined reaction volume for controlled nanoscale polymerization using either atom transfer polymerization (ATRP) or a series of copper catalyzed azide-alkyne click reactions.^{47,48,69,72,75} Here we report the use of ATRP for directed polyacrylate and

polyacrylamide synthesis initiated in a site specific manner on the virus like particle derived from the bacteriophage P22 (Figure 2.1). Additionally, copolymerization with a novel $[\text{Ru}(\text{5-methacrylamido-phenanthroline})_3]^{2+}$ monomer (abbreviated as $\text{Ru}(\text{meth-phen})_3^{2+}$) utilizes the three-fold functionality of the coordination complex to crosslink the polymer and results in significant incorporation of the photoactive $\text{Ru}(\text{meth-phen})_3^{2+}$ derivative into the encapsulated polymer.^{32,76-81}

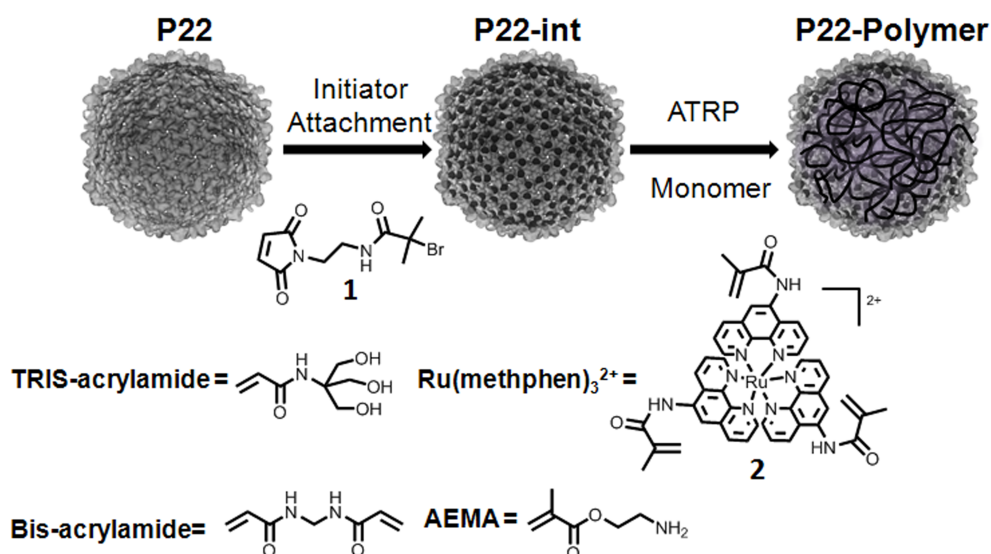


Figure 2.1 Reaction scheme for the synthesis of P22 protein-polymer hybrid using ATRP. P22 (cysteine mutant) is first modified with a cysteine reactive ATRP initiator (**1**) to create a macroinitiator for ATRP inside of the virus like particle (VLP). Monomers for these reactions include N-[Tris(hydroxymethyl)methyl]acrylamide (TRIS-acrylamide), N,N'-Methylenebisacrylamide (Bis-acrylamide), Aminoethyl methacrylate (AEMA) and Ruthenium(5-methacrylamido-phenanthroline) $_3^{2+}$ ($\text{Ru}(\text{meth-phen})_3^{2+}$).

Results and Discussion

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.^{31,36,73,82} Production of the P22 VLP in *E. coli* requires co-expression of the coat protein and a scaffold protein, which self-assemble in vivo and can be easily isolated in high yield. This VLP is capable of transformation into a series of distinct morphologies including the procapsid (PC) morphology, which contains scaffold protein, an empty shell (ES) form, where the scaffold protein has been removed and an expanded form (EX).^{73,83} The EX morphology most closely mimics the morphology found in the DNA-containing infectious virion and is the form used in this study.²⁰ To obtain the EX form, the PC form of the VLP is heated to 65 °C for 20 minutes, which generates the expanded capsid devoid of the scaffold protein.

Two different mutants of the P22 capsid, introducing unique addressable thiol groups, were explored for initiation and polymerization of an acrylamide monomer. Each mutation contains a single amino acid change in the wild-type P22 coat protein to introduce a unique cysteine residue, K118C or S39C, for attachment of an ATRP initiator (**1**). Based on a structural model, the P22_{S39C} mutant cysteine is located on the interior of the capsid while it appears that the cysteine in the P22_{K118C} mutant is partially exposed to the exterior of the capsid (Figure A.1).³⁴

To make the P22 macroinitiators for ATRP, both P22 mutants (K118C and S39C) were labeled with initiator **1**, chosen because of its good labeling and initiation, and demonstrated compatibility with biomolecules.^{63,84} It was synthesized according to established protocols and selectively reacted with P22_{K118C} and P22_{S39C} to make the macroinitiators, P22_{K118C}-int and P22_{S39C}-int, respectively. Both mutants reacted with

near quantitative single labeling of the introduced cysteine as observed by subunit mass spectrometry, although some variability in the degree of labeling was observed in different batches (Figure A.2).

Initial polymerization from the P22_{S39C}-int macroinitiator construct was accomplished using [Tris(hydroxymethyl)methyl]acrylamide (TRIS-acrylamide) under standard ATRP biomolecule conditions using a Cu(I)/bpy catalyst.⁵ Polymerization conditions of 2,600 monomers/subunit and temperature of 23°C were selected based on previous work. After 3 hr reaction the synthesis was halted by exposing the sample to air and the protein-polymer conjugate (P22_{S39C}-TRIS) was purified away from remaining TRIS monomer and the copper catalyst by spin column purification of the P22-polymer composite.

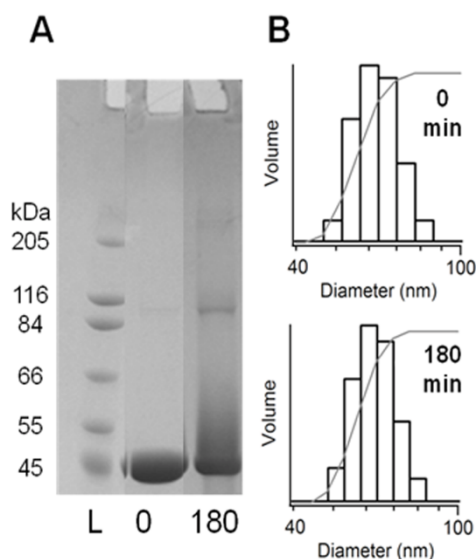


Figure 2.2 Characterization of the products of the P22_{S39C}-TRIS polymerization reaction. A) SDS-PAGE gel showing increase in Mw with increasing reaction time from 0 to 180 min (L is the Mw ladder). B) DLS of P22 sample before and after 180 min reaction time showing an unchanged diameter (63 nm).

The resulting reaction product exhibited increased subunit mass when analyzed by denaturing SDS gel electrophoresis (Figure 2.2). By this analysis it appeared that not all of the initiator labeled subunits produced 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. When the intact P22_{S39C} constructs were analyzed using multi-angle light scattering the molecular mass of the entire composite increased from 21.7 MDa (P22_{S39C}-int) to 23.4 MDa (P22_{S39C}-TRIS), corresponding to the incorporation an average of 9,000 monomers/cage (21 monomers/subunit) (Figure 2.3), slightly less than our previously reported aminoethyl methacrylate polymerization.⁴⁷

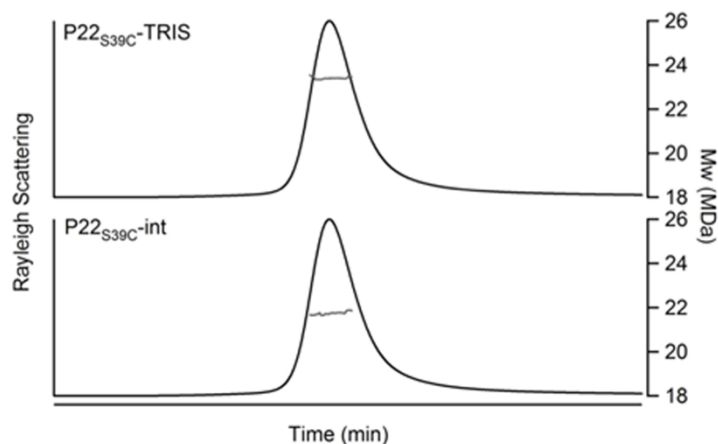


Figure 2.3 Size exclusion chromatography and multiangle light scattering of P22_{S39C} before and after TRIS-acrylamide polymerization. No change in elution volume or particle diameter was observed between the samples but an increase in Mw of 1.7 MDa was observed in the sample after the reaction.

To confirm that this polymer was confined to the interior of the P22, the size of the P22_{S39C}-TRIS construct was compared to the initial macroinitiator P22 complex. The

average hydrodynamic particle diameter was 63 ± 8 nm in the unpolymerized P22_{S39C}-int and remained the same (63 ± 7 nm) after the reaction as measured by dynamic light

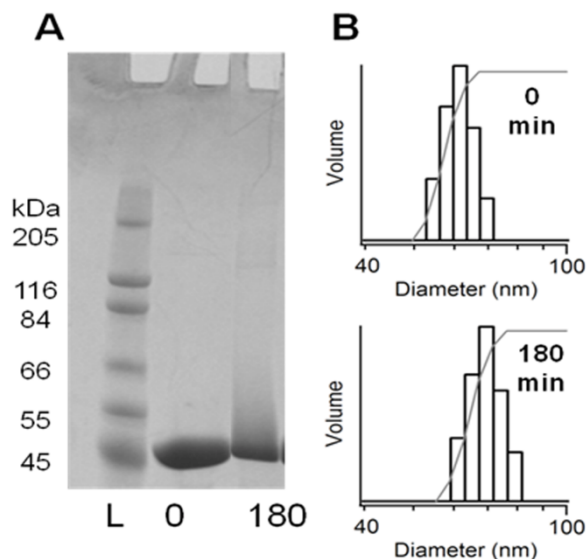


Figure 2.4 Characterization of the products of P22_{K118C}-TRIS polymerization reaction. A) SDS-PAGE gel showing increase in Mw with increasing reaction time from 0 to 180 min (L is the Mw ladder). B) DLS of P22 sample before and after 180 min reaction time showing a change in the particle diameter (60 nm to 68 nm).

scattering (DLS) (Figure 2.2B). Analysis by size exclusion chromatography (SEC) revealed that both the P22_{S39C}-int and P22_{S39C}-TRIS eluted with the same retention time, further confirming that the polymer is confined to the interior of the protein cage (Figure 2.3).

Polymerization of TRIS-acrylamide from the P22_{K118C} macroinitiator also showed increased subunit mass by denaturing gel analysis, as indicated by the shift to higher molecular mass when analyzed by denaturing gel electrophoresis (Figure 2.4A). When the P22_{K118C} native constructs were compared using multi-angle light scattering the molecular mass of the entire composite increased from 19.9 MDa (P22_{K118C}-int) to 21.0

MDa (P22_{K118C}-TRIS), corresponding to the incorporation of roughly 6,000 monomers/cage (14 monomers/subunit) into polymer strands on average (Figure 2.5).

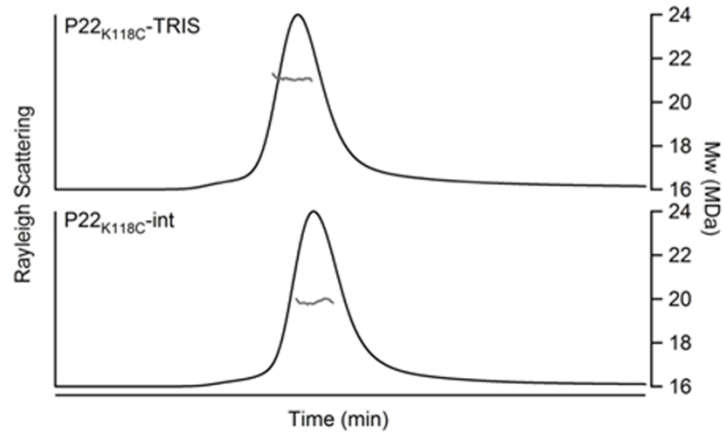


Figure 2.5 Size exclusion chromatography and multiangle light scattering of P22_{K118C} before and TRIS-acrylamide polymerization. A change in elution volume and particle diameter was observed in the sample after polymerization consistent with some polymer being able to sample the exterior of the capsid. An increase in Mw of 1.1 MDa was observed between the samples.

The polymerized P22_{K118C}-TRIS sample exhibited an increase in diameter of about 8 nm, from 60 ± 5 nm to 68 ± 6 nm, as measured by DLS suggesting partial growth of polymer on the exterior of the cage (Figure 2.4B). Upon analysis by size exclusion chromatography it was apparent that the P22_{K118C}-TRIS increased in size compared to P22_{K118C}-int as evidenced by a shift to earlier elution time indicating that the formation of a larger particle (Figure 2.5). These data suggest a model where the K118C mutation site can (at least partially) access the exterior environment of the VLP resulting in partial polymer growth on the outside of the cage. This is in contrast to the S39C mutation, which appears to behave as if it is clearly localized on the interior of the P22 cage.

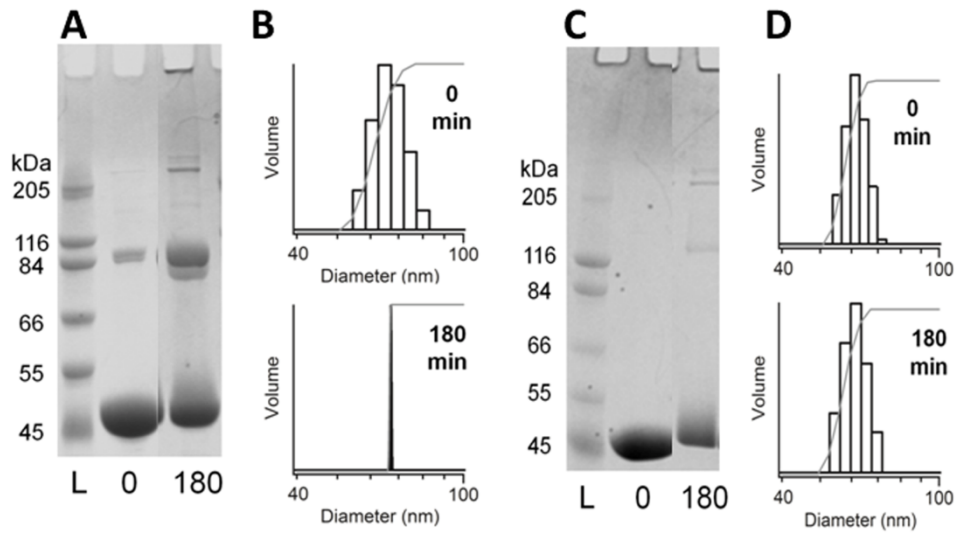


Figure 2.6 Characterization of the crosslinking polymerization reaction to form P22_{S39C}-xTRIS (A and B) and P22_{K118C}-xTRIS (C and D). A) SDS-PAGE gel showing increase in Mw with increasing reaction time from 0 to 180 min (L is the Mw ladder). B) DLS of the P22_{S39C}-xTRIS sample before and after 180 min polymerization reaction showing an unchanged diameter (67 nm). C) SDS-PAGE gel showing increase in Mw with increasing reaction time from 0 to 180 min (L is the Mw ladder). D) DLS of the P22_{K118C}-xTRIS sample before and after 180 min polymerization reaction showing an unchanged diameter (62 nm).

Polymerization of the TRIS-acrylamide using both the P22_{K118C} and P22_{S39C} constructs in the presence of the bifunctional crosslinking monomer N,N'-methylenebisacrylamide (bis-acrylamide) resulted in highly monodisperse homogeneous materials denoted P22_{K118C}-xTRIS and P22_{S39C}-xTRIS, respectively. In contrast to the linear polymerization, crosslinking polymerization from the P22_{K118C} macroinitiator resulted in a material where the polymer was apparently fully constrained within the capsid. DLS data of the P22_{K118C}-xTRIS revealed a radius of hydration of 62 ± 4 nm and for the P22_{S39C}-xTRIS a radius of hydration of 67 ± 1 nm was measured (Figure 2.6 B, D). Analysis of these materials by size exclusion chromatography and multi-angle light scattering revealed an increase in molecular mass of 2.3 MDa for the P22_{K118C}-xTRIS and

1.4 MDa for the P22_{S39C}-xTRIS sample corresponding to the incorporation of roughly 13,000 monomers/cage (32 monomers/subunit) and 8,000 monomers/cage (19 monomers/subunit) into polymer strands on average, respectively. In addition, these crosslinked polymerized materials showed identical elution from the SEC column further indicating that the polymerization was fully constrained within the capsid (Figure 2.7 A, B).

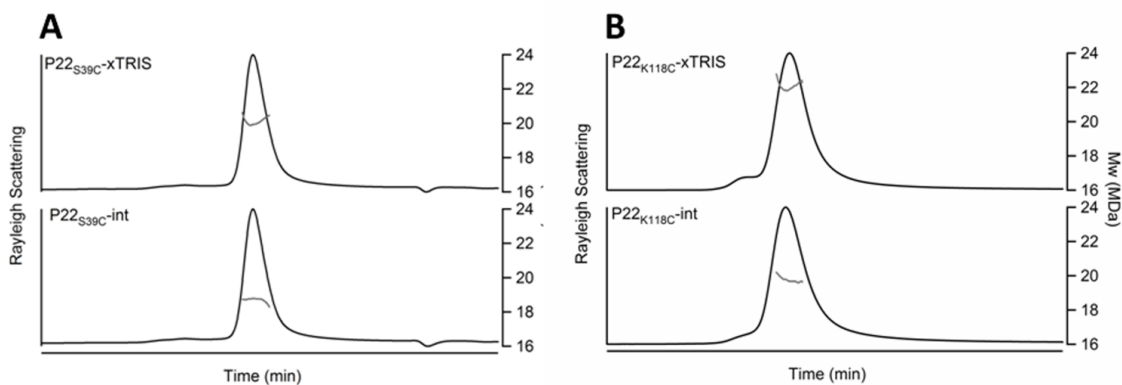


Figure 2.7 A) Size exclusion chromatography and multiangle light scattering of P22_{S39C} before and after crosslinking polymerization reaction. No change in elution volume or particle diameter was observed between the samples, but an increase in Mw of 1.4 MDa was observed between the samples. B) Size exclusion chromatography and multiangle light scattering of P22_{K118C} before and after crosslinking polymerization reaction. No shift in elution time was observed indicating that the particle diameter remains constant and an increase on 2.3 MDa mass was observed between the samples.

Based on the successful incorporation of ATRP generated polymer inside of the P22 cages, we explored this methodology to incorporate photoactive metal coordination complexes as crosslinking agents into a functionalizable polymer framework, constrained within the P22_{S39C} mutant VLP. Taking advantage of the demonstrated spatial specificity of the internal S39C mutant, we co-polymerized aminoethyl methacrylate (AEMA) with

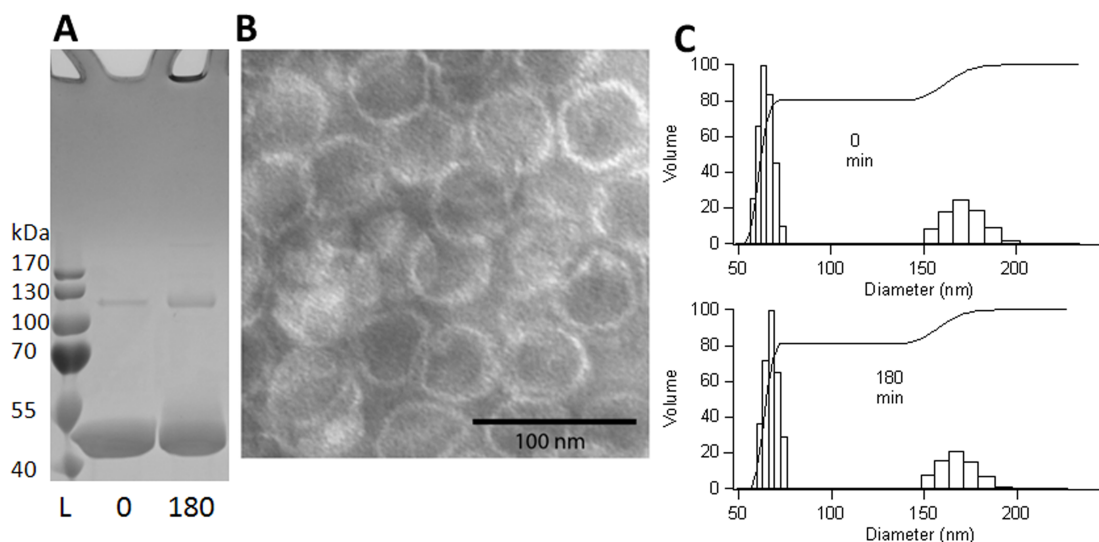


Figure 2.8 Characterization of the crosslinking polymerization reaction to form P22_{S39C}-xAEMA. A) SDS PAGE gel showing an increase in Mw (L is the Mw ladder). B) TEM image using 1% uranyl acetate stain containing properly assembled P22 VLP particles. C) DLS of P22_{S39C}-xAEMA before and after the polymerization reaction showing an unchanged diameter (61 nm).

newly synthesized monomer, Ru(meth-phen)₃²⁺, and bis-acrylamide as multifunctional crosslinkers.

The ATRP reaction of AEMA was performed under nearly identical conditions to those described above for the xTRIS samples, except for the addition of 1.2% of the Ru(meth-phen)₃²⁺ monomer to one of the samples as a new crosslinker incorporating additional photocatalytic activity. The resulting materials (P22-xAEMA and P22-xAEMA/Ru(meth-phen)₃²⁺) were subject to the same purification as for the P22 capsid (2 cycles of pelleting by ultracentrifugation and washing with buffer). The purified materials were investigated by protein denaturing SDS gel electrophoresis and a population shift to higher molecular weight was clearly visible (Figure 2.8A, 2.9A) although not as pronounced as for the TRIS-acrylamide samples described above (Figure

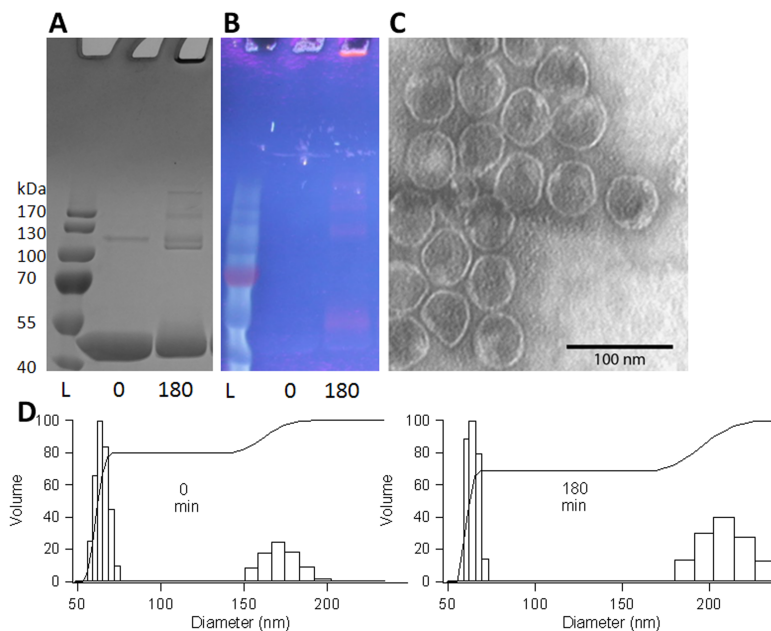


Figure 2.9 Characterization of the crosslinking polymerization reaction to form P22_{S39C}-xHEMA/Ru(meth-phen)₃²⁺. A) SDS PAGE gel showing an increase in molecular weight via streaking (L is the Mw ladder). B) SDS PAGE gel before Coomassie staining, showing the fluorescence of the Ru(meth-phen)₃²⁺ monomer during excitation with UV light. C) TEM image using 1 % uranyl acetate stain containing properly assembled P22 VLP particles. D) DLS of P22 sample before and after the polymerization reaction showing an unchanged diameter (62 nm).

2.2A). The P22-xHEMA/Ru(meth-phen)₃²⁺ samples could be visualized in these gels either using a protein (Coomassie) stain or directly by the fluorescence of the Ru(meth-phen)₃²⁺ component. As shown in Figure 2.9B, the Ru(meth-phen)₃²⁺ fluorescence on the gel co-localizes with the higher molecular weight population from the Coomassie stain.

Dynamic light scattering of these P22_{S39C}-xHEMA/Ru(meth-phen)₃²⁺ polymers revealed a hydrodynamic diameter of 62 ± 10 nm for the Ru(meth-phen)₃²⁺ and 61 ± 8 nm for the bis-acrylamide crosslinked samples (Figure 2.8C, 2.9D). This strongly suggests that these crosslinked polymers are localized within the P22 capsid. Although a larger species was present in both the unpolymerized and the polymerized samples, this

larger species was present in both samples suggesting that it is an artifact of the protein preparation rather than a result of the polymerization. Further analysis of the P22_{int} complex (unpolymerized sample) by size exclusion HPLC coupled with multi-angle light scattering showed that this complex had a hydrodynamic radius of 31.7 nm and a molecular weight of 19 MDa, which is the expected size and weight for the EX morphology of S39C with no evidence of any larger aggregated material. Determination that AEMA polymerization had occurred was accomplished by chemically labeling the amines of the AEMA with fluorescein (FITC) and using the absorbance to determine the degree of labeling. Labeling the amines with FITC provides a qualitative determination of the number of amines present in the polymerized P22. From previous work we have shown that not all AEMA amines are labeled with the FITC and this therefore underestimates the degree of polymerization.⁴⁷ The degree of Ru(meth-phen)₃²⁺ crosslinker incorporation was determined from the characteristic visible absorbance (445 nm) of the Ru(meth-phen)₃²⁺ and published molar absorptivity (20,000 M⁻¹cm⁻¹).⁸⁵ From these data we determined that each P22 capsid incorporated more than 2,000 AEMA and on average 270 ± 74 Ru(meth-phen)₃²⁺ monomers in these reactions.

Incorporation of these novel Ru(meth-phen)₃²⁺ based crosslinking agents into the encapsulated polymer imparts a unique new photo-reactivity to the P22 capsid. We probed the photocatalysis of these P22 capsids by monitoring the photoreduction of methyl viologen (MV²⁺) using either EDTA or ethanol as sacrificial reductants under visible light illumination. As shown in Figure 2.10, the Ru(meth-phen)₃²⁺ incorporated P22 samples demonstrated rapid methyl viologen reduction upon illumination as

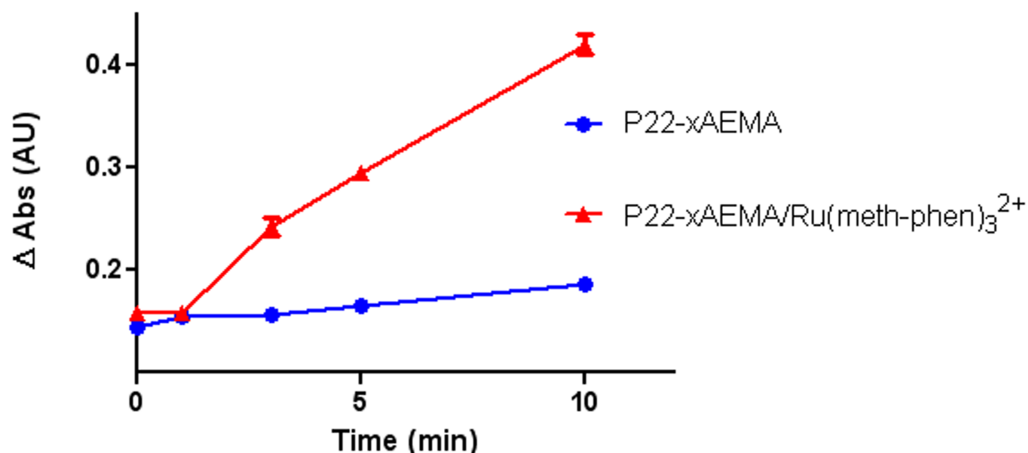


Figure 2.10 Methyl Viologen (MV^{2+}) Reduction Assay. Methyl viologen was reduced using P22_{S39C}-xAEMA/Ru(meth-phen)₃²⁺ and P22_{S39C}-xAEMA with EDTA as the sacrificial reductant under visible light illumination. The selective reduction of MV^{2+} by P22_{S39C}-xAEMA/Ru(meth-phen)₃²⁺ was observed by monitoring the absorbance of the sample at 395 nm.

evidenced by an increase in absorbance at 395 and 600 nm, characteristic of the formation of the reduced methyl viologen radical cation ($MV^{•+}$). The reduced viologen is widely used as a mediator for coupled redox reactions and we have thus created a protein-polymer composite that could act as a molecular nano-reactor for photo-induced redox chemistry.

Conclusions

Using genetic modification of a self-assembled protein cage architecture to introduce reactive cysteine residues, we have generated macroinitiators for controlled ATRP to make active protein-polymer composites. The site specific attachment of initiators to the interior surface of the virus like particle, derived from the bacteriophage P22, effectively directs polymer growth to the VLP interior resulting in confined polymer

growth as the protein shell acts as a barrier leaving only the protein shell exposed to the bulk solution. Due to the simplicity, modular nature, and potential loading capacity of the ATRP based approach this method is currently being employed to make a range of novel VLP-polymer composites. In particular, we have shown that polymers with reactive side chains can undergo further chemical modification to incorporate additional chemical functionality.

Initiation of the polymer at a site (K118C) with some external exposure results in a polymer able to sample the exterior of the capsid architecture, but incorporation of crosslinking into the reaction results in polymerization fully constrained within the capsid. Polymer initiation from a different internal site (S39C) results in constrained polymer growth in both crosslinked and un-crosslinked samples.

An expansion of the initiated ATRP approach allowed us to incorporate a novel $\text{Ru}(\text{meth-phen})_3^{2+}$ based methacrylamide monomer into the polymerization resulting in the formation of a unique metal coordination co-polymer in which the trivalent monomer crosslinks the polymer on the interior of the capsid. The resulting metal incorporated protein-polymer composite exhibits the optical and photochemical properties of the incorporated $\text{Ru}(\text{meth-phen})_3^{2+}$ and we are currently exploring its utility for coupled redox based photocatalysis.

Acknowledgements

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Experimental

Materials

All the reagents used 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 and all water was deionized using a NANOpure water purification system. Dynamic light scattering measurements were taken on a 90Plus particle size analyzer (Brookhaven Instruments).

Synthesis of 2-Bromoisobutyrate Ethoxy maleimide (1)

Compound 1 was synthesized by a modification of the procedures previously reported.^{63,84} Ethanol-maleimide (200 mg, 1.42 mmol) was mixed on ice with triethylamine (197 μ L, 1.42 mmol) in 5 ml dry dichloromethane. 2-bromo-2-methylpropionylbromide (266 μ L, 2.13 mmol) was added dropwise. The reaction was allowed to warm to room temperature and was determined by thin layer chromatography to have reached completion after 1.5 hr. The solvent was removed and the product was subsequently cleaned via column chromatography (silica gel, dichloromethane) with a yield of 76%. ¹H NMR (500 MHz, CDCl₃) δ = 1.87 (s, 6H, CH₃); 3.84 (t, J = 5.5, 2H, NCH₂); 4.31 (t, J = 5.5, 2H, OCH₂); 6.71 (s, 2H, CH_{vinyl}). ¹³C δ = 30.59 (CH₃), 36.57(NCH₂), 62.88(OCH₂), 134.26(CH_{vinyl}).

Synthesis of Ruthenium
(5-methacrylamido-phenanthroline)₃²⁺ (**2**)

Compound **2** was synthesized by a modification of procedures previously reported.⁸⁶ Ruthenium(amino-phen)₃ 2PF₆ (100 mg, 0.13 mmol) was suspended in 5 mL dry dichloromethane with triethylamine (122 μ L, 0.88 mmol). To this suspension methacryloyl chloride (84 μ L, 0.88 mmol) was added dropwise over several minutes and the solution gradually turned to a very dark black-red solution. The reaction was allowed to stir overnight at room temperature and then the solvent was removed and redissolved in 5 mL methanol. The product was mixed with 5 mL of saturated ammonium hexafluorophosphate and water, which resulted in an orange-red precipitate. The orange crystals were collected via vacuum filtration and washed three times with water and ether. The product was subsequently purified via column chromatography (silica gel, 90 % acetonitrile, 9 % water, 1 % sat. potassium nitrate) with a yield of 77%. ¹H NMR (300 MHz, ACN-d₃) δ = 7.66-7.80 (m, 11H); 8.01 (s, 3H); 8.08-8.26 (m, 10H); 8.38-8.71 (m, 12 H).

P22-int Macroinitiator Synthesis

P22_{S39C} (100 mM phosphate, 50 mM NaCl, pH 7.6, 23.6 ml, 5 mg/ml) was infused with 316 μ L of **1** (40 mM in DMSO, 5 fold excess per subunit). The mixture was allowed to react for three hours at room temperature. After 3 hrs the reaction was quenched with DTT (316 μ L, 40 mM in water). To remove excess DTT and **1**, the P22 was pelleted at 48,000 rpm for 50 minutes in an ultra centrifuge (Sorvall) followed by

resuspension into PBS, pH 7.6. Subsequent analysis by subunit mass spectrometry indicated that >95% of the subunits were labeled.

P22-TRIS Polymer Synthesis

Each sample was made in a small crimp-top vial with the addition of 2500 μ L monomer solution (2 wt% N-[Tris(hydroxymethyl)methyl]acrylamide (TRIS-acrylamide) or 2 wt% 16:4 TRIS-acrylamide:N,N'-methylenebisacrylamide (bis-acrylamide), PBS, pH 7.6) and 875 μ L PBS buffer (100 mM sodium phosphate, 50 mM NaCl, pH 7.6). To this mixture, 875 μ L of P22-int (6.0 mg/mL, 0.11 μ moles subunit, pH 7.6) was added followed by pumping and back filling with Ar 4 times to deaerate the mixture. The metal catalyst solution was made in a second vial using 10 mL deionized water degassed by bubbling Ar through the liquid for 20 minutes, to which was added 7.0 mg CuBr (0.049 mmoles) and 15.6 mg 2,2'-bipyridine (0.10 mmoles). The vial was immediately sealed and sonicated for 5 min. To the monomer-protein vial, 750 μ L of the metal catalyst solution was added and the vial was maintained at 23 °C for the remaining duration of the experiment. After 3 hrs each sample was quenched by exposure to air and the protein-polymer composite was purified away from unreacted monomer and the copper catalyst using a spin column (Biorad, Micro Bio-Spin Columns P-30, PBS, pH 7.6).

P22-xAEMA Polymer Synthesis

Each sample was made in a 10 mL crimp-top vial with the addition of 4000 μ L of 2-aminoethyl methacrylate (AEMA)/Bis-acrylamide monomer solution (4 wt% in PBS, 16:4 AEMA/bis-acrylamide, pH 7.6) and 2200 μ L PBS buffer (100 mM sodium

phosphate, 50 mM NaCl, pH 8.0), which was pH adjusted with 2 M sodium hydroxide solution back to pH 8.0. To this mixture, 1200 μ L of P22-int (8.0 mg/mL, 0.16 μ moles subunit, pH 7.6) 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 vial using 10 mL deionized water degassed by bubbling Ar through the liquid for 20 minutes, to which was added 19.2 mg CuBr (0.134 mmoles), 29.9 mg CuBr₂ (0.134 mmoles) and 83.5 mg 2,2'-bipyridine (0.535 mmoles). The vial was immediately sealed and sonicated for 5 minutes resulting in a dark-brown solution. To the monomer-protein vial, 600 μ L of the metal catalyst solution was added and the vial was maintained at 23 °C for the remaining duration of the experiment (180 min). The sample was quenched by exposure to air and the protein-polymer composite was purified away from the unreacted monomer and the copper catalyst by pelleting the protein and resuspending into 100 mM phosphate, 50 mM NaCl, pH 8.0. This was followed by treating the protein with a high salt treatment (100 mM phosphate, 250 mM sodium sulfate, pH 8.0) for 3 hours rocking gently in a 4 °C fridge and followed by untracentrifuge driven pelleting of the protein. The protein pellet was resuspended in 100 mM phosphate, 50 mM NaCl, pH 8.0. The yield was 4.22 mg (53 %).

P22-xAEMA/Ru(meth-phen)₃²⁺ Co-polymer Synthesis

Each sample was made in a 10 mL crimp-top vial with the addition of 4000 μ L of 2-aminoethyl methacrylate (AEMA) monomer solution (4 wt% in PBS, pH 7.6) and 600 μ L Ru(meth-phen)₃²⁺ (30mM in PBS/30% acetonitrile, pH 8.0) and 1600 μ L PBS

buffer/30% ethanol (100 mM sodium phosphate, 50 mM NaCl, pH 8.0), which was pH adjusted with 2 M sodium hydroxide solution back to pH 8.0. To this mixture, 1200 μ L of P22-int (8.0 mg/mL, 0.20 μ moles subunit, pH 7.6) 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 vial using 10 mL deionized water degassed by bubbling Ar through the liquid for 20 minutes, to which was added 19.2 mg CuBr (0.134 mmoles), 29.9 mg CuBr₂ (0.134 mmoles) and 83.5 mg 2,2'-bipyridine (0.535 mmoles). The vial was immediately sealed and sonicated for 5 minutes resulting in a dark-brown solution. To the monomer-protein vial, 600 μ L of the metal catalyst solution was added and the vial was maintained at room temperature for the duration of the experiment (180 min). The sample was quenched by exposure to air and the protein-polymer composite was purified away from the unreacted monomer and the copper catalyst by pelleting the protein and resuspending into 100 mM phosphate, 50 mM NaCl, pH 8.0. This was followed by treating the protein with high salt (100 mM phosphate, 250 mM sodium sulfate, pH 8.0) for 3 hours at 4 °C and re-pelleting of the protein by ultracentrifugation. The protein pellet was again resuspended in 100 mM phosphate, 50 mM NaCl, pH 8.0. The yield was 3.84 mg (48 %).

Quantification of Ruthenium via UV-vis

Protein samples were made at $\sim$ 1 mg/mL in phosphate buffer and analyzed by an UV-vis (Agilent 8453). Absorbance was monitored at 445 nm and Ru concentrations were calculated by using the molar extinction coefficient 20,000 M⁻¹cm⁻¹.⁸⁵ The

contribution of phenanthroline absorbance to protein absorbance at 280nm was accounted for by using the ratio of absorbance at 280 and 445 nm for Ru(phen)_3^{2+} .

Quantification of AEMA by FITC Labeling

343 μL of FITC (8.6 mmol, 25 mM in DMSO) was added dropwise to the vortexing protein solution, which contained 2.0 mL P22_{S39C} -xAEMA (0.086 μmol subunit, carbonate buffer, pH 9.0) at 2 mg/mL. The mixture rocked overnight for 16 hours at 4 °C followed by pelleting the protein to remove excess FITC and resuspension (carbonate buffer, pH 9.0). This process was repeated twice. Absorbance was monitored at 395 nm to determine FITC concentration with a UV-vis (Agilent 8453). The approximate AEMA per a P22 was calculated by using the extinction coefficient 69955 $\text{M}^{-1}\text{cm}^{-1}$ at 280 nm and 2233 $\text{M}^{-1}\text{cm}^{-1}$ at 495 nm for P22_{S39C} and the extinction coefficient 23711 $\text{M}^{-1}\text{cm}^{-1}$ at 280 nm and 69966 $\text{M}^{-1}\text{cm}^{-1}$ at 495 nm for FITC.

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) in 50 mM phosphate, 500 mM sodium chloride, pH 8.0. Each sample injection was 25 μL and the samples were run in triplicate. Elution profiles 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 was calculated with Astra 5.3.14 software (Wyatt Technologies) using a dn/dc of 0.185 for protein and polymer components.

Dynamic Light Scattering

Dynamic light scattering (DLS) measurements were made on a Brookhaven Instruments ZetaPals (phase analysis light scattering) particle size analyzer. DLS was measured at 90° using a 661 nm diode laser, and the correlation functions were fit using a non-negatively constrained least-squares analysis.

Transmission Electron Microscopy

TEM data were collected on a Leo 912, with Ω filter, operating at 80 keV. Samples were transferred to carbon coated copper grids and were imaged with a 1 % uranyl acetate stain.

Methyl Viologen Reduction Assay

A mixture of methyl viologen (50 mM) and EDTA (45 mM) or ethanol (10%) was made in buffer (100 mM phosphate, 50 mM NaCl, pH 8.0). For the analysis, 200 μ L of this solution was added to a cuvette as well as 50 μ L of P22-xAEMA/Ru(meth-phen)₃²⁺ (3.0 mg/mL, phosphate buffer, pH 8.0). The cuvette was sealed and de-aerated by pumping and back-filling with Ar 4 times. The samples were measured using an UV-vis (Agilent 8453) before and after being illuminated with a Luxeon Star Dental blue led light (460 nm).

Denaturing Polyacrylamide Gel Electrophoresis

P22-int macroinitiators and P22-polymer composites were analyzed using SDS-PAGE on 10-20% gradient Tris-glycine gels (Lonza). Protein was detected by staining with Coomassie blue.

CHAPTER THREE

MANGANESE (III) PORPHYRINS COMPLEXED WITH P22 VIRUS-LIKE PARTICLES AS T1-ENHANCED CONTRAST AGENTS FOR MAGNETIC RESONANCE IMAGING (MRI)

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: Shefah Qazi

Contributions: Designed and carried out the experiments and wrote the manuscript.

Co-Author: Masaki Uchida

Contributions: Assisted in experimental design.

Co-Author: Robert Usselman

Contributions: Characterized the samples by NMR and analyzed the relaxivity data.

Co-Author: Riley Shearer

Contributions: Polymerized samples for conjugation with Mn (III) porphyrin complexes.

Co-Author: Ethan Edwards

Contributions: Synthesized initiator molecule used to label P22 for polymerization and helped with polymerization reactions.

Co-Author: Trevor Douglas

Contributions: Obtained funding, coordinated the project, and assisted with the design of the experiments. Discussed the results and edited the manuscript at all stages.

Manuscript Information Page

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Trevor Douglas

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CHAPTER THREE

MANGANESE (III) PORPHYRINS COMPLEXED WITH P22 VIRUS-LIKE PARTICLES AS T1-ENHANCED CONTRAST AGENTS FOR MAGNETIC RESONANCE IMAGING (MRI)

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Abstract

Virus-like particles are powerful platforms for the development of functional hybrid materials. Here, we have grown a cross-linked polymer within the confines of the bacteriophage P22 capsid (P22-xAEMA) and functionalized the polymer with various loadings of paramagnetic manganese (III) protoporphyrin IX complexes (MnPP) for evaluation as a macromolecular MRI contrast agent. The resulting construct (P22-xAEMA-MnPP) has $r_{1,\text{particle}} = 7,098 \text{ mM}^{-1} \cdot \text{s}^{-1}$ at 298 K, 2.1 T (90 MHz) for a loading of 3,646 MnPP/capsid. The Solomon-Bloembergen-Morgan (SBM) theory for paramagnetic relaxivity predicts conjugating MnPP to P22, a supramolecular structure, would result in an enhancement in ionic relaxivity, however, all loadings experienced low ionic relaxivities ranging from $r_{1,\text{ionic}} = 1.45 \text{ mM}^{-1} \cdot \text{s}^{-1}$ to $3.66 \text{ mM}^{-1} \cdot \text{s}^{-1}$, similar to

ionic relaxivity of free MnPP. We hypothesize that intermolecular interactions between neighboring MnPPs block water access to the metal site resulting in low $r_{1,\text{ionic}}$ relaxivities. We investigated the effect of MnPP interactions on relaxivity further by either blocking or exposing water binding sites on MnPP. Based on these results, future design strategies for enhanced $r_{1,\text{ionic}}$ relaxivity are suggested. The measured $r_{2,\text{ionic}}$ relaxivities demonstrated an inverse relationship between loading and relaxivity. This results in a loading dependent r_2/r_1 behavior of these materials indicating synthetic control over the relaxivity properties making them interesting alternatives to current MRI contrast agents.

Introduction

Paramagnetic metal ions, such as gadolinium, manganese, and iron, have been extensively studied as MRI contrast agents due to their ability to efficiently relax water protons. Gadolinium-based small molecules are predominantly used in medical imaging protocols as T_1 -enhanced MRI contrast agents,^{87,88} but free gadolinium (III) ions are highly toxic and can cause nephrogenic systemic fibrosis (NSF) in patients with renal failure,^{89,90} Thus, there are considerable efforts to develop effective alternative contrast agents, and manganese is a promising candidate,⁹¹ with limited toxicity at low concentrations. High metal/ligand complex stability is an important factor in contrast agent development to avoid metal-ion exchange or release and can be provided by macrocycles such as porphyrins.⁹²

Over the years, a variety of porphyrins chelated with manganese (III) ions have been investigated for their potential use as magnetic resonance (MR) contrast agents.⁹³⁻⁹⁷ Recently, Winter et al. successfully engineered a heme protein, heme nitric oxide / oxygen-binding (H-NOX) protein to replace its native heme with manganese (III) protoporphyrin IX complexes (MnPP).⁹⁷ The engineered H-NOX exhibited enhanced relaxivity ($r_{1,\text{ionic}} = 12.0 \text{ mM}^{-1} \cdot \text{s}^{-1}$) at 60 MHz compared to the protein containing its native heme ($r_{1,\text{ionic}} = 5.9 \text{ mM}^{-1} \cdot \text{s}^{-1}$) and commercially available manganese and gadolinium-based contrast agents ($r_{1,\text{ionic}} = 3.0 \text{ mM}^{-1} \cdot \text{s}^{-1}$ for both). Although engineering of a heme protein as a scaffold of MnPP is an elegant approach, the number of MnPP per protein is limited to the number of heme binding sites, i.e. one per protein in their paper. In order to progress towards clinical application, we aim to establish a well-designed molecular system that has the capability to encapsulate such agents with much higher loading density, protect them from the external environment, and deliver them to a targeted tissue.

One approach has been to use covalent attachment of small-molecule contrast agents to macromolecular platforms.^{98,99} Various groups have explored dendrimers,¹⁰⁰ liposomes,^{101,102} protein cages,⁶⁹ and gold nanoparticles¹⁰³ as potential platforms for conjugation of small-molecule contrast agents. Virus-like particles (VLPs) derived from virus particles but devoid of their nucleic acid have an advantage over other systems because of their large macromolecular size and control over assembly, which allows for high loading density of desired cargo molecules. We, and others, have explored these

systems, such as Cowpea chlorotic mottle virus (CCMV),⁹⁰ Tobacco mosaic virus (TMV),¹⁰⁴ Cowpea mosaic virus (CPMV),¹⁰⁵ bacteriophages Q β ,⁶⁶ MS2^{106,107} and

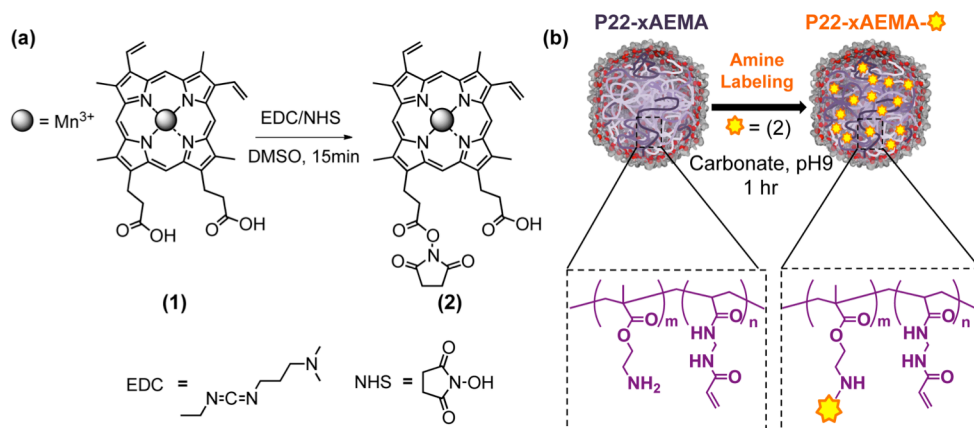


Figure 3.1 (a) Manganese (III) protoporphyrin IX MnPP (1) was reacted with EDC/NHS for 15 min in DMSO to create an activated species (2). (b) The resulting product (2) was reacted with P22-xAEMA for 1 hr in carbonate pH 9 buffer to couple MnPP to reactive amine groups on the P22-xAEMA polymer.

P22^{47,48,108} as macromolecular platforms for contrast-agent development. Although this research has resulted in the successful development of VLP-based MRI contrast agents, most of them utilized only the capsid surface to anchor imaging probes, not taking full advantage of the large interior cavity of the capsids.

Recently, we have successfully exploited the interior cavity of the P22 viral capsid and developed Gd-based MRI contrast agents with significantly high ionic (per Gd ion) and particle (per P22 capsid) relaxivities. In those studies, organic polymers were synthesized in the confined environment of the interior cavity of P22 VLPs either by a series of azide-alkyne ‘click’ reactions⁴⁸ or by atom-transfer radical polymerization (ATRP)⁴⁷ and were used as a scaffold to conjugate imaging molecules *via* a reactive

functional group on the polymer. This resulted in a significant increase in loading capacity of the cargo by taking full advantage of the capsid volume. Here, we explore the use of manganese (III) protoporphyrin IX (MnPP) complexed with P22 VLP-polymer hybrids (P22-xAEMA) as potential MRI contrast agents. MnPP was conjugated to P22-xAEMA *via* a coupling reaction between carboxyl groups of the porphyrin and pendant amine groups of the P22-xAEMA (Figure 3.1) resulting in a high per-particle relaxivity for efficient T_1 -enhanced MRI contrast-agent development. Furthermore, as ionic relaxivities of these constructs were lower than expected, we investigated MnPP aggregation, which we hypothesize is due to intermolecular interactions between neighboring MnPP that can potentially block water access to the metal site, and discuss potential solutions.

Results and Discussion

Manganese (III) protoporphyrin IX (MnPP) was attached to P22-xAEMA with various loadings by means of a simple coupling reaction.¹⁰⁹ The carboxylate groups of MnPP were activated with EDC/NHS and reacted with the amine functional groups of the P22-xAEMA polymer, encapsulated on the interior of the P22 capsid (Figure 3.1). MnPP has two carboxyl groups and both could be coupled with the amine groups of the P22-xAEMA polymer. It is difficult to differentiate between the numbers of singly labeled carboxyl groups versus doubly labeled, and so, to minimize activation of both carboxyl groups on MnPP, a 1:1 molar ratio of EDC:NHS was added per MnPP. To investigate MnPP conjugation to the surface of P22 capsid, the reaction was also conducted on

unpolymerized P22. After removal of excess MnPP and coupling agents, the concentration of protein and attached MnPP of the resulting materials (P22-xAEMA-MnPP or P22-MnPP) was determined by UV-VIS spectroscopy using known molar absorptivities.

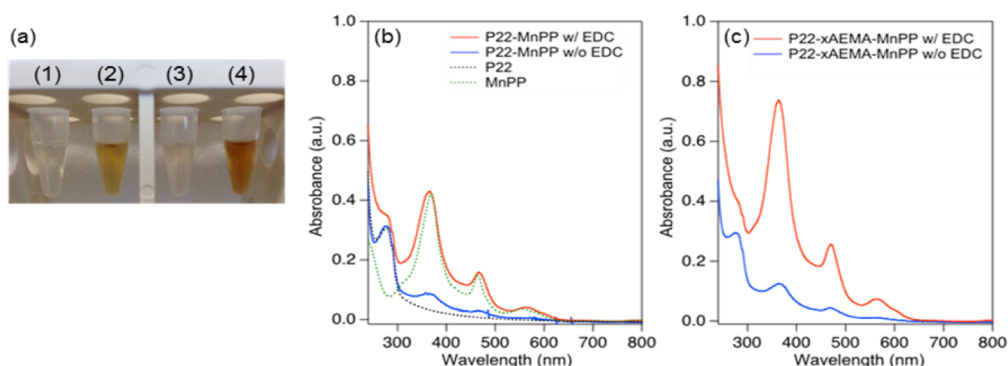


Figure 3.2 (a) Photos of each samples: (1) P22-MnPP w/o EDC, (2) P22-MnPP w/ EDC, (3) P22-xAEMA-MnPP w/o EDC and (4) P22-xAEMA-MnPP w/ EDC (1,200 MnPP/capsid). All 4 samples were incubated with 100x MnPP/subunit P22. Purification was performed using spin columns (twice) to remove unreacted manganese porphyrin from P22 and P22-xAEMA treated without (no color) and with (reddish color) EDC/NHS. UV-Visible spectra of (b) P22-MnPP w/ (red) and w/o (blue) EDC or (c) P22-xAEMA-MnPP w/ (red) and w/o (blue) EDC. Appearance of expected peaks for MnPP (green, dotted) show up at 365, 462, and 562 nm for both P22-MnPP and P22-xAEMA-MnPP with EDC/NHS (red) while peaks are less prevalent for controls without EDC/NHS (blue), and not at all for P22 (black, dotted).

To determine the extent of non-covalent interactions between MnPP and the polymer and/or P22, both P22 and P22-xAEMA were incubated with MnPP (100x MnPP/subunit P22) in the absence of EDC/NHS. Successful attachment of the MnPP *via* EDC/NHS coupling was clear from the visible characteristics of the samples (Figure 3.2a) and confirmed spectroscopically when compared to the non-covalent interaction controls (Figure 3.2b, c). After purification by spin column, the P22-xAEMA-MnPP

sample exhibited a UV-VIS spectrum (Figure 3.2c) in which the expected peaks for MnPP at 365, 472, and 562 nm⁹⁷ were clearly visible, confirming the formation of P22-xAEMA-MnPP. (Insert Figure 3.2)

The MnPP loading per P22 capsid was determined for each of the constructs from their UV-VIS spectra. As shown in Figure B.2, the MnPP loading per capsid in the P22-xAEMA-MnPP ranged from 121 MnPP/capsid to 3,646 MnPP/capsid and was tunable based on molar excess of MnPP added per capsid during the reaction. Unpolymerized

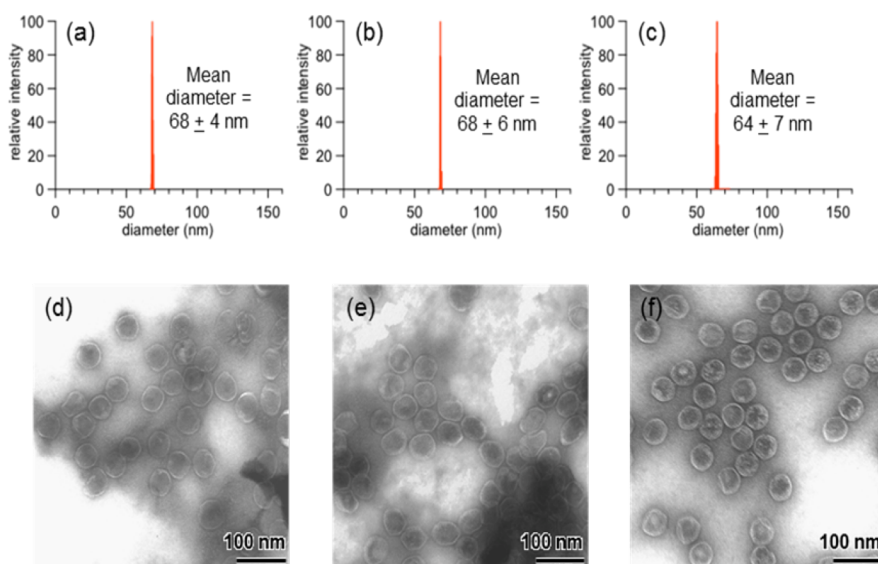


Figure 3.3 (a-c) Average diameters by dynamic light-scattering for P22 (a), P22-xAEMA (b), and P22-xAEMA-MnPP (1,200 MnPP/capsid) (c). The P22-xAEMA-MnPP construct retains its size, indicative of monodisperse population after conjugation of manganese (III) protoporphyrin IX. (d-f) Transmission electron microscopy images of P22 (a), P22-xAEMA (e), and P22-xAEMA-MnPP (1,200 MnPP/capsid) (f) shows that P22 maintains its overall morphology after conjugation to MnPP.

P22-MnPP resulted in 778 MnPP/capsid, the result of labeling endogenous lysines in the P22 capsid. There was some evidence of non-covalent interactions between MnPP and

P22, with loadings of 155 MnPP/capsid and 90 MnPP/capsid for P22-xAEMA-MnPP w/o EDC and P22-MnPP w/o EDC, respectively. All of the following characterization was done on 1,200 MnPP/capsid loading as a representative sample of P22-xAEMA-MnPP. (Insert Figure 3.3)

To confirm that the reaction with MnPP/EDC/NHS did not alter the morphology of the P22 *via* inter-particle cross-linking after EDC activation or aggregation, the size of

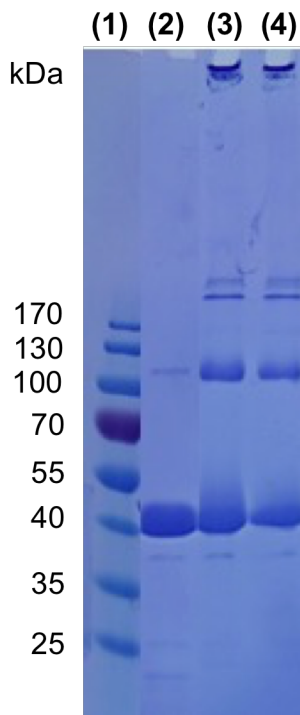


Figure 3.4 SDS-PAGE gel with marker (1), P22 (2), P22-xAEMA (3), and P22-xAEMA-MnPP (1,200 MnPP/capsid) (4).

the P22-xAEMA-MnPP was characterized. The hydrodynamic diameter, as measured by dynamic light scattering (DLS), remained essentially unchanged for the covalently-linked

MnPP samples (Figure 3.3a-c). Particle diameters 68 ± 6 nm (P22-xAEMA) and 64 ± 7 nm (P22-xAEMA-MnPP) were observed as compared to the unmodified P22 sample of 68 ± 4 nm, suggesting there was no inter-particle cross-linking mediated by the EDC reaction. When the particles were visualized by transmission electron microscopy (TEM), the morphology of the P22 appeared unchanged before and after the MnPP conjugation reaction (Figure 3.3d-f), further confirming that the cage-like structure of the P22 VLPs was unaffected by the coupling reaction. Analysis by SDS-PAGE (Figure 3.4) showed no appearance of new bands before (lane 3) and after (lane 4) modification of P22-xAEMA with MnPP. The streaking and appearance of higher molecular weight bands for P22-xAEMA (lane 3) compared to P22 (lane 2) is expected and indicative of formation of intra-particle subunit-subunit coupling due to polymer cross-linking on the interior of the capsid. Together these data suggest that the overall P22 morphology was unaffected after conjugation with MnPP. (Insert Figure 3.3)

We investigated T_1 relaxivity as a function of MnPP loading on a per Mn basis ($r_{1,\text{ionic}}$) and per P22 capsid basis ($r_{1,\text{particle}}$), over a range of loadings from 90 to 3,646 MnPP/capsid at 2.1 Tesla (90 MHz). The loading factor appeared to have almost no impact on $r_{1,\text{ionic}}$ relaxivity, which remained below $5 \text{ mM}^{-1} \cdot \text{s}^{-1}$ across the range of loadings (Figure 3.4a). However, the $r_{1,\text{particle}}$ increased with loading factor and the high loading capacity of the MnPP/capsid compensates for lower ionic relaxivities, with the highest observed $r_{1,\text{particle}} = 7,100 \text{ mM}^{-1} \cdot \text{s}^{-1}$ for this system (Figure 3.5b).

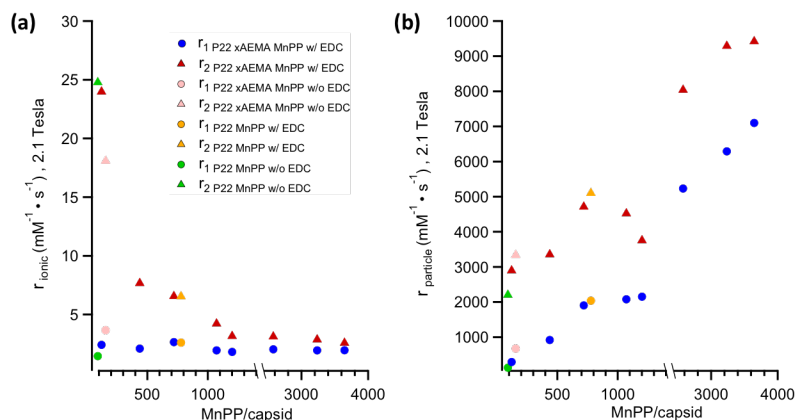


Figure 3.5 Ionic (a) and particle (b) relaxivities r_1 (blue circles) and r_2 (red triangles) of P22-xAEMA-MnPP at different loadings of MnPP, ranging from 121 to 3,646 MnPP/capsid, with P22-xAEMA-MnPP w/o EDC (155 MnPP/capsid) represented in pink, P22-MnPP w/ EDC (778 MnPP/capsid) represented in orange, and P22-MnPP w/o EDC (90 represented in green. Loading has no impact on $r_{1,\text{ionic}}$, which remains below $5 \text{ mM}^{-1} \cdot \text{s}^{-1}$, while $r_{2,\text{ionic}}$ dramatically decreases as loading increases.

The $r_{2,\text{ionic}}$ relaxivity was also evaluated for this system and was observed to have an inverse relationship with the MnPP loading factor (Figure 3.5a). The $r_{2,\text{ionic}}$ relaxivity decreased significantly with increasing MnPP loading factor at lower loading and gradually leveled off at higher loading. A similar phenomenon has been reported in an investigation of $r_{2,\text{ionic}}$ relaxivity at the initial stages of iron uptake in ferritin.¹¹⁰ In this paper, it is proposed that $r_{2,\text{ionic}}$ relaxivity decreases with increasing Fe loading factor due to the formation of antiferromagnetically coupled clusters. Similarly in our P22 system there may be more MnPP clusters at higher loading factors that can couple antiferromagnetically and which decrease the overall $r_{2,\text{ionic}}$ relaxivity of the sample. The negative impact of antiferromagnetic coupling could also affect $r_{1,\text{ionic}}$ relaxivity,¹¹¹

but we did not see a corresponding decrease in $r_{1,\text{ionic}}$ relaxivity across the MnPP loading range we examined. This phenomenon could be interesting to explore further in the future.

At lower loadings, the sample exhibits high r_2/r_1 (see Figure B.2), suggesting that this system could potentially be used as a T_2 -enhanced contrast agent. However, at higher loadings, r_2/r_1 approaches 1, thus shifting toward a T_1 -enhanced contrast agent. Therefore, P22-xAEMA loaded with MnPP can be tuned to be either a T_1 or T_2 contrast agent by controlling the loading factor. (Insert Figure 3.5)

Based on the Solomon–Bloembergen–Morgan (SBM) analytical model for understanding paramagnetic relaxivity^{32,112-114} we expected that the $r_{1,\text{ionic}}$ relaxivity would be enhanced after conjugation of MnPP to the macromolecular P22 capsid.^{47,48,115} But contrary to expectations we did not observe an enhancement. The SBM model predicts that the relaxivity is dominated by three important parameters: the number of metal-bound water molecules (q), the mean residence lifetime for metal-bound water (τ_M), and the rotational correlation time (τ_R). We, and others, have effectively used this model to predict and design new contrast agents that optimize these important parameters.^{48,69,116} Both τ_R and τ_M of free MnPP ($\tau_R = 50 - 80$ ps, $\tau_M = 10$ ns)¹¹⁷ are comparable to free Gd-DTPA ($\tau_R = 70$ ps, $\tau_M = 16$ ns).⁴⁸ We have shown previously that conjugating a Gd-DTPA to a similar P22-polymer system does not alter τ_M of the small molecule contrast agent⁴⁸ significantly, but does dramatically alter τ_R . Thus, we assumed the same τ_R for the P22-xAEMA-MnPP system and assumed that τ_M would be similar to that of free MnPP. The nuclear magnetic dispersion profile (NMRD) for MnPP

conjugated to a large particle, such as the P22-xAEMA particle (τ_R around 10 ns)^{48,69} is predicted to have enhanced relaxivity at low field (20 MHz).¹¹⁴ Thus, the ionic relaxivity of P22-xAEMA-MnPP (see Figure B.2), was measured at 0.45, 2.1, and 7 Tesla (19, 90, and 300 MHz) (Figure 3.6) with the expectation that we would see a relaxivity

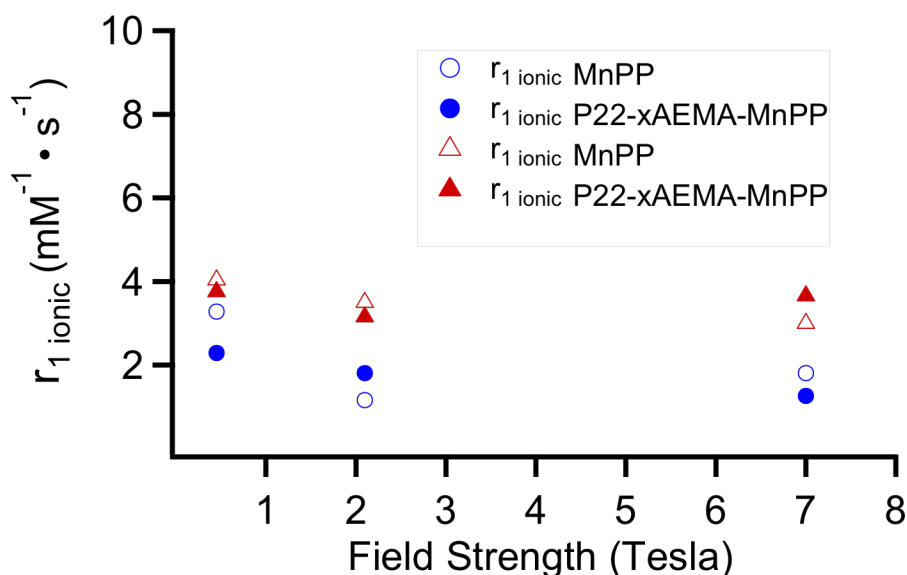


Figure 3.6 Ionic relaxivities r_1 (blue circles) and r_2 (red triangles) of P22-xAEMA-MnPP at 1,200 MnPP/capsid (closed) and free MnPP (open) at 14.5 mM (the same local concentration of its P22-xAEMA-MnPP counterpart, see Table B.1) at three different field strengths of 0.45, 2.1, and 7 Tesla. The trend shows relatively small changes in relaxivity across field strengths.

enhancement, particularly at 0.45 T (19 MHz). For comparison, the relaxivity of free MnPP at 14.5mM was also measured. The concentration of free MnPP was based on local concentration of MnPP in the P22-xAEMA capsid, i.e. the volume of the spherical 64 nm P22 and number of Mn encapsulated as the P22-xAEMA-MnPP construct (1,200 MnPP / capsid). The results show that the relaxivity changes only slightly across field

strengths, and not as significantly as expected based on NMRD profiles of other macromolecular Mn-complexes^{114,118} and our previous work with macromolecular Gd-based agents.⁴⁸ (Insert Figure 3.6)

In order to understand why no increase in $r_{1,\text{ionic}}$ relaxivity was observed for MnPP after conjugation to P22 VLPs as compared to free MnPP, we explored interactions between MnPPs further. The presence of intermolecular MnPP interactions could structurally block water access to the manganese ion, resulting in a lower effective q and thus a diminished $r_{1,\text{ionic}}$ relaxivity.¹¹⁷ This clustering of MnPPs could account for the observed lack of $r_{1,\text{ionic}}$ relaxivity enhancement for P22-xAEMA-MnPP, when compared to free MnPP. We conducted the following two experiments to explore this possibility.

To probe the effects of water access to the Mn ion on the relaxivity, we titrated free MnPP with imidazole, which is expected to bind the axial sites in MnPP and compete for water access. This competitive binding experiment was monitored by both changes in the optical spectrum and changes in the ionic relaxivity. Figure 3.7A and Figure B.3 show the changes in the UV-VIS spectrum of MnPP upon titration with imidazole.¹¹⁹ At 640 μM MnPP, imidazole was added at a range up to 1000 molar excess per MnPP. The UV-VIS spectrum shows an emerging peak at 482 nm, which increased with increasing imidazole, clearly indicating interactions between the imidazole and the MnPP complex (Figure B.3). We further investigated the effects of imidazole binding to MnPP by measuring the ^1H relaxivity at 2.1 T (90 MHz) at a range of added imidazole concentrations. We found an inverse relationship between the change in absorbance at 482 nm (imidazole binding to MnPP) and the measured relaxivity (Figure

3.7B). This is consistent with imidazole binding to both axial sites on the MnPP at high imidazole concentrations, where these sites are essentially saturated and the effective value of q approaches 0. Thus, imidazole appears to influence intermolecular MnPP interactions and blocks water access, which has a clear, dominant influence on the relaxivity. (Insert Figure 3.7)

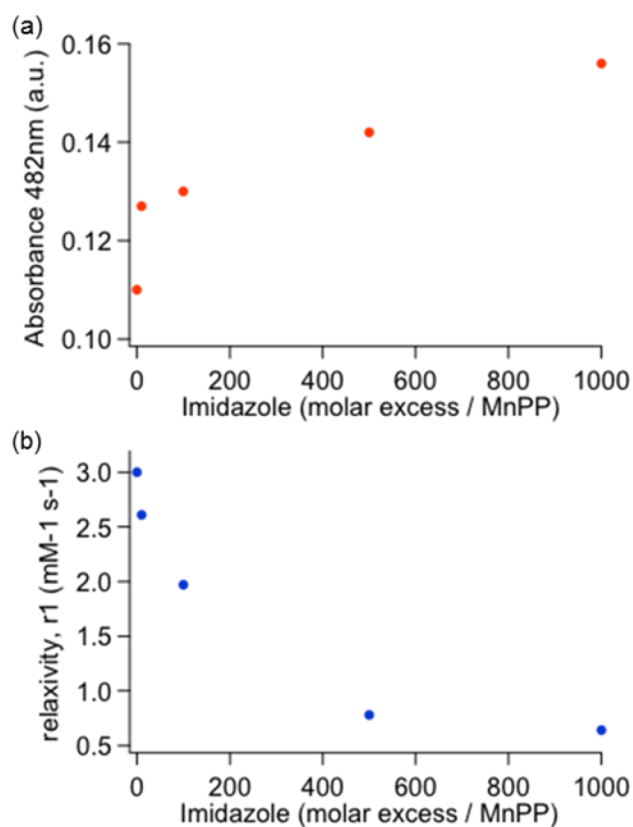


Figure 3.7 UV-VIS, absorbance at 482 nm (a) and $r_{1,\text{ionic}}$ relaxivity at 2.1 Tesla (90 MHz) (b) measurements were taken at 0, 10, 100, 500, and 1000 equivalents of added imidazole to 640 μM MnPP. There is an inverse relationship between increase in absorbance at 482 nm due to imidazole binding to MnPP and decrease in relaxivity. Even at 10 equivalents of imidazole a significant difference in relaxivity was observed.

In an attempt to disrupt intermolecular interactions between neighboring MnPPs and potentially increase water access and therefore the number of bound and exchangeable waters (q), we investigated the effects of adding acetone on the $r_{1,\text{ionic}}$

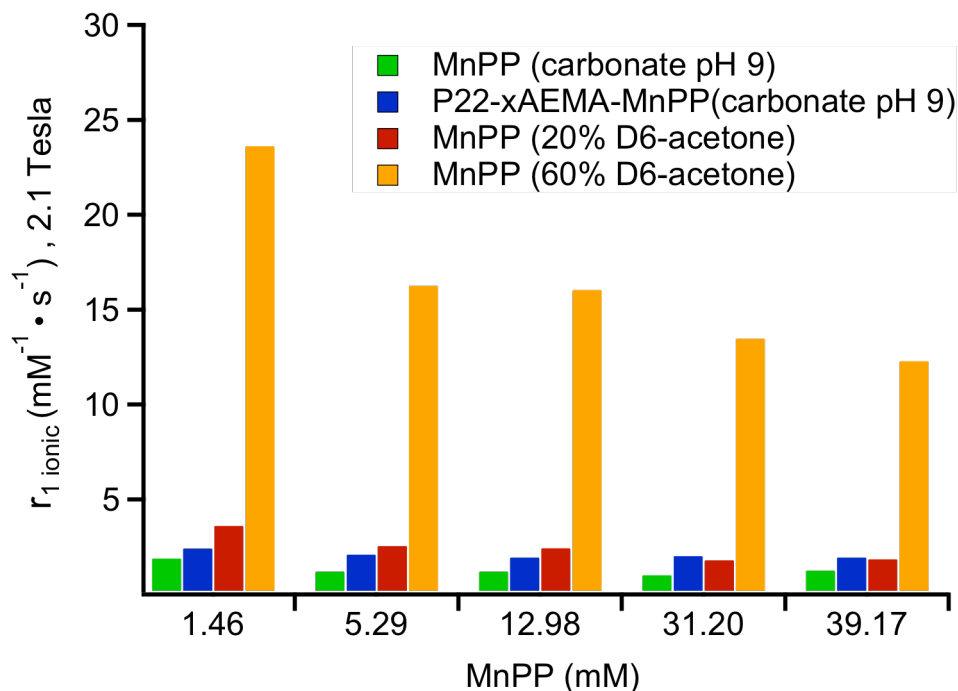


Figure 3.8 Ionic relaxivities at different concentrations of free MnPP compared to P22-xAEMA-MnPP in carbonate pH 9 buffer (See Figure B.2 for corresponding loading factors). The free MnPP was further treated with either 20% or 60% D6-acetone to try to disperse MnPPs and see impact of acetone on relaxivity. MnPP (green) at same local concentrations of MnPP for P22-xAEMA-MnPP (blue) exhibit similar $r_{1,\text{ionic}}$ values, with free being slightly smaller P22-xAEMA-MnPP. After treating free MnPP with 20% D6-acetone (red), the ionic relaxivities did not change much, only increasing slightly for the lower concentrations. At 60% D6-acetone (orange), a dramatic increase in ionic relaxivity was observed at all concentrations, with a trend toward highest increase in relaxivity for lowest concentration of MnPP.

relaxivity of free MnPP at concentrations based on the same local concentration of MnPP in our P22-xAEMA-MnPP system. First, $r_{1,\text{ionic}}$ relaxivity of MnPP and P22-xAEMA-MnPP were investigated at 2.1 Tesla (90 MHz). MnPP and P22-xAEMA-MnPP exhibited

similar $r_{1,\text{ionic}}$ values at all concentrations, with free MnPP having slightly smaller $r_{1,\text{ionic}}$ values than P22-xAEMA-MnPP (Figure 3.8). Second, $r_{1,\text{ionic}}$ relaxivity of MnPP in the presence of 20 % or 60 % D6-acetone was measured. These experiments were conducted only as a proof of concept, as acetone is neither a suitable solvent for protein stability nor for *in-vivo* applications. The free MnPP was treated with D6-acetone to disperse the MnPPs and observe the impact on relaxivity (Figure 3.8). After treating free MnPP with 20 % D6-acetone, the ionic relaxivities did not show significant change, with only slight increase in relaxivity at the lower MnPP concentrations. However, with 60 % D6-acetone, a dramatic increase in ionic relaxivity was observed at all concentrations, with a trend toward highest increase in relaxivity for the lowest concentration of MnPP. This data correlates well with published data¹¹⁷ and indicates that the acetone disrupts intermolecular MnPP interactions, resulting in a greater water exchange at the manganese centers and an increase in the effective value of q . Extrapolation from these data suggest that the high local concentrations of MnPP inside the P22-xAEMA-MnPP probably results in strong intermolecular interactions between neighboring MnPPs, resulting in a lower observed ionic relaxivity compared to when the interactions are disrupted, as in the case of free MnPP in the presence of acetone. This result implies that we could increase ionic relaxivity of MnPP by disrupting inter-molecular interactions between MnPPs. For example, modification of the porphyrin moiety, in a manner analogous to the designed picket-fence porphyrins,¹²⁰ could minimize MnPP interaction. (Insert Figure 3.8)

Conclusions

In summary, we have covalently encapsulated a large number of MnPP inside P22 VLP-polymer hybrids and demonstrated potential for this construct as a tunable MRI contrast agent. This system could be a good alternative to gadolinium-based contrast agents, which are known to be linked to nephrogenic systemic fibrosis. We can conjugate more MnPP to P22-xAEMA versus P22 alone, using simple EDC/NHS to chemically introduce MnPP to the P22 capsid up to 3,646 MnPP/capsid. The improvement in labeling is important for the delivery of contrast agents on a per-particle basis, allowing for high concentration delivery of the contrast agent. Although we do not see an improvement in $r_{1,\text{ionic}}$ relaxivity, the high per-particle relaxivity of the P22-xAEMA-MnPP, resulting from high loading density, could be beneficial when these particles are imparted with targeting cell capabilities. Our results suggest that we could also increase $r_{1,\text{ionic}}$ relaxivity of MnPP by disrupting inter-molecular interactions between MnPPs. In future design iterations of these hybrid materials we might overcome intermolecular interactions of MnPPs by altering the porphyrin moiety to minimize MnPP interactions.¹²⁰ Alternatively, incorporation of other Mn-porphyrins, which have larger $r_{1,\text{ionic}}$ relaxivity, such as Mn-TPPS,^{117,121,122} may enable us to more fully utilize the advantages of the P22-polymer platform.

Acknowledgements

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Experimental

Materials

All materials were analytical grade and purchased from either Sigma-Aldrich or Fisher Scientific and used as received unless otherwise noted. All water was deionized by use of a NANOpure water purification system. All protein samples that have been chemically modified were analyzed *via* UV-VIS (UV-Vis; Model 8453, Agilent, Santa Clara, CA) and dynamic light-scattering (DLS; 90Plus particle-size analyzer, Brookhaven Instrument, Holtsville, NY). The synthesis of 2-bromoisobutryl aminoethyl maleimide has been reported.⁴⁷ The manganese (III) protoporphyrin IX chloride was purchased from Frontier Scientific.

Mutagenesis, Protein Purification, and P22-xAEMA Formation

The P22 (S39C) point mutation was made with established polymerase chain-reaction protocols (Agilent) with pET-11a-based plasmids encoding genes for scaffolding and coat protein. The amplified DNA was transformed into *E. coli* strain BL21 (DE3) and selected for ampicillin resistance.⁴⁷ P22 (S39C) procapsid (PC), made up of 420 subunits, was produced by a heterologous expression system in *E. coli*. and purified by a previously described procedure.⁴⁸ The resulting virus pellet, after purification, was resuspended in 100 mM sodium phosphate, 50 mM NaCl, pH 7.0, and spun at 17,000 rpm for 20 minutes to remove particulates and lipid. The PC was heat treated for 20 min at 65 °C to transform the protein into its expanded (EX) form as previously described and analyzed.⁸³ The EX samples were purified by pelleting *via* centrifugation at 45,000 rpm

for 50 minutes in an ultra-centrifuge (Sorvall), followed by resuspension into 100 mM sodium phosphate, 50 mM NaCl, pH 7.6 in preparation for protein labeling. The EX was labeled with an ATRP initiator molecule and cross-linked AEMA polymer (P22-xAEMA) was synthesized inside the P22 VLP cavity, as previously described.⁴⁷ P22 procapsid (PC), expanded (EX), and polymerized (P22-xAEMA) samples were characterized with SDS-PAGE, transmission electron microscopy (TEM, Leo 912 AB), and dynamic light scattering (DLS, Brookhaven 90Plus, Brookhaven, NY).⁴⁷ The protein concentration was determined by absorbance at 280 nm where an extinction coefficient ($65,920 \text{ M}^{-1} \text{ cm}^{-1}$, subunit P22) was used. As described above, EX form of P22 was exclusively used for P22-xAEMA formation, and hereafter, we will refer to unpolymerized EX form of P22 as P22 and polymerized P22 as P22-xAEMA. All further labeling experiments with P22 and P22-xAEMA and sample analysis were conducted in sodium bicarbonate buffer (100 mM sodium bicarbonate, 50 mM NaCl, pH 9.0) because MnPP is more soluble in this buffer than in phosphate buffer at neutral pH.

Manganese (III) Protoporphyrin IX Labeling Conditions (MnPP) – without EDC/NHS

P22 and P22-xAEMA were labeled with MnPP without the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS). First, a 30 mM stock solution of MnPP was made up in DMSO and vortexed until completely dissolved. From the 30 mM MnPP stock solution, 72 μL (1.40 mg, 2.14 μmoles) MnPP was diluted with 144 μL water. Next, to 284 μL of P22 or P22-xAEMA (0.0214 μmoles subunit, 3.5 mg/mL, 100 mM sodium carbonate buffer, 50 mM NaCl, pH 9.0), 216 μL of

diluted MnPP was added dropwise (100x MnPP/subunit of P22, i.e. 42,000x MnPP/capsid of P22) while vortexing the protein solution. The mixture was allowed to react for 1 hr at room temperature followed by purification away from excess MnPP/EDC/NHS with a Micro Bio-Spin column (Bio-Rad, Hercules, CA) twice, resulting in 90 MnPP/capsid (67.7 % yield) and 184 MnPP/capsid (71.8 % yield), respectively.

Manganese (III) Protoporphyrin IX Labeling Conditions (MnPP) – with EDC/NHS

Various loadings of MnPP per P22-xAEMA (121 to 3,646 MnPP/capsid) were achieved by using ranging molar excess from 1 to 100 MnPP/subunit P22. The following describes preparation of 100 molar excess MnPP/subunit P22. To prepare P22-MnPP and P22-xAEMA-MnPP with EDC/NHS, a 1:1:1 molar ratio of MnPP:EDC:NHS was made by adding 72 μ L (30 mM in DMSO, 1.40 mg, 2.14 μ moles) MnPP to a solution of 72 μ L EDC (30 mM in water, 0.25 mg, 2.14 μ moles) and 72 μ L NHS (30 mM in water, 0.41 mg, 2.14 μ moles) and stirred for 15 min at room temperature to activate carboxyl groups on MnPP. To 284 μ L P22 or P22-xAEMA (0.0214 μ moles subunit, 3.5 mg/mL, 100mM sodium carbonate buffer, 50 mM NaCl, pH 9.0), 216 μ L of MnPP/EDC/NHS mixture was added dropwise while vortexing the protein solution. The mixture was allowed to react for 1 hr at room temperature followed by purification away from excess MnPP/EDC/NHS by use of a spin column twice, resulting in 780 MnPP/capsid (82.0 % yield) and 1,200 MnPP/capsid (92.3 % yield), respectively. It should be noted that amount of labeling varies when experiment is repeated at same molar excess (i.e for

molar excess of 100 MnPP/subunit P22, labeling ranges from 1,200-3,646 MnPP/capsid).

All NMR data is based on the output, which is labeling amount of MnPP/capsid.

Sample Analysis

P22 and P22-xAEMA samples and those with MnPP were analyzed by a variety of methods aimed at interrogating the purity, morphology, and composition of the samples. P22, P22-xAEMA, and P22-xAEMA-MnPP were imaged by transmission electron microscopy (Leo 912 AB, 100 kV) by negatively staining the sample with 1 % uranyl acetate on Formvar carbon coated grids. These samples were also analyzed with SDS-PAGE on 10-20 % gradient Tris-glycine gels (Lonza). Protein was detected by staining with Coomassie blue.

Protein and Manganese Concentration

Protein concentrations of P22 and P22-xAEMA samples were determined by absorbance at 280 nm (extinction coefficient: $65490 \text{ M}^{-1} \cdot \text{cm}^{-1}$, subunit P22). The protein concentration for P22-xAEMA-MnPP samples was determined by absorbance at 280 nm after the contribution from manganese porphyrin (extinction coefficient: $19.82 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) was subtracted. The amount of MnPP conjugated with P22 VLPs was determined using an extinction coefficient of $25 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 462 nm.¹²³

Relaxivity Measurements

T_1 and T_2 measurements were carried out on a broadband 19 MHz (0.447 T) vertical NMR spectroscopy system, Anasazi FT-NMR 90 MHz (2.1 T) and Bruker DPX 300 MHz (7.0 T) spectrometers. Each P22-xAEMA-MnPP sample was diluted with 67 %

D₂O in H₂O to yield a dilution series. The longitudinal relaxation rate constant (T_1) was measured at each dilution by use of an inversion recovery pulse sequence and the transversal relaxation rate constant (T_2) was measured by use of a Carr-Purcell-Meiboom-Gill (CPMG) sequence at 298 K, where the relaxation delay was set to six times the estimated T_1 or T_2 . For 19 MHz (0.447 T), recovery times of 30 s were used for T_1 relaxation times. Figure B.1 shows a plot of $1/T_1$ versus [Mn] (mM) for the 3 dilutions which was used to determine relaxivity values ($r_{1,\text{ionic}}$), where ionic relaxivity (relaxivity per Mn) is equal to the slope of the line, and particle relaxivity (relaxivity per P22) was calculated by multiplying the slope by Mn/capsid. Similarly, a plot of $1/T_2$ versus [Mn] (mM) was used to determine and $r_{2,\text{ionic}}$ relaxivity values.

Imidazole Titration Experiments to Block Water Access to MnPP

In order to assess the influence of water accessibility on $r_{1,\text{ionic}}$ relaxivity of MnPP, we titrated free MnPP with imidazole. Imidazole was dissolved in carbonate buffer (100 mM sodium carbonate, 50 mM NaCl, pH 9.0) to create a stock solution of 10 M, which was added to 640 μM free MnPP in molar equivalents of 0, 10, 100, 500, or 1000 excess to give a final manganese concentration of 390 μM . Samples were further characterized *via* UV-VIS spectrometer with a 40x dilution. T_1 relaxivity of the samples was measured as described above.

Addition of Acetone to Disrupt Inter-Molecular Interactions of Free MnPP

In order to evaluate the potential effect of MnPP aggregation within P22 capsid on $r_{1,\text{ionic}}$ relaxivity, we compared free MnPP in solution versus MnPP conjugated to P22-xAEMA at the same concentration. The concentration of free MnPP was based on local concentration of MnPP in the P22-xAEMA capsid, i.e. the volume of the spherical 64 nm P22 and number of Mn encapsulated as the P22-xAEMA-MnPP construct. Free MnPP was prepared in different concentrations, from 1.46 - 39.17 mM MnPP for P22-xAEMA-MnPP at loadings ranging from 121-3646 MnPP/capsid (100 mM sodium carbonate, 50 mM NaCl, pH 9.0). In addition, in order to evaluate the effect of disrupting inter-particle interactions between MnPPs, we treated free MnPPs with two different concentrations of acetone, and measured $r_{1,\text{ionic}}$ relaxivity. At all concentrations of MnPP, deuterated acetone was added to achieve a final matrix of 60 % D6-acetone, 33.33 % H₂O and 6.67 % D₂O or 20 % D6-acetone, 33.33 % H₂O and 46.67 % D₂O. For 60 % D6-acetone, 252 μL deuterated acetone (99.5 %-d) was added to 140 μL of MnPP and 28 μL of D₂O. For 20 % D6-acetone, 84 μL deuterated acetone (99.5 %-d) was added to 140 μL of MnPP and 196 μL of D₂O. This was further diluted with corresponding matrix to yield a dilution series of 1:3, 1:9, and 1:27. T_1 relaxivity was measured as described above in the relaxivity measurements section.

CHAPTER FOUR

CO-LOCALIZATION OF CATALYSTS WITHIN A PROTEIN CAGE LEADS TO
EFFICIENT PHOTOCHEMICAL NADH AND/OR HYDROGEN PRODUCTIONContribution of Authors and Co-Authors

Manuscript in Chapter 4

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Contributions: Assisted in analysis of data, and critically edited the manuscript.

Co-Author: Benjamin Schwarz

Contributions: Assisted in analysis of data, edited and helped write the manuscript.

Co-Author: Paul Jordan

Contributions: Assisted in analysis of data and critically edited the manuscript.

Co-Author: John Lisher

Contributions: Assisted in ICP-MS analysis.

Co-Author: Trevor Douglas

Contributions: Obtained funding, coordinated the project, and assisted with the design of the experiments. Discussed the results and edited the manuscript at all stages.

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CHAPTER FOUR

CO-LOCALIZATION OF CATALYSTS WITHIN A PROTEIN CAGE LEADS TO EFFICIENT PHOTOCHEMICAL NADH AND/OR HYDROGEN PRODUCTION

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Abstract

Using the interior of the P22 virus-like particle (VLP) we have co-localized and constrained multiple copies of a photosensitizer (Eosin-Y) and a NADH/Hydrogen catalyst (Cobaloxime). These small molecules were conjugated to an amine bearing polymer framework synthesized within the confines of the P22 capsid by atom transfer radical polymerization (ATRP). Using aminoethyl methacrylate (AEMA) and bis-acrylamide as the monomers we introduced a crosslinked polymer framework with addressable amines and conjugated each of the small molecules through an isothiocyanate moiety. With precise control over the average labeling stoichiometry, we conjugated the Eosin-Y and Cobaloxime catalysts to the polymer such that they were co-localized on the interior of the P22 VLP. This co-localization facilitated the photochemical production of NADH from NAD⁺ under aqueous conditions with a maximum turnover of $11.40 \times 10^{-3} \text{ s}^{-1}$. The reaction products could be switched from NADH to H₂ production by increasing the relative stoichiometry of the Cobaloxime

labeling. The co-confinement of this coupled catalytic system within the VLP P22 creates a nano-material whose turnover activity is independent of the bulk concentration. To the best of our knowledge this is the first example of using a VLP to control the efficient photochemical production of both NADH and hydrogen, by creating a co-localized, spatially arranged and protected environment.

Introduction

Controlling the spatial arrangement of catalysts in a complex multi-step reaction is a powerful tool used by natural systems to control the flux of reactants and products.^{14,44,124-126} Photosynthesis is an example of particularly well-defined structurally regulated process with photosystems arranged in complexes across a membrane interface. The biological conversion of light to high energy molecules (such as ATP or NAD(P)H) depends on the efficient intermolecular transfer of electrons through co-localized iron-sulfur centers and transport proteins within photosystem complexes.^{124,125,127} Artificial photosynthetic strategies have continued to gain attention in light of increased global awareness of energy needs, which highlights the need for efficient light-harvesting systems.^{124,125,128} Biomimetic nanoscale arrangement of catalysts is a minimally exploited design principle that may lead to more efficient target product generation in synthetic catalyst systems.

Taking inspiration from nature we have used a virus-like particle as a template for the construction of a coupled catalyst system where co-catalyst densities can be controlled. Virus-like particles (VLPs) provide highly regular nanomaterial platforms for

the attachment of molecules in highly regular arrangements.⁴⁴ Broadly, VLPs are self-assembling protein cages that are derived from viral, or non-viral, cage forming proteins. The self-assembly of VLPs leads to highly monodisperse particles, that often contain a symmetric arrangement of protein subunits. Taking advantage of the multivalency of the subunits in these VLPs, a range of bioconjugation strategies have been developed to incorporate synthetic cargos selectively within the confines of the assembled particles.^{39,47,66,72} Here we show that the VLP provides a structural framework for two catalysts, a light harvesting photocatalyst Eosin-Y (EY) and a NADH/hydrogen catalyst chloro(pyridine)cobaloxime ($\text{CoCl}(\text{dmgH})_2\text{Pyr}$), in an effort to increase the efficiency of the coupled system and provide control over the generation of NADH or hydrogen as the final products of the reaction pathway.

The VLP derived from the bacteriophage P22 has been effectively used as a nanoscale template for the encapsulation of multiple copies of synthetic polymers,^{47,48} peptides,⁴⁶ enzymes,^{49,129} small molecules,^{47,51} and nanoparticles.⁴⁶ The P22 VLP is assembled from 420 subunits of a coat protein (CP) and 100-300 subunits of a scaffolding protein (SP). A unique feature of this VLP is that it can transform into distinct morphological forms that have different sizes and mechanical properties.^{47,130} Because of the versatility of the P22 VLP and the wide range of synthetic cargos that can be incorporated, this system is an excellent template for confining and co-localizing small molecule catalysts.

We have previously demonstrated complex behavior of sequential enzymatic catalysts co-localized on the interior of the P22 VLP particle.⁵¹ Here we study the effects

of co-localization on a useful small molecule catalyst system that has the potential to benefit significantly from precise structural arrangement and co-localization.

$\text{CoCl}(\text{dmgH})_2\text{Pyr}$ is a small molecule catalyst that has been extensively studied for generating hydrogen both electro- and photochemically.¹³¹⁻¹³⁵ Recently, $\text{CoCl}(\text{dmgH})_2\text{Pyr}$ and EY together have been reported to photochemically produce NADH and hydrogen in aqueous conditions.¹³⁴

The two-catalyst system of EY/ $\text{CoCl}(\text{dmgH})_2\text{Pyr}$ requires the efficient transfer of electrons from the photoexcited EY to the $\text{CoCl}(\text{dmgH})_2\text{Pyr}$, a process that may be significantly enhanced by co-localization, reminiscent of the natural photosynthetic systems. Also, $\text{CoCl}(\text{dmgH})_2\text{Pyr}$ has been shown to catalyze the production of both hydrogen and NADH under the same bulk reaction conditions.¹³⁴ The end product of this reaction could be conceivably controlled by structural segregation of the $\text{CoCl}(\text{dmgH})_2\text{Pyr}$ catalysts, the interaction of which (homolytic pathway) is one way to form the hydrogen product.

In this study, we have polymerized the interior of the P22 capsid to form a crosslinked poly-aminoethyl methacrylate network (P22-xAEMA) with addressable labeling sites on the side chain of the polymer to which we immobilized both the light harvesting Eosin-Y Isothiocyanate (EY-NCS) and reduction catalyst chloro(3-pyridyl isothiocyanate)cobaloxime (Co-NCS) in controlled stoichiometric ratios. Using visible light to excite the EY and drive the reduction of NAD^+ to form NADH or the reduction of H^+ to form H_2 , we found that this co-catalyst system was significantly more efficient when encapsulated and co-localized inside P22 (P22-Co/EY) compared to the co-

catalysts free in solution. By controlling the relative concentrations of each catalyst during the polymer labeling we could also tune the reaction pathway from NADH formation (at low cobalt incorporation) to H₂ formation (at high cobalt incorporation) indicating control over the reaction pathway through the material design and synthesis.

Results and Discussion

We have used the virus-like particle (VLP) from the bacteriophage P22 as a hollow nanoscale template for the co-localization of multiple copies of a light harvesting catalyst and NADH/H₂ catalyst to effect two photocatalytic reduction reactions (Fig. 4.1A). The P22 VLP was recombinantly expressed in *Escherichia coli*, and assembled into a T = 7 procapsid (PC) particle. Upon heating to 65°C the capsid underwent an expansion that is structurally related to the expansion observed in the infectious virus upon packing of DNA. We used the EX form of P22 in this study because of its larger internal volume (35 % increase) and stability.¹³⁰

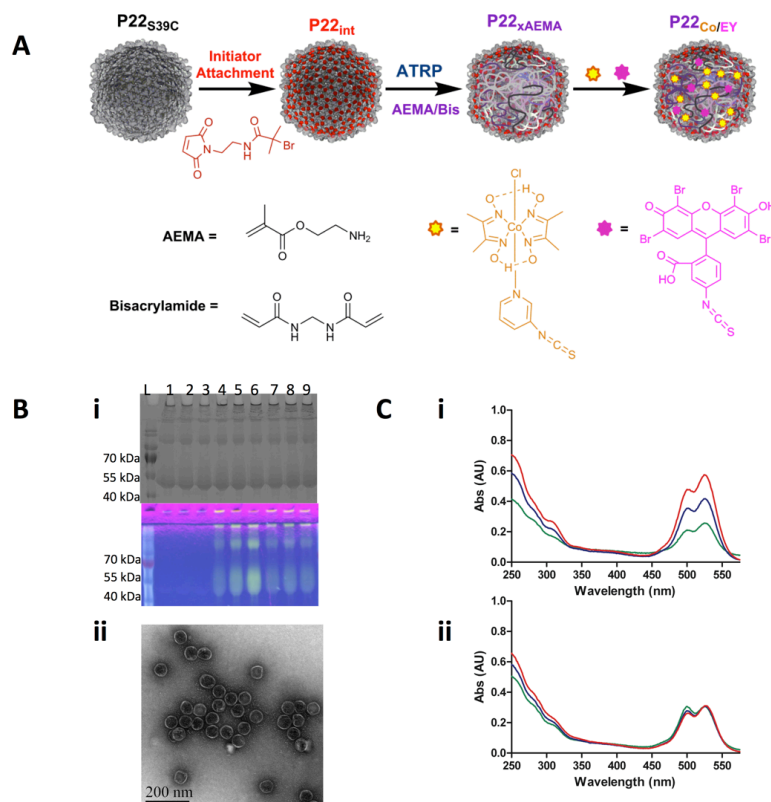


Figure 4.1: Polymerization and characterization of P22-Co/EY material. (A) Polymerization scheme for the formation of the P22-Co/EY photosynthetic material by atom transfer radical polymerization (ATRP) and isothiocyanate labeling of free amines. (B) Two different views of the same SDS-PAGE denaturing gel with samples: (L) protein ladder (10-180 kDa), (1) P22-xAEMA-control 1, (2) P22-xAEMA-control 2, (3) P22-xAEMA-control 3, (4) P22_{0.25}xCo/0.25x_{EY}, (5) P22_{0.25}xCo/0.50x_{EY}, (6) P22_{0.25}xCo/0.75x_{EY}, (7) P22_{0.25}x_{EY}/0.25xCo, (8) P22_{0.25}x_{EY}/0.50xCo, (9) P22_{0.25}x_{EY}/0.75xCo. (i) SDS-PAGE analysis shows a stained gel with a large protein band starting at the expected molecular weight (46 kDa) with streaking to higher molecular weight bands indicative of polymers of different sizes formed from the P22 subunit. Unstained gel exposed to UV light showing the fluorescence of Eosin-Y migrating with the P22 protein-polymer material. (ii) Transmission electron microscopy (TEM) image of the P22-Co/EY material showing that P22 maintains its structure after chemical modifications. (C) Two UV-vis spectra of the P22-Co/EY hybrid materials. (i) Samples 4-6. UV-vis spectra showing the absorbance change at 525 nm indicating increased labeling of Eosin-Y. (ii) Samples 7-9. UV-vis spectra showing the absorbance change at 320 nm indicating increased labeling of Cobaloxime while the absorbance at 525 nm stays the same indicating constant Eosin-Y labeling.

A P22 mutant (P22_{S39C}) was used for these studies in which a unique cysteine residue, located on the interior surface allowed for the selective attachment of a small polymer initiation molecule to the introduced thiol.^{47,48} To ensure the EX (P22_{S39C}EX)

morphology was achieved after heating, samples were analyzed by native agarose gel electrophoresis and monitored for a lower electrophoretic mobility, indicative of capsid expansion (Fig. C.1). The sample was additionally analyzed by size exclusion chromatography coupled with multi-angle light scattering (MALS) and refractive index detection to determine both the Mw and size. These data revealed a Rg of 28.9 ± 0.1 nm and the molecular weight of 19.7 MDa as compared to the PC form, which has a Rg of 24.7 ± 0.1 nm, and Mw of 23.4 MDa.

P22_{S39C}EX was reacted with the ATRP polymer initiator **1** (P22-int) through the maleimide with near-quantitative single labeling (Fig. 4.1A). Initiator **1** was synthesized by modification of a previously reported protocol^{63,84} and the small molecule attachment was monitored and confirmed by electrospray mass spectrometry, with a mass of 46,928 kDa (46,926 kDa expected).^{47,48}

By using a “grafting from” approach, atom transfer radical polymerization (ATRP) was performed to synthesize an addressable crosslinked polymer framework containing aminoethyl methacrylate (AEMA) and bis-acrylamide on the interior of the P22-int (Fig. 4.1A). Polymerization was catalyzed by a Cu(I)/bipyridine catalyst system, a standard condition for biomolecule ATRP.^{47,48,64,68} The monomer AEMA was chosen because it contains a primary amine, which allows for post polymerization modification by small molecule labeling of each monomer on the interior of the capsid. The extent of polymerization was evaluated by SDS-PAGE, which revealed streaking of the P22 coat protein subunits, indicative of polymer growth from the subunits with a range of polymer lengths (Fig. 4.1Bi).^{47,48} A large change in electrophoretic mobility was observed by

native agarose gel after polymerization indicative of a change of overall charge of the VLP, demonstrating that the P22-int had transformed to the P22-xAEMA (Fig. C.1). The size of P22-xAEMA was measured by dynamic light scattering (DLS) (Fig. C.2). The P22-xAEMA (62.34 ± 15.19 nm) revealed no significant increase in size compared to P22-int (61.39 ± 6.89 nm) confirming that the polymer was constrained on the interior of the capsid. P22-xAEMA was also imaged by TEM and showed that there was no change in the overall morphology of the P22 after polymerization (Fig. 4.1Bii).

We designed and synthesized a $\text{CoCl}(\text{dmgH})_2\text{Pyr}$ derivative that contained a 3-pyridyl isothiocyanate (pyr-NCS) as a ligand to the cobalt, which we subsequently used for covalent attachment of the cobaloxime to the pendant amines on the encapsulated AEMA polymer (Fig. 4.1A). The $\text{CoCl}(\text{dmgH})_2\text{Pyr}$ derivative (Co-NCS) was synthesized by modifications of previously published protocols.¹³⁶ Briefly, reacting compound **2** with 3-pyridyl isothiocyanate in methanol for ~30 minutes converted the green solid into a brown crystalline solid **3**, which was isolated via vacuum filtration and washed with water/ether (Fig. C.3). The compound was analyzed by NMR (^1H and ^{13}C) and electrospray mass spectrometry (ESI-MS), which revealed the expected product ($[\text{M} + \text{Na} + \text{CH}_3\text{OH}]^+$, theoretical = 515.8090 Da and experimental = 515.0291 Da) with a yield of 85%.

To make the desired photocatalytic P22 nanoparticles, which incorporated both the light harvesting and reduction catalyst, isothiocyanate derivatives of the $\text{CoCl}(\text{dmgH})_2\text{Pyr}$ (Co-NCS) and EY (EY-NCS) were attached to the amine rich polymer framework in controlled stoichiometric ratios (Fig. C.4) with either an excess of one

catalyst over the other or an equimolar amount of each catalyst. This labeling strategy allowed us to create samples (with excess or equimolar amounts of each catalyst) to investigate the Cobaloxime and EY dependence on the overall photochemical activity and to find an optimal labeling density of catalysts.

The polymer framework was labeled with Co-NCS and the cobalt concentration of the resultant material was quantified by inductively coupled plasma mass spectrometry (ICP-MS). Alternatively, the poly-AEMA on the interior of the capsid was labeled with EY-NCS, which was easily quantified by UV-vis spectroscopy from its characteristic absorbance at 525 nm (ext. coeff. = $112,000 \text{ M}^{-1}\text{cm}^{-1}$).¹³⁷ We designed two sets of samples to be sequentially labeled with different stoichiometric amounts of Co-NCS and EY-NCS. The first two sets were labeled with either 0.25x Co-NCS or 0.25x EY-NCS (relative to the number of free amines on the polymer) and analysis by ICP-MS and UV-vis revealed that on average 587 ± 44 and 751 ± 114 (Co-NCS or EY-NCS, respectively) were attached per P22. After labeling and purification these samples were equally divided into 3 sample subsets, that were then re-labeled with the other corresponding co-catalyst (EY-NCS or Co-NCS respectively), allowing us to vary the amount of the second catalyst. These resulting samples were labeled with 0.25x (P22_{0.25xCo/0.25xEY}, P22_{0.25xEY/0.25xCo}), 0.50x (P22_{0.25xCo/0.50xEY}, P22_{0.25xEY/0.50xCo}), or 0.75x (P22_{0.25xCo/0.75xEY}, P22_{0.25xEY/0.75xCo}) of the second catalyst and after purification and analysis the samples were found to have on average 583 ± 9 and 537 ± 2 , 1112 ± 17 and 1067 ± 9 , or 1521 ± 16 and 1506 ± 21 EY-NCS and Co-NCS attached respectively (Table C.1). The differences in labeling ratios of these small molecules can be visualized in the absorbance

profiles of the different materials (Fig. 4.1C). All samples were analyzed by SDS-PAGE and visualized under UV light before staining with Coomassie blue to reveal the fluorescence of the EY-NCS dye co-migrating with the protein bands (Fig. 4.1B_i), a clear indication that each P22-xAEMA particle was labeled with both the Co-NCS and EY-NCS. To verify that the morphology of the P22 samples were not disrupted during the labeling procedure, samples were analyzed by dynamic light scattering (DLS) and transmission electron microscopy (TEM). DLS revealed little to no change in the size (~62 nm) (Fig. C.2) of the particles and TEM showed that the overall morphology of P22 was retained after the labeling of the polymer network (Fig. 4.1B_{ii}).

After verifying polymer formation and synthetic modification of the polymer framework through labeling with the catalysts, the materials were tested for the photo-induced production of NADH in aqueous solution (Fig. 4.2A). The reaction was performed at pH 7.0 (100 mM phosphate, 50 mM NaCl, 0.5 mM NAD⁺, 0.2 M TEOA) in a sealed

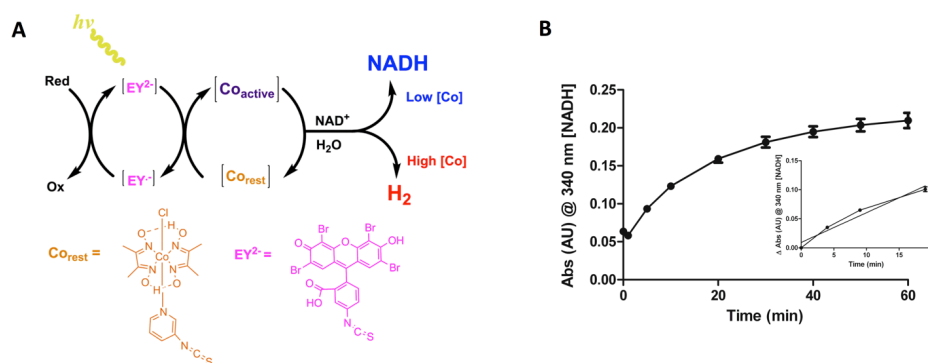


Figure 4.2 Proposed catalytic scheme and photochemical production of NADH monitored by UV-vis. (A) Proposed catalytic scheme showing a bifurcated pathway at different concentrations of Cobaloxime. (B) An example of an activity plot of the photochemical production of NADH monitored by changes in the spectrum at 340 nm. (Inset) Initial rate for the photochemical production of NADH.

degassed cuvette and changes in the characteristic absorbance of NADH at 340 nm (ext. coeff. = $6,220 \text{ M}^{-1}\text{cm}^{-1}$) were monitored (Fig 4.2B).⁴⁹ No photochemical production of NADH was observed when either Co-NCS or EY-NCS were absent or if they were labeled on separate P22 particles. This was the first indication that co-localization of these catalysts on the interior of P22 was needed to ensure electron transfer for efficient photochemical NADH production and that by labeling separate P22 capsids with the catalysts no catalytic communication between particles was possible. The most efficient P22-Co/EY synthesized as part of these studies had a catalytic turnover for NADH of $11.40 \times 10^{-3} \text{ s}^{-1}$, which was ~ 1.2 times higher than the catalysts free in solution, supporting the hypothesis that co-localizing the catalysts increases catalytic efficiency (Table 4.1).

Table 4.1 Cobaloxime and Eosin-Y concentrations and turnovers for NADH and Hydrogen for both P22-Co/EY and catalysts free in solution. Cobaloxime concentrations were calculated from ICP-MS analysis and Eosin-Y concentrations were calculated from the characteristic absorbance at 525 nm, using an extinction coefficient of $112,000 \text{ M}^{-1}\text{cm}^{-1}$. All turnovers were calculated with respect to the cobaloxime concentration and all measurements were performed in triplicate. All uncertainty reflects one standard deviation.

Sample	[Co] ($\text{M} \times 10^{-6}$)	[EY] ($\text{M} \times 10^{-6}$)	$k_{\text{cat-NADH}}$ ($\text{s}^{-1} \times 10^{-3}$)	$k_{\text{cat-Hydrogen}}$ ($\text{s}^{-1} \times 10^{-9}$)
Free Solution 1	200	100	0.44 ± 0.05	37.68 ± 2.07
Free Solution 2	130	100	2.52 ± 0.16	10.51 ± 4.79
Free Solution 3	65	50	4.51 ± 0.38	11.88 ± 4.03
Free Solution 4	13	10	9.89 ± 1.14	N/D
P22 _{0.25xCo/0.25xEY}	1.51 ± 0.04	1.56 ± 0.02	11.40 ± 2.90	N/D
P22 _{0.25xCo/0.50xEY}	1.70 ± 0.01	2.98 ± 0.05	8.10 ± 0.52	N/D
P22 _{0.25xCo/0.75xEY}	1.65 ± 0.02	4.09 ± 0.04	8.90 ± 0.88	N/D
P22 _{0.25xEY/0.25xCo}	1.43 ± 0.01	2.08 ± 0.02	10.83 ± 0.78	N/D
P22 _{0.25xEY/0.50xCo}	2.86 ± 0.02	2.20 ± 0.01	2.67 ± 0.32	N/D
P22 _{0.25xEY/0.75xCo}	4.04 ± 0.05	2.22 ± 0.01	2.43 ± 0.19	N/D
P22 _{1xCo/1xEY}	5.12 ± 0.15	12.43 ± 0.24	4.04 ± 0.50	N/D
P22 _{5xCo/1xEY}	32.41 ± 1.64	7.81 ± 0.09	2.18 ± 0.01	101.12 ± 7.49

From our sequential sub-stoichiometric labeling strategy we also observed that samples labeled with equal or more EY-NCS (than Co) had higher catalytic turnovers (Table 4.1). These results indicate that stoichiometry plays an essential role in the overall reaction and that an equal or excess of EY-NCS is necessary for efficient electron transfer, consistent with the proposed mechanism where 2 EY are needed to reduce one $\text{CoCl}(\text{dmgH})_2\text{Pyr}$ (Fig C.5A).^{78,134}

To test the concentration dependence of the catalysis in the P22-Co/EY constructs, we evaluated the photochemical production of NADH at different bulk dilutions. As shown in Fig. 4.3A and 4.B, there was little to no detectable change in the

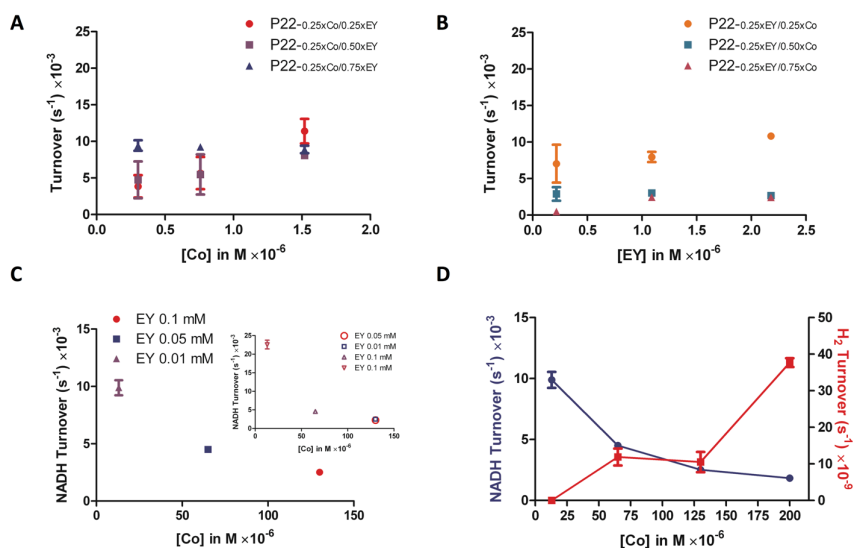


Figure 4.3 Concentration dependence of catalytic turnover with respect to Cobaloxime for catalysts free in solution and encapsulated within P22 (P22-Co/EY). (A) Concentration dependence for samples P22_{0.25xCo/0.25xEY}, P22_{0.25xCo/0.50xEY}, P22_{0.25xCo/0.75xEY} showed no significant change on overall turnover. (B) Concentration dependence for samples P22_{0.25xEY/0.25xCo}, P22_{0.25xEY/0.50xCo}, P22_{0.25xEY/0.75xCo} showed no significant change in turnover. (C) Concentration dependence for catalysts free in solution showing a significant change in turnover. (Inset) Eosin-Y concentration dependence of catalytic turnover. (D) Concentration dependence of free cobaloxime for the photochemical production of NADH (blue) and hydrogen (red). At low concentrations of Co the highest turnover for NADH was observed and at high concentrations of Co the highest turnover for hydrogen was observed, suggesting a bifurcated pathway.

NADH turnover efficiency for the P22-Co/EY material over a 5-fold change in bulk Co-NCS or EY-NCS concentration. By comparison, in the free Co-NCS/EY-NCS a significant change in catalytic turnover was observed with changes in bulk concentration and we observed an inverse dependence of the turnover on Co-NCS and EY-NCS concentration. At low concentrations of free Co-NCS/EY-NCS the turnover for NADH was $9.89 \times 10^{-3} \text{ s}^{-1}$ (Fig. 4.3C). Upon diluting the free Co-NCS, while maintaining EY-NCS at 0.1 mM, we observed the most efficient turnover for NADH of $22.58 \times 10^{-3} \text{ s}^{-1}$ (Fig. 4.3C inset) showing that in bulk solution an excess of EY is required for efficient reactivity. This confirms the hypothesis that the encapsulated P22-Co/EY behaves fundamentally differently from the catalysts free in solution (Co-NCS/EY-NCS) and exhibits no bulk concentration dependence because the local concentration on the interior of the P22 capsid remains fixed. It is this local concentration that determines the rate of the reaction (ie. the intermolecular interaction between the two co-catalysts) and by co-localizing these molecules on the interior of the VLP we have created a nanoparticle catalyst that has catalytic turnover that is bulk concentration independent.

There are reports that the $\text{CoCl}(\text{dmgH})_2\text{Pyr}$ and EY co-catalyst system are able to photochemically generate hydrogen by proton reduction, in addition to NADH production (Fig. 4.2A).¹³⁴ After noticing the inverse dependence of the control reaction for NADH formation we evaluated the Co-NCS/EY-NCS free in solution for hydrogen production and noticed a direct dependence on Co-NCS concentration. At high Co-NCS concentrations we observed a higher turnover for hydrogen production (Fig. 4.3D). This Co-NCS dependence on hydrogen production was mirrored by the corresponding

decrease in NADH production with increasing Co-NCS concentrations (Fig. 4.3D).

These results suggest a bifurcated reaction pathway from a common intermediate (Fig. 4.2A). Previous reports have suggested that H_2 production arises from a homolytic pathway from a $CoCl(dmgh)_2Pyr$ hydride species, which is produced through a bimolecular reaction between two $CoCl(dmgh)_2Pyr$ species. This is consistent with our observed Co-NCS concentration dependence for H_2 production (Fig. C.5B).^{78,134}

We evaluated the encapsulated P22-Co/EY materials for hydrogen production but could not detect any H_2 with the materials described above, which were highly active for NADH production. We hypothesized that this was the result of the P22-Co/EY particle design where the local Co-NCS concentration is fixed by the labeling stoichiometry and the Co-NCS molecules are relatively immobile within the P22 capsid therefore limiting the possibility of the required bimolecular interaction between $CoCl(dmgh)_2Pyr$ species required for H_2 production.

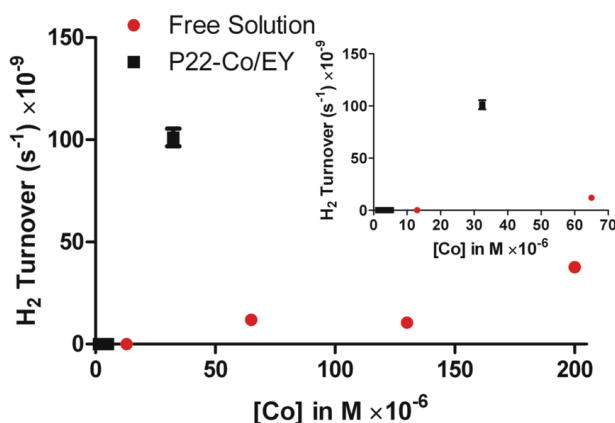


Figure 4.4 Photochemical Hydrogen Production. Hydrogen turnover with respect to cobaloxime concentration for P22-Co/EY and the catalysts free in solution. P22-Co/EY produced hydrogen only for the highest labeled sample and had a higher turnover than catalysts free in solution. (Inset) Hydrogen turnover plot showing that P22-Co/EY samples with lower Co labeling produced no detectable hydrogen.

To test this P22-Co/EY materials were labeled with high levels of Co-NCS (P22_{5xCo/1xY}). The high excess labeling achieved 3706 ± 187 Co-NCS per cage, roughly 6 times more Co-NCS than the samples described above (Table C.1) labeled at lower Co density. These P22-Co/EY materials were able to catalyze H₂ production with a turnover of $101.12 \times 10^{-9} \text{ s}^{-1}$, which is approximately 3 times greater than the turnover for the free catalysts (Co-NCS/EY-NCS) in solution, $37.68 \times 10^{-9} \text{ s}^{-1}$ (Fig. 4.4). This supports our hypothesis and proposed mechanism for this coupled co-catalyst system where, at high Co-NCS concentrations, hydrogen production is favored via a homolytic pathway where a Co-NCS-hydride species produces H₂ through a bimolecular reaction (Figure C.5B).^{78,134} It also highlights the versatility of the P22-Co/EY platform where labeling of the internal polymer can be used to affect the predominant pathway (NADH or H₂ production) through control over the stoichiometry and labeling density.

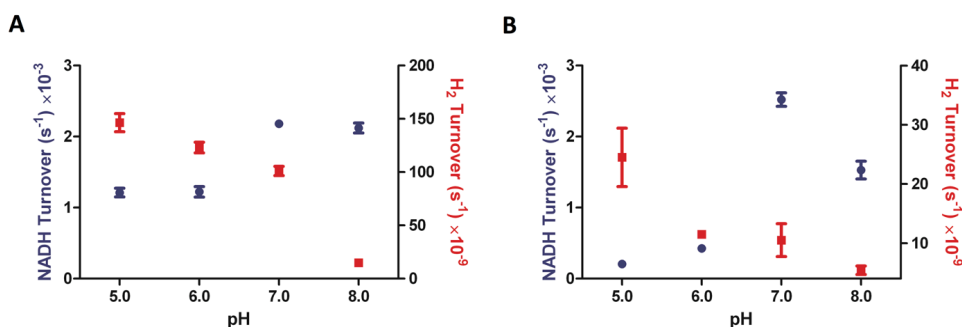


Figure 4.5 pH dependence of the photochemical production of NADH and hydrogen. (A) pH dependence of P22-Co/EY for the photochemical production of NADH and hydrogen. At pH 7.0 and pH 5.0 the highest turnover for NADH and hydrogen respectively was observed. (B) pH dependence for the catalysts free in solution. At pH 7.0 and 5.0 the highest turnover for NADH and hydrogen, respectively was observed.

Having detected hydrogen from both P22-Co/EY (P22_{5xCo/1xEY}) and free Co-NCS/EY-NCS we investigated the pH dependence for both the NADH and H₂ photochemical production. As expected, and consistent with the proposed mechanism for NADH production (Fig. 4.2A) and previous reports¹³⁴, we found that the optimal pH for photochemical NADH production was 7.0 for both P22-Co/EY (P22_{5xCo/1xEY}) and Co-NCS/EY-NCS free in solution (Fig. 4.5). The pH dependence for photochemical H₂ production was shifted to a lower pH and the optimal conditions for H₂ production was found to be pH 5.0 for both P22-Co/EY and Co-NCS/EY-NCS free in solution (Fig. 4.5, Table C.2), consistent with expectations for H⁺ reduction to form H₂.

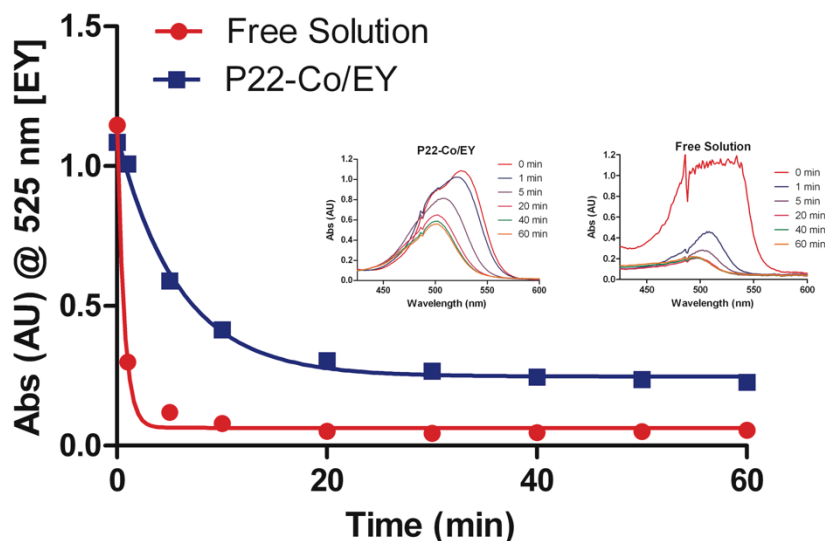


Figure 4.6 Photodegradation of Eosin-Y in P22-Co/EY (P22_{1xCo/1xEY}) as compared to the catalysts free in solution. Eosin-Y encapsulated in P22 has a slower 1st order decay rate suggesting that P22 protects and slows Eosin-Y's photodegradation. (Insets) UV-vis data of the Eosin-Y absorbance peak decaying over time.

The natural function of the protein capsid in the P22 bacteriophage is the delivery and protection of the nucleic acid cargo of the infectious virus. The P22 capsid in this

synthetic system also has the ability to protect the encapsulated cargo - an added benefit for this biomimetic nano-platform. We have observed that the encapsulation of EY-NCS within P22 appears to slow its photodegradation. EY-NCS in bulk solution exhibited a significantly faster photoinduced decay than EY-NCS labeled on the inside of P22, with decay constants of 1.52 min^{-1} and 0.17 min^{-1} respectively (Fig. 4.6). All of the P22-Co/EY labeled samples exhibited similar decay rates indicating that the EY-NCS is in a similar environment in all encapsulated samples (Fig. C.6).

An additional consequence of using the bioconjugation approaches outlined here is that it allows for the conjugation of the relatively water insoluble Co-NCS catalyst to the interior of the P22. Once these molecules are labeled onto the P22 platform their solubility increases considerably, by virtue of the capsid solubility. The P22-Co/EY materials can be concentrated in solution up to $\sim 50 \text{ mg/mL}$, which corresponds to a bulk Co-NCS of up to $\sim 32 \text{ }\mu\text{M}$. Co-NCS is insoluble in water by itself, highlighting the versatility of this biomimetic encapsulation approach.

Conclusions

Inspired by the compartmentalization and spatial arrangement of photosynthetic machinery, we have designed and synthesized a co-localized two-catalyst system on the interior of the VLP P22 for the photochemical production of NADH and/or hydrogen. By confining and co-localizing the catalysts on the interior space of the P22 VLP we have created a photocatalytic nanoparticle that is more efficient than the bulk catalysts. Because of the conjugation of the small molecule catalysts on the interior of the VLP we

have synthesized a nanoparticle catalyst whose turnover is independent of the bulk concentration and thus behaves quite differently from the free catalysts in bulk solution.

The labeling density of the catalysts allows for significant control over the photochemical reactivity, as well as control over the reaction pathway and the final reaction product. This controlled stoichiometric labeling strategy demonstrates the first step towards creating control over the spatial arrangement and density of catalysts. This approach emphasizes the importance of the precise nanoscale arrangement for complex reactions, which is an under utilized design principle. Because of the increased global awareness of energy needs creating green methods such as this one for generating useful molecules/fuels is essential for future energy needs. Therefore creating artificial photosynthetic systems that can control the final products of important high-energy molecules, like NADH and H_2 , is highly desirable. Implementing these biomimetic design principles will be important for creating complex and efficient catalytic materials.

Using biomaterials such as the VLP P22 allows for large-scale production of monodisperse nanoparticles that are amenable to post synthetic modifications, such as ATRP, allowing for the high loading density of catalysts that other bio-conjugation techniques cannot achieve. Because of the stability of these particles they remain active for months and their monodisperse characteristics facilitate their characterization. This study highlights the versatility of biomaterials such as VLPs as useful scaffolds for material design and bioconjugation.

Acknowledgements

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Experimental

Materials

All reagents used 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 and all water was deionized using a NANOpure water purification system. Dynamic light scattering measurements were taken on a Malvern, Zetasizer Nano-S.

Protein Purification

P22 (S39C) was expressed, purified and analyzed by size exclusion chromatography with multi-angle light scattering and refractive index detection by following procedures previously reported.⁴⁷

Synthesis of 2-bromoisobutyrate Ethoxy Maleimide (1)

Compound 1 was synthesized by a modification of the procedures previously reported.^{47,48}

Synthesis of Dichloro(dimethylglyoxime)
(dimethylglyoximate)Cobalt(III) (2)

Compound 2 was synthesized by a modification of procedures previously reported.¹³⁶ $\text{Co}(\text{Cl})_2 \cdot 6\text{H}_2\text{O}$ (2.18 g, 9.17 mmol) was dissolved into 50 mL of acetone while stirring. Dimethyl glyoxime (2.20 g, 19.0 mmol) was added and dissolved with gentle stirring. The solution turned dark purple at this time, and a gentle stream of nitrogen was applied over the solution. A green solid began to deposit, after ~ 30 minutes after the solution was chilled on ice. The product was collected by vacuum filtration and washed with two 15 mL aliquots of cold acetone. The yield was 2.95 g (89%).

Synthesis of Chloro(3-pyridyl
isothiocyanate)Cobaloxime (3)

Compound 3 was synthesized by a modification of previously reported procedures.¹³⁶ $\text{Co}(\text{dmgH})(\text{dmgH}_2)\text{Cl}_2$ (400mg, 1.14mmol) was dissolved in 10 mL of methanol. 3-pyridyl isothiocyanate (300 μl , 2.67 mmol) was added slowly by syringe while the mixture was stirring. This reaction mixture was stirred for ~30 minutes until the green solid disappeared and was replaced by a brown crystalline solid. 15 mL of water was added while stirring and the suspension was cooled on ice for 10 minutes. The solid was collected by vacuum filtration and washed with a 2:1 mixture of H_2O and methanol (5 mL) three times, followed by two washes with diethyl ether (5 mL). The yield was 440 mg (85%). ^1H NMR (300 MHz, DMSO) δ = 2.32 (s, 12H, CH_3); 4.02 (s, 2H, OH); 7.41 (t, 1H, CH); 7.78 (d, 1H, CH); 8.55 (d, 1H, CH); 8.64 (s, 1H, CH) ppm. ^{13}C NMR δ = 13.51 (CH_3), 126.61 (NCS), 134.12 (CH), 134.88 (CH), 146.66 (CH),

148.81 (CH), 153.37 (CN), 153.91 (C) ppm. MS (ESI) m/z : $[M + Na + CH_3OH]^+$
calculated for $[C_{14}H_{18}ClCoN_6O_4S] + [Na + CH_3OH]$, 515.8090; found, 515.0291.

P22-int Macroinitiator Formation

P22-int was synthesized following procedures previously reported.^{47,48,63,84}

P22_{S39C} (100 mM phosphate, 50 mM NaCl, pH 7.6, 23.6 ml, 5 mg/ml) was infused with 316 μ L of 1 (40 mM in DMSO, 5 fold excess per subunit). The mixture was allowed to react for three hours at room temperature. After 3 hrs the reaction was quenched with DTT (316 μ L, 40 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.

P22-xAEMA Polymer Formation

P22-xAEMA polymer material was synthesized by following procedures previously reported.^{47,48} Each sample was made in a 10 mL crimp-top vial with the addition of 4000 μ L of 2-aminoethyl methacrylate (AEMA)/Bis acrylamide monomer solution (4 wt% in PBS, 16:4 AEMA/Bis-acrylamide, pH 7.6) and 2200 μ L PBS buffer (100 mM sodium phosphate, 50 mM NaCl, pH 8.0). To this mixture, 1200 μ L of P22-int (8.0 mg/mL, 0.20 μ moles subunit, pH 7.6) was added followed by pumping and back filling with Argon four times to de-aerate the mixture. The metal catalyst solution was made in a second vial using 10 mL deionized water degassed by bubbling Ar through the liquid for 20 minutes, to which 19.2 mg CuBr (0.134 mmoles), 29.9 mg CuBr₂ (0.134 mmoles) and 83.5 mg 2,2'-bipyridine (0.535 mmoles) were added. The vial was

immediately sealed and sonicated for 5 minutes resulting in a dark-brown solution. To the monomer-protein vial, 600 μL of the metal catalyst solution was added under Ar atmosphere and the vial was maintained at room temperature for the duration of the experiment (180 min). The sample was quenched by exposure to air and the protein-polymer composite was purified away from the unreacted monomer and the copper catalyst by pelleting the protein by ultracentrifugation and resuspended in 100 mM phosphate, 50 mM NaCl, pH 8.0. This was followed by treating the protein with a high salt buffer (100 mM phosphate, 250 mM sodium sulfate, pH 8.0) for 3 hours while rocking gently at 4 °C followed by pelleting of the protein. The protein pellet was resuspended in 100 mM phosphate, 50 mM NaCl, pH 8.0. The yield was 4.22 mg (53 %).

Labeling P22-xAEMA with Co-NCS and EY-NCS

P22-xAEMA was labeled by following procedures previously reported.⁴⁷ P22-xAEMA was prepared at 1 mg/mL in pH 9.0, (2 mL) sodium bicarbonate (100 mM). A 20 mM solution of either Cobaloxime-NCS or Eosin-Y-NCS was prepared in DMSO. This solution was slowly added to the P22-xAEMA at stoichiometric labeling conditions (1 mol AEMA : 0.25 mol, 0.50 mol or 0.75 mol Co/Eosin). The solution was allowed to react for 16 hours at 4°C while rocking, wrapped in tin foil. The P22 reaction product was purified by pelleting the protein by ultra centrifugation and resuspension into pH 9.0 sodium bicarbonate buffer twice. The yield was 1.70 mg (85 %).

Quantification of Co-NCS
Labeled on P22-xAEMA by ICP-MS

Following previously reported procedures Cobalt was analyzed by Inductively coupled plasma mass spectrometry.^{129,138} Samples were diluted into a 2.5% solution of Nitric Acid and pH 7.0 PBS. Cobalt was analyzed by using a Perkin Elmer ELAB DRCII ICP-MS with a Microflow PFA-ST concentric nebulizer. It was equipped with a 100 $\mu\text{L}/\text{min}$ self-aspiration capillary, cyclonic spray chamber, a quartz torch and platinum sample and skimmer cones. For cobalt analysis the machine was used in DRC mode using ammonia for the reaction gas and an EzyFit glass mixing chamber. Germanium (50ppb) was used as an internal standard and a SmartTune (Elan DRC/Plus/II) as an external standard (Perkin Elmer, Waltham, MA).

Quantification of EY-NCS by UV-vis

Samples were analyzed by an UV-vis (Agilent 8453 UV-vis) monitoring the absorbance at 525 nm. Eosin-Y concentrations were calculated by using the extinction coefficient $112,000 \text{ M}^{-1}\text{cm}^{-1}$ at A_{525} for Eosin-Y.¹³⁷

SDS Polyacrylamide Gel
Electrophoresis Denaturing Gel Assay

P22 samples (10 μL , 1 mg/mL) were mixed with 4x loading dye with DTT (5 μL) and boiled for 5 minutes. Samples were analyzed using a 12% SDS-PAGE acrylamide gel run at 35 milliamperes for 60 minutes with a prestained PageRuler protein ladder (10-180 kDa). Protein was detected by staining with InstantBlue™ stain, rinsed with water and imaged with a UVP MultiDoc-It Digital Imaging System.

Native Agarose Gel Assay

P22 samples (10 μ L, 2 mg/mL) were mixed with 4x loading dye (5 μ L) and analyzed by running on a 1 % native agarose gel. Gels were run for 3 hours at 65 volts using a running buffer that contained 40 mM Tris-base, 5 mM sodium acetate, 1 mM EDTA, pH 8.2. Detection of protein was done by staining with InstantBlue™ stain, rinsed with water and imaged with a UVP MultiDoc-It Digital Imaging System.

Dynamic Light Scattering

Protein samples were prepared at ~ 0.5 mg/mL at 100 μ L and spun on a benchtop centrifuge at 17,000 rpm for 10 minutes to remove any dust particles. Samples were placed into a DLS cuvette (Hellma Analytics, ZEN2112) and analyzed by dynamic light scattering, on a Malvern, Zetasizer Nano-S.

Transmission Electron Microscopy

Samples were imaged by transmission electron microscopy using a JEOL JEM 1010 (100 kV accelerating voltage) by negatively staining the sample with 2% uranyl acetate on a formvar and carbon coated Cu grid.

Photochemical Production of NADH

A reaction buffer containing, pH 7.0, 100 mM PO_4^{3-} , 50 mM NaCl, 0.5 mM NAD^+ , 0.2 M TEOA was freshly prepared. 240 μ L of the reaction buffer was added to a sealable sterna cuvette and blanked on an UV-vis (Agilent 8453 UV-vis). 10 μ L of the reaction solution was added to the cuvette, and degassed by multiple rounds of pumping and back filling with argon (3X, 3 min. per cycle). The sample was irradiated by a LED

light (Luxeon Star Dental Blue, 460 nm) and monitored by the UV-vis (Agilent 8453 UV-vis) at specific time points. NADH concentrations were calculated using an Extinction coefficient of $6,220 \text{ M}^{-1}\text{cm}^{-1}$ at A_{340} .⁴⁹

Photochemical Production of Hydrogen

A reaction buffer containing, pH 7.0, 100 mM PO_4^{3-} , 50 mM NaCl, 0.5 mM NAD^+ , 0.2 M TEOA was freshly prepared. 240 μL of the reaction buffer was added to a septum sealed cuvette (Starna Cells, GL14, 6Q). 10 μL of the reaction solution was added to the cuvette, and degassed by multiple rounds of pumping and back filling with argon (3X, 3 min. per cycle). The sample was irradiated by a LED light (Luxeon Star Dental Blue, 460 nm) and monitored by GC (Shimadzu Gas Chromatography GC-8A) at specific time points. Hydrogen concentrations were calculated by integration of the GC peak and comparing to a Hydrogen standard response curve.

CHAPTER FIVE

EXPLORING THE ROLE OF THE ELECTROSTATICS FOR POLYMERIZATION
ON THE INTERIOR OF THE VIRUS-LIKE PARTICLE P22Introduction

Protein cage-polymer hybrid materials have had increased interest for the development and synthesis of new biomaterials because of their unique architectures. Protein cage nanoparticles and virus-like particles are amenable to easy and site-specific synthetic modification with water-insoluble small molecules, thermally stable, produced at high yields with homogenous particle size.^{37,44,139} Investigating the mechanism of atom transfer radical polymerization (ATRP) grafted from protein subunits, specifically in the confines of protein-cage architectures like the P22 virus-like particle (VLP), will allow for advances in this biomaterial field helping create complex hybrid materials with improved functionality.

Inspired by the electrostatic encapsulation of RNA in viral systems, like cowpea chlorotic mottle virus (CCMV) from the *Bromoviridae* family,^{24,25} we have tested the role that electrostatics play when ATRP is performed in a viral capsid. Because the P22 VLP has a highly net negative charge we hypothesized that electrostatic interactions could play a crucial role in the transport of individual monomers into the capsids interior. We believe that there are two possible electrostatic interactions, monomer-capsid and monomer-polymer that could substantially affect polymerization. *Bromoviridae* viruses, such as CCMV/BMV, use an electrostatically driven RNA encapsulation process, which

has led to a variety of biomaterials, by electrostatically encapsulating a variety of foreign cargo, while maintaining the wt CCMV characteristics and morphology.²⁶⁻²⁹

Understanding the rationale behind how electrostatic contributions affect the growth of polymer at the interior of the capsid, is key to making advances in this field. With a better understanding of this complex bio-polymerization process we can design new materials taking advantage of the natural highly negatively charged P22 VLPs, like has been done with CCMV and BMV.

The P22 VLP has previously been used as a size constrained vessel for spatially control polymer synthesis.^{47,48,58} The applications for these protein-polymer materials range from a high cargo loaded nanoparticle for improving MRI imaging,⁴⁷ a T1 imaging agent,⁵⁸ incorporation of an active photocatalytic crosslinker,⁴⁸ and branched coordination polymers.⁴⁷ In this chapter the P22 VLP is used to test the role that electrostatics have for site-specific polymerization on the interior of the particle. Using the P22 VLP system for directed polymerization by atom transfer radical polymerization (ATRP), we can directly see the effects of cationic, anionic and neutral monomers in the incorporation in the P22-polymer system (Figure 5.1 & 5.2).

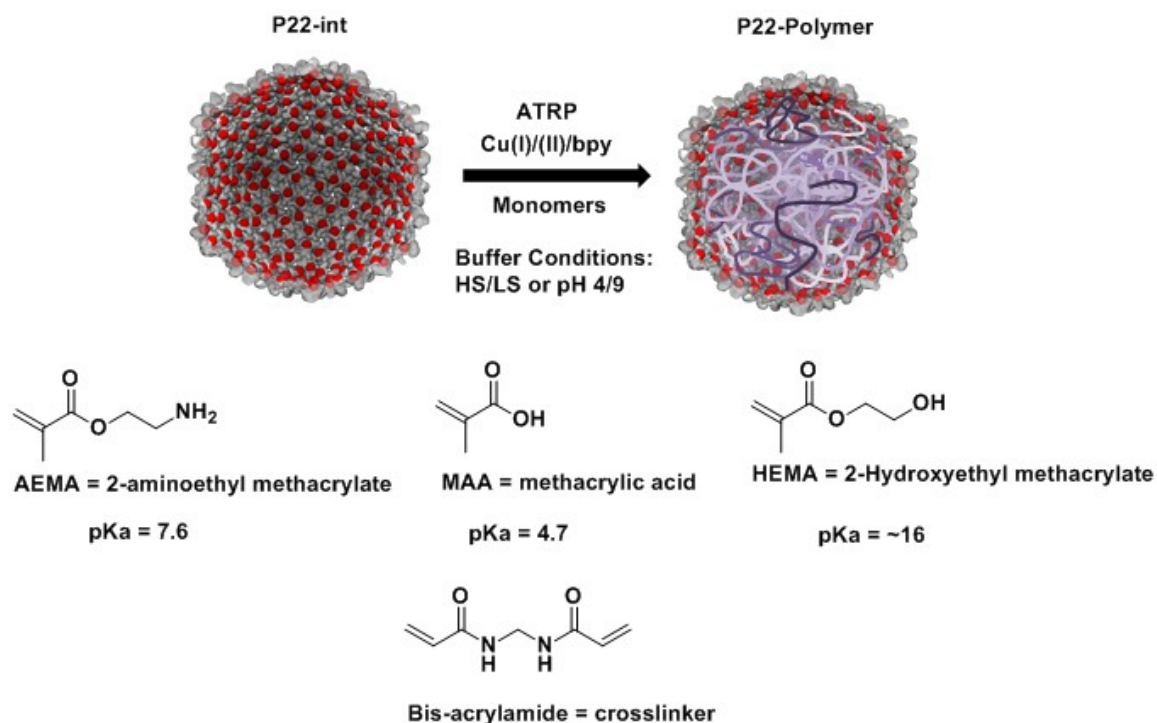


Figure 5.1 Polymerization scheme and monomers chosen for testing the effects of electrostatics with ATRP polymerization on the inside of the P22 VLP. Three monomers were used to probe the effects of charged side chains with ATRP (2-aminoethyl methacrylate (AEMA, pKa 7.6), methacrylic acid (MAA, pKa 4.7), 2-hydroxyethyl methacrylate (HEMA, ~16) and a crosslinker bis-acrylamide. Buffer conditions consisted of high (300 mM NaCl) and low salt (1 mM NaCl) for electrostatic screening and high (pH 9) and low pH (pH 4) to vary pH above and below monomer side chain pKa's.

Results and Discussion

The virus-like particle (VLP) from the bacteriophage P22 was used as a hollow nanoscale template for testing the effects of electrostatics on polymerization (Figure 5.1). The S39C mutant of P22 VLP was recombinantly expressed and assembled in *Escherichia coli* into T=7 procapsid (PC) VLP. The VLP was transformed into the EX morphology by heating the capsid to 65°C, analyzed by native agarose gel and purified by ultracentrifugation. The S39C position was labeled with a small molecule initiation

molecule for polymerization (P22-int) and was monitored by subunit mass spectrometry revealing near quantitative single labeling, as previously described in Chapters 2, 3 and 4.^{47,48,58}

First, specific monomers were selected that allow for electrostatic interactions during polymerization to be studied. Three monomers were chosen for this study, 2-aminoethyl methacrylate (AEMA), methacrylic acid (MAA), and 2-hydroxyethyl methacrylate (HEMA). These were chosen because each had a different functionalized end group, AEMA contains an amine, MAA contains a carboxylic acid, and HEMA contains a hydroxyl group (Figure 5.1). Each of these functional groups have different pK_as (7.6, 4.7, ~16, respectively)^{140,141} allowing the charge of the monomer's side chain to be varied by changing pH. Presumably, charged monomers should have either attractive or repulsive interactions with the highly negatively charged P22 VLPs while neutral monomers should not interact with the capsid, therefore should not influence the extent of polymerization.

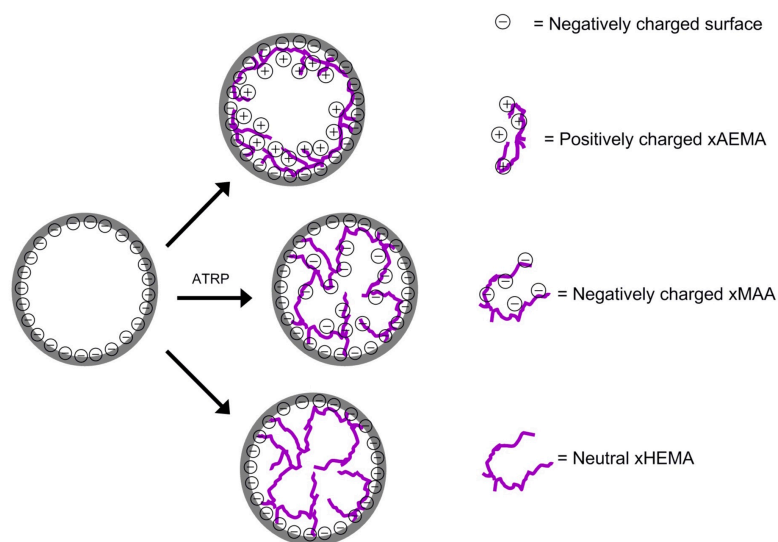


Figure 5.2 Schematic Representation of the Effect of Monomer Charge on Polymerization. The capsid of the P22 VLP is negatively charged therefore it is expected that polymerization with a positively charged 2-aminoethyl methacrylate (AEMA) will lead to polymer capsid interaction (top capsid), whereas polymerization with the negatively charged methacrylic acid (MAA) will lead to polymer capsid repulsion, and polymerization with the neutrally charged 2-hydroxyethyl methacrylate (HEMA) should not lead to significant capsid interaction.

Two different ionic strength buffers were chosen, low salt (LS, 1 mM NaCl), high salt (HS, 300 mM NaCl), to probe the effects that electrostatic screening has on polymerization loading. ATRP was performed as previously described (Chapters 2,3 and 4),^{47,48,58} in a pH 8.0 100 mM PO_4^{3-} and either LS or HS buffer. Bis-acrylamide was also incorporated to constrain the polymer network on the interior of the P22 VLP, as shown in previous Chapters 2, 3, and 4.^{47,48} Polymerization was performed with each aforementioned monomer in the high and low salt containing buffers respectively, creating six samples for the ionic strength screening (HS/LS-xAEMA, HS/LS-xMAA, and HS/LS-xHEMA). The samples were run on a denaturing SDS-PAGE gel alongside a

non-polymerized sample (EX-int). All samples exhibited streaking of the P22 coat protein subunit, indicating that polymer strands have grown off of the P22 subunit (Figure 3, lanes 1-7). P22-xAEMA had a dramatic shift in electrophoretic mobility monitored by agarose gel, while the other samples had similar mobility as the EX-int control. This indicated that the positive charged monomer AEMA substantially alters the P22 VLP's net charge, while both the negative (MAA) and neutral (HEMA) monomer do not (Figure 4). The samples in high salt showed increase precipitation and about a 50 % reduction in overall yield (LS yield ~65 %, HS ~35 %) indicating that higher salt conditions are not suitable and detrimental to overall VLP stability. A new study just published reveals a similar affect that VLP stability decreases in high salt when tested by atomic force microcopy (AFM).¹³⁰ These initial results indicate that ionic strength doesn't affect the polymerization chemistry because all samples successfully grew polymers.

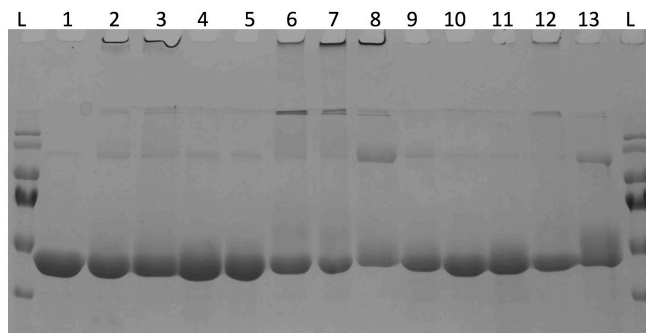


Figure 5.3 SDS PAGE Denaturing Gel of Polymerized Samples in Different Ionic Strength and Different pH buffers. For different ionic strengths samples were in a high salt (HS) buffer or a low salt (LS) at pH 8.0, and for different pH buffers samples were in a pH 9.0 or pH 4.0. All samples had polymerization occur evident by the streaking of the protein subunits. Samples are (1) EX-int (2) HS AEMA/Bis (3) LS AEMA/Bis (4) HS MAA/Bis (5) LS MAA/Bis (6) HS HEMA/Bis (7) LS HEMA/Bis (8) pH 9-AEMA/Bis (9) pH 4-AEMA/Bis (10) pH 9-MAA/Bis (11) pH 4-MAA/Bis (12) pH 9-HEMA/Bis (13) pH 4-HEMA/Bis

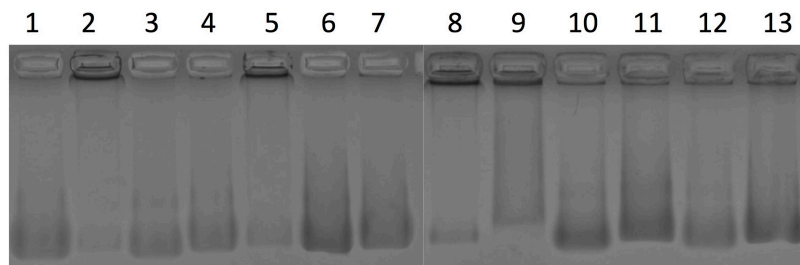


Figure 5.4 Native Agarose Gel of Polymerized Samples in Different Ionic Strength and Different pH Buffers. Samples are (1) EX Control (2) LS AEMA/Bis (3) LS MAA/Bis (4) LS HEMA/Bis (5) HS AEMA/Bis (6) HS MAA/Bis (7) HS HEMA/Bis (8) pH 9-AEMA/Bis (9) pH 4-AEMA/Bis (10) pH 9-MAA/Bis (11) pH 4-MAA/Bis (12) pH 9-HEMA/Bis (13) pH 4-HEMA/Bis

Next, two different pH buffers were chosen, pH 4, 100 mM sodium acetate, and pH 9, 100 mM sodium bicarbonate, (50 mM NaCl background) to alter the charge of the monomer's side chain by choosing buffers above and below their pKa's. ATRP was performed as previously described,^{47,48} with bis-acrylamide (crosslinker), in the two new buffer conditions pH 4 and pH 9, with the three different monomers, creating six more samples to test the pH dependence (4/9-xAEMA, 4/9-xMAA, 4/9-xHEMA). These P22-polymer samples were run on a denaturing gel with all samples showing streaking of the P22 coat protein band. The mobility of the samples were similar on a native agarose gel as seen before (Figure 3 & 4). Like the HS conditions the pH 4 samples lost a substantial amount of protein (pH 9 ~55 % yield, pH 4 less than 25% yield), presumably due to protein precipitation due to the low pH. Both of these results indicate that varying pH or ionic strength did not affect the overall polymerization chemistry. This is evidence for the robustness and versatility of ATRP towards bio-polymerization in vastly different conditions.

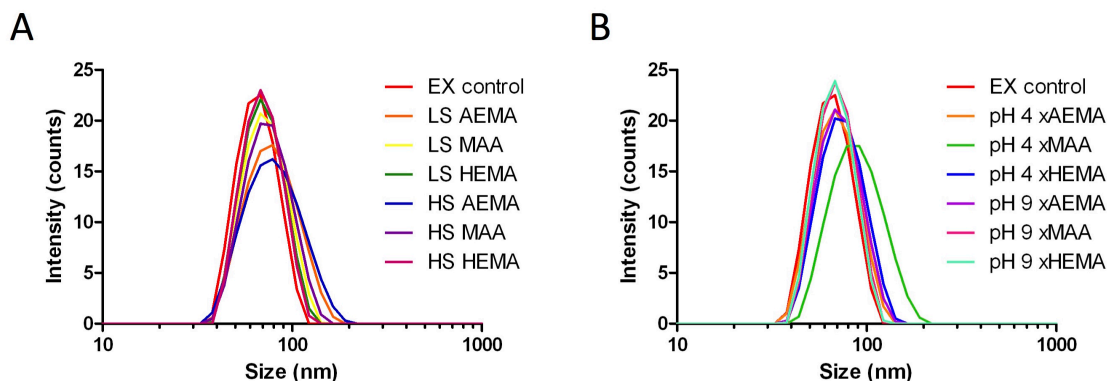


Figure 5.5 Dynamic Light Scattering (DLS) of Polymerized Samples Measuring the Hydrodynamic Radius. (A) Samples polymerized in different ionic strength buffers. (B) Samples polymerized in different pH buffers. Overall very small changes occur, mainly slight broadening of the peaks after polymerization. pH 4 xMAA has the biggest change in hydrodynamic radius.

By two different qualitative gel electrophoretic techniques all samples showed evidence of polymerization. The particles size and molecular weight were measured to ensure that polymer growth was constrained to the interior of the P22 VLP and quantitatively determine the loading of polymer cargo.

First samples were analyzed by dynamic light scattering, a solution phase technique used to determine the hydrodynamic size of nanoparticles. The samples polymerized in different ionic strength conditions (LS and HS) resulted in a monodisperse peak that had similar hydrodynamic diameters (~64 nm) compared to the non-polymerized control sample (~64 nm) (Figure 5.5A). This indicates that polymer formation was constrained on the interior of the VLP. A similar result was observed for the samples polymerized in different pH buffers. All the samples had the same hydrodynamic size except for the pH 4-xMAA sample which exhibited a significant increase in size to ~80 nm (Figure 5.5B). This indicated that at pH 4 the xMAA polymer

network was extending outside the particle. At pH 4 (below the pKa of carboxylic acid side chain) the monomer is expected to be protonated and neutrally charged, therefore reducing the electrostatic repulsive forces between the polymer network and the P22 VLP allowing polymer strands to sample the exterior environment. This increase in size for the pH 4-xMAA can't be attributed to protein aggregation because both pH4 -xAEMA and xHEMA VLPs remained the same size (Figure 5.5B). From these results pH might play a bigger factor in the ATRP polymerization process, but a quantitative analytical method is needed to determine the exact amount of loaded polymer.

To determine the molecular weight of incorporated polymers another solution phase technique was used called multi-angle light scattering (MALS), which has been used for the analysis of a variety nanoparticles, polymers and proteins.^{49,142-144} All samples except P22-xAEMA were analyzed by MALS, because P22-xAEMA has strong interactions with the chromatography step that separates these P22-polymer materials before analysis by MALS. Further work needs to focus on determining a reliable and facile method for MALS analysis of xAEMA. There are other analysis methods for MALS that bypass the chromatography step, but these have major limitations as well. Performing these types of measurements requires large amounts of material (at least 25 mg), exact protein concentration and extinction coefficients, and experimentally determined dn/dc (also requires a large amount of sample, at least 25 mg). Therefore 8 of the 12 polymerized samples were compatible with our size-exclusion multi-angle light scattering (SEC-MALS) analysis (Figure 5.6). These samples were analyzed by the simplest data processing method that only uses a single refractive index increment (dn/dc

for protein, 0.185) because the dn/dc for these polymers is very similar (0.16-0.19) to the proteins dn/dc .^{48,145}

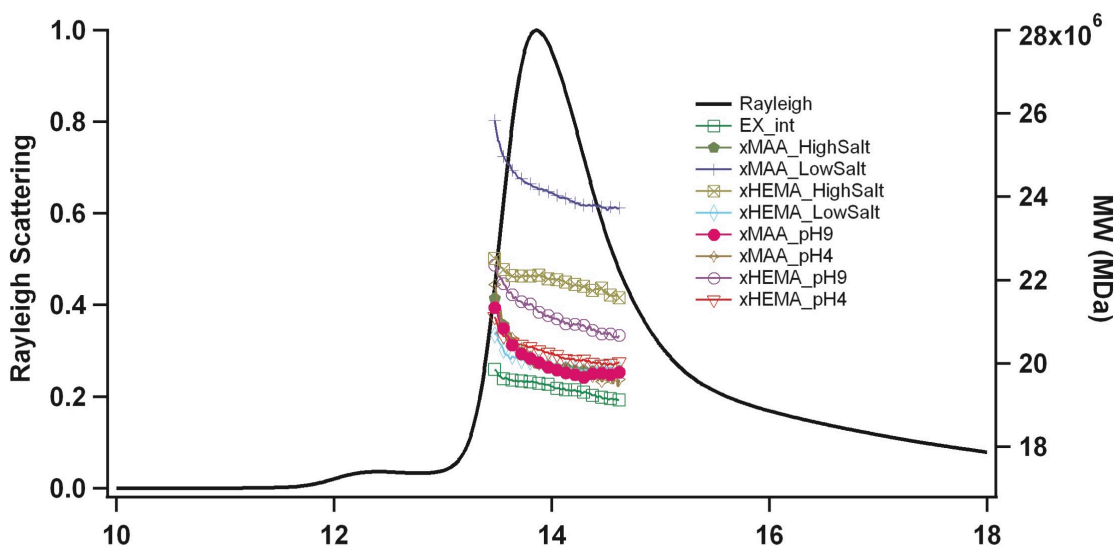


Figure 5.6 Multi-Angle Light Scattering (MALS) of Polymerized Samples Measuring the Molecular Weight. Molecular weight (Mw) information is provided in Table 5.1.

The samples polymerized in varying salt concentrations had the biggest effect on polymer loading. P22-xMAA-LS incorporated the most polymer with a Mw increase of 4.87 MDa and the incorporation of about 56,588 monomers/cage or 137 monomers/subunit (Figure 5.6 & Table 5.1). This is the highest loading of polymeric cargo that we have achieved to date in the P22 ATRP system. The next sample that had a high polymer loading was P22-xHEMA-HS which had a Mw increase of 2.49 MDa and incorporated about 19,133 monomers/cage or 45 monomers/subunit. These results indicate two points: the result of varying ionic strength are unclear (monomer dependent) and the streaking of the P22 subunit visualized by SDS-PAGE gel is misleading. This is

evident by very small differences in protein subunit streaking visualized by gel for lanes 5 and 6 (Figure 5.3), but an enormous increase in Mw (Figure 5.6 & Table 5.1), therefore streaking of subunits should not be used as an indicator of polymer loading. We did not expect these results and hypothesized that the high ionic strength conditions would screen electrostatic interactions (repulsive/attractive from monomer side chains) and facilitate monomer transport through the P22 capsid and the polymer framework allowing for increased loading. Likewise the P22-xHEMA was suppose to act as a positive control and remain unaltered in either salt condition because its monomer has a neutral side chain

Table 5.1 Multi-angle light scattering (MALS) polymer loading data. N/D = not determined because of material issues

Sample	MW (MDa)	Polymer MW (MDa)	Monomers per Cage
P22 EX-int	19.35 ± 0.11	0	0
P22-xMAA HS	19.95 ± 0.15	0.60	6,971
P22-xMAA LS	24.22 ± 0.18	4.87	56,588
P22-xMAA pH 9	19.47 ± 0.48	0.12	1,394
P22-xMAA pH 4	19.98 ± 0.82	0.63	7,320
P22-xHEMA HS	21.84 ± 0.28	2.49	19,133
P22-xHEMA LS	19.85 ± 0.17	0.50	3,842
P22-xHEMA pH 9	21.18 ± 0.39	1.83	14,061
P22-xHEMA pH 4	20.01 ± 0.22	0.66	5,071
P22-xAEMA (HS/LS/9/4)	N/D	N/D	N/D

therefore there should be less capsid and monomer electrostatic interactions. The role of ionic strength is unclear and is more complicated then expected. Ionic strength conditions need to be optimized for each monomer to find the perfect conditions for optimal polymer loading. Unfortunately the MALS data for xAEMA could not be obtained, which could have given more insight into this question. Further experiments

need to be performed before there is a definitive answer for the role that ionic strength plays in screening electrostatic interactions.

The P22-xMAA pH 9 and pH 4 samples had a significant difference in loading by MALS, 1,394 vs. 7,320 monomers respectively (Figure 5.6 and Table 5.1). At a pH of 4 the carboxylic acid functional group is predominantly protonated neutralizing its negative charge. Therefore we believe P22-xMAA-pH 4's increased polymer loading is due to the decreased repulsive interaction with the P22 capsid, agreeing with our hypothesis that neutralizing the monomer charge can help the transport of monomers into the P22 VLP. P22-xHEMA pH 9 incorporated close to three times that of P22-xHEMA pH 4, 14,061 vs. 5,071 respectively. Once again this result disagreed with our hypothesis that this neutrally charged monomer's polymer loading should remain constant, whether ionic strength or pH were adjusted. At this point it is unclear why HEMA's polymerization was affected by the different reaction conditions. Additionally the SEC profiles for all of the P22-polymer composites were identical to P22-int verifying that the crosslinked polymer network was constrained on the interior of the P22 VLP. These results once again verify that polymerization conditions need to be optimized for each monomer. The effects of pH and ionic strength may be more complicated than we expected, and can affect the monomer, the capsid, and the polymerization reaction.

Conclusions

Inspired by the electrostatic packaging of genetic material by RNA viruses, we set out to explore the role electrostatics have on the loading of VLP's with different charged

monomers by a controlled radical polymerization, ATRP. Further studies will be needed to decipher the effects that electrostatics have on polymerization. From the initial studies we did notice some dramatic differences in polymer loading with the highest polymer loading seen in the P22 ATRP system with about ~56,000 monomers of methacrylic acid (MAA) incorporated in the low salt condition. There was also a significant loading difference between the pH 4 and pH 9 samples, which is attributed to neutralization of charge repulsion between the carboxylic acid functional groups and the P22 capsid. The neutral monomer 2-hydroxyethyl methacrylate (HEMA) loading into the P22 VLP was affected by both ionic strength and pH, which was unexpected because electrostatic interactions should not be present. Unfortunately, we don't have quantitative polymer loading data for the xAEMA sample set, which could help justify some of these results. Further studies need to be conducted determining the proper conditions for xAEMA MALS analysis.

These results are evidence that each monomer's polymerization conditions need to be carefully optimized for optimal loading. These studies provide an initial platform/guide for conditions that affect polymerization, expand the knowledge of this VLP polymerization process, demonstrate the versatility (range of monomers and polymerization conditions extremely large) and potentially help increase its use in biomaterial synthesis. Understanding the factors that affect this bio-polymerization process allows for further development of this technique for the synthesis of complex bio-polymer materials with improved functionality for a wide range of biomedical, catalytic and structural biomaterial applications.

Experimental

Materials

All reagents used 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 and all water was deionized using a NANOpure water purification system. Dynamic light scattering measurements were taken on a Malvern, Zetasizer Nano-S.

Protein Purification

P22 (S39C) was expressed, purified and analyzed by size exclusion chromatography with multi-angle light scattering and refractive index detection by following procedures previously reported.^{47,48}

Synthesis of 2-bromoisobutyrate Ethoxy Maleimide

2-bromoisobutyrate was synthesized by a modification of the procedures previously reported.^{47 48}

P22-int Macroinitiator Formation

P22-int was synthesized following procedures previously reported.^{47,48}

P22-Polymer Formation

P22-Polymer material was synthesized by following procedures previously reported.^{47,48} Each sample was made in a 2 mL crimp-top vial with the addition of 500

μ L of Monomer of Choice/Crosslinker solution (4 wt% in PBS, 16:4 (AEMA, MAA, or HEMA)/Bis-acrylamide, pH 7.6) and 275 μ L PBS buffer (100 mM sodium phosphate, 50 mM NaCl, pH 8.0). To this mixture, 150 μ L of P22-int (8.0 mg/mL, pH 7.6) was added followed by thorough degassing. The metal catalyst solution was made in a second vial using 10 mL deionized water degassed with Ar. The vial was immediately sealed and sonicated for 5 minutes resulting in a dark-brown solution. To the monomer-protein vial, 75 μ L of the metal catalyst solution was added under Ar atmosphere and the vial was maintained at room temperature for the duration of the experiment (180 min). The sample was quenched and purified by pelleting the protein by ultracentrifugation and resuspended in 100 mM phosphate, 50 mM NaCl, pH 8.0. The samples were treated with a high salt buffer (100 mM phosphate, 250 mM sodium sulfate, pH 8.0) for 3 hours while rocking gently at 4 °C followed by pelleting of the protein. The protein pellet was resuspended in 100 mM phosphate, 50 mM NaCl, pH 8.0.

SDS Polyacrylamide Gel Electrophoresis Denaturing Gel Assay

P22 samples (10 μ L, 1 mg/mL) were mixed with 4x loading dye with DTT (5 μ L) and boiled for 5 minutes. Samples were analyzed using a 12% SDS-PAGE acrylamide gel run at 35 milliamperes for 60 minutes with a prestained PageRuler protein ladder (10-180 kDa). Protein was detected by staining with InstantBlue™ stain, rinsed with water and imaged with a UVP MultiDoc-It Digital Imaging System.

Native Agarose Gel Assay

P22 samples (10 μ L, 2 mg/mL) were mixed with 4x loading dye (5 μ L) and analyzed by running on a 1 % native agarose gel. Gels were run for 3 hours at 65 volts using a running buffer that contained 40 mM Tris-base, 5 mM sodium acetate, 1 mM EDTA, pH 8.2. Detection of protein was done by staining with InstantBlue™ stain, rinsed with water and imaged with a UVP MultiDoc-It Digital Imaging System.

Dynamic Light Scattering

Protein samples were prepared at ~ 0.5 mg/mL at 100 μ L and spun on a benchtop centrifuge at 17,000 rpm for 10 minutes to remove any dust particles. Samples were placed into a DLS cuvette (Hellma Analytics, ZEN2112) and analyzed by dynamic light scattering, on a Malvern, Zetasizer Nano-S.

Multi-Angle Light Scattering

Samples were analyzed using a Dawn 8 instrument (Wyatt, Santa Barbara, CA) for SEC-MALS analysis. P22 samples were separated over a WTC-0200S (Wyatt Technologies) size exclusion column on an Agilent 1200 HPLC at 0.7 mL/min flow rate and monitored by a UV-Vis detector (Agilent), a Wyatt HELEOS Multi Angle Laser Light Scattering (MALS) detector, a quasi-elastic light scattering detector (QELS), and an Optilab rEX differential refractometer (Wyatt). The number average particle molecular weight (M_n) and radius of gyration (R_g) were determined by calculating across each peak half max with Astra 6 software (Wyatt) using a previously calculated dn/dc value of 0.185 mL/g.

CHAPTER SIX

HYBRID NANOREACTORS: COUPLING ENZYMES AND SMALL-MOLECULE
CATALYSTS WITHIN VIRUS-LIKE PARTICLES

Contribution of Authors and Co-Authors

Manuscript in Chapter 6

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Contributions: Assisted in experimental design, carried out experiments, edited and help write the manuscript.

Co-Author: Trevor Douglas

Contributions: Obtained funding, coordinated the project, and assisted with the design of the experiments. Discussed the results and edited the manuscript at all stages.

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CHAPTER SIX

HYBRID NANOREACTORS: COUPLING ENZYMES AND SMALL-MOLECULE CATALYSTS WITHIN VIRUS-LIKE PARTICLES

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Abstract

Virus like particles (VLPs) provides unique scaffolds for the construction of coupled catalytic systems by attachment and encapsulation of catalysts within their hollow interiors. The interior of VLPs provides an environment where catalysts of biologic or synthetic origins can be confined, protected, and co-localized in close proximity with catalysts of different types. Here we utilize the P22 VLP as a scaffold to construct a synthetic hybrid catalyst by attachment of a small organometallic catalyst to the interior co-localized with an encapsulated enzyme. This produces a complex and active coupled biomimetic catalyst system. By combining both enzymatic and synthetic catalysts together, new biological-synthetic hybrid materials can be produced incorporating the best of both catalytic systems.

Introduction

Catalytic materials, either biological or synthetically derived, have the ability to perform a vast array of chemical transformations. Biological catalysts, typified by enzymes and optimized by evolutionary adaptation, are synthesized at the level of gene expression. This makes production relatively simple through host growth, by either native or recombinant expression in more efficient host systems. Synthetic catalysts on the other hand require more extensive design and synthetic development but can possess unique physical and functional properties, such as simpler chemical structure, greater thermal stability, and tunable properties, that are not found in their biological counterparts. Organometallic catalysts provide additional complexity but, like their metalloenzyme counterparts, can be difficult to obtain and their metal centers can be sensitive to degradation. Much research has been dedicated to incorporating organometallic catalysts into architectures that mimic those of enzymes to create synthetic metalloenzymes for catalyzing reactions in aqueous environments. As such, constructing synthetic metalloenzymes provide an opportunity not only to develop more robust catalysts with unique properties from natural enzymes, but catalysts that can be utilized in tandem with enzymes to create coupled catalytic systems (metabolic pathways)^{108,146}. By combining both enzymatic and synthetic small molecule inorganic catalysts together, new biological-synthetic hybrid materials can be produced incorporating the best of both catalytic systems. The inspiration for the catalyst coupling approach described here derives from cellular environments, where biocatalysts (enzymes) are often grouped and separated into sub-cellular compartments or complex

multi-protein assemblies, forming components of metabolic pathways (metabolons) that coordinate and enhance the catalytic activities through multi-enzyme interactions.^{14,126,147,148} New catalytic materials can be generated by combining and coupling the catalytic capabilities of synthetic catalysts and enzymes onto a single nanoparticle scaffold that localizes the catalysts in close proximity to one another. Here, we describe a biomimetic approach for constructing new catalytic hybrid nanomaterials that harness and couple the catalytic properties of synthetic small molecule catalysts and biological (enzyme) catalysts.

Nature utilizes a number of strategies to separate enzymes into discrete compartments including assembly and sequestration of enzymes into protein-based microcompartments, which are cage-like subcellular structures typified by the carboxysome.^{14,126,147} Biomimetic design, inspired by these structures, has enormous potential for synthetic materials applications.^{44,149} A number of cage-like architectures have been identified with the ability to encapsulate non-native guest molecules within their hollow interior, including synthetic polymers, inorganic nanoparticles, and proteins.^{27,139} Virus like particles (VLPs), a class of protein cages, assemble from viral coat proteins into hollow structures similar to the native virus, but devoid of the viral genome normally sequestered in the interior. VLPs are often robust, with tolerance against thermal and chemical denaturation, protease digestion, and dehydration. Their self-assembly produces regular homogenous structures that can be used as templates for chemical and genetic modification. Incorporation of protein cargoes onto the interior of VLPs has been demonstrated by either *in vitro* disassembly-reassembly of coat proteins

mixed with cargo proteins or *in vivo* by heterologous co-expression of viral coat protein with fusion proteins of the cargo protein with a protein/peptide tag that directs incorporation into the mature VLP.^{46,49,50,150-159} Many VLPs can be obtained in high yield, are easily modified, on the interior, and therefore represent excellent candidates for constructing hybrid biocatalysts. In particular, we have explored their utility as synthetic catalyst nanomaterials by combining and localizing both enzyme and synthetic inorganic catalysts within the confines of the VLP.

Using the bacteriophage P22 VLP, we have constructed a hybrid catalyst nanomaterial through the co-incorporation of a redox enzyme and a small organometallic catalyst. As the redox enzyme, we chose the alcohol dehydrogenase D (AdhD) enzyme from *Pyrococcus furiosus*, which utilizes NADH to reduce 3-hydroxy-butan-2-one (acetoin) to 2,3-butanediol.¹⁶⁰ Alcohol dehydrogenases, such as AdhD, present useful industrial biocatalysts with considerable interest in the food, pharmaceutical, and fine chemical industries, which makes them particularly appealing targets for catalysis.¹⁶⁰ In addition, many commercially relevant enzymes utilize the NADH cofactor and small molecule catalysts that can regenerate this cofactor would be advantageous. We chose to use a rhodium catalyst, $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$, known to convert NAD^+ to NADH using formate as a reductant.¹⁶¹ Coupling of the two activities provides an illustration of initial steps towards the design of a semi-synthetic metabolic pathway. We have previously shown that the monomeric AdhD enzyme can be incorporated into P22 by heterologous expression in *E. coli*, producing nanoparticles with a high-density packaging of enzymes that retain their overall high catalytic efficiency for reducing the 3-hydroxy-butan-2-one

to 2,3-butanediol (Figure 6.1).⁴⁹ The $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ catalyst is water soluble and with minor modification to the phenanthroline group can be readily incorporated onto the protein architecture making a synthetic metalloenzyme-like hybrid material.

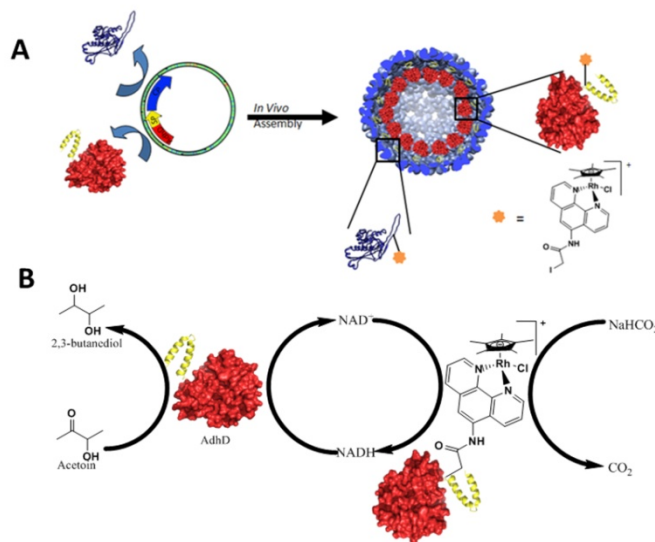


Figure 6.1 (A) Cartoon representing the *in vivo* recombinant expression and encapsulation of the fusion product AdhD-SP inside a properly assembled P22 capsid. The assembly of P22 is initiated by the C-terminus of the truncated scaffold protein (yellow) and the engineered cysteine residue on both the coat and scaffold protein for the attachment of a small molecule catalyst is shown. (B) The reaction catalyzed by AdhD and the Rhodium catalyst. On the left is the reaction catalyzed by AdhD, which oxidizes NADH and converts acetoin into 2,3-butanediol. The reaction on the right is the reaction catalyzed by the Rh catalyst, which reduces NAD⁺ and converts sodium formate into carbon dioxide.

Results and Discussion

For incorporation of the $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ catalyst into the hybrid material a slight modification was made to the phenanthroline ligand. Starting with a 5-aminophenanthroline derivative of the parent complex, $\text{Cp}^*\text{Rh}(5\text{-NH}_2\text{-phen})\text{Cl}^+$, the -NH_2 on the phenanthroline was converted to an iodoacetamide group. Details of the

synthesis and complete analysis of the iodoacetamide derivative ($\text{Cp}^*\text{Rh}(\text{I-phen})\text{Cl}^+$) is provided in the supporting information. The iodoacetamide allows direct conjugation to the free thiol groups of cysteine residues. Cysteine residues were engineered in positions adjacent to the encapsulated AdhD in the scaffold protein (S141C) and in the coat protein (K118C) a site predicted to be on the interior of the assembled P22 capsid based on available structural models.⁷³

AdhD-P22 containing the engineered cysteine residues was obtained by co-expression of the fusion protein of AdhD and P22 scaffolding protein (AdhD-SP) and P22 coat protein (CP) and purification as previously described.⁴⁹ Purified AdhD-P22 was analyzed by SDS-PAGE, size exclusion chromatography coupled to multi-angle light scattering and refractive index detectors (SEC-MALS/RI), and TEM (Figure 6.2), which showed co-purification of AdhD-SP and CP and formation of VLPs containing densely packed interiors with the expected size of P22. Molar masses of AdhD-P22 determined from SEC-MALS/RI showed that on average ~250 AdhD enzymes were encapsulated per capsid, consistent with results found previously.⁴⁹ After purification and characterization, AdhD-P22 was labeled with the $\text{Cp}^*\text{Rh}(\text{I-phen})\text{Cl}^+$ and purified by ultracentrifugation to yield an orange colored pellet, as opposed to the opaque pellet observed for unlabeled AdhD-P22. Analysis of the re-suspended AdhD-P22 pellet by size exclusion chromatography revealed a single peak in which the absorbance at 280nm (protein) and the absorbance at 360 nm (from the $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$) co-eluted at the same elution volume as the unlabeled AdhD-P22 capsid (Figure D.1). This strongly suggests that the $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ was covalently conjugated to the AdhD-P22.

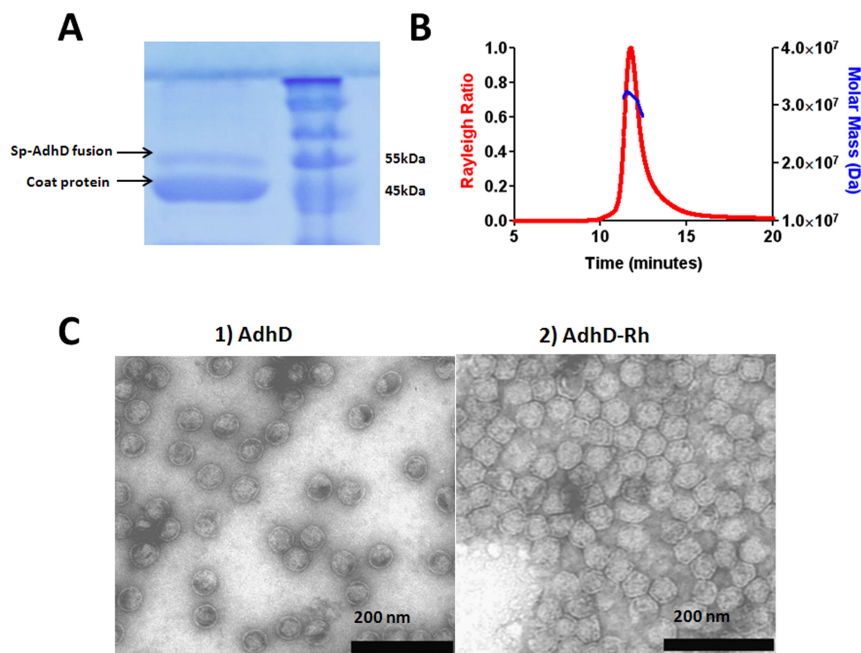


Figure 6.2 Characterization of P22 encapsidated with AdhD and labeled with Rh catalyst. (A) SDS-PAGE gel of purified P22 AdhD-SP-Rh, top band corresponds to AdhD-SP (expected 50 kDa) and bottom band corresponds to CP (expected 46.6 kDa). (B) Size exclusion chromatography profile and molecular weight analysis from multi-angle light scattering (MALS) of procapsid P22 AdhD-SP labeled with Rh catalyst showing the Raleigh scattering (red trace) and corresponding molar mass fraction (blue) across the elution profile. (C) Transmission electron micrograph images of unlabeled and labeled P22 AdhD-SP: (1) AdhD unlabeled; (2) AdhD labeled with Rh catalyst.

Conjugation of $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ to the coat protein (CP) was confirmed by electrospray ionization (ESI) mass spectrometry, which showed a quantitative mass increase of 508 for the labelled CP over the unlabeled CP (Figure D.2) along with additional species corresponding to decomposition products of the Cp^*Rh complex. In particular, two decomposition products are consistent with the loss of the Cp^* ligand and coordination of phosphate anions (from the buffer) to the Rh complex (Figure D.2, D.3). Loss of Cp^* has been reported from related complexes during catalysis.^{162,163} Despite concerted efforts, we were unable to detect the AdhD-SP fusion by either LC-ESI or

matrix assisted laser desorption ionization (MALDI) mass spectrometry and so could not confirm the $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ complex attachment to C141-SP. Investigation of the unlabelled and labelled AdhD-P22 by transmission electron microscopy confirmed that attachment of the organometallic $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ catalyst did not affect the morphology of the VLPs, which appear as ~58 nm diameter particles (Figure 6.2C) in both samples. To determine the extent of labelling, we analyzed the rhodium and sulfur content by inductively coupled plasma-mass spectrometry (ICP-MS). As noted earlier, we were able to determine the number of AdhD-SP per capsid (250 AdhD-SP per capsid or 420 CP) from multi-angle light scattering (MALS), allowing us to account for sulfur content identified by ICP-MS distributed in both the CP and AdhD-SP proteins through their methionine and cysteine residues. The ratio of sulfur, exclusively from the cysteine and methionine residues of AdhD-P22, to rhodium provides an accurate measure of the degree of labeling. Results from ICP-MS revealed 2120 equivalents of Rh per cage. This was greater than the expected 670 Rh per cage, corresponding to labeling of each exposed cysteine of the CP and AdhD-SP. Multiple analyses of Rh labeling by ICP-MS was found to yield the same result as the determination by UV-Vis spectroscopy (using molar absorption values for the free $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$). It is possible that the $\text{Cp}^*\text{Rh}(\text{I-phen})\text{Cl}^+$ could interact non-covalently with the AdhD-P22 or react with other protein nucleophiles. We performed extensive purification using mild detergents and/or high ionic strength buffers to try and remove any non-covalently attached $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$. However, after several washes no change in $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ content was observed, suggesting it was bound tightly and could not be removed any further. In addition,

labeling of wild type P22 (with no additional cysteines for labeling) showed that only a small fraction of the wild type P22 CP was labeled with the $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ complex (Figure D.2, D.4), indicating that the high value of Rh observed by ICP-MS is not due to covalent modification of the CP at alternative sites. However, these samples did show the orange color associated with the Rh catalyst and catalytic regeneration of NADH (Figure D.5) was observed.

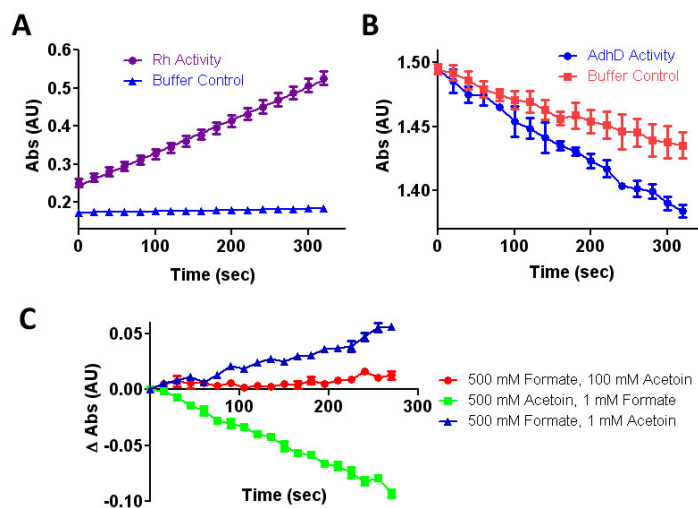


Figure 6.3 Kinetic plots monitoring the formation or depletion of NADH at 340 nm using an UV-Vis, showing the activity of the fusion product AdhD-SP-Rh. All trials done in triplicate. (A) Kinetic plot showing the Rhodium catalyst's activity after labelled to SP-AdhD. (B) Kinetic plot showing the activity of AdhD after labelled with the Rhodium catalyst. (C) Changing the concentrations of substrates dramatically changes the rates of consumption and formation of NADH, therefore allowing us to tune the reaction to produce or deplete NADH as desired.

The catalytic activity of the $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ conjugated AdhD-P22 (AdhD-P22-Rh) was investigated for NADH formation using formate as a reductant, in the absence of substrate for the AdhD. AdhD-P22-Rh had a turnover rate of 0.0115 s^{-1} for NADH formation, monitored by the increase in NADH absorption at 340 nm, which is slower

than was observed for free $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$, 0.556 s^{-1} (Figure 6.3A). The significantly lower rate suggests that not all the $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ adhering to the cage is active. This finding, in conjunction with the mass spectrometry and ICP data, suggests there is a decomposition of the catalyst that associates with the protein cage, resulting in excessively high concentrations of observed Rh by ICP, thus producing lower than expected overall activity for the concentration of catalyst. To evaluate this further, we labelled AdhD-P22 with several different stoichiometric ratios of the $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ complex to determine the degree of labelling and any side products that were formed and examined the resulting materials by mass spectrometry (Figure D.2, D.4). The data confirmed that some of the $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ complex decomposes, forming new complexes with the phosphate anion that is present in the phosphate buffer (Figure D.3). However, the catalytic activity of the $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ labelled AdhD-P22 showed an increase in the rate of NADH formation with an increase in labelling, which is also accompanied by the increase in degradation products. This data suggests that even though the $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ complex degrades, these degradation products, in whole or in part, are still active towards the formation of NADH (Figure D.5). By contrast, the AdhD enzyme activity in the labeled construct AdhD-P22-Rh was also evaluated for reduction of 3-hydroxy-butan-2-one (acetoin) in the presence of NADH (but in the absence of substrate for the $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$, formate) and was found to have a rate identical to that of unlabeled AdhD-P22 as previously found,⁴⁹ indicating that $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ attachment does not interfere with AdhD activity (Figure 4.3B). The observed decrease

in [NADH] in buffer controls is due to thermal degradation and was accounted for in all rate calculations.

After verifying that each of the catalytic components were independently active in the hybrid AdhD-P22-Rh particles, we investigated the coupling between the two catalysts. This was achieved by probing the coupled assay in the presence of the substrate for AdhD (acetoin), NAD^+ , and formate. As shown in the reaction scheme in Figure 4.1B, the attached Cp^*Rh complex catalyzes the reduction of NAD^+ to form NADH, which could then be utilized by the AdhD enzyme in the reduction of acetoin to 2,3-butanediol. The overall reaction was monitored spectroscopically by the change in absorbance at 340 nm due to NADH. The production of 2,3-butanediol from 3-hydroxybutan-2-one was additionally confirmed by NMR and could be followed through the formation of a doublet at 1.05 ppm corresponding to the protons on the methyl group (Figure C.6). Initial results indicated that the production of NADH by $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ coupled with the consumption of NADH by AdhD was dependent upon the concentrations of formate and acetoin (Figure 6.3C). Adjusting substrate concentrations to high formate concentrations (500 mM) and low acetoin (1 mM) lead to the reaction by $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ to dominate, resulting in build up the NADH. Reversing the concentrations of formate (1 mM) and acetoin (100 mM) lead to dominance of the reduction reaction by AdhD, resulting in a net decrease of NADH. Additionally, a steady-state condition could be achieved in which the production and consumption of NADH was balanced, under conditions of 500 mM formate and 100 mM acetoin (Figure

6.3C). This is hugely advantageous because it demonstrates that the system can be tuned to either produce or consume NADH, by adjusting substrate levels, as desired.

Conclusions

These results illustrate the ability to use protein cage scaffolds for constructing new hybrid catalytic materials taking advantage of the best in biological evolution and small molecule catalyst design. The P22 platform is especially unique in its ability to encapsulate enzymes completely in vivo. This approach presents unique benefits for sustainability in that *E. coli* can be used as biofactories for producing the catalytic nanoparticles in high yields requiring relatively few steps for purification. As shown here, synthetic catalysts can be readily appended to the P22 framework with high labeling efficiency to construct synthetic metalloenzyme-like nanoparticles, broadening the synthetic utility of the system. We have also shown that the system can be tuned to produce or consume NADH by altering substrate concentration, allowing for easy manipulation of the system. Future development of hybrid catalysts utilizing synthetic small molecule inorganic catalysts with the ability to harvest light for regenerating enzyme cofactors could address problems faced in sustainability. We have already shown that enzymes encapsulated within P22 can be embedded within gel matrices and other immobilization methods using reactive amino acid residues on the surface for conjugation.^{49,164} The protein cage platform presents a useful scaffold that can be used to immobilize the catalysts contained on its interior, eliminating direct linkage to surfaces, which can negatively affect catalysis of enzymes and allowing reuse of the catalysts.

Acknowledgements

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Experimental

Materials

All manipulations requiring dry solvents were performed with freshly distilled solvents. All other reagents were commercially available and were not purified any further unless otherwise stated. Preparation of buffers used analytical quality chemicals and ultrapure (Milli-Q) water. NMR spectra were taken with a 300 MHz, 500 MHz, or 600 MHz Bruker spectrometer using D₂O as the internal standard. Mass spectra were obtained in positive-ion mode with a microTOF mass spectrometer (Bruker Daltonics). UV spectra were obtained on an Agilent 8453 UV-Vis spectrometer fitted with a temperature controlled cuvette holder linked with a RC 20 Lauda temperature control module.

Preparation of 5-Iodoacetamido-1,10-phenanthroline (I-phen)

I-phen was prepared by following previously published methods^{39,165}. Briefly, five grams (0.024 mol) of DCC and 10 grams (0.054 mol) of iodoacetic acid were dissolved in 150 mL of ethyl acetate. The solution was stirred at room temperature for about 3 hours. The dicyclohexylurea was removed by filtration and the filtrate was evaporated to dryness. 5-amino-1,10-phenanthroline (1.6 g, 0.0082 mol) was dissolved

into 200 mL of acetonitrile by gentle heating. Once this solution cooled to room temperature the iodoacetic anhydride was dissolved in 75 mL of CH₃CN and added to the main solution. The reaction was allowed to stir overnight at room temperature. The crystalline solid was collected by vacuum filtration with a coarse frit and washed with 5% cold sodium bicarbonate, followed by washing with water. The final product was further dried *in vacuo*. Similar yields were obtained to the published method.

Preparation of Chlorido
Complex $[(\eta^5\text{-C}_5\text{Me}_5)\text{Rh}(\text{I-phen})\text{Cl}]\text{Cl}$

Two equivalents 112.5 mg (0.30 mmol) of the (I-phen) were added to a suspension of $[(\eta^5\text{-C}_5\text{Me}_5)\text{RhCl}_2]_2$ 93.7 mg (0.15 mmol) in dichloromethane (30 mL). This mixture was stirred for 3 hr at room temperature, and the color changed from orange to a bright orange-red. The mixture was evaporated to dryness, re-dissolved in water and filtered. The filtrate was concentrated to dryness, which gave the product as an orange-yellow powder in good yield. Yield: 86 %, 178.3 mg. ¹H NMR (300 MHz, D₂O, δ): 1.50-1.70 (m, CH₃), 4.32 (d, ³*J*_{H,H} = 4.8 Hz, CH₂), 7.73-7.91 (m, CH), 7.94 (s, CH), 8.33-8.43 (m, CH), 8.51 (d, ³*J*_{H,H} = 8.4 Hz, CH), 9.02 (dd, ³*J*_{H,H} = 23.1 Hz, ³*J*_{H,H} = 5.2, CH) ppm. ¹³C NMR (300 MHz, D₂O, δ): 8.32 [C₅(CH₃)₅], 42.94 (CH₂), 97.9 [C₅(CH₃)₅], 126.39 (C), 126.67 (CH), 129.43 (CH), 131.53 (CH), 134.34 (CH), 138.20 (C), 143.13 (C), 144.98 (C), 152.31 (C), 153.08 (CH), 168.57 (CO) ppm. MS (ESI) *m/z*: [M]⁺ calculated for C₂₄H₂₅ClIN₃ORh, 635.98 ; found, 635.97.

Preparation of AdhD-P22

The preparation of AdhD-P22 has been previously described.⁴⁹ Briefly, the AdhD-SP and P22 coat protein were co-expressed in BL21(DE3) *E. coli* cells using a pETDuet-1 assembler vector by induction with 0.5 mM of IPTG for 4 hours after cells had reach an OD₆₀₀ of 0.6. Cells were removed from media by centrifugation and resuspended in PBS buffer pH 7.0 and lysed by sonication, then the insoluble cellular lysate was removed by centrifugation. The supernatant was spun in an ultracentrifuge over a sucrose cushion, resulting in a highly pure pellet containing AdhD-P22 VLPs, which was further purified over an S-500 size exclusion column to remove any aberrant assemblies.

Preparation of Modified Protein with Small Molecule Catalyst

A 20 mM solution of rhodium (III) catalyst was prepared in deionized water. To a 7-8 mg/mL solution of P22 (1 mL) protein, 50 μ L of the 20 mM solution of rhodium catalyst was added. The reaction was agitated gently at room temperature for ~3 hours and the protein solution was concentrated by ultra centrifugation at 45,000 rpm, at 4 °C for 50 minutes. The pellet was re-suspended in phosphate buffer 100 mM PO₄, 50 mM NaCl pH 7.

Sample Preparation for Mass Spectrometry

Samples were prepared at 1 mg/mL in phosphate buffer (100 mM PO₄, 50 mM NaCl pH 7). Samples were spun at 13,200 rpm for 10 minutes to remove any small aggregates and placed into mass spectrum vials. A polyLC SEC

(PolyHYDROXYETHYL A, 100×4.6 -mm; $5 \mu\text{m}$ $500 \text{ \AA}$) was used to separate the protein and electrospray LC-MS were obtained in positive-ion mode with a microTOF mass spectrometer (micro-TOF-LC Bruker Daltonics equipped with an ESI orthogonal electrospray source coupled to an Agilent 1100 series LC pump).

Catalytic NADH Regeneration

A $99 \mu\text{L}$ solution containing NAD^+ ($300 \mu\text{M}$), sodium formate (200 mM) and phosphate buffer ($\text{pH } 7$) was incubated for 5 minutes at 60°C and reduced by adding $1 \mu\text{L}$ $[(\eta^5\text{-C}_5\text{Me}_5)\text{Rh}(\text{Iphen})\text{Cl}]\text{Cl}$ labeled to P22-sp-AdhD. The formation of NADH was monitored by UV-Vis absorption at 340 nm . (E for NADH = $6220 \text{ M}^{-1}\text{cm}^{-1}$)

Chemoenzymatic Hydrogenation of Ketones

The enzymatic conversion of acetoin (100mM) to 2,3-butanediol was carried out in phosphate buffer ($\text{pH } 6.1$), 200 mM sodium formate, $300 \mu\text{M}$ NAD^+ and $300 \mu\text{M}$ NADH at 60°C . P22-SP-AdhD labeled with $[(\eta^5\text{-C}_5\text{Me}_5)\text{Rh}(\text{Iphen})\text{Cl}]\text{Cl}$ ($1 \mu\text{L}$ added with internal concentration of AdhD $\sim 66.8 \mu\text{M}$) was added to the reaction mixture after incubating for five minutes. The rhodium catalyst and sodium formate served as the NADH-regenerating catalyst and hydrogen source, respectively. Formation of 2,3-butanediol was monitored by nuclear magnetic resonance and mass spectrometry.

Modulating Reaction Pathway Fluxes by Varying Substrate Concentrations

Reaction pathway flux was modulated by varying the concentration of formate and/or acetoin in the reaction mixture. Briefly, all reactions were carried out in phosphate

buffer (pH 6.1) with 300 μM NAD^+ and 300 μM NADH at 60 $^{\circ}\text{C}$. Initial substrate concentrations of 500 mM formate and 1 mM acetoin were evaluated and subsequently modified, by lowering formate and increasing acetoin concentrations respectively, to obtain desired pathway fluxes. P22-SP-ADHD labeled with $[(\eta^5\text{-C}_5\text{Me}_5)\text{Rh}(\text{Iphen})\text{Cl}]\text{Cl}$ (1 μL added with internal concentration of AdhD ~ 66.8 μM) was added to the reaction mixture after incubating the for five minutes to stabilize the temperature of the reaction mixture. Formation of NADH was monitored by UV-Vis absorption at 340 nm ($\epsilon_{\text{NADH}} = 6220 \text{ M}^{-1}\text{cm}^{-1}$).

CHAPTER SEVEN

CARGO-SHELL AND CARGO-CARGO COUPLINGS GOVERN THE MECHANICS
OF ARTIFICIALLY-LOADED VIRUS-DERIVED CAGES

Contribution of Authors and Co-Authors

Manuscript in Chapter 7

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Contributions: Carried out experiments, edited and help write the manuscript.

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CHAPTER SEVEN

CARGO-SHELL AND CARGO-CARGO COUPLINGS GOVERN THE MECHANICS
OF ARTIFICIALLY-LOADED VIRUS-DERIVED CAGES

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Abstract

Nucleic acids are the natural cargo of viruses and key determinants that affect viral shell stability. In some cases the genome structurally reinforces the shell, whereas in others genome packaging causes internal pressure that can induce destabilization. Although it is possible to pack heterologous cargoes inside virus-derived shells, little is known about the physical determinants of these artificial nanocontainers stability. Atomic force and three-dimensional cryo-electron microscopies provided mechanical and structural information about the physical mechanisms of viral cage stabilization beyond

the mere presence/absence of cargos. We analyzed the effects of cargo-shell and cargo-cargo interactions on shell stability after encapsulating two types of proteinaceous payloads. While bound cargo to the inner capsid surface mechanically reinforced the capsid in a structural manner, unbound cargo diffusing freely within the shell cavity pressurized the cages up to ~30 atm due to steric effects. Strong cargo-cargo coupling reduces the resilience of these nanocompartments in ~20% when bound to the shell. Understanding the stability of artificially loaded nanocages will help to design more robust and durable molecular nanocontainers.

Introduction

Synthetic biomimetic nanostructures are revolutionizing materials design at the nanoscale. Inspired by nature, where intracellular structures act as optimized nanocompartments, new self-assembling biological systems have been generated with growing interest in biology, chemistry, and materials science.^{13,46,47,49,126,166} Virus-like particles (VLP) show a controlled hierarchical assembly, are easy to produce, and their structure can accommodate modifications of their inner and/or outer surfaces. These attributes enable the incorporation of artificial cargos in the VLP, such as small molecules,^{44,47} metal nanoparticles^{44,46} or proteins,⁴⁴ to produce hybrid materials that can be used for a broad range of applications. The confinement of proteins inside VLP permits the modulation, spatial control and protection of their enzymatic activity in a variety of environments, and has evident interest for pharmaceutical and nanotechnological applications.^{50,167,168}

The development of robust, stable nanocages able to maintain their structure is crucial for their durability in harsh environmental conditions, and some successful strategies can be adopted from nature. Genetic material can reinforce natural viral shells after packing via structural interaction with the viral shell, similar to the way beams buttress the structure of a building.^{169,170} In other cases, the genome destabilizes the viral shell by inducing outward pressurization that stiffens the viral particles.¹⁷¹⁻¹⁷³ Therefore, the stability of protein-derived cages loaded with heterologous proteins depends not only on the presence of its internal cargo, but also on the mutual cargo-shell and cargo-cargo interplay, as in the case of their natural counterparts.

Virus-like particles (VLP) derived from the *Salmonella typhimurium* bacteriophage P22 are suitable models to address some of these questions, as it is a versatile and well-characterized system in virology and nanomaterials synthesis.^{73,174} The P22 VLP capsid is built of 420 copies of a coat protein (CP) that assembles into a T=7 icosahedral shell with the aid of 100-300 copies of scaffolding proteins (SP); the SP C-terminal helix-loop-helix motif interacts with the CP. In contrast to authentic phages, these VLPs have 12 identical pentons with no portal.¹⁷⁵ P22 VLP procapsids (PC) undergo a series of well-defined structural transitions after heating that generate mature viral shells (EX particles), emulating bacteriophage P22 maturation.^{73,174,176} This capsid transition (PC→EX) involves an increase in internal volume of ~35%, as well as capsid shell thinning and a decrease in its porosity.

Heterologous expression of CP and N-terminal-truncated SP fused to other gene products results in self-assembly of the PC (59.6 nm outer diameter, 46,450 nm³ internal

volume); heating at 65°C for 20 min yields the EX (64.8 nm, 71,900 nm³) (Figure 7.1A). We used the SP fusion strategy to incorporate enhanced green fluorescence protein (EGFP) or β -glucosidase from the hyperthermophile *Pyrococcus furiosus* (CelB) into the VLP interior when expressed in *Escherichia coli* together with CP (Figure 7.1B). As the interaction between EGFP monomers is anticipated to be weaker than those between CelB monomers, which form spontaneous tetramers inside the shell,⁴⁹ we used these two proteins to study the effects of cargo-cargo coupling. The ability of PC structure to mature into the EX

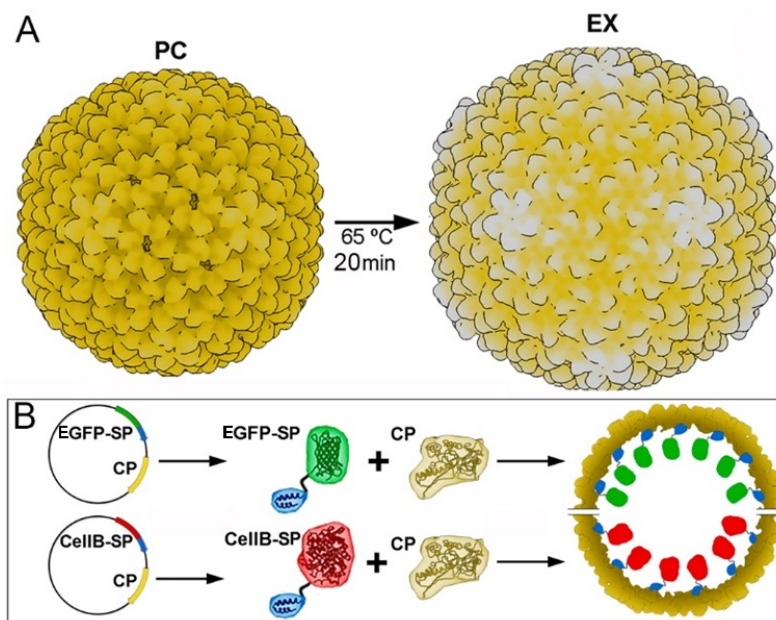


Figure 7.1. Synthesis of P22 nanocages and the expanded morphology of the P22 capsid. (A) P22 capsid maturation. P22 viral-like particles (VLP) are produced as procapsids (PC), which are transformed to an expanded shell form (EX) by heating at 65°C for 20 min. (B) Assembly of P22 VLP with different cargos. Co-expression of coat protein (CP, yellow) with N-terminal truncated scaffold protein (SP, blue) fused to EGFP (green) or CelB monomers (red) leads to VLP assembly as PC with encapsulated cargo. VLP assembly is facilitated by interaction of the essential SP C-terminal domain and CP subunits, which leads to encapsulation of SP-fused proteins. For clarity, EGFP and CelB are shown as monomers.

morphology while maintaining its cargo allowed us to address the effects of cargo-shell coupling. During maturation, the SP C-terminal segment of the SP-cargo protein is unbound from the interior surface shell, resulting in soluble cargo molecules inside the EX.^{36,174,176-182}

Here we combined mechanical and structural information obtained from atomic force microscopy (AFM) and three-dimensional cryo-electron microscopy (3D cryo-EM) to describe physico-chemical mechanisms that influence the stability of synthetic P22 protein cages. Our results show that the interplay between the cargo and the shell determines whether the mechanical reinforcement is structure- or pressure-induced, as happens in natural viral cages. Cargo bound to the shell provides structural reinforcement to the P22 procapsid, although a strong cargo-cargo interaction renders particles more brittle. In the expanded form, cargo-shell interactions are removed and the SP-associated cargo proteins remain inside the particle in a “free” soluble state that pressurizes the expanded structure up to 3 MPa.

Results and Discussion

We used 3D cryo-EM to analyze the structures of two morphologies of heterologous bacteriophage P22 T=7 VLP, termed PC and EX, loaded with a cargo of EGFP or CelB. Empty PC and EX were included as controls (Figure 7.2). We used HPLC size exclusion chromatography with multi-angle light scattering (MALS) to determine the number of cargo copies per particle, and found 128 ± 1 CelB and 220 ± 5 EGFP monomers. Figure 7.2A shows cryo-EM images of these purified P22 VLP with different

morphologies, imaged at -170°C in a 200 kV cryo-electron microscope. A 3D reconstruction (3DR) was calculated for each of the six sets of particle types (Figure 7.2B-D). Based on a Fourier shell correlation (FSC) coefficient, the resolutions achieved were between 12-16 Å (Figure F.1). Particle diameters were determined from radial density profiles from the 3DR (Figure 7.2E). Whereas spherical PC (empty, EGFP- or CelB-loaded) had a 298 Å outer radius, EX form measured 324 Å. This capsid expansion range is similar in bacteriophages P22, λ and HK97.¹⁸³

Cargo-loaded PC and EX had the same size and general morphology as empty PC and EX. The P22 PC were T=7 isometric structures formed by 72 capsomers, 60 of which are skewed CP hexamers and 12 are CP pentamers, as they lack the portal structure

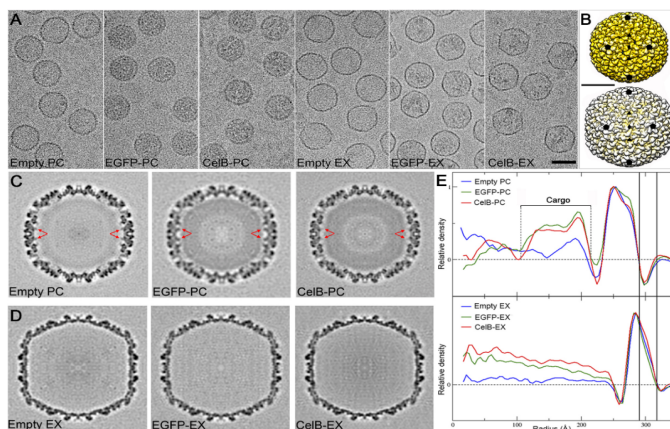


Figure 7.2. Three-dimensional cryo-EM reconstructions of empty, EGFP- and CelB-loaded P22 capsids. (A) Cryo-EM of (left to right) empty PC, EGFP-loaded PC (EGFP-PC), CelB-loaded PC (CelB-PC), empty EX, EGFP-loaded EX (EGFP-EX) and CelB-loaded EX (CelB-EX). Bar, 50 nm. (B) Surface-shaded representations of the outer surfaces, viewed along an icosahedral twofold axis, of P22 PC (top) and EX (bottom). Outer surfaces of empty and loaded PC and EX are similar at this resolution. Symbols indicate icosahedral symmetry axes. Bar, 25 nm. (C) Central sections from the 3DR viewed along a twofold axis of T=7 empty PC (left), EGFP- (center) and CelB-PC (right). Darker shading indicates higher density. Arrows indicate some densities due to the SP C-terminal region in the PC internal surface (note that these densities are reduced in the CelB-PC central section). (D) Central sections from the 3DR viewed along a twofold axis of T=7 empty EX (left), EGFP- (center) and CelB-EX (right). (E) Radial density profiles of 3DR of empty PC, EGFP- and CelB-PC, empty EX, and EGFP- and CelB-EX. PC (top) and EX shells (bottom) are essentially superimposable. In PC, a cargo shell spans radii from 107 to 216 Å. Vertical lines indicate PC (298 Å) and EX external radii (324 Å).

(Figure 7.2B, top).⁷³ Empty PC showed densities at the inner surfaces of the hexamers, due to SP C-terminal residues, which cannot be accounted for by the CP (Figure 7.2C, arrows), as reported.^{73,174,176}

Images of EGFP- or CelB-loaded PC (EGFP-PC or CelB-PC) were darker than empty counterparts (Figure 7.2A), which indicated the presence of packed cargos. After imposing icosahedral symmetry, the 3DR showed that cargo density (within a 220 Å radius) was organized as a thick shell beneath the PC shell, with numerous connections to the PC inner surface (Figure 7.2C middle [EGFP-] and right [CelB-PC]). As in *in vivo*-matured virions, P22 EX were more angular than PC, and the hexons became symmetrical hexamers (Figure 7.2D). Heat-induced capsid maturation untethered EGFP- or CelB-fused SP from the EX interior surface and cargos were observed as free densities in the capsid interior (Figure 7.2A), in concordance with wild-type P22 procapsid release of SP after heating.¹⁸⁴

Fixation of the protein shells to a flat substrate via hydrophobic¹⁸⁵ and/or electrostatic interactions¹⁸⁶ is a requisite for AFM. Each protein shell has individual features such as hydrophobic patches or local charge densities, resulting in distinct attachment forces. For a specific shell, these forces can even reduce the height of the softest virus-like morphologies¹⁸⁷ although this is not always the case.^{173,188}

The precise combination of factors that leads to this partial collapse remains undefined, but so far a decrease in height has been always accompanied with a decrease in the mechanical stiffness of the specimen.

Representative AFM images of each VLP type adsorbed on glass (see Materials & Methods) showed partial collapse (Figure 7.3A), which allowed us to carry out a comparative study of particle deformability. Whereas empty VLP showed a height decrease of ~10% compared with their native size in cryo-EM, cargo-loaded VLP maintained heights near 100% of the cryo-EM value (Figure 7.3B). The limited shell deformation of cargo-loaded VLP suggested that these particles were more rigid than their empty counterparts.

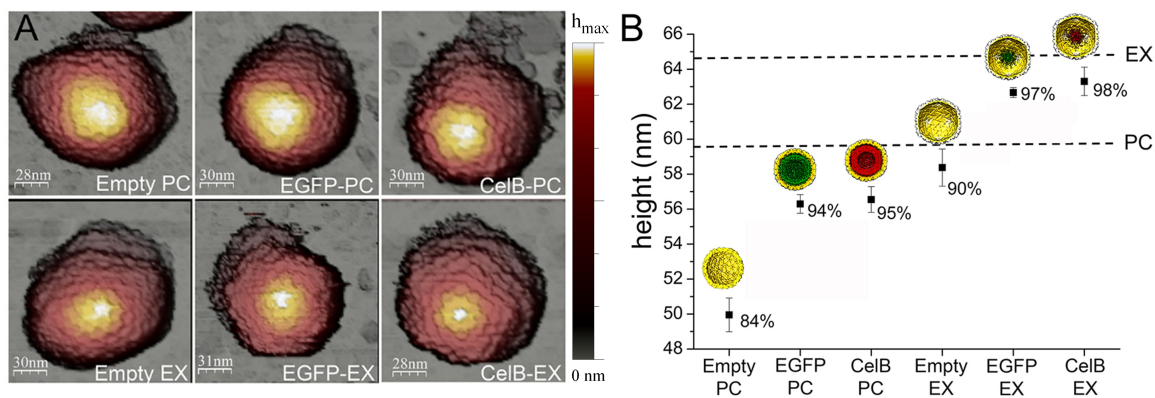


Figure 7.3. AFM topographies of P22 particles. (A) AFM images of empty PC, EGFP-PC, CelB-PC, empty EX, EGFP-EX and CelB-EX. Each image was normalized to its maximum height. (B) Height of empty PC, EGFP-PC, CelB-PC, empty EX, EGFP-EX and CelB-EX after adsorption. Percentages indicate the ratio between average height as measured by AFM and nominal height based on 3D cryo-EM reconstructions. Dashed lines indicate nominal PC and EX outer diameters.

VLP mechanical stability can be assessed systematically using nano-indentation curves of individual particles. This procedure involves deformation of single particles with an AFM tip until the specimen breaks. The force vs. indentation curve (FIC) obtained in these experiments provides two mechanical parameters that are linked to the rigidity and brittleness of the particle probed.^{189,190} Figure 7.4A shows a characteristic FIC

performed on an intact EGFP-PC. AFM images taken before and after the FIC demonstrated the rupture of the shell, which in this case caused a crack associated with a reduction in height from 57 to 47 nm (Figure 7.4A, right). Other examples of this sort of experiments can be found in Figure F.2.

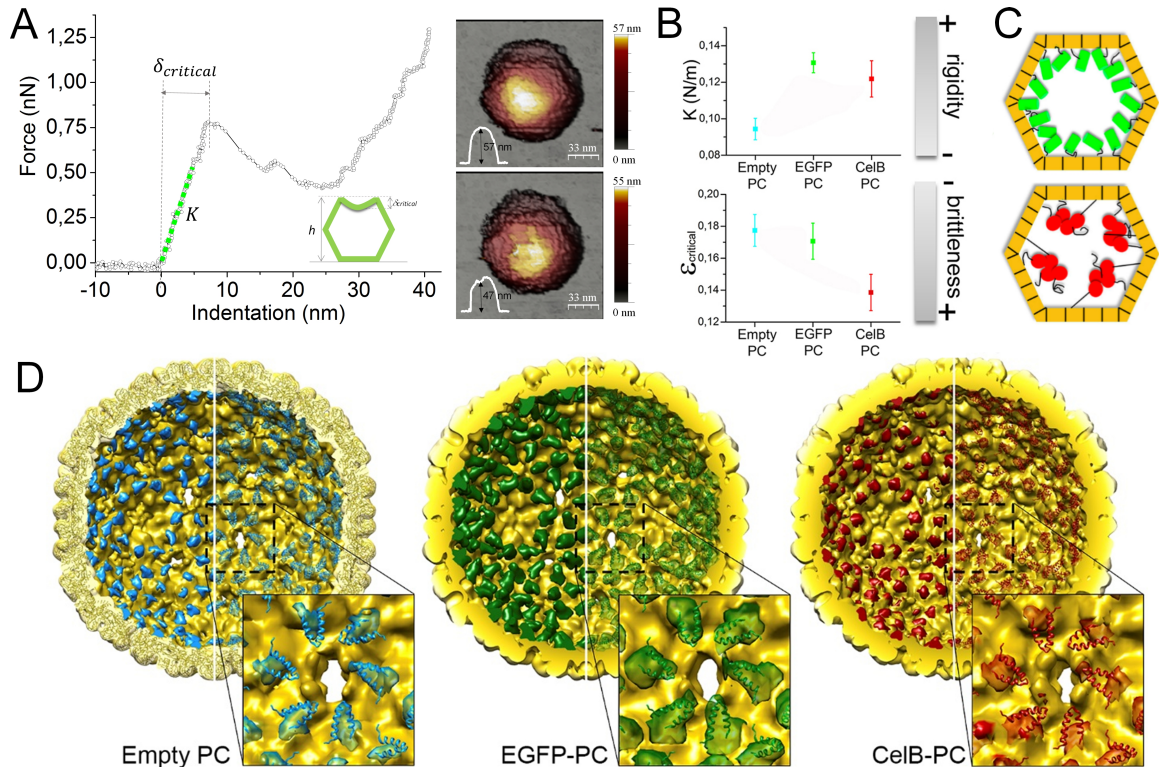


Figure 7.4. Structure and mechanical properties of P22 PC. (A) (Left) Typical force-vs-indentation curve (FIC) of an EGFP-PC. Particle rigidity (k) and critical deformation ($d_{critical}$) can be calculated from the nanoindentation. Critical strain ($\epsilon_{critical}$), which provides information about particle deformation before breakage, is defined as the ratio between the critical indentation and particle height (inset). (Right) AFM topographies of the PC before (top) and after nanoindentation (bottom). A profile of the particle along its center is inset in each image (bottom left). (B) Comparison of the average rigidity (top) and fragility (bottom) of empty and loaded PC. (C) Analysis of CP-SP interactions in empty and EGFP- and CelB-PC. PC viewed down a twofold axis from inside, with docked SP helix-loop-helix motif (right half). Empty PC shows the CP atomic model (yellow) in the shell and the SP motif in the SP density (blue; inset); the SP density is shown in EGFP-PC (green; center) and in CelB-PC (red; right). (D) Scheme of PC showing the organization of CelB tetramers (red, top) and EGFP monomers (green, bottom) fused to SP (black lines).

Particle rigidity is related to the slope of the curve (also termed spring constant, k) and the maximum deformation the particle can withstand before rupturing defines the critical indentation (δ_{critical}), a measure of brittleness.¹⁹¹ The ratio between this critical deformation and particle height (h) before deformation accounts for critical strain,

$$\varepsilon_{\text{critical}} = \frac{\delta_{\text{critical}}}{h} \text{ (Figure 7.4A, inset), which is the parameter that we used in our experiment}$$

to establish differences in particle brittleness.¹⁹¹ Our AFM data showed that EGFP- and CelB-PC were more rigid than empty PC (Figure 7.4B top, Table E.1), in agreement with our initial topographical analysis (Figure 7.3). To determine the cause of this difference in stiffness we analyzed the cryo-EM maps in detail. Comparison of the average radial densities of empty and full PC particles indicated that most cargo localized in a shell beneath the capsid wall within a 216-107 Å radius (Figure 7.2E, top). Whereas the empty PC structure showed a single spherical layer corresponding to the capsid shell, full PC presented an extra concentric layer corresponding to cargos. These data suggested that the stiffening was caused by a structural reinforcement that could be evaluated by continuous elasticity modeling. Simplification of these complex structures to homogeneous shells^{169,190,192,193} permits estimation of the ratio of Young's modulus of the capsid (E_s) and the cargo (E_c). The radial density derived from the cryo-EM data indicated that empty- and loaded-PC had effective thicknesses of ~7.5 and ~17.5 nm, respectively (Figure 7.2E). Finite element modeling estimated E_s/E_c of 7 and 10 for EGFP and CelB cargos, respectively (Figure E.3A), indicating that the capsid shell is approximately ten times more rigid than the cargo “shell”. Therefore, the container might be able to offer protection to the contents because of its higher Young's modulus.

Whereas both packed cargos has similar influence on the rigidity of the particles, there was a difference in brittleness between EGFP- and CelB-loaded PC compared to empty shells (Figure 7.4B bottom, Table E.1). To evaluate this difference in strain, we performed docking analysis of the P22 CP model [Ca model, Protein Data Bank (PDB) ID 2XYY,¹⁷⁴ and the SP C-terminal region (residues 238-303, PDB ID 2GP8)¹⁹⁴] in the empty and loaded PC cryo-EM density maps. The SP C terminus fitted well in the triangular density at the internal surface of the PC skewed hexamers (Figure 7.4C left, blue). This SP-related density was also well preserved in the EGFP-PC at the same radial position, and showed the connections between CP shell and cargo (Figure 7.4C middle, green). CelB-PC were more disordered in this region than empty and EGFP-PC, and SP-mediated connections were irregular and less defined (Figure 7.4C right, red). This scenario is compatible with the existence of fewer SP-mediated connections for CelB-PC than for EGFP-PC. Whereas EGFP molecules remain as monomers, CelB monomers strongly interact to form tetramers⁴⁹ whose assembly could affect SP-CP interaction. CelB monomers are connected to CP by a 123 amino acid linker that joins the cargo with the SP-CP binding domain. We propose that CelB tetramers inside the PC shell would tighten some of these SP-mediated connections, leading to an additional geometrical constraint that might reduce capsid subunit mobility during nanoindentations (Figure 7.4D, top). In contrast, the monomeric condition of EGFP would not impose any constraint on the SP linkers (Figure 7.4D, bottom). The reduced mobility of the CelB-PC structural subunits would render more brittle particles, resulting in lower critical strain for CelB-PC ($\epsilon_{\text{critical}} = 0.14$) than for EGFP-PC ($\epsilon_{\text{critical}} = 0.17$) (Table E.1).

Beyond the breaking force, indentation curves (Figure E.2, top) undergo an abrupt decay informing about how fast the tip penetrates the virus. Although with high variability, post-fracture curves show that the cantilever decays to low deflection values quicker on empty PC than on filled PC, thus revealing that there are less obstacles impairing the tip indentation inside the fractured empty PC. We tentatively hypothesize that that cargo, well bound to the PC capsid, prevents the tip from indenting inside the virus.

During the transition from PC to EX, the SP domains release the CP and escape from the capsid, resulting in empty EX particles.¹⁷⁶ However, in the case of loaded particles the SP fused to EGFP or CelB structures cannot escape and become free inside the EX after detaching of the capsid wall. We analyzed the mechanics of three expanded protein cages (empty, CelB- and EGFP-EX) to establish differences between them. Figure 7.5A shows a representative nanoindentation curve performed on a CelB-EX, with the AFM images before (Figure 7.5A, top right) and after the tip-induced breakage (Figure 7.5A, bottom right). Statistical analysis of the mechanical properties of the three EX types showed that whereas cargo increased rigidity, $\epsilon_{\text{critical}}$ did not vary (Figure 7.5B).

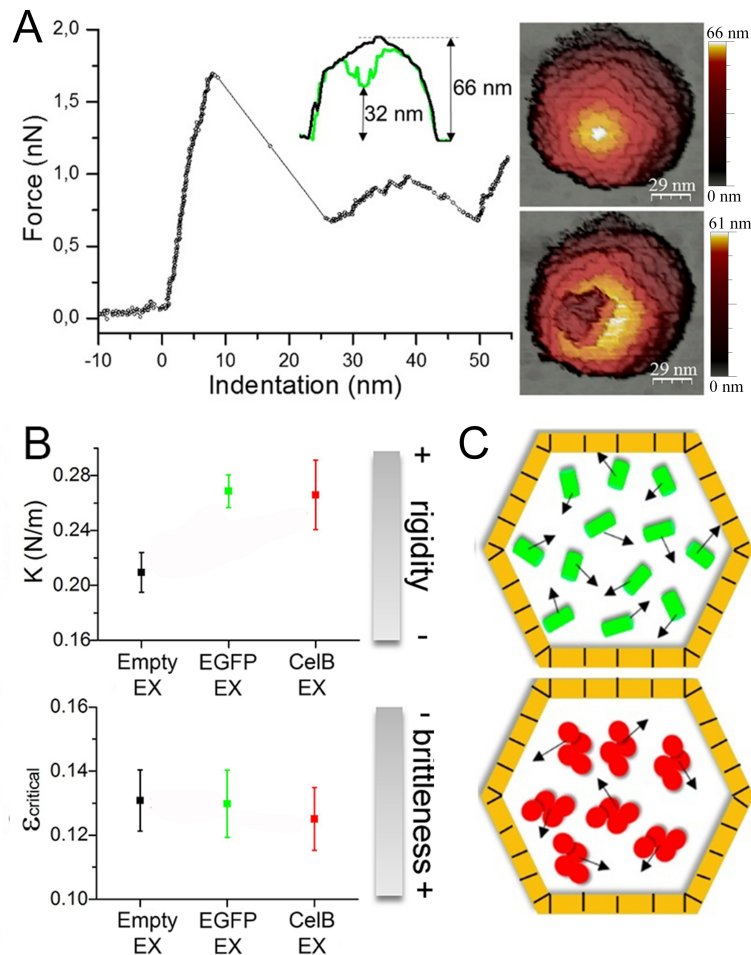


Figure 7.5. Mechanical characterization of EX capsids. (A) Typical FIC of a CelB-EX. Inset, Aligned profiles of particle topographies before (black) and after (green) breakage. Right, CelB-EX images before (top) and after breakage (bottom). (B) Comparison of the average rigidity (top) and fragility (bottom) of empty and loaded EX. (C) Scheme of EX showing cargo units as diffusing entities within the protein shell (colors as in Fig. 7.4).

Compared with the AFM data for CelB-PC, the lack of influence of this cargo on EX brittleness is likely related to the detachment of SP domains that connected CelB tetramers to the shell, which no longer represent a geometrical constraint. EGFP monomers and CelB tetramers remained unanchored, free to diffuse within the EX cage (Figure 7.5C). This might justify why the full EX particles do not differentiate of the empty ones in the post-fracture nanoindentation patterns: both particles reach low

deflection values as soon as they are broken (Figure E.4). In contrast with PC structures, the cargo is unbound of the capsid shell. We tentatively hypothesize that proteins can diffuse out as soon as the capsid is cracked and they do not impair the tip indentation in the fractured shell. In addition, the mechanical properties (i.e., breaking force and rigidity) of EX increased significantly in comparison with PC, as a consequence of the stabilization gained by the VLPs during capsid maturation (Table E.1, Figure E.2 and E.4).⁷³

Because no permanent structural contacts exist between the cargo and the capsid shell in EX capsids, the increased rigidity in EX (Figure 7.5B, top) could be due to an osmotic pressure arising from the different concentration between the inner shell and the surroundings. This difference would drive water molecules into the shell and increase the pressure of the cages. Alternatively, it might be caused by electrostatic repulsion between cargo molecules retained within the capsid. To determine which of these mechanisms was responsible for the capsid reinforcement we modeled our system and performed new AFM experiments.

Regardless of its physical origin, we can estimate the magnitude of the internal pressure in loaded EX using the continuous elastic prediction for rigidity of a pressurized thin spherical shell indented by a point force.¹⁹⁵

$$k_1 = \frac{\pi}{2} k_0 \frac{(\tau^2 - 1)^{\frac{1}{2}}}{\arctan h \left[(1 - \tau^{-2})^{\frac{1}{2}} \right]} \quad (1)$$

Here, $\tau = pR_l / k_0$ is a dimensionless parameter that compares the relative relevance of pressure p against the elastic constant of the unpressurized shell, k_0 , and R_l the internal

radius of the P22 EX, considered effectively as a sphere ($R_I = 29.1$ nm). Taking $k_0 = k_{EX} = 0.21$ N/m, $k_1 = k_{EGFP-EX} = k_{CeIB-EX} = 0.27$ N/m, and solving Eq. (1) for p , an estimate of 3 ± 1 MPa is obtained for the increase in internal pressure after cargo internalization, a value that was corroborated by Finite Element Simulations (Figure E.3B).

The osmotic pressure generated from a freely moving cargo constrained to the EX interior depends on the nature of the interactions between the cargo molecules and between the cargo and the inner capsid wall. The simplest influence stems from steric interactions between cargo molecules and the fact that they cannot escape from the capsid. The irregular geometry of the cargo, enhanced by the presence of the SP linker and the helix-loop-helix motif attached to it, makes accurate quantification difficult. Its magnitude can nonetheless be estimated by modeling the cargo molecules as uncharged hard spheres with an effective radius r_{HS} . In this approach, the expression for this “hard spheres” contribution to the osmotic pressure is^{196,197}
$$p_{HS} = k_B T \rho \frac{(1 + \eta + \eta^2 - \eta^3)}{(1 - \eta)^3} \quad (2)$$
 where $k_B = 1.38 \times 10^{-23} \text{ m}^2 \text{ kg s}^{-2}$, K is the Boltzmann constant, $T = 300$ K is the temperature, ρ is the number density of the cargo inside the particle, and $\eta = \frac{V_{CARGO}}{V_{EX}} = \frac{N \times 4/3\pi R_{HS}^3}{V_{EX}}$ is the packing fraction, defined as the volume occupied by the cargo divided by the available volume in the capsid (Tables E.1 and E.2). This assumption allowed us to evaluate the effective hard sphere radius (R_{HS}) needed to obtain the pressure values estimated in the experiments (Eq. 1).

Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) indicated that, on average, the mass of loaded particles decreased slightly after

expansion, as reported in previous studies.⁴⁶ In our case, loaded EX lost 20 CelB (16%) and 64 EGFP (30%) monomers in comparison with their PC forms. This loss of material was probably related to defective particles. Shell defects, such as missing pentamers, allow cargo molecules to leave the cavity, and thus only particles flawed or broken during thermal expansion would be the responsible for this loss of average mass measured by SEC-MALS. AFM permits topographic characterization of each EX particle and resolves capsomeric defects, thus allowing to select intact particles that have presumably not lost any cargo molecules. Therefore, we assumed that in our AFM experiments PC and EX capsids contained the same amount of monomers, and we used the PC values to calculate the different packing factors and the effective radii.

In the case of CelB-EX, in which $N=128\pm 1$ monomers were encapsulated per capsid and the estimated pressure was $p=3\pm 1$ MPa, the effective radius was $R_{HS}(\text{CelB})=7.8$ nm. For EGFP-EX, with $N = 220 \pm 5$ monomers encapsulated and the same pressure as CelB-EX, the effective radius was $R_{HS}(\text{EGFP})=3.9$ nm. Although we approached the irregular geometry of the cargos as hard spheres, the model reasonably predicted an effective radius appropriate to the size of the CelB tetramer ($10.1 \times 10.1 \times 5.7 \text{ nm}^3$)¹⁹⁸ and of the EGFP monomer ($4.8 \times 3.3 \times 3.5 \text{ nm}^3$)¹⁹⁹ (Table E.2). A second contribution to the pressure could arise from the electrostatic repulsion between the cargo molecules or/and the cargo and the shell. The magnitude of this influence could be estimated in two ways, by considering a solution of N effective charged spheres in an electrolyte⁷⁷ or through the concept of Donnan equilibrium.²⁰⁰ Both calculations led to theoretical pressure values that were negligible compared with the values obtained in our

experiments (Supporting Information). The electrostatic contribution of natural cargos such as dsDNA has been detected in natural viral cages by altering the ionic strength or using a condensing agent such as spermidine to screen DNA-DNA repulsion, which reduces virus rigidity.^{172,173,201} To assess the relevance of the electrostatic effects experimentally, we carried out AFM nanoindentations on loaded EX at low ionic strength and in the presence of spermidine (Figure E.5). In both cases, particle stiffness did not change, which supported the hypothesis used in theory that the electric nature of the cargo made no significant contribution (SI).

Conclusions

Our data indicate that the presence of a cargo stiffens the cages in two different ways. In the case of PC, when the cargo is linked to the shell through the C-terminal SP motif, the capsid is reinforced structurally. For EX capsids, however, cargo-shell interaction are lost and the increase in rigidity is due to the different concentration of osmolyte (CelB tetramers or EGFP monomers) between the inside of the capsid shell and the surroundings. This different concentration drives water molecules inside the particle creating an osmotic pressures of ~ 30 atm, a value comparable to the DNA-induced pressurization in natural *viruses* (40-60 atm for phi29,^{172,202} 20 atm for lambda,²⁰³ 10 atm for P22 phages,^{32,204} and 30 atm for human adenovirus¹⁷³). We also found that geometrical constraints imposed by the tetrameric structure of CelB cargo makes PC brittle.

Overall, our results show the critical interplay between the cargo and the shell contributes to the reinforcement of the capsid via different physical mechanisms. Understanding these mechanisms and their molecular determinants would permit the optimization of their performance and robustness in harsh environments and it is fundamental for their development as protective shields against proteases, desiccation and/or thermal destabilization.

Acknowledgements

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Experimental

Biochemical and Genetic Analyses

Proteins were cloned, expressed and purified, and analyzed by size exclusion chromatography with multi-angle light scattering and refractive index detection was performed as described.^{46,47,49} Plasmids containing the genes for coat protein and cargo proteins of interest fused to scaffold protein (EGFP-SP⁴⁶, CelB-SP⁴⁹) were transformed in BL21 (DE3) *Escherichia coli* (Novagen) for protein expression. The transformed *E. coli*

cells were grown to an $OD_{600} = 0.6$, induced with isopropyl b-D-thiogalactopyranoside (IPTG), and grown for an additional 4 h before cells were collected by centrifugation at 4,500 rpm. Cells were resuspended in PBS pH 7, lysed by sonication, centrifuged (at 12,000 rpm) to remove cell debris, and virus particles were isolated by ultracentrifugation through a sucrose cushion. The resuspended pellet was further purified on a Sephacryl (S-500; GE Healthcare) size exclusion column in PBS pH 7 (1 ml/min). For SEC-MALS analysis, samples were analyzed on a Dawn 8 instrument (Wyatt Technologies, Santa Barbara, CA). Samples were separated by Agilent 1200 HPLC on a WTC-0200S size exclusion column (Wyatt Technologies) and monitored with a UV-Vis detector (Agilent), a Wyatt HELEOS Multi Angle Laser Light Scattering (MALS) detector, a quasi-elastic light scattering detector (QELS), and an Optilab rEX differential refractometer (Wyatt Technologies). The Wyatt Astra 6 software determined average molecular weight (M_w) and radius of gyration (R_g) values.

Cryo-EM and Image Processing

Samples (5 ml) were applied to Quantifoil R 2/2 holey grids, blotted, and plunged into liquid ethane. Cryo-EM images were recorded in low-dose conditions with a FEI Eagle CCD camera in a Tecnai G2 electron microscope equipped with a field emission gun operating at 200 kV and at a detector magnification of 69,444X (2.16 Å/pixel sampling rate).

General image processing operations were performed using Xmipp,²⁰⁵ and graphics were produced using UCSF Chimera.²⁰⁶ The Xmipp automatic picking routine was used to select particles. Defocus was determined with CTFfind²⁰⁷ and CTF phase

oscillations were corrected in images by flipping them in the required lobes.

Homogeneous populations were selected by two-dimensional classification using the Xmipp CL2D reference-free clustering routine.²⁰⁸ Published structures of P22 PC and EX (PDB 2XYY and 2XYZ)¹⁷⁴ were filtered to 30 Å, size-scaled and used as initial models for their respective samples. The Xmipp iterative projection matching routine²⁰⁹ was used to determine and refine particle origin and orientation. For the CelB tetramer, an artificial noise model was used as starting reference for parallel iterative angular refinement using the EMAN program.²¹⁰ Once converged, the resulting model was selected and refined using the Xmipp iterative projection matching routine. For PC, EX and CelB, 90% of the particles were included in the final 3DR, and resolution was assessed by FSC between independent half dataset maps (Figure F.1). The final map of the CelB tetramer included 11,432 particles, and resolution for the 0.5 and 0.3 criteria was 16.4 and 14.5 Å, respectively. The UCSF Chimera fitting routine was used to dock the crystallographic models of PC, EX, SP and Pyrococcus b-glucosidase CelB (PDB 2XYY, 2XYZ, 2GP8 and 3APG, respectively) in the cryo-EM maps. The 3D reconstructions are deposited in the Electron Microscopy Data Bank (<http://www.ebi.ac.uk/pdbe/emdb>) with accession no. EMD-3171 (empty PC), EMD-3172 (EGFP-PC), EMD-3173 (CelB-PC), EMD-3174 (empty EX), EMD-3175 (EGFP-EX), EMD-3176 (CelB-EX) and EMD-3177 (CelB tetramer).

AFM

AFM experiments were performed as described,¹⁹³ using a Nanotec Electrónica microscope (Madrid, Spain) operating in jumping mode plus.²¹¹ Imaging forces were maintained below 150 pN. Rectangular silicon-nitride cantilevers (RC800PSA, Olympus, Center Valley, PA) with a nominal spring constant of 0.05 N/m were calibrated before each measurement by Sader's method.²¹² Experiments were carried out in standard buffer conditions (100 mM phosphate, 50 mM NaCl, pH 7) at a controlled temperature of 17°C. A 20 ml drop of diluted stock solution was incubated on previously silanized glass coverslips. Cleaning and functionalizing of glass surfaces was as described.¹⁶⁹ After 30 min, the sample was washed with buffer solution to a volume of 90 ml. AFM images were processed with WSxM software. AFM control experiments were performed in two conditions, at low ionic strength (50 mM phosphate, 25 mM NaCl, pH 7) or with 1 mM spermidine (100 mM phosphate, 50 mM NaCl, 1 mM spermidine, pH 7). Details of the procedure are found in the SI.

Nanoindentation was done at a loading rate of 60 nm/s with forward elongation of 100 nm. Force-vs-indentation curves (FIC) were obtained from force-vs-Z-piezo curves as reported.¹⁹³ The elastic constant was obtained by fitting the initial linear part of each FIC. Breaking force and critical indentation were measured with WsxM.^{65,213} A summary of the number of particle and average values of the mechanical properties analyzed are detailed in the SI.

CHAPTER EIGHT

TWO-DIMENSIONAL CRYSTALLIZATION OF P22 VIRUS-LIKE PARTICLES

Contribution of Authors and Co-Authors

Manuscript in Chapter 8

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Contributions: Carried out experiments, edited and help write the manuscript.

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Contributions: Assisted in experimental design, carried out experiments, edited and helped write the manuscript.

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Co-Author: Rajarshi Roychoudhury

Contributions: Assisted with protein production and purification.

Co-Author: Benjamin Schwarz

Contributions: Assisted in experimental design, carried out experiments, edited and help write the manuscript.

Co-Author: Dustin Patterson

Contributions: Obtained funding, coordinated the project, and assisted with the design of the experiments. Discussed the results and edited the manuscript at all stages.

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Contribution of Authors and Co-Authors (Continued)

Contributions: Obtained funding, coordinated the project, and assisted with the design of the experiments. Discussed the results and edited the manuscript at all stages.

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CHAPTER EIGHT

TWO-DIMENSIONAL CRYSTALLIZATION OF P22 VIRUS-LIKE PARTICLES

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Abstract

Virus like particles (VLPs) are well established platforms for constructing functional biomimetic materials. The VLP from the bacteriophage P22 can be used as a nano-container to sequester active enzymes, at high concentration, within its cavity through a process of directed self-assembly. Construction of ordered 2D assemblies of these catalytic VLPs can be envisioned as a functional membrane. To achieve this, it is important to establish methods to fabricate densely packed monolayers of VLPs. Highly ordered assemblies of P22 can also be utilized as a two-dimensional (2D) crystal for electron crystallography to get precise structural information of the VLP. Here we report 2D crystallization of different P22 morphologies: P22 procapsid (PC), enzyme encapsulated PC (β -glycosidase and enhanced green fluorescent protein), empty shell (PC without scaffold proteins, ES), the expanded form of P22 (EX), and enzyme encapsulated

EX (NADH oxidase). The 2D crystals of P22 VLPs were formed on a positively charged lipid monolayer at the water-air interface with a subphase containing 1% trehalose. A P22 solution, injected underneath the lipid monolayer, floated to the surface because of the density difference between the subphase and protein solution. The lipid monolayer, with adsorbed P22, was transferred to a holey carbon grid and was examined by electron microscopy. 2D crystals were obtained from a subphase containing 100 mM NaCl, 10 mM MES (pH 5.0) and 1% trehalose. The diffraction spots from the transferred film extended to the 6th order in negatively stained samples and the 10th order in cryo-electron microscopy samples.

Introduction

Virus like particles (VLPs) are well established platforms for constructing functional biomimetic materials. VLPs are robust protein cages composed of multiple copies of protein subunits. The VLP derived from the *Salmonella typhimurium* bacteriophage P22 adopts an approximately 58 nm T=7 icosahedral structure assembled from 420 copies of the 46 kDa coat protein (CP) and as many as 300 copies of the 34 kDa scaffolding protein (SP). The VLPs spontaneously assemble *in vivo* after heterologous co-expression of the two proteins. The SP can be truncated to an essential C-terminal helix-turn-helix domain, while still retaining assembly capabilities, allowing for genetic fusion of guest proteins to the SP which directs the self-assembly of the capsid and results in their encapsulation.^{45,182} The P22 VLP has thus been modified to encapsulate many copies of a wide range of active proteins within the cavity of the VLP capsid.⁴⁹

Initially the VLP adopts a spherical procapsid (PC) morphology but upon heating to 65 °C undergoes a structural transformation to an expanded form (EX), which closely resembles the expansion of the infectious virus during DNA packaging.^{45,83,184} In the case of encapsulated enzymes, the cavity can be used as a nano-size reaction chamber, able to undergo the same structural transformations, while the exterior surface of the VLPs can also be modified, using genetic or chemical means, to link capsids together potentially forming assemblies of coupled catalytic particles.²¹⁴

To achieve this multidimensional molecular engineering it is important to know the structure of the VLP precisely in order to implement molecular level design. The structure of the P22 procapsid has been investigated by single particle analysis, cryo-electron microscopy (cryo-EM)^{73,174,215,216} and while these structural models have provided a valuable foundation for engineering P22⁴⁷, a consistent atomic resolution structure has not yet been obtained.²¹⁷ Since VLP's have low contrast in cryo-EM, complimentary methods may be required to achieve high-resolution structural information. A related bacteriophage, HK97, was investigated by both cryo-EM²¹⁸ and X-ray crystallography^{127,219} resulting in an atomic resolution (3.44 Å) structure. One way to obtain crystallographic information of the P22 VLP is through the formation of 2D crystalline arrays because efforts at obtaining high quality 3D crystals of the VLP alone have been unsuccessful.

Additionally, the development of ordered 2D arrays and 2D crystals using VLPs as building blocks has gathered significant interest from the materials science community, because of the potential to make functional nanoparticle arrays from the

assembly of materials at length scales from the nano- to macroscale.^{183,220} We, and others, have successfully demonstrated encapsulation of various cargo molecules inside of the P22 to develop functional nanoparticles.^{46,47,49,51,108,129,214} Therefore developing methods to make and study 2D arrays of P22 VLP would pave the way to create functional P22 arrays.

To make 2D crystals of soluble proteins, or protein assemblies, a substrate with molecular level flatness is required. Freshly cleaved mica surfaces,^{221,222} silicon wafers²²³ and clean mercury surfaces^{224,225} have all previously been used as templates. In these cases, a lateral immersion force during the drying process from water²²⁶ is employed for assembling the protein arrays. Lipid monolayers,²²⁷⁻²³³ polypeptides,²³⁴ and denatured protein films²³⁵ formed at the water-air interface have all been used as crystallization templates. Nonspecific electrostatic interactions or specific ligand-receptor interactions have also been utilized to assemble proteins on the lipid monolayer. Recently, 2D crystallization of Cowpea mosaic virus (CPMV) and turnip yellow mosaic virus (TYMV) under lipid monolayers was reported^{108,236} where electrostatic interactions between CPMV and the positively charged lipid monolayer was utilized for assembling the particles. Assembly was monitored in situ by grazing-incidence small-angle X-ray scattering (GISAXS). Ordered 2D array formation was observed in a narrow pH range just above the CPMV isoelectric point.

Here we report two-dimensional (2D) crystallization of bacteriophage P22 procapsid for electron microscopy to gain structural insights and an understanding of the conditions needed to make 2D crystal arrays. We used a similar strategy for the 2D

crystallization of P22 VLPs under lipid monolayers. The lipid monolayers, with adsorbed P22, were transferred to TEM grids and observed by conventional TEM or cryo-EM. Regular hexagonally packed arrays of P22 VLPs were obtained under a narrow range of conditions and from these data we could extract structural information regarding the P22 capsids.

Results and Discussion

To check the appearance of the transferred lipid monolayers, 2, 3, and 4 μL of DMPC in chloroform (200 μM) were spread on the surface of the 177 mm^2 trough. The area density of DMPC molecules was calculated to be 0.8, 0.6, 0.4 $\text{nm}^2/\text{molecule}$, respectively. The monolayer at the water-air interface was transferred to holey carbon grids and examined by TEM (**Fig. 8.1**). At a density of 0.4 $\text{nm}^2/\text{molecule}$, monolayers were successfully transferred to the holey carbon grids and homogeneous films covering the holes were observed. On the other hand, at densities of 0.8 and 0.6 $\text{nm}^2/\text{molecule}$, most of the monolayers were ruptured and did not form uniform layers in the holes of the carbon support film. According to the π -A isotherm (surface tension vs. molecular area density) of DMPC at room temperature, a density of 0.4 $\text{nm}^2/\text{molecule}$ corresponds to the liquid-condensed phase and the lower density corresponds to the liquid-expanded

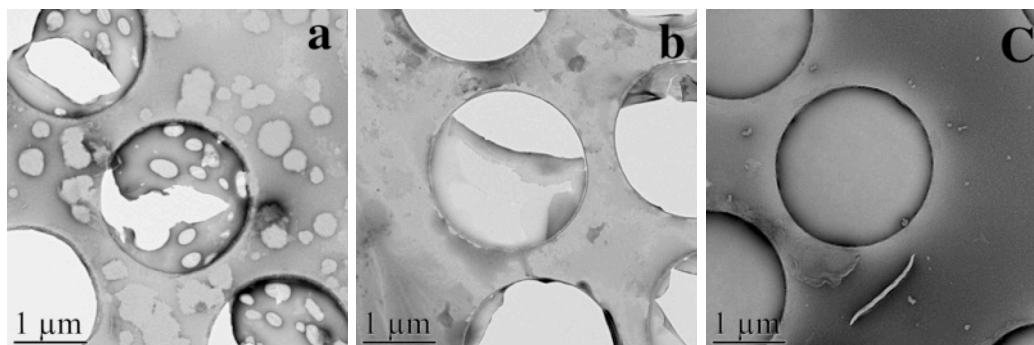


Fig. 8.1 TEM images of transferred DMPC monolayer. DMPC monolayers spread with an area density of (a) 0.8, (b) 0.6 and (c) 0.4 nm²/molecule were transferred to the holey carbon grids and stained by uranyl acetate.

phase.²³⁷ The density of 0.7 nm²/molecule, which corresponds to the liquid expanded phase, was used for CPMV crystallization.²³⁶ In that case, assembly of VLPs was monitored by GISAXS in situ with no need to transfer the monolayer. The liquid expanded phase is optimal because higher lateral diffusion is expected. However, because the transferred monolayer from the liquid-expanded phase showed inhomogeneous and fragile features in TEM, we used the liquid-condensed phase for these experiments. The lateral diffusion coefficient of the phospholipid monolayer in this phase is on the order of 10⁻¹⁴ m²/s,²³⁸ and this mobility would be enough to allow for rearrangement of neighboring VLPs in the crystallization process.

In the case of CPMV crystallization, mixtures of DMPC and DMTAP monolayers were used and the ordering was observed in a narrow pH range (pH 4.3 -5.0) just above the isoelectric point (pI) of CPMV (pI=4.3).²³⁶ On the other hand, ordering of CPMV was not sensitive to the positive charge density of the monolayer, and only the presence of DMTAP was required.²³⁶ Here we changed the pH of the subphase between 4.5 to 6.8 (at ionic strength of 100 mM NaCl) and also used mixtures of DMTAP and DMPC

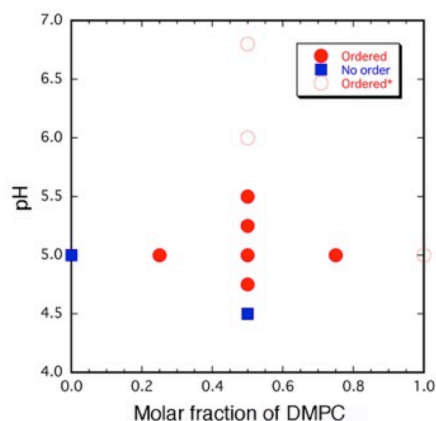


Fig. 8.2 Effect of subphase pH and molar fraction of the monolayer on P22 ordering. All the conditions contain 100 mM NaCl and 1% trehalose. Well ordered arrays (red closed circles), ordered arrays (closed red triangles), and no arrays (blue closed squares) were found under these conditions. The density of adsorbed VLPs on the lipid monolayer was small at pH 4.5 and also under monolayers without DMTAP.

monolayers. Ordered arrays (the arrays which have diffraction spots extending past the 3rd order) were observed above pH 4.75 and particularly well-ordered arrays (the arrays which have diffraction spots extending past the 5th order) were found in the range of pH 4.75 to pH 5.5 (**Fig. 8.2**). The pI of the assembled P22 PC is not known experimentally but the calculated pI value, from the amino acid sequence of the coat protein, is 4.97. This is higher than the pI of CPMV, and therefore it is reasonable that the optimum pH for the P22 crystallization was higher than for CPMV. In the case of CPMV crystallization, a series of sharp Bragg rods were observed in GISAXS, arising from the ordered hexagonal packing of the virus particles under low ionic strength and narrow range of pH conditions (pH 4.4- 5.0).²³⁶ The Bragg rods diffused into circular ring-like features upon increasing the subphase pH. This indicated that there was a reduction of the

crystal domain size or ordering at higher pH conditions. In our case, the transferred P22 PC also showed less ordering at higher pH conditions.

The content of DMTAP and DMPC in the monolayer was also varied and ordered arrays were obtained only in the presence of DMTAP. The DMPC monolayer, without DMTAP, adsorbed small amounts of P22 VLPs and did not form arrays. Ordered arrays were found in the pure DMTAP monolayer, however this film was fragile and less of the total monolayer area was successfully transferred to the holey carbon grids. In conclusion, the quantity of the surface charge density is not crucial for the crystallization and the lipid monolayer merely required a positive charge to adsorb the virus particles.

The NaCl concentration in the subphase was adjusted in the range from 0, 50, 100, to 200 mM at pH 5.0. Well-ordered arrays were found with a NaCl concentration of 100 mM, whereas ordered arrays were not observed at NaCl concentrations of 0 and 200 mM. In previous studies on protein 2D crystallization on a lipid monolayer,²²⁸⁻²³³ relatively high ionic strength (50 mM to 300 mM) or the presence of a divalent cation was needed. Substantial screening of the electrostatic inter-particle repulsion would be required for the 2D crystallization. The outer shell of P22 is constructed from 420 subunits of CP that contains many basic and acidic amino acids. While the VLPs have a negative net charge at a pH higher than the pI, the VLPs also have local regions of effective positive and negative charges. Ordering of particles is strongly influenced by the distribution of the charges that are involved in the inter-particle interactions.²³⁹ To control the inter-particle interactions, adjustment of pH and ionic strength for specific particles is required. The ideal conditions for P22 were found to have a salt concentration

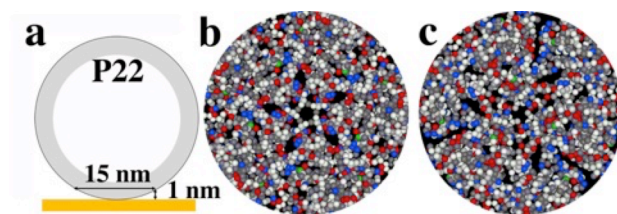


Fig. 8.3 Distribution of acidic and basic amino acids on the outer surface of P22. (a) Schematic presentation of P22 placed on a flat lipid monolayer. The surface of the sphere will have a 1 nm elevation from the monolayer at the point 7.5 nm from the contact center. (b) Distribution of acidic amino acids (red), basic amino acids (blue), histidine (green), and others (gray) around the 5-fold symmetry axis with 15 nm diameter. (c) Amino acid distribution around the 3-fold symmetry axis. The ball model was generated by 2XYY structure from the Protein Data Bank (PDB) showing only the α -carbons of the protein.

of 100 mM at pH 5.0. On the other hand, adsorption of VLPs would be regulated by the average charge. The calculated Debye screening length is about 1 nm under 100 mM salt concentration. If we assume a 58 nm VLP sphere placed on a flat substrate (lipid monolayer), then a 1 nm elevation of the sphere surface can be obtained at a 7.5 nm distance from the center of the contact (**Fig. 8.3a**). Therefore, an area derived from a 15 nm diameter on the VLP surface is considered to have an interaction with the lipid monolayer surface. Within this 15 nm diameter cross section of the VLP's outer surface around the 5-fold symmetry (**Fig. 8.3b**) and the 3-fold symmetry (**Fig. 8.3c**) axes, the number of acidic amino acids at each area is 80 and 60, respectively, whereas the number of basic amino acids is 50 and 36, respectively. Therefore both areas likely have a net

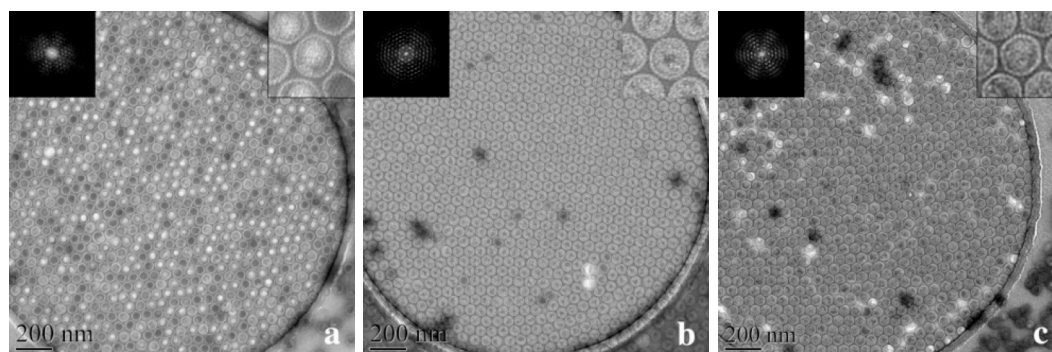


Fig. 8.4 TEM images of transferred 2D arrays stained with uranyl acetate. (a) PC with full-length scaffold protein. (b) CelB incorporated PC. (c) EGFP incorporated PC. Left insets present the Fourier power spectrum from the central area of each image. Right inset presents a magnified image of the individual particles.

negative charge and would determine the interaction between the VLP and lipid monolayer.

An EM image of a transferred film of P22 PC is shown in **Fig. 8.4a**. The array exhibited a well-ordered hexagonal lattice with diffraction spots (from the Fourier transform) extending to the 6th order. The particle-particle distance was found to be 62 nm. The diameter of P22 CP is known to be 58 nm, therefore the gap between particles is calculated to be 4 nm. P22 VLPs are large, thus some distortion or collapse of the particle is expected during the drying and staining process and from the images it can be seen that the central part of the VLPs was not uniform.

P22 PC, with encapsulated CelB or EGFP, also formed well-ordered hexagonal arrays with diffraction spots extending to the 8th and 6th order, respectively (**Fig. 8.4b, c**). The hexagonal lattice constant was the same as PC. Stained images of individual VLPs (with cargo) in each array were much more uniform compared to the PC (without cargo), likely because the high density of incorporated proteins is expected to

mechanically reinforce the PC structure. About 2-3 % of mis-folded particles or empty particles were found distributed in the array. Despite repeated efforts at purification, it was difficult to eliminate these particles completely from the preparation, even using density gradient ultracentrifugation.

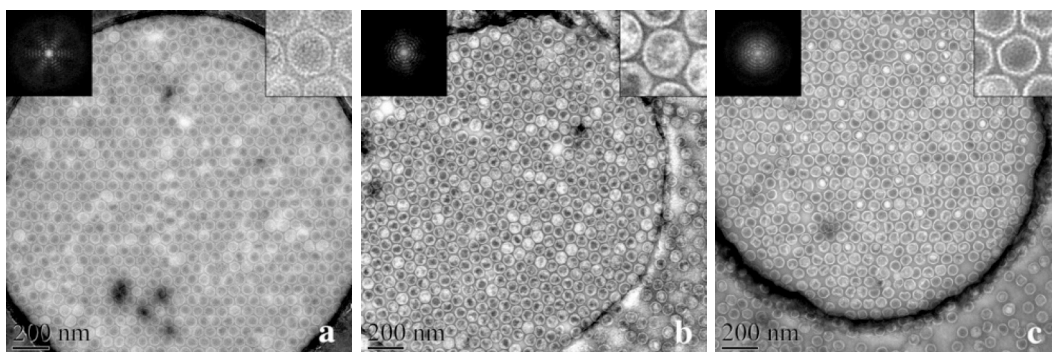


Fig. 8.5 TEM images of transferred 2D arrays stained with uranyl acetate. (a) EX form of P22. (b) NOX incorporated EX form of P22. (c) ES of P22. Left insets present the Fourier power spectrum from the central area of each image. Right inset presents a magnified image of the individual particles.

The expanded (EX) morphology of P22 VLPs also formed ordered arrays under the same subphase conditions. The particle-particle distance was found to be 66 nm with diffraction spots extending to the 6th order (**Fig. 8.5a**). Since the diameter of the EX form is known to increase to 62 nm, a particle-particle distance of 66 nm is reasonable. In addition, the EX structure of P22 VLP with NADH oxidase (NOX) encapsulated in the interior cavity also formed ordered arrays (**Fig. 8.5b**). The particle-particle distance was 68 nm with diffraction spots extending to the 4th order (**Fig. 8.5b**). P22 particles with encapsulated enzymes formed ordered arrays from both the procapsid and expanded

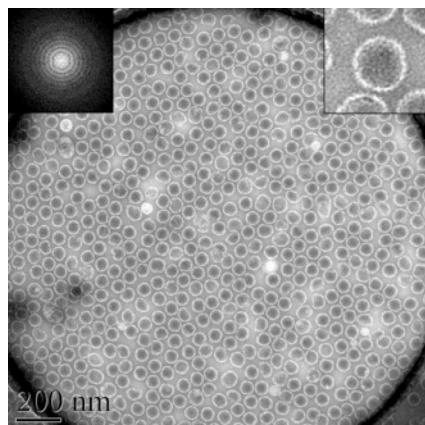


Fig. 8.6 TEM image of transferred 2D array of the wiffle ball (WB) structure of P22, stained with uranyl acetate. Left insets present the Fourier power spectrum from the central area of each image. Right inset presents a magnified image of the individual particles.

forms. This suggests that the enzymes, encapsulated within the P22 capsid, do not affect the ability of the P22 to form arrays under the same conditions as mentioned in **Fig. 8.2**.

The empty shell (ES) form of P22 was also investigated for 2D crystallization. The ES has the same morphology as the PC but lacks any scaffold protein on the interior. The ES also formed hexagonal array from the same subphase conditions as PC (**Fig. 8.5c**). However, compared to the other structures, individual particles in the stained sample were heavily deformed. In the ES form, the capsid shell is comprised of only the coat protein without any supporting interior cargo. Particles without interior cargo seem to be mechanically weaker under the drying and staining conditions, and this is consistent with the mechanical properties of ES in comparison with PC with cargo encapsulated, assessed by AFM (data not shown).¹³⁰ The particle-particle distance for ES arrays was found to be 69 nm with diffraction spots extending to the 4th order.

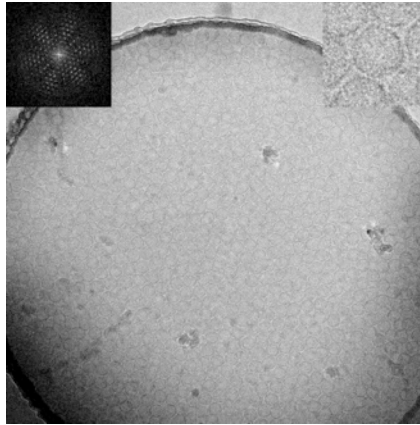


Fig. 8.7 The cryo-EM transferred 2D array of PC form of P22. Left insets present the Fourier power spectrum from the central area of each image. Right inset presents a magnified image of the individual particles.

Interestingly, the wiffle ball (WB) morphology, where the capsid has lost the 12 pentameric vertices and has large 10 nm pores at these sites, did not make ordered arrays under the same conditions (**Fig. 8.6**). The WB P22 gathered to make small clusters of hexagonal packing but did not grow into long range ordered arrays under the conditions listed in **Fig. 8.2**. Since the key difference between WB and the other morphologies examined is the absence of the pentons, it appears that these vertices play an important role in fixing the orientation of the other morphological forms of the P22 VLPs.

To obtain more detailed structural information from the ordered P22 arrays, samples were examined by cryo-electron microscopy. The transferred specimen of the PC form of P22 VLP was rapidly frozen in liquid ethane and studied by cryo-EM.

A cryo-EM image is shown in **Fig. 8.7**. From the hexagonal lattice, diffraction spots extended to the 10th order and gave a particle-particle distance of 62 nm. No

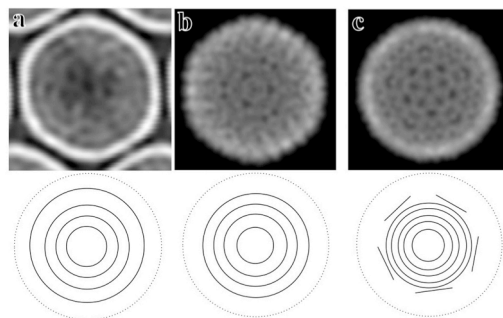


Fig. 8.8 Reconstructed images. (a) Cryo-EM of PC reconstructed from 2D crystal, (b) projection image along the icosahedral 3-fold axis calculated from PDB file 2XYY of P22, (c) projection image view along the icosahedral 5-fold axis. The concentric rings below the reconstructed images show the arrangement of the lower density areas of each image. The outermost ring represents the exterior surface of each particle. The concentric ring pattern of (a) resembles that of (b), implying the PC is arranged on a 3-fold axis perpendicular to the array plane.

heavily deformed individual VLPs were seen in the micrographs and therefore it is likely that the deformed VLPs found in stained images were an artifact of the staining procedure. However, the extended lattice in **Fig. 8.7** was deformed at the periphery, thus further trials will be required to correct this.

The reconstructed image of PC (cryo-EM) at 5 nm resolution is shown in **Fig. 8.8a**. The lower density areas (holes) can be seen at the center of the particle and are also concentrically distributed around the center. The projection images of P22 procapsid viewed from the icosahedral threefold symmetry axis and the pentameric vertex were generated using 2XYY structure from the Protein Data Bank (PDB) and are shown in

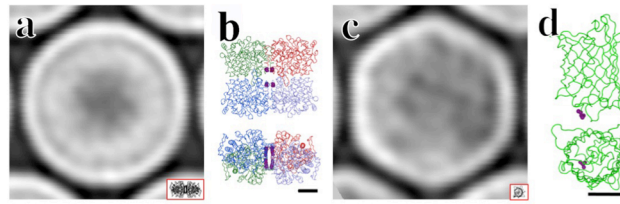


Fig. 8.9 Reconstructed images of enzyme incorporated PC and molecular models of enzymes. (a) Reconstructed image of CelB incorporated PC, (b) top view (upper) and side view (lower) of the tetrameric CelB molecular model with the C-termini highlighted in purple, (c) reconstructed image of EGFP incorporated PC, and (d) side view (upper) and top view (lower) of the molecular model of EGFP with the C-termini highlighted in purple. Scale bars are 2nm in (b) and (d). Insets in (a) and (c) showing the CelB and EGFP molecular models at the same magnification as the reconstructed images.

Fig. 8.8b and **8.8c**. The orientation of the particle was not clear at this resolution however the arrangement of the holes was similar to the view from the threefold axis.

The reconstructed images of CelB and EGFP incorporated within the PC (stained) at 7 nm resolution are shown in **Fig. 8.9a** and **8.9c**. The C terminal domain of the SP forms a helix-loop-helix motif and binds tightly to the CP,¹⁷⁴ however the structure of N terminal domain of the SP is not defined. This domain is considered to be flexible,¹⁹⁴ thus the orientation of the fused enzymes in the cavity cannot be defined.

The reconstructed image of PC-CelB reveals a dense area at the interior lumen of the capsid with a less dense region in the center of the capsid (**Fig. 8.9a**). The molecular weight of the CelB capsid measured by MALS was 29 MDa. This corresponds to 128 CelB monomers incorporated within the capsid. CelB is known to form a tetramer, thus 32 tetramers are expected to be in the capsid. The structure of the PC inner surface is not clear, therefore we assumed that the radius of the inner surface with the truncated SP is

25 nm by measuring the rigid area (well ordered area) of the reconstructed image presented by Chen et.al.¹⁷⁴ The molecular models of the tetrameric form of CelB using 3APG of PDB are shown in **Fig. 8.9b**. The tetramer is roughly a $11 \times 9 \text{ nm}^2$ rectangle with a depth of 7 nm. The C-termini of the CelB molecules are located at the center of the tetramer (**Fig. 8.9b**) and these parts are connected to the N-termini of the SPs. The structure of the junction between SPs and the enzymes is not defined, therefore we assume the $11 \times 9 \text{ nm}^2$ base of the tetramer faces towards the inner surface of the PC shell, then the radius of the inner shell will be reduced to 18 nm when CelB binds to the inner surface. Thus the area of innermost surface of the P22 cavity is calculated to be about 4100 nm^2 , and 41 tetramers can be incorporated in the cavity. Even if the CelB tetramer binds to the PC surface by the other side base, the possible number of protein molecules is almost the same. Regardless, the calculated number is larger than the experimentally estimated number of the incorporated CelB tetramers. This means there will be some free space where the CelB tetramers are not bound. If the distribution of the CelB is not uniform, and not located around the threefold symmetry axes, then the density at the center of the reconstructed image becomes diminished in the 2D arrays.

In contrast, reconstructed image of PC-EGFP (**Fig. 8.9c**) showed a dense area at the center of the reconstructed image. The molecular weight of PC-EGFP measured by MALS was 30 MDa, corresponding to about 220 monomers in the capsid cavity. The molecular models of EGFP calculated from 2YOG of PDB (**Fig. 8.9d**) shows that the C-terminus of EGFP is located at the base of the cylindrical form. This molecule will likely orient itself with the cylindrical side vertical to the inner surface of P22. The height of the

cylinder is about 5 nm, thus the innermost radius of the EGFP bound PC is estimated at 20 nm. Consequently the surface area is calculated to be about 5000 nm^2 . Since the radius of cylinder is about 1.8 nm, about 450 molecules can be incorporated in the cavity if the cylinder base forms a hexagonal packing on the surface of 20 nm radius spherical surface. There will be much more free space in the EGFP packing than the CelB, and the cargo molecules are expected to be distributed evenly around the three fold symmetry axes. Therefore the distribution of the EGFP molecules should be different from the distribution of the CelB molecules.

Conclusions

2D crystals of P22 VLPs were successfully obtained on a positively charged lipid monolayer at the water-air interface regardless of the cargo encapsulated inside of P22, and the morphology of the P22 VLPs, procapsid, expanded, or empty shell. Individual particles in an array were more uniform if the P22 VLPs had encapsulated cargo molecules of CelB or EGFP, likely because these VLPs are mechanically more robust. By analyzing the stained sample images, the difference of the encapsulated protein distribution could be visualized. Cryo-electron microscopy revealed the PC structure at 5 nm resolution, however this can be improved because these cryo-electron microscopy experiments are still in their preliminary stages. To increase the resolution we need to improve the crystallinity of the 2D arrays and take hundreds of electron micrographs. We are also limited by the pixel size of the CCD camera. In our case, the pixel size was $1916 \text{ nm}/4096 \text{ pixels} = 0.46 \text{ nm/pixel}$. The highest resolution in our case is $0.46 \times 2 = 0.92 \text{ nm}$.

Therefore to obtain atomic resolution (0.3 nm) we need a CCD camera with three times smaller pixel size. The combination of these techniques could produce higher resolution structure of the P22 VLP using the approaches outlined above. This will aid in the synthetic efforts towards using this VLP as a biomaterial. Similar strategies could be employed for other VLPs that are hard to crystallize.

Acknowledgements

We are grateful to Dr. Barry Stein and Dr. David G. Morgan of Indiana University Electron Microscopy Center for useful suggestions in electron microscopy. This work was supported by grants from the National Science Foundation (NSF-BMAT DMR-1507282) and the JSPS KAKENHI (Grant Number 25390020).

Experimental

Materials

The zwitterionic lipid 1,2-dimyristoyl-sn-glycero-3-phospho-choline (DMPC) and cationic lipid 1, 2-dimyristoyl-3-trimethyl-ammonium-propane (DMTAP) were purchased from Avanti Polar Lipids, Inc. All other chemical reagents used in this study were analytical grade and purchased from either Sigma-Aldrich or Fisher Scientific and used as received. All water was deionized using a Milli-Q Advantage water purification system (EMD Millipore).

Expression and Purification of P22 Capsid

The procapsid (PC) form of P22 VLP was produced by co-expression of the coat protein (CP) and scaffold protein (SP) in *E. coli* and purified as previously described.⁵¹ The CelB (a homotetrameric β -glycosidase enzyme) and NADH oxidase (NOX) genes from the hyperthermophile *Pyrococcus furiosus* were fused to the truncated SP (residue 141-303) gene and expressed in *E. coli* as previously described.^{49,51} Enhanced green fluorescent protein (EGFP) was also fused to the truncated SP with the same procedure.⁴⁶ The molecular weight of PC-CelB and PC-EGFP incorporated were measured by Multi Angle Light Scattering (MALS) using a Wyatt HELEOS detector. The expanded (EX) form of P22 was prepared by heating the PC at 65 °C for 20 min as previously described.¹⁸⁴ The wiffle ball (WB) form of P22, in which 12 pentons of EX are lost from the capsid, was prepared by heating the PC at 75 °C for 20 min. The empty procapsid shells (ES) were prepared by repeated extraction of SP from PC with GuHCl (0.5 M) at 4 °C.²⁴⁰ The P22 EX, WB and ES samples were centrifuged at 45,000 rpm for 50 minutes, and the pellet was resuspended in a phosphate buffer (Sodium phosphate 50mM, Sodium chloride 100mM pH 7.0). The sample was further purified by CsCl (1.2 g/cm³-1.4 g/cm³) or sucrose (5%-20%) density gradient centrifugation.²⁴¹

Electron Microscopy

Holey carbon grids with 2 μ m holes (CF405, C-flat carbon grids) were purchased from Electron Microscopy Sciences and were reinforced by coating carbon from the backside. The lipid monolayer was transferred to a holey carbon grid by attaching and detaching the grids from the interface. The transferred specimen was immediately stained

with 2 % uranyl acetate or vitrified by rapid freezing using Vitrobot (FEI). Stained samples were examined with a JEM 1010 (JEOL Ltd.) at an acceleration voltage of 80 kV. The Cryo-EM was performed with a JEM 3200FS at an acceleration voltage of 300 kV with an energy slit of 30 eV on the in-column energy filter. The cryo-transfer holder (Gatan 626) at liquid nitrogen temperature was used for imaging. Images were recorded on a Gatan Ultrascan 4000 CCD camera under minimal-dose conditions (maximum 20 e/A^{o^2}) at a nominal magnification of 25,000.

2D Crystallization

A Teflon trough with 15 mm diameter and 3 mm depth was used for crystallization. The trough was filled with 500 μ L of subphase solution containing buffer (2-(N-morpholino) ethanesulfonic acid, MES), sodium chloride and trehalose (or glucose). A mixture of zwitterionic lipid DMPC and cationic lipid DMTAP in chloroform was spread on a subphase. One hour after lipid spreading, 12 μ g (3 μ L of 4 mg/mL solution) of P22 VLP solution was injected beneath the lipid monolayer. This is about 3 times larger than the amount of VLPs needed to cover the 177 mm² surface of the trough in a close packed state. The protein solution floated to the surface because the density of the subphase was larger than the protein solution. Initially the protein solution localized just under the lipid monolayer but eventually diffused into the subphase to give a uniform solution of 0.024 mg/mL. After 15 hours, the lipid monolayer with adsorbed P22 was transferred to a holey carbon grid and was stained by uranyl acetate or vitrified in liquid ethane.

Image Reconstruction

The 2D crystal images were processed using the software “2dx” (<http://www.2dx.unibas.ch/>).²⁴² The alignment of the crystal lattice was refined using the “Unbending” process in the 2dx software. Reconstructed molecular images were obtained from the improved diffraction spots by an inverse FFT process.

CHAPTER NINE

BACTERIOPHAGE P22 IS A LEAKY CONTAINER FOR SCAFFOLDING
PROTEINS

Contribution of Authors and Co-Authors

Manuscript in Chapter 9

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Contributions: Designed studies and carried out the experiments and wrote the manuscript.

Co-Author: Ethan Edwards

Contributions: Designed studies, carried out experiments, edited and co-wrote the manuscript.

Co-Author: Ranjit Koliyatt

Contributions: Assisted in purification of the scaffold protein and assisted in the multi-angle light scattering measurements.

Co-Author: Trevor Douglas

Contributions: Obtained funding, coordinated the project, and assisted with the design of the experiments. Discussed the results and edited the manuscript at all stages.

Co-Author: Martin Jarrold

Contributions: Obtained funding, coordinated the project, and assisted with the design of the experiments. Discussed the results and edited the manuscript at all stages.

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CHAPTER NINE

BACTERIOPHAGE P22 IS A LEAKY CONTAINER FOR SCAFFOLDING PROTEINS

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Abstract

Bacteriophage P22 is a widely studied tailed, double-stranded DNA phage. The T=7 capsid of bacteriophage P22 can be assembled as a virus-like particle (VLP) with a consisting of 420 copies of the coat protein and a distribution of hundreds of scaffolding proteins (SPs), giving it a total mass ranging from 20 to 30 MDa. The VLP is morphologically versatile. It can be chemically converted to an empty shell form (ES) and thermally converted to an expanded form (EX) or a ‘wiffle ball’ form (WB), where the twelve five-fold vertices of the capsid are ejected. The VLP is stable under a wide range of conditions, and its internal cavity is large enough to incorporate hundreds of proteins as cargo. P22 VLPs are therefore frequently used in nano-engineering applications. Here, we investigate the long-term stability of P22 VLPs using charge detection mass spectrometry (CDMS), a single-particle technique which is ideally suited for measuring the masses of extremely heavy, heterogeneous ions. Over the course of

weeks, we observed gradual mass loss corresponding to initially hundreds of SPs per VLP leaking out to final numbers of 15-34 SPs per VLP. After successfully re-stuffing VLPs with an excess of SPs, significant mass loss occurred once again over several weeks. These results are consistent with degradation and aggregation of SPs, which continuously removes SPs VLPs from the equilibrium between free and bound SPs until only a tightly bound population of SPs remain.

Introduction

Bacteriophage P22 is a Salmonella-infecting, widely studied member of the most common class of all viruses: tailed, double-stranded DNA phages.⁷³ Before the mature, DNA-containing phage forms, P22 first assembles into a procapsid.⁷³ The procapsid is a T=7 icosahedral capsid (the protein shell of a virus) consisting of 415, coat proteins, a portal protein complex, multiple copies of several ejection proteins, and typically several hundred copies of the assembly chaperone scaffolding protein (SP). The procapsid contains only 415 coat proteins—rather than the expected 420 coat proteins for a T=7 capsid—because the portal complex takes the place of one of the twelve, five-fold vertices of the capsid. As DNA is actively packaged, the capsid expands, increasing its diameter by about 10%, and SPs exit through pores in the capsid.²⁴³

When only coat protein and scaffolding protein are present, P22 forms virus-like particles (VLPs) with high fidelity.²⁴⁰ VLPs are T=7 capsids consisting of 420, 46,621-Da coat proteins and a distribution of several hundred 33,565-Da SPs. SPs can be removed from the VLPs by washing with the chaotrope guanidine hydrochloride

(GuHCl)²⁴⁰ creating an empty shell (ES) or heating to about 65 °C.¹⁸⁴ The heating process also causes the capsid to expand into the expanded shell (EX), similar to the morphological change that occurs when DNA is packaged. Further heating to about 75 °C causes the twelve pentons of the VLP to eject,⁸³ leaving a stable capsid referred to as a wiffle ball (WB) with regularly spaced, 10-nm holes.

P22 VLPs are commonly used for nano-engineering applications—in which enzymes, imaging agents, or reactive centers are incorporated into the protein shells—due to their morphological versatility, stability, and size.^{44,46,49,50,108,175} The VLPs can adopt three separate morphologies as described above, each of which might be used to serve particular purposes. Even the WB form of P22 VLPs can survive diverse biological conditions, demonstrated by the uses of WBs as potential MRI contrast conjugates¹⁰⁸ and drug-delivery platforms.¹⁷⁵ Finally, as ~55 nm, hollow shells, P22 VLPs have ample internal volume to hold hundreds of fluorescent proteins⁴⁶ or enzymes.⁴⁹ One way to incorporate these foreign cargos is to fuse them onto truncated SPs,^{46,49,50} in which case the cargo is naturally incorporated during VLP assembly. There are several advantages to this method: there is little chance of negatively affecting assembly since the native coat protein and coat protein binding site of the SP can be used, and synthesis of the nanoparticles is simplified since there is no need for linkers or affinity tags. However, in some applications the method would only be useful if the SPs are stable inside the VLPs over long periods. It has previously been reported that SPs can remain bound to VLPs for months,²⁴⁴ though little evidence was presented to support that statement. Here, to evaluate that long-term stability, we use charge detection mass spectrometry (CDMS)²⁴⁵⁻

²⁴⁸ to track the masses of VLPs containing wild type SPs over the course of several weeks.

CDMS is a single-particle technique where the mass of each ion is obtained by multiplying its mass-to-charge ratio (m/z) and charge, which are measured simultaneously. Compared to conventional MS, where the mass is obtained by assigning charge states to peaks in an m/z spectrum of an ensemble of ions, CDMS can be used to analyze much more massive and heterogeneous species for which charge state peaks are not resolved. These advantages come at the cost of speed, since thousands of ions must be measured one at a time. An empty P22 VLP weighs 19.58 MDa, and with SPs they typically weigh 20-30 MDa. Moreover, VLPs typically have a mass distribution that is tens of SPs wide.²⁴⁹ Ions of all of those different masses would have overlapping charge state peaks. Consequently, current conventional MS could not possibly make these measurements. CDMS, however, is well-suited to make them. CDMS is one of several single-particle techniques, which might be capable of making these measurements.²⁵⁰

Results and Discussion

Freshly purified P22 VLPs were divided into two samples sets that were transferred into 25 mM ammonium acetate and 150 mM ammonium acetate. The total protein concentration in each sample was 1 mg/mL and both samples were immediately analyzed by CDMS. Half of each sample was stored at room temperature, and the other half was stored at 4 °C. All four samples (two ionic strengths at two temperatures each) were measured by CDMS multiple times over the course of about six weeks. Our mass

measurements are typically $\sim 1\%$ above the theoretical mass²⁵⁰ because of incomplete desolvation and salt adducts.^{201,251} Therefore, all mass measurements reported here have been reduced by 1% to obtain better estimates of the true number of scaffolding proteins (SPs) per VLP.

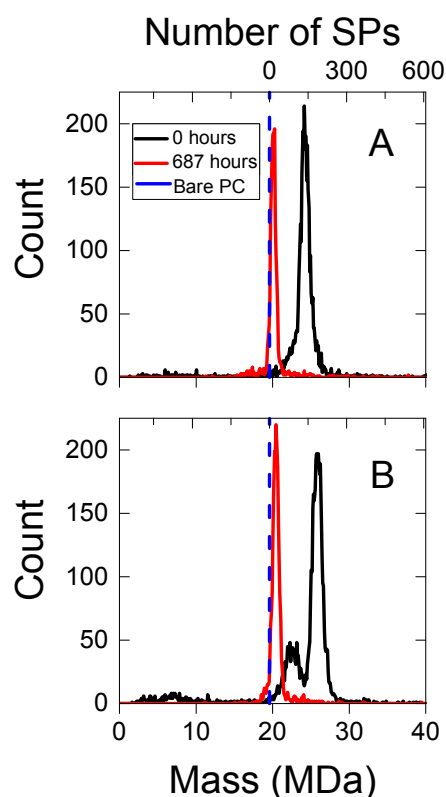
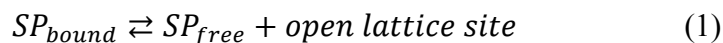


Figure 9.1 Representative mass spectra of P22 VLPs held at room temperature at initial time point (black curves) and nearly a month later (red curve). Blue dashed lines show the mass of an empty VLP consisting of 420 coat proteins and no SPs. A) Samples kept in 25 mM ammonium acetate (AA). B) Samples kept in 150 mM AA.

Figure 9.1 shows representative mass spectra from this experiment after adjusting the masses by 1%. The black curves in Figure 10.1A and B are the mass spectra measured immediately after purification of the samples held at 25 mM and 150 mM salt, respectively. The average molecular weights measured by CDMS were 24.5 MDa at 25

mM salt and 26.1 MDa at 150 mM salt, corresponding to VLPs with 139 and 186 SPs, respectively. This data correlates to multi-angle light scattering (MALS), a solution-phase technique for measuring molecular weights of these particles (Figure 9.2, traces labeled ‘As Purified’). The molecular weights measured by MALS were 23.5 +/- 0.1 MDa and 26.8 +/- 0.1 MDa for 25 mM and 150 mM, respectively. In general, the CDMS measurements should be considered more accurate, but the MALS data confirms that the VLPs at 150 mM are initially at higher mass even in solution. In other words, the transfer to the gas phase for CDMS is not the cause of the difference in masses. The red curves are mass spectra measured after 687 hours (just under a month) for samples kept at room temperature. The dashed blue line is at the mass of an empty VLP: a P22 particle of exactly 420 coat proteins and no SPs. All the measured spectra are at higher masses than that because the actual VLPs consist of 420 coat proteins plus distributions of SPs. The 687 hour stabilized samples showed similar masses by MALS, indicating a distribution of SPs that remain in the particles (Figure 9.2, traces labeled ‘As Purified Stabilized’). The peaks measured after a month show a loss of several MDa, representing a substantial loss of SPs over time. The reaction, which only involves breaking non-covalent bonds, is given by,



where SP bound represents an SP bound to a coat protein in a VLP, SP free represents an SP which is unbound and therefore free in solution, and open lattice site represents an empty binding site of SP to a VLP.

The black curve in Figure 9.1A is made of a single peak, while the black curve in Figure 9.1B clearly consists of two peaks which indicates a bimodal distribution of encapsidated SPs. Both samples came from the same cell culture at the same time and

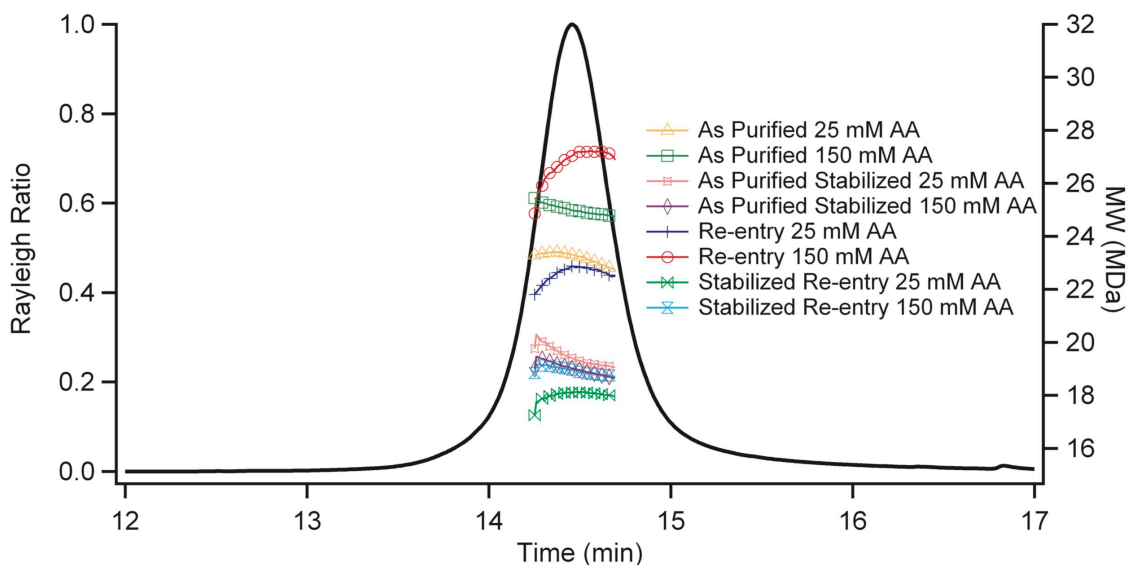


Figure 9.2 Multi-angle light scattering (MALS) plot verifying the mass loss of the samples by a solution-phase technique. The MALS data correlates well with the CDMS data. Samples are as follows: As purified 25 mM AA (orange triangles), As purified 150 mM AA (green squares), As purified stabilized 25 mM AA (peach stars), As purified stabilized 150 mM AA (purple diamonds), Re-entry 25 mM AA (blue lines), Re-entry 150 mM AA (red circles), Stabilized Re-entry 25 mM AA (green sideways hourglass), Stabilized Re-entry 150 mM AA (cyan hourglass).

were only separated during purification. The coat protein:SP interaction is electrostatic,²⁵² so the binding of SP to the capsid is not as strong at 150 mM salt as at 25 mM. Therefore, some SPs were likely removed from a subpopulation of the VLPs at 150 mM salt during purification because of this weaker binding. The lower-mass peak had essentially disappeared by the next measurement about 13 hours later, so it perhaps arose from an

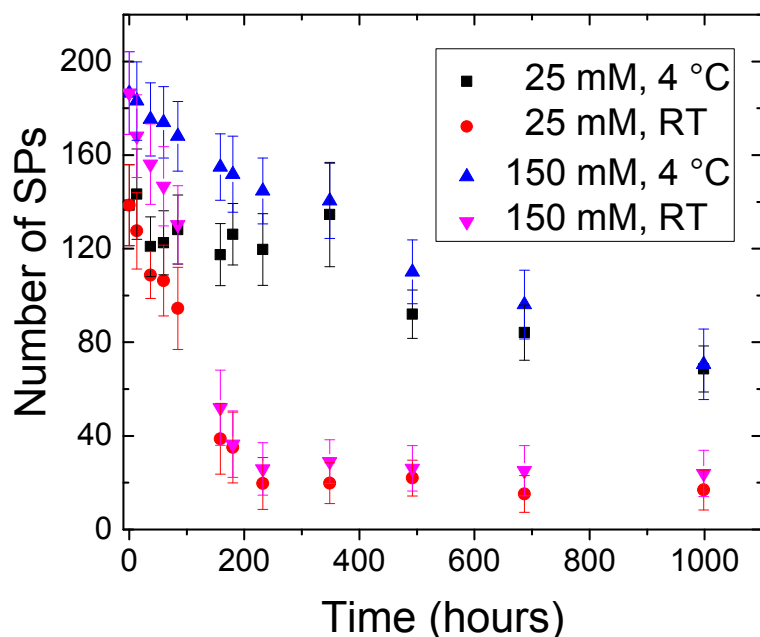


Figure 9.3 Plots of average number of SPs per VLP as a function of time for samples kept in 25 mM ammonium acetate at 4 °C (black squares) and at room temperature (red circles) and for samples kept in 150 mM ammonium acetate at 4 °C (blue triangles) and at room temperature (upside-down pink triangles). Bars represent width of the measured mass peaks from distribution of SPs (W_{IH} in equation 2).

unstable subpopulation of VLPs that fell apart or precipitated out of solution within half a day.

Figure 9.3 is a plot of average number of SPs per VLP over time for all four samples (both temperatures and both ionic strengths). The number of SPs was calculated by subtracting the mass of an empty VLP from the 1%-adjusted, average measured mass and dividing by the mass of an SP. Measured masses were determined by fitting a Gaussian to the peak in the mass spectrum. The center of the Gaussian was used as the average mass. The bars on Figure 9.3 show the widths of the SP distributions in the VLPs, calculated by equation 2. This equation implies that the measured width of a peak

comes from a combination of finite instrumental resolution, distributions of solvent and salt adducts, and intrinsic heterogeneity from distributions of SPs.

$$W_{IH} = \sqrt{W_{MEAS}^2 - W_{RES}^2 - W_{ADD}^2} \quad (2)$$

In equation 2, W_{IH} is the root-mean-square deviation from the mean (RMSD) of the intrinsic mass distribution from SPs (the full width at half maximum divided by 2.35482), W_{MEAS} is the RMSD of the Gaussian fit to the measured peak, W_{RES} is the RMSD from the mass resolution, and W_{ADD} is the RMSD due to adduct formation. W_{RES} and W_{ADD} were calculated as previously described.²⁵⁰

Figure 9.3 clearly shows that all VLPs lose SPs over time. The VLPs in 150 mM ammonium acetate start with an average of 186 SPs per VLP (only the larger peak in the black curve of Figure 9.1B is included in Figure 9.3), whereas the VLPs in 25 mM ammonium acetate begin with only 139 SPs per VLP. Again, both samples were purified from the same cell culture at the same time, so this difference must be a result of the different ionic strengths. Although the VLPs in 150 mM ammonium acetate start with significantly more SPs than those in 25 mM ammonium acetate, both samples held at room temperature drop to 15-26 SPs by about 230 hours, just under 10 days, after which the masses remain fairly constant. In contrast, the samples held at 4 °C lost scaffolding much more slowly. After about 1000 hours, the average number of SPs per VLP had dropped to about 70 for both 4 °C samples, and there was no sign that they had not reached a steady state yet. For most samples, masses drop monotonically until they reach their steady state. At that point, the apparent bouncing around of the masses probably represents scatter in the measurement rather than real shifts in mass. For the sample in 25

mM ammonium acetate held at 4 °C, however, the masses jump around with no clear trend in mass until after about 350 hours. For a given time point, all four samples were run consecutively with the same instrumental settings; therefore, the odd behavior of this particular sample is probably real rather than a measurement artifact. The cause of this behavior is unclear.

In general, the SP leakage from all VLPs is not simply a result of the VLPs being stored in ammonium acetate. We have observed similar SP leakage from VLPs stored in a phosphate buffer and exchanged into ammonium acetate immediately before analysis by CDMS.

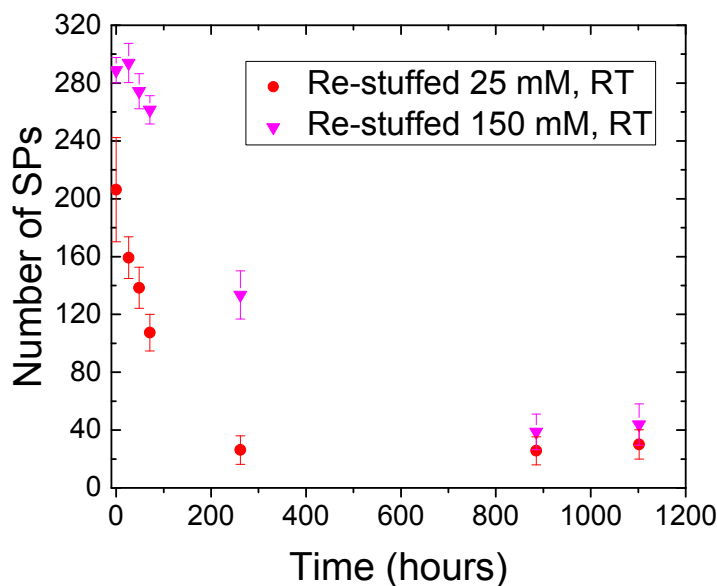


Figure 9.4 Plots of average number of SPs per VLP as a function of time from re-stuffing VLPs after last time point of Figure 2 by adding SPs to a concentration of 1.1 SP/coat protein. Red circles are for sample kept in 25 mM ammonium acetate at room temperature, and upside-down pink triangles are for sample kept in 150 mM ammonium acetate at room temperature. Bars represent width of the measured mass peaks from distribution of SPs (W_{IH} in equation 2).

We initially suspected that we were observing an equilibrium process between SPs bound to coat protein and SPs free in solution, and that purifying the VLPs from cells removed any free SPs, which might have been present in the cells. To test this hypothesis, we added SPs to the room temperature samples after the last time point on Figure 9.3 to a concentration of 1.1 SPs per coat protein. Results are shown in Figure 9.4. If this were a simple equilibrium process, we would expect some of the scaffolding to reenter the VLPs and bind to coat protein and for the VLP masses to stay constant after that. However, the experimental results were qualitatively similar to the room temperature data on Figure 9.3. Nearly 300 SPs in 150 mM salt and over 200 SPs in 25 mM salt reentered the VLPs, but rather than the masses reaching equilibrium, the SPs leaked out to final numbers of 25-44 SPs over the course of weeks. MALS data agrees qualitatively with CDMS data (Figure 9.2, traces labeled 'Re-entry' and 'Stabilized Re-entry').

To ensure that the above results were reproducible, we generated another batch of VLPs, also purified into 25 and 150 mM ammonium acetate. These samples were kept at room temperature. Again, the VLPs began with hundreds of SPs immediately after purification, but the SPs leaked out to final numbers of 26-34 SPs per VLP (slightly higher numbers than in Figure 9.3 with the first batch of VLPs, but within the range in Figure 9.4 for re-stuffed VLPs) over the course of weeks. To see if we could draw out even more SPs from the VLPs, we removed the free scaffolding from the solution by centrifugation and then diluted the total protein concentration back down to 1 mg/mL. However, the VLP masses did not noticeably change after removal of the free SPs.

A common method to remove SPs from P22 VLPs or procapsids is to heat the VLPs¹⁸⁴ or to wash them 3-4 times in the chaotrope guanidine hydrochloride (GuHCl).²⁴⁰ It is typically assumed that these steps remove all SPs (with some exceptions²⁵³); however, CDMS shows that this is not the case. Figure 9.5 shows representative mass spectra of P22 VLPs heated to 65 °C (black curve) or washed several times with GuHCl (red curve). The dashed blue line is at the mass of an empty VLP consisting of only 420

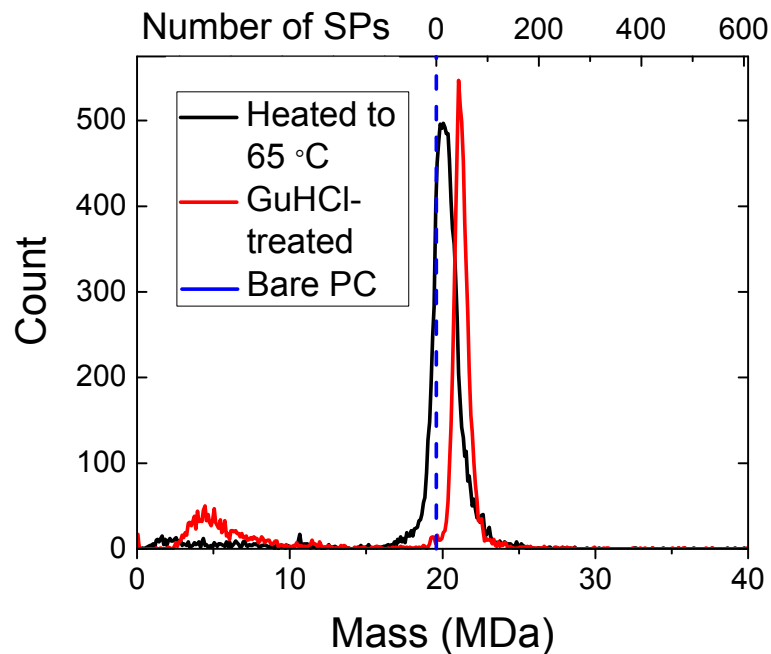


Figure 9.5 Representative mass spectra of P22 VLPs that were heated to 65 °C (black curve) or washed several times with GuHCl (red curve) to attempt to remove all SPs. Blue dashed line shows the mass of an empty VLP consisting of 420 coat proteins and no SPs.

coat proteins. Before heating or GuHCl-washing, the VLPs had 130 SPs on average.

Heating to 65 °C led to 17 SPs on average, and washing with GuHCl led to 48 SPs on average. In other cases, these SP-removing treatments still left over 100 SPs in the VLPs.

We have never observed heating or GuHCl-washing to remove SPs to below the range

achieved by leaving the VLPs at room temperature and allowing the SPs to leak out, even when GuHCl-washing and heating steps are combined.

To summarize the results from these experiments, P22 VLPs consisting of exactly 420 coat proteins and a distribution of hundreds of scaffolding proteins (SPs) gradually leak SPs until 15-44 remain after several weeks. This SP leakage occurred even after adding an excess to VLPs which transiently drove the reentry of hundreds of SPs into VLPs. Once 15-44 SPs remain encapsidated, they could not be extracted by removing free SPs in solution in an attempt to drive the reaction equilibrium given by equation 1 towards free SPs.

Equation 1 is an over-simplified model for the coat protein:SP interaction because it implies that all interactions are equivalent and that all SPs are monomers, and it ignores any steric effects which might block SPs from binding at available sites in a VLP if too many other SPs are present. In reality, there are multiple classes of coat protein:SP binding strengths,²⁵⁴⁻²⁵⁶ and SPs are known to form dimers and even tetramers which participate in interactions with coat proteins.^{251,257} Even so, it is possible to measure an effective dissociation constant (K_D) that washes out all of these effects to simplify analysis.²⁵² Equation 3 defines the K_D for the simplified reaction given by equation 1.

$$K_D = \frac{[SP_{free}] [open\ lattice\ sites]}{[SP_{bound}]} \quad (3)$$

No previously measured values of the K_D of this reaction are consistent with our final results. The initial, total concentration of protein for all of these samples represented in Figure 9.3 was 1 mg/mL, corresponding to 16.3-17.3 μ M of coat protein and 7.2-5.7 μ M of SP (the ranges account for samples at both 25 and 150 mM salt, which had

different initial ratios of coat protein to SP as evident in Figure 9.3). The concentration of open lattice sites is somewhere within 4.4-11.6 μM . The range of open sites is broad because the number of available binding sites for SP on each VLP is not precisely known, but it lies somewhere between 300 and 420.²⁵³ Even assuming that there are only 300 SP binding sites per VLP, and even with only 138 initially bound SPs per VLP (which was the lower initial case on Figure 9.3), the K_D would have to be over 65 μM to end up with the experimental result of about 20 SPs per VLP. However, the highest reported K_D for this reaction is 28 μM ,²⁵² and more recent estimates of the K_D are under 15 μM .²⁵³ Moreover, this equilibrium model completely falls apart for the experiment in which we

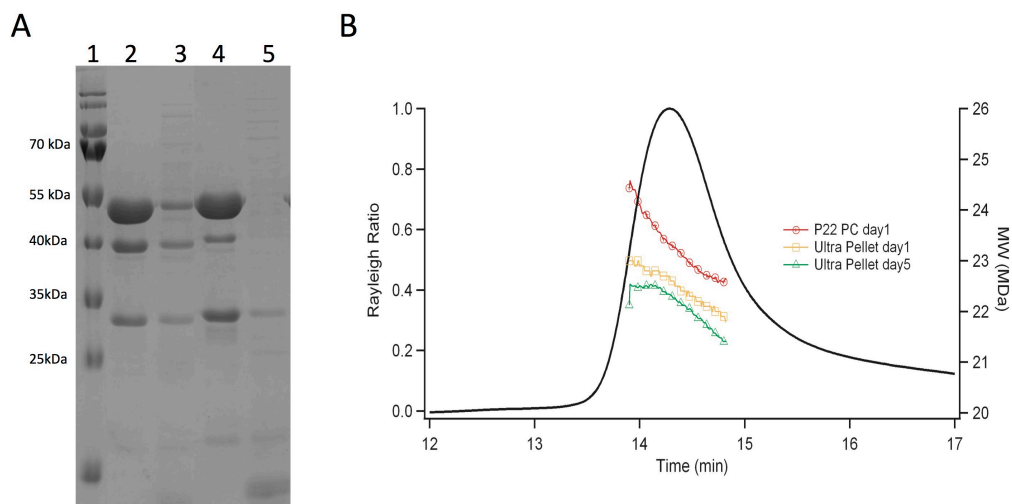


Figure 9.6 SDS PAGE gel showing SPs degradation and Multi-Angle Light Scattering showing SPs loss from P22. A) SDS PAGE denaturing gel showing the degradation and loss of SPs from the P22 VLP. (1) Protein Ladder (2) P22 PC pellet after ultracentrifugation spin day 1, showing the coat protein band (~46 kDa), the scaffold protein band (~33 kDa) as well as a cleavage product of the scaffold protein (~28 kDa) (3) Supernatant from the ultracentrifugation spin day 1, showing loss of primarily SPs protein (4) P22 PC pellet after ultracentrifugation spin day 5, showing an enhancement of the SPs cleavage band (5) Supernatant from the ultracentrifugation spin day 5, showing a complete loss of SPs protein band and more cleavage products, indicative of SPs degradation over time. B) Multi-angle light scattering (MALS) plot verifying the mass loss of SPs over this 5 day period. Samples are as follows: P22 PC day1 (red circles), Ultra Pellet day1 (yellow squares), and Ultra Pellet day5 (green triangles). Mass loss is seen after the first ultracentrifugation spin and more mass loss after 5 days correlating well with the gel data.

re-stuffed the VLPs with SPs. If this model were valid, then the VLP masses would have risen as SPs reentered them, and then the VLP masses would have leveled off once equilibrium was reached. The fact that the masses first increased but then fell back to their initial values shows that this is not a simple equilibrium. Even if we had incorporated SP oligomers and multiple binding classes of SPs to coat proteins into the equilibrium model, it could not have predicted the re-stuffing followed by leaking back to initial values.

The most likely explanation of our results is degradation and aggregation of free SPs, which would continuously deplete the concentration of free SPs. This degradation event and the loss of SPs can be visualized by running the samples at different time points on a SDS-PAGE denaturing gel (Figure 9.6A). By running initial samples (day 1) versus samples that have sat for 5 days (day 5) we can see the degradation and loss of SPs from the samples. This degradation or loss of SPs was also shown by multi-angle light scattering (MALS) where the mass of the PC drops after ultracentrifugation (Figure 9.6B), and further mass loss is seen after 5 days, consistent with the SDS-PAGE gel (Figure 9.6). The equilibrium would keep driving bound SPs to be released, and the cycle would continue. This would explain the results presented in Figure 9.3, and it also explains the results from the re-stuffing experiment, shown in Figure 9.4. In that experiment, the average mass of VLPs initially increased as equilibrium drove SPs to enter VLPs, but the slow degradation of SPs gradually shifted equilibrium until most of the SPs had left the VLPs once again. After several weeks, the VLPs reached a steady state of 26-44 SPs per VLP. At that point, equilibrium was probably reached, and the solution contained mostly

degraded SPs. Figure 9.7 shows results from dynamic light scattering (DLS), a technique to determine the hydrodynamic radius of small particles, to confirm that aggregates are also forming. Before removing aggregates by spinning the samples down, samples in both 25 and 150 mM ammonium acetate show a large peak around 60 nm, corresponding to VLPs, and they also reveal large micron sized aggregates. After centrifugation, only VLPs remain. While Figure 9.7 does not prove that the SPs are aggregating, the result shows that over time there is significant aggregates forming, supporting our hypothesis.

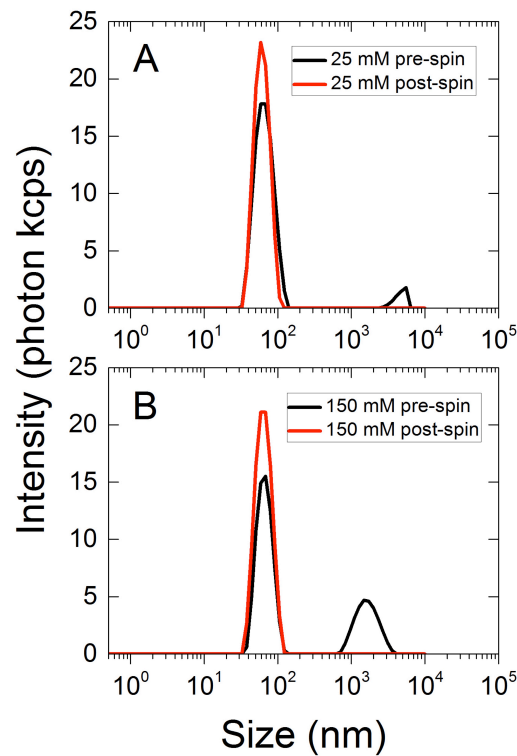


Figure 9.7 Dynamic light scattering results from VLP samples after their masses had reached their steady states. Black curves are before centrifugation to remove precipitates; red curves are after centrifugation. A) Samples kept in 25 mM ammonium acetate. B) Samples kept in 150 mM ammonium acetate.

In the experiment represented by the room temperature plots of Figure 9.3, we removed free SPs from solution by centrifugation after the steady state of 26-34 SPs had been reached, and we did not detect any more SPs leaving the VLPs afterwards. The remaining SPs likely represent the more tightly bound class of SPs. The most recent estimate of the K_D for the tightly bound population of SPs is $0.77 \mu\text{M}$.²⁵³ At our concentration of VLPs, and starting with 26-34 SPs, a K_D of $0.77 \mu\text{M}$ dictates an equilibrium of 24-32 SPs bound to each VLP at our total protein concentration. This represents a loss of 67.1 kDa per VLP from the initial conditions of 26-34 SPs, a difference which could be difficult for our instrument to reliably detect in this 20-MDa regime over a long time period. Anyway, the K_D depends on the solution that the VLPs are in;²⁵¹ the $0.77 \mu\text{M}$ K_D estimate was for a solution of 20 mM sodium phosphate, 50 mM sodium chloride. The K_D in ammonium acetate is unknown, but it appears to be small enough that the vast majority of the tightly bound SPs remain bound even in the near absence of free SPs.

The results of all of these experiments can be explained by degradation/aggregation of free SPs along with a small population of 15-44 more tightly bound SPs which cannot completely be removed by any known in vitro methods. Other CDMS experiments have shown that matured, infectious phage P22 has no SPs,²⁵⁰ so the in vivo maturation of P22 is much more efficient at removing SPs. Moreover, in an infection, the SPs are only required for minutes to hours before mature phage forms in enough numbers to lyse the infected cell,²⁵⁸ so SP degradation probably does not occur significantly in cells. On the other hand, P22 capsids are commonly used as containers or

reaction vessels in nano-engineering. One way to accomplish this is to fuse an enzyme or reactive species onto a truncated form of the SP and then to assemble VLPs using the fused SPs as the assembly chaperone, forming a nanoreactor.^{46,49,50} If these constructs will ever be used for extended periods, SP degradation must be prevented. This might naturally be accomplished in cases where the fused SP-enzyme is too large to leak through pores in the capsid.^{46,49}

Conclusions

P22 procapsid-like particles (VLPs) consist of 420 coat proteins and a distribution of hundreds of scaffolding proteins (SPs); they weigh 20-30 MDa. Charge detection mass spectrometry (CDMS) was used to measure the masses of VLPs over the course of weeks, during which the average VLP mass dropped from gradual loss of SPs. For samples held at room temperature, the masses eventually reached a steady state of 15-34 SPs per VLP. Adding a molar excess of SPs to the VLPs temporarily increased the masses as hundreds of SPs reentered the VLPs, but over the course of weeks, the SPs were lost to the 26-44 SPs per VLP range. These results are best explained by slow degradation of unbound SPs, which would continuously draw bound SPs out of the VLPs to reestablish equilibrium. Degradation and aggregation of SPs has implications for the long-term viability of P22 VLPs used for nano-engineering applications in which enzymes or other functional molecules are fused onto SPs and incorporated into VLPs.

Acknowledgements

We gratefully acknowledge the support of the NSF through awards CHE-1531823 to MFJ and BMAT DMR-1507282 to TD.

Experimental

Protein Purification

P22 (S39C) was expressed, purified and analyzed by size exclusion chromatography with multi-angle light scattering (MALS) and refractive index detection by following procedures previously reported.⁴⁷ Briefly, plasmids containing the genes for coat protein and scaffolding protein (SP) were transformed into BL21 (DE3) E. coli (Novagen) for protein expression. The transformed E. coli cells were grown to an OD₆₀₀ = 0.6, induced with isopropyl β -D-thiogalactopyranoside (IPTG, 0.5 mM), and grown for an additional 2.5 hours before cells were concentrated and collected by centrifugation at 4500 rpm. Cells were resuspended in pH 7.0 PBS buffer (50 mM PO₄³⁻, 100 mM NaCl) and frozen overnight at -80 °C. Cells were defrosted and incubated with (100 μ L/liter of culture) lysozyme (15 mg/mL), DNase (20 mg/mL) and RNase (30 mg/mL) for 30 minutes, then lysed by sonication and centrifuged at 12,000 rpm to remove cell debris. The virus particles were isolated by ultracentrifugation through a sucrose cushion, resuspended in either a pH 8.2, 25 mM ammonium acetate or 150 mM ammonium acetate and analyzed by SEC-MALS. For further purification samples were run over a sephacryl (S-500) size exclusion column (GE Healthcare) running at 1 mL/min in pH 8.2,

ammonium acetate buffer (25 mM or 150 mM) and analyzed by both SEC-MALS and CDMS.

For re-entry experiments an N-terminal histidine tagged wild-type scaffold protein variant was used. Plasmids containing the genes for scaffold protein were transformed into BL21 (DE3) *E. coli* (Sigma-Aldrich). Transformed cells were grown to an OD₆₀₀ = 0.6, induced with Arabinose, and grown for an additional 4 hours. Cells were concentrated by centrifugation at 4500 rpm and then frozen at -80 °C. Cells were defrosted and incubated with Pierce™ Protease Inhibitor tablets (Thermo Fisher), lysozyme (15 mg/mL), DNase (20 mg/mL), and RNase (30 mg/mL) for 30 minutes. After incubation cells were lysed by sonication and centrifuged at 12,000 RPM to remove cell debris. The supernatant was loaded onto a Nickel-Nitriloacetic acid (Ni-NTA) column (Roche) and scaffold protein was eluted with a gradient of imidazole buffer at pH 7.8. Fractions were collected and analyzed by SDS PAGE gel. For further purification selected fractions after Ni-NTA were run over a Superose 12 column (GE Healthcare). Fractions were collected and analyzed by SDS PAGE. Prior to re-entry experiments samples were dialyzed into a pH 8.2 ammonium acetate buffer (25 mM or 150 mM).

Scaffolding Protein Reentry

Once P22 VLPs masses had stabilized (see Results section), VLPs were re-stuffed with SPs by following procedures previously reported.⁴⁵ Wild-type SP (1.1-fold molar excess, 0.45 mg/mL final concentration) was added to a solution of nearly empty VLPs (0.6 mg/mL final concentration) and allowed to rock for 4 hours at room temperature. Reentry experiments were performed at 25 mM ammonium acetate and 150 mM

ammonium acetate, pH 8.2. Samples were analyzed by SDS-PAGE, SEC-MALS and CDMS.

Free Scaffolding Protein Removal

Once P22 VLPs masses had stabilized (see Results section), VLPs were concentrated by ultracentrifugation (50 min, 45,000 rpm) to remove any free SPs and resuspended in pH 8.2, ammonium acetate buffer (25 mM or 150 mM). Samples were analyzed by CDMS.

Scaffolding Protein Removal from VLPs

To remove SPs from VLPs by the chaotrope guanidine hydrochloride (GuHCl), previously reported procedures were followed.⁴⁷ After purification VLP samples were concentrated by ultracentrifugation (50 min, 45,000 rpm) and resuspended in 2 mL of pH 7.0 PBS buffer (100 mM PO₄³⁻, 50 mM NaCl). Samples were mixed with ~23 mL of 0.5 M GuHCl buffer (pH 7.0, 100 mM PO₄³⁻, 50mM NaCl) for a final volume of 25 mL and rocked for 2 hours at 4°C. VLPs were concentrated by ultracentrifugation and resuspension into pH 7.0 PBS. This process was repeated 3 times and they were resuspended in pH 8.2, ammonium acetate buffer (25 mM or 150 mM). To remove SPs by heating, P22 VLPs at 1 mg/mL were heated to 65°C for 20 minutes to obtain the P22 expanded morphology. Samples were concentrated by ultracentrifugation and resuspended in pH 8.2, ammonium acetate buffer (25 mM or 150 mM).

Multi-Angle Light Scattering

Samples were analyzed using a Dawn 8 instrument (Wyatt, Santa Barbara, CA) for SEC-MALS analysis. P22 samples were separated over a WTC-0200S (Wyatt Technologies) size exclusion column on an Agilent 1200 HPLC at 0.7 mL/min flow rate and monitored by a UV-Vis detector (Agilent), a Wyatt HELEOS Multi Angle Laser Light Scattering (MALS) detector, a quasi-elastic light scattering detector (QELS), and an Optilab rEX differential refractometer (Wyatt). The number average particle molecular weight (M_n) and radius of gyration (R_g) were determined by calculating across each peak half max with Astra 6 software (Wyatt) using a previously calculated dn/dc value of 0.185 mL/g.

Dynamic Light Scattering

Protein samples were prepared at ~0.5 mg/mL at 100 μ L and spun on a benchtop centrifuge at 17,000 rpm for 10 minutes to remove any dust particles. Samples were placed into a DLS cuvette (Hellma Analytics, ZEN2112) and analyzed by dynamic light scattering, on a Malvern, Zetasizer Nano-S.

Charge Detection Mass Spectrometry

The instrument and data analysis used here have been described thoroughly.^{246,247,259-261} In brief, ions are generated by electrospray ionization and are transferred through several differentially pumped regions with RF ion guides. They are energy-selected with a dual hemispherical deflection energy analyzer. Ions with the desired energy are passed into an electrostatic ion trap which contains a conductive tube

in the center. When the voltages on the trap's end caps are raised, any ion within the trap is forced to oscillate back and forth through the detector tube. Each time it passes through the tube, a charge is induced on the tube that is picked up by a cryogenically cooled JFET (2SK152) at the input of a charge-sensitive preamplifier (Amptek A250). The resulting signal is sent to a computer and analyzed with fast Fourier transforms (FFTs). The ion's m/z is determined from its oscillation frequency, and its charge is determined by summing the magnitudes of the first two harmonic peaks in the FFT. The m/z and charge are multiplied to obtain mass. After a predetermined trapping time, the end cap voltages are lowered, and a new ion is allowed to enter the trap. The trapping time here was 91 ms; only ions trapped for the full duration are included in the results. This process is repeated for thousands of ions, and the masses are binned into a histogram to produce a mass spectrum.

CHAPTER 10

CONCLUSION

Inspired by nature, the P22 virus-like particle (VLP) was used to create a suite of biomaterials with a wide range of applications. The P22 VLP is a model system for both viral assembly and biomaterial synthesis due to its well-defined and relatively simple assembly as well as its ability to be amendable by both genetic and synthetic means. This system also has multiple morphologies, and is thermally/chemically resilient making it a nanomaterial with little limitations. From these and previous studies, new complex protein-polymer composites were developed for applications ranging from energy production to the fundamental understanding of both synthetic and protein composites.

In Chapters 2, 3, 4 and 5 the interior of the P22 VLP was used for the constrained synthesis of a variety of polymers including acrylamides, acrylates, crosslinkers, photocrosslinkers, testing the limits of this protein-polymer system. In Chapter 2, the incorporation of a photocrosslinker created a photocatalytic VLP that was active towards the reduction of methyl viologen. Chapter 3 showed the labeling of the addressable amine on the P22-polymer network to create a T_1 -enhanced contrast agent with the high loading of manganese porphyrin as an alternative to the much more toxic gadolinium. In Chapter 4 the P22-polymer's addressable amine was labeled with a two-catalyst system constructing a confined and co-localized photocatalytic particle that efficiently produced NADH and hydrogen photochemically. By controlling labeling density of the catalyst we could tune the reaction and favor either NADH or hydrogen production, giving rise to the

importance of nano-scale arrangements of catalysts. The role of electrostatics in the P22-ATRP system was explored in Chapter 5. The work in Chapter 5 needs further studies to validate a consistent model, but it was evident that pH had a significant affect on the incorporation of methacrylic acid, by changing the charge on the monomer side chain. Because of it's simplicity, modular nature, promiscuity of monomers, and high loading capacity, makes the P22-ATRP system highly desirable for the synthesis of complex biomaterials.

In Chapter 6 a new bio-hybrid catalytic material was synthesized using both genetic and synthetic approaches. By coupling an enzyme, AdhD, with a small molecule catalyst, $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$, a bio-hybrid nanoreactor was synthesized. This system could be controlled by altering substrate concentrations allowing for NADH to be consumed, produced and or behave in a steady state manner. This work sets a precedence for the design and synthesis of more complex hybrid materials which could give rise to unexpected properties.

In Chapter 7 the biophysical properties of the P22 VLP system were tested with atomic force microscopy, revealing that cargo and protein cage interactions can substantially change the materials physical properties. 2-D crystal arrays were formed in Chapter 8, indicating that the P22 VLP can form ordered crystalline arrays with all morphologies except the wiffleball, giving insights into the VLP's structure. This is a preliminary step towards designing and synthesizing functional P22 arrays as biomaterials. In Chapter 9, the dynamics of the scaffolding protein were monitored by charge-detection mass spectrometry, revealing the dynamic and unstable nature of this

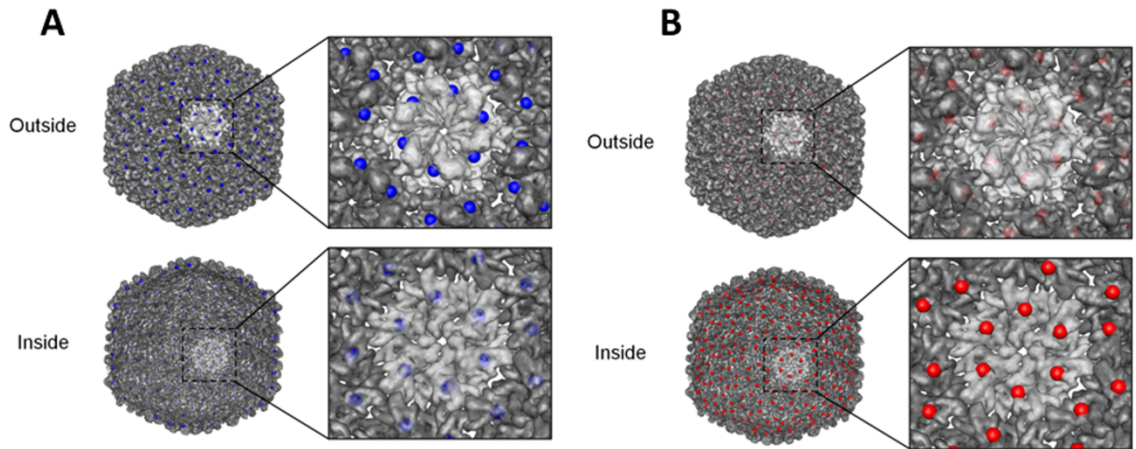
small essential protein for the VLP assembly. These results give insights into the stability for future biomaterial studies.

In conclusion, the bacteriophage P22 VLP was used as a platform for biomaterial synthesis both synthetically and genetically. Several new biomaterials were designed, developed and synthesized using the P22 VLP system, expanding the range and complexity. The work here shows the versatility of this platform for its diverse use as a nanomaterial, for applications that range from MRI imaging agents to photocatalytic nanoreactors. Additionally, VLP platforms allow for the fundamental biochemical and biophysical properties of these VLPs to be explored. In general, each of the genetic and synthetic approaches described here are easily translatable into other protein systems for the development of more sophisticated and efficient materials for energy and medicinal applications.

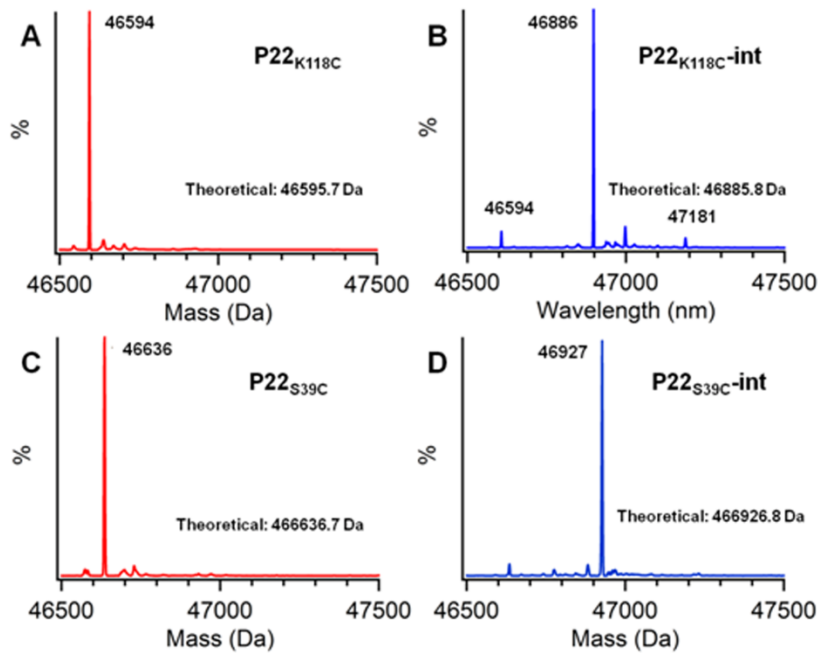
APPENDICES

APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER TWO



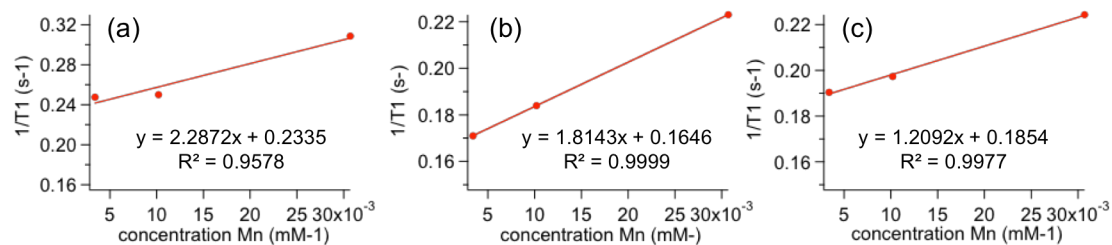
Supplementary Figure A.1 A) Structural models of the expanded morphology of the P22 capsid that shows the location of the introduced cysteine mutations, K118C and S39C derived from a structural model of P22 using coordinate data deposited as Protein Data Bank file 2XYZ. A) The location of the K118C (in blue) showing both a view of the exterior of the capsid (top) and a half shell cut-away view revealing the interior (bottom) of the capsid. B) The location of the S39C mutation (in red) showing both a view of the exterior of the capsid (top) and a half shell cut-away view revealing the interior (bottom) of the capsid.



Supplementary Figure A.2 Representative subunit mass spectrometry analysis of the of the P22_{S39C} and P22_{K118C} macroinitiators.

APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER THREE



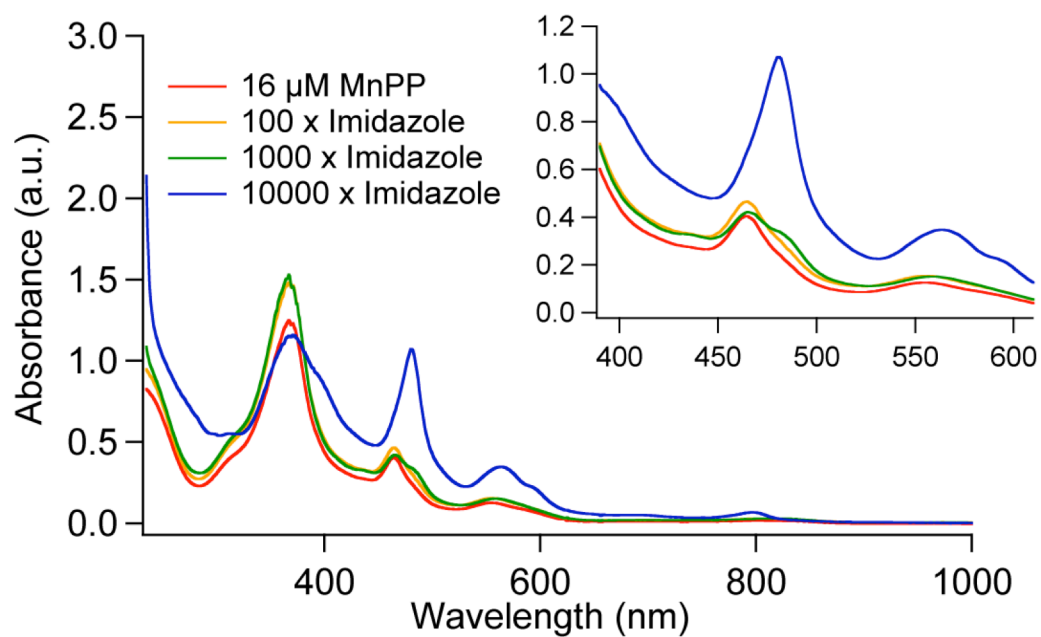
Supplementary Figure B.1 NMR T_1 -measurements for P22-xAEMA-MnPP (1,200 MnPP/capsid) at 19 MHz (a), 90 MHz (b), and 300 MHz (c) showing R^2 value close to 1 indicating a good fit, with slope equal to ionic relaxivity.

MnPP/capsid	Conc. MnPP inside capsid (mM)	$r_{2,ionic} / r_{1,ionic}$
90	1.09	17.09
121	1.46	9.91
155	1.88	4.94
437	5.29	3.65
719	8.70	2.48
778	9.42	2.51
1,072	13.0	2.18
1,201	14.5	1.75
2,578	31.2	1.54
3,236	39.2	1.48
3,646	44.1	1.32

Supplementary Table B.1 P22-VLPs conjugated to MnPP with calculated concentrations of MnPP inside P22 and r_2 / r_1 ratio based on different loading factors.

Green = P22-MnPP w/o EDC, Pink = P22-xAEMA-MnPP w/o EDC, Orange = P22-MnPP w/EDC,

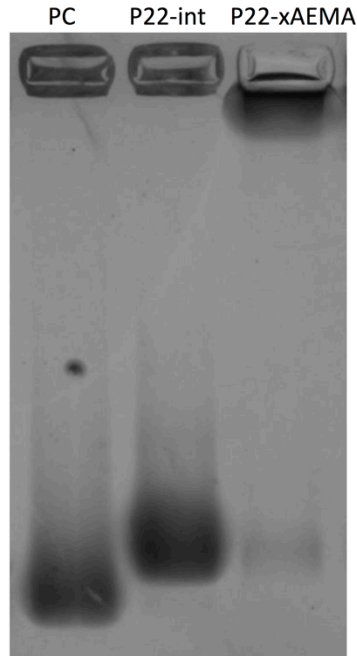
White = P22-xAEMA-MnPP w/EDC



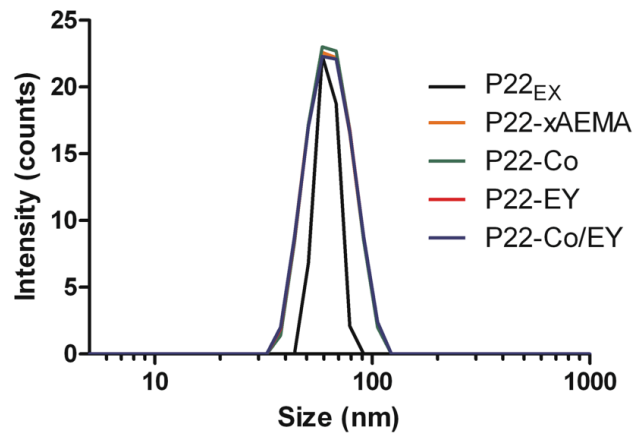
Supplementary Figure B.2 UV-VIS spectrum of imidazole added to free MnPP (red) at molar excess 100 (orange), 1000 (green), and 10000 (blue) excess. Inset: The appearance of the peak at 482 nm indicates binding of imidazole to MnPP.

APPENDIX C

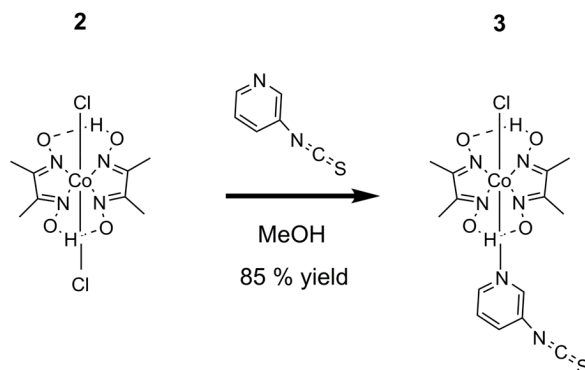
SUPPORTING INFORMATION FOR CHAPTER FOUR



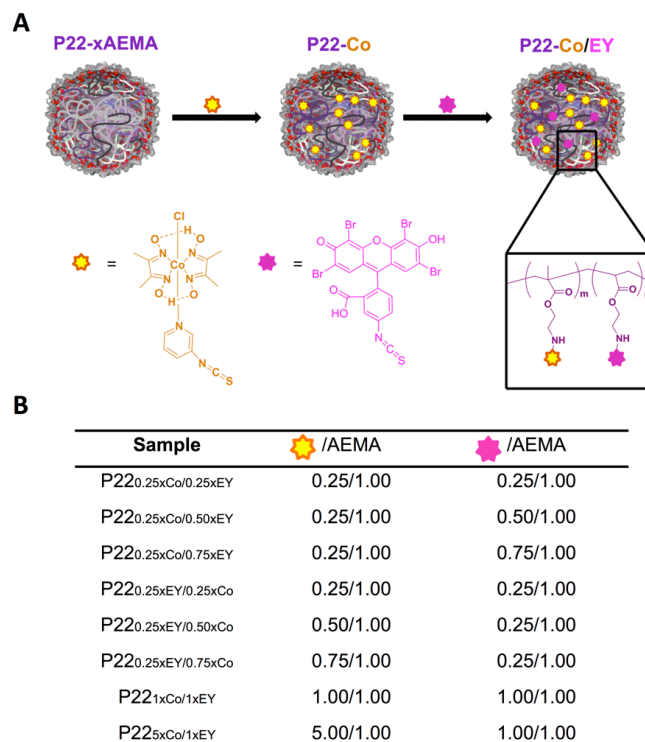
Supplementary Figure C.1 Native agarose gel of P22 PC, P22-int, P22-xAEMA. The observed shift in electrophoretic mobility shows the temperature induced transformation of P22 PC to EX, and the large shift in mobility shows the transformation of P22-int to P22-xAEMA because of the net positive charge of the poly(AEMA) in the running-buffer.



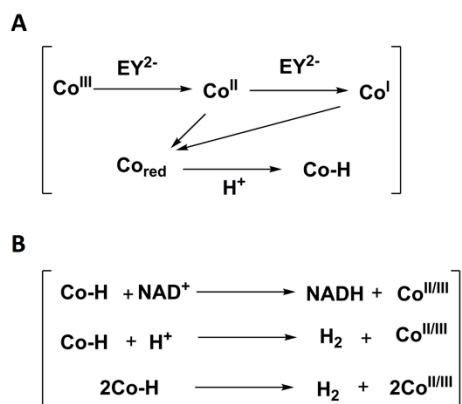
Supplementary Figure C.2 Dynamic light scattering (DLS) of P22 EX-int, P22-xAEMA, P22-xAEMA-Co, P22-xAEMA-EY, P22-xAEMA-Co/EY. The measured hydrodynamic diameter for the constructs are P22 EX-int (61.39 ± 6.89 nm), P22-xAEMA (62.34 ± 15.19 nm), P22-Co (61.72 ± 13.99 nm), P22-EY (62.10 ± 15.33 nm), P22-Co/EY (61.93 ± 15.36 nm).



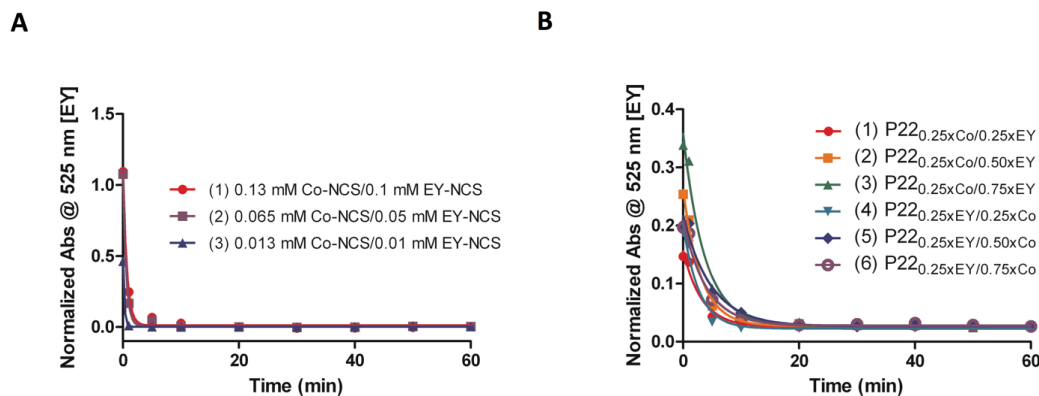
Supplementary Figure C.3 Synthesis of chloro(3-pyridyl isothiocyanate)cobaloxime. $\text{Co}(\text{dmgh})(\text{dmgh}_2)\text{Cl}_2$ (**2**) was reacted with 3-pyridyl isothiocyanate in methanol, which yielded the desired product chloro(3-pyridyl isothiocyanate)cobaloxime (**3**) with a yield of 85 %.



Supplementary Figure C.4 Sub-stoichiometric labeling strategy of Co-NCS and EY-NCS. (A) Sequential labeling allows for precise control over the amount of each small molecule attached to the internal polymer. (B) Table of the sub-stoichiometric labeling amounts in relation to the estimated amount of amines on the xAEMA polymer.



Supplementary Figure C.5 Proposed mechanism for the reduction of cobaloxime and the photochemical production of NADH and hydrogen. (A) The reduction of Co^{III} to Co^{I} requires two photoexcited EY, this reduced Co (Co_{red}) can react with protons to give a Co-H species. (B) The Co-H species can react in three manners in these reaction conditions, either with NAD^+ , with a H^+ or with itself, leading to NADH or H_2 production, respectively.



Supplementary Figure C.6 EY-NCS photodegradation monitored by UV-vis. (A) Three different concentrations of free in solution EY-NCS photodegradation monitored by tracking the characteristic absorbance at 525 nm. Decay constants for free in solution samples (1), (2) and (3) are 1.521 min^{-1} , 1.076 min^{-1} , and 4.092 min^{-1} respectively. (B) EY-NCS photodegradation monitored by tracking the characteristic absorbance at 525 nm of the samples: (1), (2), (3), (4), (5), and (6) with decay constants: 0.1561 min^{-1} , 0.2614 min^{-1} , 0.3578 min^{-1} , 0.2004 min^{-1} , 0.2163 min^{-1} , 0.2085 min^{-1} , respectively.

Supplementary Table C.1 Co-NCS and EY-NCS catalysts per cage. Cobaloxime concentrations were calculated from ICP-MS analysis and Eosin-Y concentrations were calculated from the characteristic absorbance at 525 nm, using an extinction coefficient of $112,000 \text{ M}^{-1}\text{cm}^{-1}$. All measurements were performed in at least duplicate and all uncertainty reflects one standard deviation.

Sample	Co per cage	EY per cage
P22 _{0.25xCo/0.25xEY}	564 ± 15	583 ± 9
P22 _{0.25xCo/0.50xEY}	633 ± 4	1112 ± 17
P22 _{0.25xCo/0.75xEY}	615 ± 10	1521 ± 16
P22 _{0.25xEY/0.25xCo}	537 ± 2	775 ± 9
P22 _{0.25xEY/0.50xCo}	1067 ± 9	821 ± 3
P22 _{0.25xEY/0.75xCo}	1506 ± 21	826 ± 6
P22 _{1xCo/1xEY}	586 ± 18	1422 ± 28
P22 _{5xCo/1xEY}	3706 ± 187	893 ± 10

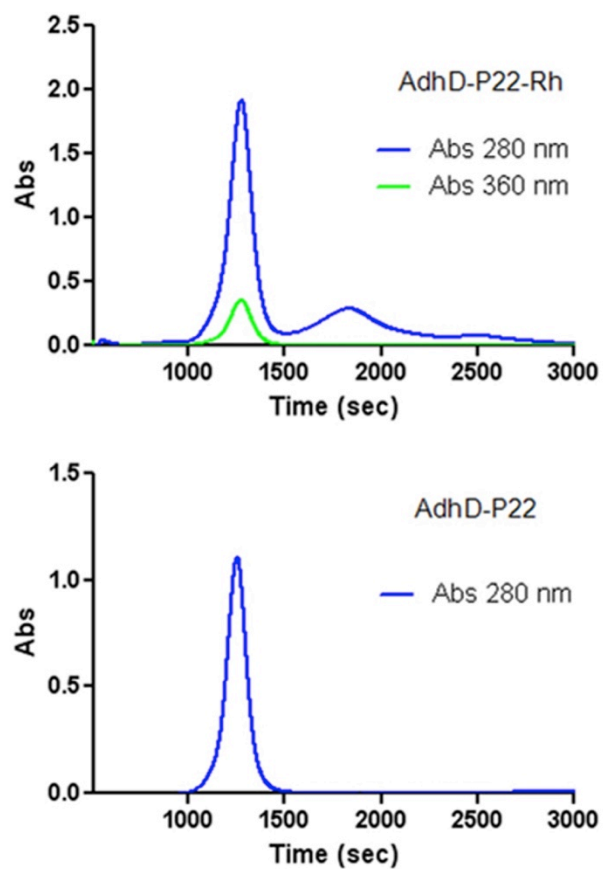
Supplementary Table C.2 pH dependence on turnovers for NADH and hydrogen for both P22-Co/EY and catalysts free in solution. All turnovers were calculated with respect to the cobaloxime concentration and all measurements were performed in triplicate.

Sample	$k_{\text{cat-NADH}} (\text{s}^{-1} \times 10^{-3})$ pH 5.0	$k_{\text{cat-NADH}} (\text{s}^{-1} \times 10^{-3})$ pH 6.0	$k_{\text{cat-NADH}} (\text{s}^{-1} \times 10^{-3})$ pH 7.0	$k_{\text{cat-NADH}} (\text{s}^{-1} \times 10^{-3})$ pH 8.0
Free Solution 2	0.21 ± 0.04	0.43 ± 0.06	2.52 ± 0.16	1.53 ± 0.22
P22 _{5xCo/1xEY}	1.21 ± 0.10	1.22 ± 0.13	2.18 ± 0.01	2.12 ± 0.12

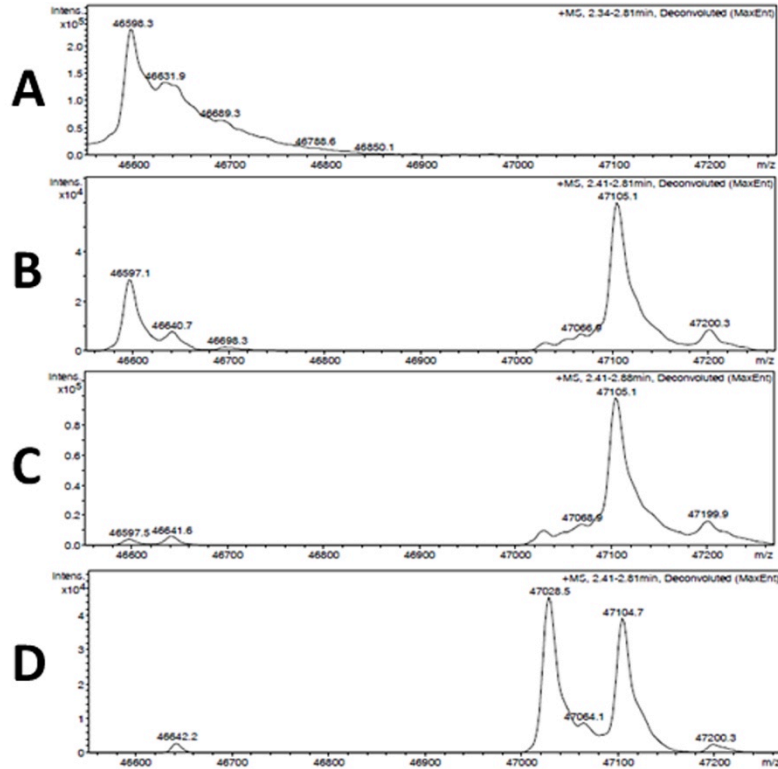
Sample	$K_{\text{cat-Hydrogen}} (\text{s}^{-1} \times 10^{-9})$ pH 5.0	$K_{\text{cat-Hydrogen}} (\text{s}^{-1} \times 10^{-9})$ pH 6.0	$K_{\text{cat-Hydrogen}} (\text{s}^{-1} \times 10^{-9})$ pH 7.0	$K_{\text{cat-Hydrogen}} (\text{s}^{-1} \times 10^{-9})$ pH 8.0
Free Solution 2	24.49 ± 8.57	11.49 ± 0.79	10.51 ± 4.79	5.43 ± 1.27
P22 _{5xCo/1xEY}	146.49 ± 14.70	123.08 ± 8.54	101.12 ± 7.49	17.86 ± 1.69

APPENDIX D

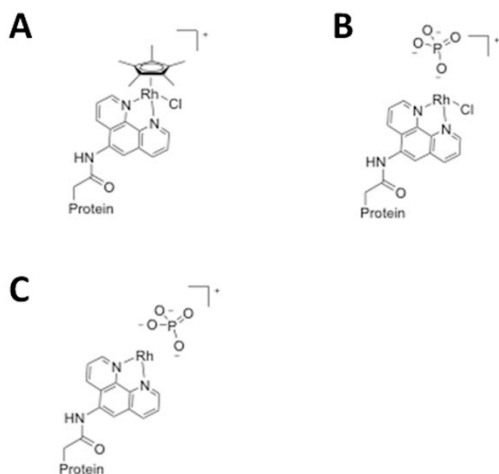
SUPPORTING INFORMATION FOR CHAPTER SIX



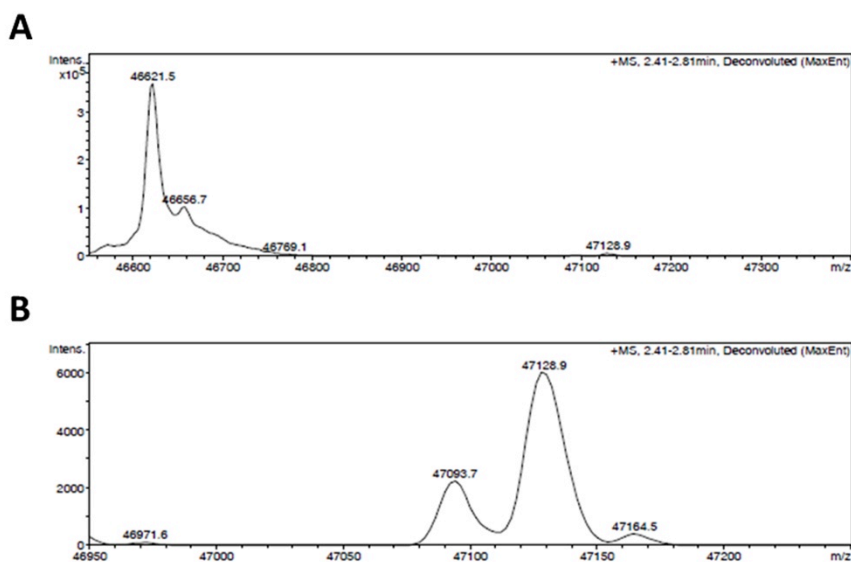
Supplementary Figure D.1: Size exclusion chromatography elution profiles of $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ labeled AdhD-P22 (top) and unlabeled AdhD-P22 (bottom). Both samples show the same elution time and signal at 360 nm shows co-elution of the $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ with the P22 cage in the AdhD-P22-Rh sample.



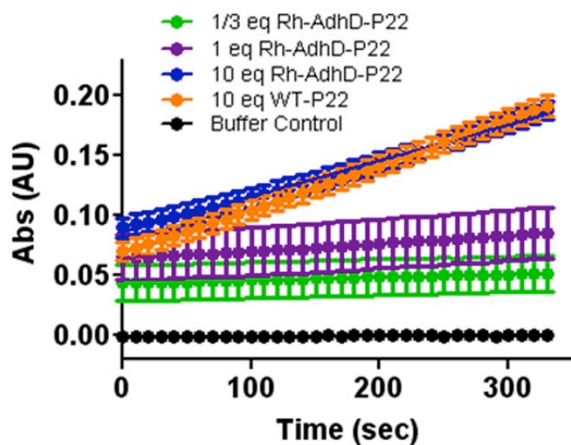
Supplementary Figure D.2: Evaluation of protein labeling by electrospray ionization mass spectrometry. (A) Unlabeled P22-K118C coat protein (expected 46595.6 Da). (B) Labeled P22-K118C coat protein labeled with 1/3 eq. $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ (expected 47106.4 Da). (C) Labeled P22-K118C coat protein with 1 eq. $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ (expected 47106.4 Da). (D) Labeled P22-K118C coat protein with 10 eq. $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ (expected 47106.4 Da). Refer to Supplemental Figure 3 for $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ degradation products that are responsible for other labeled peaks.



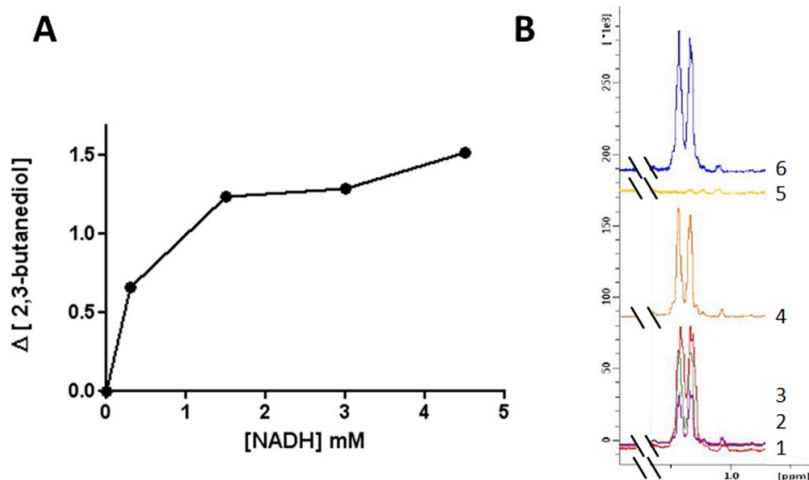
Supplementary Figure D.3: Proposed structures of $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ catalyst and degradation products labeling P22 (coat protein). (A) $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ (B & C) $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ degradation products from labeling P22 coat protein.



Supplementary Figure D.4: Evaluation of wild type P22 labeling by electrospray ionization mass spectrometry. (A) Wild type P22 coat protein labeled with 10 eq. $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$ (expected 47131.4 Da for labeled wild type coat protein and 46620.6 Da for unlabeled wild type coat protein). (B) Zoomed in view of the wild type P22 coat protein labeled with 10 eq. $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$, showing only a small fraction of coat protein labeled with the Rh complex (expected 47131.4 Da).



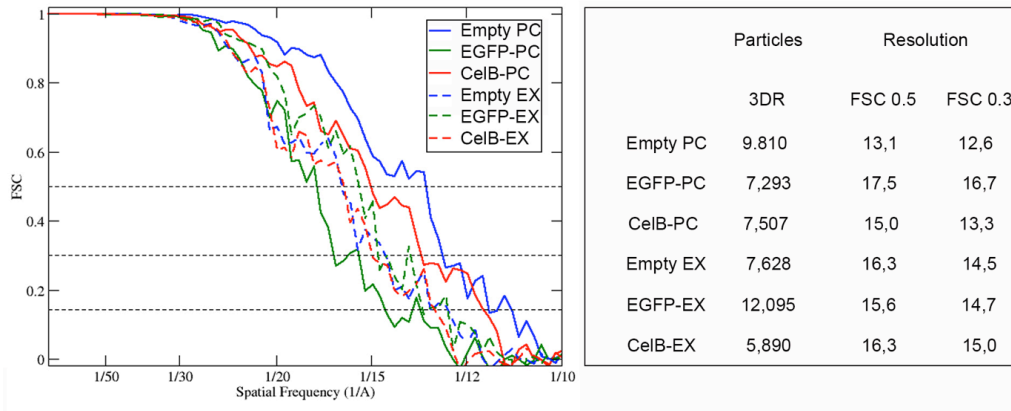
Supplementary Figure D.5: Kinetic plots monitoring the formation of NADH at 340 nm using an UV-Vis spectrometer, showing the activity of the fusion product AdhD-SP-Rh. All trials were performed in triplicate. After labeling with different stoichiometric ratios of $\text{Cp}^*\text{Rh}(\text{phen})\text{Cl}^+$, the kinetics of NADH formation increases with increasing labeling.



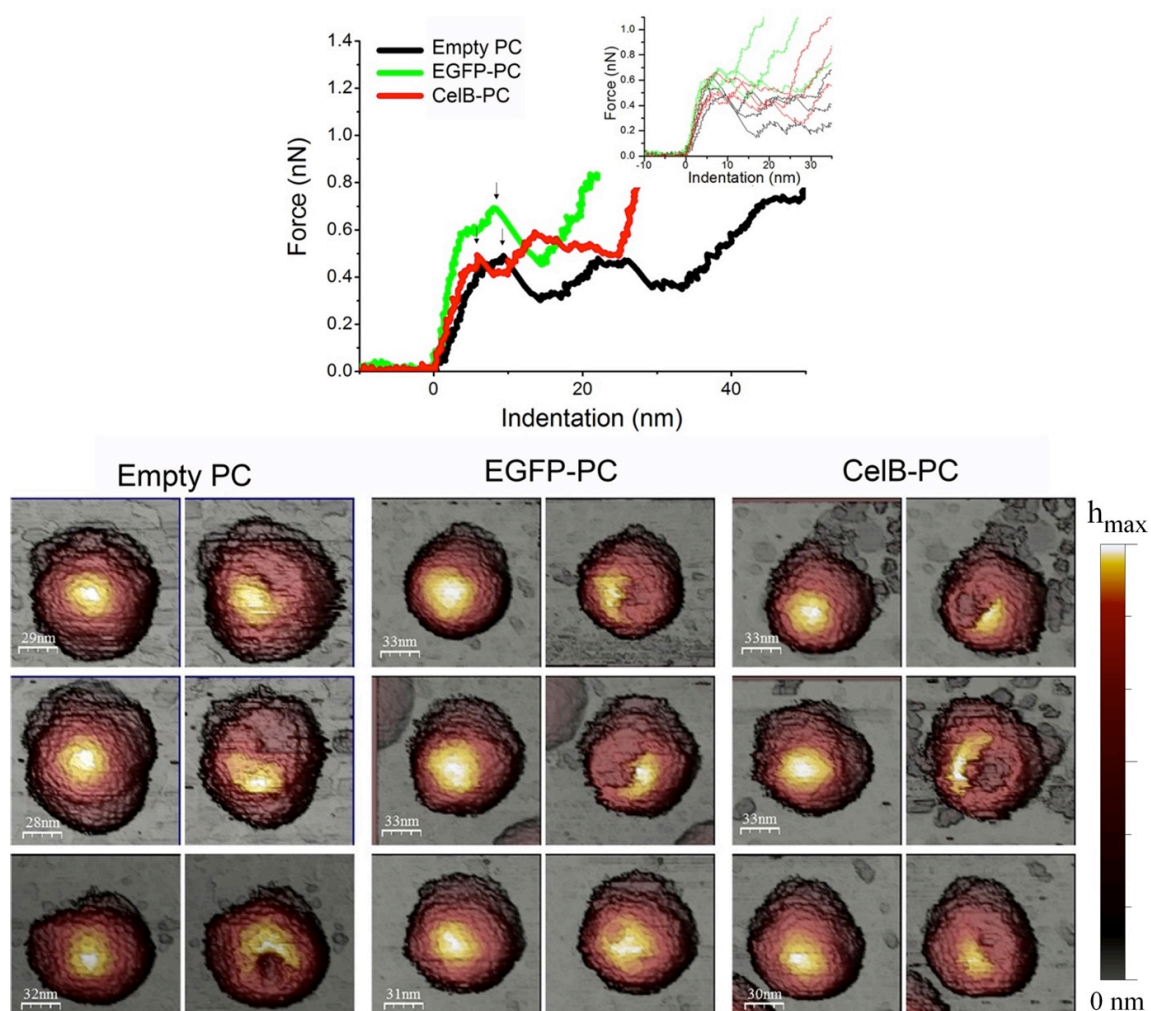
Supplementary Figure D.6: ^1H NMR monitoring of 2,3-butanediol product formation. (A) Plot showing the change in 2,3-butanediol formation with increasing concentration of NADH as determined from ^1H NMR spectrum monitoring the formation of a doublet peak corresponding to the methyl protons of 2,3-butanediol. (B) Zoomed in view of ^1H NMR spectrum showing the formation of a doublet peak corresponding to the methyl protons of 2,3-butanediol (x-axis scale increments 0.05 ppm): (1) Normal reaction conditions (AdhD-Rh, $\sim 300\ \mu\text{M}$ NADH, 100 mM acetoin, pH 6.1, 50 mM phosphate) (2) 5x NADH (3) 10x NADH (4) 15x NADH (5) Buffer control (10x NADH, 10x NAD^+ , 100mM Acetoin, 200 mM Sodium Formate, pH 6.1, 50 mM phosphate) (6) Coupled Reaction (AdhD-Rh, 10x NADH, 10x NAD^+ , 100mM Acetoin, 200 mM Sodium Formate, pH 6.1, 50 mM phosphate).

APPENDIX E

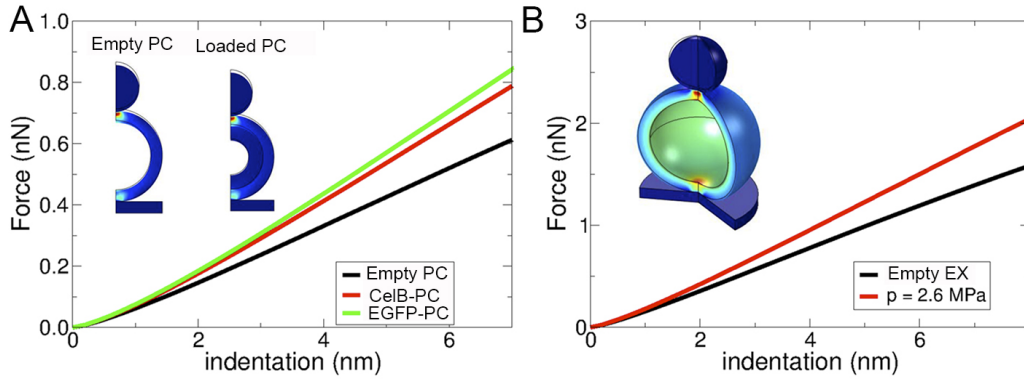
SUPPORTING INFORMATION FOR CHAPTER EIGHT



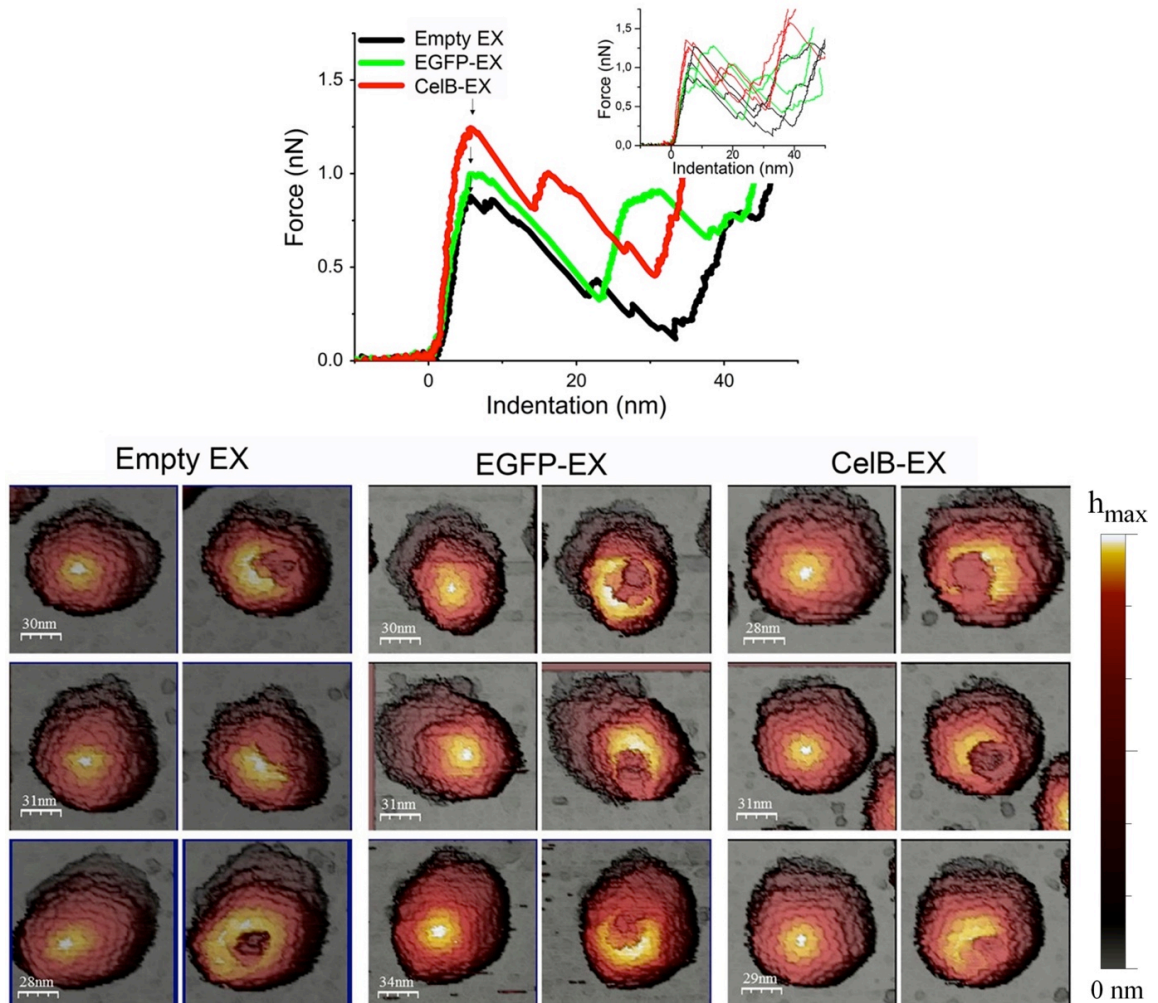
Supplementary Figure E.1: Resolution of empty procapsid (PC), EGFP- and CelB-PC, empty expanded particles (EX), EGFP- and CelB-EX. Fourier shell correlation (FSC) resolution curves were calculated for empty PC (blue), EGFP-PC (green), CelB-PC (red), empty EX (dashed blue), EGFP-EX (dashed green) and CelB-EX (dashed red). Number of particles included in the 3DR, and resolutions based on 0.5 and 0.3 criteria are indicated.



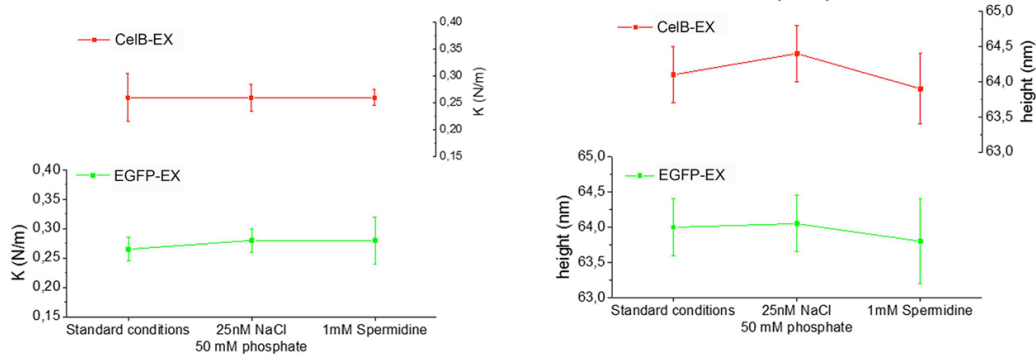
Supplementary Figure E.2: AFM nanoindentation on empty and loaded PC. (Top) Comparison of three typical nanoindentation curves for an empty PC (black), EGFP-PC (green) and CelB-PC (red). We observed a non-linear regime before particle breakage (arrows). (Inset) Other examples of PC FIC. (Bottom) AFM images of PC before (left) and after a single nanoindentation (right). Images show clear damage to the particle shell. Each image was normalized to its maximum height.



Supplementary Figure E.3: (A) Force-indentation curves obtained from Finite Element Simulations that mimic AFM experiments for PC. The empty PC is represented as a spherical shell with an external radius $R = 29.8$ nm and 7.5 nm thickness, indented on a hard substrate by a spherical tip with radius $R_{in} = 15$ nm. The Young modulus of the PC was $E = 58$ MPa, chosen to yield the same spring constant value as in experiments for empty PC. The cargo effect is modeled by addition of a second, 10-nm-thick layer (2D transverse sections, inset). The graph shows the values for the empty PC (black line); CelB-PC (red) and EGFP-PC (green) are shown with a second internal layer of $E = 6$ and $E = 8$ MPa, respectively. (B) Force-indentation curves obtained from Finite Element Simulations that mimic the AFM experiments for EX. The graph shows the values for an unpressurized empty EX (black line) and an EX (red) with an internal pressure of 2.6 MPa. The inset shows the model used for simulations, where EX is shown as a 6.6-nm-thick spherical shell with an external radius $R = 32.4$ nm, indented on a hard substrate by a spherical tip with a radius $R_{in} = 15$ nm. The Young modulus of the capsid was $E = 178$ MPa, chosen to yield the same spring constant value as in experiments for empty EX.



Supplementary Figure E.4: AFM nanoindentation on empty and loaded EX. (Top) Comparison of three typical nanoindentation curves for an empty EX (black), EGFP-EX (green) and CelB-EX (red). Particle breakage is indicated (arrows). (Inset) Other examples of EX FIC. (Bottom) AFM images of EX before (left) and after a single nanoindentation (right). Images show loss of some capsid subunits. Each image was normalized to its maximum height.



Supplementary Figure E.5: AFM indentations of CelB- and EGFP-EX in different buffers. We tested the effect of electrostatic interactions on cargo-loaded EX rigidity at low ionic strength (50 mM phosphate, 25 mM NaCl, pH 7) or in the presence of spermidine (100 mM phosphate, 50 mM NaCl, 1 mM spermidine, pH 7) compared to standard conditions (100 mM phosphate, 50 mM NaCl, pH 7). EGFP- and CelB-EX were tested similarly; each sample was incubated in standard buffer and its elastic constant measured by AFM, followed by buffer exchange first to lower ionic strength and then to spermidine buffer. No significant differences were found (Table S3). (A, B) Average elastic constant (A) and average height (B) of CelB-EX (red) and EGFP-EX (green) in different buffer conditions.

Supplementary Table E.1: Mechanical characterization of empty and loaded P22 PC and EX

	Particles (n°)	Breaking force (nN)	K(N/m)	$\epsilon_{\text{critical}}$	Height (nm)	Relative deformation
Empty PC	32	0.56 ± 0.02	0.094 ± 0.006	0.18 ± 0.01	50 ± 1	0.86
EGFP-PC	35	0.68 ± 0.03	0.131 ± 0.005	0.17 ± 0.01	56.3 ± 0.5	0.97
CelB-PC	25	0.54 ± 0.03	0.12 ± 0.01	0.14 ± 0.01	56.5 ± 0.7	0.98
Empty EX	24	1.02 ± 0.05	0.21 ± 0.01	0.13 ± 0.01	58 ± 1	0.91
CelB-EX	24	1.20 ± 0.07	0.27 ± 0.03	0.13 ± 0.01	63.3 ± 0.8	0.98
EGFP-EX	21	1.11 ± 0.05	0.27 ± 0.01	0.13 ± 0.01	62.7 ± 0.3	0.98

The values show mean $\pm$ SE of single particles. Breaking force is the maximum force reached before breakage. The elastic constant was determined by linear adjustment of the initial part of the FIC. Critical strain ($\epsilon_{\text{critical}}$) was calculated by dividing critical indentation by height $\epsilon_{\text{critical}} = \frac{\delta_{\text{critical}}}{\text{height}}$. Particle height was determined from a profile of the center of its image. Relative deformation is the ratio of AFM height to nominal size from 3DR cryo-EM.

Supplementary Table E.2: Volume and charge of P22 PC, EX and their cargos

	Volume ^a (nm ³)	Total cargo volume (nm ³)	Shell thickness (nm)	Net surface charge ^b (e)
PC	46,452 ^c		7.5	-
EX	71,936 ^d		6.6	-840 ^j
CelB (tetramer)	581 ^e		-	-37 ^k
EGFP	55 ^f		-	0 ^l
C _t helix-loop- SP helix	4.2		-	5 ^m
N _t linker	15.8 ^g		-	2 ⁿ
CelB & 4 SP	661	21,152 ^h	-	-288
EGFP & 1 SP	75	16,800 ⁱ	-	+1586

^aMeasurements were obtained by 3D cryo-EM analysis

^bNet surface charges were calculated as indicated in the VIPERdb server

(<http://viperdb.scripps.edu>); basically, net surface charge is calculated by adding the charges of the inner surface-exposed positive (Lys, Arg, His) and negative (Glu, Asp) residues.

^cVolume when a perfect sphere is assumed, PC inner radius = 223 Å (outer radius = 298 Å).

^dVolume when a perfect sphere is assumed, EX inner radius = 258 Å (outer radius = 324 Å).

^eCelB tetramer dimensions: 101 x 101 x 57 Å

^fEGFP monomer dimensions: 48 x 33 x 35 Å

^gVolume occupied by the SP segment 141-263

^hVolume for 32 CelB tetramers

ⁱVolume for 224 EGFP monomers

^jThe EX asymmetric unit inner surface has a total charge of -14 e (39 Asp, 62 Glu, 23 Lys, 64 Arg).

^kThe CelB tetramer outer surface has 169 Asp, 283 Glu, 238 Lys, 92 Arg, and 85 His

^lThe EGFP outer surface contains 12 Asp, 12 Glu, 16 Lys, 6 Arg and 2 His

^mThe SP C_t helix-loop-helix motif has 7 Asp, 1 Glu, 7 Lys and 3 Arg

ⁿThe SP N_t linker (residues 141-263) has 6 Asp, 10 Glu, 9 Lys, 8 Arg and 2 His

Supplementary Table E.3: Elastic constant and height of CelB- and EGFP-EX in distinct ionic conditions

Buffer	CelB-EX			EGFP-EX		
	Particles (n°)	K (N/m)	Height (nm)	Particles (n°)	K (N/m)	Height (nm)
50 mM NaCl, 100 mM phosphate	9	0.26 ± 0.045	64.1 ± 0.4	4	0.27 ± 0.02	64 ± 0.4
25 mM NaCl, 50 mM phosphate	14	0.26 ± 0.025	64.4 ± 0.4	13	0.28 ± 0.02	64.05 ± 0.4
1 mM spermidine	17	0.26 ± 0.015	63.9 ± 0.5	7	0.28 ± 0.04	63.8 ± 0.6

Finite Element Simulations of P22 PC and EX

Finite elements simulations of AFM indentation of P22 were performed using the COMSOL Multiphysics 4.3 program (Comsol, Stockholm, Sweden). In all cases, the capsid wall was considered to be made of a homogenous material, using Young Modulus E and Poisson ratio $\nu = 0.3$ (a standard value for protein-like materials). This model capsid was placed on a hard flat substrate and indented by a hard spherical object with radius $R_{in} = 15$ nm to mimic the AFM tip. The system was simulated using a 2D axisymmetric model meshed with over 3000-6000 triangular elements. Contacts were implemented between the shell and the tip as well as the supporting surface during indentation with a contact-penalty stiffness method according to manufacturer's protocols. A parametric, non-linear solver was used to simulate the stepwise lowering of the tip onto the capsid. The spring constant was derived from a linear fit of force vs indentation for small indentations in the linear region between 2 and 6 nm.

The empty PC was modeled as a thick spherical shell with an external radius $R = 29.8$ nm and thickness $h = 7.5$ nm (Figure E.2A, inset). The presence of cargo attached to the PC was modeled by adding a second layer beneath the capsid layer with a different Young's modulus and 10 nm thick (estimated from 3DR cryo-EM radial density profiles). The indentation curves obtained for the model PC with a $R_{in} = 15$ nm tip and Young modulus value $E = 58 \pm 4$ MPa, chosen to reproduce the experimental value of the empty PC spring constant (0.094 ± 0.006 N/m), is shown in Figure E.2A. The indentation curves for two PC with the internal second (cargo) layer showed Young's modulus values of 6 MPa and 8 MPa, yielding spring constants of $k = 0.12$ and $k = 0.131$ N/m, identical to the experimental values measured for CelB-PC and EGFP-PC, respectively. The effective Young's modulus for the cargo is thus nearly 10 times smaller for CelB and 7 times smaller for EGFP than that of the PC shell.

The empty EX was modeled as a thick spherical shell with an external radius $R = 32.4$ nm and thickness $h = 6.6$ nm (Figure E.2B, inset). Finite elements simulations were used to corroborate pressure estimates based on the Vela formula. Indentation curves for the model EX capsid with a $R_{in} = 15$ nm tip and a Young modulus value $E = 0.178 \pm 0.009$ GPa, chosen to reproduce the experimental spring constant value of empty EX (0.21 ± 0.01 N/m), are shown in Figure E.2B. The indentation curve when EX internal pressure is 2.6 MPa, which yields a spring constant $k = 0.27$ N/m, is identical to the experimental value measured for the cargo-loaded EX. The estimated pressure in finite element simulations is thus 2.6 ± 1.1 MPa, compatible with the value obtained from the Vela formula (Eq. 1, main text).¹⁹⁵

Electrostatic Contributions to Osmotic Pressure in EX

The cargo can be considered a solution of N effective charged spheres in an electrolyte with a Debye length given by the salt concentration of the buffer (100 mM phosphate, 50 mM NaCl, yielding $\kappa^{-1} = 0.796$ nm). Electrostatic repulsion between the cargo molecules is screened by the buffer salt and is described using the electrostatic repulsion part of the DLVO potential between two charged spheres; this allows calculation of the second virial coefficient B_2 , which quantifies the leading electrostatic contribution to the osmotic pressure. In the simplest approximation of treating the cargo as point charges,⁷⁷

$$B_2 = \frac{z^2}{2\Sigma}$$

Where Σ is solvent ionic strength and z is the charge of cargo molecules, in elementary charge units. The resulting contribution to the osmotic pressure is

$$\Pi_{elec} = k_B T \rho^2 B_2$$

For CelB, with an estimated charge of 9 electrons ($Z = -9$; [Table E.2](#)) per tetramer (which includes the SP charge),

$$p \approx k_B T \rho^2 B_2 = 180 Pa$$

For EGFP, with an estimated positive charge of 7 ([Table E.2](#)),

$$p \approx k_B T \rho^2 B_2 = 5000 Pa$$

In both cases, this contribution is negligibly small and cannot explain the large pressure values derived in the nanoindentation experiments.

The electrostatic contribution to the osmotic pressure can also be estimated through the Donnan equilibrium, by which the chemical equilibrium between the capsid interior and exterior, with a fixed charge that cannot escape from the capsid, leads to a higher interior counterion concentration that is responsible for the osmotic pressure. The osmotic pressure here can be evaluated as described,²⁰⁰ to yield

$$\Delta\Pi = k_B T \left(\sqrt{n_0^2 - n_b^2} - 2n_b \right)$$

where n_0 is net charge density of the cargo and n_b is bulk salt density (~150 mM here).

The interior charge density can be estimated as $n_0 = \frac{zN}{V_{capsid} - V_{cargo}}$, where we subtract the

volume occupied by the cargo. For CelB with $z = -9$ per tetramer:

$$\Delta\Pi = k_B T \left(\sqrt{n_0^2 - n_b^2} - 2n_b \right) = 370 Pa$$

For EGFP with $z = 7$ per molecule:

$$\Delta\Pi = k_B T \left(\sqrt{n_0^2 - n_b^2} - 2n_b \right) = 9800 Pa$$

In this case, the electrostatic contribution is thus also negligibly small.

To summarize, the osmotic pressure is dominated by packing effects, since the electrostatic contribution is negligible. This was confirmed by AFM nanoindentations, which showed no change in the elastic constants, independently of the presence of spermidine (which further screens electrostatic interactions) or when ionic strength was reduced.

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