

BIOMIMETIC SYNTHESIS OF CATALYTIC MATERIALS

by

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## ABSTRACT

Supramolecular proteins assemblies have been used as platforms for the synthesis of catalytic nanomaterials. These supramolecular structures are assembled from a limited number of subunits that provide a unique structurally defined platform for the synthesis of catalytic nanomaterials. Small heat shock protein (Hsp) and ferritin (Fn) are 12 nm protein cage-like assemblies of 24 subunits that have been used as platforms for the synthesis of noble metal nanoparticles through the *in vitro* reduction of corresponding ions.

Protein encapsulated metal nanoparticles were used as catalysts for photochemical reduction of protons to H<sub>2</sub> gas. The maximum catalytic rates of the protein encapsulated platinum nanoparticles are an order of magnitude better than for similarly sized platinum nanoparticles described in the literature. The protein cage increases the activity of the nanoparticles compared to other passivating layers by only minimally coating the particle. Fn was also used as the platform for the synthesis of catalytic platinum alloys of zinc and nickel. The alloys synthesized in this method showed an increase in the catalytic production of H<sub>2</sub> gas per platinum atom.

The Hsp protein cage was tested as a potential platform for use as a drug delivery vehicle for the targeted delivery of photodynamic therapy agents (PTA). The PTA, a Ru(bpy)<sub>3</sub><sup>2+</sup> derivative, was attached to the interior and exterior of the protein cage to determine the effect of the protein cage on reactive oxygen species (ROS), specifically singlet oxygen, generation by the PTA. While the Hsp was oxidized by ROS, the PTA production of ROS was not significantly quenched by the protein cage displaying its potential as a delivery vehicle for PTA.

*Thiocapsa roseopersicina* hydrogenase that is a a supramolecular was used in the synthesis nickel metal nanoparticles. The enzymatic oxidation of H<sub>2</sub> gas was used as the source of reducing equivalents. The hydrogenase was shown to specifically mineralize nickel metal nanoparticles on the interior surface revealing the reductive active site.

## INTRODUCTION

### Biom mineralization

Bone, teeth, pearls, and seashells are all macroscopic composite structures that are synthesized by organisms. These macromolecular structures are synthesized by controlling the microscopic interactions between the hard inorganic portion and the soft organic portion of the composite. The processes that control the formation of the macroscopic materials are called biomineralization. The hard/soft interaction provides the context for controlling morphology, polymorph selection, and spatial localization in biological systems.

The earliest studies on biomineralization quickly identified the importance of the interaction between the soft biological materials (proteins, lipids, and carbohydrates) and the hard materials (calcium carbonate and calcium phosphate). Early studies into the structure of bone showed that dissolved bone contained large amounts of organic material (proteins) that was intricately associated with the hard inorganic material hydroxyapatite. From that point on, a major focus of studies on biomineralization has been trying to understand the interaction between the hard and soft materials.

The biomineralization of hard materials is critical to almost all higher organisms. Biomineralization processes are used in everything from teeth to eggshells to diatom skeletons. If proper control was not exerted in the biomineralization of eggshells, the detrimental effects would be readily identified. A bird eggshell that is too thick or too hard will not allow the chick to escape from the protection of the shell, while a shell that is too thin provides little protection from the environment rendering the shell useless.

Another example of the necessity for strict biological control is teeth. Teeth that are too soft or brittle would be quickly damaged and lose their usefulness. The shape and size control of tooth growth is important for the intended use of the tooth; a long pointy tooth would not be a benefit for cow that needs flat, wide teeth for grinding.

The study of biomineralization identified three main types of biologic processes: covalent polymerization, such as the formation of  $\text{SiO}_2$ ; inorganic salt formation, exemplified by  $\text{CaCO}_3$  formation in seashells; and metal oxide formation, such as the formation of  $\text{Fe}_3\text{O}_4$  in magnetotactic bacteria. Diatoms are a remarkable example of the covalent biomineralization of amorphous silica.<sup>1</sup> Diatoms form extensive exoskeletons (frustule) of amorphous  $\text{SiO}_2$ .<sup>2-4</sup> Kroger and coworkers have shown that there are two distinct types of proteins used by diatoms to regulate silica formation; one readily promotes silica mineralization and one influences silica structure *in vitro* and does not precipitate silica.<sup>1</sup> The  $\text{SiO}_2$  is formed within specialized vesicles, silica deposition vesicles, during cell division.<sup>4</sup> These preformed particles are then assembled into the frustule.<sup>3</sup> While complete function of the  $\text{SiO}_2$  frustule is not understood, it is believed that it protects the diatom against grazing.

In higher organisms, inorganic salt formation is a widely utilized biomineralization process. In this type of mineralization, minerals form through the controlled precipitation of inorganic salts by organic matrices that make up small amount of the final complex. In the cases of mollusk shells, the organic matrix (proteins and polysaccharides) make up less than five percent of the mostly calcium carbonate shell while playing a critical role in controlling the mineral formation.<sup>5</sup> The calcium carbonate crystal (aragonite) formed in the inner surface of mollusk shells has 3000 times the

fracture resistance of synthetic aragonite.<sup>6</sup> The inner aragonite layer is also less thermodynamically stable than the calcium carbonate crystal, calcite, exterior. The control over crystal growth in the layers of the mollusk shells is controlled by the proteins that make up the organic matrix in each layer.<sup>6</sup>

The last major process of biomineralization is the formation of metal oxides. Magnetotactic bacteria's biomineralization of a magnetic iron oxide particle, magnetite, is an example of the formation of an inorganic oxide. The magnetic particle that is formed in a vesicle, magnetosome, that has a membrane nearly identical to the cytoplasmic membrane.<sup>7</sup> Proteins that promote the formation of magnetite under mild synthetic conditions are localized in the magnetosome membrane.<sup>8</sup> Magnetotactic bacteria have shown the ability to "feel" magnetic fields that allow the bacteria to more efficiently find and stay in optimum growth conditions in a vertical gradient.<sup>9</sup>

#### Biological Mediated Mineralization

The biologically controlled formation of mineral structures can also occur in a less precisely defined way known as biologically mediated mineralization.<sup>10</sup> In this type of mineral formation, minerals are formed as a by product of respiration as a terminal electron acceptor or the reductive mineral formation is used in the detoxification of a toxic, soluble metal.<sup>10</sup>

The formation of reduced biologically mediated minerals as a by product of respiration arises from either the direct electron donation to the metal or through the reduced sulfur mediated mineral formation. All organisms obtain energy by transferring electrons during respiration. Energy is extracted from the electron transport chain as electrons move "down" the chain. A terminal electron acceptor is the compound that

accepts the electron at the end of cellular respiration. Using the reduced sulfur mediated mineral formation, a number of sulfate-reducing bacteria have been found to mineralize iron sulfur particles on the exterior of the cells.<sup>11</sup> The iron reacts with the reduced sulfur to form an insoluble iron-sulfur particle. Rickard and coworkers showed that the sulfur-reducing bacteria *Desulfovibrio desulfuricans* mineralizes three different types of iron sulfur minerals; including pyrite, the largest geologic sulfur sink.<sup>11</sup> Sulfur-reducing bacteria have formed insoluble sulfide particles of Ag<sup>12</sup> and Cd.<sup>13</sup>

The formation of biologically mediated reduced metal particles through the direct reduction of metals occurs through the donation of electrons from respiration or the biologically mediated reductive detoxification. Iron(III)-reducing bacteria donate electrons to insoluble iron(III) oxide forming soluble iron(II).<sup>14</sup> This electron donation by iron(III)-reducing bacteria has been shown to reduce soluble AuCl<sub>3</sub> to insoluble Au<sup>0</sup>.<sup>15</sup>

The direct biologically mediated reduction of toxic metals for the purpose of detoxification is not completely understood in all cases; however, there have been a few systems where some light has been shed on the proteins involved. In the bacteria *Desulfovibrio fructosovorans*, technetium(VII), a highly soluble waste product of nuclear fission, was reduced with the formation of a black precipitate when hydrogen was used as an electron donor suggesting that a hydrogenase was involved in the reduction of technetium(VII).<sup>16</sup> Hydrogenases are enzymes that are able to reduce protons to H<sub>2</sub> gas or oxidize H<sub>2</sub> gas to protons. The reduction of technetium(VII) was decreased to one fifth when hydrogenase inhibitors were added or the hydrogenase was genetically knocked out.<sup>16</sup> In experiments on purified hydrogenase, the technetium(VII) was not reduced in the absence of hydrogenase. These experiments and others using

hydrogenases from *Thiocapsa roseopersicino* and *Lamprobacter modestohalophilus* reveal that hydrogenases do play a role in the direct reductive detoxification of heavy metals.<sup>17, 18</sup>

### Model for Biomineralization

To expand our molecular level understanding of biomineralization processes, the biomineralization of the iron storage protein ferritin is used as a model system. Ferritin is a unique biomineralization system that is composed of a single protein in solution which allows it to be probed by both solution techniques and surface techniques. It is a conceptually simple system with only a single protein component, which directs biomineralization of iron oxide at the protein-solution interface.

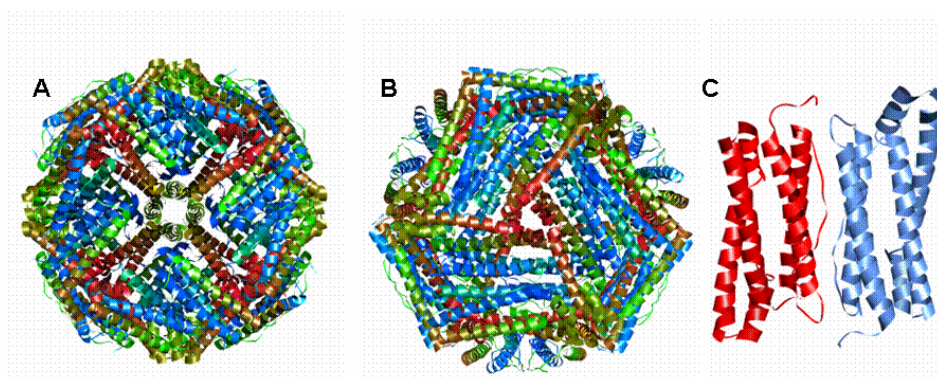


Figure 1.1. Ribbon diagrams of human ferritin. A. Four fold axis of assembled 24 subunit protein cage, B. Three fold axis assembled 24 subunit protein, C. Dimer of protein subunits arranged as in the two fold axis assembled cage.

The ferritin protein (Figure 1.1) is an assembly of subunits that forms a sphere-like architecture which incorporates all the control elements necessary for biomineralization. These include an enzymatic catalyst for molecular transformation of precursor ions, a mineral nucleation site, and an architecture that determines the overall

morphology of the biomineral. In addition, the colloidal nature of the protein cage renders the final biomineral soluble and mobile, yet biochemically isolated.<sup>19</sup>

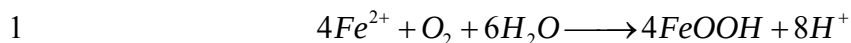
Ferritin is a spherical protein cage architecture that is nearly ubiquitous in biology where it functions to direct the biomineralization of iron as a mechanism for maintaining iron homeostasis.<sup>20, 21</sup> All ferritins are composed of 24 structurally similar subunits that assemble into a very robust protein cage of 432 symmetry.<sup>22-24</sup> The external diameter of these assembled protein cages is 12 nm and the architecture defines an internal cavity that is 6-8 nm in diameter.

Mammalian ferritin is assembled from two classes of subunits that are nearly identical in structure, but, differ in primary sequence. The two forms of ferritin subunits, H-chain (heavy) and L-chain (light), self assemble to form hetero-ferritins with different ratios of each subunit dependant on the organ of its origin. The designations H and L were made based on their differences in subunit electrophoretic mobility with molecular masses of 21 and 19 kDa, respectively.<sup>20</sup> H-chain ferritin has a conserved enzymatic site, known as the ferroxidase site, that is able to catalyze the oxidation of  $\text{Fe}^{2+}$  using molecular  $\text{O}_2$  more rapidly than L-chain. L-chain subunit has a more negative charge on the interior surface of the assembled Fn as clusters of acidic residues (glu and asp) that comprise the mineral nucleation site.<sup>25</sup> H-chain ferritin has a nucleation site that is in close proximity to the ferroxidase site with one glutamate residue shared between the two sites.

While iron is a necessary element for life, it has to be tightly regulated because of its ability to form reactive oxygen species. When iron is stored as a nanoparticle of iron

oxide (ferrihydrite) inside the protein cage ferritin (Fn), it is completely sequestered and rendered inert.

*In vivo*, ferritin is responsible for sequestering and storing toxic iron as a mineral of iron oxide (ferrihydrite) through an overall protein mediated reaction represented in reaction 1.



The biological process of iron oxidation and sequestration is considerably more complex than reaction 1 indicates and lacks a complete detailed understanding. In the presence of ferritin, potentially toxic iron is encapsulated and stored on the interior of the protein cage. The mineralized particles are electron dense and are the approximate dimensions of the interior of the protein cage (5-7 nm diameter). In the absence of Fn, an uncontrolled precipitation of bulk iron oxide results from the reaction of iron with oxygen.

The mechanism by which iron is incorporated into Fn *in vitro* can be described by four major events; iron entry, iron oxidation, iron oxide nucleation, and iron oxide particle growth. Iron entry into the cage-like architecture occurs via the channel at the 3-fold axis of symmetry.<sup>26, 27</sup> Fe<sup>2+</sup> oxidation is enzymatically catalyzed at the ferroxidase center resulting in the formation of Fe<sup>3+</sup>.<sup>28</sup> The nucleation of an iron oxide material from this insoluble ion is facilitated at the interior protein interface, and the particle grows from this nucleation site until the protein limits the particle growth.

There are two major aspects of mineral formation that control the formation of iron oxide in ferritin; nucleation and particle growth.<sup>29</sup> Nucleation is the process that brings together the starting materials to form a nucleus from which the particle grows. Particle growth is the process after nucleation that expands the particle nucleus into a

larger particle by addition of atoms. If nucleation is the limiting step in the iron-oxide particle synthesis inside ferritin; then, once a nucleus is formed the particle will quickly grow to filling the interior of ferritin. If particle growth is the limiting step in the iron-oxide particle synthesis inside ferritin; then, the iron oxide particles will be smaller than the interior of the ferritin because the nucleation events will take place faster than growth limiting the final size of the particle. Because the interior of ferritin is filled in the iron oxide formation, the mineralization is believed to be nucleation limited.<sup>30</sup>

#### Model for Synthetic Nucleation Driven Mineralization

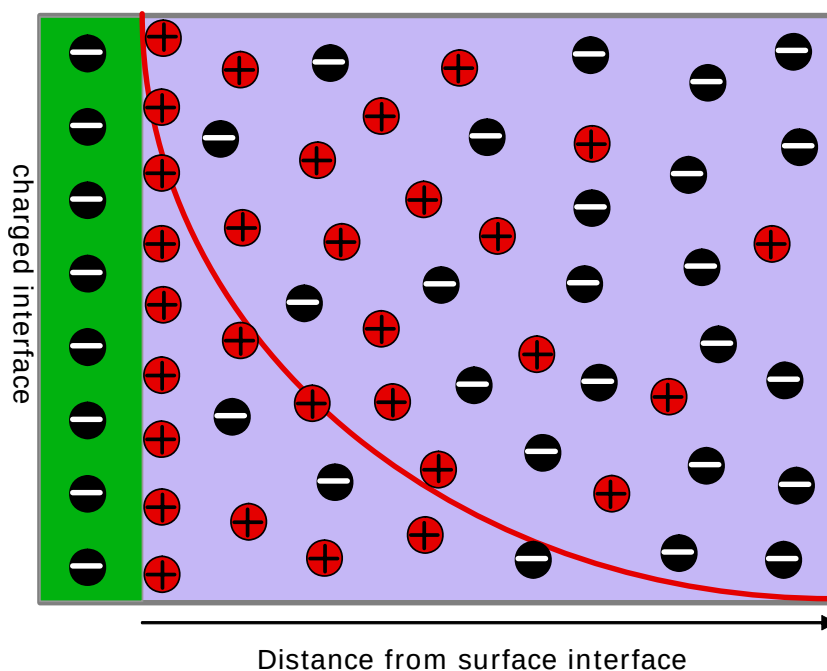


Figure 1.2. A schematic of a charged surface showing the double layer, according to the Gouy-Chapman model, and the distribution of counter ions relative to surface. The exponential decrease in counter ion concentration is shown by the red curve.

Iron oxide mineralization in ferritin suggests that the mineralization reaction may not exhibit a high degree of specificity for Fe, which would allow a range of transition metal ions to be mineralized within Fn. This can be approximated using Gouy-Chapman theory of charged surfaces (Figure 1.2). Briefly, Gouy-Chapman theory states that the

charge on a surface influences the ion distribution of electrolyte adjacent to the surface through Coulombic interactions.<sup>31, 32</sup> The surface charge potential decays exponentially as a function of distance from surface according to equation 2.

$$\psi_x = \psi_o e^{-\kappa x} \quad (2)$$

Where  $\psi_x$  is the potential at a distance  $x$ ,  $\psi_o$  is the surface potential, and  $\kappa$  is a Debye parameter measured in reciprocal distance. The distance ( $x$ ) from the surface where  $\psi_x$  is  $(1/e)\psi_o$  defines the thickness of the diffuse double layer. The width of the double layer is a function of ionic strength of the media and the charge of the interacting ion. According to this model, the very negatively charged interior surface of Fn will accumulate counter ions ( $\text{Fe}^{2+}$ ), at concentrations significantly higher than bulk concentration and in close proximity to its surface. This model for surface charged directed oxidative mineralization states that there is no specificity for Fe

Using the surface directed electrostatic model, Fn has been used as a template for the synthesis of non-native minerals including cobalt oxides ( $\text{CoOOH}$ ,  $\text{Co}_3\text{O}_4$ ),<sup>33, 34</sup> manganese oxides ( $\text{Mn}_2\text{O}_3$ ,  $\text{Mn}_3\text{O}_4$ ),<sup>30, 35</sup> amongst other materials. These mineralization reactions suggest that the electrostatic character of the interior surface of the protein cage plays an important role in mineralization. This model has provided a rational approach for synthetic mineralization that relies on the electrostatic character of the interior of a protein cage to direct material synthesis.

### Biomimetic Mineralization

Biological organisms have been the inspiration for man for a long time. The Wright brothers designed their first airplane wings after birds' wings. Biomimetic is the

abstraction of good design from nature. In biomimetic mineralization, design principles used in biological mineralizations are mimicked by synthetic chemists in an attempt to improve current synthetic practices.

Mimicking control that biological systems exhibit over the synthesis of macromolecular structures is of great interest to synthetic chemists. Biological synthesis of minerals is both simple and highly controlled. Biological mineralizations are controlled by the manipulation of biological organic materials, proteins, lipids, and sugars, to control or regulate the formation of biominerals.<sup>2</sup> A unique feature of biomineralization is the limited range of conditions that are available for the formation of the minerals. The conditions must be aqueous and within the habitable temperature range of life. These limitations are overcome, in part, by the compartmentalization of areas within organisms where distinct conditions can be created for the mineralization process.<sup>7</sup>

The past twenty years have seen a convergence of chemistry, biology, and materials science.<sup>29, 36</sup> Hierarchical assembly, a common feature in biological processes, has been recognized to be an important aspect for designed functional materials. Detailed analysis of macromolecular structures and biological processes has provided valuable information for materials scientists to use or mimic in a new generation of synthetic materials, example ferritin. Merging of biology and materials science at the level of basic science is well underway;<sup>36</sup> however, challenges remain in the development of applied materials.<sup>5, 37</sup> One of the major focuses of current research is in the area of biomimetic synthesis of materials. Biomimetic synthesis is the modeling of synthetic chemistry after a biological system. In this work, biomineralization of ferritin was used as a model for protein encapsulated materials synthesis.

### Biomimetic Mineralization in Ferritin

In a biomimetic synthesis, Mann and co-workers showed that  $\text{Fe}_3\text{O}_4$  (or  $\gamma\text{-Fe}_2\text{O}_3$ ) nanoparticles could be artificially synthesized within empty ferritin (apo-ferritin) cages at elevated temperature and pH.<sup>38-41</sup> Transmission electron microscopy (TEM) revealed homogeneously-sized nanoparticles commensurate in size with the interior diameter of ferritin ( $7.3 \pm 1.4$  nm).

Other inorganic nanoparticles can be prepared within the apo-ferritin using similar biomimetic strategies. Using similar biomimetic strategies,  $\text{Mn(O)OH}$  and  $\text{Mn}_3\text{O}_4$ ,<sup>30, 35</sup>  $\text{Co(O)OH}$  and  $\text{Co}_3\text{O}_4$ ,<sup>33, 34</sup>  $\text{Cr(OH)}_3$ ,<sup>42</sup>  $\text{Ni(OH)}_3$ ,<sup>42</sup>  $\text{In}_2\text{O}_3$ ,<sup>43</sup>  $\text{FeS}$ ,<sup>30, 44</sup>  $\text{CdS}$ ,<sup>45</sup>  $\text{CdSe}$ ,<sup>46</sup>  $\text{U}$  oxide,<sup>47</sup> and  $\text{ZnSe}$ <sup>48</sup> have all been synthesized within the protein cage ferritin. The synthesis of  $\text{ZnSe}$  is interesting because the nanoparticles were synthesized by first incubating with the protein cage with  $\text{Zn}^{2+}$  before the addition of  $\text{Se}^{2-}$ . This synthesis supports the hypothesis that the electrostatics on the interior of the protein concentrate cations on the interior, which are complexed with  $\text{Se}^{2-}$  allowing for spatially selective  $\text{ZnSe}$  particle formation on the inside the cage.<sup>48</sup> Metallic nanoparticles such as  $\text{Pd}$ ,<sup>49</sup>  $\text{Ag}$ ,<sup>50</sup>  $\text{Ni}$ ,<sup>51</sup>  $\text{Cu}$ ,<sup>52</sup> and  $\text{Co}$ <sup>51</sup> have also been synthesized within ferritin by the reduction of a metal salt by a strong reducing agent (such as  $\text{NaBH}_4$ ). In order to synthesize  $\text{Ag}$  nanoparticles on the interior of  $\text{Fn}$ , protein encapsulation was combined with  $\text{Ag}$  binding peptides identified by phage display techniques to selectively nucleate minerals.<sup>50</sup> A silver-binding peptide was genetically inserted on the interior surface of the ferritin cavity, which facilitated the reduction of silver ions to metallic silver in a spatially selective manner within the protein cage.<sup>50</sup>

### Biomimetic Mineralization in Protein Cages

Biomimetic mineralization on the interior of protein cages requires at least three essential features. 1. Pores in the protein shell that allow access of particle precursors to the interior the protein cage from the bulk solution. 2. Chemically distinct interior and exterior surfaces. 3. Stability of the protein cage under synthetic conditions required for the synthesis.

Viral coat proteins have many of features for required for biomimetic materials synthesis. Structural analysis has revealed that all viruses are composed of a protein shell architecture comprised of a limited number of subunits that package and protect the viral genome similar to the encapsulation of iron oxide by ferritin.<sup>36, 53, 54</sup> These nanoscale composites facilitate the transportation, protection, and delivery of nucleic acids between diverse chemical environments. Viral protein cages protect the nucleic acid from a range of environmental stresses including extreme desiccation, high temperatures, extremes in pH, and host defense systems, which makes the viral protein cage stable to a wide range of synthetic conditions.<sup>55, 56</sup> These systems lend themselves to the synthetic supramolecular chemistry goals of designing of systems that assemble into well defined architectures. Combining the advantages of both the biological and synthetic approaches to mineral formation can provide a pathway for the controlled assembly of functional architectures.<sup>36</sup>

Using ferritin and viral structures as inspiration has enabled the exploration of a diverse collection of protein cage architectures as bio-templates for materials applications. Viral capsids, and other protein cages, are increasingly being used as multivalent, multifunctional nano-containers.<sup>36</sup> These protein cage architectures are

precise assemblies of protein subunits that can be viewed as molecular containers providing precisely defined exterior and interior surfaces. Typically these highly symmetrical architectures are based on helical, icosahedral, cubic, or tetrahedral symmetries. In nature, these protein cage architectures serve a diversity of roles from nucleic acid storage and transport in viruses to iron mineralization and sequestration in ferritins. However, they all share the common features of being assembled from a limited number of subunits to form robust nanostructures. Our goal continues to be imparting new function to these architectures for direct application in materials synthesis, drug encapsulation and delivery, and catalysis.

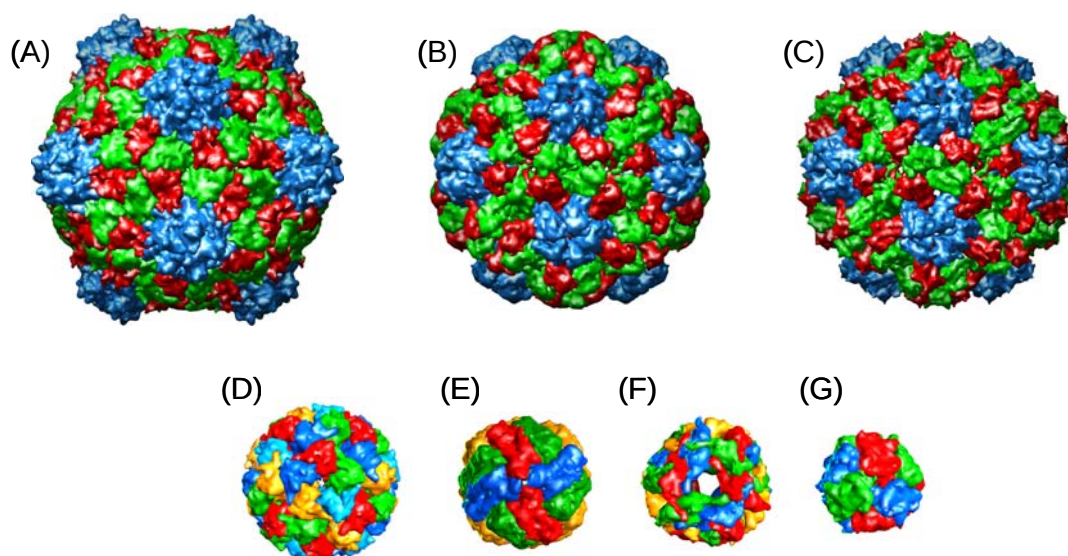


Figure 1.3. Chimera representations of protein cage architectures. A. Cowpea mosaic virus (CPMV) 31 nm in diameter; B. Brome mosaic virus (BMV), 28 nm in diameter; C. Cowpea chlorotic mottle virus (CCMV), 28 nm in diameter; D. Lumazine synthase (LS), 15 nm in diameter; E. Ferritin, 12 nm in diameter; F. small heat shock protein (Hsp), 12 nm in diameter; G. DNA binding protein from starved cell (DPS), 9 nm in diameter. Images A-C are from VIPER (<http://viperd.b.scripps.edu>).

Protein cage architectures offer simultaneous control over size, shape, biocompatibility, and the ability to alter functionality using both chemical and genetic means. However, no single protein cage architecture has all the requisite properties of

size, chemical/thermal stability, and functional group presentation. Therefore, a selection of protein cage architectures has emerged that can serve as robust size-constrained ‘spherical’ nanocontainers, 9-31 nm in diameter (Figure 1.3).<sup>24, 57-62</sup> This selection of protein cage containers has been used as platforms for the biomimetic synthesis of a wide range of materials.

Recent studies have shown considerable interest in the development of container-like protein cage architectures for material synthesis from Cowpea chlorotic mottle virus (CCMV), Cowpea mosaic virus (CPMV), Brome mosaic virus (BMV), lumazine synthase (LS), small heat shock protein from *Methanococcus jannaschii* (Hsp), and a DNA binding protein (DPS).

Conceptually, these container-like cage architectures have chemically distinct surfaces, interior and exterior, that can be manipulated in order to impart functionality by design. Remarkably, regions not directly involved in the assembly process of the protein cage architectures are generally amenable to modifications without loss of the cage-like architecture.<sup>36</sup> By combining both chemical and genetic modification of the subunits, novel function can be incorporated into different surfaces of the cage. An achievable goal is now the modification of protein cages to achieve multiple functionalities in a single architecture through encapsulation of a synthetic cargo and incorporation of targeting to a specific surface or cell.

### Using the Interior of the Protein Cage Architectures

Protein cages are large molecular structures that are amenable to both chemical and genetic manipulation. These cages assemble from protein subunits into a supramolecular architecture with a defined interior cavity that serves as an ideal

nanocontainer for encapsulation of molecular or nanomaterial cargo. Through genetic or chemical modification of the interior surfaces, protein cages can be selectively altered to control nucleation and/or sites for molecular attachment for encapsulation within the cage-like architecture. Any material encapsulated within the cage is protected from the external environment, while allowing limited access to the cargo.

Ferritin Family of Proteins: DPS proteins (DNA-binding proteins from starved cells) are members of the ferritin protein family. They have smaller cage-like architectures capable of mineral encapsulation similar to ferritins.<sup>57</sup> Dps protein cages are assembled from 12 subunits with an exterior diameter of 9 nm and interior diameter of 5 nm. Several different DPS or DPS-like proteins have been discovered in both the bacterial and archeal domains of life.<sup>63-68</sup> The true biological function of the DPS proteins has not been revealed; however, a DPS from the bacterium *Listeria innocua* (LDPS) has been used as a size constrained reaction vessel for biomimetic nanomaterials synthesis of ferrimagnetic ( $\text{Fe}_3\text{O}_4$ )<sup>69</sup> and antiferromagnetic ( $\text{Co}_3\text{O}_4$ ) nanoparticles<sup>70</sup>. The LDPS encapsulated magnetic nanoparticles reveal size dependent magnetic properties which differ from the larger ferritin encapsulated materials. The redesign and construction of a Dps protein cage with a hydrophobic interior surface, evidences the extreme versatility of protein cages.<sup>71</sup>

Icosahedral viral capsids: The cavity of icosahedral viral capsid architectures is another versatile size constrained environment where the interior surface can be used for nucleation and synthesis of nanomaterials and attachment of small molecules. The initial attempts at mineral formation using a virus as platform for materials synthesis utilized

cowpea chlorotic mottle virus (CCMV) a RNA-containing plant virus. CCMV is composed of 180 identical coat protein subunits that self-assemble to form an icosahedral cage architecture approximately 28 nm in diameter with a 24 nm diameter interior cavity. In the wild-type protein, the interior surface carries a high positive charge density, which interacts with the negatively charged viral RNA. The positively charged interior surface has been used for directing nucleation during mineralization reactions to form spatially constrained nanoparticles of polyoxometalate salts (tungstates  $\text{H}_2\text{W}_{12}\text{O}_{42}$ , molybdates, and vanadates  $\text{V}_{10}\text{O}_{28}^{6-}$ ).<sup>72</sup> Figure 1.4<sup>72</sup> A and B shows 2% uranyl acetate stained and the unstained transmission electron microscopy (TEM) analysis of the polyoxotungstate encapsulated by CCMV.

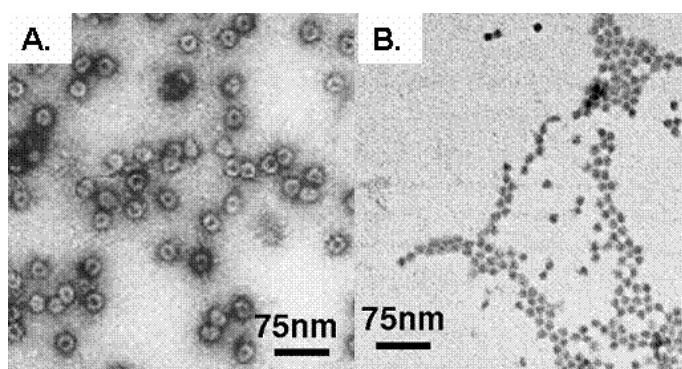


Figure 1.4. TEM characterization of CCMV with a polyoxotungstate. A. Stained TEM of CCMV mineralized with polyoxotungstate showing the protein encapsulation of electron dense cores. B. Unstained TEM of electron dense polyoxotungstate cores.

CCMV was subsequently used as the platform for other nanomaterial syntheses. Through a combination of protein design and genetic engineering, the interior surface charge of the CCMV capsid was changed from positive to negative (by glutamic acid substitution), without disrupting the ability of the CCMV cage to assemble. The interior interface of this engineered cage architecture provides allowed the nucleation of transition metal oxides, such as  $\text{Fe}_2\text{O}_3$ ,  $\text{Fe}_3\text{O}_4$ ,  $\text{Co}_2\text{O}_3$ ,<sup>73</sup> which precede though cationic

precursors that are stabilized at the interior surface of the engineered cage. A similar mineralization of  $\text{Fe}_2\text{O}_3$  has also been demonstrated using the icosahedral protein cage of lumazine synthase (LS),<sup>74</sup> which while not a virus, assembles into a unique 60 subunit icosahedral protein cage architecture. These virus like protein cages (VLP) were shown to nucleate and grow iron oxide nanoparticles within the confines of the cage.

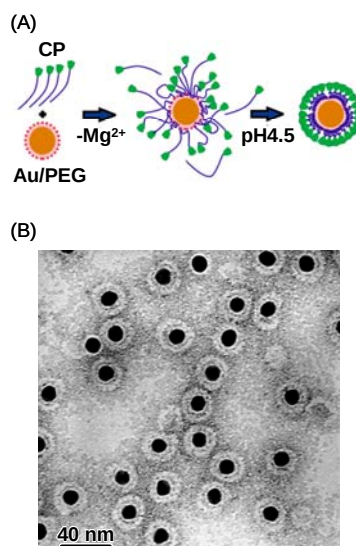


Figure 1.5. Description of the formation of BMV around preformed gold nanoparticles. A. Proposed mechanism of BMV capsid (CP = capsid protein) formation around preformed nanoparticles: The first step is electrostatic interaction leads to the formation of disordered protein - Au nanoparticle complexes. The second step is a crystallization phase in which the protein-protein interactions lead to the formation of a regular capsid, B. Transmission electron micrograph of negatively stained virus-like particles assembled around functionalized gold nanoparticles (black centers, 12 nm in diameter).

An alternative approach to the synthesis of protein cage encapsulated nanoparticles has been to direct the assembly of viral capsid subunits around a preformed nanoparticle. Subunits of brome mosaic virus (BMV) were assembled around preformed Au nanoparticles with a citrate surface layer, presumably due to complementary charge interactions between citrate and the subunit.<sup>75, 76</sup> To improve the encapsulation efficiency, Au particles coated with a carboxyl terminated

poly(ethyleneglycol) were demonstrated to reassemble and encapsulate with near perfect efficiency. The carboxyl groups interact with the subunit interface, while the poly(ethyleneglycol) provides a mimic for a disordered hydration layer in the RNA packaged BMV (Figure 1.5<sup>75</sup>).

A variety of organic chemical modifications have been demonstrated on the interior of the viral capsid using existing functional groups (cys (-SH), lys (-NH<sub>2</sub>)) as reactive sites for chemical attachment. The ability to manipulate the chemical structure allows the tailoring of unique chemical and physical properties for targeted material synthesis, drug delivery, and heterogeneous catalysis.

Small Heat Shock Protein (Hsp): Small heat shock protein, originally isolated from the hyperthermophilic archaeon *Methanococcus jannaschii*, is another cage-like protein that has been utilized for nanoparticle and molecular encapsulation.<sup>58</sup> The 24 subunit Hsp cage has an exterior diameter of 12 nm with a 6.5 nm interior cavity, and assembles with the same 432 symmetry as ferritin. Hsp differs from ferritin in that it has 3 nm pores allowing ready access to the interior of the cage. *In vitro*, the Hsp cage mineralizes iron oxide nanoparticles selectively on the interior of the cage while *in vivo* has not been shown to mineralize iron oxide.<sup>77</sup> The Hsp platform has been genetically modified to incorporate polymorph specific peptides (KTHEIHSPLLHK) for CoPt (identified from phage display) on the N-terminus of the subunit, which is thought to present itself on the interior of the cage.<sup>78</sup> This Hsp mutant was shown to direct growth of the tetragonal L1<sub>0</sub> phase of CoPt within the cage. In contrast, control reactions with Hsp lacking the CoPt peptide produced the disordered CoPt phase. The magnetic

properties of these two phases differ in that the  $L1_0$  phase is ferromagnetic at room temperature while the disordered phase is superparamagnetic.

The interior of these cage architectures provides a well-defined, constrained molecular container that isolates any encapsulated cargo from the external environment. However, these protein cage architectures are not rigid, inert reaction vessels, but rather dynamic systems that allow molecular exchange between inside and outside. The cage therefore acts not only as a size constrained environment but also as a dynamic scaffold for chemical reactivity.

Chaperonins: Chaperonins are hollow cylinder-like protein assemblies, composed of two stacked supramolecular protein rings. In biological systems, the chaperonins encapsulate denatured proteins within the cylindrical cavity to direct the protein folding process. After refolding, the chaperonins undergo a conformational change, initiated by ATP hydrolysis, and release the encapsulated protein. This process was effectively mimicked using pre-formed CdS nanoparticles which were encapsulated by the chaperonins (GroEL from *Escherichia coli* and *T.th* cpn from *Thermus thermophilus* HB8) and released from the ring upon ATP hydrolysis.<sup>79</sup> The encapsulated CdS exhibited enhanced photoluminescent stability suggesting that the protein ring provides additional stability to the nanoparticles.<sup>80</sup>

#### Using the Exterior Surface of Protein Cages for Multivalent Ligand Display

The exterior surfaces of protein cage architectures provide an exposed platform for chemical and genetic functionalization. These modifications include taking advantage of endogenous or genetically introduced functional groups (thiols, phenols,

amines, and carboxylic acids) as attachment sites for multivalent presentation of attached ligands. These reactive sites can be used to couple organic and inorganic ligands such as organic molecules, metal coordination complexes, metal colloids, peptides, and large proteins. The high symmetry of these protein cage architectures is advantageous because every functional group (with or without an attached ligand) is presented on each subunit in a spatially defined manner over the entire cage resulting in a functional nanomaterial with highly controlled labeling.

Icosahedral Viral Capsids: A range of icosahedral viral particles have emerged as robust platforms that can be chemically and genetically modified for multivalent ligand display on the exterior surface. CCMV and Cowpea mosaic virus (CPMV) are examples of icosahedral cage architectures that have been utilized as platforms for multivalent ligand display of modifications on the exterior surface.

Genetic modification of CCMV subunits results in multivalent presentation of 180 copies of any engineered functional group. These engineered functional groups can be used as attachment sites for ligand presentation on the exterior surface. It has been shown that amines, carboxylic acids, and thiol groups naturally present or genetically inserted on to the exterior surface of CCMV can react with activated small molecules. Using activated fluorescein (maleimide or N-hydroxysuccinimide), quantitative labeling of these sites can be achieved without disrupting the supramolecular cage architecture.<sup>81</sup> In addition, small peptides (11 amino acids) can be chemically linked to the exterior surface of CCMV, illustrating that chemical modification is a generic approach to surface modification of the virus.<sup>81</sup>

Other viruses, such as CPMV are composed of two subunit types allowing for site discrimination within the icosahedral cage. CPMV capsid is a 31 nm exterior diameter icosahedron formed from sixty copies of one of these subunits that form the 12 pentameric regions and 60 copies of the other subunit that form the 20 pseudo-hexameric regions of the icosahedron.<sup>59</sup> By introducing different functional groups onto the subunits, ligands can be spatially arranged on the CPMV architecture allowing or preventing ligand interaction.<sup>81-87</sup> CPMV provides an illustration of control over both the spatial distribution and multivalency on a highly symmetric platform.

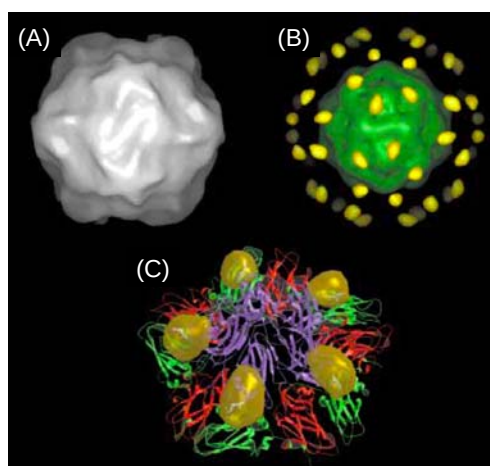


Figure 1.6. Cryo electron microscopy analysis of derivitized CPMV mutant in which cysteine was inserted between residue 98 and 99 (CPMV-CYS). A. Three-dimensional reconstruction of CPMV particles at 29 Å resolution labeled with 1.4 nm gold nanoparticles, B. Difference electron density map generated by subtracting density of CPMV X-ray structure from the density shown in A. Since the computed native CPMV density was made from only protein, the nucleic acid (shown in green) is visible in the difference map as well as the gold particles, C. A pentameric section of the difference electron density map around the five-fold axis superimposed on the atomic model of CPMV showing that the gold is attached at the site of the CYS mutation.

A conducting network of around CPMV particles was created by linking surface attached Au nanoparticles (2 or 5 nm) (Figure 1.6<sup>87</sup>) through conducting organic molecules.<sup>87</sup> Individual CPMV particles, decorated with both Au nanoparticles and the

conducting organic matrix, were placed onto a self-assembled monolayer of undecanethiol on an Au/mica substrate with a dithiol-functionalized conducting molecule inserted. The conductance of these individual viral particles was then measured using a scanning tunneling microscope. The current vs. voltage (I/V) curves for the viruses showed a dramatic increase in current when the conducting organic molecules were placed on the viral capsid.<sup>87</sup> If the organic molecules could no longer bridge between the gold particles, the I/V curves were the same between the virus particles with and without the conducting organic ligands. The exterior surface of CPMV has been used as a platform for extensive chemical modification and attachment of ligands including redox active moieties, antibodies, peptides, fluorophores, poly(ethylene glycol), and DNA. Many of these attachments have utilized coupling via endogenous amines and thiols.

Genetic or chemical inclusion of peptides to the exterior of viral protein cages is a novel strategy for applications including vaccine development,<sup>88-91</sup> and cell targeting.<sup>92</sup> For example, it has been shown that an epitope derived from human immunodeficiency virus type (HIV-1) can be expressed on the surface of particles of CPMV and this construct can elicit the production of neutralizing antibodies in mice.<sup>90</sup>

Others have modified polyoma virus derived cages (VLP) with ligands such as foreign proteins, peptides, fluorophores and utilized them for cell targeting for *in vivo* imaging.<sup>93-96</sup> The trajectories of fluorescent-labeled VLPs on cell surfaces were successfully tracked at the single-particle level using total internal reflection fluorescence microscopy. Development of this technique would provide a powerful tool to understand cell biology.

Using the exterior surface to target foreign pathogens is of considerable interest in the development of new MR contrast agents, drug delivery vehicles, detection, and imaging. Combining the ability to functionalize the exterior surface with the capacity to encapsulate an imaging agent and/or therapeutic agents is of great importance in designing a multifunctional platform.

Small Heat Shock Protein: Hsp has also proven to be a versatile nanoscale platform for genetic and chemical modification.<sup>77, 97, 98</sup> The Hsp has large (~ 3 nm) pores at the 3-fold axes thus allowing free exchange between the interior and bulk solution. Endogenous lysine residues and genetically engineered cysteines have been chemically modified for the attachment of fluorescent probes and cell specific targeting antibodies. An anti-CD4 monoclonal antibody conjugated to fluorescently labeled Hsp mutant enabled targeting of the Hsp to CD4<sup>+</sup> lymphocytes within a population of splenocytes.<sup>98</sup> In addition, cancer cell specific targeting peptides have been genetically incorporated onto the exterior of Hsp and shown to effectively target tumor cells *in vitro*. These cell specific targeted protein cages would be useful vessels to deliver imaging and therapeutic agents to desired tissue and highlight the potential biomedical importance of these architectures.

### Research Goals

By drawing on the strengths of biological systems and synthetic chemistry techniques, one goal of this research is to develop protein cages as feasible platforms for catalysis. Proteins assemble into secondary, tertiary, and quaternary structures that can be manipulated to direct the assembly of inorganic materials. The quaternary structure

can then be directed to assemble at the surface of a cell or substrate. The control and precision exhibited by biological systems in the assembly of protein cage architectures combined with the synthetic techniques to have the potential to develop a new class of composite materials that have characteristics that are not seen when using traditional synthetic methods. Understanding the processes of biomineral formation allows the control seen in biological systems to be utilized by synthetic chemists. Another goal of this research is to develop the general synthetic routes that can be applied to other materials and systems for the synthesis of materials beyond the scope of this dissertation. These routes can be used for the continued development of the protein cages as platforms for nanomaterial synthesis.

In this chapter, biomimetic synthesis and its potential for materials synthesis was introduced. Also, protein cages as versatile platforms for the synthesis of functional materials were presented. Fn has been widely used as a platform for the synthesis of nanomaterials under the hypothesis that the electrostatically distinct interior and exterior surface is of critical importance to the mineralization on the interior of Fn. In the next chapter, a protein cage system (Hsp) without an electrostatically distinct interior and exterior will be discussed in comparison with Fn to probe the role of the electrostatically distinct interior surface in mineralization of a reduced noble metal nanoparticle. The first hypothesis tested was do the processes of biomineralization would still occur in systems without an electrostatic driving force and if so to gain insights into these processes. Several different protein cages were used to determine if protein cages could be used as platforms for the mineralization of reduced noble metal nanoparticles and the effect the protein cage has on the mineralization. A second hypothesis tested is can the hierarchical

protein cage and ring assemblies direct the mineralization and act as viable platforms for active catalysis. In the third chapter, the catalytic activity of the protein encapsulated noble metal nanoparticles were determined to study the protein cages architectures platforms for nanoparticle catalytic materials. The fourth chapter presents the use of a protein cage as a drug delivery vehicle and the detailed effects of the protein cage architecture on the photogenerated reactive oxygen species. This research set out to determine if the hierarchical assembly allows for the catalytic release of reactive species produced by a catalytic moiety attached to the cage. The fifth chapter presents the use of a hydrogenase enzyme as the platform for mineralization of reduced metal nanoparticles. In the mineralization, the site of enzymatic reduction by the protein determines point of mineral growth. Chapter 2 and 4 are descriptions of biomimetic synthesis of biologically mediated mineralization and Chapter 3 is the characterization of the catalytic activity of these mineralizations where the products of the catalysis are stable. Chapter 4 is the characterization of the catalytic production of a reactive species associated with the protein cage. These studies highlight the use biomimetic synthesis and the subsequent catalytic activity of hierarchical protein structure encapsulated catalysts.

### Introduction

Biological systems display precise control over the synthesis of hard materials or minerals. These hard materials,  $\text{CaCO}_3$ ,  $\text{CaPO}_4$ , etc., when combined with soft materials, protein, lipids, carbohydrates, are manipulated for a wide range of functions. Bones are the product of a biological mineralization that is used to provide structure and protection for animals. Egg shells are biomineralized to provide protection to the developing fetus. Intracellular magnetic particles enclosed in lipid bilayer membranes act as compasses for magnetotactic bacteria guiding them to and helping to maintain optimal position in vertical nutrient gradients.<sup>9, 99</sup> With all of these examples of biomineralization, the synthesis of the products must be controlled in both morphology and location within the organism. For example, magnetotactic bacteria that synthesize a non-magnet particle will not have the ability to “sense” the magnetic field from the earth. Similarly, bone mineralization that is not controlled and forms in the soft tissue fusing the skeleton has devastating results (fibrodysplasia ossificans progressiva). These examples reveal the importance of controlling biological mineralization. Mimicking the control over mineralization seen in biology is the driving force behind synthetic chemists’ interest in biomineralization. Biomimetic mineralization is the synthesis of minerals using processes that are modeled after biological systems.

The control over mineral growth displayed in biological systems is derived from manipulating the interaction of soft materials, proteins and lipids, and hard materials, minerals.<sup>29</sup> Soft materials provide a platform for the formation of a mineral nucleus,

nucleation, from which the final particle grows. Interactions of soft materials with the mineral nucleus are one way biological system manipulate mineral growth.<sup>3</sup> This interaction along with the environment surrounding the growing mineral is where biological systems are able to influence where minerals are grown,<sup>8, 100</sup> what minerals are grown,<sup>6</sup> and the size and shape of the mineral.<sup>1, 2, 4</sup> Detailed analysis of these macromolecular structures and their associated biological processes has provided valuable insights for materials scientists to utilize or mimic in a new generation of synthetic materials.<sup>3, 29</sup>

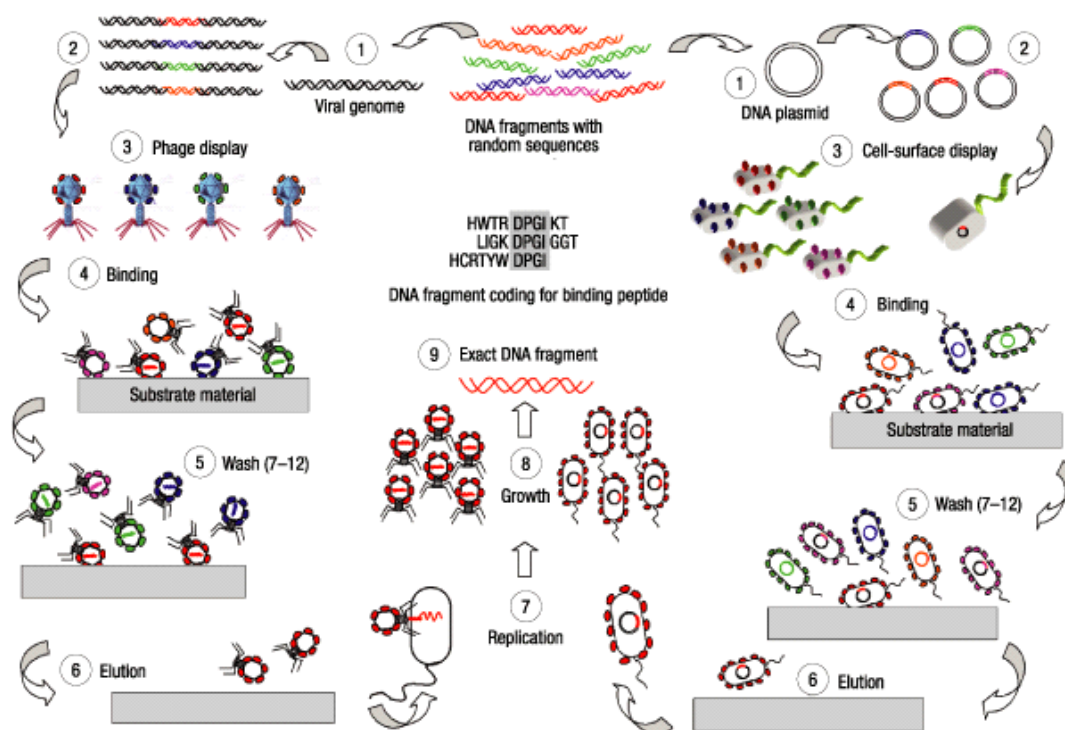


Figure 2.1. Description of the use of phage display and cell display for identifying substrate binding peptides. 1. Isolation of virus or cell site for insertion of random DNA fragments to be displayed on surface, 2. Insertion of random DNA fragments into the virus or cell DNA, 3. Expression of random DNA fragments on phage of cell surface, 4. Binding of phage or cell to surface, 5. Washing of surface to remove virus and cells with non-specific and weak binding peptides, 6. Elution of tight binding virus and cells from surface, 7. Replication of cells and virus, 8. Growth of cells and virus, 9. Extraction of DNA and identification of peptide sequence.

One approach to mimicking the control displayed by biological systems has been to employ material binding peptides.<sup>101, 102</sup> These peptides, often identified through phage display techniques, act as a nucleation site from which a specific mineral can be formed.<sup>103</sup> Figure 2.1<sup>104</sup> shows how surface binding peptides are isolated using phage and cell display techniques.<sup>104</sup> Development and use of surface binding peptides reveals the utility of controlling the nucleation of the mineral.<sup>102, 105</sup> Peptides that will strongly bind to a mineral surface can be used to influence the crystal growth; however, the nature of the interaction of between biological minerals and proteins is not well understood. There is no indication that the nucleation site must specifically bind to the final form of the mineral. Nucleation sites can be a morphologically distinct starting point which is poised to influence the synthesis of the mineral by arranging the bound metals to facilitate the growth of the final mineral.<sup>6</sup>

Mineral specific peptides have been used to aid in the synthesis of a number of materials that would not be formed in the absence of the peptide. Belcher and coworkers have used phage display techniques to synthesize CoPt,<sup>103</sup> FePt,<sup>37</sup> ZnS,<sup>37</sup> CdS,<sup>106</sup> and Au-Co<sub>3</sub>O<sub>4</sub>.<sup>5</sup> Manipulating the nucleation site of the mineral has proven to be an effective way to influence material and the morphology of the material formed.

In biological systems, the most common forms of biomineralizations are inorganic salts and oxide materials.<sup>3, 6</sup> Biological formation of metal particles is not technically a mineralization; it is a biologically mediated mineral formation.<sup>10</sup> Biologically mediated reduction can take a soluble toxic metal or metalloid to an insoluble reduced metal particle that limits or removes the toxicity to the cell. Gold,<sup>15</sup> silver,<sup>12</sup> and palladium<sup>10</sup> have been shown to be reduced into nanoparticles by cells to

reduce the toxicity both inside and outside the cell. While these systems have not been widely studied, the potential for detoxification of toxic, soluble metals is enormous.<sup>13</sup>

The general synthesis of noble metal nanoparticles is the reduction of noble metal ions into particles in the presence of a stabilizing or passivating agent, which prevents particle aggregation. These nanoparticles are used as catalysts in a wide range of reactions from methanol oxidation in fuel cells<sup>107</sup> to the hydrogenation of aldehydes and vinylic compounds.<sup>49, 108, 109</sup> Using a biological template, the synthesis and the catalytic properties of noble metal nanoparticles can be manipulated in a way that standard nanoparticle approaches can not match.

Protein cages are biological platforms ideal for the synthesis of reduced metal nanoparticles. The structure of the protein cage provides a unique platform for the mineralization for materials. By synthesizing a nanoparticle on the interior of a protein cage, the protein cage is acting like a passivating layer that prevents particle aggregation; but, does not coat the entire surface of the nanoparticle the way a typical passivating layer does. Table 2.1<sup>75, 110-117</sup> lists the particle size and passivating layer used in Pt nanoparticle synthesis. In this current study, small heat shock protein (Hsp),<sup>58</sup> horse-spleen ferritin (Fn), *Listeria innocua* DPS (LDPS),<sup>118</sup> and *Pyrococcus furiosus* DPS-like (PfDPS)<sup>65</sup> are used as the platform for noble metal nanoparticle synthesis. Hsp and ferritin are both 24 subunit 12 nm diameter protein cages. However, the structure of Hsp is much more open allowing greater access to interior than the ferritin. The DPS proteins from *Listeria* and *Pyrococcus* are much smaller protein cages having a 9 nm external diameter. These cages were chosen as the platforms for the mineralization of

nanoparticles to determine the effect that the protein cage has on the particle formation and the catalytic activity of the synthesized nanoparticles.

| Size (nm) | Passivating layer                                      | Author    |
|-----------|--------------------------------------------------------|-----------|
| 2.8-1.9   | Vinyl Polymers with amide side chains                  | Chen      |
| 4.8-7.1   | polyvinylpyrrolidone                                   | Narayanan |
| 1.9-3.0   | organic thiols                                         | Eklund    |
| 4.0-5.5   | polyvinylpyrrolidone or polyacrylic acid               | Teranishi |
| 4.0-11.0  | Sodium di-2-ethyl hexylsulfosuccinate reverse micelles | Chen      |
| 1.02-2.2  | poly(1-vinylpyrrolidine-co-acrylic) acid               | Tu        |
| 10.0-50.0 | nanodendrites                                          | Song      |
| 3-3.5     | carbodawax                                             | Brugger   |
| 1.2-1.6   | poly(amidoamine) dendrimers                            | Esumi     |

Table 2.1. Table of particle size and passivating layers used for Pt nanoparticle synthesis.

## Materials and Methods

### Materials

All materials were obtained from Sigma-Aldrich and used as received without further purification. All water used was purified through a Nanopure system to 18.2 MΩ resistivity.

### Transmission Electron Microscopy (TEM)

TEM data were obtained on a Leo 912 AB, with  $\Omega$  filter, operating at 100 keV. The samples were concentrated using microcon ultrafilters (Microcon YM-100) with 100 kDa nominal molecular weight cutoff and transferred to carbon coated copper grids. Samples were imaged unstained or negatively stained with 2% uranyl acetate. Electron

diffraction patterns were collected on samples and the d-spacings were calculated and compared with the powder diffraction file for Pt after calibrating the diffraction camera with an Au standard.

#### Dynamic Light Scattering (DLS)

DLS measurements were carried out on a Brookhaven Instrument Corporation 90-PALS at 90 degrees using a 661 nm diode laser, and the correlation functions were fit using a non-negatively constrained least-squares analysis.

#### Size Exclusion Chromatography (SEC)

SEC was performed on a Biologic Duo-Flow fast protein liquid chromatography system equipped with a Quad-Tec UV-Vis detector (BioRad) or an ÄKTA Pharmacia 900 using a superose 6 SEC column (Amersham Pharmacia) size exclusion chromatography column.

#### Hsp Expression and Purification

One liter cultures of *E. coli* (BL21(DE3) B strain) containing pET-30a(+) WtHsp plasmid were grown overnight in M9 salts + 10 g NaCl + 10 g Bactotryptone + kanamycin medium (37 °C, 220 rpm). Cells were harvested by centrifugation 3700×g for 20 minutes (Heraeus #3334 rotor, Sorvall Centrifuge) and re-suspended in 80 mL of 50 mM MES 100 mM NaCl, pH 6.5. Lysozyme, RNase A, and DNase I were added to final concentrations of 0.041 mg/mL, 0.055 mg/mL and 0.08 mg/L respectively and incubated for 30 minutes on ice. The sample was French pressed (American Instrument Co., Inc) and sonicated (Branson Sonifier 250, Power 4, Duty cycle 50%, 3 × 5 minutes with 5 minute rest intervals). Bacterial cell debris was removed via centrifugation for 45

minutes at 12,000×g. The supernatant was heated for 10 minutes at 60 °C and centrifuged for 20 minutes at 12,000×g thereby removing many heat labile *E. coli* proteins. The remaining cell extract was purified by gel filtration chromatography (Superose-6, Amersham-Pharmacia; BioRad Duoflow). The 16.5 kDa subunit molecular weight (Hsp16.5) was verified by SDS poly-acrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry (Waters MicroMass Q-TOF). The assembled protein was imaged by transmission electron microscopy (TEM) (LEO 912 AB) (stained with 2% uranyl acetate on formvar carbon coated grids), and analyzed by dynamic light scattering (DLS) (90 plus Brookhaven Instruments). Protein concentration was determined by absorbance at 280 nm divided by the published extinction coefficient (9322 M<sup>-1</sup>cm<sup>-1</sup>).

#### Listeria Dps Expression and Purification

Primers were designed to amplify the coding region of the Dps from *Listeria innocua* (Genbank Accession AJ244014). The forward primer (5' ggaagatacatatgaaaacaatcaactcag 3') included an *NdeI* restriction endonuclease site directly upstream of the start codon for insertion into a pET-30a(+) expression vector (Novagen, Madison, WI). The reverse primer (5' gatggatccttattctaattggagcttttcc 3') included the stop codon for the gene and a *BamHI* site, again for insertion.

Lyophilized *Listeria innocua* bacteria were purchased from ATCC (#51742). Genomic DNA was extracted from the cells by mechanical lysis of the cells and a routine phenol/chloroform/isoamyl alcohol extraction followed by an ethanol precipitation. The DNA pellet was resuspended in 50 µl of sterile ddH<sub>2</sub>O and 1 µl was used in a standard polymerase chain reaction (PCR) with the primers above to amplify the *Listeria innocua* Dps. The resulting PCR product was digested with *NdeI* and *BamHI* restriction

endonucleases and cleaned up with a Qiaquick PCR purification kit (Qiagen). This product was ligated into a *NdeI/BamHI* linearized pET30a vector that had been treated with calf-intestinal phosphatase. The ligation was transformed into XL-2 blue ultracompetent *E. coli* (Stratagene) and screened for the presence of insert. The insert sequence was confirmed by sequencing using Big Dye Termination protocols on an ABI 310 automated capillary sequencer (Applied Biosystems).

The positive DNA was transformed into BL21 *E. coli* (Novagen) for protein expression. Colonies were screened for expression after IPTG induction and analyzed on a Coomassie stained SDS-PAGE gel. Cultures of *E. coli* were grown, in 1.0 L of LB media overnight at 37 °C. Cells were harvested by centrifugation at 12,000×g for 30 minutes.

The harvested *E. coli* pellet from 1.0 L of culture was suspended in 30mL of lysis buffer (50mM Tris, pH 8.0). Lysozyme (1 mg/ml) and DNase (1 mg) were added and incubated for 30 minutes at room temperature. The suspension was sonicated for 10 min in 30 sec intervals and then centrifuged at 12,000×g for 20 minutes at 4 °C. The supernatant was heated at 65 °C for 10 minutes then cooled rapidly on ice before centrifuging again at 12,000×g for 20 minutes at 4 °C. The supernatant was then applied to an anion exchange column (Mono-Q) equilibrated with the MES buffer (50 mM, pH 6.5) and the protein was eluted using a salt gradient (0 to 2.0 M NaCl in 50 mM Tris, pH 6.5). Fractions eluting from the anion exchange column were pooled and loaded onto a size exclusion chromatography column (Superose 6) equilibrated with MES buffer (50 mM MES, 100 mM NaCl, pH 6.5). Elution of the protein was monitored at 280 nm.

Protein concentration was determined by the molar absorbance at 280nm of  $2.59 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ .

### Pyrococcus Dps Expression and Purification

Cultures of *E. coli* were grown, in 1.0 L of LB media overnight at 37 °C. Cells were pelleted from one-liter cultures by centrifugation 3700×g and resuspended in 30 mL of lysis buffer (50 mM MES, 100 mM NaCl, pH 6.5, 0.002 mg/mL DNaseA, 0.05 mg/mL RNaseI, 1 mg/mL lysozyme). The slurry was French pressed, sonicated (3 × 5 min), and centrifuged to remove cellular debris. The resulting supernatant was heated at 85 °C for 10 min, cooled on ice, and then centrifuged to remove denatured proteins. The recovered supernatant was passed through a 0.2 mm filter and loaded onto a Superose 6 size exclusion column (Amersham Biosciences) equilibrated with MES buffer (50 mM MES, 100 mM NaCl, pH 6.5). Elution of the protein was monitored at 260, 280, and 350 nm.

### Genetic Engineering of Pt-Hsp

*Methanococcus jannaschii* genomic DNA was obtained from ATCC (43607). As described previously, the gene encoding the small Hsp (WtHsp) was polymerase chain reaction (PCR)-amplified and cloned into NdeI/BamHI restriction sites of the PET-30a(+) vector (Novagen, Madison, WI) for expression of the full-length protein with no additional amino acids. PtBP3

(5'gtttaactttaagaaggagatatacatatgttcataaggatcctcattaaaaccattatttgaaagaatgtttaaagagtttttgc 3')

was the primer used to insert the peptide IGSSLKP into the N-terminus of WtHsp. These inserts were subsequently ligated into an alkaline phosphatased, BamHI digested vector overnight at 17 °C and transformed into XL-2 ultracompetant *E. coli* (Stratagene,

La Jolla, CA). Transformants were screened for the presence of the IGSSLKP insert and confirmed by sequencing the PCR amplified product on an ABI 310 automated capillary sequencer with BigDye Chain termination sequence technology (Applied Biosystems, Foster City, CA).

#### Horse-Spleen Ferritin Demineralization

Native ferritin was dialyzed into 1 L of 100 mM acetate with EDTA pH 4.5 while gently bubbling nitrogen gas to prevent reoxidation of reduced iron. After solution is deoxygenated, 1-2 mLs of thioglycolic acid was added. After 2 hours of dialysis, the dialysis tubing was transferred to a new liter of acetate/EDTA buffer with thioglycolic acid. The native ferritin solution should change from orange to clear. After dialyzing against 3 total liters of acetate/EDTA buffer with thioglycolic acid, the dialysis tubing was transferred to 1 liter of 100 mM Tris, 100mM NaCl pH 8.5. This step was repeated as many times as necessary until the solution no longer smells like thioglycolic acid.

#### Synthesis of Iodoacetoamido-1,10-phenanthroline

Iodoacetic acid (3.72 g, 24 mmol) and dicyclohexylcarbodiimide (DCC) (1.86 g, 54 mmol) in 60 mL of ethyl acetate were stirred together for 3 hrs.<sup>119</sup> The solution was then filtered to remove the resulting urea and then solution was concentrated to dryness by rotary evaporation. The residue was redissolved in 30 mL of acetonitrile and added to 40 mL of acetonitrile containing 5-amino-1,10-phenanthroline (1.6 g, 8.6 mmol). The solution was stirred overnight at room temperature. The product was collected by centrifugation and washed once with cold 5% sodium bicarbonate, once with cold water,

and dried in a desiccator under vacuum overnight. The product was confirmed by mass spectrometry.

#### Labeling of Hsp G41C With Iodoacetoamido-1,10-phenanthroline

Hsp G41C (5 mg,  $1.3 \times 10^{-5}$  mmol) in 5 mL of deoxygenated buffer (25 mM HEPES 50 mM NaCl pH 8.0) was reacted with 0.55 mg of iodoacetoamido-1,10-phenanthroline (Iphen) (0.075 mL, 0.55 mg, 0.002 mmol), from a 20 mM stock of Iphen in DMF, at 40 °C for 2 hours. The reaction was purified by SEC (50 mM MES 100 mM NaCl pH 6.5) after the column was washed with EDTA and dithionite to remove free metals, and equilibrated with buffer (50 mM MES 100 mM NaCl pH 6.5).

#### Sonicators Used for Mineralization

Two bath sonicators were used in the mineralization of Pt alloys. A Branson B200 Ultrasonic Cleaner and a Fisher Scientific Ultrasonic Cleaner FS20. A Branson Sonifier 250 was used as the tip sonicator.

#### Photo-mineralization Light Source

A Xe arc lamp (175 W, Lambda-LS, Sutter Instruments) was used as the light source. The light flux was measured using an Extech Instrument EasyView light meter to be 511,700 lux. IR radiation was removed by passing the beam through a 10 cm water filter and the UV was removed using UV-absorbing glass. The temperature was controlled using a circulating water bath.

## Results

### Optimal Mineralization Conditions

Below is a list of the optimal conditions for the synthesis of reduced noble metal catalysts encapsulated with in varying protein cage architectures.

Optimal Platinum Mineralization Conditions in Hsp G41C: The optimal mineralization conditions for the mineralization of platinum nanoparticles inside Hsp G41C was Hsp G41C at a final concentration of 1 mg/mL in buffer (25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5) at 65°C with 5x DMAB and  $\text{K}_2\text{PtCl}_4$  in a 20 mM stock solution continuously added over 15 minutes. The mineralization reaction was not specific for the interior of Hsp G41C with a significant amount (1/3) on the exterior.

Optimal Platinum Mineralization Conditions in Hsp With Pt Binding Peptide Inserted on the Interior: The platinum mineralization on the interior of Pt-Hsp was never at a point where there was an optimum reaction condition. Each mineralization had significant bulk mineral formation and nanoparticle growth on the exterior of the protein cage.

Optimal Platinum Mineralization Conditions in Hsp With a Phenanthroline Covalent Attached: The optimal mineralization of Hsp Phen was the photo-mineralization of 1000  $\text{Pt}^{2+}$  with a 4-fold excess citrate added continuously over 45 minutes to Hsp Phen at 0.5 mg/mL final concentration in buffer (50 mM MES pH 6.5) at 40°C. UV/Vis and SEC confirm the color change indicative of platinum metal nanoparticle formation. SEC and DLS confirm that the mineralization is on the interior of the protein cage. TEM analysis reveals no detectable particles

mineralized indicating the formation of reduced platinum clusters of a size too small to be imaged by TEM mineralized in association with the Hsp Phen.

Optimal Platinum Mineralization Conditions in WtHsp: The optimal conditions for the mineralization of platinum nanoparticles inside WtHsp was WtHsp at final concentration of 1 mg/mL in buffer (25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5) at 65°C with 5x DMAB and  $\text{K}_2\text{PtCl}_4$  in a 20 mM stock solution continuously added over 15 minutes. The mineralization reaction was nearly (95%) specific for the interior of WtHsp with minimal bulk mineral formation.

Optimal Platinum Mineralization Conditions in Fn: The optimal conditions for the mineralization of platinum nanoparticles inside Fn was Fn at 1 mg/mL final concentration in buffer (25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5) at 65°C with 5x DMAB and  $\text{K}_2\text{PtCl}_4$  in a 20 mM stock solution continuously added over 15 minutes. The mineralization reaction was specific for the interior of Fn with no bulk mineral formation.

Optimal Platinum Alloy Mineralization Conditions in Fn: The final optimized conditions for mineralizing Fn with Zn or Ni-Pt 1:3 is Fn (10 mg/mL in 150 mM NaCl) added to buffered solution (200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5) with a final concentration of 1 mg/mL at 15°C using a tip sonicator (20% duty, 1 power). 20 mM metal ( $\text{Zn}^{2+}$  or  $\text{Ni}^{2+}$  and  $\text{PtCl}_4^{2-}$ , mixed before addition) was added in of aliquots of 25  $\mu\text{L}$  metal with 10 minutes in between aliquot additions with half of the metal added each hour while adding 300  $\mu\text{L}$  of 3 mg/mL DMAB over 2 hours using a syringe pump.

### Optimal Palladium and Palladium Alloy

#### Mineralization Conditions in Fn: The optimized conditions for mineralizing Fn

with Pd or Zn-Pd 1:3 is 1 mg/mL (10 mg/mL in 150 mM NaCl) in 10 mM HEPES 150 mM NaCl pH 8.5 at 15°C using a tip sonicator (20% duty, 1 power). 20 mM metal ( $\text{PdCl}_4^{2-}$  or  $\text{ZnCl}_2$  and  $\text{PdCl}_4^{2-}$ , mixed before addition) was added in of aliquots of 25  $\mu\text{L}$  metal with 10 minutes in between aliquot additions with half of the metal added each hour while adding 300  $\mu\text{L}$  of 3 mg/mL DMAB over 2 hours using a syringe pump.

### Optimal Platinum and Platinum Alloy

#### Mineralization Condition in PfDPS: The product of the mineralizations of Pt and

Zn-Pt nanoparticles using PfDPS were never specific for the interior of the protein cage under varying buffer, salt, sonication, and pH conditions. PfDPS was stable under a broad range of reducing conditions. All the conditions attempted were dominated by the formation of aggregates and precipitation. The mineralizations could be manipulated to form minimal bulk precipitation; however, the analysis revealed that the minerals were not forming on the interior of the protein cage.

### Optimal Platinum and Platinum alloy

#### Mineralization Condition in LDPS: The LDPS protein cage was not stable under

a broad range of reducing conditions required for the optimization of the platinum mineralization. The mineralization reactions were cloudy after the reaction with temperatures higher than 40°C. Using conditions where the protein was stable, the platinum and platinum alloys were not specific for the interior of the protein.

### Hsp as a Platform for Nanoparticles Synthesis

In most solution-based colloidal systems, a passivating layer is needed to keep the particle in solution and to prevent aggregation. However, in the case of catalytically active colloids, the passivating layer interacts with the surface of the particle decreasing substrate access to the catalyst which can result in the loss of activity. By using a platform with a defined structure and clear interior cavity, such as the protein cage architecture, a particle smaller than interior of the cage can be grown on the interior surface giving a catalytic particle with a minimum amount of surface coating shown in figure 2.2. This in turn maximizes the exposed surface area and may result in an increase activity of the catalyst.

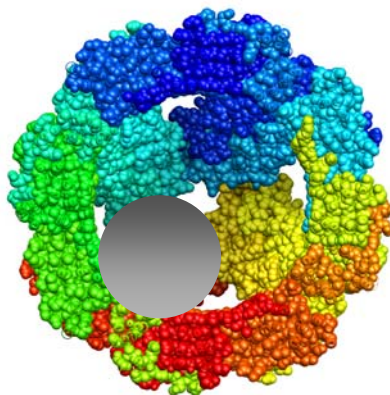


Figure 2.2. A cut-away depiction of Hsp with a nanoparticle in the interior cavity showing the minimal interaction between the nanoparticle and the protein cage.

Hsp was chosen as a synthetic platform because of its many native attributes which lend themselves to the synthesis of highly catalytic nanoparticles. The native structure of Hsp has a quaternary structure that allows for ample access of substrates to the interior through the pores of the protein shell.<sup>58</sup> In order to maximize the catalytic activity of a particle on the interior of the protein cage, substrates must be able to

efficiently interact with the surface of the particle and the products must be allowed to move away from the reactive surface. Allowing unhindered access to and from the reactive particle surface, the catalytic activity of the nanoparticle can be maximized by reducing transport limitations. By synthesizing a nanoparticle on the interior surface of Hsp the nanoparticle is not completely coated by the protein, which would decrease the catalytic activity of the nanoparticle.

Optimization of Pt<sup>0</sup> mineralization in Hsp G41C: Cysteines have been shown to bind to platinum ions which could allow cysteines to act as nucleation sites from which a platinum nanoparticle could grow. To that end, site directed mutagenesis is a powerful tool to introduce reactive site for specific chemical transformations. Using site directed mutagenesis, a specific nucleation site was engineered on the interior surface of the protein cage by replacement of a glycine with a cysteine residue at position 41. WtHsp has no endogenous cysteines which allows for the insertion of a specific cysteine without the background reactivity of the endogenous cysteines to consider or remove by mutagenesis.<sup>58</sup> In this mutant (Hsp G41C), the glycine at position 41 was changed to a cysteine.<sup>77</sup> The mutation was confirmed by LC/MS giving a mass of 16497.71 daltons as compared to the calculated mass of 16498 daltons for this mutant, which confirms that the mutation is correct. The protein was additionally characterized by SEC, DLS, and TEM (Figure 2.3 A, B, and C, respectively) to determine if the mutation destabilized or prevented the formation of intact particles. The chemical accessibility and reactivity of the thiol, in G41C engineered into the protein cage was determined by the labeling with a fluorescent molecule through a thiol maleimide reaction.

The synthesis of platinum nanoparticles on the interior of Hsp G41C was performed in a jacketed reaction vessel with a theoretical stoichiometric loading of the desired metal ions added (50, 150, 250, 500, and 1000  $\text{Pt}^{2+}$ ) per assembled Hsp cage.

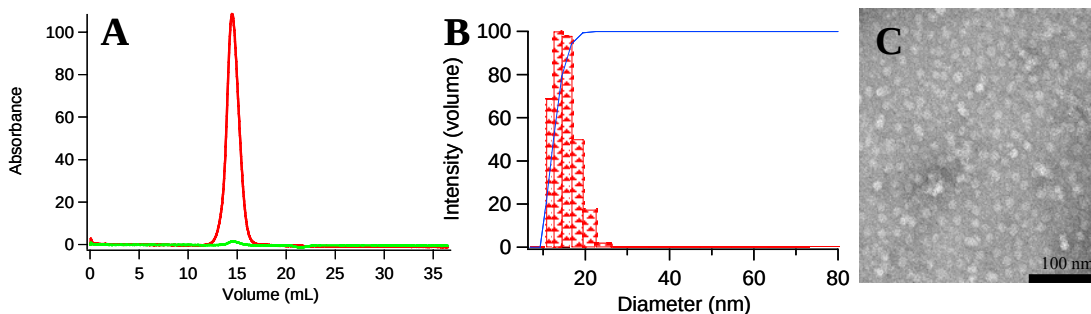


Figure 2.3. Characterization of Hsp G41C. A. SEC of Hsp G41C, -280 nm -350 nm; B. DLS of Hsp G41C; C. TEM of Hsp G41C.

Synthetic conditions were 2.5 mL solution of the Hsp G41C ( $0.5 \text{ mg}$ ,  $1.3 \times 10^{-6} \text{ mmole}$ ) which was brought to temperature by flowing water through the jacketed reaction flask. To achieve the desired theoretical loading of metal ions per cage,  $\text{K}_2\text{PtCl}_4$  (20 mM stock) was continuously added with a syringe pump (Kd Scientific) at varying rates depending on loading and duration of addition. The reduction of  $\text{Pt}^{2+}$  to Pt was carried out *in situ* by the simultaneous addition of dimethylamineborane (DMAB) to the reaction mixture. DMAB was chosen as the standard reducing agent for the Pt mineralization in protein cages over borohydride because the DMAB reaction with water is slower forming little to no  $\text{H}_2$  over 2 hours while with borohydride evolved considerable  $\text{H}_2$  gas after 10 minutes. The lack of  $\text{H}_2$  formation by DMAB allows more precisely controlled addition of the reducing agent.

After the platinum mineralization reaction, the reactions were centrifuged at  $12,000\times g$  for 5 minutes to remove any bulk mineral that had formed. The supernatant was applied to a size exclusion chromatography column as a first examination of the

reaction product. By running SEC, it was possible to determine if the protein cage was still intact (same elution volume) or aggregated after the reaction (smaller elution volume) and if there was co-elution of the protein cage (absorbance 280 nm), and

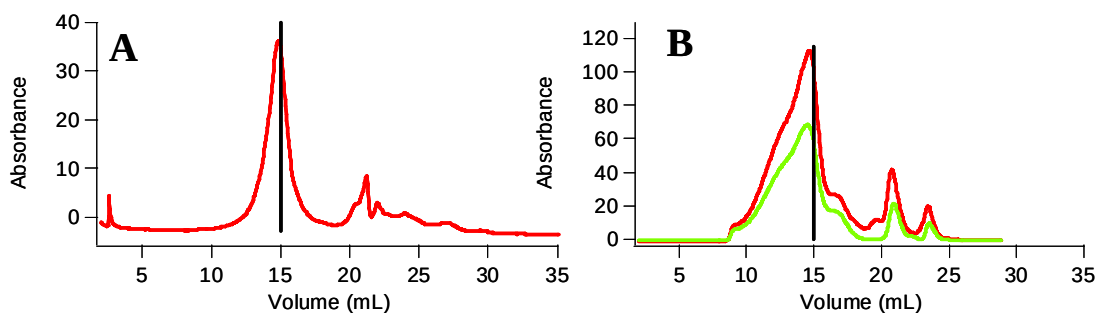


Figure 2.4. The SEC of Hsp G41C in mineralization conditions with and without the addition of  $\text{Pt}^{2+}$ . A. SEC of Hsp G41C control (5x DMAB, no  $\text{Pt}^{2+}$ ); B. SEC of 1000 Pt Hsp G41C -280 nm -350 nm. Black line indicates elution volume of untreated Hsp G41C.

mineral (absorbance 350 nm). The size exclusion chromatography was used to determine the level of aggregation in the sample or if the platinum mineral forms of the exterior of the cage, instead of the interior, both would be evident by an increase in the size of the protein and earlier retention volume compared to the unmineralized protein. Figure 2.3A shows the SEC of unmineralized Hsp G41C which clearly shows no absorption at 350 nm. Thus, any absorption at 350 nm can be attributed to mineral formation.

To determine if the Hsp protein cage was denatured under the reaction conditions, Hsp G41C was first exposed to dimethylaminoborane (DMAB) equal to the amount that would be added for a 1000  $\text{Pt}^{2+}$  per cage mineralization, with a 5-fold molecular excess, at 40 °C over two hours in 50 mM MES 100 mM NaCl pH 6.5 but without the addition of platinum. SEC of the protein (Figure 2.4A) after this reaction showed that the protein was still assembled into 12 nm particle. Figure 2.4 B is the SEC of the 1000 Pt Hsp G41C mineralization at 40 °C over two hours in 50 mM MES 100 mM NaCl pH 6.5 with

the addition 5x DMAB. The size increase indicated by the decrease in the retention volume from 15 mL (Hsp G41C) to ~12 mL (1000 Pt Hsp G41C) in the SEC shows that the platinum mineralization had not occurred specifically on the interior of the Hsp G41C protein cage.

#### Effect of DMAB on the $Pt^0$ Mineralization in Hsp G41C

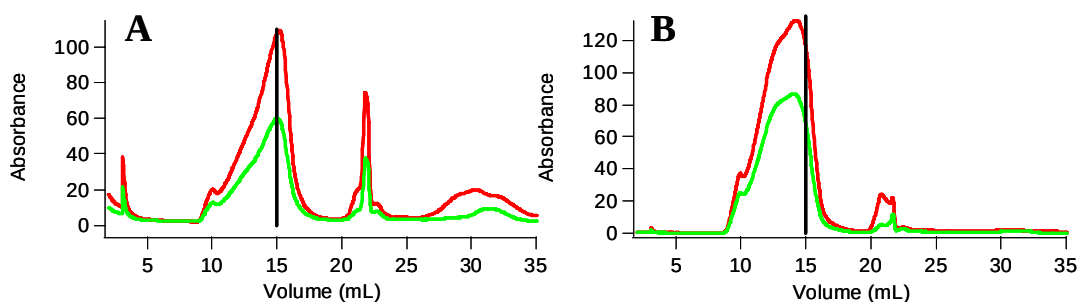


Figure 2.5. Comparison of the SEC of 4000 Pt Hsp G41C with different excesses of DMAB. A. SEC of 4000 Pt Hsp G41C with 2x DMAB; B. SEC of 4000 Pt Hsp G41C with 4x DMAB, ■280 nm ■350 nm. Black line indicates elution volume of untreated Hsp G41C.

The molecular excess of DMAB was varied because the amount of DMAB added to the solution influences the rate of reduction with the rate of reduction increasing with the increasing molecular excess. Using standard conditions of 40°C with the addition of  $Pt^{2+}$  and DMAB over two hours in 50 mM MES 100 mM NaCl pH 6.5, the molecular excess was 2x (Figure 2.5 A) or 4x (Figure 2.5 B). SEC of both reactions after shows in both cases that there is a size increase while the size increase is more significant for the 4-fold DMAB over the 2-fold reaction.

#### Effect of Addition Rate of $Pt^{2+}$ and DMAB on the $Pt^0$ Mineralization in Hsp G41C:

In an attempt to force the mineralization to occur specifically on the interior of Hsp G41C, the rate of addition of 1000  $Pt^{2+}$  (with 2-fold DMAB) was varied from 15 min to 2 hrs while holding the buffer and the

temperature conditions constant. Addition rate was varied to test whether platinum in solution was limiting the reaction rate or if the excess platinum was causing aggregation. The different addition rates did exhibit different amounts of aggregation and Pt mineral formation on the exterior of the cage, but did not however significantly change the specificity of the mineralization for the interior of the cage under the standard conditions.

#### Effect of Loading Factor on the $Pt^0$

Mineralization in Hsp G41C: The loading factor of Pt per cage was also varied to try and optimize the reaction specificity. The loading factor was varied from 50  $Pt^{2+}$  per

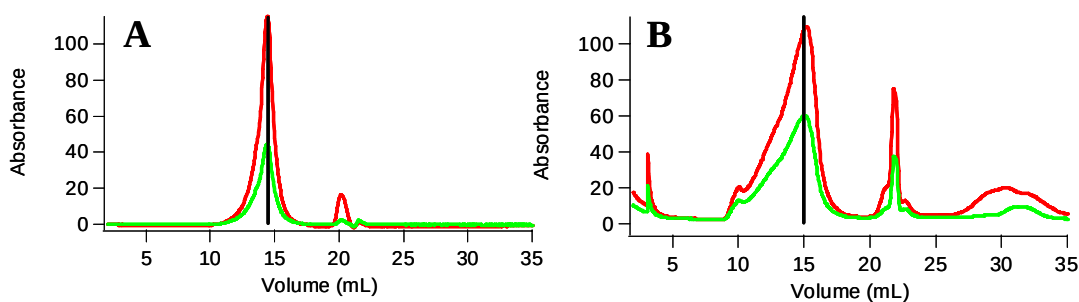


Figure 2.6. Comparison of the SEC for different Pt per Hsp G41C loading. A. SEC of 150 Pt Hsp G41C with 2x DMAB; B. SEC of 2000 Pt Hsp G41C with 2x DMAB, -280 nm -350 nm. Black line indicates elution volume of untreated Hsp G41C.

cage to 4000  $Pt^{2+}$  per cage. The loading per cage was varied because protein cage mineralizations have displayed a maximum loading factor, which when that loading factor is exceeded bulk mineral formation is increased. It was observed that the loading factor had a large effect on the amount of bulk mineralization that occurred, exterior to the protein cavity. Figure 2.6 A, B shows a SEC of 2000 Pt Hsp G41C and 250 Pt Hsp G41C at 40 °C with the addition of Pt and DMAB over two hours in 50 mM MES 100 mM NaCl pH 6.5. The SEC shows an increase in the amount of mineralization on the exterior surface of the cage with increasing Pt loading. With the loading factors of 50 to 250 Pt per cage, there was little to no bulk precipitate observed, as observed by the

narrow SEC elution profile (Figure 2.6 B) and retention time identical to unmineralized Hsp. Upon inspection of the product by TEM, the Pt particle size was determined to be at the limit of detection, ~1 nm diameter or slightly above. The TEM analysis showed that there were a number of particles on the exterior surface of the protein cage. With a particle size of near 1 nm, size exclusion chromatography becomes ineffective for determining where the Pt nanoparticle is in relationship to the cage. The resolution of the SEC is such that the addition of a 1 nm particle on the exterior of a 12 nm protein cage would not be able to be separated from protein cages without a nanoparticle on the exterior. Thus, the SEC makes the mineralization look more specific for the interior while that is not the case.

#### Effect of Buffer Conditions on Mineralization

of Pt<sup>0</sup> in Hsp G41C: In an attempt to prevent or minimize the mineralization on the exterior of the protein cage, the buffer concentrations, the buffer composition, the

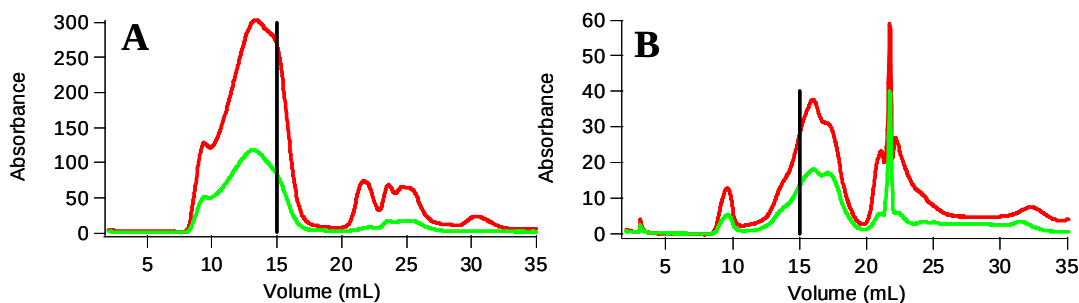


Figure 2.7. SEC of Pt<sup>0</sup> mineralization under varying buffer conditions. A. SEC of Hsp G41C 4000 Pt 50x NH<sub>4</sub>Cl 2x DMAB; B. SEC of 4000 Pt Hsp G41C with 2x DMAB and Titrino pH controlled, -280 nm -350 nm. Black line indicates elution volume of untreated Hsp G41C.

NaCl concentration in solution, and the pH was varied to determine the effect on the mineralization. These conditions were varied in an attempt to optimize the interaction of the interior of the protein cage with the platinum ions while stabilizing the ions against

bulk mineral formation and interactions with the exterior of the cage. The pH was varied from 6.5 to 7.0 in MES and 7.0 to 8.0 in HEPES to determine the optimal pH for the mineralization. Using buffered solutions, the optimal pH was determined to be pH 6.5. When the titrino was used, the Hsp G41C was dialyzed into 100 mM NaCl. Using the titrino for the mineralization did not show any improvement on the specificity of the mineralization for the interior of the cage. However, it was observed that lower concentration of buffer ~25 mM in the mineralization reaction resulted in cleaner reactions. Figure 2.7 A and B SEC of Hsp G41C 4000 Pt in 50 mM Mes pH 6.5 with 50x  $\text{NH}_4\text{Cl}$  over platinum and 2x DMAB and SEC of 4000 Pt Hsp G41C with 2x DMAB with the pH controlled by a titrino, respectively, show the variation in the mineralization with buffer content and concentration.

The concentration of the buffer and the NaCl concentration was determined to be critical to the mineralization process. NaCl concentration is one of the major factors in the specificity of the mineralization. At high NaCl concentrations ~ 0.5- 1 M the reaction formed almost all bulk precipitate with little, if anything, protein left in solution. It was determined NaCl aided the mineral formation on the exterior of the cage. To determine if the sodium or the chloride was adding in the interaction with exterior of the cage,  $\text{NH}_4\text{Cl}$  was used in the mineralization buffer. When 50-fold per  $\text{Pt}^{2+}$  excess of  $\text{NH}_4\text{Cl}$  was added to the mineralization buffer a slight improvement over NaCl was seen in the mineralization. The platinum mineralization was then conducted in  $\text{NH}_4\text{OH}$  to see if the ammonium ion was responsible for the increased specificity for the interior of the protein cage. The addition of  $\text{NH}_4\text{OH}$  to the reaction conditions also showed a slight improvement in the specificity for the interior of the protein cage.

### The Optimized Reaction Conditions for

Pt<sup>0</sup> in Hsp G41C: Using 25 mM MES 10 mM NH<sub>4</sub>OH pH 6.5 as the buffer

conditions the temperature increased to 65 °C. Both 15 and 30 minute (Figure 2.8 A, B, respectively) addition times were attempted to optimize the specific mineral formation on

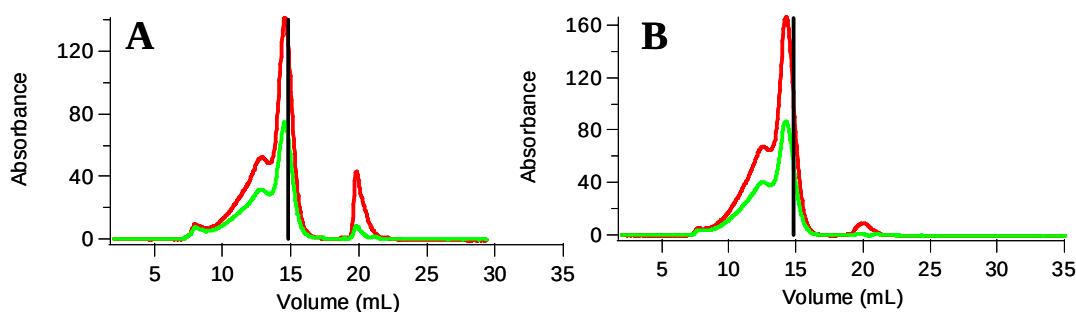


Figure 2.8. Comparison of the SEC of Hsp G41C Pt<sup>0</sup> mineralization at 65 °C with different additions times. A. SEC of 1000 Pt Hsp G41C in 25 mM MES 10 mM NH<sub>4</sub>OH pH 6.5, 65 °C, 5x DMAB added over 30 minutes; B. SEC of 1000 Pt Hsp G41C in 25 mM MES 10 mM NH<sub>4</sub>OH pH 6.5 at 65 °C with 5x DMAB added over 15 minutes, -280 nm -350 nm. Black line indicates elution volume of untreated Hsp G41C.

the interior. Under these conditions, the mineralization on the exterior of the cage was minimized. The 15 minute addition time was found to be the optimal addition rate for the mineralization. Under the high temperature conditions, the maximum loading achieved was 1000 Pt<sup>2+</sup>/cage. At higher loading factors, the amount of precipitation increased dramatically.

### Rationale and Characterization of the Insertion

of a Pt Binding Peptide Inside Hsp: Protein/biomineral interactions have been

shown to be an important part of the biomineral formation and stabilization. The interactions of biominerals with proteins can direct the locality, the morphology, the composition, and the polymorph of the resulting mineral.<sup>2</sup> In an attempt to mimic this interaction, peptides that bind to specific mineral surfaces have been identified using

phage display techniques, which is the process of identifying peptides from a library of random peptides displayed on the exterior of a phage that when they bind specifically to a site of interest, mineral, that can be selected and identified, and have been shown to aid in the mineral formation.<sup>78</sup> By inserting a peptide specific for platinum metal into the Hsp protein cage architecture, we tried to direct the specific formation of platinum metal nanoparticles on the interior of Hsp.

The platinum specific peptide (IGSSLKP) was inserted in the N-terminus of WtHsp. The N-terminus is not seen in the crystal structure due to structured disorder. However, the cryoEM reconstruction indicates that the N-terminus is in the interior of the protein cage. The platinum specific peptide was inserting into the WtHsp by replacing amino acids 3-9 (GRDPFDS) with the IGSSLKP. The Hsp with the Pt-specific peptide inserted (Pt-Hsp) was expressed and purified from *E. coli*. The insertion was confirmed by LC/MS giving a mass of 16359.15 daltons as compared to a calculated mass of 16360 daltons, the differences in mass a well within the acceptable error for protein mass spectrometry. After purification, the protein was characterized using SEC, DLS, and TEM (Figure 2.9 A, B, and C respectively) which confirmed the formation of assembled 12 nm particles and morphologically indistinguishable from the WtHsp protein.

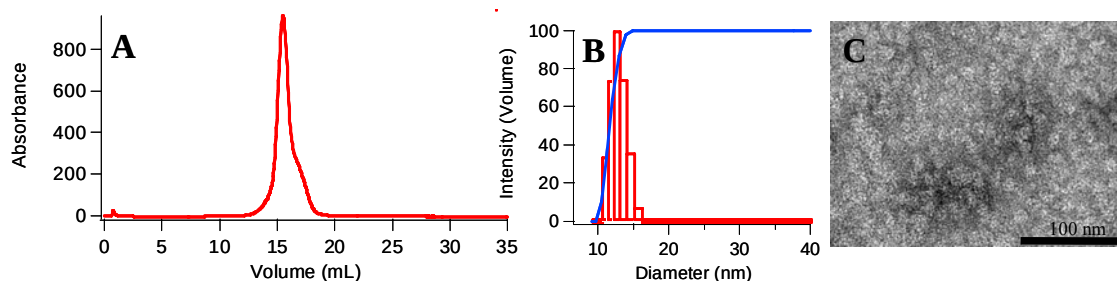


Figure 2.9. Characterization of Pt-Hsp. A. SEC of Pt-Hsp  $\sim$ 280 nm; B. DLS of Pt-Hsp; C. TEM of Pt-Hsp.

### Mineralization of Pt-Hsp Under Varying Buffer,

### Temperature, and DMAB Conditions: Synthesizing platinum nanoparticles on the

interior of Pt-Hsp was performed in a jacketed reaction vessel with a theoretical stoichiometric loading of the desired metal atoms added per assembled Hsp cage. Standard synthetic conditions were 2.5 mL solution of the Pt-Hsp (0.5 mg,  $1.3 \times 10^{-6}$  mmoles) which was brought to temperature by flowing water through the jacketed reaction flask. To achieve the desired theoretical loading of metal atoms per cage,  $K_2PtCl_4$  (20 mM stock) was continuously added with a syringe pump (Kd Scientific) at different rates depending on the loading factor per cage and the duration of the addition.

The mineralization of Pt-Hsp was performed under conditions similar to the mineralizations in Hsp G41C. Pt-Hsp was tested under the mineralization conditions that showed the least bulk precipitation in Hsp G41C because conditions that promote bulk mineralization were presumed to occur independent of changes on the interior of the protein cage. Under most conditions, the Pt-Hsp mineralizations had more precipitation than the comparable reactions in Hsp G41C. Figure 2.10 A, B shows the SEC of 2000 Pt Pt-Hsp mineralized in 25 mM MES pH 6.5 at 40°C with 2x DMAB added over 1 hour and SEC of 2000 Pt Pt-Hsp in 25 mM MES pH 6.5 at 40 °C with 4x DMAB added over 1 hour, respectively. Pt mineralizations in Pt-Hsp show much more aggregation and mineralization on the exterior of the protein cage than the identical reaction using Hsp G41C. To determine if the Pt binding peptide need to first bind  $Pt^{2+}$  ions through a kinetically slow step in order to act as nucleation site, Pt-Hsp was first incubated with 50 atoms of  $Pt^{2+}$  per cage for two different periods (one hour and half hour) in 25 mM MES

pH 6.5 then the solution was mineralized with 1000 Pt<sup>2+</sup> per cage at 40 °C. Under both conditions, extensive bulk precipitation was observed.

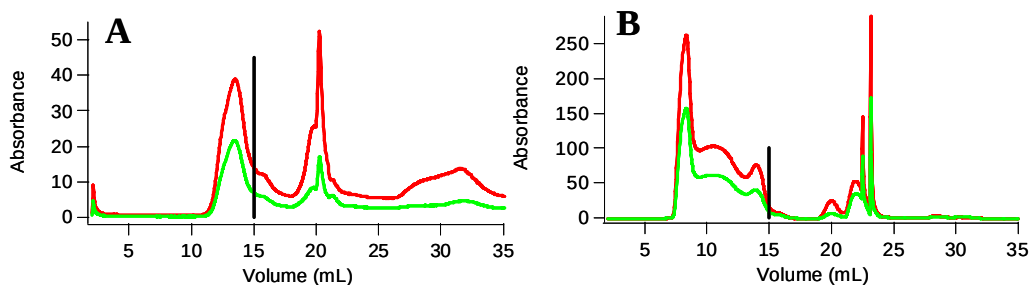


Figure 2.10. Characterization of Pt<sup>0</sup> mineralization in Pt-Hsp with different excesses of DMAB. A. SEC of 2000 Pt Pt-Hsp in 25 mM MES pH 6.5 at 40°C with 2x DMAB added over 1 hour; B. SEC of 2000 Pt Pt-Hsp in 25 mM MES pH 6.5 at 40°C with 4x DMAB added over 1 hour, -280 nm, -350 nm. Black line indicates elution volume of untreated Pt-Hsp.

Using the conditions previously optimized for Hsp G41C, 1000 Pt Pt-Hsp in 25 mM MES 10 mM NH<sub>4</sub>OH pH 6.5 at 65 °C in 5x DMAB over 20 min was synthesized and SEC performed (Figure 2.11). After the reaction, bulk precipitation was removed by

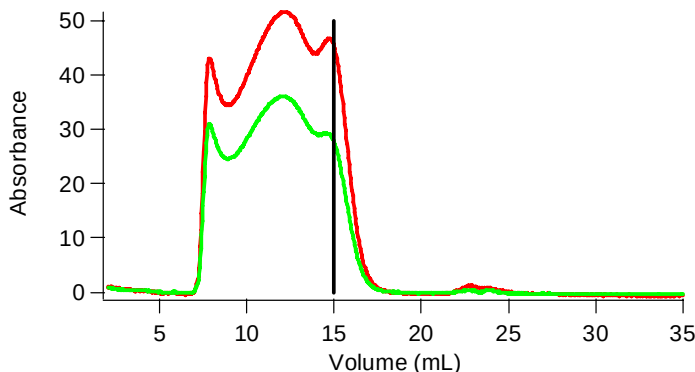


Figure 2.11. Mineralization of Pt-Hsp under conditions optimized for Hsp G41C. SEC of 1000 Pt Pt-Hsp in 25 mM MES 10 mM NH<sub>4</sub>OH pH 6.5 at 65 °C with 5x DMAB over 20 min, -280 nm -350 nm. Black line indicates elution volume of untreated Pt-Hsp.

centrifugation. The SEC profile showed that a majority of the protein in the solution had formed aggregated particles evidenced by earlier elution volume in the SEC. All attempts to mineralize platinum nanoparticles specifically on the interior of Pt-Hsp failed

to be an improvement over Hsp G41C and all most cases the mineralizations had an increased amount of aggregation and bulk precipitation.

#### Rationale and Characterization of Phenanthroline

Attached to Hsp G41C: Phenanthroline was attached to the interior of the Hsp G41C in order to chemically insert a nucleation site for platinum mineralization (Figure 2.12). Phenanthroline was chosen to use as the nucleation site to attach to the protein surface because platinum binds strongly to the nitrogen of the phenanthroline chelate.<sup>120</sup> The binding of  $\text{Pt}^{2+}$  could allow for the photochemical synthesis of platinum particles through a reduction of bound  $\text{Pt}^{2+}$ .

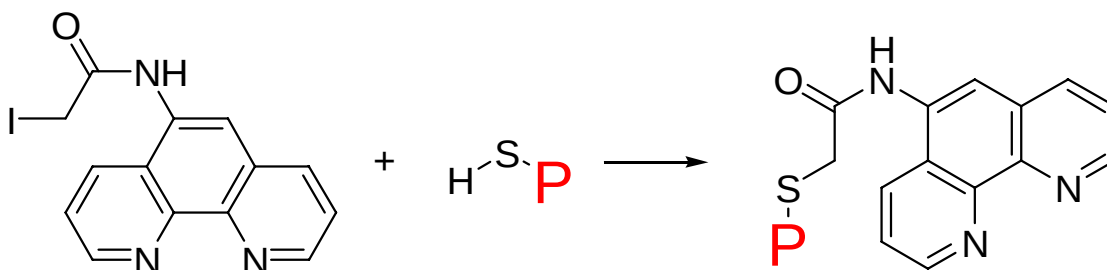


Figure 2.12. The attachment of Iphen to a protein (P).

The photochemical synthesis of  $\text{Pt}^0$  is driven by the photo reduction of  $\text{Pt}^{2+}$  by citrate. Citrate is used as the source of electrons for the reduction of  $\text{Pt}^{2+}$  to  $\text{Pt}^0$ . The photolysis of  $\text{Pt}^{2+}$  and citrate could cause the formation of bulk mineral  $\text{Pt}^0$ .<sup>121</sup> The phenanthroline-Pt could photocatalytically reduce the reduce  $\text{Pt}^{2+}$  to  $\text{Pt}^0$  to act as a nucleation site.<sup>122, 123</sup> Using the phenanthroline attached to the cage as both as a nucleation site and a photo-catalytic site, we attempted to control reduction of  $\text{Pt}^{2+}$  on the interior of the protein cage.

After Hsp G41C was labeled with Iphen (Hsp Phen), it was characterized by SEC, DLS, TEM, and LC/MS. The SEC, DLS, and TEM (Figure 2.13 A, B, and C,

respectively) confirmed that the reaction conditions or the labeling did not disrupt the assembled Hsp architecture. By attaching the Iphen to the Hsp G41C, the extinction

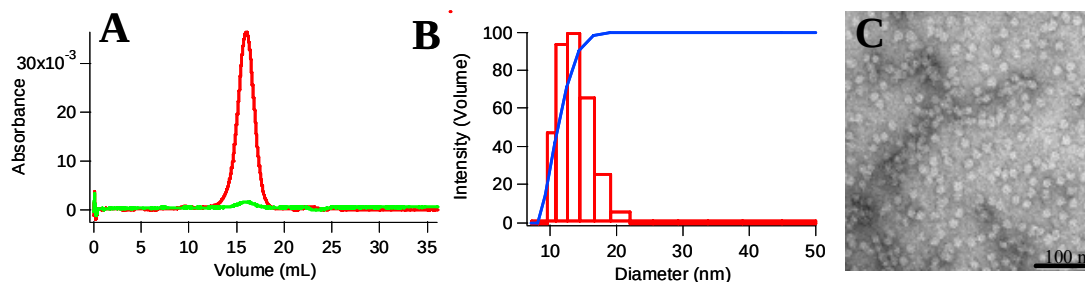


Figure 2.13. Characterization of the Hsp G41C after the attachment of phenanthroline. A. SEC of Hsp Phen ■280 nm ■350 nm; B. DLS of Hsp Phen; C. TEM of Hsp Phen stained with 2% uranyl acetate.

coefficient of Hsp is altered because the Phen contributes strongly to the absorbance at 280 nm. When the Phen has been attached to the cage, a Bradford assay was used to determine the protein concentration. The attachment of the phenanthroline was confirmed by LC/MS giving a mass of 16733.6 daltons as compared to a calculated mass of 16733.2 daltons.

#### Characterization of Control Reactions Using

Buffer, WtHsp, and Hsp G41C: We attempted to determine if attaching the phenanthroline to the cage could direct the photo-mineralization of platinum nanoparticles. Platinum photo-mineralization control reactions were first performed using buffer and Hsp G41C. In the buffer control, 64  $\mu$ L of 20 mM Pt<sup>2+</sup> and 64  $\mu$ L of 40 mM sodium citrate was added to 2 mL of 25 mM MES pH 6.5 in a 4 mL cuvette before illumination at 40°C for 2 hours. Figure 2.14A shows the UV/Vis monitoring of the Pt photo-mineralization. The product of this reaction revealed a broad absorption over the entire UV/Vis spectrum, indicative of light scattering by large particles in solution. DLS

of the reaction (Figure 2.14B) revealed the presence of large (150-250 nm) particles which confirmed the findings from the UV/Vis analysis.

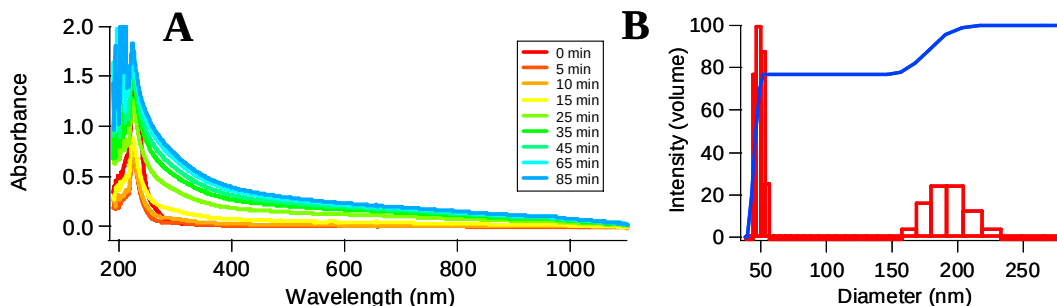


Figure 2.14. Characterization of the protein-free control Pt photo-mineralization. A. UV/Vis of buffer control of Pt photo-mineralization; B. DLS of buffer control of Pt mineralization.

The Hsp G41C control was run to determine if the attachment of the phen was necessary to direct the mineralization on to the interior surface. In the Hsp G41C control, 32  $\mu\text{L}$  of 20 mM  $\text{Pt}^{2+}$  and 32  $\mu\text{L}$  of 40 mM sodium citrate was added to a 4 mL cuvette containing Hsp G41C (0.5 mg,  $1.3 \times 10^{-6}$  mmoles) in 2 mL of 25 mM MES pH 6.5 before illumination at 40  $^{\circ}\text{C}$  for 2 hours. Figure 2.15A shows the UV/Vis monitoring of

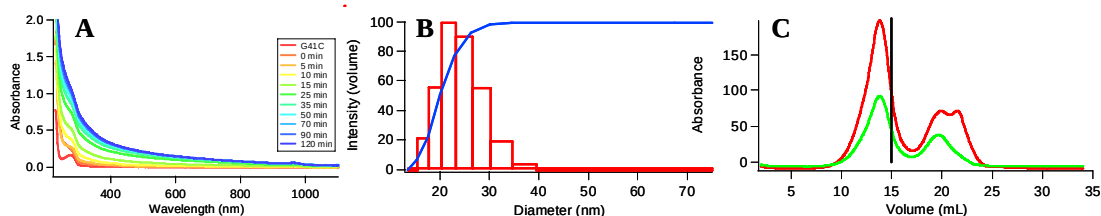


Figure 2.15. Characterization of the Hsp G41C Pt photo-mineralization. A. UV/Vis of Hsp G41C control of 500 Pt photo-mineralization; B. DLS of Hsp G41C control of 500 Pt photo-mineralization; C. SEC of Hsp G41C control of 500 Pt photo-mineralization, ■280 nm ■350 nm. Black line indicates elution volume of untreated Hsp G41C.

the 500 Pt per cage photo-mineralization in Hsp G41C. The broad absorption over the entire UV/Vis spectrum indicates the formation of large particles in the solution which causes light scattering. DLS of the reaction (Figure 2.15B) products indicated the

presence of particles larger (18-30 nm) than the diameter of Hsp G41C indicating either bulk mineralization of  $\text{Pt}^0$  or aggregation of Hsp particles and confirming the findings from the UV/Vis analysis. Before the SEC analysis, the reaction was centrifuged to remove large particles that would plug the size exclusion column and a dark pellet was separated from the supernatant. The SEC (Figure 2.15C) confirmed the presence of particles larger than that of the Hsp protein assembly.

**Photo-mineralization of Hsp Phen Using  $\text{Pt}^{2+}$  and Citrate:** In the 500 Pt Hsp Phen photo-mineralization reaction, 32  $\mu\text{L}$  of 20 mM  $\text{Pt}^{2+}$  and 32  $\mu\text{L}$  of 40 mM sodium citrate was added to a 4 mL cuvette containing Hsp Phen (0.5 mg,  $1.3 \times 10^{-6}$  mmoles) in 2 mL of 25 mM MES pH 6.5 before illumination at 40°C for 2 hours. Figure 2.16A shows the UV/Vis monitoring of the Pt

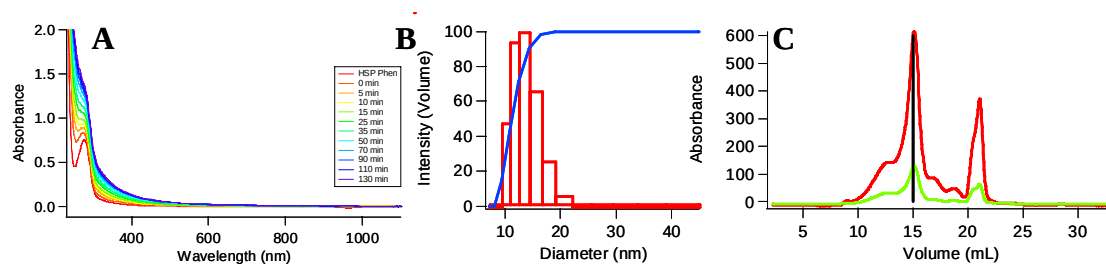


Figure 2.16. Characterization of Hsp Phen 500 Pt photo-mineralization. A. UV/Vis of Hsp Phen 500 Pt photo-mineralization; B. DLS of Hsp Phen 500 Pt photo-mineralization; C. SEC of Hsp Phen 500 Pt photo-mineralization, ■280 nm ■350 nm. Black line indicates elution volume of untreated Hsp Phen.

photo-mineralization in Hsp Phen. The broad absorption that characterized the control reaction was completely lacking in the Hsp Phen photo-mineralization indicating that there was no formation of large particles that were seen in the control reactions. DLS of the reaction products (Figure 2.16B) indicated a particle size of 13 nm, as compared to the 12 nm diameter of the Hsp cage itself suggesting that a majority of the platinum has been encapsulated by the Hsp Phen confirming the findings from the UV/Vis analysis.

The slight increase in the hydrodynamic diameter is indicative of a small percentage of  $\text{Pt}^0$  on the exterior of the protein particle giving a broader envelope to the DLS. Before the SEC analysis, the reaction was centrifuged to remove large particles and no pellet was observed suggesting either no, or very little, bulk mineralization or aggregation. The SEC (Figure 2.16C) confirms the presence of a minority of particles larger than that of the Hsp protein assembly while the majority of the sample eluted with the same retention time as the Hsp cage alone.

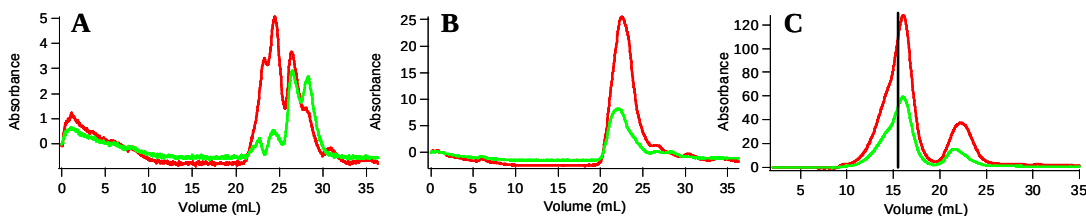


Figure 2.17. Pt photo-mineralization controls. A. SEC of 500 Pt photo-mineralization no light no protein control; B. SEC of 500 Pt photo-mineralization no protein control; C. SEC of Hsp Phen 1000 Pt photo-mineralization, -280 nm -350 nm. Black line indicates elution volume of untreated Hsp Phen.

In order to examine origin of the low molecular weight peak observed in the SEC (Figure 2.16 C), two control reactions were performed. First,  $\text{Pt}^{2+}$  and sodium citrate were mixed, at the same concentrations as in the photo-mineralization reactions and run over SEC (Figure 2.17A). Second, the protein-free photo-mineralization was conducted, centrifuged, and then run over SEC (Figure 2.17B). The control reactions show that the low molecular weight peak on the SEC is consistent with a byproduct of the photo-mineralization reaction.

In the 1000 Pt Hsp Phen photo-mineralization reaction (Figure 2.17C), 64  $\mu\text{L}$  of 20 mM  $\text{Pt}^{2+}$  and 64  $\mu\text{L}$  of 40 mM sodium citrate was continuously added to a 4 mL cuvette containing Hsp Phen (0.5 mg,  $1.3 \times 10^{-6}$  mmoles) in 2 mL of 25 mM MES pH

6.5 during illumination at 40 °C over 2 hours. The SEC analysis of the product (Figure 2.17C) indicated the majority of the material had a retention time equivalent to Hsp. The photo-mineralization of 1000 Pt<sup>2+</sup> per Hsp Phen does have more of a shoulder on the peak indicating some mineralization in the exterior.

Optimization of the Photo-mineralization of Hsp Phen Using Pt<sup>2+</sup> and Citrate: In order to optimize the photo-mineralization, reaction conditions such as Pt<sup>2+</sup> and citrate loadings, rate of addition, and buffer were varied. The platinum metal loadings were increased so that if every phenanthroline was an effective nucleation site each site would be able to form a particle large enough to be

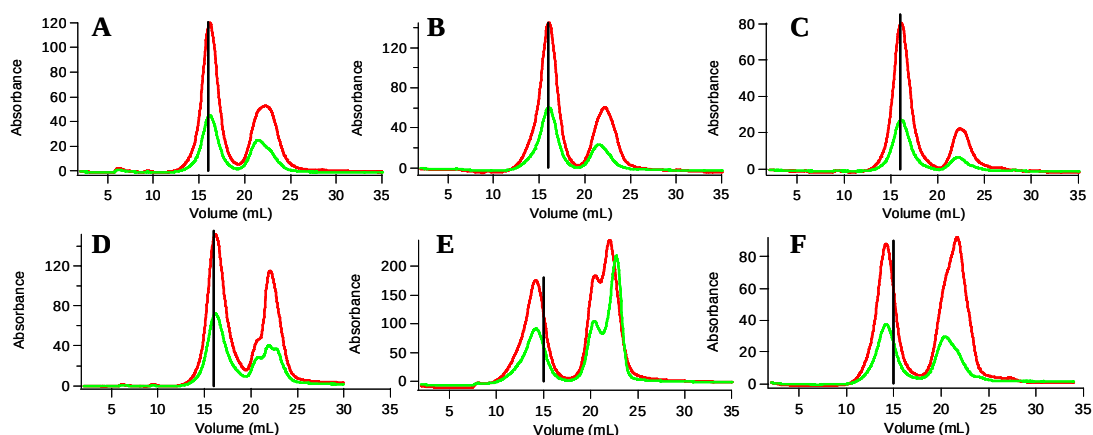


Figure 2.18. SEC characterization of varying photo-mineralization reaction conditions. A. SEC of Hsp Phen 1000 Pt photo-mineralization with 2x citrate over 4 hours; B. SEC of Hsp Phen 1000 Pt photo-mineralization with 4x citrate over 2 hours; C. SEC of Hsp Phen 1000 Pt photo-mineralization with 4x citrate 45 minutes in 10 mM MES 50 mM NaCl pH 6.5; D. SEC of Hsp Phen 1000 Pt photo-mineralization with 4x citrate 45 minutes; E. SEC of Hsp Phen 4000 Pt photo-mineralization with 4x citrate 2 hours; F. SEC of Hsp Phen 2000 Pt photo-mineralization with 4x citrate Pt and citrate added before 2 hour illumination, All reaction in 50 mM MES pH 6.5 unless otherwise noted, -280 nm -350 nm. Black line indicates elution volume of untreated Hsp Phen.

seen on TEM. Citrate excess per platinum was varied to determine if too large of an excess of citrate would prevent the desired interaction with the phenanthroline and the growing particle. Using the standard conditions, 20 mM Pt<sup>2+</sup> and 40 mM sodium citrate

solutions were continuously added to a 4 mL cuvette containing Hsp Phen (0.5 mg,  $1.3 \times 10^{-6}$  mmoles) in 2 mL of 50 mM MES pH 6.5 during illumination at 40 °C. Figure 2.18A shows the SEC of Hsp Phen mineralized with 1000  $\text{Pt}^{2+}$  using a 2-fold molar excess of citrate over 4 hours. Figure 2.18B shows the SEC of Hsp Phen mineralized with 1000  $\text{Pt}^{2+}$  using a 4-fold molar excess of citrate over 2 hours. Figure 2.18C shows the SEC of Hsp Phen mineralized with 1000  $\text{Pt}^{2+}$  using a 4-fold molar excess of citrate over 45 minutes. Figure 2.18D shows the SEC of Hsp Phen mineralized with 1000  $\text{Pt}^{2+}$  using a 4-fold molar excess of citrate over 45 minutes. Figure 2.18E shows the SEC of Hsp Phen mineralized with 4000  $\text{Pt}^{2+}$  using a 4-fold molar excess of citrate over 2 hours. Figure 2.18F shows the SEC of Hsp Phen mineralized with 2000  $\text{Pt}^{2+}$  using a 4-fold molar excess of citrate added before 2 hours of illumination. The addition of  $\text{Pt}^{2+}$  and citrate over 45 minutes in 10 mM MES 50 mM NaCl (Figure 2.18C) reveals the most uniform peak shape in the SEC. However, the absorbance at 280 and 350 nm was half the absorbance of the other continuous addition reactions. The SEC of Hsp Phen 1000  $\text{Pt}^{2+}$  photo-mineralization with a 4-fold excess citrate over 45 minutes (Figure 2.18D) was determined to be the optimal synthesis because of the uniform elution peak, the high 280 to 350 ratio, and peak height comparable to the other reactions. However, while the product formation by the photomineralization approach appeared promising, the TEM analysis of all the Pt photomineralizations showed no detectable particles by either staining or with unstained analysis. The UV-Vis confirms the formation of  $\text{Pt}^0$  particles and SEC determines the coelution of the particles with the protein cages. This suggests that the particles synthesized have a diameter below the detection level ( $\sim 1$  nm) of the TEM.

### Pt<sup>0</sup> Mineralization of Hsp Phen Using

DMAB as a Reductant: After the inconclusive photo-mineralization of Hsp phen,

Hsp phen was used in the DMAB reduced platinum mineralization. The phenanthroline could act as a nucleation site on the interior of Hsp for the mineralization Pt<sup>0</sup> particles

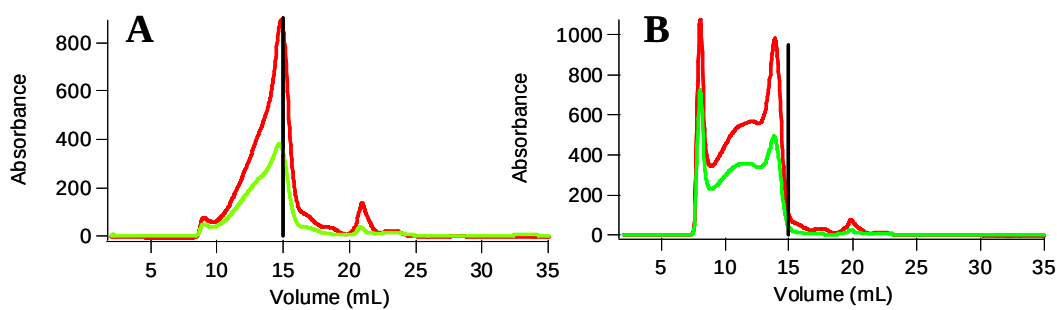


Figure 2.19. Characterization of Hsp Phen mineralized with varying loading of Pt<sup>2+</sup> using DMAB as the reductant. A. SEC of 1000 Pt Hsp Phen reduced with DMAB; B. SEC of 2000 Pt Hsp Phen reduced with DMAB, ■280 nm ■350 nm. Black line indicates elution volume of untreated Hsp Phen.

making the mineralization specific for the interior of the protein cage. Figure 2.19 shows SEC of Hsp Phen (0.5 mg,  $1.3 \times 10^{-6}$  mmoles) mineralized in 2.5 mL of 50 mM MES 100 mM NaCl pH 6.5 at 40 °C using a 2-fold molar excess DMAB over Pt<sup>2+</sup>. Reagents (Pt<sup>2+</sup> and DMAB) were added over 1 hour. Figure 2.17A shows the SEC elution profile of the 1000 Pt<sup>2+</sup> per Hsp Phen and Figure 2.19B shows the SEC elution profile for 2000 Pt per Hsp Phen reaction. To achieve the desired theoretical loading of metal atoms per cage, K<sub>2</sub>PtCl<sub>4</sub> (20 mM stock) and DMAB was continuously added with a syringe pump (Kd Scientific). The SEC of the DMAB reduced mineralizations of Hsp Phen showed no improved selectivity for the interior of the protein cage over the mineralization in Hsp G41C, indicating that there was no added benefit to attaching the phenanthroline to the interior of the Hsp cage.

Pt<sup>0</sup> Nanoparticle Mineralization in WtHsp: The platinum nanoparticles mineralization inside WtHsp (Figure 2.20) was performed in a jacketed reaction vessel with a theoretical stoichiometric loading of the desired metal ions added per assembled

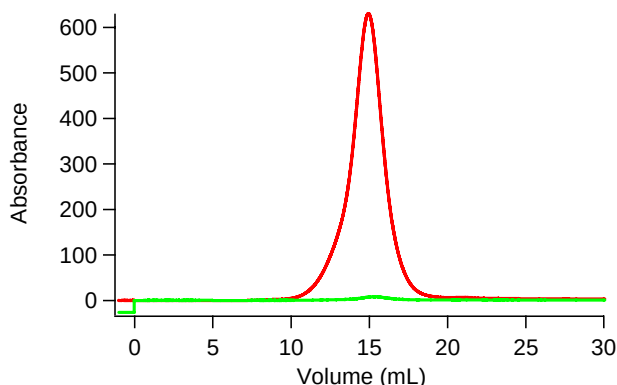


Figure 2.20. SEC characterization of WtHsp, -280 nm -350 nm.

Hsp cage. Synthetic conditions were 2.5 mL solution of the WtHsp (0.5 mg,  $1.3 \times 10^{-6}$  mmoles) which was brought to temperature by flowing water through the jacketed reaction flask. To achieve the desired theoretical loading of metal ions per cage, K<sub>2</sub>PtCl<sub>4</sub> (20 mM stock) was continuously added with a syringe pump (Kd Scientific) with the rate varying depending on the addition time and loading factor. The reduction of Pt<sup>2+</sup> to Pt<sup>0</sup> was carried out *in situ* by the simultaneous addition of DMAB to the reaction mixture.

The mineralization of WtHsp was developed using information derived from Hsp G41C (described previously). WtHsp mineralization was tested using a limited number of conditions. In general, the mineralization of WtHsp had less precipitate and less aggregation evident in the SEC under the same reaction conditions as compared to Hsp G41C, Pt-Hsp, and Hsp phen. The maximum loading for WtHsp was determined to be 1000 Pt<sup>2+</sup> per cage and that 25 mM MES 10 mM NH<sub>4</sub>OH pH 6.5 were the optimized reaction buffer conditions for the specific mineralization on the interior of WtHsp.

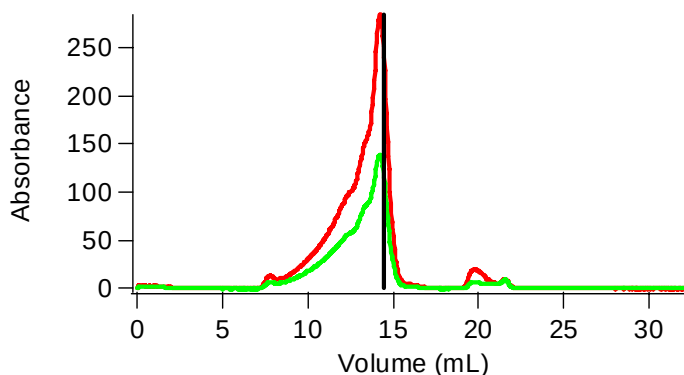


Figure 2.21. SEC characterization of 1000 Pt WtHsp 1 mg/mL in 25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5 at 55 °C with 5x DMAB added over 15 minutes, -280 nm -350 nm. Black line indicates elution volume of untreated WtHsp.

The final adjustment to the reaction conditions was to increase the protein concentration from 0.2 mg/mL to 1.0 mg/mL. After adjusting the concentration of protein in the synthesis, the reaction could be affected such that almost all of the platinum mineralized on the interior of the protein cage with very little exterior mineralization. To determine if elevated temperature was needed, figure 2.21 shows the SEC of the mineralization of 1000 Pt WtHsp in 25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5 at 55 °C.

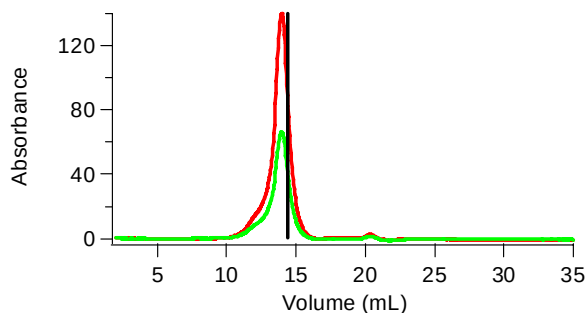


Figure 2.22. The SEC characterization of the final optimized  $\text{Pt}^0$  mineralization in WtHsp. SEC of 1000 Pt WtHsp 1 mg/mL in 25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5 at 65 °C with 5x DMAB added over 15 minutes, -280 nm -350 nm. Black line indicates elution volume of untreated WtHsp.

Figure 2.22 shows the SEC of 1000 Pt WtHsp at 1 mg/mL in 25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5 at 65 °C with 5x DMAB added over 15 minutes displaying the ability to

vary the loading factor of the reaction. The ten degree temperature increase had a significant beneficial effect on the mineralization. The 10 mM  $\text{NH}_4\text{OH}$  in the final solution was found to be an important factor to minimize mineralization on the exterior of the WtHsp cage. Loadings of 150, 250, and 1000  $\text{Pt}^{2+}$ /cage were synthesized and further characterized.

Visualization of the mineralized 1000 Pt WtHsp by transmission electron microscopy (TEM) revealed electron dense cores identified as Pt metal by electron diffraction (Figure 2.23 A inset). Intact 12 nm protein cages were clearly visible when negatively stained (Figure 2.23 B) with uranyl acetate. In the stained samples, the  $\text{Pt}^0$  particles can clearly be seen as small dark particles above the background stain and are localized within the cage structure. For average loadings of 1000 Pt/cage, metal particles of  $2.6 \pm 0.2$  nm were observed (Figure 2.23 C), in all cases size distribution

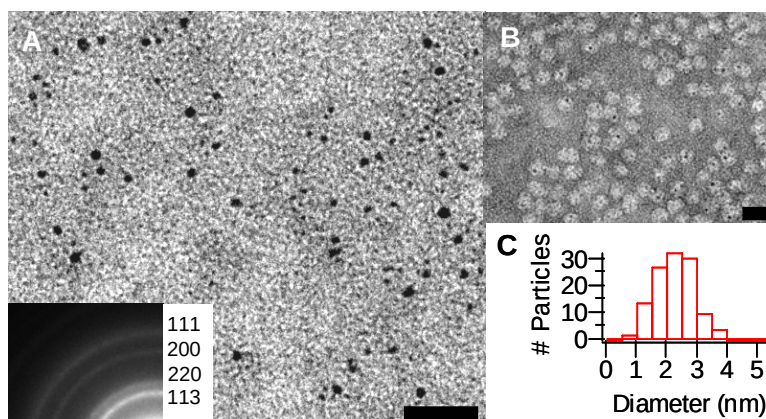


Figure 2.23. TEM characterization of 1000 Pt WtHsp. A. TEM of 1000 Pt WtHsp unstained, the inset shows electron diffraction of  $\text{Pt}^0$  from 1000 Pt WtHsp; B. TEM of 1000 Pt WtHsp stained with 2% uranyl acetate; C. Histogram of Pt particle diameters in 1000 Pt WtHsp, Average  $2.6 \pm 0.7$  nm, Scale bar = 20 nm.

measurements were based on a minimum of 100 particles. At theoretical loadings of 250 Pt/cage, particles of  $1.0 \pm 0.2$  nm were observed (Figure 2.24 A and B), while at loadings

of 150 Pt/cage, no particles could be distinguished due to the limitation of the transmission electron microscope.

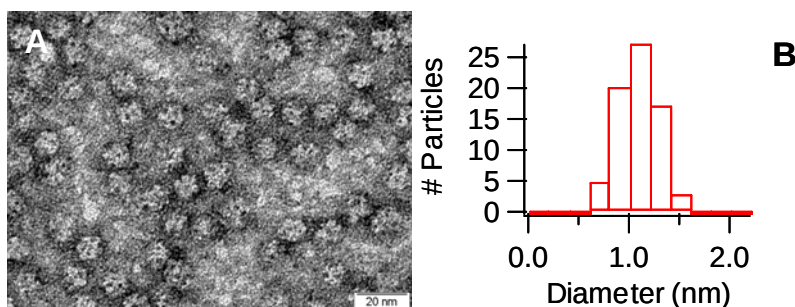


Figure 2.24. TEM characterization of 250 Pt WtHsp. A. TEM of 250 Pt WtHsp stained with 2 % uranyl acetate; B. Histogram of Pt particle diameters in 250 Pt Wt Hsp, Average  $1.0 \pm 0.2$  nm, Scale bar = 20 nm.

### Summary of Pt<sup>0</sup> Mineralization in Hsp

Attempts to genetically engineer platinum binding sites into the Hsp cage to aid the specific mineralization on the interior of the protein cage turned out to be unbeneficial. The covalent attachment of a platinum binding site for the photomineralization of platinum nanoparticles revealed inconclusive results, which leaves the products of the mineralization not fully characterized. Mineralization using the native Hsp structure was the cleanest most interior specific reaction architecture. The WtHsp at 1 mg/mL in 25 mM MES 10 mM NH<sub>4</sub>OH pH 6.5 at 65°C with 5x molar excess of DMAB added over 15 minutes was the optimal mineralization conditions for the Hsp.

### Pt<sup>0</sup> Mineralization in Horse-Spleen Ferritin

In an attempt to further probe the role of the protein cage in the mineralization process, ferritin was mineralized with platinum nanoparticles. Horse spleen ferritin was used because it has a cage-like architecture similar to WtHsp. Both protein architectures are 12 nm exterior diameter while ferritin has a interior diameter of 8 nm compared to the

6.5 nm of WtHsp. While ferritin and WtHsp have similar stabilities, ferritin is more thermally stable and is more stable at low pH. The WtHsp architecture has large pores (3 nm) in the cage structure allowing ready access to the interior; while, ferritin has a more closed structure limiting access to the interior.

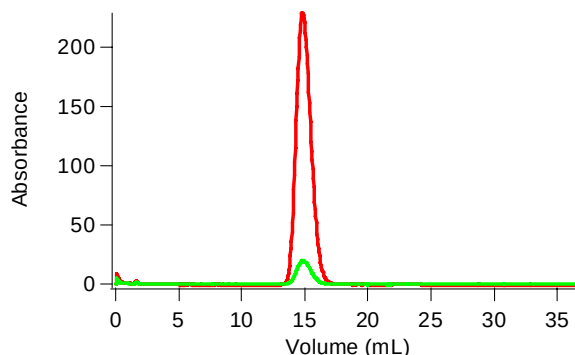


Figure 2.25. SEC of demineralized horse-spleen apo-ferritin, ■280 nm ■350 nm.

Demineralized horse-spleen ferritin (Fn) (Figure 2.25) was used for all of the mineralizations involving ferritin. Using the reaction conditions optimized for WtHsp, Fn was mineralized with both 250 and 1000  $\text{Pt}^{2+}$  per Fn. Apo-ferritin (Fn at 2.34 mg/mL in 50 mM MES 100 mM NaCl pH 6.5) was added to a solution of 25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5 for a final concentration of 1 mg/mL ( $5 \times 10^{-6}$  mmol) in 2.5 mL. A

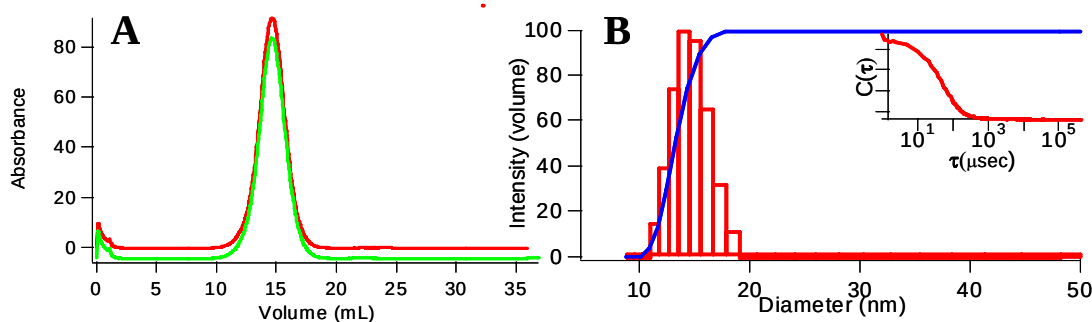


Figure 2.26. Characterization of 1000 PtFn. A. SEC of 1000 PtFn, ■280 nm ■350 nm; B. DLS of 1000 PtFn.

temperature of 65 °C was maintained using a jacketed reaction vessel with water circulating around the reaction. The reaction was stirred vigorously throughout the

reaction with a magnetic stir bar. Platinum(II) and 5-fold excess of DMAB per Pt was simultaneously added over 15 minutes using a syringe pump.

The mineralization of 1000 Pt<sup>2+</sup> inside of Fn (1000 PtFn) exhibits very little bulk mineralization or aggregation. Centrifugation after the reaction revealed almost no precipitation. The SEC of 1000 PtFn (Figure 26 A) shows that the mineralization is completely on the interior of the protein cage with no material eluting early in the chromatography. DLS revealed particles with an average diameter of nm consistent with

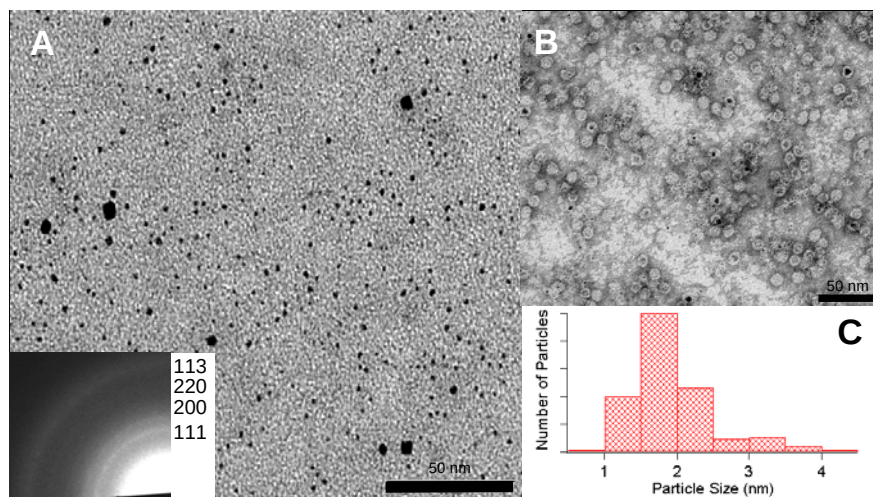


Figure 2.27. TEM characterization of 1000 PtFn. A. TEM of 1000 PtFn unstained, the inset shows electron diffraction of Pt<sup>0</sup> from 1000 PtFn; B. TEM of 1000 PtFn stained with 2% uranyl acetate; C. Histogram of Pt particle diameters in 1000 PtFn; Average 1.9 ± 0.8 nm, Scale bar = 50 nm.

the dimension of the Fn cage. Figure 26A shows co-elution of the 280 nm (protein) and 350 nm (mineral) at the same elution volume as unmineralized Fn (Figure 25). The unstained TEM analysis of this material (Figure 27 A) displays dense cores with a diameter of 1.9 ± 0.8 nm (Figure 2.27 C), in all cases size distribution measurements were based on a minimum of 100 particles. The electron diffraction of the cores (Figure 27A inset, Table I) displays a diffraction pattern consistent with Pt<sup>0</sup> nanoparticles. The

uranyl acetate stained TEM (Figure 27 B) clearly shows the electron dense  $\text{Pt}^0$  cores surrounded unstained regions which indicate encapsulation by protein.

| Miller Index | d-spacing | Measured d-spacing |
|--------------|-----------|--------------------|
| 111          | 2.265     | 2.304              |
| 200          | 1.961     | 1.965              |
| 220          | 1.387     | 1.140              |
| 113          | 1.183     | 1.195              |

Table 2.2. d-spacings of Pt-Fn.

The mineralization of 250  $\text{Pt}^{2+}$  per Fn (250 PtFn) was performed under identical conditions with the exception of 250  $\text{Pt}^{2+}$  per Fn instead of 1000. The mineralization of

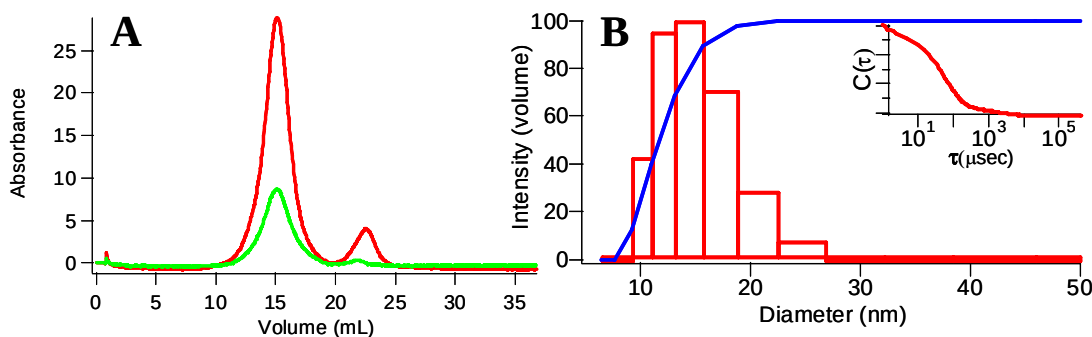


Figure 2.28. Characterization of 250 PtFn. A. SEC of 250 PtFn, -280 nm -350 nm; B. DLS of 250 PtFn.

250  $\text{Pt}^{2+}$  inside Fn was also a very clean reaction and showed no bulk mineralization or aggregation. Centrifugation after the reaction revealed no pellet. The SEC and DLS of 250 PtFn (Figure 28 A and B, respectively) show that the mineralization occurred completely on the interior of the protein cage. Figure 28A shows co-elution of the 280 nm (protein) and 350 nm (mineral) at the same elution volume as unmineralized apo-ferritin (Figure 25). The DLS showed an average diameter of nm consistent with the size

of the Fn cage. The uranyl acetate stained TEM (Figure 29 A) clearly showed the electron dense  $\text{Pt}^0$  cores, with an average diameter of  $1.8 \pm 1.2$  nm (Figure 29 B), surrounded by unstained regions which indicate encapsulation by protein.

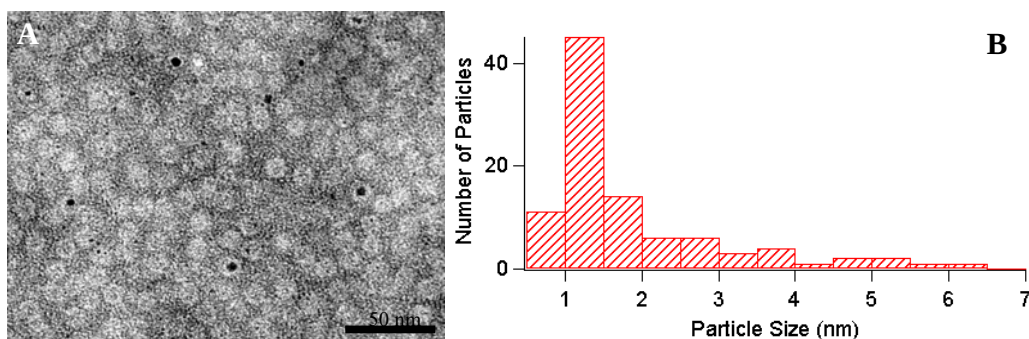


Figure 2.29. TEM characterization of 250 PtFn. A. TEM of 250 PtFn stained with 2 % uranyl acetate; B. Histogram of Pt particle diameters in 250 PtFn, Average  $1.8 \pm 1.2$  nm, Scale bar = 50 nm.

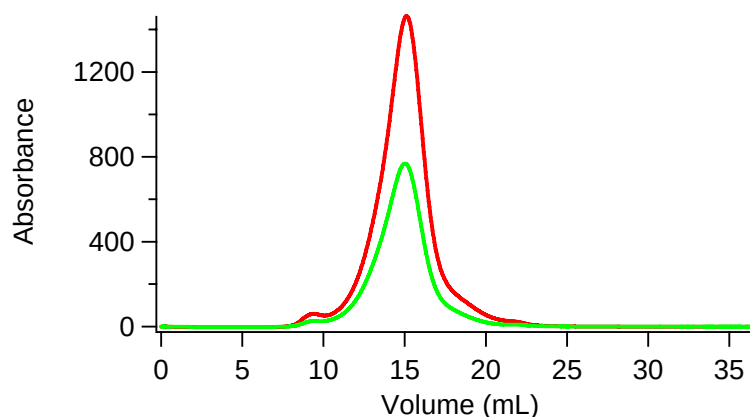


Figure 2.30. SEC characterization of 500 PtFn, ■280 nm ■350 nm.

The mineralization of 500  $\text{Pt}^{2+}$  per Fn (500 PtFn) was done under identical conditions with the exception of 500  $\text{Pt}^{2+}$  per Fn instead of 1000. The mineralization of 500 Pt inside of Fn was also very clean with almost no bulk mineralization or aggregation. Centrifugation after the reaction revealed no pellet. The SEC of 500 PtFn (Figure 30) show that the mineralization is completely on the interior of the protein cage.

Figure 28 shows co-elution of the 280 nm (protein) and 350 nm (mineral) at the same elution volume as unmineralized Fn (Figure 25). The uranyl acetate stained TEM (Figure 31 A) clearly shows the electron dense  $\text{Pt}^0$  cores, with an average diameter of  $2.8 \pm 1.3$  nm (Figure 31 B), surrounded by unstained regions which indicate encapsulation by protein.

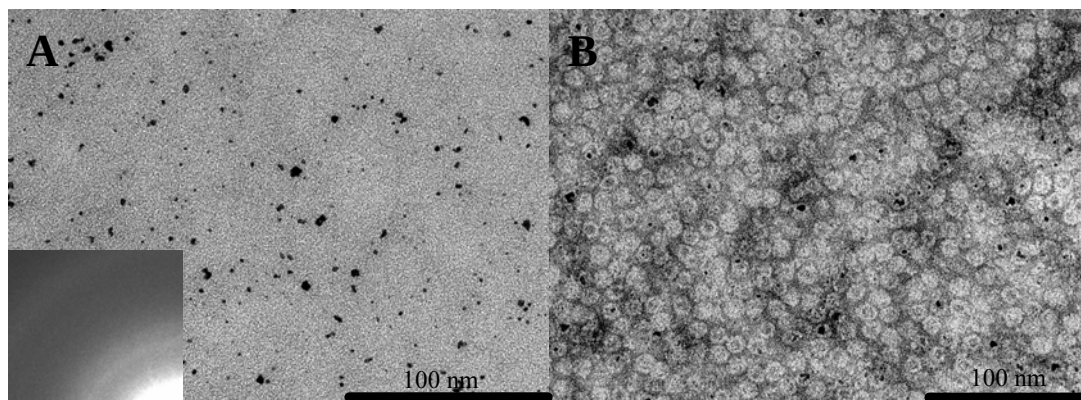


Figure 2.31. TEM characterization of 500 PtFn. **A.** TEM of 500 PtFn unstained, the inset shows electron diffraction of  $\text{Pt}^0$  from 500 PtFn; **B.** TEM of 500 PtFn stained with 2% uranyl acetate, Average particle size =  $2.8 \pm 1.3$  nm, Scale bar = 100 nm.

### Pt Alloy Synthesis in Horse-Spleen Ferritin

After determining that mineralization in Fn was more efficient and more specific for the interior of the protein cage than the Hsp platform, further studies were conducted to synthesize Pt alloys ( $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ru}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Cu}^{2+}$ ) to determine their effect on the mineralization and catalytic properties. In the first series of reactions, alloys were synthesized in a three to one ratio of Pt to alloying metal (3 Pt:1 M). The initial reaction conditions for the 1000 per cage 3:1 Pt/M mineralization in Fn were: Apo-Fn (50 mM MES 100 mM NaCl pH 6.5) was added to a buffer solution (25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5) for a final concentration of 1 mg/mL ( $5 \times 10^{-6}$  mmole) in 2.5 mL. A

temperature of 65 °C was maintained using a jacketed reaction vessel with water circulating around the reaction. The reaction was stirred vigorously throughout the reaction with a magnetic stir bar. The  $\text{Pt}^{2+}$ , dopant metal, and 5-fold excess of DMAB per metal (Pt + dopant metal) was simultaneously added over 15 minutes using a syringe pump.

The SEC of 1000 Cu-Pt, Ru-Pt, Fe-Pt, and Zn-Pt reactions are shown in Figure 2.32 A, B, C, and D respectively. The products of the mineralization with 1000 Ni-Pt and Co-Pt were pelleted upon centrifugation preventing the characterization by SEC. All of

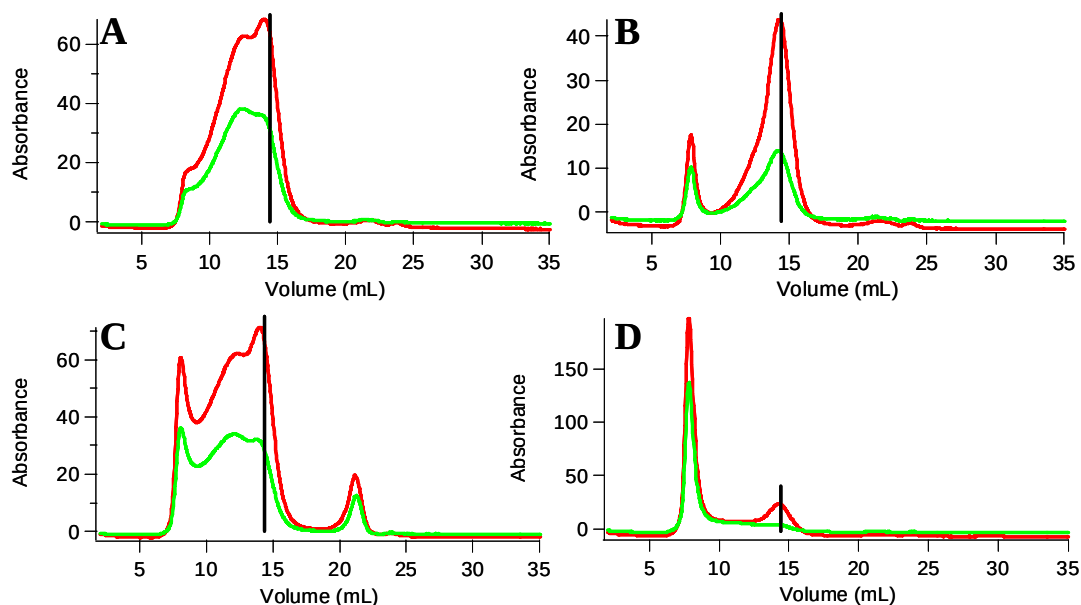


Figure 2.32. First SEC characterization of Pt alloys in Fn. A. SEC of 1000 Cu-Pt Fn; B. SEC 1000 Ru-Pt Fn; C. SEC 1000 Fe-Pt Fn; D. SEC 1000 Zn-Pt Fn, ■280 nm ■350nm. Black line indicates elution volume of untreated Apo-Fn.

the mineralization reaction products had a considerable pellet upon centrifugation and the SEC of Cu-Pt, Ru-Pt, and Fe-Pt clearly show significant mineralization on the exterior surface of the protein cage. The product of the Ru-Pt Fn synthesis is the most similar to the undoped Pt mineralization. Under these conditions the product of the synthesis with the 1000 Zn-Pt Fn shows that there is almost no unaggregated protein after the reaction.

### Optimization of Zn-Pt Mineralization in Ferritin While Stirring

After screening the platinum alloys synthesized in ferritin for catalytic activity (see Chapter 3, H<sub>2</sub> production assay for alloys of PtFn), Zn-Pt was chosen as an alloy of interest to further pursue and optimize the mineralization on the interior of the Fn cage. The typical initial reaction conditions used: 2 mg Fn (2.34 mg/mL in 50 mM MES 100 mM NaCl pH 6.5) in 2 mL of a buffered solution with the temperature maintained at 65 °C using a jacketed reaction vessel attached to a water bath.

A number of reaction conditions were initially attempted to determine the a starting point for optimization. The initial series of optimization reactions including varying NaCl concentration from zero to 500 mM because of work by Ensign and coworkers which showed the native Fn could be mineralized with copper metal under high NaCl conditions.<sup>124</sup> NH<sub>4</sub>OH concentrations were varied from zero to 100 mM, varying buffers (acetate, MES, and HEPES) and pH was varied from 5 to 8 in an attempt to minimize the metal interaction with the exterior of the cage. Temperature was adjusted from 40 to 65 °C to control the rate of reduction. All attempts had little impact on the specificity of the mineralization for the interior of Fn. The product of the Zn-Pt reaction was mostly aggregation and precipitate.

The next series of reaction conditions included using 25 mM MES as the base buffer with the addition of 10 mM of secondary buffer (EDTA, citrate, acetate, glycine, imidazole, and phosphate). These secondary buffers were added to chelate and stabilize the metals in solution. The buffers with various metal binding properties were tested to find the optimal buffer to prevent the mineralization on the exterior of the cage. These

attempts also had very little influence on the Zn-Pt reaction product which was mostly aggregate and bulk precipitation.

The initial and secondary reaction conditions showed little change in the SEC profile of the reaction product under varying conditions. Figure 2.33 shows the SEC profile for two different reaction conditions with remarkably similar SEC reaction profiles. Figure 2.33A shows the SEC profile for the product of the 1000 Zn-Pt Fn reaction in 25 mM MES 10 mM glycine pH 6.5 at 65 °C add 50  $\mu$ L of 20 mM  $\text{ZnCl}_2$  and 150  $\mu$ L  $\text{PtCl}_4^{2-}$  and 200  $\mu$ L 3 mg/mL DMAB over 15 min. Figure 2.33B shows the SEC profile for the product of the 1000 Zn-Pt Fn reaction in 25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5 at 65 °C where 200  $\mu$ L of 20 mM metal ( $\text{ZnCl}_2$  and  $\text{PtCl}_4^{2-}$  mixed before addition) was added in ten additions (20  $\mu$ L of Zn/Pt then 10  $\mu$ L of DMAB (3 mg/mL)) with three

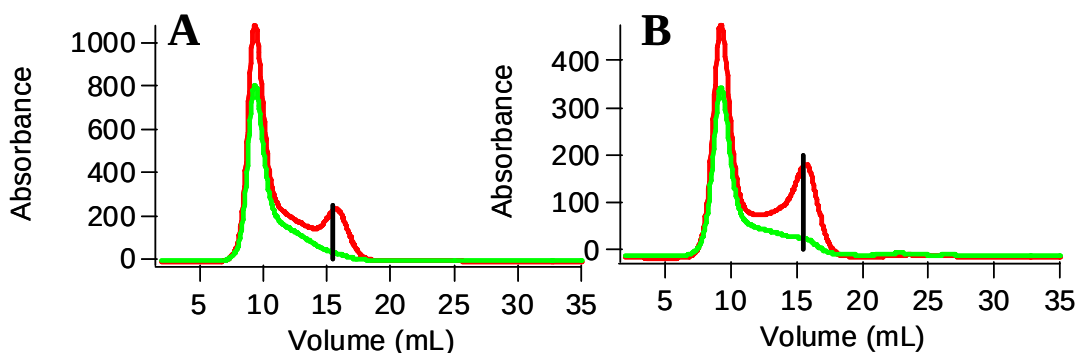


Figure 2.33. Typical SEC of high temperature Zn-Pt synthesis in Fn with varying buffer conditions. A. SEC of 1000 ZnPt Fn in 25 mM MES 10 mM glycine pH 6.5; B. SEC 1000 ZnPt Fn in 25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5, metal added in additions, ■280 nm ■350 nm. Black line indicates elution volume of untreated Apo-Fn.

minutes intervals in between additions then 5 minutes after the final addition 100  $\mu$ L of DMAB was added (Figure 2.33 B).

### Optimization of Zn-Pt Mineralization in Ferritin Using Sonication

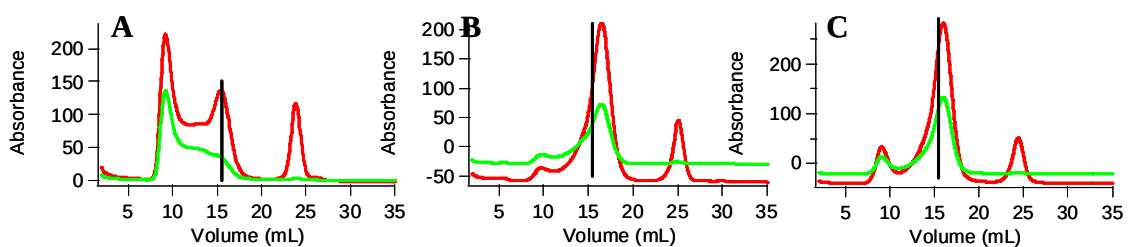


Figure 2.34. The effect of sonication on the mineralization of 500 Zn-Pt in Fn shown by SEC of 500 Zn-Pt mineralization in Fn. A. SEC of 500 ZnPt Fn in 200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5 at  $65^\circ\text{C}$ ; B. SEC 500 ZnPt Fn in 200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5 in Branson B200; C. SEC 500 ZnPt Fn in 200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5 in Fisher Scientific sonicator,  $\text{■}$ 280 nm  $\text{■}$ 350 nm. Black line indicates elution volume of untreated Apo-Fn.

Tsukamoto and coworkers mineralized tobacco mosaic virus (TMV) with Co-Pt and Fe-Pt under sonicating conditions.<sup>125</sup> This reaction procedure was probed using a series of Zn-Pt in Fn reactions under various sonication techniques. First, Fn was mineralized with 500 Zn-Pt 1:3 in 1 mg/mL 200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5 with a final volume of 2 mL while stirring vigorously at  $65^\circ\text{C}$ . 50  $\mu\text{L}$  of 20 mM metal ( $\text{ZnCl}_2$  and  $\text{PtCl}_4^{2-}$  mixed before addition) and 50  $\mu\text{L}$  of 3 mg/mL DMAB were added using a syringe pump over 15 min (Figure 2.34 A). Second, Fn was mineralized with 500 Zn-Pt 1:3 in 1 mg/mL 200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5 with a final volume of 2 mL at room temperature using a Branson B200 sonicator. 50  $\mu\text{L}$  of 20 mM metal ( $\text{ZnCl}_2$  and  $\text{PtCl}_4^{2-}$  mixed before addition) and 50  $\mu\text{L}$  of 3 mg/mL DMAB was added by 10  $\mu\text{L}$  additions of metal followed 3 minutes later by 10  $\mu\text{L}$  DMAB, 3 minutes after the DMAB addition the process was repeated 5 times while sonicating (Figure 2.34 B). Fn was mineralized with 500 ZnPt 1:3 in 1 mg/mL 200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5 with a final volume of 2 mL at room temperature using a Fisher Scientific sonicator. 50  $\mu\text{L}$  of 20 mM metal ( $\text{ZnCl}_2$  and

PtCl<sub>4</sub><sup>2-</sup> mixed before addition) and 50 µL of 3 mg/mL DMAB was added by 10 µL additions of metal followed 3 minutes later by 10 µL DMAB, 3 minutes are the DMAB addition the process was repeated 5 times while sonicating (Figure 2.34 C).

An examination of Figure 2.34 A, B, and C, clearly reveals that the sonication greatly improves the specificity of the mineralization for the interior of Fn. With the use of the sonication during the reaction, the SEC profile shows a dramatic decrease in the amount of aggregation. The Fisher Scientific sonicator provides more sonic power than the Branson B200 sonicator which caused the reaction to heat up considerably. Without a simple way to control the temperature in the sonic bath, the use of a tip sonicator, which can be used with a water bath to control the temperature, was explored.

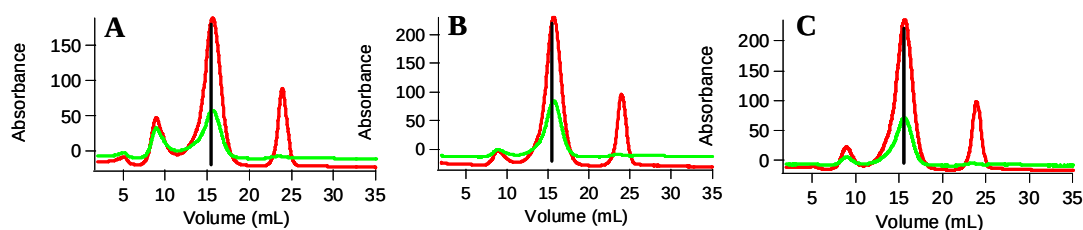


Figure 2.35. The dependence of temperature on the sonic mineralization of Zn-Pt in Fn analyzed by SEC. A. SEC of 500 ZnPt Fn in 200 mM NH<sub>4</sub>(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>) pH 5 at 40 °C; B. SEC 500 ZnPt Fn in 200 mM NH<sub>4</sub>(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>) pH 5 at 25 °C; C. SEC 1000 ZnPt Fn in 200 mM NH<sub>4</sub>(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>) pH 5 at 10 °C -280 nm -350 nm. Black line indicates elution volume of untreated Apo-Fn.

Fn was mineralized with 500 Zn-Pt 1:3 in 1 mg/mL 200 mM NH<sub>4</sub>(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>) pH 5 with a final volume of 2 mL at 40 °C using a tip sonicator (50% duty, 1 power). 50 µL of 20 mM metal (ZnCl<sub>2</sub> and PtCl<sub>4</sub><sup>2-</sup> mixed before addition) and 50 µL of 3 mg/mL DMAB was added by adding 10 µL of metal mix then waiting 3 minutes then 10 µL DMAB and after waiting three minutes repeating 5 times while sonicating (Figure 2.35 A). Fn was mineralized with 500 Zn-Pt 1:3 in 1 mg/mL 200 mM NH<sub>4</sub>(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>) pH 5 with a final

volume of 2 mL at 25 °C using a tip sonicator (50% duty, 1 power). 50 µL of 20 mM metal ( $\text{ZnCl}_2$  and  $\text{PtCl}_4^{2-}$  mixed before addition) and 50 µL of 3 mg/mL DMAB was added by 10 µL additions of metal followed 3 minutes later by 10 µL DMAB, 3 minutes are the DMAB addition the process was repeated 5 times while sonicating (Figure 2.35 B). Fn was mineralized with 500 ZnPt 1:3 in 1 mg/mL 200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  50 µL of 20 mM metal ( $\text{ZnCl}_2$  and  $\text{PtCl}_4^{2-}$  mixed before addition) and 50 µL of 3 mg/mL DMAB was added by 10 µL additions of metal followed 3 minutes later by 10 µL DMAB, 3 minutes are the DMAB addition the process was repeated 5 times while sonicating (50% duty, 1 power). A total of 50 µL of 20 mM metal ( $\text{ZnCl}_2$  and  $\text{PtCl}_4^{2-}$  mixed before addition) and 50 µL of 3 mg/mL DMAB was added by 10 µL additions of metal followed 3 minutes later by 10 µL DMAB, 3 minutes are the DMAB addition the process was repeated 5 times while sonicating (Figure 2.35 C). In an attempt to optimize the specific mineralization of ZnPt on the interior of the Fn, the temperature was varied from 40 °C to 10 °C. Even though the SEC of the 25 °C and 10 °C syntheses look similar (Figure 2.35 B and C respectively), the 10 °C synthesis has a smaller pellet after centrifugation. The optimized temperature was determined to be 15 °C.

During the optimization of the Zn-Pt in Fn synthesis, fresh Fn was prepared and dialyzed into 150 mM NaCl to determine if the buffer from the protein stock was interfering with the specificity of the mineralization for the interior of the protein cage. The stock protein concentration and buffer condition was has a major effect on the specificity of the Zn-Pt mineralization for the interior of the protein cage.

### Characterization of Alloy Mineralizations in Ferritin

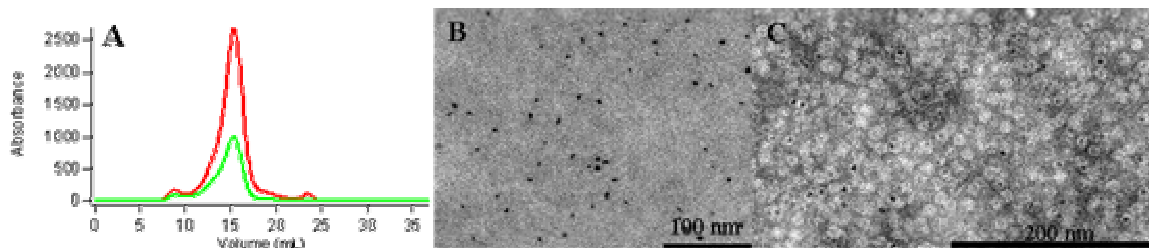


Figure 2.36. Characterization of 250 ZnPt Fn. **A.** SEC of 250 ZnPt Fn in 200 mM  $\text{NH}_4\text{Acetate}$  pH 5 at 15 °C, **-280 nm** **-350 nm**; **B.** TEM of 250 ZnPt Fn unstained, scale bar = 100 nm; **C.** TEM of 250 ZnPt Fn stained with 2% uranyl acetate, scale bar = 200 nm. Average particle size =  $3.4 \pm 1.4$  nm.

The optimized conditions for mineralizing Fn with 250 ZnPt 1:3 is Fn (10 mg/mL in 150 mM NaCl) added to buffered solution (200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5) with a final concentration of 1 mg/mL and volume of 4 mL at 15 °C using a tip sonicator (20% duty, 1 power). 100  $\mu\text{L}$  of 20 mM metal ( $\text{ZnCl}_2$  and  $\text{PtCl}_4^{2-}$  mixed before addition) was added in of aliquots of 25  $\mu\text{L}$  metal at 0, 10, 60, and 70 minutes while adding 300  $\mu\text{L}$  of 3 mg/mL DMAB over 2 hours using a syringe pump. Centrifugation after the reaction revealed minimal pellet. The SEC of 250 ZnPt Fn (Figure 2.36 A) shows that the mineralization is almost completely on the interior of the protein cage. Figure 2.36A shows co-elution of the 280 nm (protein) and 350 nm (mineral) at the same elution volume as unmineralized Fn (Figure 2.25). The unstained TEM (Figure 36 B) depicts electron dense cores with an average diameter of  $3.4 \pm 1.4$  nm (76 particles measured). The uranyl acetate stained TEM (Figure 2.36 C) clearly shows the electron dense  $\text{Pt}^0$  cores surrounded unstained regions which indicates encapsulation by protein.

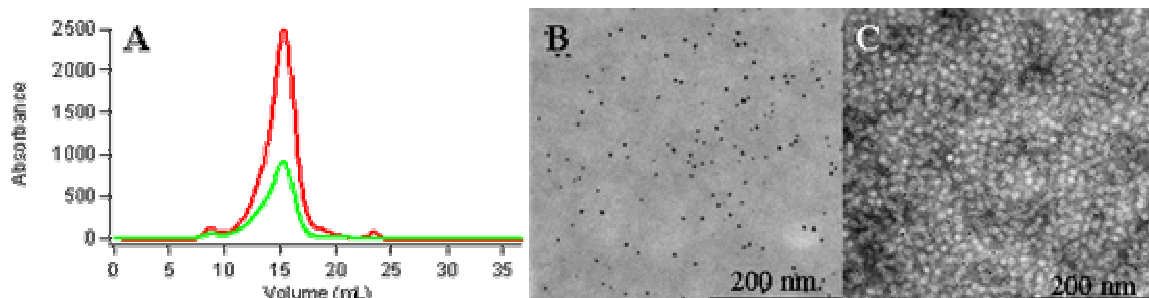


Figure 2.37. Characterization of 250 NiPt Fn. A. SEC of 250 NiPt Fn in 200 mM  $\text{NH}_4\text{Acetate}$  pH 5 at 15 °C, -280 nm -350 nm; B. TEM of 250 NiPt Fn unstained; C. TEM of 250 NiPt Fn stained with 2% uranyl acetate; Scale bar = 200 nm. Average particle size =  $3.1 \pm 1.1$  nm.

The optimized conditions for mineralizing Fn with 250 NiPt 1:3 is Fn (10 mg/mL in 150 mM NaCl) added to buffered solution (200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5) with a final concentration of 1 mg/mL and volume of 4 mLs at 15 °C using a tip sonicator (20% duty, 1 power). 100  $\mu\text{L}$  of 20 mM metal ( $\text{Ni}(\text{NO}_3)$  and  $\text{PtCl}_4^{2-}$  mixed before addition) was added in of aliquots of 25  $\mu\text{L}$  at 0, 10, 60, and 70 minutes while adding 300  $\mu\text{L}$  of 3 mg/mL DMAB over 2 hours using a syringe pump. Centrifugation after the reaction revealed minimal pellet. The SEC of 250 NiPt Fn (Figure 2.37 A) shows that the mineralization is almost completely on the interior of the protein cage. Figure 2.37A shows co-elution of the 280 nm (protein) and 350 nm (mineral) at the same elution volume as unmineralized Fn (Figure 2.25). The unstained TEM (Figure 2.37 B) depicts electron dense cores with an average diameter of  $3.1 \pm 1.1$  nm (76 particles measured). The uranyl acetate stained TEM (Figure 2.37 C) clearly shows the electron dense  $\text{Pt}^0$  cores surrounded unstained regions which indicates encapsulation by protein.

The optimized conditions for mineralizing Fn with 500 ZnPt 1:3 is Fn (10 mg/mL in 150 mM NaCl) added to buffered solution (200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5) with a final concentration of 1 mg/mL and volume of 2 mL at 15 °C using a tip sonicator (20% duty,

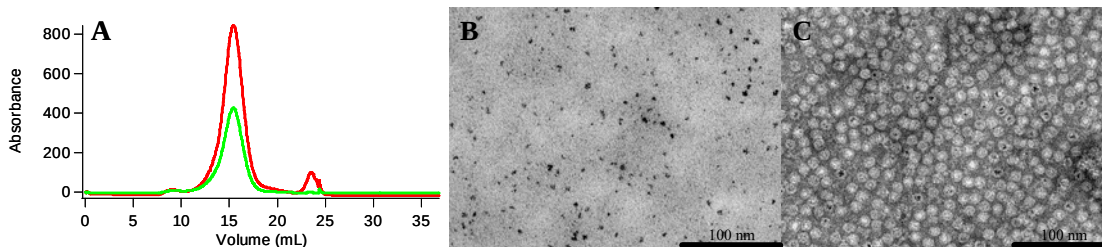


Figure 2.38. Characterization of 500 ZnPt Fn. A. SEC of 500 ZnPt Fn in 200 mM  $\text{NH}_4\text{Acetate}$  pH 5 at 15 °C, -280 nm -350 nm; B. TEM of 500 ZnPt Fn unstained, the inset shows electron diffraction of  $\text{Pt}^0$  from 500 ZnPt Fn; C. TEM of 500 ZnPt Fn stained with 2% uranyl acetate; Scale bar = 100 nm. Average particle size =  $3.1 \pm 1.1$  nm.

1 power). 100  $\mu\text{L}$  of 20 mM metal ( $\text{ZnCl}_2$  and  $\text{PtCl}_4^{2-}$  mixed before addition) was added in of aliquots of 25  $\mu\text{L}$  at 0, 10, 60, and 70 minutes while adding 300  $\mu\text{L}$  of 3 mg/mL DMAB over 2 hours using a syringe pump. Centrifugation after the reaction revealed minimal pellet. The SEC of 500 ZnPt Fn (Figure 2.38 A) shows that the mineralization is completely on the interior of the protein cage. Figure 2.38A shows co-elution of the 280 nm (protein) and 350 nm (mineral) at the same elution volume as unmineralized Fn (Figure 2.25). The unstained TEM (Figure 2.38 B) depicts electron dense cores with an average diameter of  $3.1 \pm 1.1$  nm (133 particles measured). The uranyl acetate stained TEM (Figure 2.38 C) clearly shows the electron dense  $\text{Pt}^0$  cores surrounded unstained regions which indicates encapsulation by protein.

The optimized conditions for mineralizing Fn with 500 NiPt 1:3 is Fn (10 mg/mL in 150 mM NaCl) added to buffered solution (200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5) with a final concentration of 1 mg/mL and volume of 2 mL at 15 °C using a tip sonicator (20% duty, 1 power). 100  $\mu\text{L}$  of 20 mM metal ( $\text{Ni}(\text{NO}_3)$  and  $\text{PtCl}_4^{2-}$  mixed before addition) was added in of aliquots of 25  $\mu\text{L}$  at 0, 10, 60, and 70 minutes while adding 300  $\mu\text{L}$  of 3 mg/mL DMAB over 2 hours using a syringe pump. Centrifugation after the reaction

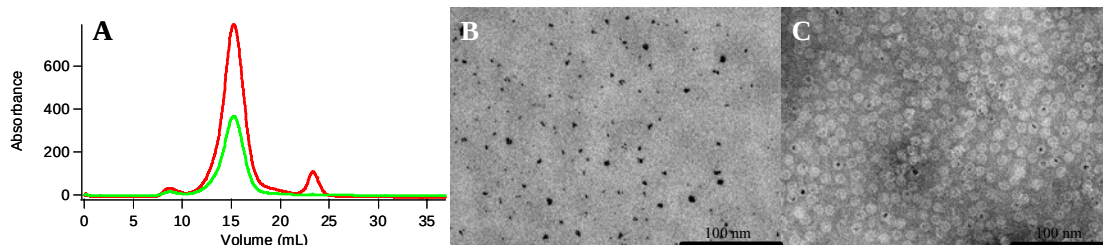


Figure 2.39. Characterization of 500 NiPt Fn. A. SEC of 500 NiPt Fn in 200 mM  $\text{NH}_4\text{Acetate}$  pH 5 at 15 °C, -280 nm -350 nm; B. TEM of 500 NiPt Fn unstained, the inset shows electron diffraction of  $\text{Pt}^0$  from 500 NiPt Fn; C. TEM of 500 NiPt Fn stained with 2% uranyl acetate; Scale bar = 100 nm. Average particle size =  $2.8 \pm 1.1$  nm.

reveals minimal pellet. The SEC of 500 NiPt Fn (Figure 2.39 A) shows that the mineralization is completely on the interior of the protein cage. Figure 2.39A shows co-elution of the 280 nm (protein) and 350 nm (mineral) at the same elution volume as unmineralized Fn (Figure 2.25). The unstained TEM (Figure 2.39 B) depicts electron dense cores with an average diameter of  $2.8 \pm 1.1$  nm (139 particles measured). The uranyl acetate stained TEM (Figure 2.39 C) clearly shows the electron dense  $\text{Pt}^0$  cores surrounded unstained regions which indicates encapsulation by protein.

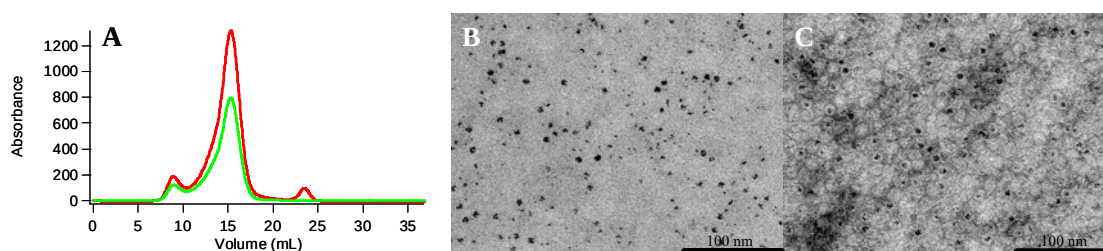


Figure 2.40. Characterization of 1000 ZnPt Fn. A. SEC of 1000 ZnPt Fn in 200 mM  $\text{NH}_4\text{Acetate}$  pH 5 at 15 °C, -280 nm -350 nm; B. TEM of 1000 ZnPt Fn unstained, the inset shows electron diffraction of  $\text{Pt}^0$  from 1000 ZnPt Fn; C. TEM of 1000 ZnPt Fn stained with 2% uranyl acetate; Scale bar = 100 nm. Average particle size =  $4.0 \pm 1.4$  nm.

The optimized conditions for mineralizing Fn with 1000 ZnPt 1:3 is Fn (10 mg/mL in 150 mM NaCl) added to buffered solution (200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5) with a final concentration of 1 mg/mL and volume of 2 mL at 15 °C using a tip sonicator (20%

duty, 1 power). 200  $\mu\text{L}$  of 20 mM metal ( $\text{ZnCl}_2$  and  $\text{PtCl}_4^{2-}$  mixed before addition) was added in of aliquots of 25  $\mu\text{L}$  at 0, 10, 20, 30, 60, 70, 80, and 90 minutes while adding 300  $\mu\text{L}$  of 3 mg/mL DMAB over 2 hours using a syringe pump. Centrifugation after the reaction reveals minimal pellet. The SEC of 1000 ZnPt Fn (Figure 2.40 A) shows that the mineralization is almost completely on the interior of the protein cage. Figure 38A shows co-elution of the 280 nm (protein) and 350 nm (mineral) at the same elution volume as unmineralized Fn (Figure 25). The unstained TEM (Figure 2.40 B) depicts electron dense cores with an average diameter of  $4.0 \pm 1.4$  nm (123 particle measured). The uranyl acetate stained TEM (Figure 2.40 C) clearly shows the electron dense  $\text{Pt}^0$  cores surrounded unstained regions which indicates encapsulation by protein.

The conditions for mineralizing Fn with 1000 NiPt 1:3 is Fn (10 mg/mL in 150 mM NaCl) added to buffered solution (200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5) with a final concentration of 1 mg/mL and volume 2 mL at 15°C using a tip sonicator (20% duty, 1

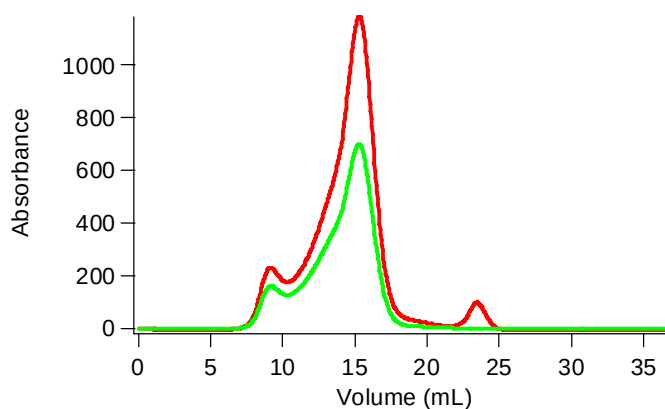


Figure 2.41. SEC of 1000 NiPt Fn in 200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5 at 15 °C. -280 nm  
-350 nm.

power). 200  $\mu\text{L}$  of 20 mM metal ( $\text{Ni}(\text{NO}_3)$  and  $\text{PtCl}_4^{2-}$  mixed before addition) was added in of aliquots of 25  $\mu\text{L}$  metal at 0, 10, 20, 30, 60, 70, 80, and 90 minutes while adding 300  $\mu\text{L}$  of 3 mg/mL DMAB over 2 hours using a syringe pump. Centrifugation after the

reaction reveals significant pellet. The SEC of 1000 NiPt Fn (Figure 2.41) shows that there is significant mineralization on the exterior of the protein cage. The 1000 NiPt Fn was reaction was not a clean reaction and was not pursued any further.

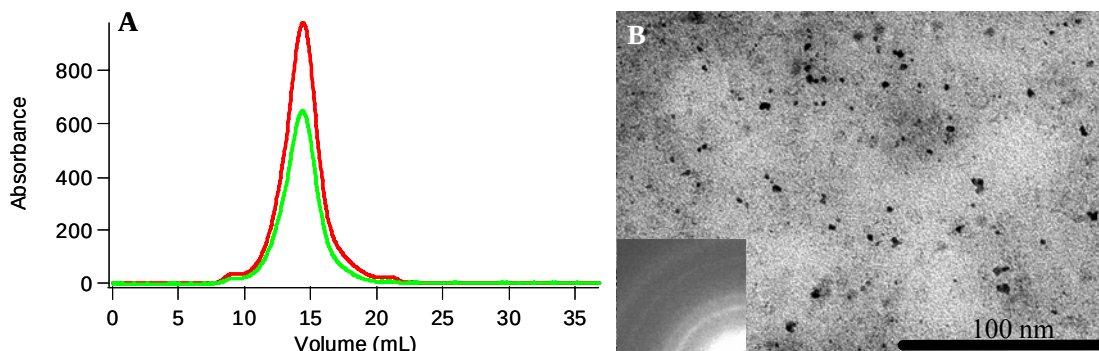


Figure 2.42. Characterization of 500 Pt+Zn Fn. A. SEC of 500 PtFn with  $\text{Zn}^{2+}$  added. -280 nm -350 nm, B. Electron diffraction of  $\text{Pt}^0$  from 500 PtFn with  $\text{Zn}^{2+}$  added the inset shows electron diffraction of  $\text{Pt}^0$  from 500 Pt+Zn Fn. Average particle size =  $2.8 \pm 0.8$  nm.

In an attempt to determine if the  $\text{Zn}^{2+}$  is incorporated into the  $\text{Pt}^0$  nanoparticle or is coating the surface,  $\text{Zn}^{2+}$  was added to previously synthesized 500 PtFn at 125 Zn per cage and allowed to incubate for one hour (500 Pt+Zn Fn). The sample was purified by SEC to determine if there was any  $\text{Zn}^{2+}$  induced aggregation (Figure 2.42 A). After SEC, electron diffraction was used to analyze the 500 PtFn with  $\text{Zn}^{2+}$  added (Figure 2.42 B). The unstained TEM (Figure 42 B) depicts electron dense cores with an average diameter of  $2.2 \pm 0.8$  nm (113 particle measured). The diffraction was indexed to  $\text{Pt}^0$ .

#### The Optimized Reaction Conditions for the Zn-Pt and Ni-Pt Mineralization in Ferritin

The final optimized conditions for mineralizing Fn with Zn-Pt and Ni-Pt 1:3 is Fn (10 mg/mL in 150 mM NaCl) added to buffered solution (200 mM  $\text{NH}_4(\text{C}_2\text{H}_3\text{O}_2)$  pH 5) with a final concentration of 1 mg/mL at 15 °C using a tip sonicator (20% duty, 1 power).

20 mM metal ( $\text{ZnCl}_2$  and  $\text{PtCl}_4^{2-}$  mixed before addition) was added in of aliquots of 25  $\mu\text{L}$  metal with 10 minutes in between aliquots with half of the metal added each hour while adding 300  $\mu\text{L}$  of 3 mg/mL DMAB over 2 hours using a syringe pump.

#### Palladium and Zinc/Palladium Alloys Mineralized in Ferritin

In addition to the development of protein based platinum catalysts, the synthesis of protein encapsulated palladium and palladium alloy nanoparticles was undertaken to determine their catalytic activity. We also wanted to determine if the doping of Zn into palladium particles would display an effect on the catalytic activity similar to that seen in Zn-Pt.

The reaction conditions for the initial screen of the palladium/platinum mineralization in Fn were Fn 2.34 mg/mL in 50 mM MES 100 mM NaCl pH 6.5 was added to a solution of 25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5 for a final concentration of 1 mg/mL ( $5 \times 10^{-6}$  mmole) in 2.5 mL. A temperature of 65 °C was maintained using a jacketed reaction vessel with water circulating around the reaction. The reaction was stirred vigorously throughout the reaction. Palladium/platinum ions and 5-fold excess of DMAB per total metal were simultaneously added over 15 minutes using a syringe pump. Figure 2.43A is the SEC of 1000 Pd per Fn mineralization (1000 PdFn). The SEC shows that a half of the reaction still in solution is aggregated. Figure 2.43B is the SEC of 1000 metal (75% Pd 25% Pt) per Fn mineralization (75/25 1000 PdPt Fn). The SEC revealed that a significant amount of mineralization occurred on the exterior of the protein cage. Figure 2.43C is the SEC profile of 1000 metal (50% Pd 50% Pt) per Fn mineralization (50/50 1000 PdPt Fn), which indicates that a small amount of mineralization occurs on

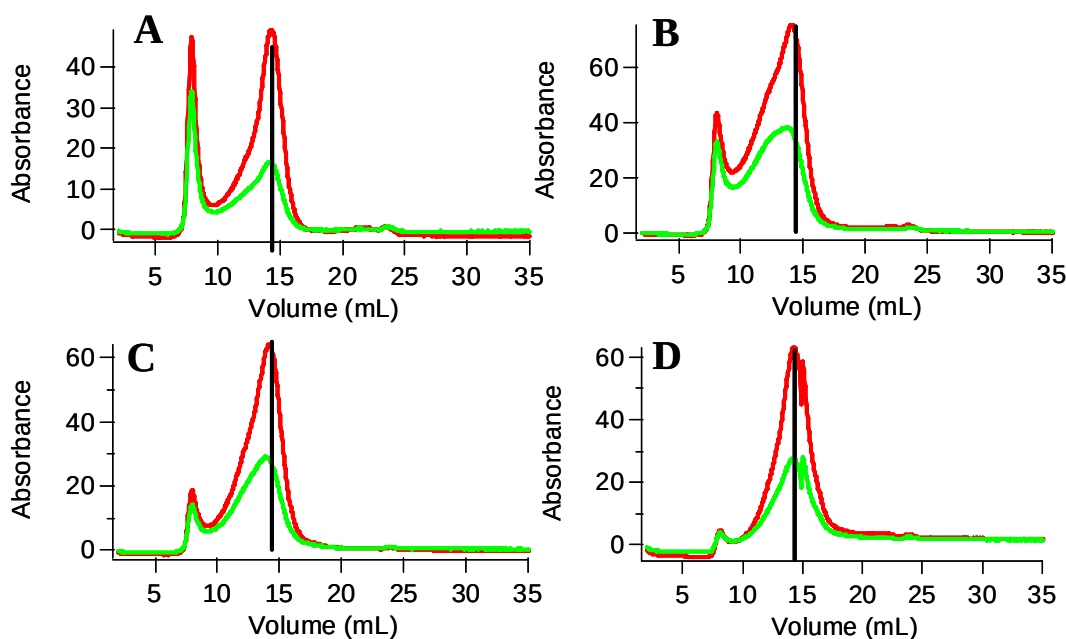


Figure 2.43. SEC characterization of Pt/Pd alloys at different ratios. A. SEC of 1000 PdFn; B. SEC of 75/25 1000 PdPt Fn; C. SEC 50/50 1000 PdPt Fn; D. SEC 25/75 1000 PdPt Fn, All reactions in 25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5 at 65 °C, -280 nm -350 nm. Black line indicates elution volume of untreated Apo-Fn.

the exterior of the protein cage. Figure 2.43D is the SEC of 1000 metal (25% Pd 75% Pt) per Fn mineralization (25/75 1000 PdPt Fn). The SEC shows that there is a small amount of mineralization on the exterior of the protein cage. In general, the reaction conditions described here for the initial palladium/platinum mineralization are poor conditions for the spatially selective mineralization of Pd and Pt/Pd alloys on the interior of Fn.

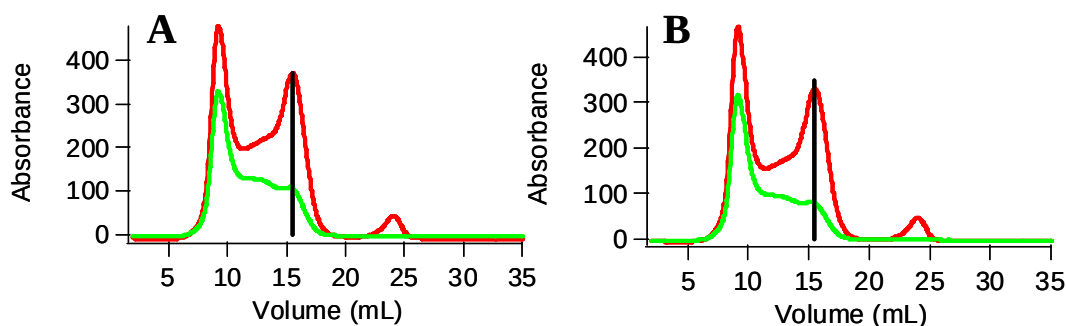


Figure 2.44. SEC profile of Pd and Zn-Pd mineralized in Fn under the conditions optimized for ZnPt Fn. A. SEC of 500 PdFn; B. SEC of 500 ZnPd Fn, -280 nm -350 nm. Black line indicates elution volume of untreated Apo-Fn.

The results of the H<sub>2</sub> production assay determined that the Pd/Pt alloying did not positively effect the H<sub>2</sub> production from Pt<sup>0</sup> nanoparticles in ferritin. However, the catalytic rate of Pd<sup>0</sup> nanoparticles and the effect of Zn alloying on that rate was of interest to determine if the Zn alloying had an effect on a broad range of minerals. As a second attempt to mineralize Pd in ferritin, the reaction conditions optimized for the mineralization of Zn-Pt inside ferritin were used. Ferritin was mineralized with 500 Pd (500 PdFn) at 1 mg/mL (2.34 mg/mL in 50 mM MES 100 mM NaCl pH 6.5) in buffered solution (200 mM NH<sub>4</sub>(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>) pH 5) with a final volume of 2 mL at 15 °C using a tip sonicator (20% duty, 1 power). 100 µL of 20 mM PdCl<sub>4</sub><sup>2+</sup> was added in of aliquots of 25 µL metal at 0, 10, 60, and 70 minutes while adding 300 µL of 3 mg/mL DMAB over 2 hours using a syringe pump. The SEC of 500 PdFn (Figure 2.44 A) shows that a majority of the protein is aggregated and almost all the absorbance at 350 nm is associated with the aggregation peak. Fn was mineralized with 500 Zn-Pd 1:3 (500 ZnPd Fn) at 1 mg/mL (2.34 mg/mL in 50 mM MES 100 mM NaCl pH 6.5) in buffered solution (200 mM NH<sub>4</sub>(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>) pH 5) with a final volume of 2 mL at 15°C using a tip sonicator (20% duty, 1 power). 100 µL of 20 mM metal (ZnCl<sub>2</sub> and PdCl<sub>4</sub><sup>2-</sup> mixed before addition) was added in of aliquots of 25 µL at 0, 10, 60, and 70 minutes while adding 300 µL of 3 mg/mL DMAB over 2 hours using a syringe pump. The SEC of 500 ZnPd Fn (Figure 2.44 B) shows that a majority of the protein is aggregated and almost all the absorbance at 350 nm is associated with the aggregation peak which is nearly identical to the 500 PdFn.

### The Optimized Conditions for the Mineralization of Pd and Zn-Pd in Ferritin

After attempting and failing to mineralize Pd inside of Fn under the conditions determined by Ueno,<sup>49</sup> the mineralization conditions used by and the Zn-Pt optimized conditions were blended together to in an attempt to specifically mineralize Pd and Zn-Pd on the interior of the ferritin cage. The combined conditions that were chosen were to use the sonication and addition procedures from the Zn-Pt mineralization and buffer conditions similar to those used by Ueno.<sup>49</sup>

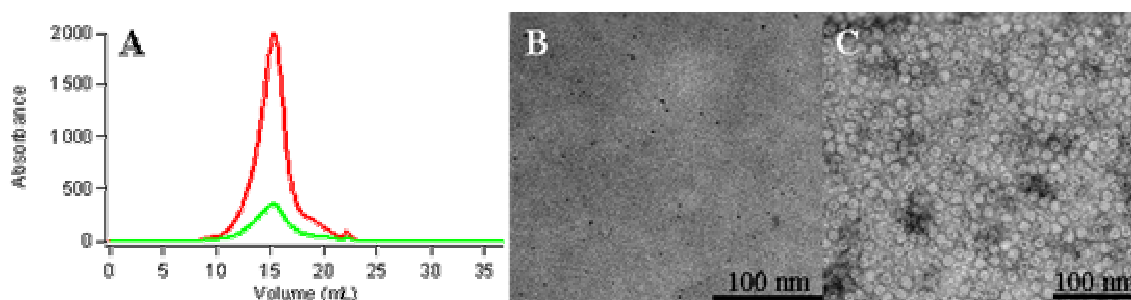


Figure 2.45. Characterization of 250 PdFn. A. SEC of 250 PdFn in 10 mM HEPES 150 mM NaCl pH 8.5 at 15 °C, -280 nm -350 nm; B. TEM of 250 PdFn unstained; C. TEM of 250 PdFn stained with 2% uranyl acetate; Scale bar = 100 nm, Average particle size =  $2.1 \pm 0.5$  nm.

The optimized conditions for mineralizing Fn with 250 Pd (250 PdFn) is 1 mg/mL (10 mg/mL in 150 mM NaCl) in 10 mM HEPES 150 mM NaCl pH 8.5 with a final volume of 4 mL at 15 °C using a tip sonicator (20% duty, 1 power). 100  $\mu$ L of 20 mM  $\text{PdCl}_4^{2-}$  was added in of aliquots of 25  $\mu$ L metal at 0, 10, 60, and 70 minutes while adding 300  $\mu$ L of 3 mg/mL DMAB over 2 hours using a syringe pump. Centrifugation after the reaction revealed no pellet. The SEC of 250 PdFn (Figure 2.45 A) shows that the mineralization is almost completely on the interior of the protein cage. Figure 45A shows co-elution of the 280 nm (protein) and 350 nm (mineral) at the same elution volume as unmineralized Fn (Figure 2.25). The unstained TEM (Figure 2.45 B) depicts

electron dense cores with an average diameter of  $2.1 \pm 0.5$  nm (110 particles measured).

The uranyl acetate stained TEM (Figure 2.45 C) clearly shows the electron dense  $\text{Pt}^0$  cores surrounded unstained regions which indicates encapsulation by protein.

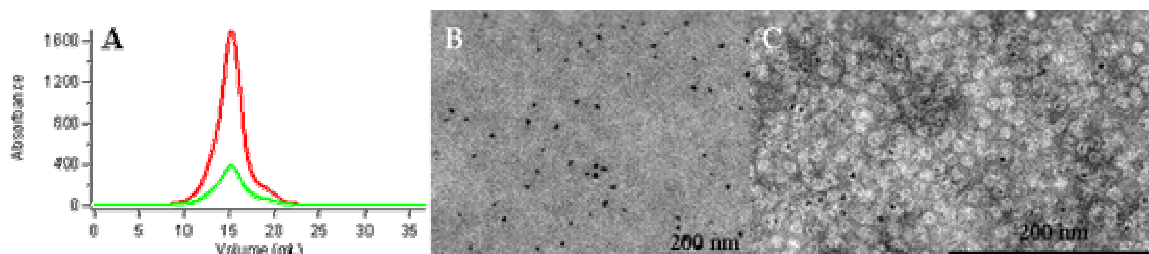


Figure 2.46. Characterization of 250 ZnPd Fn. A. SEC of 250 ZnPd Fn in 10 mM HEPES 150 mM NaCl pH 8.5 at 15 °C, -280 nm -350 nm; B. TEM of 250 ZnPd Fn unstained; C. TEM of 250 ZnPd Fn stained with 2% uranyl acetate; Scale bar = 200 nm, Average particle size =  $3.4 \pm 1.4$  nm.

The optimized conditions for mineralizing Fn with 250 Zn-Pd 1:3 (250 ZnPd Fn) is 1 mg/mL (10 mg/mL in 150 mM NaCl) in 10 mM HEPES 150 mM NaCl pH 8.5 with a final volume of 4 mL at 15 °C using a tip sonicator (20% duty, 1 power). 100  $\mu\text{L}$  of 20 mM metal ( $\text{ZnCl}_2$  and  $\text{PdCl}_4^{2-}$  mixed before addition) was added in of aliquots of 25  $\mu\text{L}$  metal at 0, 10, 60, and 70 minutes while adding 300  $\mu\text{L}$  of 3 mg/mL DMAB over 2 hours using a syringe pump. Centrifugation after the reaction reveals no pellet. The SEC of 250 ZnPd Fn (Figure 2.46 A) shows that the mineralization is almost completely on the interior of the protein cage. Figure 2.46A shows co-elution of the 280 nm (protein) and 350 nm (mineral) at the same elution volume as unmineralized Fn (Figure 2.25). The unstained TEM (Figure 2.46 B) depicts electron dense cores with an average diameter of  $3.4 \pm 1.4$  nm (83 particles measured). The uranyl acetate stained TEM (Figure 2.46 C) clearly shows the electron dense  $\text{Pt}^0$  cores surrounded unstained regions which indicates encapsulation by protein.

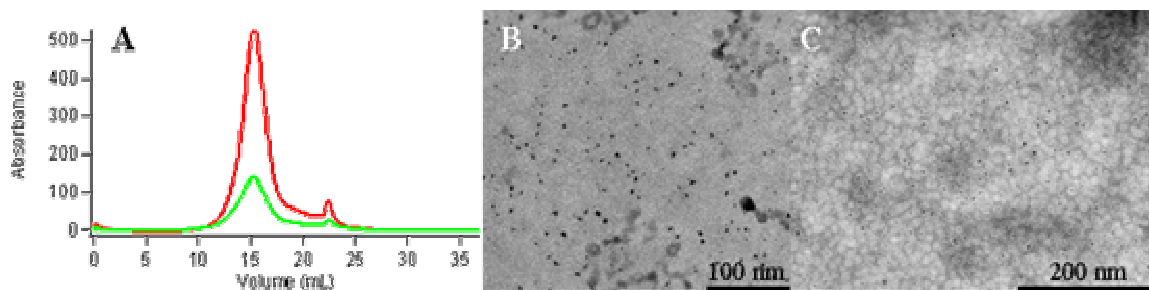


Figure 2.47. Characterization of 500 PdFn. A. SEC of 500 PdFn in 10 mM HEPES 150 mM NaCl pH 8.5 at 15 °C, -280 nm -350 nm; B. TEM of 500 PdFn unstained, scale bar = 100 nm; C. TEM of 500 PdFn stained with 2% uranyl acetate, scale bar = 200 nm; Average particle size =  $2.8 \pm 1.2$  nm.

The optimized conditions for mineralizing Fn with 500 Pd (500 PdFn) is 1 mg/mL (10 mg/mL in 150 mM NaCl) in 10 mM HEPES 150 mM NaCl pH 8.5 with a final volume of 2 mL at 15 °C using a tip sonicator (20% duty, 1 power). 100  $\mu$ L of 20 mM metal  $\text{PdCl}_4^{2-}$  was added in of aliquots of 25  $\mu$ L metal at 0, 10, 60, and 70 minutes while adding 300  $\mu$ L of 3 mg/mL DMAB over 2 hours using a syringe pump. Centrifugation after the reaction reveals minimal pellet. The SEC of 500 PdFn (Figure 2.47 A) shows that the mineralization is almost completely on the interior of the protein cage. Figure 2.47A shows co-elution of the 280 nm (protein) and 350 nm (mineral) at the same elution volume as unmineralized Fn (Figure 2.25). The unstained TEM (Figure 2.47 B) depicts electron dense cores with an average diameter of  $2.8 \pm 1.2$  nm (109 particles measured). The uranyl acetate stained TEM (Figure 2.47 C) clearly shows the electron dense  $\text{Pt}^0$  cores surrounded unstained regions which indicates encapsulation by protein.

The optimized conditions for mineralizing Fn with 500 Zn-Pd 1:3 (500 ZnPd Fn) is 1 mg/mL (10 mg/mL in 150 mM NaCl) in 10 mM HEPES 150 mM NaCl pH 8.5 with a final volume of 2 mL at 15 °C using a tip sonicator (20% duty, 1 power). 100  $\mu$ L of 20 mM metal ( $\text{ZnCl}_2$  and  $\text{PdCl}_4^{2-}$  mixed before addition) was added in of aliquots of 25  $\mu$ L

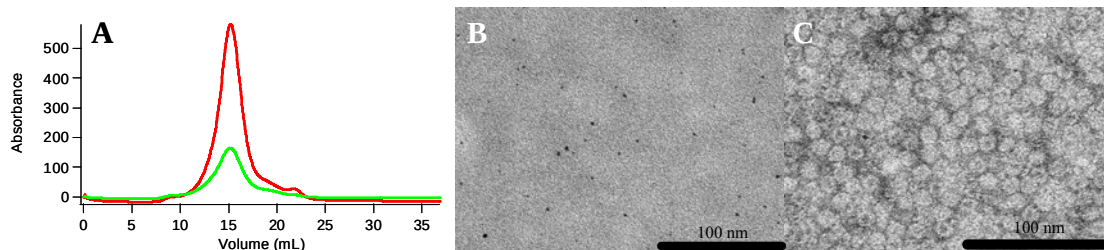


Figure 2.48. Characterization of 500 ZnPd Fn. A. SEC of 500 ZnPd Fn in 10 mM HEPES 150 mM NaCl pH 8.5 at 15 °C, -280 nm -350 nm; B. TEM of 500 ZnPd Fn unstained, the inset shows electron diffraction of Pd<sup>0</sup> from 500 ZnPd Fn; C. TEM of 500 ZnPd Fn stained with 2% uranyl acetate; Scale bar = 100 nm, Average particle

metal at 0, 10, 60, and 70 minutes while adding 300  $\mu$ L of 3 mg/mL DMAB over 2 hours using a syringe pump. Centrifugation after the reaction reveals no pellet. The SEC of 500 ZnPd Fn (Figure 2.48 A) shows that the mineralization is almost completely on the interior of the protein cage. Figure 2.48A shows co-elution of the 280 nm (protein) and 350 nm (mineral) at the same elution volume as unmineralized Fn (Figure 2.25). The unstained TEM (Figure 2.48 B) depicts electron dense cores with an average diameter of  $2.4 \pm 0.7$  nm (82 particles measured). The uranyl acetate stained TEM (Figure 2.48 C) clearly shows the electron dense Pt<sup>0</sup> cores surrounded unstained regions which indicates encapsulation by protein.

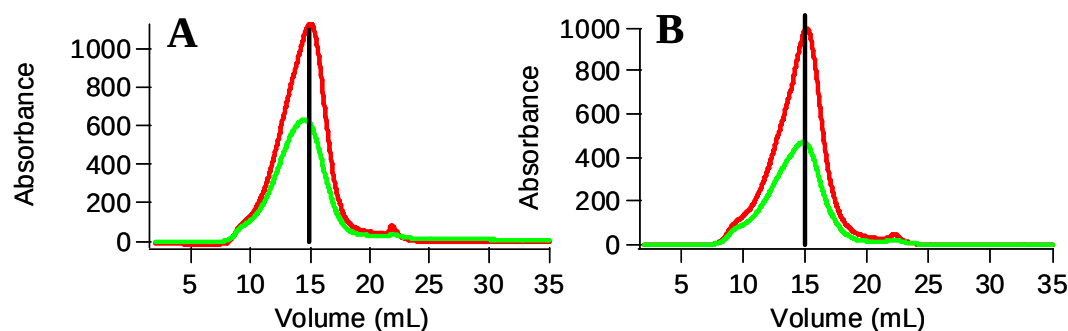


Figure 2.49. SEC profiles of 1000 Pd and ZnPd in Fn. A. SEC of 1000 PdFn in 10 mM HEPES 150 mM NaCl pH 8.5 at 15 °C; B. SEC of 1000 ZnPd Fn in 10 mM HEPES 150 mM NaCl pH 8.5 at 15 °C, -280 nm -350 nm. Black line indicates elution volume of untreated Apo-Fn.

Ferritin was mineralized with 1000 PdFn and 1000 ZnPd Fn 1:3 in 10 mM HEPES 150 mM NaCl pH 8.5 by adding 200  $\mu\text{L}$  of 20 mM metal ( $\text{PdCl}_4^{2-}$  or  $\text{ZnCl}_2$  and  $\text{PdCl}_4^{2-}$  mixed before addition), which was added by 25  $\mu\text{L}$  metal at 0, 10, 20, 30, 60, 70, 80, and 90 minutes while adding 300  $\mu\text{L}$  of 3 mg/mL DMAB over 2 hours using a syringe pump. The SEC of 1000 PdFn (Figure 2.49 A) and 1000 ZnPd Fn (Figure 2.49 B) show that the mineralization loses its specificity for the interior of the cage when then loading is 1000 metal per cage.

#### Summary of Optimized Synthesis of Pd and Zn-Pd in Ferritin

The optimized conditions for mineralizing Fn with Pd or Zn-Pd 1:3 is 1 mg/mL (10 mg/mL in 150 mM NaCl) in 10 mM HEPES 150 mM NaCl pH 8.5 at 15°C using a tip sonicator (20% duty, 1 power). 20 mM metal ( $\text{PdCl}_4^{2-}$  or  $\text{ZnCl}_2$  and  $\text{PdCl}_4^{2-}$  mixed before addition) was added in of aliquots of 25  $\mu\text{L}$  metal with 10 minutes in between aliquots with half of the metal added each hour while adding 300  $\mu\text{L}$  of 3 mg/mL DMAB over 2 hours using a syringe pump.

#### Platinum and Zinc/Platinum Mineralization in PfDPS

In order to further study the effects of the protein cage on the mineralization and catalytic properties of the noble metal nanoparticles, PfDPS was chosen as another platform for the mineralization of platinum to determine if a smaller cage 9 nm exterior diameter vs. 12 nm of Hsp and Fn would have a difference in catalytic rate which could reveal insights in the catalysis in protein cage architectures. Another advantage of PfDPS is that the protein cage is stable up to 80 °C which allows the synthesis of platinum

nanoparticles at higher temperatures to determine if the increased synthetic temperature displays any effect in the catalytic activity.

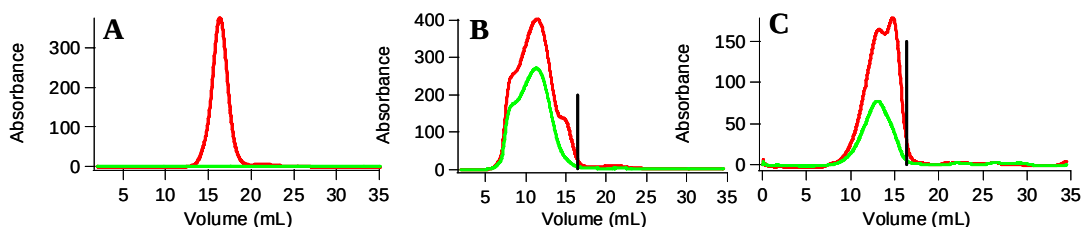


Figure 2.50. Comparison of the SEC of PfDPS to 500 Pt mineralized in PfDPS at different temperatures. A. SEC of PfDPS; B. SEC of 500 PtPfDPS in 25 mM MES 10 mM NH<sub>4</sub>OH pH 6.5 at 65 °C; C. SEC of 500 PtPfDPS in 25 mM MES 10 mM NH<sub>4</sub>OH pH 6.5 at 80 °C, -280 nm -350 nm. Black line indicates elution volume of untreated PfDPS.

The SEC of unmineralized PfDPS is shown in Figure 2.50A. The reaction conditions for platinum mineralization of 500 Pt in PfDPS were PfDPS at 7.14 mg/mL in 50 mM MES 100 mM NaCl pH 6.5 was added to a solution of 25 mM MES 10 mM NH<sub>4</sub>OH pH 6.5 for a final concentration of 1 mg/mL in 2.5 mL. A temperature of 65 °C (Figure 2.50 B) or 80 °C (Figure 2.50 C) was maintained using a jacketed reaction vessel with water circulating around the reaction. The reaction was stirred vigorously with magnetic stir bar throughout the reaction. Platinum and 5-fold excess of DMAB per Pt was simultaneously added over 15 minutes using a syringe pump. Figure 2.50 A and B show the platinum mineralization is either on the exterior or forming aggregates of mineralized cages. The temperature of the reaction has an effect on the mineralization; however, neither case shows specific mineralization in the interior of PfDPS.

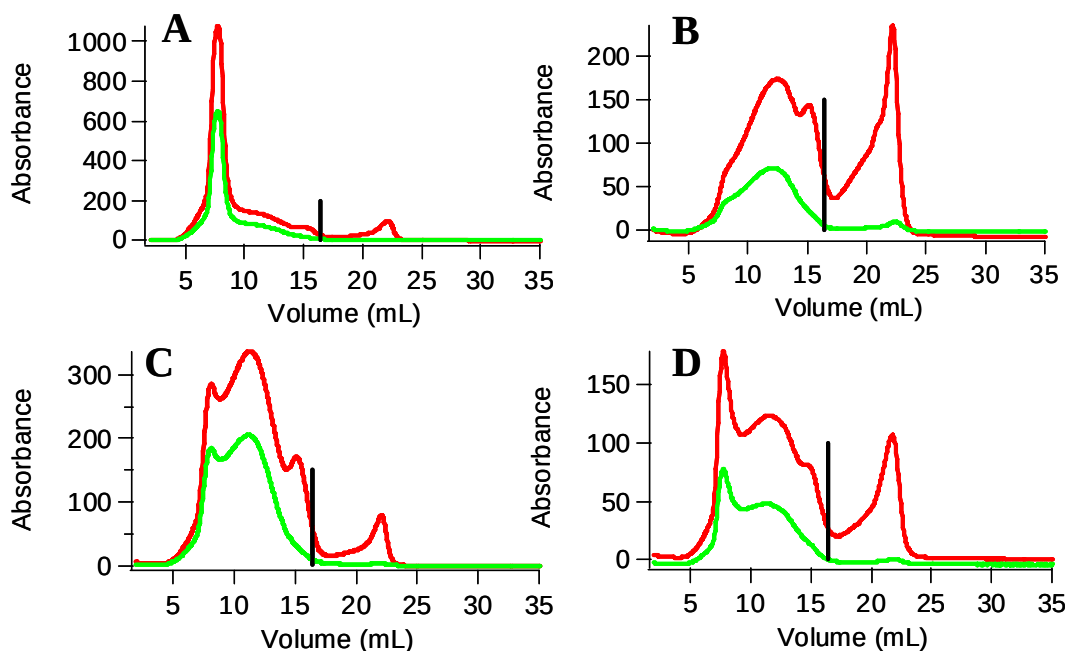


Figure 2.51. The SEC profiles of 500 PtPfdPS mineralization in a range of reaction conditions at 65 °C. A. SEC of 500 PtPfdPS in 25 mM MES 10 mM EDTA pH 6.5 at 65 °C; B. SEC of 500 PtPfdPS in 25 mM phosphate 10 mM NH<sub>4</sub>OH pH 6.5 at 65 °C; C. SEC of 500 PtPfdPS in 25 mM TRIS 10 mM NH<sub>4</sub>OH pH 7 at 65 °C; D. SEC of 500 PtPfdPS in 25 mM MES 10 mM Citrate pH 6.5 at 65 °C, -280 nm -350 nm. Black line indicates elution volume of untreated PfdPS.

The standard reaction conditions for platinum mineralization of 500 Pt in PfdPS were PfdPS at 7.14 mg/mL in 50 mM MES 100 mM NaCl pH 6.5 was added to a buffered solution for a final concentration of 1 mg/mL in 2.5 mL. A temperature of 65 °C was maintained using a jacketed reaction vessel with water circulating around the reaction. The reaction was stirred vigorously throughout the reaction. Platinum and 5-fold excess of DMAB per Pt was simultaneously added over 15 minutes using a syringe pump. Using the standard conditions above, the reaction buffer was varied to determine the optimal conditions for the specific mineralization of platinum nanoparticles on the interior of the protein cage. Buffer, salt, and pH were varied to manipulate the interaction between the protein cage and the metals ions in solution in an attempt to facilitate the specific interaction of platinum with the interior of the cage. Figure 2.51 shows the SEC

of the 500 PtPfDPS in 25 mM MES 10 mM EDTA pH 6.5 (Figure 2.51 A), 25 mM phosphate 10 mM  $\text{NH}_4\text{OH}$  pH 6.5 (Figure 2.51 B), 25 mM TRIS 10 mM  $\text{NH}_4\text{OH}$  pH 7 (Figure 2.51 C), and 25 mM MES 10 mM Citrate pH 6.5 (Figure 2.51 D). The SEC of these reactions is typical of any platinum mineralization condition for PfDPS. While the SEC profile would change with reaction conditions, the mineralization was never close to being specific for the interior of PfDPS using these standard conditions.

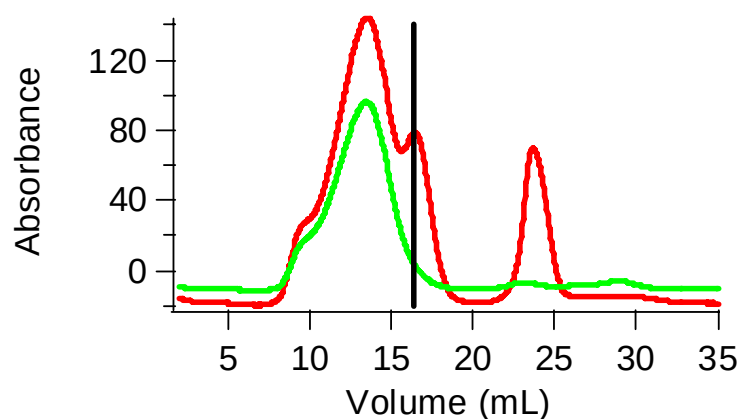


Figure 2.52. SEC profile of 400 ZnPt PfDPS in 25 mM MES 100 mM  $\text{NH}_4\text{OH}$  pH 6.5 at 25 °C, -280 nm -350 nm. Black line indicates elution volume of untreated PfDPS.

Figure 2.52 shows SEC of PfDPS mineralized with 400 Zn-Pt. PfDPS was mineralized with 400 Zn-Pt (1:3) at 1 mg/mL in buffer (25 mM MES 100 mM  $\text{NH}_4\text{OH}$  pH 6.5) at 25 °C with a final volume of 2 mL at room temperature by additions of 10  $\mu\text{L}$  of 20 mM metal ( $\text{ZnCl}_2$  and  $\text{PtCl}_4^{2-}$  mixed before addition) wait 3 minutes then add 10  $\mu\text{L}$  DMAB (3 mg/mL) then wait 3 minutes and add 20  $\mu\text{L}$  of 20 mM metal wait 3 minutes then add 20  $\mu\text{L}$  DMAB (3 mg/mL) then repeat the 20  $\mu\text{L}$  additions 4 times while sonicating using a Fisher Scientific sonicator.

### Conclusion of Pt and Zn-Pt Mineralization on the Interior of PfDPS

The mineralizations of Pt and Zn-Pt with PfDPS were never specific for the interior of the protein cage under varying buffer, salt, sonication, and pH conditions. All the conditions attempted were dominated by the formation of aggregates and precipitation with little to no free PfDPS eluting off the SEC.

### Platinum and Zinc/Platinum Mineralization in LDPS

LDPS was chosen as another platform for the mineralization of platinum after PfDPS was proved to be unable to specifically mineralize platinum on the interior surface. LDPS was chosen because it has proved to be a robust platform for other mineralizations in our lab.

The SEC of unmineralized LDPS is shown in Figure 2.53A. Figure 2.53B shows the SEC of the platinum mineralization of 400 Pt in LDPS where LDPS at 3.7 mg/mL in 50 mM MES 100 mM NaCl pH 6.5 was added to a solution of 25 mM MES 100 mM

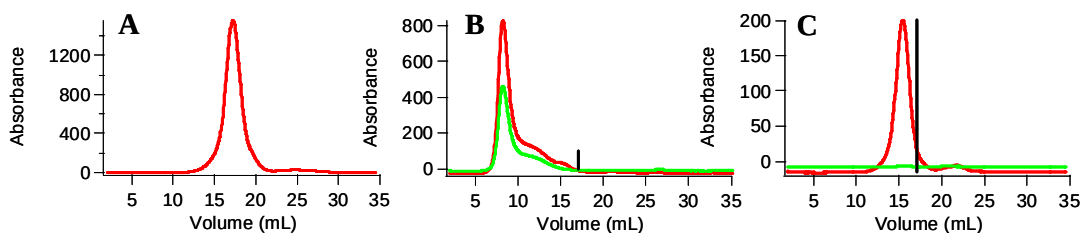


Figure 2.53. SEC characterization of LDPS, 400 PtLDPS mineralization, and LDPS under reducing conditions. A. SEC of LDPS; B. SEC of 400 PtLDPS in 25 mM MES 100 mM  $\text{NH}_4\text{OH}$  pH 6.5 at 65 °C; C. SEC of LDPS in 25 mM MES 100 mM  $\text{NH}_4\text{OH}$  pH 6.5 at 65 °C in reducing conditions, -280 nm -350 nm. Black line indicates elution volume of untreated LDPS.

$\text{NH}_4\text{OH}$  pH 6.5 for a final concentration of 1 mg/mL in 2.5 mL. The temperature was maintained at 65 °C using a jacketed reaction vessel and was stirred vigorously

throughout the reaction. Platinum and 5-fold excess of DMAB per Pt was simultaneously added over 15 minutes using a syringe pump. After the reaction, the reaction was cloudy and there was a significant pellet upon centrifugation. SEC reveals that all of the protein is aggregated after the reaction. The no platinum control, where the reaction is done under identical conditions except no platinum is added to the reaction, forms a white precipitate during the reaction that is pelleted by centrifugation after the reaction. Figure 2.53C is the SEC of the Pt free control after spinning. The SEC revealed that the elution volume is decreased in control reaction indicating an increase in the size of the particle. This also indicates that the LDPS protein is not stable under the reaction conditions.

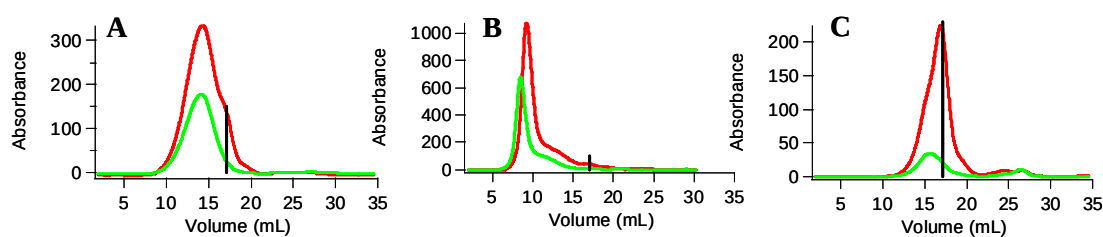


Figure 2.54. The SEC profiles of 400 Pt LDPS mineralization in a range of reaction conditions at 40 °C. A. SEC of 400 PtLDPS in 25 mM MES 10 mM NH<sub>4</sub>OH pH 6.5 at 40°C; B. SEC of 400 PtLDPS in 25 mM MES 10 mM NH<sub>4</sub>OH pH 5.0 at 40 °C; C. SEC of 400 PtLDPS in 25 mM MES 10 mM NH<sub>4</sub>OH pH 8.0 at 40 °C, -280 nm -350 nm. Black line indicates elution volume of untreated LDPS.

After the initial attempts to mineralize LDPS at 65 °C, the stability of LDPS in reducing conditions was probed and was determined to be stable under the reducing conditions at a maximum of 40 °C. The standard reaction conditions for platinum mineralization of 400 Pt in LDPS (400 PtLDPS) were LDPS at 3.7 mg/mL in 50 mM MES 100 mM NaCl pH 6.5 was added to a buffered solution for a final concentration of 1 mg/mL in 2.5 mL. The temperature was maintained at 40 °C using a jacketed reaction vessel and was stirred vigorously throughout the reaction. Platinum and 5-fold excess of DMAB per Pt was simultaneously added over 15 minutes using a syringe pump. The

SEC of 400 PtLDPS mineralization reaction in 25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5 is shown in Figure 2.54A. SEC reveals that mineralization completely forms a product that is larger than the unmineralized LDPS. The SEC of 400 PtLDPS mineralization reaction in 25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 5.0 is shown in Figure 2.54B. By lowering the pH of the reaction, the reaction forces the complete formation of larger aggregates. The SEC of the mineralization of 400 PtLDPS in 25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 8.0 is shown in Figure 2.54C. The higher pH reaction forms only a small amount of  $\text{Pt}^0$  nanoparticles indicated by the low absorbance at 350 nm. The 350 nm absorption is at a retention volume that is larger than the unmineralized LDPS indicating that the particles that are formed are on the exterior. Under these standard conditions, the mineralization of Pt nanoparticles is not specific for the interior of the LDPS.

In an attempt to make the mineralization of LDPS specific for the interior of the cage, LDPS was mineralized while sonicating. Figure 2.55A is SEC of the product of

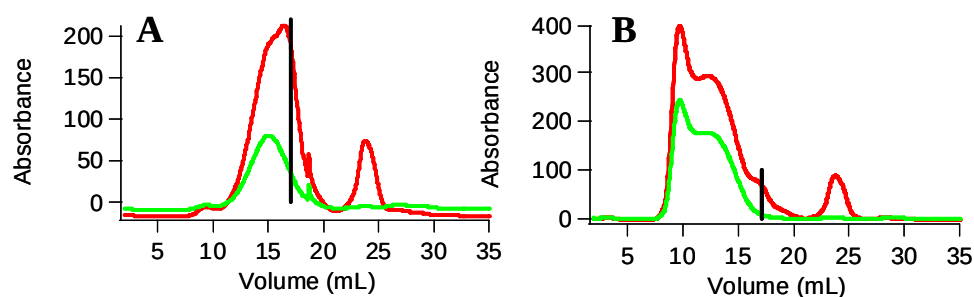


Figure 2.55. The SEC profiles of 400 ZnPt LDPS mineralization in a range of reaction conditions at 25 °C. A. SEC of 400 ZnPt LDPS in 200 mM  $\text{NH}_4\text{Acetate}$  pH 5 at 25 °C; B. SEC of 400 ZnPt LDPS in 25 mM MES pH 6.5 at 25 °C, -280 nm -350 nm. Black line indicates elution volume of untreated LDPS.

LDPS mineralization with 400  $\text{Pt}^{2+}$  at 1 mg/mL in buffer (25 mM MES 100 mM  $\text{NH}_4\text{OH}$  pH 6.5) at 25 °C with a final volume of 2 mL at room temperature by additions of 10  $\mu\text{L}$  20 mM  $\text{PtCl}_4^{2-}$  wait 3 minutes then add 10  $\mu\text{L}$  DMAB (3 mg/mL) then wait 3 minutes and

add 20  $\mu\text{L}$  of 20 mM metal wait 3 minutes then add 20  $\mu\text{L}$  DMAB (3 mg/mL) then repeat the 20  $\mu\text{L}$  additions 4 times while sonicating using a Fisher Scientific sonicator. The SEC of LDPS mineralized with 400 Zn-Pt 1:3 in 1 mg/mL 25 mM MES 100 mM  $\text{NH}_4\text{OH}$  pH 6.5 at 25  $^\circ\text{C}$  with a final volume of 2 mL at room temperature by additions of 10  $\mu\text{L}$  20 mM metal ( $\text{ZnCl}_2$  and  $\text{PtCl}_4^{2-}$  mixed before addition) wait 3 minutes then add 10  $\mu\text{L}$  DMAB (3 mg/mL) then wait 3 minutes and add 20  $\mu\text{L}$  of metal wait 3 minutes then add 20  $\mu\text{L}$  DMAB (3 mg/mL) then repeat the 20  $\mu\text{L}$  additions 4 times while sonicating using a Fisher Scientific sonicator is shown in figure 2.55B. The SEC profile shown in figure 2.55B was typical of Zn-Pt mineralizations with LDPS during sonication.

#### Conclusion of Pt and Zn-Pt Mineralization on the Interior of LDPS

Mineralizations of LDPS with Pt and Zn-Pt were not specific for the interior of the protein cage under varying buffer, salt, sonication, and pH conditions. All the conditions attempted were dominated by the formation of aggregates and precipitation with little to no free LDPS eluting off the SEC. The LDPS was semi-stable with every reaction becoming slightly cloudy upon the addition of reducing agent (DMAB). Elevated temperatures (45  $^\circ\text{C}$  and above) caused almost total protein precipitation upon the addition of reducing agent (DMAB or  $\text{H}_2$ ).

### Discussion

#### Rationale for the Use of Protein Cages

In enzyme catalysis, the protein architecture is essential for the catalytic efficiency of the enzyme. The overall protein structure plays a number of roles in the

catalysis mediated by enzymes. The protein is a structural platform that defines the active site, shuttles molecules in and out of the active site, and protects the active site from the environment. The protein can be involved by defining the overall geometry of the active site, by being an electron mediator, by activating the active site, or by providing or accepting protons in the active site. One clear example where the protein architecture plays a critical role in the catalytic activity of the enzyme is FeFe hydrogenase.<sup>126-128</sup> A number of model complexes have been synthesized that are structurally analogous to the active site of the protein<sup>129, 130</sup> but they do not have the catalytic activity of the protein encapsulated active site.

With this idea in mind, protein cages were used as a platform for the synthesis of noble metal nanoparticles, and alloys thereof, to determine the catalytic properties of the metal nanoparticles encapsulated by a protein cage. The insertion of a catalytic nanoparticle in the protein cage architecture mimics the protein stabilized activity of the enzymes. The supramolecular structure of the protein cage also provides the means of manipulating the synthesized catalyst through use of the geometrically and chemically distinct exterior surface. The exterior of the protein cage can be modified independently of mineralization in the interior cavity. The exterior can be used to attach the catalyst to a surface or for attachment of a small molecule coenzyme.

Protein cage architectures, such as ferritin, DPS, Hsp, and CCMV, provide unique platforms for the synthesis of nanoparticles. The protein cages provide a well defined architectural framework structure for biomimetic synthesis; in contrast to other passivating layers that coat the surface of the synthesized particle. Traditional surface coating of the nanoparticles has an effect on the substrate access to the catalytic surface,

which influences the overall catalytic rate.<sup>110</sup> The protein cage has only a minimal interaction with the nanoparticle leaving the rest of the nanoparticle surface uncoated and easily accessible for catalytic activity (Figure 2.1), while at the same time protecting the particle from aggregation.

### Description of Pt Mineralization in Protein Cages

In order to form a nanoparticle on the interior of the protein cage, there has to be a nucleation event specifically on the interior of the cage. Nucleation forms a precursor to the nanoparticle from which the particle grows to its final form. The specificity of the nucleation event for the interior of the protein cage is controlled, in part, by the reaction conditions of the mineralization. In order for nucleation to occur specifically on the interior of the protein cage, there has to be a favorable interaction on the interior surface with the protein cage and the precursor molecules. The generally accepted model for this interaction in mineralization is an electrostatic interaction. The electrostatic interaction of the interior surface with the mineralization precursors concentrates the precursors at the surface, which facilitates the aggregation of precursor ions resulting in the nucleation event specifically of the interior surface (see Introduction).

After the nucleation event, the nanoparticles must grow from that nucleation surface to form the final particle, this growth is called the growth phase. In the growth phase, the mineralization precursors add to the surface of the growing particle. This interaction and addition to the particle is what determines the rate of the growth phase.<sup>29</sup> The balance between the nucleation and the growth phase determines the size of the particle.<sup>29</sup> For example, if in the mineralization of a protein cage the nucleation is the slow step, the particle would be expected fill the cage once a nucleation event has

occurred. On the other hand if growth were rate limiting and nucleation was fast, many small particles would be observed with sizes well below the total size of the protein cage. In the case of  $\text{Pt}^0$  mineralization in Fn, it appears that growth is limiting because the particles do not fill the cage, suggesting that nucleation occurs readily while growth limits the particle size. This is in contrast to manganese oxide mineralization in horse spleen ferritin, which fills the interior of the protein cage under a range of Mn to protein ratios.<sup>35</sup>

Another important aspect of the mineralization of  $\text{Pt}^0$  in protein cages is the reduction of  $\text{Pt}^{2+}$  to  $\text{Pt}^0$ . The reduction of  $\text{Pt}^{2+}$  to  $\text{Pt}^0$  occurs synthetically by the addition of a reducing agent, DMAB, borohydride, or  $\text{H}_2$  gas, to the  $\text{Pt}^{2+}$  with or without protein cages. In their analysis of platinum cluster nucleation, Ciacchi and coworkers suggested that in the nucleation of platinum nanoparticles the formation of a  $\text{Pt}^{1+}$  and a  $\text{Pt}^{2+}$  dimer is the precursor to nanoparticle growth. The formation of the  $\text{Pt}^{1+}$  species is the slow step in the mineralization.<sup>131</sup> A point of note is that isolated zerovalent platinum atom intermediates have never been observed, indicating that the reduction to platinum metal is associated with clustering of atoms.<sup>131</sup> The nucleation event has to be kinetically faster on the interior of the protein cage compared to the non-specific nucleation of particles on the exterior of the protein cage or bulk mineralization in solution. Once there is a nucleation event on the interior of the cage, the particle growth from that nucleus also must be kinetically faster than the bulk mineral growth or once there is a bulk nucleation all the  $\text{Pt}^{2+}$  will be sequestered to the growth of a few bulk minerals.

The initial  $\text{Pt}^0$  mineralization efforts were focused on Hsp because of the porous structure, the amenability to mutations, and the high stability of the protein cage. The

porous quaternary structure was appealing because it allowed for ready access of the chemical precursors to the interior surface of the protein cage for nanoparticles synthesis and entry and removal of substrates and products during catalysis. Amenability to mutations is important for the manipulation of the interior surface of the protein cage to direct and aid the nucleation of  $\text{Pt}^0$  nanoparticles on the interior surface through insertion of nucleation sites. In addition, the Hsp protein cage stability was essential for testing a broad range of pH and temperature conditions for  $\text{Pt}^0$  mineralizations.

#### Engineering Pt Specific Nucleation Sites into Hsp

The concept behind the insertion of a nucleation site into the interior of Hsp is that by binding  $\text{Pt}^{2+}$  or  $\text{Pt}^0$  to the interior the engineered nucleation site will act as a catalyst for nucleation. The interaction of the protein cage surface and the precursor molecules can aid in the nucleation through a number of routes such as concentrating or binding the starting materials at the surface to facilitate the formation of a nanoparticle nucleus<sup>2, 103</sup> or to stabilize the formation of cluster that acts as the nucleus for nanoparticle growth.<sup>29</sup> The concept of an engineered nucleation site has been demonstrated to facilitate the mineralization of  $\text{Ag}^0$  nanoparticles on the interior of ferritin.<sup>50</sup>

The initial mineralization attempts described in this study were focused on the Hsp mutant G41C because it was thought that the introduced cysteines would bind platinum(II) and act as a nucleation site for the mineralization of  $\text{Pt}^0$  nanoparticles. In the native Hsp cage, the interior and exterior of the cage do not have clear electrostatic differences that could be used to direct the mineralization on the interior surface. By inserting the cysteine, the idea was that a nucleation site had been inserted onto the

interior of the cage that could be used to direct the formation of a nanoparticle nucleus on the interior of the cage because of the preferred interaction between the soft SH ligand and the soft Pt metal. In the native host, Hsp does not appear to have a role in the encapsulation of any metal or metal-oxide nanoparticles, which lead us to believe that a nucleation site insertion would be necessary for specific mineralization under synthetic conditions.

The genetically inserted cysteine did not act as a specific nucleation site aiding the mineralization of  $\text{Pt}^0$  nanoparticles. A range of reaction conditions failed to produce the required spatial selectivity of the mineralization reaction within the protein cage. The conditions that were manipulated included buffer, pH, salt, and temperature for the Pt mineralization in Hsp G41C, which were varied extensively determine the optimal conditions for specific mineralization on the interior of Hsp G41C. The optimal conditions of the mineralization were 1 mg/mL Hsp G41C in buffer (25 mM MES 10 mM  $\text{NH}_4\text{OH}$  pH 6.5) at 65°C with  $\text{PtCl}_4^{2-}$  and a five fold excess of DMAB added over 15 minutes (Figure 2.7 B). The optimized mineralization conditions still showed considerable mineral formation on the exterior of the protein cage revealing that the mineralization of Hsp G41C is not entirely specific for the interior of the protein cage.

The inability of the Hsp G41C to direct the formation of nanoparticles on the interior of Hsp could have many explanations. One is that the binding of  $\text{Pt}^{2+}$  does not occur during the reaction.  $\text{Pt}^{2+}$  is inert and the process of ligand exchange and binding to cysteine could take a longer time to occur.  $\text{Pt}^{2+}$  could also be prevented from binding to the cysteine by the protein environment surrounding cysteine. Alternatively, the  $\text{Pt}^{2+}$  may bind to the cysteine but in this bound state might be unable to act as a nucleation

site. By binding to cysteine, the reduction of  $\text{Pt}^{2+}$  to  $\text{Pt}^0$  could be retarded considerably. Henglein and coworkers showed that the reduction of  $\text{Pt}^{2+}$  to  $\text{Pt}^0$  was prevented by the coordination of hydroxide to  $\text{Pt}^{2+}$ <sup>132</sup> and a similar inhibition through coordination could be occurring in this case.

A  $\text{Pt}^0$  specific binding peptide was engineered onto the interior of Hsp to act as a site specific nucleation catalyst. The platinum specific peptide (IGSSLKP), identified by phage display, was inserted in the N-terminus of WtHsp to be a  $\text{Pt}^0$  specific nucleation site. The insertion of material specific peptides into protein architectures allows the spatial control of locality, morphology, composition, and polymorph of the resulting mineral and has been established as a way to induce the formation of a product that could otherwise not be synthesized under mild conditions. The insertion of a mineral binding peptide onto the interior of Hsp has been shown to influence the crystal phase of the CoPt nanoparticles synthesized.<sup>78</sup> However, the genetically engineered insertion of a  $\text{Pt}^0$  binding peptide did not facilitate the site specific mineralization of  $\text{Pt}^0$  on the interior of Pt-Hsp. While a narrower screen of reaction conditions was used in the synthesis of Pt nanoparticles in Pt-Hsp, compared to Hsp G41C, the Pt-Hsp was extensively tested for the ability mineralize Pt nanoparticles of the interior of the cage. Previous work has suggested that the formation of a bound precursor prior to mineralization could greatly facilitate the reaction. For example, in the mineralization of ZnSe in ferritin, the  $\text{Zn}^{2+}$  was added before the  $\text{Se}^{2-}$ , which allowed the  $\text{Zn}^{2+}$  to concentration on the interior surface of the ferritin before the  $\text{Se}^{2-}$  was added to form ZnSe.<sup>48</sup> Similarly, in the synthesis of CoPt on the exterior of M13 bacteriophage expressing a  $\text{Co}^{2+}$  binding peptide, Belcher and coworkers preloading the M13 bacteriophage with  $\text{Co}^{2+}$  before added  $\text{Pt}^{2+}$ .<sup>103</sup> In an

attempt to utilize this synthetic approach, the Pt-Hsp was pre-incubated with 50  $\text{Pt}^{2+}$  ions before the particle mineralization to determine if preloading the peptide with  $\text{Pt}^{2+}$  before the mineralization would increase the specificity of the mineralization for the interior of the cage. However, the preloading of  $\text{Pt}^{2+}$  caused the complete formation of bulk precipitate. Under all of the condition attempted, Pt-Hsp mineralizations were less specific for the interior of the cage than Hsp G41C (Figure 2.9 – 2.10).

The engineering of a platinum binding peptide into Hsp as a nucleation catalyst revealed that peptides which bind to minerals are not universally able to induce particle formation. The insertion the Pt binding peptide into Hsp made the  $\text{Pt}^0$  mineralization in the interior of Hsp less specific. The use of surface binding peptides as nucleation catalysts has been shown in some cases to aid in mineralization; however, the interaction of biomacromolecules, proteins and lipids, and nucleation precursor to particle formation is poorly understood. The nucleus to mineral formation can be an amorphous phase of the mineral or have minute defects that are important to mineralization.<sup>133</sup> Formation of amorphous nuclei as precursors to particle formation is believed to occur because of the instability of the amorphous particle. The amorphous particle is formed kinetically faster than the more stable crystal phases. Once the amorphous nucleus is formed the more stable crystal phases grow from the surface of the nucleus.<sup>29</sup> In the cases of the  $\text{Pt}^0$  mineral formation, Ciacchi and coworkers state that the nucleus in the nanoparticle formation of  $\text{Pt}^0$  particle is a non-zerovalent platinum.<sup>131</sup> However, Ciacchi also states that silver nanoparticles have a nucleus formation similar to that of Pt.<sup>131</sup> This raises the question of why the insertion of a silver binding peptide into Fn appeared to facilitate the mineralization of silver nanoparticles and the insertion of a platinum binding peptide in

Hsp did not aid in the mineral formation. Further studies are needed into the nature of the nucleation site structure, morphology, and role in particle formation to understand the why surface binding peptides are able, in some cases, to aid in particle formation.

Our final attempt at engineering a nucleation site onto the interior of Hsp was the covalent attachment of phenanthroline to the interior of the Hsp G41C. Phenanthroline was used as a nucleation site because it will bind  $\text{Pt}^{2+}$  with high affinity. In addition, Islam has shown platinum(II)(4,7-dicarboxy-1,10-phenanthroline)(1,2-benzenedithiolate) to be able to photochemically donate electrons into nanocrystalline titanium dioxide suggesting that a photoreduction and subsequent use as a nucleation site with  $\text{Pt}^{2+}$ -phenanthroline bound to a protein cage could be possible.<sup>123</sup>

This mineralization plan also incorporates work on the photochemical reduction of  $\text{Fe}^{3+}$ -citrate done by Dodge and Francis.<sup>121</sup> They mineralized iron oxide using the  $\text{Fe}^{3+}$ -citrate reduction to  $\text{Fe}^{2+}$  (Figure 2.56<sup>121</sup>) through a ligand to metal charge transfer upon illumination.<sup>121</sup>  $\text{Fe}^{2+}$  is then reoxidized by oxygen to form an iron oxide particle. The  $\text{Pt}^{2+}$ -citrate was shown in figure 2.14 to be photochemically active, presumably by a

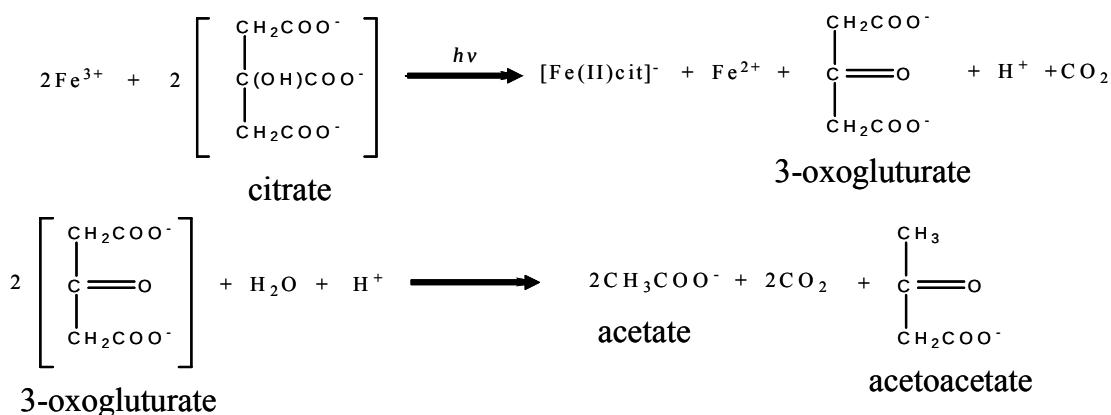


Figure 2.56. The photoreduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  using citrate.

similar ligand to metal charge transfer. The photochemical reduction of  $\text{Pt}^{2+}$  in the presence of Hsp G41C showed significant increase in the diameter of the particles in

solution. UV-Vis analysis showed an absorbance indicative of the formation of bulk precipitate. Pt-phenanthroline complex was used as a photo-nucleation site where a reduced Pt could bind and act as the nucleus for particle formation.  $\text{Pt}^{2+}$ -citrate photochemical reduction reaction acted as the means of nanoparticle growth.

The characterization of the Pt photomineralization using Hsp Phen was inconclusive. The coelution of the absorption at 350 nm with the protein is a clear indication that there is particle formation associated with the Hsp protein cage. The characteristic broad absorbance in the visible region from the formation of  $\text{Pt}^0$  nanoparticles was present after the reaction; however, TEM revealed that there was no detectable particle formation. The TEM analysis can be explained by the formation of particles that were below the detection limit of the TEM analysis. The SEC, DLS, and the UV/Vis gave indications that  $\text{Pt}^0$  forms and was associated with the Hsp cage. If the particles were too small to image with TEM, SEC and DLS are not valid characterizations of Hsp Phen Pt because DLS and SEC would not be able to determine resolve a Hsp with a  $\sim 1$  nm particle on the exterior from one with a  $\sim 1$  nm particle on the interior. However, the selective attachment of the phenanthroline to the interior clearly plays a role in the mineralization process indicated by the absence of large particle formation in seen in the Hsp G41C protein control.

While the engineered Hsp cages had limited success in mineralizing  $\text{Pt}^0$  in the interior of the Hsp cage, the WtHsp cage specifically mineralized Pt on its interior. The interior and exterior surfaces of Hsp are remarkably similar with little electrostatic characteristic defining either surface that would suggest areas for the nucleation on to the interior surface. However, WtHsp has been used to mineralization both platinum metal

and iron oxide on the interior surface.<sup>77</sup> Even though the nucleation site is not known in WtHsp, the negative impact of the mutation of glycine 41 to cysteine suggests that glycine 41 is involved in the nucleation site. The optimal reaction conditions for the Pt<sup>0</sup> mineralization in Hsp were 1 mg/mL WtHsp in buffer (25 mM MES 10 mM NH<sub>4</sub>OH pH 6.5) at 65°C with Pt<sup>2+</sup> and 5 fold excess DMAB added over 15 minutes while vigorously stirring (Figure 2.21 and 22).

### All Hsp Mineralization Conclusions

The insertion of nucleation sites onto the interior surface of Hsp through genetic or chemical means did not have the intended result. Hsp with the insertion of a cysteine or a Pt<sup>0</sup> binding peptide displayed less specificity for internal mineralization than the WtHsp protein cage structure. The WtHsp protein cage proved to be an effective platform for the mineralization of the platinum metal nanoparticles. The mineralization on the inside Hsp is poorly understood with little to no rationale for why there is specific mineralization on the interior surface.

### Discussion of Pt<sup>0</sup> Mineralization in Fn

After synthesizing Pt<sup>0</sup> nanoparticles inside Hsp, Pt nanoparticles were synthesized in ferritin to determine if new insights into the mineralization of Pt<sup>0</sup> could be revealed. Another reason to move to a different protein cage system was to probe the role played by the protein cage in the catalytic activity of the Pt<sup>0</sup> nanoparticle; whether active or merely acting a platform for the catalyst. Ferritin was chosen as the second cage to mineralize Pt<sup>0</sup> inside because even though the Fn and Hsp have the same exterior diameter and similar interior diameters they are quite different. Ferritin has a more

closed structure than Hsp which could limit the molecular access to the catalytic particle. While Hsp is a protein chaperone, Fn is an iron storage protein with a chemically distinct interior and exterior.

Pt<sup>0</sup> nanoparticles were mineralized inside ferritin under identical conditions to WtHsp Pt<sup>0</sup> mineralization, which were 1 mg/mL Fn in buffer (25 mM MES 10 mM NH<sub>4</sub>OH pH 6.5) at 65°C with Pt<sup>2+</sup> and 5 fold excess DMAB added over 15 minutes while vigorously stirring. The Pt<sup>0</sup> mineralization in Fn is a more specific reaction for interior than the same reaction in Hsp.

The platinum nanoparticle mineralization in ferritin was a cleaner reaction compared to the WtHsp with no particle formation on the exterior and no bulk mineral formation. The clear difference between the Fn and the Hsp protein cages is the Fn has a clear electrostatic difference on the interior and exterior surface of the cage. However in this mineralization, the platinum precursor is most probably PtCl<sub>2</sub>(H<sub>2</sub>O)<sub>2</sub><sup>132</sup>. The lack of a positive charge on the precursor makes the standard electrostatic model for the mineralization in ferritin unlikely. This suggests that there are other processes directing the nucleation of nanoparticles in biomineralization and under biomimetic synthetic conditions. The nucleation site for metallic nanoparticle formation (Pd, Pt, Au) and the reason for the cleaner synthesis in Fn compared to Hsp is unknown.

#### Discussion of the Mineralization of Pt Alloys in Fn

After demonstrating that protein cages are effective platforms for the mineralization of Pt<sup>0</sup> nanoparticles, Pt<sup>0</sup> alloys were synthesized to determine if the catalytic rates of Pt nanoparticles could be improved by doping other metals. If the catalytic activity calculated on a per metal basis can be maintained or increased, which

means the same catalytic activity from an alloyed metal as an all platinum particle is the same or greater, then the use of platinum in the catalyst can be decreased by the amount of the dopant.

The alloying metal can play a number of different roles in the mineralization inside Fn. Conceptually, the simplest way is that the metal could be incorporated in the Pt mineral forming a metal/platinum alloy.<sup>78, 134</sup> Alternatively, the dopant metal could affect the mineralization by binding to the nucleation site or nucleation surface on the inside the protein. One example of this is in the mineralization of CoPt on M13 bacteriophage. The M13 bacteriophage that expresses a  $\text{Co}^{2+}$  binding peptide was loaded with  $\text{Co}^{2+}$  before the addition of  $\text{Pt}^{2+}$ .<sup>103</sup> The preloaded  $\text{Co}^{2+}$  presumably binds to the  $\text{Co}^{2+}$ -binding peptide as part of the nucleation site, thereby influencing the particle formation. Dopant metal bound to the protein could affect the growth of the crystal, the nucleus formation, or the morphology of the mineral by changing the interaction with the protein surface. The dopant metal could act in an entirely passive way by influencing the crystal formation without incorporation into the mineral.<sup>135, 136</sup> Xie and coworkers showed that the addition of  $\text{Mg}^{2+}$  to a calcium carbonate mineralization reaction, resulted in the calcium carbonate phase of aragonite to be formed instead of calcite that was formed in the absence of  $\text{Mg}^{2+}$ .<sup>136</sup> The aragonite formed had no inclusion of  $\text{Mg}^{2+}$  revealing that  $\text{Mg}^{2+}$  influenced the mineralization without being incorporated into the mineral.

Once the Pt alloys of interest were determined, the synthesis was optimized and the reaction products characterized. The synthesis of Pt alloys was not specific for the interior of ferritin under the conditions similar for Pt mineralization in ferritin. Using

synthetic procedures similar to the conditions developed by Tsukamoto,<sup>125</sup> the mineralization of Pt alloys was then specific for the interior of Fn. The optimized conditions for the mineralization of Pt alloys in ferritin was 1 mg/mL ferritin in buffer (200 mM NH<sub>4</sub>Acetate pH 5.0) at 15°C while sonicating.

The optimized mineralization conditions for the Zn-Pt and Ni-Pt reactions in Fn are drastically different than the mineralization conditions used for the mineralization Pt<sup>0</sup> in Fn. These differences in the mineralization arise from the addition of a metal species (nickel and zinc) that in aqueous solution have a positive charge. Positively charged metals in solution will be concentrated on the interior surface of Fn through the electrostatic interaction described in the Introduction. This provides an interesting situation where the dopant metal is directed to the interior surface of the protein cage and the platinum in solution is not because it lacks a positive charge. Using low temperature slows down the reduction of the metal particles and results in an improvement in the mineralization efficiency. There are many unknowns in the synthesis of platinum alloys in Fn. The role that the sonication is playing is unknown but presumably it aids in the reduction of the metal because without the sonication the reduction is considerably slower at 15°C. The biggest unknown that would lead to more question than answers is what role is the dopant metal playing in the mineralization. If the dopant is only playing a role in the nucleus formation then valuable insights into the mineralization in Fn could be extracted. The incorporation of the dopant metal into the crystal is could also provide valuable insights into when the nucleation incorporated the dopant metal or if the nucleation has been shifted to only the dopant metal from which the alloyed metal is able to grow. Continued studies into the mineralization of platinum alloys have potential to

provide valuable insights into the mineralization of Pt and other none charged species inside Fn.

#### Discussion the Mineralization of Pd and Zn-Pd in Fn

Palladium nanoparticles have been synthesized in Fn by Ueno,<sup>49</sup> which in addition to what was learned from the Pt alloy experiments, allowed us to find an optimal synthesis in at 1 mg/mL ferritin in buffer (10 mM HEPES 150 mM NaCl pH 8.5) at 15°C while sonicating. The catalytic H<sub>2</sub> production activity of Pd nanoparticles was of interest and whether or not Zn doping was universally able to increase catalytic rate in noble metal nanoparticles. Pd<sup>0</sup> could not be mineralized in the interior surface of Fn under the same conditions as Pt<sup>0</sup>, which suggests that the nucleation during the palladium mineralization is different than the platinum and platinum alloys. One important difference between platinum(II) and palladium(II) is that palladium is far more labile in its ligand exchange compare to platinum. Zn-Pd reactions carried out under the same conditions as the Pd mineralization in Fn suggest that the Zn does not change the mineralization reaction in the same way that it does in the platinum alloys.

#### Conclusions From Noble Metal and Noble Metal Alloy Synthesis Inside Ferritin

Ferritin is a robust, versatile platform for the mineralization of noble metal and noble metal alloy synthesis. Ferritin is stable under a wide range of mineralization conditions making it an ideal platform for the synthesis of reduced metal particles. The mineralizations are specific for the interior of the cage with very little if any bulk precipitation. The ferritin mineralizations were much cleaner than the mineralization in

the Hsp cage under identical conditions. The mineralization of platinum and platinum alloys suggests that there are other mechanisms for the formation nuclei in addition to the electrostatic model.

#### Pt and Pt Alloy Mineralization in PfDPS and LDPS

PfDPS and LDPS mineralization with Pt nanoparticles was attempted to determine the effect a smaller protein cage would have on the catalytic activity of the Pt nanoparticles. PfDPS and LDPS have smaller interior diameters (4 nm) than Fn (8 nm), which if particle sizes similar to those in Fn could be synthesized in PfDPS and LDPS the Pt<sup>0</sup> nanoparticle would fill the entire interior of the cage. PfDPS under no conditions could be mineralized specifically on the interior of the protein cage with Pt<sup>0</sup> or Zn-Pt (Figure 2.46, 47, and 48). LDPS was very unstable and aggregated readily under reducing conditions limiting its use for the mineralization of reduced metal particles.

#### Conclusion

With the exception of LDPS and PfDPS, protein cages are robust platforms for the reductive mineralization of metals on the interior surface. The protein cages provide uniquely structured architectures for the synthesis of a range of reduced metal nanoparticles. Insertion of engineered nucleation sites, Pt<sup>0</sup> binding peptide and G41C, into Hsp provided no advantage to the interior specific mineralization of Pt<sup>0</sup>; however, they provided valuable insights into the mineralization of Pt<sup>0</sup> in Hsp. Hsp Phen mineralization reaction was unable to be conclusively characterized leaving the results of the reaction inconclusive. WtHsp mineralized Pt<sup>0</sup> more specifically on its interior than

PtHsp and Hsp G41C. Ferritin mineralization of  $\text{Pt}^0$  is more specific than on the interior of Hsp. The mineralization of  $\text{Pt}^0$  in Fn suggests that there are other mechanisms for mineralization than the electrostatic models. Alloying of Pt with other metals has the potential to increase the catalytic activity of platinum catalysts and decrease the dependence of on platinum for efficient catalysis.

## CHARACTERIZATION OF THE CATALYSIS OF PROTEIN CAGE ENCAPSULATED NOBLE METAL NANOPARTICLES

### Introduction

Recently, there has been considerable interest and investment in the development of non-fossils fuel based energy sources. While considerable attention has been given to the development of biomass based fuels (ethanol from corn, switchgrass etc.), a recent Department of Energy study stated that all of the arable land on the Earth would have to be planted with switchgrass to provide the current power needs of the world.<sup>137, 138</sup> However, covering just 0.16% of the land of the Earth with 10% efficient solar conversion would provide enough energy for the current demand.

In a photoenergy gathering system, three processes the must occur; capture of light energy, conversion of the gathered energy into a usable form, and storage of that energy. In all of the processes used in solar energy conversion, efficient catalysts are needed for the practical implementation of a light harvesting system.<sup>137, 139-141</sup> One approach for the utilization and storage of photoenergy is the production of H<sub>2</sub> gas.<sup>142, 143</sup> In this approach, photoenergy gathered by antennae, small molecule dye,<sup>144</sup> semiconductor,<sup>145</sup> etc., is transferred to a H<sub>2</sub> producing catalysts which then converts the collected energy to H<sub>2</sub> gas that can be used as an alternative to fossil fuels. This clean burning fuel can be stored and transported allowing for its utilization as a fuel for transportation.<sup>143</sup>

The current industrial production of hydrogen gas is steam reformation of natural gas at high temperature. This production does little to reduce dependence on the fossil fuels. The current catalysts for the low temperature photomediated production of H<sub>2</sub> gas

is platinum metal. While the price of platinum makes its use less appealing, the real disadvantage of platinum based catalysts is that the wide spread implementation of platinum catalysts could exhaust the global supply.<sup>146</sup> With the knowledge of this limitation, two approaches have been pursued; one is the maximization of the catalytic activity of each platinum atom that is used<sup>147</sup> and second is to develop catalysts that use limited amounts of platinum or are non-platinum based catalysts.<sup>107, 148-150</sup>

To maximize the catalytic efficiency per atom of platinum, platinum nanoparticles have been synthesized to maximize catalytic activity. Nanoparticles maximize the surface area and to decrease the number of platinum atoms that are not surface exposed and therefore catalytically inactive. A number of studies have focused on the optimizing the passivating layer to allow the maximum catalytic activity.<sup>110, 115, 151</sup> Another approach to maximizing the catalytic activity of platinum based catalysts is the addition of other metals (dopants) into the crystal structure to increase the catalytic activity and decrease amount of platinum. Some examples of dopants used are Ni,<sup>107, 152</sup> Re,<sup>153</sup> Mo,<sup>153</sup> Ti,<sup>134</sup> Co,<sup>152</sup> Ru,<sup>154</sup> and Cr.<sup>155</sup>

In an attempt to move away from platinum as a catalyst entirely, the use of enzymes or synthetic mimics of enzymes for the conversion reducing equivalents and protons to H<sub>2</sub> gas has been an area of intense research.<sup>126, 129, 130</sup> These enzymes, called hydrogenases (H<sub>2</sub>ase) have tremendous potential for use in the conversion of the solar energy into H<sub>2</sub>.<sup>156-158</sup> These enzymes and their synthetic analogs are FeFe or FeNi based with no dependence on platinum.<sup>159, 160</sup> The hydrogen production rate for each hydrogenase is 6000-9000 H<sub>2</sub> per sec.<sup>128, 161</sup> While the synthetic analogs are structurally analogous to the active sites of hydrogenases, the H<sub>2</sub> production activity of the analogs

has been low because the protein plays an essential role in the catalytic activity of the enzyme.<sup>130, 162, 163</sup>

In an attempt to mimic the protein encapsulated inorganic metal cluster of the H<sub>2</sub>ase enzyme and utilize the benefits of synthetic chemistry, protein cage architectures were used as platforms for the synthesis of catalytic noble metal nanoparticles. By synthesizing the nanoparticles on the interior of the protein cage, the protein acts as a passivating layer for the nanoparticle. The protein cage passivating layer is a unique platform for catalysis because the surface of the catalyst is not coated with a small molecule or polymer. The architecture of the protein cage is determined by its own structure and does not conform to the structure nanoparticle, which allows for a large portion of the catalytic particle to be uncoated. In this approach, the activity of the platinum is maximized by synthesizing nanoparticles which have high surface to volume ratios and minimize the surface coating on the nanoparticle while keeping the nanoparticle from aggregating.

. After synthesizing protein encapsulated noble metal nanoparticles (Pt, Pd, and Pt and Pd alloys), the catalytic activity of these encapsulated nanoparticles was characterized. H<sub>2</sub> production was chosen as the reaction of interest because of current interest, given the rising costs of fossil fuel based energy. One of the technological limitations in moving towards a H<sub>2</sub> based economy is the limited platinum availability.<sup>146</sup> We have attempted to maximize the catalytic H<sub>2</sub> production per Pt by synthesizing nanoparticles of the interior of protein cages to achieve the smallest particle size to maximize catalytic activity. In a particle based system, the coating of the particle has been demonstrated to have a dramatic effect on the catalytic properties of the

nanoparticles.<sup>110</sup> We set out to test utility of the protein architecture as a platform for catalytic H<sub>2</sub> production.

## Methods

### Light Induced Hydrogen Production

The reaction was performed in a septum sealed glass cuvette containing a 2 mL solution of 500 mM acetate, 200 mM EDTA, 0.5 mM methyl viologen, and 0.2 mM Ru(bpy)<sub>3</sub><sup>2+</sup> at pH 5.0 deaerated with nitrogen. A Xe arc lamp (175 W, Lambda-LS, Sutter Instruments) was used as the light source. The light flux was measured using an Extech Instrument EasyView light meter to be 511,700 lux at high light and 167,600 lux at low light. IR radiation was removed by passing the beam through a 10cm water filter and the UV was removed using UV-absorbing glass. The production of H<sub>2</sub> was determined by gas chromatography (see below).

### Chemical Reduction of Methyl Viologen and Hydrogen Production

Zinc powder (0.5 g) was added to 10 mL of a 2% solution of mercury (II) chloride in water<sup>164</sup>. The solution was mixed and allowed to stand for 10 min after which the supernatant was removed. The resulting amalgam was added to a sealable glass cuvette containing 2 mL of argon deaerated solution of 500 mM EDTA, and 0.5 mM methyl viologen at pH 5.0. The cuvette was sealed and purged with argon before the Pt was added. The production of H<sub>2</sub> was determined by gas chromatography.

### Gas Chromatography

Hydrogen production was measured using gas chromatography. All experiments were carried out on a Shimadzu GC-8A TCD with a 6-ft. x 1/8-in. 80/100 Porapak Supelco column using argon as carrier gas. Bulk H<sub>2</sub> gas was used as a standard to quantitate the H<sub>2</sub> production.

### Electrochemistry

All experiments were conducted on a Bioanalytical Systems, Inc. CV-50W Voltammetric Analyzer. A Bioanalytical Systems, Inc. Glassy Carbon Electrode (3.0 mm dia.) was used as the working electrode. Before using the electrode it was polished with polishing alumina (0.05  $\mu$ m) and extensively sonicated in 20% ethanol. The protein catalyst was adsorbed on to the electrode in 50 mM MES 100 mM NaCl pH 6.5. 10  $\mu$ L of the sample placed directly of the surface of the electrode and allowed to stand for 5 minutes. The electrode was then inserted into the reaction vessel with a Ag/AgCl reference electrode and a Pt wire auxiliary electrode.

### Results

#### Key Results From Protein Cage Encapsulated Noble Metal Catalysis

Listed below are the highlights of the H<sub>2</sub> production results from noble metal catalysts encapsulated with in protein cage architectures.

#### Characterization of H<sub>2</sub> Production from WtHsp

Encapsulated Pt<sup>0</sup> Nanoparticles: Catalytic H<sub>2</sub> production from WtHsp revealed that the platinum particles synthesized on the interior surface are catalytically active at

the reduction of protons to  $H_2$  gas. WtHsp mineralized with 150 platinum atoms per cage did not catalytically production  $H_2$  gas. WtHsp encapsulated platinum nanoparticle are an order of magnitude more active than platinum nanoparticles synthesized in the absence of the protein cage.

#### Characterization of $H_2$ Production From Fn

Encapsulated  $Pt^0$  Nanoparticles: Fn encapsulated protein platinum nanoparticles also catalytically reduced protons to  $H_2$  gas. The ferritin encapsulated platinum nanoparticles were more active for the production  $H_2$  when calculated per cage, per platinum, and per surface area than the platinum encapsulated by WtHsp.

#### Characterization of the $H_2$ Production From

Platinum Alloys Encapsulated by Fn: Platinum alloys of Ni and Zn mineralized inside Fn showed a significant increase in the  $H_2$  production per platinum compared to the  $H_2$  production rates of the platinum only catalysts synthesized on the interior of Fn. Zn-Pt alloys showed a greater increase in the  $H_2$  production rate that the Ni-Pt alloys.

#### Characterization of the $H_2$ Production From Palladium

and Palladium Alloys Encapsulated by Fn: Palladium catalysts mineralized on the interior surface of the Fn revealed that protein encapsulated palladium nanoparticles are 3-4 times less active than the similar catalyst. Doping Zn into Pd did not show the same increase in  $H_2$  production that was seen in the Pt doping of Zn.

#### CV Characterization of the Fn Encapsulated Catalysts: Absorbing Fn

encapsulated catalysts to the surface of a glass carbon electrode was use to characterize the catalytic properties protein encased catalysts. The absorbed catalysts displayed CV wave characteristic of a catalysis absorbed of the surface. After cycling the CV, a

number of times hydrogen bubbles form on the surface of the electrode. CV has the potential to be used further characterize the catalytic activity of the ferritin encapsulated nanocatalysts, especially the long term durability of the catalysts.

### Characterization of the Photo-mediated $H_2$ Production Assay

The  $H_2$  production assay used to characterize the catalytic activity of the protein encapsulated noble metal nanoparticles uses a photocatalyst to reduce an electron relay which is able to donate an electron to the nanoparticles. Noble metal nanoparticles utilize the electrons to reduce protons to  $H_2$ , while the photocatalyst is regenerated by oxidizing the sacrificial electron donor.<sup>110, 151, 165</sup> The standard conditions for the  $H_2$  production assay were based on the methodology originally described by Gratzel.<sup>166</sup> However, these

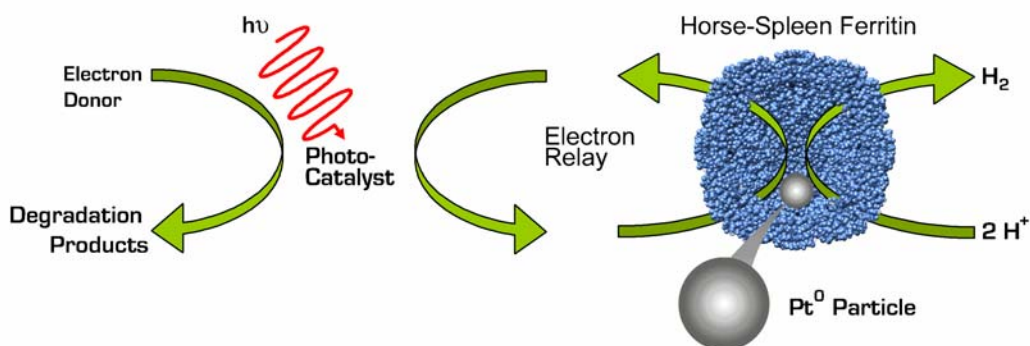


Figure 3.1. Reaction schematic of the  $H_2$  production assay based on the methodology described by Gratzel.

conditions for  $H_2$  production assay were varied to determine the range of photocatalysts, sacrificial electron donors, and electron mediators that could be used to test the  $H_2$  production ability of the Pt<sup>0</sup> nanoparticles (Figure 3.1). The standard reaction conditions called for the use of acetate (proton source), EDTA (sacrificial electron donor), methyl viologen ( $MV^{2+}$ ) (electron mediator), and  $Ru(bpy)_3^{2+}$  (photocatalyst) at pH 5.0.

Sacrificial Electron Donors: Using  $\text{Ru}(\text{bpy})_3^{2+}$  as the photocatalyst, the sacrificial electron donor was varied to determine if more simple organics could be used to regenerate the  $\text{Ru}(\text{bpy})_3$ . Citrate, ascorbate, methanol, ethanol, sulfite, formate, dimethylamine borane, hypophosphate, glucose, sucrose, NADPH,  $\text{I}_3/3 \text{I}^-$ , and acetate were all used as sacrificial electron donors in the  $\text{H}_2$  production assay. However, none produced  $\text{H}_2$  during the course of the assay or formed the reduced methyl viologen (blue color). EDTA was the only sacrificial electron donor tested that allowed for the evolution of  $\text{H}_2$ .

Photocatalysts: In an effort to determine the most efficient and durable photocatalyst for  $\text{H}_2$  production a number of previously described photocatalysts from the literature were tested. Acridine orange was used as a photocatalyst in the  $\text{H}_2$  production assay under the same conditions as the standard conditions for  $\text{Ru}(\text{bpy})_3^{2+}$  and the same  $\text{Pt}^0$  concentration. The acridine orange was able to produce reducing equivalents under these and  $\text{H}_2$  was evolved, but at a significantly slower rate (half) than the  $\text{Ru}(\text{bpy})_3^{2+}$  based assay. Green fluorescent protein (GFP) was also used as the photocatalyst for the  $\text{H}_2$  production assay. GFP was not stable at the standard reaction conditions; the characteristic green color was lost and precipitate was formed, so the reaction pH was raised 5 to 7. The GFP  $\text{H}_2$  production assay at pH 7 did produce low levels of  $\text{H}_2$  but was unable to match the performance of the  $\text{Ru}(\text{bpy})_3^{2+}$  based system (10% of  $\text{Ru}(\text{bpy})_3^{2+}$ ).

H<sub>2</sub> Production Dependence on Light Intensity: Varying light conditions were tested to determine the optimal conditions for the photoassay. Two light conditions were

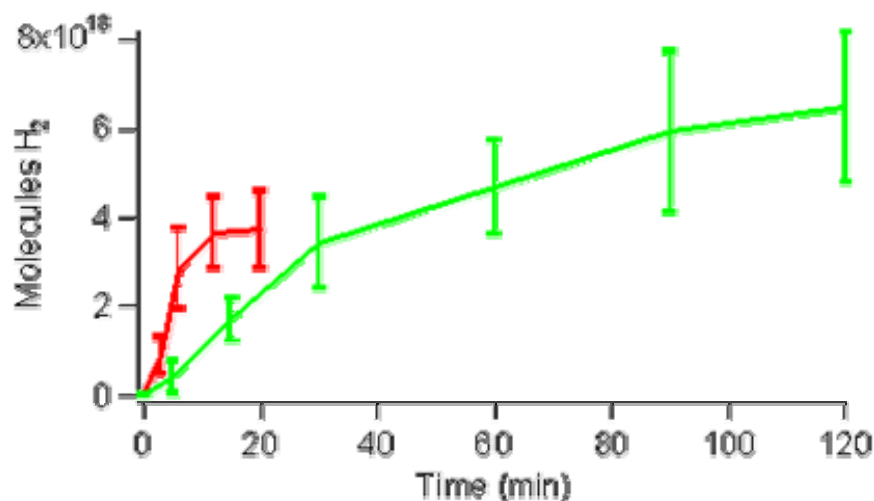


Figure 3.2. The H<sub>2</sub> production curves from **high** and **low** light at standard conditions and the same amount of platinum in each reaction.

chosen high (511,700 lux) and low (167,600 lux). Figure 3.2 shows the difference between the high and low light H<sub>2</sub> production reactions. The high light has a much faster initial rate but the photocatalyst is degraded after 20 minutes (Figure 3.2). Under the low

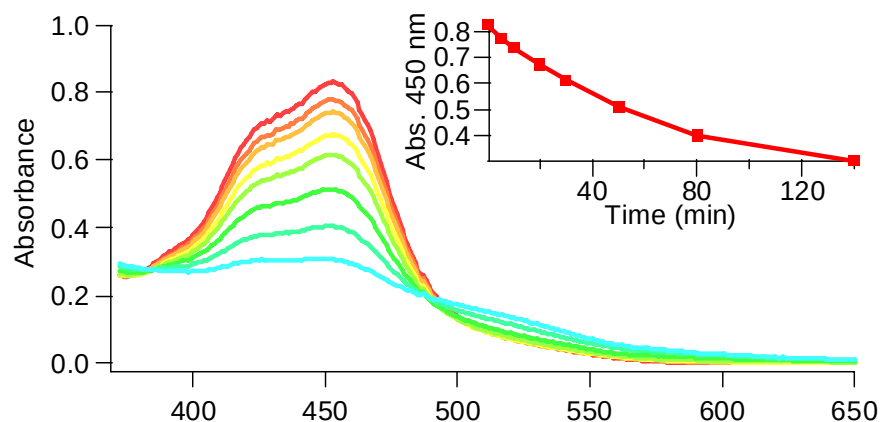


Figure 3.3. The UV/Vis time course of Ru(bpy)<sub>3</sub><sup>2+</sup> under high light conditions showing the degradation upon illumination; Inset: the absorption at 450 nm over time displaying the degradation.

light condition, the photocatalyst is degraded much slower and the total amount of H<sub>2</sub> produced is increased; however, the maximum H<sub>2</sub> production rate is significantly lower.

The high light condition was chosen as the optimal condition to determine the maximum rate for the catalysts.

Chemical Reduction: A chemical source for the reduction of  $MV^{2+}$  was sought because the photodegradation of  $Ru(bpy)_3^{2+}$  prevents the long-term (hours or days) characterization of the protein encapsulated noble metal nanoparticles.<sup>167-169</sup> In an attempt to chemically generate the reducing equivalents for the  $H_2$  production assay, the  $H_2$  production assay used to determine the catalytic rates of hydrogenase was used.<sup>170</sup> In this assay, sodium dithionite reduces  $MV^{2+}$  which then donates an electron to the hydrogenase to use for the reduction of protons. The reaction conditions for the dithionite reaction assay was 1 mM  $MV^{2+}$  and 20 fold molecular excess of sodium dithionite in 500 mM acetate pH 5.0. The  $H_2$  production assay showed no  $H_2$  production but the  $MV^{2+}$  was clearly reduced. The dithionite is assumed to poison the  $Pt^0$  catalyst because sulfur in the dithionite tightly binds to the  $Pt^0$  coating the surface blocking the reactive sites on the surface. In an attempt to prevent the poisoning of the  $Pt^0$  nanoparticles by dithionite, the  $Pt^0$  catalysts were first incubated with 50 mM citrate pH 6.0 (a common coating for the synthesis of  $Pt^0$  nanoparticles that does not poison the catalytic surface) at room temperature and 60°C for 30 minutes. The dithionite assay was then run to determine the effect of the coating. The citrate coating provided no increase in the  $H_2$  production using dithionite as a reductant.

In an effort to find a chemical reduction of  $MV^{2+}$  that does not poison the platinum catalyst, a Jones reductor (Zn amalgam) was used to reduce the  $MV^{2+}$ .<sup>164</sup> The Jones reductor was chosen as a chemical reductant because the Zn amalgam is a solid that

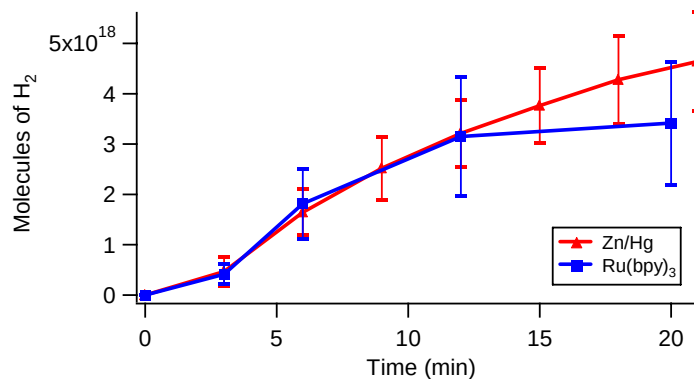


Figure 3.4. Comparison of the H<sub>2</sub> production assay using the Ru(bpy)<sub>3</sub><sup>2+</sup> and the Jones reductor. The H<sub>2</sub> production curves from 4.21 × 10<sup>-9</sup> moles Pt using ■ Ru(bpy)<sub>3</sub><sup>2+</sup> and ▲

could reduce the MV<sup>2+</sup> without negatively interacting with the surface of the catalyst.

The Jones reductor had to be run with EDTA in the assay or the reaction produced minimal H<sub>2</sub>. One problem with the Jones reductor is that without the addition of Pt<sup>0</sup> there was H<sub>2</sub> production. While a direct comparison of the Jones reductor and the Ru(bpy)<sub>3</sub><sup>2+</sup> photoassay (Figure 3.4) appears to be similar, the Jones reductor produces 40% less H<sub>2</sub> when the background production is taken into account.

### H<sub>2</sub> Production From WtHsp Encapsulated Nanoparticles

The H<sub>2</sub> production assays were performed on 250 Pt WtHsp and 1000 Pt WtHsp. All of the assays were performed using the standard reaction conditions. The platinum concentrations were determined using ICP-MS conducted by Energy Laboratories in Billings, MT. Each sample was run at least three times at three concentrations of platinum. The reaction were run at three different concentrations of platinum because the assay

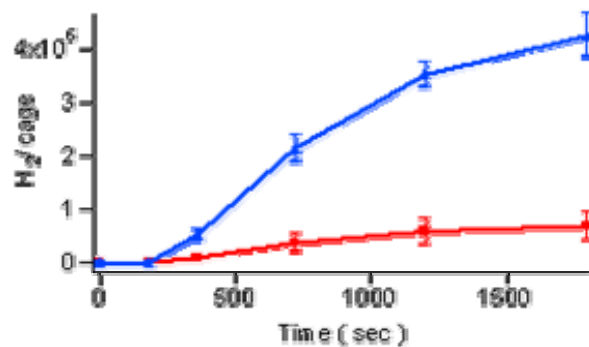


Figure 3.5.  $\text{H}_2$  production from 250 Pt and 1000 Pt mineralized WtHsp in 0.2 mM  $\text{Ru}(\text{bpy})_3^{2+}$ , 0.5 mM methyl viologen, 200 mM EDTA, and 500 mM acetic acid pH 5.0.  $\blacktriangle$  1000 Pt WtHsp  $5.1 \times 10^{-10}$  moles Pt;  $\blacksquare$  250 Pt WtHsp  $8.2 \times 10^{-10}$  moles Pt.

Figure 3.5 shows the  $\text{H}_2$  production curves of 250 Pt WtHsp and 1000 Pt WtHsp in  $\text{H}_2/\text{cage}$  vs. seconds. The  $\text{H}_2$  production was showed as molecules of  $\text{H}_2$  per protein cage to compare the protein cage activity to the  $\text{H}_2$  production activity of the  $\text{H}_2$ ase. When calculated on a per cage basis, the initial rate of  $\text{H}_2$  formation was  $4.47 \times 10^3 \text{ H}_2 \cdot \text{sec}^{-1}$  ( $\pm 394 \text{ H}_2 \cdot \text{sec}^{-1}$ ) for a loading factor of 1000 Pt per Hsp and  $7.63 \times 10^2 \text{ H}_2 \cdot \text{sec}^{-1}$  ( $\pm 405 \text{ H}_2 \cdot \text{sec}^{-1}$ ) for a loading factor of 250 (Figure 3.5). The rates of the 1000 Pt WtHsp are more 4 times greater than the rates of 250 Pt WtHsp, which is to be expected because there is 4 times more platinum per cage in the mineralization. These rates are comparable to those reported for hydrogenase enzymes ( $4 \times 10^3 \text{ H}_2/\text{sec}$  to  $9 \times 10^3 \text{ H}_2 \cdot \text{sec}^{-1}$ ).

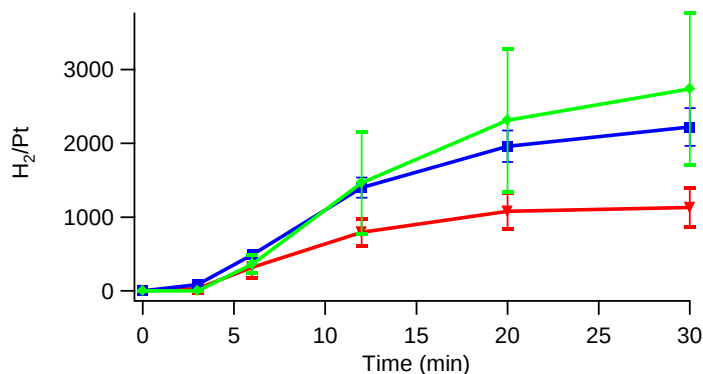


Figure 3.6.  $\text{H}_2$  production curves from 250 Pt WtHsp in standard conditions,  $\blacktriangle$   $8.2 \times 10^{-10}$  moles Pt;  $\blacksquare$   $1.7 \times 10^{-9}$  moles Pt;  $\blacklozenge$   $4.9 \times 10^{-9}$  moles Pt.

Figure 3.6 shows the  $\text{H}_2$  production curves for the 250 Pt WtHsp at varying concentrations of platinum displayed as  $\text{H}_2 \cdot \text{Pt}^{-1}$ . By converting the  $\text{H}_2$  production rates to  $\text{H}_2 \cdot \text{Pt}^{-1} \text{min}^{-1}$ , the catalytic rates of the protein encapsulated noble metal nanoparticles can be compared to literature values for other noble metal nanoparticles and the  $\text{H}_2$  per Pt rate can then be used to determine which synthesis is the most efficient at optimizing the  $\text{H}_2$  production per platinum. Figure 3.7 shows the  $\text{H}_2$  production curves for the 1000 Pt WtHsp displayed as  $\text{H}_2 \cdot \text{Pt}^{-1}$  at different concentrations of platinum. The maximum rate for the 1000 Pt WtHsp is  $268 \text{ H}_2 \cdot \text{Pt}^{-1} \text{min}^{-1}$ . The maximum rate for the 250 Pt WtHsp is  $183 \text{ H}_2 \cdot \text{Pt}^{-1} \text{min}^{-1}$ .

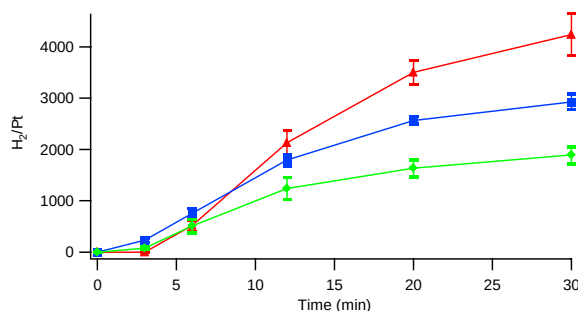


Figure 3.7.  $\text{H}_2$  production curves from 1000 Pt WtHsp in standard conditions,  $\blacktriangle$   $3.9 \times 10^{-9}$  moles Pt;  $\blacksquare$   $1.4 \times 10^{-9}$  moles Pt;  $\blacklozenge$   $5.1 \times 10^{-10}$  moles Pt.

Figure 3.8 shows the  $\text{H}_2$  production curves for the protein free Pt control, which is the 1000 Pt in WtHsp reaction run without protein in the mineralization reaction, at varying concentrations of platinum displayed as  $\text{H}_2 \cdot \text{Pt}^{-1}$ . The maximum rate from the control reactions is  $15.7 \text{ H}_2 \cdot \text{Pt}^{-1} \text{min}^{-1}$ .<sup>171</sup>

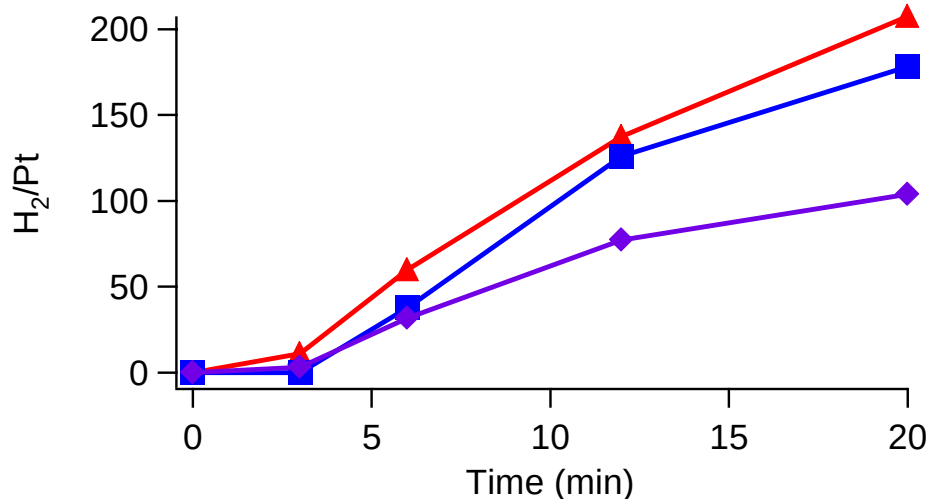


Figure 3.8. H<sub>2</sub> production curves from Pt control synthesis in standard conditions, ▲  $2.4 \times 10^{-9}$  moles Pt; ■  $4.5 \times 10^{-9}$  moles Pt; ◆  $1.1 \times 10^{-8}$  moles Pt.

#### H<sub>2</sub> Production From Ferritin Encapsulated Nanoparticles Using Photocatalysis

The H<sub>2</sub> production assays were performed on 250 Pt Fn and 1000 Pt Fn. All of the assays were performed using the standard reaction conditions. The platinum concentrations were determined using ICP-MS conducted by Energy Laboratories in Billings, MT. Each sample was run at least three times at three concentrations of platinum.

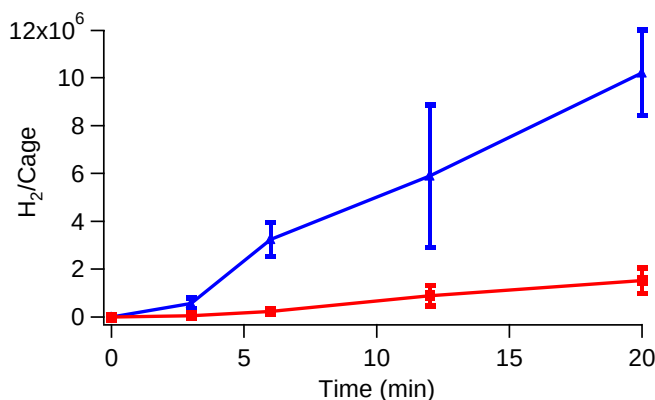


Figure 3.9. H<sub>2</sub> production curves from 1000 and 250 PtFn in standard conditions. ▲ 1000 Pt Fn,  $1.1 \times 10^{-9}$  moles Pt, ■ 250 Pt/ferritin,  $3.3 \times 10^{-10}$  moles Pt.

Initial rates of  $H_2$  formation, when calculated on a per cage basis, were  $14.9 \times 10^3 H_2 \cdot Fn^{-1} sec^{-1}$  ( $\pm 2.77 \times 10^3 H_2 \cdot Fn^{-1} min^{-1}$ ) for a loading factor of 1000 Pt per ferritin and  $1.82 \times 10^3 H_2 \cdot Fn^{-1} min^{-1}$  ( $\pm 8.47 \times 10^2 H_2 \cdot Fn^{-1} min^{-1}$ ) for a loading factor of 250 (Figure 3.9). The rate per cage for the loading of 1000 PtFn is 7 times higher than the rate of 250 PtFn. The higher rate for the 1000 loading compared to the 250 loading is expected because of the 4 fold increase in platinum per cage.

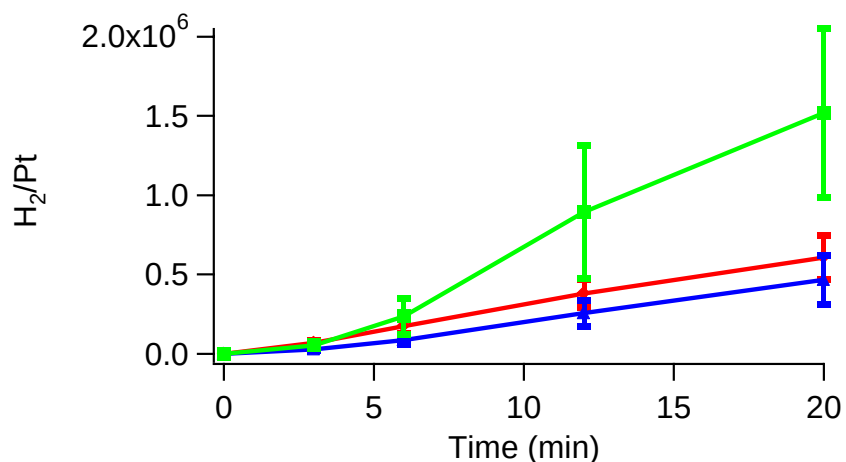


Figure 3.10.  $H_2$  production curves from 250 PtFn in standard conditions,  $\blacktriangle$   $9.2 \times 10^{-11}$  moles Pt;  $\blacklozenge$   $2.8 \times 10^{-10}$  moles Pt;  $\blacksquare$   $3.3 \times 10^{-10}$  moles Pt.

Figure 3.10 shows the  $H_2$  production curves for the 250 PtFn at varying concentrations of platinum displayed as  $H_2 \cdot Pt^{-1}$ . Figure 3.11 shows the  $H_2$  production curves for the 1000 Pt Fn displayed as  $H_2 \cdot Pt^{-1}$  at different concentrations of platinum. The maximum rate for the 1000 Pt Fn is  $893 H_2 \cdot Pt^{-1} min^{-1}$ . The maximum rate for the 250 Pt Fn is  $H_2 \cdot Pt^{-1} min^{-1}$ .

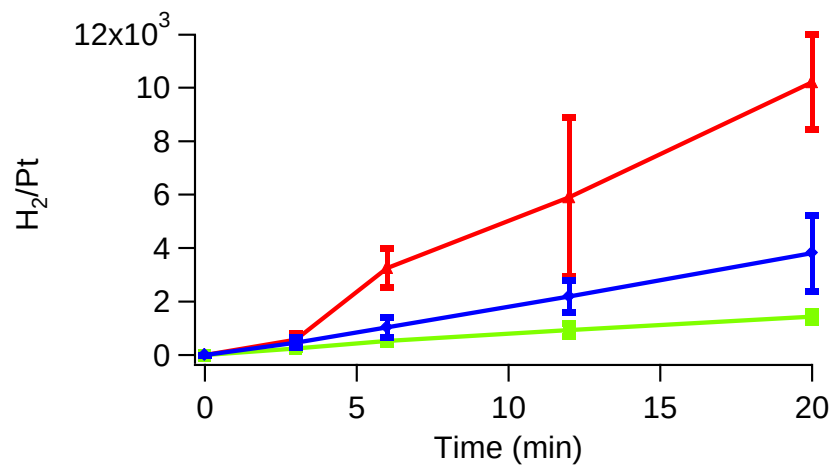


Figure 3.11. H<sub>2</sub> production curves from 1000 PtFn in standard conditions,  $\blacktriangle$   $1.1 \times 10^{-9}$  moles Pt;  $\blacklozenge$   $1.3 \times 10^{-10}$  moles Pt;  $\blacksquare$   $9.2 \times 10^{-11}$  moles Pt.

### H<sub>2</sub> Production Assay for Alloys of PtFn

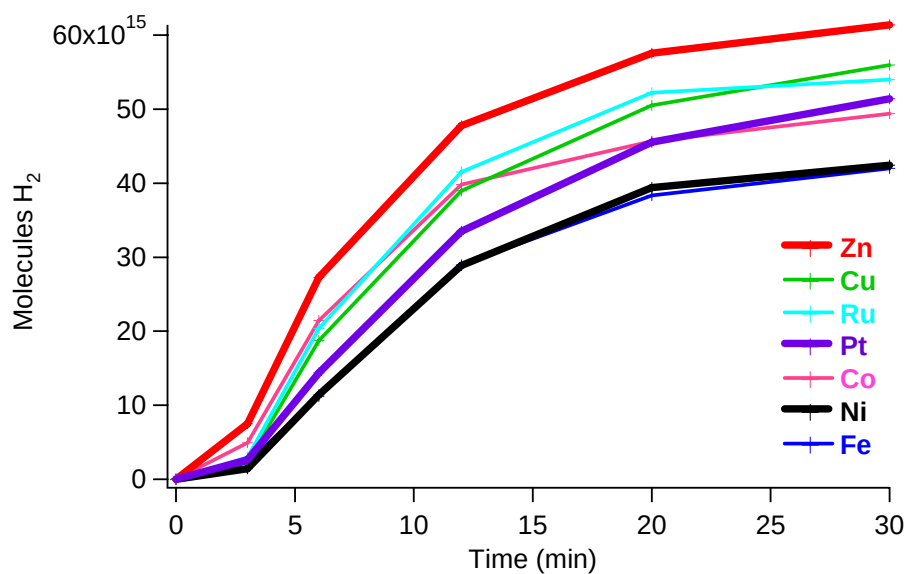


Figure 3.12. H<sub>2</sub> production curves from the initial screen of possible dopants.

Increasing the catalytic efficiency per atom of platinum can be achieved by the addition of other metals that increase or maintain the catalytic activity of the nanoparticles while decreasing the amount of platinum used. In an attempt to increase the H<sub>2</sub> production by doping other metals into the platinum nanoparticles, Fn as a

platform for the synthesis of platinum alloys of Zn, Cu, Ru, Co, Ni, and Fe in a 3:1 ratio of platinum to dopant under identical conditions. The catalytic screen was done on the reaction before any purification of the reaction to ensure consistent metal loading in the H<sub>2</sub> production assay. Figure 3.12 shows the results of the initial H<sub>2</sub> production assay to determine the dopants of interest. In this assay, the total metal added to the reaction was the same which allows for the comparison of the platinum only to the platinum alloys. Any curve equal to the platinum only curve would mean that the same amount of H<sub>2</sub> was produced from 75% of the amount of platinum. Any curve greater than the platinum only curve would mean that more H<sub>2</sub> was produced from 75% of the amount of platinum. The ZnPt alloy is clearly the most active alloy that was synthesized. The NiPt alloy was also chosen because it is a well researched platinum alloy.

| Synthesis | Ratio (Pt/Metal) |
|-----------|------------------|
| 250 ZnPt  | 11.5             |
| 500 ZnPt  | 3.7              |
| 1000 ZnPt | 33.1             |
| 250 NiPt  | 19.3             |
| 500 NiPt  | 297.4            |

Table 3.1. The ratio of Pt per metal in each synthesis determined by ICP-MS.

Before the H<sub>2</sub> production assays, ICP-MS was run on the 250 PtFn, 500 PtFn, 250 ZnPt Fn, 500 ZnPt Fn, 1000 ZnPt Fn, 500 NiPt Fn, and 250 NiPt Fn to determine platinum concentration and platinum to dopant ratio. Table 3.1 shows the ratios of Pt to dopant for the different syntheses. Interestingly, the different syntheses have varying

levels of dopants incorporated into or associated with the alloys, which allows us to examine the effect of the doping level.

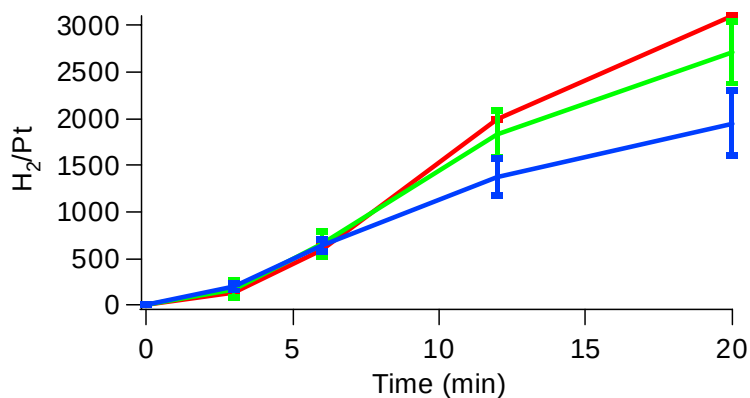


Figure 3.13. H<sub>2</sub> production curves from 1000 ZnPt Fn in standard conditions, ■  $3.8 \times 10^{-9}$  moles Pt and  $1.2 \times 10^{-10}$  moles Zn; ■  $1.9 \times 10^{-9}$  moles Pt and  $5.8 \times 10^{-11}$  moles Zn; ■  $9.6 \times 10^{-10}$  moles Pt and  $2.9 \times 10^{-11}$  moles Zn.

Figure 3.13 shows the H<sub>2</sub> production assay of 1000 ZnPt Fn at three different concentrations of catalyst added to the assay and each concentration was run three times. The Y-axis is in number of molecule of H<sub>2</sub> produced per platinum in the reaction. The maximum rate of H<sub>2</sub> production for the synthesis catalyst is  $234 \text{ H}_2 \cdot \text{Pt}^{-1} \text{ min}^{-1}$ .

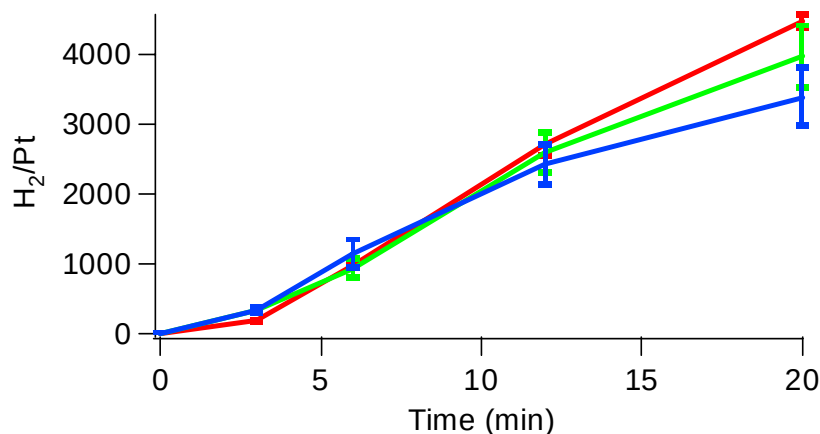


Figure 3.14. H<sub>2</sub> production curves from 500 ZnPt Fn in standard conditions, ■  $5.5 \times 10^{-10}$  moles Pt and  $1.5 \times 10^{-10}$  moles Zn; ■  $1.1 \times 10^{-9}$  moles Pt and  $3.0 \times 10^{-10}$  moles Zn; ■  $2.2 \times 10^{-9}$  moles Pt and  $2.9 \times 10^{-12}$  moles Zn.

Figure 3.14 shows the  $H_2$  production assay of 500 ZnPt Fn at three different concentrations of catalyst added to the assay and each concentration was run three times. The Y-axis is in number of molecule of  $H_2$  produced per platinum in the reaction. The maximum rate of  $H_2$  production for the synthesis catalyst is  $290 H_2 \cdot Pt^{-1} \min^{-1}$ . Figure 3.18 shows the  $H_2$  production assay of 500 PtFn at three different concentrations of catalyst added to the assay and each concentration was run three times. The Y-axis is in number of molecules of  $H_2$  produced per platinum in the reaction. The maximum rate of  $H_2$  production for the synthesis catalyst is  $255 H_2 \cdot Pt^{-1} \min^{-1}$ .

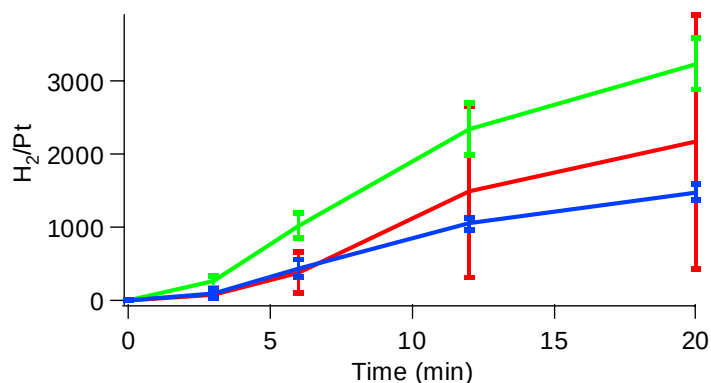


Figure 3.15.  $H_2$  production curves from 500 NiPt Fn in standard conditions,   
■  $9.1 \times 10^{-10}$  moles Pt; ■  $1.8 \times 10^{-9}$  moles Pt; ■  $3.6 \times 10^{-9}$  moles Pt.

Figure 3.15 shows the  $H_2$  production assay of 500 NiPt Fn at three different concentrations of catalyst added to the assay and each concentration was run three times. The Y-axis is in number of molecules of  $H_2$  produced per platinum in the reaction. The maximum rate of  $H_2$  production for the synthesis catalyst is  $255 H_2 \cdot Pt^{-1} \min^{-1}$ .

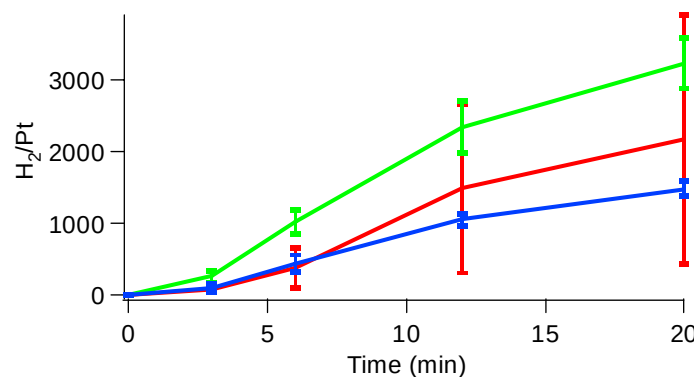


Figure 3.16. H<sub>2</sub> production curves from 500 PtFn in standard conditions,   
 ■ 6.1 x 10<sup>-10</sup> moles Pt; ■ 1.2 x 10<sup>-9</sup> moles Pt; ■ 2.4 x 10<sup>-9</sup> moles Pt.

Figure 3.16 shows the H<sub>2</sub> production assay of 500 PtFn at three different concentrations of catalyst added to the assay and each concentration was run three times. The Y-axis is in number of molecules of H<sub>2</sub> produced per platinum in the reaction. The maximum rate of H<sub>2</sub> production for the synthesis catalyst is 254 H<sub>2</sub>•Pt<sup>-1</sup>min<sup>-1</sup>.

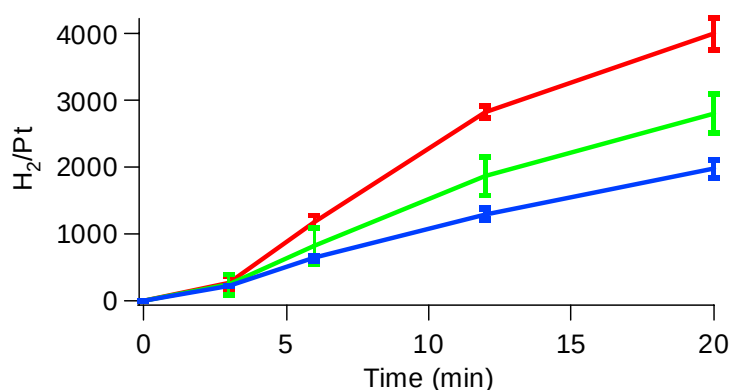


Figure 3.17. H<sub>2</sub> production curves from 250 ZnPt Fn in standard conditions,   
 ■ 1.2 x 10<sup>-9</sup> moles Pt and 1.0 x 10<sup>-10</sup> moles Zn; ■ 2.3 x 10<sup>-9</sup> moles Pt and 2.0 x 10<sup>-10</sup> moles Zn; ■ 4.6 x 10<sup>-9</sup> moles Pt and 4.0 x 10<sup>-10</sup> moles Zn.

Figure 3.17 shows the H<sub>2</sub> production assay of 250 ZnPt Fn at three different concentrations of catalyst added to the assay and each concentration was run three times. The Y-axis is in number of molecules of H<sub>2</sub> produced per platinum in the reaction. The maximum rate of H<sub>2</sub> production for the synthesis catalyst is 300 H<sub>2</sub>•Pt<sup>-1</sup>min<sup>-1</sup>.

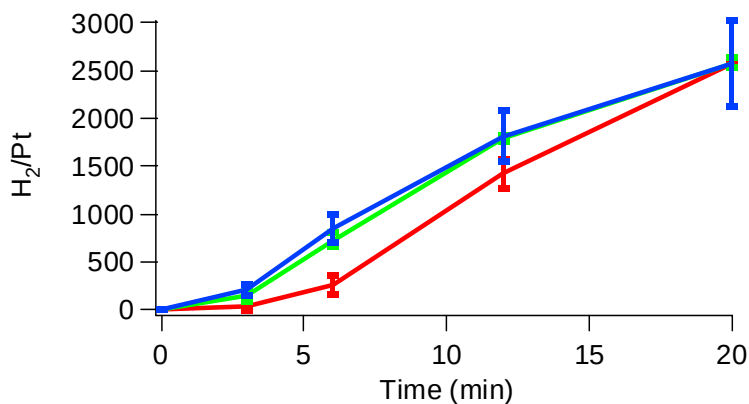


Figure 3.18. H<sub>2</sub> production curves from 250 PtFn in standard conditions,   
 ■ 4.0 x 10<sup>-10</sup> moles Pt; ■ 8.1 x 10<sup>-10</sup> moles Pt; ■ 1.6 x 10<sup>-9</sup> moles Pt.

Figure 3.18 shows the H<sub>2</sub> production assay of 250 PtFn at three different concentrations of catalyst added to the assay and each concentration was run three times. The Y-axis is in number of molecules of H<sub>2</sub> produced per platinum in the reaction. The maximum rate of H<sub>2</sub> production for the synthesis catalyst is 216 H<sub>2</sub>•Pt<sup>-1</sup>min<sup>-1</sup>.

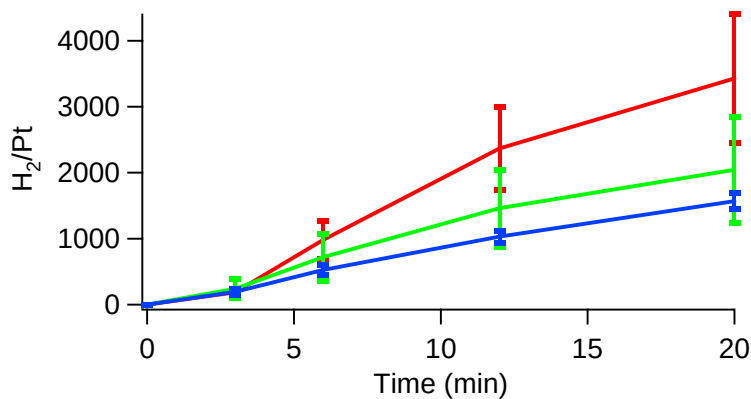


Figure 3.19. H<sub>2</sub> production curves from 250 NiPt Fn in standard conditions,   
 ■ 1.1 x 10<sup>-9</sup> moles Pt and 5.7 x 10<sup>-11</sup> moles Ni; ■ 2.9 x 10<sup>-9</sup> moles Pt and 1.1 x 10<sup>-10</sup> moles Ni; ■ 4.4 x 10<sup>-9</sup> moles Pt and 2.3 x 10<sup>-10</sup> moles Ni.

Figure 3.19 shows the H<sub>2</sub> production assay of 250 NiPt Fn at three different concentrations of catalyst added to the assay and each concentration was run three times. The Y-axis is in number of molecules of H<sub>2</sub> produced per platinum in the reaction. The maximum rate of H<sub>2</sub> production for the synthesis catalyst is 265 H<sub>2</sub>•Pt<sup>-1</sup>min<sup>-1</sup>.

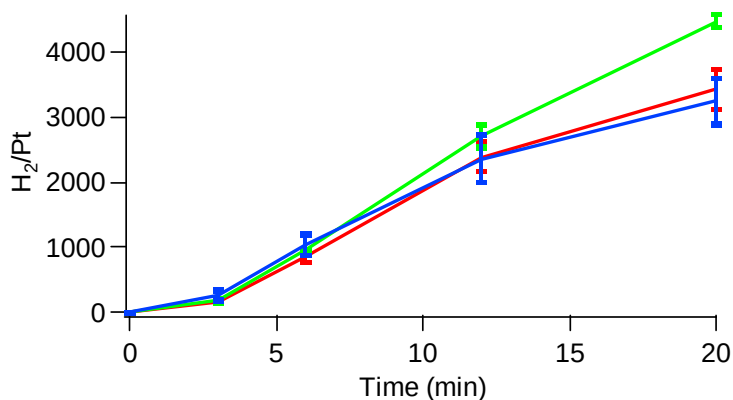


Figure 3.20. H<sub>2</sub> production curves from 500 ZnPt Fn, 500 NiPt Fn, and 500 PtFn in standard conditions, ■ 500 PtFn; ■ 500 ZnPt Fn; ■ 500 NiPt Fn

Figure 3.20 is a comparison of the H<sub>2</sub> production curves from 500 ZnPt Fn, 500 NiPt Fn, and 500 PtFn per platinum. The maximum rates for the catalysts are 290 H<sub>2</sub>•Pt<sup>-1</sup> min<sup>-1</sup> for 500 ZnPt Fn, 255 H<sub>2</sub>•Pt<sup>-1</sup> min<sup>-1</sup> for 500 PtFn, and 255 H<sub>2</sub>•Pt<sup>-1</sup> min<sup>-1</sup> for 500 NiPt

| Synthesis       | Rate (H <sub>2</sub> •Pt <sup>-1</sup> min <sup>-1</sup> ) |
|-----------------|------------------------------------------------------------|
| 250 Pt WtHsp    | 183                                                        |
| 1000 Pt WtHsp   | 268                                                        |
| 250 PtFn First  | 437                                                        |
| 1000 PtFn First | 893                                                        |
| 250 Pt          | 216                                                        |
| 250 NiPt        | 265                                                        |
| 250 ZnPt        | 300                                                        |
| 500 Pt          | 255                                                        |
| 500 NiPt        | 255                                                        |
| 500 ZnPt        | 290                                                        |
| 1000 ZnPt       | 234                                                        |

Table 3.2. Maximum rates of H<sub>2</sub> production for the different synthesis of platinum and platinum alloys in Fn and Hsp in H<sub>2</sub>•Pt<sup>-1</sup> min<sup>-1</sup>.

Fn

(Table 3.2). The rate difference between the 500 PtFn and 500 ZnPt Fn corresponds to a rate increase of 14% per platinum in the 500 ZnPt Fn. The maximum H<sub>2</sub> production rate for 500 PtFn and 500 NiPt Fn are almost identical.

Figure 3.21 shows the comparison of the  $\text{H}_2$  production curves from 250 ZnPt Fn, 250 NiPt, and 250 PtFn per platinum. The maximum rates for the catalysts are  $300 \text{ H}_2 \cdot \text{Pt}^{-1} \text{ min}^{-1}$  for 250 ZnPt Fn,  $265 \text{ H}_2 \cdot \text{Pt}^{-1} \text{ min}^{-1}$  for 250 NiPt Fn, and  $216 \text{ H}_2 \cdot \text{Pt}^{-1} \text{ min}^{-1}$  for 250 PtFn (Table 3.2). The rate difference between the 250 PtFn and 250 NiPt Fn corresponds to a rate increase of 23% per platinum. The rate difference between the 250 PtFn and 250 ZnPt Fn corresponds to a rate increase of 39% per platinum.

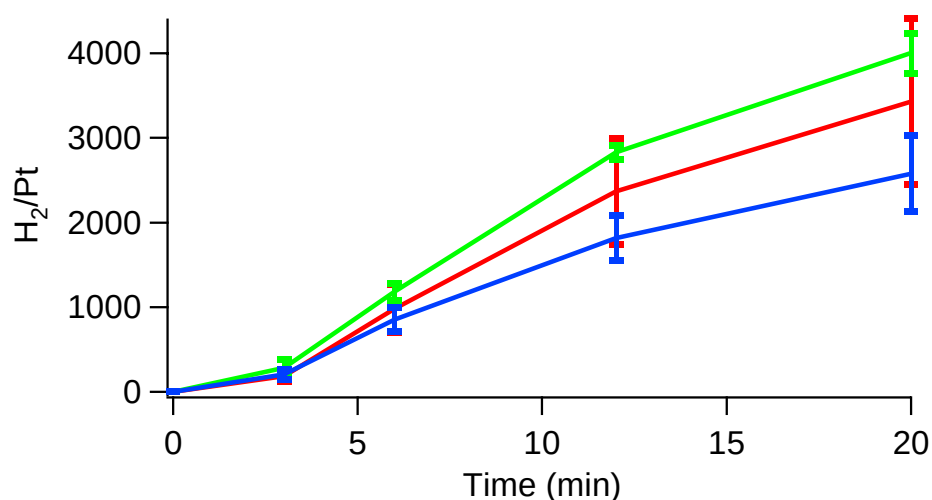


Figure 3.21.  $\text{H}_2$  production curves from 250 NiPt Fn, 250 ZnPt Fn, and 250 PtFn in standard conditions, ■ 250 NiPt Fn; ■ 250 ZnPt Fn; ■ 250 PtFn.

Table 3.3 displays the maximum  $\text{H}_2$  production rates of the different syntheses in Fn and Hsp based on the surface area of the particles. The surface area was calculated by using the average particle diameter determined by TEM. The total surface area in the assay was calculated by assuming that each particle has the same structure as  $\text{Pt}^0$ . The number of platinum per particle was then calculated using the unit cell of  $\text{Pt}^0$  and the total number of particles was determined from the total metal concentration in the reaction (total metal concentration was used instead of platinum concentration).

| Synthesis       | Rate ( $\text{H}_2 \cdot (\text{\AA}^2)^{-1} \text{min}^{-1}$ ) |
|-----------------|-----------------------------------------------------------------|
| 250 Pt WtHsp    | 20                                                              |
| 1000 Pt WtHsp   | 66                                                              |
| 250 PtFn First  | 87                                                              |
| 1000 PtFn First | 187                                                             |
| 250 Pt          | 45                                                              |
| 250 NiPt        | 101                                                             |
| 250 ZnPt        | 73                                                              |
| 500 Pt          | 55                                                              |
| 500 NiPt        | 55                                                              |
| 500 ZnPt        | 55                                                              |
| 1000 ZnPt       | 71                                                              |

Table 3.3. Maximum rates of  $\text{H}_2$  production for the different synthesis of platinum and platinum alloys in Hsp and Fn based on the total surface area of the nanoparticles in  $\text{H}_2 \cdot (\text{\AA}^2)^{-1} \text{min}^{-1}$ .

#### $\text{H}_2$ Production Assay for ZnPd Fn and PdFn

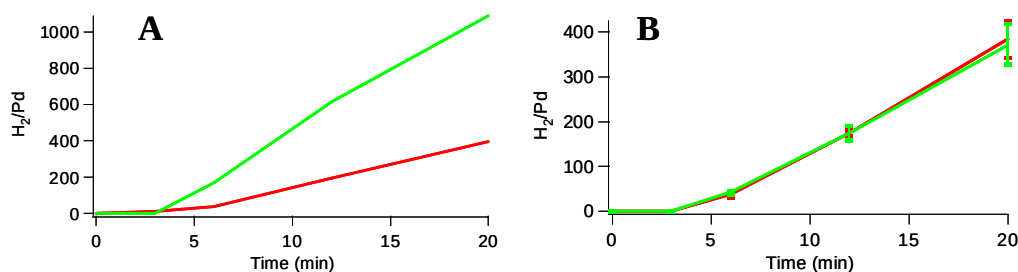


Figure 3.22.  $\text{H}_2$  production curves from PdFn and ZnPd Fn. A.  $\text{H}_2$  production curves from 500 PdFn and 250 PdFn in standard conditions, ■ 500 PdFn; ■ 250 PdFn; B.  $\text{H}_2$  production curves from 500 ZnPd Fn and 250 ZnPd Fn in standard conditions, ■ 500 ZnPd Pt Fn; ■ 250 ZnPd Fn.

Figure 3.22A shows the hydrogen production curves for 500 PdFn and 250 PdFn (only one assay run for 500 PdFn and 250 PdFn). Which can be compared to 500 ZnPd Fn and 250 ZnPd Fn  $\text{H}_2$  production curves shown in figure 3.22B. A comparison of the graphs shows that there is minimal benefit to doping Zn into Pd nanoparticles even

though there is considerable doping (16.2 Pd per Zn in 250 ZnPd Fn and 4.6 Pd per Zn in 500 ZnPd Fn). The  $H_2$  production rate of 500 PdFn compared to 500 PtFn is 3.4 times slower.

### $H_2$ Production From Ferritin Encapsulated Nanoparticles Using Electrochemistry

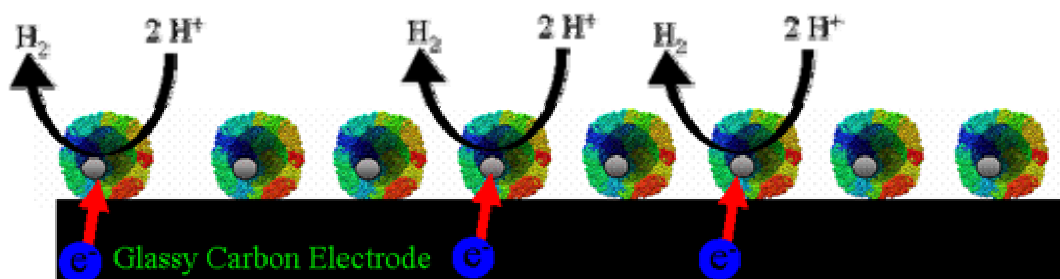


Figure 3.23. Diagram of the  $H_2$  production of noble metal ferritin catalysts absorbed on the surface of a glassy carbon electrode.

In order to further characterize the catalytic activity of the by Pt and Pd nanoparticles on the interior of Fn, cyclic voltammetry (CV) was used. The synthesized

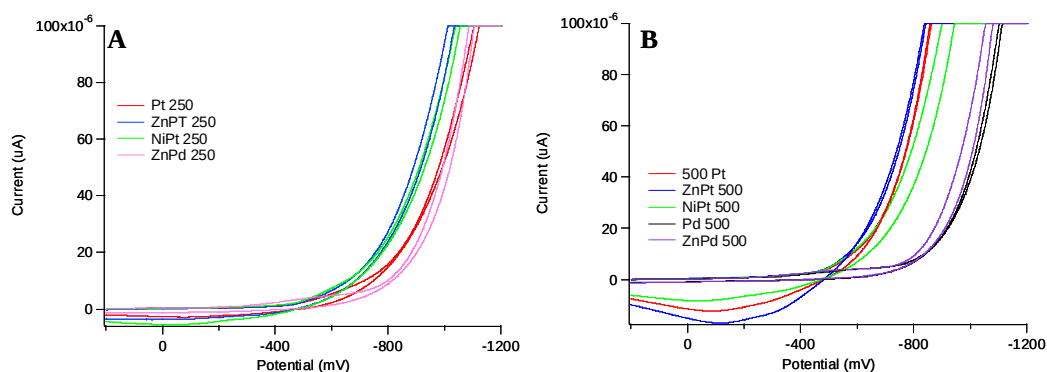


Figure 3.24. Cyclic voltammogram characterization of Pt and Pt alloy nanoparticles. A. Cyclic voltammogram of 250 PtFn, 250 ZnPt Fn, 250 NiPt Fn, and 250 ZnPd Fn adsorbed onto a glassy carbon electrode; B. Cyclic voltammogram of 500 PtFn, 500 ZnPt Fn, 500 NiPt Fn, 500 Pd, and 500 ZnPd Fn adsorbed onto a glassy carbon electrode.

catalyst encased in Fn was absorbed on to the surface of the of a polished glassy electrode as shown in figure 3.23.<sup>172-175</sup> CV was run in 2 M Acetate pH 4.0 on each sample.

Figure 3.24A shows the CVs of 250 PtFn, 250 ZnPt Fn, 250 NiPt Fn, and 250 ZnPd Fn and figure 3.24B shows the CVs of 500 PtFn, 500 ZnPt Fn, 500 NiPt Fn, 500 Pd, and 500 ZnPd Fn. Upon cycling the CV a number of times ( $\sim 10$ ), bubbles can be seen on the surface of the electrode. Using GC analysis, it was determined that  $H_2$  gas was evolved. Figure 3.25 is the CV of a platinum wire run under the same conditions. In these initial studies, little quantitative data can be extracted; however, there is a clear catalytic wave. These initial results show that CV could be used as a powerful tool to further analyze the catalysis of these nanoparticles.

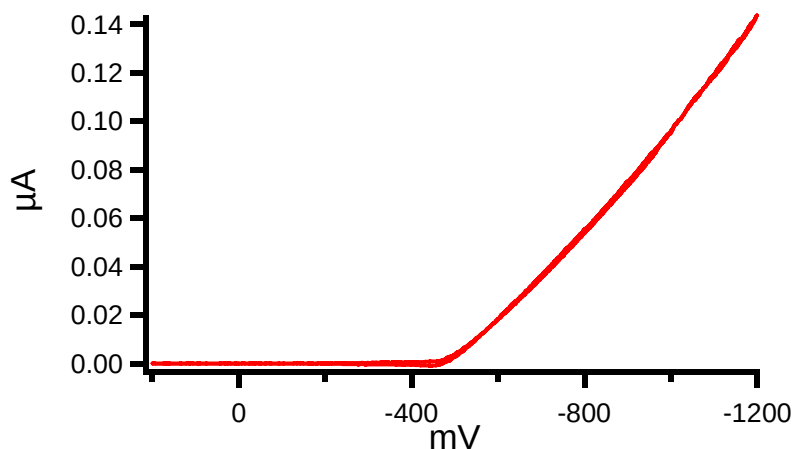


Figure 3.25. Cyclic voltammogram of a platinum wire.

## Discussion

### The $H_2$ Production Assay

$H_2$  production assays used to probe the catalytic activity of a  $H_2$  production catalyst or enzyme can have many variations. The general role of the assay is to provide protons and electrons to the catalysts in a way that they can be efficiently utilized. In this study, electrons are generated photocatalytically and transferred to the protein

encapsulated nanoparticle through an electron relay. Presently, optimization of the photo-induced electron generation portion of the  $H_2$  assay was not a major goal of this

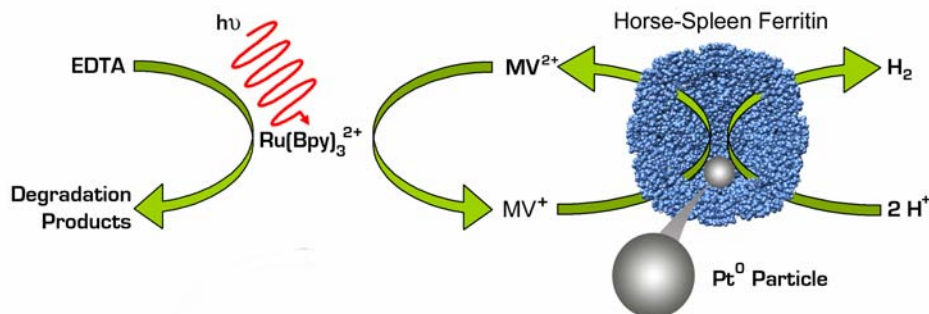


Figure 3.26. Schematic of the light mediated  $H_2$  production from Pt-Fn. Methyl viologen ( $MV^{2+}$ ) is used as an electron transfer mediator between the  $Ru(bpy)_3^{2+}$ , photocatalyst, and the Pt-Fn responsible for  $H_2$  production.

research. However, there are number of other light harvesting molecules that have been used as photocatalysts for the generation electrons including  $Ir^{176, 177}$  and porphyrin based systems.<sup>178, 179</sup> Nanoparticles have also received much attention recently as potential light harvesting agents.<sup>180-182</sup> By probing our catalysts with a wide variety of electron sources, the versatility of the protein encapsulated catalysts and the utility of the cage was displayed. A number of other electrons sources, sacrificial electron donors, photocatalysts, and chemical reductants, were tested to determine the range of conditions that could be used for the  $H_2$  production assay.  $Ru(bpy)_3^{2+}$  based system (Scheme 3.26) was selected as the assay to probe the  $H_2$  production of protein encapsulated Pt nanoparticles. A Jones reductor was also an effective electron donor (Figure 3.4); however, it proved to be too inconsistent to assay the  $H_2$  production. The high light conditions were chosen to more accurately determine the maximum rate of the synthesized nanoparticles (Figure 3.2). Cyclic voltammetry (CV) is a promising way to

more broadly characterize the catalytic properties of the nanoparticles. Using CV, studies on the effective lifetime of the catalysts could be conducted to characterize the durability of the protein encapsulated catalysts. The protein encapsulated nanoparticles' catalytic properties for the oxidation of simple organics could be characterized to determine the catalytic versatility of the nanocatalysts.

### H<sub>2</sub> Production From Pt WtHsp

WtHsp was the first system developed because of the porous nature of the protein cage was hypothesized to allow maximum access to the catalytic particle. Access to the interior of the protein cage was considered important to facilitate efficient catalysis by the encapsulated nanoparticles. After the synthesis of platinum nanoparticles using a number of variations of Hsp, WtHsp was chosen as the particle to study the H<sub>2</sub> production of because the WtHsp synthesis was the most specific for the interior for the Hsp proteins.

The 250 and 1000 Pt WtHsp mineralizations of Pt<sup>0</sup> were assayed using the standard Ru(bpy)<sub>3</sub><sup>2+</sup> H<sub>2</sub> production conditions. Figure 3.5 shows the 250 and 1000 Pt WtHsp H<sub>2</sub> production curves graphed as production per protein cage versus time. When calculated on a per cage basis, the initial rate of H<sub>2</sub> formation was  $4.47 \times 10^3 \text{ H}_2 \cdot \text{sec}^{-1}$  ( $\pm 394 \text{ H}_2 \cdot \text{sec}^{-1}$ ) for a loading factor of 1000 Pt per WtHsp and  $7.63 \times 10^2 \text{ H}_2 \cdot \text{sec}^{-1}$  ( $\pm 405 \text{ H}_2 \cdot \text{sec}^{-1}$ ) for a loading factor of 250. The hydrogen production numbers from the 1000 Pt loading per cage are expected to be more active than the 250 Pt per cage when compared on the per cage basis because there is more Pt added to each cage. H<sub>2</sub>ase maximum rate is 6000- 9000 H<sub>2</sub>/sec per protein. On a per protein basis, the maximum rate for 1000 Pt WtHsp is on the same order of magnitude as H<sub>2</sub>ase and ten fold less for 250 Pt WtHsp.

1000 Pt WtHsp is more catalytically active per platinum than the 250 Pt WtHsp. Figure 3.6 and 3.7 are the 250 and 1000 Pt WtHsp (respectively) H<sub>2</sub> production curves graphed as the H<sub>2</sub> molecule produced per Pt molecule. The maximum rate for the 1000 Pt WtHsp is 268 H<sub>2</sub>•Pt<sup>-1</sup>min<sup>-1</sup>. The maximum rate for the 250 Pt WtHsp is 183 H<sub>2</sub>•Pt<sup>-1</sup>min<sup>-1</sup>. Another important fact is that 150 Pt WtHsp has no detectable H<sub>2</sub> production. The TEM of this reaction shows that there are no detectable Pt particles. Using the structure of Pt<sup>0</sup> to calculate the diameter of a Pt particle with 150 atoms, the particle size is 1.8 nm, which is clearly visible on the TEM. From this calculation, it was determined that smaller particles with fewer than 150 Pt per particle were being synthesized. A report by Greenbaum<sup>183</sup> stated that there is a minimum threshold for the production of H<sub>2</sub> from platinum nanoparticles of 50 atoms of Pt. This, in addition to the TEM, and the H<sub>2</sub> production results, lead to the conclusion that the Pt<sup>0</sup> nanoparticles synthesized in the mineralization of 150 Pt WtHsp was below the 50 atoms of Pt threshold for the H<sub>2</sub> production.

Catalytic activity of a particle is dependant on the surface area. An increase in the surface area increases the potential catalytic activity. Surface area to volume ratio is described by equation 3.1. As the diameter of the particle decreases, the surface

Equation 3.1.

$$\frac{4\pi r^2}{\frac{4}{3}\pi r^3} = \frac{6}{(\text{diameter})}$$

area increases thereby increasing the catalytic surface. In the case of the 250 and 1000 Pt WtHsp, the 250 Pt WtHsp has less surface area per particle but more total surface area than the 1000 Pt WtHsp using the same amount of platinum. However, the 250 Pt WtHsp is not more catalytically active than the 1000 Pt WtHsp. This can be ascribed to 250 Pt

WtHsp having more particles that are below the 50 atoms per particle threshold than the 1000 Pt WtHsp, which lowers the  $H_2$  per Pt activity by having more inactive clusters.

Figure 3.8 shows the  $H_2$  production curves for the protein free Pt control, which is the 1000 Pt in WtHsp reaction run without protein. This synthesis forms large particles and aggregates. The maximum rate from the control reactions is  $15.7 H_2 \cdot Pt^{-1} min^{-1}$ . The synthesis of the platinum inside the WtHsp has increased the catalytic activity of the  $Pt^0$  by at least an order of magnitude. Table 3.4<sup>110, 151, 184</sup> shows the catalytic  $H_2$  production rates using other passivating layers in the synthesis of  $Pt^0$  nanoparticles.

| Synthesis | $H_2/min/Pt$ |
|-----------|--------------|
| Brugger   | 20           |
| Keller    | 16           |
| Song      | 6.5          |

Table 3.4.  $H_2$  production rates of other synthesis of  $Pt^0$  nanoparticles.

#### $H_2$ Production From PtFn

After the synthesis and characterization of Pt WtHsp, PtFn was synthesized under the same synthetic conditions to determine the role of the protein cage in the catalytic  $H_2$  production. Ferritin was hypothesized to be less active based on the more closed structure of the protein cage which could limit the access of the catalytic reagents to the nanoparticles encapsulated within ferritin. The maximum rates of  $H_2$  formation, when calculated on a per cage basis, were  $14.9 \times 10^3 H_2 \cdot Fn^{-1} min^{-1}$  ( $\pm 2.77 \times 10^3 H_2 \cdot Fn^{-1} min^{-1}$ ) for a loading factor of 1000 Pt per ferritin and  $1.82 \times 10^3 H_2 \cdot Fn^{-1} min^{-1}$  ( $\pm 8.47 \times 10^2 H_2 \cdot Fn^{-1} min^{-1}$ ) for a loading factor of 250 (Figure 3.9). The rate per cage for the loading of 1000 PtFn is 7 times higher than the rate of 250 PtFn while the loading factor is only a

4 fold increase. As discussed above, the different activity between the loadings is from the formation of fewer particles below the inactivity threshold at higher theoretical loadings. Figure 3.10 and 11 shows the  $H_2$  production curves for the 250 and 1000 Pt Fn (respectively) displayed as  $H_2 \cdot Pt^{-1}$ . The maximum rate for the 1000 PtFn is  $893 H_2 \cdot Pt^{-1} min^{-1}$ . The maximum rate for the 250 Pt Fn is  $437 H_2 \cdot Pt^{-1} min^{-1}$ .

These rates are a significant increase compared to the same synthesis in WtHsp. While 1000 PtFn has an increased surface area per platinum compared to the area of 1000 Pt WtHsp, the surface area increase can only account for a small part of the increased catalytic rate. With the 250 PtFn compared to the 250 Pt WtHsp, the 250 Pt WtHsp should have a faster catalytic rate if surface area is the only factor; however, the 250 PtFn has 2x the  $H_2$  production rate per platinum. Table 3.3 shows the catalytic rate of the 250 and 1000 Pt WtHsp and 250 and 1000 PtFn (labeled First) calculated in  $H_2 \cdot (\text{\AA}^2)^{-1} min^{-1}$ . The table shows the maximum rates of hydrogen production per  $\text{\AA}^2$  for the different syntheses. The 250 PtFn is 4 times more active per  $\text{\AA}^2$  than 250 Pt WtHsp. The 1000 PtFn is 2.8 times more active per  $\text{\AA}^2$  than 1000 Pt WtHsp. Assuming all of the atoms of platinum are in active particles, the PtFn is more active when the reactions are compared per surface area. The mineralization of platinum nanoparticles inside of Fn compared to WtHsp is more efficient at synthesizing particles that are above the 50 atom activity threshold. The porous structure of Hsp was not as large of a factor in the catalytic rate as hypothesized.

#### Screen of Pt/metal Alloys in Fn

The results of the synthesis of  $Pt^0$  in Fn and the catalytic activity of the synthesized particles determined Fn was the protein cage of choice for continued studies.

Synthesis of Pt alloys was attempted to determine if the catalytic activity of the nanoparticles could be increased on a  $\text{H}_2\cdot\text{Pt}^{-1}$  basis. Using Fn as the platform, platinum alloys of Zn, Cu, Ru, Co, Ni, and Fe in a 3:1 ratio of platinum to dopant were synthesized under identical conditions. Figure 3.12 depicts the initial  $\text{H}_2$  production curves used to determine which dopants had the largest impact on the catalytic activity. The  $\text{H}_2$  production curves of the dopants were directly compared to the platinum only control. From this assay, it was determined that Zn-Pt would be investigated further. Because of current interest and success of Ni-Pt alloys it was also investigated further in spite of our initial  $\text{H}_2$  production assay not showing an increase in catalytic rate.

#### $\text{H}_2$ Production From Pt Alloys in Fn

| Synthesis | Ratio (Pt/Metal) |
|-----------|------------------|
| 250 ZnPt  | 11.5             |
| 500 ZnPt  | 3.7              |
| 1000 ZnPt | 33.1             |
| 250 NiPt  | 19.3             |
| 500 NiPt  | 297.4            |
| 500 Pt+Zn | No Zn            |

Table 3.5. Displays the number of Pt atoms per dopant metal in the synthesis after the reaction products have been cleaned up. Determined by ICP-MS.

After the synthesis and clean up of the Pt alloys, ICP-MS was conducted to determine the amount of dopant associated with the protein cage. Table 3.5 shows the ratios of Pt per dopant for the different syntheses. The ICP-MS reveals that the different syntheses have different ratios of dopings. Figures 3.13-19 are the  $\text{H}_2$  production curves of 1000 ZnPt Fn, 500 ZnPt Fn, 500 NiPt Fn, 500 PtFn, 500 Pt+Zn, 250 ZnPt Fn, 250 PtFn, and 250 NiPt Fn, respectively. The 250 and 500 PtFn were resynthesized to use as

a comparison under the same conditions as the 250 and 1000 PtFn (labeled in Table 3.2 and 3.3 as First) detailed earlier, except that the stock protein used was 10 mg/mL Fn in 150 mM NaCl. Under the new synthetic conditions, the maximum rate for H<sub>2</sub> production was 216 H<sub>2</sub>•Pt<sup>-1</sup>min<sup>-1</sup> for 250 PtFn and 255 H<sub>2</sub>•Pt<sup>-1</sup>min<sup>-1</sup> for the 500 PtFn (Table 3.2). The H<sub>2</sub> production rates are slightly lower than the rates for the earlier synthesis, which could results from variations in the synthesis and H<sub>2</sub> production assay.

The comparison of the effects of adding dopants to the Pt<sup>0</sup> synthesis is quite interesting. Figure 3.21 is the H<sub>2</sub> production graphs for 500 ZnPt Fn, 500 Pt+Zn Fn, and 500 PtFn. The ZnPt Fn shows an increase of 14% in the H<sub>2</sub> production over 500 PtFn with 3.7 Pt per Zn. The 500 NiPt Fn had one Ni per 297.4 Pt, and had almost identical H<sub>2</sub> production compared to 500 PtFn. Figure 3.22 is a graph of the 250 NiPt Fn, 250 ZnPt Fn, and 250 Pt Fn H<sub>2</sub> production curves. The addition of one Ni per 19.3 Pt in the 250 NiPt Fn reaction increases the rate of H<sub>2</sub> production by 23% over 250 PtFn. The addition one Zn per 11.5 Pt in the 250 ZnPt Fn reaction increases the H<sub>2</sub> production by 39% over 250 PtFn.

To factor in the particle size of each synthesis, the H<sub>2</sub> production rate was calculated by H<sub>2</sub>•(Å<sup>2</sup>)<sup>-1</sup>min<sup>-1</sup> (Table 3.3). The assumption in this calculation is that each metal is the same and incorporated into the nanoparticle. Using this calculation, the 500 NiPt, 500 Pt, and 500 ZnPt Fn have virtually identical production rates (Table 3.3). The 250 NiPt Fn is the most catalytically active of the 250 loadings followed by 250 ZnPt Fn which is opposite of the per Pt calculation. Both 250 ZnPt and 250 NiPt Fn are significantly more active than 250 PtFn.

The dopant metal could be playing any number of roles in the mineralization. The dopant could be incorporating in the crystal which would affect the catalytic activity. Toda and coworkers showed that maximum catalytic activity seen by doping Ni, Co, and Fe into Pt occurred at 30, 40, and 50%, respectively, showing the effect of doping on catalytic rates and that doping is element depend.<sup>152</sup> The addition of  $\text{Zn}^{2+}$  or  $\text{Ni}^{2+}$  could also change the nucleation of the particle and cause the formation of a different crystal face to be formed much like in the insertion of phage display peptide to force the mineralization of a specific crystal. The dopant could also be acting as guiding agent which does not get incorporated into the crystal but nonetheless influences the nanoparticle formation.<sup>136</sup> In 500 NiPt Fn, the  $\text{Ni}^{2+}$  is probably not acting as a guiding agent because there is 297.4 Pt per Ni as would be the case if  $\text{Ni}^{2+}$  was acting as a guiding agent; however, there is no change in the catalytic activity that would indict an influence on the mineralization. The 250 NiPt Fn has 19.3 Pt per Ni and an increased catalytic activity which would indicate the Ni is either being incorporated or is effecting the nucleation. Either way it is unlikely that  $\text{Ni}^{2+}$  is acting as a guiding agent in the synthesis of the Ni-Pt nanoparticles. With Pt per Zn ratios of 33.1 to 3.7, the catalytic increase from the Zn dopant could be either from the incorporation into the particle or the modification of the nucleation.

In the initial screen for the catalytic  $\text{H}_2$  production from ZnPt Fn, the rate was increased by more than 25% per Pt (Figure 3.12). The final synthesis of the 500 ZnPt in Fn showed only a 14% increase in the catalytic rate. This difference in the catalytic increase could be from the synthetic conditions of the initial synthesis versus the final synthesis. The initial mineralization was at 65°C and the final mineralization was at

15°C. Annealing of minerals to induce changes in crystal phases is a widely used practice inorganic synthesis.

#### H<sub>2</sub> Production From Pd and Pd Doped Nanoparticles in Fn

Fn has been mineralized with Pd by Ueno.<sup>49</sup> However, they did not look at the H<sub>2</sub> production activity. Originally Pd was used a dopant into Pt nanoparticles but the PdPt alloys showed no increase in catalytic activity in initial assays. Pd particles themselves were pursued to determine their catalytic H<sub>2</sub> production rates and if the Zn doping could be used to increase the catalytic efficiency of non-Pt based particles. Figure 3.23A is the H<sub>2</sub> production curves from 250 Pd and 500 Pd Fn which have maximum rate of 27 and 75 H<sub>2</sub>•Pd<sup>-1</sup>min<sup>-1</sup>, respectively. Figure 3.23A is the H<sub>2</sub> production curves from 250 ZnPd and 500 ZnPd Fn which have maximum rate of 26 and 81 H<sub>2</sub>•Pd<sup>-1</sup>min<sup>-1</sup>, respectively. The Zn doping in 250 Pd has no effect while the 500 ZnPd Fn shows a slight, 8%, increase in rate compared to 500 PdFn. The Zn doping does not have the rate increasing effect that was seen in the Zn doping into Pt in Fn.

#### Characterization of Noble Metal and Noble Metal Alloys Encapsulated in Fn by CV

Using CV to characterize the catalytic activity of Fn encapsulated noble metal and noble metal alloys nanoparticles has tremendous potential to increase our understanding of the catalytic properties the nanoparticles. The Rubpy<sub>3</sub><sup>2+</sup> based H<sub>2</sub> production assay has a limited window of characterization because of the degradation of Rubpy<sub>3</sub><sup>2+</sup>, MV<sup>2+</sup>, and the EDTA during the assay. This degradation not only stops the production of the electrons used for the reduction of protons; but, also poisons the catalysts. In order to

characterize the long-term durability of the ferritin encapsulated catalysts, an alternative source of electrons needs to be used. Use of CV as the source of electrons is an ideal way to probe the long-term durability of the catalysts because the degradation products are eliminated. The CV characterization also allows for other catalytic activities, methanol and formate oxidation, to also be probed.<sup>134, 147, 155, 185</sup> The ability to probe the durability will give another dimension to the different syntheses inside ferritin. The dopants could increase or decrease the long-term durability of the catalysts.

We are only beginning to probe these particles with CV and have some fundamental considerations that need to be overcome before the analytical power of CV can be fully utilized. Currently, we are unable to conduct long-term durability studies of the ferritin encased catalysts because the catalytic signal decreases and goes to zero after sweeping the field a small number of times (~10). We are unable to determine if the decreased signal is from the poisoning of the catalyst or if it is due to the protein desorption from the surface of the electrode. However, we have been able to show the catalytic production of H<sub>2</sub> by all of the noble metal and noble metal alloys synthesized in ferritin. When compared to the CV of the bare Pt wire, the potential at which the catalytic wave begins is similar indicating that the protein cage is not a substantial barrier to the electron transfer from the electrode to the enclosed nanoparticle.

### Conclusion

Protein cages are excellent platforms for the synthesis of a wide range of catalytically active nanoparticles for the production H<sub>2</sub>. The refinement of the catalytic activity of the nanoparticles has yielded promising results and tremendous potential for

further refinement. The protein cage environment provides a unique platform for the nanoparticle catalysis that allows minimal coating of the nanoparticles. The use of CV for probing the catalytic activity of the ferritin encapsulated nanoparticles has the potential to expand the uses and our understanding of these catalysts.

### Introduction

Recently, there has been considerable interest in developing tools to kill specific cells, such as cancer cells, inside the body.<sup>186-190</sup> This interest arises from the desire to make treatments more effective and minimize drug side effects by having the drug administered only to the cells of interest and not throughout the body. Targeted drug delivery is the directed administration of drugs or other molecules to a specific cell type in the body.<sup>188, 191</sup>

One aspect of targeted drug delivery can be viewed as a mimic of cell specific infection by viruses. Viruses are a perfect example of the targeting that is desired for the cell specific drug therapy. Viruses' targeting is encoded into high specialized host specific proteins on their outer surfaces. Built in targeting enables them to infect only the cells in which they can enter and replicate.

Many targeting agents, antibodies<sup>192-194</sup>, small peptides<sup>98, 188</sup>, and small molecules<sup>195</sup>, have been developed to direct the delivery of drugs to specific cells mimicking the action of viruses. Use of these targeting agents has clearly been shown to specifically deliver imaging agents to the cells of interest with little non-specific targeting.<sup>194</sup> They have also been used to kill cells when coupled with a drug, revealing potential of targeted drug delivery.<sup>196</sup>

Use of targeting molecules to deliver drugs or imaging agents can be limited by the amount of "cargo", drug or imaging agent, that can be transported by the targeting agent.<sup>197</sup> Overloading antibodies, targeting peptides, and small molecule targeting agents

with drugs or imaging agents can cause a decrease in solubility and interfere with targeting. With few drug molecules per targeting agent, cell death only occurs when there are multiple delivery events per cell.

Drug delivery vehicles have been developed to increase the payload of the targeting molecules or protect the organism from drugs with high toxicity.<sup>198</sup> While the platform, liposomes, dendrimers, proteins, etc., used for drug delivery varies, drug delivery vehicles mimic the viral protection of RNA or DNA on the interior of a protein based architecture that stores and protects the encased nucleic acid. Once the virus enters the cell, the payload of nucleic acid is released into the cell and is able to do its work. An ideal drug delivery vehicle would be able to protect and carry a large payload of drug, allow for that easy attachment of targeting molecules in a controlled manner, allow a large amount of drug to be delivered per binding event, and the drug would be activated once delivered to the cell.<sup>199</sup> The encapsulating of the drug by a delivery vehicle also provides another level of protection from side effects by isolating the drug from the environment while being delivered.

There have been a wide range of polymer based drug delivery vehicles<sup>200-202</sup>. Liposome based drug delivery vehicles are the most common and well researched vehicles.<sup>186, 198, 200</sup> Using a protein-liposome based targeting vehicles, Rezler et. al. displayed the potential for liposome based drug delivery vehicles by targeting and killing cancer cells.<sup>203</sup> Mundargi used polysaccharide particles to encapsulate and target drugs to cells.<sup>204</sup> Montet has coated iron oxide nanoparticles with dextran and dextran functionalized with targeting molecules to of 30 nm for the targeted delivery of an MRI contrast agent.<sup>191</sup> These are just a few examples of the types of approaches the have been

used for the synthesis of a targeted drug delivery vehicle. One of the biggest disadvantages of these approaches is the lack of synthetic control. The size of the delivery vehicle and number of drug and targeting molecules is highly variable between particles.<sup>205</sup>

Use of spherical self-assembled protein architectures as drug delivery vehicles has potential to be an excellent platform for targeted drug delivery because of their innate attributes.<sup>77, 97, 98</sup> Protein cage architectures provide a platform for the controlled functionalization with targeting agents and drugs. The crystal structures of many protein cage architectures have been solved allowing for rational design through the site specific mutagenesis. The protein cages can be manipulated using mutagenesis to site specifically add an attachment point for the functionalization of the cage with targeting or drug molecules, or for the genetic insertion of protein based targeting molecules.<sup>36, 53, 81, 92, 206, 207</sup>

One promising class of drugs used for the treatment of diseases is photodynamic therapy agents (PTA).<sup>208-210</sup> PTAs are light activated molecular catalysts used in the treatment of many diseases such as skin cancer.<sup>209, 211</sup> The benefit of using a catalyst in disease treatment is that the amount of “drug” that needs to enter a cell is less because of the catalytic production of the “killing” agent. By delivering a catalytically produced “kill” effect in the cell, each PTA repeatedly produces the desired kill effect; where as, a standard drug can only have one desired effect per drug delivered. Use of a catalytic drug mimics the infection of a virus. When a virus enters a cell, its nucleic acid is used as a template to produce more viruses. From one delivery event, multiple viruses are produced, in the same way that the delivery of one catalytic drug produces multiple kill

events. When activated by light, PTAs produce reactive oxygen species (ROS) which oxidize cellular components disrupting their function, which leads to cell death. Many current uses of PTA are non-targeted treatments that use the selectivity of light exposure to limit treatment area.

PTAs can be attached to targeting molecules that will deliver and incorporate into the specific cells.<sup>212, 213</sup> Not only is the PTA targeted to specific cells but the area of ROS production is limited to where the illumination occurs giving an increased specificity to the PTA. Much lower doses of PTA can be administered for treatment because the targeted PTA that is given to the patient goes only to the desired cells and is not distributed throughout the body, which limits the side effects from untargeted PTA upon exposure to sunlight.

By using protein cages to combine the benefits of targeted drug delivery and PTAs, a virus mimic could be synthesized. This mimic has many of the characteristics that make viruses effective at targeting and invading cells. The protein cage allows for spatially controlled genetic and synthetic modification for the functionalization with targeting moieties, covalent and genetic, and covalent attachment of PTA.

For this study, we have chosen Hsp as the delivery vehicle because it is amendable to mutations and is stable under broad synthetic conditions. It has been shown to be an effect platform for the targeted delivery of drugs to specific cell through both attachment of antibodies and genetic insertion of a targeting peptide.<sup>97, 98</sup> In this study, the effects of the protein cage on ROS production when the PTA is attached to the protein cage and the effects of ROS on the protein cage will be characterized.

## Materials and Methods

### Materials

All materials were obtained from Sigma-Aldrich and used as received with no further purification. All water used was purified through a Nanopure system to 18.2 M $\Omega$  resistivity.

### Synthesis of 5-Iodoacetoamino-1,10-phenanthroline (Iphen)

Iphen was synthesized by modification of previously reported procedure.<sup>119, 214</sup> A solution of 1,3-dicyclohexylcarbodiimide (5.29 g, 26 mmol) and iodoacetic acid (4.76 g, 26 mmol) in 50 mL dry ethyl acetate was stirred for 3 hours at room temperature. The resulting solution was filtered to remove the urea. The solution was dried by rotary evaporation and redissolved in 25 mL acetonitrile. The solution was added to 25 mL of acetonitrile containing 5-amino-1,10-phenanthroline (1.0 g, 0.005 mol) and stirred overnight at room temperature. The product was collected by centrifugation and washed with cold 5% sodium bicarbonate and water. The product was dried under vacuum and confirmed by mass spectroscopy. (Yield: 1.32 g).

### Synthesis of Ru(bpy<sub>2</sub>)Cl<sub>2</sub>

Ru(bpy<sub>2</sub>)Cl<sub>2</sub> was synthesized according to literature procedures.<sup>215</sup> RuCl<sub>3</sub>·3H<sub>2</sub>O (7.8 g, 29.8 mmol), bipyridine (9.36 g, 60.0 mmol), and LiCl (8.4 g, 2.0 mmol) were refluxed in dimethylformamide (50 mL) for 8 h. The reaction was cooled to room temperature, 250 mL of acetone was added and the solution was stored at 4 °C overnight. The resultant product was filtered and washed with water and ether and dried by suction.

### Synthesis of Ru(bpy<sub>2</sub>)Iphen<sup>2+</sup>

Ru(bpy<sub>2</sub>)Iphen<sup>2+</sup> was synthesized by modification of previously reported procedure.<sup>214</sup> Ru(bpy)<sub>2</sub>Cl<sub>2</sub> (0.7 g, 1.45 mmol) and Iphen (0.5 g, 1.38 mmol) were refluxed in 50 mL MeOH for 3 h with stirring. The solution was filtered. The product was precipitated by the addition of a concentrated aqueous solution of NH<sub>4</sub>PF<sub>6</sub> to a warm solution. The orange solid was collected by filtration and washed with cold water and ether and dried in a desiccator. (Yield: 1.18 g).

### Protein Functionalization

The protein solution to be labeled (small heat shock protein mutants Hsp G41C or Hsp S121C) was dialyzed into deoxygenated buffer (50 mM HEPES 100 mM NaCl pH 8.0) overnight. The protein was transferred to a jacketed reaction vessel at 40 °C under nitrogen. The sample was diluted to ~1mg/mL with deoxygenated buffer and a 5-fold excess of Ru(bpy<sub>2</sub>)Iphen<sup>2+</sup> dissolved in minimal DMF was slowly added. The solution was protected from light and allowed to react for 3 hours. After the reaction was completed the protein was concentrated and passed over size exclusion chromatography (Dulbecco's phosphate buffered solution (DPBS) pH 7.4) to separate unreacted dye and to the exchange buffer.

### Singlet Oxygen Production Assay

In a 3 mL clear glass serum vial (Wheaton), 20 μM ruthenium (either free or attached to protein cage) and 50 mM 2,2,6,6-tetramethyl-4-piperidone (TEMP) final concentration was added to DPBS pH 7.4. The reaction was illuminated by a Xe arc lamp (175 W, Lambda-LS, Sutter Instruments) with an water filter to remove the IR

radiation and an UV-absorbing glass (<360 nm) to remove the UV radiation. The reactions are maintained at 37 °C and vigorously stirred. At each time point, a 100 µL sample is removed and added to 10 µL of 1 M sodium azide to quench the reaction. For the oxygen-free reaction, the serum vials are sealed and degassed under nitrogen. The light was quantitated using an Exttech Instrument EasyView light meter to be 511,700 lx.

#### Electron Paramagnetic Reasonance (EPR)

EPR data was collected on a Bruker X-band EMX spectrometer. The instrumental conditions were as follows: microwave frequency, 9.84 GHz; modulation frequency, 100 kHz; modulation amplitude, 1G; time constant, 81.92 msec; sweep time, 81.92 msec; sweep width, 80 G; and center field, 3510 G. 100 uL of sample was loaded into a flat-cell microslide (0.3 x 6.0 mm I.D., VitroCom Inc.) for the analysis. The results were compared to standard curve using purchased 4-oxo-TEMPO (TEMPO) using signal amplitude difference in the highest field peak.

#### Transmission Electron Microscopy (TEM)

TEM data were obtained on a Leo 912 AB, with  $\Omega$  filter, operating at 100 keV. The samples were concentrated using microcon ultrafilters (Microcon YM-100) with 100 kDa nominal molecular weight cutoff and transferred to carbon coated copper grids. Samples were imaged negatively stained with 2% uranyl acetate.

#### Dynamic Light Scattering (DLS)

DLS measurements were carried out on a Brookhaven Instrument Corporation 90-PALS at 90 degrees using a 661 nm diode laser, and the correlation functions were fit using a non-negatively constrained least-squares analysis.

### UV-Vis Spectroscopy

UV-Vis spectroscopy measurements were carried out on an Agilent 8453 UV-Vis spectrometer.

### Size Exclusion Chromatography (SEC)

SEC was performed on a Biologic Duo-Flow fast protein liquid chromatography system equipped with a Quad-Tec UV-Vis detector and using a Superose 6 size exclusion chromatography column.

### Protein Expression and Purification

One liter cultures of *E. coli* (BL21(DE3) B strain) containing pET-30a(+) Hsp16.5 G41C or S121C plasmid were grown overnight in M9 salts + 10 g NaCl + 10 g Bactotryptone + kanamycin medium (37 °C, 220 rpm). Cells were harvested by centrifugation 3700×g for 20 minutes (Heraeus #3334 rotor, Sorvall Centrifuge) and re-suspended in 80 mLs of 50 mM MES, pH 6.5. Lysozyme, RNase A, and DNase I were added to final concentrations of 0.041 mg/mL, 0.055 mg/mL and 0.08 mg/L respectively and incubated for 30 minutes on ice. The sample was French pressed (American Instrument Co., Inc) and sonicated (Branson Sonifier 250, Power 4, Duty cycle 50 %, 3 × 5 minutes with 5 minute rest intervals). Bacterial cell debris was removed via centrifugation for 45 minutes at 12,000×g. The supernatant was heated for 10 minutes at 60 °C and centrifuged for 20 minutes at 12,000×g thereby removing many heat labile *E. coli* proteins. The remaining cell extract was purified by gel filtration chromatography (Superose-6, Amersham-Pharmacia; BioRad Duoflow). The subunit molecular weight was verified by SDS poly-acrylamide gel electrophoresis (SDS-PAGE) and mass

spectrometry (Waters MicroMass Q-TOF). The assembled protein was imaged by transmission electron microscopy (TEM) (LEO 912 AB) (stained with 2% uranyl acetate on formvar carbon coated grids), and analyzed by dynamic light scattering (DLS) (90 plus Brookhaven Instruments). Protein concentration was determined by absorbance at 280 nm divided by the published extinction coefficient ( $9322 \text{ M}^{-1} \text{ cm}^{-1}$ ).

## Results

### Key Results from a PTA Attached to Hsp

Listed below are the key results from the attachment of a PTA to the Hsp protein cage and the characterization of the catalytic activity.

Attachment of RuIphen to Hsp: RuIphen was specifically attached to the engineered mutants of Hsp specifically on the interior and exterior surface. Loading of the Hsp cage could be controlled by the conditions of labeling reaction.

$^1\text{O}_2$  interaction with the protein cage: The protein cage is extensively oxidized during the  $^1\text{O}_2$  production reaction. The extent of oxidation is dependant on the amount of illumination. Even though the oxidation of the protein cage is extensive, the protein cage remains assembled after illumination.

Characterization of  $^1\text{O}_2$  production:  $^1\text{O}_2$  is the ROS produced by RuIphen. This production is not significantly quenched by the attaching the RuIphen to Hsp. Attaching the PTA to the interior or exterior of the protein cage did not significantly effect the  $^1\text{O}_2$  production nor did lower the loading factor of the PTA. These results indicate that attachment of a PTA to protein cage does not significantly effect the ROS production.

Cell killing using a PTA attached to a targeted protein cage: In a proof of concept, RuIphen was attached to a protein cage that was targeted with an antibody to *S. aureus*. The PTA alone or the untargeted protein cage labeled with RuIphen did not show significant cell killing. Only when the PTA was attached to the targeted protein cage was there significant cell death.

#### Attachment of Ru(bpy<sub>2</sub>)Iphen<sup>2+</sup> to the Protein Cage

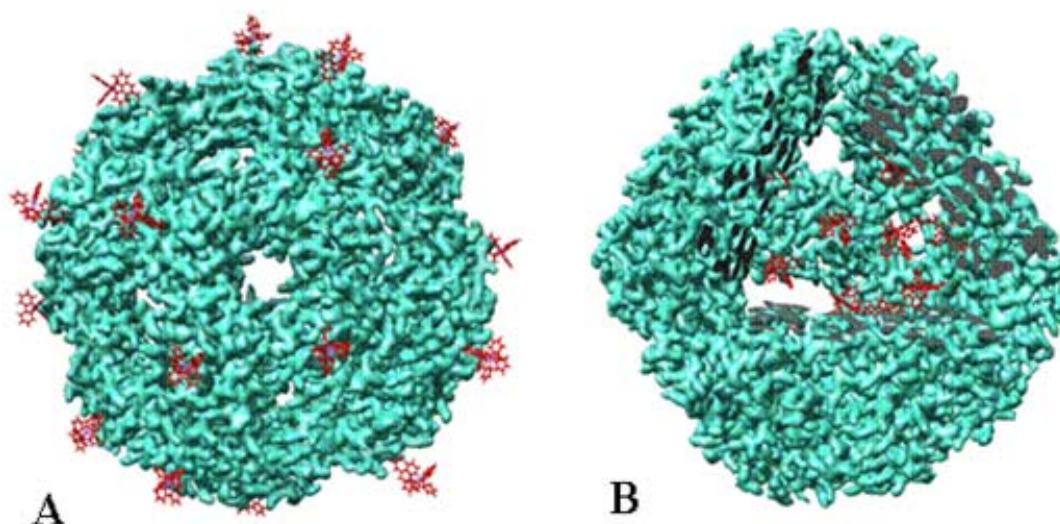


Figure 4.1. Chimera representation of the labeling of Hsp S121C and Hsp G41C with RuIphen. A. Chimera model representation of the small heat shock protein (Hsp) (pdb: 1shs) mutant S121C (Hsp S121C) labeled with RuIphen; B. Chimera model representation of cut-away view of Hsp showing the interior cavity of mutant G41C (Hsp G41C) labeled with RuIphen.

To probe the interaction of the PTA and the protein cage, we engineered two mutants of Hsp, Hsp G41C which has a cysteine inserted on the interior surface and Hsp S121C which has a cysteine on the exterior surface of the protein cage. Figure 4.1 A and B is a Chimera model representation of RuIphen attached to Hsp S121C and Hsp G41C, respectively.

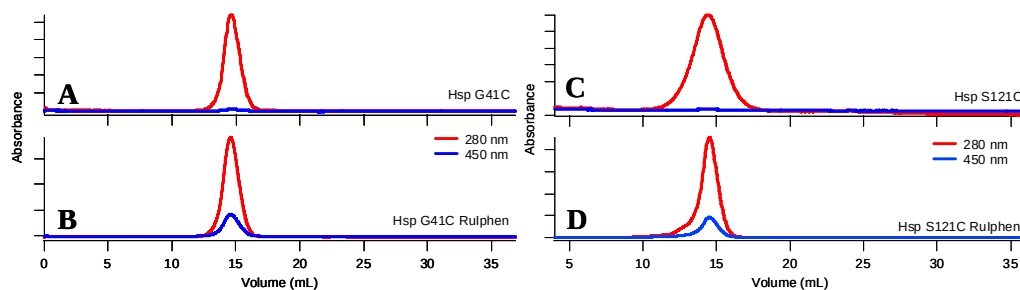


Figure 4.2. The size exclusion chromatography of Hsp G41C and S121C unlabeled and labeled with RuIphen. A. Unfunctionalized Hsp G41C; B. SEC of Hsp G41C functionalized with RuIphen showing coelution of protein (280 nm) and RuIphen (450 nm); C. SEC of unfunctionalized Hsp S121C; D. SEC of Hsp S121C functionalized with RuIphen showing coelution of protein (280 nm) and RuIphen (450 nm).

After mutagenesis, the Hsp mutants G41C and S121C were characterized by liquid chromatography/mass spectrometry (LC/MS) and SDS PAGE to confirm the mutation and size exclusion chromatography (SEC) and transmission electron microscopy (TEM) to confirm intact particles. Once the mutations were confirmed, Hsp G41C and Hsp S121C were functionalized with RuIphen. Briefly described, the functionalization reaction was done on 1-2 mg/mL Hsp in deoxygenated 50 mM Hepes pH 8.0 at 40 °C for two hours with 5 fold excess of ruthenium complex per subunit. The reaction was concentrated and purified using SEC to remove the unreacted RuIphen. SEC of the Hsp G41C and Hsp G41C functionalized with RuIphen (Hsp G41CRu) have the same retention volume showing no change in the exterior diameter of the assembled protein cage figure 4.2 A and B, respectively. The lack of absorption at 450 nm (RuIphen) by HSP G41C and the significant absorption at 450 nm by HSP G41CRu demonstrates the coelution of HSP G41C and the RuIphen. SEC of the Hsp S121C and the RuIphen functionalized Hsp S121C (Hsp S121CRu) have the same retention volume showing no perceivable change in the exterior diameter of the protein cage from attaching the PTA to the exterior figure 4.2 C and D, respectively. SEC of the Hsp S121CRu shows the coelution of intact protein (280 nm) and RuIphen (450 nm).

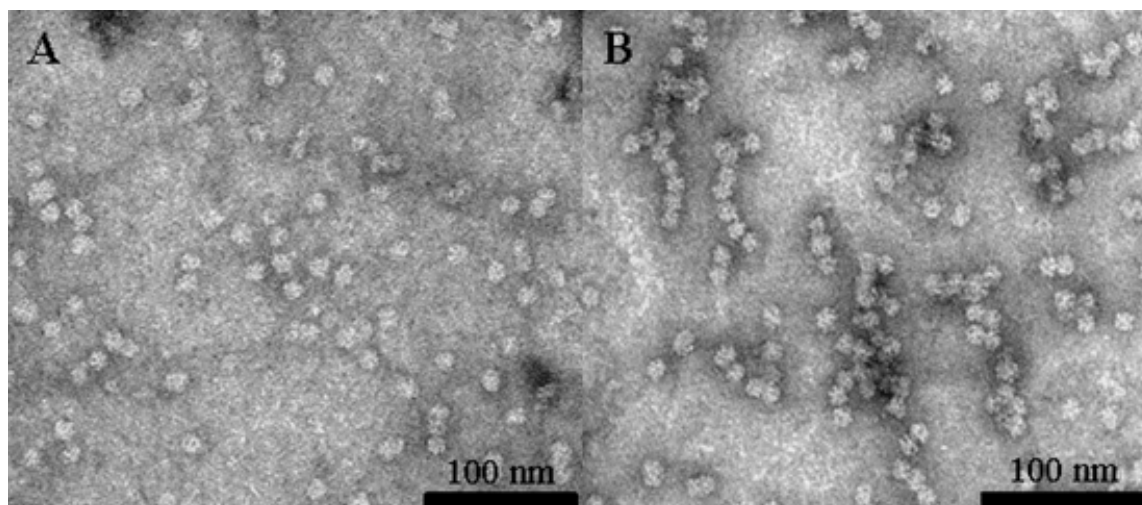


Figure 4.3. The TEM analysis of Hsp G41C and S121C labeled with RuIphen. A. TEM of Hsp G41C functionalized with RuIphen stained with 2% uranyl acetate; B. TEM of Hsp S121C functionalized with RuIphen stained with 2% uranyl acetate, Scale bar = 100 nm.

TEM analysis of Hsp G41CRu and Hsp S121CRu stained with 2% uranyl acetate displays ~12 nm voids where the electron dense stain was not present because of the protein cage on the EM grid confirming that the functionalization of both cages does not cause disassembly of the protein cage architecture figure 4.3 A and B, respectively.

#### Characterization of Effects of Illumination

SDS-PAGE analysis of the unlabeled and labeled Hsp G41C and Hsp S121C was conducted by analyzing the same gel using fluorescent imaging (RuIphen) and Coomassie staining (protein). While the labeled and unlabeled protein subunits migrate similarly, the fluorescent analysis of the gel clearly shows the fluorescence from the RuIphen associated with both HSP G41CRu and HSP S121CRu (Figure 4.4 A and B lane 3 and 6, respectively) and not associated with the unfunctionalized Hsp G41C and Hsp S121C (Figure 4.4 A and B lane 2 and 5, respectively). Included in the SDS-PAGE analysis is HSP G41CRu and HSP S121CRu after 1 hour of illumination (Figure 4.4 A

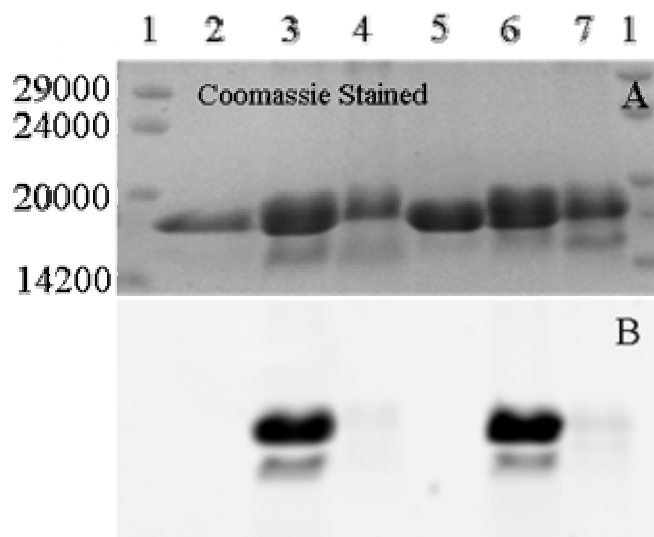


Figure 4.4. SDS-Page of Hsp G41C and Hsp S121C functionalized with RuIphen imaged using Coomassie stain A and fluorescence B on the same gel. Lanes: 1. Molecular weight standards (Da); 2. Hsp G41C; 3. Hsp G41C functionalized with RuIphen; 4. Hsp G41C functionalized with RuIphen after 1 hour illumination; 5. Hsp S121C; 6. Hsp S121C functionalized with RuIphen; 7. Hsp S121C functionalized with RuIphen after 1 hour illumination. Analysis shows that Hsp G41C (A, B: Lane 3) and Hsp S121C (A, B: Lane 6) are effectively labeled with RuIphen by the association of the fluorescence from RuIphen with the protein. The gel shows that Hsp G41C (A, B: Lane 4) and Hsp S121C (A, B: Lane 7) lose almost all fluorescence associated with the RuIphen after illumination. The lower bands in A: 2-7 and B: 3 and 6 are a degradation product, determined by mass spectrometry, of the Hsp protein that does not effect the labeling sites.

and B lane 4 and 7, respectively). The fluorescent analysis of the gel reveals minimal fluorescence associated with the conjugates after illumination compared to the labeled protein before illumination which is consistent with the UV/Vis analysis of the illumination of free RuIphen (Figure 4.5). The mass has been slightly increased by the illumination. SEC after 1 hour of illumination shows that the Hsp G41CRu and Hsp S121CRu (Figure 4.6 A and B, respectively) is still a 12 nm assemble meaning that any reaction that takes place during the illumination does not cause the cage to fall apart. The SEC shows a decrease in the absorbance a 450 nm from the RuIphen attached to the cage confirming the decrease in fluorescence seen in the SDS-PAGE analysis.

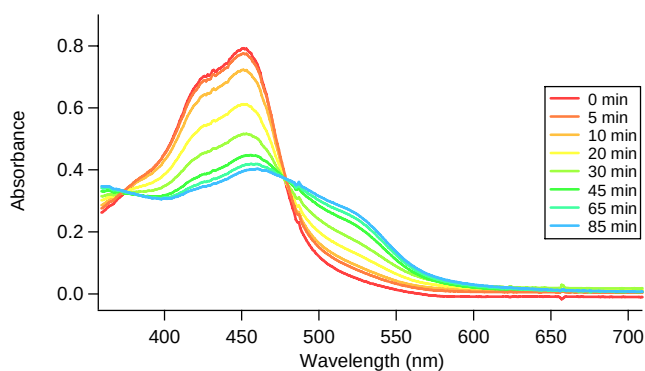


Figure 4.5. UV/Vis time course of RuIphen illumination showing the degradation of RuIphen during illumination.

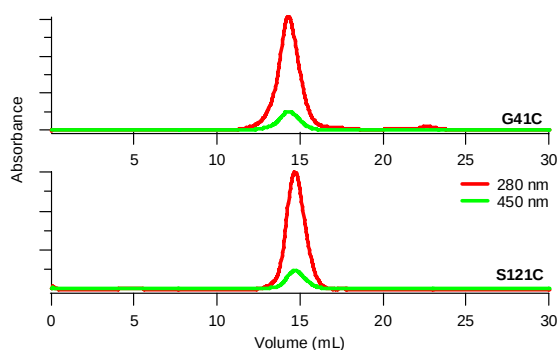


Figure 4.6. The SEC of Hsp G41C Ru and S121CRu after illumination. A. SEC of Hsp G41CRu after 1 hour of illumination, B. SEC of Hsp S121CRu after 1 hour of illumination.

The LC/MS analysis of the functionalized protein subunit determined that both Hsp G41CRu and Hsp S121CRu were labeled with one RuIphen (Figure 4.7 A and B, respectively). While the reaction was not complete, Hsp G41C and Hsp S121C could be labeled to 82 and 85% loading respectively, determined by UV-VIS analysis. To determine the effect of the  $^1\text{O}_2$  production and illumination on the protein cage and to determine the origin of the mass increase seen in the SDS-PAGE analysis, LC/MS was used to analyze the protein cage during the  $^1\text{O}_2$  production. The LC/MS shows that the protein is oxidized over the course of the illumination as indicated by the numerous

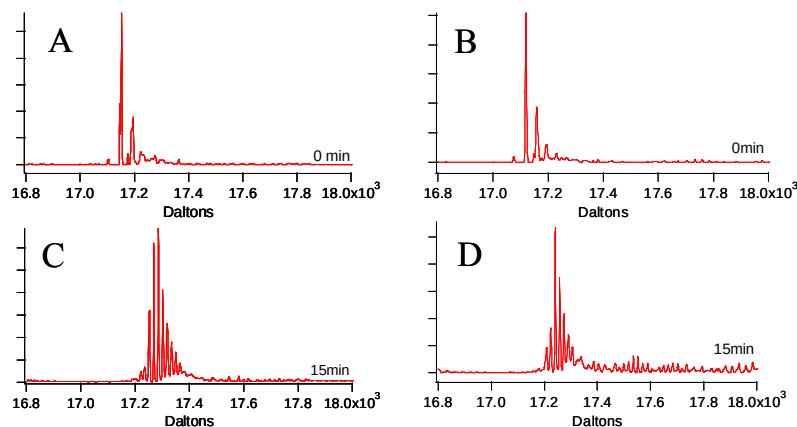


Figure 4.7. The LC/MS analysis of Hsp G41CRu and S121CRu before and after illumination. A. Hsp G41C functionalized with RuIphen calculated MW: 17140.62 ( $M + H^+$ ), experimentally determined MW: 17142.5 ( $M + H^+$ ); B. Hsp S121C functionalized with RuIphen calculated MW: 17117.68 ( $M + H^+$ ), experimentally determined MW: 17119 ( $M + H^+$ ); C. Mass spectrometry of Hsp G41C functionalized with RuIphen after 15 minutes of illumination showing the mass increase from the oxidation by  $^1O_2$ ; D. Mass spectrometry of Hsp S121C functionalized with RuIphen after 15 minutes of illumination showing the mass increase from the oxidation by  $^1O_2$ .

additions of 16 Daltons to Hsp G41CRu and Hsp S121CRu ((Figure 4.7 C and D, respectively).

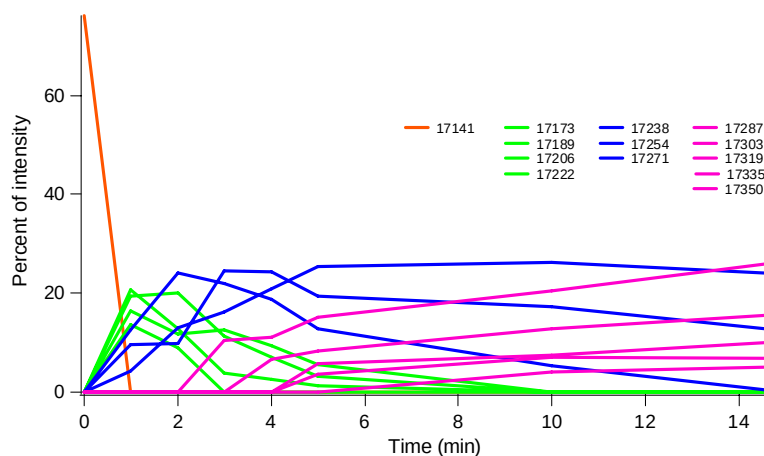


Figure 4.8. Percent mass intensity time course during illumination of Hsp G41C functionalized with RuIphen.

Using LC/MS to analyze the progressive oxidation of the protein cage, we see the mass of the subunit of Hsp G41CRu increases as the time of illumination increases.

During the time course, trends in the mass increase are seen. At one minute, the unoxidized Hsp G41CRu peak has disappeared and peaks corresponding to the addition of 1-4 oxygens per subunit grow in which corresponds to the green lines in figure 4.8. As the illumination continues, subunits with 5-7 additional oxygen begin to appear, which corresponds to the blue lines in figure 4.8, and the less oxidized peaks, green lines, begin to decrease in intensity. At the end of the illumination, subunits with 8-12 oxygen additions, which corresponds to the pink lines in figure 4.8, begin to become the dominant peaks; while the subunits with 1-4 oxygen additions, green lines, are almost gone and the subunits with 5-7 oxygen additions, blue lines, are starting to decrease.

#### Characterization of $^1\text{O}_2$ Production

In order to analyze the singlet  $\text{O}_2$  production by the protein RuIphen composite, the singlet  $\text{O}_2$  production was monitored by its reaction with TEMP 2,2,6,6-tetramethylpiperidine (TEMP) to form TEMPO, an EPR active species. Briefly, the light induced production of singlet  $\text{O}_2$  assay was conducted in a serum vial open to the air with vigorous stirring. The reaction was conducted in 50 mM TEMP with DPBS at pH 7.4. The each assay was normalized to 20  $\mu\text{M}$  PTA. At each time point, 100  $\mu\text{L}$  of the solution was removed and added to 10  $\mu\text{L}$  of 1M sodium azide, to quench the singlet  $\text{O}_2$  production. Hsp G41CRu was labeled with two different loadings of RuIphen, 51 % and 82 % (Figure 4.9 A and C, respectively), to determine the effect of the loading on  $^1\text{O}_2$ . The TEMPO production curves demonstrate that there is minimal difference in the TEMPO production meaning the protein does not significantly quench the  $^1\text{O}_2$  production. To determine if the lag in TEMPO production in the first minutes of the assay are from the reaction of  $^1\text{O}_2$  with the protein cage or are from a lag in  $^1\text{O}_2$

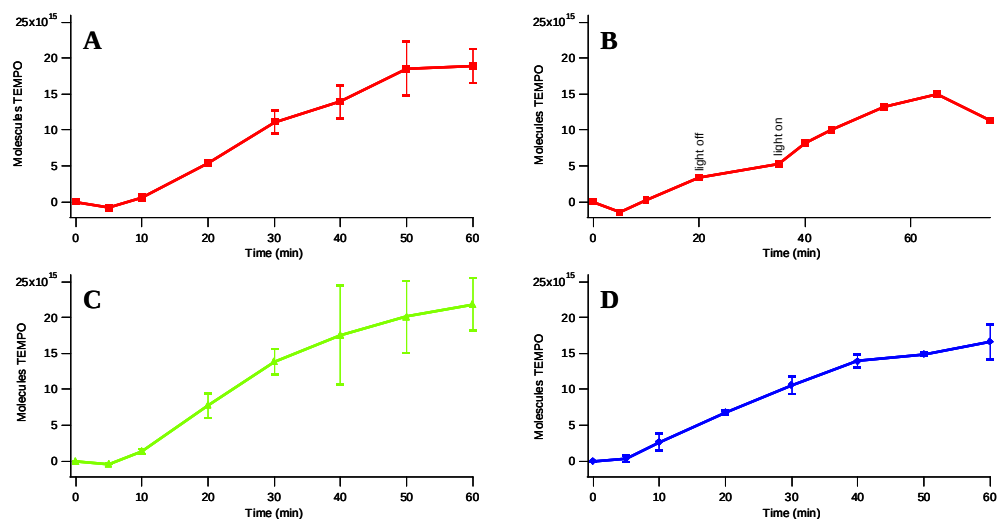


Figure 4.9. Graphs depicting the TEMPO generation by Hsp G41C and Hsp S121C functionalized with RuIphen. A. Hsp G41C functionalized with RuIphen at 82 % loading; B Hsp G41C functionalized with RuIphen at 82 % loading with the light turned off from minute 25 to minute 35; C. Hsp G41C functionalized with RuIphen at 51 % loading; D. Hsp S121C functionalized with RuIphen at 85 % loading. All reactions are normalized to 20  $\mu$ M ruthenium in 50 mM TEMP and DPBS pH 7.4 at 37 °C.

production when the illumination is started, the illumination of the HSP G41CRu was stopped for 10 min in the middle of the assay and restarted (Figure 4.9 B). This experiment demonstrates that the lag phase was not seen when the illumination was restarted determining that there is not a lag in the  $^1\text{O}_2$  production and illumination is needed to produce  $^1\text{O}_2$ . The lag phase is probably from the  $^1\text{O}_2$  reaction with the protein cage. While the TEMPO production from Hsp S121CRu (Figure 4.9 D) is slightly lower than that of Hsp G41CRu, the production curves are remarkably similar demonstrating that the position of the RuIphen on the protein cage (interior or exterior) does not dramatically effect the production of singlet  $\text{O}_2$ . Figure 4.10 displays the TEMPO production from the RuIphen attached to CCMV.

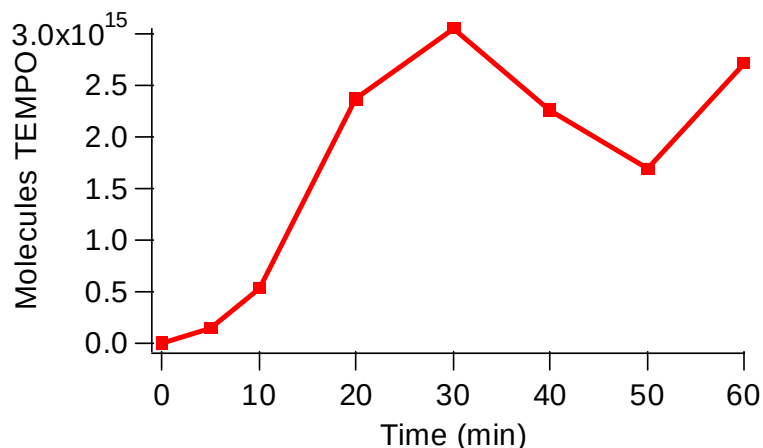


Figure 4.10. Graph depicting the TEMPO generation from RuIphen labeled CCMV. 20  $\mu\text{M}$  ruthenium in 50 mM TEMP and DPBS pH 7.4 at 37  $^{\circ}\text{C}$ .

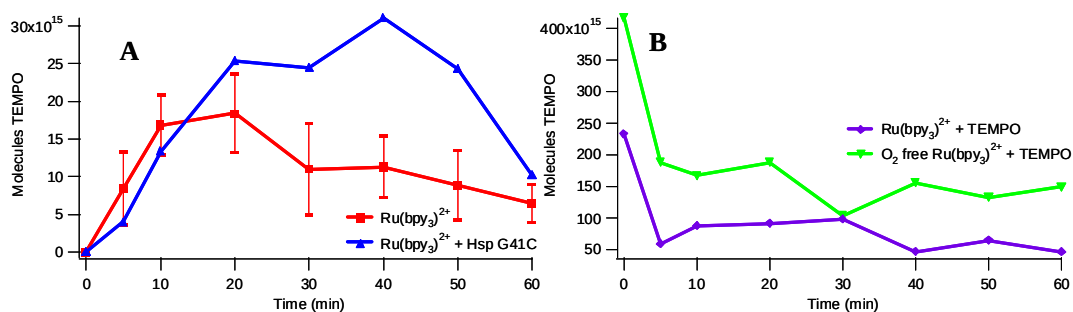


Figure 4.11. Graphs depicting the TEMPO generation and degradation by unbound  $\text{Ru}(\text{bpy})_3^{2+}$ . A.  $\blacktriangle$  Free  $\text{Ru}(\text{bpy})_3^{2+}$ ,  $\blacksquare$  Free  $\text{Ru}(\text{bpy})_3^{2+}$  with Hsp G41C to the equivalent of 85 % loading; B.  $\blacktriangledown$  Free  $\text{Ru}(\text{bpy})_3^{2+}$  with purchased TEMPO added,  $\blacklozenge$  Deoxygenated free  $\text{Ru}(\text{bpy})_3^{2+}$  with purchased TEMPO. All reactions are normalized to 20  $\mu\text{M}$  ruthenium in DPBS pH 7.4 at 37  $^{\circ}\text{C}$ . (A) has 50 mM TEMP in the solution.

Control reactions with free  $\text{Ru}(\text{bpy})_3^{2+}$  show similar TEMPO production; however, the reaction kinetics are different. Free  $\text{Ru}(\text{bpy})_3^{2+}$  quickly produces a maximum of TEMPO then the EPR signal from the TEMPO decreases (Figure 4.11 A). When the singlet  $\text{O}_2$  production assay with free  $\text{Ru}(\text{bpy})_3^{2+}$  is conducted in the presence of Hsp G41C added to the equivalent of 85 % loading (Figure 4.11 A), the reaction kinetics become more similar to that of Hsp G41CRu and Hsp S121CRu, but there is still a decrease in the EPR signal at the end of the reaction. In order to probe the loss of

EPR signal in the free  $\text{Ru}(\text{bpy})_3^{2+}$  control, free  $\text{Ru}(\text{bpy})_3^{2+}$  was illuminated in the presence of purchased TEMPO with the presence or absence of molecular  $\text{O}_2$  (Figure 4.11 B). Both of the TEMPO reaction conditions show that the free  $\text{Ru}^{2+}\text{bpy}_3$  degrades the EPR signal, which indicates an  $\text{O}_2$ -free mechanism for degradation of TEMPO.

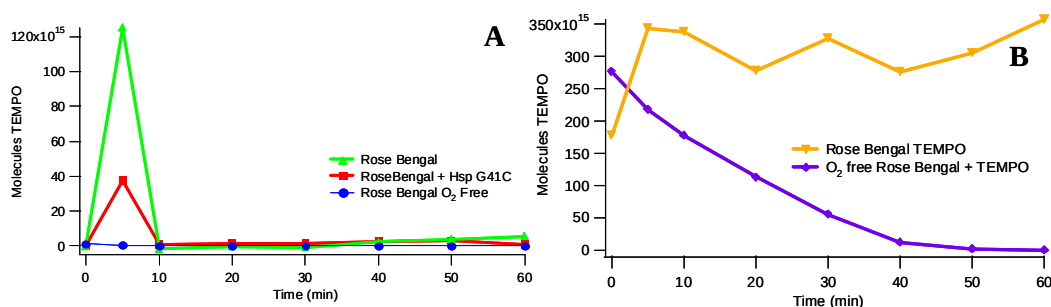


Figure 4.12. Graphs depicting the TEMPO generation and degradation by Rose Bengal. A. ▲ Rose Bengal, ■ Rose Bengal with Hsp G41C to the equivalent of 85 % loading, ● Deoxygenated Rose Bengal; B. ▼ Rose Bengal with purchased TEMPO added, ◆ Deoxygenated Rose Bengal with purchased TEMPO added. All reactions are normalized to 20  $\mu\text{M}$  Rose Bengal in DPBS pH 7.4 at 37 °C. (A) has 50 mM TEMP in the solution.

To test and calibrate the  $^1\text{O}_2$  production assay, Rose Bengal was used as a comparison. Rose Bengal showed a 4-fold increase in the amount of TEMPO generated (Figure 4.12 A); however, the EPR signal was quickly quenched comparable to the free  $\text{Ru}(\text{bpy})_3^{2+}$ . In the singlet  $\text{O}_2$  production reaction from Rose Bengal in the presence of Hsp G41C added to the equivalent of 85 % loading, the TEMPO production is suppressed; however, the reaction kinetics are almost identical (Figure 4.11 A). Analysis of the reaction between purchased TEMPO and Rose Bengal upon illumination in the presence and absence of  $\text{O}_2$  shows that Rose Bengal does not degrade TEMPO in the presence of  $\text{O}_2$ ; however, it readily degrades TEMPO in the absence of  $\text{O}_2$  (Figure 4.12 B).

## Discussion

The goal of this research was to probe whether Hsp is a suitable photodynamic therapy agent (PTA) delivery vehicle. This study focused on the interaction between the protein cage with the PTA and the ROS. If the protein quenches the ROS production or the PTA is inactivated by the protein, further studies using Hsp as a PTA delivery vehicle would be futile. For this study, a  $\text{Ru}(\text{bpy})_3^{2+}$  analog was chosen as the PTA because it is a well characterized reactive oxygen species (ROS) generator, specifically produces singlet  $\text{O}_2$ , that can be readily attached to a protein.<sup>216-220</sup>

### Attachment of $\text{Ru}(\text{bpy})_2\text{Iphen}^{2+}$

Hsp's amenability to genetic and covalent modifications make it an ideal targeted drug delivery vehicle. Figure 4.1 A and B is a Chimera representation of the  $\text{Ru}^{2+}(\text{bpy})_3$  analog, ruthenium(II) dibipyridine 5-iodoacetamido-1,10-phenanthroline (RuIphen), covalently linked to Hsp S121C and Hsp G41C, respectively. By using the Hsp cage, the site of functionalization and extent of loading on the cage can be efficiently controlled. The covalent attachment of the photo-therapeutic on the interior or the exterior did not adversely affect the protein cage stability.

### Interaction of $^1\text{O}_2$ With Hsp

The oxidation of Hsp by  $^1\text{O}_2$  was extensive but did not degrade the protein architecture.<sup>221-224</sup> SEC confirmed that the cage was still a 12 nm particle and that the RuIphen was photo-bleached. LC/MS data provided the most insight into the  $^1\text{O}_2$  interaction with the protein cage. A LC/MS time course run on Hsp G41CRu oxidation (Figure 4.8) revealed that during illumination the protein cage subunit mass is increasing

in 16 dalton increments, corresponding to the addition of an oxygen atom. The  $^1\text{O}_2$  that is produced by the RuPhen is oxidizing the Hsp during the reaction and the extent of oxidation increases with illumination. LC/MS of protease digests of the oxidized protein to determine amino acids oxidized by  $^1\text{O}_2$  were unable to reveal any protein fragments. This is easily explained by the extensive oxidation of the protein interrupting the protease sites or the increased negative charge on the protein inhibiting MS analysis.

#### Characterization of $^1\text{O}_2$ production

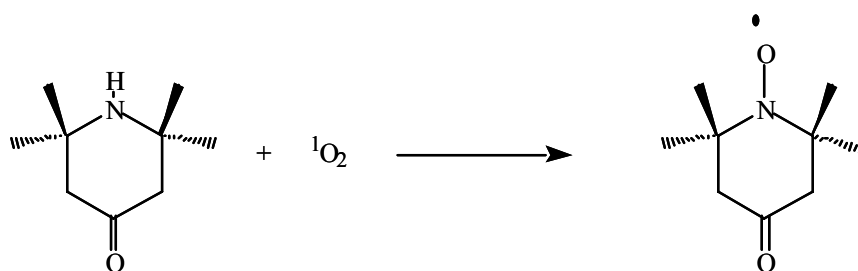


Figure 4.13. TEMP reaction with  $^1\text{O}_2$  to form TEMPO.

Using TEMP conversion to TEMPO, (Figure 4.13) to detect the production of  $^1\text{O}_2$ , we have characterized the production of  $^1\text{O}_2$  from Hsp based delivery vehicles.<sup>225-227</sup> TEMP specifically reacts with  $^1\text{O}_2$  to form TEMPO without cross reactivity from other ROS.<sup>227</sup> The TEMPO production curves from different loadings of PTA in Hsp G41C are nearly identical, suggesting that the protein does not significantly quench the production of  $^1\text{O}_2$ . TEMPO production curves in the presence of protein have a lag of production at the beginning illumination, suggesting that the protein is oxidized before the reaction with TEMP. TEMPO production did not show a lag in the production after the illumination was restarted suggesting the lag phase was caused by  $^1\text{O}_2$  interaction with the protein. Hsp S121CRu TEMPO production curves are remarkably similar to the

Hsp G41CRu curves suggesting that the protein doesn't significantly quench the  $^1\text{O}_2$  production.

The free  $\text{Ru}(\text{bpy})_3^{2+}$  control TEMPO production (Figure 4.11 A) reaction yielded an unexpected result. The total TEMPO production was similar to the protein bound RuIphen; however, the maximum was reacted with much less illumination without a lag at the beginning of the reaction. This could be explained by the fact that the free  $\text{Ru}(\text{bpy})_3^{2+}$  could react faster than the protein bound RuIphen and the free  $\text{Ru}^{2+}\text{bpy}_3$  does not oxidize the protein. The most surprising result was the fact that the TEMPO signal decreased after reaching a maximum. Free  $\text{Ru}(\text{bpy})_3^{2+}$  TEMPO production in the presence of protein revealed the protein, through an unknown mechanism, provided some protection for the TEMPO. In an attempt to understand the degradation reaction, free  $\text{Ru}(\text{bpy})_3^{2+}$  was reacted with purchased TEMPO to in the presence and absence of  $\text{O}_2$  (Figure 4.11 B). Both reactions degraded the TEMPO signal suggesting that the mechanism for degradation is an  $\text{O}_2$  free pathway with no preference to reacting with  $\text{O}_2$ . Rose Bengal was used as the PTA to further probe this interaction.<sup>223</sup> Rose Bengal produces 5 fold increase in TEMPO production compared to that  $\text{Ru}(\text{bpy})_3^{2+}$ ; but, the TEMPO signal is quickly quenched. Free Rose Bengal was reacted with purchased TEMPO in the presence and absence of  $\text{O}_2$  (Figure 4.12 B). In the presence of  $\text{O}_2$ , the Rose Bengal does not degrade the TEMPO signal; however, it quickly degrades the signal in the absence of  $\text{O}_2$  suggesting Rose Bengal degrades the TEMPO signal through an  $\text{O}_2$  free mechanism. These reactions also suggest the Rose Bengal specifically reacts with  $\text{O}_2$  if it is present instead of degradation reaction. TEMP reaction with singlet  $\text{O}_2$  is

sensitive and effective way to detect the production  $^1\text{O}_2$ . Because of the side reactions with the PTA, it cannot be used to quantitate the amount of  $^1\text{O}_2$  produced.

#### Use of Targeted Protein Cage

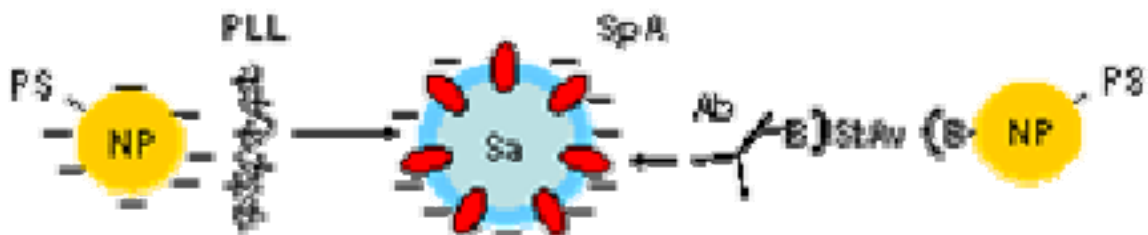


Figure 4.14. Targeting of PS functionalized CCMV nanoplatforms to *S. aureus* cells. Targeting strategies using electrostatic (left) or complementary biological interactions (right). Abbreviations are (from left to right): photosensitizer (PS); nanoplatform (NP); poly-L-lysine (PLL); *S. aureus* cell (Sa); protein A (SpA); anti-SpA-mAb-B (Ab), biotin (B) and streptavidin (StAv).

As part of an on going study within the lab on therapeutic application of protein cage architectures, the RuIphen based PTA described was tested *in vitro*. CCMV was used to create a targeted nanoplatform (NP) using an antibody as the targeting agent and RuIphen as the photo-dynamic therapy agent shown in figure 4.14 (See Appendix). The targeted NP uses many of the benefits of the protein cage architecture to make a targeted PTA delivery vehicle. CCMV coupled with RuIphen and modified with an antibody directed to *S. aureus* cells was more effective at killing cells (Figure 4.15 A) than the untargeted RuIphen modified CCMV (Figure 4.15 B) upon illumination. This system shows that the potential application of targeted PTA attached to a protein cage for targeted cell killing. The  $^1\text{O}_2$  production assay on the CCMV linked RuIphen (Figure 4.10) shows that CCMV quenches more  $^1\text{O}_2$  than Hsp. CCMV's increased quenching could be related to the fact that the interior of CCMV is filled with RNA, a known target of  $^1\text{O}_2$ . This result suggests that Hsp would be more effective protein cage delivery system.

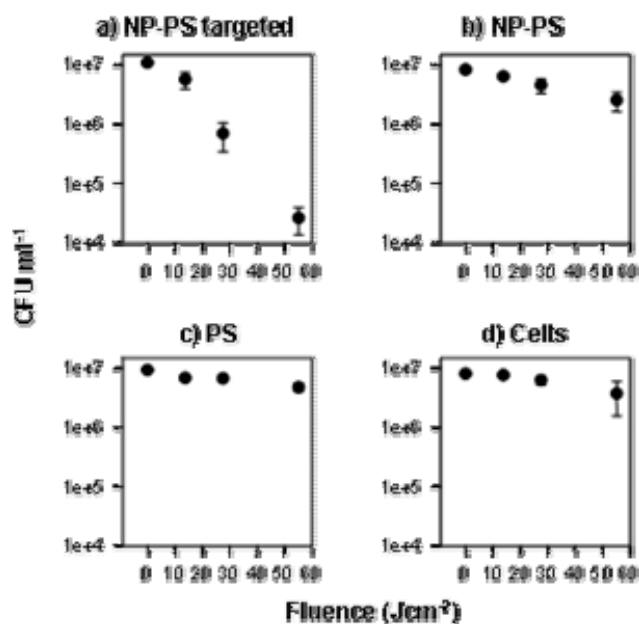


Figure 4.15. Photodynamic inactivation of *S. aureus* cells targeted with PS functionalized CCMV (NP-PS). At a fluence rate of 46 mWcm<sup>-2</sup>. Data are presented as CFU mL<sup>-1</sup> recovered after treatment. Error bars are standard errors for the mean of 3 independent replicates. a) cells targeted with S130C/K42R-PS-B at 2  $\mu$ M equivalent PS concentration; b) cells exposed to S130C/K42R-PS at 2  $\mu$ M equivalent PS concentration (non-targeted); c) cells exposed to 2  $\mu$ M PS; d) cells targeted with anti-SpA-mAb-B and StAv but not with the PS functionalized nanoplatform. An unpaired t-test indicated that the means for the targeted cells were significantly different from means for all other conditions at fluences of 27.6 and 55.2 Jcm<sup>-2</sup> at the 5% level.

### Conclusions

We have demonstrated that Hsp is a robust protein cage architecture for potential use in the delivery of PDT drugs. Both Hsp and CCMV can be genetically modified specifically on the interior or exterior surface and can be subsequently covalently modified by RuIphen to form a catalytically active composite structure. The protein cage is oxidized during the singlet O<sub>2</sub> production but does not significantly quench the production. The singlet O<sub>2</sub> production is independent of the attachment on the interior or exterior of the protein cage. Ease of controlled modification, genetic or covalent, and stability to a broad range of synthetic conditions make protein cages ideal for multi-

functionalized synthesis as drug delivery vehicles. The TEMP to TEMPO reaction is specific for the production of  $^1\text{O}_2$  and can be used to detect the production of  $^1\text{O}_2$ .

Because of the TEMPO degradation reactions by the PTAs, the  $^1\text{O}_2$  production cannot be quantitated using the TEMP to TEMPO reaction.

### Introduction

The cellular detoxification of soluble heavy metals by reduction to insoluble reduced heavy metal particles is of great interest for the biological remediation of waste sites that are contaminated with heavy metals from industrial processes.<sup>16, 228</sup> The use of non-biological removal of heavy metal contaminants is limited because of the non-specificity of the treatment which causes large amounts of non-toxic metals to be removed and incorporated with the toxic metals raising the costs of detoxification considerably.<sup>13</sup> The biological reduction of toxic heavy metals can specifically reduce the toxic heavy metals to insoluble metal particles thereby removing the metals from the water.<sup>15, 229, 230</sup> While some organisms can reduce toxic metals by using the metal as a terminal electron acceptor,<sup>14</sup> other organisms reduce the heavy metals in the presence of H<sub>2</sub> or other simple electron donors that they are unable to utilize as a source for growth. Michel and coworkers used a Fe-only hydrogenase from *Desulfovibrio vulgaris* strain Hildenborough to reduce Cr(VI) to the less toxic but still soluble Cr(III) using H<sub>2</sub>.<sup>231</sup> De Luca and coworkers showed that the sulfate reducing bacterium *Desulfovibrio fructosovorans* reduced technetium(VII) into a black precipitate in the absence of sulfate and under an atmosphere of H<sub>2</sub>.<sup>16</sup> The reduction of technetium to a reduced insoluble particle removes the toxic metal from the water and decreases the mobility of the toxic metal in water there by limiting the toxic effects of the metal. In the absence of H<sub>2</sub> or in a mutant where the structural proteins for the most abundant hydrogenase are deleted, the technetium(VII) reduction was reduced by more than five fold.<sup>16</sup> Hydrogenases are

enzymes that reversibly reduce protons to hydrogen gas with high efficiency. These examples and other illustrate the central role of hydrogenases in the reduction of toxic heavy metals.

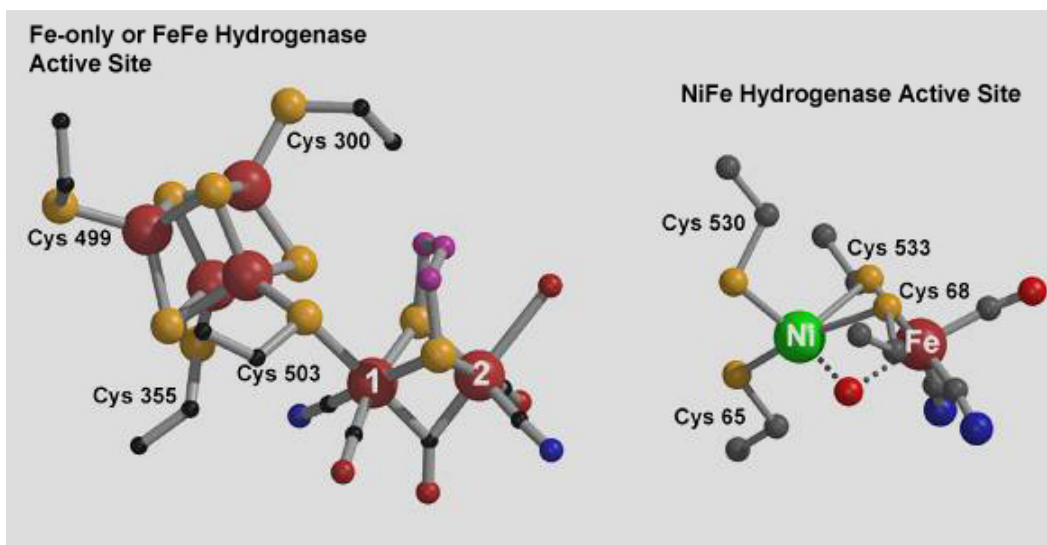


Figure 5.1. The active site of Fe-only hydrogenase and NiFe hydrogenase. Left: Fe-only, Right: NiFe. Image from [www.chemistry.montana.edu/john.peters/](http://www.chemistry.montana.edu/john.peters/).

Hydrogenase enzymes are present in a variety of microorganisms and function either to catalyze hydrogen oxidation or proton reduction. Hydrogenases contain either Ni and Fe or Fe only in the active site of the enzyme. The NiFe hydrogenases have a bimetallic NiFe active site where a Fe atom bound by bridging thiolates is also coordinated with both carbon monoxide and cyanide ligands.<sup>232</sup> In the Fe-only class of hydrogenases, the active site “H cluster” consists of a [4Fe-4S] cubane bridged to a di-iron subcluster, which is coordinated by a non-protein dithiolate bridging ligand and carbon monoxide and cyanide ligands.<sup>126</sup>

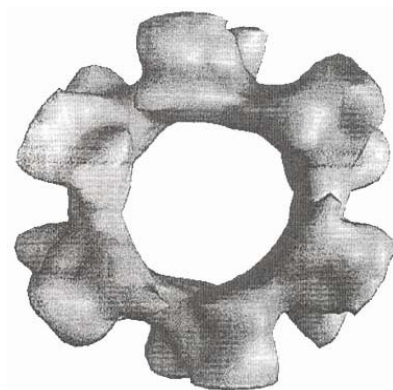


Figure 5.2. Stained TEM reconstruction of TrH<sub>2</sub>ase.

In this study, the NiFe hydrogenase from the purple sulfur bacteria *Thiocapsa roseopersicina* will be used.<sup>233</sup> This hydrogenase is a bidirectional catalyst that has been previously characterized as a supramolecular ring shaped assembly of ~650kD (Figure 5.2<sup>233</sup>) that has enhanced thermostability and oxygen tolerance compared with other hydrogenases. *T. roseopersicina* can be cultured on a large scale and large quantities of the enzyme can be obtained from the native host.

The supramolecular structure of the TrH<sub>2</sub>ase can be used as a platform for the synthesis of reduced metal nanoparticles similar to the way protein cage and protein ring architectures are used. The enzymatic activity of the protein can be used to synthesize nanoparticles that are difficult to mineralize in aqueous solution and also provide a unique platform for catalyst that cannot be matched by non-biological architectures.

By using an enzyme as the biological architecture for nanoparticle synthesis, the enzymatic properties of the protein can be coupled to the catalytic properties of the nanoparticle. Complexation of catalytic activities of the individual parts (enzyme and synthetic particle) into a new catalyst with has the potential to produce hybrid activities similar to the coupling in biological systems. For example, photosynthetic capture of light, water oxidation, and reduction of NADPH takes place in the chloroplasts. In

photosystem II, which occurs in chloroplasts, light is absorbed by the protein P680, which generates an electron that is passed through an electron relay to photosystem I. After donating its electron P680 is a powerful oxidizing agent that can then oxidize water to return to its ground state. In photosystem I, the electron from photosystem II is used to reduce  $\text{NADP}^+$  to NADPH, which is used by the organism in the fix  $\text{CO}_2$ . This photosynthetic pathway shows one way biological systems couple separate catalytic reactions into one system combining the enzymatic properties of both systems.<sup>234</sup>

Zadvorney showed that  $\text{Ni}^{2+}$  can be reduced by  $\text{TrH}_2\text{ase}$  to form large  $\text{Ni}^0$  particles.<sup>17</sup> Nickel(II) reduction to nickel metal nanoparticles was chosen as the mineralization of interest because nickel cannot be reduced by  $\text{H}_2$  in the absence of  $\text{TrH}_2\text{ase}$ . Nickel metal is also an important industrial catalyst used for the hydrogenation of acetylene at high temperature.<sup>235, 236</sup> Coupling a  $\text{Ni}^0$  and hydrogenase enzyme has the potential to catalyze the hydrogenation of acetylene to ethylene at low temperature compared to the current high temperature catalysis.

Mineralization of  $\text{TrH}_2\text{ase}$  with  $\text{Ni}^0$  has other implications besides the synthesis of a biologically coupled catalyst.  $\text{Ni}^0$  mineralization can be used to determine where the enzymatic site is located on  $\text{TrH}_2\text{ase}$ . Using the  $\text{Ni}^0$  mineralization is a simple way to determine location of the enzymatic site because the  $\text{Ni}^{2+}$  cannot be reduced by  $\text{H}_2$  (the reducing agent in the reaction) in the absence of the  $\text{TrH}_2\text{ase}$ . The  $\text{Ni}^0$  formed by the reduction is not soluble and will form a  $\text{Ni}^0$  particle at the point of reduction. Mineralization is conceptually simple, the  $\text{TrH}_2\text{ase}$  interacts with and catalyses the splitting of  $\text{H}_2$  gas into protons and electrons. The protons are released into the solution; while, the electrons are used as reducing equivalents, in this case, to reduce the  $\text{Ni}^{2+}$  to

$\text{Ni}^0$ . The location where the reducing equivalents can be accessed by the external environment could in principle be anywhere on the surface of the protein. The mineralization of  $\text{Ni}^{2+}$  to a  $\text{Ni}^0$  particle at the site where the reduction occurs will allow the visualization of the reduction site by TEM.

### Methods

#### Growth and Purification of *Thiocapsa roseopersicina* Hydrogenase (TrH<sub>2</sub>ase)

*Thiocapsa roseopersicina*, strain Syvash, was grown under anaerobic conditions on modified Pfenning medium. The cells were collected in the logarithmic growth phase and were acetone treated before DEAE-cellulose DE<sub>52</sub> (Whatman, Great Britain), phenyl-sepharose CL-4B, and Sephacryl S-300 (Pharmacia, Sweden) chromatography described previously. The hydrogenase purity was confirmed by SDS-PAGE. The activity of the TrH<sub>2</sub>ase was determined by the hydrogen gas mediated methyl viologen reduction.<sup>17, 18</sup>

#### Mineralization of TrH<sub>2</sub>ase With $\text{Ni}^0$

TrH<sub>2</sub>ase was mineralized with a theoretical loading of 1000 Ni atoms per TrH<sub>2</sub>ase ring architecture. A 50  $\mu\text{L}$  reaction was conducted in 50 mM Tris-HCl pH 9.0 at 15 mg/mL at 8°C. 6.3  $\mu\text{L}$  of 200 mM  $\text{NiCl}_2$  in 50 mM Tris-HCl pH 9.0 was in 1  $\mu\text{L}$  increment with the final addition being 1.3  $\mu\text{L}$ . After each addition, the Ni was allowed to react for 4 days before the next addition and the reaction was allowed to react for 4 days after the final addition.

### Transmission Electron Microscopy (TEM)

TEM data were obtained on a Leo 912 AB, with  $\Omega$  filter, operating at 100 and 60 keV. Samples were imaged unstained or negatively stained with 2% uranyl acetate. Electron diffraction patterns were collected on samples and the d-spacings were calculated and compared with the powder diffraction file for Ni after calibrating the diffraction camera with an Au standard.

### Dynamic Light Scattering (DLS)

DLS measurements were carried out on a Brookhaven Instrument Corporation 90-PALS at 90 degrees using a 661 nm diode laser, and the correlation functions were fit using a non-negatively constrained least-squares analysis.

## Results

### DLS Analysis of TrH<sub>2</sub>ase

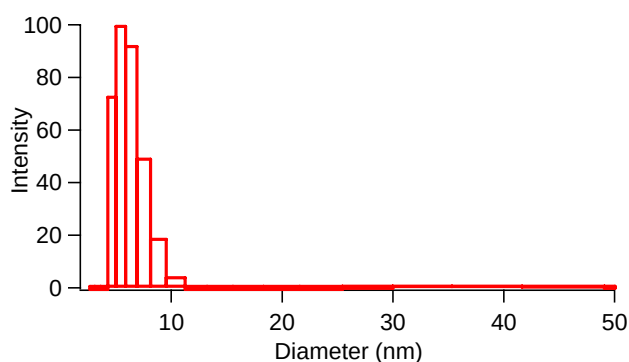


Figure 5.3. DLS of TrH<sub>2</sub>ase.

The DLS analysis of TrH<sub>2</sub>ase at a concentration of 1 mg/mL in buffer (50 mM Tris-HCl pH 9.0) indicating an average particle size of ~6 nm. The DLS characterization indicates the supramolecular structure of the TrH<sub>2</sub>ase is dependant on the concentration of the protein.

TEM Characterization of Ni<sup>0</sup> Mineralized  
TrH<sub>2</sub>ase (NiTrH<sub>2</sub>ase)

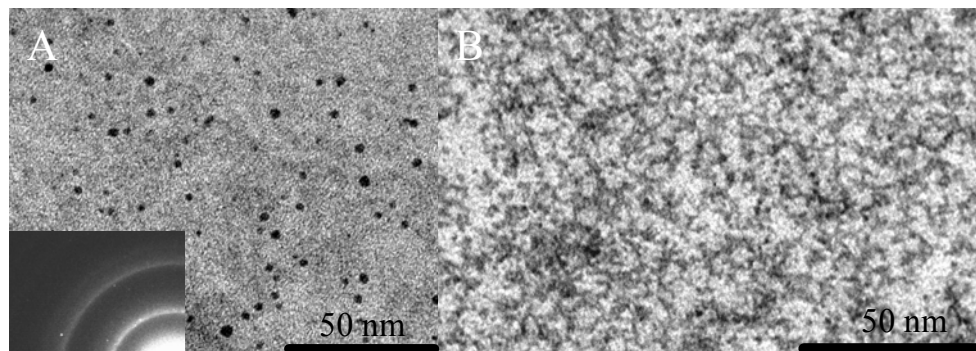


Figure 5.4. TEM analysis of NiTrH<sub>2</sub>ase. A. Unstained TEM of NiTrH<sub>2</sub>ase, inset: electron diffraction, Average diameter of  $2.6 \pm 0.6$  nm; B. TEM of NiTrH<sub>2</sub>ase stained with 2% uranyl acetate, Scale Bar = 50 nm.

The unstained TEM analysis of NiTrH<sub>2</sub>ase (Figure 5.4 A) clearly shows electron dense cores with an average diameter of  $2.6 \pm 0.6$  nm. The diffraction (Figure 5.4A

| D-Spacings |              |              |
|------------|--------------|--------------|
| Calculated | Experimental | Miller Index |
| 2.16       | 2.11         | 002          |
| 2.02       | 2.08         | 101          |
| 1.32       | 1.25         | 110          |
| 1.22       | 1.23         | 103          |
| 1.13       | 1.15         | 112          |
| 1.1        | 1.04         | 201          |

Table 5.1. Table of the measured d-spacings of the NiTrH<sub>2</sub>ase.

inset) aligns with the Miller index of Ni<sup>0</sup> (Table 5.1). TEM analysis of NiTrH<sub>2</sub>ase stained with 2% uranyl acetate reveals the Ni<sup>0</sup> particles are surrounded by white halos indicative

of protein surrounding the protein. The protein shell that surrounds the  $\text{Ni}^0$  particles has three diameter distributions  $\sim 6$ ,  $\sim 8$ , and  $\sim 11$  nm.

### Magnetic Characterization of $\text{Ni}^0$ in NiTrH<sub>2</sub>ase

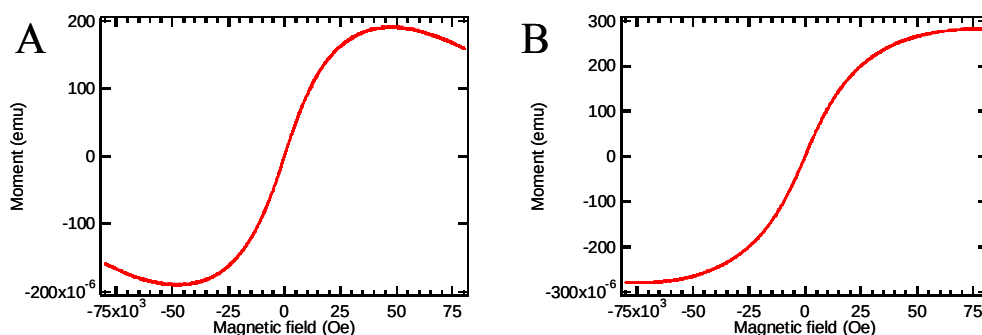


Figure 5.5. Vibrating sample magnetometry (VSM) characterization of NiTrH<sub>2</sub>ase. A. Raw VSM measurement of NiTrH<sub>2</sub>ase; B. After subtracting the diamagnetic signal at high field caused by the large amount of protein, VSM of NiTrH<sub>2</sub>ase.

VSM measurements were performed on a physical properties measurement system (Quantum Design). Sample preparation included isolation of the protein/mineral composite using a centrifugal ultrafiltration device (Microcon) with a 100k NMW cutoff. This sample was transferred into dH<sub>2</sub>O (18.2 MΩ resistivity Nanopure) followed by lyophilizing the material to form a solid pellet. A 0.3 mg pellet was weighed to an accuracy of 0.01 mg on an analytical balance (Ohaus) and immobilized on a quartz paddle using Duco cement and placed in the VSM. Sample measurement was made at 5 K and the field was swept between 80,000 Oe and -80,000 Oe while vibrating at a frequency of 40 Hz (Figure 5.5 A). After measurement the data were analyzed using IgorPro (Wavemetrics) and the diamagnetic signal at high field caused by the large amount of protein was subtracted (Figure 5.5 B).

Figure 5.6 is an illustration of the mineralization process occurring on the interior of the ring structure. The unstained TEM of the mineralization of  $\text{Ni}^0$  by TrH<sub>2</sub>ase clearly shows electron dense cores that have an average diameter of  $2.6 \pm 0.6$  nm (Figure 5.4 A). The electron diffraction of the synthesized particles (Figure 5.4A, inset; and Table 5.4) show that the particles are  $\text{Ni}^0$ . The uranyl acetate stained TEM analysis (Figure 5.4 B) confirms that the  $\text{Ni}^0$  particles are on the inside of the ring structure. Stained TEM analysis reveals that there a majority of the TrH<sub>2</sub>ase ring structures with a Ni particle encapsulated.  $\text{Ni}^0$  mineralization by TrH<sub>2</sub>ase is not an efficient reaction with less than half of the particles mineralized.

TEM images show electron dense particles of 2-3 nm that are surrounded by a protein, indicated by a white halo. However, the protein halo that surrounds the  $\text{Ni}^0$  particle has three different diameters, ~6, ~8, and ~11. The 8 and 11 nm proteins of the surrounding the  $\text{Ni}^0$  particles can be explained from the cryoEM reconstruction of the the TrH<sub>2</sub>ase.<sup>233</sup> TrH<sub>2</sub>ase ring structure is 11 nm in diameter and 8 nm deep, which could be oriented on the TEM grid with ring flat or on its side. The 6 nm protein can be explained by DLS of the TrH<sub>2</sub>ase (Figure 5.3). DLS was conducted on a 1 mg/mL TrH<sub>2</sub>ase solution. TrH<sub>2</sub>ase ring formation is concentration dependant. At high protein concentration >15 mg/mL, the TrH<sub>2</sub>ase is in the 11 nm ring conformation and a lower concentrations (~ 1 mg/mL) the ~6 nm protein complex is formed. In the uranyl acetate stained TEM analysis of NiTrH<sub>2</sub>ase, all of the sizes of the protein complexes are present.

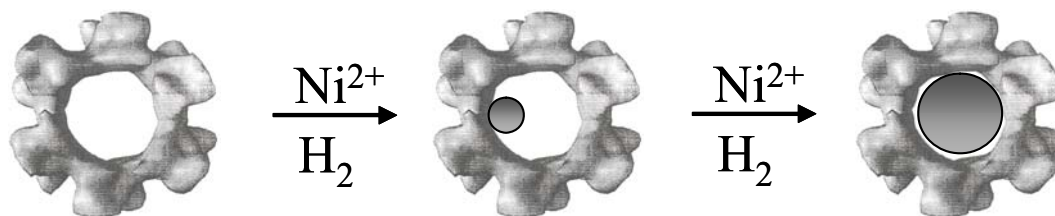


Figure 5.6. Reaction scheme for the  $\text{Ni}^0$  mineralization with TrH<sub>2</sub>ase.

Stained TEM analysis gives us valuable biochemical data. The reduction site for the TrH<sub>2</sub>ase is specifically on the interior of the protein, indicated by the mineralization on the interior of the protein. Because the mineralization is on the interior of the protein, TrH<sub>2</sub>ase has the potential to be used as a platform for nanoparticles synthesis (Figure 5.6<sup>233</sup>). By incasing the nanoparticles, the protein can be used to stabilize and prevent the aggregation of the nanoparticles. If the mineralization was on the exterior surface, the protein would be a poor platform for nanoparticles synthesis because the interaction between nanoparticles on the exterior of the TrH<sub>2</sub>ase and other TrH<sub>2</sub>ases could cause the aggregation and precipitation of both the protein and nanoparticles.

$\text{Ni}^0$  particles monitored by VSM showed a typical superparamagnetic signal similar to particles prepared using dendrimer templates.<sup>237</sup> The saturation magnetic moment is similar to those reported in dendrimer as well. Under the conditions of this experiment a  $M_s$  of 1 emu/g of material was determined. This is higher than expected due to the fact that the largest amount of the mass of the pellet was protein and not the Ni particles. This high protein to Ni ratio probably also accounts for the high diamagnetic signal that had to be subtracted (Figure 5.5 A). In the corrected loops, there was no apparent coercive field with no measurable hysteresis (Figure 5.5 B).

To characterize the catalytic properties of the NiTrH<sub>2</sub>ase, we have chosen to probe the hydrogenation of acetylene. This reaction is of critical importance to industrial

polymer synthesis because acetylene poisons the catalysts used in the polymerizing ethylene. The selective hydrogenation of acetylene to ethylene would be useful for protection of industrial polymerization catalysts. Current catalysts are unable to selectively hydrogenate acetylene to ethylene in high yields at lower temperature. At higher temperatures, the hydrogenation of acetylene is less controlled taking hydrogenating acetylene to ethane. Boudjahem and coworkers have shown the dependence of acetylene hydrogenation products on the metal phase morphology.<sup>235</sup> With these considerations, we have chosen to determine the acetylene hydrogenation products of the NiTrH<sub>2</sub>ase. The NiTrH<sub>2</sub>ase has the potential to have unique catalytic activity because of the protein-based platform of TrH<sub>2</sub>ase combined with the catalytic activity of TrH<sub>2</sub>ase. Currently, we have been unable to show catalytic activity from the NiTrH<sub>2</sub>ase.

### Conclusions

TrH<sub>2</sub>ase was used as a biomimetic platform for the synthesis of a Ni<sup>0</sup> particle. The mineral forms specifically on the interior of the ring structure of TrH<sub>2</sub>ase, which is presumably a site specific reduction site in the presence of H<sub>2</sub>. TrH<sub>2</sub>ase is not efficient at mineralizing Ni<sup>0</sup> nanoparticles. Magnetic characterization is consistent with the formation of nanoparticle Ni<sup>0</sup>.

## CONCLUDING REMARKS

Protein cages serve as molecular containers that can be manipulated genetically and covalently for use as platforms for materials synthesis. These versatile platforms have been used for in the synthesis of a wide range of materials. Using protein cages as the scaffold for the synthesis of materials provides a unique environment through a rigid but dynamic structure. With distinct interior and exterior surfaces that can be modified independently of each other, protein cages can be filled with materials then attached to a surface. The protein cages facilitate the mineralization of nanoparticles by providing a surface for nanoparticle growth. The general model for the mineralization of protein cages is that electrostatic differences between the interior and exterior surfaces of the protein cages direct the specific mineralization on the interior surface of the protein. The direction of the mineralization is facilitated by a favorable interaction of precursors of the mineralization and the interior surface of the protein which concentrates the starting materials at the interior surface.

This model was tested using the platinum nanoparticle mineralization on the interior of Hsp. Hsp does not have an electrochemically distinct interior and exterior surface that could clearly be stated to be the directing factor of the mineralization. The insertion of a platinum binding peptide and the genetic insertion of a point mutation to bind platinum ions showed no specificity for the interior of Hsp in the platinum mineralization. However, the platinum mineralization of the native Hsp protein cage was specific for the interior of the cage. With no clear electrostatic driving force for the mineralization of platinum, the electrostatic model of biomineralization is not the only process at work in this biomimetic mineralization. However, when the mineralization is

conducted under identical conditions using Fn, the mineralization is more specific for the interior of the cage and shows less bulk mineralization. The reason for the more specific mineralization is unclear because in the case of platinum the precursor does not have a charge which would make the negative interior of Fn no advantage in the mineralization.

Protein cages act as novel passivating layers that allow for maximum catalytic surface exposure while preventing the aggregation of the synthesized nanoparticles. The protein encapsulated catalytic particle is provided a chemically isolated environment allows for molecular access to the catalyst while prevent aggregation and large molecule poisoning of the catalyst. This environment allows a 10 fold increase in the catalytic activity over tradition synthetic noble metal nanoparticle syntheses. Fn encapsulated platinum nanoparticle are more active than the nanoparticles that are synthesized on the interior of Hsp. The difference arises from the synthesis of more particles that are active for the production of the  $H_2$  in Fn compared to Hsp. The protein cages have been shown to be effective platforms for nanoparticle catalysis.

The utilization of protein cage architectures in biomedical applications allows for precise control over specific functionalization not seen in other drug delivery systems. The exterior surface can be functionalized genetically or covalently with a cell recognition moiety and/or a drug or cell imaging agent. At the same interior of the protein cage can be filled with a drug or a cell imaging agent, maximizing the versatility of the protein cage as a drug delivery vehicle. The utility of using Hsp as a delivery vehicle for photodynamic therapy agents was demonstrated by characterizing the  $^1O_2$  production from a PTA covalently attached to the cage. While the protein cage was oxidized by the  $^1O_2$  produced by PTA, the  $^1O_2$  production was not significantly quenched

during by the protein cage. The attachment site of the PTA (interior or exterior) and the extent of loading of the PTA did not significantly affect the  $^1\text{O}_2$  production from the PTA providing further evidence that the protein architecture is not significantly quenching the  $^1\text{O}_2$  production.

The use of protein supramolecular structures as platforms for the synthesis of nanoparticle expands beyond the protein cage architectures. The protein ring hydrogenase from *T. roseopersicina* was used as a platform for the protein encapsulated synthesis of nickel metal nanoparticles. The enzymatic oxidation of  $\text{H}_2$  gas to protons and electrons was utilized to reduce the nickel ions to nickel metal. The location of the nickel nanoparticle in relation to the ring structure of the hydrogenase reveals that the biochemical site of reduction for the hydrogenase is on the interior of the protein ring. The use of an enzyme to mineralize a nanoparticle has the potential to combine the enzymatic activity of the hydrogenase with the catalytic activity of the synthesized nanoparticle. This potential activity is currently being pursued with the utmost interest.

APPENDIX A

TARGETING AND PHOTODYNAMIC KILLING OF A MICROBIAL  
PATHOGEN USING PROTEIN CAGE ARCHITECTURES  
FUNCTIONALIZED WITH A PHOTSENSITIZER

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### Abstract

The selectivity of antimicrobial photodynamic therapy (PDT) can be enhanced by coupling the photosensitizer (PS) to a targeting ligand. Nanoplatfoms provide a medium for designing delivery vehicles that incorporate both functional attributes. We report here the photodynamic inactivation of a pathogenic bacterium, *Staphylococcus aureus*, using targeted nanoplatfoms conjugated to a photosensitizer (PS). Both electrostatic and complementary biological interactions were used to mediate targeting. Genetic constructs of a protein cage architecture allowed site specific chemical functionalization with the PS, and facilitated dual functionalization with the PS and the targeting ligand. These results demonstrate that protein cage architectures can serve as versatile templates for engineering nanoplatfoms for targeted antimicrobial PDT.

### Introduction

The limitations of conventional antimicrobial therapy are being exposed by the manifestation of two forms of microbial resistance: acquired antibiotic resistance exhibited at the single cell level, and the intrinsic resistance of microbial biofilm communities.<sup>238, 239</sup> A promising alternative, photodynamic therapy (PDT), relies on photosensitizers (PS) which when activated by light produce reactive oxygen species (ROS). ROS react with accessible cell components, subverting their function, and finally causing cell death.<sup>240, 241</sup> Bacterial strains that have acquired resistance to conventional antimicrobials are susceptible to antimicrobial PDT.<sup>242-244</sup> In addition, antimicrobial PDT

has been successfully used to control biofilms<sup>245-247</sup>, and forms the basis for emerging adjuvant and alternative treatments for biofilm infections of the oral cavity.<sup>248</sup>

Collateral damage to host tissue is a substantial concern for all forms of PDT, and the capability to more specifically target microbes with PS would significantly expand the range of possible clinical applications.<sup>249</sup> Nanoplatforms provide a relatively large surface area that can be used to engineer the presentation of both the PS and targeting ligand to combine cell selectivity with photodynamic killing.<sup>250</sup> Self-assembling protein cage architectures offer an exceptionally versatile template for design of multifunctional nanoplatforms that optimize functional group presentation, and thus are ideally suited as delivery vehicles for selective PDT<sup>36, 43, 50, 68, 85, 251-255</sup>. The unique subunit structure of protein cages enables genetic insertion of chemically reactive amino acids or biologically reactive peptides into the monomeric protein subunits. These are subsequently presented symmetrically over the entire outer (or inner) surface of the assembled protein cage.<sup>77, 98</sup> The ability to use a combination of genetic and chemical modifications to engineer protein cages expands the possibilities for design of multifunctional nanoplatforms that can be used for selective targeting of PS. In addition, their non-specific adsorption to mammalian cells is minimal<sup>98, 256</sup>, probably due to their intrinsic negative surface charge at physiological pH.

A fundamental issue that needs to be addressed before any class of PS functionalized nanoplatforms can be effectively engineered for selective antimicrobial PDT is whether the ROS generated by PS delivered by the nanoplatforms can inactivate microbes. In contrast to mammalian cells prokaryotes have no means such as endocytosis to allow entry of nanoplatforms into the cytoplasm. Although membrane damage is

generally considered to be the primary cause of PDT induced cell death<sup>257</sup>, this has not been unequivocally established.<sup>241</sup> The relatively large molecular size of nanoplateforms may limit the proximity of PS to critical cell components. Proximity is essential for the activity of singlet oxygen ( $^1\text{O}_2$ ) which has a very short diffusion length (<50 nm) and is thought to be the primary ROS causing oxidative damage.<sup>258</sup> Here we show that a genetically modified protein cage nanoplateform functionalized with PS can be targeted to *S. aureus* cells, and that the cell-bound PS functionalized protein cages induce light activated killing under standard light fluence conditions.

### Experimental

#### Genetic Modification of CCMV/K42R and Production of the Constructs in Plants

The salt stable mutant of cowpea chlorotic mottle virus in which lysine 42 has been replaced by an arginine (CCMV/K42R) has been described previously.<sup>259</sup> Using this construct as starting material, polymerase chain reaction based site directed mutagenesis was used to produce S102C/K42R and S130C/K42R, in which serines (102 and 130, respectively) were replaced by cysteines. Complementary oligonucleotide primers were: 5' GTTGCTTCCCAGTGTTTGTGGTACCGTGAAATCCTGTG 3' (S102C/K42R, plus strand), 5'CACAGGATTTACGGTACCACAAACACTGGGAAGCAAC 3' (S102C/K42R, minus strand); 5'GCTGTGGCCGACAATTGCAAAGATGTTGTTCGC 3' (S130C/K42R, plus strand) and 5' GCGACAACATCTTTGCAATTGTGCGCCACAGC 3' (S130C/K42R, minus strand). Plasmids containing the mutated sequences were first screened by digestion with unique restriction enzymes (KpnI for S102C/K42R and MfeI

for S130C/K42R). The mutations in the CCMV coat proteins of S102C/K42R and S130C/K42R were confirmed by DNA sequencing. Constructs were produced in cowpea plants and the virus was isolated as previously described.<sup>260</sup> Purity was verified using size exclusion chromatography (SEC) (Superose 6, Amersham Biosciences) and dynamic light scattering (DLS)<sup>261</sup>.

### Synthesis of Functionalized PS

5-Iodoacetoamino-1,10-phenanthroline (phen-IA) was synthesized by modification of a previously published protocol.<sup>262</sup> A solution of 1,3-dicyclohexylcarbodiimide (5.29 g) and iodoacetic acid (4.76 g) in 50 mL dry ethyl acetate was stirred for 3 hours at room temperature. The resulting solution was filtered to remove the urea, dried by rotary evaporation and redissolved in 25 mL acetonitrile. This solution was added to 25 mL of acetonitrile containing 5-amino-1,10-phenanthroline (1.0 g, 0.005 mol) and stirred overnight at room temperature. The product was collected by centrifugation, washed with cold 5% sodium bicarbonate and water, and dried under vacuum.

$\text{Ru}(\text{bpy})_2\text{Cl}_2$  was synthesized according to literature procedures.<sup>215</sup>  $\text{RuCl}_3 \cdot 3\text{H}_2\text{O}$  (7.8 g), bipyridine (9.36 g), and lithium chloride (8.4 g) were refluxed in dimethylformamide (50 mL) for 8 h. The reaction was cooled to room temperature, 250 mL of acetone was added and the solution was stored at 4°C overnight. The resultant product was filtered and washed with water and ether and dried by suction.

$\text{Ru}(\text{bpy})_2\text{phen-IA}$  was synthesized by modification of a previously published protocol.<sup>214</sup>  $\text{Ru}(\text{bpy})_2\text{Cl}_2$  (0.7 g) and phen-IA (0.5 g) were refluxed in 50 mL MeOH for 3 h with stirring. The solution was filtered and the product was precipitated by the addition of a concentrated aqueous solution of ammonium hexafluorophosphate. The

orange solid was collected by filtration and washed with cold water and ether and dried in a desiccator.

PS Conjugation to S102C/K42R and S130C/K42R to Produce S102C/K42R-PS and S130C/K42R-PS

Genetically modified CCMV (S102C/K42R or S130C/K42R) ( $1.5 \text{ mg mL}^{-1}$ , 75  $\mu\text{M}$  monomer subunit) was dialyzed into 50 mM HEPES (pH 6.5) overnight and then transferred into fresh dialysis buffer (200 mL). Nitrogen was bubbled into the dialysis buffer for 1h. 40  $\mu\text{L}$  of a 5.5 mM solution of the PS (Ru(bpy)<sub>2</sub>phen-IA) (0.18 mM) in DMS was added to 1.2 mL of the CCMV solution. This was stirred in the dark at room temperature for 5h and then dialyzed into 50 mM HEPES (pH 6.5). SEC was used for further purification. Fractions eluting from within the CCMV peak, the position of which was predetermined using unlabeled CCMV, were combined. These were further purified by dialysis into 100 mM sodium acetate buffer (pH 4.8) and concentrated to  $1.0 \text{ mg mL}^{-1}$  protein using ultrafiltration (Amicon). Integrity of the labeled virus was confirmed by DLS and TEM as described previously.<sup>261</sup> UV-visible spectroscopy was used to determine the molar ratio of PS to monomer subunit. PS-conjugated S102C/K42R and S130C/K42R are referred to as S102C/K42R-PS and S130C/K42R-PS, respectively.

Biotinylation of S130C/K42R-PS to Produce S130C/K42R-PS-B

S130C/K42R-PS was biotinylated by reaction with a 0.5 mM solution of Sulfosuccinimidyl-6'-(biotinamido)-6-hexanamido hexanoate (Pierce) with  $1.0 \text{ mg mL}^{-1}$  S130C/K42R-PS (50  $\mu\text{M}$  monomer subunit) in 50 mM HEPES buffer, 150 mM sodium chloride at pH 7.1 at room temperature in the dark with stirring for 30 min. The reaction

was terminated by exchange into 1 mM sodium acetate buffer at pH 4.8 using SEC.

Fractions eluting from within the CCMV peak, the position of which was predetermined using unlabeled CCMV, were combined and further purified by dialysis into 100 mM sodium acetate buffer (pH 4.8), and concentrated to 1.8 mg mL<sup>-1</sup> CCMV using ultrafiltration (Centricon Microcon YM-10). Integrity of the labeled virus was confirmed by DLS and TEM. Association of the biotin functional groups with the biotinylated product (S130C/K42R-PS-B) was initially tested by a dot blot assay using alkaline phosphatase conjugated anti-biotin antibody (A-7064, Sigma-Aldrich Co.).

#### Liquid Chromatography/Electrospray Mass Spectrometry

Liquid chromatography/electrospray mass spectrometry (LC/MS) was performed on a QToF Micro instrument (Waters). CCMV injected at 0.5-1.0 mg mL<sup>-1</sup> (1-2 µL) was eluted from a Thermoelectric Biobasic size exclusion column (250 x 1 (mm)) in 80% isopropyl alcohol and 0.1 % formic acid. The virus disassembled into monomers during the ionization process that were analyzed by the detector. Mass spectra of the multiply charged ions were deconvoluted using instrument software to produce a representation of monomer mass versus intensity.

#### Cell Culturing

ATCC strain 12598 (Cowan I) was maintained at -80°C in 20% glycerol, 2% peptone. Solid medium (Nutrient agar, Difco 0001) was streaked for single colonies. A single colony was used to inoculate 5 mL of a broth culture (Nutrient broth, Difco 0003) and placed on a shaker at 37°C at 280 rev min<sup>-1</sup> overnight. Fresh broth (5 mL) was inoculated from the starter culture to achieve an OD<sub>600 nm</sub> of 0.250 and incubated as above

for 3 h. This procedure resulted in a culture that had undergone about 3 doublings in the fresh medium and was in exponential phase (Supporting Information, Figure 1S).

#### Electrostatic Targeting of S102C/K42R-PS to Cells.

Cells were cultured at 37°C in batch in nutrient broth, harvested in exponential phase, and resuspended in phosphate buffered saline (PBS) (10mM sodium phosphate, 100 mM sodium chloride, pH 7.0) to an OD<sub>600nm</sub> of 0.2 (approximately 1.5 x 10<sup>7</sup> colony forming units (CFU) per mL). To form the poly-L-lysine (PLL) interlayer the cell suspension was exposed to 6.25 µg mL<sup>-1</sup> PLL (P1274, Sigma-Aldrich Co.) for 5 min. These cells were then exposed to S102C/K42R-PS (2 µM equivalent PS concentration).

#### Targeting of Cells with S130C/K42R-PS-B Using Complementary Biological Interactions

We modified previously published protocols to react biotinylated antibody with the cells and then react streptavidin (StAv) to the antibody coated cells.<sup>263, 264</sup> Exponential phase cells were pelleted at 4500Xg for 10 min and resuspended in 5 mL PBS at an OD<sub>600nm</sub> of 0.3. The cell suspension was incubated for 30 min on ice with a 1:100 dilution of biotinylated anti-protein A (SpA) monoclonal antibody (anti-SpA mAb-B) (P3150 Sigma-Aldrich Co.). This cell suspension was washed once in PBS to achieve a 1:100 dilution of the antibody and incubated with 20 µg mL<sup>-1</sup> StAv (Sigma-Aldrich Co.) for 30 min on ice. After two wash steps in PBS (a 1:400 dilution of the StAv) the OD<sub>600nm</sub> of the cell suspension was adjusted to 0.2. These cells were then exposed to S130C/K42R-PS-B (2 µM equivalent PS concentration).

### Field Emission Scanning Electron Microscopy

PLL coated Si <100> wafers (Virginia Semiconductor Inc., Fredericksburg, Virginia) were exposed to cells fixed in 3% glutaraldehyde in PBS for 1h. The coupons were rinsed twice in Nanopure water and dried under a stream of liquid nitrogen. The coupons with adsorbed cells were coated with a thin film of iridium by exposing the sample for 15 s at 20 mA in a Emitech sputter coater. Cells were viewed with a Supra 55VP FESEM (Zeiss) using the Inlens detector at 1 kV and 3 mm working distance.

### Evaluation of Photodynamic Killing

For evaluation of photodynamic killing at the low fluence rate cell suspensions were distributed in wells of a 96-microtiter well plate (200  $\mu$ l) immediately after addition of S102C/K42R-PS, and were exposed to a bank of LEDs (peak emission at 470 nm) in a temperature controlled light box (20°C) at a fluence rate of 0.763 mWcm<sup>-2</sup> for 3.5 h yielding a total fluence of 9.6 Jcm<sup>-2</sup>. For evaluation of photodynamic killing at the more standard fluence rate (46 mWcm<sup>-2</sup>) cell suspensions were distributed into wells formed in a Teflon block (100  $\mu$ L per well) 20 min after addition of S102C/K42R-PS or S130C/K42R-PS and illuminated by light from a mercury vapor lamp coupled to a Nikon Eclipse E600 microscope using a B2A filter block (excitation 450-490 nm; emission > 515 nm). The stage height was adjusted so that the surface of the cell suspension was beyond the focal point of the 20X objective, 17 mm from the surface of the objective lens, to achieve a circular 7mm spot that covered the surface of the cell suspension in the well. Fluence rate was determined using a photometer. Times of illumination were 5, 10 and 20 min to achieve total fluences of 13.8, 27.6 and 55.2 Jcm<sup>-2</sup> which are in the

standard range for antimicrobial PDT. Viable cells numbers were evaluated by enumeration of CFU on solid medium (Nutrient agar) after incubation at 37°C for 16 h.

### Optical Microscopy

The red fluorescence from the PS associated with targeted cells was observed at 1000 X (100 X objective and 10 X ocular) using the same microscope and filter set as used to illuminate cells for photodynamic killing at standard fluence rates. The fluorescence emission under these conditions is faint and rapidly photobleaches. However, the red fluorescence from targeted cells was clearly visible. Images were acquired with an Olympus Camedia camera coupled to the microscope.

### Results and Discussion

PS conjugated nanoplatfrom was targeted to *S. aureus* cells. *S. aureus* is a prominent player in infections that are difficult to treat due to acquired resistance to antibiotics<sup>265, 266</sup> and is also one of the primary biofilm forming microbes involved in persistent biofilm infections.<sup>238</sup> In some cases *S. aureus* strains with acquired resistance, which is orchestrated at the single cell level, form biofilms, which are further protected by a community-derived transient intrinsic resistance.<sup>267, 268</sup> Thus, certain classes of *S. aureus* infections are prime candidates for PDT.

As a delivery vehicle for PS we modified a genetic construct (K42R) of cowpea chlorotic mottle virus (CCMV) that is exceptionally stable (referred to as CCMV/K42R).<sup>259</sup> CCMV is an icosahedral plant virus with a 28 nm diameter (Figure 1). The capsid is a protein cage architecture that self-assembles from 180 identical protein subunits. CCMV

has served as a template for implementing several novel approaches for spatial control of multiligand presentation.<sup>207, 269</sup>

Site specific mutagenesis was used to replace serines with reactive cysteines at surface exposed sites on each of the 180 (20 kDa) protein monomer subunits that form the assembled CCMV/K42R protein cage. These served as attachment points for the PS (Figure A.1). The PS, a ruthenium complex, was modified to react selectively with the sulfhydryls of the surface exposed cysteines on these genetic constructs (S102C/K42R

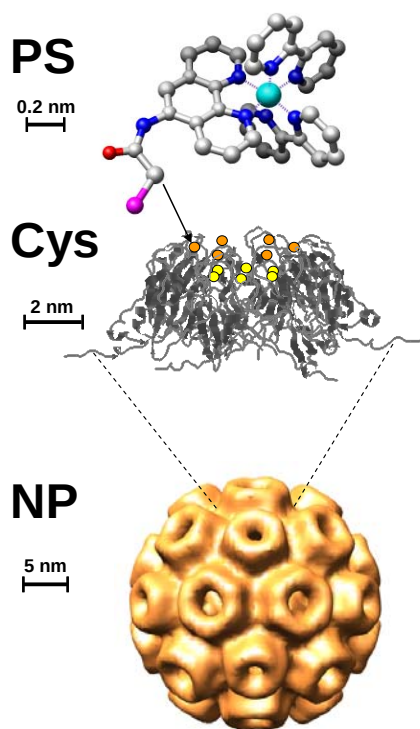


Figure A.1. The genetically modified CCMV nanoplatform (NP) was functionalized with the PS (RuIphen) at surface exposed sites. The positions of the sulfhydryl groups of the cysteines (Cys) added into the two genetic constructs are shown for a hexamer of protein subunits: S102C/K42R (orange) and S130C/K42R (yellow); the position of these sulfhydryls on the pentamers is similar. The PS was modified to promote reaction with exposed sulfhydryls by adding an iodoacetamide group to the phenanthroline moiety.

and S130C/K42R) by functionalizing the phenanthroline moiety with an iodoacetamide group (Figure A.1). PS conjugated constructs are referred to as S102C/K42R-PS and

S130C/K42R-PS, respectively. UV-visible absorption spectroscopy was used to establish the association of the PS with each construct and to determine the molar ratio of bound PS to monomeric subunits (estimated as 0.520 for S102C/K42R-PS and 0.380 for S130C/K42R-PS). In addition, LC/MS was used to confirm covalent linkage of the PS to the CCMV constructs.

We have previously used both electrostatic and complementary biological interactions to direct the assembly of functionalized CCMV into organized films.<sup>207</sup> These same two strategies were used to target PS conjugated nanoplateforms to the cells (Figure 2a). Cationic polymers, including poly-L-lysine (PLL), have been used to enhance the activity of PS.<sup>270, 271</sup> In general, cell walls tend to carry a net negative charge. Thus, we predicted that electrostatic interactions could provide a simple method to non-specifically target the nanoplateform to *S. aureus*. In addition, PLL has intrinsic antimicrobial properties<sup>272</sup> suggesting that an interlayer of PLL on the cell wall would enhance the activity of a PS functionalized nanoplateform. Whereas electrostatic interactions can be used to mediate relatively non-specific targeting to cells, complementary biological interactions offer a means to selectively target PS to specific microbes.<sup>273-275</sup> S130C/K42R-PS was specifically targeted to protein A (SpA) presented in the cell wall of *S. aureus* by using streptavidin (StAv) to couple biotinylated anti-SpA monoclonal antibody (anti-SpA mAb-B) to biotinylated nanoplateform (Figure 2a). SpA binds to the Fc region of IgG and may play a role in immune avoidance<sup>186</sup>. We used flow cytometry to characterize expression of SpA in the *S. aureus* ATCC 12598 strain cultured under our conditions.

A biotin targeting ligand was added to the endogenous lysines of S130S/K42R-PS to produce S130S/K42R-PS-B. The S130C/K42R construct was chosen for dual functionalization with PS and the biotin functional group based on the consideration of minimizing possible interference with binding of StAv to the biotin targeting ligand due to steric hindrance from the relatively large PS functional group. Preliminary results indicated that one of the most reactive endogenous lysines (K87) is located near the pseudo three fold axis of the CCMV viral capsid which is well spaced from the reactive cysteines on S130C/K42R. Covalent linkage of the biotin functional groups into the S130C/K42R-PS-B construct was confirmed by LC/MS.

The presence of PS conjugated nanoplatfrom on targeted cells was first verified by epi-fluorescence microscopy (Figure A.1b-d). Targeted cells appeared red while cells exposed to PS conjugated nanoplatfrom without targeting produced a faint green intrinsic fluorescence which was only visible in images acquired at sufficiently long exposure times. The red fluorescence from cells targeted using electrostatic interactions was clearly more faint than the fluorescence from cells targeted using complementary biological interactions. The red background in the images originates from free PS conjugated nanoplatfrom in solution. The presence of PLL probably precipitates a portion of the nanoplatfrom in solution causing the diffuse background in Figure 2b to be fainter than that in Figure 2c.

The arrangement of targeted nanoplatfrom on the cell wall was characterized directly by FESEM (Figure A.3<sup>207</sup>). The FESEM images indicated that the cell wall

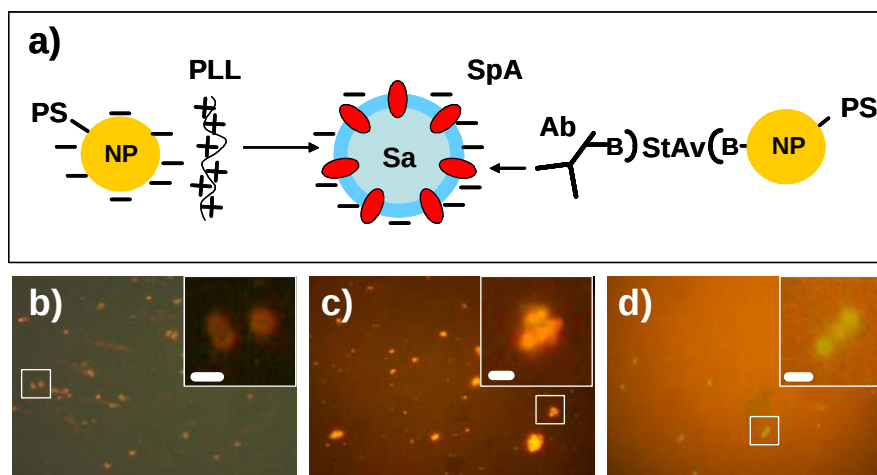


Figure A.2. Targeting of PS functionalized CCMV nanoplateforms to *S. aureus* cells. a) Targeting strategies using electrostatic (left) or complementary biological interactions (right). Abbreviations are (from left to right): photosensitizer (PS); nanoplateform (NP); poly-L-lysine (PLL); *S. aureus* cell (Sa); protein A (SpA); anti-SpA-mAb-B (Ab), biotin (B) and streptavidin (StAv). b, c, d) Epi-fluorescence images: b) Cells targeted with S102C/K42R-PS via electrostatic interactions (exposure time, 0.5 s); c) cells targeted with S130C/K42R-PS via complementary biological interactions (exposure time, 0.5 s); d) Cells mixed with S130C/K42R-PS (non-targeted) (exposure time, 1 s); the cells in d) appear green due to faint intrinsic fluorescence; the red background originates from the PS conjugated nanoplateform in solution. Scale bars in the magnified inserts are 1  $\mu\text{m}$ .

associated nanoplateforms maintained their integrity. Targeting via electrostatic interactions produced more sparse coverage than targeting via complementary biological interactions and, in addition, targeting via complementary biological interactions produced a more uniform coverage. Twenty images were acquired of cells targeted via this latter scheme. The image presented in figure A.3c is representative of the high density of coverage exhibited by cells targeted with complementary biological interactions. The level of non-specific binding of non-targeted PS functionalized nanoplateform (S102C/K42R-PS) shown in Figure 1a is representative of the low level observed for S130C/K42R-PS as well.

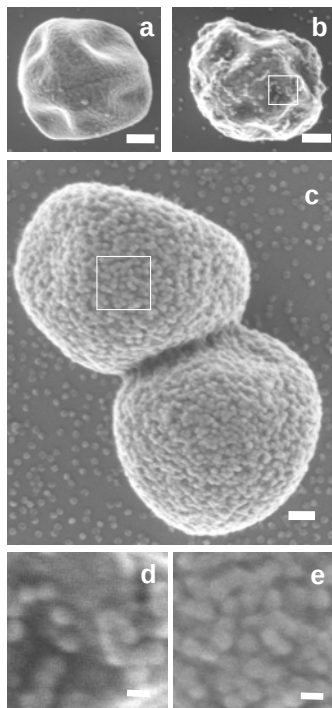


Figure A.3. FESEM images showing the arrangement of targeted nanoplateforms on the cell wall: a) cell exposed to S102C/K42R with no targeting; b) cell targeted with S102C/K42R-PS via electrostatic interactions; c) cell targeted with S130C/K42R-PS via complementary biological interactions; d) magnified view of the region indicated in b); e) magnified view of the region indicated in c). Scale bars: a ,b) 200 nm; c) 100 nm; d, e) 28 nm (the diameter of CCMV); note: CCMV in solution adsorbs strongly to the positively charged substratum used for FESEM visualization.

Cell populations targeted with the PS-conjugated CCMV constructs via both electrostatic and complementary biological interactions were photodynamically inactivated to a significant level (Figures A.4-6). Cells targeted with S102C/K42R-PS via electrostatic interactions mediated by PLL were photodynamically inactivated at a very low fluence rate (Figure A.4) as well as at a more standard fluence rate and standard total fluence levels (Figure A.5). Without the PLL interlayer PS conjugated to the nanoplateform was almost ineffectual in killing cells: non-targeted PS conjugated nanoplateforms (S102C/K42R-PS and S130C/K42R-PS) did not induce appreciable photodynamic killing under conditions in which free PS in solution reduced viable cell numbers by 5 orders of magnitude. Results presented in Figures A.4 and A.5 show that a

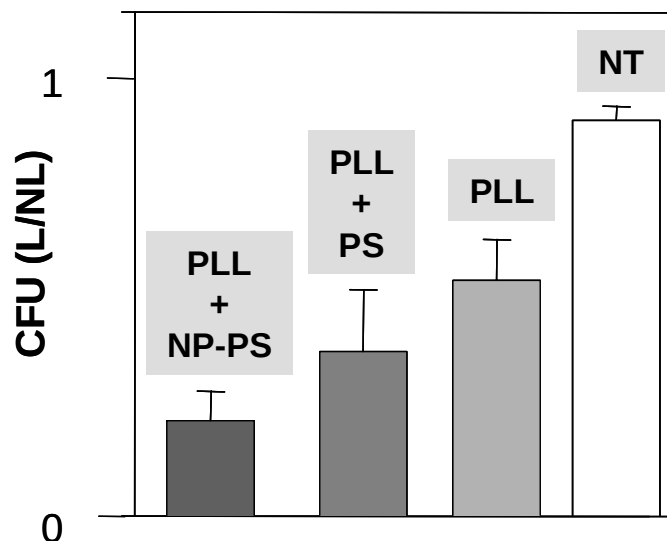


Figure A.4. Photodynamic inactivation of *S. aureus* cells targeted with PS functionalized nanoplatfrom (NP-PS) via electrostatic interactions at a fluence rate of  $0.763 \text{ mWcm}^{-2}$  (total fluence of  $9.6 \text{ Jcm}^{-2}$ ). Data are presented as the ratio of CFU for illuminated cells to CFU for cells maintained in the dark. Error bars are standard errors for the mean of 8 independent replicates: PLL + NP-PS: cells exposed to PLL and targeted with S102C/K42R-PS at  $2 \mu\text{M}$  equivalent PS concentration; PLL + PS: cells exposed to PLL and the photosensitizer ( $2 \mu\text{M}$ ); PLL: cells exposed only to PLL; NT: no treatment. A paired t-test indicated that the difference between the means for the pairs (PLL) and (PLL+NP-PS) were significant at the 1% level, whereas the difference between the means for the pairs (PLL+PS) and (PLL+NP-PS) were not significant at the 10% level. (The difference between the mean for the untreated conditions was significantly different from means for the other three conditions at the 1% level).

PLL interlayer on the cell wall renders the level of killing activity of PS conjugated to the nanoplatfrom to within the range of free PS. Under dark conditions, PLL at the concentration used for these experiments ( $6.25 \mu\text{g mL}^{-1}$ ) reduced the viable cells by about half an order of magnitude, probably by compromising the cell membrane (Figure 4 and 5c). Association of S102C/K42R-PS with cells pre-exposed to PLL significantly reduced the number of viable cells compared to exposure to PLL alone for experiments at the low fluence rate (8 independent replicates, paired t-test, p value 0.006) (Figure A.4). This same trend was observed at a more standard fluence rate and standard total fluence levels (Figures A.5a and c). The association of PLL with the cell wall also enhanced the killing

activity of free PS in solution (compare Figure A.5b and Figure A.6c) as anticipated from previous results<sup>270, 276</sup>. The dependence of the killing activity on light fluence in the absence of PS exhibited in Figure A.5c, and much less prominently in Figure A.5d,

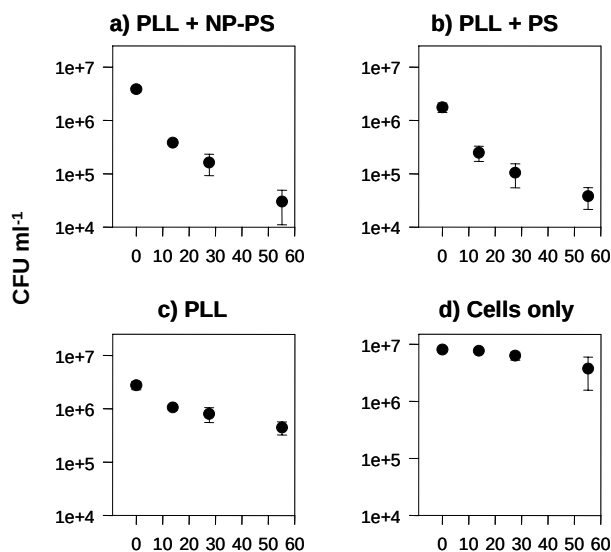


Figure A.5. Photodynamic inactivation of *S. aureus* cells targeted with PS functionalized nanoplateform (NP-PS) via electrostatic interactions at a fluence rate of 46 mWcm<sup>-2</sup>. Data are presented as CFU mL<sup>-1</sup> recovered after treatment. Error bars are standard errors for the mean of 2 independent replicates. a) cells exposed to PLL and targeted with S102C/K42R-PS at 2  $\mu$ M equivalent PS concentration; b) cells exposed to PLL and the photosensitizer (2  $\mu$ M); c) cells exposed only to PLL; d) no treatment.

suggests that there were intrinsic PS associated with the cell wall whose killing activity was also enhanced by PLL<sup>277, 278</sup>. The killing activity of S102C/K42R-PS targeted via electrostatic interactions is likely attributable to a combination of membrane disruption by PLL and proximity of the PS to the cell membrane. The results suggest that the killing activities conferred by a cationic polymer or peptide and a PS both conjugated to a nanoplateform could be combined to produce significant levels of cell inactivation.

S130C/K42R-PS-B targeted to *S. aureus* cells using complementary biological interactions produced levels of photodynamic killing that were significantly greater than the free PS in solution (2  $\mu$ M) and non-targeted S130C/K42R-PS at an equivalent PS

concentration (Figure A.6). Data presented in Figure 6 are the means and standard error for three independent experiments. The reduction in viable cells at 27.6 and 55.2 Jcm<sup>-2</sup> is significantly greater at the 5% level of confidence for cells targeted with S130C/K42R-PS-B compared with cells exposed to S130C/K42R-PS (not targeted) and cells exposed to PS alone (unpaired t-test).

The level of killing produced in cells targeted via complementary biological interactions (Figure A.6a) was approximately the same as that achieved by targeting SpA in *S. aureus* with an antibody conjugated to a PS under comparable conditions of both light fluence levels and equivalent PS concentration.<sup>275</sup> In general, nanoplateforms possess a relatively large loading capacity and large surface area compared to the other available targeting vehicles. For example, CCMV has a molecular weight that is about 25 times that of an IgG molecule. It is possible that by loading more PS onto the nanoplateform the killing activity could be enhanced. Alternatively, the killing activity might have been limited by the distribution of SpA expression in the cell population as proposed in the case of targeting with PS conjugated antibody. The Cowan I strain used in our experiments is known to express SpA at a high level.<sup>263</sup> However, according to the flow cytometry analysis the level of expression of SpA in a small portion of the cell population was very low or negligible. Our results indicate that the majority of these non-targeted cells would survive exposure to the fluence levels used in our experiments. The PS loading advantage offered by a targeted CCMV nanoplateform (compared to a smaller biomolecule such as an antibody) might have been more evident in a case in which epitope presentation in the cell wall was more sparse, but uniformly distributed among the entire cell population.

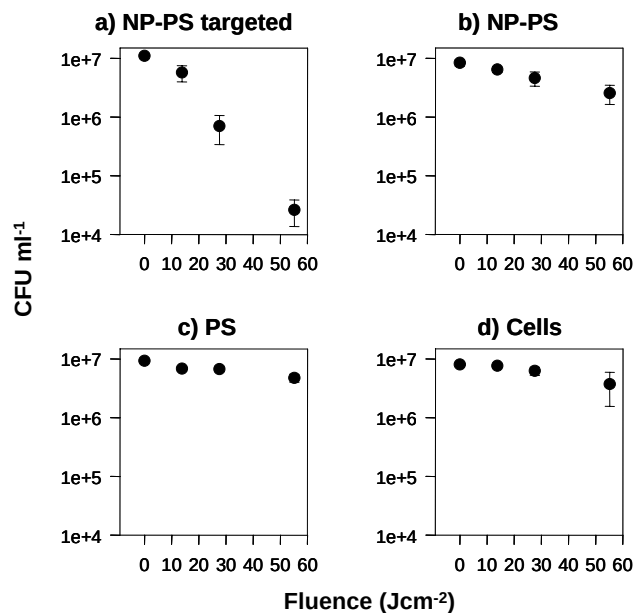


Figure A.6. Photodynamic inactivation of *S. aureus* cells targeted with PS functionalized nanoplatform (NP-PS) via complementary biological interactions at a fluence rate of 46 mWcm<sup>-2</sup>. Data are presented as CFU mL<sup>-1</sup> recovered after treatment. Error bars are standard errors for the mean of 3 independent replicates. a) cells targeted with S130C/K42R-PS-B at 2  $\mu$ M equivalent PS concentration; b) cells exposed to S130C/K42R-PS at 2  $\mu$ M equivalent PS concentration (non-targeted); c) cells exposed to 2  $\mu$ M PS; d) cells targeted with anti-SpA-mAb-B and StAv but not with the PS functionalized nanoplatform. An unpaired t-test indicated that the means for the targeted cells were significantly different from means for all other conditions at fluences of 27.6 and 55.2 Jcm<sup>-2</sup> at the 5% level.

In addition to offering the possibility of delivering substantial PS per binding event, the relatively large surface area of nanoplatforms provides space for decoration with multiple targeting ligands. The ability to engineer placement of multiple targeting ligands on a PS delivery vehicle opens up the possibility of finely tuning selectivity. This capability could be used as a means to eliminate pathogens while preserving beneficial commensal microbes for treatment of oral infections<sup>248</sup>, or to insure selective eradication of genetic or physiological variants of a pathogen causing a recalcitrant wound infection<sup>249</sup>. In the case of *S. aureus* there are at least 22 different cell wall anchored proteins that are involved in conferring virulence and could potentially be targeted with appropriately functionalized nanoplatforms.<sup>279, 280</sup>

For topical applications the indirect targeting scheme used in our experiments would be feasible. Similar strategies, involving pretargeting with StAv, have been proposed even for intravenous applications.<sup>281</sup> Protocols for biotinylation of antibodies that preserve binding affinity of the active site are well established, and our results demonstrate that site specific dual labeling of CCMV genetic constructs with both a functional biotin and an active PS is manageable. In this context, nanoplatfroms such as protein cage architectures provide an accessible template for implementing a modular approach to selective PDT. The advantage of this alternative approach is that strategies for targeting multiple ligands and optimizing PS loading per binding event could be developed independently and then coupled via the nanoplatfrom.

### Conclusion

Our results demonstrate the feasibility of using PS functionalized nanoplatfroms based on genetically modified CCMV as targeted delivery vehicles for selective antimicrobial PDT. If the requirement for selectivity is not too stringent, electrostatic interactions can be used to target PS functionalized CCMV to microbial pathogens. In this case relatively dilute concentrations of the cationic polymer significantly enhance the photodynamic killing. To achieve true selectivity, complementary biological interactions can be used. The simplest strategy in this case is to dual functionalize the nanoplatfrom with a biotin and the PS. The advantage of being able to use a combination of both chemical and genetic methods to control functional group presentation lends protein cage architectures a versatility not offered by most other nanoplatfroms. This versatility can be exploited to pursue a modular approach to design of vehicles for selective antimicrobial PDT in which PS loading and targeting functions can be independently optimized.

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