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New Synthetic Methods for Integrating Metal Nanoparticles with Biomolecules

by

Stacy Louise Capehart

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate Division of the University of California, Berkeley

Committee in charge:

Professor Matthew B. Francis, Chair
Professor Michelle C. Chang
Professor Gerard Marriott

Spring 2014
New Synthetic Methods for Integrating Metal Nanoparticles with Biomolecules

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By Stacy Louise Capehart
Abstract

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Doctor of Philosophy in Chemistry
University of California, Berkeley
Professor Matthew B. Francis, Chair

Integrated systems that incorporate biomolecules, organic small molecules, and inorganic materials with precise, nanoscale positional relationships present a great synthetic challenge yet to be mastered by ‘top down’ engineering approaches or ‘bottom up’ chemical synthetic methods. Focusing on this challenge, we have developed well defined protein-inorganic nanomaterial conjugates to understand fundamental photophysical properties and develop new materials for targeted dual fluorescence/electron microscopy. First, we designed and constructed three systems to explore the effect of metal nanoparticles on organic fluorophores. The basis of these systems was the spherical protein capsid of bacteriophage MS2, which was used to house either organic fluorophores or gold particles within its interior volume. The exterior surface of each capsid was modified with ssDNA to facilitate the placement of either a gold nanoparticle or a fluorophore of interest at a fixed distance from the capsid. Next, pairing reported external modification strategies for MS2 that target both native and unnatural amino acids with a variety of interior inorganic cargo, we sought to produce additional materials for metal controlled fluorescence (silver nanoparticles), dual fluorescence/electron microscopy (CdSe/ZnS quantum dots and CdSe/CdS dot rods), and electron microscopy (discrete gold nanoparticle assemblies). Finally, a previously reported oxidative coupling reaction between anilines and ortho-aminophenols was adapted for gold nanoparticle bioconjugation. The reaction proceeds under mild conditions with fast reaction times and little-to-no background protein adsorption to the gold nanoparticles.
Dedicated to my parents,
Tenneille Weston and Gail Capehart
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There are a number of Francis group graduate students and postdocs that I have overlapped with throughout the years (35!). They have made and continue to make the lab a fun and productive environment. Nick taught me some key initial techniques in the group, including AFM and TEM. He always had a great excitement for a diverse set of research projects in the lab, and he was easily engaged in conversations about experimental design.

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a calm demeanor in good times and bad. He’s been a great friend both inside and outside of lab. I hope someday he’ll forget about the terrible time he had on his first backpacking trip to Yosemite, and go on another trip with us. I promise I would never knowingly pack a metal shovel and metal hammer in his backpack.

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Finally, and most importantly, I have to thank my dad. I can honestly say that not a day has gone by in graduate school that I don’t think of him. I try not to focus on his absence, but embrace his continued presence in my life. Almost all of his life’s passions: science, running, and hiking have become my passions. In sharing each of these interests, I feel incredibly close to him and often imagine what he would say in these moments. Nothing brings me greater joy.
Chapter 1

Controlled integration of gold nanoparticles and organic fluorophores using synthetically modified MS2 viral capsids

Abstract

The placement of fluorophores in close proximity to metal nanoparticle surfaces is proposed to enhance several photophysical properties of the dyes, potentially leading to improved quantum yields and decreased photobleaching. It is difficult in practice, however, to establish and maintain the nanoscale distances that are required to maximize these effects. The type of metal, size, and shape of the nanoparticle, the physical distance separating the metal nanoparticle from the organic dye, and the spectral properties of the fluorophore itself are all proposed to influence the quantum yield and lifetime. This results in a complex behavior that can lead to either enhanced or quenched fluorescence in different contexts. In this report, we describe three systems that can be used to explore these effects, while physically preventing the fluorophores from contacting the nanoparticle surfaces. The basis of these systems is the spherical protein capsid of bacteriophage MS2, which was used to house either organic fluorophores or gold particles within its interior volume. The exterior surface of each capsid was modified with ssDNA to facilitate the placement of either a AuNP or a fluorophore of interest at a fixed distance from the capsid. Due to their well-defined and modular nature, these architectures allowed for the exploration of the many variables involved in metal-controlled fluorescence, leading to a better understanding of this phenomenon. The major results from each system are discussed within this chapter.

1.1. Introduction to metal controlled fluorescence

Biological structures, such as proteins\textsuperscript{1-6} and nucleic acids,\textsuperscript{7-9} are finding increasing use for the positioning of multiple chemical groups into complex assemblies that have specifically defined orientations and spacings. These approaches capitalize on the nanoscale features inherent in biomolecules, which through self-assembly can bring attached components together into a functional whole. In addition to providing structural precision, modularity, and synthetic efficiency, the rigid nature of many biomolecules allows the distance relationships to be maintained after they are established. As ever more sophisticated targets are pursued, new bioconjugation methods will be required to allow different combinations of biomolecules to be merged with an expanding number of functional small molecules. In addition, methods will be required to interface biomolecules with inorganic surfaces and particles in a well-defined manner.

One compelling synthetic target for precise nanoscale synthesis is the controlled integration of organic dyes and metal nanoparticles, as this could allow the exploitation of an interesting phenomenon in nanophotonics.\textsuperscript{10} It has been theoretically postulated and experimentally demonstrated that placing a metal nanoparticle in close proximity to an organic fluorophore significantly alters the photophysical properties of the fluorophore.\textsuperscript{8,10-30} Through coupling interactions with the metal nanoparticle, the fluorophores are predicted to exhibit both an enhanced excitation rate and an accelerated radiative decay rate, as described in Figure 1.1. This results in the desirable properties of an improved quantum yield and a decreased fluorescence lifetime, which could minimize competing photobleaching pathways.\textsuperscript{14}

![Diagram](https://example.com/diagram1.png)

\textbf{Figure 1.1.} a) A fluorophore (green star) in the absence of a metal absorbs a photon at a particular excitation rate (\(E\), green line), promoting it from the singlet ground state (\(S_0\)) to the first excited singlet state (\(S_1\)). Non-radiative vibrational relaxation (blue line) to the first vibrational level of the first excited singlet state is followed by a combination of radiative energy transfer (\(\Gamma\), red line) in the form of an emitted photon and non-radiative energy transfer (\(k_{nr}\), blue line). b) A fluorophore (green star) in the presence of a gold nanoparticle (AuNP, red sphere) results in an additional excitation rate (\(E_m\), bold green line) and radiative energy transfer (\(\Gamma_m\), bold red line). These additional energy terms are hypothesized to result in an increased quantum yield and decreased lifetime of the fluorophore.
This effect originates from the metal nanoparticle surface plasmon resonance (SPR), a strong absorbance characteristic of AuNPs larger than 5 nm and smaller than 150 nm. The SPR arises from the collective oscillation of conduction electrons on the surface of the AuNP, resulting in strongly scattered light of wavelengths close to the surface resonant frequency. The effect of the AuNPs is proposed to depend on the spectral properties of the AuNPs in close proximity to the fluorophore. The precise absorbance profile of AuNPs primarily depends on three factors: shape, size, and the electronic environment of the AuNP. Spherical AuNPs have a symmetric SPB that centers around 500-600 nm, while AuNP rods exhibit both transverse and longitudinal absorbances that vary depending on the rod aspect ratio. Spherical AuNPs of larger diameter display a red-shifted SPB. Proximity to another AuNP in solution (separation distance less than each particle’s individual diameter) also results in a red-shifted SPB.

Theoretical and experimental treatments have indicated that the type of metal, size and shape of the nanoparticle, the physical distance separating the metal surface from the organic dye, and the spectral properties of the fluorophore itself are all critical parameters for achieving a maximum effect.

There are a number of experimental demonstrations of this behavior. As examples of a positive effect, a 2.5-fold increase in fluorescence emission has been observed for fluorophores deposited on copper nanoparticle films, and an increase of up to 8-fold has been observed for organic dyes placed in close proximity (5 nm separation) to 80 nm gold nanoparticles. In addition, an enhancement of 15-fold has been observed for dyes placed ~7.5 nm from the surface of 50 nm silver nanoparticles. In contrast, quenching of up to 99.8% has been observed for fluorophores placed 1-2 nm from a gold nanoparticle.

Theoretical treatments and experimental validations have shown quenching with a $1/R^4$ distance dependence for small (∼2 nm diameter) AuNPs and enhancement for large (>30 nm diameter) AuNPs. However, many inconsistencies still exist in the literature as to the precise influence of AuNPs on organic dyes in the intermediate size regime. Some groups have reported fluorescence quenching near 5 nm and 10 nm diameter AuNPs. Other groups have reported fluorescence enhancements between 2-fold and 17-fold for similarly sized AuNPs.

These studies suggest that the interplay of the many involved variables results in an optimal metal-fluorophore distance that is platform-specific, making it difficult to probe these effects in a systematic manner. They also underscore the critical importance of physically separating the fluorophore from the particles to prevent direct contact quenching. This is a very difficult task in many cases, considering the nanoscale distances that are involved. What is needed to study these effects fully is a series of well-defined and readily adjusted architectures that can vary each of the independent parameters to modulate the fluorescence properties. To address this, we report herein two protein-based systems that can establish desired metal-fluorophore distances, while simultaneously providing a physical barrier that prevents the dyes from coming into contact with the nanoparticle surfaces. We report our observations of metal-controlled fluorescence. These studies highlight the important role that covalently modified protein assemblies can play in the synthesis of multicomponent nanoscale materials.
1.2. MS2 as a biomolecular scaffold to study metal controlled fluorescence

The biomolecular scaffold used to integrate the nanoparticles and dyes in these studies was provided by the protein capsid of bacteriophage MS2, shown in Figure 1.2.41 This structure consists of a 27 nm diameter spherical hollow protein coat that self-assembles from 180 sequence identical monomers. The capsid remains intact upon exposure to a variety of conditions, including complete solvent removal, pH values from 3-10, and temperatures up to 55 °C (in H2O).42 Site-selective modification of MS2 has allowed for the incorporation of useful chemical functionalities on both the exterior and the interior surfaces of the capsid. Interior chemical modifications are normally made possible by the diffusion of small molecules through 2 nm pores in the protein shell—a strategy that had been used to attach taxol,43 Gd-based MRI contrast agents,44,45 F-18 PET labels,46 and various small molecule fluorescent dyes47,48 to amino acid residues on the inner surface. The exterior chemical modification of MS2 has involved the targeting of both native49 and artificial50 amino acids, and has been used to display peptides,49-52 polymers,49,51 and DNA aptamers48 for use in biomedical applications. The external DNA strands have also been used to integrate the capsids into DNA origami assemblies.53

Three bacteriophage MS2 systems were explored to establish desired metal-fluorophore distances. The first design relied on the interior chemical modification of MS2 with a fluorophore of interest, and the exterior attachment of AuNPs through DNA hybridization. The second design relied on the reassembly of MS2 to incorporate a AuNP into the interior volume of the capsid. In this design, fluorophores were incorporated through chemical modification of the exterior surface of MS2. In the third design, DNA origami tiles were used to place AuNPs at fixed distances from dye-labeled MS2 capsids.

![Bacteriophage MS2](image)

*Figure 1.2. Bacteriophage MS2 is a spherical viral capsid protein ~27 nm diameter with a defined interior and exterior surface, ~2 nm thick shell, and 2 nm diameter pores. A TEM image of T19pAF N87C MS2 negatively stained with uranyl acetate is shown on the above right (scale bar = 20 nm).*

1.3. Design 1: Interior fluorophore-modified, exterior AuNP-labeled MS2

The effect of metal nanoparticles on organic fluorophores was first addressed using a viral capsid-based structure rendered in Figure 1.3-a. This design provided for the union of MS2 (externally modified with ssDNA and internally modified with multiple organic fluorophores) and a AuNP (functionalized with a complementary ssDNA). This architecture ultimately allowed for the tuning of (1) the distance between the metal
Figure 1.3. Construction of an MS2-based system for studying the effect of AuNPs on organic fluorophores. a) The structure incorporates MS2 (grey sphere internally modified with organic fluorophores, shown in green, and externally modified with ssDNA) with AuNPs (modified with cDNA). b) Method for introducing T19pAF and N87C mutations. c) SDS-PAGE gel of cell lysate and post-purification for T19pAF N87C MS2. d) Chemical modification of N87C residues with AF 594 maleimide, characterized by e) SEC HPLC, f) LC-MS, and g) RP-HPLC. h) Method for DNA modification of T19pAF residues, confirmed by i) SDS-PAGE gel electrophoresis of MS2-DNA conjugates.
nanoparticle and fluorophore (controlled by the length of the spacer DNA), (2) the SPB of the metal nanoparticle (controlled by the shape, size, and electronic environment of the AuNP), and (3) the absorption/emission properties of the organic fluorophore (intrinsic to the particular fluorophore) to systematically determine each component’s influence on the fluorescence intensity of the overall system. This MS2-AuNP conjugate design requires both interior and exterior chemical modifications of MS2 as well as an exterior chemical modification of AuNPs.

1.3.1. Interior and exterior surface modification of MS2 for design 1

T19pAF N87C MS2 incorporates an unnatural amino acid, para-aminophenylalanine in position 19 (pAF) and a cysteine mutation in position 87. T19pAF N87C MS2 functions as the molecular scaffold for the assembly of the proposed MS2-AuNP conjugate. T19pAF N87C MS2 was successfully expressed and purified according to conditions previously reported, allowing for the installation of 180 solvent accessible sulphydryl groups on the interior surface of MS2 (N87C) and 180 solvent accessible anilines (T19pAF) on the exterior surface of MS2. The N87C mutation allows for the attachment of organic dyes to the interior surface of MS2 with maleimide chemistry while the T19pAF mutation facilitates the attachment of DNA to the exterior surface of MS2 using a previously-reported sodium periodate mediated oxidative coupling strategy. Analysis of the total cell lysate and purified protein by gel electrophoresis is shown in Figure 1.3-c.

T19pAF N87C MS2 was first modified on the interior cysteine with a maleimide fluorophore, shown in Figure 1.3-d. Alexa Fluor 350 (AF 350) and Alexa Fluor 594 (AF 594) maleimide were selected to modify the interior cysteine. The maximum absorbance of AF 350 is blue shifted, while the maximum absorbance of AF 594 is red shifted from the SPB of 15 nm diameter AuNPs, as shown in Figure 1.4. T19pAF N87C MS2 was successfully modified with AF 350 and AF 594, as confirmed by LC-MS and RP-HPLC (Figure 1.3-f and Figure 1.3-g, respectively). Following modification of the interior cysteine, the MS2 capsids remained assembled, as demonstrated by SEC (Figure 1.3-e).

MS2 covalently modified on the interior surface with AF 350 or AF 594 was then modified on the exterior surface with ssDNA using a previously reported sodium periodate-mediated oxidative coupling strategy (Figure 1.3-h). To attach the N,N-diethyl-N'-acylphenylenediamine moiety to DNA, an NHS ester containing N,N-diethyl-

![Figure 1.4](image-url)

*Figure 1.4. AuNP absorbance spectra as well as Alexa Fluor 350 and Alexa Fluor 594 absorbance and fluorescence emission spectra.*
*N*-acylphenylenediamine derivative was synthesized. Amine DNA was then acylated through combination with the NHS-ester containing *N*,*N*-diethyl-*N*'-acylphenylenediamine. The phenylenediamine-containing DNA was then coupled to surface exposed anilines (T19pAF), introduced to MS2 using the amber stop codon suppression technique. T19pAF N87C MS2 conjugated to ssDNA has a lower electrophoretic mobility than unmodified T19pAF N87C MS2 and travels a shorter distance in a denaturing protein gel, shown in Figure 1.3-i. This suggests the covalent attachment of ssDNA to the exterior surface of dye-labeled MS2.

### 1.3.2. Modification of AuNPs for design 1

The next step in assembling the structure outlined in Figure 1.3-a involved modifying AuNPs with ssDNA. A 5' thiolated ssDNA complementary to the sequence attached to the exterior surface of MS2 was incubated with 15 nm diameter spherical AuNPs, yielding AuNPs that should hybridize to the ssDNA attached to the exterior surface of MS2. AuNPs were also incubated with thiolated ssDNA not complementary to the sequence attached to MS2.

The attachment of AuNPs to complementary and non-complementary ssDNA was confirmed through dynamic light scattering (DLS), as shown in Figure 1.5. Unconjugated AuNPs appeared slightly smaller by DLS (14.4 nm diameter) as compared to TEM (15 nm diameter).

![Figure 1.5. DLS measurements of size (particle diameter) distribution by number for a) unmodified 15 nm diameter AuNPs, b) AuNPs modified with ssDNA, c) AuNPs modified with non-complementary DNA, d) AuNPs from c) incubated with a mismatch sequence, resulting in no size increase, and e) AuNPs from b) incubated with a matched sequence, resulting in a 7.2 nm net size increase. The net size increase suggests the attachment of ssDNA to the AuNPs.](image)
nm diameter). Attachment of ssDNA to AuNPs resulted in a slight increase in particle diameter (a net size increase of 1.1 nm). Upon hybridization with the matched sequence, the particle size increased for the complementary sequence (leading to a net size increase of 7.2 nm in the particle diameter). Incubation of the DNA-modified AuNPs with the non-complementary sequence did not, however, lead to a further increase in particle size. This suggests the AuNPs were successfully modified with ssDNA and could still hybridize to their complement. We took these results to indicate that a high level of functionalization was achieved, and thus the particles were carried forward in our experiments.

1.3.3. Combining MS2 and AuNPs for design 1

MS2 capsids modified with AF 350 or AF 594 and ssDNA were added to solutions of AuNPs modified with complementary ssDNA. They were combined with both a low

Figure 1.6. Characterization of DNA-MS2 incubated with cDNA and ncDNA modified AuNPs. a) Representative TEM images of DNA-MS2 incubated with complementary and non-complementary DNA-modified AuNPs (15 nm diameter). Number of AuNPs attached to DNA-MS2 histograms for b) complementary AuNPs and c) non-complementary AuNPs.
concentration of AuNPs relative to MS2 and a high concentration of AuNPs relative to MS2. Transmission electron microscopy (TEM) images showed that AuNPs seemed to be bound specifically to MS2. At low concentrations of AuNPs, one AuNP was often attached per capsid while at a high concentration of AuNPs, MS2 capsids were often surrounded by a ring of AuNPs.

To distinguish specific binding from non-specific binding, the non-complementary DNA-modified AuNPs, ncDNA, were incubated with DNA-modified MS2. TEM images revealed that the non-complementary AuNPs were generally not associated with MS2 capsids, while the complementary AuNPs were often bound to the MS2 capsids (Figure 1.6-a). This was quantified by taking several wide field TEM images and counting the number of AuNPs associated with each MS2 capsid. These results are shown in Figure 1.6b-c and demonstrate that the complementary sequence resulted in 1.6 ± 0.2 AuNPs per capsid (n = 964), while the non-complementary sequence (ncDNA), resulted in 0.4 ± 0.3 AuNPs per capsid (n = 213).

Monitoring changes in the SPB also indicated specific association of the MS2-AuNP conjugates in solution. A red-shift of the SPB was observed upon incubating MS2 with a high concentration of AuNPs. This was not observed in the control samples (AuNPs, AuNPs with DNA, or AuNPs with DNA and MS2 without DNA). This provided strong evidence that several AuNPs were associated with MS2 capsids in solution because their new electronic environment significantly affected their SPR. This effect was studied systematically to determine the concentration at which the modified MS2 capsids associated with one AuNP on average, as opposed to plasmon-coupled (two or more) AuNPs attached to one AuNP. This was determined by titrating in AuNPs to MS2 and recording the maximum absorbance wavelength (SPR), shown in Figure 1.7. We took these results to indicate that the cDNA modified AuNPs were attached to the dye-labeled MS2 capsids through DNA hybridization, and that the constructs could be taken on for fluorescence studies.

![Figure 1.7. Titration of MS2 with AuNPs. The non-complementary, scrambled sequence, SPB (blue dots) centered around 522 nm, while the complementary sequence SPB (orange dots) varied depending on the ratio of MS2:AuNPs. The maximum SPB shift was observed at a ratio of 0.2 MS2 per one AuNP (n = 3).](image-url)
Figure 1.8. Normalized fluorescence intensity maximum for AF 350 or AF 594-modified T19pAF N87C MS2. Fluorophore labeled MS2 was externally modified with short, medium, and long DNA and incubated with cDNA-modified a) 5 nm AuNPs, b) 10 nm AuNPs, and c) 40 nm AuNPs.
1.3.4. Fluorescence studies of MS2-AuNP conjugates for design 1

MS2 was modified on its interior surface with AF 350 or AF 594, and externally modified with a short (14 bp, 4.6 nm), medium (27 bp, 8.9 nm), or long (43 bp, 14.2 nm) ssDNA sequence. These constructs were then incubated with a corresponding complementary or non-complementary ssDNA-modified 5 nm, 10 nm, or 40 nm diameter AuNPs, resulting in a total of six data sets, as plotted in Figure 1.8. For each data set, samples without AuNPs had the largest fluorescence intensity maximum. These measurements also revealed that for AF 350 modified samples, there was not a significant dependence of fluorescence intensity on the distance of the AuNP from the capsid surface. For samples modified with AF 594, the fluorescence intensity maximum increased as the distance to the capsid decreased. In each case, however, the fluorescence intensity maximum was less than the samples without AuNPs.

The dramatic effect of AuNPs alone on the fluorescence intensity of each sample led us to consider the influence of a similar sized material (nanometer diameter) on the fluorescence intensity measurements. A nanomaterial that does not contain a SPR in the visible range could perhaps effect the fluorescence intensity measurements similarly by preventing excitation light from reaching the sample and blocking emission light from reaching the detector. To investigate this effect, palladium nanoparticles (PdNPs) with a diameter of 4.7 \(\pm\) 1.1 nm (as measured by TEM) were synthesized according to a previously reported procedure. The characterization of the synthesized PdNPs is shown in Figure 1.9. We thought the effect of PdNPs could be used as a direct comparison to the effect of 5 nm diameter AuNPs on the fluorescence intensity of dye-labeled capsids due to their similar size. The PdNPs decreased the fluorescence intensity maximum for AF 350 modified capsids. However, PdNPs had little effect on the fluorescence intensity maximum for AF 594 modified capsids, as shown in Figure 1.8a.

![Palladium nanoparticle (PdNP) characterization](image)

*Figure 1.9.* Palladium nanoparticles synthesized to mimic the light scattering effect of AuNPs in solution. The absorbance spectra of the palladium nanoparticles is shown. The particles were synthesized with a diameter of 4.7 \(\pm\) 1.1 nm, as measured by TEM (inset, scale bar = 20 nm).

It was hypothesized the PdNPs had an effect on AF 350 modified capsids but not AF 594 modified capsids due to the absorbance profile of the PdNPs (Figure 1.9). PdNPs absorb more light at the excitation and emission wavelengths of AF 350 as opposed to...
AF 594 (where PdNPs absorb minimal light). This effect is referred to as the inner filter effect and is described in Figure 1.10. At high sample concentrations (highly absorbing samples), both the excitation and emission light are reduced as light passes through the sample. This decreases the resulting fluorescence emission intensity values.

Ultimately, it was determined that for samples containing AuNPs, it would be challenging to control or accurately correct for the large extinction coefficient of AuNPs in comparison to the extinction coefficients for AF 350 and AF 594. This was especially complicated by the inhomogeneous sample population. The ratio of AuNPs:fluorophores was not uniform across all of the samples measured. Due to the inner filter effect, it was challenging to compare results across sample types or between different AuNP-MS2 sample preparations. The inner filter effect will be a factor highlighted again for the second design detailed later in this chapter.

1.3.5. Design 1 conclusions

In summary, an MS2-based system for studying the effect of AuNPs on organic fluorophores was successfully designed and assembled. The design discussed in this section (design 1) relied on the interior chemical modification of MS2 with a fluorophore of interest, and the exterior attachment of AuNPs through DNA hybridization. Due to the large effect of AuNPs alone on the bulk fluorescence measurements, design 1 highlighted the need to obtain a more homogenous sample population, either through preparation or purification. Any corrections to fluorescence intensity maximum values to account for the inner filter effect would depend on constructing a system with a homogenous ratio of AuNPs to fluorophores. To address some of these challenges, a second MS2-based system was designed (design 2).

1.4. Design 2: AuNP encapsulation, exterior fluorophore labeling of MS2

Based on the availability of the external modification strategies for MS2, we envisioned encapsulating a metal nanoparticle of interest on the interior of MS2 and using an exterior surface chemical modification to place organic dyes at uniform fixed distances from the metal core.
1.4.1. MS2 encapsulation of AuNPs

Several other groups have reported the encapsulation of metal nanoparticles by viral capsid proteins, allowing for the incorporation of quantum dots,\textsuperscript{56} gold,\textsuperscript{57-62} and cobalt iron oxide nanoparticles.\textsuperscript{56,63} This suggests that several additional platforms could potentially be amenable to the strategy described herein, but none of these studies have combined the particle incorporation with synthetic modification of the capsid proteins.

In the case of MS2, other groups have reported the reassembly of MS2.\textsuperscript{64,65} We recently reported a strategy to encapsulate DNA oligomers and negatively charged proteins by adding them to a pool of disassembled capsid proteins stabilized by trimethylammonium oxide as an osmolyte.\textsuperscript{66} The reassembly presumably occurs due to electrostatic interactions between the anionic cargo and the building interior positive charge of forming capsids. In the current study, this methodology was also found to be applicable to encapsulation of DNA-coated AuNPs. As outlined in Figure 1.11., MS2 capsids were first disassembled in acetic acid and then reassembled in phosphate buffer.

\textbf{Figure 1.11.} a) T19pAF MS2 subjected to reassembly conditions in the presence of different sizes and concentrations of AuNPs. Transmission electron micrograph images of T19pAF MS2 reassembled around b) 10 nm AuNPs, c) 5 nm AuNPs, d) 10 nm AuNPs, e) 15 nm AuNPs, f) 20 nm AuNPs, g) one 5 nm AuNP, h) two 5 nm AuNPs, i) three 5 nm AuNPs, and j) four 5 nm AuNPs. The native gel shown in k) demonstrates similar electrophoretic mobility for MS2 reassembled around different size AuNPs (lanes 3, 5, 7, and 9) as particle-free MS2 (lane 1). The electrophoretic mobilities of the reassembled samples are different than their corresponding free AuNPs (lanes 2, 4, 6, and 8, respectively).
in the presence of AuNPs labeled with ~20-60 DNA strands (depending on the size of the AuNP). In the case of the AuNPs, the presence of the osmolyte was not required, possibly due to the very high amount of negative charge on the metal surfaces. After 15-40 h of incubation at 4 °C, the capsids were observed to reform around 5 nm, 10 nm, 15 nm, and 20 nm diameter AuNPs. In most cases, 60 to 90% of the capsids observed with TEM contained nanoparticles (see specific numbers below). The presence of the AuNPs within the structures was confirmed through transmission electron microscopy (TEM, Figure 1.11-b-j), and the encapsulated particles were found to run identically to native capsids using non-denaturing agarose gel electrophoresis (Figure 1.11-k). Dynamic light scattering (DLS, Figure 1.12.) showed minimal changes in the sizes of the assembled capsids for all four nanoparticle sizes. Interestingly, multiple particles could be captured inside some capsids when a large excess of 5 nm AuNPs was used. The encapsulation

![Dynamic light scattering](image)

**Figure 1.12.** Dynamic light scattering by number data for a) T19pAF MS2, b) 5 nm, d) 10 nm, f) 15 nm, h) 20 nm, and j) 40 nm AuNPs, as well as T19pAF MS2 subjected to reassembly conditions in the presence of R-DNA modified c) 5 nm, e) 10 nm, g) 15 nm, i) 20 nm, and k) 40 nm AuNPs. T19pAF MS2 reassembled in the presence of 5 nm, 10 nm, 15 nm, and 20 nm AuNPs resulted in average diameters similar to T19pAF MS2 not subjected to reassembly conditions. These values were different from their respective AuNPs. As a control, T19pAF MS2 was subjected to reassembly conditions in the presence of 40 nm AuNPs. This sample k) had a similar diameter to the 40 nm AuNPs j). TEM analysis of this sample revealed no protein shell surrounding the metal particles.
of 40 nm particles, which are larger than the capsid interior, proved unsuccessful.

For the studies described in the remaining section, MS2 capsids containing 10 nm AuNPs were selected. As reported by another group using a similar viral capsid system, 10 nm AuNPs encapsulated most efficiently, resulting in the fewest unencapsulated AuNPs and empty viral capsids.\(^{67}\) In order to install the exterior DNA strands, the assemblies were prepared using MS2 capsid proteins containing an unnatural amino acid, \(p\)-aminophenylalanine (\(p\)AF), introduced to position 19 using the amber stop codon suppression technique.\(^{50,68}\) This provided 180 chemically distinct aniline groups on the outer surface, which we have previously targeted for modification using oxidative coupling reactions.\(^{48,50,69}\) In this specific case, the distribution of reassembled T19pAF MS2 species around 10 nm AuNPs was characterized using TEM (Figure 1.13.). The majority of the assemblies consisted of capsids containing a single nanoparticle (77.2%), while capsids without AuNPs accounted for a few of the observed structures (2.7%). Bare AuNPs (6.6%) and potentially misformed capsids (13.5%) comprised the remaining species. T19pAF MS2 successfully reassembled in the presence of 10 nm AuNPs modified with several

![Figure 1.13.](image)

\(\text{Figure 1.13. a) - d) Additional transmission electron micrograph images of T19pAF MS2 reassembled around 10 nm AuNPs. e) The distribution of reassembled species for T19pAF MS2 subjected to oxidative coupling with DNA. Representative images are also shown for (i) T19pAF MS2 reassembled around R-DNA modified 10 nm AuNPs (77.2%), (ii) T19pAF MS2 reassembled without 10 nm AuNPs (2.7%), (iii) free 10 nm AuNPs, and (iv) 10 nm AuNPs with a potentially misformed protein shell (n = 914).}\)
different DNA sequences (Figure 1.14-a), suggesting that simple electrostatic interactions are sufficient to encourage reassembly. In addition, other MS2 mutants (T15Y N87C MS2 and T19Y N87C MS2) successfully reassembled in the presence of DNA modified 10 nm AuNPs, as confirmed by TEM, DLS, and native agarose gel electrophoresis (Figure 1.14.b). These measurements suggest the critical parameter of particle diameter is identical for capsids with and without 10 nm AuNPs.

**1.4.2. Initial bulk fluorescence emission intensity measurements on dye-modified capsids**

For our initial studies involving fluorophores, MS2 capsids containing 10 nm diameter AuNPs were chemically modified with TAMRA NHS ester (excitation 550 nm/emission 575 nm) or Oregon Green 514 NHS ester (excitation 488 nm/emission 521 nm). Excess dye was removed by gel filtration, followed by spin concentration. Most capsids contain one AuNP and are chemically modified to a similar extent with a dye of interest (TAMRA or Oregon Green 514). Due to the relatively homogenous sample population for this design (ratio of dyes:AuNPs), we thought this system would be more amenable to bulk fluorescence measurements as compared to design one (described in the earlier section). We envisioned subsequent corrections of the fluorescence emission intensity values to account for the large absorbance values of each sample at the excitation and emission wavelengths (due to AuNPs in solution).

Bulk fluorescence measurements were collected for samples with and without AuNPs at increasing concentrations of MS2. Absorbance values for each dilution were also recorded at the excitation and emission wavelengths for the dye of interest. As with design one, AuNPs in solution greatly affected the fluorescence intensity values due to the inner filter effect. Samples without AuNPs display a linear dependence of fluorescence emission intensity on MS2 (dye) concentration, as shown in Figure 1.15. Uncorrected spectra for samples with AuNPs show a small range of linear dependence, followed by a point at which higher dye concentrations do not result in larger fluorescence emission intensity values. Presumably, a large percentage of excitation and emission light is blocked when a non-linear response to increasing dye concentration is noted. Corrected fluorescence intensity maximum values that take into account the sample absorbance at the excitation wavelength.
Figure 1.15. T19pAF MS2 reassembled around 10 nm AuNPs were externally modified with TAMRA NHS ester and Oregon Green 514 NHS ester. Fluorescence emission intensity measurements were recorded at the emission maximum for MS2 monomer concentrations between 0.5 and 2.0 μM. The uncorrected values are shown in a) and c) for TAMRA NHS and Oregon Green 514, respectively. Corrected values shown in b) and d) take into account the sample absorbance at the excitation and emission wavelengths for TAMRA NHS and Oregon Green 514, respectively.
and emission wavelengths were determined using the formula shown in Figure 1.15.,
\[ F_{\text{corrected}} = F_{\text{obs}} \cdot \text{antilog}((\text{OD}_{\text{exc}} + \text{OD}_{\text{em}})/2) \]. These corrected fluorescence intensity maximum values show a linear dependence for samples with and without AuNPs.

We thought this strategy of recording, followed by calculating corrected bulk fluorescence intensity measurements could be a viable method for determining the effect of AuNPs on organic fluorophores. However, we were concerned that the measurement relied on accurately determining the sample protein concentration. At the low concentrations required for the measurements, it was difficult to ensure the samples with and without AuNPs had identical protein concentrations. Furthermore, the corrected bulk fluorescence measurement values were dramatically different from the recorded measurements. We wanted to explore a different measurement method, where the values recorded could be directly compared for samples with and without AuNPs without having to calculate corrected values. We also wanted to develop a system where the distance between the dyes and the metal center could be varied. In the case described so far, there is a fixed distance between the metal center and the organic dye. A system was designed to allow for varying the distance between the organic dye and metal center and single molecule microscopy methods were explored for measuring the fluorescence emission intensity of individual capsids.

1.4.3. Construction of a modular MS2-DNA-fluorophore system

We envisioned using an exterior surface chemical modification to attach ssDNA to the surface of the capsid. The external nucleic acid strands would facilitate the placement of organic dyes at uniform fixed distances from the metal core. The protein shell would prevent contact quenching between the metal nanoparticle and the exterior groups, and the sequence selectivity of DNA hybridization would allow a radially symmetric set of dyes to be located at desired distances.

Samples of T19pAF MS2 capsids both with and without 10 nm AuNPs were next modified to display external DNA strands. Two strategies were explored for the attachment of ssDNA to the exterior surface and subsequent display of fluorophores. In the first approach, the pAF residues were exposed to 5 mM sodium periodate for 1 hour in the presence of 39 nt ssDNA bearing a phenylenediamine moiety at their 5’ termini. This construct was subsequently incubated with a complementary, 5’ fluorophore labeled ssDNA, as shown in Figure 1.16-a. In the second approach, the pAF residues were exposed to 1 mM sodium periodate for 5 minutes in the presence of 31 nt, 38 nt, and 54 nt hairpin strands bearing aminophenol groups at their 5’ termini and an fluorescent dye on their 3’ termini, as outlined in Figure 1.16-b.48

Previous studies have shown that these conditions are compatible with the protein assembly and do not impair the hybridization ability of the DNA strands.48 No effects were observed for the AuNPs as well, as judged by the retention of their surface plasmon band centered at 520 nm. The percent DNA modification of MS2 for each construct was quantified by SDS-PAGE gel electrophoresis using optical densitometry (Figure 1.17. and Figure 1.18.), indicating similar modification levels for capsids with and without gold inside.

To explore the distance dependence of the enhancement, the synthetic procedure was used with different fluorophore-DNA sequences to position the dyes 3 bp from the
**Figure 1.16.** Overall synthetic strategy for constructing fluorophore-AuNP conjugates. For exterior surface modification, a) phenylene diamine-containing or b) aminophenol-containing DNA can be attached to exterior anilines on T19pAF MS2 using a sodium periodate mediated oxidative coupling reaction.

**Figure 1.17.** a) MS2-DNA conjugates were analyzed by SDS-PAGE followed by Coomassie staining. Samples in lanes 1 and 2 were reacted with A DNA, and display a gel shift corresponding to the MS2-DNA conjugate. Lane 1 contains MS2 without gold and Lane 2 contains MS2 with gold. Lane 3 was not reacted with A DNA. The DNA modification was determined to be approximately 15% by optical densitometry for both the plus gold and minus gold case. b) MS2-DNA conjugates incubated with complementary fluorescent DNA and a stabilizing strand were purified by native agarose gel electrophoresis. The gel was then visualized under UV-light (left), which revealed the MS2-fluorescent DNA conjugate as well as the free fluorescent DNA. The gel was also Coomassie stained to confirm the presence of MS2 in both the minus gold and plus gold case. The MS2-containing bands were extracted and these samples were visualized in c) by TIRF microscopy for the minus gold (left) and plus gold (right) case.
capsid (1 nm from protein, 9.5 nm from the AuNP), 12 bp from the capsid (4 nm from the protein, 12.5 nm from the AuNP), and 24 bp from the capsid (8 nm from the protein, 16.5 nm from the AuNP). For the 12 bp and 24 bp distances, additional stabilizing strands were included to ensure that rigid double stranded DNA separated the fluorophores from the surfaces. Stabilizing strands were not included for the 3 bp distance.70

To select a dye of interest, we referred to literature precedent. Previous work exploring single silver nanoprisms indicated that maximum fluorescence enhancement occurs with a dye that has a fluorescence emission slightly red-shifted from their surface plasmon band.25 The emission spectrum of AF 488 directly overlaps with the surface plasmon resonance peak for 10 nm AuNPs, providing a close fit to this criterion.

1.4.4. Single molecule fluorescence measurements of design 2 MS2-DNA constructs

Total internal reflection fluorescence (TIRF) microscopy was used to measure the fluorescence intensity of the individual capsids in the twelve resulting samples (two constructs with three distances for one set containing gold and three distances for another set without gold).

All TIRF microscopy measurements were taken in solution. As a result, a slide modification strategy had to be developed that promoted the association of the dye labeled

Figure 1.19. A representative image of a poly-L-lysine coated glass slide incubated with fluorophore labeled MS2.
MS2 constructs to the surface of the slide for imaging. To accomplish this, a variety of slide modification strategies were explored. The first strategy was simply preparing glass slides by coating them with poly-L-lysine. In previous experiments with directly dye labeled capsids, this strategy provided dose dependent, uniform surface coverage, as shown in Figure 1.19. For DNA-labeled capsids, however, we observed non-uniform surface coverage. We hypothesize that the uneven surface coverage was a result of a strong electrostatic attraction between the fluorophore labeled DNA and the positively charged poly-L-lysine surface. It was hypothesized that this strong attraction could be problematic for future measurements because any excess fluorophore labeled DNA would associate strongly with the surface, significantly affecting the fluorescence intensity measurements (only fluorophore labeled ssDNA would cover the surface). To promote specific attachment of the constructs to the glass slide, a second slide modification strategy was explored. Reductive amination for a variety of amine DNA: amine PEG-OMe ratios were explored on aldehyde coated glass, as previously reported and detailed in Figure 1.20. This strategy should promote the specific interaction of the MS2-DNA constructs with the glass surface through DNA hybridization while the PEG should shield any repulsive electrostatic interactions between the negatively charged MS2 construct and negatively charged glass surface. While this strategy seemed to promote a specific interaction of the MS2-DNA constructs with the surface and tunable surface coverage, the strategy often resulted in non-uniform surface coverage. Regions with presumably little DNA coverage and regions with high DNA coverage often resulted in clear pattern edges and large bright areas (Figure 1.21). The final strategy that was explored used neutravidin coated glass slides.

**Figure 1.20.** MeO-PEG/ssDNA coated glass slides were prepared as outlined above. Following oxidation using an oxygen plasma cleaner, aldehyde functional groups were introduced using an aldehyde silanation reagent. The aldehyde coated glass slide was then immersed in 5' amine ssDNA and amine PEG-OMe, followed by heating to promote imine formation. An aqueous solution of sodium borohydride was then used to reduce the imines to amines and any unreacted aldehydes to alcohols. Representative fluorescence images are shown on the right of 100% PEG-OMe, 75%:25% PEG-OMe:ssDNA, and 25%:75% PEG-OMe:ssDNA coated glass slides.
incubated with a biotinylated cDNA strand. This allowed for a controlled, uniform surface coverage of cDNA, and promoted even surface coverage. The MS2-AuNP-fluorophore samples were incubated with glass slides bearing DNA strands that were complementary to those attached to the capsids, leading to the capture of particles. Once a reasonable surface density was reached, a set of TIRF images was collected for each sample. A diagram outlining the TIRF setup and surface modification can be found in Figure 1.22. Representative TIRF images for both constructs are shown in Figure 1.17.b and Figure 1.18.b.

![TIRF setup and surface modification diagram](image)

**Figure 1.21.** Images of 1:1 PEG-OMe: ssDNA modified slides incubated with AF 594 MS2 ± AuNPs. As highlighted by the yellow dotted lines, the pattern was not consistent across the slide, with uneven coverage and spotted areas.

**Figure 1.22.** Total internal reflection fluorescence (TIRF) microscope setup for a) the fluorophore labeled hairpin DNA, and b) fluorophore labeled complementary ssDNA strategies. Microscope slides were modified as described in the materials and methods section.
1.4.5. Analysis of single molecule fluorescence intensity measurements

Each set of images was analyzed separately, as described in the materials and methods section. A distribution of intensities was anticipated due to the differences in the levels of fluorescence labeling between individual capsids. In our initial measurements, little difference was observed when samples without AuNPs were compared to samples with AuNPs. The reason the samples had little difference became evident when we plotted spot area versus mean fluorescence intensity, shown in Figure 1.23., Figure 1.24., and Figure 1.25 for the 3 bp, 12 bp, and 24 bp distances, respectively. For the 3 bp and 12 bp distances, it was evident that two populations were present in the sample. One population was brighter than the other population. For the longest distance (24 bp) the fluorescence intensity plots for samples with and without gold overlapped, and they each displayed one population. It was hypothesized that excess fluorophore DNA was still present in the sample upon imaging. The excess fluorophore could be disrupting the measurements by decreasing the average fluorescence intensity for samples with AuNPs. Single fluorophore sample contamination became more evident after taking time lapsed movies of the samples bleaching over time. Single dye traces bleached within a few frames, while capsid samples that displayed multiple fluorophores bleached gradually over time, as plotted in Figure 1.26.
Figure 1.23. Mean intensity versus area plots for AF 594 spaced 3 base pairs from the MS2 capsid surface for samples a) without AuNPs, and b) with AuNPs. An overlay of the data for samples with and without AuNPs is shown in c). Samples with AuNPs display two populations. The first population overlays with the data from samples without AuNPs. The second population contains brighter particles.
Figure 1.24. Mean intensity versus area plots for AF 594 spaced 12 base pairs from the MS2 capsid surface for samples a) without AuNPs, and b) with AuNPs. An overlay of the data for samples with and without AuNPs is shown in c). Samples with AuNPs display two populations. The first population overlays with the data from samples without AuNPs. The second population contains brighter particles.
Figure 1.25. Mean intensity versus area plots for AF 594 spaced 24 base pairs from the MS2 capsid surface for samples a) without AuNPs, and b) with AuNPs. An overlay of the data for samples with and without AuNPs is shown in c). Unlike the samples where AF 594 was 3 bp and 12 bp from the capsid surface, samples both with and without AuNPs overlay and display a single population.
1.4.6. Correcting single molecule fluorophore contamination for fluorescence intensity measurements

To address this issue, excess fluorescently-labeled DNA was removed through successive centrifugal filtrations with multiple 100 kD molecular weight cutoff spin filters, followed by native agarose gel electrophoresis. DLS, and fluorescence correlation spectroscopy (FCS) were used to characterize the resulting constructs (Figure 1.27. and Figure 1.28., respectively). Each of these techniques suggested that the MS2 capsids were assembled, fluorescently labeled, and accompanied by few if any unattached fluorescently labeled DNA strands.

The TIRF data plotted in Figure 1.29-a and Figure 1.29-b are represented as mean intensity histograms, allowing the average brightness per particle to be compared in the presence and absence of the gold particles. A 1.8-fold enhancement for a 3 bp separation, a 1.5-fold enhancement for a 12 bp separation, and no effect was observed for a 24 bp separation when the gold particles were added to the capsids for the fluorophore labeled DNA hybridization construct. A 2.2-fold enhancement for a 3 bp separation, a 1.2-fold enhancement for a 12 bp separation, and no effect was observed for a 24 bp separation when the gold particles were added to the capsids for the fluorophore labeled DNA hairpin construct. For the DNA hairpin construct, images were also collected using confocal microscopy. The confocal microscopy results agreed with the TIRF microscopy results and are shown in Figure 1.29-c.

Although one might anticipate the fluorophore brightness for each of the three gold-free samples to be identical, we attribute the changes in mean intensity among the gold-free samples to differences in fluorescence labeling. Higher modification levels may not necessarily result in increased sample brightness. Interactions between dyes attached to the capsid as well as interactions between the attached dyes and aromatic residues on the protein surface may affect the quantum yield of the fluorophore. Consequently, the most accurate comparisons are drawn only by comparing the samples with and without gold for each separation distance.

For the case of the hairpin DNA construct, it is important to note that we observed
Figure 1.27. Dynamic light scattering by number data for a) 3 bp hairpin MS2, b) 3 bp hairpin MS2 plus gold, c) 12 bp hairpin MS2, d) 12 bp hairpin MS2 plus gold, e) 24 bp hairpin MS2, and f) 24 bp hairpin MS2 plus gold. Dynamic light scattering by number for MS2 samples incubated with a complementary hairpin sequence for g) 3 bp hairpin MS2, h) 3 bp hairpin MS2 plus gold, i) 12 bp hairpin MS2, j) 12 bp hairpin MS2 plus gold, k) 24 bp hairpin MS2, and l) 24 bp hairpin MS2 plus gold.
significant differences between hairpin DNA structures that were and were not preformed prior to capsid attachment. The number of fluorescently labeled hairpin DNA strands attached to the capsids also seemed to affect precise value of fluorescence enhancement.

Lower levels of fluorophore modification resulted in larger fluorescence intensity differences between capsids with and without AuNPs. For example, in the case described in the above text, the MS2 modification levels were ~60 strands per capsid (33%), ~45 strands per capsid (25%), and ~34 strands per capsid (19%), for 3 bp, 12 bp, and 24 bp separation, respectively. For these modification levels, a 2.2-fold enhancement, 1.2-fold enhancement, and no effect was observed for the 3 bp, 12 bp, and 24 bp separations, respectively. In a separate experiment 3.9-fold, 3.4-fold, and 2.0-fold enhancements were observed for the 3 bp, 12 bp, and 24 bp distances. In that case, the MS2 modification levels were ~34 strands per capsid (19%), ~9 strands per capsid (5%), and ~9 strands per capsid (5%) for 3, 12, and 24 bp separation, respectively. We attribute these differences to decreased interaction of AF 488 for low levels of modification.

1.4.7. Fluorescence lifetime measurements of design 2 MS2-DNA constructs

Fluorescence lifetime data were also collected for the AF 488 dye, three analogous DNA constructs, three MS2 samples without AuNPs, and three MS2 samples with AuNPs, as tabulated in Figure 1.30-a and Figure 1.30-b. A representative fluorescence lifetime trace overlaid with the instrument response function is plotted in Figure 1.30-b. A decrease in fluorescence lifetime was observed when the fluorophores were placed close to the
**Figure 1.29.** Mean intensity histograms determined from TIRF microscopy images for a) the phenylenediamine complementary fluorophore-DNA hybridization constructs, and b) the o-aminophenol hairpin DNA constructs. Additionally, mean intensity histograms determined from confocal microscopy images for c) the o-aminophenol hairpin DNA constructs outlined in the text. For each set of histograms, the separation distances are 3 bp, 12 bp, and 24 bp from left to right. All samples with AuNPs are shown in red. All samples without AuNPs are shown in blue.
a. fluorescence lifetimes for phenylene diamine cDNA constructs

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b. fluorescence lifetimes for o-aminophenol hairpin DNA constructs

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Figure 1.30. Fluorescence lifetime analysis for MS2-DNA conjugates, including a) phenylenediamine cDNA hybridization constructs, and b) o-aminophenol hairpin DNA constructs.
gold-free capsid surface, with the shortest lifetime observed for the smallest separation between the fluorophore and protein. We attribute this decrease in lifetime to interactions between the AF 488 and either other dyes attached to the capsid or aromatic residues on the capsid. A further decrease in fluorescence lifetime was observed for each of the samples with AuNPs on the interior surface, with the shortest lifetime at the smallest separation. This decrease in lifetime was in accordance with other groups that have reported fluorescence enhancement.\textsuperscript{15,18-21,23,24} We attribute this effect to an increased radiative decay rate as a result of the AuNP being in close proximity to the fluorophores.

Fluorescence lifetime measurements were also recorded for the hairpin DNA-MS2 constructs after incubation with ssDNA complementary to the hairpin loop. These data are shown in Figure 1.30-c. We hypothesize that the complementary hairpin ssDNA moves the fluorophore away from the capsid, reducing the effect of the capsid and the interior AuNP on the fluorescence lifetime for all distances.

A control lifetime experiment was conducted where 10 nm AuNPs were modified with multiple DNA strands and then incubated with complementary fluorescently-labeled DNA strands that placed fluorophores 9.5 nm, 12.5 nm, and 16.5 nm from the AuNP surface.\textsuperscript{72} We were not able to obtain reasonable fits of fluorescence lifetimes for these data at concentrations similar to those used for capsid samples, due to low photon counts. This suggests the dyes are able to contact the surface of the AuNP directly, quenching their fluorescence. These results have been described in bulk fluorescence measurements for a similar system.\textsuperscript{72} This is in sharp contrast to the results we see where the metal nanoparticle is encapsulated in the viral capsid.

1.5. Design 3: Origami tiles for placing AuNPs at fixed distances from dye-labeled MS2

Four rectangular origami tiles were designed to place 10 nm AuNPs at fixed distances from interior fluorophore, exterior DNA labeled MS2 capsids. Each design displayed A20 capture strands for hybridization with complementary, T\textsubscript{20} DNA labeled MS2. The four designs placed AuNP capture strands at increasing distance (labeled 1, 2, 3, and 4) from the MS2 capsids, as shown in Figure 1.31. As described previously, T19pAF N87C MS2 was modified on the interior surface with AF 532 maleimide dyes, at nearly quantitative conversion, as determined by ESI-MS. The exterior surface of AF 532 MS2 was then modified with phenylenediamine containing T\textsubscript{20} DNA strands using a sodium periodate-mediated oxidative coupling. Attachment of the T\textsubscript{20} DNA strands to AF 532-labeled MS2 was confirmed through SDS PAGE gel electrophoresis. Subsequent incubation of T\textsubscript{20} DNA, AF 532 MS2 with DNA origami tiles containing AuNPs gave four types of origami tiles with AuNPs at increasing distances from the capsid surface. These structures were characterized through TEM and high resolution AFM, revealing capsid to AuNP surface separations of 12-30 nm. Scanning confocal fluorescence microscopy images were acquired on a few of the AuNP/MS2 containing origami tiles, and high resolution AFM imaging was used to correlate the tile components with the fluorescence images. Little to no fluorescence enhancement was observed for these samples. We hypothesize these results are due to the large variety of dye to AuNP surface distances. Since AF 532 dyes coat the interior surface of MS2, at the shortest distance of 12 nm, half of the dyes are at > 32 nm from the AuNP surface. These large distances might results in
the little to no observed total effect of AuNPs on the AF 532 dyes. Perhaps larger diameter AuNP particles would result in a measurable effect of the AuNPs on AF 532 labeled MS2 capsids.

1.6. Conclusions

These studies demonstrate the utility of nanoscale protein assemblies for the integration of multiple components into complex systems. The precise dimensions of the viral capsids, in addition to the distinct chemically addressable exterior and interior surfaces, were crucially important for positioning of the dyes without allowing metal surface contact. In ongoing studies, we are using this synthetic system to explore an expanded range of metals, nanocrystal sizes, and fluorophore spectral properties. We are also exploring the use of additional protein scaffolds for the construction of fluorophore-nanoparticle structures with different geometric relationships. In particular, larger viral capsid proteins would be able to house bigger gold and silver nanoparticles that have been shown to give larger enhancement values. In addition to providing experimental tests of metal-enhanced fluorescence, the availability of these systems will provide valuable synthetic routes to access these structures for use in future applications.

Figure 1.31. Strategy for using DNA origami to arrange fluorophore labeled MS2 at fixed distances from AuNPs. a) Four rectangular origami tiles were designed that spaced AuNPs at four distances from MS2 (labeled 1, 2, 3, and 4). b) MS of unmodified T19pAF N87C MS2. c) MS of AF 532 maleimide modified T19pAF N87C. d) MALDI TOF MS of unmodified and phenylene diamine NHS modified DNA. The dye labeled MS2 was attached to phenylene diamine containing DNA via oxidative coupling with periodate to yield e) T20DNA, AF 532 labeled MS2, shown in Coomassie-stained and fluorescence SDS PAGE gel images.

1.6. Conclusions

These studies demonstrate the utility of nanoscale protein assemblies for the integration of multiple components into complex systems. The precise dimensions of the viral capsids, in addition to the distinct chemically addressable exterior and interior surfaces, were crucially important for positioning of the dyes without allowing metal surface contact. In ongoing studies, we are using this synthetic system to explore an expanded range of metals, nanocrystal sizes, and fluorophore spectral properties. We are also exploring the use of additional protein scaffolds for the construction of fluorophore-nanoparticle structures with different geometric relationships. In particular, larger viral capsid proteins would be able to house bigger gold and silver nanoparticles that have been shown to give larger enhancement values. In addition to providing experimental tests of metal-enhanced fluorescence, the availability of these systems will provide valuable synthetic routes to access these structures for use in future applications.
1.7. Materials and methods

1.7.1. General procedures and materials

Unless otherwise noted, all chemicals were purchased from commercial sources and used as received without further purification. Analytical thin layer chromatography (TLC) was performed on EM Reagent 0.25 mm silica gel 60-F254 plates with visualization by ultraviolet (UV) irradiation at 254 nm and staining with potassium permanganate. Purifications by flash chromatography were performed using EM silica gel 60 (230-400 mesh). Chromatography solvents were used without distillation. All organic solvents were removed under reduced pressure using a rotary evaporator. Water (dd-H$_2$O) used in all procedures was deionized using a NANOpureTM purification system (Barnstead, USA). All unconjugated gold colloid solutions were purchased from Ted Pella, Inc (Redding, CA). Bis(p-sulfonatophenyl)phenylphosphine dehydrate dipotassium salt (BSPP) was purchased from Sigma Aldrich (St. Louis, MO). All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Coverslips (25 mm, number 1.5 thickness) were purchased from Warner Instruments (Hamden, CT), catalog number: 64-0715 (CS-25R15). Lab-tek chamber slides (8-well, glass) were obtained from Nunc (Rochester, NY). Neutravidin was purchased from Invitrogen (Carlsbad, CA) and stored as 1 mg/mL aliquots. Biotin-PEG-maleimide 5 kDa was purchased from Nanocs (Boston, MA). Poly-L-Lysine (20 kDa) grafted with PEG (2 kDa) and PEG-Biotin (3.4 kDa) was purchased from SuSoS (Dübendorf, Switzerland) and stored at 1 mg/mL in HEPES buffer, pH 5.3.

1.7.2. Instrumentation and sample analysis

**NMR.** ¹H and ¹³C spectra were measured with a Bruker AVB-400 (400 MHz) spectrometer. ¹H NMR chemical shifts are reported in δ in units of parts per million (ppm) relative to CDCl$_3$ (δ 7.26, singlet). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), p (quintet), or br-s (broad singlet).

**Mass Spectrometry.** Electrospray Ionization Liquid Chromatography/Mass Spectrometry (LC/MS) analysis was performed using an API 150EX system (Applied Biosystems, USA) equipped with a Turbospray source and an Agilent 1100 series LC pump. Protein chromatography was performed using a Phenomenex Jupiter™ 300 5µ C18 300 Å reversed-phase column (2.0 mm x 150 mm) with an MeCN: dd-H$_2$O gradient mobile phase containing 0.1% formic acid (FA). Protein mass reconstruction was performed on the charge ladder with Analyst software (version 1.3.1, Applied Biosystems). Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) was performed on a Voyager-DETM system (PerSeptive Biosystems, USA). Prior to MALDI-TOF MS analysis, samples were desalted using C18 ZipTip® pipet tips (Millipore, USA). Oligonucleotide samples were co-crystallized using a 3-hydroxypicolinic acid: ammonium citrate solution (45 mg/mL:5 mg/mL in 4.5:5.5 MeCN:dd-H$_2$O).

**Gel Analyses.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Mini-Protean apparatus from Bio-Rad (Hercules, CA) with
10-20% gradient polyacrylamide gels (BioRad, CA), following the protocol of Laemmli. All samples were mixed with SDS loading buffer in the presence of 1,4-dithiothreitol (DTT) and heated to 95 °C for 10 min to ensure complete denaturation. Gels were run at 120 V for 80 min. Commercially available molecular weight markers (Bio-Rad) were applied to at least one lane of each gel for assignment of molecular masses. For fluorescent protein conjugates, a UV-backlight was used for visualization. Gels were then stained with either Coomassie Brilliant Blue (Bio-Rad) or SYPRO® ruby protein gel stain from Molecular Probes™ (Eugene, OR). Coomassie stained protein gels were imaged on an EpiChem3 Darkroom System (UVP, USA). SYPRO® ruby stained gels were visualized on a Typhoon 9410 variable mode imager (Amersham Biosciences) (exc. 532 nm and em. 610 nm).

Dynamic Light Scattering (DLS). DLS measurements were obtained using a Malvern Instruments Zetasizer Nano ZS. Samples were filtered through a 0.22 μm centrifugal filter unit (Millipore Corporation, Billerica, MA) prior to data collection. Data plots are shown as size distribution by number, which weighs large and small particles equally. Diameters were calculated from an average of three measurements.

Transmission Electron Microscopy (TEM). TEM images were taken at the University of California Berkeley Electron Microscope Laboratory (UCB EML) using a FEI Tecnai 12 transmission electron microscope (TEM) with 120 kV accelerating voltage. Samples were prepared by pipetting 5 μL onto Formvar-coated copper mesh grids (400 mesh, Ted Pella, Redding, CA) for 5 min, followed by rinsing with 8 μL of dd-H2O. The grids were then exposed to 8 μL of a solution of uranyl acetate (15 mg/mL in dd-H2O) for 90 s as a negative stain. Excess stain was then removed and the grids were allowed to dry in air.

UV-Vis Spectroscopic Measurements. UV-Vis spectroscopic measurements were conducted on a Varian Cary 50 scan benchtop spectrophotometer (Varian Inc., USA). All measurements were taken in absorbance mode at a medium scan rate and recording measurements from 200 nm – 800 nm.

Fluorescence Emission Measurements. Fluorescence emission measurements were obtained on a Fluoromax-2 spectrofluorometer (ISA Instruments, USA). For experiments with gold nanoparticles, the excitation wavelength was set to 520 nm and fluorescence emission was monitored from 570 nm – 720 nm with a 0.1 s integration time. Recorded values were the average of three scans per sample. The slit widths were set to 2.50 nm for both excitation and emission. For experiments with silver nanoparticles, the excitation wavelength was 400 nm and fluorescence emission was monitored from 520 nm – 720 nm with a 0.1 s integration time. Recorded values were the average of three scans per sample. The slit widths were set to 2.50 nm for both excitation and emission.

Total Internal Reflection Fluorescence Microscopy. Single particle fluorescence imaging was performed on a Nikon Ti-E/B (Tokyo, Japan) inverted microscope equipped with a Nikon 100x Apo TIRF 1.49 NA objective lens and an Epi/TIRF illuminator. Static images were recorded with a Hamamatsu (Hamamatsu City, Japan) Orca-R2 interline charge coupled device (CCD) camera. The sample was illuminated with the 488 nm line.
of a Spectra Physics (Santa Clara, CA) 177g argon-ion laser, which was controlled using an acousto-optic tunable filter from Solamere (Salt Lake City, UT). The excitation light was directed to a Chroma (Bellows Falls, VT) ZT488rdc dichroic mirror, which directed the light to the sample. Emission light was filtered by a Chroma ET500lp long-pass filter and a Chroma ET525/50m band-pass filter. Images were acquired using Micro-Manager microscopy software.74

**Spinning Disk Confocal Microscopy.** A spinning disk confocal head (Yokogawa CSU-X1-M1N-E, Solamere, Salt Lake City, UT) was custom fit to the microscope and camera described in the previous section. A T405/488/568/647 multiline dichroic (Semrock, Rochester, NY) in the spinning disk head was used to direct excitation light to the sample. Emission light was filtered with an ET525/50M (Chroma, Bellows Falls, VT) in a custom-mounted filter wheel (ASI FW-1000, Eugene, OR). Images were captured using a 1024x1024 pixel electron-multiplying CCD camera (Andor iXon3 888, Belfast, Ireland) using MicroManager software, as above.74

**Fluorescence Lifetime Measurements and Fluorescence Correlation Spectroscopy.** Fluorescence lifetime and fluorescence correlation spectroscopy measurements were acquired on a customized Nikon TE2000E inverted fluorescence microscope with a Nikon 100X Apo TIRF NA 1.49 objective lens. The microscope was modified to include an additional dichroic mirror (Chroma Technology Corp., Rockingham, VT) and a Nikon PFS system, which maintains the focal position. Illumination was provided with a 479 nm pulsed diode laser (LDH-P-C-485, PicoQuant, Berlin, Germany) set to a repetition rate of 10 MHz. Fluorescence emission light was collected through the objective and passed through a custom notch filter (Semrock, Rochester, NY) to remove any scattered laser light. The emitted light was then passed through a 50 μm confocal pinhole (Thorlabs, Newton, NJ), a 492 nm long pass filter and a 550 nm short pass filter. The emission light was split by a 50/50 beamsplitter onto two avalanche photodiodes (APDs) (SPCM-AQRH-16, Perkin-Elmer, Canada). This configuration allowed cross-correlation of the signal to remove contribution of the after-pulsing effect of the detectors to the correlation function.

**Samples were deposited on Lab-tek chamber slides as droplets.** A focal point was chosen at which fluorescence intensity did not change despite significant changes in the focus knobs, indicating that we were observing fluorescence emission from the solution.

The detectors and the pulsed laser were connected to a PicoQuant TimeHarp 200 time-correlated single photon-counting (TCSPC) card, which collects signal from the APDs through a universal router (PicoQuant PRT 400, TTL SPAD router). The power of each laser was measured at 5 μW before entering the optical path of the microscope and was checked periodically. No observable change in excitation power was noted throughout the course of the experiment. The autocorrelation function was calculated using a multiple-tau algorithm.75
1.7.3. Experimental procedures

**Cloning and expression of MS2 mutants.** Bacteriophage MS2 T19pAF plasmid production and growth has been previously reported. The Peter Schultz lab (Scripps Research Institute, La Jolla, CA) provided the tRNA- and tRNA-synthetase-encoding plasmids necessary for p-aminophenylalanine (pAF) incorporation. A yield of ~10 mg/L was obtained for MS2-pAF19 following two purification rounds. For the T19pAF N87C MS2, position 87 was mutated into a cysteine using the following forward and reverse primers to allow for the incorporation of a maleimide dye through a cysteine alkylation reaction.

Forward: 5’ – AGCCGCATGCGTTCGTACTTATGTATGGAACTAACCATT – 3’  
Reverse: 5’ – GAATGGTTAGTTCCATACATAAGTACGAACGCCATGCGGCT – 3’

Growth and purification of T19pAF N87C MS2 was identical to that of T19pAF MS2.

**Interior Surface Modification of T19pAF N87C MS2.** The general procedure for the cysteine alkylation reaction of T19pAF N87C MS2 with an Alexa Fluor 350 c5-maleimide (Invitrogen, Carlsbad, CA) and AF 594 c5-maleimide (Invitrogen, Carlsbad, CA) was performed as reported previously.

**General procedure for the quantification of oligonucleotide coverage on AuNPs.** A typical reaction proceeded as follows. To a solution of AuNPs modified with DNA (concentration of AuNPs determined by absorbance at 520 nm) was added a solution of fluorescently-labeled complementary DNA (the identity of the sequence is given below) at a final concentration of 12.5 μM in 10 mM phosphate buffer pH 7.0 with 0.3 M NaCl. The resulting solution was allowed to incubate overnight at rt while protected from light. Samples were subsequently centrifuged for 15 min at 14,000 rpm. Fluorescence emission spectra were then taken of the resulting supernatant. The difference in fluorescence intensity maximum between the control sample and the cAuNP sample (corresponds to the DNA attached to the AuNPs) was recorded. To determine the concentration of fluorescently labeled DNA, solutions of fluorescently-labeled complementary DNA were made at a concentration of 1 μM, 10 μM, 25 μM, 40 μM, and 50 μM. A fluorescence calibration curve was then made from these samples. The calibration curve allowed for the determination of DNA concentration in the unknown samples (control sample, cAuNP sample, and ncAuNP sample). The oligonucleotide surface coverage was then determined by dividing the concentration of DNA attached to AuNPs (control sample minus the cAuNP sample) by the concentration of AuNPs in solution (previously determined from absorbance). This value corresponded to the average number of DNA per AuNP in solution.

The sequence identity of fluorescently labeled ssDNA complementary to sequence attached to AuNP is as follows:

5’– 56-FAM– ATGTTAGCGTAATGTGAATTTGTGTGT – 3’
**General procedure for incubation of AuNP-DNA and MS2-cDNA for design 1.** The concentration of T19pAF N87C MS2 and AuNPs were estimated by measuring their absorbances at 280 nm, and 520 nm, respectively. The desired amounts of MS2 and AuNPs were then combined and allowed to incubate in the dark at rt for 1.5 h. For TEM images within the text, a high concentration of AuNPs relative to MS2 (0.12 MS2 capsids: AuNPs) corresponds to 2 μM MS2 monomer and 0.09 μM AuNPs while a low concentration of AuNPs relative to MS2 (1.85 MS2 capsids: AuNPs) corresponds to 30 μM MS2 monomer and 0.09 μM AuNPs.

The sequence identity of ssDNA attached to AuNPs - complement to MS2-DNA is as follows:

5’ – 5ThioMC6-D – ACACACAAATTTCACATTACGCTAACAT – 3’

The sequence identity of ssDNA attached to MS2 - complement to AuNP-DNA is as follows:

5’ – 5AmMC6 – ATGTTAGCGTAAATGTGATTTGTGTGT – 3’

The sequence identity of ssDNA attached to AuNPs - not complementary to MS2-DNA is as follows:

5’ – 5ThioMC6-D – TTACGCAATTTCACATAACATACACG – 3’

**Design 1 DNA sequences for fluorescence intensity results.**

The sequence identities of oligonucleotides attached to MS2 are as follows:

- Short: 5’-/5AmMC6/GTG-AAT-TTG-TGT-GT-3’
- Medium: 5’-/5AmMC6/ATG-TTA-GCG-TAA-TGT-GAA-TTT-GTG-TGT-3’

The sequence identities of oligonucleotides attached to AuNPs are as follows:

- Complementary short: 5’-/5ThioMC6-D/ACA-CAC-AAA-TTC-AC-3’
- Complementary medium: 5’-/5ThioMC6-D/ACA-CAC-AAA-TTC-ACA-TTA-CGC-TAA-CAT-3’

**Synthesis of PVP-stabilized Pd nanoparticles.** Palladium nanoparticles (PdNPs) were prepared according to a previously reported procedure. A series of four seeding and growth reactions were used to produce the final PdNPs. PdCl₂ (0.6 mmol), 0.2 M HCl (6.0 mL), and dd-H₂O (294 mL) were combined to afford an aqueous solution of
H₂PdCl₄ (2.0 mM). The 2.0 mM PdCl₄ solution (15 mL) was combined with dd-H₂O (21 mL), ethanol (14 mL), and polyvinylpyrrolidone (0.0667 g, average Mₗ = 25,000) and refluxed for 3 h. The resulting solution was then used to seed a subsequent growth of the nanoparticles by combining the above PVP-Pd solution (25 mL) with 2.0 mM H₂PdCl₄ (7.5 mL), dd-H₂O (9.2 mL) and ethanol (8.3 mL). The resulting solution was then refluxed for 3 h. The seeding and growth steps were repeated twice more to afford the final PdNP solution.

Preparation of poly-L-lysine coated glass slides for TIRF images. Glass slides were first cleaned for 1 h with a 1% HCl solution in 70% ethanol. A 0.01% poly-L-lysine solution was prepared via a 1:10 dilution of 0.1% poly-L-lysine solution (Sigma Aldrich) in dd-H₂O. Cleaned slides were then incubated with a freshly prepared 0.01% poly-L-lysine solution for 1 h. Following incubation, the poly-L-lysine solution as removed, and the slide was rinsed once with dd-H₂O.

DNA and PEG surface patterning for slide images shown in Figure 1.20 and 1.21. DNA and PEG-modified glass were prepared as previously described. Briefly, glass slides were oxidized in an O₂ plasma cleaner (100 W) for 2 min and then immediately submerged in a 2% trimethoxysilane aldehyde solution. The slides were then rinsed in a 8.6% glacial acetic acid/methanol solution (v/v) and dried in a 120 °C oven for 10-20 min. 5'-amine DNA and methoxypolyethylene glycol amine (as 80 μM solutions) in pH 7 sodium chloride sodium citrate (SSC) buffer were then added to the aldehyde-coated glass, in the ratios described within the text. The solutions were then dehydrated at 120 °C for 30 min to drive imine formation and then rinsed in a 0.4% SDS aqueous solution, followed by dd-H₂O. The resulting slides were then exposed to a solution of sodium borohydride (66 mM in 1:3 EtOH: phosphate buffer pH 7) for 15 min with periodic stirring and then rinsed in a 0.4% SDS aqueous solution, followed by dd-H₂O, and dried under a stream of N₂.

Preparation of gold nanoparticles (AuNPs) for reassembly

Phosphination of AuNPs (5 nm and 10 nm diameter). Phosphination of AuNPs (5 nm and 10 nm diameter) was carried out following a previously reported procedure. In a typical reaction, bis(µ-sulfonatophenyl)phenylphosphine dehydrate dipotassium salt (BSPP, 5 mg) was mixed with AuNPs (10 mL) and vigorously stirred at rt overnight. Solid NaCl was then added to the AuNP solution until the color turned from deep red to purple. The solution was then centrifuged (5000 rpm) for 10 min, yielding a pellet of nanocrystals. The supernatant was carefully removed and the pellet redissolved in an aqueous solution of BSPP (25 mg in 100 mL dd-H₂O). The redissolved nanocrystal solutions were combined and then precipitated through the addition of methanol, until the color again changed from deep red to purple. The solution was again centrifuged (5000 rpm) for 10 min and the supernatant was removed. The nanocrystal pellet was again dissolved in an aqueous solution of BSPP, and stored at room temperature until use. The AuNP concentration was determined by recording the absorbance at 520 nm, and relating the absorbance values to concentration via Beer’s Law (ε₅₂₀ nm = 9.3*10⁶ M⁻¹cm⁻¹ and ε₅₂₀ nm = 8.1*10⁷ M⁻¹cm⁻¹ for 5 nm and 10 nm AuNPs, respectively).
Phosphination of AuNPs (15 nm, 20 nm and 40 nm diameter). BSPP (5 mg) was added to a solution of AuNPs (10 mL) and stirred vigorously overnight. The AuNP solution was then concentrated through multiple rounds of centrifugal filtration with a 100 kD MWCO centrifugal filter (Millipore), and diluted in an aqueous solution of BSPP (25 mg in 100 mL dd-H$_2$O). The AuNP concentrations were determined by recording their absorbance at 524 nm (15 nm AuNPs), 525 nm (20 nm AuNPs), and 528 nm (40 nm AuNPs), and relating their absorbance values to concentration via Beer’s Law ($\epsilon_{524\text{ nm}} = 2.4 \times 10^8$ M$^{-1}$cm$^{-1}$, $\epsilon_{525\text{ nm}} = 6.4 \times 10^8$ M$^{-1}$cm$^{-1}$, and $\epsilon_{528\text{ nm}} = 7.7 \times 10^8$ M$^{-1}$cm$^{-1}$ for 15 nm, 20 nm, and 40 nm AuNPs, respectively).$^{79-81}$

Alkanethiol oligonucleotide modification of BSPP-stabilized AuNPs. Alkanethiol oligonucleotide modification of BSPP-stabilized AuNPs was achieved using a modified version of a previously reported procedure.$^{77,80}$

The oligonucleotide sequence identity was as follows:

5’-/5ThioMC6-D/TTT-TTT-TTA-CAT-GGG-TAA-TCC-TCA-TGT-3’

Lyophilized oligonucleotides containing a 5’ thiol modifier [5’-(CH$_2$)$_6$-S-S-(CH$_2$)$_6$-phosphodiester bond-oligonucleotide-3’] purchased from IDT technologies (Coralville, IA) were resuspended in dd-H$_2$O to yield a final concentration of 100 μM. The disulfide functionality was cleaved by incubation with DTT (0.1 M) at rt in 100 mM phosphate buffer pH 8.0 for 1 h. Excess DTT was removed by passing the solution through a commercially available gel filtration column pre-equilibrated with dd-H$_2$O (NAP 5 column, GE Healthcare). It is important to note that 90% of the recommended volume was applied to the column for elution, to prevent excess DTT from remaining in the oligonucleotide solution. Fractions collected from the gel filtration column that contained oligonucleotides were combined and immediately added to BSPP-stabilized AuNPs (9 nM) to yield a final oligonucleotide concentration of 10 μM. The resulting solution was then incubated at rt for 20 min. The concentration of NaCl was increased by increments of 0.05 M to a final concentration of 0.1 M through successive additions of 2 M NaCl. Following each addition of 2 M NaCl, the alkanethiol oligonucleotide-modified AuNP solution was sonicated for 10 s, followed by agitation at rt for 20 min. After the final concentration of 0.1 M NaCl was reached, the AuNP solution was allowed to incubate at rt overnight. Excess oligonucleotides were removed through multiple rounds of centrifugal filtration with a 100k MWCO centrifugal filter (Millipore), and diluted in 50 mM phosphate buffer, pH 7.0. The final AuNP concentration was approximated by measuring the absorbance at 520 nm, 520 nm, 524 nm, and 528 nm for 5 nm, 10 nm, 15 nm, and 20 nm diameter AuNPs, respectively.

Disassembly and reassembly of T19pAF MS2 around AuNPs (10 nm diameter). Bacteriophage MS2 T19pAF was first subjected to disassembly conditions, using a method previously described.$^{54,66}$ In a typical disassembly reaction, 150 μM T19pAF MS2 in 50 mM phosphate buffer, pH 7.2 (200 μL) was combined with two volumes of cold glacial acetic acid (400 μL) and kept on ice for 30 min. The mixture was then centrifuged for 20 min (13,000 rpm at 4 °C). Following centrifugation, the supernatant was removed and applied to a gel filtration column (NAP-5, GE Healthcare) pre-equilibrated with 1 mM acetic acid. Fractions were collected from the gel filtration column and their absorbance
values at 280 nm were recorded. The fractions containing MS2 were combined on ice and used immediately.

For reassembly, the final MS2 coat protein dimer concentration was approximated by recording the absorbance at 280 nm and assuming eighty percent reassembly efficiency. The AuNP concentration was approximated by measuring the maximum absorbance and relating that value to the concentration via Beer’s law. In a typical reassembly reaction, AuNPs (final ratio of 1:1 AuNPs: MS2 capsids) were combined with two thirds volume of 100 mM phosphate buffer, pH 7.2 containing 100 mM NaCl. The final one third volume comprised MS2 coat protein dimer solution. The solution was then kept at 4 °C for 40 h to allow for reassembly. Following reassembly, a saturating amount of NaCl was added directly to the solution. The reassembly reaction was then applied to a gel filtration column (NAP-5, GE Healthcare), pre-equilibrated with 10 mM phosphate buffer, pH 7.2. Samples were then concentrated through multiple rounds of centrifugal filtration with a 100k MWCO centrifugal filter (Millipore), and diluted in 10 mM phosphate buffer, pH 7.2. The reassembly was confirmed through dynamic light scattering, transmission electron microscopy, and native agarose gel electrophoresis (2.5% agarose, 50% glycerol loading buffer, 0.5x TBE running buffer, pH 8.0, 2 h at 40 V, on ice). A portion of the sample was then run on an SDS-PAGE gel with standard concentrations of MS2. The concentration of the reassembled sample was then determined by optical densitometry using ImageJ software.

If desired, the MS2 samples containing 10 nm AuNPs were also able to be purified via PEG precipitation (0.5 M NaCl, 10 w/v% PEG-6k) for 1 h at 4 °C. MS2 samples were isolated through centrifugation at 5000 rpm for 20 min. The supernatant was carefully removed and the precipitate was resuspended in 50 mM phosphate buffer, pH 7.0. The resuspended sample was then passed through a gel filtration column pre-equilibrated with 50 mM phosphate buffer, pH 7.0 (NAP-5, GE Healthcare).

Preparation of MS2 hairpin DNA constructs - alkylation of thiol oligonucleotides. Oligonucleotides were purchased with thiol groups installed at either the 5’ or 3’ end as designated below. The oligonucleotide sequence identities were as follows:

3 bp hairpin: 5’-/5AmMC6/-TTT-ATG-GCA-CGC-CCT-TTT-TTC-CGC-GTG-CCA-/Thio-C3-SS/-3’

12 bp hairpin: 5’-/5AmMC6/-TTT-TTT-TTT-TTT-CGG-CAC-GCC-CTT-TTT-TCC-GCG-TGC-CG-/Thio-C3-SS/-3’

24 bp hairpin: 5’-/AmMC6/-TTT-TTT-TTT-TTT-CCC-TTT-CCC-TTT-GTC-GGC-ACG-CCC-TTT-TTT-CGG-CGT-GCC-GAC-/Thio-C3-SS/-3’

complementary hairpin: 5’-/5ThioMC6-D/-GGC-ACG-CGG-AAA-AAA-GGG-CGT-GCC-3’

Alkylation of thiol oligonucleotides was performed as previously described.14 Alexa Fluor 488 c5 maleimide (Invitrogen) was stored as a 20 mM stock in DMSO at -20 °C until
use. The following sequences were modified with Alexa Fluor 488 c5 maleimide: 3 bp hairpin, 12 bp hairpin, and 24 bp hairpin. Aliquots of 10 mM biotin maleimide 5k PEG in DMSO were stored at -20 °C until use. The following sequences were modified with biotin maleimide 5k PEG: complementary hairpin. Commercially available gel filtration columns (NAP 5, GE healthcare) were used to separate the small molecule or PEG from DNA (3 bp hairpin, 12 bp hairpin, 24 bp hairpin, and complementary hairpin). The DNA samples were then lyophilized and re-suspended in 50 mM phosphate buffer, pH 8.0. All reactions were monitored by MALDI-TOF-MS.

**General procedure for the addition of ortho-nitrophenol to oligonucleotides.** DNA (3 bp hairpin, 12 bp hairpin, and 24 bp hairpin) was labeled with o-nitrophenol following labeling of the 3' thiol with AF 488 maleimide dyes. A typical reaction was as follows: DNA at a concentration of 300 μM was reacted with ortho-aminophenol succinimidyl ester (60-120 eq.) in 1:1 solution of DMF and 50 mM phosphate buffer, pH 8.0. The reaction mixture was briefly vortexed and allowed to react at rt for 1.5 h. DNA samples were then filtered through commercially available 0.22 μm centrifugal filter units (Millipore Corporation, Billerica, MA). Commercially available gel filtration columns (NAP 5, GE healthcare) were then used to separate the small molecule from DNA. A 100 mM stock of Na₂S₂O₄ in 200 mM phosphate buffer, pH 6.5 was added to the purified DNA mixture at a final concentration of 10 mM and allowed to react for 10 min. This yielded the ortho-aminophenol labeled oligonucleotides which were further purified through multiple rounds of gel filtration columns, lyophilized, and resuspended in 50 mM phosphate buffer, pH 7.0 to a final concentration of ~1 mM. DNA hairpin formation was then promoted by heating the DNA samples to 95 °C followed by cooling to 4°C at 1°C/5 min. DNA samples were held at 4 °C prior to attachment to MS2.

**General procedure for ortho-aminophenol-DNA conjugation to MS2.** The general procedure for the conjugation of DNA to MS2 was followed as reported previously. Reassembled T19pAF MS2 (20 μM) and ortho-aminophenol-containing oligonucleotide (200 μM) were combined in 50 mM phosphate buffer, pH 7.0, containing 150 mM NaCl. The solution was then combined with NaIO₄ (1 mM), briefly vortexed and allowed to react at rt for 5 min. For a 50 μL reaction, the reaction was then quenched by the addition of tris(2-carboxyethylphosphinehydrochloride (TCEP, 10 μL of 330 mM). The pH of the TCEP solution was adjusted to 7.0 prior to addition. The resulting sample was then purified using a gel filtration column (NAP-5, GE healthcare). The eluent from the column was then concentrated through successive centrifugal filtrations using 100k molecular weight cutoff filters (Millipore) to remove excess DNA. It is critical for all TIRF, confocal, and lifetime measurements that excess fluorescently-labeled DNA is removed; this can either be achieved through successive centrifugal filtrations or purification on a non-denaturing agarose gel. A portion of the sample was then run on an SDS-PAGE gel. Optical densitometry was then used to determine the percent DNA modification.

**Incubation of MS2 and MS2 (10 nm AuNPs) for 12 bp hairpin and 24 bp hairpin sequences with stabilizing strands.** Stabilizing oligonucleotide sequences were dissolved in 50 mM phosphate buffer pH 7.0 to a concentration of 300 μM.
The sequence identities of the stabilizing sequences were as follows:

12-s hairpin: 5’-AAA-AAA-AAA-AAA-3’

24-s hairpin: 5’-AAA-GGG-AAA-GGG-AAA-AAA-AAA-AAA-3’

In a typical incubation, MS2-DNA (~10 μM monomer), NaCl (300 μM), and stabilizing DNA (~50 μM) were combined to a final volume of 30 μL in 50 mM phosphate buffer, pH 8.0. This solution was cycled from 37 °C to 4 °C at 0.5 °C/min, and then held at 4 °C for 2-10 h.

**Synthesis of ortho-nitrophenol acid.** The synthesis of ortho-nitrophenol acid was performed as reported previously.82 Spectral data agreed with those previously reported.82-84

**Synthesis of ortho-nitrophenol succinimidyl ester.** The synthesis of ortho-nitrophenol succinimidyl ester was performed as reported previously. Spectral data agreed with those previously reported.83,84

**Synthesis of 4-(4-diethylamino-phenylcarbamoyl)-butyric acid succinimidyl ester.** The synthesis of this compound was performed as reported previously. To an oven-dried round bottom flask equipped with a Teflon stir bar was added 4-(N,N-diethylamino) aniline hydrochloride (500 mg, 2.49 mmol) and dichloromethane (30 mL) followed by triethylamine (0.348 mL, 2.49 mmol). Glutaric anhydride (284 mg, 2.49 mmol) was then added and the resulting solution was stirred at rt for 30 min. N,N’-dicyclohexylcarbodiimide (479 mg, 2.49 mmol) and N-hydroxysuccinimide (287 mg, 2.49 mmol) were then added, and the stirring was continued for 1 h at rt. The reaction was then gravity filtered to remove precipitate and concentrated under reduced pressure. The resulting black oil was dissolved in dichloromethane, gravity filtered, and applied directly to a silica gel column (20% - 100% EtOAc: Hexanes). Fractions were collected and concentrated yielding a light green oil, 217 mg (23 %) that was dissolved in 2.3 mL of dimethylsulfoxide and frozen for storage until use. 1H NMR (400 MHz, CDCl3): δ 8.13 (s, 1H), δ 7.18 (d, 2H, J = 9 Hz), δ 6.43 (d, 2H, J = 9 Hz), δ 3.15 (q, 4H, J = 7 Hz), δ 2.63 (br-s, 4H), δ 2.52 (t, 2H, J = 7 Hz), δ 2.23 (t, 2H, J = 7 Hz), δ 1.97 (p, 2H, J = 7 Hz), δ 1.07 (t, 6 H, J = 7 Hz). 13C NMR (400 MHz, CDCl3): δ 169.6, 169.4, 168.4, 144.8, 127.1, 121.9, 112.2, 44.4, 34.9, 30.6, 25.5, 20.8, 12.3.

**General procedure for the addition of phenylenediamine to oligonucleotides.**

The general procedure for the addition of phenylenediamine to oligonucleotides was reported previously.48 The oligonucleotide sequence identity for design 2 was as follows:

A: 5’-/5AmMC6-D/TTT-TTT-TTT-TAT-AGC-GTA-ATG-TGA-ATT-TGT-GTG-3’

A typical reaction was as follows: DNA at a concentration 300 μM was reacted with 4-(4-diethylaminophenylcarbamoyl) butyric acid succinimidyl ester (60-120 eq.) in 1:1 solution of DMF and 50 mM pH 8.0 phosphate buffer. The reaction mixture was briefly
vortexed and allowed to react at rt for 1.5 h. Commercially available gel filtration columns (NAP 5, GE healthcare) were then used to separate the small molecule from the DNA. The DNA sample was then lyophillized overnight and re-suspended in 10 mM phosphate buffer, pH 7.2.

**General procedure for phenylenediamine-DNA conjugation to MS2.** The general procedure for the conjugation of DNA to MS2 was followed as reported previously. Reassembled T19pAF MS2 (20 μM) and phenylenediamine-containing oligonucleotide (200 μM) were combined in 50 mM phosphate buffer, pH 7.0, containing 150 mM NaCl. The solution was then combined with NaIO₄ (5 mM), briefly vortexed and allowed to react at rt for 1 h. For a 100 μL reaction, the reaction was then quenched by the addition of tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 10 μL of 500 mM). The pH of the TCEP solution was adjusted to 7.0 prior to addition. The resulting sample was then purified using a gel filtration column (NAP-5, GE healthcare). The eluent from the column was then concentrated through successive centrifugal filtrations using 100k molecular weight cutoff filters (Millipore) to remove excess DNA. A portion of the sample was then run on an SDS-PAGE gel. Optical densitometry was then used to determine the percent DNA modification.

**Alkylation of thiol oligonucleotides.** Oligonucleotides were purchased with thiol groups installed at either the 5’ or 3’ end as designated below.

The oligonucleotide sequence identities were as follows:

- 3 bp A’: 5’-CAC-ACA-AAT-TCA-CAT-TAC-GCT-AAC-ATA-AAA-AAA-AAA’-/Thio-C3-SS/-3’
- 12 bp A’: 5’-CAC-ACA-AAT-TCA-CAT-TAC-GCT-AAC-ATA-/Thio-C3-SS/-3’
- 24 bp A’: 5’-CAC-ACA-AAT-TCA-CAT-/Thio-C3-SS/-3’
- surface 24 bp A’: 5’-/5ThioMC6-D/-CAC-ACA-AAT-TCA-CAT-3’

Alkylation of thiol oligonucleotides was performed as previously described. Alexa Fluor 488 c5 maleimide (Invitrogen) was stored as a 20 mM stock in DMSO at -20 °C until use. The following sequences were modified with Alexa Fluor 488 c5 maleimide: 3 bp A’, 12 bp A’, and 24 bp A’. Aliquots of 10 mM biotin maleimide 5k PEG in DMSO were stored at -20 °C until use. The following sequences were modified with biotin maleimide 5k PEG: surface 24 bp A’. DNA (3 bp A’ and 12 bp A’ sequences) was isolated using the ethanol precipitation protocol of Shah, et al. Commercially available gel filtration columns (NAP 5, GE healthcare) were used to separate the small molecule or PEG from DNA (24 bp A’ and surface 24 bp A’ sequences). The DNA samples were then lyophilized overnight and re-suspended in 50 mM phosphate buffer, pH 8.0. All reactions were monitored by MALDI-TOF-MS.

**Incubation of MS2 (10 nm AuNPs)-A, MS2-A with (3 bp A’ + AF 488, 12 bp A’ + AF 488, 24 bp A’ + AF 488).** Stabilizing oligonucleotide sequences were dissolved in 50
mM phosphate buffer pH 7.0 to a concentration of 100 μM.

The sequence identities of the stabilizing sequences were as follows:

12-s: 5’-AAA-AAA-AAA-AAA-3’

24-s: 5’-TAC-GCT-AAC-ATA-AAA-AAA-AAA-3’

In a typical incubation, MS2-DNA (~10 μM monomer), NaCl (300 μM), DNA-fluorophore (~30 μM), and stabilizing DNA (~20 μM) were combined to a final volume of 30 μL in 50 mM phosphate buffer pH 8.0. This solution was cycled from 37 °C to 4 °C at 0.5 °C/min 5 times, and then held 4 °C for 2-10 h. Following incubation, the solution was concentrated into 0.5x TBE buffer, pH 8.0, in 100k MWCO spin filters (Millipore) at 4 °C to remove some excess DNA-fluorophore and buffer exchange for agarose gel electrophoresis. These samples were then run on an agarose gel (1% agarose, 50% glycerol loading buffer, 0.5x TBE running buffer pH 8.0, 80 V, 1 h, on ice). The gel was then visualized under ultraviolet light, and bands containing MS2 were extracted, cut into small pieces and placed into a gel extraction spin column (Freeze ‘N Squeeze, Biorad, Hercules, CA). Following the addition of 200 mM phosphate buffer pH 7.0 (200 μL), the extraction spin columns were kept at 4 °C for 1 h. The spin columns were then centrifuged for 10 min (5000 rpm). The flow through was then applied to 100k MWCO spin columns and spun for 10 min (5000 rpm). This process was repeated three additional times; the combined samples were then stored at 4 °C and imaged within 12 h.

Preparation of glass coverslips for TIRF and confocal microscopy. Coverslips were cleaned by sonication in a 50:50 (v/v) mixture of isopropanol and water. They were then dried and further cleaned by plasma treatment for 5 min in a Harrick Plasma PDC-32G plasma cleaner. They were then assembled with an Attofluor cell chamber (Invitrogen, Carlsbad, CA) and 0.25 mL poly-L-lysine PEG with PLK-PEG-biotin (500:3 ratio of the two solutions by volume) was added. After 30 min, the samples were rinsed five times with 5 mL of 1xTAE-Mg2+ (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, and 12.5 mM magnesium acetate, pH 8.0) buffer each time. Neutravidin was added to a final concentration of 0.1 mg/mL and incubated for 10 min. Excess neutravidin was rinsed with ten 5 mL rinses with 1xTAE-Mg2+ buffer. The sample was incubated for an additional 30 min and then rinsed with five 5 mL portions of 1xTAE-Mg2+ buffer. PEG-biotin labeled DNA was added to approximately 10 nM final concentration and incubated for 10 min. Again, excess DNA was rinsed away with five 5 mL portions of 1xTAE-Mg2+ buffer. Capsid solutions were added until an appropriate density was achieved.

TIRF image collection. Samples were checked for fluorescence contamination by finding focus using reflection interference contrast microscopy and then examining the sample with the same imaging conditions as described below. In general, the level of observable particles before adding our capsid sample was very low: most fields of view contained at most 1-2 particles.

TIRF angle was empirically optimized for the first sample we examined on a given day, and kept constant between samples. The angle was adjusted until few diffusing
species could be seen during stream acquisition. Since coverslips may differ in thickness (although the observations did not indicate that re-optimization was necessary), images were sometimes collected from the same sample at de-optimized TIRF angles and little difference was observed in molecular brightness. The stage was then moved to a new area and a series of images was collected with an exposure time of 240 ms. The camera was set to bin pixels on chip 2 by 2, providing a pixel size of 0.1284 μm.

These settings provided sufficiently high signal to noise ratio to resolve individual capsids, as described in the image analysis section. Regions of interest were chosen without prior inspection to avoid photobleaching before data collection.

**TIRF image analysis.** Each image set was analyzed separately using ImageJ software. Images were loaded into the program as a stack, background subtracted, and the threshold range was determined (image\adjust\threshold\default). The threshold range for each stack was set as twenty five percent of the smallest maximum through the largest maximum. Images that contained few or no particles (less than 10) were removed from each stack. The particles were then analyzed by selecting the size as greater than or equal to 3 square pixels and the circularity was selected to be 0.90 – 1.00 (analyze\ analyze particles). The area, mean, and integrated intensity were recorded for each particle spot in the stack of images. Mean intensity histograms were fit to a Gaussian curve, as shown as a black curve in the plots using OriginPro 8 software.

**Preparation of glass plates for lifetime measurements.** Glass 8-well plates were incubated with 0.5 M NaOH for 1 h at rt. The NaOH solution was then removed, and a solution of BSA in phosphate buffered saline was added to the wells. Following overnight incubation at 4 °C, the BSA solution was removed, the plates were rinsed once with 1x TAE-Mg²⁺ buffer, and dried prior to sample addition. All prepared glass plates were used within 3 h of preparation.

**Analysis of fluorescence lifetime data.** The instrument response function was measured at approximately one nanosecond, while the measured decay curves have lifetimes on the order of a few nanoseconds. Consequently, a simple semi-log plot with linear regression was not sufficient to extract lifetime information. Thus, the decay curves were fit via an iterative non-linear least squares method that takes into account the instrument response function as measured from a colloidal sample. The samples containing AuNPs scattered light strongly, and the signal due to scattering was incorporated into the fitting to correctly account for the signal shape. Measurement error was calculated as the standard deviation between three successive acquisitions. The error of the fitting procedure was also evaluated and included in the stated errors by calculating the standard deviation of the fitted time constants derived from randomized starting parameters. The following is the fitting function used.

\[
f(t) = g(t) * \left[ A \cdot H(t - t_0) \cdot e^{\frac{-(t - t_0)}{\tau}} + B \cdot \delta(t - t_0) \right]
\]

Where \( g(t) \) = the measured instrument response function, \( H(t) \) = the Heaviside step function that defines \( t_0 \) for the fluorescence decay, \( \delta(t) \) = the Dirac delta function that accounts for the scattered light, \( A \) = amplitude of the fluorescence component, \( t_0 \) = time
zero for the fluorescence decay, $\tau =$ decay time for the fluorescence component, $B =$ amplitude of the scattered light, and the * denotes a numerical convolution.

**Effect of TIRF microscopy versus confocal microscopy on brightness measurements.** The effect of small differences in the distance of capsids from the surface as a result of gold functionalization was considered in regards to the effect of these differences on measured fluorescence intensity, since the intensity of excitation light decays exponentially as a function of distance from the surface. The following equation relates intensity of the excitation light to the distance from the surface:88

$$I(z) = I(0)e^{-z/d}$$

$I(z)$ is the intensity at a given distance from the surface. $I(0)$ refers to the intensity at the surface. The distance from the surface is represented as $z$, and the penetration depth is represented by $d$. The penetration depth is given by:88,89

$$d = \lambda / 4\pi \left(1 / (n_1^2 \sin^2(\alpha) - n_2^2)\right)^{1/2}$$

The critical angle for the glass/water ($n_1 = 1.52, n_2 = 1.33$) interface is approximately 61°. This gives a penetration depth by the above calculation of 216 nm and would result in a difference of $e^{-1/216}$ or 0.5% in excitation light intensity for a difference in distance of 1 nm. Of course, it has been suggested that the depth of the evanescent field is not the only contribution to the $z$-selectivity of TIRF microscopy.88,90 In addition, the collection light efficiency of fluorophores nearer the surface is enhanced if a very high numerical aperture objective (NA > 1.4) is used. This behavior can be approximated using a single exponential with lower penetration depth. In a system similar to ours, but using an objective with a NA of 1.45 (instead of 1.49 in our study), the penetration depth was found to be 125 nm. The resulting difference of intensities between fluorophores separated by 1 nm would be $e^{-1/125}$ or 0.8%. Assuming an even smaller penetration depth of 60 nm, the difference in intensities between the aforementioned fluorophores would be expected to be approximately 1.6%. These differences cannot explain the differences in fluorescence intensity reported in the manuscript.

1.8. References


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(84) Obermeyer, A. C., University of California, Berkeley, 2013.


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Chapter 2

Expanding the types of inorganic materials for bacteriophage MS2 encapsulation

Abstract

Housing inorganic nanomaterials inside spherical viruses represents an area of particular interest because inorganic materials have interesting magnetic, catalytic, and optical properties. Taking advantage of the self-assembly of bacteriophage MS2, we recently reported the reassembly of MS2 for the encapsulation of RNA, DNA, poly(acrylic acid), negatively tagged enzymes, and DNA-labeled AuNPs. We sought to expand the types of inorganic materials housed inside MS2 to include AgNPs, CdSe/ZnS quantum dots, CdSe/CdS dot rods, and discrete assemblies of AuNPs. Pairing reported external modification strategies for MS2 that target both native and unnatural amino acids with a variety of interior inorganic cargo could produce materials for metal controlled fluorescence (AgNPs), dual fluorescence/electron microscopy (CdSe/ZnS and CdSe/CdS), and electron microscopy (discrete AuNP assemblies). Progress towards the construction of these materials is discussed.
2.1. Introduction: spherical protein cages as templates for the construction of inorganic materials

Viruses provide unique, nanometer-scale scaffolds for the construction of materials important for applications as diverse as materials synthesis, catalysis, and biomedicine. There exists a diverse library of protein cages, ranging in size from 10s to 100s of nanometers in diameter. These structures self-assemble from repeating subunits, providing templates that are often amenable to production in large quantities with low polydispersity, and high chemical stability. Each protein cage provides three unique interfaces for modification: the exterior surface, the interface between protein subunits, and the interior surface. Modification of the exterior surface can allow for the multivalent display of ligands. Altering protein subunit interfaces can modulate the structure or stability of the resulting capsid. Interior chemical modification can produce protein cages that house inorganic nanomaterials or contain small molecule cargo for applications in drug delivery.1-4

Housing inorganic nanomaterials inside spherical viruses represents an area of particular interest because inorganic materials have interesting magnetic, catalytic, and optical properties.1 There are two main strategies to produce protein cages that house inorganic nanomaterials, as highlighted in Figure 2.1. The first approach relies on metal nanoparticle growth inside a protein cage. In this strategy, the nucleation and growth of

Figure 2.1. Existing strategies for housing inorganic nanomaterials inside spherical viral capsids.
the inorganic materials is dictated by the charge of the interior residues of the protein. For example, Cowpea Chlorotic Mottle Virus (CCMV), which contains nine basic residues on the interior surface of each protein subunit (positive native interior charge), was used to nucleate polyoxometalate species (paratungstate and decavanadate). A more common approach for this method is to take advantage of a protein cage with either a native or engineered negative interior charge. Proteins with negative interior charges have been used to grow Fe$_3$O$_4$, Fe$_2$O$_3$, Mn(O)OH, Mn$_3$O$_4$, Co(O)OH, Co$_3$O$_4$, Cr(OH)$_3$, Ni(OH)$_3$, In$_2$O$_3$, FeS, CdS, CdSe, ZnSe, palladium nanoparticles, silver nanoparticles, CoPt, and platinum nanoparticles. In each case, the growth of the inorganic material is confined to the proteins interior volume. In the second approach, disassembled protein subunits are incubated with ligand-coated metal nanoparticles of interest. This approach takes advantage of the self-assembly of viruses to promote the encapsulation of negatively charged materials in their interior volume. This strategy has been used to encapsulate AuNPs and CdSe/ZnS inside Brome Mosaic Virus (BMV), as well as AuNPs, CoFe$_2$O$_4$, and CdSe inside Red Clover Necrotic Mosaic Virus (RCNMV).

We recently reported the reassembly of bacteriophage MS2 around RNA, DNA, poly-(acrylic acid), negatively tagged enzymes, and DNA-labeled AuNPs, taking advantage of the self-assembly of MS2. We sought to expand the types of inorganic materials housed inside MS2 to include AgNPs, CdSe/ZnS quantum dots, CdSe/CdS dot rods, and discrete assemblies of AuNPs. Pairing reported external modification strategies for MS2 that target both native and unnatural amino acids with a variety of interior inorganic cargo could produce materials for metal controlled fluorescence (AgNPs), dual fluorescence/electron microscopy (CdSe/ZnS and CdSe/CdS), and electron microscopy (discrete AuNP assemblies). Progress towards the construction of these materials is discussed in the following chapter.

2.2. AgNPs for bacteriophage MS2 encapsulation

Reports of metal controlled fluorescence (discussed in chapter 1) describe a larger effect of silver nanoparticles (AgNPs) on nearby fluorophores as compared to similarly sized AuNPs. As discussed in chapter 1, we observed moderate to no enhancement of the fluorescence for dyes placed at fixed distances from 10 nm diameter AuNPs. We envisioned housing AgNPs in MS2 and placing dyes at fixed distances from the capsid surface to improve the magnitude of the enhancements we observed with 10 nm AuNPs. Two synthetic methods were tested to produce either water-soluble or organic-soluble AgNPs of ~10 nm diameter, as shown in Figure 2.2. The first method involved the reduction of silver nitrate with sodium borohydride or sodium citrate to yield water soluble AgNPs ~9 nm in diameter, as determined by UV-Vis absorbance, TEM, and DLS (characterization shown in Figure 2.3.). The second method involved the reduction of silver acetate with oleylamine to produce organic soluble AgNPs ~8 nm diameter, as determined by DLS.

AgNPs are known to be less stable than AuNPs to changes in pH and ionic strength. To render the AgNPs water soluble and stable to reassembly conditions, four different ligands (methoxy-PEG-thiol, thiol DNA, mercaptoundecanoic acid, and carboxylic acid-PEG-thio) were screened to replace the citrate/borohydride or oleylamine ligands (as shown in Figure 2.2.).
Following ligand exchange, AgNPs were tested for their overall charge (as determined by their mobility on a native agarose gel) and stability to reassembly buffer conditions (pH ranges of 6-8 and NaCl concentrations of 100-200 mM). For a successful reassembly, it was hypothesized that AgNPs would need to be negatively charged and stable to reassembly buffer conditions. Ligand 1 (methoxy-PEG-thiol) was found to produce neutrally charged AgNPs stable to reassembly buffer conditions. Ligand 2 (thiol DNA) and Ligand 3 (mercaptoundecanoic acid) gave negatively charged AgNPs that precipitated in reassembly buffer. Ligand 4 (carboxylic acid-PEG-thiol) resulted in negatively charged AgNPs that were stable in reassembly buffer.

Due to their exceptional stability to changes in ionic strength and pH, Ligand 1-stabilized AgNPs were subjected to MS2 reassembly conditions in the presence and absence of TMAO to determine if any amount of reassembly would occur regardless of the neutral charge on the AgNPs. Reassembly products were not observed. As shown in Figure 2.4-a, native agarose gel revealed ligand 1 AgNPs subjected to reassembly
conditions do not travel with the same electrophoretic mobility as native MS2. No reassembly products were observed by TEM.

A 1:1 ratio of Ligand 1:Ligand 2 stabilized AgNPs were also subjected to MS2 reassembly. Although these AgNPs were found to be negatively charged and stable to changes in ionic strength and pH, no reassembly products were observed by native gel (Figure 2.4-b) or TEM.

Ligand 4 stabilized AgNPs were also subjected to MS2 reassembly conditions. Native agarose gel revealed potential reassembly products. However, these products appeared to be misformed by TEM, often displaying partial capsid assembly. The most successful ligand combination for AgNP reassembly was found to be a 1:1 ratio of ligand 2:ligand 4 stabilized AgNPs, which gave AgNPs that traveled with the same electrophoretic mobility as MS2 by native gel and provided some MS2 capsids that appeared to be reassembled by TEM, as shown in Figure 2.4-c.

The difficulty in obtaining reproducibly stable and reassembled AgNPs encapsulated in MS2 informed our decision to abandon this project. The general instability of these materials would prohibit their applications in future metal controlled fluorescence studies.

**Figure 2.4.** AgNPs subjected to reassembly conditions coated with a) mPEG, b) mPEG/DNA, and c) carboxylic acid-PEG/DNA ligands. Native agarose gels shown in a) and b) demonstrate an unsuccessful reassembly of mPEG and mPEG/DNA coated AgNPs, as AgNPs subjected to reassembly conditions do not travel with the same electrophoretic mobility as native MS2. TEM images negatively stained with uranyl acetate in c) show MS2 capsids reassembled around carboxylic acid-PEG/DNA coated AgNPs.
2.3. CdSe/ZnS quantum dots and CdSe/CdS dot rods for bacteriophage MS2 encapsulation

Fluorescence-based methods, such as flow cytometry, are often used to evaluate the binding ability of cell-targeted imaging agents. These fluorescence-based methods rely on the assumption that a greater number of dyes attached per cell will give more fluorescence response per cell. For bacteriophage MS2-based agents, multiple targeting groups can be attached per capsid. The binding ability of these constructs can then be compared to their monomeric targeting groups, to determine if multiple targeting groups per capsid enhance or impair cell binding. Previous work in our group has noted that increased dye labeling per capsid does not result in increased capsid brightness. A set of DyLight 650-modified MS2 capsids containing between ~1 dye/capsid and >100 dyes/capsid were measured for their per particle brightness by SEC HPLC, as shown in Figure 2.5-a. It was found that the fluorescence quenching dominates at as little as ~15% dye modification. We think that the dyes inside MS2 quench due to the close proximity of the N87C residues on the interior assembled capsid, as shown in Figure 2.5-b. This non-linear dye brightness response makes it difficult to evaluate the binding of monomeric targeting groups versus MS2 constructs.

To address the issue of fluorescence quenching of dyes attached to the interior

![Figure 2.5](image)

**Figure 2.5.** Increased interior dye labeling of N87C MS2 results in fluorescence quenching of the attached dyes. a) absorbance/fluorescence of MS2 capsids with DyLight 650/capsid labeling as determined by HPLC SEC. A280 (blue diamonds) indicate similar total protein concentration for each measurement. A649 (red squares) show increased dye labeling corresponds to decreased total fluorescence (FL/A649 x 1000, purple circles). Fluorescence quenching is thought to occur due to the proximity of N87C residues when MS2 is assembled as a full capsid. A MS2 dimer shows b) the proximity of N87C residues on the interior surface.
of MS2, we sought to encapsulate CdSe/ZnS quantum dots and CdSe/CdS dot rods. Encapsulation of these materials should produce capsids with predictable brightness. CdSe/ZnS quantum dots and CdSe/CdS dot rods were chosen due to their superior fluorescence properties in comparison to traditional organic dyes. They exhibit long fluorescence lifetimes, fluorescence stability upon continuous excitation, a broad absorbance spectrum (Figure 2.6) and a sharply defined emission peak. Their broad absorbance allows for single excitation multicolor analysis for multiple quantum dots or dot rods. Furthermore, these materials are electron dense, and could potentially be used as dual multi-color fluorescence/electron microscopy imaging agents.

Multiple strategies were explored to house CdSe/ZnS or CdSe/CdS inside MS2 capsids (Figure 2.7-a). As in the case of AuNPs and AgNPs, these materials needed to be rendered water soluble, negatively charged, and stable to reassembly buffer conditions. CdSe/ZnS quantum dots and CdSe/CdS dot rods are synthesized in organic solvents, and stabilized by trioctylphosphine oxide (TOPO) ligands. These organic soluble materials were rendered water soluble through three approaches: incubation with an amphiphilic 40% octadecylamine polyacrylic acid polymer (Figure 2.7-b), ligand exchange with thiol-PEG-carboxylic acid/mercaptpropionic acid (Figure 2.7-c), and incubation with a hydrophobic anhydride-containing polymer (Figure 2.7-d). In the final approach, crosslinking the anhydride polymer with a diamine exposed carboxylic acid residues on the coating polymer. All three approaches gave water soluble, negatively charged quantum dots.

Next, these materials were subjected to MS2 reassembly conditions and evaluated by native agarose gel electrophoresis. As shown in Figure 2.8., the polyacrylic acid, 40% octadecylamine CdSe/ZnS quantum dots subjected to reassembly conditions did not travel with the same electrophoretic mobility as native MS2 at any of the reassembly pHs or salt concentrations screened. We hypothesize the small amount of reassembly product visible in the Coomassie-stained gel lane 4 is due to MS2 reassembly around excess polyacrylic acid polymer. No reassembly products were observed by TEM.

Mercaptpropionic acid (MPA) CdSe/ZnS quantum dots were also subjected to MS2 reassembly conditions and evaluated by native agarose gel electrophoresis. As
Figure 2.7. Strategies explored to house a) CdSe/ZnS quantum dots and CdSe/CdS dot rods inside reassembled MS2 capsids. Quantum dots or dot rods stabilized with hydrophobic TOPO ligands were rendered water soluble through b) incubation with an amphiphilic 40% octadecylamine polyacrylic acid polymer, c) ligand exchange with thiol-PEG-carboxylic acid/mercaptopropionic acid, and d) incubation with a hydrophobic anhydride containing polymer, followed by crosslinking with a diamine to yield exposed carboxylic acid residues.
Figure 2.8. Native agarose gel of 40% octadecylamine, polyacrylic acid CdSe/ZnS quantum dots (Emax 610 nm) subjected to MS2 reassembly conditions reveals the quantum dots are not encapsulated in MS2. As shown by the fluorescence image in a), quantum dots subjected to reassembly conditions travel with the same electrophoretic mobility as the quantum dots that were not subjected to reassembly conditions. In the Coomassie-stained gel, b), the small amount of MS2 reassembly product shown in lanes 3 and 4 most likely results from reassembly of MS2 around excess amphiphilic polymer.
shown in Figure 2.9-b, MPA quantum dots subjected to reassembly conditions were found to travel with the same electrophoretic mobility as native MS2. The reassembled sample was evaluated by TEM (Figure 2.9-b). Some assembled MS2 capsids were observed by TEM, but the quantum dots did not provide enough contrast to determine whether they were housed on the interior of MS2 or non-specifically attached to the exterior of the capsids.

To further elucidate whether or not the quantum dots were inside the capsids, reassembled samples were incubated with silver nitrate, followed by silver nitrate reduction with sodium borohydride (Figure 2.10), to yield 1-5 nm diameter AgNPs. We hypothesized the silver nitrate would preferentially nucleate on the negatively charged quantum dots. If the quantum dots were housed on the interior of the capsid, then silver NP growth should be clearly visible on the interior of the capsid via TEM. However, TEM images showed the silver nanoparticles grew either free in solution or on the exterior surface of the capsid. This indicated the quantum dots were possibly not housed inside the capsids, but rather non-specifically attached to the exterior capsid surface.

Reassembly experiments with CdSe/CdS dot rods were explored due to their superior TEM contrast (Figure 2.11) in comparison to CdSe/ZnS quantum dots. However, all reassembly experiments with these materials were not successful.

Figure 2.9. Native agarose gel a) fluorescence image and b) Coomassie-stained image of mercaptopropionic acid CdSe/ZnS quantum dots (E\text{max} 610 nm) subjected to MS2 reassembly conditions reveals that reassembled QDs travel with the same electrophoretic mobility as native MS2. TEM images of the reassembled sample shown in c) demonstrates that some MS2 capsids were reassembled, but the QDs did not provide enough contrast to clearly visualize whether or not they were housed inside the capsids.
Although MPA quantum dots subjected to MS2 reassembly conditions appeared to travel with the same electrophoretic mobility as MS2, suggesting MS2 encapsulation, a further control of co-injecting the MPA coated quantum dots with MS2 revealed a large amount of non-specific interaction between the MS2 and QDs (Figure 2.12, lane 4). This was not observed for DNA-coated AuNPs co-injected with MS2 (Figure 2.12, lane 3). The significant amount of non-specific interaction between MPA quantum dots and MS2 as well as the difficulty we had in visualizing the materials by electron microscopy informed our decision to abandon this project.

**Figure 2.10.** a) Mercaptopropionic acid CdSe/ZnS quantum dots subjected to reassembly conditions were incubated with silver nitrate followed by reduction with sodium borohydride to yield AgNPs. If the QDs were housed in MS2, the AgNPs should grow preferentially on the encapsulated quantum dots (top). However, as shown in the TEM image b) the AgNPs were observed to grow on the capsid surface.

**Figure 2.11.** TEM image of CdSe/CdS dot rods.
**Figure 2.12.** A native agarose gel of ssDNA coated AuNPs (lane 1) and mercaptopropionic acid QDs (lane 2). Co-injections of MS2 with ssDNA-AuNPs (lane 3) and mercaptopropionic acid QDs (lane 4) revealed non-specific interactions between MS2 and MPA QDs.
2.4. Multiple AuNP encapsulation in bacteriophage MS2

MS2 reassembly in the presence of excess 5 nm diameter DNA-labeled AuNPs gave MS2 capsids that contained between 0 and 5 AuNPs. Reassembly at ratios of 2:1, 3:1, 6:1, 10:1, and 15:1 gave a distribution of free AuNPs, 0, 1, 2, 3, 4, and 5 AuNPs per MS2 capsid, as well as misformed capsids, as shown in the histogram in Figure 2.13. We envisioned producing capsids with discrete numbers of AuNPs for use as electron microscopy contrast agents.

We sought to pre-form AuNP dimers and trimers so they could be added to a pool of disassembled capsids and yield MS2 housing either two or three AuNPs (Figure 2.14). There are three existing strategies to produce discrete AuNP clusters, as highlighted in Figure 2.15. First, the assembly of AuNP trimers has been mediated through complementary DNA hybridization. While this method produces negatively charged, high purity AuNP trimers, the high cost of thiol-DNA, the low efficiency of trimer formation, and low yielding purification limits the methods scalability. The resulting AuNP trimers are also too large in diameter for MS2 encapsulation. A second reported method utilizes the self-assembly of PEG/alkane thiol modified AuNPs to yield discrete AuNP clusters. These clusters, however, are neutrally charged and therefore not suitable for MS2 encapsulation. In a third reported method, >15 nm diameter AuNPs can be encapsulated in a silica shell and separated by ultracentrifugation to yield AuNP monomers, dimers, and trimers. Due to

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**Figure 2.13.** MS2 reassembly in the presence of excess 5 nm diameter AuNPs. a) Reassembly in the presence of excess 5 nm AuNPs results in MS2 capsids that contain between 0 and 5 AuNPs. b) Reassembly at ratios of 2:1 (blue), 3:1 (red), 6:1 (green), 10:1 (purple), and 15:1 (light blue) of AuNPs:MS2 capsids gives a distribution of free AuNPs, 0, 1, 2, 3, 4, and 5 AuNPs per MS2 capsid, as well as misformed capsids, as detailed in the bar graph (n is number of species identified via TEM). c) A representative TEM image of a reassembly sample of 2:1 AuNP:MS2 capsid is shown.

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Figure 2.14. Method for producing MS2 capsids housing a discrete number of AuNPs. The strategy relies on pre-forming AuNP dimers and trimers and subjecting them to MS2 reassembly conditions.

a.

b.

PEG thiol
alkane thiol

self-assembly

AuNP dimer inside reassembled capsids

AuNP trimer inside reassembled capsids

AuNP s dimer


c.

Figure 2.15. Existing strategies for producing discrete AuNP clusters. a) The assembly of AuNP trimers has been mediated through DNA hybridization. b) self-assembly of PEG/alkane thiol modified AuNPs can yield neutrally charged, water soluble discrete AuNP clusters. c) >15 nm diameter AuNPs can be encapsulated in a silica shell and separated by ultracentrifugation to yield AuNP monomers, dimers, and trimers.
the large size of AuNPs needed for the purification of these silica encapsulated particles, the resulting dimers and trimers would be too large for MS2 encapsulation.

As an alternative to these reported strategies, we sought to template AuNP dimers and trimers through the site specific attachment of AuNPs to TMV (a dimer) and Mth 1491 (a trimer), as shown in Figure 2.16. Taking advantage of the high affinity of thiols for AuNPs, thiols were introduced to the protein surface of Mth 1491 and TMV. Solvent accessible cysteines were introduced to Mth 1491 (S43C Mth 1491, Figure 2.17). Chemical modification of T104K TMV with succinimidyl-3-(2-pyridyldithio) propionate (SPDP), followed by cleavage of the disulfide with TCEP, gave solvent-accessible thiols for attachment to AuNPs (Figure 2.18). Upon incubation of these samples with either BSPP or citrate stabilized AuNPs, we observed a mixture of products by TEM. These products could not be separated by native gel. Furthermore, incubation of Mth 1491 and T104K TMV without the introduced thiols showed significant levels of background attachment of AuNPs by native gel. This made it difficult to distinguish whether the constructs were held together by a stable AuNP-thiol interaction or simply an electrostatic interaction.

To promote the purification of these materials, a DNA-mediated assembly strategy was explored (Figure 2.19). This strategy would produce constructs with a greater negative charge, which would allow for better separation of monomers, dimers, and trimers by native gel. In this approach, ssDNA modified Mth 1491 incubated with cDNA-AuNPs would yield DNA-mediated Mth AuNP trimers. DNA-modified 5 nm AuNPs were produced according to a previously reported procedure, yielding samples with 1, 2, or 3 ssDNA per particle. S43C Mth 1491 was modified with a linker, maleimide-PEG₆-NHS. Upon removal of unreacted maleimide-PEG₆-NHS, the sample was immediately subjected to amide bond formation with 5’ amine cDNA. DNA modification of Mth 1491 was confirmed by SDS-PAGE gel electrophoresis. Unfortunately, the highest level of DNA
Modification observed for Mth 1491 constructs was ~50%. This modification level was not high enough to promote AuNP trimer formation. We hypothesize the DNA attachment did not go to complete conversion due to electrostatic repulsion between the protein surface and ssDNA as well as possible hydrolysis of the NHS moiety on the linker. As a result, this strategy was no longer pursued.

**Figure 2.18.** Modification of T104K TMV with SPDP, and subsequent reduction with TCEP yielded TMV double disks with solvent accessible thiol for attachment to AuNPs.

**Figure 2.19.** a) A cDNA-mediated strategy to produce Mth 1491 AuNP trimers. b) S43C Mth 1491 modified with maleimide PEG6 NHS, followed by the addition of amine DNA yielded Mth-ssDNA conjugates, shown in the coomassie-stained SDS PAGE gel. c) 5 nm diameter AuNPs incubated with 1:1 cDNA:AuNPs gave AuNPs modified with discrete numbers, as shown in the native gel.
In our final strategy, we wanted to coat the AuNPs with negatively charged peptides that contained a functional handle for further bioconjugation. The negatively charged particles should allow for purification by native gel and ultimately promote MS2 reassembly. Furthermore, a bioconjugation handle that is stable to hydrolysis should promote the efficient attachment of the particles to the protein surface.

Two negatively charged peptides were synthesized, terminated on one end with a disulfide (for AuNP attachment), and terminated on the opposite end with an ortho-aminophenol (for oxidative coupling to aniline residues on the protein surface), as shown in Figure 2.20. The peptide was synthesized using standard solid phase peptide synthesis chemistry. A C-terminal tyrosine residue was modified with 4-nitrobenzenediazonium tetrafluoroborate to afford an azo peptide. The azo peptide was then cleaved from the

![Diagram showing the synthesis of peptides](image)

**Figure 2.20.** Overall strategy for the synthesis of an AuNP-binding, o-aminophenol containing peptide. The peptide was synthesized using standard solid phase peptide synthesis chemistry. A C-terminal tyrosine residue was modified with 4-nitrobenzenediazonium tetrafluoroborate to afford an azo peptide. The azo peptide was then cleaved from the resin with base and reduced to the o-aminophenol by sodium dithionite. MALDI-TOF-MS was used to confirm the mass of the peptides.
resin with base and reduced to the ortho-aminophenol with sodium dithionite. The peptide masses were confirmed by MALDI-TOF MS.

The coupling of these peptides to proteins was first tested using an aniline-containing protein on hand, T19pAF MS2. The peptide was found to modify T19pAF MS2, as confirmed by SDS PAGE gel electrophoresis (Figure 2.21). No conjugate was observed for T19W MS2 or for T19pAF MS2 in the absence of oxidant. Furthermore, the peptide-modified MS2 capsids were incubated with AuNPs. Imaging these samples with TEM revealed MS2 structures with AuNPs decorating the capsid surface (Figure 2.22). These structures are presumably held together via AuNP-thiol interactions.

**Figure 2.21.** Modification of T19pAF MS2 with [LA]-GDDD-oAP-COOH peptide analyzed by SDS PAGE gel. Increasing peptide equivalents (2-8) resulted in increased modification of T19pAF MS2. A peptide-MS2 conjugate was not observed for T19W MS2 or in the absence of oxidant.

![Figure 2.21](image)

**Figure 2.22.** Incubation of [LA]-GDDD-oAP peptide modified T19pAF MS2 with AuNPs provided MS2 capsids coated with AuNPs, as shown in the TEM images negatively stained with uranyl acetate.

![Figure 2.22](image)

To test this peptide-mediated strategy on the proteins of interest, TMV and Mth 1491, TMV and Mth 1491 were modified with isatoic anhydride, to yield aniline-TMV and aniline-Mth, as confirmed by mass spectrometry (Figure 2.23). These isatoic anhydride constructs did not modify with either ortho-aminophenol containing peptide using the same number of equivalents that successfully modified T19pAF MS2. Since the peptides of interest did not modify these constructs, a larger spacing was introduced between the aniline and protein by modifying A30C Mth 1491 and T104K TMV with EDC and NHS activated 3-(4-aminophenyl) propionic acid (Figure 2.24). This gave aniline-Mth and aniline-TMV. The oxidative coupling of aniline-TMV and aniline-Mth to the short and long negative ortho-aminophenol peptides, as well as oAP 5k PEG gave discrete conjugates, as confirmed by SDS-PAGE gel electrophoresis.
Figure 2.23. a) Mth 1491 and T104K TMV modified with isatoic anhydride gave b) 1-2 anilines per Mth 1491 and TMV, as shown by mass spectrometry. c) SDS-PAGE gel showed no Mth 1491-[LA]-GDDD-oAP peptide conjugate, but the aniline-Mth was reactive towards oAP 5K PEG. T19pAF MS2 was reactive to both [LA]-GDDD-oAP peptide and oAP 5K PEG.
After confirming peptide attachment to the proteins of interest, the next step was to attach the oAP peptides to AuNPs and subsequently couple the AuNPs to aniline-TMV and aniline-Mth. A shift in electrophoretic mobility in native gel electrophoresis indicated oAP peptide attachment to the AuNPs. Unfortunately, simply incubating the peptide modified AuNP samples with aniline-Mth and aniline-TMV at various pHs resulted in a substantial background interaction of the peptide-labeled AuNPs with the protein (Figure 2.25). As described previously, significant levels of non-specific AuNP attachment would make it difficult to distinguish covalent AuNP attachment from non-specific absorption. As a result, it seemed unlikely this multimeric protein approach would work for the construction of AuNP dimers and trimers, and was subsequently abandoned.

Figure 2.24. a) Modification of A30C Mth1491 and T104K TMV with EDC and NHS activated 3-(4-aminophenyl) propionic acid gave b) aniline-Mth and aniline-TMV, as shown by mass spectrometry. c) The oxidative coupling of aniline-Mth and aniline-TMV with [LA]-GDDD-oAP, [LA]-GDDDGAGGAGG-oAP, and oAP 5K PEG gave discrete conjugates by SDS-PAGE gel.

After confirming peptide attachment to the proteins of interest, the next step was to attach the oAP peptides to AuNPs and subsequently couple the AuNPs to aniline-TMV and aniline-Mth. A shift in electrophoretic mobility in native gel electrophoresis indicated oAP peptide attachment to the AuNPs. Unfortunately, simply incubating the peptide modified AuNP samples with aniline-Mth or aniline-TMV at various pHs resulted in a substantial background interaction of the peptide-labeled AuNPs with the protein (Figure 2.25). As described previously, significant levels of non-specific AuNP attachment would make it difficult to distinguish covalent AuNP attachment from non-specific absorption. As a result, it seemed unlikely this multimeric protein approach would work for the construction of AuNP dimers and trimers, and was subsequently abandoned.
2.5. Conclusions

Taking advantage of the self-assembly of bacteriophage MS2, we sought to expand the types of inorganic materials housed inside MS2 to include AgNPs, CdSe/ZnS quantum dots, CdSe/CdS dot rods, and discrete AuNP assemblies. Pairing reported external modification strategies of MS2 that target both native and unnatural amino acids with a variety of interior inorganic cargo could produce materials for metal controlled fluorescence (AgNPs), dual fluorescence/electron microscopy (CdSe/ZnS quantum dots and CdSe/CdS dot rods), and electron microscopy (discrete AuNP assemblies).

Water-soluble AgNPs were found to be unstable in high ionic strength buffers and intolerant to changes in solution pH. AgNPs coated with a 1:1 ratio of carboxylic acid terminated thiolated PEG: thiolated ssDNA were found to be stable to reassembly conditions and result in some reassembled MS2 capsids. Difficulty in obtaining reproducibly stable and reassembled AgNPs encapsulated in MS2, however, prevented their further use in metal controlled fluorescence studies.

CdSe/ZnS quantum dots and CdSe/CdS dot rods were rendered water soluble through both polymer wrapping and ligand exchange strategies. Mercaptopropionic acid coated CdSe/ZnS quantum dots subjected to reassembly conditions resulted in some reassembled capsids, as determined by native agarose gel electrophoresis and transmission electron microscopy. However, it was difficult to determine if the quantum dots were non-specifically adsorbed to the surface of the capsid or housed within the interior volume of the capsid. Similar results were observed for CdSe/CdS dot rods subjected to MS2 reassembly conditions.

TMV and Mth 1491 were explored as protein templates for producing discrete AuNP dimers and AuNP trimers, respectively. Thiol-AuNP interactions, cDNA hybridization, and an oxidative coupling reaction between aniline-containing proteins and AuNPs coated with negatively charged, ortho-aminophenol containing peptides were explored to produce the AuNP-protein conjugates of interest. Significant levels of background adsorption of the AuNPs to proteins of interest made it difficult to distinguish covalent AuNP attachment from non-specific AuNP adsorption.

Attempts at expanding the types of inorganic materials for MS2 encapsulation or covalent attachment to proteins of interest highlighted the general instability of most inorganic materials in buffered aqueous solutions. Instability of these inorganic materials resulted in their non-specific adsorption to proteins. The limited strategies for stabilizing and attaching inorganic materials site-specifically to proteins of interest informed our work on producing discrete AuNP-protein conjugates, described in detail in chapter 4.
2.6. Materials and methods

2.6.1. General procedures and materials

Unless otherwise noted, all chemicals were purchased from commercial sources and used as received without further purification. Water (dd-H$_2$O) used in all procedures was deionized using a NANOpure® purification system (Barnstead, USA). All unconjugated gold solutions were purchased from Ted Pella, Inc (Redding, CA). Bis(p-sulfonatophenyl)phosphine dehydrate dipotassium salt (BSPP) was purchased from Sigma Aldrich (St. Louis, MO). All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). CdSe/ZnS quantum dots were purchased from Ocean Optics, Inc (Dunedin, FL). CdSe/CdS dot rods were provided courtesy of Bruce Cohen, Ph.D. 40% octadecylamine-modified poly-(acrylic acid) polymer was provided courtesy of Bruce Cohen, Ph.D. Poly(maleic anhydride-alt-1-tetradecene), average MW 7,300 was purchased from Sigma Aldrich (St. Louis, MO) and received courtesy of Prof. Paul Alivisatos.

2.6.2. Instrumentation and sample analysis

**NMR.** $^1$H and $^{13}$C spectra were measured with a Bruker AVB-400 (400 MHz) spectrometer.

**Mass Spectrometry.** Matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a Voyager-DE system (PerSeptive Biosystems, USA) and data were analyzed using Data Explorer software. Peptide samples were co-crystallized with α-cyano-4-hydroxycinnamic acid in 1:1 acetonitrile (MeCN) to H$_2$O with 0.1% trifluoroacetic acid (TFA). Electrospray ionization mass spectrometry (ESI-MS) of peptides was performed using an Agilent 1260 series LC pump outfitted with an Agilent 6224 Time-of-Flight (TOF) LC/MS system.

**High Performance Liquid Chromatography.** HPLC was performed on Agilent 1100 Series HPLC Systems (Agilent Technologies, USA). Sample analysis for all HPLC experiments was achieved with an inline diode array detector (DAD) and inline fluorescence detector (FLD). Analytical and preparative reverse-phase HPLC of peptides was accomplished using a C18 stationary phase and a MeCN/H$_2$O with 0.1% TFA gradient mobile phase.

**UV-Vis Spectroscopic Measurements.** UV-Vis spectroscopic measurements were conducted on a Varian Cary 50 scan benchtop spectrophotometer (Varian Inc., USA). All measurements were taken in absorbance mode at a medium scan rate and recording measurements from 200 nm – 800 nm.

**Gel Analyses.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Mini-Protean apparatus from Bio-Rad (Hercules, CA) with 10-20% gradient polyacrylamide gels (BioRad, CA), following the protocol of Laemmli.$^{42}$ All samples were mixed with SDS loading buffer in the presence of 1,4-dithiothreitol (DTT)
and heated to 95 °C for 10 min to ensure complete denaturation. Gels were run at 120 V for 80 min. Commercially available molecular weight markers (Bio-Rad) were applied to at least one lane of each gel for assignment of molecular masses. For fluorescent protein conjugates, a UV-backlight was used for visualization. Gels were then stained with Coomassie Brilliant Blue (Bio-Rad). Coomassie stained protein gels were imaged on an EpiChem3 Darkroom System (UVP, USA). Native agarose gel electrophoresis was carried out on a Mini-Sub Cell GT System from Bio-Rad (Hercules, CA) with 1 or 1.5% agarose gels. All samples were mixed with 50% glycerol (2:1 sample:50% glycerol) prior to loading on the gel. Gels were typically run at 60 V for 30 min. For fluorescent protein conjugates, a UV-backlight was used for visualization. Gels were then stained with Coomassie Brilliant Blue (Bio-Rad). Coomassie stained agarose gels were imaged on an EpiChem3 Darkroom System (UVP, USA).

**Dynamic Light Scattering.** DLS measurements were obtained using a Malvern Instruments Zetasizer Nano ZS. Samples were filtered through a 0.22 μm centrifugal filter unit (Millipore Corporation, Billerica, MA) prior to data collection. Data plots are shown as size distribution by number, which weighs large and small particles equally. Diameters were calculated from an average of three measurements.

**Transmission Electron Microscopy.** TEM images were obtained at the UC Berkeley Electron Microscope Lab (UCB EML) using a FEI Tecnai 12 transmission electron microscope with 120 kV accelerating voltage. Protein samples were prepared for TEM analysis by pipetting 5 μL of the samples onto Formvar-coated copper mesh grids (400 mesh, Ted Pella, Redding, CA), after 3 min of equilibration the samples were then wicked with filter paper. The samples were then rinsed with dd-H2O. Subsequently, the grids were exposed to 8 μL of a 1% (w/v) aqueous solution of uranyl acetate for 90 s as a negative stain. Excess stain was then removed and the grids were dried in air.

### 2.6.3. Experimental procedures

**Inorganic nanomaterial synthesis and modification**

**Synthesis of water-soluble AgNPs.** Prior to AgNP synthesis, it is important to note all glassware was cleaned in a base bath. AgNPs were synthesized according to a previously reported procedure. An aqueous 1 mM AgNO₃ solution (10 mL) was added dropwise to vigorously stirred, ice cold, 2.0 mM aqueous solution of NaBH₄ (30 mL). The clear, colorless solution turned pale yellow and later bright yellow upon the addition of AgNO₃. Following the addition of AgNO₃, the stirring was stopped. The resulting solution was stored at room temperature.

**Synthesis of organic-soluble AgNPs.** Prior to AgNP synthesis, it is important to note all glassware was cleaned in a base bath. AgNPs were synthesized following a variation of a previously reported procedure. Silver acetate (50 mg, 0.30 mmoles) was dissolved in oleylamine (2.5 mL, 7.5 mmol), and quickly injected into refluxing toluene (50 mL). The resulting solution was refluxed overnight, yielding a dark brown solution which was concentrated in vacuo to a volume of ~10 mL. Addition of methanol (~200 mL)
resulted in a black precipitate that was isolated by centrifugation. The black solid was re-dispersed in hexanes and precipitated with ~100 mL of methanol. The black solid was again collected, dispersed in hexanes to facilitate transfer to a round bottom flask, and dried in vacuo.

**Ligand exchange of organic-soluble AgNPs.** Solid AgNPs (4 mg) were dissolved in toluene (2.0 mL) and combined with ligands 1-4, Figure 2.2 (100 equivalents per nanoparticle). The resulting solution was stirred at rt for 30 min, yielding a black solid precipitate. Water was then added to the stirring solution, yielding a yellow aqueous solution, which was then washed twice with toluene, and stored at rt until use.

**Synthesis of ligand 4 shown in Figure 2.2.** Ligand 4 was synthesized according to a previously reported procedure. 1H NMR spectral data agreed with those previously reported.37

**Poly(maleic anhydride alt-1-tetradecene) polymer wrapping as shown in Figure 2.7.d.** Polymer wrapping was achieved following a previously reported procedure.45 Briefly, a solution of poly(maleic anhydride alt-1-tetradecene) was dissolved in chloroform and combined with a solution of CdSe/ZnS or CdSe/CdS particles in chloroform (100 polymer equivalents per nm² surface area). The resulting solution was stirred at rt for 2 h and the solvent was evaporated under a gentle stream of nitrogen. To the dried polymer-nanocrystal solution was added bis(6-aminohexyl)amine in chloroform (0.1 equivalents relative to polymer units). The resulting solution was then sonicated at rt for 20 mins and the solvent was again evaporated under a gentle stream of nitrogen. The resulting solid was then re-dispersed in 100 mM borate buffer, pH 9.0. Excess polymer was removed through successive rounds of centrifugal filtration with 100 kD MWCO filters.

**3-mercaptopropionic acid ligand exchange for CdSe/ZnS quantum dots.** Ligand exchange with 3-mercaptopropionic acid was achieved following a previously reported procedure.46 Briefly, to a dispersion of nanocrystals in toluene was added 3-mercaptopropionic acid dissolved in formamide (200 equivalents). The combined solutions were stirred to promote the transfer of nanocrystals into the formamide phase. The formamide phase was extracted, and washed with toluene three times. To the washed formamide solution was then added acetonitrile to precipitate out the nanocrystals. The precipitate was redispersed in 100 mM borate buffer, pH 8.5. Excess polymer was removed through successive rounds of centrifugal filtration with 100 kD MWCO filters.

**40% octadecylamine, poly-(acrylic acid) polymer wrapping of quantum dots.** Coating of CdSe/ZnS quantum dots with 40% octadecylamine, poly-(acrylic acid) polymer was achieved as reported previously.47 Nanocrystals dispersed in chloroform were combined with a solution of 40% octadecylamine, poly-(acrylic acid) polymer in chloroform (200 equivalents of polymer). The resulting solution was stirred at rt for 1 h, followed by solvent evaporation under a gentle stream of nitrogen. The resulting solid was then dissolved in 100 mM borate buffer, pH 8.5, and sonicated at rt for 30 min, followed by sonication at 75 °C for 10 min to disperse the nanocrystals in solution. Aggregates were removed by passing the solution through a 0.22 μm filter. Excess polymer was removed
Phosphination of AuNPs (5 nm and 10 nm diameter). Phosphination of AuNPs (5 nm and 10 nm diameter) was carried out following a previously reported procedure. In a typical reaction, bis(p-sulfonatophenyl)phenylphosphine dehydrate dipotassium salt (BSPP, 5 mg) was mixed with AuNPs (10 mL) and vigorously stirred at rt overnight. Solid NaCl was then added to the AuNP solution until the color turned from deep red to purple. The solution was then centrifuged (5000 rpm) for 10 min, yielding a pellet of nanocrystals. The supernatant was carefully removed and the pellet redisolved in an aqueous solution of BSPP (25 mg in 100 mL dd-H2O). The redissolved nanocrystal solutions were combined and then precipitated through the addition of methanol, until the color again changed from deep red to purple. The solution was again centrifuged (5000 rpm) for 10 min and the supernatant was removed. The nanocrystal pellet was again dissolved in an aqueous solution of BSPP, and stored at room temperature until use. The AuNP concentration was determined by recording the absorbance at 520 nm, and relating the absorbance values to concentration via Beer’s Law ($\epsilon_{520 \text{ nm}} = 9.3 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon_{520 \text{ nm}} = 8.1 \times 10^7 \text{ M}^{-1}\text{cm}^{-1}$ for 5 nm and 10 nm AuNPs, respectively).

Alkanethiol oligonucleotide modification of BSPP-stabilized AuNPs for reassembly. Alkanethiol oligonucleotide modification of BSPP-stabilized AuNPs was achieved using a modified version of a previously reported procedure.

The oligonucleotide sequence identity was as follows:

5’-/5ThioMC6-D/TTT-TTT-TTA-CAT-GGG-TAA-TCC-TCA-TGT-3’

Lyophilized oligonucleotides containing a 5’ thiol modifier [5’-(CH2)6-S-S-(CH2)6-phosphodiester bond-oligonucleotide-3’] purchased from IDT technologies (Coralville, IA) were resuspended in dd-H2O to yield a final concentration of 100 μM. The disulfide functionality was cleaved by incubation with DTT (0.1 M) at rt in 100 mM phosphate buffer pH 8.0 for 1 h. Excess DTT was removed by passing the solution through a commercially available gel filtration column pre-equilibrated with dd-H2O (NAP 5 column, GE Healthcare). It is important to note that 90% of the recommended volume was applied to the column for elution, to prevent excess DTT from remaining in the oligonucleotide solution. Fractions collected from the gel filtration column that contained oligonucleotides were combined and immediately added to BSPP-stabilized AuNPs (9 nM) to yield a final oligonucleotide concentration of 10 μM. The resulting solution was then incubated at rt for 20 min. The concentration of NaCl was increased by increments of 0.05 M to a final concentration of 0.1 M through successive additions of 2 M NaCl. Following each addition of 2 M NaCl, the alkanethiol oligonucleotide-modified AuNP solution was sonicated for 10 s, followed by agitation at rt for 20 min. After the final concentration of 0.1 M NaCl was reached, the AuNP solution was allowed to incubate at rt overnight. Excess oligonucleotides were removed through multiple rounds of centrifugal filtration with a 100k MWCO centrifugal filter (Millipore), and diluted in 50 mM phosphate buffer, pH 7.0. The final AuNP concentration was approximated by measuring the absorbance at 520 nm, 520 nm, 524 nm, and 528 nm for 5 nm, 10 nm, 15 nm, and 20 nm diameter AuNPs, respectively.
Cloning, expression, and purification of T19pAF MS2. Bacteriophage MS2 T19pAF plasmid production and growth has been previously reported.35,51 The Peter Schultz lab (Scripps Research Institute, La Jolla, CA) provided the tRNA- and tRNA-synthetase-encoding plasmids necessary for p-aminophenylalanine (pAF) incorporation.52 A yield of ~10 mg/L was obtained for MS2-pAF19 following two purification rounds.

General procedure for disassembly and reassembly of T19pAF MS2 around inorganic nanomaterials. Bacteriophage MS2 T19pAF was first subjected to disassembly conditions, using a method previously described.31,53 In a typical disassembly reaction, 150 μM T19pAF MS2 in 50 mM phosphate buffer, pH 7.2 (200 μL) was combined with two volumes of cold glacial acetic acid (400 μL) and kept on ice for 30 min. The mixture was then centrifuged for 20 min (13,000 rpm at 4 °C). Following centrifugation, the supernatant was removed and applied to a gel filtration column (NAP-5, GE Healthcare) pre-equilibrated with 1 mM acetic acid. Fractions were collected from the gel filtration column and their absorbance values at 280 nm were recorded. The fractions containing MS2 were combined on ice and used immediately.

For reassembly, the final MS2 coat protein dimer concentration was approximated by recording the absorbance at 280 nm and assuming eighty percent reassembly efficiency. The AuNP concentration was approximated by measuring the maximum absorbance and relating that value to the concentration via Beer’s law. In a typical reassembly reaction, AuNPs (final ratio of 1:1 AuNPs: MS2 capsids) were combined with two thirds volume of 100 mM phosphate buffer, pH 7.2 (or 100 mM tris, pH 7.0 for quantum dots or dot rods, which are unstable to phosphate buffer) containing 100 mM NaCl. As indicated in the figures, 100 mM TMAO (final concentration) was withheld or added to the reassembly solution. The final one third volume comprised MS2 coat protein dimer solution. The solution was then kept at 4 °C for 40 h to allow for reassembly. Following reassembly, a saturating amount of NaCl was added directly to the solution. The reassembly reaction was then applied to a gel filtration column (NAP-5, GE Healthcare), pre-equilibrated with 10 mM phosphate buffer, pH 7.2. Samples were then concentrated through multiple rounds of centrifugal filtration with a 100k MWCO centrifugal filter (Millipore), and diluted in 10 mM phosphate buffer, pH 7.2 (or 10 mM tris buffer for quantum dots and dot rods, which are unstable to phosphate buffer). The reassembly was confirmed through dynamic light scattering, transmission electron microscopy, and native agarose gel electrophoresis (1.5% agarose, 50% glycerol loading buffer, 0.5x TBE running buffer, pH 7.6, 45 min at 60 V). A portion of the sample was then run on an SDS-PAGE gel with standard concentrations of MS2. The concentration of the reassembled sample was then determined by optical densitometry using ImageJ software.

If desired, the MS2 samples containing inorganic nanomaterials were also able to be purified via PEG precipitation (0.5 M NaCl, 10 w/v% PEG-6k) for 1 h at 4 °C. MS2 samples were isolated through centrifugation at 5000 rpm for 20 min. The supernatant was carefully removed and the precipitate was resuspended in 50 mM phosphate buffer, pH 7.0 (or 50 mM tris for quantum dots and dot rods, which are unstable to phosphate buffer). The resuspended sample was then passed through a gel filtration column (NAP-5, GE Healthcare).
AgNP growth on MPA-coated quantum dots subjected to MS2 reassembly. AgNP growth was performed following a variation of a previously reported procedure. MPA quantum dots subjected to MS2 reassembly conditions were run on a native agarose gel and the band corresponding to MS2 was extracted from the gel. The extracted sample was then incubated in an aqueous solution of AgNO$_3$ (1 mM final concentration) at rt for 2 h. An aqueous solution of sodium borohydride (1 mM final concentration) was then added and immediately vortexed to ensure mixing of the solutions. The reduction proceeded for 10 min, yielding a yellow solution. The solution was directly spotted on a TEM grid for imaging.

Expression and purification of T104K TMV. The TMV coat protein had the following mutations: K53R, K68R (RR-TMV), and T104K TMV. This TMV mutant was expressed and purified as described previously.

General procedure for thiolation of T104K TMV. A 1.5 mL Eppendorf tube was charged with a solution of RR T104K TMV (100 μL of 100 μM solution in 10 mM phosphate buffer, pH 8.0). To this solution was added succinimidyl 3-(2-pyridyldithio) propionate (SPDP, 5.7 μL, 10 eq., 100 mM solution in DMSO). The resulting solution was briefly vortexed and the reaction was allowed to proceed at rt for 2 h. The reaction was then quenched through the addition of tris(2-carboxyethyl)phosphine, TCEP (5 μL of 500 mM solution in water, pH adjusted to 7.0). The mixture was briefly vortexed and incubated at rt for 20 minutes. The final thiolated TMV was purified by passing the solution through a size exclusion column, and concentrated through successive centrifugal filtrations with 100 kD MWCO filters.

Isatoic anhydride modification of T104K TMV. A 1.5 mL Eppendorf tube was charged with a solution of T104K TMV (99 μL of 100 μM solution in 10 mM phosphate buffer, pH 8.0). To this solution was added isatoic anhydride (1 μL, 10 eq., 100 mM in DMF). The resulting solution was briefly vortexed and the reaction was allowed to proceed at rt for 2 h. The reaction mixture was then purified by passing the solution through a gel filtration column, and concentrated through successive centrifugal filtrations with 100 kD MWCO filters.

3-(4-aminophenyl) propionic acid N-hydroxysuccinimide ester modification of T104K TMV. A 1.5 mL Eppendorf tube was charged with a solution of T104K TMV (99 μL of 100 μM solution in 10 mM phosphate buffer, pH 8.0). To this solution was added the NHS ester of 3-(4-aminophenyl) propionic acid (1 μL, 10 eq., 100 mM in DMF). The resulting solution was allowed to proceed at rt for 2 h. The reaction mixture was then purified by passing the solution through a gel filtration column, and concentrated through successive centrifugal filtrations in 100 kD MWCO filters.

Expression and purification of S43C Mth1491. Mth1491 was expressed and purified according to a literature procedure.

Generation of A30C Mth1491. The QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) was first used to mutate the Mth1491-S43C plasmid to produce
the wild type Mth1491-43S using the following forward and reverse primers:

Forward: 5’ –CGTATCGAGGTGGTTGCGTACAGTATGGGCGTTAATGTGCTGC– 3’
Reverse: 5’ –CTGACGACGCAGCACATTAACGCCATCCTGACGCAACCACCTCG– 3’

The alanine at position 30 was converted to a cysteine using the following forward and reverse primers:

Forward: 5’ –GCAACGTCCGTAATCTGATGTGCGATCTGGAATCGGTACGTATC– 3’
Reverse: 5’ –CCTCGATACGTACCGATTCCAGATCGCACATCAGATTACGGACG– 3’

A30C Mth1491 expression and purification was identical to that of S43C Mth1491.

Isatoic anhydride modification of A30C Mth 1491. A 1.5 mL Eppendorf tube was charged with a solution of A30C Mth 1491 (99.9 μL of 100 μM solution in 10 mM phosphate buffer, pH 8.0). To this solution was added isatoic anhydride (0.1 μL, 1 eq., 100 mM in DMF). The resulting solution was briefly vortexed and the reaction was allowed to proceed at rt for 2 h. The reaction mixture was then purified by passing the solution through a size exclusion column, and concentrated through successive centrifugal filtrations with 10 kD MWCO filters.

3-(4-aminophenyl) propionic acid N-hydroxysuccinimid ester modification of A30C Mth 1491. A 1.5 mL Eppendorf tube was charged with a solution of A30C Mth 1491 (99.9 μL of 100 μM solution in 10 mM phosphate buffer, pH 8.0). To this solution was added the NHS ester of 3-(4-aminophenyl) propionic acid (0.1 μL, 1 eq., 100 mM in DMF). The resulting solution was briefly vortexed and the reaction was allowed to proceed at rt for 2 h. The reaction mixture was then purified by passing the solution through a size exclusion column, and concentrated through successive centrifugal filtrations in 10 kD MWCO filters.

General procedure for solid-phase peptide synthesis. Peptides were synthesized using standard Fmoc-based chemistry on Tentagel S-OH resin (Advanced ChemTech, Louisville, KY). The side chain protecting groups used were: Asp(OtBu), and Tyr(tBu). The C-terminal amino acid (10 equiv) was preactivated at 0 °C with 5 equivalents of diisopropylcarbodiimide (DIC) and then coupled to the resin with 0.1 equivalents of N,N-dimethylaminopyridine (DMAP) as a catalyst. Deprotection of the Fmoc groups was performed with a 20 min incubation in a 20% v/v piperidine in dimethylformamide (DMF) solution. Coupling reactions were carried out using 20 equivalents of amino acid with 10 equivalents of 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU) and 20 equivalents of N,N-diisopropylethylamine (DIPEA) in DMF for 20 min. Side-chain deprotection was accomplished using a 1-2 h incubation with a 95:2.5:2.5 ratio of TFA to H₂O to triisopropylsilane (TIPS). Peptides were cleaved from the resin by a 30-45 min incubation with a 100 mM sodium hydroxide solution. The resulting basic solution was neutralized with 100 mM phosphate buffer, pH 6.5.
General procedure for azo coupling to peptides. To a portion of resin-bound peptide (20 mg) in 900 μL of 100 mM phosphate buffer, pH 9.0 was added 100 μL of a saturated solution of 4-nitrobenzenediazonium tetrafluoroborate (300 mM, Sigma-Aldrich) in MeCN at 4 °C. After rotation for 30 min at 4 °C, the resin was rinsed with MeCN and DMF until no color remained in solution. The peptides were then cleaved from the resin, and then purified by HPLC using a C18 Gemini column (5 micron, 250 x 7.9 mm, Phenomenex, Torrance, CA). Peptide modification was confirmed by MALDI-TOF MS.

General procedure for dithionite reduction of azo peptides. To a solution of azo-modified peptide (1 mM) in 100 mM phosphate buffer, pH 6.5 was added an equal volume of a freshly prepared solution of sodium dithionite (100 mM) in 100 mM phosphate buffer, pH 7.2. After 10 min, the solution was applied to a C18 Sep-Pak pre-conditioned with methanol then 0.1% aqueous TFA. After washing with aqueous 0.1% TFA and 5% MeCN in aqueous 0.1% TFA, the peptide was eluted with MeCN. After removing the MeCN under reduced pressure the solid peptide was dissolved in 100 μL of 10 mM phosphate buffer, pH 6.5. The ortho-aminophenol peptide was characterized by MALDI-TOF and stored at -20 °C.

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Chapter 3

Tryptophan oxidation for site-selective bioconjugation

Abstract

We sought to expand the current bioconjugation strategies for tryptophan residues by taking advantage of its electron-rich indole framework. Oxidation of the 2,3-bond of tryptophan by sodium periodate was found to produce \( N \)-formyl kynureine derivatives that could be deformylated in the presence of acid to afford aniline moieties. These aniline moieties were found to be reactive towards ortho-aminophenols and phenylenediamines in the presence of sodium periodate for some substrates. Progress towards the development of this methodology for the specific application of developing targeted electron microscopy contrast agents is discussed.
3.1. Existing tryptophan modification strategies

Bioconjugation reactions that target native amino acid residues are useful for the introduction of unique chemical functionalities to proteins such as dyes, drugs, targeting groups, or polymers for creating protein-based materials. There are a number of existing techniques for labeling lysine, cysteine, tyrosine, aspartic acid, and glutamic acid residues.\(^1\)\(^2\) However, there are few methods for the site-specific labeling of tryptophan residues. Tryptophan residues are of low abundance and are usually fully or partially buried, making them challenging to modify. Existing strategies for the site-specific modification of tryptophan residues include Koshland’s reagent and oxidation with dimethyl chlorosulfonium ions and \(N\)-bromosuccinimide (Figure 3.1). These strategies have been used to quantify the number of tryptophan residues in a protein but provide no direct method to attach functional groups of interest.\(^3\)-\(^8\) Site-selective alkylation of tryptophan residues has been reported with rhodium carbenoids\(^9\)-\(^11\) and malondialdehyde\(^12\)-\(^14\) (Figure 3.2). The later strategy introduces a uniquely reactive aldehyde, which can be further functionalized with hydrazides. A final, recently reported strategy is the oxidative coupling of tryptophan residues and phenylenediamines in the presence of cerium ammonium nitrate.\(^15\),\(^16\) However, this strategy is not site-specific as it has also been shown to modify tyrosine residues. Since there are few site-selective strategies for the modification of tryptophan residues, we sought to expand the current bioconjugation strategies by taking advantage of the electron-rich indole framework of tryptophan. We envisioned oxidation of tryptophan residues, followed by subsequent modification as a new bioconjugation strategy.

3.2. Oxidation of tryptophan residues for subsequent modification

Oxidative cleavage of the 2,3-bond of L-tryptophan by tryptophan dioxygenase has been demonstrated as an important first step in the biosynthesis of niacin.\(^17\) This oxidative cleavage and subsequent deacylation by formamidase enzymes introduces two unique functional groups, a ketone and an aniline, to the indole framework.\(^16\)

a. *Modification with Koshland’s reagent:*

\[
\text{Koshland’s reagent:} \quad \text{L-tryptophan} + \text{Koshland’s reagent} \rightarrow \text{Modified L-tryptophan}
\]

b. *Oxidation with dimethyl chlorosulfonium ions and \(N\)-bromosuccinimide:*

\[
\text{Oxidation:} \quad \text{L-tryptophan} \rightarrow \text{Oxidized L-tryptophan}
\]

*Figure 3.1.* Existing methods for the site-specific modification of tryptophan residues. a) Koshland’s reagent and b) oxidation of tryptophan residues with dimethyl chlorosulfonium ions/NBS have traditionally been used.
Previous work in our group demonstrated oxidation of the 2,3-bond of L-tryptophan with 100 eq. of sodium periodate (M + 32 adduct, Figure 3.3) at rt for 4 h on a peptide substrate, hypertrehalosemic peptide. Deformylation with 5000 eq. trifluoroacetic acid (relative to oxidized peptide) at rt for 2 h was found to afford a kynureine derivative (M + 4 adduct). The kynureine derivative provides a unique ketone and aniline for further functionalization. We envisioned that the ketone could be reactive towards alkoxyamines for oxime formation while the aniline derivative could be used in previously reported oxidative coupling reactions with ortho-aminophenols or phenylenediamines in the presence of sodium periodate or potassium ferricyanide. On a peptide substrate, it was found that the ketone was not reactive towards alkoxyamines as previously predicted. However, the aniline was reactive towards anisidine derivatives as well as ortho-aminophenols in the presence of sodium periodate, forming stable mass adducts (Figure 3.3-b,c). Product formation was observed with sodium periodate as an oxidant. No product formation was observed with potassium ferricyanide as an oxidant.
3.3. Reassembled bacteriophage MS2 as a model protein for tryptophan oxidation chemistry

To test this methodology on a protein substrate, a T19W mutant of bacteriophage MS2 was produced. Residue 19 is solvent accessible and on the exterior face of the capsid (Figure 3.4). Protein expression and purification yielded ~90 mg/L culture (compared to ~100 mg/L culture for wt MS2 and ~5-10 mg/L culture for an unnatural amino acid, T19pAF MS2).

Since the necessary reaction conditions for oxidation (100 μM peptide with 10 mM sodium periodate at rt for 4 h) and subsequent deformylation (100 μM peptide with 500 mM trifluoroacetic acid at rt for 2 h) are fairly aggressive, even a robust protein such as bacteriophage MS2 does not remain assembled under these conditions. Consequently, we envisioned either directly reassembling T19-(M+4) or subjecting T19-(M+32) capsids to acidic disassembly conditions, producing a mixture of T19-(M+32) and T19-(M+4) disassembled capsids that could be reassembled around negatively charged cargo of interest (Figure 3.5-a). The exterior, T19-(M+4) residues could then be modified with
targeting groups of interest. We were specifically interested in producing capsids that housed AuNPs and displayed exterior DNA aptamers for use as electron microscopy contrast agents.

Conversion of T19W MS2 to oxidized T19-(M+32) was achieved through the addition of sodium periodate (400 eq. relative to MS2 monomer) at rt for 48 h. Conversion was monitored using mass spectrometry (Figure 3.5-b). Following oxidation, the capsids remained assembled as confirmed by DLS and TEM. T19-(M+32) MS2 was converted to T19-(M+4) MS2 through the addition of trifluoroacetic acid (4000 eq. relative to oxidized MS2 monomer) at rt for 48 h. Following treatment with acid, a large amount of protein precipitate was observed in the reaction mixture, and the capsids did not appear to remain assembled by DLS or TEM. However, upon solubilizing the precipitated protein in a 1:1 (v/v) solution of glacial acetic acid: water, and passing the solution through a gel filtration column, a T19-(M+4) mass adduct was observed (Figure 3.5-b).

The reactivity of these substrates was then tested with anisidine or ortho-aminophenol ssDNA (Figure 3.6-a,b) in the presence of oxidant (sodium periodate or potassium ferricyanide). Reaction products were screened using denaturing SDS-PAGE gel electrophoresis (Figure 3.6-c). As a positive control, T19pAF MS2 was subjected to oxidative coupling conditions in the presence of an ortho-aminophenol labeled ssDNA, yielding a DNA-MS2 conjugate. T19-(M+4) MS2 was subjected to oxidative coupling conditions in the presence of both anisidine and ortho-aminophenol ssDNA in the presence of either sodium periodate or potassium ferricyanide. All combinations of reagents gave no observable DNA-MS2 conjugate. The band at ~26 kD corresponds to an MS2 dimer, not the desired DNA-MS2 conjugate.

It is also important to note that formation of an M+4 adduct on an alternative peptide substrate, melittin, resulted in insoluble peptide after purification from deformylation conditions (trifluoroacetic acid).21 As a result, M+4 melittin was not amenable to subsequent modification. We hypothesized that in both the case of melittin (a cationic peptide) and bacteriophage MS2, a trifluoroacetate counter-ion could potentially be interfering with the oxidative coupling reaction.

Figure 3.4. A spherical viral capsid, bacteriophage MS2, was genetically modified to introduce a solvent accessible tryptophan residue on the exterior surface of the capsid (T19W MS2).
Figure 3.5. General strategy to utilize kynurenine-containing MS2 capsids. a) Strategy for the reassembly of MS2 capsids in the presence of DNA-coated 10 nm diameter AuNPs. b) Mass spectrometry traces demonstrating the conversion of T19W MS2 to oxidized (M + 32) T19W MS2 in the presence of sodium periodate, and deformylated, (M + 4) T19W MS2, with TFA. c) Reassembly distribution of M + 4 T19W MS2 revealed a significant portion of the reassembled products did not have interior cargo. d) Representative TEM images for T19W, M + 32 T19W, and M + 4 T19W MS2 subjected to reassembly conditions in the presence of 10 nm diameter AuNPs.
Concurrently, T19W, T19-(M+32), and T19-(M+4) MS2 were subjected to reassembly conditions in the presence of DNA-labeled 10 nm diameter AuNPs. Reassembly products were observed in moderate to good yields for both T19W and T19-(M+32) MS2. For T19-(M+4) MS2, a poor yield was observed for reassembly (< 5% capsid yield). A significant fraction of the total protein and AuNPs precipitated upon purification of the reassembled species. The < 5% reassembled species were analyzed by TEM and ~43% contained no AuNPs, ~55% contained 1 AuNP/capsid, and ~2% contained 2 AuNPs/capsid. While it appeared some of the T19-(M+4) species may have reassembled to house AuNPs, we hypothesized that since the total amount of reassembled capsids was a small fraction of the original protein content, it was possible that the observed reassembled species were in fact residual T19W or T19-(M+32) MS2 species present in the reassembly solution. We were unable to obtain a mass spectrum of the reassembled species (due to the presence of AuNPs in solution) to confirm this hypothesis. Regardless, direct reassembly of T19-(M+4) MS2 was not a promising strategy, so we decided to pursue an alternative approach.

At this point, we thought a promising approach would be to disassemble T19-(M+32) MS2 species in the presence of either 66% acetic acid or a combination of acetic acid and other (stronger) acids to produce T19-(M+4) species in the disassembly process. These disassembled species could then be directly subjected to reassembly in the presence of

![Diagram](image)

**Figure 3.6.** Anisidine-ssDNA was tested for modification of T19W, M + 32 T19W, and M + 4 T19W MS2. a) 5'-amine ssDNA was modified with an anisidine NHS, providing an anisidine-containing ssDNA. b) The ssDNA modification was confirmed by MALDI-TOF MS. c) T19pAF, T19W, M + 32 T19W, and M + 4 T19W MS2 were screened for their reactivity to *ortho*-aminophenol and anisidine-modified ssDNA in the presence of sodium periodate and potassium ferricyanide. Product formation was monitored by SDS-PAGE gel electrophoresis. Modification was observed for T19pAF MS2 with *ortho*-aminophenol DNA in the presence of periodate. Modification was not observed for any of the other MS2 derivatives.
melittin (W):
GIGAVLKVLTGLPALISWIKRKRQQ

Figure 3.7. Optimization of acidic conditions for the formation of an M + 4 adduct (kynurenine derivative) on a tryptophan-containing peptide, melittin. a) Oxidation of melittin with periodate yielded near quantitative conversion to an M + 32 adduct. A variety of concentrations, acids, and reaction times were screened and the reaction products were monitored by MALDI-TOF MS. b) Acetic acid, formic acid, hydrochloric acid, and trifluoroacetic acid were all screened at concentrations ranging from 1 mM - 10 M. c) A combination of 10 M acetic acid and 1 – 500 mM TFA, HCl, and formic acid were screened for their ability to convert to the deformylated, M + 4 product. Reaction products were monitored by MALDI-TOF MS.
DNA-labeled 10 nm AuNPs to afford AuNPs housed on the interior volume of T19-(M+4) MS2 capsids.

Ideally, the stronger acid would not be trifluoroacetic acid, as it seemed to be problematic for subsequent oxidative coupling reactions on both protein and peptide substrates. To potentially avoid this interference, we screened other acidic solutions on an oxidized melittin (M + 32) substrate and recorded the relative ratio of M + 4 product formation for a variety of acids at varying concentrations and times. Acetic acid (10 mM – 10 M), formic acid (10 mM – 500 mM), hydrochloric acid (1 mM – 50 mM), and as a comparison, trifluoroacetic acid (10 mM – 500 mM) were screened for their M + 4 product formation by MALDI-TOF MS (Figure 3.7). Additionally, combinations of 10 M acetic acid and trifluoroacetic acid (10 mM – 100 mM), hydrochloric acid (1 mM – 50 mM), and formic acid (10 mM – 500 mM) were screened for product formation by MALDI-TOF MS. The most promising conditions from this screen seemed to be hydrochloric acid and

![Chemical structures and MALDI-TOF spectra](image)

*Figure 3.8.* Promising conditions tested for M + 4 product formation. a) ESI MS of T19W MS2, b) oxidized T19W MS2 (M + 32), and c) oxidized (M + 32) T19W MS2 subjected to 66% acetic acid in the presence of 25 mM – 100 mM HCl.
combinations of 10 M acetic acid and hydrochloric acid. These conditions were tested on oxidized T19W (M+32) and screened for product formation by ESI MS (Figure 3.8). Combinations of T19-(M+32) MS2 and hydrochloric acid were found to produce an insoluble protein precipitate during the course of deformylation. Combinations of 10 M acetic acid and 25 mM – 100 mM HCl were found to maintain soluble protein. Exposure of T19-(M+32) capsids to 10 M acetic acid plus 25 mM – 100 mM HCl at rt for 48 h yielded significant M + 4 product formation. However, subjecting these disassembled T19-(M+4) MS2 solutions to reassembly conditions in the presence of DNA labeled 10 nm diameter AuNPs yielded no observable reassembly products as determined by native agarose gel electrophoresis and DLS.

3.4. Conclusions and future outlook

Oxidation of tryptophan residues with sodium periodate, followed by deformylation to afford aniline residues seems to be a promising bioconjugation strategy for the site-specific modification of tryptophan residues. This methodology can introduce a uniquely reactive aniline moiety without needing to introduce an unnatural amino acid on a protein surface. However, the acidic conditions required for deformylation limit the broad applicability of this methodology and potentially interfere with subsequent aniline reactivity. Alternative approaches for deacylation, such as heat, photodeprotection22 or treatment with peptide deformylase23-25 could be milder alternatives to achieve the desired transformation.

3.5. Materials and methods

3.5.1. General procedures and materials

Unless otherwise noted, all chemicals were purchased from commercial sources and used as received without further purification. Water (dd-H2O) used in all procedures was deionized using a NANOpure® purification system (Barnstead, USA). All unconjugated gold solutions were purchased from Ted Pella, Inc (Redding, CA). Bis(p-sulfonatophenyl) phosphine dehydrate dipotassium salt (BSPP) was purchased from Sigma Aldrich (St. Louis, MO). Agarose (low-EEO/multipurpose/molecular biology grade) was purchased from Fisher Scientific (Pittsburgh, PA).

3.5.2 Instrumentation and sample analysis

**Mass Spectrometry.** Matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a Voyager-DE system (PerSeptive Biosystems, USA) and data were analyzed using Data Explorer software. Prior to MALDI-TOF MS analysis, samples were desalted using C18 ZipTip® pipet tips (Millipore, USA). Peptide samples were co-crystallized with α-cyano-4-hydroxycinnamic acid in 1:1 acetonitrile (MeCN) to H2O with 0.1% trifluoroacetic acid (TFA). Oligonucleotide samples were co-crystallized using a 3-hydroxypicolinic acid: ammonium citrate solution (45 mg/mL:5 mg/mL in 4.5:5.5 MeCN:dd-H2O). Electrospray ionization mass spectrometry (ESI-MS) of proteins was performed using an Agilent 1260 series liquid chromatograph that
was connected in-line with an Agilent 6224 Time-of-Flight (TOF) LC/MS system equipped with a Turbospray ion source (Agilent Technologies, USA). Protein chromatography was performed using an Agilent poroshell 300SB-C18 reversed-phase column (1.0 x 75 mm) with a MeCN:dd-H₂O gradient mobile phase containing 0.1% formic acid (FA). Protein mass reconstruction was performed on the charge ladder with Agilent MassHunter software.

**Gel Analyses.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Mini-Protean apparatus from Bio-Rad (Hercules, CA) with 10-20% gradient polyacrylamide gels (BioRad, CA), following the protocol of Laemmli. All samples were mixed with SDS loading buffer in the presence of 1,4-dithiothreitol (DTT), and heated to 95 °C for 10 min to ensure complete denaturation. Gels were run at 120 V for 80 min. Commercially available molecular weight markers (Bio-Rad) were applied to at least one lane of each gel for assignment of molecular masses. Gels were then stained with Coomassie Brilliant Blue (Bio-Rad). Coomassie stained protein gels were imaged on an EpiChem3 Darkroom System (UVP, USA). Native agarose gel electrophoresis was carried out on a Mini-Sub Cell GT System from Bio-Rad (Hercules, CA) with 1% or 1.5% agarose gels. All samples were mixed with 50% glycerol (2:1 sample:50% glycerol) prior to loading on the gel. Gels were typically run at 60 V for 30 min. For fluorescent protein conjugates, a UV-backlight was used for visualization. Gels were then stained with Coomassie Brilliant Blue (Bio-Rad). Agarose gels were imaged either in black and white with an EpiChem3 Darkroom System (UVP, USA) or in color with an Olympus 6.0 megapixel digital camera model number FE-120 (Shinjuku, Tokyo, Japan).

**Transmission Electron Microscopy.** TEM images were obtained at the UC Berkeley Electron Microscope Lab (UCB EML) using a FEI Tecnai 12 transmission electron microscope with 120 kV accelerating voltage. Protein samples were prepared for TEM analysis by pipetting 5 μL of the samples onto Formvar-coated copper mesh grids (400 mesh, Ted Pella, Redding, CA), after 3 min of equilibration the samples were then wicked with filter paper. The samples were then rinsed with dd-H₂O. Subsequently, the grids were exposed to 8 μL of a 1% (w/v) aqueous solution of uranyl acetate for 90 s as a negative stain. Excess stain was then removed and the grids were dried in air.

### 3.5.3. Experimental procedures

**General procedure for the formation of an N-formyl kynurenine derivative (M + 32 adduct) on melittin.** An Eppendorf tube was charged with a solution of melittin (100 μM final concentration) in 10 mM sodium acetate buffer, pH 4. A fresh 100 mM solution of sodium periodate in dd-H₂O was made and the oxidant (10 mM final concentration) was added to the reaction mixture. The resulting mixture was briefly vortexed and the reaction was allowed to proceed at rt for 4 h. A neutralized tris(2-carboxyethyl) phosphine hydrochloride (TCEP) solution in dd-H₂O (3 mM final concentration) was added to quench the reaction.

**General procedure for the treatment of melittin N-formyl kynurenine derivative with acidic solutions for M + 4 adduct formation.** An Eppendorf tube was charged
with a solution of oxidized melittin (100 μM final concentration) in 10 mM sodium acetate buffer, pH 4. An acidic solution (final concentrations 10 mM – 10 M acetic acid, 10 – 500 mM formic acid, 1-50 mM HCl, 10-500 mM TFA, 10 M acetic acid + 10-100 mM TFA, 10 M acetic acid + 1-50 mM HCl, 10 M acetic acid + 10-500 mM formic acid) was added, the resulting mixture was briefly vortexed, and the reaction was allowed to proceed at rt for 3 h – 48 h. Product formation was monitored using MALDI-TOF MS.

**Optimized procedure for the formation of a kynurenine derivative (M + 4 adduct)** on melittin with trifluoroacetic acid. Oxidized melittin (100 μM final concentrated) was added to an acidic solution (500 mM TFA). The solution was briefly vortexed and the reaction was allowed to incubate at rt for 2 h. Product formation was monitored using MALDI-TOF MS.

**Phosphination of AuNPs (10 nm diameter).** Phosphination of AuNPs (5 nm diameter) was carried out following a previously reported procedure. In a typical reaction, bis(p-sulfonatophenyl)phenylphosphine dehydrate dipotassium salt (BSPP, 5 mg) was mixed with AuNPs (10 mL) and vigorously stirred at rt overnight. Solid NaCl was then added to the AuNP solution until the color turned from deep red to purple. The solution was then centrifuged (5000 rpm) for 10 min, yielding a pellet of nanocrystals. The supernatant was carefully removed and the pellet redissovled in an aqueous solution of BSPP (25 mg in 100 mL dd-H2O). The redissovled nanocrystal solutions were combined and then precipitated through the addition of methanol, until the color again changed from deep red to purple. The solution was again centrifuged (5000 rpm) for 10 min and the supernatant was removed. The nanocrystal pellet was again dissolved in an aqueous solution of BSPP, and stored at room temperature until use. The AuNP concentration was determined by recording the absorbance at 520 nm, and relating the absorbance values to concentration via Beer’s Law \( (\varepsilon_{520\,\text{nm}} = 8.1 \times 10^7 \, \text{M}^{-1}\text{cm}^{-1} \text{ for } 10 \text{ nm diameter AuNPs}) \).

**Alkanethiol oligonucleotide modification of BSPP-stabilized AuNPs (10 nm diameter) for reassembly.** Alkanethiol oligonucleotide modification of BSPP-stabilized AuNPs was achieved using a modified version of a previously reported procedure.

The oligonucleotide sequence identity was as follows:

5’-5ThioMC6-D/TTT-TTA-CAT-GGG-TAA-TCC-TCA-TGT-3’

Lyophilized oligonucleotides containing a 5’ thiol modifier [5‘-(\(\text{CH}_2\)_6-S-S-(\(\text{CH}_2\)\(_2\)_6-phosphodiester bond-oligonucleotide-3‘)] purchased from IDT technologies (Coralville, IA) were resuspended in dd-H2O to yield a final concentration of 100 μM. The disulfide functionality was cleaved by incubation with DTT (0.1 M) at rt in 100 mM phosphate buffer pH 8.0 for 1 h. Excess DTT was removed by passing the solution through a commercially available gel filtration column pre-equilibrated with dd-H2O (NAP 5 column, GE Healthcare). It is important to note that 90% of the recommended volume was applied to the column for elution, to prevent excess DTT from remaining in the oligonucleotide solution. Fractions collected from the gel filtration column that contained oligonucleotides were combined and immediately added to BSPP-stabilized AuNPs (9 nM) to yield a final oligonucleotide concentration of 10 μM. The resulting solution was then incubated at rt for 20 min. The
concentration of NaCl was increased by increments of 0.05 M to a final concentration of 0.1 M through successive additions of 2 M NaCl. Following each addition of 2 M NaCl, the alkanethiol oligonucleotide-modified AuNP solution was sonicated for 10 s, followed by agitation at rt for 20 min. After the final concentration of 0.1 M NaCl was reached, the AuNP solution was allowed to incubate at rt overnight. Excess oligonucleotides were removed through multiple rounds of centrifugal filtration with a 100k MWCO centrifugal filter (Millipore), and diluted in 50 mM phosphate buffer, pH 7.0. The final AuNP concentration was approximated by measuring the absorbance at 520 nm diameter AuNPs.

Cloning, expression, and purification of T19pAF MS2. Bacteriophage MS2 T19pAF plasmid production and growth has been previously reported. The Peter Schultz lab (Scripps Research Institute, La Jolla, CA) provided the tRNA- and tRNA-synthetase-encoding plasmids necessary for p-aminophenylalanine (pAF) incorporation. A yield of ~10 mg/L was obtained for MS2-pAF19 following two purification rounds.

Cloning, expression, and purification of T19W MS2. The QuickChange II Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to mutate the pBAD-MS2 plasmid to generate the pBAD-MS2-T19W plasmid. The threonine at position 19 was converted to a tryptophan using the following forward and reverse primers:

Forward: 5'-GGA-ACT-GGC-GAG-GTG-TGG-GTC-GCC-CCA-3'
Reverse: 5'-GTT-GCT-TGG-GGC-GAC-CCA-CAC-GTC-GCC-3'

Growth and purification of T19W MS2 was identical to that of wild type MS2. A yield of ~90 mg/L was obtained for MS2-T19W following one round of purification.

General method for the oxidation of MS2 with sodium periodate to generate an N-formyl kynureine derivative (M + 32 adduct). An Eppendorf tube was charged with a solution of T19W MS2 (25 μM final concentration) in 25 mM sodium acetate buffer, pH 5.0. A freshly prepared solution of sodium periodate in dd-H₂O (10 mM final concentration) was added to the protein solution. The reaction mixture was briefly vortexed and allowed to sit at rt for 48 h. A neutralized tris(2-carboxyethyl)phosphine hydrochloride (TCEP) solution in dd-H₂O (15 mM final concentration) was added to quench the reaction and briefly vortexed. The reaction mixture was then purified by passing the solution through a commercially available gel filtration column (NAP-5, GE Healthcare), and concentrated through successive centrifugal filtrations in 100 kD MWCO filters.

General method for the formation of a kynureine derivative on MS2 (M + 4 adduct). An Eppendorf tube was charged with a solution of oxidized T19W MS2 (25 μM final concentration) in 10 mM sodium acetate buffer, pH 5.0. An acidic solution (final concentration 100 mM TFA, 10 M acetic acid + 25 mM HCl, 10 M acetic acid + 50 mM HCl, 10 M acetic acid + 100 mM HCl) was added. The solution was briefly vortexed and the reaction was allowed to sit at room temperature for 48 h. The reaction mixture was then purified by passing the solution through a commercially available gel filtration column (NAP-5, GE Healthcare), and concentrated through successive centrifugal filtrations in 100 kD MWCO filters.
General procedure for disassembly and reassembly of T19W MS2 around 10 nm diameter AuNPs. Bacteriophage MS2 was first subjected to disassembly conditions, using a method previously described.\textsuperscript{35,36} In a typical disassembly reaction, 150 \(\mu\)M MS2 in 50 mM phosphate buffer, pH 7.2 (200 \(\mu\)L) was combined with two volumes of cold glacial acetic acid (400 \(\mu\)L) and kept on ice for 30 min. The mixture was then centrifuged for 20 min (13,000 rpm at 4 \(^\circ\)C). Following centrifugation, the supernatant was removed and applied to a gel filtration column (NAP-5, GE Healthcare) pre-equilibrated with 1 mM acetic acid. Fractions were collected from the gel filtration column and their absorbance values at 280 nm were recorded. The fractions containing MS2 were combined on ice and used immediately.

For reassembly, the final MS2 coat protein dimer concentration was approximated by recording the absorbance at 280 nm and assuming eighty percent reassembly efficiency. The AuNP concentration was approximated by measuring the maximum absorbance and relating that value to the concentration via Beer’s law. In a typical reassembly reaction, ssDNA-modified AuNPs (final ratio of 1:1 AuNPs: MS2 capsids) were combined with two thirds volume of 100 mM phosphate buffer, pH 7.0 containing 100 mM NaCl. The final one third volume comprised MS2 coat protein dimer solution. The solution was then kept at 4 \(^\circ\)C for 40 h to allow for reassembly. Following reassembly, a saturating amount of NaCl was added directly to the solution. The reassembly reaction was then applied to a gel filtration column (NAP-5, GE Healthcare), pre-equilibrated with 10 mM phosphate buffer, pH 7.2. Samples were then concentrated through multiple rounds of centrifugal filtration with a 100k MWCO centrifugal filter (Millipore), and diluted in 10 mM phosphate buffer, pH 7.2. The reassembly material was applied to a native agarose gel for purification prior to imaging with transmission electron microscopy.

Sample extraction from native gels. Native agarose gels were visualized under white light, and bands containing AuNP-MS2 conjugates of interest were extracted, cut into small pieces (1 mm x 1 mm) and placed into a gel extraction spin column (Freeze ‘N Squeeze, Biorad, Hercules, CA) and briefly centrifuged so all agarose pieces were at the base of the spin column. Following the addition of 10 mM phosphate buffer, pH 7.0 (200 \(\mu\)L), the extraction spin columns were kept at 4 \(^\circ\)C for 1 h. The spin columns were then centrifuged for 10 minutes (7000 rpm). The flow through was then applied to 100 kD MWCO spin columns and centrifuged for 10 minutes (6000 rpm). This process was repeated three additional times and the combined samples were stored at 4 \(^\circ\)C prior to imaging by TEM.

General procedure for the addition of 2,5-dioxopyrrolidin-1-yl 6-(4-(dimethylamino)phenoxy) hexanoate to oligonucleotides. A typical reaction was as follows: 5’ amine DNA at a concentration of 300 \(\mu\)M was reacted with 2,5-dioxopyrrolidin-1-yl 6-(4-(dimethylamino)phenoxy) hexanoate (60-120 eq.), synthesized as previously reported,\textsuperscript{16} in a 1:1 solution of DMF and 50 mM phosphate buffer, pH 8.0. The reaction mixture was briefly vortexed and allowed to react at rt for 1.5 h. DNA samples were then filtered through commercially available 0.22 \(\mu\)m centrifugal filter units (Millipore Corporation, Billerica, MA). Commercially available gel filtration columns (NAP 5, GE Healthcare) were then used to separate the small molecule from DNA. This yielded
anisidine-labeled oligonucleotides which were further purified through multiple rounds of gel filtration columns, lyophilized, and resuspended in 50 mM phosphate buffer, pH 6.5 to a final concentration of ∼1 mM.

The sequence identity of ssDNA shown in Figure 3.6 is as follows:

\[
\text{T21 DNA} = 5'\text{-/5AmMC6/-TTT-TTT-TTT-TTT-TTT-TTT-TTT-3'}
\]

**General procedure for anisidine-DNA conjugation to MS2.** T19W, T19W M + 32, and T19W M + 4 MS2 (20 μM final concentration) and anisidine-containing oligonucleotide (200 μM final concentration) were combined in 50 mM phosphate buffer, pH 6.5. A freshly prepared solution of sodium periodate (1 mM) or potassium ferricyanide (1 mM) was then added. The resulting solution was briefly vortexed and allowed to react at rt for 5 min in the case of sodium periodate or 30 minutes in the case of potassium ferricyanide. The reaction was then quenched by the addition of a neutralized solution of tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 1.5 mM final concentration). The reaction mixture was then combined with loading buffer and analyzed by SDS-PAGE gel electrophoresis, as shown in Figure 3.6.

3.6. References

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Chapter 4

Oxidative coupling of ortho-aminophenols and anilines for the bioconjugation of inorganic nanoparticles

Abstract

While there are a number of methods for attaching AuNPs to biomolecules, the existing methods suffer from significant background AuNP adsorption, unstable reagents, and long reaction times. To improve the existing AuNP bioconjugation strategies, we adapted our previously reported oxidative coupling between anilines and ortho-aminophenols for AuNP bioconjugation with fast reaction times under mild conditions. Progress towards the production and attachment of ortho-aminophenol and aniline-labeled AuNPs for the attachment of AuNPs to biomolecules of interest is reported.
4.1. Introduction: existing gold nanoparticle bioconjugation strategies

Five existing strategies allow for the attachment of gold nanoparticles (AuNPs) to biomolecules (Figure 4.1). The most common method takes advantage of the strong thiol-AuNP interaction (~200 kJ/mol in bond energy) for the direct attachment of cysteine-containing peptides, thiol-DNA, and proteins with solvent accessible cysteines to AuNPs. In a second method, electrostatic adsorption, proteins are incubated with AuNPs at a pH such that the positive external charge of the protein can interact with negatively charged AuNPs. Bovine serum albumin and IgG antibodies have been attached to AuNPs with this strategy. In a third method, amines are displayed on the surface of AuNPs through a surface passivation method (either electrostatic adsorption of BSA or silica coating). Conjugation of surface amines on the passivation layer with activated carboxylic acids on a biomolecule of interest form a stable amide bond between the AuNP and biomolecule. A fourth method utilizes affinity-based, non-covalent interactions, such as a biotin-streptavidin interaction (biotinylated biomolecule and streptavidin coated AuNPs), and His6/nickel-nitrilotriacetic acid (NTA) interaction (His-6 tag on the biomolecule interacts with NTA-coated AuNPs). The final existing method for AuNP bioconjugation involves the covalent conjugation of AuNPs to a biomolecule of interest, which has been demonstrated through the conjugation of azide-AuNPs to alkyne labeled proteins. The reaction requires a Cu(I) catalyst at room temperature for three days. In addition, commercially available NHS ester and maleimide functionalized AuNPs allow for the modification of lysine or cysteine residues, respectively.

While there are a number of methods for attaching AuNPs to biomolecules, the existing methods suffer from significant background AuNP adsorption. Positively charged biomolecules can readily adsorb to negatively charged AuNPs. This presents a problem in the case of chemisorption, electrostatic adsorption, affinity-based non-covalent interactions, and some covalent conjugation strategies where the AuNP surface is unpassivated. The Cu(I)-mediated click strategy suffers from aggressive reaction conditions and long reaction times. NHS ester and maleimide functionalized AuNPs hydrolyze in aqueous solution over time. We sought to improve the existing bioconjugation strategies by adapting our previously reported oxidative coupling between anilines and ortho-aminophenols. The involved reagents are stable under aqueous conditions and the reaction proceeds quickly under mild conditions. Adapting this strategy for the bioconjugation of AuNPs should provide a significant improvement over the existing strategies.
Figure 4.1. Current bioconjugation strategies for AuNPs: a) chemisorption, b) electrostatic adsorption, c) surface passivation and subsequent functionalization, d) affinity-based, non-covalent interactions, and e) covalent conjugation.
4.2. Ortho-aminophenol gold nanoparticles

We first sought to decorate AuNPs with ortho-aminophenols for conjugation to either aniline-containing proteins or the N-terminus of proteins. To achieve this, we synthesized a AuNP-binding, ortho-aminophenol containing discrete PEG, [lipoic acid]-PEG₄-PEG₄-orthoAP-COOH, 2, as shown in Figure 4.2. The discrete PEG was synthesized using standard solid phase peptide synthesis chemistry. A C-terminal tyrosine residue was modified with 4-nitrobenzenediazonium tetrafluoroborate to afford the azo discrete PEG, 1. Compound 1 was then cleaved from the resin with base and reduced to the ortho-aminophenol, 2, with sodium dithionite. These materials were purified using RP-HPLC, and their masses were confirmed using MALDI-TOF MS.

Aniline-containing proteins or the N-terminus of proteins were then modified with 2 using conditions previously reported by our group and outlined in Figure 4.3. T19pAF MS2, aniline-(T104K) TMV, RNAse, lysozyme, and T104K TMV PAGSYS were successfully modified with compound 2, as shown by mass spectrometry reconstruction traces in Figure 4.4. The disulfide form of 2 corresponds to a mass addition of 876. The fully reduced, dithiol form of 2 corresponds to a mass addition of 878. Oxidation of 2 was observed only for reactions with sodium periodate as the oxidant.

Following confirmation of the attachment of 2, the T19pAF MS2, aniline-TMV, and T104K TMV PAGSYS modified proteins and their corresponding controls of unmodified T19pAF, T104K TMV, and T104K TMV PAGSYS were incubated with BSPP-stabilized AuNPs. If 5 nm diameter AuNPs could successfully attach to 2-labeled proteins, it would be plausible that compound 2 could first be attached to AuNPs, and then subjected to oxidative coupling conditions to afford AuNP-protein conjugates directly.

It is important to note that regardless of the oxidant used to attach 2, all reactions were quenched by the addition of TCEP. The addition of TCEP was required to reduce any oxidized disulfide species prior to incubation with AuNPs. Following incubation, these
Figure 4.3. Oxidative coupling strategies for attaching 2 or AuNPs coated with 2 to proteins of interest (at an N-terminus or introduced aniline moiety).
where $R = \text{PEG}_{4}\text{-PEG}_{4}\text{[LA]}$

Figure 4.4. Mass spectrometry traces demonstrating attachment of 2 to a) T19pAF MS2, b) aniline-(T104K) TMV, c) RNAse (N-terminus), d) lysozyme (N-terminus), e) T104K TMV PAGSYS (N-terminus). The disulfide form of 2 corresponds to a mass addition of 876. The fully reduced, dithiol form of 2 corresponds to a mass addition of 878. Oxidation of 2 was observed only for reactions with sodium periodate as the oxidant.
samples were run on a native agarose gel, as shown in Figure 4.5-a and Figure 4.6-a. All samples required incubation with pH adjusted (pH ~ 7), glutathione prior to purification to reduce non-specific adsorption of TMV or MS2 to BSPP AuNPs as observed by native gel. Samples corresponding to AuNP-protein conjugates were observed only in 2-modified MS2 or TMV. The bands corresponding to AuNP-protein conjugates were extracted from the native gel, and their average diameter was determined by dynamic light scattering, as shown in Figure 4.5-b and Figure 4.6-b. In the case of MS2, three discrete bands were observed by native agarose gel, labeled as 1, 2, and 3 in Figure 4.5-a. These bands corresponded to diameters of 40.8 ± 1.2 nm, 60.7 ± 0.5 nm and 78.0 ± 0.4 nm. Upon observing the structures by TEM, we assigned band 1 to MS2 capsids with multiple AuNPs attached to the exterior surface (expected ~37 nm diameter). We assign bands 2 and 3 to MS2 dimers and MS2 trimers with multiple 5 nm diameter AuNPs attached to their exterior surface, respectively. An MS2 dimer should correspond to a ~64 nm diameter and an MS2 trimer should be ~64 nm to ~91 nm diameter (depending on the attachment orientation in solution). Representative TEM images are shown in Figure 4.5-c,e. Multiple TEM images were compiled to provide a histogram for the number of MS2 capsids per conjugate for the middle band and top band. We attribute differences between dynamic light scattering average diameter values and the TEM histogram data to drying effects on the TEM grid.

In the case of T104K TMV PAGSYS and aniline-TMV, bands corresponding to AuNP-protein conjugates were extracted from the native agarose gel and their average diameter was determined by dynamic light scattering, as shown in Figure 4.6-b. AuNP attachment to the N-terminus of T104K TMV PAGSYS was observed on the exterior surface of TMV, providing a ring of AuNPs as shown in Figure 4.6-c. AuNP attachment to aniline-TMV was observed on the face of TMV double disks, as shown in Figure 4.6-d,f. After observing the formation of AuNP-protein conjugates upon incubation of 2-labeled proteins with unmodified AuNPs, we next sought to modify AuNPs directly with 2, and then subject the resulting solution to oxidative coupling conditions. A concern was the stability of ortho-aminophenol labeled AuNPs over time. Therefore, we incubated 1 with citrate stabilized AuNPs to afford 1-labeled AuNPs as determined by decrease in electrophoretic mobility by native gel. The 1-labeled AuNPs were then reduced to 2-labeled AuNPs through the addition of one equivalent of sodium dithionite. It is important to note that an excess of dithionite was found to dissolve the AuNPs in solution. After removing excess dithionite through multiple rounds of centrifugal filtration with 100 kD MWCO spin filters, the 2-labeled AuNPs were used immediately.

Several proteins were subjected to oxidative coupling conditions with 2-AuNPs to test their N-terminal reactivity (wt GFP, creatine phosphokinase, T104K TMV PAGSYS, and myoglobin) as well as aniline reactivity (T19pAF MS2), as shown in Figure 4.7. Results shown in Figure 4.7 were initially interpreted as stable AuNP-protein conjugate formation (AuNPs and proteins ran together on the gel) for oxidative coupling at 10 mM sodium periodate for pH 7.5 (N-terminus) and pH 6.5 (aniline). Similar AuNP-protein conjugates were not observed for the corresponding control of subjecting 2-AuNPs to oxidative coupling conditions at pH 6.0 (N-terminus) and pH 9.0 (aniline), as shown in Figure 4.7.
Figure 4.5. Characterization of 2-modified T19pAF MS2 incubated with BSPP-stabilized AuNPs. The incubated samples were separated by a) native agarose gel, revealing three discrete assemblies that were characterized by b) DLS, and c) TEM for the bottom band, d) middle band, and e) top band. In f) and g), the number of MS2 capsids per cluster as determined by TEM were tallied and are represented as histograms for the middle band and top band, respectively.
Figure 4.6. Characterization of 2-modified T104K TMV PAGSYS and aniline-TMV (T104K) incubated with BSPP-stabilized AuNPs. The incubated samples were separated by a) native agarose gel, revealing a single band for Pro-terminal TMV and aniline-TMV AuNP conjugates that were characterized by b) DLS, and TEM for c) Pro-terminal TMV, and d) aniline TMV incubated with 5 nm diameter AuNPs. Higher magnification TEM images are shown for 2-modified aniline-TMV incubated with e) 5 nm diameter AuNPs, and f) 15 nm diameter AuNPs.
However, upon extracting AuNP-protein sample from the gels shown in Figure 4.7, no AuNP-protein conjugates were observed by TEM. A size increase was not observed by DLS. Additionally, AuNP-protein conjugates were not observed by native gel at lower concentrations of periodate or with potassium ferricyanide as the oxidant. Furthermore, subjecting 1-labeled AuNPs or tyrosine-labeled AuNPs to similar oxidative coupling conditions as those shown for 2-labeled AuNPs in Figure 4.7 yielded similar co-running bands of AuNPs and proteins. The results from these controls seemed to indicate the observed AuNP-protein bands were not stable AuNP-protein conjugates. We hypothesized co-running AuNP-protein bands were observed due to the direct oxidation of the AuNP (due to the high concentration of periodate) which in turn resulted in non-

\[ R = \text{PEG}_4-\text{PEG}_4 \text{NH}_2 + \text{PEG}_4-\text{PEG}_4 \text{NH}_2 \]

Figure 4.7. Native agarose gel electrophoresis of wtGFP, creatine phosphokinase, TMV PAGSYS, myoglobin, T19pAF MS2, and T19W MS2 combined with 2-modified AuNPs and 10 mM sodium periodate. Proteins tested at these conditions were found to co-migrate with AuNPs at pH 7.5 (for N-terminal attachment) or pH 6.5 (for aniline attachment). AuNPs and proteins did not co-run on the gel at pH 6.0 (for N-terminal attachment), pH 9.0 (for aniline attachment), or for T19W MS2, which does not have a solvent accessible aniline or N-terminus. As shown in the TEM image in the lower right corner, no AuNP-T19pAF MS2 conjugates were observed.
specific adsorption of the proteins of interest to the AuNP surface. As a result, we decided to abandon this approach and explore an alternative aniline-AuNP strategy.

4.3. Aniline gold nanoparticles

Due to concerns about the long-term stability of ortho-aminophenol AuNPs, we were interested in producing aniline-AuNPs, which should be stable in aqueous conditions at room temperature for extended periods. An increased PEG length spacing should also help prevent any non-specific adsorption of proteins to the AuNP surface.

To achieve this, we synthesized a lipoic acid terminated 5K PEG aniline 3 or azide 4 for direct attachment to AuNPs, as shown in Scheme 4.1. The aniline 3 should attach to ortho-aminophenol containing biomolecules when subjected to oxidative coupling conditions. The azide 4 should serve as the corresponding control, as shown in Figure 4.8.

![Scheme 4.1. Synthesis of ligands 3 and 4.](image)

MS2, BSA, RNAse A, and lysozyme were incubated with BSPP-stabilized AuNPs and 3-stabilized AuNPs overnight, and then run on a native agarose gel to determine non-specific adsorption of proteins to AuNPs. Significant adsorption was observed for BSPP stabilized AuNPs, but no observable amount of protein was adsorbed to the 3-labeled AuNPs (Figure 4.9). With evidence that background sticking would not be a major concern for 3-stabilized AuNPs, we tested the ability of these aniline-coated AuNPs to undergo oxidative coupling with ortho-aminophenol containing DNA, peptides, and proteins.

![Figure 4.8. A second strategy explored for the attachment of 3-modified AuNPs to ortho-aminophenol containing proteins. The 4-modified AuNPs serve as a control.](image)
Amine-ssDNA was first modified with a nitrophenol NHS ester, followed by reduction with sodium dithionite to yield ortho-aminophenol ssDNA, as shown in Figure 4.10-a. The mass addition of the ortho-aminophenol was confirmed through MALDI-TOF MS (Figure 4.10-b,c). Upon subjecting the ortho-aminophenol ssDNA to oxidative coupling in the presence of 3-AuNPs and 4-AuNPs, a gel shift was observed only for 3-AuNPs in the presence of oxidant. Furthermore, a longer DNA strand resulted in a AuNP with greater electrophoretic mobility (longer ssDNA modified AuNP ran further on the gel) presumably due to the increased negative charge on the AuNP (Figure 4.10-d).

Two different ortho-aminophenol containing peptides were then tested for their attachment to 3-AuNPs. A clear shift by native gel, corresponding to attachment of the peptide was observed for lipoic acid-GDDD-oAP-COOH (produced as discussed in Chapter 2) peptides subjected to oxidant in the presence of 3-AuNPs and 4-AuNPs, a gel shift was observed only for 3-AuNPs in the presence of oxidant. No gel shift was observed for the corresponding controls, including incubation of 3-AuNPs, as well as the incubation of 4-AuNPs with the peptide of interest in the presence and absence of oxidant (Figure 4.11a). An additional peptide, fibrin binding, GPRPPGSGSK-[oAP]-COOH was tested similarly for its attachment to AuNPs. The peptide was found to attach to 3-AuNPs in the presence of 10 mM potassium ferricyanide in 30 minutes, as confirmed by a change
A protein, lysozyme, was also tested for attachment to 3-AuNPs. In a similar strategy as that for amine ssDNA, lysozyme was modified with an ortho-nitrophenol NHS as confirmed by ESI-MS (Figure 4.12-a). Reduction with sodium dithionite afforded an ortho-aminophenol labeled lysozyme. A discrete shift by native gel was observed only for ortho-aminophenol lysozyme with 3-AuNPs in the presence of ferricyanide or sodium periodate (Figure 4.12-b). Additionally, 3-AuNPs and 4-AuNPs combined with ortho-aminophenol lysozyme in the presence of potassium ferricyanide were applied to a denaturing SDS PAGE gel, confirming the attachment of 3 as shown by the 5K PEG gel shift in lane 2. A gel shift was not observed for 4-AuNPs combined with ortho-aminophenol lysozyme in the presence of potassium ferricyanide, as shown in lane 3 (Figure 4.12-c).

A similar strategy was also used to produce AuNP-antibody conjugates. Ortho-aminophenol modified antibodies were subjected to oxidative coupling conditions in the presence of 3-AuNPs and 4-AuNPs. A discrete gel shift was observed only for 3-AuNPs with ortho-aminophenol antibodies in the presence of oxidant, as shown in Figure 4.13-a. This strategy could presumably be extended to any protein that contains solvent accessible lysine residues, such as horseradish peroxidase. A reconstructed mass spectrum shows the addition of a nitrophenol NHS ester to horseradish peroxidase in Figure 4.13-b.
Figure 4.11. Peptide attachment to 3-AuNPs. a) LA-GDDD-oAP-COOH peptide attached only to 3-AuNPs in the presence of oxidant, as shown by a shift in native gel. b) An oAP-containing GPR-peptide (fibrin binding) was attached to 3-AuNPs in 30 min at 10 mM potassium ferricyanide, as confirmed by c) a shift in native gel.
Figure 4.12. Lysozyme attachment to [3]-AuNPs. a) mass spectrometry traces demonstrating 1-3 modifications of lysozyme with ortho-nitrophenol NHS. b) Following reduction of ortho-nitrophenol lysozyme with dithionite, a discrete shift by native gel was observed only for ortho-aminophenol lysozyme with 3-AuNPs in the presence of ferricyanide. c) 3-AuNPs and 4-AuNPs combined with AP-lysozyme in the presence of ferricyanide were run on a denaturing gel, confirming the attachment of 5 K PEG in the case of 3-AuNPs. No PEG-lysozyme conjugate was observed for 4-AuNPs.
Figure 4.13. Antibody attachment to 3-AuNPs. a) native agarose gel reveals a shift for o-aminophenol Ab combined with 3-AuNPs in the presence of periodate. b) nitrophenol NHS modification of horseradish peroxidase as confirmed by mass spectrometry.
4.4. Conclusions and future outlook for aniline inorganic materials

We think this aniline-AuNP oxidative coupling strategy presents a promising alternative for the attachment of AuNPs to any ortho-aminophenol labeled biomolecule of interest. The reaction proceeds under mild conditions with fast reaction times and little-to-no background protein adsorption to the AuNPs. In the future, we hope to use this strategy to attach a AuNP to a fluorophore labeled antibody and determine the binding ability of the AuNP-antibody construct using flow cytometry (Figure 4.14-a). We also want to test the effect of AuNP attachment on the activity of certain enzymes, such as horseradish peroxidase (Figure 4.14-b). Finally, we’re interested in extending this strategy for other thiophilic inorganic nanomaterials. We envision this strategy would be useful for silver nanoparticles, CdSe/ZnS quantum dots, CdSe/CdS dot-rods, and magnetic nanoparticles, such as Au@Fe₃O₄ and Au@Fe₂O₃ nanoparticles.

4.5. Materials and methods

4.5.1. General procedures and materials

Unless otherwise noted, all chemicals were purchased from commercial sources and used as received without further purification. Water (dd-H₂O) used in all procedures was deionized using a NANOpure® purification system (Barnstead, USA). All unconjugated gold solutions were purchased from Ted Pella, Inc (Redding, CA). Bis(p-sulfonatophenyl)phosphine dehydrate dipotassium salt (BSPP) was purchased from Sigma Aldrich (St. Louis, MO). Fmoc-N-amido-dPEG4-acid was purchased from Quanta BioDesign, Ltd (Powell, Ohio, USA). Glutathione was purchased from Nutritional Biochemicals Corporation (Cleveland, OH). Agarose (low-EEO/multipurpose/molecular biology grade) was purchased from Fisher Scientific (Pittsburgh, PA).
4.5.2. Instrumentation and sample analysis

**NMR.** $^1$H and $^{13}$C spectra were measured with a Bruker AVB-400 (400 MHz) spectrometer.

**Mass Spectrometry.** Matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a Voyager-DE system (PerSeptive Biosystems, USA) and data were analyzed using Data Explorer software. Prior to MALDI-TOF MS analysis, samples were desalted using C18 ZipTip® pipet tips (Millipore, USA). Peptide samples were co-crystallized with α-cyano-4-hydroxycinnamic acid in 1:1 acetonitrile (MeCN) to H$_2$O with 0.1% trifluoroacetic acid (TFA). Oligonucleotide samples were co-crystallized using a 3-hydroxypicolinic acid: ammonium citrate solution (45 mg/mL:5 mg/mL in 4.5:5.5 MeCN:dd-H$_2$O). Electrospray ionization mass spectrometry (ESI-MS) of proteins was performed using an Agilent 1260 series liquid chromatograph that was connected in-line with an Agilent 6224 Time-of-Flight (TOF) LC/MS system equipped with a Turbospray ion source (Agilent Technologies, USA). Protein chromatography was performed using an Agilent poroshell 300SB-C18 reversed-phase column (1.0 x 75 mm) with an MeCN:dd-H$_2$O gradient mobile phase containing 0.1% formic acid (FA). Protein mass reconstruction was performed on the charge ladder with Agilent MassHunter software.

**High Performance Liquid Chromatography.** HPLC was performed on Agilent 1100 Series HPLC Systems (Agilent Technologies, USA). Sample analysis for all HPLC experiments was achieved with an inline diode array detector (DAD) and inline fluorescence detector (FLD). Analytical and preparative reverse-phase HPLC of peptides was accomplished using a C18 stationary phase and a MeCN:H$_2$O with 0.1% TFA gradient mobile phase.

**Gel Analyses.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Mini-Protean apparatus from Bio-Rad (Hercules, CA) with 10-20% gradient polyacrylamide gels (BioRad, CA), following the protocol of Laemmli. All samples were mixed with SDS loading buffer in the presence of 1,4-dithiothreitol (DTT), with the exception of Figure 4.12-c where no DTT was added to the loading buffer, and heated to 95 °C for 10 min to ensure complete denaturation. Gels were run at 120 V for 80 min. Commercially available molecular weight markers (Bio-Rad) were applied to at least one lane of each gel for assignment of molecular masses. For fluorescent protein conjugates, a UV-backlight was used for visualization. Gels were then stained with Coomassie Brilliant Blue (Bio-Rad). Coomassie stained protein gels were imaged on an EpiChem3 Darkroom System (UVP, USA). Native agarose gel electrophoresis was carried out on a Mini-Sub Cell GT System from Bio-Rad (Hercules, CA) with 1% or 1.5% agarose gels. All samples were mixed with 50% glycerol (2:1 sample:50% glycerol) prior to loading on the gel. Gels were typically run at 60 V for 30 min. For fluorescent protein conjugates, a UV-backlight was used for visualization. Gels were then stained with Coomassie Brilliant Blue (Bio-Rad). Agarose gels were imaged either in black and white with an EpiChem3 Darkroom System (UVP, USA) or in color with an Olympus 6.0
megapixel digital camera model number FE-120 (Shinjuku, Tokyo, Japan).

**Dynamic Light Scattering.** DLS measurements were obtained using a Malvern Instruments Zetasizer Nano ZS. Samples were filtered through a 0.22 μm centrifugal filter unit (Millipore Corporation, Billerica, MA) prior to data collection. Data plots are shown as size distribution by number, which weighs large and small particles equally. Diameters were calculated from an average of three measurements.

**Transmission Electron Microscopy.** TEM images were obtained at the UC Berkeley Electron Microscope Lab (UCB EML) using a FEI Tecnai 12 transmission electron microscope with 120 kV accelerating voltage. Protein samples were prepared for TEM analysis by pipetting 5 μL of the samples onto Formvar-coated copper mesh grids (400 mesh, Ted Pella, Redding, CA), after 3 min of equilibration the samples were then wicked with filter paper. The samples were then rinsed with dd-H₂O. Subsequently, the grids were exposed to 8 μL of a 1% (w/v) aqueous solution of uranyl acetate for 90 s as a negative stain. Excess stain was then removed and the grids were dried in air.

### 4.5.3. Experimental procedures

**General procedure for solid-phase peptide synthesis and solid-phase synthesis of 1,2.** Peptides were synthesized using standard Fmoc-based chemistry on Tentagel S-OH resin (Advanced ChemTech, Louisville, KY). The side chain protecting groups used were: Arg(Pbf), Lys(Boc), Ser(tBu), Asp(OtBu), and Tyr(tBu). The C-terminal amino acid (10 eq.) was pre-activated at 0 °C with 5 equivalents of diisopropylcarbodiimide (DIC) and then coupled to the resin with 0.1 equivalents of N,N-dimethylaminopyridine (DMAP) as a catalyst. Deprotection of the Fmoc groups was performed with a 20 min incubation in a 20% v/v piperidine in dimethylformamide (DMF) solution. Coupling reactions were carried out using 20 equivalents of amino acid with 10 equivalents of 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) and 20 equivalents of N,N-diisopropylethylamine (DIPEA) in DMF for 20 min. Side-chain deprotection was accomplished using a 1-2 h incubation with a 95:2.5:2.5 ratio of TFA to H₂O to triisopropylsilane (TIPS). The resulting basic solution was neutralized with 100 mM phosphate buffer, pH 6.5.

**General procedure for azo coupling to peptides.** To a portion of resin-bound peptide (appx. 20 mg) in 900 μL of 100 mM phosphate buffer, pH 9.0 was added 100 μL of a saturated solution of 4-nitrobenzenediazonium tetrafluoroborate (300 mM, Sigma-Aldrich) in MeCN at 4 °C.19 After rotation for 30 min at 4 °C, the resin was rinsed with MeCN and DMF until no color remained in solution. The peptides were then cleaved from the resin with base, and then purified by HPLC using a C18 Gemini column (5 micron, 250 x 7.9 mm, Phenomenex, Torrance, CA). Peptide modification was confirmed by MALDI-TOF MS.

**General procedure for dithionite reduction of azo peptides.** To a solution of HPLC purified azo-modified peptide (appx. 1 mM) in 100 mM phosphate buffer, pH 6.5
was added an equal volume of a freshly prepared solution of sodium dithionite (100 mM) in 100 mM phosphate buffer, pH 7.2. After 10 min, the solution was applied to a C18 Sep-Pak pre-conditioned with methanol then 0.1% aqueous TFA. After washing with aqueous 0.1% TFA and 5% MeCN in aqueous 0.1% TFA, the peptide was eluted with MeCN. After removing the MeCN under reduced pressure the solid peptide was dissolved in 100 μL of 10 mM phosphate buffer, pH 6.5. The ortho-aminophenol peptide was characterized by MALDI-TOF and stored at -20 °C.

**Cloning and expression of T19pAF MS2.** Bacteriophage MS2 T19pAF plasmid production and growth has been previously reported. The Peter Schultz lab (Scripps Research Institute, La Jolla, CA) provided the tRNA- and tRNA-synthetase-encoding plasmids necessary for p-aminophenylalanine (pAF) incorporation. A yield of ~10 mg/L was obtained for MS2-pAF19 following two purification rounds.

**Expression and purification of T104K TMV.** The TMV coat protein had the following mutations: K53R, K68R (RR-TMV), and T104K TMV. This TMV mutant was expressed and purified as described previously.

**Expression and purification of T104K TMV PAGSYS.** The TMV coat protein had the following mutations: K53R, K68R (RR-TMV), T104K TMV, and an extended N-terminus from SYS to PAGSYS. This TMV mutant was expressed and purified as described previously.

**3-(4-aminophenyl) propionic acid N-hydroxysuccinimide ester modification of T104K TMV.** A 1.6 mL Eppendorf tube was charged with a solution of T104K TMV (99 μL of 100 μM solution in 10 mM phosphate buffer, pH 8.0). To this solution was added the NHS ester of 3-(4-aminophenyl) propionic acid (1 μL, 10 eq., 100 mM in DMF). The resulting solution was briefly vortexed and the reaction was allowed to proceed at rt for 2 h. The reaction mixture was then purified by passing the solution through a gel filtration column, and concentrated through successive centrifugal filtrations in 100 kD MWCO filters.

**Modification of T19pAF MS2 and aniline (T104K) TMV with LA-PEG₄-PEG₄-oAP-COOH, 2, shown in Figure 4.4.** A 1.5 mL Eppendorf tube was charged with a solution of T19pAF MS2 or aniline TMV (20 μM final concentration, phosphate buffer, pH 6.5) and HPLC purified 2 (100 μM final concentration, phosphate buffer, pH 6.5). To this solution was added either sodium periodate (1 mM final concentration) or potassium ferricyanide (1 mM final concentration). The resulting mixture was briefly vortexed and the reaction was allowed to proceed at rt for 5 minutes (in the case of sodium periodate) or 30 minutes (in the case of potassium ferricyanide). The reaction mixture was then purified by passing the solution through a commercially available gel filtration column (NAP 5, GE Healthcare), and concentrated through successive centrifugal filtrations in 100 kD MWCO filters. If indicated on the mass spectrum, the reaction mixture was quenched by the addition of TCEP (50 mM final concentration, pH 7.0), briefly vortexed, and allowed to sit at rt for 20 minutes prior to application to a gel filtration column and concentration through successive centrifugal filtrations.
Modification of RNAse A, lysozyme, and TMV PAGSYS with LA-PEG_4-PEG_4-oAP-COOH, 2, shown in Figure 4.4. A 1.6 mL Eppendorf tube was charged with a solution of RNAse A (Sigma), lysozyme (Sigma) or TMV PAGSYS (20 μM final concentration, phosphate buffer, pH 7.5) and HPLC purified 2 (100 μM final concentration, phosphate buffer, pH 7.5). To this solution was added potassium ferricyanide (1 mM final concentration). The resulting mixture was briefly vortexed and the reaction was allowed to proceed at rt for 30 min. The reaction mixture was then purified by passing the solution through a commercially available gel filtration column (NAP 5, GE Healthcare), and concentrated through successive centrifugal filtrations in 100 kD MWCO filters. If indicated on the mass spectrum, the reaction mixture was quenched by the addition of TCEP (50 mM final concentration, pH 7.0), briefly vortexed, and allowed to sit at rt for 20 minutes prior to application to a gel filtration column and concentration through successive centrifugal filtrations.

Phosphination of AuNPs (5 nm diameter). Phosphination of AuNPs (5 nm diameter) was carried out following a previously reported procedure. In a typical reaction, bis(p-sulfonatophenyl)phenylphosphine dehydrate dipotassium salt (BSPP, 5 mg) was mixed with AuNPs (10 mL) and vigorously stirred at rt overnight. Solid NaCl was then added to the AuNP solution until the color turned from deep red to purple. The solution was then centrifuged (5000 rpm) for 10 min, yielding a pellet of nanocrystals. The supernatant was carefully removed and the pellet redissolved in an aqueous solution of BSPP (25 mg in 100 mL dd-H_2O). The redissolved nanocrystal solutions were combined and then precipitated through the addition of methanol, until the color again changed from deep red to purple. The solution was again centrifuged (5000 rpm) for 10 min and the supernatant was removed. The nanocrystal pellet was again dissolved in an aqueous solution of BSPP, and stored at room temperature until use. The AuNP concentration was determined by recording the absorbance at 520 nm, and relating the absorbance values to concentration via Beer’s Law (ε=9.3*10^6 M^-1 cm^-1 for 5 nm diameter AuNPs).

Incubation of 2-labeled T19pAF MS2, aniline (T104K) TMV, TMV PAGSYS with 5 nm diameter AuNPs, shown in Figure 4.5 and Figure 4.6. To a solution of 2-modified T19pAF MS2, aniline TMV or TMV PAGSYS (10 μL of 2 μM in 10 mM phosphate buffer pH 7.0) was added BSPP stabilized 5 nm diameter AuNPs (10 μL of 2 μM). The solutions were allowed to incubate at rt for 20 h. Following incubation, a solution of glutathione was added (5 μL of 10 mg GSH/1 mL 200 mM phosphate buffer, pH 6.5), the resulting mixture was briefly vortexed and allowed to sit at rt for 30 minutes prior to separation by native agarose gel electrophoresis.

Sample extraction from native gel, shown in Figure 4.5 and Figure 4.6. A native agarose gel was visualized under white light, and bands containing AuNP-protein conjugates of interest were extracted, cut into small pieces (1 mm x 1 mm) and placed into a gel extraction spin column (Freeze ‘N Squeeze, Biorad, Hercules, CA) and briefly centrifuged so all agarose pieces were at the base of the spin column. Following the addition of 10 mM phosphate buffer, pH 7.0 (200 μL), the extraction spin columns were kept at 4 °C for 1 h. The spin columns were then centrifuged for 10 minutes (7000 rpm).
The flow through was then applied to 100 kD MWCO spin columns and centrifuged for 10 minutes (6000 rpm). This process was repeated three additional times and the combined samples were stored at 4 °C prior to imaging by TEM.

**Preparation of 1-(LA-PEG₄-PEG₄-azo-COOH)-AuNPs.** A 1.5 mL Eppendorf tube was charged with a solution of 1 (200 μL of 100 μM). To this solution was added 0.5 M TCEP pH 7.0 (5 μL). The resulting solution was briefly vortexed and the reduction was allowed to proceed for 30 minutes. To a solution of unconjugated gold colloids (10 mL of 0.05 μM, Ted Pella) was added phosphosate buffer pH 8.0 (1 mL of 100 mM) and the solution of 1 pre-incubated with TCEP. The resulting solution was stirred at rt overnight. The final solution was stored at 4 °C.

**Preparation of 2-(LA-PEG₄-PEG₄-oAP-COOH)-AuNPs.** To a suspension of 1-(LA-PEG₄-PEG₄-azo-COOH)-AuNPs (1 mL) was added a freshly prepared solution of sodium dithionite (20 μL of 100 mM) in 100 mM phosphate buffer, pH 7.2. After 10 min, the solution was applied to a commercially available gel filtration column (NAP 5, GE Healthcare) and concentrated through successive centrifugal filtrations with 100 kD MWCO filters into 10 mM phosphate buffer, pH 7.0.

**Oxidative coupling of 2-labeled AuNPs with wt GFP, creatine phosphokinase, TMV PAGSYS, and myoglobin, shown in Figure 4.7.** A 0.6 mL Eppendorf tube was charged with a solution of wt GFP, creatine phosphokinase, TMV PAGSYS, or myoglobin (10 μM final concentration), and 2-labeled AuNPs (0.25 μM final concentration). The reaction mixtures were in phosphate buffer pH 7.5 (sample) or pH 6.0 (control). To this solution was added sodium periodate (10 mM final concentration). The resulting mixture was briefly vortexed and the reaction was allowed to proceed at rt for 2 h. The reaction was quenched through the addition of excess 100 mg/1 mL dd-H₂O mannose solution (5 μL for a typical 10 μL reaction volume). Samples were then applied to a 1.5% agarose gel and run at 60 V for 35 min at rt.

**Oxidative coupling of 2-labeled AuNPs with T19pAF MS2 and T19W MS2, shown in Figure 4.7.** A 0.6 mL Eppendorf tube was charged with a solution of T19pAF MS2 or T19W MS2 (10 μM final concentration), and 2-labeled AuNPs (0.25 μM final concentration). The reaction mixtures were in phosphate buffer pH 6.5 (sample) or pH 9.0 (control). To this solution was added sodium periodate (10 mM final concentration). The reaction was quenched through the addition of excess 100 mg/1 mL dd-H₂O mannose solution (5 μL for a typical 10 μL reaction volume). Samples were then applied to a 1.5% agarose gel and run at 60 V for 35 min at rt.

**Lipoic acid N-hydroxysuccinimide ester.** To a solution of lipoic acid (1.02 g, 4.94 mmol, 1.0 equiv) and NHS (563 mg, 4.94 mmol, 1.0 equiv) in 8 mL of DCM at 0 °C was added a solution of DCC (960 mg, 4.66 mmol, 0.94 equiv) in 6 mL of DCM dropwise. The reaction mixture was stirred on ice for 1 h, transferred to the cold room and stirred for an additional hour before the stirring was stopped. Precipitation was allowed to continue and complete overnight in the cold room. The next morning, the reaction mixture was placed in a -20 °C freezer for 30 min to ensure full precipitation of DCU, and the
cold reaction mixture was quickly filtered on a Buchner funnel and washed with 2x5mL portions of DCM that had been pre-cooled to -20 °C. Concentration of the filtrate delivered the crude product (1.5 g) as a yellow solid. 'H NMR reveals the product contains ca. 5% of unreacted lipoic acid and NHS. This material was pure enough to be taken forward without any additional purification. 'H NMR spectral data agree with those previously reported.28

Valeric acid-PEG5k-Lipoamide. To a room temperature solution of valeric acid-PEG5k-amine (mw 5000, 100 mg, 0.020 mmol, 1.0 equiv) and lipoic acid NHS ester (30 mg, 0.10 mmol, 5 equiv) in DCM (0.5 mL) in a 1 dram screw cap vial was added triethylamine (27 uL, 0.20 mmol, 10 equiv). The reaction was capped and stirred overnight at room temperature. The next morning, the solution was concentrated under a gentle stream of nitrogen, and 1 mL of water was added to precipitate residual lipoic acid NHS ester. The suspension was then filtered through a 0.22 uM spin filter. The aqueous solution was then repeatedly spin concentrated in a 4 mL 3 kDa MWCO filter with water. The recovered material was then lyophilized to afford 102 mg of crude product as a very pale yellow powder.

N-(2-aminoethyl)-3-(4-azidophenyl)propanamide. DCC (108 mg, 0.523 mmol, 1.0 equiv) in 1 mL of DCM was added dropwise to a 1 mL suspension of 3-(4-azidophenyl)propionic acid (100 mg, 0.523 mmol, 1.0 equiv) and NHS (60 mg, 0.523 equiv, 1.0 equiv) at 0 °C. After 45 minutes, ethylenediamine (314 mg, 350 uL, 5.23 mmol, 10 equiv) was added in one portion with vigorous stirring. The reaction was allowed to come to room temperature over 10 minutes, and the reaction was stirred for an additional 30 min at this temperature before being placed in a freezer for 2 h to precipitate DCU. The DCU was filtered through a small pad of cotton/celite in a pasteur pipette, and an aqueous workup with water and brine (3:1) was performed, with successive extractions performed with DCM. The combined organic extracts were dried over magnesium sulfate, filtered, and concentrated to give the desired product (95 mg) as a pale yellow solid.

Aniline-PEG5k-lipoamide, 3. The same procedure for the preparation of azide-PEG5k-lipoamide was followed on the same scale, except using 11 mg of 2-(4-aminophenyl)ethylamine that afforded 18 mg of product as a white powder.

Azide-PEG5k-lipoamide, 4. A suspension of valeric acid-PEG5k-lipoamide (20 mg, 0.004 mmol, 1.0 equiv), EDC (7.6 mg, 0.04 mmol, 10 equiv) and NHS (4.6 mg, 0.04 mmol, 10 equiv) in 0.5 mL of DCM in a 1 dram screwcap vial was allowed to react at room temperature for 1 hour. To this suspension was added N-(2-aminoethyl)-3-(4-azidophenyl)propanamide (18 mg, 0.08 mmol, 20 equiv), and the reaction mixture vigorously stirred at room temperature overnight. The DCM was removed under a gentle stream of nitrogen, and the residue was suspended in 1 mL of water to precipitate unreacted small molecule starting materials. The suspension was filtered through a 0.22 uM spin filter, and the filtrate was repeatedly spin concentrated with water in a 4 mL 3 kDa mwco spin concentrator to remove soluble small molecules. The aqueous solution was recovered and lyophilized to afford 20 mg of azide-PEG5k-lipoamide as a white powder.
Preparation of 3 (aniline-PEG5k-lipoamide)-AuNPs and 4 (azide-PEG5k-lipoamide)-AuNPs. To a solution of unconjugated gold colloids (5 mL of 0.05 μM, Ted Pella) was added phosphate buffer pH 8.0 (1 mL of 100 mM) and either aniline-PEG5k-lipoamide (1 mg) or azide-PEG5k-lipoamide (1 mg). The resulting solution was stirred at rt for 10 h. The final 3- and 4- labeled AuNPs were purified and concentrated through successive centrifugal filtrations with 100 kD MWCO filters into a 10 mM phosphate buffer pH 7.0 solution.

Background interaction of AuNPs with proteins of interest, as shown in Figure 4.9. T19pAF MS2 (2 μM final concentration), Bovine Serum Albumin (Sigma, 2 μM final concentration), RNAse A (Sigma, 2 μM final concentration), and lysozyme (Sigma, 2 μM final concentration) were incubated with either BSPP or 3-stabilized 5 nm diameter AuNPs (0.5 μM final concentration) in the presence of 100 mM NaCl with 10 mM phosphate buffer, pH 7.0 overnight at rt. The sample (10 μL) was then combined with 50% glycerol (5 μL) and applied directly to a 1.5% agarose gel. The gel was run at a constant 60 V for 40 min at rt.

Synthesis of ortho-nitrophenol acid. The synthesis of ortho-nitrophenol acid was performed as reported previously. Spectral data agreed with those previously reported.

Synthesis of ortho-nitrophenol N-hydroxysuccinimide ester. The synthesis of ortho-nitrophenol succinimidy ester was performed as reported previously. Spectral data agreed with those previously reported.

General procedure for the addition of ortho-aminophenol to oligonucleotides. A typical reaction was as follows: 5’ amine DNA at a concentration of 300 μM was reacted with o-nitrophenol succinimidyl ester (60-120 eq.) in 1:1 solution of DMF and 50 mM phosphate buffer, pH 8.0. The reaction mixture was briefly vortexed and allowed to react at rt for 1.5 h. DNA samples were then filtered through commercially available 0.22 μm centrifugal filter units (Millipore Corporation, Billerica, MA). Commercially available gel filtration columns (NAP 5, GE healthcare) were then used to separate the small molecule from DNA. A 100 mM stock of sodium dithionite in 200 mM phosphate buffer, pH 6.5 was added to the purified DNA mixture at a final concentration of 10 mM and allowed to react for 10 min. This yielded the o-aminophenol labeled oligonucleotides which were further purified through multiple rounds of gel filtration columns, lyophilized, and resuspended in 50 mM phosphate buffer, pH 6.5 to a final concentration of ~1 mM.

The sequence identities of ssDNA shown in Figure 4.10 are as follows:

short DNA = 5’/-/5AmMC6/-GTG-AAT-TTG-TGT-GT-3’

MUC DNA = 5’/-/-GCA-GTT-GAT-CCT-TTG-GAT-ACC-CTG-G-3’

Oxidative coupling between 3-AuNPs or 4-AuNPs and ortho-aminophenol DNA, as shown in Figure 4.10. A 0.7 mL Eppendorf tube was charged with a solution of
3- or 4-labeled AuNPs (0.4 μM final concentration) and ortho-aminophenol ssDNA (100 μM final concentration, 5 eq. assuming 50 anilines or azides/AuNP) in phosphate buffer, pH 6.5. To this solution was added potassium ferricyanide (10 mM final concentration). The resulting solution was briefly vortexed and the reaction was allowed to proceed at rt for 2 h. The reaction mixture was then applied directly to a 1% agarose gel and run at a constant 60 V for 40 min.

Oxidative coupling between 3-AuNPs or 4-AuNPs and ortho-aminophenol peptides, as shown in Figure 4.11. A 0.7 mL Eppendorf tube was charged with a solution of 3- or 4-labeled AuNPs (0.4 μM final concentration) and ortho-aminophenol peptide (100 μM final concentration for LA-GDDD-oAP-COOH or GPRPPGSGKG-oAP-COOH) in phosphate buffer, pH 6.5. To this solution was added potassium ferricyanide (10 mM final concentration). The resulting solution was briefly vortexed and the reaction was allowed to proceed at rt for the indicated time (0.5 h – 2.5 h). The reaction mixture was then applied directly to a 1% agarose gel and run at a constant 60 V for 30 min (LA-GDDD-oAP-COOH, Figure 4.11-a) or 90 min (GPRPPGSGKG-oAP-COOH, Figure 4.11-c).

Ortho-aminophenol NHS ester modification of lysozyme. A 1.6 mL Eppendorf tube was charged with a solution of lysozyme (50 μM final concentration) in phosphate buffer, pH 8.0. To this solution was added ortho-nitrophenol NHS ester (100 μM final concentration, 2 eq.). The resulting solution was briefly vortexed and the reaction was allowed to proceed for 1 h at rt. The reaction mixture was then purified by passing the solution through a gel filtration column, and concentrated through successive centrifugal filtrations in 10 kD MWCO filters, affording ortho-nitrophenol lysozyme. A freshly prepared 100 mM solution of sodium dithionite in 200 mM phosphate buffer, pH 6.5 was added to the purified ortho-nitrophenol lysozyme at a final concentration of 10 mM and allowed to react for 10 min. This yielded ortho-aminophenol labeled lysozyme that was further purified by passing the solution through a gel filtration column, and concentrated through successive centrifugal filtrations in 10 kD MWCO filters.

Oxidative coupling between 3-AuNPs or 4-AuNPs and ortho-aminophenol lysozyme, as shown in 4.12. A 0.7 mL Eppendorf tube was charged with a solution of 3- or 4-labeled AuNPs (0.4 μM final concentration) and ortho-aminophenol lysozyme (20 μM final concentration) in phosphate buffer, pH 6.5. To this solution was added potassium ferricyanide (5 mM final concentration). The resulting solution was briefly vortexed and the reaction was allowed to proceed at rt for 1.5 h. The reaction mixture was then applied directly to a 1% agarose gel and run at a constant 60 V for 60 min. For SDS-PAGE gel analysis, it is important to note that DTT was not included in the loading buffer.

Ortho-nitrophenol NHS ester modification of horseradish peroxidase. A 1.5 mL Eppendorf tube was charged with a solution of peroxidase from horseradish (Sigma, 4 mg/mL final concentration) in phosphate buffer, pH 8.0. To this solution was added ortho-nitrophenol NHS ester (0.4 mM final concentration, 10 eq.). The resulting solution was briefly vortexed and the reaction was allowed to proceed for 1 h at rt. The reaction mixture was then purified by passing the solution through a gel filtration column, and concentrated through successive centrifugal filtrations in 10 kD MWCO filters.

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4.6. References


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