Tuning Quantum Dot Interfaces for Biofunctionalization with expressed Haptocorrin and Fluorescent Proteins: Applications in Targeted Delivery, Cellular Imaging, Self-Assembly, and Energy Transfer

Joshua Zylstra

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Abstract

Semiconductive quantum dots (qdots) have emerged as a valuable material for energy transfer and cellular imaging due to its unique optical properties such as tunable emission, broad absorption, and long fluorescent lifetimes. In order to take advantage of these properties, the surface chemistry of the qdots must first be changed in order to facilitate transfer out of organic solvents and into aqueous buffers. In my dissertation research, I developed a qdot phase transfer method using the amino acid histidine that quickly render qdots soluble in aqueous buffers, retains quantum yield (QY), allows for subsequent ligand exchange to tailor qdot surface chemistry, and is easily functionalized by biomaterials. A polymer wrapping method using poly(styrene-co-maleic anhydride) (PSMA) functionalized with Nα,Nα-Bis(carboxymethyl)-L-lysine (NTA) has also been developed. I also expressed and purified a red fluorescent protein (tagRFP) with a N-terminal hexa-histidine tag. TagRFP was utilized for initial fluorescence resonance energy transfer studies (FRET) with both histidine and polymer wrapped qdots as well as sequential bioluminescence resonance energy transfer (BRET)-FRET using firefly luciferase as an energy donor, qdots or qrods as an intermediate acceptor/donor, and tagRFP as the final energy acceptor.

The second focus of my dissertation research was the development of an expression system for human haptocorrin (HC). This process is an important part of the complex vitamin B₁₂ (B_{12}) uptake pathway, specifically as it provides protection for B₁₂ from the mouth through the stomach, and into the intestine where is degraded. At the beginning of this work, there was no recombinant expression system for HC and its study was limited to purification from saliva and serum. I have cloned, expressed, and purified HC in the methylotrophic yeast *P. pastoris*, and
Human Embryonic Kidney Cells, as well as using it to target the asialoglycoprotein receptor for cellular imaging studies.
Tuning Quantum Dot Interfaces for Biofunctionalization with expressed Haptocorrin and Fluorescent Proteins: Applications in Targeted Delivery, Cellular Imaging, Self-Assembly, and Energy Transfer

By

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Department of Chemistry
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Dissertation

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1) First absorption maxima (l_A), and calculated extinction coefficient (e). 2) Calculated using Ppy QY of 10 %, QR(556) QY of 12 %.

Table 4.3: Optical and FRET parameters for BRET - FRET nanosystem using QD(530).
1) First absorption maxima (l_A), and calculated extinction coefficient (e). 2) Calculated using Ppy QY of 10 %, QD(530) QY of 27 %.

Table 4.4: Optical and FRET parameters for BRET - FRET nanosystem using QD(530).
1) First absorption maxima (l_A), and calculated extinction coefficient (e). 2) Calculated using Ppy QY of 32 %, QD(530) QY of 27 %.
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AM</td>
<td>Amnionless</td>
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<tr>
<td>Ar</td>
<td>Argon</td>
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<tr>
<td>B$_{12}$</td>
<td>Vitamin B$_{12}$</td>
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<tr>
<td>BtLH2</td>
<td>Benzothiophene analogue to firefly luciferin</td>
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<tr>
<td>BRET</td>
<td>Bioluminescence resonance energy transfer</td>
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<tr>
<td>BR</td>
<td>BRET ratio</td>
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<tr>
<td>CD320</td>
<td>Transcobalamin II receptor</td>
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<tr>
<td>CdO</td>
<td>Cadmium oxide</td>
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<tr>
<td>CdS</td>
<td>Cadmium sulfide</td>
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<tr>
<td>CdSe</td>
<td>Cadmium selenide</td>
</tr>
<tr>
<td>Cip</td>
<td>Calf intestinal phosphitase</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>CB</td>
<td>Cubilin</td>
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<tr>
<td>CUBAM</td>
<td>Cubilin amnionless</td>
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<tr>
<td>DHLA</td>
<td>Dihydrolipoic acid</td>
</tr>
<tr>
<td>DDA</td>
<td>Dodecylamine</td>
</tr>
<tr>
<td>$Q_D$</td>
<td>Donor quantum yield</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
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<tr>
<td>$E$</td>
<td>Energy transfer efficiency</td>
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<td>LH2</td>
<td>Firefly luciferin</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FRET</td>
<td>Förster (or fluorescence) resonance energy transfer</td>
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<tr>
<td>R&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Förster distance</td>
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<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
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<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
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<tr>
<td>HEPG2</td>
<td>Human hepatocellular liver carcinoma</td>
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<td>hHC</td>
<td>Human haptocorrin</td>
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<td>6xHis</td>
<td>Hexa-histidine tag</td>
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<td>HDA</td>
<td>Hexyldecylamine</td>
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<td>His</td>
<td>Histidine</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>IF</td>
<td>Intrinsic Factor</td>
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<tr>
<td>IMAC</td>
<td>Immobilized metal affinity chromatography</td>
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<tr>
<td>IPTG</td>
<td>β-D-1-thiogalactopyranoside</td>
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<tr>
<td>Luc</td>
<td>Luciferase</td>
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<tr>
<td>MALDI-MS</td>
<td>Matrix assisted laser desorption/ionization mass spectrometry</td>
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<tr>
<td>MRP1</td>
<td>Multidrug resistant protein 1</td>
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<tr>
<td>nIR</td>
<td>Near infrared</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>ODE</td>
<td>Octadecene</td>
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<tr>
<td>OAc</td>
<td>Oleic Acid</td>
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<tr>
<td>OAm</td>
<td>Oleylamine</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline with TWEEN</td>
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<td>Symbol</td>
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<tr>
<td>$k_p$</td>
<td>Polarization parameter</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>Ppy</td>
<td>Photinus pyralis</td>
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<td>PL</td>
<td>Photoluminescence</td>
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<td>Qdots</td>
<td>Quantum dots</td>
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<td>Qrods</td>
<td>Quantum rods</td>
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<td>QY</td>
<td>Quantum yield</td>
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<tr>
<td>$\eta_D$</td>
<td>Refractive index</td>
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<tr>
<td>RLuc</td>
<td>Renilla reniformis</td>
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<tr>
<td>RET</td>
<td>Resonance energy transfer</td>
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<tr>
<td>S</td>
<td>Sulfer</td>
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<tr>
<td>Se</td>
<td>Selenium</td>
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<tr>
<td>$J$</td>
<td>Spectral overlap</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SILAR</td>
<td>Successive ion layer adsorption and reaction</td>
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<tr>
<td>TCII</td>
<td>Transcobalamin II</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>TBP</td>
<td>Tributylphospine</td>
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<tr>
<td>TOP</td>
<td>Trioctyphospine</td>
</tr>
<tr>
<td>TOPO</td>
<td>Trioctylphosphine oxide</td>
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<tr>
<td>UV-vis</td>
<td>Ultraviolet-visible</td>
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<td>ZnS</td>
<td>Zinc sulfide</td>
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<td>------------------------------------------------</td>
</tr>
<tr>
<td>2</td>
<td>Histidine</td>
</tr>
<tr>
<td>3</td>
<td>Dihydrolipoic acid</td>
</tr>
<tr>
<td>4</td>
<td>11-Mercaptoundecyl-tetra(ethylene glycol)</td>
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Chapter 1

Introduction to Cellular Imaging, Energy Transfer, Vitamin B$_{12}$ and the Vitamin B$_{12}$ Uptake Pathway

1.1 Cellular Imaging

Scientists understand biological processes best when they can be directly detected and visualized. Despite advances, this is still a challenge, as a target of interest must first be identified, synthesized, purified, tagged with a method of detection (such as fluorescence), and then visualized to determine cellular localization.$^{1-2}$ Over the past several decades cellular imaging has become a vital tool to elucidate cellular processes as well as protein localization in living cells.$^3$ A prominent method used to achieve this goal is to utilize fluorescence or phosphorescence processes. Compared to other techniques for following molecular influx, such as radiolabeled compounds$^{4-5}$ or gold nanoparticles,$^{6-7}$ fluorescence has the advantage of incorporating a host of desirable traits. These include fast signal acquisition, a low detection limit, multiple target detection, the ability to visualize ligand-analyte interactions, small label size, as well as being able to calculate spatial distances.$^{1-3}$ In the following sections I give a brief overview of the popular probes used for fluorescent imaging, including their general properties, advantages, and disadvantages.

1.1.1 Fluorescent Organic Dyes

The first molecule used for immunofluorescence labeling was fluorescein in 1953, with rhodamine quickly following.$^8$ Figure 1.1 shows the structure of rhodamine (a) and fluorescein (b). A key component of these structures are a large conjugation system, in this case consisting
of benzene and its derivatives, but can also consist of an alkene bridge, such as in the cyanine series of dyes\textsuperscript{9} as shown in Figure 1.1(c).

![Figure 1.1](image)

**Figure 1.1:** The chemical structures of rhodamine (a), fluorescein (b), and the cyanine dye cy3 (c).

While these molecules facilitated early cell studies\textsuperscript{10-11} advances were needed, and the last few decades have seen the development of a diverse and readily available array of fluorescent organic dyes.\textsuperscript{12} These molecules are now easily obtainable through commercial vendors, cover wavelengths throughout the UV-visible and infrared spectra, and are functionalized to be easily conjugated to a purified protein of interest. For example, the Alexa Fluor\textsuperscript{®} series of organic dyes have emission peaks at: 442, 519, 572, 603, 617, 643, and 672 nm and can be purchased functionalized with a thiol, amine, carbonyl, acetylene, or alkyne reactive group, to label a protein/molecule of interest.\textsuperscript{13} Common properties of these molecules are a small size (~1 nm per molecule), high extinction coefficients ($10^4 - 10^5$ M$^{-1}$cm$^{-1}$), high quantum yields (0.5 to 1.0) and fluorescence lifetimes ranging from 1-5 ns.\textsuperscript{14}

While these dyes are user friendly and easily available, they still require a purified substrate in order to be effective. To this end, researches have developed methods for specifically attaching a dye to a protein being expressed in live cells,\textsuperscript{15-21} avoiding the necessity to purify a large amount of substrate to fluorescently tag and reintroduce back to the cellular environment. To this end, a few different systems have been developed into order to specifically label proteins.
One class of these tags relies on the recombinant fusion of an enzyme to later tag the protein of interest. For example, Wood et al. reported a protein labeling method called a HaloTag,\textsuperscript{15} which uses a modified haloalkane dehalogenase to covalently bond halogenated molecules to a protein of interest. This has facilitated fluorophores such as carboxytetramethylrhodamine (TMR) and carboxyfluorescein (FAM) that have been successfully conjugated to the nuclear factor (NF)-κB under physiological conditions.\textsuperscript{15} In addition, this methodology can also be used to link the protein of interest to a solid support. Similar results have been achieved using a variety of different enzyme mediated processes. For example, the SNAP-tag and CLIP-tag systems utilize O\textsubscript{6}-alkylguanine-DNA alkyltransferase to specifically add dyes attached to O\textsubscript{6}-benzylguanine and O\textsubscript{2}-benzylcytosine derivatives respectively.\textsuperscript{16-17} While these systems work for fluorescent tagging, they also induce steric hindrance, which disrupts protein function and cellular trafficking, as has been shown with green fluorescent protein constructs of similar molecular weight.\textsuperscript{18-19}

Other systems utilize a small peptide for attachment, specifically FlAsH\textsuperscript{20} and PRIME.\textsuperscript{21} The PRIME system is interesting in itself, as it requires a fluorophore ligase genetically engineered from lipoic acid ligase that provides specific addition of either an azide or coumarin with the addition of a 13 amino acid peptide recognition sequence.

**1.1.2 Fluorescent Proteins**

One way to overcome the challenge of site specific labeling is to use fluorescent proteins. Fluorescent proteins are another class of fluorophores that are prevalent in cellular imaging. Even though its discovery and purification\textsuperscript{22-23} date back to 1962, green fluorescent protein (GFP) wasn’t utilized for cellular imaging until 1994 when it highlighted sensory neurons in
nematodes. Since then the color pallet using the *Aequorea* architecture has expanded to include blue, cyan, and yellow, along with other variants. Generation of desirable variants in the red weren’t developed until the discovery of a natural analog from the nonbioluminescent coral reef, and recently the first naturally occurring vertebrate florescent protein was discovered in the Japanese eel. Due to the interest in the field, there are now clones available that cover the majority of the visible spectra (424 nm-649 nm) with new analogs even reaching the infrared region. The common physical properties of these protein are a size of ~27-28 kDa with a hydrodynamic diameter of ~5 nm, a extinction coefficient in the range of $10^4 - 10^5 \, \text{M}^{-1}\text{cm}^{-1}$, lifetimes of 1-5 ns, and quantum yields that range from 0.1 to 0.8.

The common structural component of fluorescent proteins is shown in Figure 1.2, and consists of a beta-barrel structure that facilitates a cyclo-addition of three amino acids that construct the functional fluorophore. While other amino acids in the protein structure can play a role in the emission spectrum, such as $\pi$ stacking in yellow fluorescent protein, the main determining factor is the amino acid trimer. For instance, the main difference between the blue, cyan, and green analogs is the first amino acid being a histidine, tryptophan, or tyrosine respectively. By changing the aromatic amino acids in the trimer (increasing or decreasing the conjugation in the system) the fluorescent spectra will be shifted, with larger conjugation systems leading to less energetic (more red shifted) emission. This is exemplified by red fluorescent protein, which is the result of a larger conjugated system from the addition of an acylimine group to the standard $p$-hydroxybenzylideneimidazolinone green fluorescent protein chromophore.
Figure 1.2: The crystal structure of green fluorescent protein (PDB ID: 1KP5) (A) and the chromophores for blue fluorescent protein (B), cyan fluorescent protein (C), emerald green fluorescent protein (D), and red fluorescent protein (E).

The main advantage of this system is that the fluorescent protein can be fused directly to a protein of interest during expression to easily visualize organelles when expressed with a protein or peptide that is known to localize in a particular compartment of a cell, help elucidate the localization of a protein of interest, or even help to discern the complicated nervous system of the brain. These systems can also be readily expressed together to make Förster Resonance Energy Transfer (FRET) sensors that can be used to follow intercellular traffic of ions/molecules such as copper or 3',5' cyclic monophosphate (cAMP). Conformational changes of a protein of interest may also be elucidated by monitoring the change in the FRET signal.

1.1.3 Fluorescent Metal Complexes

In addition to conjugated systems such as organic dyes and fluorescent proteins that take advantage of conjugated \( \pi \) systems, metals that have \( d^6 \) or \( d^8 \) electron configurations or
lanthanides with $f^6 f^8$, where there is valence electron mobility, can also achieve fluorescence. Another class of compounds used for cellular imaging are chelated metal ion complexes. Using the lanthanide series offers some advantages over traditional dyes in that they have narrow emissions bands, ~20 nm, a large stokes shift, and have exceptionally long florescence lifetimes\(^{44}\) (in the millisecond range) which allow for time-resolved microscopy to differentiate between the complex of interest and cellular background fluorescence.\(^{45-46}\) While dyes and fluorescent proteins can be directly excited, lanthanide ions have a negligible extinction coefficient due to f-f transitions,\(^{47}\) as apposed to the transition metals, where d-d transitions absorb in the visible spectrum. This issue has been resolved by building a molecular antenna that absorbs in the ultraviolet-visible region into the chelator ligand’s architecture\(^{48-49}\) as shown in figure 1.3(a). This system works by first exciting an electron to the excited state followed by intersystem crossing to reach a long lived ligand centered triplet state from which the triplet to lanthanide energy transfer occurs.\(^{50}\) The electron will then relax to the lanthanide emitting state, and then relax to the ground state releasing a photon.\(^{47}\)

![Figure 1.3](image)

**Figure 1.3:** An example of lanthanide (europium) macrocycle (a) with an attached UV-Vis antenna and a rhenium(1) thiazole complex (b).
The emission of the lanthanide complex is controlled by the metal center chosen, as well as the macrocycle used for chelation. For example, terbium(III) and europium(III) fluoresce with a maximum at ~550 nm and ~615 nm respectively, and using other metal centers allow for the fluorescence signal to be pushed into the infrared such as neodymium at ~1180 nm and ytterbium at ~1550 nm using 340 nm excitation. In addition to stabilizing the metal center, the ligand structure also dictates the cellular destination of the complex. It has been demonstrated that the macrocycle structure can also direct the destination of the complex to an area of interest such as the mitochondria, ribosomes, lysosomes and endosomes.

In addition to the lanthanides, heavy metals have also been used for the construction of phosphorescent probes using rhenium, iridium, ruthenium, rhodium, and platinum. These probes differ from the lanthanides in that they can be directly excited, without the need for a molecular antenna. For example, the rhenium(I) thiazole complex shown in Figure 1.3(b) has been utilized as an imaging probe for the detection of the folate and cubilin receptors in mammalian cell lines.

1.1.4 Quantum Dots

Another class of inorganic fluorophores are semiconductive quantum dots (qdots). Qdots are nanometer sized semiconductive material with size dependent optical properties. Figure 1.4 shows an illustration of the size dependent nature of qdots. Due to quantum confinement effects, smaller qdots have a larger Fermi band gap than their larger counterparts, and therefore a higher energy emission. Increasing the size of qdots results in a red shift of the emission spectra until the size of the semiconductive material exceeds the Bohr radius, the material loses its quantized properties, and acquires the properties of the bulk material. By changing the semiconductive core
and particle size (diameter of 1-10 nm), qdot fluorescence can be tuned to have spectral coverage from 400-1800 nm, making them a very useful imaging material.\textsuperscript{14}

**Figure 1.4:** An illustration of the quantum spectral properties of qdots, specifically CdSe. Smaller particles have a larger band Fermi band gap, corresponding higher energy emission. As particles approach the Bohr exciton radius the energy of the photon decreases (red shifts), unit quantized bands are lost, and the particles display bulk properties.

In addition to the large emission coverage, Figure 1.5 shows the symmetrical emission bands that generally have a full width half max (FWHM) between 25-35 nm in the visible spectra, and up to 90 nm in the infrared. The absorption is broad, with high molar absorption coefficients ($10^{5}-10^{6}$ M$^{-1}$cm$^{-1}$) at the first absorption peak, which make qdots excellent candidates for multiple color imaging, as a single laser can excite multiple qdots emitting at a variety of wavelengths. Qdots also posses long fluorescent lifetimes, (10-100 ns) making them useful for time resolved spectroscopy since the qdots signal can be distinguished from biological background fluorescence and organic dyes.\textsuperscript{14}
Figure 1.5: An illustration of a CdSe and CdSe/ZnS qdots and an example of an absorbance (dashed line) and fluorescence (solid line) spectra.

Many different strategies have been employed to harness qdot emission properties for cellular imaging. One challenge however is that most qdots first need to be transferred into aqueous media by either ligand exchange or polymer wrapping methods (*vide infra*), due to synthesis in non-polar media.\(^{66-69}\) After the qdots have been effectively phase transferred into aqueous buffers, researchers have attached a variety of biological molecules to make them effective cellular probes. Some of the first cellular uptake studies relied on simple electrostatic interactions between a negatively charged qdot that facilitated transported into the cell by endocytosis\(^ {70}\) or the attachment of antibodies for immunolabeling.\(^ {71}\) Since the initial reports, a variety of methods for cellular uptake had been developed to transport qdots across the cellular membrane. One class involves functionalizing qdots with cell penetrating peptides and proteins to allow for biological recognition. Some examples of peptide moieties used for cellular import are the TAT peptide (arginine and/or lysine rich) from human immunodeficiency virus,\(^ {72-76}\) a
RGD peptide motif that allows for labeling of cellular integrins, as well as insect neuropeptide (APSGAQRLYGFGL) that directs qdot transport into the nucleus. Many proteins have also been used to drive qdot cellular transport, such as epidermal growth factor (EGF), transferrin, and antibodies.

1.2 Resonance Energy Transfer

In addition to using fluorescent molecules for imaging, they also can be paired together to make intuitive FRET systems that can be used to elucidate inter-molecule distances between acceptor and donor molecules. FRET is a nonradiative process where energy is transferred from a donor molecule through dipole-dipole interaction to a lower energy acceptor occurring in the distance range of 10 to 100 Å. These systems consist of a higher energy donor and a lower energy acceptor where the emission profile of the donor overlaps with the absorbance profile of the acceptor. Figure 1.6 shows the absorption and emission spectra of an example qdot-tagRFP FRET system (a), along with an idealized Jablonski diagram (b). FRET efficiency can be determined experimentally by the measured decrease in donor fluorescence, but to elucidate the specific distance between the two probes, it is necessary to calculate the overlap integral ($J$) using the equation: $J = \int f_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda$ where $f_D$ is the donor fluorescence intensity, $\varepsilon_A$ is the acceptor extinction coefficient, and $\lambda$ is the wavelength. In the example given in Figure 1.6, a large $J$ value would be expected, as the emission spectra of the qdot has a $\lambda_{max}$ at 556 nm, which matches well with the absorbance spectra of tagRFP ($\lambda_{max}$ 555), as well as a large $\varepsilon_A$ of $10^5$ M$^{-1}$cm$^{-1}$. After calculating $J$, it can be used to calculate the Förster Distance ($R_0$) using the equation: $R_0 = 8.8 \times 10^{23} k_p^2 \eta_D^{-4} Q_D J$, where $k_p$ is the dipole orientation, $\eta_D$ is the refractive index of the media, $Q_D$ is the donor quantum yield, and $J$ is the overlap integral. By calculating
the $R_0$ value it is possible to calculate the distance between the donor and acceptor based on an experimentally derived FRET efficiency value ($E$), and using the FRET efficiency equation:

$$E = 1 - \frac{F_{DA}}{F_D} = 1 - \frac{\tau_{DA}}{\tau_D} = \frac{R_0^6}{R_0^6 + r^6} = \frac{nR_0^6}{nR_0^6 + r^6},$$

where $F_{DA}$ is the fluorescence of the donor in the presence of the acceptor, $\tau$ is lifetime, $R_0$ is the Förster distance, and $r$ is the distance between the donor and acceptor.\(^{65}\)

**Figure 1.6:** An example of a FRET pair composed of a qdot-tagRFP pair. The Qdot has an emission maxima of ~556 nm, and tagRFP which has an absorption maximum of ~555 nm and an emission maxima of ~584 nm (a). A representative schematic of the band gap alignment of the qdot 556 and tagRFP\(^{90}\) (PDB ID: 3M24) band gaps with 400 nm excitation (b).

There have been many different sets of FRET pairs that have been utilized for a variety of processes. The staple of FRET analysis has been two organic dyes which have been used to study a variety of biochemical processes such as cAMP production,\(^{91}\) phosphodiesterase activity,\(^{92}\) β-lactamase,\(^{93}\) and integrin binding.\(^{94-95}\) FRET systems have also been developed between fluorescent proteins\(^{40-43}\) and qdot-fluorescent proteins.\(^{96-98}\)
More recently, another energy transfer system, bioluminescence resonance energy transfer (BRET), has begun to explore using firefly protein (RLuc) as an emission donor. The advantage of this system is that it doesn’t require an external energy source, such as laser excitation, due to the protein’s bioluminescent properties. To date, there have systems reported using RLuc with fluorescent proteins, near IR dyes, and qdot/qrod systems.

1.3 Vitamin B_{12} and its transport proteins

The second focus of my research was the expression and characterization of the vitamin B_{12} transport protein haptocorrin. In the following section I will describe vitamin B_{12}, its importance as a coenzyme, the role of haptocorrin, and the complicated three-protein transport system that has evolved to maintain vitamin B_{12} homeostasis.

1.3.1 Vitamin B_{12} History, Structure, and Function

Vitamin B_{12} (B_{12}), also know as cobalamin (Cbl), is a complex molecule that has a rich history. Termed “Nature’s most beautiful cofactor”, B_{12} is a water-soluble vitamin that has been involved in many milestones in chemistry. Its story begins in the early 20\textsuperscript{th} century when physicians noticed that pernicious anemia was caused by a decrease in red blood cells, that it appeared to be a caused by a nutritional deficiency, and could be treated by an increase in liver consumption. In 1948 a red compound was successful isolated and crystallized from liver extract and was given the name vitamin B_{12}. The crystal structure of B_{12} as cyanocobalamin was later solved by Dorothy Hodgkin in 1956 by X-ray crystallography, and was eventually made via organic synthesis methodology in 1972.

The molecule’s structure in itself is intriguing as it is naturally occurring, stable, organometallic compound with a complex architecture. Figure 1.7 shows the structure of B_{12} consisting of three major regions, the first is a cobalt(III) metallocenter stabilized by 4 nitrogen
atoms in a corrin ring that forms an equatorial plane. The second region is a 5,6-dimethylbenzimidazole moiety that coordinates to the β-axial position of the metallocenter, which is connected by the third region, a nucleotide linker that extends down from the D-ring of the corrin. Another important feature of B₁₂ is the variable X group on the α-axial position, which can exist as cyanocobalamin, methylcobalamin, adenosylcobalamin and aquocobalamin, allowing for a variety of reactivity from this molecule.

Figure 1.7: The structure of vitamin B₁₂, along with its substituent X groups. B₁₂ exists as cyanocobalamin and aquacobalamin, as well as the biologically active methylcobalamin and adenosylcobalamin.

While the X group does vary, only methylcobalamin and adenosylcobalamin are believed to be active biological cofactors, with other moieties being converted to methyl- and
adenosylcobalamin by the trafficking chaperone MMACHC inside cells. 111-113 B12 plays major roles in several pathways, including red blood cell formation and nervous system development and maintenance, 114 but is absolutely necessary as a cofactor for two processes. The first process uses adenosylcobalamin as a cofactor with mitochondrial L-methylmalonyl-CoA mutase to covert methylmalonyl-CoA to succinyl-CoA. 115-116 The second process uses methylcobalamin as a cofactor for methionine synthase as it catalyses the methylation of homocystein in its conversion to methionine. 115-116

1.3.2 B12 Transport Proteins

B12 is only synthesized by bacteria, 117 so mammals must obtain this molecule through the consumption of meat, cheese, and dairy products in their diet. 118 The utilization of dietary B12 is a complex pathway tightly controlled by three immunologically distinct proteins: haptocorrin (HC), intrinsic factor (IF), and transcobalamin II (TCII). Figure 1.8 shows a schematic of the B12 uptake pathway. The first transport protein that B12 encounters in the mouth is HC, a 47 kDa glycoprotein secreted by the salivary glands. HC initially binds B12 and protects the molecule as it is transported through the stomach to the duodenum. In the duodenum B12 is released by HC due to an increase in pH and proteolytic degradation of the protein. 119 B12 is then bound by IF and travels down the intestine until it is endocytosed by the cubilin amnionless (CUBAM) receptor for transport across the ileal epithelium. B12 then dissociates from IF in a lysosome, where it is picked up by TCII and secreted into the blood serum or transported across the cell membrane through multi drug resistant protein 1 (MRP1), and then binds to receptor CD320 (TCII receptor) to deliver Cbl to deficient cells. 120-121
Figure 1.8: A scheme of the B$_{12}$ internalization pathway. Abbreviation: (HC) haptocorrin, (IF) intrinsic factor, (TCII) transcobalamin II, (CB) cubilin receptor, (AM) amnionless, (MRP1) multidrug resistant protein 1, (CD320) TCII receptor.

It has been shown that B$_{12}$ can be modified by the conjugation of proteins,$^7,^{122-124}$ peptides,$^{125-126}$ and fluorescent compounds$^{62-64}$ through the 5’ ribose hydroxyl group and maintain transport protein binding. To the best of our knowledge, there has been no attempt to directly use the transport proteins as a method of target delivery, such as a qdot for cellular imaging, or a moiety to elicit an immune response, such as rotavirus. HC is an attractive platform for such a system as it is can be endocytosed through the asialoglycoprotein receptor in the liver$^{127-128}$ to monitor cellular imaging when attached to a qdot probe, and is readily degraded in the small intestine so as not to allow viral particles into the blood stream, while providing protection to gastric degradation.

TCII and IF have previously been expressed in Picha pastoris from 1-10 mg/L,$^{129-130}$ which has facilitated the elucidation of the crystal structure for each protein,$^{131-132}$ as well as the
IF-Cubilin complex. At the beginning of my work, there was no recombinant expression system for HC, and its study is limited by the necessity to purify it from saliva and rabbit or human serum, limiting its use in fundamental research.

1.4 Summary Overview

My thesis is split into two projects. In the first project, the overall goal is to develop new chemistry to facilitate the functionalization of qdots for use in assembly, energy transfer, and imaging. The first obstacle that needed to be overcome was changing the qdot surface chemistry to render it soluble in aqueous buffers. I developed a method using the amino acid histidine (Chapter 2), which I show can modify the qdot surface, allowing for phase transfer and direct exchange with biomaterials. I will also describe the functionalization and characterization of a polymer wrapped qdot with a nitrilotriacetic acid (NTA) surface chemistry (Chapter 3).

I then tested the qdots surface reactivity towards biomolecules using FRET. For FRET tagRFP was cloned, recombinatly expressed in E. coli, purified via immobilized metal affinity chromatography (IMAC), and assembled onto the qdot surface (Chapter 4).

The second focus of my research was the expression of recombinant human HC (Chapter 5). HC is an important part of B12 transport system, has been hypothesized to have antimicrobial activity, as well as being a potential platform for the delivery of therapeutics. The purpose for this recombinant expression system was to allow for further insights into HC transport, and for its use as a biological delivery platform.

In order to express recombinant HC, a codon optimized gene was designed with a C-terminal hexa-histidine tag for purification and later qdot conjugation. I cloned the HC gene into the pPIC9 vector, transformed it into P. pastoris, and conducted expression and purification trials...
both on the bench-top and using a bioreactor. In addition to working with yeast, I also expressed HC using a mammalian cell system, Human Embryonic Kidney (HEK) cells, and used that HC for cellular internalization studies targeting the asialoglycoprotein receptor in liver carcinoma (HEPG2) cells using a fluorescent tag.

1.5 References


5. Lensbouer, J. J.; Li, Q. W.; Estlinbaum, M.; Doyle, R. P., R161, K452 and R460 residues are vital for metal-citrate complex transport in Cit(Sc) from Streptomyces coelicolor. Metallomics 2010 2 (5), 342-347.


Chapter 2

A Modular Phase Transfer and Ligand Exchange Protocol for Quantum Dots

In this chapter I describe a quantum dot phase transfer protocol that I developed that uses the amino acid histidine. Histidine was chosen in order to first displace the organic encapsulation ligands, and secondly to provide a weakly chemisorbing intermediate at the qdot ionic interface. This work is reproduced with permission from Zylstra, J.; Amey, J.; Miska, N. J.; Pang, L.; Hine, C. R.; Langer, J.; Doyle, R. P.; Maye, M. M. A Modular Phase Transfer and Ligand Exchange Protocol for Quantum Dots. Langmuir 2011, 27, 4371-4379. Copyright 2011 American Chemical Society. This work is also reproduced in part with permission from Zylstra, J.; Alam, A.; Han, H.; Doyle, R. P, Maye. M. M. Tailoring Quantum Dot Interfaces for Improved Biofunctionality and Energy Transfer. In Functional Nanoparticles for Bioanalysis, Nanomedicine, and Bioelectronic Devices Volume 1; ACS Symposium Series 1112 American Chemical Society, Washington D.C., 2012; pp 59-79.  

2.1 Introduction to Quantum Dot Synthesis and Direct Ligand Exchange Phase Transfer

The synthetic methodology for colloidal nanoparticles, such as for semiconductive quantum dots (qdots), is an interesting combination of organic and inorganic wet-chemical reactions, with solid-state high temperature annealing, nucleation and growth, and epitaxial deposition.1-6 Much work has been accomplished since the seminal reports,1-2, 7 and the knowledge base for qdot synthesis and the remarkable final photophysical properties has grown considerably.3-7 The fabricated qdots are crystalline, monodisperse, and highly hydrophobic due
to dense shells of self-assembled monolayers.\textsuperscript{7} Such encapsulation is a key focus for promoting utility of the qdots. The surface chemistry of the organic encapsulating shells is thus a prominent area of colloidal research.\textsuperscript{6-16} In particular, the design and implementation of chemical functionalization at qdot interfaces is particularly useful for studies and applications under aqueous conditions, such as the buffers and ionic strengths required for studies related to bioimaging and biosensing.\textsuperscript{9-12, 16-24} These functionalization protocols often occur in multiple steps, and typically require rendering the organic shell hydrophilic.\textsuperscript{9-20}

Along these lines research effort has focused on the development of new functionalization protocols that endow the qdots with a tailorable hydrophilic nature, colloidal stability, and ease of functionalization.\textsuperscript{8-16} The functionality change must protect the inorganic core from degradation, but also provide solubility in modest ionic strengths, as well as act as either an anchoring group for additional functional groups, or be easily exchanged with ligands or biomaterials.\textsuperscript{16-27} Moreover the perfect surface chemistry should provide a soft-binding to the qdot interface, or organic encapsulation, and not alter surface electronics, such as the introduction of electron or hole traps.\textsuperscript{28-31} Such surface modification is especially crucial for studies related to biodiagnostics, and biological imaging. For instance, a number of studies have utilized the broad absorption, tunable color, high brightness and photostability of qdots for both in-vitro and in-vivo monitoring of biomaterials or biosystems,\textsuperscript{16-71} including carcinoma cells\textsuperscript{32} and organ targeting.\textsuperscript{36-38} In these studies, it has been shown that the biodistribution of qdots is highly related to qdot stability at high ionic strength, culture media, and serum composition. Another critical parameter that determines qdot transport or uptake is the individual qdot hydrodynamic diameter ($D_h$). Researchers tailor these properties towards specific functions by designing and implementing new phase transfer and functionalization protocols.
Our design falls into the phase transfer and functionalization class utilizes alternative protocols that employ monolayer exchange between the initial hydrophobic encapsulation of the qdots (e.g. TOPO) with small hydrophilic self-assembled monolayers (SAMs). This approach allows for smaller $D_h$, but often decreased QY values due to direct binding to the qdot interface. These protocols include the use of short-chain mercapto acids, thiolated dendrimers, as well as other functional moieties that utilize thiol for attachment. Multidentate ligands, such as the dithiol dihydrolipoic acid (DHLA), have also been used extensively for both phase exchange, as well as an anchor for further modification. For instance, a poly(ethylene) glycol modified DHLA has proven especially useful, with much improved long term stability and biocompatibility over an extended pH range. Additionally, a DHLA based zwitterionic ligand has been developed which maintains the stability associated with PEG derivatized ligands, while also maintaining small hydrodynamic diameters. Recently it has been shown that utilizing tetradentate binding of PEG-based ligands, qdots are able to withstand extreme solvent conditions by maintaining solubility and quantum yield over a wide pH range under high salt conditions. A number of small molecules have also proven to be effective for qdot phase transfer. The amino acid cysteine was recently shown to exhibit characteristics of small $D_h$, stability to a range of pH, and cellular imaging capabilities due to its zwitterionic surface. In addition, a phosphonic acetic acid ligand was also recently employed for phase transfer. Recent advances in this field have been summarized in table 2.1.

<table>
<thead>
<tr>
<th>Ligand Exchange and Functionalization</th>
<th>Preparation Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single Thiol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mercaptoacetic Acid</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Mercaptopropinoic Acid</td>
<td>24, 73-76</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1: Phase transfer strategies employing self-assembled monolayers (SAMs)

In this chapter, I describe a modular approach that utilizes a small molecule to first induce phase transfer and second to act as an intermediate encapsulation that facilitates further functionalization while in aqueous media. For this, we employed the amino acid L-histidine (His, 2) to readily ligand exchange the dense hydrophobic shells at CdSe/ZnS qdot interfaces. The novelty of this approach lies in the interesting ability of 2 to displace the initial triocylphosphineoxide (TOPO) and oleylamine (OAm) organic encapsulation, leading to phase transfer within minutes, while at the same time providing a weakly chemisorbing encapsulation that can undergo additional ligand exchange. The resulting qdots show QYs values comparable
to other monolayer-based phase transfer strategies, as well as $D_h < 12$ nm. The 2-modified qdots are shown to undergo further homogeneous ligand exchange with a number of monolayers, allowing for fine control of surface coverage and charge.

2.2 Materials and Methods

2.2.1 Materials Cadmium oxide (CdO, 99.99%), sulfur (S, 100 mesh), zinc acetate (ZnAc, 99.99%), trioctylphosphine oxide (TOPO, 90%), 1-octadecene (ODE, 90%), olelyamine (OAm, 90%), oleic acid (OAc, 90%), tributylphosphine (TBP, 97%), L-histidine (His, 2, >99%) α-lipoic acid (>99%), 11-Mercaptoundecyl-tetra(ethylene glycol) (PEG, 4, 95%), chloroform (CHCl₃, >99.8%), methanol (MeOH >99.8%), acetone (99.5%), sodium borohydrate (NaBH₄ >96%), sodium tetraborate (99.998%), Boric Acid (≥ 99.5%), chloroform-d (CDCl₃, 99.8 atom % D), and were obtained from Sigma-Aldrich and used without further purification. Agarose were obtained from Acros organics. Deuterium oxide (D₂O, 99.9 atom% D) was obtained from Cambridge Isotope Laboratories, Inc. A Vacuum Atmospheres Omni glovebox provided an inert atmosphere for synthesis. Ultrapure water (18.2 MΩ) was provided from a Sartorius Stedim Arium 61316 reverse osmosis unit combined with a Arium 611DI polishing unit.

2.2.2 Instrumentation

UV-visible Absorption (UV-vis): The UV-vis measurements were collected on a Varian Cary100 Bio UV-vis spectrophotometer between 200-900nm. The instrument is equipped with an 8-cell automated holder with high precision Peltier heating controller.
**Photoluminescence (PL):** The PL emission and excitation measurements were collected on a Fluoromax-4 photon counting spectrofluorometer (Horiba Jobin Yvon). The instrument is equipped with a 150W xenon white light excitation source and computer controlled monochromator. The detector is a R928P high sensitivity photon counting detector that is calibrated to emission wavelength. All PL emission and excitation spectra were collected using both wavelength correction of source intensity and detector sensitivity. The excitation wavelength is 400 nm using 3nm excitation and emission slits unless otherwise noted, and excitation spectra were collected at the qdot emission peak using 1nm excitation and emission slits. The instrument is equipped with a computer-controlled temperature controller provided by a Thermo NESLAB temperature recirculator (Thermo Scientific).

**Dynamic Light Scattering (DLS) and Zeta-Potential (ζ-Pot):** DLS measurements were performed on a Malvern Zetasizer Nano Series instrument utilizing 173° backscatter. Size was calculated using CONTIN analysis. Samples were filtered through a 0.2 μM filter prior to analysis and were averaged over 6 data sets. ζ-Pot measurements were performed on the same instrument using a Malvern folded U-shaped capillary cuvette.

**Transmission Electron Microscopy (TEM):** TEM measurements were performed on either a FEI T12 Twin TEM operated at 120 kV with a LaB6 filament and Gatan Orius dual-scan CCD camera (Cornell Center for Materials Research), or a JEOL 2000EX instrument operated at 120 kV with a tungsten filament (SUNY-ESF, N.C. Brown Center for Ultrastructure Studies). Particle size was analyzed manually by modeling each qdot as a sphere, with statistical analysis performed using ImageJ software on populations of at least 100 counts.
\textit{Nuclear Magnetic Resonance (NMR)}: Qdot $^1$H NMR spectra were acquired on a Bruker DRX-500 equipped with a qx probe. Monomer ligand $^1$H NMR were acquired on a Bruker DPX-300 equipped with a qnp probe. Spectra were generated and analyzed using TopSpin NMR software. 1-qdots were prepared for NMR by multiple acetone precipitations to remove free ligand from solution. After precipitation, 1-qdots were redispersed in CHCl$_3$ and rotary evaporated to a dry film. The dry film was then redispersed in CDCl$_3$. 2-qdots were phase transferred as described above and then underwent 6 acetone precipitations to remove free 2. After the final precipitation the 2-qdots were redispersed in H$_2$O and rotary evaporated to form a thin film. The film was then dissolved in D$_2$O. After NMR the 2-qdots were mixed with 3 (DHLA) and incubated at 50° C for 90 minutes on a shaker. After incubation the 3-qdots were cleaned by four acetone precipitations, redispersed in H$_2$O, rotary evaporated to a film, and the redispersed in D$_2$O. Typical qdot concentrations during NMR analysis were in the 70-140 mM range.

\textit{Fourier Transform Infrared Spectroscopy (FTIR)}: FTIR spectra were acquired on a Nicolet 860 Spectrophotometer equipped with a liquid N2 cooled MCT detector. Qdot samples were first cleaned with acetone precipitation, were dried to a film under air, and were then formed into KBr pellets using a hydraulic pellet press. After pellet formation samples were placed under vacuum for 48hrs. Spectra were averaged over 128 scans and baseline corrected.
**Agarose Gel Electrophoresis:** Gel Electrophoresis experiments were performed using a conventional gel electrophoresis box with a VWR voltage regulator. Qdot samples were loaded to 1% agarose gels in 1X TBE running buffer, with applied voltages typically of 50-70V.

**2.2.3 Calculations**

**Qdot Concentration:** The qdot concentration were calculated based on UV-vis optical absorption measurements of the qdot first band edge absorption (1s-1s) intensity using qdot size dependent optical extinction coefficients ($\varepsilon_{\text{qdot}}$). Qdot size was correlated to absorption wavelength using the calibration methods described by Peng\textsuperscript{98-99} and Mulvaney,\textsuperscript{100} which was then used to estimated $\varepsilon_{\text{qdot}}$. For instance, a CdSe qdot with band edge absorption of 555 nm corresponds to a core diameter $\approx$3.17 nm, which in turn determines the $\varepsilon_{\text{qdot}}$=1.86x10$^5$ cm$^{-1}$M$^{-1}$. The final qdot concentration was then obtained using the Beer-Lambert relationship $Abs = \varepsilon bc$; where $\varepsilon$ is the estimated extinction coefficient (M$^{-1}$cm$^{-1}$), $b$ is the path length, and $c$ is concentration.

**Qdot Quantum Yield:** The qdot photoluminescence quantum yields ($QY$) were calculated based on comparison to a reference dye using standard methods,\textsuperscript{101} using equation 3:

$$QY_{\text{qdot}}(\%) = QY_R \left( \frac{Abs_R}{Abs_{\text{qdot}}} \right) \left( \frac{PL_{\text{qdot}}}{PL_R} \right) \left( \frac{\eta^2_{\text{qdot}}}{\eta^2_R} \right)$$

(3)

where $QY_R$ is the reference dye quantum yield (Rhodamine=31%, Rhodamine 6G=95%), $Abs_R$ and $Abs_{\text{qdot}}$ are the optical absorption at specific excitation for the reference dye and qdot samples respectively. Here, careful attention was paid to prepare samples with optical absorption below 0.05-0.10 in order to limit inner filter effects,\textsuperscript{101} and $PL_R$ and $PL_{\text{qdot}}$ correspond to the total area of the PL emission after wavelength dependent calibration of both the excitation source, and photoluminescence detector, as well as after PL spectra baseline.
correction. The emission is fit to a Gaussian profile. For samples exhibiting a trap-state emission (lower energy), only the band-edge emission PL area is used in QY calculations. The refractive index of the reference and qdot solvent, \( n_R \) and \( n_{\text{qdot}} \), where also taken into account when required.

2.2.4 Quantum Dot Synthesis

The CdSe/ZnS qdots were synthesized in house following traditional methods with slight modification\(^{102-104}\) to ligand concentrations. Scheme 2.1 shows a typical synthesis, CdO (0.025 g) was dissolved in oleic acid (OAc) (3.0 mL) and heated to 230°C under Ar purging until the solution original maroon color suspension became optically clear and transparent. Then a molten mixture of TOPO (0.5 g) and oleylamine (OAm) (range from 0.0-5.0 mL) in ODE (5 mL) was added to the cooled Cd precursor solution under Ar. The solution was stirred and heated to the nominal injection temperature of 280°C upon which the Se precursor (0.118 g Se dissolved in 1.0 mL TBP) was injected. The TBP-Se precursor was prepared under inert atmosphere in a glovebox. After injection, we observed immediate color change from colorless to bright orange, indicating CdSe growth. The reaction flask was removed from the heating mantle and allowed to cool to room temperature under purging and stirring. A small amount (~3 mL) of hexane was added to prevent solidification around 60°C.
Scheme 2.1: A scheme showing a typical qdot synthesis. The cadmium precursor is first prepared by dissolving CdO in OAc at 230 °C (a). OAm and TOPO are then added to the solution and the temperature is increased to 280 °C (b). A selenium precursor is prepared by dissolving Se in TBP under argon. At 280 °C, the Se precursor is then injected, leading to qdot nucleation and growth.

The resulting TOPO/OAm capped qdots (1-qdots) were first purified free of excess ligands first by multiple methanol extractions until the methanol layer appeared clear, indicating the lack of excess ligand. After the excess ligand was removed, the 1-qdots were precipitated from the solution by addition of dry acetone and centrifuged. The resulting TOPO/OAm rich supernatant was decanted, and this procedure was repeated at least three times. The 1-qdots were then redispersed and stored in dried chloroform before undergoing shell addition.

After cleaning, the size and concentration of the CdSe core were calculated based on UV-vis optical absorption measurements of the qdot first band edge absorption (1s-1s) intensity using qdot size dependent optical extinction coefficients ($\varepsilon_{qdot}$). Qdot size was correlated to absorption wavelength using the calibration methods described by Peng$^{98-99}$ and Mulvaney,$^{100}$ which was then used to estimated $\varepsilon_{qdot}$. For instance, a CdSe qdot with band edge absorption of 555 nm corresponds to a core diameter $\approx$3.17 nm, which in turn determines the $\varepsilon_{qdot}$=1.86x10$^5$ cm$^{-1}$M$^{-1}$.

The final qdot concentration was then obtained using the Beer-Lambert relationship $A = \varepsilon bc$;
where $\varepsilon$ is the estimated extinction coefficient ($\text{M}^{-1}\text{cm}^{-1}$), $b$ is the path length, $c$ is concentration, and $A$ is absorbance.

The CHCl$_3$ was then removed from the core via rotary evaporation and the qdots were redispersed in ODE and heated to 200 °C under Ar in a 4 neck round bottom flask. Additional OAm (one to five mL) was also added to the mixture to limit core growth. The sulfur precursor (200 mM sulfur dissolved in ODE) and the zinc precursor (200 mM zinc acetate dissolved in OAm) were injected sequentially, allowing a minimum of 10 minutes between injections to allow for shell annealing. The final zinc injection was in excess to injected sulfur to insure a zinc monolayer on the outermost surface of the qdot shell. After the final injection the qdots were annealed for an additional thirty minutes to insure shell quality. Upon the completion of shell addition the reaction mixture was allowed to cool to room temperature and cleaned in the same manner as the core. The 1-qdots were then redispersed and stored in dried chloroform before undergoing ligand exchange, as described in the next section.

### 2.2.5 Quantum Dot Phase Transfer and Functionalization

The 1-qdots were initially made hydrophilic by a direct ligand exchange using a ratio, $r$ ($r = [\text{Ligand}] / [\text{qdot}]$) of 3,000-fold excess of L-histidine (His, 2) dissolved in basic MeOH ([2] = 100 mM). The 2-qdots were phase transferred via a homogeneous step, which avoided precipitation. In this process a 100 mM 2 solution was dissolved in a 3:1 basic MeOH: H$_2$O solution. The 3,000-fold excess of 2 was added to 1-qdots in chloroform and vortexed for two minutes. After vortexing, the 2-qdots collected in the aqueous phase and additional 10mM borate buffer (pH 8.3) was added to bring the total volume of the aqueous layer up to 500µL. Excess chloroform was then added, the sample was vortexed, and the organic layer was decanted (x3) to clean the system of excess TOPO and OAm. The 2-qdots were then cleaned of excess 2
molecules via buffer rinsing with a 100 kDa molecular weight centrifugal filter (Millipore) and 3-5mL of 10mM borate buffer. The 2-qdots were highly photostable when stored in their stock solutions (10 mM borate buffer, [2-qdot] > 2mM), with ~50% loss in QY over one year.

The 2-qdots were further functionalized in a homogenous ligand exchange step with a second ligand, such as DHLA (3). Briefly, α-lipoic acid was first reduced with a 2x solution of sodium borohydride in H2O to form the dithiol functionalized 3, ([3]= 100mM). The reduced 3 was then extracted out of aqueous solution by first making the solution acidic (pH = 2) and then extracted with 3x volume ratio of CHCl3, followed by rotary evaporation. The freshly reduced 3 was then dissolved in dry MeOH.

The 2-qdots were then incubated with 3 at r ~ 6,000 at a pH~8 and mixed at 50°C for 1h. We observed no change to the qdot suspension, indicating the lack of precipitation. Next, the 3-modified qdots (3-qdot) were then purified by buffer exchange via the use of a 100kDa centrifugal molecular weight cut-off filter (Millipore), to remove excess ligand without the need for precipitation. An RPM of 5,000 was employed, and a 3-5 mL of pure borate buffer was used. Finally, a centrifugal filter with pore size of 200 nm was used to remove potential aggregates. Alternatively, the 3-qdots could be purified via acetone precipitation, however this approach was only employed for samples undergoing NMR analysis, due to the need for higher purity. We did not observe appreciable differences in colloidal stability between the purification methods.

An analogous procedure was followed for the functionalization of the 2-qdots with an array of thiol SAMS, including PEG (4). For example, mixed monolayers, containing a tailored 3/4-shell composition of fraction \( f_3 = \frac{[3]}{[3]+[4]} \) were prepared. For this, a solution of controlled \( f_3 \) is calculated and added sequentially to a 2-qdot solution at \( r = \frac{[3]+[4]}{[2\text{-qdot}]}=3,000 \) in a mixture of DMSO:H2O and allowed to incubate for 1 hr at 50 °C after the addition of each
ligand. As described above, we observe no change to the colloidal solution during this process. The resulting $3/4$-qdots are then purified as described above.

2.3 Results and Discussion

In this section we first describe the histidine (2) based phase transfer and functionalization strategy employed. We then show that the resulting 2-shell can be exploited for further ligand exchange, resulting in a number of surface chemistries, as well as the fabrication of well defined mixed monolayer qdots. The phase transfer process is characterized by UV-visible spectrophotometry (UV-vis), photoluminescence (PL) emission spectroscopy, and Transmission Electron Microscopy (TEM). The ligand exchange is characterized by Fourier Transform Infrared (FTIR) spectroscopy, $^1$H-NMR spectroscopy (NMR), dynamic light scattering (DLS), zeta-potential analysis (z-Pot), and agarose gel electrophoresis.

Scheme 2.2: An idealized schematic illustrating the qdot functionalization steps used in this study. OAm/TOPO-capped CdSe/ZnS (1-qdot) are first phase transferred (A) from chloroform to aqueous buffers using His (2), resulting in 2-qdots. Next, the 2-capping can undergo homogeneous ligand exchange (B) with an array of potential monolayers, such as DHLA (3), PEG (4), and mixed 3/4 monolayers.
The general strategy employed for qdot phase transfer and further modification is illustrated in Scheme 2.2. First, CdSe/ZnS core/shell qdots were synthesized via standard organometallic methods with slight modification\textsuperscript{102-104}. The as-synthesized qdots were capped with a trioctylphosphine oxide (TOPO) and oleylamine (OAm) (1-qdot), and stored in dry chloroform.

![Figure 2.1: A series of photographs under UV excitation for the histidine (2) mediated phase transfer. Here, two as-synthesized 1-qdots with QY of 47 and 33\% in toluene (a) are mixed with 2 ([2]/[1-qdot] = 3000) in borate buffer (10 mM, pH = 8.3) causing the immediate phase transfer (b). After vortexing for 2 min (c), the now 2-qdots are readily dispersed in the borate buffer. Notice that no precipitation occurs during this process and each solution is optically clear. Finally (d), the 2-qdots are washed free of excess toluene by decanting, and of excess 2 by buffer exchange with pure borate buffer using centrifugal molecular weight cut off filters (100 kDa, 5000 RPM, not shown).](image)

The histidine mediated phase transfer process is shown in Figure 2.1. To initiate phase transfer to buffers, an excess ratio \( r \), \( r = [2]/[1\text{-qdot}] = 3000 \), of 2 in 3:1 basic methanol/water mixture (pH = 13.0) is added to a solution of 1-qdots ([1-qdots]=1~5 mM) and vortexed for 1-5 min. Using this biphasic approach, the 2-qdot immediately undergo phase transfer into buffer (10 mM borate buffer, pH = 8.3) in only minutes, without precipitation. This decreases the tendency for non-reversible aggregation, which is particularly useful, since the lack of aggregates allowed an immediate optically clear product that can be used without additional centrifugation,
filtration, or sonication. If required, the 2-qdot can then be purified free of excess 2 either by acetone precipitation, or via molecular weight cut off filters and associated buffer rinsing. The resulting 2-qdots are then quantified by measuring the first band edge absorption via UV-vis, and compared using the size dependent extinction coefficients using the calibration method recently described.\textsuperscript{98-100}

![Figure 2.2: A representative set of UV-vis absorption (a) and PL emission (b) for the as-synthesized 1-qdot (i), the phase transferred 2-qdot (ii), and ligand exchanged 3-qdot (iii). (c) The comparison of qdot quantum yield (QY) values before and after phase transfer and ligand exchange for an identical CdSe/ZnS qdot.](image)

This 2-mediated phase transfer step has a number of important qualities. First, the biphasic phase transfer and collection of the 2-qdot is immediate, and does not require long incubation times, excessive concentrations, or elevated temperatures. Second, this process results in high phase transfer mass yields (75-90%). A third attribute of this approach is the modest preservation of optical properties and quantum yields (QY) of the qdots. For instance, Figure 2.2 shows the UV-vis (a) and PL emission (b) spectra for as-synthesized 1-qdot (i) and 2-qdot after phase transfer (ii). No obvious changes, such as spectral shift are observed, indicating the integrity of the qdot itself during phase transfer. The 1-qdots had QY of \( \approx 53\% \) directly after synthesis, while the 2-qdot showed a QY \( \approx 30\% \) after phase transfer. This QY loss was also influenced by core size, as well as ZnS-shell thickness and quality, thus batch to batch values varied. However, over the course of multiple phase transfer reactions and 1-qdot batches, a
typical QY loss of $\approx 47\%$ was observed (Fig. 2.2c). This loss in QY is much lower than if directly modified with thiols, but higher than polymer-wrapped systems. This modest QY decrease is likely due to the weak chemisorbing nature of 2 to Zn cations at the interface of the qdot, and increased interaction of the qdot with the polar media. Moreover, the lack of spectral shift suggests little change to qdot morphology itself.

This was further reinforced by visualization of the qdots via TEM before and after phase transfer. Figure 2.3 shows representative TEM micrograph for the as-synthesized 1-qdots (a,c) and the phase transferred 2-qdots (b,d), with measured diameters of 4.6 ± 0.5 and 4.4 ± 0.6 nm respectively.

![Figure 2.3: A representative set of TEM micrographs for the as-synthesized 1-qdot (a), with statistical analysis revealing diameter of 4.6 ± 0.5 nm (b), and for the phase transferred 2-qdot (c), statistical analysis revealing diameter of 4.4 ± 0.6 nm (d).](image)

The ability of 2 to induce such effective phase transfer while maintaining a large proportion of QY is notable, considering its small size, lack of alkyl chains for monolayer formation, and presumably weak coordination to the qdots ZnS shell. In biology, 2 is known to
be key cation binding sites in metalloproteins, which coordinate via the N(2)-position at the imidazole ring. It is this binding to divalent cations, such as Ni\(^{2+}\), that is the basis for polyhistidine based purification of engineered proteins. Moreover, in solution, 2 has been shown to form a bis(L-Histidinato-N,N’) complex with Zn\(^{2+}\) cations, which also chelate at the \(\alpha\)-amino group and the N(2) of the imidazole ring, as shown by crystal structure analysis. Moreover, such histagged binding has also been shown by Mattoussi and co-workers to bind strongly to qdots dispersed in aqueous media, presumably due to the coordination of the imidizole ring to free Zn\(^{2+}\) sites at the ZnS-lattice interface.

An added novelty of this histidine phase transfer method is that the 2-capping can undergo ligand exchange with an assortment of self-assembled monolayers (SAMs). To illustrate this ability, we chose DHLA (3) as a model. For instance, when 3 is added to a solution of purified 2-qdots at \(r \sim 3000\) (\(r = [3]/[\text{qdot}]\)) in buffer and let to incubate for 1 h at 50 °C, the 2 is place exchanged by the more favorable dithiol 3. During this reaction, the solution remains optically clear, indicating lack of aggregation. The UV-vis (a) and PL spectra (b) of the resulting 3-qdot is shown in Figure 2.2(iii), which reveals little to no change compared to either 2- or 1-qdots. The QY of the qdot is also largely maintained (Fig. 2.2c). The resulting 3-qdot were then purified and quantified as described above. This 3 modification is in contrast to protocols that use 3 itself to induce precipitation of 1-qdots in non-polar solvents, followed by collection, and purification. The extent of 3 exchange with 2-qdots, as well as the 2 exchange with 1-qdots was characterized in a number of ways, as described next.
Figure 2.4: FTIR results characterizing the ligand encapsulation for the as-synthesized 1-qdot (i), the phase transferred 2-qdot (ii), and ligand exchanged 3-qdot (iii).

Figure 2.4 shows the FTIR spectra for the purified 1-qdot (i), 2-qdot (ii), and 3-qdot (iii). The 1-qdots show FTIR signatures consistent with their long alkyl chains and methyl termination, namely –CH₂ and –CH₃ stretching at v₁ and v₂ respectively. Upon 2-modification, we observe the dramatic loss of v₁ stretching, and decrease in v₂. Such spectral change is consistent with the loss of the alkyl chains of 1, and the modification with 2 (Scheme 2.1). The emergence of a carboxylate stretch v₅ at ~1600 is associated with the –CO₂⁻ of 2, and the presence of the v₅ shoulder for 2 suggests the presence of protonated –COOH, likely in a hydrogen bonding motif.¹⁰⁹ Taken together, these results show the highly effective ligand exchange of both OAm and TOPO in 1 by 2. Upon modification with 3, we observe an increase in v₂ stretching, indicative of the –CH₂ of 3, as well as the v₅ increase from the carboxylate. These results suggest that 2 is effective at exchanging a large percentage of 1 at the qdot interface, likely the main reason for such high phase exchange ability. Moreover, FTIR also shows that the 2 can easily be replaced after phase transfer via 3. These results were further investigated using ¹H-NMR, as discussed next.
Further evidence for ligand exchange was provided by $^1$H-NMR analysis. NMR analysis has emerged as a powerful analytical tool to investigate the organic-encapsulating shells of nanomaterials, particularly when studying the multiple surface chemistries, and ligand exchange dynamics at qdot interfaces.\textsuperscript{110-115} For instance, Hens and co-workers have utilized both 1D and 2D NMR techniques to characterize qdot interfaces, and to observe ligand exchange in-situ.\textsuperscript{110-113} Figure 2.5 shows a typical 1D $^1$H-NMR spectra for purified solutions of 1-qdot (i), 2-qdot (ii),
and 3-qdot (iii). The first observation is the significantly broadened $^1$H resonances compared to the molecules themselves (Fig. 2.6-2.8).

Figure 2.6: The $^1$H-NMR results for 1-qdot (i), OAm (ii), and TOPO (iii) after purification and redispersion in CDCl$_3$. Insets: Illustration for 1 with labeled $^1$H assignments.
These broad resonances are characteristic of surface bound molecules, particularly those at nanoparticle interfaces.\textsuperscript{110-115} The dense or close packing of the ligands at the surface leads to a molecularly crowded environment, leading to inter-ligand resonances, and more solid-state like characteristics, such as lacking free rotation, for instance. Added to this is the fact that the ligands themselves adopt the diffusion characteristics of the qdot itself, which have orders of magnitude slower diffusion constants ($D$).\textsuperscript{110-113}

For NMR characterization, the 1-qdot was first precipitated in acetone, dried under vacuum, and resuspended in deuterated chloroform, whereas the 2-qdot and 3-qdot were precipitated in acetone following phase transfer, dried under vacuum and transferred into D\textsubscript{2}O. Typical qdot concentrations during NMR analysis were in the 70-140 µM range. Figure 2.5-i shows the 1-qdot $^1$H resonances at $\approx$0.88 and $\approx$1.26 ppm, which is consistent with the alkyl backbone of the 1-capping, namely methyl ($1a$) and methylene ($1b$) protons respectively (see
inset, Fig. 2.5). In addition, the 1c and 1d resonance are observed at \( \approx 5.4 \) (not shown) and \( \approx 2 \) ppm respectively. Upon ligand exchange and phase transfer by 2, the 2-qdots show four new resonances; 2a, 2b, 2c, and 2d that are characteristic of the 2-capping (Fig. 2.5-ii). There is a considerable decrease (>98%) in resonance intensity at \( \approx 1.28 \) ppm (1b), indicating the exchange of practically all the initial 1-capping. Second, we observe resonances at \( \approx 3.00, \) and \( \approx 3.80 \) corresponding to the 2b- and 2a-proton positions of 2, and resonances at \( \approx 6.97 \) (2c) and \( \approx 7.67 \) ppm (2d) that corresponds to the protons from the imidazole ring, which show broadened and decreased resonance intensities compared to the neat compound. Interestingly, the resonance signature for 2-qdots is very similar to that observed for the 2-coordination to Zn\(^{2+}\) cations in solution (Fig. 2.6), indicating that both the amine and the imidazole ring to be participating in qdot coordination, whose increased sterical environment and crowding lead to resonance broadening.

**Figure 2.8:** The \(^{1}\text{H}-\text{NMR}\) results for 3-qdot (i), \( \alpha \)-Lipoic Acid (ii), and DHLA (iii) after purification and redispersion in D\(_2\)O. Insets: Illustration for 3 with labeled \(^{1}\text{H}\) assignments.
After ligand exchange of the 2-qdots by 3, the resulting 3-qdots show a change in $^1$H resonance indicative again of a highly effective displacement. For example, an increased number of protons (Fig. 2.5-iii) are observed, with resonances 3$d$ to 3$g$ corresponding to the alkyl chains of 3 (see inset). Interestingly however is that the 3$a$, 3$b$, and 3$c$ resonances, those closest to the thiolate coordination sites, show resonances of $\approx$3.18 (3$a$), $\approx$1.97 and $\approx$2.46 (3$b$), and $\approx$3.67 ppm (3$c$). If we compare this resonance structure to that of the disulfide a-lipoic acid (i.e. closed ring configuration), and the reduced dihydrolipoic acid, we find that the 3-qdot resonance agrees well with that of a closed-ring configuration (Figure 2.8).\textsuperscript{116} This suggests that each thiolate may be chelating to a single Zn$^{\text{II}}$ site within the ZnS-shell lattice. Taken together, these results suggest that 2 is surprisingly effective at the removal of the initial hydrophobic 1-capping, while at the same time providing a facile surface chemistry that allows for effective ligand exchange with 3. Moreover, these results suggest the 2 molecules are coordinating to the qdot interface in a manner similar to metal cations, whereas the 3 molecules adopt a closed ring configuration at the interface.
To further investigate the binding of the 2-qdot, 2D NMR experiments were performed. Figure 2.9a shows the NOESY spectra for 2-qdots, which provides two important insights into the system, firstly that the histidine molecules are binding onto the qdot surface, which can be seen by the negative NOE cross peaks indicative of slow moving (bound) ligands. If there was no interaction the small 2 molecule would show fast motion (unbound) positive NOE crosspeaks.\textsuperscript{112} The second piece of information that can be gained from this spectrum is that the 2a proton at 2.8ppm shows a NOE cross peak with the 2c proton at 6.97ppm, but not with the 2d proton at 7.67 ppm. Since the NOESY probes interactions through space this data support our hypothesis of how histidine binds to the qdot interface, namely that the distance between the 2a and 2d proton is greater than the distance between the 2a and 2c protons, (as shown by the NOE crosspeaks) consistent with the crystal structure.\textsuperscript{107} To further investigate this, 2D DOSY can
provide information regarding whether or not the 2 is a static (high affinity) or dynamic (low affinity) interface. The DOSY of the 2-qdot is shown in Figure 2.9b, and reveals one main diffusion band with a diffusion constant of \( <D> = 0.54 \times 10^{-9} \text{ m}^2/\text{s} \) which strong evidence for a dynamic bond due to the similar diffusion coefficient,\(^{112}\) when compared to the 0.57 \( \times 10^{-9} \text{ m}^2/\text{s} \) diffusion coefficient of pure histidine in an aqueous solution (data not shown). Interestingly, a small amount of residual organic capping ligand is still attached to the particle, with a \( <D> = 0.083 \times 10^{-9} \), which is consistent with the diffusion of a particle through solution.\(^{110}\)

Figure 2.10: An optical micrograph of a representative agarose gel electrophoresis result for 2-qdot before (1,4), and after ligand exchange with 3 (2, 5), with comparison to 3-qdots prepared via direct 3 attachment (3,6) using CdSe/ZnS (1-3) and CdSe/CdS/ZnS (4-6) qdots. (1% Agarose, 70 V, 30 min, 1x TBE)

The effective 3 exchange with 2-qdots was also observed qualitatively using agarose gel electrophoresis. Figure 2.10 shows a representative gel image that compares 2-qdot with that of further modified 3-qdots for two different qdot cores (CdSe/ZnS (1-3), CdSe/CdS/ZnS (4-6)). In contrast to the 2-qdot, which does not penetrate the agarose gel due to precipitation in the presence of the running buffer (1x TBE) and voltages applied (30-75V), the 3-qdot was shown both stable, and highly charged, as indicated by the high mobility and uniform bands observed.
The 3-qdots prepared via the 2-mediated phase transfer method (lanes 2, 5) were compared to those prepared via direct attachment of 3 to 1-qdots,\textsuperscript{33-35} followed by precipitation, redispersion and sonication (lanes 3,6). As is shown in Figure 5, both preparation routes result in 3-qdots with similar mobility, and QY of 27.5 and 31.7\% respectively. These gel results provide two main qualitative findings. First, the 2-qdots are best utilized as an intermediate encapsulation; one that preserves mass and optical yields during initial phase transfer. This is evident due to the lack of stability at high ionic strengths. Second, the 2-qdots are easily functionalized with additional monolayers, such as 3, without need for a second precipitation step, or loss of yields.

\textbf{Figure 2.11:} (a) DLS results for the 1-qdot (i), 2-qdot (ii), and 3-qdot (iii) respectively. (b) Zeta-potential results of for the 2-qdot (ii) and 3-qdot (iii).

The hydrodynamic properties of the qdots were then characterized using dynamic light scattering (DLS) and zeta-potential analysis (\(\zeta\)-Pot). DLS probes the hydrodynamic diameter (\(D_h\)) of the qdots, which is a function of inorganic core size (CdSe/ZnS), organic shell thickness
(i.e. 2, 3), charge, and ionic double layer. Zeta-potential on the other hand measures surface charge at the qdot and ionic double layer interface, which can be measured in-situ to collecting hydrodynamic results, allowing for average charges per nanoparticle to be measured. Figure 2.11 shows representative results of the DLS (a) and ζ-Pot (b) results for the 1-, 2- and 3-qdots respectively. The 1-qdots have $D_h \approx 8$ (a, i), whereas the 2-qdots showed $D_h \approx 11$ nm (a, ii), and the 3-qdot showed $D_h \approx 7$ nm (a, iii). The 2-qdot and 3-qdot have charges of $\zeta \approx -34$ mV (c) and $\zeta \approx -68$ mV (d), respectively. This suggests some degree of clustering for the 2-qdots, however the values agree closely with a simple model of 3-qdot. Furthermore, the ~2x increase in surface charge for the 3-qdot case indicates a higher surface coverage.

Given the ease of 3 exchange at the 2-qdot interface, a number of potential advantages and future functionalization strategies emerge. One is that the 2-qdots may serve as a favorable substrate for tuning ligand coverage with mixed monolayers. Such tunability can include multifunctional interactions, surface domains, charge domains, or overall qdot charge. Moreover, since the 2-qdot exchange takes place in aqueous buffers, with controllable ionic strengths, a number of screening effects can be explored. This is a limitation in many systems where ligands are added to the 1-qdot while in non-polar solvents, and which can suffer from rapid and irreversible aggregations. To demonstrate this utility, we have controllably added a mixed 3/4 monolayer to the surface of 2-qdots, where 4 is a neutral PEG thiol, and effectively controlled the surface charge of the particles, shown ideally in Scheme 2.2.
Figure 2.12: An optical micrograph under UV illumination of a representative agarose gel electrophoresis result for 3/4-qdot mixed monolayer qdots with varied fractions of 3 ($f_3=\frac{3}{3}+\frac{4}{4}$) indicating the decreased in electrophoretic mobility with decreasing qdot surface charge due to increased concentrations of 4, $f_3=1.0$ (2), 0.8 (3), 0.6 (4), 0.4 (5), 0.2 (6), 0.0 (7). (0.75% Agarose, 30 V, 60 min, 1x TBE)

Briefly, a mixed 3/4 composition of fraction ($f_3=\frac{3}{3}+\frac{4}{4}$) was added sequentially to a 2-qdot solution at $r = \frac{3+4}{2}$-qdot=3,000 and allowed to incubate for 1 h at 50 °C. As described above, we observe no change to the colloidal solution during this process. The resulting 3/4-qdots are then purified as describe above. The difference in charge between 3 and 4 serves as a convenient basis for discerning surface coverage, and was analyzed via agarose gel electrophoresis. Figure 2.12 shows a representative gel electrophoresis image for the 3/4-qdot at decreasing $f_3$, where $f_3=1.0$, 0.8, 0.6, 0.4, 0.2 and 0.0. For example, compared to the $f_3=1.0$ qdot (i.e. 3-qdot), the $f_3=0.8$ shows decreased mobility, due in large part to a decrease in charge, and increased $D_h$ provided by the longer and neutral 4. A systematic decrease in mobility is shown for increase $f_3$, illustrating the ability to tailor surface charge by ligand coverage. This ability to use the 2-qdots as intermediates for controlled mixed monolayers serves as a useful basis for improving our pH and ionic stability future work. This approach will also aid in our
development of single qdots with multiple binding motifs for biomimetic self-assembly and FRET studies.

2.4 Summary and Conclusions:

In summary, we have demonstrated the use of an intermediate qdot functionality to promote both phase transfer and ligand exchange under aqueous conditions. The FTIR and NMR results show that the amino acid L-histidine is effective for the complete removal of the initial organic capping of CdSe/ZnS qdots, and can be further exchanged by DHLA. The NMR results in particular supports a model for the chelating of histidine to Zn$^{2+}$ cations of the ZnS interface, as well as similar binding by DHLA. A novelty of the approach is that the histidine provides a modest chemisorption to the qdot interface, and it can readily undergo further ligand exchange with an assortment of alkanethiol monolayers, allowing for surface functionality and charge to be easily tailored in a homogeneous environment. This utility was shown by the preparation of mixed monolayer qdots, allowing for tailoring qdot surface charge. This may aid in future studies by use of an assortment of surface chemistries, charges, and compositions.

2.5 References:


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89. Nann, T., Phase-transfer of CdSe@ZnS quantum dots using amphphilic hyperbranched polyethylenimine. *Chem Commun (Camb)* 2005 (13), 1735-1736.


Chapter 3

Nitrilotriacetic Acid-capped Qdots via Polymer Wrapping

In this chapter I describe the use of a "polymer wrapping" method to encapsulate qdots and render them hydrophilic. The purpose of these qdots were to use the multiple carboxylic acid moieties of Nα-Nα'-Bis(carboxymethyl)-L-lysine (NTA) to chelate Ni^{2+} ions. Such qdots would then be used as a point of attachment for hexa-histidine tagged proteins. This work is reproduced in part with permission from Zylstra, J.; Alam, A.; Han, H.; Doyle, R. P, Maye. M. M. Tailoring Quantum Dot Interfaces for Improved Biofunctionality and Energy Transfer. In Functional Nanoparticles for Bioanalysis, Nanomedicine, and Bioelectronic Devices Volume 1; ACS Symposium Series 1112 American Chemical Society, Washington D.C., 2012; pp 59-79.

3.1 Introduction to Qdot Phase Transfer Through Encapsulation:

In addition to direct ligand exchange, another class of qdot phase transfer and functionalization protocols is the polymer wrapping method.\textsuperscript{1-24} This method is particularly important as it leads to qdots with stability at a wide range of pH and ionic strengths. This approach is diverse, and a number of strategies have emerged recently.\textsuperscript{5-24} For instance, researchers have used a poly(maleic anhydride alt-1-tetradecene) polymer to phase transfer and functionalize an assortment of hydrophobic nanomaterials, including; Au, Fe\textsubscript{2}O\textsubscript{3}, and CdSe/ZnS particles.\textsuperscript{10-14} A similar approach can be used to fabricate qdot/FRET pairs of controlled ratios and FRET distances,\textsuperscript{12-13} and to integrate qdots into polymer capsules.\textsuperscript{14} The use of amphiphilic polymers, as well as bilayers, to investigate cellular uptake and to probe biocompatibility has also been explored in detail.\textsuperscript{17-20} In addition, biodegradable polymers have been used for qdot
cellular uptake. These amphiphilic polymer wrapped qdots also show colloidal stability when subjected to a wide range of pH or increased ionic strength, as recently demonstrated by the use of a poly(styrene-co-maleic anhydride) polymer tailored with PEG derivitatives. These PEG modified polymer-qdots in particular show colloidal stability between pH of 3-13 in biologically relevant buffers, allow for the control of surface charge, as well as the tailoring of individual qdot hydrodynamic diameter ($D_h$). In addition, there have recently been advances using amphiphilic polymers that use multidentate binding directly to the ZnS surface. For instance, an imidazole-functionalized polymer was able to provide qdot stability and solubility in aqueous media while maintaining a small $D_h$ of 10 to 12 nm. Moreover, a thiol/amine based polymer showed similar characteristics with a $D_h$ of 5.6 to 9.7 nm has also been prepared. In an analogous approach, researchers have also utilized lipids for phase transfer. In addition to these polymer based approaches, researchers have also encased qdots with hydrophillic inorganic shells for improved aqueous stability, such as in silica shells. These recent advances are characterized in table 3.1.

<table>
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<th>Encapsulation and Functionalization</th>
<th>Preparation Method</th>
<th>Reference</th>
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<td>Poly(maleic anhydride) (Block) Copolymers</td>
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<td>Silica Encapsulation</td>
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<td></td>
<td>Biotinylated Polymer</td>
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**Table 3.1:** Phase transfer methods that involve qdot encapsulation

In this section I describe the phase transfer and functionalization of qdots utilizing the
copolymer Poly(styrene-co-maleic anhydride) (PSMA), and Nα,Nα-Bis(carboxymethyl)-L-lysine (NTA) to create a qdot platform for histidine-tagged protein conjugation. These qdots where then characterized by NMR, FTIR, TEM, and DLS.

3.2 Materials and Methods

3.2.1 Materials: The majority of reagents are discussed in Chapter 2.2.1. Nα,Nα-Bis(carboxymethyl)-L-lysine (NTA, 97%), Ethanol Amine (EA, 99.5%), and Poly(styrene-co-maleic anhydride), cumene terminated (PSMA) were purchased from Sima-Aldrich. Jeffamine M-1000 (Jeffamine) was provided by the Huntsman chemical company, and Nickel Chloride (NiCl) was purchased from Matheson Coleman & Bell and Zinc Chloride was purchased from Fisher Scientific.

3.2.2 Qdot Synthesis

Qdots were synthesized via the methods described in Chapter 2.2.4 with one additional step. After synthesis and purification by precipitation the TOPO/OIA qdots were resuspended in 3 mL of ODE and heated overnight at 80 °C in an argon atmosphere with excess TOP (3 mL). The qdots were then cleaned of excess TOP by acetone precipitation and suspended in chloroform.

3.2.3 Qdot Functionalization via Polymer Wrapping

The TOP qdots were then wrapped with PSMA using a ratio, r (r = [Polymer]/[qdot]) of 200-500 fold excess of PSMA suspended in dry CHCl₃ and mixed for three hours. The qdots where then made hydrophilic by the addition of primary amine ligands to open up the maleic anhydride ring such as NTA, Jeffamine, and ethanolamine. For ethanol amine, a solution of 20 µL of ethanol amine was added to 0.5 mL of H₂O, added to the chloroform mixture, and then allowed to mix for an additional 30 minutes. The qdots would migrate into the aqueous layer.
were then collected and dialyzed against H$_2$O to remove excess polymer. For Jeffamine, the ligand was dissolved in chloroform in a 100-200 mM solution, and then added in excess and allowed mix with the PSMA qdots overnight. After mixing, H$_2$O was added to the solution and mixed until an emulsion was formed. The chloroform was then removed from the emulsion in vacuo, resulting in polymer-wrapped qdots in the aqueous layer. The qdot solution was then back extracted with chloroform and dialyzed to remove excess polymer and ligand. NTA was dissolved with NiCl$_2$ or ZnCl$_2$ in DMSO, 100 μL of N,N-diisopropylethylamine was then added to deprotinate the primary amine, and the solution was then added to PSMA qdots in chloroform and reacted for >4 hours. The chloroform was then removed in vacuo and the aqueous solution was cleaned similar to Jeffamine qdots. For mixed ligand constructs, the amount of anhydride rings in solution were calculated by multiplying moles of PSMA by 5.8 and the first ligand, such as NTA, was added and allowed to react for >4 hours. The second ligand was then added, such as jeffamine or ethanolamine, allowed to react overnight, and then cleaned as described above.

3.3 Results and Discussion

In this section we describe a polymer encapsulation strategy using PSMA as a phase transfer and functionalization agent for qdots. PSMA is a useful polymer due to its small size (M.W. 1,600), hydrophobic phenyl groups, and easily modified maleic anhydride rings.\textsuperscript{7, 47} We then show that primary amine ligands can be exploited to functionalize the qdots by opening the maleic anhydride rings, resulting in tailorable surface chemistries, as well as the fabrication of well defined mixed monolayer qdots. The resulting qdots are characterized by UV-visible spectrophotometry (UV-vis), photoluminescence (PL) emission spectroscopy, and Transmission
Electron Microscopy (TEM), Fourier Transform Infrared (FTIR) spectroscopy, $^1$H-NMR spectroscopy (NMR), and dynamic light scattering (DLS).

Scheme 3.1: An idealized schematic of wrapping TOP organic qdots with PSMA, followed by the addition of primary amine ligands to open up the anhydride rings and render the qdots hydrophilic. Primary amines that have been utilized are (a) Jeffamine M-1000, (b) $N\alpha,N\alpha$-Bis(carboxymethyl)-L-lysine hydrate, and (c) ethanol amine.

The general strategy employed for this method is to first take as synthesized TOPO-qdots and heating them with TOP, resulting in a monolayer of TOP/TOPO on the qdot surface. These dots are then mixed with PSMA, as shown in Scheme 3.1, allowing the phenyl groups on the polymer to intercalate into the qdot surface through hydrophobic interactions. Primary amines are then added to the mixture, opening the maleic anhydride rings, functionalizing the qdot surface, rendering the qdots hydrophilic.

After phase transfer, the qdots were analyzed via $^1$H NMR to elucidate their surface chemistry. Figure 3.1 shows that we can successfully attach Jeffamine and NTA to qdots through a polymer interface, as well as the attachment of mixed ligand systems. To prepare qdots for NMR, the samples were phase transferred as described earlier, (NTA was added mixed with
ZnCl₂ for NMR samples) dialyzed against H₂O for 24 hours with 3 buffer changes, dried under vacuum, and transferred into D₂O. Typical concentration for NMR were ~100 μM.

**Figure 3.1:** The ¹H-NMR results for PSMA wrapped qdots after purification and redispersion in D₂O. Insets: Illustration for Jeffamine and NTA with labeled ¹H assignments with (ii) showing a qdot functionalized with NTA, (iii) mixed NTA and PEG) and (iv) PEG.

Figure 3.1-ii shows a typical NMR spectrum of NTA functionalized dots with broad ¹H resonances at ~1.4, 1.55, 1.6, and 1.8 ppm corresponding to resonances from protons corresponding to methylene protons (1b, 1c, and 1d) forming the lysine side chain backbone, a resonance at ~2.9 ppm from the protons on the carbon adjacent to the primary amine (1a), and resonances at ~3.1, 3.3, and 3.5 ppm from the protons on the carbons adjacent to the carboxolate groups (1e and 1f). Figure 3.1-iv shows the typical resonances associate with jeffamine, mainly
the methyl groups at ~1.1, 1.2, and 3.3 ppm (2b and 2f) and the protons from the ethylene glycol units exhibiting a broad peak at ~3.6 ppm (2a, 2c, 2d, and 2e). We were also able to facilitate the addition of a mixture of ligands on the surface as illustrated in Figure 3.1-iii, clearly showing the presence of the NTA peaks at ~1.4, 1.55, 1.6, 1.8, and 2.9 ppm, as well as the large jeffamine peaks at ~1.1 and 3.3 ppm.

Figure 3.2: The 2D NMR results for the NTA-qdots. A HMBC $^1$H-$^13$C (a) and a TOSCY $^1$H-$^1$H (b) with labeled assignments.

The NTA-qdots were also characterized with Heteronuclear Multiple Bond Correlation (HMBC) and Total Correlation Spectroscopy (TOSCY) NMR. The advantage of running a HMBC for this system is that there are 3 carboxylate groups, and looking for $^1$H-$^13$C correlations through 2 bonds allowed for the determination of the protons adjacent to the quaternary carbons of the NTA center. Figure 3.2(a) shows three peaks at ~180 ppm, showing the presence of the carboxylate carbons, and that they correlate to protons at ~3.1, 3.3, and 3.5 ppm. The TOSCY, shown in Figure 3.2(b) allows for through bond $^1$H-$^1$H correlations, allowing for identification of
how the protons that correlate with the carboxylate groups, interact with each other. From the
TOSCY cross peaks, it is evident that the protons at 3.1 and 3.5 ppm do not interact with the 1b,
1c, and 1d protons of the lysine side chain (~1.4, 1.55, 1.6, and 1.8 ppm) and therefore are likely
the 1f protons (see Fig. 3.2(b) inset). The other carboxylate adjacent proton (3.3 ppm) does
interact with the lysine side chain residues, identifying it as the 1d proton.

In addition to NMR, the purified qdots were also analyzed via FTIR. Figure 3.3 shows
the FTIR spectra of the NTA (ii) NTA and Jeffamine (iii), and Jeffamine (vi) qdots analyzed by
NMR, as well as qdot with the PSMA polymer before further functionalization (i). The
vibrational modes are summarized in Table 3.1. The PSMA qdots show FTIR signatures
consistent with their long alkyl chains and methyl termination, namely –CH₂ and –CH₃
stretching at ν₂ and ν₄ respectively as well as peaks at ν₅ and ν₆ corresponding to the anhydride
rings in the polymer. The addition of NTA (Figure 3.3-ii) and Jeffamine (Figure 3.3-iv) resulted
in the complete loss of ν₄ and ν₅ stretching, indicative of the amine mediated ring opening. In the
case of NTA, there was also an appearance of a strong band at ν₈ and ν₉ due to the emergence of
symmetric and asymmetric carboxylate stretches respectively, as well as a broad band at ν₁
indicative of the O-H stretch of the carboxylic acid. Using Jeffamine as a ring-opening agent
resulted in signature peaks at ν₁₀, ν₁₁, and ν₁₂ consistent with carbon-oxygen bonds. Importantly,
similar to the NMR, figure 2-iii shows characteristics of both Jeffamine and NTA, showing we
have successfully functionalized the particle with a mixture of ligands.
Figure 3.3: FTIR results characterizing the polymer encapsulation for the organic qdot with PSMA (i), qdot phase transferred with NTA (ii), qdot phase transferred with NTA and Jeffamine (iii), and qdot phase transferred with Jeffamine (iv).

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</tr>
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</tr>
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</tr>
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</tr>
<tr>
<td>ν₁₂</td>
<td>1220</td>
<td>C-O stretch</td>
</tr>
</tbody>
</table>

Table 3.1: A table identifying the origins of the major vibration intensities shown in Figure. 3.3
To determine the thickness of the polymer shell of the NTA qdots, TEM images were taken of the Core/Shell qdot, as well as Core/Shell/Polymer qdot stained with phosphotungstic acid (Figure 3.3b). The stain does not associate with the charged polymer, allowing for it to be visualized via TEM. The polymer appears as a white halo around the particle against the stained grid. Using this technique we are able to calculate the ligand/polymer shell to be 2 nm thick, showing a modest increase in particle size. In addition to TEM, the hydrodynamic size of the qdots was calculated using DLS to be 13.5 nm, agreeing with the counted particle size from TEM.

![Figure 3.4](image)

**Figure 3.4:** A representative set of TEM micrographs for the as-synthesized 1-qdot (a), with statistical analysis revealing diameter of 8.0 ± 0.6 nm and stained NTA polymer wrapped qdot (b), statistical analysis revealing diameter of 11.9 ± 1.5 nm. DLS (c) of the NTA polymer wrapped qdots, calculating a size of 13.5 nm.

### 3.4 Summary and Conclusions

In this chapter I have shown that I can successfully functionalize qdots with a PSMA copolymer with a Ni²⁺ chelated NTA, as well NTA/Jeffamine mixed functionalities. The 1D and 2D NMR, as well as the FTIR showed the presence NTA on the qdot surface in both the single and mixed ligand systems. A modest hydrodynamic diameter (~12 -13 nm) was also maintained, as shown by TEM and DLS. These qdots are used to bind to fluorescent proteins in Chapter 4.
3.5 References


Chapter 4

Expression of Recombinant tagRFP, and its use in FRET and sequential BRET-FRET Energy Transfer

In this chapter, I describe the cloning, expression, and purification of a red fluorescent protein (tagRFP) expressed with a N-terminal hexa-histidine tag for purification and qdot conjugation. After purification, tagRFP was mixed with both histidine-capped (Chapter 2), and NTA qdots (Chapter 3). The interaction was analyzed with fluorescence resonance energy transfer (FRET). This work is reproduced with permission from Alam, R.; Zylstra, J.; Fontaine, D. M.; Branchini, B. R.; Maye, M. M. Novel multistep BRET-FRET energy transfer using nanoconjugates of firefly proteins, quantum dots, and red fluorescent proteins. *Nanoscale* 2013, 5, 5303-5306. Copyright 2013 The Royal Society of Chemistry.

4.1 Introduction to FRET and BRET using Quantum Dots and Recombinantly Expressed Fluorescent Proteins

Quantum dots (qdots) and rods (qrods) are powerful energy donors in resonance energy transfer (RET) designs due to their broad absorption profile, size, tailored emission, photostability, and long excited state lifetimes (>10 ns).1-3 Another attribute of qdots is the ability to conjugate terminally hexahistidine-tagged proteins that bind to qdot/qrod interfaces by coordination to the Zn²⁺ or Cd²⁺ rich interfaces.4-10 A number of fluorescent proteins have been used as acceptors from qdots recently, including Yellow,3 mOrange,11 and mCherry.12

On the other hand, qdots are limited as energy acceptors, because of the likelihood of co-excitation, which makes analysis challenging.2-3,13-15 However in the absence of direct excitation
qdots can indeed act as highly efficient acceptors. For instance, when bioluminescent proteins are used as donors, bioluminescence resonance energy transfer (BRET) can occur. This has led to a number of novel BRET molecular- and nano-conjugates, with acceptors ranging from fluorescent proteins, dyes, qdots, and qrods. To date, qdot-based BRET nanosystems have used luciferase expressed with multiple copies of surface exposed lysine residues, which were conjugated to polymer wrapped qdots. These qdot-BRET nanosystems have produced a wide range of light. In addition to these one-step processes, researchers recently investigated novel multi-step FRET processes (FRET-FRET), or combinations of BRET and FRET (BRET-FRET). Medintz et al. recently showed a novel FRET-FRET system using two red-emitting dye acceptors attached to a singe qdot donor. Branchini and co-workers recently used luciferase-red protein florescent protein fusions in combination with covalently bound nIR organic dyes for BRET-FRET. Franco and co-workers reported BRET-FRET with three proteins expressed in succession, and BRET-FRET between Luc and nIR dyes using doped polymer nanoparticles has also been described.

In this chapter I describe the cloning and expression of a N-terminal hexa-histidine tagged red fluorescent protein (tagRFP) in E. coli and use it to probe energy transfer of the histidine capped qdots (Chapter 2), and the polymerwrapped NTA qdots (Chapter 3) by FRET analysis. A BRET-FRET system will also discussed using firefly luciferase with a modified substrate, a histidine capped qdot, and tagRFP.
4.2 Materials and Methods

4.2.1 Materials

*Chemicals & Materials:* Chemicals were obtained from the suppliers as described in Chapter 2. Additional chemicals include: Octadecylphosphonic acid (ODPA, 98%) and hexylphosphonic acid (HPA, 98%) were purchased from Strem Chemicals. The Mg-ATP (bacterial source) was purchased from Sigma-Aldrich, and restriction endonucleases from New England Biolabs (Beverly, MA). Firefly luciferin (LH₂) was a generous gift from Promega (Madison, WI).

4.2.2 Qdot and Qrod Synthesis and Phase Transfer

*Synthesis of CdSe Quantum Dot:* CdSe qdots were synthesized as described in Chapter 2.2.4

*Synthesis of CdSe/ZnS Quantum Dot with dot-in-dot Morphology:* A Thin layer of ZnS was grown on CdSe qdots following the SILAR approach as described in Chapter 2.2.4.

*Synthesis of CdSe/Cds Quantum Rod with dot-in-rod Morphology:* Qrod samples were made by Rabeka Alam, see reference²⁶ for details.

*Histidine-Mediated Phase transfer:* Synthesized qdot and qrods were phase transferred in aqueous buffers using the histidine mediated phase transfer method described in Chapter 2.2.5.
4.2.3 Protein Expression and Purification

TagRFP Expression: TagRFP was cloned using the pPA-TagRFP-N vector as template DNA. The gene was ligated into pET15b for expression, as well as the addition a N-terminal hexa-histidine tag. The constructed vector was then transformed into BL21 Gold (DE3) E. coli and expressed overnight under 0.01 mM IPTG induction. The cells were then harvested, sonicated, and the soluble fractions was purified utilizing immobilized metal affinity chromatography (IMAC). The samples were analyzed using SDS-PAGE, western blotting, and concentrations were calculated using the extinction coefficient at 555 nm.27

Ppy Expression: The Ppy WT was provided by Dr. Branchini and was expressed as a GST-fusion protein, purified by affinity chromatography, and stored as described in detail previously.28 The plasmids for 6xHis-Ppy GRTS was constructed by excising the corresponding genes for Ppy GRTS28 from the pGEX-6P-2 vector and ligating them into a modified pQE30 expression vector using previously described procedures.29 The His-tagged proteins were expressed, purified and stored using procedures described elsewhere.29 The found molecular masses (Da) of the proteins not previously reported were within the allowable experimental error (0.01%) of the calculated values (in parenthesis): 6xHis-Ppy GRTS, 61 996 (62 002).

Protein concentrations were determined with the Bio-Rad Protein Assay system using BSA as the standard. DNA sequencing to verify the ligations was performed at the W. M. Keck Biotechnology Laboratory at Yale University. Specific activity and steady state kinetics measurements were determined as previously reported,29-31 except that the final LH2 concentration was 0.3 mM and integration times were 15 min. Bioluminescence emission spectra were obtained using methods and equipment previously described.29 Mass spectral analyses were
performed by tandem HPLC-electrospray ionization mass spectrometry (LC/ESIMS) using a ThermoFinnigan Surveyor HPLC system and a ThermoFinnigan LCQ Advantage mass spectrometer and previously developed conditions for protein mass determinations.\textsuperscript{32}

BtLH\textsubscript{2}: The firefly luciferin analog BtLH\textsubscript{2} was prepared using previously described method.\textsuperscript{33}

4.2.4 Qdot/Qrod Conjugation and BRET Measurement and Analysis

\textit{PpyGRTS-QD-RFP Conjugation:} To construct the PpyGRTS-QD-RFP BRET-FRET nano-conjugates, the His-functionalized QDs were incubated with the hexa-histidine tagged PpyGRTS in 10 mM borate buffer at a 1:1 ratio on ice. Then RFP was added to the mixture at varying loading ratios \((L) = [\text{tagRFP}]:[\text{Ppy}]\) and incubation was allowed to proceed for at least 15 minutes before BRET analysis.

\textit{BRET Measurement and Analysis:} In a typical BRET experiment, a mixture of 100 \(\mu\)L of 500 \(\mu\)M BtLH\textsubscript{2} (modified firefly luciferin) and 30 \(\mu\)L of 8.66 mM Mg-ATP in 50 mM 2-Amino-2-methyl-1, 3-propane buffer (pH 9.1) or a mixture of 100 \(\mu\)L of 91 \(\mu\)M LH\textsubscript{2} (firefly luciferin) and 30 \(\mu\)L of 8.66 mM Mg-ATP in 25 mM gly-gly buffer (pH 7.8) is quickly added to the Ppy-QD conjugate solution in a 96-well plate and bioluminescence emission is immediately collected. The bioluminescence and BRET were collected on a Varian Cary-Eclipse spectrophotometer in bioluminescence /chemiluminescence mode using a 96-well plate reading accessory. White 96-well plates were employed, with volumes ranging from 50-200 mL. Bioluminescence spectra were collected every 15 seconds for 7.5 minutes. The instrument was
corrected for detector sensitivity by comparison of fluorescence standard emission intensities (500–800 nm) with the corrected detector on the Fluoromax-4 spectrophotometer. The presented BRET results are the average of the first five spectra collected over 1.5 min after addition of BtLH$_2$ or LH$_2$. Control experiments showed that the BRET ratio did not change over the course of the typical BRET decay. Finally, the BRET efficiencies of the systems were calculated as BRET ratio ($BR$), which is defined as the ratio of peak area of the acceptor and donor emission respectively. Peak area was calculated by spectral deconvolution of each spectrum using the data analysis package in Igor Pro (Wavemetrics Inc.).

4.2.5 Calculations

*Qdot and Qrod Concentration:* The concentrations of the Qdot cores were calculated based on UV-vis optical absorption measurements of the QD first band edge absorption as described in Chapter 2.2.3.

*Quantum Yield (QY):* The QR photoluminescence quantum yields (QY) were calculated based on comparison to a reference dye using standard methods as described in Chapter 2.2.3

*Förster Resonance Energy Transfer (FRET) Calculations:* In this study, the bioluminescence resonance energy transfer (BRET) constants were calculated in the identical manner to FRET. In FRET, the Förster distance ($R_0$) is calculated using equation 1$^{34-35}$:

$$R_0 = 8.8 \times 10^{-23} k_p^2 \eta D^{-\frac{1}{4}} Q_D J$$ (1)
where \( \eta_D \) is the refractive index of the medium (\( \eta_D = 1.33 \)), \( k_p \) is the polarization parameter (\( k_p = 2/3 \)), \( Q_D \) is the donor quantum yield \( QY(Ppy(BtLH_2) \approx 10\% \), and \( J \) is the spectral overlap integral. The \( J \) value can be calculated using equation 2:

\[
J = \int f_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda
\]  (2)

where \( \lambda \) is the defined wavelengths of the donor-acceptor spectral overlap (\( 1 = 450–650 \) nm), and \( f_D(\lambda) \) is the integrated donor emission and \( \varepsilon_A(\lambda) \) represents the integrated acceptor absorption using the acceptor extinction coefficient (\( \varepsilon_A = 1.00 \times 10^5 \) M\(^{-1}\)cm\(^{-1}\)). The values of \( R_0 = 4.2 \) nm, and \( J = 3.2 \times 10^{-13} \) cm\(^6\) were calculated using equations 2 and 3, as well as the software PhotoChemCAD for Ppy and tagRFP.

Using the \( R_0 \) values calculated above, the FRET efficiency, \( E \), was calculated using equation 3\(^{34-35}\):

\[
E = 1 - \frac{F_{DA}}{F_D} = \frac{R_0^6}{R_0^6 + r^6}
\]  (3)

where \( F_{DA} \) is donor fluorescence in the presence of acceptor, and \( F_D \) is fluorescence of the donor without acceptor.

4.3 Expression of Recombinant tagRFP in E. coli

4.3.1 Insertion of the tagRFP gene into pET15b plasmid

Primers used for polymerase chain reaction (PCR) included a forward (5’-GATC CATATG AGCGAG CTGATT AAGG-3’) and reverse (5’-GATC GGATCC TTAATT AAGCTT GTGCC-3’). The template DNA was pPA-TagRFP-N and was generously provide by the Hougland lab (Syracuse University) and originally obtained from Evrogen (Moscow, Russia). PCR conditions were as follows: 95 °C for 5 min, followed by 35 cycles of a 95 °C
denaturing step for 1 min, a 53 °C annealing step for 1 min, and a 74 °C elongation step for 1 min, and then held 16 °C.

The reaction was then run down a 1 % agarose gel at 70 mV and the bands corresponding to the full-length tagRFP gene were extracted (Figure 4.1a). The PCR product was double digested with NdeI and BamHI. The pET15b_CitSc vector was digested using the same conditions with the addition of calf intestinal phosphatase (CIP). The pET15b vector was chosen as it incorporates a N-terminal hexa-histidine tag for ease in purification, as well as a platform for qdot conjugation. The pET15b_CitSc vector was used as the CitSc gene was incorporated using the same NdeI and BamHI restriction sites. Agarose gel electrophoresis was run on both digestion mixtures, and bands corresponding to the tagRFP gene and linearized pET15b were extracted from the gel (Figure 4.1b). The tagRFP gene and pET15b vector were ligated at 22 °C for 90 minutes and transformed into chemically competent DH5α E. coli.

![Figure 4.1](image-url) **Figure 4.1**: DNA gels of the construction of pET15b_tagRFP. Cloning tagRFP using the pPA_tagRFP_N vector as a DNA template with DNA template concentrations of 5, 10, 25, and 50 ng/μL (a), the triple digestion of pET15b_CitSc and double digestion of the tagRFP gene with NdeI and BamHI (b), and diagnostic digest of 7 E. coli cultures following ligation (A-G) to confirm insertion of the tagRFP gene into pET15b (c).

Single colonies of the transformed E. coli were picked and used to inoculate 5 mL LB broth with 5 μL of ampicillin (amp) [50 mg/mL], and incubated at 37 °C for 16 hours. The
plasmids from the overnight cultures were then isolated and digested with *NdeI* and *BamHI* and were analyzed by gel electrophoresis. A plasmid sample (Figure 4.1c lane B) that yielded a clean band at ~700 base pairs was sent for DNA sequencing and confirmed to be the correct construct (Figure 4.1c).

### 4.3.2 tagRFP Expression and Purification

The pET15b_tagRFP vector was transformed into chemically competent BL21 Gold (DE3) *E. coli*. Single colonies were used to inoculate 5 mL LB broth with 5 µL of amp [50 mg/mL], and incubated on a shaker at 250 rpm at 37 °C for 16 hours. After the initial growth, two 500 µL aliquots were used to inoculate two 250 mL flasks containing 50 mL of LB broth with 50 µl of amp [50 mg/mL]. The cultures were grown until an optical density at 600 nm of 0.4-0.6 was reached. The cultures were then induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of [0.01 mM] and [0.1 mM], and the incubation temperature was reduced to 30 °C. The cultures were then incubated overnight with time points taken at 1, 4 and 22 hrs. The cells were then harvested, lysed via sonication, and analyzed via SDS-PAGE (Figure 4.2).
After expression trials, it was determined that the ideal expression conditions were inducing with [0.01 mM] IPTG overnight, see lane D (Figure 4.2). To this end, tagRFP was expressed and then purified by immobilized metal affinity chromatography (IMAC) using a Ni\(^{2+}\) column on an Akta Prime (GE Akta). Buffer A was 10 mM phosphate-buffered saline (PBS) and 0.5 M NaCl (pH 7.4), and buffer B was 10 mM PBS, 0.5 M NaCl, and 1 M imidazole (pH 7.4). Binding was done at 1 mL/min with 100% buffer A. The column was then rinsed at 1 mL/min with 90% buffer A and 10% buffer B, with the tagRFP eluted at 50% buffer A and 50% buffer B. After elution, the protein was dialyzed against 10 mM borate buffer (pH 8.3) with 3 changes over 24 hours. The fractions were then analyzed by 12% SDS-PAGE (Figure 4.3a). The purified sample was also analyzed by western blot using an anti His 6x primary antibody conjugated to HRP. Absorbance and fluorescence spectroscopy was also performed to confirm tagRFP’s optical properties (Figure 4.3b).
4.4 Results and Discussion

4.4.1 Qdot-tagRFP FRET

Having expressed tagRFP, the first FRET system we studied consisted of the His-qdot (Chapter 2) acting as energy donors and the NTA-qdots (Chapter 3) with PL emission at 530 nm and QY of 25.5% and 7% respectively, and tagRFP with emission at 584 nm serving as the FRET acceptor. The tagRFP was chosen to sufficiently separate donor-acceptor emission spectra, while maintaining an acceptable overlap integral. The spectral properties and FRET efficiency plot is shown in Fig. 4.4.
Figure 4.4: Normalized PL emission and absorption spectra for 530 nm emitting qdot donor (green) and tagRFP acceptor (red) (a). The FRET efficiency curve was calculated with an $R_0$ of 51.7 Å for the His-qdots (Green line) and 33.5 Å for the NTA-qdots (Red line) at $n = 1$ (b).

A typical FRET result between the His-qdot (i) or NTA-qdot (ii) and tagRFP at acceptor/donor ratios ($n = [\text{tagRFP}]/[\text{qdot}]$ of 0 ~ 3) is shown in Fig. 4.5a. The FRET efficiency ($E$) was calculated to be 76% and 8% for $n=1$ respectively. With this measured $E$ value, and spectral overlap integral ($J = 3.8 \times 10^{-13}$ M$^{-1}$ cm$^3$, Fig. 4.4a), we estimate the qdot-to-RFP distance ($r$, center-to-center) of $r \sim 4.2$ nm for the His-qdot and $r \sim 5.0$ for the NTA-qdot system. This estimate fits well with ideal models, since the radius of the qdot is 1.8 nm and the distance from the $N$ terminus to the active site of tagRFP is $\sim 2.3$ nm, giving a shortest acceptor to donor distance of $\sim 4.1$ nm which fits well with the experimental data. The NTA-qdots have a larger hydrodynamic radius resulting from the encapsulation of the organic capping ligands within the polymer shell. This greater distance is shown in an increase in the $r$ value of 0.8 nm.
These results show that we are able create qdot-tagRFP constructs for non-radiative energy transfer through self-assembly. As expected, the FRET efficiency is much greater for the His-qdot system, due to shorter r distance, as well as the His-qdot sample having a higher quantum yield. For these reasons, we chose the His-qdot platform as the basis for the BRET-FRET system described next. The NTA-qdots had a larger r value, as expected, and the FRET efficiency data fit well as shown in Fig. 4.5b, showing successful attachment of tagRFP onto the polymer surface.

**4.4.2 Firefly Luciferase-qdot-tagRFP BRET-FRET**

We then moved onto a more complex series of novel BRET-FRET conjugates that employ either qdot or qrod as an inorganic linker and energy transfer step between luciferase and
these fluorescent proteins. To the best of our knowledge, such qdot/qrod based BRET-FRET has not been shown previously. The advantage of using qdot linkers in energy transfer is that energy can be transferred between bioluminescent proteins and fluorescent proteins that exhibit small or no spectral overlap, due to the qdots acting as an energy transfer step, as well as an inorganic support.

In this study, the BRET-FRET process was initiated using a novel thermostable firefly protein variant of *Photinus pyralis*, (denoted here as Ppy) and a recently developed benzothiophene analogue to firefly luciferin (BtH2), which when combined with Ppy, ATP and O2 emits at ~520 nm. This energy is then non-radiatively transferred by BRET to either a qdot or qrod acceptor, which then acts as a donor in a second non-radiative FRET step to a red fluorescent protein (tagRFP), with final emission at ~584 nm. Thus, no direct excitation occurred in this process other than the addition of the BtLH2. Both the Ppy and tagRFP were expressed with hexa-histidine tags (6xHis) at the N terminus. To facilitate such attachment, the qdots/qrods were first transferred from chloroform to borate buffer using the histidine mediated phase transfer protocol described in chapter 2. The resulting His-capped qdots/qrods were then incubated with Ppy at [qdot/qrod] : [Ppy] = 1 : 1 molar ratios. Next, the tagRFP was added at a 1 : 1 ratio with the adsorbed Ppy. To avoid excess Ppy or tagRFP in the solution, nickel-loaded colloids could be added to adsorb excess 6xHis-Ppy or 6xHis-tagRFP, however this resulted in little to no change in emission properties, suggesting the lack of excess free protein in the conjugate solutions.

Figure 4.6a shows an idealized illustration of the Ppy-qdot-tagRFP conjugates, with a corresponding scheme of an energy level diagram. Figure 4.6b shows a transmission electron micrograph (TEM) showing the CdSe/ZnS qdots used, with diameter (d) of 3.8 ± 0.3 (denoted as QD(556)). Figure 4.6c-i shows the corresponding spectral overlap for the BRET process,
between the QD(556) acceptor and the Ppy-BtLH$_2$ donor. Quantification of the overlap integral ($J$) (Table 4.1) indicates a Förster distance ($R_0$) of 4.3 nm for BRET.

<table>
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<th>Acceptor Absorption$^1$</th>
<th>FRET Calculation$^2$</th>
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<td>$\lambda_A$ (nm)</td>
<td>$\varepsilon$ (M$^{-1}$ cm$^{-1}$)</td>
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<td>Ppy – tagRFP</td>
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<tr>
<td>Ppy – QD</td>
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<tr>
<td>QD – tagRFP</td>
<td>555</td>
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Table 4.1: Optical and FRET parameters for BRET-FRET nanosystem using QD(556).
1) First absorption maxima ($\lambda_A$), and calculated extinction coefficient ($\varepsilon$). 2) Calculated using Ppy QY of 10 %, QD(556) QY of 10 %.

The overlap between the QD(556) donor, and tagRFP acceptor for the second FRET step is shown in figure 4.6c-ii, which indicates a $R_0 = 4.7$ nm. These values are comparable to estimated donor acceptor distances of $r_{\text{BRET}} = 5.4$ and $r_{\text{FRET}} = 4.2$ nm, based on the qdots size and the protein crystal structures. Figure 4.6d shows the BRET emission spectra for the Ppy-qdot conjugates (i), and the BRET-FRET spectra for the Ppy-qdot-tagRFP conjugates (ii). Indication of BRET is observed by the emission of QD(556), and of BRET-FRET due to the emission of tagRFP. The BRET efficiency was quantified by calculating the so-called BRET ratio ($BR$), which is the ratio of the integrated emission of the acceptor divided by the donor. For the BRET process, a $BR = 1.3$ was measured, whereas for the BRET-FRET process the BR was found to increase to 4.3. The increase in $BR$ is contributed by both the asymmetric emission of tagRFP, as well as the close physical and optical proximity of each component within the conjugate.
To better understand the role that spatial distance between Ppy and tagRFP play in this process (Figure 4.7a), we next changed the qdot morphology without changing spectral properties by using CdSe/CdS *dot-in-rod* qrods (denoted as QR(556)). A TEM of the QR(556) is shown in figure 4.7b, in which an aspect ratio (*l/w*) of 12.6 is measured. In general, the spectral properties were similar to the qdot case. For instance, Figure 4.7c shows the BRET overlap between QR(556) acceptor and the Ppy-BtLH$_2$ donor (i), and FRET between QR(556) donor...
with tagRFP acceptor (ii), in which a $R_0 = 5.6$ and 4.7 nm were calculated (Table 4.2).

<table>
<thead>
<tr>
<th></th>
<th>Acceptor Absorption$^1$</th>
<th>FRET Calculation$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_A$ (nm)</td>
<td>$\varepsilon$ (M$^4$ cm$^{-1}$)</td>
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<tr>
<td><strong>Ppy – tagRFP</strong></td>
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<td>1.00 x 10$^5$</td>
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<td><strong>Ppy – QR</strong></td>
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<td>8.20 x 10$^4$</td>
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<tr>
<td><strong>QR – tagRFP</strong></td>
<td>555</td>
<td>1.00 x 10$^5$</td>
</tr>
</tbody>
</table>

Table 4.2: Optical and FRET parameters for BRET - FRET nanosystem using QR(556). 1) First absorption maxima ($\lambda_A$), and calculated extinction coefficient ($\varepsilon$). 2) Calculated using Ppy QY of 10 %, QR(556) QY of 12 %.

In these Ppy-qrod-tagRFP conjugates; the Ppy-to-CdSe, tagRFP-to-CdSe, or Ppy-to-tagRFP distances can vary, since the CdSe core is ~ 3 nm in diameter, and is located approximately 1/3 of the rod length,$^{1,4}$ which should result in lower energy efficiencies on average. Figure 4.7d shows the BRET emission for the Ppy-QR(556) conjugates (i), and BRET-FRET emission for the Ppy-QR(556)-tagRFP conjugates (ii). In the BRET step, a decrease in $BR$ to 0.9 is observed compared to the dot case (Figure 4.6), which is also observed for the BRET-FRET step, $BR = 2.1$. This loss of efficiency is likely due to increased $r_{BRET}$ and $r_{FRET}$, due to the rod morphology. It is interesting however that the BRET-FRET observed is rather efficient ($BR > 1$), and suggests that the Ppy and tagRFP may organize close to one another on the rod.
Figure 4.7: (a) Scheme of Ppy-QR(556)-tagRFP conjugate and idealized energy transfer diagram. (b) TEM micrograph of QR(556). (c) Spectral overlaps for BRET between Ppy and QR(556) (i), and FRET between QR(556) and tagRFP (ii). (d) BRET emission spectra for Ppy-QR(556) conjugate at [Ppy] : [QR(556)] = 1 : 1 (i), and for BRET-FRET of Ppy-QR(556)-tagRFP conjugate at [tagRFP] : [Ppy] : [QR(556)] = 1 : 1 : 1.

An alternative to this morphology consideration is the possibility that radiative energy transfer effects may be playing a role. To address this, control experiments using free Ppy and tagRFP in the absence of qdots or qrods (i.e. no linking) at similar concentrations were performed\textsuperscript{26}. These experiments revealed much lower energy transfer ($BR \sim 0.4$), due to radiative energy transfer (i.e. inner filter effects), thus suggesting BRET-FRET in the conjugates.
In Figure 4.6 and 4.7, the qdot or qrod acts as both the inorganic linker (i.e. support) and as an electronic step in the BRET-FRET process. We next studied a more electronically “transparent” qdot connection for BRET-FRET. To achieve this, qdots that emit at 530 nm (denoted as QD(530)) were synthesized. Figure 4.8 shows the schematic of these conjugates and energy considerations with the corresponding $R_0$ values shown in Table 4.3.

**Figure 4.8:** (a) Scheme of Ppy-QD(530)-tagRFP conjugate and idealized energy transfer diagram. (b) TEM micrograph of QD(530). (c) Spectral overlaps for BRET between Ppy and QD(530) (i), and FRET between QD(530) and tagRFP (ii). (d) BRET emission spectra fro Ppy-QD(530) conjugate at [Ppy] : [QD(530)] = 1 : 1 (i), and for BRET-FRET of Ppy-QD(530)-tagRFP conjugate at [tagRFP] : [Ppy] : [QD(530)] = 1 : 1 : 1 (ii).
Since the Ppy-BtLH\textsubscript{2} system emits at \(\sim 520\) nm\textsuperscript{33}, this qdot has a much lower overlap with Ppy (Figure 4.8c). However, since Ppy and tagRFP are brought close to one another on the qdot support, energy transfer from Ppy to tagRFP is possible. Figure 4.8d shows representative BRET spectra for Ppy-QD(530) (i), and BRET-FRET spectra for Ppy-QD(530)-tagRFP (ii). BRET does occur in this system, but deconvolution is challenging due to considerable emission. Interesting, BRET-FRET occurred at high efficiency, with \(BR\) of 2.9 being measured.

To further study the transparent qdot linker idea, we employed Ppy with the standard firefly luciferin (LH\textsubscript{2}), which produces bioluminescence at \(\sim 547\) nm\textsuperscript{4,21,37,39}. Here, Ppy-LH\textsubscript{2} and QD(530) have an even smaller overlap (Table 4.4), since QD(530) absorbs at energy higher than Ppy emission (Figure 4.9c), Ppy-LH\textsubscript{2}-QD(530) BRET does not occur.

### Table 4.3: Optical and FRET parameters for BRET - FRET nanosystem using QD(530).

1) First absorption maxima (\(\lambda_A\)), and calculated extinction coefficient (\(\varepsilon\)). 2) Calculated using Ppy QY of 10\%, QD(530) QY of 27\%.

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To further study the transparent qdot linker idea, we employed Ppy with the standard firefly luciferin (LH\textsubscript{2}), which produces bioluminescence at \(\sim 547\) nm\textsuperscript{4,21,37,39}. Here, Ppy-LH\textsubscript{2} and QD(530) have an even smaller overlap (Table 4.4), since QD(530) absorbs at energy higher than Ppy emission (Figure 4.9c), Ppy-LH\textsubscript{2}-QD(530) BRET does not occur.
Table 4.4: Optical and FRET parameters for BRET-FRET nanosystem using QD(530).
1) First absorption maxima ($\lambda_A$), and calculated extinction coefficient (e). 2) Calculated using Ppy QY of 32 %, QD(530) QY of 27 %.

<table>
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Figure 4.9d shows Ppy-LH$_2$-QD(530) emission, which consists of just Ppy-LH$_2$ emission. However, when the tagRFP is attached, the Ppy-LH$_2$-QD(530) conjugate shows high BRET. Since the qdot is not participating in the energy transfer, we refer to this system as BRET instead of BRET-FRET. These conjugates were found to be remarkably efficient, with BRs = 9.2 being measured. These BR are higher than those for the Ppy-QD(556)-tagRFP conjugate described in figure 4.6. This suggests that the inefficient energy step in these BRET-FRET processes may be the Ppy-qdot BRET step. This is likely related to much longer lifetimes of the qdot/qrod (>10 ns)$^{1-3}$ compared to tagRFP (~3 ns),$^{27}$ which results in a significant bottleneck in the process.
**Figure 4.9:** (a) Scheme of Ppy-QD(530)-tagRFP conjugate and idealized energy transfer diagram using LH2 substrate. (b) TEM micrograph of QD(530). (c) Spectral overlaps for BRET between Ppy + LH2 and QD(530), showing much decreased overlap (i), and FRET between Ppy + LH2 and tagRFP (ii). (d) BRET emission spectra for Ppy + LH2-QD(530) conjugate (i), and for BRET of Ppy + LH2-tagRFP conjugate at \([\text{tagRFP]} : [\text{Ppy + LH2}] : [\text{QD(530)}] = 1 : 1 : 1\) (ii).

### 4.5 Summary and Conclusions

In summary, we have shown that by simultaneously conjugating Ppy and tagRFP to qdots or qrods, that sequential BRET-FRET energetransfer can easily be produced. This approach has a number of advantages, including the ease of attaching 6xHis-tagged proteins to the qdot or qrod, which allows for conjugation with multiple donors or acceptors without the need for multistep linking, purification, or fusion expression. Additionally, the qdot morphology can be
tuned to tailor energy transfer, by changing either spatial properties via morphology changes, or electronic changes, via size. Moreover, the qdots can be prepared to act as so-called transparent substrates in multi-step energy transfer processes. This work may lead to future studies utilizing multiple qdot/qrod acceptors, or nIR emitting proteins; thus opening up new paradigms in sensing, bio-diagnostics, and imaging applications.

4.6 References


Chapter 5

Expression of Recombinant Human Haptocorrin in \textit{P. pastoris} and Human Embryonic Kidney Cells, and Using it to Target the Asialoglycoprotein Receptor for Cellular Imaging Studies

In this chapter I describe the cloning, expression, and purification of recombinant human haptocorrin (hHC) in the methylotrophic yeast \textit{P. pastoris}, as well as in Human Embryonic Kidney (HEK) cells using immobilized metal affinity chromatography (IMAC), and vitamin B_{12} affinity chromatography. The production of HC was confirmed with western blotting (both for the hexa-histidine tag, as well as by a primary hHC antibody) and mass spectrometry. Purified HC was then used to target the asialoglycoprotein receptor in human carcinoma (HEPG2) cells.

5.1 Introduction

The uptake of dietary vitamin B_{12} (cobalamin, Cbl) is a complex pathway tightly controlled by three immunologically distinct proteins: haptocorrin (HC), intrinsic factor (IF), and transcobalamin II (TCII). HC is a \( \sim 65 \) kDa glycoprotein (47 kDa without glycosylation) secreted by the salivary glands that initially binds Cbl and is responsible for transport from the mouth, through the stomach, and to the duodenum where Cbl is released due to an increase in pH and proteolytic degradation of HC.\(^1\) Cbl is then bound by IF (\( \sim 50\)kDa) and endocytosed by the cubilin amnionless (CUBAM) receptor for transport across the ileal epithelium. Cbl is released from degraded IF in the lysosome where it is either picked up by TCII and released into the bloodstream, or released into the bloodstream through the multidrug resistant protein receptor and then picked up by TCII. The Cbl-TCII complex then binds to receptor CD320 (TCII receptor) to deliver Cbl to deficient cells.\(^2,3\)
The name haptocorrin became standard nomenclature in the 1980’s to consolidate proteins with identical protein backbones (TCI, TCIII, R-binder, salivary binder, and cobalophilin). HC has the least substrate specificity of the known Cbl binding proteins. While IF and TCII will only bind cobalamins, HC is also able to bind other corrinoids; Cbl analogs unable to function as coenzymes, which has been suggested to constitute 40% of plasma corrins. HC is evolutionarily the youngest of the trio of Cbl binding proteins having evolved from a gene duplication of IF, which evolved from a gene duplication of TCII. HC presence has been shown in a number of different higher mammals, and interestingly mouse TCII has recently been shown to have HC characteristics. A single Cbl binding protein has been identified in the rainbow trout.

The interest in HC in the scientific community has greatly increased after it was noted that many tumors de novo express surface bound HC, making it an attractive imaging target, due to its ability to recognize Cbl analogs. There are also conflicting reports of HC being an antimicrobial agent, with positive results looking at its potential role in breast milk against E. coli, but minimal effect shown against a panel of commercial pathogens. Depending on its origin, different glycoforms of HC have been identified that only differ in sialic acid content. While there is no known receptor for HC with high sialic acid content, HC that lacks sialic acid is rapidly cleared by the asialoglycoprotein receptor in the liver.

While knowledge of HC has increased over the past 30 years, a major hurdle in fully investigating the protein has been a readily available source. At the beginning of this work, there was no recombinant expression system for HC, and its study was limited by the necessity to purify it from saliva and human or rabbit serum. TCII and IF have previously been expressed in Pichia pastoris at 1-10 mg/L, which has facilitated the elucidation of the crystal
structure for each protein,\textsuperscript{22-23} as well as Cubilin (Cub) domains 5-8 of the IF-Cub complex.\textsuperscript{24} To this end, in this chapter I will describe the cloning, expression, and purification of HC in \textit{P. pastoris}, as well as the expression of HC in HEK cells,\textsuperscript{19} which were developed while we were working on the \textit{P. pastoris} system, and ultimately allowed the HC crystal structure to be solved.\textsuperscript{25}

5.2 Materials and Methods

5.2.1 Materials:

Enzymes were purchased from New England Biolabs. Ampicillin, yeast extract, and bacto peptone were purchased from EMD. His-6x and TCI antibodies were purchased from Abcam (Cambridge, MA). NaOH, and KH$_2$PO$_4$ were purchased from BDH. Antifoam C was purchased from J.T. Baker. Biotin was purchased from Sigma-Aldrich. Yeast nitrogen base was purchased from Invitrogen. Chemiluminescent substrate was purchased from Thermo scientific. Fetal Bovine Serum (FBS) and Penn-Strep were purchased from Invitrogen. Dulbecco’s Modified Eagle Medium (DMEM), F12K Medium, and Chinese Hamster Ovary (CHO) Cells were purchased from the American Type Culture Collection (ATCC). Liver Hepatocellular carcinoma (HepG2) cells were obtained from the Hougland lab at Syracuse University. Human Embryonic Kidney (HEK)293 GnTI, HEK293 GnTI hHC, and HEK293 hHC cell lines were obtained from Dr. Schibli at the University of Zurich, Switzerland.

\textit{Strains, plasmids, and culture media:} \textit{E. coli} DH5\textalpha were used for plasmid propagation. \textit{P. pastoris} KM71 cells and the pPIC9 plasmid were purchased from Invitrogen. The \textit{P. pastoris} tRNA optimized HC gene with a C terminal hexa-histidine tag was purchased from GenScript in a pUC57 plasmid (pUC57_hHC). Luria-Bertani medium was used for the growth of \textit{E. coli} in liquid culture. Yeast extract peptone dextrose (YPD), buffered glycerol-complex (BMGY), and
buffered methanol-complex (BMMY) media were prepared according to the *Pichia* expression manual.

### 5.2.2 Methods:

*Construction of expression plasmids:* The pUC57_HC was transformed into chemically competent DH5α cells, plated on LB ampicillin (Amp) plates, and grown overnight. A colony was then grown in five mL of LB Amp for 16 hours. After growth, the plasmid was extracted using a Wizard DNA extraction kit. The isolated plasmid was then double digested with *EcoRI* and *NotI* and purified by gel electrophoresis. pPic9 was triply digested with *EcoRI*, *NotI*, and calf intestinal phosphatase (CIP) and purified in the same manner. The digested DNA was then extracted using a Qiagen gel extraction kit. The pPic9_HC plasmid was then constructed using T4 ligase at 15 °C for four hours in 1:1, 3:1, 5:1, and 10:1 gene to plasmid ratios. The ligation mixture was then transformed into chemically competent DH5α *E. coli*, plated on LB Amp plates, and grown for 16 hours at 37 °C. Colonies were then picked, the plasmid was isolated and double digested with *EcoRI*, and *NotI* to identify correct gene insertion. Plasmids that showed a diagnostic cut were sent to Genewiz for sequencing and confirmed correct gene insertion.

*Transformation of *P. pastoris*: The pPIC9_hHC expression plasmid was linearized with SacI and then transformed into *P. pastoris* KM71 by electroporation. The transformed cells were then plated and selected based on slow grown on histidine deficient plates. Colony PCR was conducted to select colonies contained the HC gene.

*Flask Expression of Haptocorrin in *P. pastoris*: *P. pastoris* transformed with pPic9_hHC was cultured in shake flasks. Colonies were inoculated into 10 mL BMGY medium in a 125 mL baffled flask and grown at 28 °C in a shake incubator (300 rpm) for 16 hours. 750 μL of the culture was then used to inoculate 750 mL of BMGY medium with 100 μL of antifoam in a 2 L
baffled flask. The culture was then grown for 48 hours. The cells were then harvested by centrifugation at 5,300 rpm for 5 minutes and transferred into 150 mL of BMMY medium in a 1 L baffled flask. Methanol was added to a final concentration of 0.5% every 24 hrs to maintain induction for 48-72 hrs.

*Fermentation Expression of Haptocorrin in P. pastoris:* Expression cultures were initially grown in 100 ml of BMGY for 16 hrs at ~28 °C to obtain cell density. Fifty mL of the initial culture were then used to inoculate 700 mL of fermentation basal salts in a one-liter bioreactor and allowed to grow until the initial glycerol was exhausted (shown by a sharp spike in dissolved oxygen). Fifty percent glycerol was then added at 13.65 mL/hr for 4-8 hrs to increase cell density. The glycerol feed was then stopped and after a second spike in the dissolved oxygen a 0.75 mL/hr methanol feed was initiated. The culture was then grown on 0.7-2.1% methanol per liter per hour for 88-100 hours.

*Mammalian Cell Culture Conditions:* All cell cultures were incubated in a mammalian cell incubator maintained at 37 °C with 5% CO₂ and 95% humidity. Cell cultures were grown in BD Falcon cell culture bottle with vented lids. Subcultures were made as follows: At ~80% confluence the complete growth media was removed and discarded. The adherent cells were then harvested using five mL of Cellstripper and incubated for 25 minutes. The flask was then rinsed, and the cells were transferred to a 15 mL conical tube, and were collected via centrifugation. Resuspension of the cells was achieved by gentle aspiration using a pipette in one mL of complete medium. 100-250 μL of this solution were then added to a new flask containing 30 mL of complete growth medium. Cell stocks were prepared by making a 5 % v/v DMSO solution containing 1-2 million cells.
The Chinese Hamster Ovary (CHO) cell line was cultured as an adherent monolayer in F12K Ham’s media. HEK293 GnTI and HEPG2 cell lines were cultured in DMEM. All media was supplemented with 10,000 units penicillin, 10 mg/mL streptomycin, and 10 % (v/v) FBS.

*Expression of Haptocorrin in HEK293 hHC:* HEK293 and HEK293 GnTI cells stably transfected with the Haptocorrin gene were provided by Dr. Schibli at the University of Zurich. These cells were cultured in DMEM as described above with the addition of 400 μg/mL of Zeocin. The cells were cultured as described above until the cells reached a density great enough to seed 20x 250 mL flasks (600 mL of complete medium) at which time the cells were incubated for 7-8 days to allow for HC expression.

*Purification of Haptocorrin from P. pastoris:* After expressing hHC as described, the culture was centrifuged at 10,500 g for 30 minutes. The supernatant was filtered through a 0.45 μm membrane and the filtrate was then purified by either immobilized metal affinity chromatography (IMAC) on a Ni$^{2+}$ column, or vitamin B$_{12}$ affinity chromatography using an ATKAprime (GE Akta).

For IMAC purification the filtrate was loaded over a five mL Ni$^{2+}$ column at 2 mL/min. Buffer A was 0.5 M NaCl 10 mM phosphate buffered saline (PBS) (pH 7.4). Buffer B was 0.5 M NaCl 10mM PBS, and 250 mM imidazole (pH 7.4). The sample was loaded using a 150 mL super loop (GE Atka). Binding conditions were 2 mL/min with 100% buffer A. Elution conditions were initially 5 mL/min with 90 % A, 10 % buffer B, followed by elution with 100% buffer B. The elutant was then dialyzed against PBS and concentrated using a 30 kDa spin filter.

For B$_{12}$ affinity chromatography the sample was loaded over a column loaded with B$_{12}$ agarose at 2 mL/min. Buffer A was 0.5 M NaCl 10 mM (PBS) (pH 7.4). Buffer B was 0.5 M NaCl 10 mM PBS, and 6 M urea (pH 7.4). Binding conditions were 2 mL/min with 100% buffer
A. Elution conditions were initially 5 mL/min with 84 % A, 16 % buffer B, followed by elution with 100% buffer B. The eluant was then dialyzed against 4.5, 3, 1.5, and 0 M urea in PBS and concentrated using a 30 kDa spin filter.

Purification of Haptocorrin from HEK293 and HEK293 GnTI cells: Haptocorrin was purified from the media of stably transfected HEK cells. This purification was accomplished by first filtering the media through a 0.45 μm filter, the filtrate NaCl concentration was increased to ~0.5 M though the addition of PBS with 5 M NaCl to limit non-specific binding during IMAC or B12 affinity chromatography.

Initial IMAC purification was achieved by loading the filtrate over a five mL Ni²⁺ column at 2.5 mL/min. Buffer A was 1.0 M NaCl 10 mM phosphate buffered saline (PBS) (pH 7.4). Buffer B was 1.0 M NaCl 10mM PBS, and 500 mM imidazole (pH 7.4). The sample was loaded using a 150 mL super loop (GE Atka). Binding conditions were 2.5 mL/min with 100% buffer A. Elution conditions were initially 2.5 mL/min with 96 % A, 4 % buffer B, followed by elution with 100% buffer B. The eluant was then dialyzed against PBS and subjected to subsequent B12 affinity or gel permeation chromatography.

For vitamin B12 affinity chromatography the sample was loaded over a column loaded with B12 agarose at 2 mL/min. Buffer A was 1.0 M NaCl 10 mM sodium phosphate (pH 7.4). Buffer B was 0.5 M NaCl 10 mM sodium phosphate, and 6 M urea (pH 7.4). Binding conditions were 2 mL/min with 100% buffer A. Elution conditions were 2 mL/min with 100% buffer B. The elutant was then dialyzed against 4.5, 3, 1.5, and 0 M urea in PBS and concentrated using a 30 kDa spin filter.

For gel permeation chromatography the elution from the initial IMAC purification was first concentrated to ~500 μL using a 30 kDa molecular weigh cut off filter. The concentrated
sample was then loaded over S-100 and S-200 sephacryl columns (GE Healthcare) connected in series to increase separation at 0.3 mL/min in 100 mM NaCl 10 mM PBS (pH 7.4).

**Western Blot Conditions:** Samples were run on a 12% acrylamide gel and then transferred to a nitrocellulose membrane using an iBlot (Invitrogen) dry blotting system. The membrane was then blocked in a 5% nonfat powdered milk PBS-T (100 mM NaCl 100 mM sodium phosphate 0.5% v/v TWEEN® 20) solution (w/v) for one hour at room temperature. For the HRP-His 6x antibody, the primary antibody was then diluted in fresh 5% nonfat milk PBS-T at 7500:1 and rocked overnight at 4 °C. The membrane was then rinsed 6 times for 15 min with fresh PBS-T. For the TCI antibody, the primary antibody was diluted in fresh 5% nonfat milk PBS-T at 1000:1 and rocked overnight at 4 °C. The membrane was then rinsed 6 times for 15 min with fresh PBS-T. The membrane was then rinsed with HRP-secondary antibody for one hour and then washed as before. The asialoglycoprotein receptor western blot was done in the same manner as the TCI. After each respective method the membrane was visualized by chemiluminescence.

**5.2.3 Instrumentation**

**Circular Dichroism:** Circular dichroism (CD) of HC was preformed on a Jasco J-715 spectrophotometer with the following parameters: 1.0 nm; scan rate, 20 nm/s; time constant, 8 s; room temperature, 23 °C; scan range, 190 to 260 nm. Protein concentrations were quantified by a Bradford assay (Bio-Rad). Secondary structural analysis was performed using CDPro software and the CONTIN analysis program.
5.3 Results and Discussion

5.3.1 Expression of Recombinant Human Haptocorrin in the Methyotropic Yeast *P. pastoris*

*Insertion of the C-terminal Hexa-histidine Haptocorrin gene into the pPIC9 plasmid:* The expression of haptocorrin (HC) was briefly attempted in *E. coli* with the addition of a N-terminal chitin-binding domain. This expression ultimately proved to be unsuccessful, and expression trials were moved into an Eukaryotic system. The methylotropic yeast *P. pastoris* has been shown to be an effective tool for the expression of proteins not compatible with traditional *E. coli* expression. The advantages of switching to a yeast host include the ability to create disulfide bonds, (*E. coli* has a high reduction potential in the cytoplasm of greater then -500 mV) as well as the incorporation of carbohydrates onto the protein backbone increasing solubility/stability. The advantage of specifically using *P. pastoris* is that it tends to not hyper-glycosylate proteins and has a strongly controlled promoter tied to its ability to survive on a single carbon source.

A HC gene isolated from the cDNA of *Sus scrofa* was generously provided by Dr. Alpers of the Washington University School of medicine. This gene was used for initial expression attempts in *P. pastoris*, but ultimately proved to be unsuccessful. Therefore, a *P. pastoris* tRNA optimized human HC (hHC) gene with a C-terminal hexa-histidine tag was purchased from GenScript (pUC57_hHC) in the pUC57 plasmid. This plasmid was transformed into DH5α chemically competent cells, plated, and grown overnight on LB-amp agar plates. The pUC57_hHC plasmid was then extracted as before. pUC57_hHC was then double digested with *EcoRI* and *NotI* at 37 °C for one hour (Figure 5.1). The pPIC9 plasmid was triple digested with *EcoRI*, *NotI*, and CIP. The digested DNA was then run down an agarose gel, visualized, cut out, and extracted using a gel extraction kit. The extracted, digested DNA was then ligated together
using T4 ligase at 15 °C overnight. The ratios of HC to pPIC9 were 1:1, 3:1, 5:1, and 10:1 based on the molar concentrations. After ligation, the ligation mixture was transformed into DH5α chemically competent cells, plated, and grown overnight on LB-amp agar plates. A single colony was found after incubation. This colony was picked and grown in a 5 mL LB-amp culture overnight. The pPIC9_hHC plasmid was then extracted using a Wizard DNA extraction kit. This extraction was then linearized with SacI and transformed into *P. pastoris* via electroporation.

**Figure 5.1:** Cloning of hHC and insertion into the pPIC9 shuttle vector: (Left) Triple digestion of pPIC9. (Center) Double digestion of pUC57_hHC. (Right) Double digestion of pPIC9_hHC.

**Insertion of the N-terminal Hexa-histidine Tagged Human Haptocorrin in the pPIC9 Plasmid:** Primers were designed for the cloning of HC out of pUC57_hHC into the pPIC9 plasmid with an N-terminal hexa-histidine tag. The forward and reverse primers were 5’-GAT CGA ATT CAT GCA TCA CCA TCA CCA TCA CCA CCG TCA GTT GTC TCA T-3’ and 5’-GAT CGC GGC CGC TTA GTG ATG GTG ATG GTG ATG GTA TTT GCT CC-3’ respectively. The PCR reaction was run for 35 cycles with a 1 minute 95 °C melting temperature, a one minute 63 °C annealing temperature, and one minute 15 second 74 °C elongation temperature. PCR was conducted with 1, 3, 5, and [10 ng/µL] of DNA in solution with deep vent DNA polymerase
(Figure 5.2 bands A-D). PCR bands were isolated and ligated with the pPIC9 vector as before to construct the pPIC9 NhHC plasmid (Figure 5.2 bands E-L). The pPIC9 NhHC vector was then transformed into \textit{P. pastoris} in the same manner as pPIC9_hHC.

![Figure 5.2: Cloning of N-terminal His hHC and insertion into the pPIC9 shuttle vector: PCR of N-terminal His6xHC (A-D), triple digestion of pPIC9 with \textit{EcoRI}, \textit{NotI}, and CIP (E), and the double digestion of hHC with \textit{EcoRI} and \textit{NotI} (F) of pUC57_hHC. The diagnostic digest of plasmids isolated from colonies after ligation using \textit{EcoRI} and \textit{NotI} (G-K).]

### 5.3.2 Haptocorrin Benchtop Expression Trials in \textit{P. pastoris}

\textit{Trials with pPIC9\_hHC in P. pastoris}: After transfecting \textit{P. pastoris} with pPIC9_hHC, expression trials were conducted with two colonies that showed evidence of gene insertion with colony PCR. Expression was attempted at a pH of 3 or 6 to determine the optimal conditions for protein production. The pH three expressions were attempted due to the natural environment of HC, being able to survive in the low pH environment of the stomach, as well low pH inhibition of proteases that lead to protein degradation.

Initial cell density was achieved by growing a colony in 25 mL of BMGY in a 125 mL baffled flask for 40 hrs. The media was then centrifuged to pellet the cells. The cells were then
resuspended in 200 mL of BMMY (pH 3) in a 1 L baffled flask and allowed to express for 48 hrs at ~28 degrees with additional MeOH added at 24 hrs. After expression, the media was centrifuged to pellet the cells, the media was then decanted, the pH was adjusted to 7.4 with ammonium hydroxide, and then the media was filtered to remove any precipitate. The filtered media was then loaded on a 1 mL IMAC column at 0.3 mL/min using IMAC binding buffer and then eluted with 250 mM imidazole. After elution, a twelve percent acrylamide gel was run, and bands were sent for mass spectrometric analysis to the laboratory for biological mass spectrometry at Texas A&M University (Figure 5.3). They were then analyzed by first being digested with trypsin, an enzyme that cleaves peptide bonds following arginine or lysine residues, allowing for a more reliable signature then looking for a single parent peak. Getting mass spectrometry of the digested hHC is also beneficial in that P. pastoris can glycosylate proteins, masking the true mass of the protein backbone.

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**Figure 5.3:** pH 3 expression of hHC purified by IMAC: The SDS-PAGE gel of a 48hr pH 3 expression. The band shown in red was sent for MALDI analysis and showed evidence of HC expression as 6 bands were found that matched the theoretical MW of HC fragments following
trypsin digestion. Gel lanes are: (A) the ladder, (B) the PIC, (C) FPLC flow through, and (D) the eluted fraction.

Expression was then scaled up and allowed to express for 48 hrs in BMMY (pH 6). A colony was picked and grown in 25 mL BMGY for 22 hrs. Ten mL of this culture was then transferred to 100 mL of fresh BMGY and allowed to grow for an additional 16 hrs. One hundred mL was then centrifuged to pellet the cells, the media was decanted, and then the cell pellet was resuspended in BMMY (200 mL medium in a 1 L baffled flask x 2), and allowed to express for 48 hrs. The media was then centrifuged, filtered, purified via IMAC, and a protein band at ~40 kDa was sent to Texas A&M to see if the percent mass coverage could be improved (Figure 5.4). This resulted in us achieving 29% mass coverage, including the C-terminal his-tag, providing strong evidence for hHC expression.
Figure 5.4: 400 mL expression, purification, and MALDI MS Confirmation of HC: (Top) An FPLC trace showing the purification of HC via IMAC (top). A SDS-PAGE gel showing hHC expression and purification. Lane A is the ladder, B is the PIC, C is the crude media, D is the FPLC flow through, and E is the imidazole eluted fraction (bottom left). The dark band pointed out by the arrow was sent to Texas A & M for mass spectrometry identification and the identified fragments are shown (bottom right), with the corresponding protein sequence with detected portions highlighted in bold (bottom right).

Another purification method that was used was purifying the media over B12 agarose in a glass column. The column was first equilibrated with binding buffer (10 mM sodium phosphate, 1 M NaCl). The media was then flashed over the column; the column was rinsed with six 10 mL aliquots of binding buffer, and then eluted off of the column with 6 M urea. (Figure 5.5) The eluted fraction shows two prominent bands around 66 kDa.
**Figure 5.5:** SDS PAGE gel of B$_{12}$ agarose purified hHC: 1) Ladder, 2) PIC, 3) Crude Media, 4) Column flow through, 5) 1$^{st}$ rinse, 6) 2$^{nd}$ rinse, 7) 3$^{rd}$ rinse, 8) 4$^{th}$ rinse, 9) 6 M Urea elution 1.5 hrs on column, 10) 6 M urea elution 18 hrs on column

After the successful use of B$_{12}$ to purify hHC, a column was constructed to better control the purification conditions. A culture was purified over an in-house constructed B$_{12}$ based affinity column, using 6 M urea as eluent, as described before (Figure 5.6)
Figure 5.6: FPLC B$_{12}$ purification of hHC: After constructing a B$_{12}$ purification column (upper right) media from a 450 mL expression was passed over the column using the FPLC resulting in the above UV trace (Upper right). The corresponding SDS-PAGE gel (bottom) shows similar results to the initial glass column purification. A) Ladder, B) PIC, C) Crude Media, D) Column flow through, E) 6 M urea elution.

This method was then utilized two more times, the samples were dialyzed, and then concentrated using a 30 kDa MWCO filter to concentrate the samples, and 5 μg of each run was used for western blotting with both His 6x and TCI primary antibodies. (Figure 5.6) Conditions for the His 6x primary antibody were a 5000:1 dilution of the primary antibody with a one hour room temperature wash. Conditions for the TCI primary antibody were a 2000:1 dilution of the primary antibody at room temperature, and a 5000:1 dilution of the secondary antibody. All membranes were blocked in PBS-T with 5% fat free milk, and visualized using chemiluminescence (Figure 5.7).
Figure 5.7: His 6x and TC1 western blots of hHC: A) Ladder, B-D) 3 different purifications that were eluted off of a B_{12} column and concentrated with a spin filter.

For HC expression optimization, *P. pastoris* was grown in BMGY, the cells were harvested, and then transferred into pH 6 and pH 3 BMMY to induce express. The cultures were grown for 96 hours with time points being taken every 24 hours. (Figure 5.8) The western shows that the dominant hHC bands at ~66 and 75 kDa are the most prominent after 72 hours of growth when cultured in pH 6 BMMY, and that negligible hHC production is observed when cultured at pH 3.
Figure 5.8: His 6x western HC time course: 1 – Pre induction control, 2 – pH 6 24 hours, 3 – pH 6 48 hours, 4 – pH 6 72 hours, 5 – pH 6 96 hours, 6 – pH 3 24 hours, 7 – pH 3 48 hours, 8 – pH 3 72 hours, 9 – pH 3 96 hours.

For circular dichroism studies, media was purified via fast protein liquid chromatography (FPLC) on either a B12 column or IMAC column (Figure 5.9). The his 6x western shows that our recombinant his-tagged hHC is able to be purified and concentrated from the medium utilizing both B12 affinity and IMAC techniques and results in major bands at approximately 66 and 75 kDa. In addition to the His 6x antibody, western blotting was also conducted using an HC primary antibody to confirm protein production. The western band appears between 50 and 75 kDa, consistent with the bands that were imaged using the His 6x antibody.
**Figure 5.9:** Top left: An FPLC trace of hHC purified via B_{12} affinity chromatography. Top right: An FPLC trace of HC purified by IMAC. Bottom: His6x western blot of B_{12} purification. 1 – Pre induction control, 2 – crude media, 3 – column flow through, 4 – 1 M urea elution, 5 – 6 M urea elution. His6x western blot of IMAC purification. 6 – crude media, 7 – column flow through, 8 – 25 mM imidazole, 9 – 250 mM imidazole

Purified HC was analyzed by circular dichroism to better understand its secondary structure. Analysis of the eluted fractions (Figure 5.10) identifies the B_{12} eluted fraction to contain: 6.6% alpha-helical, 41.8% beta-pleated sheet, 19.9% turn, and 31.7% undefined. The IMAC eluted fraction contains: 6% alpha-helical, 40.3% beta-pleated sheet, 24.1 turn, and 29.6% undefined.
Figure 5.10: Circular dichroism spectra of hHC [~10 μg/mL] after purification (B₁₂ column – blue, IMAC column – green). The samples were dialyzed from PBS (pH 7.4) using 50 kDa molecular weigh cut off tubing into distilled water prior to analysis.

Due to the degradation witnessed in the western blots, optimization of the expression conditions was attempted by changing expression time, pH, and temperature. Four cultures were set up with media and pH 6.2 and 7.2 and temperatures at 15 and 30 °C. A western blot corresponding to these trials can be seen in Figure 5.11. While there appears to be many ghost bands, the broad bands in G and H around 75 kDa in the pH 7 media at 15 °C seemed to be the most encouraging on a small scale.

Figure 5.11: A western blot of hHC temp and pH trials: Expression of hHC was attempted at pH 6.2 (A-E) and 7.2 (F-J) at different time points and temperatures. The PIC is show in lanes A and F with 15 °C time points at 24 hrs (B and G), 48 hrs (C and H) and 30 °C time points at 24 hrs (D and I), and 48 hrs (E and J).

Trials with pPIC9 NhHC in P. pastoris: While working on this project a paper was published on the expression of recombinant human haptocorrin in HEK293 cells. This system expressed using the pcDNA4/myc-His A vector which expresses the protein of interest with a C-
terminal thrombin cleavage site, myc epitope, and a hexa-histidine tag. While this expression system worked well, the resulting HC had a reduced binding affinity to HC for B₁₂ by ~ two orders of magnitude. Following cleavage of the tags using thrombin, an extra 4 amino acids remain on the C-terminus, which we believe is what is responsible for the reduced binding affinity. In order to see if our hypothesis was correct, and N-terminal His-tag HC vector was constructed and transformed into *P. pastoris*.

Due to the degradation that was shown in the C-terminal His-tagged western blots, I attempted to optimize the expression of both constructs by changing the expression time, expression temperature, and the pH of the media (Figure 5.12 pH 6 and 5.13 pH 7).

**Figure 5.12:** Expression of C and N terminal His-tagged HC at pH 6: HC expression was attempted at pH 6 at different temperatures to optimize expression conditions. Protein eluted off of an IMAC column and visualized by western blot using both the HC primary antibody and His-6x primary. The C-terminal His-tagged HC is shown expressed at 15 °C (A) and 30 °C (B) and N-terminal his-tagged HC expressed at 15 °C (C) and 30 °C (D).
5.3.3 Fermentation of hHC to increase yield:

One advantage of *P. pastoris* over higher Eukaryotes is the ability to do protein expression under fermentation conditions, vastly increasing the cell density over shake flask expression. To this end the hHC has been expressed and purified in a one-liter bioreactor to see if fermentation is a feasible means for its expression (Figure 5.14).
Figure 5.14: Fermentation and purification of hHC: The left trace shows real time feedback of the fermenter conditions: Dissolved oxygen is shown in blue and agitation is shown in red. A SDS-PAGE gel of a fermentation run A) Pre induction control, B) Crude Media, C) Media flowthrough, D) 25 mM imidazole rinse, and E) 250 mM imidazole elution.

It has been shown in the literature that changing the temperature and pH of the expression media can help with proteolytic degradation. To this end, the pH of the media was manipulated in order to try and optimize expression conditions. For these studies, 50 mL of shake flask culture was used to inoculate the bioreactor as before. For pH 3 expressions, the pH of the bioreactor was set at 3 during the glycerol feed phase, allowing the pH to drift down due to the growth of *P. pastoris*. For pH 7 expression, after the glycerol feed phase was terminated, the pH was increased by the addition of ammonium hydroxide. The fermentation media was then purified via FPLC using Vitamin B$_{12}$ affinity chromatography for the pH 3 samples, and IMAC chromatography for the samples expressed at pH 5 and 7 as described earlier (Figure 5.16).
Figure 5.16: Fermentation and purification of hHC at different pH: Samples purified via B_{12} affinity chromatography at pH 3 (A), and IMAC chromatography at pH 5 (B) and pH 7 (C). A TCI primary antibody was used for the western blot.

While none of the conditions produce the desired result (a narrow single band above 50 kDa), it is interesting that the pH 3 sample does correspond to intact protein shown by a broad band above 50 kDa. This is most likely due to the deactivation of neutral proteases due to the pH decrease. We also see a sharp band at ~60 kDa in the pH 7 sample that most likely corresponds to the intact protein, but is a minor species compared to the large streak between 30-50 kDa. While both of these media changes seem to have improved the quality of protein, they also greatly reduced the yield, so we moved on to expression in a mammalian cell host.

5.3.4 Expression of recombinant hHC in Human Embryonic Kidney Cells

*Construction of a N-terminal hexa-histidine Haptocorrin Expression Vector:* A mammalian expression vector was constructed using the optimized HC gene and the pPA-tagRFP-N expression vector. For this construct PCR primers were designed for the insertion of
EcoRI and BamHI digestion sites on the 5’ and 3’ ends of the gene respectively with a hexa-histidine tag added into the forward primer. PCR conditions were as follows: 95 °C denature for 1 min, 65.0 °C annealing for 1 min, and 74 °C elongation for 1.5 min for 35 cycles (Figure 4.21). The PCR product was then purified with agarose gel electrophoresis, and double digested with EcoRI and BamHI. The pPA-tagRFP-N_hHC construct was then prepared. This vector has been transfected into HEK293 cells and is currently undergoing G418 selection.

**Figure 5.17:** Cloning of N-Terminal His hHC and insertion into the pPA-tagRFP-N shuttle vector: PCR of N-terminal His6xJZHC (A-D), undigested pPA-tagRFP-N (E), triple digestion of pPA-tagRFP-N with EcoRI, BamHI, and CIP (F), and the double digestion of hHC with EcoRI and BamHI (G) of pUC57_hHC. The diagnostic digest of plasmids isolated from colonies after ligation using EcoRI and BamHI (H-L).

**Expression in HEK293 and HEK293 GnTI cells:** While I was working on the expression of HC in *P. pastoris*, the expression of HC was published by the Schibli lab using HEK293 cells\textsuperscript{19}. These cells, including HEK293 GnTI and HEK293 GnTI cells transfected with HC were generously provided from their lab. Similar to our expression in *P. pastoris*, the HC is secreted from the cells and purified from the cellular media. Of the two cells lines, we initially expressed HC from the HEK293 GnTI cell line because they have had N-acetylg glucosaminyltransferase I
knocked out, resulting in the expression of proteins without complex glycans. This knock out has proved to be successful when obtaining protein crystal structures, and we believed that this limited glycosylation would aid in later studies targeting the asialoglycoprotein receptor, which clears glycoproteins that do not posses terminal sialic acid residues.

For initial protein expression attempts, the HEK293 GnTI HC cells were grown and passed in DMEM media with 20 mM HEPES and 400 μg/mL Zeocin. Once the cells were passed into 12 flasks (30 mL of media/flask) the cells were stimulated with sodium butyrate and allowed to grow for 7 days. The media was then collected, clarified using a 0.45 μm filter, and then purified via IMAC (Figure 5.18).
Figure 5.18: Purification of HC from HEK293 GnTI cells: HEK293 GnTI cells were stimulated with sodium butyrate for 7 days. The media was then purified by IMAC (top) and visualized via SDS-PAGE and western blot. The lanes are: Crude media (A), flow through (B), 20 mM imidazole rinse (C), and 500 mM imidazole elution (D).

While the HC is present at ~60 kDa as one band, there are still a few bands of undesired proteins that co-eluted from the IMAC column. The eluted fraction was therefore concentrated with a 35 kDa spin filter and loaded on an S-200 gel permeation column to attempt to separate out the protein bands based on molecular weight (Figure 5.19).
The expression of HC from the HEK293 cells was also undertaken. These cells express HC with the native glycosylation pattern so it is expected to have a larger molecular weight due to the presence of more carbohydrate. The expression was conducted as described earlier, and the media was purified using B₁₂ affinity chromatography (Figure 4.24). After the media was loaded over the column, the B₁₂ column was rinsed with 1 M urea, and then HC was denatured with 6 M urea and eluted off of the column. Following the elution, the protein was dialyzed against 4.5, 3 and 1.5 M urea, followed by 1 mM phosphate buffer. The sample was then lyophilized and analyzed (Figure 5.20).
Figure 5.20: B$_{12}$ Purification and analysis of hHC expressed from HEK293 cells: HC was purified from the media using B$_{12}$ affinity chromatography (top) and the corresponding silver stained SDS-PAGE gel. The purification was then analyzed with western blotting using both the His 6x and TCI primary antibodies.

While the B$_{12}$ column does give a more pure product than the initial IMAC conditions, as well as showing that the HC is functional, it is a slow process that requires the denaturing of the protein. Therefore, it would be beneficial to be able to utilize the histidine tag for purification. To this end, the salt concentration (NaCl) of the binding and elution buffer was increased from 500 mM to 1 M, the NaCl concentration of the media was raised from ~100 mM to 500 mM, and
imidazole was added to the media up to a concentration of 10 mM. In addition, the column was rinsed with 40 mM imidazole instead of 20 mM imidazole before the final elution (Figure 5.21).

**Figure 5.21:** IMAC Purification of hHC from HEK293 cells: IMAC purification HC: Crude (A), FT (B), 40 mM imidazole rinse (C), and 500 mM imidazole elution (D). Fraction C and D were also analyzed by TCI western blot.

To further purify the recombinant haptocorrin it was subjected to a second purification step using $\text{B}_{12}$ affinity chromatography (Figure 5.22).
In addition to purification via B$_{12}$ affinity chromatography, gel permeation chromatography was also used for subsequent purification after initial IMAC purification. Since minimal success was seen using a S-200 column with HC expressed in the HEK293 GnTI cells (Figure 4.19), a S-100 and S-200 were connected in series to provide better separation based on size, and then analyzed by SDS-PAGE and His 6x western blot (Figure 4.23). Briefly, 600 mL of HEK293_hHC media was loaded over an IMAC column, rinsed with 20 mM imidazole, and then eluted with 500 mM imidazole. The eluted fraction was then concentrated with a 35 kDa spin filter from 30 mL to ~450 μL, and was then loaded onto the gel permeation column. While the western blot shows that hHC is present in all the eluted fractions, it is apparent that all the bands present in the SDS-PAGE ranging between 65 and 75 kDa in fractions 32-34 are present in the western blot (Figure 4.23).
Figure 5.23: Purification of HC from HEK293 cells using IMAC and gel permeation chromatography. (C) Concentrated IMAC elution and (26-34) fractions from the gel permeation column. Western blot conditions: 5000:1 His6x primary.
Figure 5.24: A SDS-PAGE gel and western blot showing the purification of hHC using IMAC and gel permeation chromatography. (A) HEK293 crude media, (B) IMAC Flowthrough, (C) 20 mM imidazole rinse, (D) 500 mM imidazole elution, (E) Concentrated elution, (F) Fraction 33, (G) Fraction 33 concentrated, (H) Fraction 33 concentrated and treated with neuroaminase.

5.3.5 Cellular Internalization Studies using Alexa Fluor® 680 tagged HC

In addition to the stably transfected cells, we were also able to obtain a small amount of purified hHC from the Schibli group. This has allowed for studies using hHC to target the asialoglycoprotein receptor, which is expressed in liver cells. While not a specific receptor for hHC, it is responsible for the clearance of proteins marked for destruction by the removal of their terminal sialic acid, exposing galactose residues, which the receptor recognizes. In order to confirm that HEPG2 cells expressed the asialoglycoprotein receptor, the cell lysate of HEPG2, HEK293 GnTI, and CHO cells were tested using a western blot (Figure 5.26). Interestingly, we see a faint band in the HEK cells, which have been a common negative control for such studies, as well as a strong band in the CHO cells.
Figure 5.25: A western blot of cellular lysate of (A) HEPG2, (B) HEK293 GnTI, and (C) CHO cells (right). A scheme showing an example of complex glycans common in mammalian proteins, and the effect of treatment with neuraminidase (right).

In order for HC to be recognized by the asialoglycoprotein receptor it must first be treated with the enzyme neuraminidase (nHC) to remove the terminal sialic acid residues (figure 5.25). After digestion, the HC and nHC were then dialyzed in carbonate buffer (pH 8.3) in 50 kDa molecular weigh cut off tubing to increase the pH as well as the removal of the 44 kDa enzyme. The HC and nHC were then mixed with amine reactive Alexa Fluor® 680 (AF680) for one hour at room temperature, and then dialyzed into PBS (pH 7.4) for removal of non-conjugated AF680. The samples were then analyzed via SDS-PAGE and western blotting (Figure 5.26).
Figure 5.26: SDS-PAGE gels of HC (A), nHC (B), HCAF680 (C), and nHCAF680. Gels are: An unstained SDS-PAGE gel showing the the AF tagged HC and nHC including an inset of the AF680 excitation and emission (left), the same gel coomassie stained (middle), and a western blot using the HC antibody.

HC and nHC tagged with AF680 were then used for cellular internalization studies. For this study HC and nHC ~[250 nM] in DMEM cell media was mixed with HEPG2 cells for 2 hrs. These samples where then washed three times with PBS (pH 7.4) and used for imaging (Figure 5.26). In these images we can see no fluorescence in the HEPG2 control, minimal fluorescence in the HC control, and pockets of fluorescence present in the nHC sample, which should be readily internalized by the asialoglycoprotein receptor.
Figure 5.27: Confocal images of cells with a laser excitation at 633 nm and detection from 680-800 nm (spectra show in upper right). A HEPG2 control with no AF680 (a), HEPG2 HC AF680 (b), and nHC AF680 (c).

5.4 Summary and Conclusions

In summary, hHC has been successful expressed in the Eukaryotic hosts *P. pastoris* and HEK cells and has been characterized by SDS-PAGE, western blot, circular dichroism, and mass spectrometry. While hHC has been expressed in *P. pastoris* at 0.5-1.0 mg/L, ultimately it proved
to be readily degraded. Expression in HEK293 and HEK293 GnTI cells has been shown to be more stable, and has been purified from cellular media using IMAC and B₁₂ affinity, along with gel filtration chromatography. While a HC receptor has not yet been identified, the asialoglycoprotein receptor has been targeted with HC treated with neuraminase and tagged with AF 680 to visualize cellular internalization.

5.5 References


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Chapter 6

Conclusion and Outlook

Taken together this work has contributed to two different research fields and made a number of discoveries. First, I demonstrated that histidine can be utilized as an efficient molecule to facilitate phase transfer by complete removal of the organic capping ligands used in qdot synthesis, while retaining quantum yield, limiting qdot aggregation, and allowing for subsequent functionalization. 1D and 2D NMR gave insight into the binding mechanism of histidine and DHLA on the qdot surface, as well as the dynamic binding nature.

In addition to direct ligand exchange, I have also shown that polymer wrapping with a NTA analogue can bind with divalent metal cations (Ni$^{2+}$ and Zn$^{2+}$), facilitating the attachment of biomaterials.

I also showed the ability to clone, express, and purify recombinant tagRFP with a hexa-histidine tag in *E. coli*. TagRFP was then used to probe qdot surface chemistry with both the histidine- and NTA-qdots to determine the qdot-to-tagRFP distance through FRET studies. In addition, a two-step energy transfer system was designed and implemented that used a bioluminescent protein firefly luciferase, in this donor system the qdot/qrod acted as an energy acceptor/donor, and tagRFP acted as the final energy acceptor. By varying they qdot emission, we were able to show BRET-FRET.

Finally I showed the progress made on the expression and purification of human haptocorrin (HC). HC was cloned into the pPIC9 vector with both a C and N hexa-histidine tag, and expressed and purified in *P. pastoris*. After trying many different expression conditions, varying time, temperature, pH, and media, it was determined that while HC was being expressed
in 0.5-1.0 mg/L amounts, and verified by western blot and mass spectrometry data, it was being readily degraded. HEK 293 and HEK 293 GnTI cells transfected with the human HC gene were donated, and the hHC was expressed and purified with both the native (HEK293) and minimal (HEK293 GnTI) glycosylation variants using immobilized metal affinity chromatography (IMAC), B_{12} affinity chromatography, and gel permeation chromatography. Natively glycosylated HC was treated with neuraminidase, covalently tagged with Alexa Fluor® 680, and then exposed to human hepatocellular liver carcinoma (HEPG2) cells to visualize HC uptake through the asialglycoprotein receptor.

In the future, the two-step energy system will be explored using qrods with rod in rod morphology, which have previous been shown to be more efficient BRET acceptors. This will be accomplished by using a more red shifted fluorescent protein (E2-Crimson), which I have expressed and purified, in order to better understand and improve the BRET-FRET process. In addition, the expression of hHC will allow for further fundamental study of the protein, such as working towards the discovery of an HC receptor. Other work stemming from this project include ongoing work looking at HC’s potential to have bacteriostatic properties and the potential to be used as a scaffold for drug/antigen delivery through the gastrointestinal track, such as delivering a rotavirus vaccine into the intestine to illicit an immune response, or Zn^{2+} to fight zinc deficiency.
Curriculum Vitae

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Research Experience

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Quantum Dot Synthesis and Functionalization, UV-vis and Fluorescence Spectroscopy,
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Dynamic Light Scattering (DLS), Fourier Transform Infrared Spectroscopy (FTIR),
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Thin Layer Chromatography.

Publications

   M. A Modular Phase Transfer and Ligand Exchange Protocol for Quantum Dots. Langmuir,
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### Presentations


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References

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