PHOTOCLEAVABLE QUANTUM DOT-GOLD NANOPARTICLE SYSTEMS FOR SUPER-RESOLUTION IMAGING

Undergraduate Honors Research Thesis

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By

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Abstract

The ability to directly see structures and observe biological processes below the 200 nm resolution limit of traditional optical imaging methods would enable substantial advances in biology. Super-resolution imaging techniques use the bright/dark states of fluorescent probes and computer algorithms to image beyond that barrier by imaging probes in small groups rather than all at once. Quantum dots (QDs) have advantages over the florescent dyes and proteins currently used because of their increased brightness, stability, and resistance to photobleaching. However, they cannot be turned on and off stochastically. Here, we describe a QD-gold nanoparticle (AuNP) system that uses Förster (fluorescence) nonradiative energy transfer (FRET) for potential application in super-resolution imaging. When the composite is formed using a linker, it is dark, but when the linker is cleaved by light energy, QD fluorescence is restored. The initial conjugation was not successful because of instability of the AuNP. The AuNP was stabilized by using triethylene glycol mono-11-mercaptoundecyl ether, but no FRET was detected. Analysis with a transmission electron microscope demonstrated very few conjugated samples. FRET was tested using high concentrations of AuNPs and QDs, but without clear quenching. Future work would include using a shorter, reversible linker, such as molecules that change conformation upon UV light or shrinkable/stretchable polymers.

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Field of Study

Major Field: Chemical and Biomolecular Engineering

Minor Field: Entrepreneurship

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Chapter 1: Introduction

It would be very beneficial to see structures and observe important biological processes that current optical imaging methods cannot adequately image. For example, because of advances in microscopy and the utilization of fluorescent tagging, much information has been elucidated regarding the role of protein structures in transporting various cargos within the cell.[1] Yet there is still much missing information regarding how these transport vesicles navigate the vast cytoplasmic distribution network and supply chain in order to deliver the right material to the proper destinations.[1] Super-resolution imaging techniques can enable researchers to observe these small transport vesicles beyond the current limits of imaging technology.

A number of recent imaging techniques have been developed (e.g.: Stochastic Optical Resolution Microscopy [STORM] and fluorescence photoactivation localization microscopy [FPALM]) that have the ability to see objects as small as 20-60 nm.[2] The goal is to reduce that limit even further (e.g., 1-5 nm^2) and image *in vivo*, where light scattering in tissues and other limitations of fluorescent dyes and proteins have hampered progress. It is possible to view small structures with other imaging methods, such as with electron microscopy. But these techniques have limitations in live biological systems, such as requiring the sample to be fixed.[2] Fluorescence imaging, however, is much more biologically friendly.[2-3]

Our modification of STORM, QSTORM, utilizes the STORM imaging algorithms but uses (QDs) instead of fluorescent dyes and proteins. QDs are better because they are brighter and more resistant to photobleaching than conventional dyes.[4] These advantages would allow researchers to use QD-based imaging systems for longer periods of time (because more photons are collected) and obtain greater useful information than traditional fluorescence imaging agents.

Chapter 2: Background

2.1: Overview of STORM, QSTORM, and our nanoparticle system

Traditional fluorescent imaging techniques are limited to a resolution of no more than 200 nm (lateral direction).[5] The diffraction of light prohibits imaging below this diffraction limit, causing smaller spots to be seen as large blurs.[5] However, it possible to image below this physical diffraction limit by using super-resolution imaging techniques, such as the ones briefly reviewed below.

Stimulated emission depletion (STED) is capable of 20-45 nm resolution. Two lasers (one with its intensity focused on the center, the other with the intensity focused on the edges) excite the sample and non-liner emission occurs almost instantly. A zero-intensity region from the superimposing of the two laser pulses creates a donut hole and allows imaging below the diffraction barrier.[2]

Photoactivated localization microscopy (PALM), fluorescence photoactivation localization microscopy (FPALM), and STORM image single molecules.[2, 6] Such techniques are powerful, with potential to detect the location of a single molecule ~1nm in size if enough photons collected and if there are no emission interference within 200 nm from similar molecules.[2] This has been demonstrated by Gelles *et al* in their ability to

track kinesin-coated beads at 1-2 nm.[2,7] These super-resolution techniques rely on stochastic fluorescence.[2]

STORM, the technique used for this thesis, relies on the stochastic bright/dark states of particles and computer algorithms to reconstruct the image. The technique pinpoints the centers of blurry dots and thus gives a high–resolution image, even below 200 nm. STORM has been successfully used in the field. For example, one group imaged mammalian cells at 20-30 nm resolution.[3] For an example of superior STORM images compared to traditional fluorescence imaging methods, see Figure 1 below.

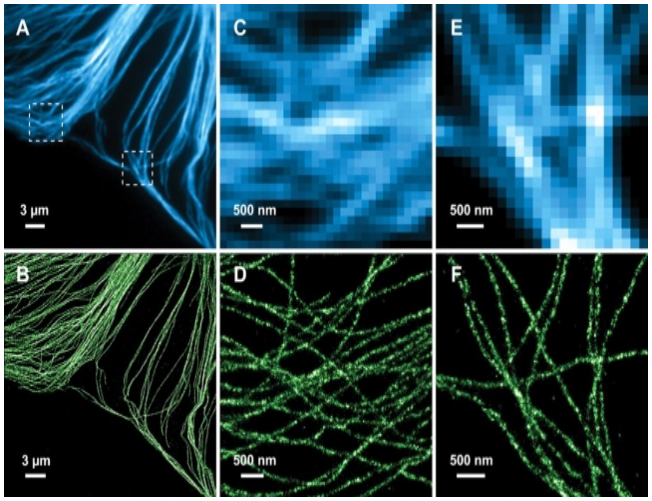

Figure 1: Example of STORM images compared to traditional fluorescence imaging methods[3]

One way in which a super-resolution image is reconstructed is similar to recording the lights on Eiffel Tower for a period of time and then overlapping the frames into a single frame. Individually, the lights on the Eiffel Tower (left image in Figure 2) give only a vague schematic of the Eiffel Tower, but when all of the lights have been overlapped, they reconstruct the structure of the Eiffel Tower (right side of Figure 2).

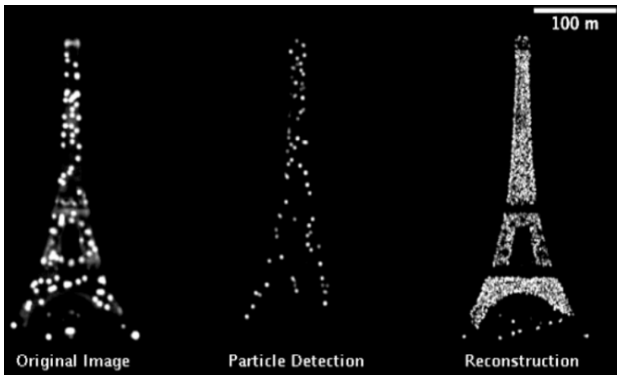

Figure 2: An example of image reconstruction using stochastic blinking[8]

The second way in which a super-resolution image is reconstructed is by locating the centers of two particles close together (see Figure 3 below). If the particles are sufficiently close, it is no longer possible to distinguish between the two particles; all that is seen is one blurry spot. However, if one of the dots was turned off while the other one is left on and vice versa, it is possible to locate the centers of the particles and thus distinguish between them. This results in higher-resolution images since much smaller centers, not large blurry dots can now be seen.

Figure 3: An example of super-resolution imaging by locating the centers

QSTORM utilizes the STORM imaging algorithms but uses QDs instead of fluorescent dyes and proteins in order to develop *in vivo* super-resolution imaging techniques on par with, if not better than PALM, FPALM, STORM, and other super-resolution imaging methods. QDs are better because they are brighter and more resistant to photobleaching than conventional dyes, allowing more photons to capture the image.[4]

The QSTORM nanoparticle system works via Förster (fluorescence) nonradiative energy transfer (FRET). FRET works when an energetically excited "donor" molecule is able to transfer its energy to a nearby "acceptor" molecule.[4a] For instance, when a fluorescent QD (the donor) is close to a gold nanoparticle (AuNP), its energy is transferred and the normally bright QD becomes quenched (dark). But as soon as the distance increases beyond 10 nm, the QD is fluorescent again because the energy is no longer transferred to the second acceptor molecule. Although the FRET working distance varies between each donor/acceptor pair, generally, the optimum distance is between 1-10 nm.[9] It is important that the wavelengths of energy emitted by the donor corresponds with the wavelengths of energy that the acceptor molecule can absorb.[4a] Note, however, that because the FRET affects are proportional to $1/R^6$, the FRET energy transfer efficiency declines rapidly at larger distances (see Figure 4 below).[4a] For one dye conjugate, quenching efficiency was below 25% at 6 nm.[4a]

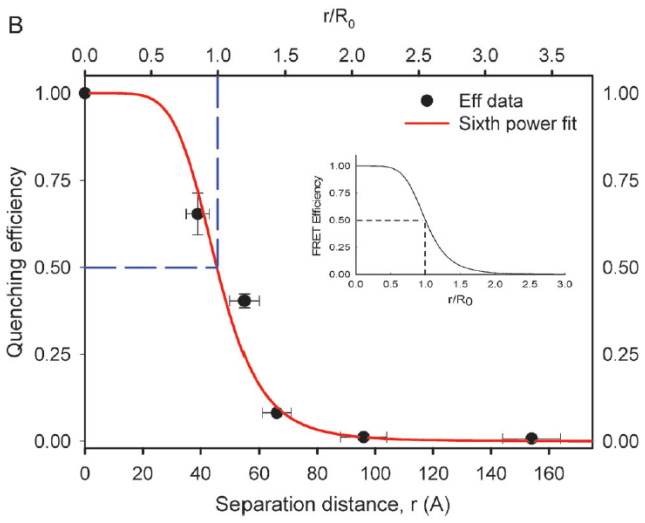

Figure 4: Quenching as a function of distance for one dye molecule[4a]

The larger the AuNP, the better the FRET efficiency may become.[10] However, if a particle is too large (e.g. silver NP around 100 nm), then it is more likely to enhance fluorescence rather than to quench it.[11] A 22 nm AuNP is more likely to quench[11], so a 15 nm NP should do very well in terms of quenching. There is, however, a downside to AuNP usage. Though AuNPs exhibit fluorescence quenching, they can also actually enhance the fluorescence a few nanometers from the QD. [11-12] This is because at short distances, the excited electrons in the AuNPs do not have time to nonradiatively decay after the rapid energy transfer from the QDs, resulting in scattering of the emitted plasmons from the excited QDs and thus an enhancement of fluorescence.[12] The photocleavable linker used in this study should be sufficiently long to be outside of this enhancement range.

2.2: Similar strategies

The idea to utilize QDs and other nanoparticles for fluorescence quenching and possible imaging applications is not new. For example, Hell's group used continuous-wave optical intensities of about 1.9 MW cm^2 to achieve a reversible effect in Mn-doped ZnSe QDs with efficiencies above 90%.[13] They used visible light to excite the QD (440 nm) and control it (676 nm).[13] In contrast, our approach uses UV light (~365 nm) to excite the QD and then examines the resulting fluorescence (quenched and unquenched because of FRET) at ~520 nm. We are also using a photocleavable linker. Other groups have used various dyes and/or proteins attached to QDs to control QD emission.[4b, c, 14]

At present, however, there are no known publications utilizing various photocleavable linkers with QDs and AuNPs (reversible or nonreversible) for FRET applications. There are, however, some similarities in published literature[15]. For instance, one group showed that photoactivatable and photoconvertible fluorophores (not QDs) can be connected to a quencher (not an AuNP) using a photocleavable linker.[16] A key contribution to the referenced articles, however, is the study of their FRET system both *in vivo* and in cells. Another team has used a QD-linker-AuNP system as a glucose sensor where the linker is displaced upon the presence of glucose, resulting in fluorescence.[17] Yet another group used a QD and either dabcyl or an AuNP as quencher for DNA detection. The linkers either amide-linked or linked via streptavidin and biotin groups.[18]

Additionally, one research team described a reversible photoswitchable QD that exhibited up to 52% quenching efficiency via photochromic polymer coatings on the QDs.[19] However, it is uncertain whether their strategy is applicable for super-resolution

imaging via STORM or QSTORM. First, their quenching, while good, may not be strong enough for STORM applications (the quenching efficiency should be very high, as close to 100% as possible). Furthermore, it is unclear if the quenching/unquenching occurs randomly or not. Only stochastic changes are useful for STORM/QSTORM. Yet it is clear that there is significant work being done in the field to improve or devise new super-resolution imaging techniques.

2.3: Streptavidin

Streptavidin (necessary for conjugation between the QD and the linker) binds to biotin very well (K_d~10^{-13}-10^{-14} M).[20] Streptavidin is a fairly large protein (60 kDa[20b]), consisting of 159 amino acids, though the smaller versions of streptavidin are also common.[20a] Some of the stability of the biotin-streptavidin interaction can be attributed to the several hydrogen-bonding side chains at the binding site, as well as the closing of the biding site after conjugation, thereby protecting the site from the aqueous surroundings.[20a] The streptavidin on the QDs is a tetramer, allowing it bind up to four biotin molecules.[20b]

However, because of the steric interference[20b] from the 15 nm AuNPs attached to a biotin linker, it is likely that there is only one biotin molecule per each streptavidin, particularly at the low concentration of 1nM (as concentration increases, some of the steric hindrance may be forcefully overcome because of increased pressure of the more abundant biotin molecules to bind to a single streptavidin).[20b] There are 5-10 streptavidin groups located on the QD.[21]

The streptavidin distance (in addition to the length of the linker) has an effect on QD quenching. In one literature study, amide-linked beacons outperformed streptavidin-linked beacons, having 57% greater fluorescence increase.[18] However, there is some ambiguity about the size of the conjugated streptavidin complex. Upon the conjugation of biotin, the streptavidin-biotin complex has a volume of 133±2 nm^3.[20b] This corresponds to about 5.1 nm per edge length, assuming the tetramer is approximately cubic. Using the Pythagorean Theorem, this would imply that at the diagonal of the cube (the longest side), the largest distance from the QD to the linker would be about 7.2 nm (this includes biotin).

A more accurate diameter is provided by the literature to be 11.1±0.1 nm.[20b] However, the actual length may be slightly smaller because the conformation of the protein is likely different in its conjugated form on the QD. This is suggested by another literature study, where the researchers cited a paper by Pazy et. al. suggesting that streptavidin was about 4 nm long.[18, 22]

2.4: Quantum Dots

QDs are nanoparticles capable of being fluorescent over many different wavelengths.[6] They are useful for many biological and medical applications, such as imaging tumors because QDs accumulate at tumor sites because of factors like permeability in tumor membranes and the binding of QDs to antigens in the tumor cells.[23] The small size of the QDs (and also of the AuNPs) enables them to better penetrate tumor

cells.[24] QDs have been widely used for a number of other imaging applications and thus can be used for QSTORM.[23, 25]

Quantum dots generally consist of a semiconductor core (e.g., CdSe or CdTe) with polymer coatings or other surface ligands to stabilize the QD and to make it useful for biological applications (such as being water-soluble and possessing functional groups for conjugation applications).[26]

For the imaging applications in this study, organisms need to survive long enough for the imaging process. However, for other applications, the use of QDs may be a concern because QDs are generally considered toxic (in some sense, this is a precaution since there are no set standards for testing the toxicity of the many types of QDs currently available, not to mention literature discrepancies[26]). One cause for the toxicity concern in QDs is because they contain elements like cadmium (Cd)[27] and lead (Pb)[4a] (for instance, Cd^{2+} is both a neurotoxin and a carcinogen[25b]).

However, a study published in *Nature Nanotechnology* had concluded that QDs were not harmful to non-human primates after a period of 90 days.[27] Long-term effects, however, still need to be studied because the removal of QDs from the body appears to be slow (there is accumulation in the liver, spleen and kidneys).[27] Furthermore, though there has been some observed toxicity in cell cultures[28], studies with small animals *in vitro* showed no significant toxicity[29]. The data is ambiguous at present, and as such, QDs are treated as toxic. But there is potential that the QDs are not toxic and could successfully be used for biological and medical applications. In any case, it is possible that nontoxic QDs can be synthesized, such as carbon-based QDs (provided they do not aggregate).[25b]

Chapter 3: Methods and Materials

3.1: Overview

Figure 5 below shows a summary of the key steps in this experiment. Because of AuNP stability, FRET and stability testing were done almost concurrently.

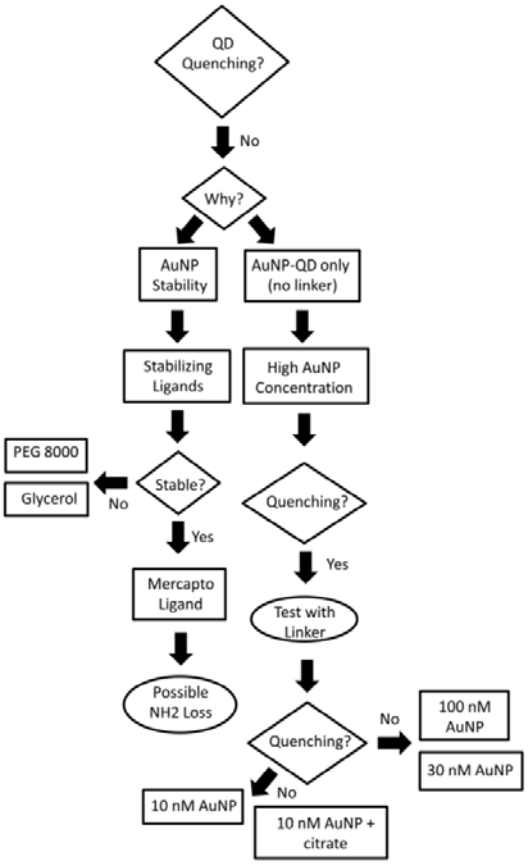

Figure 5: Summary of Key Steps in This Study

The synthesis strategy was to start with the AuNPs, conjugate the linker (L), purify the complex from excess linker and NHS byproducts via filter centrifugation (FC), and then conjugate the QD. Once the sample has been fully conjugated, the AuNP-L-QD complex was examined under a spectrofluorometer and the fluorescence measured.

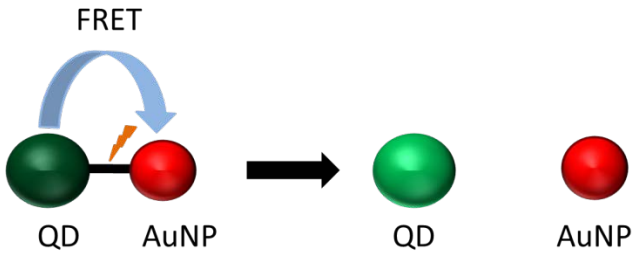

Figure 6: How FRET works in our nanoparticle system

For fluorescent measurements, two key trends were looked for: (1) initial quenching of fluorescent signal. The QD emission should have been significantly lower for the complex than for controls consisting of QDs by themselves and conjugated QDs and AuNPs floating free in solution, and (2) increase of fluorescence over time. If the system was quenched and the linker then cleaved by the laser, then the two NPs should have moved sufficiently away for an increase in the fluorescence signal intensity.

3.2: AuNP Stability

Fluorescent measurements were taken primarily at AuNP and QD concentrations of 1 nM each. One nM was sufficient to clearly distinguish between quenched and

unquenched samples. Also, using less sample was both more cost effective and resulted in lower waste generation.

However, at 1nM concentration, the AuNPs were found to be very unstable, particularly in buffer (necessary for AuNP-L conjugation). As determined from the UV-Vis absorbance plots, the AuNPs decreased in concentration within 24 hours. If there was visible aggregation, one would expect that the AuNP peak would broaden and shift and one would also expect visible sediments on the bottom of the vial. Neither events occurred to a significant degree. Likely, the polymer coating on the AuNP came off, resulting in the AuNP breaking up into smaller, possibly even elemental Au compositions.

At first, Nanocs AuNPs were used, but the stock solutions were very dilute and the batch-to-batch stability variability was high. For a similar price, we switched to Nanopartz AuNPs. These showed much greater batch consistency and were much more concentrated, enabling us to use less stock sample for the experiments.

Both Nanocs and Nanopartz exhibited some stability in water, but were very unstable in buffer solutions. Different buffer solutions were tried (e.g. phosphate-bases (PB), borate, PBS), as well as different buffer molarities. All resulted in unstable AuNPs within 24 hours. QD buffer (provided with the QDs by the manufacturer for cell imaging) performed better, but its bovine serum albumin BSA additives resulted in noisy fluorescence signals (and possible steric hindrance during conjugation). Furthermore, since BSA is a protein, it has numerous amide groups that could react with the linker, thus reducing the conjugation efficiency.

Subsequently, a stabilizing ligand approach was tested. Twenty percent glycerol was recommended by the Nanocs provider, but did not help. One percent poly(ethylene glycol) PEG 8000 did well in water, but did not result in enough stability in buffer. A mercapto-based (ME) ligand was used, giving sufficient AuNP stability for use within one working day (~8 hours), but there was no clear FRET signals. The final approach used 30 and 100 nM AuNPs (and 27 and 90 nM QDs, respectively) to perform the conjugation, with the samples being diluted to 1 nM prior to testing the fluorescence.

3.3: Materials/Equipment/Chemicals:

3.3.1: AuNPs

Nanocs AuNPs:

Amine functional AuNPs (15 nm) were purchased from Nanocs (Cat. No. GP015-AM-1; New York, NY).

Nanopartz AuNPs:

Amine functional AuNPs (15 nm) were purchased from Nanopartz (Prod. No. C11-15-TA-50; Loveland, CO). Stock concentration of 1st batch was 205.122 nM, stock concentration of 2nd batch was 307.683 nM.

3.3.2: QDs

Streptavidin QDs:

Approximately 20 nm streptavidin-conjugated QDs from Invitrogen™ (Cat. No. Q10041MP; Grand Island, NY) (now Life Technologies and soon to be acquired by Thermo Fisher Scientific) with fluorescence emission at 520 nm, used as provided by the manufacturer, without any further modification, washing, or purification. See Figure 7 below for the QD absorption and fluorescence emission spectra:

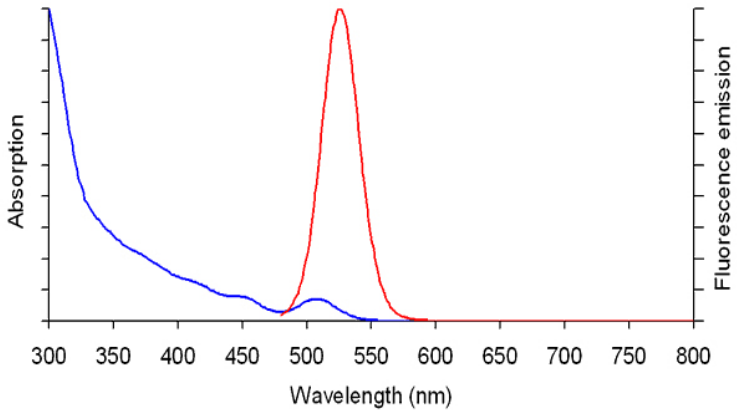

Figure 7: Quantum Dot absorption-emission curve[30]

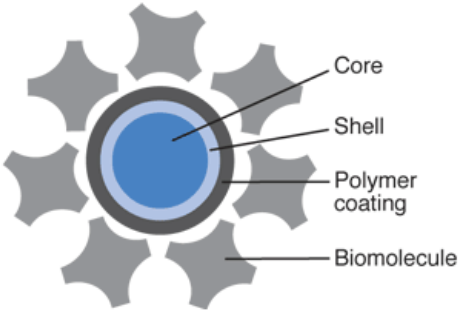

Figure 8: Schematic of the QD structure[21]

Carboxyl QDs:

Carboxyl QDs were purchased from Life Technologies (Qdot 525 ITK, ref # Q21241MP; Grand Island, NY) (soon to be acquired by Fisher Scientific).

3.3.4: Equipment

Rotavapor:

Conjugation reactions occurred on a rotation setting of 3 on the Buchi Switzerland Rotavapor R-210 (Flawil, Switzerland).

UV-VIS absorbance:

The relative absorbance is directly proportional to the concentration of the sample by Beer's law. Here, if the peak decreased significantly, this implied that the concentration of the sample also decreased significantly. The UV-Vis used was a Genesys6 (Thermo Electron Corporation [now Thermo Fisher Scientific]: West Palm Beach, FL).

FC Machine:

The FC was an Eppendorf Centrifuge 5417 R (Eppendorf North America: Hauppauge, NY). FC 4000 rpm for 10 min (2-3x, depending on experiment), and 1 min reverse FC for 1 min. The only exception was for the control in 4th FRET (see section 4.2.7). The mass of the centrifuge tube and the centrifuge tube and filter was weighed

prior to adding solution. The tube and filter was weighed again after addition of sample and after the removal of the filtrate solution. The final reverse FC used the mass of the tube only as a reference point. Samples then were diluted to the original concentration.

FC Filters:

Filter centrifuge filters were purchased from Millipore (Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-50 membrane, MWCO 50K [EMD Millipore Corporation-Billerica, MA]). Centrifugation occurred at 4000 rcf for 10 min, then reverse filter centrifuge for 1 min at 4000 relative centrifugal force (rcf). At this point, the FC was done 3x; later on, it was reduced to 2x to decrease the instability of the final product (forces from FC always destabilize some AuNPs; so less FC implies less AuNPs becoming unstable).

UV Lamp:

The UV lamp is a UVGL-58 Handheld UV Lamp from UVP [No. 10101.1-92; Upland, CA]. The lamp could use 254 and 365 nm light (the 365 nm light was used in experiments) at 6 watts, 115V, ~60 Hz, 0.12 amps.

TEM:

The TEM is an FEI Tecnai G2 Spirit Biotwin from FEI Company (Hillsboro, OR).

pH meter:

The pH meter is an Accumet Basic ABIS pH Meter (Fisher Scientific: Pittsburgh, PA).

Sonicator:

The sonicator was a FS30 by Fisher Scientific (Pittsburgh, PA).

Spectofluorometer:

The spectrofluorometer system consists of a large central box with one tag, SN 2298 and a second tag Master SN 2747, 814 Photomultiplier Detection System, A101B Arc Lamp, 3 electronics boxes, LPS-220B Lamp Power Supply, MD-5020 Motor Driver, BryteBox. The supplier is Photon Technology International (PTI) (Birmingham, NJ).

3.3.5: Buffers

QD Buffer:

From Invitrogen™ (now Life Technologies) and part of the QD kit (Cat. No. Q10041MP; Grand Island, NY). The buffer is composed of 2% BSA in 50 mM borate buffer with 0.05% sodium azide. The pH was 8.3.

PBS:

Phosphate Buffered Saline (PBS) Tablets were purchased from MP Biomedicals (Cat. # 2810305; Solon, OH).

PB Buffer:

Phosphate-based (PB) buffer consisted of:

1. Sodium phosphate monobasic dehydrate, Bio-ultra purchased from Sigma Life Science (71505-250G; St. Louis, MO).

2. Sodium phosphate dibasic purchased from Sigma Life Science (S5136-500G; St. Louis, MO).

Borate Buffer:

Sodium Borate Buffer, ultra-pure grade was purchased from Amresco (Code: 1B1117-100G; Solon, OH).

3.3.6: Other Chemicals

Linker:

The photocleavable linker (NHS-PC-LC_{WS}-Biotin, MW 793.856 Da) was bought from Ambergen (Watertown, MA) and consists of an N-hydroxysuccinimide (NHS) ester functional group for the linker-amine AuNP conjugation, a photocleavable nucleus (a 1-(2-nitrophenyl)ethyl group), a water-soluble spacer, and a biotin group for the linker-QD conjugation.[31]

The NHS ester reacts with primary aliphatic amine (NH_2) groups[31], like the ones on the AuNP. The linker will photocleave at the carbamate bond (between the photocleavable nucleus and the NHS group) upon the application of 300-365 nm UV

light.[31] If the sample is well mixed, essentially all of the linker can be cleaved at 365 nm within 10 minutes (see Figure 9 below).

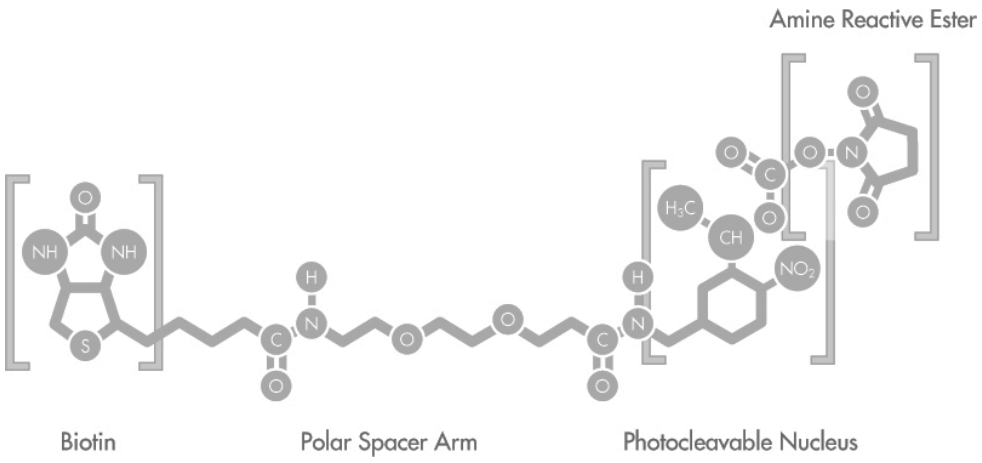

Figure 9: Structure of linker:[32]

The manufacturer recommends that the linker be stored in 20°C or -70°C for up to 6 months in *anhydrous* dimethylformamide (DMF) because the linker easily hydrolyzes in water. Store with desiccant. Before use, allow samples to equilibrate to 4°C, then to room temperature (RT). The manufacturer also recommends using PBS around pH 7.5 or 100 to 200mM of sodium bicarbonate buffer (pH as is). Although normal light should not cause the linker to cleave, the linker stocks and samples were kept covered in foil for maximum protection against photocleavage.

The linker manufacturer suggested using 10-25 molar excess linker, though other conjugation experiments had at least ~100 fold excess.[33] The final FRET test used 150x excess linker (because of volume considerations).

Jmol[34] was used to roughly estimate the length of the linker to be about 3 nm:

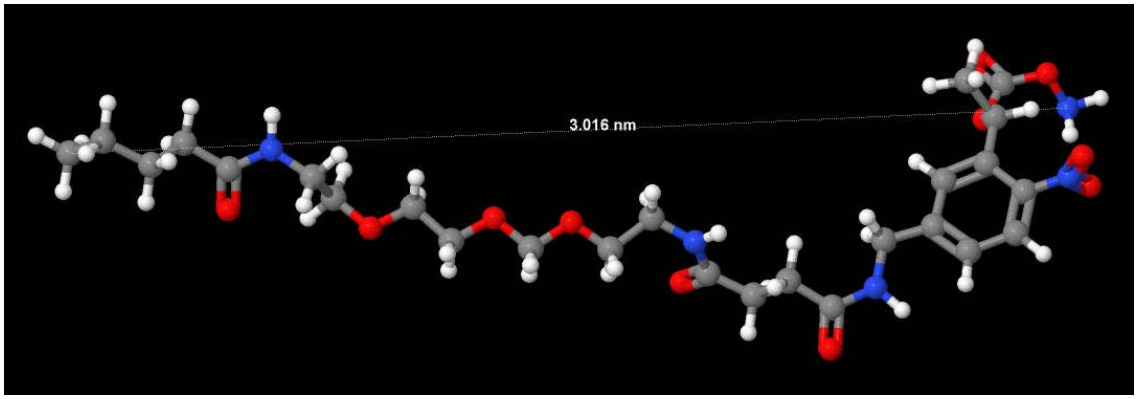

Figure 10: Length Estimate of Linker Using Jmol[34]

Linker stock solutions were as follows:

Sample	Concentration	Equivalent Concentration
Stock 1	50 mM	1 mg in 25 uL
Stock 2	5 mM	5,000,000 nM
Stock 3	0.5 mM	500,000 nm
Stock 4	0.05 mM	50,000 nM
Stock 5	$5x10^{-6}$ M	5,000 nM

Table 1: Linker Stock Solutions

Mercapto-based Ligand:

Excellent stability of AuNPs has been reported with the Tri(ethylene glycol) mono-11-mercaptoundecyl ether (ME) ligand.[35] The ME ligand is small (~300 Da) and should not interfere with the conjugation reactions.

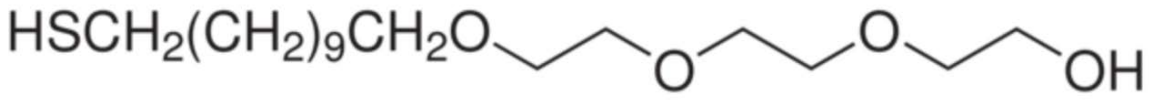

Figure 11: Structure of Mercapto Ligand[36]

Initially, the original stock sample was divided into much smaller samples in centrifuge tubes in an Ar atmosphere. Samples were stored in -20 °C freezer in a secondary container with desiccant. Approximately 24,000 nM ME was enough for AuNP stability for one working day (~8 hours).

Citrate:

Sodium Citrate Dihydrate was purchased from Fisher Scientific (BP327-500; Pittsburgh, PA).

Glycerol:

Glycerol, for molecular biology, minimum 99% was purchased from Sigma Life Science (G 5516-100 mL; St. Louis, MO).

PEG:

Poly(ethylene glycol), MW 8000 was purchased from Sigma Aldrich (P5413-500G; St. Louis, MO).

NHS:

N-Hydrosuccinimide (NHS) was purchased from Thermo Scientific (prod # 24500; West Palm Beach, FL).

EDC:

1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) was purchased from Thermo Fisher (cat. No. 22980; Rockford, IL).

Chapter 4: Results and Discussion

4.1: Stability

4.1.1: AuNP Stability in Water and Buffers

Nanocs AuNPs Stability in water:

37.5 uL of stock Nanocs AuNPs solution (at that time, presumed to be 10 nM) was added to 262.5 uL of distilled (DI) water. After a brief vortex, absorbance readings were taken at 0 hours and at 24 hours. The sample was kept at room temperature (RT) and sealed in Parafilm.

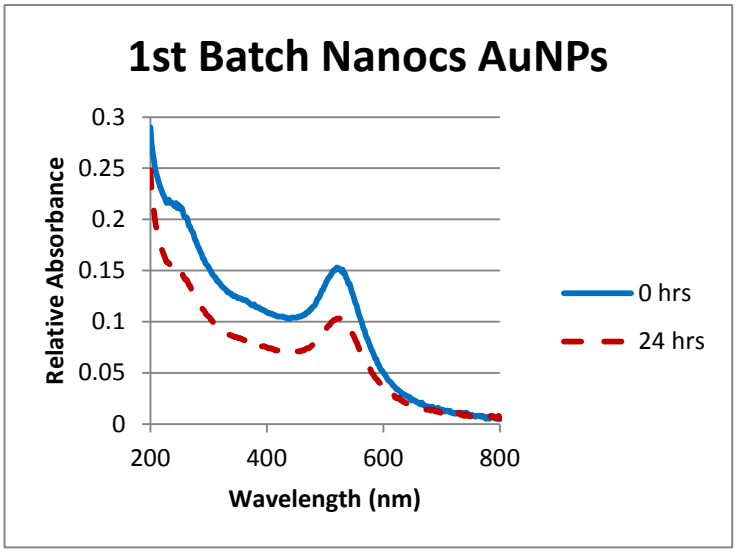

Figure 12: 1st Batch of Nanocs AuNPs

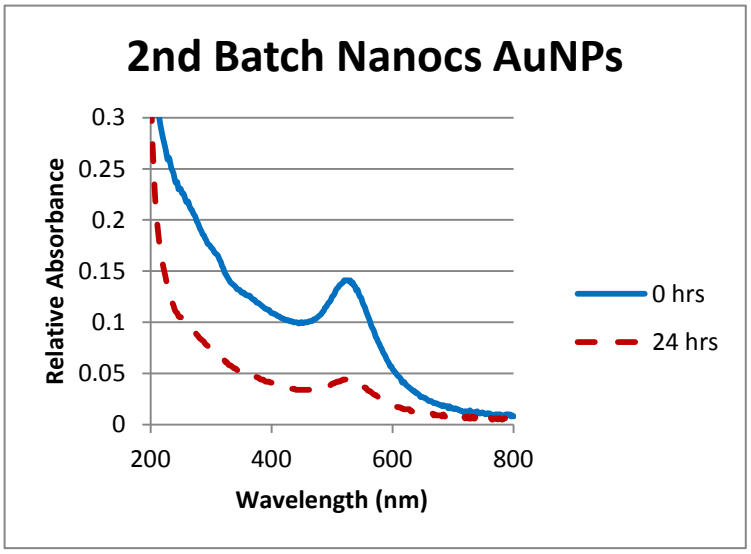

Figure 13: 2nd Batch of Nanocs AuNPs

Because of fluorescence sensitivity, cost minimization, and wise stewardship of stock samples, dilute concentration of AuNPs were used (typically around 1 nM). However, AuNPs at that concentration proved to be very unstable and efforts were made to stabilize the AuNPs, first for 24 hours and then, because of difficulties, for just ~6-8 hours (enough for one working day). AuNPs were more stable in water, but the chief stability criteria was in buffer, which was needed for successful AuNP-L conjugation.

The first AuNPs were from Nanocs. The chief problem with these AuNPs was the very dilute stock solution. Based on prior tests done in the lab, the stock concentration was assumed to be ~10 nm (and later corrected to ~4.5 nM based on absorbance data from the more concentrated and accurate Nanopartz AuNPs).

Another problem with Nanocs AuNPs was their poor batch-to-batch variability. Figures 12 and 13 above show the large difference in absorbance.

Nanopartz stability in Water:

Nanopartz AuNPs (1.34 uL) were added to 273.66 uL of water. After a brief vortex, sample absorbance was tested at 0, 2.5, 6.5, and 9.75 hours.

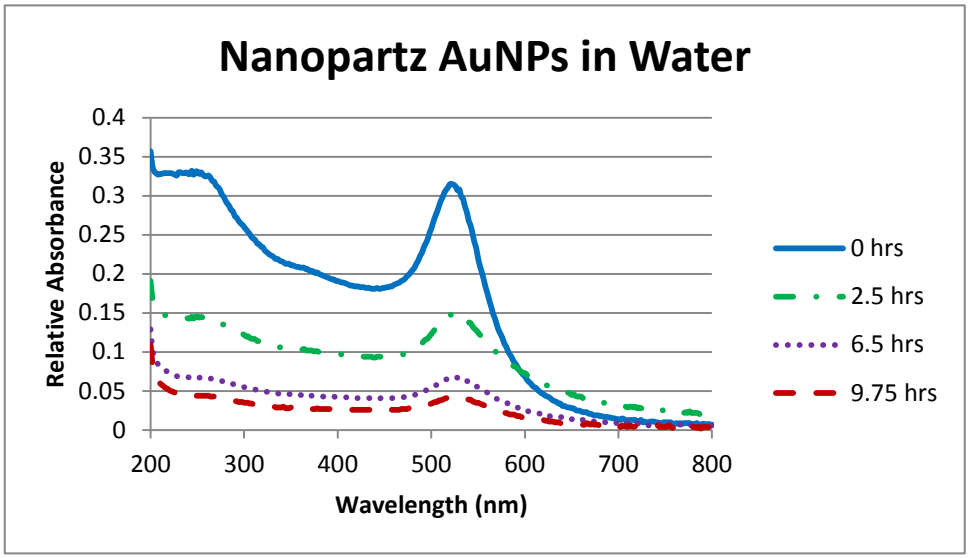

Figure 14: Nanocs AuNPs Stability in Water

Once Nanopartz AuNPs began to used, their stability was also examined in water. They were not more stable than Nanocs AuNPs, but they were a lot more concentrated (for example, the concentration of the 1st batch was 205.122 nM), their concentration were known from the manufacturer, and the batch-to-batch variability was low (not shown). At this point, the target for AuNP stability shifted from 24 hours to ~6-9 hours, or one working lab day. Figure 14 shows the stability of the 1st batch of Nanopartz AuNPs in water. Clearly, their stability needed to be improved prior to use in conjugation experiments.

Figure 14 was at an initial AuNPs concentration of 1 nM. We used this data to estimate the comparable concentration of stock Nanocs AuNPs to be about 4.5 nM by comparing the absorbance data. For the same price, Nanocs required much less sample (1-2 uLs for most tests) compared to ~30 uL or more for most Nanocs tests. Clearly, if Nanopartz AuNPs could be made reasonably stable, they would be the better AuNP choice for future experiments.

Nanopartz AuNPs resuspension:

Nanopartz AuNPs (1.34 uL) were added to 273.66 uL water. After a brief vortex, the absorbance was taken and the solution was allowed to sit for ~24 hours. Then, resuspension was attempted in 3 ways (in order, until resuspension or all of the attempts failed at resuspension): (1) 3000 rpm vortex for 2 min, (2): sonication for 5 min followed by 2 min vortex at 3000 rpm, and (3) repeating (2) if needed.

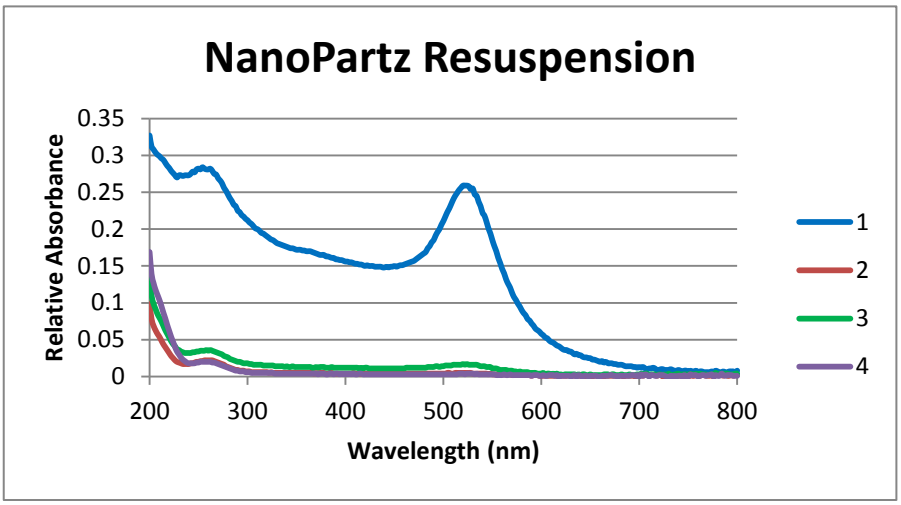

Figure 15: Nanopartz AuNPs Resuspension Attempts

On occasion, Nanopartz might settle to the bottom (the manufacturer warned about this and suggested sonication to resuspend the nanoparticles). Resuspension was performed, but it did not improve stability. This result and the observation that the Nanopartz usually did not settle (especially when in buffer) combined with no broadening and shift of absorbance peaks suggests not a visible aggregation of AuNPs, but a loss of protective polymer coatings of the AuNPs and subsequent decrease in AuNP concentration. Figure 15 shows the results of an attempt at Nanopartz AuNPs resuspension.

AuNPs Stability in Buffer:

For the PB buffer, a Sigma-Aldrich buffer reference was used.[37] The actual buffer formualtion was 0.0749 NaH_2PO_4 * $2H_2O$, 1.3444 g Na_2HPO_4. Briefly, 43.35 mL of DI water was added to a beaker containing Na_2HPO_4, and 2.7 mL was added to a beaker with NaH_2PO_4 * $2H_2O$. The solutions were then combined until a pH 8.0 was reached (as measured by a pH meter). In this case, the volumes were almost perfect and no pH adjustment was needed. The strength of the buffer was 0.2 M.

Then, for the actual Nanocs AuNP tests, 37.5 uL of Nanocs AuNPs were added to 262.5 uL of the 0.2 M PB buffer and the absorbance tested at 0 and 19 hours.

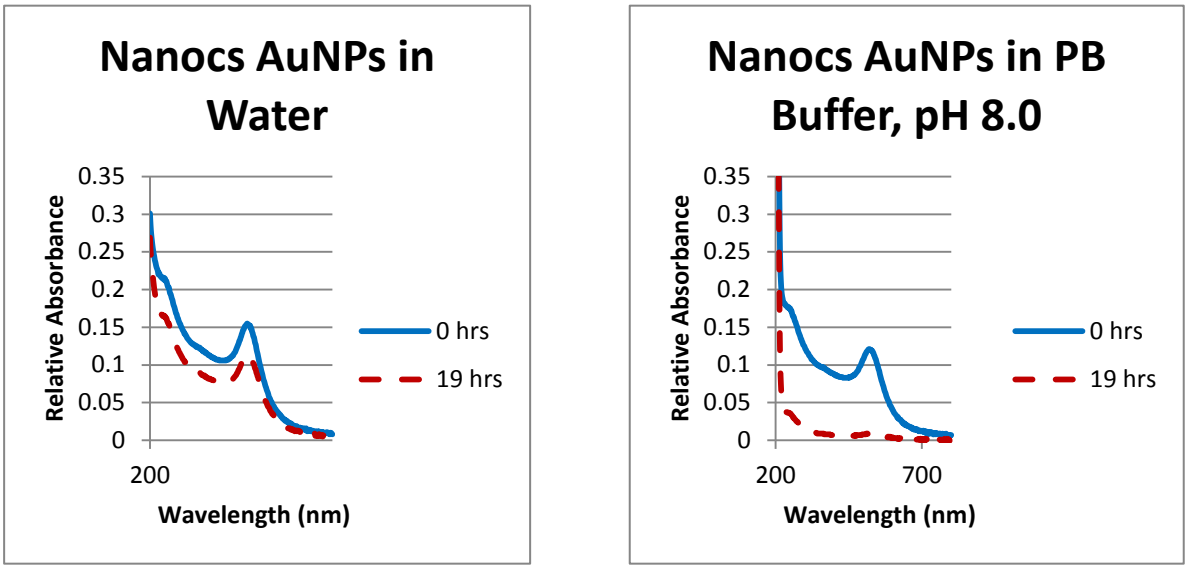

Figure 16: Nanocs AuNPs Stability in Water and in Buffer

Since the AuNP-L conjugation is the first step in the process, it is important that the AuNPs be stable. The AuNP-L conjugation reaction occurs between an amine group and an amine-reactive NHS ester on the linker. The conditions of this reaction occur at slightly basic conditions.[38] More specifically, in the handout accompanying the linker shipment, the manufacturer of the linker mentioned that "reactions must be performed in amine free buffers (e.g. Tris buffers are not compatible). PBS, pH 7.5 or 100 to 200 mM sodium bicarbonate (no pH adjustment) with 100 to 200 mM NaCl are suitable."[39] Several buffers and at different concentrations were tried to see if any of them would result in more stable AuNPs.

Whereas the first batch of Nanocs AuNPs exhibited stability in water, they were unstable in a phosphate-based (PB) buffer at pH 8.0. See Figure 16.

PBS and borate buffers (not shown) had similar AuNP stability issues.

Nanocs AuNPs stability in 0.075M buffer:

PB buffer (0.075 M) was made by adding 84uL of the 0.2 M buffer to 224 uL of DI water. Then, 37.5 uL of Nanocs AuNPs were added to 262.5 uL of 0.075 M buffer and samples were tested at 0, 1, 2, and 3 hours.

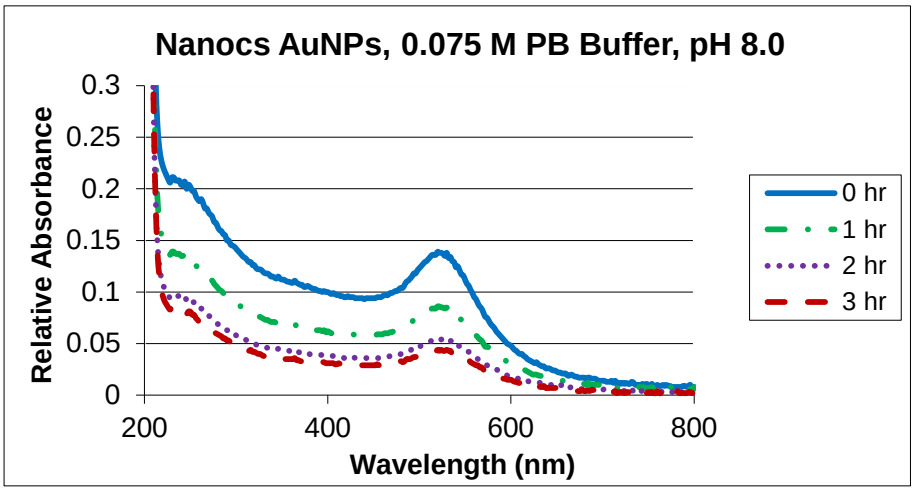

Figure 17: Nanocs AuNPs stability in 0.075 M PB Buffer

Since the PB buffer resulted in less stable AuNPs than water, a reduction in the buffer concentration was attempted (reduced salt concentration results in more stable AuNPs because of charge screening). PB buffer strength was reduced to 0.1 M. As seen from Figure 17 above, that did not help either. After 3 hours, the AuNP absorbance was sufficiently low and the experiment was stopped. Better stability was needed.

QD Buffer:

Nanocs AuNPs (37.5 uL) were added to 262.5 uL of cold QD buffer. The absorbance was tested at 0 hours and 2 days.

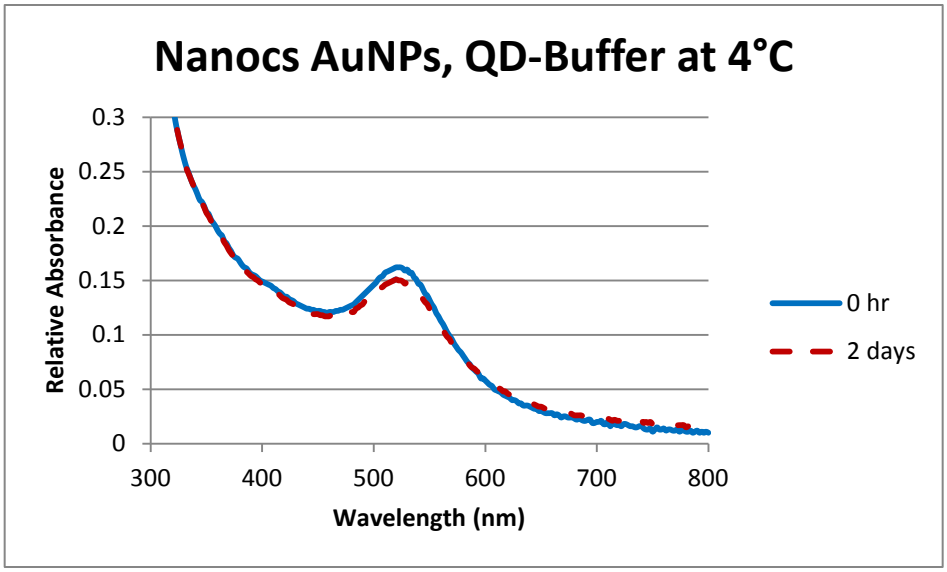

Figure 18: Nanocs AuNPs in QD-Buffer

AuNPs were very stable in QD buffer, even after 4 days. Note: here, temperature was kept at 4 °C, but it was later shown that temperature had no significant effect on AuNP stability for such short time periods. Consequently, all other samples were kept at room temperature (RT).

FC:

To a centrifuge tube wrapped in foil, 37.5 uL of NP, 261.9 uL of QD buffer, and 0.6 uL of Stock 5 linker solution were added. The resultant solution was then rotated for about 4 hours on setting 3 on the rotavapor. Absorbance readings were taken before and after FC. For FC, samples for filter centrifuged for 10 min at 4000 rcf, then reverse filter centrifuged (filter flipped upside down) for 1 min at 4000 rcf.

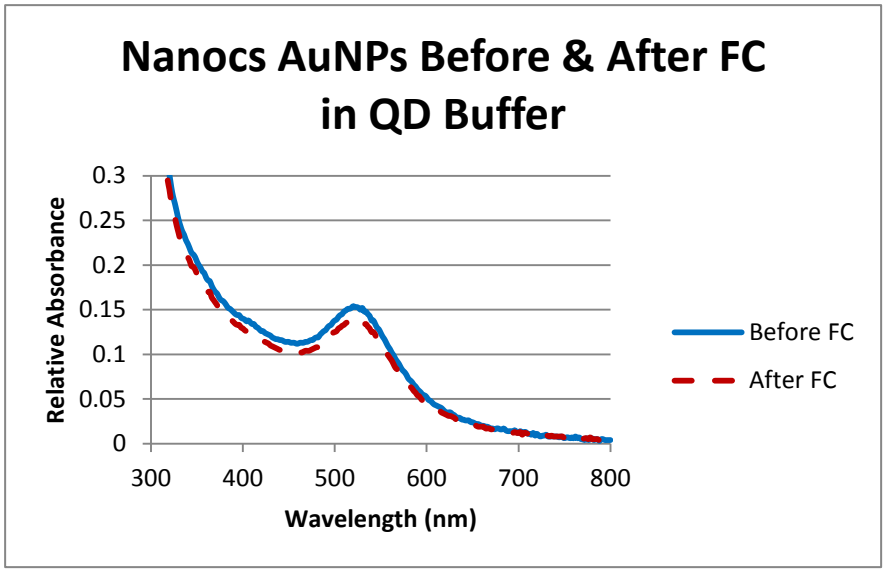

Figure 19: Nanocs AuNP Stability After FC

Filter centrifugation (FC) generally results in AuNP instability because of the high forces involved. FC was necessary to purify the AuNP-L from excess linker and other byproducts before conjugating the complex to QDs. Figure 19 shows that AuNPs in QD buffer were very stable even after FC.

4.1.2: AuNP Stability with stabilizing ligands

L-AuNP and QD-L-AuNP complexes:

20QD3:

Stock QDs (1 uL) were added to 99 uL of DI water.

AuNP-L-QD:

DI water (239.6 uL), 12.5 uL PB buffer, 32.3 uL Nanocs AuNPs, and 0.6 uL of Stock 5 linker solution were added to a centrifuge tube covered in foil. Then, added 15 uL of "20QD3." Let solution react for 60 minutes in the dark, then quenched via 3x FC. Absorbance reading was taken after 1 hour.

AuNPs:

Nanocs AuNPs (26.88 uL), 93.75 uL PB buffer, and 129.37 uL water were added to a centrifuge tube. Absorbance readings were taken at 0 hours and 1 hour.

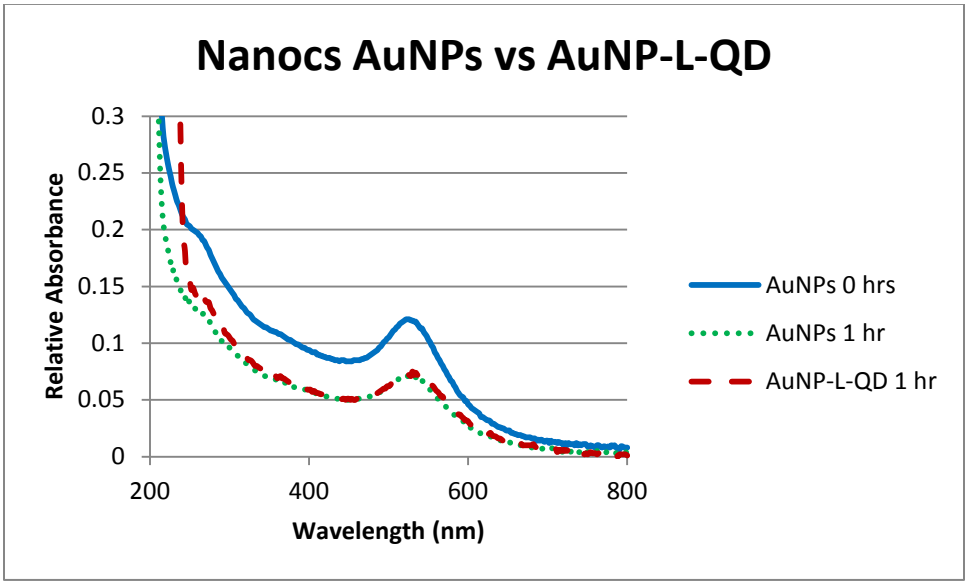

Figure 20: AuNP-L-QD stability vs. just AuNPs

Before using stabilizing ligands, a test was conducted to determine if the conjugated AuNP-L-QD complex would be more stable than AuNPs alone. As Figure 20 shows, the complex was not more stable than just plain AuNPs. Consequently, different stabilizing ligands were tested next.

Glycerol:

PB buffer (15 uL, 0.2 M), 60 uL glycerol, 195 uL DI water, and 30 uL Nanocs AuNPs were added to a centrifuge tube. After vortexing, absorbance readings were taken at 0 and 24 hours

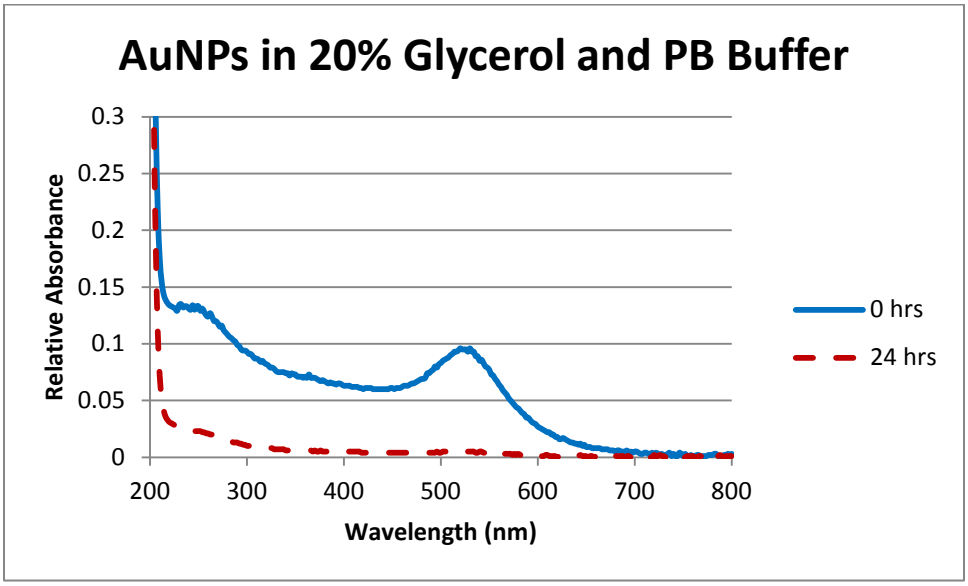

Figure 21: Nanocs AuNPs in 10mM PB buffer with 20% glycerol

Despite the success of the QD buffer, BSA, the stabilizing protein in the buffer, was very bulky and resulted in obscure fluorescence signals (perhaps because of scattering). Furthermore, the large size of the protein would likely have interfered with the conjugation reaction. A different stabilizing ligand was needed. The AuNPs manufacturer, Nanocs, suggested using 20% glycerol. That did not improve AuNP stability.

PEG:

25% PEG solution:

PEG 8000 (13.76 g) and 41 mL DI water were added to a 100 mL beaker.

10% PEG:

Twenty-five percent PEG solution (120 uL) and 32.3 uL of Nanocs AuNPs were added to 147.7 uL of water. After a brief vortex, the sample absorbance was tested after 0 hours, 24 hours, and 5 days.

1% PEG:

Twenty-five percent PEG solution (12 uL) and 32.3 uL of Nanocs AuNPs were added to 255.7 uL of water. After a brief vortex, the sample absorbance was tested after 0 hours, 24 hours, and 5 days.

0.1% PEG:

Twenty-five percent PEG solution (1.2 uL) and 32.3 uL of Nanocs AuNPs were added to 266.5 uL of water. After a brief vortex, the sample absorbance was tested after 0 hours, 24 hours, and 5 days.

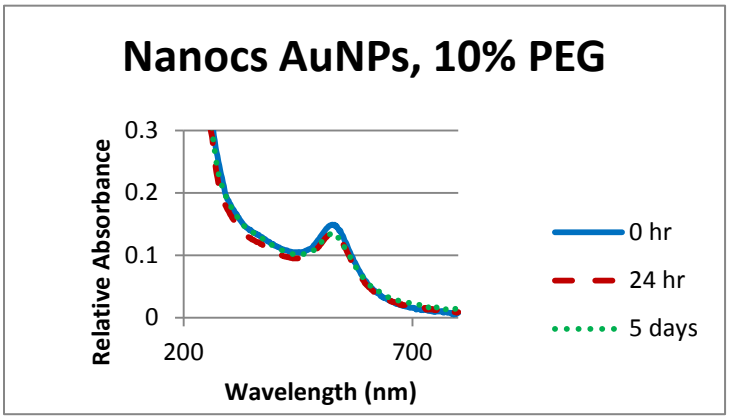

Figure 22: AuNPs Stability, Water, 10% PEG

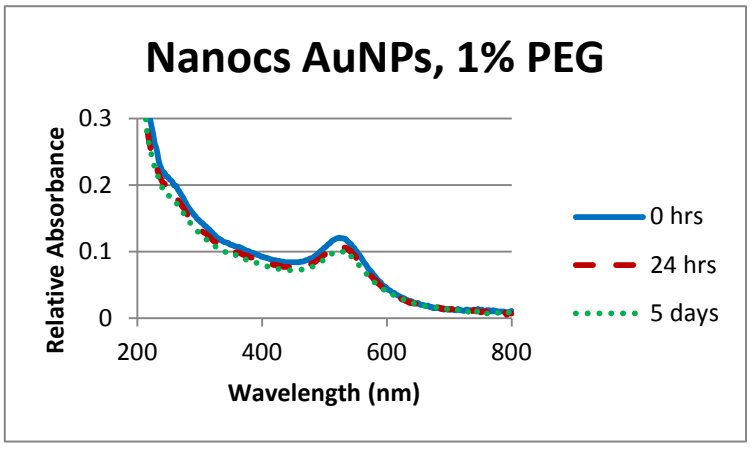

Figure 23: AuNPs Stability, Water, 1% PEG

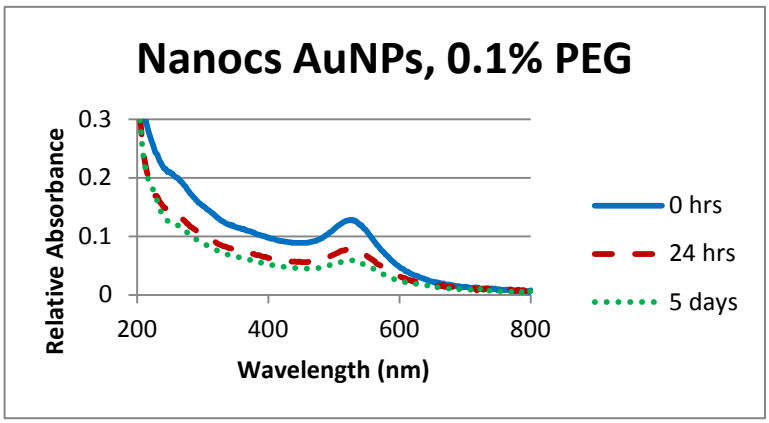

Figure 24: AuNPs Stability, Water, 0.1% PEG

The stability of Nanocs AuNPs was tested with PEG 8000 at 10%, 1%, 0.1% PEG concentrations with 1nM AuNPs in water. Figures 22-24 show the stability results after 5 days.

At least in water, PEG did help improve stability. PEG at 0.1% was not successful, but 1% and 10% did help improve the stability of AuNPs. Since 10% PEG did not offer significant improvement over 1% PEG, 1% PEG was chosen for further stability tests.

1% PEG Buffer Procedure:

Nanocs AuNPs (32.3 uL), 12 uL 25% PEG, 112.5uL PB buffer (pH 8.0), and 143.2 uL DI water were added to a centrifuge tube. The sample absorbance was tested at 0, 1, 2 and 3 hours.

As can be seen from Figure 25 below, Nanocs AuNPs with 1% PEG were unstable in water. A better stabilizing ligand was needed.

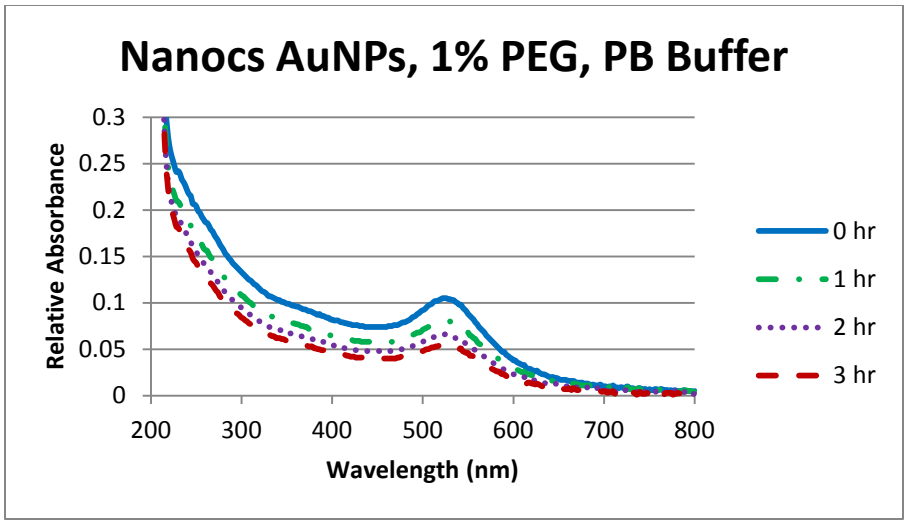

Figure 25: Nanocs AuNPs Stability, Buffer, 1% PEG

ME:

Water only control:

Nanopartz AuNPs (0.701 uL) and 6 uL of PB buffer (pH 8.0) were added to 293.299 uL water. The sample absorbance was tested at 0, 6.5, 9.5, and 23.4 hours.

ME solutions:

DI, without oxygen (deO_2) water (991.357 uL) was added to a centrifuge tube labeled "2.1" with 8.6 mg of stock ME. Next, to a centrifuge tube labeled "2.2," the ME concentration was further diluted by adding 100 uL of "2.1" and 900 uL of DI deO2 water.

DI deO2 water (988.3 uL) was added to a centrifuge tube labeled “3.1” with 11.6 mg of stock ME. Next, to a centrifuge tube labeled “3.2,” the ME concentration was further diluted by adding 100 uL of “3.1” and 900 uL of DI deO2 water.

8000 ME:

Nanopartz AuNPs (0.701 uL), 6 uL of PB buffer (pH 8.0), and 0.939 uL of “2.2” ME solution were added to 292.36 uL DI deO_2 water. The sample absorbance was tested at 0, 6.5, 9.3, and 23.25 hours.

24,000 ME:

Nanopartz AuNPs (0.701 uL), 6 uL of PB buffer (pH 8.0), and 2.09 uL of “3.2” ME solution were added to 291.21 uL DI deO_2 water. The sample absorbance was tested at 0, 6.75, 9.3, and 23.5 hours.

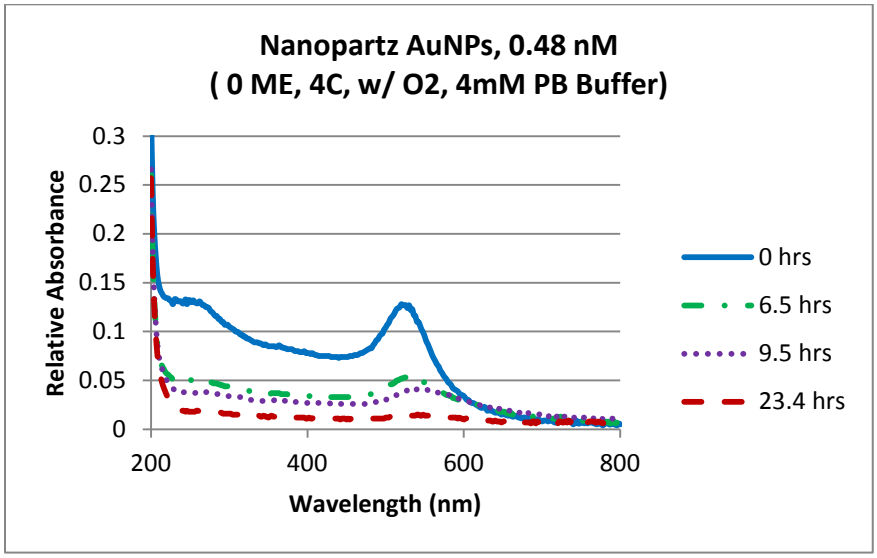

Figure 26: Nanopartz AuNPs only, no ME, 0-23.4 hr

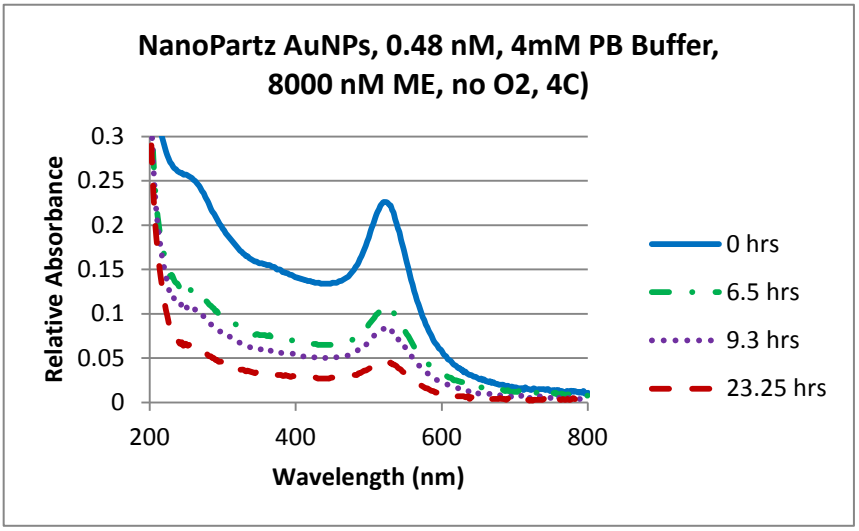

Figure 27: 8000 nM ME, Nanopartz AuNPs Stability

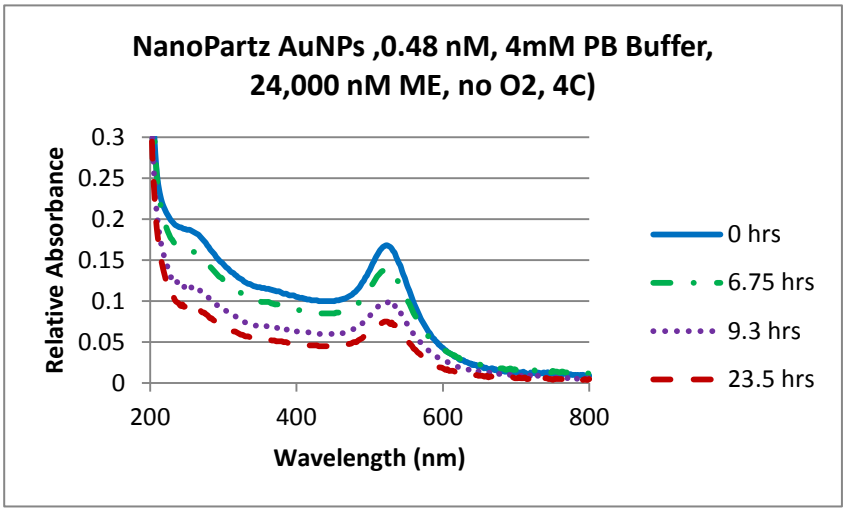

Figure 28: 24,000 nM ME, Nanopartz AuNPs Stability

For the remainder of the experiments the more concentrated (and more reliable) Nanopartz AuNPs were used. Also, a new stabilizing ligand, ME, was tested with the Nanopartz AuNPs. As seen from the Figures 26-28, Nanopartz AuNPs in buffer with

24,000 nM ME were the most stable. Also, at this point, the stability at ~6-9 hours was considered good enough for conjugation tests. Note: some of these samples are without oxygen because QDs were more stable in solutions without oxygen (shown later). However, the presence or absence of oxygen had no significant effect on the AuNP stability.

Figure 26 is interesting because there was some aggregation (slight peak broadening and shift). This did not usually occur with Nanopartz samples, especially when they were in buffer (here, AuNPs are in water).

ME after FC:

No ME, FC:

The sample without ME and FC was already described above (*24,000 ME* on pg. 41).

No ME:

Nanopartz AuNPs (0.702 uL) were added to 299.298 uL deO_2 DI water. FC was performed 3x, with absorbance readings taken immediately before and after FC.

ME:

First, “4.2” ME solution was made by adding 11.6 mg of ME and 988.34 uL of deO_2 DI water to a centrifuge tube. Then, 100 uL of “4.1” and 900 uL of deO_2 DI water were added to a centrifuge tube labeled “4.2”. Following this, 0.702 uL Nanopartz

AuNPs and 2.089 uL “4.2” were added to 297.209 uL deO_2 DI water. FC was performed 3x, with absorbance readings taken immediately before and after FC.

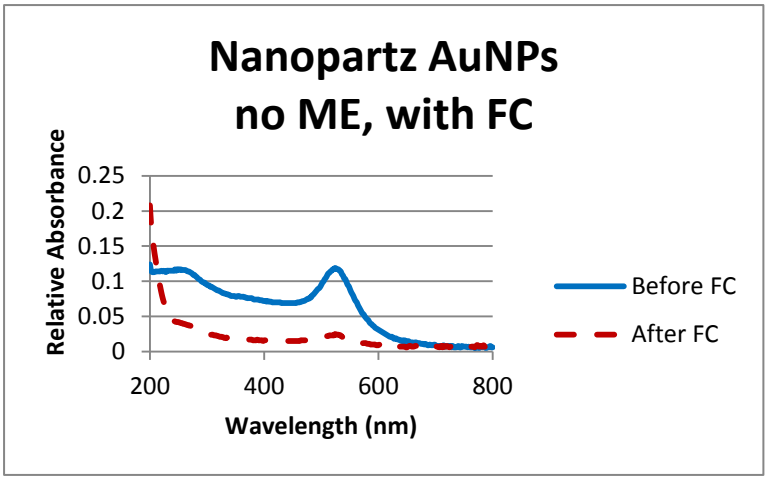

Figure 29: AuNPs after FC with no ME

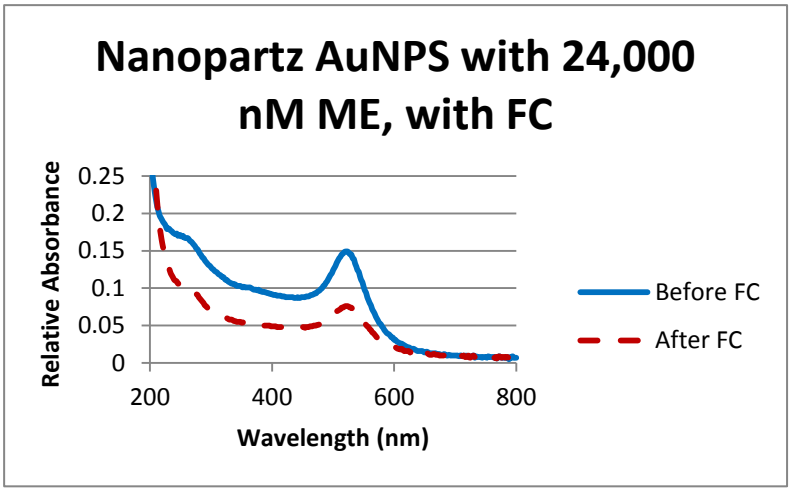

Figure 30: AuNPs after FC with ME

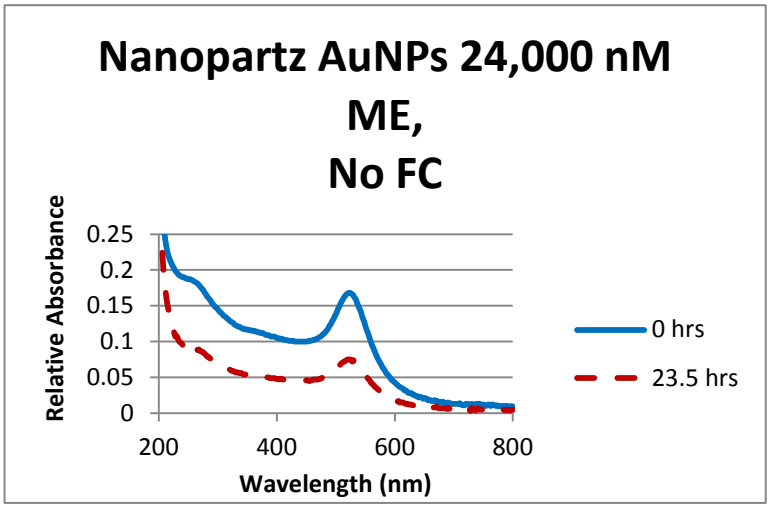

Figure 31: AuNPs in Buffer, no ME, no FC

Because of the stability benefits of ME on the Nanopartz AuNPs, stability was tested before and after FC (it is known that the forces from centrifugation can make AuNPs unstable). The sample without ME was very unstable after FC, while the sample with 24,000 nM ME was stable after FC. Its stability was comparable to AuNPs just sitting in buffer after 23.5 hours, without any FC.

4.1.3: QDs Stability

All samples were tested in DI water and QDs were at 1 nM concentrations. Each sample was exposed for about 10 min at 365 nm exposure and fluorescence signals recorded over that time (and then averaged for the final plots). The samples without O_2 had their water spurged with Ar for about 5 minutes prior to adding QDs. A 400 uL stock was made for O_2 and DeO_2 samples each. 90 uL samples were used for tests at the day of synthesis (0 hours or 1st working lab day), the next day (24 hours or 2ndworking lab

day), the third day (36 hours or 3rd working lab day), and the fourth day (48 hours or 4th working lab day).

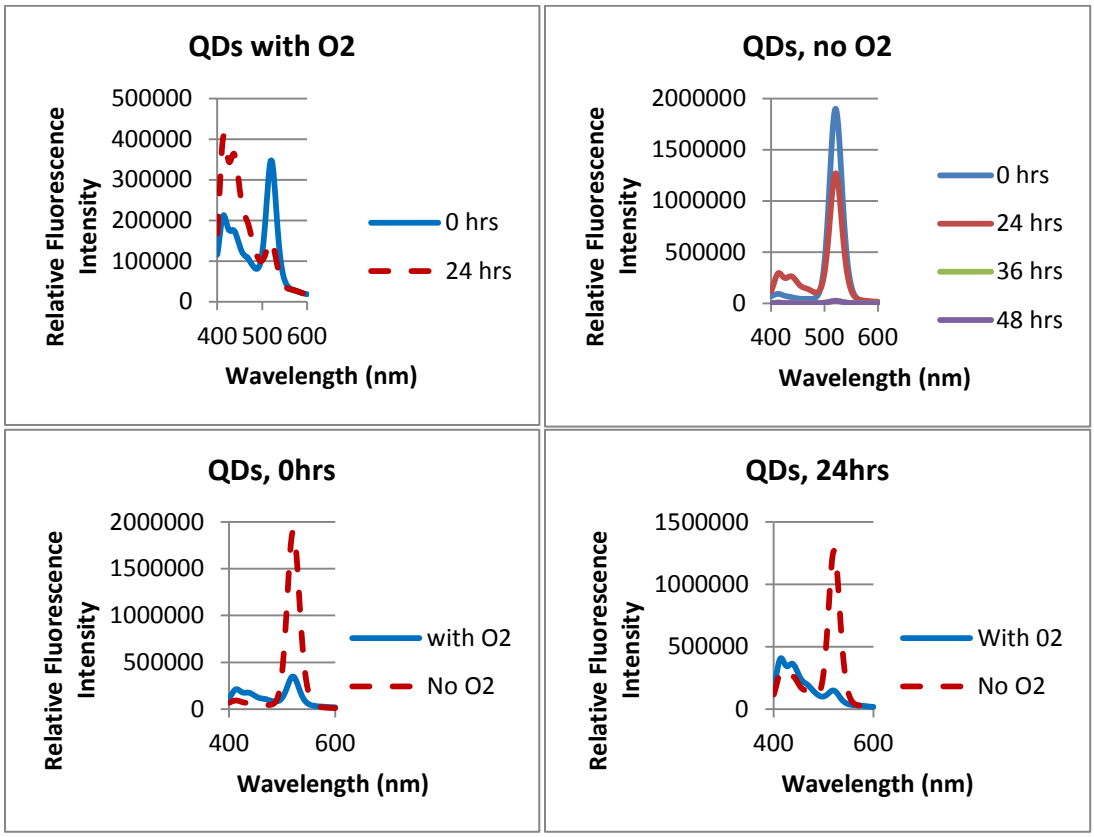

Figure 32: QD stability with and without O2 over time

There was also concern of slight instability in the QDs. From prior work, it was known that the presence or absence of oxygen could affect stability. QD samples in water with and without oxygen were tested and their fluorescence spectra compared over time. See Figure 32 above.

Clearly, QDs with no O_2 were more stable, and both samples were best within one working day (24 hours). In fact, the QDs with O_2 were sufficiently unstable that no

further tests were performed, while the QDs without O_2 were observed for 48 hours. Also, when comparing 0 and 24 hours, the QDs without O_2 had significantly stronger peaks.

4.1.4: ME

DI water (292.36 uL), 6 uL of PB buffer (pH 8.0), 0.939 uL of "2.2" ME solution (see pg. 40) and 0.701 uL Nanopartz NPs were added to a centrifuge tube. Absorbance was taken at 0, 6.3, 9 and 23.3 hours.

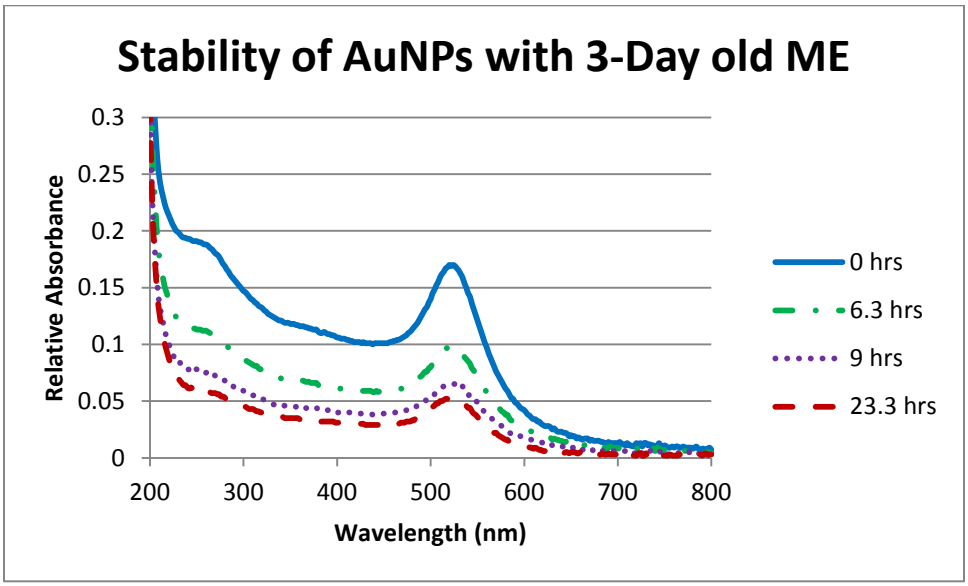

Figure 33: AuNP stability with 3-day old ME

At first, ME samples were used only for one day, but positive stability results suggested that samples could be employed longer. Three-day old ME was tested to see if it could still stabilize Nanopartz AuNPs for about ~24 hours. See Figure 33 above. ME was still good after three days.

Linker cleavage UV-VIS:

DI water (999 uL) and 1 uL stock 2 linker were added to a centrifuge tube and briefly vortexed. A 300 uL sample was exposed to UV light over time, with absorbance readings at total exposure times of 0, 2.5, 5, and 10 min (after each time point, sample was again exposed until the next overall time point).

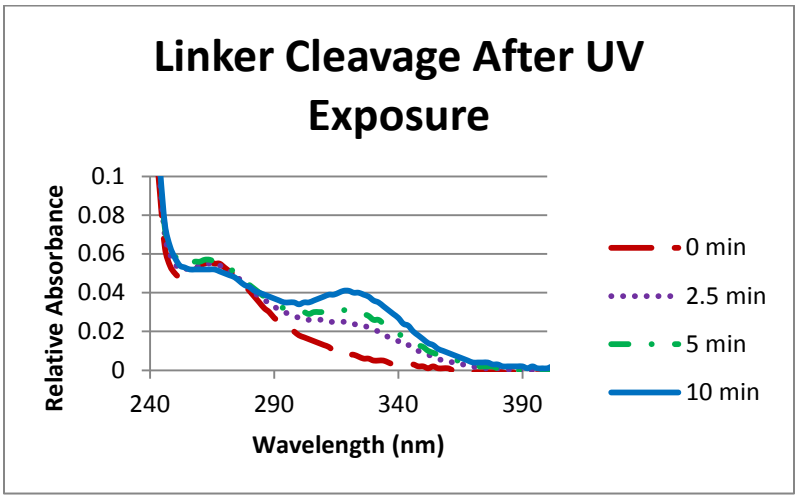

Figure 34: UV-Vis of Linker after UV Exposure

Another important test done was to make sure that the linker could photocleave. Using water as the baseline and testing the UV-Vis absorbance of the linker after 10 min UV light exposure in a handheld lamp, there was a slight peak at about 325 nm (see Figure 34 above). Unfortunately, this peak had low intensity, so it is obscured once AuNPs and/or QDs are added to the sample:

4.2: FRET Tests

4.2.1: Preliminary FRET attempts

Using QD buffer for both QDs and Nanocs AuNPs, AuNP-QD and AuNP-L-QD fluorescence data was taken at 365 nm excitation wavelength after 1 hour (not shown) and 4 hour conjugation. QD and AuNP control were at 1nM each.

35 nM QD solution:

QD buffer (98.25 uL) and 1.75 uL QDs were added to a centrifuge tube.

18.75KL linker solution:

One uL of stock 2 linker solution was added to 265.7 uL of DI water.

AuNP-QD:

QD buffer (87.14 uL), 2.86 uL of the premade 35 nM QD solution, and 10 uL of Nanocs AuNPs solution were added to a centrifuge tube. The tube was sealed with Parafilm, briefly vortexed, and the sample fluorescence was tested using 365 nm excitation.

AuNP-L-QD:

Note: this is for the 4 hour conjugation, but the 1 hour conjugation had a similar procedure, just a shorter (1 hour) reaction time.

QD buffer (447.87 uL), 50 uL of Nanocs AuNP solution, and 2.13 uL of the "18.75KL" linker solution were added to a foil-covered centrifuge tube and briefly vortexed. The sample was allowed to react for 1 hour on the "3" setting of the rotavapor. The resultant concentrated sample (134.4 uL for the 4 hour conjugation sample) was then rediluted back to 1 nM AuNPs (and also 1nM QDs) by adding 253.2 uL QD buffer and 11.4 uL 35 nM QD solution. Note: During FC, QD buffer was used as the washing fluid. After QDs were added, samples were taken for fluorescence testing (QDs had about 1hour to conjugate to the AuNP-L complex).

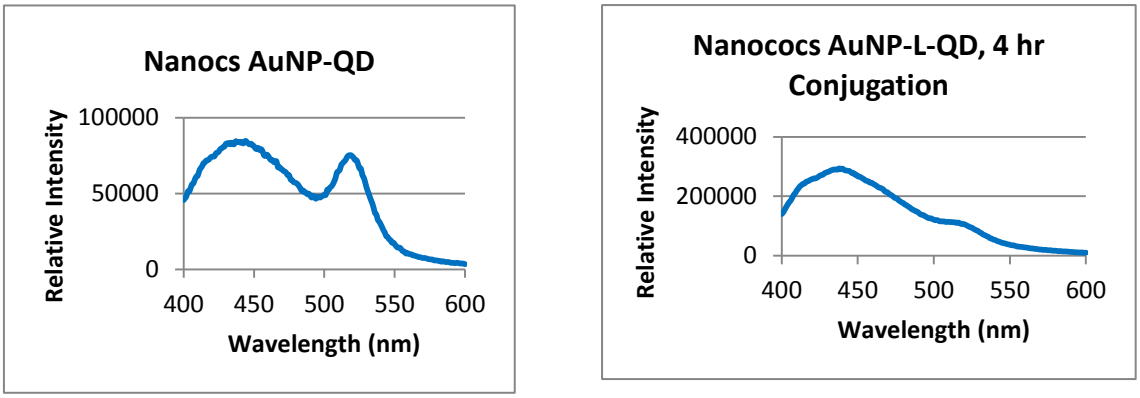

Figure 35: First Fluorescent Data, QD Buffer

These results showed that the BSA was severely obscuring QD signals (we expected a strong peak at 520 nm, not very blurry "noise" that overpowered the 520 peak). This can be seen by comparing the intensity of the AuNP-QD sample (AuNPs and QDs alone in solution, no conjugation), with the AuNP-L-QD samples (conjugated, with

3x FC using QD buffer). Since BSA was too bulky of a protein to pass through the filter membranes, it accumulated, likely resulting in the noise obscuring the QD peak. This was one of the reasons that QD-buffer was rejected for AuNPs stability.

QD Buffer BSA Procedure:

PEG:

~18% PEG Procedure:

Here, buffer and water refer to the solvent in which the AuNP-L conjugation occurred. PEG solutions were water-based.

Buffer Sample:

PB buffer (112.5 uL), 142.6 uL DI water, 12 uL 25% PEG solution, 32.3 uL Nanocs AuNPs, and 0.6 uL of stock 5 linker solution were added to a centrifuge tube covered in foil and briefly vortexed. The sample was allowed to reaction on setting "3" on the rotavapor for one hour, then 3x FC. The sample was then rediluted by adding 52.68 uL DI water and, after 24 hours 16 uL 20 QDs (formulation in one of sections above). The QDs were allowed to conjugate for about 1 hour prior to testing fluorescence.

Water Sample:

DI water (255.1 uL), 12 uL 25% PEG solution, 32.3 uL Nanocs AuNPs, and 0.6 uL of stock 5 linker solution were added to a centrifuge tube covered in foil and briefly

vortexed. The samples were allowed to react for one hour on setting “3” on the rotavapor, then 3x FC. The sample was rediluted by adding 62.24 uL DI water and, after 24 hours, 15 uL 20 QDs (formulation shown previously). The QDs were allowed to conjugate for about 1 hour prior to testing fluorescence.

Buffer and water comparison of fluorescence of AuNP-L-QD with 18% PEG (1% was also tested, but the data was similar and is not shown. In fact, for the QD and AuNP-L-QD in buffer, the fluorescence decreased, not increased over time, perhaps because of photobleaching.

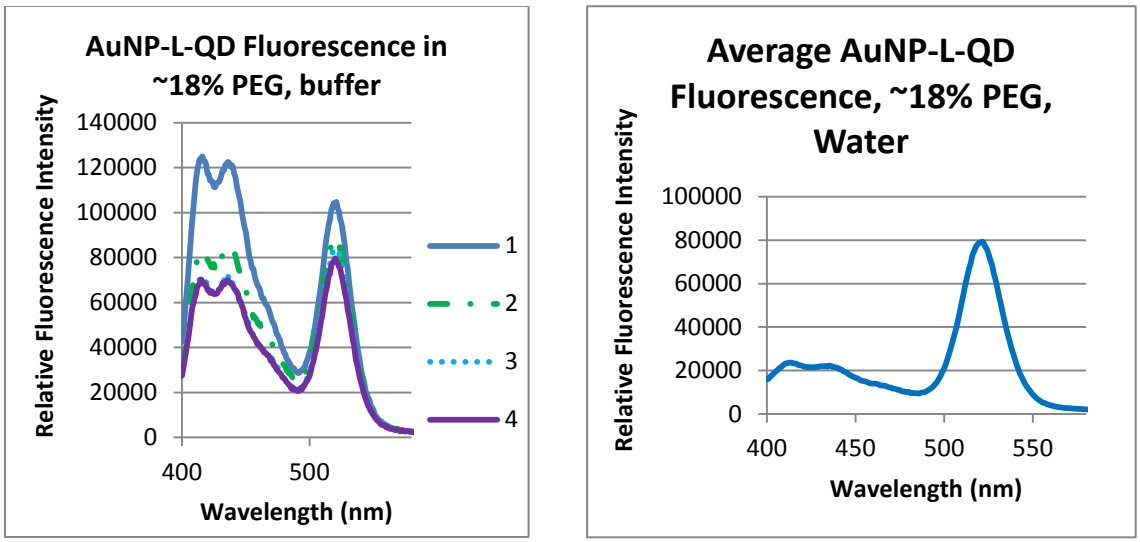

Figure 36: ~18% PEG with buffer and water

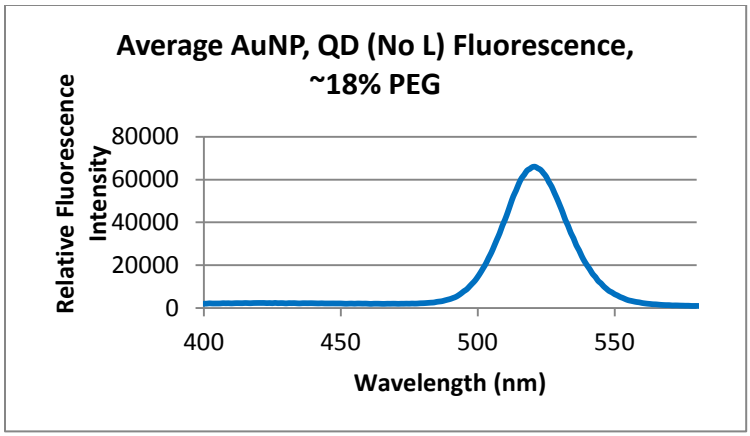

Figure 37: QDs and AuNPs only

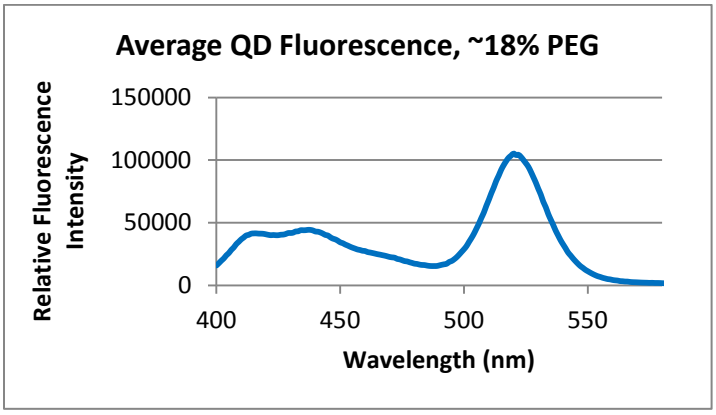

Figure 38: QDs only

The data was slightly ambiguous as to whether FRET occurred or not. Clearly, there was no increase in fluorescence after light cleavage of the linker molecule. Another issue with quenching is the ambiguity in the fluorescence intensities (because of variability in the samples). Except for the first reading in the buffer sample, both buffer and water AuNP-L-QD complexes had similar intensities that were greater than controls of AuNPs and QDs in solution (non-conjugated). Had there been FRET, the conjugated

samples should have been considerably quenched compared to the nonconjugated samples. Yet the QD only solution is more intense and may allow for some quenching when compared to the conjugated samples. The data is vague, and more FRET experiments will follow, but at this point, it did not seem that FRET was occurring.

4.2.2: 1st FRET

1st FRET Procedure:

24 ME solution:

"4.1" ME solution (1.044 uL) was added to 1498 uL deO_2 DI water. This was repeated if more washing solution was needed.

Conjugated sample:

DI deO_2 water (483.081 uL), 10 uL of PB buffer, 2.438 uL Nanopartz AuNPs, 1 uL of stock 5 linker solution, and 3.481 uL of "4.2" ME solution (formulation for 4.2 ME has already been given in the ME section above) were added to a foil-covered centrifuge tube. The sample was covered with Parafilm, briefly vortexed, and set to rotate on "3" for 1 hour. The sample was then FC 2x (washing solvent: "24 ME" solution), and the concentrated sample was rediluted to the original concentration by adding 11.6 uL "20QD," 441.8 uL "24 ME" solution. The final sample with QDs was allowed to react for about 1 hour.

QD Control:

The “20QD” formulation (7.2 uL) (procedure shown in prior section) was added to 292.8 uL of deO_2 DI water and its fluorescence was tested.

AuNP-QD control (non-conjugated):

Nanopartz AuNPs (0.876 uL) and 2.089 uL “4.2” were added to 297.035 uL deO_2 DI water and its fluorescence was tested.

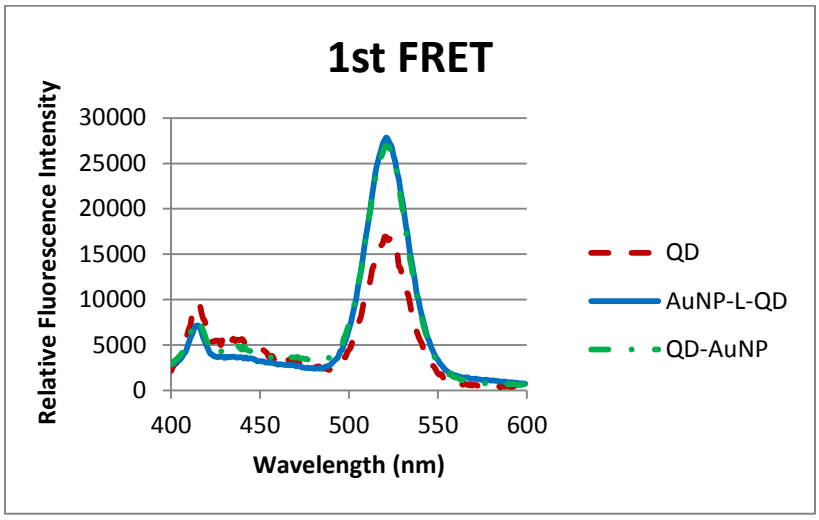

Figure 39: 1st FRET Attempt

This was the first official FRET test using the most stable linker, ME, with Nanopartz AuNPs. The figure above shows the results of the test, where there was no quenching (no FRET). There was also no significant increase in fluorescence of the conjugated sample over time, so the plot shows the average of these exposures.

4.2.3: 2nd FRET

2nd FRET Procedure:

L5000:

One uL of stock 3 linker was added to 99 uL anhydrous DMF.

5.2 Me Solution:

DI deO_2 water (991 uL) was added to a centrifuge tube labeled "5.1" with 9.3 mg of stock ME. Then, 100 uL of "5.1" and 900 uL deO_2 DI water were added to a centrifuge tube labeled "5.2".

1 nM AuNP-L-QD:

One uL of L5000, 4.2343 uL "5.2" solution, 2.348 uL AuNPs, and 473uL deO_2 PBS were added to a centrifuge tube covered in foil. The sample was allowed to rotate for 1 hour on setting "3" of the rotavapor, after which 12 uL 20QD was added.

4 nM AuNP-L-QD:

One uL of L5000, 4.2343 uL "5.2" solution, 9.75 uL AuNPs, and 480 uL deO_2 PBS were added to a centrifuge tube covered in foil. The sample was allowed to rotate for 1 hour on setting "3" of the rotavapor, after which 12 uL 20QD was added.

1nM AuNP-QD (non-conjugated):

"5.2" ME solution (0.869 uL), 0.488 uL Nanopartz AuNPs, and 2.4 uL 20QD were added to 96.6 uL deO_2 PBS. Fluorescence spectra were then taken.

4nM AuNP-QD (non-conjugated):

"5.2" ME solution (0.869 uL), 0.488 uL Nanopartz AuNPs, and 2.4 uL 20QD were added to 94.6 uL deO_2 PBS. Fluorescence spectra were then taken.

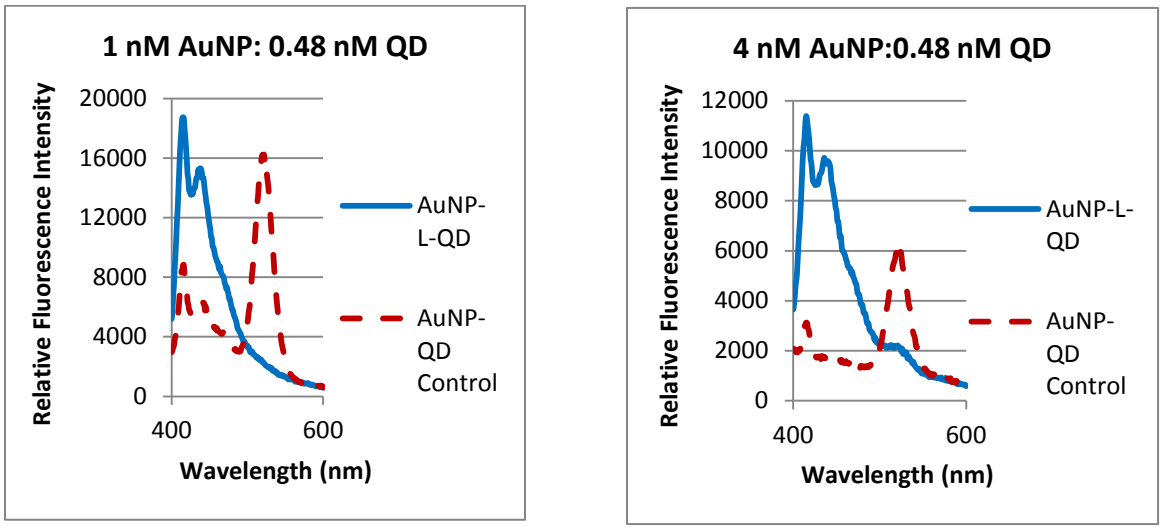

Figure 40: Results from 2nd FRET

Since there was no clear FRET in the prior test, several changes were made. First, FC was not used to decrease the chances of AuNPs become unstable because of centrifugation forces. Second, per the linker manufacturer's recommendation, pH 7.5 PBS was used from this point on as the conjugation buffer. Both AuNP-L and the conjugation of QDs to the AuNP-L complex lasted 1 hour each. Also, while the QDs

were at 0.48 nM, AuNPs were tested at 1 nM and 4 nM concentrations to test if more particles were needed for quenching. Stock 3 linker was used.

The QD control is not shown because the sample was bad (there was no QD signal, when there should have been a 520 nm peak). Figure 40 above does suggest possible quenching as a result of FRET since the conjugated samples have weaker 520 peaks than the AuNPs-QDs (non-conjugated) control.

However, at such large AuNP:QD ratios, it is also possible that nonFRET quenching occurred, such as via inner filter effects. In any case, although there is some possible FRET in these samples, future FRET tests were never as clear, suggesting that this was either a FRET anomaly (though there was no increase in fluorescence upon linker cleavage, as we would expect if photocleavage after FRET occurred) or there was some other reason for the quenching (e.g. inner filter effects, or because QDs were not stable/accidently weren't added to solution because of small pipette volumes).

4.2.4: 3rd FRET

3rd FRET Procedure:

L5000.2:

One uL of stock 2 linker and 9 uL of anhydrous DMF were added to centrifuge tube. The sample was stored in fridge (in desiccant) until use.

“6.2” ME solution:

DI deO_2 water (987.437 uL) was added to a centrifuge tube labeled “6.1” with 12.5 mg stock ME. Then, 100 uL “6.1” and 900 uL deO_2 DI water were added to a centrifuge tube labeled “6.2”.

2:1 AuNP-L-QD, Laser & Lamp, and FC:

Nanopartz AuNPs (1.463 uL), 1.938 uL of “6.2” ME solution, and 0.6 uL of L5000.2, were added to 281 uL deO_2 PBS. The sample was FC 2x with washing by “24” ME solution. Then, the sample was rediluted by adding 220.7 uL “24 ME” solution and 15 uL 20QD. Fluorescent spectra were then taken.

2:1 AuNP-L-QD, Laser & Lamp, FC:

Nanopartz AuNPs (2.925 uL), 1.938 uL of “6.2” ME solution, and 0.6 uL of L5000.2, were added to 287.54 uL deO_2 PBS. The sample was FC 2x with washing by “24” ME solution. Then, the sample was rediluted by adding 225.4 uL “24 ME” solution and 15 uL 20QD. Fluorescent spectra were then taken.

2:1 AuNP-L-QD, Laser & Lamp, no FC

Nanopartz AuNPs (2.925 uL), 1.938 uL of “6.2” ME solution, 0.6 uL of L5000.2, and 15 uL 20QD were added to 287.54 uL deO_2 PBS. Fluorescent spectra were then taken.

QD:

20QD solution (2.4 uL) was added to 97.6 uL deO_2 PBS. Fluorescent spectra were then taken.

1 nM AuNP-QD (non-conjugated):

Nanopartz AuNPs (0.488 uL), 0.869 uL 5.2 ME solution, and 5 uL 20QD were added to 94 uL deO_2 PBS.

2 nM AuNP-QD (non-conjugated):

Nanopartz AuNPs (0.975 uL), 0.869 uL 5.2 ME solution, and 5 uL 20QD were added to 93.16 uL deO_2 PBS.

1:1 Results:

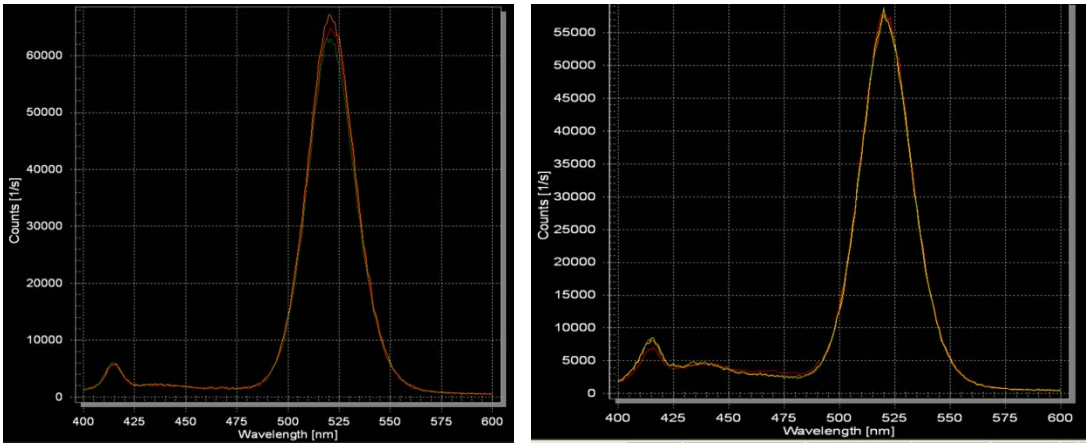

Figure 41: 1:1 AuNP-L-QD, Laser (left) and Lamp (right), FC

2:1 results:

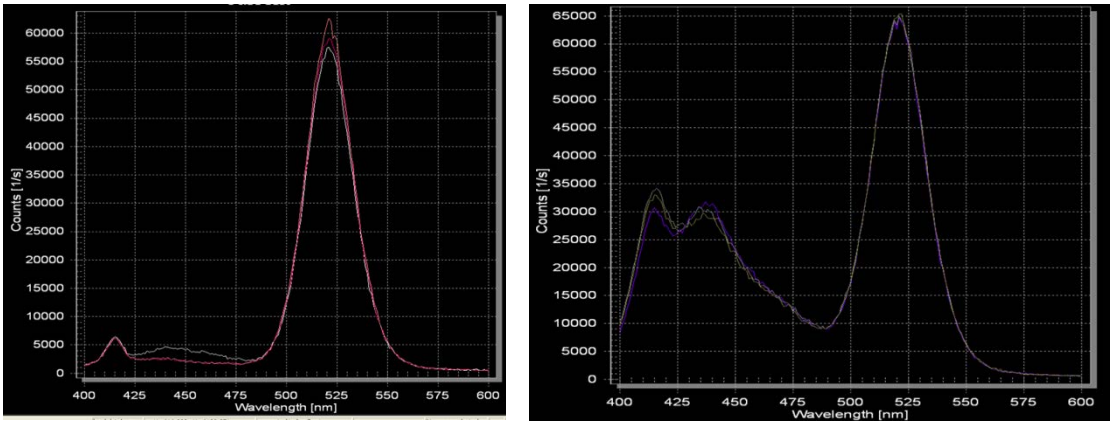

Figure 42: 2:1 AuNP-L-QD, Laser (left) and Lamp (right), FC

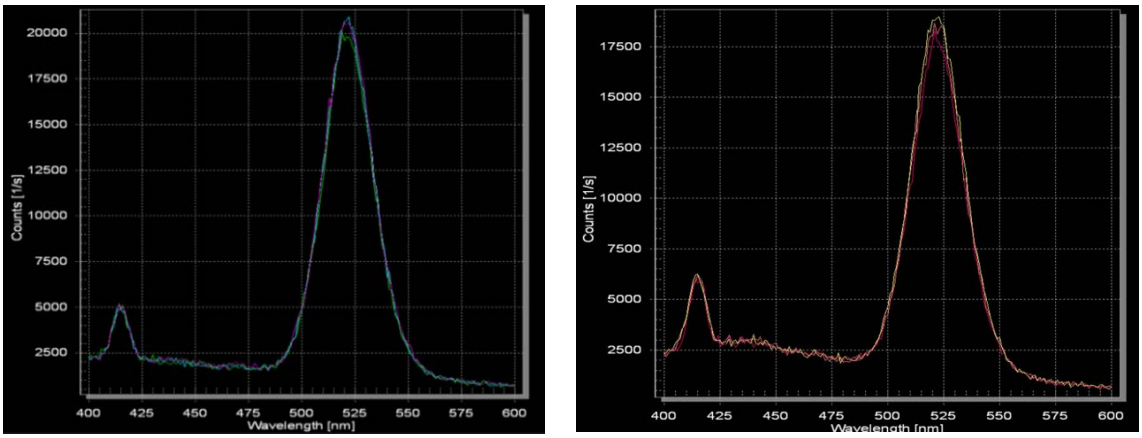

Figure 43: 2:1 AuNP-L-QD, Laser (left) and lamp (right), no FC

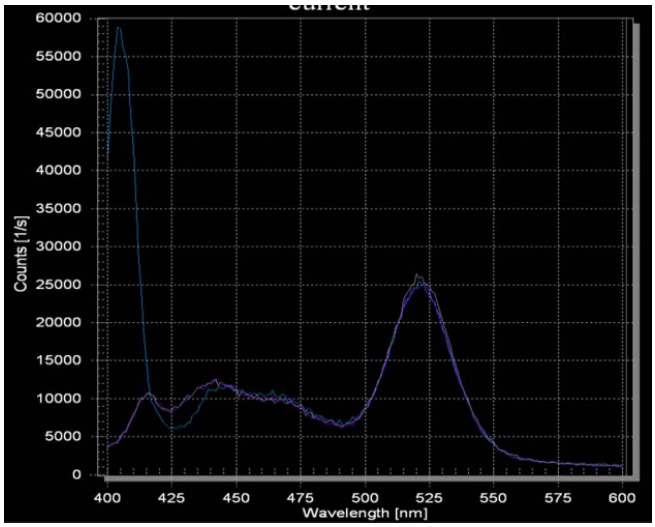

Figure 44: 2:1 Au:QD (no L)

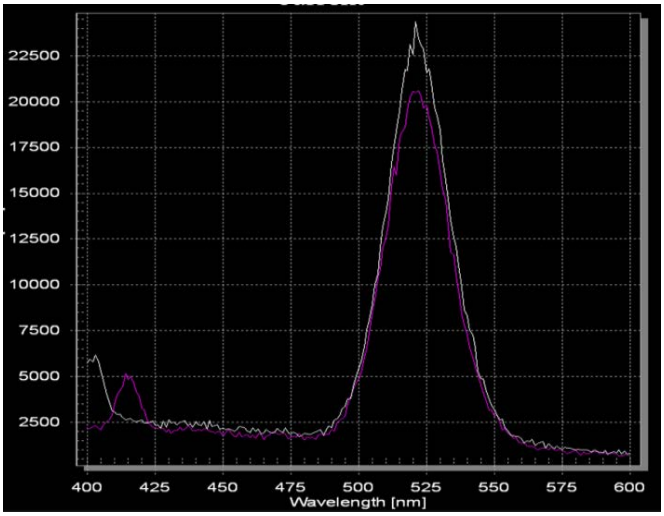

Figure 45: Raman Peak Shift (not from QD)

Adding twice as much AuNPs as QDs did not yield much improvement. As before, there was no increase in fluorescence, but the laser and lamp samples were more consistent this time (likely better redilution). No FC gave a weaker sample. The QD-AuNP control was greater than the no FC samples, so it is possible some quenching may

have occurred. But the conclusion is weak given that there was no increase in fluorescence upon illumination. The variance of intensities makes solid conclusions difficult to achieve.

One other major concerns from past FRET is that even if there is some quenching, there was no subsequent increase in fluorescence upon linker cleavage, as should be occurring if the AuNP-L-QDs in fact conjugated and then photocleaved. One hypothesis was that the laser power was not sufficient to cleave enough linker for detectable restoration of fluorescence after FRET. Although this is unlikely because the spectrofluorometer is very sensitive, the 3rd FRET attempt in part compared the FRET of the AuNP-L-QD complexes after exposure to laser and to a hand-held UV lamp. Although it is possible that the UV-lamp might also not have had the power to cleave samples, it should have been sufficient, since signs of cleavage were detected using UV-Vis absorbance in prior experiments (see pg. 48). This also tested if the "quenching" seen in 2nd FRET was reproducible under slightly different conditions (with less inner-filter effects).

The general setup was as follows: 5 min lamp exposure to select samples, 5 min laser exposure for all samples, test if the peaks shift at a different excitation wavelengths, FC and no FC, and 1:1 and 2:1 AuNP:QD ratios (not all of the data shown).

As the Figure 41 above shows, the sample with the laser were not significantly quenched compared to the lamp sample. Oddly, the control (QD-AuNPs, non-conjugated) was very weak. The fluorescence of "conjugated" samples was not significantly weaker (i.e., experiencing FRET) than the controls (it was, in fact, more intense).

4.2.5: TEM Images

DI deO_2 water (990.452 uL) was added to a centrifuge tube labeled “7.1” with 9.5 mg of stock ME. Then, 100 uL “7.1” and 900 uL deO_2 DI water were added to a centrifuge tube labeled “7.2”.

TEM samples:

Nanopartz AuNPs (0.488 uL), 96.662 uL deO_2 PBS, 0.85 uL of “7.2,”, and 2 uL of stock 4 linker were added to a centrifuge tube covered in foil. The samples were allowed to react for 1 hour on setting “3” of rotavapor, then 2x FC using “24 ME” solution as the washing fluid. The samples were then rediluted with “24 ME” to get the original concentrations, with 5 uL of 20QD added as part of the redilution. The samples were then conjugated with QDs for about an hour before TEM images were taken.

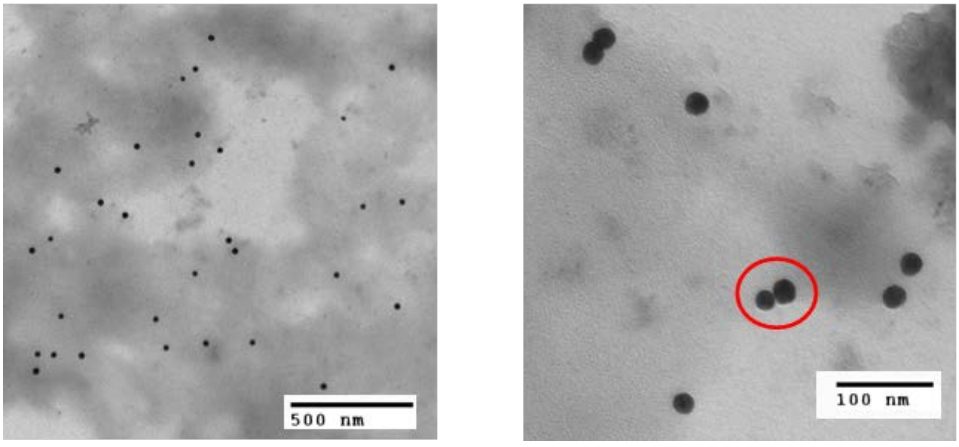

Figure 46: TEM Images of “Conjugated” AuNP-L-QD Complexes

TEM was also used to measure the success of conjugation. As Figure 46 shows, there was very little conjugation, with only a few successes. It should be noted, though, that from the lab's experience, TEM does not provide a very accurate way of proving conjugation; fluorescent samples can still appear unconjugated under TEM, likely because the linker was broken off during the sample drying process (required for TEM). In the end, fluorescence is the best way to see if FRET occurred or not.

4.2.6: High AuNP concentration:

10 nM AuNP Procedure:

10 nM AuNPs (no citrate):

Nanopartz AuNPs (12.188 uL) were added to 237.812 uL 1x Dulcetto's PBS (pH 7.5). Sample absorbance was taken at 0 and 24 hours.

10 nM AuNPs (with citrate):

Nanopartz AuNPs (13.407 uL) and 6.4 mg of sodium citrate dehydrate were added to 261.593 uL 1x Dulcetto's PBS (pH 7.5). Sample absorbance was taken at 0 and 23 hours.

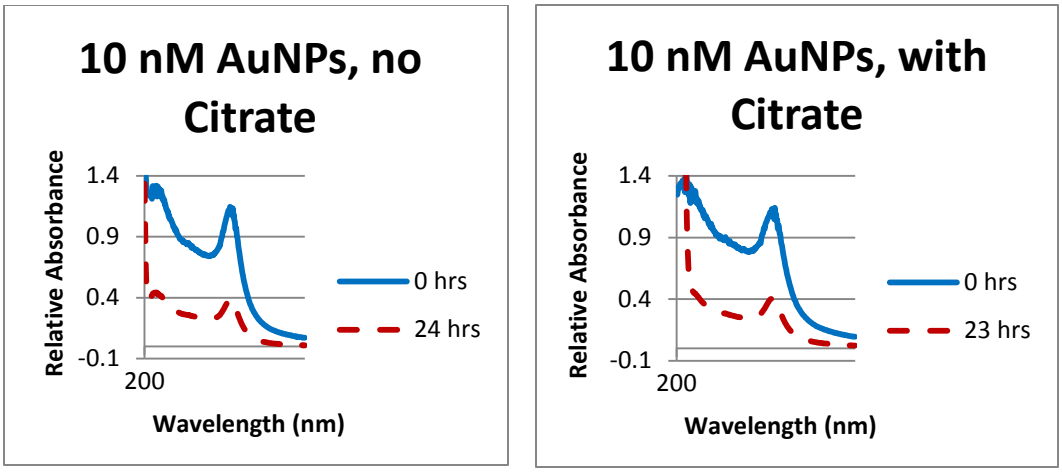

Figure 47: 10 nM AuNPs, without citrate (left) and with citrate (right)

Since AuNPs are stable in their concentrated stock forms, and since a control quenching test was successfully performed (see next section) at higher AuNP concentrations, some AuNP stability tests were conducted with higher AuNP concentrations (>1 nM). As seen from Figure 47 above, a 10 nM AuNP concentration did not improve stability over what was observed with ME. Since much of AuNPs are citrate-based, citrate was added in an attempt to improve stability, but without any noticeable effect (see Figure 47 above). Thirty nM was too concentrated for UV-VIS and its fluorescence spectra were not much different than the 100 nM FRET results. To directly compare the FRET results to the control (conjugation without linker), only the AuNP concentration at 100 nM will be shown and discussed below.

4.2.7: 4th FRET

Procedure for quenching control:

All water samples were DI water and all water, PBS, and MeOH samples were without oxygen. Though solutions were made at the concentration of 100 nM, they were rediluted to 1 nM before testing (e.g. fluorescence, TEM).

Made four samples:

The four samples made were: AuNP-QD in buffer, AuNP-QD in water, QD-EDC/NHS in water, and QD-EDC/NHS in buffer. Note: QDs, NHS, EDC were added quickly to preexisting solution volumes and quickly combined because NHS and EDC are unstable for long exposures in water.

Preparation of QDs:

Methanol (MeOH) (4 uL) and 3 uL QDs were each added to centrifuge tubes labeled “BQ” and “WQ”. The centrifuge tubes were sealed with Parafilm until use.

Preparation of EDC:

MeOH (500 uL) was added to the tube with 2.6 mg EDC and was then briefly vortexed. MeOH (2,314 uL) and 28.5 uL of the stock EDC were added to a 4 mL glass vial “E2”. The solution was briefly mixed. Then, 4 uL of the solution was added to BQ and WQ each.

Preparation of NHS:

MeOH (500 uL) was added to the tube with 5.4 mg NHS, and vortexed briefly. MeOH (2,657 uL) and 28.6 uL of the stock NHS was added to a 4 mL glass vial "H2." After the resultant solution was briefly mixed, 4 uL of the solution was added to BQ and WQ each. BQ and WQ were allowed to react for 30 min.

AuNP-QD in buffer:

After the 30 min reaction, further added 117 uL AuNPs, 24.74 uL pH 8.37, 0.485M phosphate buffer and 83.25 uL water to BQ.

AuNP-QD in water:

After the 30 min reaction, further added 117 uL AuNPs and 108 uL water to WQ.

QD-EDC/NHS control in buffer (for fluorescence only):

MeOH (4 uL), 3 uL QDs, 4 uL EDC solution, 4 uL NHS solution, 24.74 uL phosphate buffer (pH 8.37, 0.485M) and 200.25 uL water were added to CB1.

QD-EDC/NHS control in water (for fluorescence only):

MeOH (4 uL), 3 uL QDs, 4 uL EDC solution, 4 uL NHS solution, and 225 uL water were added to CW1. The solution was allowed to react for ~4 hours. Then, FC 3x at 5000 rcf, 10 min. After measuring the weight of the initial sample, water was added and then the 1st FC was performed. FC samples were rediluted to 100 nM right before examining fluorescence. All redilutions and washings were with water.

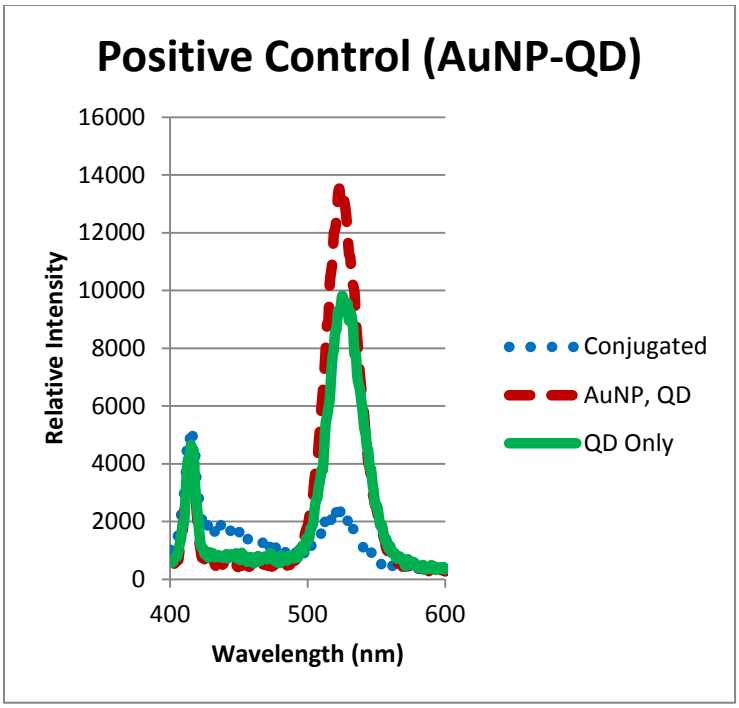

Figure 48: Quenching from Conjugated AuNP-QD control

It was possible that difficulties in FRET and consistent quenching were because of AuNP instability. Even with ME ligand, the NH_2 groups may have detached from the AuNP, resulting in unconjugated AuNP-L-QD complexes. To test if the AuNPs may have been the cause of no reproducible FRET, a control quenching test was performed between AuNPs and carboxyl QDs, conjugating them directly to each other as a positive control (no linker involved).

The key principles of the procedure were followed from a bioconjugation paper.[33] Here, it was very important to use proper volumes/ratios of solutions. For example, MeOH was not allowed to be more than 5% of the solution volume, NHS was added in 100x excess, EDC in 55x excess. Furthermore, the carboxyl QDs were unstable at lower molarities, so a 100 nM QD (and also Nanopartz AuNP) concentration was used.

Furthermore, because pipettes had limits to how much volume they could transfer, 4 uL was the minimum volumes pipetted from samples.

As seen from Figure 48, clear quenching of the conjugated AuNPs-QDs were achieved. Given a sufficiently short linker, a similar strategy with 100 nM AuNPs (diluted to 1 nM before testing) should show clear FRET.

100 nM AuNPs with Linker FRET:

Procedure:

In all cases, deO_2 DI water, and deO_2 PBS were used. Samples were also made with 30 nM, but these did not give better results than 100 nM, so only a result for 100 nm and its procedure are shown.

Note: Because it was expected that FC would destabilize some AuNPs, the target concentration was 90 nM QDs. However, there were no FC filters available at the time of experiment, so testing was done without FC, but still at 90 nM QDs.

100 nM AuNP-L-QD:

PBS (18 uL), 9.75 uL of the 2nd batch of Nanopartz AuNPs and 1.35 uL Stock 3 linker were added to a centrifuge covered in foil. The sample was allowed to react on rotation setting “3” for 3 hours. Then, 1.35 uL QDs were added.

100 nM AuNP, QD (nonconjugated):

PBS (18.9 uL) and 9.75 uL of the 2nd batch of Nanopartz AuNPs were added to a centrifuge tube. 1.35 uL QDs were added to the centrifuge tube after the 100 nM AuNP-L-QD sample had reacted for 3 hours.

QD only:

Water (28.65 uL) was added to a centrifuge tube. QDs (1.35 uL) were added to the centrifuge tube after the 100 nM AuNP-L-QD sample finished its 3-hour reaction, added.

Tested fluorescence of all three samples by diluting them to 1nM: 99 uL water and 1uL solution

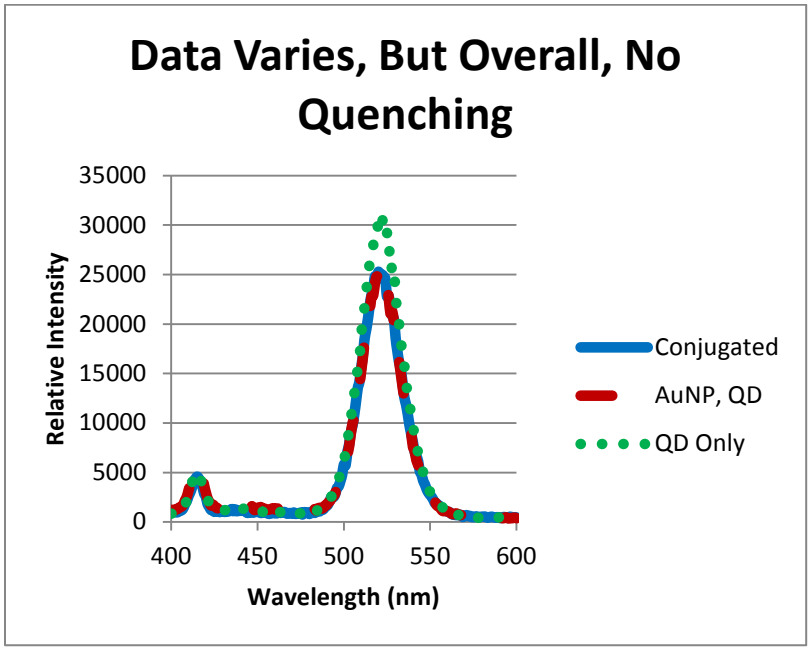

Figure 49: One of the results from 100 nM AuNP Experiment

Since 100 nM AuNPs can achieve quenching with just a QD, 100 nM AuNP (and QD) concentration was attempted with the linker. Figure 49 shows one of the results from the experiment:

The variability of the fluorescence intensities was a concern in these spectra. Sometimes, the controls were slightly more quenched than the conjugated samples, sometimes, they were slightly more intense, and occasionally, as in Figure 49, they were almost identical. If clear quenching from FRET was observed, the procedure could have been optimized to reduce variability. One sample seemed to have quenching, but when it was tested two more times, the quenching could not be replicated, leading to the conclusion that maybe it had less QDs in that particular trial. In the end, there were no clear sings of quenching nor increase in fluorescence as would be expected if FRET had indeed occurred.

Chapter 5: Conclusions and Further Work

Both Nanocs and Nanopartz AuNPs were unstable in buffer, which was required for AuNP-L conjugation. It is possible to add stabilizing ligands, but that could lead to conjugation interference and possible loss of the NH_2 groups on the AuNP. Thus, the best stability strategy was to use high concentrations of AuNPs (~100nM) and dilute to 1nM right before use.

TEM provided helpful information, but it did not accurately reflect results of quenching experiments. This is likely a result of the linker breaking when the solutions were dried for TEM. Fluorescence measurements are the surest way to confirm if FRET occurred or not.

There was great variability in the fluorescence spectra, making strong conclusions difficult. Although some samples showed potential quenching compared to control (2^{nd} FRET), there was never a significant increase in fluorescence upon application of light, which should have cleaved the linker and increased the fluorescent intensities of the samples. Furthermore, though positive FRET results may have been occasionally obtained, they were not reproducible, and most likely indicated a lack of QDs (due to experimental error or possible QD instability).

It should also be noted that from estimates of streptavidin and linker length and the $1/R^6$ dependence of FRET on distance, possible quenching was on the borderline of

the FRET boundary. In fact, one paper using just streptavidin-biotin calculated a maximum FRET efficiency for streptavidin was 14%.[18] Since our length is likely beyond 5 nm, we could expect no more than 50% quenching efficiency.[18] The combination with the linker and streptravidin distance may have increased the QD-AuNP separation distance to one too large for FRET to occur. Most of the experimental data showed no significant quenching, with only one FRET test having some quenching, which was not repeated in any further FRET tests (though slightly different methods were used).

Because the carboxyl-QD and amine AuNPs did exhibit clear quenching, the best recommendation for future work would be to choose a much shorter linker. In particular, the streptavidin protein should be avoided because of its large size. Some possibilities are linkers that change conformations[40] upon UV light and polymers that can stretch and shrink in response to photoactivation or other stimuli. Larger NPs may also be helpful.

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