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Protein Nanoparticles for Use in Photodynamic Therapy

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Protein Nanoparticles for Use in Photodynamic Therapy

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B.S., Indiana University of Pennsylvania, 2015

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Protein Nanoparticles for Use in Photodynamic Therapy

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**Scheme 1:** Py-Phe and its derivatives. Binding constants (M\(^{-1}\)) for the probe binding to BSA are listed in blue.
Chapter 1: Introduction to photochemistry of proteins

The study of photochemistry observes the electronic changes that occur when a molecule absorbs a photon of light and the interactions that the active molecule has with other molecules. This information can be used to create a wide array of applications including solar cells, biomedical applications such as protein cleavage and photodynamic therapies, as well as industrial applications such as automotive coatings. The following chapter highlights some of the basic principles and uses for photochemistry.

1.1 Basics of photochemistry

Photochemistry is governed by two main laws. The first law of photochemistry, Grotthus-Draper law, indicates that for a photochemical reaction to occur a photon of light must be absorbed by a molecule. Most photochemical reactions are completed at wavelengths in the visible light region of the electromagnetic spectrum (Figure 1). The electromagnetic spectrum orders wavelengths of light in terms of their photon energies and the visible range consists of the wavelengths between 400-750 nm. The wavelengths in this region are responsible for the colors observed by the human eye, which are a direct result of the transmitted wavelengths of white light that are not absorbed by an object. Additionally, this region is useful to photochemical reactions because it allows for increased specificity of the reaction. If the chemical species of interest does not absorb light at the wavelength of interest, no reaction will occur.
The probability (rate) of a photon being absorbed by a molecule is governed by two principles; Beer law and Lambert law. Beer law states that the concentration of the solution is proportional to the amount of radiation that is absorbed. Lambert law states that the fraction of incident radiation (power) that is absorbed by the medium is independent of the intensity of the incident radiation and, that each cross section of the medium will absorb an equal fraction of the incident light; that the absorbance is proportional to the light path. Combined together, Beer-Lambert law can be used to derive equation 1.\(^7\)

\[
A = \varepsilon b C \quad \text{eq. (1)}
\]

Where $A$ is the absorbance, $\varepsilon$ is the molar absorptivity ($\text{L mol}^{-1} \text{ cm}^{-1}$); $\varepsilon$ is the molar absorptivity; $b$ is the path length of the cuvette, and $C$ is the concentration. The absorbance value needed can be obtained using absorbance spectroscopy, which measures the absorbance of a sample as a function of wavelength.\(^6\)

To maintain the linear relationship between the variables the absorbance value of the
dilute solution needs to be between 0.1 and 1. Using too concentrated of a solution can cause shift in the spectrum.

The second law of photochemistry (Stark-Einstein law) states that for each photon (quantum) of light absorbed, only one molecule is activated.\textsuperscript{7} When photons are absorbed by the system, a transition from a lower energy to a higher energy state occurs. The efficiency of the reaction is called the quantum yield.

While in this higher energy (excited) state, various chemical and physical processes can occur. The electronic states and the various transitions that can occur are schematically represented using a Jablonski (energy level) diagram (Figure 2).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{jablonski_diagram.png}
\caption{Jablonski diagram. $S_0$ indicates the ground state, $S_1$ is the first singlet excited state, $S_2$ is the second singlet excited state, $T_1$ and $T_2$ is the triplet excited states\textsuperscript{8}}
\end{figure}

In the Jablonski diagram, $S_0$ denotes the ground (lowest) energy state and $S_n$ and $T_1$ (Figure 3, bold horizontal lines) denote the singlet and triplet excited (higher) energy states.\textsuperscript{8} The designation between the singlet and triplet states and what makes one more...
accessible than the other is based upon the pairing of the electrons (Figure 3). In the Singlet excited state, all of the electrons are paired and the Pauli exclusion principle is upheld. In the triplet state, the electrons are no longer paired, they are parallel. Since the Pauli exclusion principle is no longer upheld, the transition is considered “forbidden”.

Within the different energy states, several vibrational levels exist (non-bolded horizontal lines). Radiative processes, such as fluorescence are denoted with vertical bold lines. Non-radiative processes, such as inter-system crossing, are denoted with vertical dotted lines. These processes do not require the transfer of energy.

Radiative processes such as absorbance or fluorescence are processes in which a photon’s energy is transferred to the electrons in a molecule. Many radiative processes provide photophysical information that is useful to characterizing a molecule. This is due to their non-destructive nature; the reactions are fast and concentration dependent, emitting or absorbing energy at an amount equal to that of the energy associated with the difference between the ground and excited states. Radiative processes include absorbance, fluorescence, and phosphorescence. Absorbance is a process in which a photon of light is captivated by a molecule, sending electrons from the ground state to the vibrational levels of the singlet excited states (Figure 2, blue arrow). Fluorescence (Figure
2, yellow arrow), is the opposite of absorbance. Instead of absorbing photons of light, sending the molecule into the excited state, the excited electrons are emitted, sending them back towards the ground state. The intensity at which the light is emitted from the sample is proportional to the intensity of the incident radiation. The other main radiative process is phosphorescence (Figure 2, red arrow), where light is emitted from the molecule on a slower time scale because the return to the ground state from the triplet state is spin forbidden.  

Conversely, nonradiative processes such as internal conversion or intersystem crossing are transitions that redistribute the available energy. Internal conversion (Figure 2, green wavy arrow) occurs as a result of vibrational relaxation to the lowest excited state level, a necessity to uphold Kasha’s rule. Intersystem crossing involves the transition from the excited single state to the excited triplet state (Figure 2, dark green wavy arrow), changing the spin multiplicity. Conversion to the triplet state also provides the necessary energy to obtain some photochemical products.

Unlike light activated photochemical reactions, thermal reactions require a change in temperature change the energy therefore, do not require a light source to initiate the reaction. Furthermore, thermal chemical reactions focus on the exchange of energy between the sample and its surroundings (endothermic and exothermic). Opposite of photochemical reactions, the temperature has a significant effect on the rate of reaction.

1.2 Model Protein
Serum Albumin (SA) was utilized as a model protein because it is inexpensive, abundant, and well characterized. This 66 kDA protein (Figure 4) is a waste product of the meat industry. Its major function is as a carrier protein in the circulatory system giving it the ability to bind numerous small molecules, fatty acids, metal ions, amino acids, peptides, and hormones. This heart shaped protein is made of three homologous domains each with the ability to support hydrogen bonding, electrostatic interactions and hydrophobic interactions. Furthermore, it also contributes to the overall colloidal osmotic pressure, the pressure exerted by proteins in blood plasma.

Figure 4: Crystal structure of Bovine Serum Albumin (BSA) (PDB ID: 3VO3).

1.3 Applications of photochemical reactions
There a wide variety of applications that utilize light to initiate or terminate the reaction due to its benign reaction conditions. Several applications that are relevant to protein are outlined below.

1.3.1: Protein photocleavage

Reagents with the ability to photocleave protein have a wide variety of applications including structural investigations,\textsuperscript{16} therapeutic design,\textsuperscript{17} or footprinting ligand binding sites on large biological structures.\textsuperscript{18} Use of light to initiate and terminate these reactions not only provides an element of control over the reaction but, does not create harmful byproducts because it is a “green” reagent.\textsuperscript{19} A further advantage to this method is its ability to be completed in solution, providing insight into the relevant structures of biomolecules in this phase.

In designing a photocleaving agent several factors need to be considered, including the size and shape reagent, its hydrogen bonding capabilities, chirality, and charge. These features work together to selectively bind one site on a protein, minimizing the number of protein fragments created. The approach to synthesize these photoreagents is based on the assumption of ‘Complementarity’, in which the features of the photocleaving reagent should “complement” the binding site of the target protein.\textsuperscript{19} The design of Py-Phe (Figure 5) features several structural aspects that are necessary for protein photocleavage reaction to occur. Py-L-Phe, a photocleaving reagent, has both hydrophobic and hydrophilic regions that are separated by a short peptide linker. These features work in concert to provide specificity for the binding site. The hydrophobic and hydrophilic regions are composed of a pyrenyl chromophore (Py) that would be
buried within the protein and a Phenylalanine (Phe) residue that would reside at the protein/solution interface as a recognition element. The 6 atom linker region serves to connect the two moieties while keeping them separate enough that they fulfill the requirements of the binding site. The chromophore needs to be carefully chosen to ensure that photons of the desired wavelength are absorbed ensuring the initiation of the reaction. Pyrene has a high extinction coefficient in the near UV region (>40,000 M<sup>-1</sup>cm<sup>-1</sup> at 343 nm). Carbodiimide chemistry was used to put the three components together. In a normal EDC coupling reaction, the primary amines are covalently attached to carboxylic acids with the help of a coupling agent.

![Figure 5: Structural features of Py-L-Phe.](image)

Organic photocleaving agents like Py-Phe and its various derivatives (Scheme 1) have strong absorption in the near visible region, a long lived excited state, and high affinity for hydrophobic sites on proteins, making it an excellent reagent for protein photocleavage at a single site.
Each molecule within this family of photocleaving agents has the ability to selectively bind to BSA and cleave it. However, each derivative has different binding abilities that affect the cleavage yields. Within the family of agents there molecules with reversed charge, different recognition elements, different linker lengths, and varied chirality. The binding abilities of each molecule provides insight to the contribution of the different pieces of the reagent. For example, the difference between the binding constants for Py-L-Phe and Py-D-Phe indicate the importance of the chirality of phenylalanine (the recognition element). Also, based on the 10 fold decrease in the binding abilities of PMA-L-Phe and PMA-D-Phe, it can also be concluded that the charge of the molecule is also important. Lastly, experiments in which the linker length is systematically changed indicates that the length is also important. Analysis of the ligands shows that most of them bind to the protein site non-competitively and, with single occupancy.
The reaction for protein photocleavage required the addition of a Co(III) metal complex such as Co(III) Hexammmine (CoHA). Co(III) complexes are known to quench the pyrene excited state and are needed in order to produce the necessary cation radical.\textsuperscript{19} In a typical reaction, photoreagent, protein, tris-acetate buffer, and CoHA were hit with 343 nm light for a desired time period. After the designated period of time, the sample was removed from the light and analyzed with SDS/PAGE (Figure 6).

\textit{Figure 6:} SDS/PAGE gel of BSA photoreaction. Lane 1: Molecular weight markers in kDa, Lane 2: BSA, Lanes 3-7: BSA+Py-L-Phe+CoHA, all irradiated for 0 (dark control), 10, 20, 30 and 60 minutes, Lane 8: BSA, Lane 9 BSA+Py-L-Phe, Lane 10: BSA + CoHA, Lanes 8-10 irradiated for 30 minutes

The cation radical formed during the reaction is hypothesized to cause hydrogen atom abstraction from the peptide backbone, causing photocleavage to occur (Figure 7). The mechanism for photocleavage was elucidated by synthesizing various derivatives of Py-Phe as well as through flash photolysis studies. Cationic probes in which the polarity of the amide bond was reversed, as a result of this reversal, the photoproduct yield was <5\%.\textsuperscript{19} Probes in which the linker length was changed and probes containing a different recognition element also shows significantly less photoproduct as compared to Py-L Phe and Py-D-Phe.\textsuperscript{2,17}
Using a light activated cleavage agent it is possible to site specifically cleave protein. This application is advantageous because it allows for simple binding site mapping and makes larger proteins easier to sequence.

Computational modeling of Py-Phe bound to BSA (Figure 8) was completed and was in good agreement with the experimental data determined through amino acid sequencing.
Photocleavage of BSA by a photocleaving agent is well characterized with computational and experimental methods. Using this same method (Figure 9) the binding site for the fluorescent dyes on the protein nanoparticles and the cross link density of the BSA proteins that make up the particles can be elucidated.

![Diagram](image)

**Figure 9: Possible scheme for photocleavage of BSA protein nanoparticle.**

There is also the chance that the particle will not be evenly cleaved, at which case the particle may form two fragments of different sizes. If the cleavage site is at a point in which not all the cross-links are destroyed, no fragments may be observed. If that is the case, it might take multiple reactions to see cleavage of the protein.

**Chapter 2: Testing the photochemistry parameters of GlowDots**

Photobleaching of a fluorophore is a dynamic process in which the molecule is destroyed after exposure to an excitation light source. Commonly, this occurs when the fluorophore undergoes a photo-oxidation reaction while in the singlet or triplet excited state. Once a fluorephore molecule has been bleached, it no longer participates in emission, thereby creating a loss in signal intensity. A low photobleaching quantum yield indicates that several excitation-emission cycles are necessary before a loss in signal occurs, indicating that the rate of intensity loss will be
The goal of this study is to determine the photobleaching quantum yields of GlowDots, to help support their experimental use as an imaging agent.

2.1 Introduction

The study of photobleaching is important for a number of reasons, mainly in order to test the rate of fluorophore destruction in a sample. One application of a fluorescent sample is as an imaging agent. Molecules of this nature require a low photobleaching quantum yield to ensure that they will last for 2-3 hours at a time (a normal confocal microscopy session). For the quantification of a photobleaching experiment, a common method is chemical actinometry. A chemical actinometer is a system that has been well characterized and for which the quantum yield is known. The system should be sensitive and thermally stable, with additional advantages being use of simple analytical methods for analysis. These systems undergo light induced reactions that involve either a reversible or irreversible chemical reaction, when irradiated with a specific wavelength of light, allowing the user to directly observe the number of photons absorbed as a function of time. Commonly, an actinometer is used to calibrate a detector or, to determine the quantum yield for a photochemical reaction using equation 2. Specifically, an actinometer can be used to determine the number of einsteins (photons per unit time) for a photochemical reaction.

\[
\Phi = \frac{\text{number of moles consumed or produced per unit time}}{\text{number of photons absorbed per unit time}}
\]  

\text{eq (2)}

Tris (2, 2'-bipyridyl) ruthenium(II) chloride (Ru(bpy)$_3$Cl$_2$) (Figure 10A), a precious metal catalyst, can be utilized as a chemical actinometer within the visible light
spectrum for calibration of a light source. Use of the visible portion of the electromagnetic spectrum uses milder conditions, allowing for a "greener" reaction.\textsuperscript{24} Additionally, \textit{Ru(bpy)}$_3$\textit{Cl}_2 is a diamagnetic molecule with a long lived excited state lifetime. Utilization of the singlet oxygen chemistry \textit{Ru(bpy)}$_3$\textit{Cl}_2 is well known allows the actinometer to monitor the oxidation of 1,9-diphenlyanthracene (DPA) (Figure 10B) to its corresponding endoperoxide using Uv/Vis spectroscopy. The oxidation reaction is monitored specifically because the corresponding peak is not obscured by the metal-to-ligand charge transfer band of the \textit{Ru(bpy)}$_3$\textit{Cl}_2. This system was chosen instead of the well-known ferrioxalate actinometer because of its ease of use.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structures}
\caption{Structures of \textit{Ru(bpy)}$_3$\textit{Cl}_2 and DPA.}
\end{figure}

Photodynamic therapy (PDT) is a type of cancer treatment that uses light and oxygen to damage cells, sending them into apoptosis.\textsuperscript{25,26} In this system, singlet oxygen is produced upon the interaction of the light beam with a photosensitizer, commonly a fluorescent dye, creating free radicals which has a destructive effect on cells. In the absence of light, PDTs are benign.\textsuperscript{34} In a normal treatment, the PDT is injected into the diseased tissue in the first step and exposed to a specific wavelength of light in the second.\textsuperscript{27} The main limitations of this methodology include low tumor
targeting efficacy and poor water solubility.\textsuperscript{34} However, use of nanoparticles, micelles, and other types of nanomaterials have been integral in overcoming these issues.

GlowDots are made from bovine serum albumin (BSA), a waste product from the meat industry. BSA is also non-toxic\textsuperscript{28} and has been approved by the Food and Drug Administration for medicinal applications; making it an excellent material for biological nanotechnologies. This protein is also natural drug-delivery vehicle that has the capability to bind a number of small molecules, dyes, peptides, hormones, and drugs.\textsuperscript{29} BSA has 99 COOH groups from glutamate and aspartate residues, and 59 amine groups from lysine side chains which can be used for self-crosslinking to build particles of any desired size. Furthermore, any leftover amine and carboxyl groups as well as other surface functions can be used for the exquisite control of particle surface chemistry, unlike QDs which require surface passivation and modification.\textsuperscript{30} GlowDots do not require any specialized modification of their surface to be soluble in aqueous solutions, unlike QDs or polymer nanoparticles.

In the current studies, we have examined the quantum yields of emission and quantum yields of photodegradation of GlowDots, which are reported in this chapter.

\textbf{2.2 Experimental}

\textbf{2.2.1 nBSA Synthesis}

Protein solution was prepared by stirring 150 mg of BSA in 1 mL of de-ionized water (\(d\)H\(_2\)O). EDC (1M, \(d\)H\(_2\)O) was added in 10 mM aliquots and stirred for 20 min between additions. Particle growth was monitored by dynamic light scattering (DLS). Reaction was quenched by adding 2 mL of 20 mM CO\(_3^{2-}\)/HCO\(_3^-\) pH 9.3 buffer. Sample was then diluted into 100 mM Phsophate 150 mM Sodium Chloride buffer pH 7.0 and
heated to 85 C for 10 minutes. Solutions were allowed to cool back to room temperature. Taurine powder was added to the nanoparticle solution (100x concentration of protein) and 100 mM EDC added dropwise to the solution. This was allowed to stir for 30 min. A solution of fluorescent dye, (0.5 % (w/w)) in DMSO was added to the protein solution and stirred for 4 h in the dark at room temperature. Samples were filtered in Amicon 100 kDa cutoff centrifuge filter tubes with 10 mM Na₂HPO₄ pH 7.0 buffer until filtrate was clear of fluorescent dye evidenced by absorption spectrum of the run through (approximately 10 x sample volume). This method was repeated for making all 6 varieties of GlowDots: 1- Pyrene butanoic acid (340), 7-methoxycoumarin-3-hydroxy (350), Diethylaminocoumarine-3-carboxylic acid (432), fluorescein isothiocyanate (494), tetramethylrhodamine -5-(and-6)-isothiocynate (543), and 5-(and-6)-carboxy-x-rhodamine (576). All samples were denoted as GlowDotX where X is the emission wavelength of the dye.

2.2.2. Absorbance measurements

Absorption spectra were measured on an HP 8450 diode array spectrophotometer (Varian Inc., Santa Clara, CA). Samples were diluted in 10 mM Na₂HPO₄ pH 7.0 and the baseline averaged from 700-900 nm was subtracted.

2.2.3. DLS measurements

Hydrodynamic radius of GlowDots was monitored by photon correlation spectroscopy with Precision Detectors (Varian Inc., part of Agilent Technologies), CoolBatch+ dynamic light scattering apparatus with 1 x 1 cm² square plastic cuvette and a 658 nm excitation laser source with a 90° geometry. Data collection was done at room temperature, for 1 s, 3 repetitions with 200 accumulations. All samples were filtered with 0.22 µm filter (PDVF,
13 mm, Restek). Precision Ellucidate v 1.1.0.9 and Precision Deconvolve v 5.5 were used to collect and analyze the data respectively.

2.2.4. Transmission electron microscopy measurements

GlowDots solution (1 ng/mL) was applied to a carbon-coated Cu grid (400-mesh) and blotted with filter paper (Whatman #4). After an hour of drying, the grids were imaged using a Techni T12 S/TEM.

2.2.5. Agarose gel

Agarose gels were prepared by dissolving agarose (0.5 % w/v, Sigma electrophoresis grade) in heated Tris acetate (40 mM, pH 7.0). The gel was poured on a horizontal electrophoresis apparatus (Gibco model 200, Life Technologies Inc., MD) and Tris acetate (40 mM, pH 7.0) was used as the running buffer. Samples were loaded into the wells at the center of the gel with 50 % (v/v) loading buffer (50 % v/v glycerol, 0.01 % m/m bromophenol blue). Electrophoresis was carried out for 30 min at 100 mV at room temperature. The gel was stained overnight with 0.02 % m/m Coomassie Blue, 10 % v/v acetic acid and then destained overnight with 10 % v/v acetic acid.

2.2.6. Photobleaching experiments

To determine the photobleaching quantum yields of each of the particles, samples were irradiated using a monochrometer and tested against a standard. Standards used in the experiment were Ru(bpy)$_3$Cl$_2$ actinometer or free dye in solution. Samples were irradiated until a 10% loss in absorbance was observed. Wavelengths used were standard confocal laser lines (405 nm, 488 nm, and 561 nm). In the cases where the dye
conjugated to the surface of the protein did not absorb in any of those regions, 340 nm and 365 nm were used. Before irradiation, the standard was matched to the sample of interest at the aforementioned wavelengths to ensure that the same number of photons were present.

2.2.7. Circular Dichroism

CD spectra were measured on a Jasco J-710 CD spectrometer. A concentration of 3 µM protein in 10 mM Na₂HPO₄ buffer pH 7.0 was used. Spectra were obtained using a 0.05 cm path length quartz cuvette in the region of 260 – 195 nm. Other operating parameters were: sensitivity 100 mdeg, data pitch 0.5 nm, continuous scanning mode, 50 nm/min scanning speed, 1 s response, 1.0 nm bandwidth and 3 accumulations. CD spectra were corrected by subtracting buffer signal from sample signal. Enzyme structure retention was assessed by calculating the change in ellipticity where BSA in 10 mM Na₂HPO₄ buffer pH 7.0 was taken as 100 % ellipticity.

2.3 Results/Discussion

In this method, the carboxylic groups of bovine serum albumin (BSA) molecules were cross-linked with the amine groups of neighboring BSA molecules. Particle formation was monitored via dynamic light scattering (DLS). Once 60% or more of the particles reached 35 nm diameter, the particle formation was quenched with the addition of carbonate buffer. To achieve a monodispersed size, particles were annealed at 85 °C for 5 minutes. Protein nanoparticles were then decorated with reactive fluorescent labels to provide them with a tunable emission. Final products were characterized by DLS, agarose electrophoresis, TEM, circular dichroism, as well as, absorbance spectroscopy.
Subsequently, each particle was named using the term ‘GlowDot’ and the peak absorbance wavelength associated with the attached dye. For example, protein nanoparticles labeled with fluorescein isothiocyanate are designated as GlowDot494.

2.3.1. Synthesis of nBSA

To synthesize the protein nanoparticles (GlowDots) used in the photochemical studies carbodiimide chemistry was used to slowly cross-link carboxylic acid and primary amines from a 150 mg/mL BSA solution together using 1 M EDC (Figure 1).

Figure 1: Synthesis of GlowDot protein nanoparticles.

Throughout the synthesis, dynamic light scattering (DLS) was used to check the size of the particles. An aliquot of the 10 mM EDC was added to the BSA solution every 20-30 minutes until the DLS showed sizes of 9 nm and 40 nm. At this point, the solution was quenched by adding 2x the sample volume of 100 mM phosphate, 500 mM sodium chloride buffer, pH 7.2 and the size distribution was allowed to equilibrate for 24 hours to obtain a 50/50 distribution of 9 and 35 nm. To achieve a monodispersion of particles, the sample was diluted to 1 mg/mL and heated at 85°C for 5 minutes. After cooling to room
temperature, the particles were labeled with 0.5% w/w of one of six reactive fluorescent dyes (Table 1) and allowed to stir in the dark, at room temperature for 4 hours. To purify the samples, filter centrifugation was utilized to remove any free dye, free protein, or salt from the solution. Samples were washed with 10 mM phosphate buffer, pH 7.2 until the run through showed no absorbance peak at 280 nm.

Table 1: Fluorescent dyes used in the synthesis.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Particle Abbreviation</th>
<th>Structure</th>
<th>Abs/Em</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Pyrenebutanoic acid, succinimidyl ester</td>
<td>GlowDot340</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>340/376 nm</td>
</tr>
<tr>
<td>7-Methoxycoumarin-3-carboxylic acid, succinimidyl ester</td>
<td>GlowDot350</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>358/410 nm</td>
</tr>
<tr>
<td>7-Diethylaminocoumarin-3-carboxylic acid, succinimidyl ester</td>
<td>GlowDot432</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>432/472 nm</td>
</tr>
<tr>
<td>Fluorescein Isothiocyanate</td>
<td>GlowDot494</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>494/520 nm</td>
</tr>
<tr>
<td>Tetramethylrhodamine-5 (and 6)-isothiocyanate</td>
<td>GlowDot543</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>543/571 nm</td>
</tr>
<tr>
<td>5-(and-6)-Carboxy-X-rhodamine, succinimidyl ester</td>
<td>GlowDot576</td>
<td><img src="image6.png" alt="Structure" /></td>
<td>576/601 nm</td>
</tr>
</tbody>
</table>

2.3.2. Characterization of nBSA
To characterize the GlowDot samples, several techniques were used. To better understand the optical properties of the particles, absorbance spectroscopy was used. All of the particles show a change in the dye absorbance peak in comparison with the free dye (Figure 12A). GlowDot340 and GlowDot543 had an additional peak while a peak shift (compared to free dye) was observed for all other GlowDots. Changes in absorbance spectra suggest that the dyes are buried inside the protein nanoparticle with limited solvent exposure.

![Absorbance and Fluorescence spectra of GlowDot particles](image)

*Figure 12: A) Absorbance spectra and B) Fluorescence spectra of the GlowDot particles.*

To demonstrate the size of the particles, DLS and transmission electron microscopy (TEM) were used. All particles were annealed to have a size of approximately 40-50 nm, which closely matches particle sizes seen in the transmission electron microscopy image of the protein nanoparticles (Figure 13B). A single size distribution was observed after purification of the nanoparticle that was labeled with a reactive fluorescent dye.
Agarose gel electrophoresis was used to confirm that the reactive fluorescent dye is actually binding to the protein. Agarose gel electrophoresis of GlowDot340, GlowDot350, GlowDot432, GlowDot494, GlowDot543 and GlowDot576 initially imaged using UV light (254 nm, Figure 14A) clearly show five fluorescent bands directly matching the protein bands viewed in the coomassie stained gel (Figure 14B). The lack of a second fluorescent band in the lanes of the gel confirms there is no free dye in the samples. All of the GlowDots migrated a shorter distance towards the positive electrode compared to unreacted BSA. This is due to the increase in size of the particle and the possibility of small changes in the net charge of the protein due to crosslinking. While every conjugation event consumes a positively charged amine and a negatively charged carboxylic acid, resulting in no net change in overall charge, cross-linking could result in the exposure of residues that are normally buried, hence changing the net charge of the protein.
Figure 14: A) Agarose gel electrophoresis (160 mM Tris Acetate, pH 8.0) demonstrated the decreased mobility of GlowDot (lane 2) compared to BSA (lane 1). Addition of the fluorescent dyes 1-pyrenebutanoic acid, succinimidyl ester (340), 7-methoxycoumarin-3-hydroxy (350), Diethylaminocoumarine-3-carboxylic acid (432), fluorescein isothiocyanate (494), tetramethylrhodamine-5-(and-6)-isothiocynate (543), and 5-(and-6)-carboxy-x-rhodamine (576) (lanes 3-7 respectively). B) Coomassie Blue stain gel of fluorescent protein nanoaparticle gel in B.

2.3.3 Photobleaching Experiments

Photostability experiments were completed to ensure the stability of the particles upon exposure to light. Most of the particles were photostable for at least 30 minutes. GlowDot 494 and GlowDot543 were the most photostable, not losing 10% of their absorbance intensity for 1 hour. GlowDot340, GlowDot350, and GlowDot432 was the least photostable, losing 10% of their intensity in less than 10 minutes.

Photobleaching quantum yields were calculated using either Ru(bpy)$_3$Cl$_2$ actinometer or the free dyes in solution as standards to help quantify the light intensity. GlowDot494 was calculated by determining the number of photons absorbed in the
system using chemical actinometry. Actinometer used for experiments was Ru(bpy)$_3$Cl$_2$ as outlined by Pitre, et al. 2015. In a normal experiment, actinometer absorbance was matched to GlowDots at a specific wavelength (340, 365, 405, 488, 561 nm). After matching, each sample was irradiated using a light beam passed through a monochromator (Figure 15) for time increments ranging from 30 seconds - 30 minutes depending on the particle. Conversely, the actinometers were irradiated for increments ranging from 5 seconds to 60 seconds.

The particle intensity decrease was monitored by absorbance spectroscopy. In a typical experiment, samples were irradiated at room temperature in the dark until a 10% drop in the absorbance at the monitored wavelength was observed. In many cases, photoirradiation caused chemical cross-linking of the particles. To remove these large aggregates, samples were centrifuged at 10,000 rpm for 2 minute increments before
taking the absorbance. Samples containing pyrene (Figure 16A and B) were monitored at 340 nm. Methoxycoumarin (Figure 16C and D) were irradiated and monitored at 365 nm. Samples containing DEAC (Figure 16E and F) were irradiated and monitored at 405 nm. FITC samples (Figure 16G and H) were monitored at 488 nm. Samples containing TRITC (Figure 16I and J) and ROX (Figure 16K and L) both were monitored at 561 nm.
Using the absorbance data and equations 3-5\textsuperscript{24}, the photobleaching quantum yields could be calculated. First, the moles decomposed by irradiation of the sample need to be calculated (eq. 3).

\[
\text{# moles consumed} = \frac{\text{Absorbance}_{\text{initial}} - \text{Absorbance}_{\text{final}}}{\varepsilon l} \times V \quad \text{.......................... (eq 3)}
\]

Where Absorbance\textsubscript{initial} is the absorbance value at the appropriate wavelength from the matched samples, Absorbance\textsubscript{final} is the value at the same wavelength after the 10% drop in intensity is absorbed, \(\varepsilon\) is the extinction coefficient calculated for the free dye at that wavelength, \(l\) is the pathlength of the cuvette (1 cm) and, \(v\) is the volume of the sample (0.6 mL). The dilution factor of the sample also needs to be considered. Next, to determine the intensity of the light source equation 4 was used.

\[
\frac{N\hbar \nu}{\tau} = \frac{\text{moles consumed}}{\Phi I} \quad \text{........................... (eq 4)}
\]
Where $\frac{N_{hv}}{t}$ is number of photons per unit time, moles consumed is the number calculated in equation 3, $\Phi$ is the relative quantum yield of the free dye (table 2) or the calculated quantum yield of Ru(bpy)$_3$Cl$_2^{24}$ and $t$ is the irradiation time in seconds. Finally, to calculate the photobleaching quantum yield for the irradiated particles, equation 5 was used.

$$\Phi = \frac{\text{Number of moles consumed or produced}}{t} \times \left(\frac{N_{hv}}{t}\right)^{-1} \quad \ldots \quad \text{(eq 5)}$$

Where the number of moles consumed or produced was calculated using equation 3, $t$ is irradiation time in seconds, and $\left(\frac{N_{hv}}{t}\right)^{-1}$ is the reciprocal of the value found in equation 4.

**Table 2: Summary of photobleaching quantum yield data.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Photostability (Minutes) 10% loss</th>
<th>Relative Quantum Yield</th>
<th>Photobleaching Quantum Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlowDot 340</td>
<td>&lt; 10 minutes (Irradiated at 340 nm)</td>
<td>37.0</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>GlowDot 350</td>
<td>&lt; 10 minutes (Irradiated at 365 nm)</td>
<td>1.2</td>
<td>5.7 x 10$^{-3}$ ± 0.01</td>
</tr>
<tr>
<td>GlowDot 432</td>
<td>&lt; 10 minutes (Irradiated at 405 nm)</td>
<td>12.7</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>GlowDot 494</td>
<td>30 Minutes (Irradiated at 488 nm)</td>
<td>0.4</td>
<td>5.5 x 10$^{-3}$ ± 0.003</td>
</tr>
<tr>
<td>GlowDot 543</td>
<td>60 Minutes (Irradiated at 561 nm)</td>
<td>1.6</td>
<td>3.1 x 10$^{-3}$ ± 0.004</td>
</tr>
<tr>
<td>GlowDot 576</td>
<td>25 Minutes (irradiated at 561 nm)</td>
<td>0.9</td>
<td>$4.4 \times 10^{-4} \pm 1.5 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

Ideally, the lowest photobleaching quantum yield possible is going to be optimal. After completing each measurement in triplicate, it was determined that GlowDot576 has the lowest photobleaching quantum yield, $4.4 \times 10^{-4}$ (Table 2 and Figure 17) which indicates that its rate of photodestruction is the slowest. The rate of fastest photodestruction occurs with GlowDot432 (Table 2). The experimental value obtained for GlowDot494 in 10 mM phosphate buffer, pH 7 is $5.5 \times 10^{-3}$ which is comparable to literature, in which the photodestruction quantum yield of free FITC in water at pH 9 is $1.2 \times 10^{-4}$.\textsuperscript{31}
Overall, the values obtained in the study are in good agreement with the values shown in literature. Furthermore, the primarily low values indicate that GlowDots would be excellent for use in confocal microscopy because they would be able to sustain the high intensity laser.

2.3.4. Analysis of irradiated products

Circular dichroism was used to determine whether or not the native structure of BSA was retained after irradiating the samples with certain wavelengths of light. The
structure of the BSA in GlowDots and the irradiated GlowDots were analyzed and compared to free BSA to determine whether any of the secondary structure was lost due to cross-linking. To normalize the protein concentrations of each sample a Bradford assay was carried out.
As shown in Figure 18, irradiated samples show a significant loss in alpha helical structure (less than 50%). By setting BSA to depict 100% ellipticity, structure retentions for GlowDots and their irradiated counterparts were calculated using the signal intensity at 220 nm. Non-irradiated GlowDot sample’s secondary structure retention of at least 50%. The irradiated samples showed structural retention < 50% (Table 3), indicating that the samples were destroyed upon light irradiation. GlowDot350 showed the highest amount of structural retention upon irradiation (47%) and GlowDot494 showed the least amount of retention (31%).
Table 3: Summary of the % structural retention calculated for the irradiated and non-irradiated samples of GlowDots.

<table>
<thead>
<tr>
<th>Sample:</th>
<th>% retention:</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlowDot340</td>
<td>45%</td>
</tr>
<tr>
<td>Irradiated</td>
<td>40%</td>
</tr>
<tr>
<td>GlowDot340</td>
<td></td>
</tr>
<tr>
<td>GlowDot350</td>
<td>53%</td>
</tr>
<tr>
<td>Irradiated</td>
<td>47%</td>
</tr>
<tr>
<td>GlowDot350</td>
<td></td>
</tr>
<tr>
<td>GlowDot432</td>
<td>53%</td>
</tr>
<tr>
<td>Irradiated</td>
<td>35%</td>
</tr>
<tr>
<td>GlowDot432</td>
<td></td>
</tr>
<tr>
<td>GlowDot494</td>
<td>61%</td>
</tr>
<tr>
<td>Irradiated</td>
<td>31%</td>
</tr>
<tr>
<td>GlowDot494</td>
<td></td>
</tr>
<tr>
<td>GlowDot543</td>
<td>46%</td>
</tr>
<tr>
<td>Irradiated</td>
<td>35%</td>
</tr>
<tr>
<td>GlowDot543</td>
<td></td>
</tr>
<tr>
<td>GlowDot576</td>
<td>52%</td>
</tr>
<tr>
<td>Irradiated</td>
<td>43%</td>
</tr>
<tr>
<td>GlowDot576</td>
<td></td>
</tr>
</tbody>
</table>

2.3.5 Cell uptake studies

To test the ability of GlowDots as imaging agents, cell uptake studies have been completed. To boost their ability to be taken up by cells, a taurine label was coupled to...
the nanoparticle surface. Taurine was chosen for this task because of its known ability to increase uptake of D-peptides by cells tenfold.\textsuperscript{32} As shown in Figure 19, white emitting particles were successfully taken into Hela cells. Based on the cell uptake studies and the photobleaching studies, GlowDot particles have the potential to be useful as a PDT.

![Image](image.png)

*Figure 19: Overlay of red, green, and blue channels of HeLa cell uptake of nBSAW405 particles decorated with the natural amino taurine and the cell under transmitted light.*

Presently in literature, there are several serum albumin PDTs in the works.\textsuperscript{26,33,34} For example, in a paper by Preub, A. et al.\textsuperscript{26} Human serum albumin (HSA) nanoparticles with diameter 100-300 nm were synthesized using a well-known solvation method before being loaded with one of two porphyrin based photosensitizers (Figure 20) which are known to interact with organelles such as lysozomes, and mitochondria.\textsuperscript{26}
The main experiments completed were to understand the drug release and phototoxicity as a function of incubation time as well as to understand the uptake mechanism and photophysical properties of the two systems in Jurkat cells. To understand the uptake mechanism, nanoparticle degradation and drug release were monitored with confocal microscopy. They also investigated the ability of the photosensitizer molecules (Figure 20) to generate singlet oxygen. In their experiments, mTHCP loaded particles had a higher phototoxicity than mTHPP loaded ones, based on a higher absorbance of the excitation wavelength.\textsuperscript{26} mTHPC particles have a higher ability to produce singlet oxygen due to its amphipathic nature it also has a faster release rate.

Based on the results shown above and the literature data, it may be possible to use the protein particles to create a PDT. Based on the photostability data collected the dyes embedded in the protein react with singlet oxygen to create free radicals in a photooxidation reaction. These radicals then cause irreversible damage to the cell and eventually leading to apoptosis.\textsuperscript{35} For the reaction to optimally occur, it important that the
protein vehicle, in this case BSA, be in its monomeric form because induces an increase in singlet oxygen production.\textsuperscript{36} By exchanging the visible range fluorescent dye currently used on the GlowDot particle’s surface for a Near Infrared (NIR) dye, it would be possible to create a PDT with a significant penetration depth. An organic NIR dye, like the visible range ones could be used as a photosensitizing agent. Furthermore, since BSA has the ability to bind a large number of molecules, it is possible to attach a targeting agent to the surface of the protein to increase specificity for tissues or cells of interest. It is necessary to ensure that the targeting mechanism works correctly, so as to keep healthy cells from being destroyed. If the experiments work, a novel PDT can be synthesized (Figure 19) containing folic acid as a specificity element, a NIR florescent dye as a photosensitizer and, a biocompatible protein as a vehicle.

\textit{Figure 21: Reaction for synthesis of GlowDot494 conjugated with folic acid and taurine for cell uptake and selectivity.}

One possible pathway would be to label the particles with folic acid to increase the specificity for MCF-7 (breast cancer).\textsuperscript{37} Folate receptors overexpress at the surface of cancer cells but produce low levels of folate in normal cells (such as A549), providing a simple way to distinguish between the two types of cells.\textsuperscript{37,38} Using this cell targeting method, the receptor-mediated endocytosis pathway can be used for uptake.\textsuperscript{39} Upon uptake, hitting the area with a light source would enact the PDT, killing the cells of interest (Figure 22).
2.4 Conclusions

Since the photochemical reaction was completed in the presence of oxygen, it can be hypothesized that destruction of the fluorophore occurs as a result of a photooxidation reaction. However, more experiments are needed to confirm the hypothesis. Circular dichroism experiments show that adding a fluorescent dye to the protein nanoparticles does not significantly affect the protein secondary structure. However, hitting the protein nanoparticles with a concentrated light beam does significantly impact the secondary structure. Additionally, most of the particles have low photobleaching quantum yields; signifying that the rate of intensity loss is slow. This is further confirmed based on the length of time for which the particles showed a 10% loss in the absorbance intensity at peak associated with the confocal laser line. Most of the particles did not see a noticeable change until 30-60 minutes of irradiation. Since a common confocal microscopy session is 2-3 hours, it is likely that the particles would make decent imaging agents. Due to the crosslinking of the particles observed upon irradiation, the method of photobleaching is photooxidation. This is further supported based on the presence of oxygen during the
experiment. This feature opens the particles up to a whole new realm of possible applications, including use in photodynamic therapy.

Chapter 3: Towards the synthesis of protein nanoparticle protected gold nanoclusters

One potential direction for the protein nanoparticles is to replace the reactive fluorescent dye with gold nanoclusters. At their small size, gold nanoclusters fluoresce in the near IR region, an advantageous trait for biological applications. Fluorescence in the near IR region allows for a higher penetration depth and decreased background noise.

3.1 Introduction

Gold nanoclusters (AuNCs) are groupings of gold atoms held together by metal-metal bonds having a diameter of less than 3 nm. Within this realm of clusters exist gas phase clusters, condensed (solution) phase clusters, and organometallic clusters. Due to their small size, they usually require a capping agent, such as a protein or organic ligand to keep them from aggregating together in solution. Furthermore, their electronic properties differ from those associated with larger nanomaterials made of the same metals. For example, AuNCs are not able to support the surface plasmon resonance (SPR) band that is associated with bulk gold and gold nanoparticles. The absence of this band is due to the inadequate density of the gold atoms to merge the conduction and valence bands (they are very close to the Fermi energy/ de Broglie wavelength). They
also are able to exhibit size dependent fluorescence in the visible and near Infrared region of the electromagnetic spectrum.

The emission wavelength of the nanoclusters are dependent on the number of atoms that make up the gold nanoclusters and are proportional the Fermi energy of gold \( (E_{\text{Fermi}} = 5.53 \text{ eV for gold}) \). Using the spherical jellium approximation, the number of atoms can be calculated using the equation \( E_{\text{emission}} = E_{\text{Fermi}}/N^{1/3} \); Where \( N \) is the number of atoms per cluster.\(^{41}\) This model explains the relation between the delocalized free conduction electron behavior relative to the atomic core size.\(^{41}\) Literature indicates that a wide range of cluster sizes have been formed experimentally; forming clusters made of anywhere between 3\(^{42}\) and 55 gold atoms and, emission wavelength is dependent on the number of atoms present per cluster. Generally, as the size increases the fluorescence emission red shifts, until the size of the clusters exceeds 30 atoms. At this point, the clusters no longer are fluorescent. AuNCs have fluorescence emission wavelengths ranging from 400 - Near IR range, where Au\(_{25}\) clusters have a fluorescence peak around 650-700 nm. Furthermore, photophysical properties of the clusters are also affected by the pH, temperature and, capping agent used in the synthesis. The pH of the solution is important for controlling the power of the reducing agent as well as the conformation of the protein. The temperature is also important in controlling the reaction rate and the conformation of the protein capping agent. Lastly, the capping agent is important to the Ligand-metal-charge transfer (LMCT) that occurs. A capping agent capable of donating a large number of electrons is optimal.

Chemical reduction, photoreduction, and chemical etching are the main ways to synthesize AUNCs.\(^{43}\) Because they can be made in several fashions, they also have a
wide variety of capping agents at their disposal, making them advantageous to a wide realm of applications including detection and dual-modality imaging agents. Namely, that the NIR emission of the particles gives them an increased penetration depth and deals with less background noise. Furthermore, their ultrasmall size and low cytotoxicity are additional advantages. Use of chemical reduction allows for the reduction of $\text{Au}^{3+}$ to $\text{Au}^{0}$ with the help of a reducing agent, for example, Bovine serum albumin or sodium borohydride.\textsuperscript{43}

Among the many possible capping agents used in literature to stabilize the clusters, Bovine serum albumin (BSA) is one of the most common.\textsuperscript{40} BSA is well characterized and known to be nonhazardous. Other capping agents have also been used to synthesis gold nanoclusters through chemical reduction including MES and other small molecules,\textsuperscript{44} polymers,\textsuperscript{45} and other proteins.

Gold nanoclusters are of interest because of their high quantum yields\textsuperscript{41} nonphotobleachability as compared to organic fluorophores\textsuperscript{46} and, longer lifetime.\textsuperscript{46} They have the ability to be useful in several applications including catalysis, sensing, and drug delivery.

3.2 Experimental

To characterize the BSA protected gold nanoclusters, herein called Au-nBSA, DLS, Fluorescence and absorbance spectroscopy were used.
3.2.1. Au-nBSA synthesis

Au-nBSA particles are synthesized using an abridged method of nBSA synthesis in conjunction with a well-known synthesis for gold-BSA particles.\textsuperscript{40} In this method, nBSA particles were cross-linked with EDC, quenched with 2x the volume of either Deionized water or 100 mM phosphate 500 mM sodium chloride buffer (pH 7.2), and allowed to equilibrate overnight.\textsuperscript{47} The sample was then diluted to 50 mg/mL and combined with an equal volume of 10 mM HAuCl\textsubscript{4} and a small volume of 1 M NaOH before being heated overnight at 37°C. After heating, the solution was centrifuged at 11,000 rpm for 15-20 minutes to remove large aggregates.

3.2.2. DLS

Hydrodynamic radius of Au-nBSA was monitored by photon correlation spectroscopy with Precision Detectors (Varian Inc., part of Agilent Technologies), CoolBatch+ dynamic light scattering apparatus with 1 x 1 cm\textsuperscript{2} square plastic cuvette and a 658 nm excitation laser source with a 90° geometry. Data collection was done at room temperature, for 1 s, 3 repetitions with 200 accumulations. All samples were filtered with 0.22 µm filter (PDVF, 13 mm, Restek). Precision Ellucidate v 1.1.0.9 and Precision Deconvolve v 5.5 were used to collect and analyze the data respectively.
3.2.3. Absorbance Spectroscopy

Absorption spectra were measured on an HP 8450 diode array spectrophotometer (Varian Inc., Santa Clara, CA). Samples were diluted in 10 mM Na$_2$HPO$_4$ pH 7.0 and the baseline averaged from 700-900 nm was subtracted.

3.2.4. Fluorescence Spectroscopy

Fluorescence Spectra were recorded on Cary Eclipse Fluorimeter. All spectral measurements were done in a 1 x 1 cm$^2$ quartz cuvette.

3.3 Results/Discussion

Small introduction

3.3.1. Synthesis of Au-nBSA particles

To show the diverse possibilities that are available with nBSA, particles containing gold nanoclusters were synthesized using a modified version of a well-known BSA protected gold nanocluster method (Xie, J et al).\textsuperscript{40} Figure 23 illustrates the synthesis. Using the protein nanoparticle synthesis described previously, \textsuperscript{47} BSA is cross-linked together and the reaction is quenched with either deionized water or 100 mM, 500 mM sodium chloride buffer, pH 7.2. After allowing the sample to equilibrate overnight, 10 mM HAuCl$_4$ is added to the solution and allowed to stir for 2 minutes before the addition of 1 M NaOH, which gives BSA to act as a reducing agent for the gold. The solution is then heated overnight to obtain a solution that is deep red in color.
3.3.2 Characterization of Au-nBSA

Shown in Figure 24, the sample changes from a deep red (before centrifugation) to a light brown after centrifugation. The weaker fluorescence seen in Figure 21B can be attributed to the lower concentration of gold nanoclusters embedded in the protein; as centrifugation removed a large number of particles from the sample. This difference might also be attributed to the ionic strength of the buffer used, which could affect the formation of the clusters in the protein due to shielding with the ions. Dynamic light scattering (DLS) was also used to show the change in size upon centrifugation. Figure 24A shows that when deionized water quenched protein nanoparticles are used to make Au-nBSA, the
size of the particles shows a trimodal, broad set of sizes. When 100 mM phosphate, 500 mM sodium chloride buffer is used to quench the protein nanoparticle synthesis, the size becomes more defined (Figure 24B). To further attempt to control the size, the incubation temperature will be manipulated.

The absorbance spectrum obtained from the quenched particles show little to no peak in the SPR band for gold (Figure 25A), which is consistent with literature. The lack of an SPR band is indicative of the gold nanoclusters embedded in the protein being less than 3 nm.
Fluorescence spectroscopy was used to determine the sizes of the gold clusters embedded in the protein using the spherical jellium model $E_{\text{emission}} = E_{\text{Fermi}}/N^{1/3}$. To do this, $E_{\text{emission}}$ and $E_{\text{Fermi}}$ need to be converted to wavelengths using $E = \frac{hc}{\lambda}$.\(^{49}\) In this model, the Fermi wavelength for gold is 0.7 nm.\(^{49}\) Using the peak wavelengths from the fluorescence data (Figure 25B), the clusters sizes for BSA protected gold nanoclusters in water and in 100 mM phosphate, 500 mM sodium chloride buffer are ~10 atoms per
cluster. 10 atoms per cluster is bigger than those reported in literature (25 atoms/cluster is common)\(^{40}\), indicating that the cross-linked protein nanoparticles may change the availability of the amino acid side chains that the clusters are sequestered around.

![Figure 26: Agarose gel of Au-nBSA. Lane 1: Au-nBSA, Lane 2: nBSA, Lane 3: BSA. A) shows gel under 365 nm UV light. B) shows gel after Coomassie Blue stain for 24 hours.](image)

Agarose gel electrophoresis was used to ensure that the gold nanoclusters are actually being formed with the protein. Figure 24 shows that the protein band and the fluorescence associated with the nanoclusters each traveled the same distance, indicating that they are either in close proximity to each other or that they are attached. The clear spot seen in lane 1 of Figure 24B is due to overloading of the gel.

The DLS spectra of the particles show that there is a wide size distribution when initially synthesized (Figure 23 A and C). To remedy this the samples were centrifuged at 11,000 rpm for 20 minutes to remove the large particles. After centrifugation, the
particles quenched with deionized water showed a trimodal distribution (Figure 23B). Using a higher ionic strength solution to quench the protein nanoparticles significantly decreased the size distribution (Figure 23D), although the size is larger than what is considered to be a nanoparticle, making it not useful for drug delivery.\textsuperscript{50}

\textit{Figure 27:} A) Au-nBSA particles made in Deionized water before centrifugation. B) Au-nBSA particles made in Deionized water after 15 minutes centrifugation at 11,000 rpm. C) Au-nBSA particles made in 100 mM phosphate, 500 mM salt buffer before centrifugation. D) Au-nBSA particles made in 100 mM, 500 mM sodium chloride buffer after centrifugation.
3.4 Conclusions

Based on the data accumulated, it is possible to apply the nanoparticle synthesis method to other systems, showing its versatility. The nanoparticles synthesized in this work were in good agreement with the photophysical data previously described in literature. By changing the ionic strength of the solution or the incubation temperature of the synthesis it is possible to control the size distribution of the particles. Furthermore, changes to the pH and to the temperature of the reaction can be used to control the number of atoms per cluster. To fully understand the mechanism behind the formation of nanoclusters and how to control the synthesis of Au-nBSA, more work needs to be done.

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