Development of Novel Biological Nanomaterials Through Protein Chemical Modification

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Enzymes are capable of catalyzing many different reactions, some of which are not possible through conventional organic chemistry methods. However, their sensitive nature to environmental conditions such as pH, temperature, solvents, and denaturants limits their applications. One method of stabilizing protein against denaturing conditions is to adsorb them onto the surface of nanomaterials such as 3D nanoparticles, 2D inorganic nanodisks or even creating carrier free protein nanoparticles with no support system present. However, even these methods have drawbacks of low enzyme loadings, loss in activity and cost of production.

The object of this dissertation was to develop novel nanomaterials. First, to better understand the physical properties that effect protein adsorbing to nanoparticles, enzymes were modified chemically to alter net protein charge so that the relationship of charge on the enzyme to charge on α-ZrP could be better understood. It was concluded that protein charge and ions present in solution play an important governing role in how much enzyme can be adsorbed onto a surface.

Development of facile surface modification method for controlling the physical properties of nanoparticles for enhanced enzyme adsorption. Protein was modified chemically to have a net positive charge, resulting in favorable binding between the positively charge protein and negatively charge silica nanoparticle surface. Anionic catalytic enzymes were adsorbed onto this nanomaterial and their percent loading and catalytic activity assessed. For both enzymes investigated, percent loading (m/m) and catalytic activity retention were among the highest reported to date.

Lastly a nanoparticle made of protein with independently tunable emission, size and surface properties was synthesized. These particles are biodegradable, nontoxic and capable of white emission. White
emitting particles are highly sensitive to cellular local environment. The methods of investigation and interpretation of the results are discussed in full in this work.
Development of Novel Biological Nanomaterials through Protein Chemical Modification

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B.S., University of Nebraska at Kearney, 2011

A Dissertation

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APPROVAL PAGE

Doctor of Philosophy Dissertation

Development of Novel Biological Nanomaterials through Protein Chemical Modification

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(There are no bad ideas, only ideas meriting death – Klingon Proverb)
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Chapter 1.
Introduction

1.1. Biological nanomaterials

Chemical modification of natural materials derived from proteins, carbohydrates, lipids and nucleic acids can control the properties of these natural materials in a rational manner. Such bio-derived materials would be sustainable, green, biodegradable, but for practical applications, we need to demonstrate materials of very high value in terms of function, durability, stability and price. A significant advantage of these materials is that these are chemically modified, not biochemically, and hence we can access infinite chemical space and not limited to natural amino acids or nature itself. One reason for this effort has been the concern regarding the extensive accumulation of synthetic polymers in our environment at an alarming rate, which is causing extensive damage to the life on this planet. This problem needs urgent attention from scientists, engineers as well as the general public to find acceptable solutions. Our approach is to use proteins as raw materials and modify them chemically, such that these biological materials are rapidly degraded in a pre-programmed manner into harmless components when released into the environment after use. Naturally occurring materials may not have the necessary characteristics and properties for the intended purpose, or for device applications, and hence, chemical modification of the natural materials is a logical route to access these materials.

One major advantage of such biological materials is that they can be rationally programmed to degrade when exposed to the environmental conditions over pre-determined time scales and hence, they would not accumulate in the environment or generate toxic waste during synthesis, use or disposal. Another advantage of biological materials is that these can be rationally designed based on fundamental principles of chemistry to make conducting, insulating or semiconducting materials. Similarly, their size, refractive index as well as hydrophobicity or lipophilicity can be controlled at the molecular level via well-known chemical methodologies.

A clear understanding of the properties of the biological materials with respect to their composition, surface chemistry, morphology, assembly, stability as well as interactions with other kinds of matter is
essential to tailor their properties for specific applications. Examples of chemical modification of ordinary proteins to make protein fluorescent nanoparticles of controlled size are described in this thesis. A comparison is made between these bio-derived materials and the current state-of-the-art quantum dot equivalents. The examples illustrated here show the possibility of making biodegradable materials, which are functional, programmable, inexpensive, novel and also generate new and exciting chemistry. In this chapter, an introduction to such materials will be provided with few specific examples to demonstrate the strategy, scope and versatility of the approach and philosophy of making bio-derived materials for the long term sustainability of our standard of living on this planet.

1.2. Synthesis of nanoparticle and types of composite nanoparticles.

Nanomaterials have applications in many different areas such as environment, energy, diagnostics, therapeutics, electronics, and sensing just to name a few. A nanomaterial is any material that is 1 to 1000 nanometers in length in at least 1 dimension. There are many different types of nanomaterials from biological virus capsid to man-made quantum dots, each exhibiting its own set of unique properties that make it suitable for particular functions.
Nanoparticles Synthetic Strategy

Top-down method

Bottom-up method

Vapor phase synthesis

Liquid phase synthesis

Use of solid precursors

Liquid/Vapor precursors

Co-precipitation
Sol-gel methods
Microemulsions

Inert gas condensation
Pulsed laser ablation
Spark discharge generation
Ion sputtering

Chemical vapor synthesis
Spray pyrolysis
Laser pyrolysis
Plasma synthesis
Flame synthesis

Figure 1. Two methods for synthesizing nanoparticles: top down and bottom up. Top down method is physically separating bulk into small pieces while there are many chemical ways to synthesis nanoparticles from bottom up.

Nanoparticle can be synthesized two different ways; top down or bottom up. The top down method begins with the bulk item that is physically separated it to smaller and smaller pieces until left with nano sized particles. Physical separation of the bulk material can be accomplished via milling, grinding and volatilization but results in a wide distribution of sizes of the particles. Further methods must then be deployed to get a more precise batch of material with controlled, narrowly distributed sizes. The bottom up method starts with the smallest building block the nanomaterial and then uses chemical approaches to build up the particle. There are many different methods for synthesizing nanomaterials by bottom up method (Figure 1). This work will focus on particles synthesized by bottom up methods.
In order to create versatile nanoparticles with multiple functions, composite nanoparticles of two or more chemical phases are created. These materials are expected to have properties of all of the phases in the particles and in some cases may even exhibit new properties not seen in any of the individual components arising from the unique interfaces or defect sites created in the particle. Different types of nanocomposite materials are shown in Figure 2.¹ The most common type of composite nanoparticles are core@shell structure where the two materials are distinctly separated with one completely covering the other. Other methods include heterostructure, where two joined particles have a single common interface, intermetallic and alloy particles that form brand new compounds with new characteristics, and lastly matrix dispersed nanoparticles where one component is dispersed inside the other material(s). This chapter will also discuss the adsorption of biological catalysts on these nanomaterials to as an example of making bioactive, biological materials that match or exceed the performance of conventional chemical catalysts. This is an important current industrial application and may also play an important role in synthesizing novel biological materials described above.

1.3. Advantages of enzyme adsorption
Figure 3. (A) Free energy (G) of native (N) vs. denatured (D) states of enzymes. When enzyme interact either the difference between G for the N and D state of the enzyme will become larger, leading to enzyme stabilized by the particle or the free energy (G) between the N and D state will become smaller, occurring when the enzyme is destabilized by the nanoparticle. (B) Goal of adsorption of protein onto nanoparticles is to stabilize enzyme by lowering the free energy of the native state and raising the free energy of the denatured states (center), however sometimes adsorption onto nanoparticles can result in destabilization of the nanoparticles; raising of the native state free energy and lower of the denatured state free energy.

Enzymes are nature’s catalysts and highly versatile for the synthesis of new biological materials under benign conditions of room temperature, atmospheric pressure and near-neutral pH, conditions that are favored to decrease the effect on the environment. Enzymes, however, are unstable under the conditions of most chemical reaction conditions, expensive, and function on only a limited set of substrates. To address these important issues, our lab and many other around the world have been developing rational approaches to stabilize enzymes and broaden their selectivities such that one enzyme could work on multiple substrates at reasonable rates.

One rational approach to stabilize enzymes has been to adsorb them in the galleries of inorganic layered solids and preserve the biological activities of enzymes which are important for industrial catalysis, drug delivery and sensing applications. Enzymes operate best under specific temperature, pH, and when they can maintain their native structure. Such limitations causes many headaches when
trying to use them in most applications. Enzyme denaturation requires a certain amount of free energy. When extreme environmental factors such as increasing temperature, for example, is applied then the free energies of the native state and the denatured states are equalized at the denaturation temperature. Further increase in temperature favors the denatured state, biologically inactive protein. A good way to counter this problem is to create a system in which the difference in free energy of the native state and denatured state is greater when compared to that in solution. This can be achieved by placing the enzyme on a nano solid support which acts to lock in a part of the enzymes structure, thus increasing the free energy gap between the native and denatured states. However, enzyme binding must be done with care and avoid denaturing the structure during the process. Some nano solid surfaces also have the reverse effect, where they lower the free energy gap between the native and denatured states, thereby facilitating enzyme denaturation which is often not desirable.

Another challenge of working with enzymes is separating them from their products and recycling them for more than a single cycle. Particularly when using enzymes for industrial catalysis, it is necessary to separate the enzyme from the product. Free enzyme in solution with its product is difficult to separate requiring lengthy and potentially costly methods. In addition to difficulty reclaiming the enzyme, their recyclability is low. Binding on solid supports can make it easier to reclaim enzyme and because of its stabilizing effects on the enzyme, allow for more uses of the enzyme before all activity is lost.

There are four main methods of enzyme binding; adsorption and deposition, physical entrapment, covalent attachment, and bio conjugation. With each method having its own set of advantages and disadvantages (Table 1). An ideal support will have high enzyme loading, high retention in activity of enzyme after loading, stable to mechanical and enzymatic degradation and should be feasibly priced. Reported binding methods to date offer only a random selection of these traits leaving the rest to behind. For example, deposition of enzyme onto solids while showing high activity retention does not offer high enzyme loadings. There is work to be done in enzyme binding to improve the methods to give high loading, high enzyme activity all the while keeping the process facile and feasible for large scale production.
Table 1. Advantages vs disadvantages of different methods of immobilizing enzyme onto a solid surface.\(^5\)

<table>
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<th>DISADVANTAGE</th>
<th>APPLICATION</th>
<th>REFERENCE NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPOSITION ON SOLID</td>
<td>Retaining almost all activity</td>
<td>Low enzyme loading</td>
<td>Inversion of carbohydrates</td>
<td>6</td>
</tr>
<tr>
<td>ADSORPTION ON MESOPOROUS SILICATES</td>
<td>Support is chemically and mechanically stable and resistant to microbial attach</td>
<td>Variable pore size preparation in harsh conditions causing denaturation of Enzyme</td>
<td>Scaffold for mesoporous carbon materials</td>
<td>7,8</td>
</tr>
<tr>
<td>IMMobilization ON POLYKETONE BY HYDROGEN BONDS</td>
<td>Easy Immobilization, high binding capacity, extraordinary stable immobilization</td>
<td>Only small increase in Km value</td>
<td>Applicable for large enzymes, peroxidase and amine oxidases</td>
<td>9</td>
</tr>
<tr>
<td>CLASSICAL COVALENT IMMobilization</td>
<td>Relatively stable to hydrolysis at neutral pH</td>
<td>Esters are unstable in aqueous Conditions</td>
<td>Immobilization of antibodies, proteases, and oxidases</td>
<td>10,11</td>
</tr>
<tr>
<td>Physical Entrapment</td>
<td>Avoid Negative Influence on enzymes surfaces, thermally and mechanically very stable</td>
<td>Diffusion of substrate to the enzyme is restricted</td>
<td>Applicable for most enzymes and antibodies, development of biosensors</td>
<td>12</td>
</tr>
<tr>
<td>IMMobilization USING AFFINITY TAB</td>
<td>Possibility of in situ immobilization</td>
<td>Relatively low selectivity</td>
<td>Capture of proteins during purification in affinity chromatography</td>
<td>13</td>
</tr>
<tr>
<td>ENCAPSULATION WITH LIPID VESICLES (LIPOSOMES)</td>
<td>High degree of reproducibility</td>
<td>Enzymes inactivation by shear force</td>
<td>Medical, biomedical fields, enzyme replacement</td>
<td>14</td>
</tr>
<tr>
<td>IMMobilization ON BIODEGRADABLE POLYMERS</td>
<td>Longer circulation in the blood stream</td>
<td>Low entrapment efficiencies, burst release, instability of encapsulated Enzyme</td>
<td>Control release for enzyme replacement therapy</td>
<td>15</td>
</tr>
</tbody>
</table>

1.4. Types of Supports
Just as the method of binding of the enzyme it is important, the support material is also important. The support must have properties suitable for its applications. For example, supports with low mechanical strength cannot be used for industrial applications.¹⁶

**Table 2** Different types of support materials⁵

<table>
<thead>
<tr>
<th></th>
<th>Flexible</th>
<th>Covalent</th>
<th>Adsorption</th>
<th>Reversible</th>
<th>Single Protein Molecule</th>
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<td><strong>Synthetic Polymer</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><strong>Biopolymers</strong></td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td><strong>Hydrogels</strong></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Inorganic Supports</strong></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Smart Polymers</strong></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Magnetic Nanoparticles</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Crosslinked Enzyme Aggregates (CLEAs)</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

1.5. 2D nanoparticles of alpha zirconium (IV) phosphate (αZrP)
Alpha zirconium phosphate, $\alpha$-ZrP, is an inorganic solid that forms nanodisks which stack to form nanocomposites. Previously, it was shown that positively charged enzymes could be efficiently intercalated between the nanodisks with little to no harm done to enzymes such as glucose oxidase and hemoglobin while maintaining a high enzymatic activity. Further studies showed that anionic proteins could be cationized with the polyamine triethylenetetramine to create a favorable binding scenario between the protein and $\alpha$-ZrP. The enzyme had high retention of activity and could be used for more cycles before losing 50% of its activity compared to free protein.

Guest species can be accommodated by the layered solids through expansion of the interlamellar spacings. Electrostatic field from the ionizable functions of the solid and its ability to expand facilitate the intercalation of metal ions, metal complexes, organic molecules, hydrophobic organic cations, cyclodextrins semiconductor particles, metal clusters and biological macromolecules. Because of the large size of enzymes, (30 – 100 Å), the $\alpha$-ZrP stacks must be exfoliated with large cations like tet-
rabutylammonium hydroxide (TBAOH) to increase the kinetics of enzyme intercalation. Exfoliation, or separation, of the nanodisks occurs due to a large excess of negative charge when TBAOH counter ions pair with only some of the phosphate groups. When the stacks are not exfoliate, no binding of enzyme is observed. Powder XRD confirmed enzyme binding by demonstrating the increase in d-spacings of the layers when enzyme was intercalated between the nanodisks.

The retention of catalytic activity is important for bound enzymes and requires (a) retention of native-like structure, (b) access to the active site, (c) facile diffusion of reagents into the out of the enzyme location and (d) flexibility of the enzyme to assume conformation states necessary for its activity. Direct monitoring of the enzyme’s activity is the best method to determine if these requirements are met after the enzyme has intercalated into α-ZrP. Activities are measured by recording the initial velocity as a function of substrate concentration. For example, hemoglobin (Hb) has well known peroxidase activity which is easily measured by monitoring the product formation at 470 nm. Hb intercalated into α-ZrP had retention of activity and structure, providing evidence that α-ZrP is an excellent material for supporting enzymes without destabilizing them.\textsuperscript{32} Chapter two of this work will investigate more closely the relationship between protein charge, ions present and ionic strength as a function of enzyme binding to α-ZrP.

1.6. 3D Silica nanoparticles

Silica nanoparticles are common in many different nanomaterials. Their sizes can easily be controlled and they have high surface area, increasing the total amount of ligands that can be loaded. Much work has gone into immobilization protein on the surface of silica or burying it in the pores. However, there are drawbacks to these methods. Most forms of surface immobilization require extensive modification first be done to the surface of the silica nanoparticle, requiring extra steps and additional materials. Furthermore, most methods of protein immobilization result in low loadings of proteins on the surface. Cationized bovine serum albumin is known to work as a sacrificial protein glue for loading anionic pro-
teins in the galleries of alpha zirconium phosphate. In chapter 3 of this work, the method of using protein glue is examined for protein immobilization on silica surface. The extent of cationization of the protein glue and loading of the protein glue are all examined to determine the optimal conditions for facile immobilization of anionic proteins on silica nanoparticles.

1.7. Protein Nanoparticles

Carrier free protein nanoparticles are composed of pure protein that has been crosslinked together. These particles are excellent for biological and environmental applications due to their ability to be degraded by proteases, a feature that silica and αZrP do not offer. Because of their biological compatibility, these particles are excellent candidates for cell imaging so it is necessary they also be fluorescent. There are many different methods for synthesizing these particles including free-spray drying, supercritical fluid, emulsification method and desolvation however all of these methods involve extreme conditions such as temperature or organic solvents that can result in loss of protein structure. An alternative method of protein nanoparticle formation is denaturing of the protein leading to rearranging of the pi stacking of fluorescent amino acids in the protein. The denatured protein is then crosslinked via carbodiimide. The loss of protein structure prevents the use of any catalytic proteins that may have desirable activity. Furthermore, binding pockets of the protein that could have been used for drug delivery have been destroyed.
Development of a synthesis method that eliminates potentially damaging conditions was recently developed. Fluorescent reactive dyes were used to increase aggregation of protein which is then cross-linked by carbodiimide to form protein nanoparticles. The size of these particles was determined by the protein used and the reactive fluorescent dye leading to very little control over sizes and emissions independent from each other. However, retention in enzyme structure and activity was achieved.
(Figure 6A and B). Particles were rapidly taken up by the cells when incubated in the presence of glucose oxidase and imaged in confocal microscopy (Figure 6). Chapter 4 of this work will describe new way of synthesizing protein nanoparticles with independently controllable size and emission wavelengths. Furthermore, chapter 5 will discuss the synthesis of white emission protein nanoparticles for advanced, multimodal cellular imaging.
Chapter 2. Controlled Chemical Modification and Ions Effect on Enzyme Binding to α-ZrP

2.1. Introduction

The binding of enzymes to a solid surface is controlled by electrostatic interactions.\textsuperscript{23} By systematically controlling these interactions, it is possible to develop a better understanding of enzyme adsorption onto solid surfaces and also improve practical applications of solid-bound enzymes.\textsuperscript{24,25,26,27,28,29} Previous work has shown that binding enthalpies do not have a linear correlation with the net charge of enzyme.\textsuperscript{30} It is necessary to find the correct balance of electrostatic interactions; too weak of interactions results in proteins leaching from the solid surface while too strong of interactions can result in the protein being denatured.\textsuperscript{31} Electrostatic interactions involved in enzyme adsorption on to a negatively charged solid was observed by using a set of modified proteins, each with a different net charge. This work suggests that the ions present in solution play a significant role in the binding of enzymes to solids.

Many solid-bound enzyme applications have a small range of pH, temperature or ionic strength in which there is optimum activity.\textsuperscript{32} It is therefore important to find other ways to optimize these enzymatic reactions. One of the approaches being developed is altering of the protein’s net charge via chemical modification.\textsuperscript{33,34,35} By changing the net charge of a protein, it is possible to create a favorable electrostatic binding situation where, as without modification, binding is not favorable. Conversion of glutamate and aspartate residue carboxylic acid groups to amides is used to change the net charge of the protein, in some cases even changing the sign of the protein charge. This can be a powerful tool for optimizing protein binding to solid and better understanding the electrostatic interactions between enzymes and solids. Here, we test the hypothesis that electrostatic binding is not the sole governing property in enzyme binding to solids, but rather that ions play an important role.

The negatively charged Zr(IV) phosphate (α-Zr(HPO\textsubscript{4})\textsubscript{2}•H\textsubscript{2}O, abbreviated as α-ZrP, is used as the model solid. Many protein and enzymes bind to α-ZrP with high affinities under benign ambient
conditions\textsuperscript{36} while maintaining a significant amount of their activity and structure,\textsuperscript{37,38} in some cases with increased thermal stability despite free enzymes rapidly denaturing under the same conditions.\textsuperscript{39} In some cases, the enzyme showed reversible thermal denaturation only when bound to α-ZrP.\textsuperscript{40,41,42,43}

At pH 7, α-ZrP has a negative charge resulting in low affinities for negatively charged enzymes due to unfavorable charge repulsions. The affinities of these enzymes can be tuned by chemically modifying the protein to have a net positive charge. While it is possible to modify the charge of the solid, it is simpler to modify the individual proteins as needed. This paper will focus on the controlled modification of proteins and how their binding affinities change as a function of the enzyme’s net charge.

Figure 7. Carboxyl groups on the surfaces of proteins can undergo amidation with polyamines to decrease their net negative charge at specific pHs. It is possible to produce positively charged enzymes for favorable binding to negatively charged α-ZrP.

Due to having negative charge at pH 7, glucose oxidase (GOx) is used to test the binding affinities with α-ZrP after controlled modification. GOx has 132 carboxylic acid groups.\textsuperscript{44} These groups present a substantial number of sites for chemical modification with the polyamine tetraethylene-pentamine (TEPA). The charge can be systematically modified in steps between -70 (native) and +20 at pH 7.2. The degree of modification can be controlled by controlling the amount of COOH groups modified.
Current glucose-sensing technologies employ the use of solid-bound GOx. A highly active and stable form of GOx-bound solids are practical for sensor design and fabrication. One of the uses of GOx is for the catalysis of the oxidation of glucose gluconic acid by ambient oxygen forming hydrogen peroxide by-product.

The polyamine tetraethylenepentamine (TEPA) has been chosen to modify the protein’s net charge. This polyamine is appealing due to being able to lower the protein’s negative charge by multiple units depending on the pH of the enzymatic solution and the number of TEPA molecules covalently attached. Amidation of the COOH groups via carbodiimide chemistry is well established. The terminal group is expected to be reactive in protein modification despite there being multiple amino groups present in TEPA.

2.2. Experimental

2.2.1. Materials.

Glucose Oxidase (GO, Aspergillus niger), tetraethylenepentamine (TEPA), glucose, sodium phosphate, sodium chloride, and tris base were purchased from Sigma-Aldrich Co (St. Louis, MO). 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) was obtained from TCI America (Portland OR).

2.2.2. Chemical Modification and Synthesis of Cationized Enzyme.

Using various concentrations of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), the carboxyl groups of the enzyme were activated and allowed to react with tetraethylenepentamine (TEPA) as previously reported. TEPA was made into a stock solution of 1.5M and the pH adjusted to 5.0 with HCl. This solution was added to aqueous GO so that the final concentrations were 10 mg/mL GO and 150 mM TEPA. This solution was allowed to stir for 30 min. A small aliquot of aqueous EDC was added and the solution was allowed to stir for 30 min. Aliquots were continually added followed by 30 min of
stirring until the desired EDC concentration was achieved. Samples were dialyzed into 10 mM Na2HPO4 50 mM NaCl pH 7.2 in 10x the sample volume for 3 20 min cycles to remove any un-reacted amine and by products.

2.2.3. Agarose Electrophoresis.

The degree of modification of the enzyme was monitored by agarose electrophoresis. This technique uses differences in charge to separate molecules. A 0.5% agarose gel was prepared in 40 mM Tris-Acetate pH 7.2 buffer and ran in the same buffer at 100 V for 30 min. Each well contained 5 mg/mL of the glucose oxidase sample. The comb was inserted into the middle of the gel so that proteins could migrate towards either electrode. The final gel was stained with 20% acetic acid 0.02% Coomassie blue for 12 to 24 h and then destained with 10% acetic acid for 12 to 24 h. A CanonScan Lide200 scanner was used to create a digital image of the gel. The charge of the modified enzyme was determined by measuring the distance the modified enzyme migrated and comparing it to the distance GO migrated. The charge of GO is known at pH 7.2 and therefore could be used to calculate the charge of modified samples by using the relationship distance migrated is proportional to the charge of the sample.

2.2.4. Synthesis of Layered Crystalline $\alpha$-Zr(IV) Phosphate.

$\alpha$-ZrP was synthesized according to previously reported methods.$^{51,52}$ Zr(IV) oxychloride solution was mixed with phosphoric acid (9 M) and then heated to 70 °C for 24 h. The resulting white crystalline powder was filtered, washed with acetone and dried. By XRD analysis, it was found that the material had a layer spacing of 7.6 Å, matching well known values. The metal phosphate plates were exfoliated by an aqueous solution of tetrabutylammonium hydroxide until the sample became translucent. The pH of the solution was adjusted to 7.5 using HCl. The nanodisks were exposed to the enzyme solutions which resulted in the formation of enzyme/$\alpha$-Zr(IV) phosphate complexes.
2.2.5. Binding Studies of Modified Enzymes with $\alpha$-ZrP.

Binding affinities were determined by centrifugation to separate the bound protein (pellet) from un-bound protein (supernatant). Increasing concentrations of protein were added to 3 mM exfoliated $\alpha$-ZrP in 10 mM Na2HPO4 50 mM NaCl pH 7.2 and allowed to sit for 2 h at room temperature. Samples were then centrifuged to separate bound protein from protein free in solution at 12,000 rpm for 12 min. The absorbance at 280 nm of the supernatant was recorded and used to calculate the concentration of free protein still in solution by Beer's Law. The concentration of protein bound to the $\alpha$-ZrP was calculated by difference between total protein in solution and free protein in the supernatant.

2.2.6. Circular Dichroism (CD) Studies.

CD spectra for all samples were collected on a Jasco J-710 spectrometer. All samples were collected at a concentration of 1 $\mu$M enzyme in a 0.5 cm path length quartz cuvette over the 260 – 200 nm range. Other operating parameters were sensitivity 20 mdeg, bandwidth 1.0 nm, response time 4 s, resolution 0.5nm, speed 50 nm/min, and average of three scans. The CD spectra of 10 mM Na2HPO4 50 mM NaCl pH 7.2 was collected and used as the reference signal. Each spectrum was analyzed by subtracting the reference signal from the sample and normalizing each spectrum at 260 nm. The percent structure retention was assessed by determining the CD intensity of each sample at 222 nm and comparing it to GO, which was set as 100% structure.

2.2.7. Glucose Oxidase Activity.

Glucose oxidase activity was monitored before and after binding of the samples to $\alpha$-ZrP. Activity was observed at room temperature by reported methods in Molecular Devices FlexStation3. A final concentration of 0.5 mM Glucose was added to samples containing 0.5 $\mu$M GO sample, 8.4 $\mu$M Horse Rad-
dish Peroxidase and 10 mM Guaiacol in 10 mM Na2HPO4 50 mM NaCl pH 7.2 buffer. The absorbance at 470 nm was recorded for the first 60 s after glucose addition. Activity was determined from the linear portion of the curve during the first 30 s of the reaction.

2.3. Results

Tetraethylpentamime (TEPA) was covalently attached to the surface carboxyl groups of GO by established carbodiimide chemistry. Controlling the concentration of EDC added to the reaction can control the enzyme reaction with TEPA. When modification is mild, all of the enzyme is converted to slightly higher charge enzyme. When modification is heavy, the enzyme is mostly converted to the high charge. The modified enzymes are denoted by the enzymes the net charge. For example, GOx chemically modified with TEPA resulting in a net charge of +33 will be referred to as +33. When sample is bound to α-ZrP, the samples will be denoted as net charge/α-ZrP, for example +33 bound to α-ZrP is +33/α-ZrP.

2.3.1. Synthesis and Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to monitor the degree of modification of the reaction between GOx, TEPA, and EDC. The gel (0.5% agarose, 40 mM Tris pH 7.2) indicated that as the concentration of EDC is increased, the degree of modification also increased (Figure 8). The net charge of the modified samples was calculated by distance migrated in the gel being proportional to charge. GO is known to have a charge of -70 at pH 7.2 and therefore is used as the standard. All Charges are estimated within ± 5.
Figure 8. Agarose gel showing the modification of glucose oxidase by TEPA. All lanes contain 5 mg/mL of glucose oxidase. Lanes 2 through 7 contain GO samples modified with 150 mM TEPA and increasing concentrations of EDC, respectively. The final concentrations of EDC added to each sample during synthesis can be seen in the table along with the samples’ calculated net charge after modification. A box has been added to the lane where samples were loaded to aid the eye.

<table>
<thead>
<tr>
<th>Lane</th>
<th>EDC (mM) added</th>
<th>Net Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0, Native</td>
<td>-70</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>-40</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>-25</td>
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<tr>
<td>4</td>
<td>3</td>
<td>-10</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>+10</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>+20</td>
</tr>
<tr>
<td>7</td>
<td>7.5</td>
<td>+30</td>
</tr>
<tr>
<td>8</td>
<td>0, Physical Mixture</td>
<td>-70</td>
</tr>
</tbody>
</table>

The first lane in Figure 8 contains GOx, which visibly migrates toward the positive electrode. This is due to GOx having a -70 charge at pH 7.2. Lanes 2 through 7 contain reactions of 10 mg/mL GOx, 150 mM TEPA and EDC concentrations of 1, 2, 3, 4, 5, and 7.5 mM respectively. With each increasing concentration of EDC, the migration of the modified sample towards the positive electrode becomes less resulting in a set of modified samples ranging from -70 to +30. As the EDC concentration was increased in the reaction, the degree of modification of the enzyme is also increased. Thus, higher concentrations of EDC resulted in more positively modified enzymes. As previously reported by this lab, it is known that EDC is responsible for initiating the chemical reaction between carboxylic acid groups on GOx and the amines of triethylenetetramine (TETA); the reaction of GOx with TEPA is expected to be the same. When GOx and TEPA were allowed to stir for the entire reaction time with no EDC added, no modification of GOx occurred (Figure 8, lane 8). In the agarose gel, it is known that migration distance is proportional to the net charge on the enzyme. Using the known charge of GOx (-
70) at pH 7.2 and the distances GOx traveled, the charges of the samples in lanes 2-7 were calculated to be -40, -25, -10, +10, +20, and +30. The smearing of samples in the lanes suggest that each sample has a certain distribution of charges, rather than a single charge present and therefore the charge calculated refers to the largest charge in the sample. The charge of GOx can easily be controlled by the amount of EDC added to the reaction.

Figure 9. (A) Binding plot of GOx-TEPA samples to α-ZrP (3 mM) in 10 mM Phosphate 50 mM NaCl buffer pH 7.2. (B) Plot of percent bound as a function of net charge on the protein.

2.3.2. Binding Studies.

Each modified sample of GOx was bound to α-ZrP as part of our investigation of the relationship between charge and binding affinity. The binding data was analyzed testing the hypothesis that the net charge does not solely affect the binding affinity. The solid α-ZrP was prepared for binding to the enzyme by first exfoliating the plates with tetrabutylammonium hydroxide (TBAOH), as described by our previous papers. The enzyme samples were then exposed to the exfoliated nanodisks and allowed to equilibrate. After equilibration, the samples were centrifuged to separate the protein bound to α-ZrP from the free protein. The supernatant was analyzed via UV adsorption at 280 nm and the concentration of bound protein calculated by the difference between total protein and free protein. From this da-
ta, binding plots were constructed by plotting the bound protein on the y axis vs. the total protein in the sample and can be seen in Figure 9A-C.

Binding plots have been analyzed for the 6 modified samples; -55, -30, -20, -5, 10, and 20. The lowest binding was seen for samples -55 and -30, the red and orange line in Figure 2 respectively. At the highest protein concentration (12 μM), both samples have about 25% of the total protein in the reaction bound. The next highest binding samples are 10 and -5, purple and blue respectively, binding about 38% of the total protein in the samples. A slight improvement in binding is seen for sample 20 (pink) with roughly 45% of the total protein being bound to the solid. The highest binding sample is easily -20 (green) having 75% of the total protein bound.
Figure 10. (A) Bar graph of each GOx-TEPA charge $K_b$ for comparison. (B) Scatchard plot of -55. (C) Scatchard plot of -30. (D) Scatchard plot of -20. (E) Scatchard plot of -5. (F) Scatchard plot of +10. (G) Scatchard plot of +20

The binding constant ($K_b$, Figure 8A) for each charge of GOx-TEPA was calculated from the scatchard equation shown in Figure 9B-G. Low binding constants were calculated for charges -55, -30, +10 and
+20 while the highest binding constants were for -20 and -5. This bell curve trend in binding constants as a function of GOx-TEPA charge matches the binding relationships where -20 had the highest percent bound at 12 μM concentrations protein. Though the general trend of binding constants did follow the trend of binding, there is a discrepancy between the -20 and -5 samples. In the binding plot, -20 show significantly higher binding for samples containing 6, 8, 10 and 12 μM protein samples however the calculated binding constant of -20 is lower. This is most likely due to the large error in the binding plot of -20 and -5. These two samples had the lowest R value (value representing how well a linear trend line fits the data were values close to one are a good fit and values close to 0.1 are a bad fit) of all the samples tested. The poor fit of the scatchard equation could be due to samples not having reached equilibrium at 12 μM total protein yet, as the other samples appear to have done (noted by the leveling off of the bound protein vs total protein curve).

Figure 11. Estimation of optimal ratio between protein charge per surface area and solid charger per surface area. Plotting the percent protein bound at high concentration (12 μM) as a function of the charge per surface area ratio concludes the highest binding affinity is in samples with a ratio of -0.02.
The percent protein bound was plotted as a function of the ratio of charge per surface area unit of the protein to charge per surface area unit of the solid to determine the optimal charge ratio of protein to solid for binding (Figure 11). The charge per surface area of α-ZrP is -1 charge per 25 Å². The surface area of glucose oxidase was calculated from the protein radius to be $21.6 \times 10^3$ Å², assuming the protein is a sphere. The charge per unit area for the modified samples was calculated by dividing the enzyme charge by the calculated surface area. The ratios span from -0.06 to 0.02. At low charge per surface area protein to solid ratios, the percent bound is around 30%. When the charge per surface area charge is at -0.02, binding is at its highest with 73% of the total protein being bound. As the ratio continues to increase, the binding drops down to 41% for ratios -0.005 and 0.01. Binding slightly increases when the ratio is 0.02.

2.3.3. Circular Dichroism (CD) Studies.

CD spectra were collected for all samples after modification and again after binding to α-ZrP for the assessment of structural changes. Alpha helices have a characteristic double minima at 222 nm and 208 nm and a positive maximum at 195 nm. Spectra of β-sheets have a broad negative peak at 212 nm and random coils have a distinct strong negative peak at 195 nm. Chemical modification as well as binding to α-ZrP can potentially cause changes in structure and activity.
Circular dichroism (CD) was used to determine if any changes occurred in GOx structure as a result of the modification reaction and binding to α-ZrP. Enzyme structure retention is important for application of nanomaterials as the structure of enzymes is directly related to the activity of the enzyme. CD records the ellipticity of enzyme, which is directly correlated to the structure. The CD spectra of GOx, GOx-TEPA charges and all GOx-TEPA charges bound to α-ZrP samples have been recorded under
the same conditions of protein concentration, pH, ionic strength and buffer. Superimposing these spectra as seen in Figure 12A shows that while the intensity of all modified samples varies greatly, the shape of the spectra all remain the same. The double minima seen in all samples was indicative of the presence of alpha helices. The variation in intensities is most likely due to minor differences in protein concentrations and/or scattering of samples due to aggregation. This confirms that there is little change to the enzyme upon modification with TEPA. CD studies of the GOx-TEPA charges bound to α-ZrP show significant loss in ellipticity of the samples.

To quantitate the amount of ellipticity lost for each sample, the ellipticity retained at 220 nm was calculated for each GOx-TEPA charge and plotted as a function of the protein to solid ratio of charge per surface area to assess if there is an optimal charge ratio for structure retention of enzymes binding solids (Figure 12D). At the lowest charge, -55, there is 47% structure retention of enzyme when bound to α-ZrP. The structure retention then decreases when the charge is -30 to 39% retention. The highest structure retention of all GOx-TEPA samples was observed for -20, with 50% structure retention. As the net charge of the samples increased to -5, +10 and +20 the structure retention gradually decreases to 42%, 36%, and 35% respectively.
2.3.4. **Activity Studies.**

![Image](image1.png)

Figure 13. Activity Retention of modified samples after binding with $\alpha$-ZrP. Unmodified GO (-70, black) is set to have 100 % activity. The highest activity retention is seen for -30/$\alpha$-ZrP (orange) while 10/$\alpha$-ZrP (purple) showed the lowest activity retention.

The activities of modified samples bound to $\alpha$-ZrP were collected (bold colors) and compared with modified unbound enzyme (light colors) and unmodified unbound enzyme (black), Figure 13. The activity of the samples after modification decreased compared to that of unmodified enzyme (-70). Upon binding to $\alpha$-ZrP, these samples retained roughly 25 – 50% of the unmodified enzyme activity. The best activity retention was seen for -30/$\alpha$-ZrP (orange), which maintained 53 % activity. The lowest retention in activity was seen for 10/$\alpha$-ZrP with only 24 % retention. The remaining samples, -55, -20, -5, and 20 all showed a decrease in activity retention maintaining 48, 40, 37, and 30 % retention in activity respectively. The percent activity retention of the modified samples bound to $\alpha$-ZrP was compared to the percent retention in ellipticity. As the percent retention in ellipticity at 220 nm decreases with charge, so does the activity, as expected due to enzyme activity being closely related to the enzymes structure.
2.3.5. Ions effect on binding

Specific ions can have a significant impact on the binding of protein to solid surfaces. Therefore the effect of ions was tested on the GOx-TEPA charge ladder. Buffers were made so that the effect of NaCl, Na$_2$HPO$_4$ and tris could be assessed. Binding was lowest for 37 mM Tris 50 mM NaCl pH 7.2 (Figure 14, red) with the highest percent bound reaching 20%. The sodium chloride concentration in this buffer matched that in the originally used buffer, 10 mM Phosphate 50 mM NaCl buffer pH 7.2. The next buffer tested was 71 mM Tris 10 mM Phosphate buffer pH 7.2 (Figure 14, orange). The phosphate concentration reflected the concentration in the buffer used in the first binding studies and the concentration of Tris was adjusted to bring the ionic strength of the buffer closer to the ionic strength of 37 mM Tris 50 mM NaCl pH 7.2. Improvement of binding was seen for all concentrations tested with the highest total protein sample showing approximately 30% binding. Binding was also completed in 1.1 Tris 10 mM Phosphate pH 7.2 and 1.9 M Tris 10 mM Phosphate buffer pH 7.2 (Figure 14, green and blue respectively) to consider if the differences seen between phosphate and
NaCl were due to increasing concentrations of Tris. The phosphate concentration was held constant while Tris concentration increased. As the Tris concentration increased, so did the concentration of bound protein. The highest Tris concentration (1.9 M Tris 10 mM Phosphate buffer pH 7.2) showed the highest binding for all total protein concentrations tested with the highest percent bound being approximately 90%.

2.3.6. Ionic Strength effect on binding

![Binding plots of 12 μM total enzyme of increasing charges of GO-TEPA under different ionic strengths of Tris-HCl. As ionic strength of the buffer was increased, the percent of protein bound decreased for all charges. Highest binding was seen for -20 in 0.1 M Tris HCl pH 7.2 with approximately (98.5 % bound).](image)

Figure 15. Binding plots of 12 μM total enzyme of increasing charges of GO-TEPA under different ionic strengths of Tris-HCl. As ionic strength of the buffer was increased, the percent of protein bound decreased for all charges. Highest binding was seen for -20 in 0.1 M Tris HCl pH 7.2 with approximately (98.5 % bound).

The effect of ionic strength of the buffer on binding trend was investigated. The binding of -55, -30, -20, -5, 10, and 20 was studied under different ionic strengths of tris-HCl buffer pH 7.2 (Figure 15). Highest
percent bound was observed for -20 at 0.1 M tris-HCl buffer pH 7.2, matching the trend observed when binding was done in 10 mM phosphate 50 mM sodium chloride buffer pH 7.2. All samples showed highest binding in 0.1 M Tris-HCl buffer pH 7.2 and lowers binding at 2.0 M Tris HCl buffer pH 7.2. The ionic strength of the buffers were 0.05 (0.1 M Tris-HCl pH 7.2), 0.5 (1 M Tris-HCl pH 7.2), and 1.0 (2.0 M Tris-HCl pH 7.2).

2.4. Discussion.

The crystal structure from protein data base was used to estimate the number of possible sites what could be modified on glucose oxidase. Crystal structure indicated 132 glutamic and aspartic acid residues, most of which are available for modification, though it should be noted that not all available residues need modified to see a shift in the net charge. The reaction of carboxylic acids with amine groups of TEPA resulted in an amide bond converted one carboxyl and one amine on the polyamide chain leaving four basic nitrogens on TEPA for protonation. The pKa values of TEPA range from 2.7 to 9.9 and therefore may be protonated after amidation at pH 7, meaning each TEPA contributes up to 4 charge units to the overall net charge of the modified enzyme. Agarose gel electrophoresis indicated reduced mobility of modified proteins towards the positive electrode as a function of the degree of modification. The amount of modification of glucose oxidase by TEPA can successfully be controlled by the controlling the amount of EDC used in the synthesis of the GO-TEPA samples. With higher concentrations of EDC, a second reaction also occurred in the vial; crosslinked enzyme. This was partially controlled by the slow addition of EDC and low concentrations of enzyme during synthesis.

Binding plots of the modified samples to α-ZrP showed high binding for samples with a net charge of -20, with samples of higher and lower net charges having less binding. Thus it can be concluded that the relationship between charge and binding affinities is not linear when in 10 mM Phosphate 50 mM NaCl buffer pH 7.2. Scatchard plots indicated that the highest binding affinity is for samples that are slightly negatively charged. However, these numbers have large error due to equilibrium not being
reached in the binding curves at the concentrations worked with. If concentrations could be pushed higher and true equilibrium reached where binding is maximum and bound and free protein are 50/50, it would be possible to obtain more accurate binding constants with smaller error. Due to the poor estimation from scatchard plots, the percent protein bound at the highest concentration (12 μM) was plotted as a function of charge to get a better estimate of how the binding constants for different net charged proteins compare. From this graph, it was seen that maximum binding occurs at -20. Samples with more positive and more negative charges than -20 decrease in binding affinities.

To further look at maximum binding, the percent protein bound at highest concentration (12 μM) was plotted as a function of the ratio of protein charge per surface area to solid charger per surface area. From this plot we can see that the optimal charge per surface area ratio of protein to solid is at -0.02 (1/50). This means that to obtain maximum binding for this system, there should be 1 charge on the enzyme for every 50 charges on the solid. It should be noted that this ratio occurs at the -20 charged enzyme. As the charge per surface area ratio continues to increase, the binding affinities become less respectively.

It is important in biocatalyst applications that there is significant retention of secondary structure. Analysis of the enzyme via CD showed that there was structure loss upon binding with α-ZrP, however not complete loss. When the protein is modified with TEPA, there is little change in the shape of the CD spectra, indicating that modification has little to no effect on the enzyme structure. This is consistent with other reports from our lab in which proteins have been modified with polyamines. Upon binding modified samples to α-ZrP, there is loss in structure. Plotting of the ratio of surface area charge of the enzyme to solid vs the retention in ellipticity showed that a charge ratio of -0.02 (-20 charge) had the highest structure retention. This is the same charge ratio seen for the highest binding suggesting that this particular scenario is ideal for loading the maximum amount of highly active enzyme onto the solid. Activity studies of these samples conformed that the structure was not completely lost. The percent structure retention of each modified sample after binding with α-ZrP was plotted as a function of the ratio of protein charge per surface area to solid charge per surface area.
Due to the lack of linear binding correlation, binding studies were done to determine the effect of phosphate, chloride and Tris ions on the binding. When binding studies were done in 10 mM Phosphate 50 mM NaCl pH 7.2, the binding correlation between highest percent bound and charge was bell shaped. To determine if the trend was the result of an interfering ion, binding was done in 37 mM Tris 50 mM NaCl pH 7.2 to look at the effect of chloride ion, 71 mM Tris 10 mM Phosphate pH 7.2 to look at the effect of phosphate ion, and 1.1 M Tris 10 mM Phosphate pH 7.2 and 1.9 M Tris 10 mM Phosphate pH 7.2 to determine the effect of Tris concentration. The Tris buffer containing NaCl showed the lowest binding or all concentrations of total enzyme tested. Tris buffer with phosphate showed improved binding compared to the Tris-NaCl buffer however, because the Tris concentration was changed to account for ionic strength of the buffer, it was unknown if the increase was due to the absence of NaCl or increase in Tris concentration. Two buffers with constant phosphate concentration and increasing Tris concentration showed more favorable binding between +33 and \( \alpha \text{-ZrP} \), however the effect of buffer ionic strength could not be ruled out. The activities of the Tris NaCl and Tris Phosphate buffers were collect but not significant different in activities between samples in the two buffers was seen.

Binding of +33 to \( \alpha \text{-ZrP} \) was done in increasing Tris HCl buffer pH 7.2. Two trends were discovered in the data set. The first being that the trend of change vs binding was the same as seen in 10 mM Phosphate 50 mM NaCl buffer pH 7.2, with -20 having the highest percent binding. However, where binding in 10 mM phosphate 50 mM NaCl pH 7.2 was in the ranges of 25 – 75 % total protein bound, Tris-HCl buffers all showed 80 – 98% total protein bound. The second discover was that as the concentration of Tris-HCl was increased, the percent bound protein decreased. All charges showed the lowest percent bound at 2.0 M Tris pH 7.2, the highest ionic strength buffer tested. The highest percent bound was observed for 0.1 M Tris pH 7.2, the lowest ionic strength buffer tested. From these data it can be concluded that the Tris ion plays a very important role in creating favorable binding between +33 and \( \alpha \text{-ZrP} \).
2.5. Conclusion

Glucose oxidase has been modified with TEPA to create a set of enzymes with a range of net charges from -55 to +20. These modified enzyme samples were used to determine the impact of electrostatics on enzyme binding to solids. We have determined that there are more factors influencing these binding interactions in addition to pure electrostatic interactions between the enzyme and solid. An optimal ratio of charger per surface area of protein to charge per surface area solid was found to be -0.02; for every 1 charge on the protein, there should be 50 charges on the solid to maximize binding. This optimized ratio appears to be correlated to structure retention of the enzyme upon binding $\alpha$-ZrP. The bell shape relationship between charge and binding affinities works to show the presence and importance of ions in the binding process.
Chapter 3. Silica Nanoparticles for immobilization of catalytic proteins.

3.1. Introduction

Immobilization of proteins onto solid surfaces have potential applications in drug delivery\textsuperscript{54,55}, biosensors,\textsuperscript{56,57} and industrial catalysis.\textsuperscript{58} The delicate nature of proteins limits their uses in most applications. Proteins have specific set of conditions under which they are most efficient. Immobilization of proteins onto solid surface can increase the durability of enzymes in what would normally be considered harsh conditions. Current immobilization techniques have many draw backs such as low enzyme loading, expensive tedious techniques and loss of enzyme activity. This work presents a novel, facile method for immobilization of catalytic enzymes onto silica nanoparticles with high loadings and activity retention.

High surface area to volume ratios and high surface activity give nanomaterials unique advantages over other nanomaterials for protein immobilizations.\textsuperscript{59} One key issue that needs be addressed when using nanomaterials for protein immobilization is the compatibility of the materials with biological molecules such as enzymes. Herein, we report the facile stabilization of two catalytic proteins, glucose oxidase and catalase, on silica nanoparticles by using a protein glue developed in our lab. Silica was chosen as an ideal solid for testing due to its strength, commercial availability and controllable sizes. The silica nanoparticles used in this study have a radius of approximately 50 nm and a surface area of 180-600 m\textsuperscript{2}/g and are amorphous.

The carboxylic functional groups on proteins can be readily converted to amide bonds through well-established EDC chemistry. Previously in our lab, the method was used to modify glucose oxidase for the intercalation into alpha zirconium phosphate sheets.\textsuperscript{60} However, cationization of the solid is a more universal approach for achieving high loading and activity retention for any anionic protein.\textsuperscript{21}
vine serum albumin (BSA) is a cheap and readily available protein and therefore is the ideal sacrificial protein. It has 99 carboxylic acid groups in the protein and has been shown to be easily modified with polyamines.\textsuperscript{61,21} In this paper, BSA has been modified using EDC chemistry to attach pentaethylenetetramine to carboxylic groups of the protein. This modification results in BSA have a large net positive charge.

Glucose oxidase is a common protein with applications in sensing\textsuperscript{62} and food science.\textsuperscript{63} The increased stability and recyclability of this protein could further advance technologies in this field. Catalase is another common protein used for biosensors\textsuperscript{64} Stabilization of this protein has proved to be difficult. Previous works show that catalase usually is loaded at low % loadings and maintains a small amount of their catalytic activity. We herein report the synthesis of a novel nanomaterial in which catalytic protein is easily loaded onto the surface of silica via means of a protein glue, BSA\textsubscript{PEHA}.

3.2. Materials and Methods

3.2.1. Materials.

Sodium phosphate and glucose oxidase from aspergillus niger were purchased from Sigma Aldrich Co. Catalase was purchased from Worthington enzyme. Silica nanoparticles were purchased from US Research Nanomaterials. Bovine Serum Albumin was purchased from Equine-Tech Co. Pentaethylenetetramine (PEHA) was purchased from TCI America.

3.2.2. Modification of BSA.

A 1 M solution of pentaethylenetetramine was made by adding the appropriate amount of amine to its equal amount of water. The solution was kept in an ice bath while the pH was slowly adjusted to 5.5 using concentrated HCL. The resulting solution was brought up to volume using DI H2O. In a clean glass vial, 1.5 g of BSA was dissolved into 4 mL of DI H2O by stirring for 1 h. 1 M aqueous amine (5
mL) was added to the solution and allowed to stir for 45 m. Finally, 1 mL of 1 M EDC was added to the vial and the reaction was allowed to stir for 4 h under nitrogen. The solution was then dialyzed into 10 mM NaH2PO4 pH 7.0 buffer.

3.2.3. *Agarose Gel.*

A 0.5% agarose gel was poured using 40 mM Tris-Acetate pH 7.0 as the buffer. Gel samples were prepared by adding 10 uL of protein to 10 uL of loading buffer (50% glycerol, 0.01% bromophenol blue) and centrifuging for 20 s. In to the gel, 19 uL of sample were loaded. The gel was run in 40 mM Tris-Acetate for 30 m at 100 V. The gel was stained for 3 h in Stain II (10 % acetic acid v/v and 0.02% coomassie) and destained in 10 % acetic acid overnight. The gel image was analyzed using ImageJ software.

3.2.4. *Binding of BSAPEHA to SiO2.*

Silica nanoparticles were suspended in 10 mM NaH2PO4 pH 7.0 buffer to give a stock solution with concentration of 24 mg/mL. Increasing amounts of BSAPEHA was added to 6 mg/mL silica in 10 mM NaH2PO4 pH 7.0 to give final concentrations of 2-1000 uM. The reactions were allowed to sit for 1.5 h at room temperature. The concentration of bound BSAPEHA was determined by centrifugation method. The concentration of free BSAPEHA in the supernatant was calculated by the Abs280.

3.2.5. *Desorption Studies.*

Supernatant was removed from the pellets of SiO2 with BSAPEHA bound to the surface (bSiO2). The pellets were re-suspended in enough 10 mM NaH2PO4 buffer to bring the silica concentration to 6 mg/mL. The re-suspended bSiO2 was allowed to sit at room temperature for 1.5 h. The amount of BSAPEHA desorbed from the surface of the silica was calculated by the centrifugation method.

A standard solution of bSiO2 was made by stirring 1 mM BSAPEHA and 6 mg/mL SiO2 in 10 mM NaH2PO4 pH 7.0 buffer for 2 h. bSiO2 was separated from free BSAPEHA by centrifugation and the solid was re-suspended in enough buffer to give a 6 mg/mL concentration of silica. Protein concentrations were varied from 2 – 1000 uM in solution containing 1 mg/mL bSiO2 in 10 mM NaH2PO4 and allowed to sit at room temperature for 1.5 h. Centrifuging the samples, measuring the free enzyme concentration in the supernatant and then subtracting it from the total concentration of protein in the sample determined the concentration of bound enzyme. The percent loading of enzymes on bSiO2 was calculated by dividing the mg of enzyme bound to the bSiO2 by the mg of bSiO2 in the sample and multiplying by 100.

3.2.7. *Circular Dichroism.*

All CD spectra were collected on a Jasco J-710 CD spectrophotometer (Easton, MD). Proteins both free and bound, were diluted in 10 mM NaH2PO4 pH 7.0 buffer to appropriate concentrations for measuring CD. For example, glucose oxidase (free sample and bound sample) was diluted to 2.5 uM while catalase (both free sample and bound sample) was diluted to 1.25 uM protein concentration. All samples were scanned from 260 nm to 190 nm in a 0.5 cm pathlength quartz cuvette with a data pitch of 0.5 nm, continuous scanning mode, 50 nm/min scanning speed, 1 s response time, 10 nm bandwidth and 3 accumulations. All spectra were normalized at 260 nm and the blank (10 mM NaH2PO4 pH 7.0) subtracted from the spectra. The percent structure retention of the bound protein vs the free protein was calculated at 222 nm.

3.2.8. *Activity of Enzyme/bSiO2.*
Activities were collected on an Agilent UV Vis spectrophotometer in a quartz cuvette while being stirred. Glucose oxidase activity was monitored by previously reported methods. To a cuvette containing 1 uM of glucose oxidase, 10 mM guaiacol, and 2 uM HRP in 10 mM NaH2PO4 pH 7.0 buffer, glucose was added so that the final concentration was 0.2 mM. Immediately after addition, the change in absorbance at 470 nm was monitored for 60 s. The activity of the protein was considered to be the slope of the linear portion of the resulting curve. The percent activity retention of each protein was found by comparing the free protein (considered 100 % activity) to the bound protein.

3.3. Results

![Diagram showing biophilization of silica nanoparticles](image)

Figure 16. Biophilization of silica nanoparticles makes favorable binding scenario between silica and anionic protein. However, when silica has not be biophilized, there is no binding of ionic proteins.

A simple method for immobilization of anion protein on silica nanoparticles is presented here. BSA, an inexpensive protein capable of chemical modification with polyamines, is adsorbed electrostatically onto the surface of silica nanoparticles. This results in a symbol change of the surface charge of the
nanoparticle, making it favorable for anionic (negatively charged) enzymes to adsorb to the surface (Figure 16).

3.3.1. Synthesis of BSA\textsubscript{PEHA}

Figure 17. (B) BSA\textsubscript{PEHA} moves a large distance towards the negative electrode due to its positive charge (+20). (B) SDS gel of BSAPEHA (lane 2) shows a small band at 66.3 kDa with a large band of protein remaining in the gel well and a moderate smear of protein between the two bands indicating some crosslinking is occurring.

By using established EDC chemistry, the COOH groups of BSA were modified with pentaethylenehexammine (PEHA). The resulting product, denoted as BSAPEHA was dialyzed to remove any un-reacted
polyamine and EDC. BSA and BSAPeHA were run on 0.5% agarose gel for 30 m to determine the net charge of BSAPeHA. As seen in Figure 17B, the lane containing BSA shows a tight band that migrated towards the positive electrode due to the net negative charge of BSA at pH 7. In lane 2, BSAPeHA migrated as a smear over a large distance towards the negative electrode, indicating a net positive charge. The large smear of BSAPeHA indicates that there is a large distribution of charges ranging from 0 to +40, with a median charge of +20.

SDS PAGE of BSAPeHA (seen in Figure 17B, lane 2) has a moderate smear throughout the lane between two distinct bands at 66.3 kDa the well of the gel. This indicates that some of the BSAPeHA in the sample is too large to enter the gel, most likely due to cross-linking as no precipitate was seen while loading the samples into the gel. The first lane (unmarked in gel) contains molecular weight markers, which have been identified to the left of the gel in the figure. BSA (lane 1) shows to have a molecular weight of 66.3 kDa, as expected. Circular
dichroism (CD) was used to determine if there was any loss in structure due to modification with PEHA or binding to the silica nanoparticle (Figure 17C). Both the intensity and shape of the spectrum of BSA\textsubscript{PEHA} were completely retained (green) compared to that of native BSA (red). After binding to silica nanoparticles (blue) there is an almost total loss in the intensity of ellipticity however, close examination of the spectrum revels that the shape of the protein remains unchanged after binding. The retention of the shape of the curve indicates that BSA\textsubscript{PEHA} is not denatured upon binding silica nanoparticles. The loss of intensity is due to the silica nanoparticles causing the polarized light to scatter, therefore decreasing the signal of the instrument detector.
3.3.2. Synthesis of biophilized silica (bSiO$_2$)

Figure 18. (A) Scheme of biophilized silica (bSiO$_2$) by adsorbing BSA$_{PEHA}$ onto the surface of silica nanoparticles. (B) Binding isotherm of BSA$_{PEHA}$ to silica nanoparticles. As the concentration is increased from 0 to 1000 $\mu$M BSA$_{PEHA}$ (10 mM phosphate buffer pH 7.0), no saturation in binding is seen. At 1000
μM BSA\textsubscript{PEHA}, a maximum loading of 250% is seen. (C) Resuspension (10 mM phosphate buffer pH 7.0) followed by centrifugation determined no BSA\textsubscript{PEHA} desorbed from the surface of SiO\textsubscript{2}. (D) CD spectra of BSA (red, 10 mM phosphate buffer pH 7.0), BSA\textsubscript{PEHA} free in solution (green, 10 mM phosphate buffer pH 7.0), and BSA\textsubscript{PEHA} bound to SiO\textsubscript{2} (blue, 10 mM phosphate buffer pH 7.0), showed no loss in ellipticity after modification with PEHA (green) or binding to SiO\textsubscript{2} np (blue). Loss in intensity after binding SiO\textsubscript{2} np due to particles scattering light, lowering signal read by the detector.

Electrostatic binding between negatively charge SiO\textsubscript{2} nanoparticles and positively charge BSA\textsubscript{PEHA} was used to synthesize biophilized SiO\textsubscript{2}, denoted as bSiO\textsubscript{2} (Figure 18A). The binding of BSA\textsubscript{PEHA} to silica nanoparticles was tested over a large concentration range of BSA\textsubscript{PEHA}. As the concentration of BSA\textsubscript{PEHA} added to the silica was increased, the concentration of BSA\textsubscript{PEHA} bound to the silica also increases, seen as the red line in Figure 18B. The binding continues to increase linearly as higher concentrations are reached up to 1000 μM. At the highest concentration of BSA\textsubscript{PEHA} added to silica able to be tested, the concentration of BSA\textsubscript{PEHA} is 225 μM giving a mass/mass loading of BSA\textsubscript{PEHA} on silica of 250 %. The pellets of SiO\textsubscript{2} with bound BSA\textsubscript{PEHA}, which will be denoted as bSiO\textsubscript{2} from here on out, were re-suspended in fresh buffer (10 mM phosphate buffer pH 7.0) and allowed to sit at room temperature for 1.5 h to determine if any of the BSA\textsubscript{PEHA} desorbs from the silica surface. After centrifugation, it was found that no BSA\textsubscript{PEHA} desorbed from the surface (Figure 18C).

Zeta potential was used to monitor the change in surface charge of the particles as increasing BSA\textsubscript{PEHA} was added to SiO\textsubscript{2} (Figure 17D). Initially, SiO\textsubscript{2} (10 mM phosphate buffer pH 7.0) had a zeta potential of -1.75 per mg of SiO\textsubscript{2}. After the addition of 50 μM BSA\textsubscript{PEHA} (10 mM phosphate buffer pH 7.0), the zeta potential quickly increases to 0.5 per mg SiO\textsubscript{2}. The change in net charge begins to plateau and maximum positive zeta potential of +1 per mg SiO\textsubscript{2} is seen for 100 μM BSA\textsubscript{PEHA} (10 mM phosphate buffer pH 7.0) and higher BSA\textsubscript{PEHA} concentrations.
The structure of BSA_{PEHA} was tested before and after binding to the silica nanoparticles (Figure 18E). BSA CD spectra have a double minima at 222 nm and 207 nm with one maxima at 192 nm. BSA_{PEHA} shows no loss in structure after modification when compared to BSA. Both minima and the maxima are present in the spectra though there is a slight increase in intensity of the signal after modification (12% increase at 222 nm). After BSA_{PEHA} has been bound to silica nanoparticles, there is almost a complete loss in intensity of the modified protein, however the spectral shape of two minima at 222 and 207 nm and one maxima at 192 nm is still retained. It is concluded that BSA_{PEHA} had little loss in ellipticity and the drop in signal intensity is due to the nanoparticles scattering light. It was expected that BSA_{PEHA} would denature on the surface of the silica nanoparticles due to similar work done previously in this lab where BSA_{TETA} was bound to α-ZrP.\textsuperscript{21}

### 3.3.3. Binding anionic protein to bSiO\textsubscript{2}

![Binding isotherm for glucose oxidase (A) and catalase (B) binding to bare silica (blue) and bSiO\textsubscript{2} (red). Glucose oxidase and catalase both show improved binding to bSiO2 compared to SiO2.](image)

Figure 19. Binding isotherm for glucose oxidase (A) and catalase (B) binding to bare silica (blue) and bSiO\textsubscript{2} (red). Glucose oxidase and catalase both show improved binding to bSiO2 compared to SiO2.
Silica was loaded with the highest amount of BSA_{PEHA} possible for testing anionic protein binding to bSiO$_2$. Glucose oxidase or catalase was bound to the bSiO$_2$ at increasing concentrations. Binding plots were created using the Langmuir model (Figure 19). From these plots, it can be seen that both GO and Cat fit the Langmuir isotherm nicely when binding to bSiO$_2$ giving an exponential increase. However, when GO and Cat is bound to bare silica, binding remains around 0 and the isotherm fit is linear. Catalase had a binding constant of 2.4 x 10$^4$ M$^{-1}$ for bSiO$_2$ while no binding to SiO$_2$ was observed. GOx binding to bSiO$_2$ had a binding constant of 2.9 x 10$^5$ M$^{-1}$ while no binding to SiO$_2$ was observed for GOx.

Figure 20. Highest achieved percent loadings (mass protein / mass SiO$_2$) for glucose oxidase and catalase. Glucose oxidase and catalase showed improved binding to bSiO$_2$ over SiO$_2$.

A standard solution of bSiO$_2$ was synthesized and characterized to have 200 uM BSA$_{PEHA}$ bound to the surface, giving a loading of 226% loading. To this bSiO$_2$, increasing concentrations of protein was added and centrifugation used to determine the amount of protein bound to bSiO$_2$. Anionic proteins
tested are glucose oxidase and catalase (pI of 4.6, 5.4 respectively). At 100 µM total protein, glucose oxidase and catalase both showed improved binding to bSiO₂. Glucose oxidase showed an improved binding of 169% over SiO₂, which had a loading of 0%. The highest loading of acidic protein onto bSiO₂ was seen in the case of catalase. The loading onto bSiO₂ was 800% while loading on SiO₂ was 0%.

3.3.4. Structure of proteins bound to bSiO₂

Figure 21. A. Plots showing the change in glucose oxidase structure retention when bound to bSiO₂. The spectrum of GO bound to bSiO₂ becomes less intense and shows distortion of the double minima peaks at 222 and 208 nm and shift of the maxima at 192 nm B. The structure retention of each protein bound to bSiO₂ was calculated. The highest structure retention is seen for glucose oxidase with 56% while catalase shows the lowest structure retention of 10%.

The structure of proteins bound to bSiO₂ was investigated and compared to the structure of the free protein in buffer. The CD spectra of glucose oxidase had two distinct minima at 222 nm and 207 nm and a maxima at 192 nm (Figure 21A). After binding to bSiO₂ the structure became less intense and slightly distorted, having only one distinct minima at 222 and the maxima being slightly shifted up to 198 nm. The catalase CD spectrum Figure 21B) looked similar to glucose oxidase in buffer with two
minima at 220 nm and 207 nm and a maxima at 192 nm. However, after binding to bSiO2 the intensity of the spectra greatly decreased while maintaining the characteristic double minima and one maxima. The percent structure retention was calculated for both proteins using each respective protein in buffer at 100% structure (Figure 21B). Using the minima at 222 nm, it was calculated that glucose oxidase retained 58 % of the proteins native structure while catalase retained only 11 % of its original structure.

Figure 22. A. Kinetic traces of GO, GO/bSiO2. B. Activity retention of proteins bound to bSiO2 where free enzyme was considered 100% activity. The highest activity retention was seen for glucose oxidase while catalase showed no activity retention after being bound to bSiO2.

3.3.5. Activities of proteins bound to bSiO2

Activities were collected for proteins bound to bSiO2 and compared to free enzyme activity in buffer. The free enzyme activity was considered to be 100 % activity. Linear portions of the kinetic traces used to calculate the specific activity of Glucose oxidase activity can be seen in Figure 22A. The black curve is free glucose oxidase in buffer while the red curve is activity of glucose oxidase bound to bSiO2 in buffer. Due to the 0% or very low loadings of proteins onto SiO2, those activities could not be tested for comparison. Glucose oxidase maintained 80% activity compared to free GO in buffer while catalase retained no activity at all compared to catalase in buffer solution (Figure 22B).
3.3.6. Optimizing catalase binding.

Due to the total loss of activity when loading 800% catalase onto bSiO₂, further work was done to determine what was causing the activity loss and determine if bSiO₂/catalase could be optimized for high loading and activity retention. The effect of BSA_{PEHA} loading and catalase loading were both considered. Catalase (100 μM, 10 mM phosphate buffer pH 7.0) was added to bSiO₂ in which the BSA_{PEHA}
percent loading was varied (Figure 23A). The lowest loading of BSA_{PEHA} (20 μM), 60% loading of catalase was achieved. When the loading of BSA_{PEHA} was increased to 200 μM, and increase in loading of catalase (95%) was observed. For all other loadings of BSA_{PEHA} (400, 600, 800, and 1000 μM) BSA_{PEHA} loading of catalase was 110%. CD spectra of the samples (Figure 23B) show approximately 50% loss in ellipticity however the spectral shape is maintained suggesting that any loss in intensity is due to scattering of light by silica, not by loss in protein structure. Peroxidase activity of catalase was assessed to determine the effect of BSA_{PEHA} on activity retention of the protein (Figure 23C). Considering the error in the experiment, it was observed that the loading of BSA_{PEHA} had little effect on the activity retention of catalase immobilized on the surface. At low loading of BSA_{PEHA} (10%) approximately 20% activity retention was observed. At the highest loading of BSA_{PEHA} (120%) activity retention was calculated to be 30%. Though loadings between the highest and lowest showed higher activity retention on average, the error bars are large and therefore not much can be concluded from that data.

The effect of percent loading of catalase was also investigated. bSiO$_2$ (220% BSA_{PEHA} m/m, 10 mM phosphate buffer pH 7.0) was loaded with increasing amount of catalase (Figure 23D). The percent loading of catalase onto bSiO$_2$ steadily increased as 20, 40, 60 and 80 μM catalase (10 mM phosphate pH 7.0) was added to the sample. Addition of 100 μM catalase (10 mM phosphate buffer pH 7.0) resulted in the same percent loading catalase on bSiO$_2$ as seen for 80 μM. The CD spectra showed retention in spectral shape with a change in ellipticity intensity, again suggesting that there was not a significant loss in structure but that intensity lost was due to bSiO$_2$ scattering the polarized light (Figure 23E). Peroxidase activity was assessed for each sample and found that as the percent loading of catalase was increased, the percent activity retention became lower (Figure 23F).

3.4. Discussion.

Modification of BSA using EDC chemistry to covalently attach pentaethylenehexamine to the surface is supported by agarose gel electrophoresis. The smear indicates the sample is composed of a distribution of charges ranging from 0 to +40, giving a mean net charge of +20. This high positive charge in-
interacts with silica’s inherent net negative charge to drastically change the net charge of the complex between the two, bSiO2. No desorption is seen when bSiO2 is re-suspended in water. This suggest a very strong interaction between BSA_{PEHA} and silica and that more than electrostatic equilibrium mandates the binding. Because no saturation of binding is seen, it is not possible to calculate the binding affinity of BSA_{PEHA} to silica. However, we can assess that the binding constant of BSA_{PEHA} binding SiO₂ is not large due never seeing more than 20 % of the total protein in solution bind the solid. Zeta potential confirmed the sign change in net charge on the particles after the addition of BSA_{PEHA}. Because of the now net positive charge of bSiO₂, it then possible to bind negatively charged proteins to the surface.

Anionic catalytic protein glucose oxidase (GOx) solution (10 mM phosphate buffer pH 7.0) was added to bSiO₂ and the bound protein was separated from the free protein by centrifugation. GOx showed high binding with a maximum percent loading of 170 % (mass GOx/mass bSiO₂) and had 80 % retention in activity. The CD spectra of GOx bound to bSiO₂ showed a significant change in ellipticity note by the change in spectral shape. GOx free in solution had a double minima at 222 and 208 nm indicative of alpha helices, however after binding to bSiO₂, a single minima at 224 nm is observed with a maximum at 200 nm. These peak indicated that the protein has β-turn structure. The change in structure of GOx is possible opening up the active site making it more accessible thus a more significant loss in activity was not observed despite the change in structure. Activity dependence upon the BSA_{PEHA} loading on bSiO₂ were considered and it was concluded that the loading of BSA_{PEHA} on bSiO₂ had little effect on the retention of activity as all GOx samples retained approximately 80 % activity.

Comparison of bSiO₂ for GOx immobilization to other recently reported method shows that bSiO₂ can achieve the highest loading of those reported to date for silica platforms, and has comparably high activity retention (Table 3).
Table 3. Compilation of different published works on glucose oxidase immobilization. The immobilization method, % loading (m/m) and activity are listed for easy comparison.

<table>
<thead>
<tr>
<th>Author</th>
<th>Platform</th>
<th>Mode of immobilization</th>
<th>Loading</th>
<th>% activity retention</th>
<th>Reference Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current Work</td>
<td>Mesoporous silica nm (35 nm diameter)</td>
<td>Adsorption</td>
<td>1.7 mg/mg solid</td>
<td>80 %</td>
<td></td>
</tr>
<tr>
<td>Cao, S., et al 2013</td>
<td>Hollow silica</td>
<td>Adsorption</td>
<td>0.24 x10^{-3} mg/mg solid</td>
<td>62 %</td>
<td>65</td>
</tr>
<tr>
<td>Khan, A. Y., et al 2014</td>
<td>Amine-functionalized microporous silica</td>
<td>Adsorption</td>
<td>0.513 mg/mg solid</td>
<td>17 %</td>
<td>66</td>
</tr>
<tr>
<td>Khan, A. Y., et al 2014</td>
<td>Amine-functionalized mesoporous silica</td>
<td>Adsorption</td>
<td>0.634 mg/mg solid</td>
<td>2 %</td>
<td>66</td>
</tr>
<tr>
<td>Tian, F., et al 2016</td>
<td>(3-aminopropyl) trimethoxysilane</td>
<td>Encapsulation</td>
<td></td>
<td>92 %</td>
<td>67</td>
</tr>
<tr>
<td>Yang, Y. et al 2016</td>
<td>Fe_{3}O_{4} nanoparticles with silica shell</td>
<td>Immobilized/ Encapsulation</td>
<td>0.4 mg/ mg Fe_{3}O_{4}</td>
<td>81 %</td>
<td>68</td>
</tr>
</tbody>
</table>

Anionic catalytic protein catalase solution (10 mM phosphate buffer pH 7.0) was added to bSiO_{2} and centrifuged to separate free from bound protein. The percent loading was calculated at its highest to be 800 % (mass catalase/mass SiO_{2}). This was the best binding of any protein tested with this system including those not reported. CD spectra of catalase on bSiO_{2} showed 20 % structure in ellipticity intensity while the spectral shape remained unchanged compared to that of free catalase. The double minima at 222 and 208 nm indicated alpha helices present in the enzyme structure. Peroxidase activity assay determined that the catalase immobilized onto bSiO_{2} had no retention in activity. The effect of percent loading of BSA_{PEHA} on SiO_{2} and effect of percent loading catalase on bSiO_{2} was investigated to determine if it was possible to immobilize high amounts of catalase while maintaining some of its activity. Increasing loading of BSA_{PEHA} on SiO_{2} has little effect on the amount of catalase that could be immobilized onto bSiO_{2}. At low BSA_{PEHA} concentrations of 20 – 120 % loading resulted in 105 % loading catalase ± 10 %. CD spectra of catalase bound to bSiO_{2} with different BSA_{PEHA} loadings showed
retention in spectral shape and the decreased in ellipticity intensity, which is due to silica scattering. From this, it was concluded that all samples retained a significant amount of structure. Peroxidase activity studies showed an increase of activity with increased loading of BSA_{PEHA}. The highest loading and highest achieved activity retention for varied bSiO_{2} was 110 % loading catalase (1.1 mg/mg solid) with 50 % retention in activity.

The effect of catalase loading was also considered. bSiO_{2} (220% loaded with BSA_{PEHA}) was loaded with increasing amounts of catalase. As the catalase concentration increased, so did the percent loaded catalase on bSiO_{2}. CD of catalase immobilized on bSiO_{2} showed loss in ellipticity intensity, most likely due to scattering, with retention of spectral shape suggesting that catalase was not suffering from drastic loss in structure. Peroxidase activity of bSiO_{2} with increasing catalase loaded onto it showed that as the percent of catalase loaded onto bSiO_{2} increased, the activity decreased. This would suggest that the total loss of catalase when loaded at 800% was due to the amount of catalase on the particles. From this data, it was concluded that the highest achievable loading with retention in activity is 108% (1.08 mg/mg SiO_{2}) with a 50 % retention in activity. Comparison of this method with other recently published methods of catalase immobilization on silica supports shows bSiO_{2} to have the highest percent loading while the activity retention, though towards the bottom end of listed works, is still respectable (Table 4).
Table 4. Collection of recent publications on the immobilization of catalase on silica. Immobilization support, method, % loading (m/m) and activity retention are listed for comparison.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Support</th>
<th>Mode of Immobilization</th>
<th>Loading</th>
<th>% Activity</th>
<th>Reference Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current Work</td>
<td>Biophilized silica nanoparticles</td>
<td>Adsorption</td>
<td>1.08 mg/mg solid</td>
<td>50 %</td>
<td></td>
</tr>
<tr>
<td>Itoh, T. et al 2009</td>
<td>FSM-16</td>
<td>Adsorption</td>
<td>0.15 mg/mg solid</td>
<td>83.8 %</td>
<td>69</td>
</tr>
<tr>
<td>Baryamoglu, G. et al 2016</td>
<td>p(VMK)-M coated silica particles</td>
<td>Amide coupling</td>
<td>0.064 mg/mg solid</td>
<td>73.6 %</td>
<td>70</td>
</tr>
<tr>
<td>Singh, H. P. et al 2013</td>
<td>Hollow silica nanoparticles</td>
<td>Entrapment</td>
<td>19 %</td>
<td></td>
<td>71</td>
</tr>
<tr>
<td>Na, W. et al 2013</td>
<td>Siliceous mesocellular foam</td>
<td>Adsorption</td>
<td>0.372 mg/mg solid</td>
<td>79.2 %</td>
<td>72</td>
</tr>
<tr>
<td>Yang, X. et al 2012</td>
<td>(3-aminopropyl) trimethoxysilane</td>
<td>Entrapment</td>
<td>0.22 mg/mg solid</td>
<td>90 %</td>
<td>73</td>
</tr>
</tbody>
</table>

In conclusion, we have created a universal nanomaterial for the immobilization of anionic catalytic proteins. High loadings for glucose oxidase and catalase were achieved while activity retention of 50 % or greater was possible. This method proves to be the best thus far reported for glucose oxidase immobilized on silica and competitive with recent methods for catalase immobilization of silica.
Chapter 4. Multicolored, Size-Controlled Fluorescent Protein Nanoparticles (GlowDots)

4.1. Introduction

Imaging agents for research and medical applications have become a popular field in the last several years. While many different materials have emerged, there are a lot of fundamental problems with all of them, ranging from toxicity, solubility and performance. Fluorescent protein nanoparticles (GlowDots) solve many issues while maintaining the desirable photophysical qualities of the most popular imaging agent; quantum dots (QDs). GlowDots offer tunable emission, sharp emission spectra, high brightness and high molar absorptivity while adding the benefit of independently tunable emission, size and surface chemistry. Quantum dots (QDs) made from different materials such as graphene, heavy metals and silica have been well studied for their biological applications. Their tunable emission wavelengths are directly related to the particles size, high brightness, high molar absorption coefficients and narrow, sharp emission spectra. QDs on the other hand, still are faced with the challenges of toxicity, solubility, and ability to undergo surface modification. They are limited to sizes between 2-10 nm and sizes are not independent of the emission wavelength.

The emission dependence on the size of the QDs causes varying brightness values for the particles, requiring extra steps to equalize the brightness of each quantum dot. We have synthesized protein nanoparticles in which the size, emission wavelength, and brightness can be independently tuned and are capable of single or multi-emission spectra.

Protein nanoparticles can be synthesized by freeze-drying, spray drying, desolvation, supercritical fluid methods and enzymatic crosslinking. The disadvantage to most of these methods is the use of harsh organic solvents, drying or dehydration; all of which can damage the protein structure. Supercritical fluid methods have been developed to avoid the use of these harsh methods but it is limited in the sizes of nanoparticles it can produce in addition to have wide distributions. The method de-
scribed herein uses protein friendly conditions during synthesis, provides control over the sizes, and has a high retention of protein structure; important for use with catalytic proteins.

Protein nanoparticles are composed of bovine serum albumin (BSA), a protein purified from cow blood. BSA is non-toxic, digested by proteases and has been approved by the Food and Drug Administration for many different medicinal applications making it an excellent material for building biological nanotechnologies. Toxicity of QDs remains a major concern for biological and environmental uses.\textsuperscript{92,93} GlowDots do not require any specialized modification of the surface to be soluble in aqueous solutions unlike heavy metal QDs. The solubility of QDs must be addressed with modification of the surface post particle synthesis.\textsuperscript{95} In a manner that also results in a surface that can be modified for targeting, uptake or other specific purposes. The use of soluble protein as a nanoparticle eliminates extra steps and provides a surface with many different functional groups that can be used to modify the particle with aptamers, small molecules, and antibodies for tuning the particles specificity.

Nanoparticles that possess the advantageous photophysical properties of QDs while simultaneously addressing toxicity, biocompatibility, synthesis requirements, solubility, cost, and independently tuned parameter are reported in this paper. BSA nanoparticles (GlowDots) are synthesized via crosslinking and decorated with fluorescent labels. Size and emission of GlowDots are independently controlled by synthesis parameters. GlowDots had molar absorptivities and brightness intensities comparable with those of previously reported QDs.

4.2. Experimental Section

4.2.1. Materials.

Bovine serum albumin (fatty acid) was purchased from Equitech Bio. (Kerrville TX). 1-pyrenebutanoic acid ($\lambda_{\text{em}}=376$ nm), 7-methoxycoumarin-3-hydroxy ($\lambda_{\text{em}}=410$ nm), Diethylaminocoumarine-3-carboxylic acid ($\lambda_{\text{em}}=472$ nm), fluorescein isothiocyanate ($\lambda_{\text{em}}=519$ nm), tetramethylrhodamine -5-
(and-6)-isothiocynate (λem= 571 nm), and 5-(and-6)-carboxy-x-rhodamine(λem= 601 nm) were all purchased from Anaspec (Fremont, CA).

4.2.2. **Synthesis of GlowDots.**

Protein solution was prepared by stirring 150 mg of BSA in 1 mL of de-ionized water (dH2O). EDC (1M, dH2O) 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 1 mL of 20 mM CO32-/HCO3- pH 9.3 buffer. A solution of 1-pyrenebutanoic acid, (P, 0.5 % (m/m), 0.75 mg/mL, 1.9 mM) in DMSO was added to the protein solution and stirred for 2 h. 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 dyes (approximately 6 x sample volume). This method was repeated for making GlowDots with the following fluorescent labels (0.5 % m/m): 7-methoxycoumarin-3-hydroxy (4), Diethylaminocoumarine-3-carboxylic acid (472), fluorescein isothiocyanate (519), tetramethylrhodamine -5-(and-6)-isothiocynate (576), and 5-(and-6)-carboxy-x-rhodamine (601). All samples were denoted as GlowDot (#) where # is the emission wavelength of the dye.

4.2.3. **Dynamic Light Scattering (DLS).**

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.

4.2.4. **Agarose Gel Electrophoresis.**
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. This procedure was repeated with 160 mM Tris acetate buffer pH 9.0 for looking at the modification of GlowDots with Taurine and triethylene-pentamine (TEPA).

4.2.5. **Circular Dichroism (CD).**

CD spectra were measured on a Jasco J-710 CD spectrometer. A concentration of 1.25 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 – 190 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.

4.2.6. **SDS PAGE.**

A 7 % separating with 5 % stacking gel was used. Samples were prepared by adding loading buffer (10 μL, 2 % SDS, 10 % BME) to the sample then boiled for 2 minutes. Samples were loaded into the gel so that each well contained 6 μg of protein. The gel was run in SDS running buffer at 200 V constant in Bio-Rad Mini Protean Electrophoresis apparatus until the dye front was 1 cm from the bottom of gel plate. Gel was stained in Stain I (10 % v/v acetic acid, 10 % v/v isopropanol, 0.02 % coomassie
blue) for 1 h. Gel was then placed in Stain II (10 % v/v acetic acid, 0.02 % coomassie blue) overnight. The gel was destained in 10 % v/v acetic acid until bands were clearly distinguished from clear background of gel.

4.2.7. Transmission Electron Microscopy (TEM).

GlowDots solution (1 ng/mL) was applied to a carbon-coated Cu grid (400-mesh) after treating the grid with a plasma cleaner (Harrick PDC-32G). Aliquots of 3 µL were incubated on the grid for 60 s, blotted with filter paper (Whatman #4), and stained with 3 µL of 1 % uranyl acetate for 30 s followed by blotting. After an hour of drying, the grids were imaged using a FEI Tecnai Spirit TEM with an operating voltage of 80 kV and a mounted digital camera.

4.2.8. Absorbance.

Absorption spectra were measured on an HP 8450 diode array spectrophotometer (Varian Inc., Santa Clara, CA). Samples were diluted to 0.412 mg/mL in 10 mM Na2HPO4 pH 7.0 and the baseline averaged from 700-800 nm was subtracted.

4.2.9. Fluorescence.

Fluorescence Spectra were recorded on Cary Eclipse Fluorimeter. GlowDots were diluted to approximately 0.4 mg/mL in 10 mM Na2HPO4 pH 7. All spectral measurements were done in a 1 x 1 cm2 quartz cuvette.

4.3. Results and Discussion
In this method, the carboxylic groups of bovine serum albumin (BSA) molecules were cross-linked with the amine groups of neighboring BSA molecules (Figure 24). High concentrations used in synthesis result in aggregation of the protein that were then cross-linked via carbodiimide chemistry. This particle formation was monitored via dynamic light scattering (DLS). Once the desired sizes and ratios were achieved, the particle formation was quenched by the addition of carbonate buffer. To achieve controlled single size, particles were annealed at 85 °C for 5 minutes. Protein nanoparticles were then decorated with reactive fluorescent labels (Figure 24). Final products were characterized by DLS, agarose electrophoresis, TEM, polyacrylamide electrophoresis, circular dichroism, and absorbance and fluorescence spectroscopy.

Table 5. Table of reactive fluorescent dyes, name of dye-labeled protein nanoparticles, chemical structures of reactive dyes, and absorbance and emission wavelengths of each dye.
4.3.1. Agarose Gel Electrophoresis

Agarose gel (40 mM Tris-acetate pH 5.2) was used to confirm the formation of protein nanoparticles (GlowDot) and successful labeling of the particles with fluorescent dyes, to be here forth designated as GlowDot[Emittance Wavelength]. For example, protein nanoparticles labeled with fluorescein isothiocyanate will be called GlowDot519. Dyes used in synthesis were 1-pyrenebutanoic acid (λem= 376 nm), 7-methoxycoumarin-3-hydroxy (λem= 410 nm), Diethylaminocoumarine-3-carboxylic acid (λem= 472 nm).
nm), fluorescein isothiocyanate (λem= 519 nm), tetramethylrhodamine -5-(and-6)-isothiocyanate (λem= 571 nm), and 5-(and-6)-carboxy-x-rhodamine(λem= 601 nm) (Table 5).

Figure 25. (A) Agarose gel electrophoresis (40 mM Tris Acetate, pH 7.0) demonstrates decreased mobility of GlowDot (lane 2) compared to BSA (lane 1). Addition of the fluorescent dyes does not significantly change the mobility (lanes 3-7, GlowDot340, GlowDot350, GlowDot494, GlowDot543 and GlowDot576, respectively). (B) Coomassie stained image of gel in A showing protein presence in samples. (C). Fluorescent nanoparticles in solution as seen when excited at 254 nm (GlowDot340, GlowDot350, GlowDot432, GlowDot494, GlowDot543 and GlowDot576).

Agarose gel (0.5 %, 40 mM Tris Acetate pH 7.0) of GlowDot and GlowDot340, GlowDot350, GlowDot494, GlowDot543 and GlowDot576 imaged using UV light (254 nm, Figure 25A) clearly showed all six fluorescent labels’ bands directly matching with the protein bands viewed in the coomassie stained gel (Figure 25B). The lack of a second fluorescent band in the lanes of the gel confirms there is no free dye in the samples. All six GlowDots migrated a shorter distance towards the positive electrode compared to 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 of the protein. While every conjugation reaction consumes a positive amine and a negatively charged carboxylic acid, resulting in a net 0 change in charge. However, crosslinking could result in residues that are normally buried in the protein to be unburied and brought out to the surface, changing the net charge of the protein.
4.3.2. Dynamic Light Scattering

Figure 26. Dynamic light scattering data of BSA in deionized water (A) with a radius of approximately 2.74 nm and larger soluble aggregates at 64 nm. DLS of protein nanoparticles after crosslinking protein with EDC. Bimodal distribution of 11 nm and 54 nm diameters is observed (B).

Dynamic light scattering (DLS) was used to monitor particle formation. Bovine serum albumin (BSA, 150 mg/mL) dissolved in deionized water had multiple sizes present in solution (Figure 26A). The smallest was a 3.5 nm diameter particle, corresponding to free protein and a larger 65 nm diameter aggregate. Upon the stepwise addition of EDC, DLS showed the shift to a bimodal system where the smallest peak was approximately 3.5 nm and the larger was approximately 40 nm in diameter. As EDC was continually added, crosslinking increased and the size of the smallest particles in solution became larger. While concentration had immediate influence on the particle sizes present in solution, after equilibrium of the system was established all solutions contain 10 nm and 40 nm diameter particles. (Figure 26B). Particles were annealed in 10 mM carbonate buffer pH 9.3 at approximately 1 mg/mL to achieve 43 nm diameter
Figure 27. Dynamic light scattering of particles made by annealing 0.5 mg/mL (A), 1 mg/mL (B), 3 mg/mL (C), 5 mg/mL (D) and 7 mg/mL (E) BSA nanoparticles in 100 mM phosphate 500 mM sodium chloride buffer pH 7.0 at 85 C for 10 minutes. Changes in particle size as a function of protein concentration during the annealing process (F).

Particle size was controlled by the concentration of protein nanoparticles during the annealing 100 mM phosphate 500 mM sodium chloride buffer pH 7.0. The smallest size of 20 nm in diameter was achieved by annealing nBSA at a concentration of 0.5 mg/mL nBSA. Largest single size was synthesized 5 mg/mL resulting in 80 nm diameter particles (Figure 27D). Annealing particles at 7 mg/mL resulted in 66 nm particles (31 %) and 196 nm (62 %) particles. When particles are annealed in low ionic
strength buffers or DI water, this control over size dependent upon the GlowDot concentration is not observed.

![DLS Graphs](image)

Figure 28. Dynamic light scattering of purified GLowDot494 and GlowDot576. Single distribution is observed and particle diameter is approximately 35 nm.

After labeling nanoparticle with reactive fluorescent dyes and purification, particles have a single size present in solution. For example, purified GlowDot494 had mono-distributed particles with diameter of 43 nm (Figure 28A) and purified GlowDot576 had mono-distributed particle sizes of 55 nm (Figure 28B). Analysis of the flow through from the filter centrifugation step showed that protein was present in the flow through. The filters had a cutoff of 100 kDa meaning that only single, un-cross linked protein would have been able to move through the filters. Thus the final products had only crosslinked protein. This was confirmed by SDS gel in the next section.

4.3.3. Polyacrylamide Gel Electrophoresis
Figure 29. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to determine if particles were physically or covalently linked. After annealing (lane 2), free protein can be seen at approximately 66 kDa matching the band seen in free BSA (lane 2). At the top of lane 2 is a smear and visible protein that remained in the well due to its large size after extensive crosslinking. After labeling with a dye and purifying the sample in an Amicon centrifuge filtration 100 kDa cutoff tube, no free protein remains and most of the protein remains in the well due to its large size after thorough crosslinking.

SDS polyacrylamide gel electrophoresis was used to better understand the composition of the Glow-DotX samples. SDS PAGE (Figure 29) showed wells containing GlowDot (lane 2) had both crosslinked (top band and smear, too large to calculate) and free protein (bottom band, approximately 66 kDa). After labeling of the dye and purification, GlowDot541 only crosslinked protein (top band, too large to calculate). Smearing is common in samples that are crosslinked. Free BSA was shown in lane 1 for comparison. From SDS PAGE, agarose gel electrophoresis, and DLS, it is concluded that GlowDot particles are composed of both crosslinked protein and free protein strongly associating with the cross-
linked protein after annealing step. Upon labeling and purification by filter centrifugation in Amicon 100kDa cutoff filters, samples are composed of only crosslinked protein.

4.3.4. **Circular Dichroism**

![Circular dichroism graphs](image)

Figure 30. Circular dichroism of GlowDot494 (A, green) and GlowDot576 (B, red) compared to native BSA under identical condition (black). Retention in shape of the spectra concludes no loss in ellipticity of the protein after synthesis of the GlowDots.

Native structure of protein is important for biological activity retention. The structure of the BSA in GlowDots was analyzed via circular dichroism and compared to free BSA to determine if any loss in structured occurred (Figure 30). Protein ellipticity of GlowDot494 and GlowDot576 showed no change in spectral shape or intensity. CD confirmed protein retained its structure after particle formation and particle labeling which is important for considering particle formation with catalytic proteins.

4.3.5. **Absorbance measurements**
The optical properties of GlowDots were characterized by absorbance and fluorescence spectroscopy. Absorbance data of GlowDots show that the excitation data can be tuned for each particle through careful selection of the fluorescent reactive dye. Using the absorbance data and the approximate number of BSA molecules per particle calculated from DLS (136 BSA/nanoparticle), the number dyes were calculated per particle. Each particle contained $35 \pm 10$ dye molecules, depending upon the fluorescent label. Extinction coefficients for the particles were then calculated by multiplying the number of dye molecules per particle by the extinction coefficient of the free dye (Figure 2D). All particles had very high extinction coefficients due to the large number of dye molecules per particle making the particles 25 x or more brighter than the respective individual dye molecule. These brightness values are very comparable with recently reported brightness values of CdS QDs that have been modified for equal brightness between different emission particles. \[83\]
Figure 31. Absorbance spectra of free dye (grey) compared to dye bound to the protein nanoparticle for GlowDot340 (A), GlowDot350 (B), GlowDot432 (C), GlowDot494 (D), GlowDot543 (E), GlowDot576 (F).

Comparison of the absorbance spectra of GlowDots to their respective free dyes provide evidence of the location of dyes in the particles. All GlowDots have a change in absorbance spectrum compared to their respective free dyes (Figure 31B-G) GlowDot 340 and GlowDot543 had an additional peak while all other GlowDots showed a shift in peak wavelength of the dye. Changes in absorbance spectra provide evidence that the dyes are buried inside the protein nanoparticle and not hanging of the outside of the GlowDot free in the buffer. This burying of dyes results in other changes in optical properties as discussed below.
Figure 32. (A) Emission spectra of GlowDot particles with tuned emissions. (B) Chromaticity plot showing diverse emission colors of different GlowDot particles.

4.3.6. Fluorescence

The emission spectra of each GlowDots was collected (Figure 32A) and plotted together. Each GlowDot and a unique emission profile and the emission wavelengths spanned the ultraviolet and visible spectrum. The emission wavelengths synthesized were 340 nm (purple), 350 nm (blue), 432 nm (light blue), 494 nm (green), 543 nm (orange), and 576 nm (pink). GlowDot340 had two emission peaks, which is not uncommon with 1-pyrenebutanoic acid, the fluorescent probe used to label the particle. All other GlowDots had singular, uniform peaks. The chromaticity coordinates were calculated from each emission spectra (Figure 32B). Chromaticity coordinates are values used to show what color the sample will appear to the human eye. GlowDots colors as appearing to the human eye were be tuned shades of blue, green, orange and red.
The relative quantum yields of the dyes attached to GlowDots were calculated using fluorescence spectroscopy (Figure 33). QDs are known for having high quantum yield and high brightness thus it is important that GlowDots are also capable of high quantum yields and brightness values to be considered as an alternative to heavy metal QDs. The quantum yield of each GlowDot was calculated by the following equation:

$$\Phi_f = \Phi_r \left( \frac{Abs_r}{Abs_f} \right) \left( \frac{I_f}{I_r} \right)$$

Where $\Phi$ is the quantum yield, $A$ is the absorbance at a specific wavelength, and $I$ is the fluorescence intensity. Subscript $f$ refers to values of the GlowDots while subscript $r$ references to the values of the reference samples, which in all cases was the free dye in buffer (10 mM Phosphate pH 7.2).
Table 6. Table of photophysical properties of GlowDots.

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\varepsilon$ (cm$^{-1}$ M$^{-1}$) Dye</th>
<th>$\varepsilon$ (cm$^{-1}$ M$^{-1}$) particle</th>
<th>Relative Quantum Yield, $\Phi$</th>
<th>Relative Brightness (M$^{-1}$ cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlowDot340</td>
<td>4.3 x 10^4</td>
<td>2.8 x 10^6</td>
<td>37.0</td>
<td>1.0 x 10^8</td>
</tr>
<tr>
<td>GlowDot350</td>
<td>2 x 10^4</td>
<td>9.8 x 10^5</td>
<td>1.2</td>
<td>1.1 x 10^6</td>
</tr>
<tr>
<td>GlowDot432</td>
<td>3.4 x 10^4</td>
<td>1.3 x 10^6</td>
<td>12.7</td>
<td>1.7 x 10^7</td>
</tr>
<tr>
<td>GlowDot494</td>
<td>7.5 x 10^4</td>
<td>2.0 x 10^6</td>
<td>0.4</td>
<td>7.5 x 10^5</td>
</tr>
<tr>
<td>GlowDot543</td>
<td>6.5 x 10^4</td>
<td>2.1 x 10^6</td>
<td>1.6</td>
<td>3.3 x 10^6</td>
</tr>
<tr>
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<td>1.9 x 10^6</td>
<td>0.9</td>
<td>1.7 x 10^6</td>
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</tbody>
</table>

The QY of most GlowDots either remained unchanged or saw a drastic increase with the exception of GlowDot494. The decrease in quantum yield of fluorescein isothiocyanate bound to the particle vs free dye molecule may be due to change in the charge of the fluorescein molecule. The quantum yield of the fluorescein is known to decrease for the anionic form. For GlowDot350 and GlowDot576, the quantum yield remained relatively unchanged compared to the free dye in buffer. GlowDot340 and GlowDot543 saw a drastic increase in quantum yield compared to their free dye constituents in buffer. We believe that this is due the microenvironments the dye experiences after being attached to the particle. These microenvironments could possibly be more hydrophobic than the environment of the buffer around the free dye, resulting in a larger quantum yield, confirming the explanation for spectral changes observed in the absorbance of the dyes when bound to GlowDots.

The brightness of each GlowDot was calculated by multiplying the particles’ extinction coefficient by its relative quantum yield. The brightness of the particles are all 105 or larger. These values are comparable with recent literature in which the brightness of QDs was equalized.
2.4 Adaptive surface for modification

A. POSITIVE

B. NEGATIVE
Figure 34. (A) Modification of GlowDots with Taurine. (B) Cellular uptake of GlowDot519+Taurine by HeLa Cells. (C) Montage of images focusing through HeLa cell. GlowDot494 can be seen throughout the entire cell as the microscope focuses from bottom of the cell to the top.

4.3.7. **Cellular Uptake Studies**

The adaptability of particles was tested by modifying the surface through different means and testing the cellular uptake. Two different methods of modification were used: addition of Taurine to surface and addition of polyamine to surface. Taurine, known to increase the cellular uptake of small particle96 Taurine was chemically linked to the surface of the particles by EDC conjugation. GlowDot494 particles labeled with taurine were imaged in HeLa cells after co-incubation for 3 hours at 37 C and 5% CO2. Green fluorescence could be seen throughout the cells (Figure 34A) at different focal lengths of
the microscope (Figure 34C). Control studies concluded that taurine had to be attached to the particle in order to facilitate uptake.

Figure 35. (A) Uptake of GlowDot494 by MDAMB human breast cancer cells after 3 h. incubation. (B) Uptake of GlowDot494 by PC3 human prostate cancer cells after 3 h. incubation. (C) Uptake of GlowDot494 by L6 rat myoblast cells after 3 h. incubation.
Other cell lines were tested to determine if taurine labeling could be used as a general method for decorating particles. Rat myoblast (L6), Breast cancer cells (MDAMB), prostate cancer (PC3) cell lines were also tested. Cellular uptake of GlowDot494 coated with taurine was observed for all four cell lines tested (Figure 35). The facile synthesis of particles modified to be taken up by different cell lines shows the viability of GlowDots as a suitable non-toxic imaging agent.

4.4. Conclusions

Figure 36. Table comparing different imaging properties of GlowDots vs other popular imaging agents polymer dots, liposomes, quantum dots and small molecule nanoparticles.

<table>
<thead>
<tr>
<th></th>
<th>Polymer Dots</th>
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<th>Quantum Dots</th>
<th>Small Mols</th>
<th>Protein NP</th>
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<td>+</td>
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</tr>
<tr>
<td>High Absorption</td>
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<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>n.d</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Good Stability</td>
<td>+</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
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<td>+</td>
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</tr>
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<tr>
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<td>n.d</td>
<td>-</td>
<td>n.d</td>
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</tr>
</tbody>
</table>

We have reported the facile synthesis of protein nanoparticles with properties much like those of quantum dots. The emission spectrum of GlowDots is easily controlled during synthesis through the selection of the reactive fluorescent probe. Furthermore, this emission tuning is completely independent of the particles size and can have one or more emission peaks in the spectrum. The molar absorptivity and brightness values were determined to be similar to those reported for heavy metal quantum dots. GlowDots are bright, highly absorptive fluorescent protein nanoparticles with narrow emission spectra, biocompatibility and can be easily biodegraded. No special steps are required for solubilizing Glow-
Dots, and their surface provides many different locations for modification with small molecules, aptamers, peptides, and antibodies. These particles possess the same properties of quantum dots while addressing some of the areas that QD technology has yet to find a simple solution to. The amazing properties of GlowDots could be utilized in cellular imaging, drug delivery and other biological nanotechnologies.
Chapter 5.

White-Emitting Protein Nanoparticles for Cell-entry and pH Sensing

5.1. Introduction

Nanoparticle-based pH sensors are currently being developed for biological and chemical applications\textsuperscript{97} but they have certain drawbacks such as toxicity, limited range, difficult synthesis or not suitable for biological applications.\textsuperscript{98-100,101,102,103,104} This paper reports a facile, green and modular synthesis of pH sensing, fluorescent, metal-free, white-emitting nanoparticles derived from a common, inexpensive protein, bovine serum albumin (BSA). The use of protein matrix for the particle synthesis renders them biodegradable at pre-programmed rates, and the presence of a variety of multiple functional groups on the surface are convenient for further manipulation to attach other ligands, dyes or drugs. These particles provide sensitive probes for sensing pH, temperature or other changes in their local environment.

The choice of BSA for nanoparticle synthesis is justified by multiple reasons. BSA has 82 lysine and 99 aspartate/glutamates for intensive covalent crosslinking of the protein to form nanoscale particles,\textsuperscript{106} and BSA binds a number of small molecule, metal ions and signal peptides, and therefore, particles derived from BSA can potentially serve as carriers for these ligands.\textsuperscript{106,107} BSA-derived particles also have a high potential for controlled degradation by proteases\textsuperscript{108,109} The presence of a large number of different pKa groups on BSA provides greater flexibility in designing a pH-sensing platform due to known matrix effects on dye properties.\textsuperscript{110,111} BSA also serves as a model protein for protein transduction into cells, where the protein is directly imported into the cells to trigger specific biological responses. The method is less sensitive to hydrolysis by cellular environment than DNA/RNA transfection.\textsuperscript{112,113,114,115} Certain cells have specific receptors for SA and can promote receptor-mediated endocytosis. Hence, nanoparticles derived from BSA (nBSA) are expected to be versatile, biocompatible
and also biologically interesting for pH sensing and cellular imaging. Thus, we tested if BSA is a versatile material for building nanoparticles for biological applications.

White emitting nanomaterials are of current interest for use in organic light emitting diodes\textsuperscript{116-117,118,119} pollution monitoring,\textsuperscript{120} and detection of metal ions\textsuperscript{121} and anions.\textsuperscript{122} Sensors based on white emitting particles are expected to be more sensitive to analytes due to the ability to monitor three different excitation/emission wavelengths simultaneously for robust ratiometric sensing.\textsuperscript{123} This approach minimizes interference from background emission and provides greater flexibility in choosing the excitation/emission wavelengths. The presence of three or more pKa groups on them may contribute to increased sensitivity of the particles to pH.

We report here the first examples of white-emitting BSA nanoparticles (GlowDots) with chemical control over their size, surface chemistry and optical properties. The GlowDot emission is sensitive to pH over a range of 7 pH units making them convenient for sensing applications.\textsuperscript{124} They readily enter cells for protein transduction into live cells, and facilitate live cell imaging. Our particles are tested here, for sensing at different parts of the cell, for the first time as a proof of concept.

The chemical novelty of the current approach is the ability to independently tune the particle size, color, extinction coefficient, spectral width and quantum yields, while keeping the synthetic methodology modular, simple, efficient and capable of being executed in a resource-limited setting. These are significant advantages over other nanoparticles such as quantum dots (QDs). The size and color of QDs cannot be independently tuned, special modifications are required to make them water soluble, and they could be toxic to sensitive biological systems.\textsuperscript{125} Thus, GlowDots have specific advantages and complement QDs in many aspects.

5.2. Experimental

5.2.1. Materials.
Bovine Serum Albumin was purchased from Equitech Bio. (Kerrville TX). 7-methoxycoumarin-3-hydroxy, succinimidyl ester (M), fluorescein isoethiocyanate (F), 5-(and-6)-carboxy-x-rhodamine, succinimidyl ester (R), and 7-dimethylaminocoumarin-3-carboxylic acid, succinimidyl ester (D), purchased from Anaspec (Fremont, CA). 1-Ethyle-3-(3-dimethylaminopropyl)carbodiimide was purchased from TCI America (Portland, OR).

5.2.2. Synthesis of GlowDots.

Protein solution was prepared by stirring 150 mg of BSA in 1 mL of de-ionized water (dH2O). EDC (1M, dH2O) 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 15 mM CO32-/HCO3- pH 9.3 buffer. Uniform particle sizes was achieved by slowly heating 1 mg/mL solution to 85°C and allowing to slowly cool back to room temperature. White fluorescent particles (nBSAW) where synthesized by adding F (0.19 mg/mL, 0.49 mM), M (2.15 mM, 0.68 mg/mL) and R (0.34 mg/mL, 0.54 mM) to 1 mL of nBSA in 10 mM CO32-/HCO3- pH 9.3. Mole ratios of dyes were adjusted as needed until white fluorescence was observed. Reaction was stirred for 2 h and purified by ultracentrifiltration (Amicon, 100 kDa) until filtrate was clear. White emitting particle solution was also synthesized by mixing molar ratios of nBSA350, nBSA494 and nBSA576. Ratios of GlowDots were adjusted as needed until white fluorescence was achieved. nBSAW405T was synthesized by labeling the particles with Taurine (1 mg/mL BSA, 1.5 mM Taurine, and 160 mM EDC added last) before the annealing step. During the labeling process, M was replaced with 6-diethylaminocoumarin-3-carboxylic acid, succinimidyl ester (D). All other parameters were the same.

5.2.3. Dynamic Light Scattering (DLS).

Hydrodynamic radii of nBSAW265 particles were monitored by means of photon correlation spectroscopy with Precision Detectors (Varian Inc.), CoolBatch+ dynamic light scattering apparatus with 10 x
10 mm2 square cuvette, 658 nm excitation laser source with a 90° geometry. Data collection was done at room temperature, for 1 s, 3 repetitions with 200 accumulations. The GlowDots, nBSA and BSA 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.

5.2.4. *Agarose Gel Electrophoresis.*

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. This procedure was repeated with 40 mM Tris acetate buffer with lower pH to determine the pI of GlowDots.

5.2.5. *Circular Dichroism (CD).*

CD spectra were measured on a Jasco J-710 CD spectrometer. A concentration of 1.25 µM protein in 10 mM Na2HPO4 pH 7.0 was used. Spectra were obtained using a 0.05 cm path length quartz cuvette in the region of 260 – 190 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 pH 7.0 was taken as 100 % ellipticity.
5.2.6. **SDS PAGE.**

A 7 % separating with 5 % stacking gel was used. Samples were prepared by adding loading buffer (10 μL, 2 % SDS, 10 % BME) to the unheated sample then boiled for 2 minutes. Samples were loaded into the gel so that each well contained 6 μg of protein. The gel was run in SDS running buffer at 200 V constant in Bio-Rad Mini Protean Electrophoresis apparatus until the dye front was 1 cm from the bottom of gel plate. Gel was stained in Stain I (10 % v/v acetic acid, 10 % v/v isopropanol, 0.02 % Coomassie blue) for 1 h. Gel was then placed in Stain II (10 % v/v acetic acid, 0.02 % Coomassie blue) overnight. The gel was destained in 10 % v/v acetic acid until bands were clearly distinguished from clear background of gel.

5.2.7. **Transmission Electron Microscopy (TEM).**

The nanoparticle suspension (0.2 mg/mL) was applied to a carbon-coated Cu grid (400-mesh) after treating the grid with a plasma cleaner (Harrick PDC-32G). Aliquots of 3 μL were incubated on the grid for 60 s, blotted with filter paper (Whatman #4), and stained with 3 μL of 1 % uranyl acetate for 30 s followed by blotting. After an hour of drying, the grids were imaged using a FEI Tecnai Spirit TEM with an operating voltage of 80 kV and a mounted digital camera.

5.2.8. **Spectral Measurements.**

Absorption spectra were measured on an HP 8450 diode array spectrophotometer (Varian Inc., Santa Clara, CA). Samples were diluted to 0.412 mg/mL protein in 10 mM Na2HPO4 pH 7.0 and the baseline averaged from 700-800 nm was subtracted. Fluorescence Spectra were recorded on a Cary Eclipse Fluorimeter. Samples were diluted to approximately 0.4 mg/mL protein, in 10 mM Na2HPO4 pH 7.0 and a filter with cutoff below 300 nm used on the emission side. All spectral measurements were done in a 1 x 1 cm2 quartz cuvette.
5.2.9. *Cell Imaging Studies.*

HeLa cells were grown in DMEM at 37°C 5% CO2 for 24 hours in an 8 well chamber with cover plate bottoms. To each well of cells, 0.3 mg/mL nBSAW405T was added. Samples were incubated at 37°C and 5% CO2 for 3 hours. Imaging was done on a Nikon A1R confocal microscope. Blue channel was excited by a 405nm diode laser and monitored at 461 nm. The green channel was excited with 488 argon laser and monitored at 525 nm. The red channel was excited by a 588 nm argon laser and monitored at 595 nm. All images were processed with FIJI (Fiji Is Just ImageJ).
5.3. Results

5.3.1. Particle Synthesis and Characterization

White-emitting GlowDots of controlled size were prepared by crosslinking BSA molecules using carbodiimide chemistry and subsequent labeling with reactive dyes, 7-methoxycoumarin-3-carboxylic acid, succinimidyl ester (M), fluorescein isothiocyanate (F), and 5(6)-carboxy-X-rhodamine, succinimidyl ester (R) in appropriate mole ratios (Figure 37a-c). The following notation will be used for the react-
tion products, nBSAW254, where n represents nanoparticles, BSA is the protein used, W denotes white light emission, and 254 is the excitation wavelength (nm) that results in white light emission.

Figure 38 (A) Agarose gel of nBSAW254 (40 mM Tris acetate, pH 6.5) imaged under 254 nm excitation, BSA (lane 1), nBSAW254 (lane 2) and lysine derivatives of M (lane 3), F (lane 4), and R (lane 5). (B) Same gel as in (a) but stained with Coomassie Blue to visualize the protein bands. (C) Agarose gel of nBSAW254 compared to free reactive dyes M (lane 2), F (lane 3), R (lane 4) run in 40 mM Tris acetate pH 7.0. (D) Agarose gel of same samples in (C) run in 40 mM Tris acetate pH 5.5.

After purification of the reaction mixture by ultrafiltration followed by resuspension of the particles, the agarose gels nBSAW254, (40 mM Tris- acetate, pH 6.5) showed a single band of white-emission (Figure 38A, lane 2), imaged under 254 nm light. Lanes 3-5 contained dyes M, F, and R reacted with L-lysine, as model systems and there was no indication of the presence of free dyes (M, F or R) in lane 2. The same gel was stained with Coomassie Blue (Figure 38B) to view the corresponding protein bands and the band corresponding to BSA (Lane 1) is absent in lane 2, containing the GlowDots. nBSAW254 migrated slower than BSA due to its larger size when compared to that of BSA. Every conjugation event between the BSA molecules consumes one carboxylic acid group and one amine group, and therefore, the net charge on nBSAW254 is expected to be similar to that of BSA (discussed below).
In a separate experiment, the mobilities of free dyes without the reactive groups were also examined. No two components had the same mobility (Figure 38C,D). Thus, nBSAW254 had no free protein or free dyes, and there has been a single band of white emitting sample. The presence of crosslinked pro

![Image](image1.png)

Figure 39. A) 7% separate SDS gel shows nBSA (lane 3) is composed of cross linked particles (smear at top of gel and protein still in well) with no free BSA (shown in lane 1 for comparison). (B) Trypsin digestion reveals that nBSA particles are digested (lane 4) at a slower rate compared to BSA (lane 3) (C) Dynamic light scattering of nBSAW254 (10 mM phosphate buffer pH 7.0). (D) TEM of nBSAW254. (E) Circular Dichroism shows no loss in ellipticity for nBSA (red) and nBSAW254 (blue) compared to BSA (black). All samples show a double minima at 207 and 222 nm with one maximum at 195 nm. This spectral shape corresponds to an alpha helix structure.
tein in the particles was also confirmed by SDS PAGE (Figure 39A) which clearly showed extensive crosslinking of BSA as these bands moved very slowly in the gel but remained at the top of the gel (Figure 39, lane 3). nBSA particles were treated with trypsin to determine if proteases would degrade the particles. After 6 h of incubation at 37°C with 1:20 trypsin:BSA ratio, BSA showed extensive digestion (Figure 39B, lane 3) as evidenced by the presence of many bands smaller than that of free BSA (Figure 39B, lane 2). nBSA did not show digestion under similar conditions (Figure 39B, lane 4). However after 9 h incubation with trypsin under the same condition, peptides could be seen in the SDS PAGE gel.

GlowDot size was determined by dynamic light scattering (DLS) and confirmed by TEM analysis. DLS indicated peaks with diameters of 36 (92%) and 5 nm (8%) (10 mM phosphate buffer pH 7.2, Figure 39C). From these data and the known size of BSA,126 Up to 135 BSA molecules are present in each nBSAW254 particle. The DLS data were also supported by TEM micrographs (Figure 39D) which indicated particles of ~40 nm in diameter. Combined together, the agarose gels, SDS PAGE, DLS, and TEM confirmed the successful formation of nanoparticles. Next, the secondary structure of BSA in the particles was examined by circular dichroism (CD) spectroscopy. The far UV-CD spectrum of nBSAW254 (10 mM NaH2PO4 pH 7.2) had minima at 222 and 208 nm and a maximum at 195 nm (Figure 39E), which is nearly superimposable with that of BSA. Thus, crosslinking chemistry did not influence protein structure to a significant extent.
5.3.2. **Optical Properties**

![Absorption spectra](image)

Figure 40. (A) Absorption spectrum of nBSAW254 (10 mM Na2HPO4 pH 7.2) with maxima at 280, 350, 495, and 576 nm. (B) Absorbance spectra of nBSAW254 (black) compared to reactive fluorescent dyes M (blue), F (green), and R (red).

The optical properties of nBSAW254 particles were characterized by absorbance and fluorescence spectroscopy. The absorbance spectrum of nBSAW254 (Figure 40A, 10 mM Na2HPO4 pH 7.2) showed four peaks at 280, 345, 503, and 582 nm which corresponded to those of BSA, M, F, and R, respectively.

Using the absorbance data of the linked dyes, we estimate that each nBSAW254 particle has 67 M, 7 F and 12 R dye molecules. Therefore, the molar extinction coefficients of nBSAW254 at these peak positions are very high, which resulted in intense light absorption. Absorption peaks of M and F bound to nBSAW254 were shifted when compared to those of the free dyes due to changes in their microenvironments upon linking to the particles. However, no change in the absorbance peak position was noted for R present in nBSAW254 (Figure 40B).
Figure 41. (A) White emission of nBSAW254 when excited at 254 nm. (B) Multi-mode excitation and emission of the nBSAW254. (C) Emission spectra of nBSAW254 when excited at 350, 495 and 576 nm. (D) Excitation spectrum of nBSAW254 when monitored at 400 nm (dye M). (E) Excitation spectrum of nBSAW254 when monitored at 527 nm (dye F). (F) Excitation spectrum of nBSAW254 when monitored at 607 nm (dye R).

Fluorescence spectra of the dyes linked to the particles strongly depended on the excitation wavelength. nBSAW254 excited at 254 nm had 4 emission peaks: 348, 400, 520, and 601 nm (10 mM phosphate buffer pH 7.2) (Figure 41A), which correspond to those of BSA, M, F, and R, respectively. The emission color, however, strongly depended on the excitation wavelength (Figure 41B,C). Therefore, dyes bound to the particles had little or no cross-talk among their corresponding excited states, and the excitation spectra confirmed this conclusion (Figure 41D-F). This kind of multidimensional emission which depended on the excitation wavelength, is not usually possible for simpler molecular systems, which is another unique advantage of GlowDots for imaging applications. One could monitor
the emission of M or F or R, depending on the excitation wavelengths chosen or multiple, simultaneous excitation/emission wavelengths to suppress background emission.

<table>
<thead>
<tr>
<th></th>
<th>( \lambda_{\text{Ex}} ) (nm)</th>
<th>( \Phi_{\text{Free Dye}} )</th>
<th>( E_{\text{Particle}} ) (M(^{-1})cm(^{-1}))</th>
<th>( \Phi_{\text{nBSA}_{W254}} )</th>
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<tr>
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</tr>
</tbody>
</table>

Figure 42. (A) Quantum yields and color coordinates of nBSA\(_{W254}\). (B) Chromaticity plots of nBSA\(_{W254}\) when excited at specific wavelengths.

The relative quantum yields of nBSA\(_{W254}\) at specific wavelengths of the dyes were estimated by comparing the particle emission with those of the corresponding free dyes, under otherwise identical conditions. The quantum yield of nBSA\(_{W254}\) at 350 nm (excitation at 336 nm) was 0.82, and at 580 nm (excitation at 576 nm) has been 0.89 which are close to those of the corresponding free dyes,\(^{127,128}\) while the quantum yield at 495 nm (excitation at 492 nm) has been substantially lowered from 0.79\(^{129}\) to 0.29 (Figure 42A, third column). Decrease in quantum yield of F is most likely due to quenching of its excited state by amino acid residues at the binding site or a change in the equilibrium concentrations of the anionic, neutral and cationic forms of the dye, which differ greatly in terms of their quantum yields. The latter is unlikely, as the absorption spectra of the bound F does not indicate substantial changes in these compositions.\(^{[130]}\) The brightness, defined as the product of extinction coefficient and the quantum yield, was calculated for each of the three dyes on the particle and found to be 1.1 x
106 (M), 1.6 x 105 (F), 3.8 x 105 (R), which is much higher than that of reported quantum dots (Figure 42A, fourth column).

Next, we examined the quality of the white emission of the particles by analyzing the chromaticity co-ordinates, which indicated values of 0.30,0.35 (Figure 42B, star) when excited at 254 nm,

![Image](image.png)

Figure 43. (A) Chromaticity of nBSAW254 emission as a function of pH. (B) Plot of the ratio of fluorescence intensities at 524 to 608 nm (F/R) (black) of nBSAW254 particles, as a function of pH from 4-11. (C) Chromaticity plot as a function of pH. (D) Table comparing slope and range of linear sensitivity of nBSAW254 and nBSAW405 vs other types of recently published pH sensors designed for cellular imaging. (E) Ratiometric sensing of nBSAW405 for the M/R intensity (blue) and F/R (red). (F) Color change of nBSAW405 as the pH is raised from 5 to 10. Color does not change after pH 7, matching the ratiometric data.
While pure white light would have chromaticity coordinates of 0.33, 0.33 which confirmed the high quality of white emission from nBSAW254. To demonstrate the versatility of the current approach to generate white light under a different excitation wavelength, we replaced M by 7-Diethylaminocoumarin-3-carboxylic acid (D) to produce particles (35 nm in diameter) that emitted white light when excited at 405 nm (nBSAW405). nBSAW405 had a composition of 41 D, 27 F, and 41 R, per particle and had chromaticity coordinates of 0.30, 0.36, when excited at 405 nm. This demonstrates the versatility of the current approach to tune the optical properties of the particles without compromising other properties.

Figure 44. (A) pH sensitivity of nBSA405W in solution when irradiated at 365 nm. (B) Ratiometric sensitivity of the intensity at 475 nm / 605 nm (blue/red, blue line) and 524 nm / 605 nm (green/red, green line). (C) pH sensitivity of nBSA365W when synthesized to have the red dye in the interior of the protein (ROX Core) when in solution and irradiated at 365 nm, (C) Log of the ratio of 520 nm intensity/601 nm intensity resulting in a linear trend line (green/red intensities)
5.3.3. *pH Sensitivity*

The white emission of nBSAW254 at pH 7 changed to purple at pH 1-2, yellow at pH 11, dull green at pH 13 and nearly quenched at pH 14 (Figure 43A). The ratio of the intensity of 524 nm band to that of 608 nm vs pH was linear over pH 4-11 (Figure 43B), and had a slope of 0.13/pH unit and the corresponding chromaticity plot shown in Figure 43C. nBSAW254 was sensitive to pH over a much broader pH range than Fe3O4-ANNA (4-5.7), Naphthalimide-rhodamine (4.5-5.25), small molecules(2-3.8), and hemicyanin small molecule (6.5-8) (Figure 43D).124,131,132,133,134,135 The response of nBSAW405 particles was linear over the pH range of 5-7 (Figure 43E). The color of nBSAW405 changes from purple to a cool white as the pH increases from 5 to 7 and remains the same color from pH 7 to 10 (Figure 43F). Color change slightly for nBSAW405 compared to nBSAW254 due to different pH sensitivity of 7-diethylaminocoumarin-3-carboxylic acid, succinimidyl ester, the dye used to replace 7-methoxycoumarin-3-carboxylic acid, succinimidyl ester. These data clearly supported the utility of our particles for pH sensing applications.
Figure 45. (C) Uptake of nBSAW405 particles decorated with taurine monitored at 461 (blue, excitation 405 nm), 525 (green, excitation 488 nm), 595 (red, excitation 561 nm) nm and transmitted light (grey). (D) Overlay of red, green and blue channels showing dynamic changes in fluorescence from live cell imaging. (E) Bar graph of ratios of blue/red, blue/green, and green/red emission from specific parts of the cell (green, blue and pink areas).

5.3.4. **Cellular Imaging Studies**

As a proof of concept of the utility of GlowDots for cellular probing, we examined GlowDots’ ability to enter the cells and probe the local environment by exposing nBSA254W to Hela Cells. Cells were co-incubated with nBSA254W (0.3 mg/mL, 10 mM Phosphate buffer pH 7.2) and glucose oxidase (0.3 mg/mL, 10 mM Phosphate buffer pH 7.2) for three hours, similar to previously reported methods. After washing away free particles not taken up by the cells (3x, 10 mM Phosphate buffer pH 7.2),...
cells were imaged on the confocal microscope. Cells untreated with particles were imaged for background comparison (Figure 45A). The blue channel image was obtained by monitoring at 450 nm while exciting at 405 nm (Figure 45A and B, top left panel). The green channel was monitored at 525 nm while exciting at 488 nm (Figure 45A and B, top right panel) and the red channel emission was recorded at 595 nm while exciting at 561 nm. Figure 45A and B, bottom left). Finally, the fourth channel (Figure 45A and B, bottom right) is the image of the cells under transmitted light. Overlaying of the three colored panels clearly showed particle uptake (Figure 45C) with variations in specific color intensities. The only color observed in the blue channel was determined to be from the cells’ autofluorescence. Color was not seen due to the blue dye (7-methoxycoumarin-3-carboxylic acid) having an emission wavelength lower (400 nm) than the excitation wavelength available on the instrument (405 nm).

Next, nBSAW405 was exposed to HeLa cells. These particles were modified with taurine, and taurine has been known to promote cellular uptake of short peptides. Cells were incubated for 3 h, washed 3x with 10 mM phosphate buffer (pH 7.2) and imaged on a confocal fluorescence microscope. The blue channel image was obtained by monitoring at 450 nm while exciting at 405 nm (Figure 45D, top left panel). The green channel was monitored at 525 nm while exciting at 488 nm (Figure 45D, top right panel) and the red channel emission was recorded at 595 nm while exciting at 561 nm. Figure 45D, bottom left). Finally, the fourth channel (Figure 45D, bottom right) is the image of the cells under transmitted light. Overlaying of the three colored panels clearly showed particle uptake (Figure 45E) with variations in specific color intensities.

The red, green and blue emission intensities of the particles in the cells were used to analyze the changes in the emission color from location to location inside the cell. Each ratio of histogram values generated from the software FIJI (Fiji Is Just ImageJ) from the captured confocal images of the particles in the cells was investigated for its sensitivity to different portions of the cells. The ratio of
blue/green color intensity (Figure 45C, center set of data) showed the most diverse set of ratios for the green, blue and pink areas of the cell. Each area had a vastly different ratio of blue to green pixel intensity, with the blue areas showing the highest ratio of 2.91 +/- 0.19 and green areas showing the lowest 0.30 +/- 0.09. The ratio of blue/green pixels in the pink area fell in the middle with a ratio of 1.02 +/- 0.12. The next most diversely identifying ratio was ratio of blue pixels to red pixels (Figure 45C, left set of data). Again, the blue area had the highest ratio of 1.5, pink had 0.45 +/- 0.06 and finally green had ratio of 0.29 +/- 0.08. The ratio of green/red showed the least sensitivity to the three different colored areas of the cell analyzed. Green area had the highest ratio of 0.99 +/- 0.07, followed by blue with a ratio of 0.52 +/- 0.04 and pink had the lowest ratio of 0.44 +/- 0.02. While each area of the cell did show to have statistically unique ratios of green/red pixels, and these differences reflect changes in the environment around the chromophores and their potential application as cellular probes with further work.

5.4. Discussion

Protein nanoparticles have been synthesized via chemical crosslinking of protein aggregates. This method resulted in a solution with 10 nm and 35 nm particles present. SDS gel of this sample showed that the particles are composed of both free protein (66 kDa, native BSA) and crosslinked protein (132 kDa, dimer) or larger. Free, non-crosslinked protein was removed from the sample during filter centrifugation, the purification method which also removed any by products from the crosslinking step, unreacted dye and small molecules and replaced any remaining carbonate buffer with 10 mM phosphate buffer pH 7.0.

From the particle diameter and hydrodynamic radius of BSA (3.45 Å), it was calculated that each nanoparticle contained up to 135 molecules of BSA. Of these 135 molecules, approximately 90 of them are on the surface, available for reaction with the dye molecules. Each particle had approximately 100 total dye molecules total, making the surface protein to dye ratio near 1 to 1. Due to BSA having multi-
ple surface carboxylic acid groups from lysine and arginine residues, it was expected that each protein could have multiple dyes attached, however that does not seem to be the case. This could be due to the orientation of each molecule of BSA on the surface limiting access to available carboxylic acids. However, the number of dye molecules on the particles resulted in high brightness, high extinction coefficients and high quantum yields making them excellent candidates for sensing and imaging applications.

White emission was achieved by adjusting the mole ratio of red, blue and green dyes so that each had near equal emission when excited at 254 nm. Chromaticity coordinates showed that near pure white light was achievable with the system. Because white emission was made up of red, green and blue emission, it was possible to have four unique emission profiles and thus emission colors from the same sample by changing the excitation wavelength. Because of the varying pH sensitivity of R, F, and M, and the difference on local environments sensed by each individual dye molecule, the sensitivity of the particles to pH was found to be very high. R, a rhodamine derivative is known to have high stability over a large pH range, therefore, very little change was observed in the emission of R from pH 1 to 14. F, fluorescein derivative, is known to be stable over more basic pH ranges and thus it had greatest changes in fluorescence at low pHs. Lastly, M, a derivative of coumarin, is known to have stability in more acidic pH, resulting in little changes over low pH range but large sensitivity to higher pH ranges. These three dyes combined with their sensitivities resulted in linear ratiometric sensitivity from 4 – 11, with pH range of 2-13 being visually responsive.

Taurine, a naturally occurring small molecule facilitated the uptake of particles into HeLa. Inside the cells, different color were observed due to changes in local environment around the chromophores. Analysis of the red, green and blue pixel intensities in different portions of the cell showed that nanoparticles’ emission was significantly changed in different parts of the cell. The difference in ratios between parts of the cell is concluded to be from changes in local environment and not protease degra-
dation of the particles due to the length of time required to degrade the particles (>6 h) and particles were imaged within 4 h after addition to the cell media.

Ultimately these particles are controllable, white emitting and capable of sensing changes in cellular environments. Comparison of these particles vs other common imaging particles can be seen in Table 7. Comparison of GlowDots properties vs those of quantum dots and fluorescent dyes. Table 7.

Table 7. Comparison of GlowDots properties vs those of quantum dots and fluorescent dyes.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>GlowDots</th>
<th>Quantum Dots</th>
<th>Fluorescent Dyes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption Spectra</td>
<td>Sharp</td>
<td>Broad</td>
<td>Sharp</td>
</tr>
<tr>
<td>Emission Spectra</td>
<td>Narrow or Broad</td>
<td>Narrow</td>
<td>Narrow or Broad</td>
</tr>
<tr>
<td>Photobleaching</td>
<td>Slow</td>
<td>Slow</td>
<td>Fast</td>
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<tr>
<td>Fluorescence lifetimes</td>
<td>1-10 ns</td>
<td>1-10 ns</td>
<td>&lt; 5 ns</td>
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<tr>
<td>Blinking</td>
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<td>Yes</td>
<td>No</td>
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<tr>
<td>Brightness</td>
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<td>Very high</td>
<td>Low</td>
</tr>
<tr>
<td>Tunable Size</td>
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<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Molar extinction</td>
<td>Very high</td>
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<td>Low</td>
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<td>Stokes shift</td>
<td>10-100 nm</td>
<td>10-400 nm</td>
<td>10-100 nm</td>
</tr>
<tr>
<td>Size</td>
<td>5-100 nm</td>
<td>5-100 nm</td>
<td>&lt; 2 nm</td>
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<tr>
<td>Surface functions</td>
<td>Naturally abundant</td>
<td>Need to attach</td>
<td>Few</td>
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<tr>
<td>Biological activity</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Built-in receptors</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
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<tr>
<td>Water-solubility</td>
<td>Natural</td>
<td>Need modification</td>
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<tr>
<td>Enzymatic activity</td>
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<td>Storage Stability</td>
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<td>Yes</td>
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<tr>
<td>Biodegradation</td>
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<td>Cost</td>
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<tr>
<td>White-emission</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

5.5. Conclusion
Here, we report the very first protein fluorescent nanoparticles that emit white light upon excitation at 254, or 405 nm, with chromaticity coordinates close to that of pure white light. Particle formation was under chemical control, and their surface chemistry, absorption spectra and emission characteristics are tuned as desired, while keeping the particle size the same. Due to the large number of copies of the 3 dyes present in each particle, these have very strong absorption and intense emission characteristics that are suitable for cell imaging applications. Sensitivity of emission to pH further enhanced their utility to image cell interior. The surface of the particles modified with taurine entered HeLa cells very readily and provided preliminary assessment of cellular local environment on these length scales in live cells. Properties of BSA make it an ideal material for building nanoparticles for biological applications. BSA has specific binding sites for a number of dyes, drugs, ions, peptides and hormones, and serves as an excellent carrier for cellular uptake. White emission can be a very useful tool for biological applications and also for high sensitivity to different local environments in the cell for more robust measurements. White emitting, biocompatible, cost effective protein nanoparticles have a high potential for applications for sensing.
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