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Effects of glycation on blood protein interactions with nanomaterials: a biophysical and cytotoxicity study

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Effects of glycation on blood protein interactions with nanomaterials: a biophysical and cytotoxicity study

By

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Abstract

When engineered nanoparticles (NPs) enter into a biological system, the proteins and biomolecules in the system adsorb to the NP surface to form a "protein corona" (PC). The PC greatly influences NP transformations, biological interactions, and cell response. Further complicating the development of NPs for biomedical applications, disease states alter the population of proteins and changes the biophysical features of individual proteins through post-translational modifications. Here-in, we aim to understand how glycation of the PC, as in uncontrolled diabetes, alters the NP-PC interaction and toxicity by conducting biochemistry and cell toxicity experiments. We focus upon 40 and 80 nm citrate-coated silver nanoparticles and glycated and unmodified human serum albumin (HSA) as a model system. To investigate the impact of glycation on NP-PC interactions, we evaluate changes to NP size and surface charge, HSA secondary structure, and binding constants for the complex. Results indicate a stable system with no particle agglomeration or protein unfolding, an increase in surface charge, and dynamic binding. To investigate the impact of the PC and glycation on NP interactions with human cells, we investigate changes in proliferation of HepG2 cells with addition of the complex. Results indicate the protein corona increases the cytotoxicity of the NPs. Results will contribute to the body of research exploring how nanoparticles change when they enter human systems. In the long term this research will assist in developing NPs for medical applications, especially in a diabetic population.

Acknowledgements

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1. Introduction

1.1 Background and Motivation

Cardiovascular disease is the number one cause of death worldwide; it contributes to one-third of all global deaths. In the United States, 610,000 annual deaths are due to heart disease.[1] 28.1 million Americans currently have heart disease.[2] Patients with cardiovascular disease may eventually require surgical intervention and implantation of a medical device such as a heart valve or stent. However, implanting any device into the body leads to concerns about rejection of the device. One of the ways to potentially improve the rejection rates of implants is to seed silver nanoparticles in the implant. Silver nanoparticles have notable antimicrobial and anti-inflammatory properties, so their addition to the device may mitigate some concerns about rejection of the device.[3] Integration of silver nanoparticles into implantable medical devices shows significant promise in addressing some of the most pressing concerns associated with these devices.

Silver nanoparticles are presently not widely used in implantable medical devices. Although research into these applications is active; more research is needed to determine the long-term toxicity of the particles.[3] Studying nanoparticle (NP) toxicity is extremely complicated because nanoparticles do not retain their original character when they enter the human body. The protein corona (PC), a coating of proteins that forms around the NP once it enters the bloodstream, has significant impacts on both the NP and cell response. It can affect the physicochemical properties of NPs, including size, surface charge, and agglomeration state, as well as biological fate, including pharmacokinetics, biodistribution, and therapeutic efficacy.[4,5] Complicating research, the properties of the PC are unique according to the disease state of the patient. The PC can be composed of different constituent proteins or contain proteins with

unique post-translational modifications. This variation in the PC between individuals with different disease states has led to the idea of the "personalized protein corona," the notion that diseases, habits, age, sex, and other factors can influence the formation of the PC.[6] The influence of disease state on the protein population found in the protein corona is shown in the figure below.

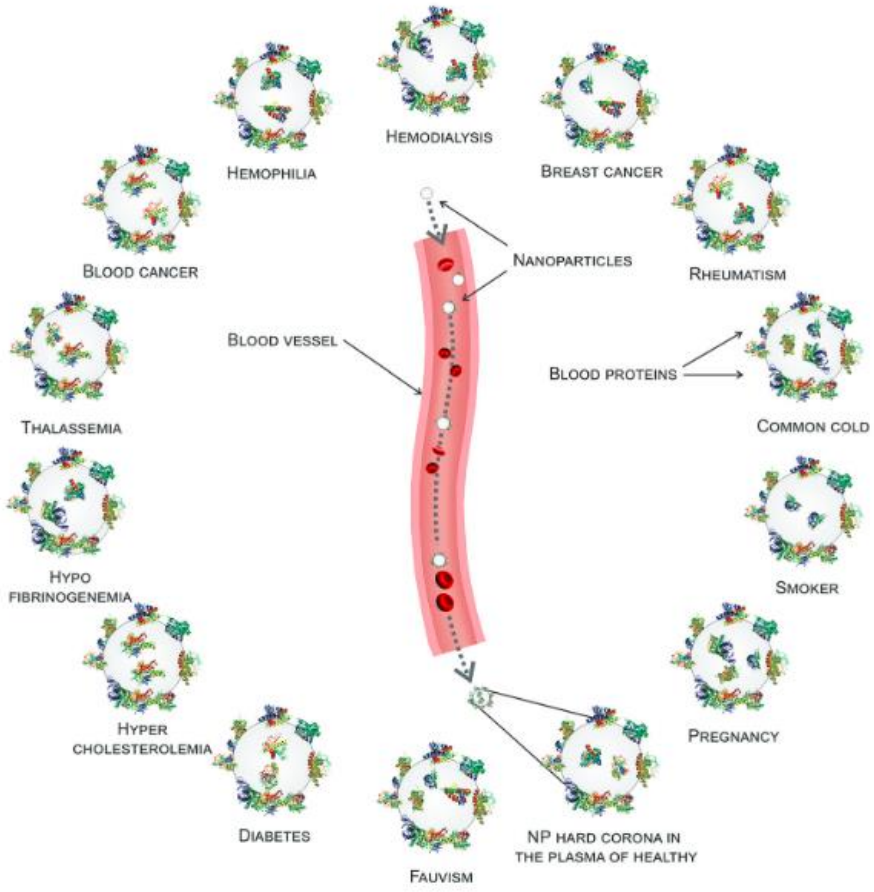

Figure 1. Personalized protein coronas in various disease states.[6]

Understanding the influence of the personalized protein corona is critical for the advancement of nanomedicine. Nanoparticles have many exciting applications in medicine, including targeted drug delivery, radiosensitizers in radon or proton therapy, in bioimaging and biosensing applications, or as fungicides (Klebowski 2018). The disease-dependent biological

factors that influence nanoparticle toxicity must be understood in order to utilize nanoparticles as a powerful tool in medicine.

Our Senior Design project is focused on a single disease that influences the properties of the personalized protein corona: diabetes. Diabetes has many comorbidities, or other diseases that have higher risk of incidence and morbidity in patients with diabetes. One of the most prominent is cardiovascular disease. In 2014, a total of 1.5 million people with diabetes were admitted to the hospital for major cardiovascular diseases.[7] Diabetes is presently a significant health concern in the United States. Over 30 million Americans have diabetes, which is nearly ten percent of the population.[8] 45% of patients with type 2 diabetes are unable to control their disease adequately, leading to excess sugar in the blood. This excess sugar can randomly attach to blood proteins; this process is called glycation of blood proteins. There is currently a large gap in the literature regarding the role of PC glycation in the biological fates of NPs. This area of research has tremendous relevance in the medical field, as it will influence the design of NP drug delivery systems in patients with existing conditions, such as diabetes, that affect the PC. Moreover, patients with diabetes are over 200% more likely to develop diseases like coronary artery disease, myocardial infarction, stroke, congestive heart failure, and peripheral vascular disease than a person without diabetes.[9] Our Senior Design Project aims to explore the impact of glycation of blood proteins on the fate and toxicity of silver nanoparticles. We will investigate the influence of both an engineered property of the nanoparticles, size, as well as the glycation state of the blood protein human serum albumin, on NP-PC characteristics and toxicity in human liver cells.

1.2 Relevant Literature Review

In this section, we will review the literature relevant to the background of our thesis. We will begin by discussing cardiovascular disease, since silver nanoparticles can be embedded in implants which may be needed in patients with cardiovascular disease. We will then discuss diabetes. Diabetes is one of the risk factors for cardiovascular disease; those with diabetes are more likely to require surgical intervention for heart disease compared to the general population. Untreated diabetes leads to excess blood sugar, which can glycate the protein corona, so the disease state of diabetes is immediately relevant to the work in our thesis.

Next, we will discuss the background to nanomaterials and their biological applications, which provides important context to the current and future uses of the nanoparticles investigated in our thesis. We give a broad overview of the protein corona followed by sections about how the protein corona influences NP fate and interactions at the nano-bio interface. These sections are necessary to provide context for how our thesis fits into the literature surrounding protein coronas. Finally, we discuss how diabetes influences the protein corona, which is important to establishing the motivation for our thesis. Relevant papers in the field will be reviewed in Section 1.3.

1.2.1 Cardiovascular Disease

Cardiovascular disease is the number one cause of death both globally and in the United States. The rate of heart disease death per county is shown in Figure 2 below. 85% of the deaths associated with heart disease are due to heart attack or stroke.

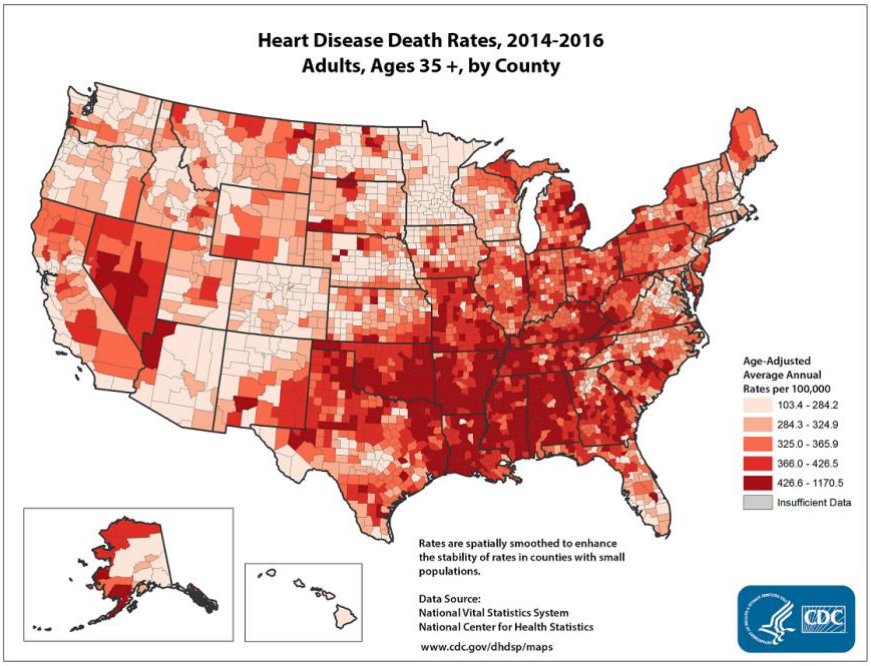

Figure 2. Heart disease death rates in the US.[1]

There are a variety of treatments and interventions for heart disease, including pharmaceuticals, changes in diet, increasing physical activity, quitting smoking, and maintaining a healthy body weight. However, some patients with cardiovascular disease will require a surgical intervention. There are several types of implants which can reduce the danger of heart disease. Valve replacement can be necessary if there is a defective valve in the heart. When heart valves are unable to work properly, there is additional strain on the heart that can lead to heart failure.[10] For patient with atherosclerosis, or a narrowing of the arteries, a stent may be a necessary medical intervention. Coronary arteries become narrowed due to a buildup of plaque in the heart. This can eventually lead to a heart attack. Stents are tiny wire mesh tubes that can be permanently surgically implanted in coronary arteries to prop open the artery and prevent blockage and a heart attack.[11]

There are several medical conditions and lifestyle choices that put people at risk for heart disease. These include diabetes, overweight and obesity, poor diet, physical inactivity and

excessive alcohol use. Diabetes in particular greatly increases the risk of developing cardiovascular disease.

1.2.2 Diabetes

Diabetes is not a single disease, but rather a set of diseases characterized by high blood sugar (hyperglycemia) and insulin insufficiency. Normal fasting blood glucose concentrations are defined as less than 110 mg/dL, whereas patients with diabetes have fasting blood glucose concentrations greater than 125 mg/dL.[12] As of 2017, 30.3 million Americans, or 9.4% of the population, have diabetes, a number that has been rising steadily (see Figure 3). Of those 30.3 million Americans with diabetes, 90 to 95% have type 2 diabetes, or roughly 27 to 29 million Americans.[7] Of those with type 2 diabetes, at least 45% fail to achieve adequate glycemic control, indicating that they chronically have high blood sugar levels, or excess glucose in their blood.[13] In 2014, 108,000 Americans with diabetes had lower limb amputations, or about 0.5% of all Americans with diabetes.[7]

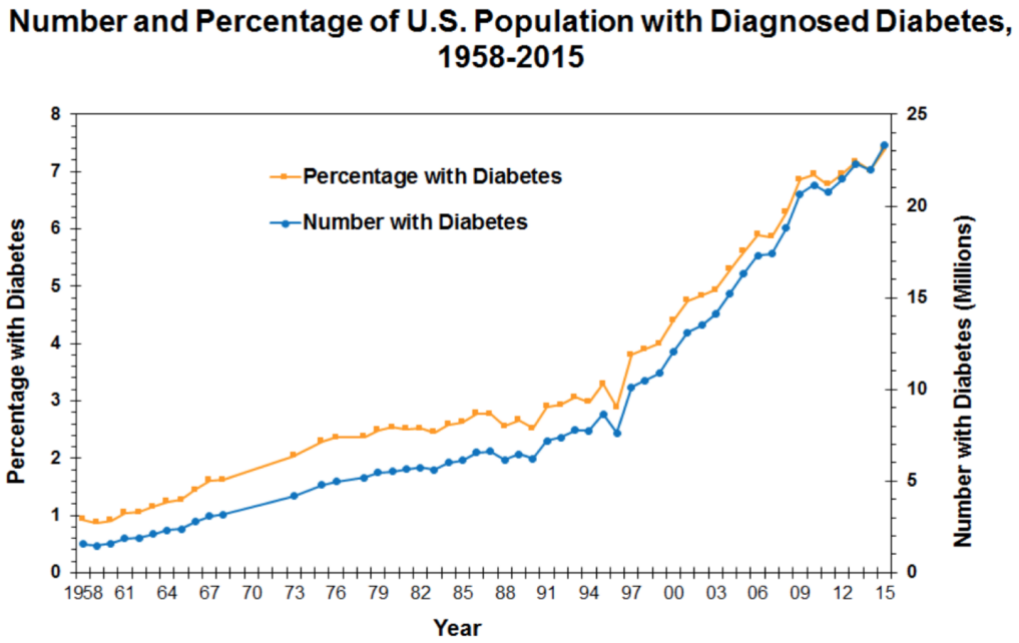

Figure 3. Prevalence of diagnosed diabetes in the US.[8]

Diabetes also has many associated comorbidities. Macrovascular complications include cardiovascular disease, which manifests in coronary artery disease, myocardial infarction, stroke, congestive heart failure, and peripheral vascular disease. Those with diabetes are two to four times as likely to develop cardiovascular disease as the average person without diabetes.[9] Because nanoparticles show promise in treating these comorbidities of diabetes, improved characterization of nanoparticle interactions in a diabetic patient's body could greatly improve treatment.

1.2.3 Introduction to Nanomaterials and their Biomedical Applications

Nanomaterials, including nanoparticles, are engineered materials with a diameter between 1-100 nanometers, or around 1-100 billionths of a meter. They possess unique size-dependent properties that differentiate nanomaterials from the bulk scale, including high surface area to volume ratio, unique optical properties, chemical reactivity, and tunability of properties.[14] These unique properties of nanomaterials make them applicable in a variety of fields, including optical, electrical, and biomedical fields. They are especially relevant in medical applications because the nanoscale is the scale at which biology occurs. Nanomaterial use in the biomedical field focuses primarily on development in two areas: medical devices and drug delivery. For medical devices, nanomaterials can improve the functioning of the device by improving sensitivity of a device by increasing the surface area to volume ratio of the sensor, or giving it antimicrobial properties. In the field of drug delivery, the materials can encapsulate drugs and carry payloads to targeted areas in order to effectively treat diseases.

Engineered properties of nanoparticles can be adjusted in order to make them appropriate for a variety of applications, as outlined in Figure 4 below.[15] Depending on the desired application and chemistry, particles can be synthesized with varied composition and surface

chemistries. The core of particles can be made of organic, inorganic, biochemical, and metallic materials.

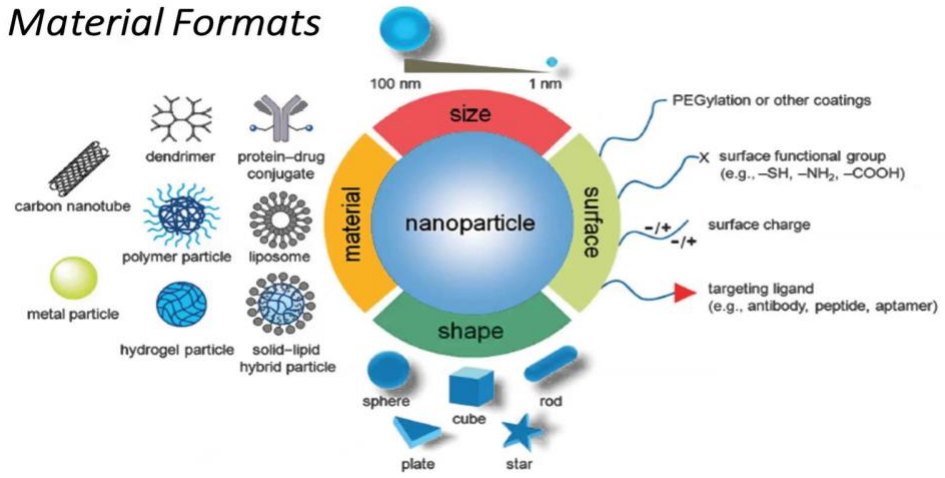

Figure 4. Overview of nanoparticle engineered properties. Nanoparticle size, shape, material and surface coating can be adjusted in order to meet desired function, making them exciting for application in drug delivery in a variety of disease states and contexts.[16]

This core can then be surrounded by various surface coatings, such as citrate, polyethylene glycol (PEG), branched polyethylenimine (bPEI), or polyvinylpyrrolidone (PVP) (Figure 5).

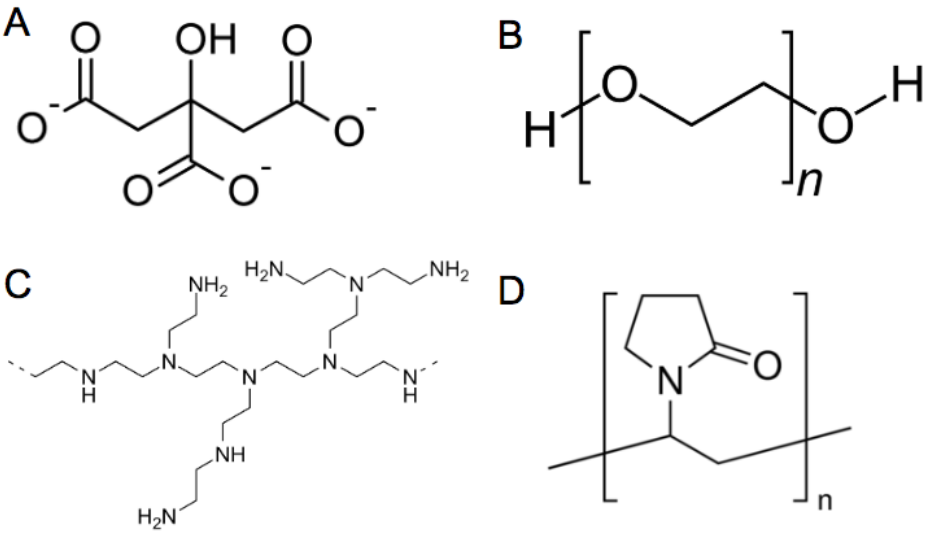

Figure 5. Structures of common nanoparticle coatings. A. Citrate. B. Polyethylene glycol (PEG). C. Branched polyethylenimine (bPEI). D. Polyvinylpyrrolidone (PVP).[17–20]

With the right combination of core and surface chemistry, scientists and engineers are able to create nanomaterials with desired properties for a particular application. For example, a silver core could be used for the antimicrobial properties of silver. Silver nanoparticles coated with citrate have a negative surface charge and are less toxic than particles with other surface coatings, such as bPEI. Thus, if a scientist was trying to create nanoparticles with antimicrobial properties that was non-toxic to humans, they might choose a silver material and a citrate coating.

Silver nanoparticles (AgNPs) have well-documented antimicrobial properties. In fact, Hippocrates used elemental silver in order to treat ulcers thousands of years ago. Their unique antimicrobial properties have led to AgNPs being widely used in commercial products. AgNPs have been incorporated into clothing like exercise gear and socks in order to prevent bad smells, as well as toothbrushes, food storage containers, and humidifiers. Because of their anti-inflammation and wound-healing properties, AgNPs could also one day be incorporated into medical devices. It is generally accepted that AgNPs release silver ions which interact with three major areas of a bacterial cell in order to kill it: the peptidoglycan cell wall and cell membrane, the bacterial DNA, and proteins within the bacteria including enzymes incorporated in vital metabolic processes such as the electron transport chain.[3]

AgNPs can be used in cardiovascular implants, such as heart valves and stents, as well as venous catheters.[3] Coating the implanted devices with AgNPs can lead to antibacterial and anti-inflammatory effects, which is significant in surgery where rejection of an implant is a major concern. Several silver-coated (not silver NP) implants underwent clinical trials in the late 1990s, but the silver species led to inhibition of fibroblasts, and use of silver was discontinued in cardiovascular implants. However, AgNPs have a large surface area to volume ratio and produce

antimicrobial effects with lower concentrations of silver ion due to the controlled release of the silver ion. AgNPs are promising for use as an antimicrobial and anti-inflammatory agent in implanted devices that will likely have a less toxic response in human cells compared to cationic silver. Silver nanoparticles can also slow the growth of biofilms on shorter-term devices used in surgery and recovery, such as venous catheters. Studies show that this application is non-toxic and clinical trials are underway for applications of this technology in hospitals.[3] This potential future application of AgNPs in cardiovascular implants and surgical implants is relevant in our study because many patients with diabetes have heart disease and may require surgical intervention for their illness.

Silver nanoparticles also have potential to be used in cancer treatment. AgNPs can scatter and absorb light, which can be useful in both the imaging and killing of tumors. Absorbed light can lead to thermal killing of cancer cells, and scattered light can assist in imaging cancer cells, which can be useful for the diagnosis of the disease. Additionally, AgNPs promote the agglomeration of reactive oxygen species (ROS) which induces an inflammatory response which may lead to apoptosis of the cancer cells.[21] Cancer is also a comorbidity of diabetes, as discussed in Section 1.2.1.

1.2.4 Protein Corona Overview

Despite rapid advances in nanoparticle technology in the past few years, very few nanoparticles have successfully made it to clinical trials or to into clinical use. This is because the nanoparticle undergoes significant changes when it is introduced to a biological system, which may cause the particle to lose the identity it had in a laboratory setting. The most significant change that alters the identity of the nanoparticle upon entry into a biological system is called the protein corona.[22]

When a nanoparticle is introduced to a biological system, a layer of proteins and other biomolecules adsorb onto the nanoparticle surface. This layer is called the protein corona (PC). The PC alters the nanoparticle's physicochemical characteristics, functionality, biodistribution, and toxicity.[4] According to Ke et al., the PC has several effects on the nanoparticle:

1. The PC may increase nanoparticle solubility and therefore uptake in an aqueous environment.
2. The proteins surrounding the nanoparticle may misfold and agglomerate, and these changes may bring about an immune response from the biological host to remove the nanoparticle.
3. The protein corona may hinder, or mask altogether, the biological or chemical properties and functionalities that the nanoparticle was engineered to possess.[5]

These three factors indicate the significant and varied impact of the protein corona on nanoparticles. Nanoparticles that were engineered to have certain properties *in vitro* could have entirely different properties *in vivo*. Shannahan states that an understanding of the protein corona is imperative for the safe and effective use of nanoparticles in biomedical applications.[4]

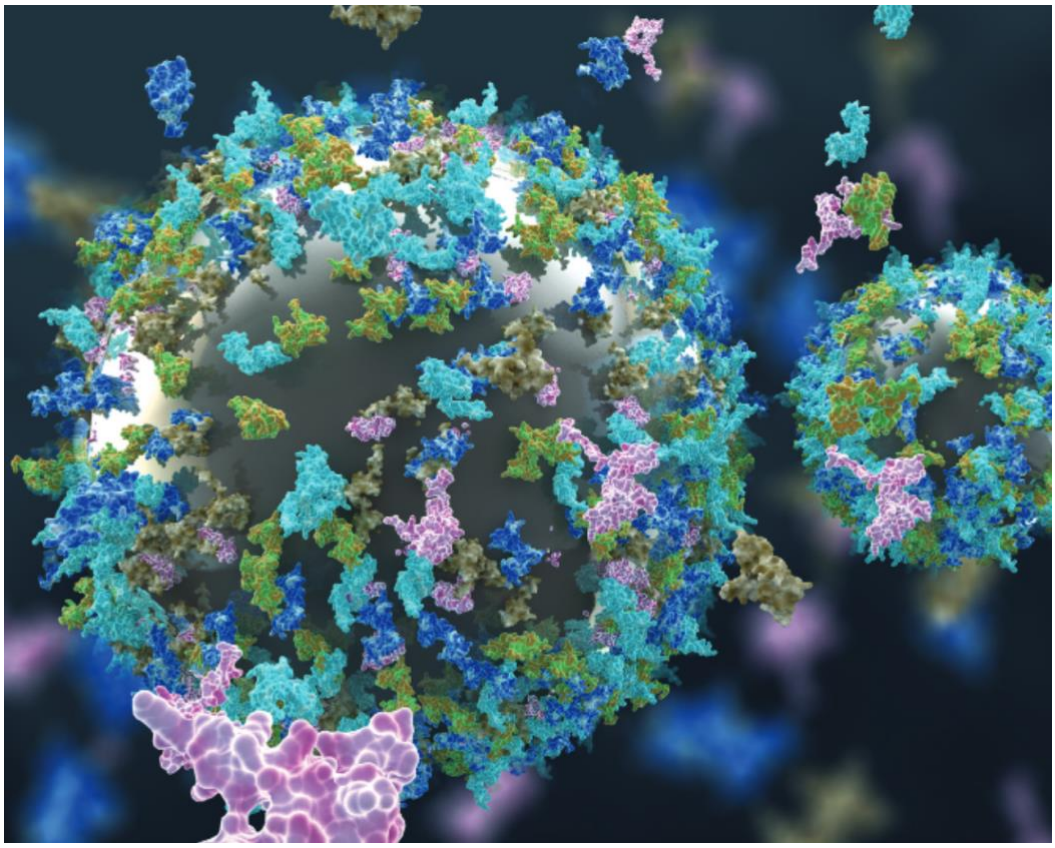

Figure 6. Artistic rendition of the protein corona on a nanoparticle (from Ella Maru Studios). Although the relative dimensions of proteins and nanoparticles are a bit misleading in this image (both are typically similar in size), the image nicely portrays the diversity of proteins that coat the surface of nanomaterials.

Recently, focus has turned to the "personalized" protein corona, the idea that the protein corona varies depending on characteristics of the biological host, such as gender, habits, age, and disease states.[23] Plasma taken from humans with different diseases, such as hemophilia, hypercholesterolemia, diabetes, and cancer, led to different protein compositions in the protein corona. This study also showed that pregnancy or habits like smoking could lead to similar changes in the protein corona composition.[6] This realization that the protein corona varies greatly between patients necessitates a vast field of research to draw out the subtleties of nanoparticle-protein interactions.

1.2.5 Impacts of Protein Corona on Nanoparticle Fate

The protein corona significantly alters the fate of the nanoparticle in the biological system. Nanoparticles have been shown to dissolve in biological solutions. The dissolution of silver ions from silver nanoparticles is known to be related to the antimicrobial properties of silver nanoparticles.[4] Nanoparticles also undergo significant changes in size and surface charge upon binding of the protein corona.[23] One study looking at the interactions of human serum albumin (HSA) with citrate-coated gold nanoparticles (AuNPs) found that the presence of HSA caused AuNPs to agglomerate significantly, increasing the observed diameter of the AuNPs up to 15 times their original size.[24] Another study looking at HSA and citrate-coated AuNPs also found that the surface charge of the AuNPs became less negative upon addition of HSA.[25]

These changes are extremely important to how the nanoparticle behaves in the body. Cells do not "see" the nanoparticle itself but rather interact with the entire nanoparticle-protein corona complex. Thus, instead of only the properties carefully given to the nanoparticles by scientists by way of different sizes, core materials, and surface chemistries, the protein coating on the nanoparticles is a key factor of how the body responds to nanoparticles, as will be discussed below.

1.2.6 The Protein Corona and the Nano-bio Interface

The protein corona has a significant impact on the nano-bio interface, that is, the interaction of nanoparticle with cells in a biological system. Unexpected changes can occur at the nano-bio interface as a result of changes to nanoparticle fate, as discussed in Section 1.2.4. It is critical to understand the potential influence of the protein corona on the way that nanoparticles interact with cells in order to safely develop nanoparticle-based technologies for human health.

The PC can influence cellular uptake of the NPs which in turn affects biodistribution of the NPs, as well as immune response and possible toxicity of the particles in the body.

The protein corona can affect cellular uptake, which in turn affects biodistribution of nanoparticles in the body and can diminish the ability to target nanoparticles to an area of interest, such as a tumor. Changes in the protein corona can significantly alter the uptake pathway of nanoparticles into cells.[26] Additionally, changes in the protein corona can change the cells to which the nanoparticles are targeted. When particles are actively targeted to a certain site, it is possible to engineer them with specific surface coatings and ligands that target them to a specific cell type. When the protein corona develops around the particle, it may block the the targeting ligand, thus reducing the targeting abilities of the particle and potentially reducing its effectiveness as a drug delivery method.[27]

The formation of the protein corona can also lead to an immune response towards the particle, rendering them toxic to humans. The presence of the PC can induce an immune response by making the particles recognizable to macrophages and other immune cells. This can lead to inflammation and toxicity. Additionally, the proteins can undergo conformational changes when they adsorb onto the particle, which can also induce an immune response.[27]

1.2.7 Diabetes and the Protein Corona

As discussed in Section 1.2.4, the protein corona can be "personalized" based on the age, sex, habits, and disease state of the individual. Two factors can contribute to this personalization: the population of proteins present in the protein corona and the post-translational modifications performed on the proteins. Glycation is the post-translational modification which is relevant in our study. Glycation is the non-enzymatic addition of sugar (usually glucose) molecules to a

protein. Glycosylation, on the other hand, is the enzyme-mediated modification of a protein with a sugar molecule.[28]

In patients with diabetes, it is not likely that glycosylation would be increased relative to a healthy individual because glycosylation is enzyme-mediated. However, because glycation is non-enzymatic, it is more random and therefore more dependent on the concentration of glucose in the solution surrounding the protein.

Specific to human serum albumin (HSA), it is known that glycation can occur at the *N*-terminus, as well as at the 59 lysine residues of the protein (see Figure 7 below).[29]

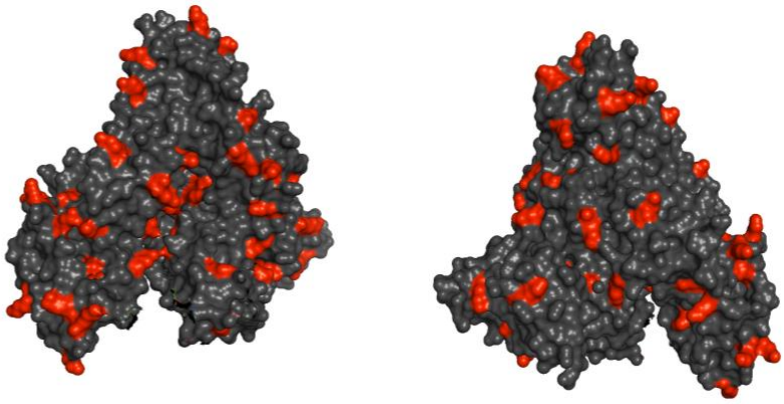

Figure 7. Human serum albumin structure, viewed from two angles. Lysine residues (possible glycation sites) are highlighted in red.

The mechanism of glycation of HSA involves the reversible formation of a Schiff base between a nitrogen on the protein (as found in lysine residues) and a glucose molecule. This Schiff base can then form a more stable Amadori product, or a ketoamine.[29] The mechanism of this formation is shown below in Figure 8.

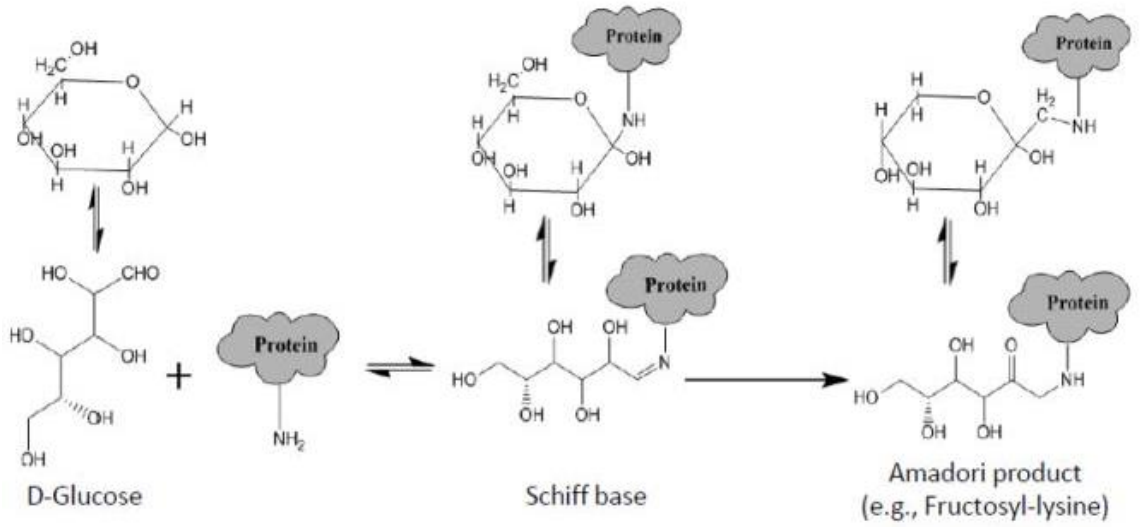

Figure 8. Mechanism of formation of the glycated protein. The first reaction to form the Schiff base is fairly reversible, while the Amadori product is more stable and thus less easily reversed.[29]

Because glycation is non-enzymatic and therefore fairly dependent on glucose concentration, when a patient has consistently elevated blood glucose levels (as in untreated diabetes), we would expect a higher percentage of proteins in their blood to be glycated. Over 45% of patients with type 2 diabetes fail to achieve adequate glycemic control, and this indeed causes their blood proteins to be glycated at a higher rate than those of a healthy individual.[13] The current literature indicates that patients with diabetes have 2 to 5-fold greater concentrations of glycated HSA in their blood than a healthy person.[29] This would lead to a protein corona that has a much higher amount of glycated HSA compared to the protein corona of a healthy patient. The impact of this modification of the protein corona is largely unknown.

Worldwide, 382 million individuals have type 2 diabetes, a number that is expected to rise to 417 million by 2035.[13] Considering the high prevalence of this disease and its comorbidities, such as cardiovascular disease, cancer, and possible need for eventual amputation, as well as the vast changes in nanoparticle fate and toxicity caused by changes in factors such as disease state, a sound understanding of the effect of excess blood glucose on the nanoparticle-

protein corona complex is vital to the effective administration of nanoparticle-based drug delivery as well as the safe use of silver nanoparticles for antimicrobial applications.

1.3 Current Research

Very little research has been done about the impacts of glycation of the protein corona. More work in this area is necessary, given both the prevalence of the diabetes disease state as well as the prevalence of glycosylation as a post-translational protein modification in healthy people as well. Only two papers have been published that address the impacts of a glycated protein corona on nanoparticles. We intend to help begin to fill this large gap in the literature with our work.

In the paper "The 'Sweet' Side of the Protein Corona: Effects of Glycosylation on Nanoparticle-Cell Interactions" by Wan et al., the authors studied the effects of deglycosylation of the protein corona after the corona had already formed. Silica nanoparticles were incubated with human plasma to form a protein corona. This corona was then deglycosylated by an enzyme. These deglycosylated nanoparticle-protein complexes were found to have decreased colloidal stability and increased uptake by two types of macrophages compared to their glycosylated counterparts. The deglycosylated forms also had increased pro-inflammatory properties. The authors concluded that further studies were needed to determine the role of glycans in nanoparticle-protein interactions.[30]

In the paper "Disease-related metabolites affect protein-nanoparticle interactions" by Tavakol et al., proof-of-concept simulations were performed in order to test the impact of various glucose concentrations in the bloodstream on the protein corona. They tested the impact of three concentrations of glucose: no glucose, healthy levels of blood glucose, and diabetic levels of

blood glucose. It was found that there is increased fibrinogen binding to the spherical particles due to bridging as the number of surface glucose molecules increases with glucose concentration. Excessive glucose on the particle surface can also lead to fibrinogen structural changes, which may lead to attack by red blood cells, thus negatively affecting potential therapeutic benefits of the particles that result from long circulation time. The concentration of glucose was also shown to change the Vroman effect. The Vroman effect states that proteins that have high concentrations but low binding affinities to the particle will be replaced by proteins which have lower concentrations but higher binding affinities over time. The simulation found that with high glucose concentrations, the Vroman effect was accelerated. This indicates that glucose levels have have significant impacts on the final biological identity of the particle.[31]

These papers leave many important questions about the impacts of the glycated protein corona in human cells. The paper by Wan et al. included some cell studies and inflammatory responses to glycosylation (enzyme-mediated addition of sugar molecules to a protein) of the protein corona. However, the paper did not consider the impacts of high levels of glycation that may be found in diabetes.[30] The paper by Tavakol et al. did consider high levels of blood glucose, but still has significant limitations. The study was merely a proof-of-concept simulation, and no wet lab work was performed. Additionally, only corona-nanoparticle interactions were explored, rather than nanoparticle-cell interactions.[31] More work needs to be done in order to close this gap and consider the impact of high sugar conditions on both particles and the nano-bio interface. There are additionally no papers that explore the impact of the protein corona on both the biological fate of the particles as well as the cytotoxicity of the particles. Our approach will provide insight into the downstream effects of the glycation of the protein corona.

1.4 Project Goals and Experimental Design

We identified the following knowledge gap in the protein corona literature: How do changes in blood serum proteins, as affected by disease state, impact nanoparticle-cell interactions? We know that the protein corona is "personalized," meaning that it can vary from person to person based on the individual's age, sex, habits, or disease state. These changes can manifest in two ways. First, there could be a different population of proteins present in the protein corona. Second, there could be different post-translational modifications on those proteins. We identified a gap in the literature regarding how glycation, a post-translational modification, impacts nanoparticle-protein corona interactions and thus, the fate and toxicity of nanoparticles. It is from this knowledge gap that our project goal was defined: *We intend to understand how the biophysical interactions of glycated and non-glycated proteins with silver nanoparticles influence human cell response.*

In order to fill this knowledge gap, we explored the relative influence of both engineered and biological properties of the protein corona. The experiments were designed based on our project goal. There are two major arms of our experiments in order to address the two major aims of the project: biochemical and cell culture experiments. Biochemistry experiments which elucidated the biophysical interactions of glycated and non-glycated proteins. These experiments provided context and insight into the results found in the second arm of experiments. Cell culture experiments using HepG2 cells determined the impact of the protein corona on cell toxicity. This is a novel approach because it includes both cell-based experiments as well as extensive particle characterization.

1.4.1 Design Rationale

Silver Nanoparticles

Silver nanoparticles were chosen due to their relevance in diabetes comorbidities, their pervasiveness in the literature, and because they make an excellent model system for metallic nanomaterials. Silver nanoparticles show extreme promise for medical applications, and are widely studied for their potential value to the development of nanomedicine. Due to their antimicrobial properties, silver nanoparticles are promising for incorporation into medical devices to treat cardiovascular disease, such as stents or heart valves. Patients with diabetes are at particular risk for heart disease. Diabetes is commonly poorly controlled leading to excess glucose in the blood and glycated blood proteins. Therefore, studying silver nanoparticles with glycated proteins as a post-translational modification is relevant for study.

Silver nanoparticles are often studied in the literature, and there have been many studies about their behavior in biological systems.[32,33] Thus, we have a strong foundation of knowledge to build upon as we design our experiments. Furthermore, there is plenty of context for the behavior of silver nanoparticles in biological systems, so we can focus solely on glycation.

Silver nanoparticles have been shown to oxidize and dissolve in biological media, which contributes to their toxicity.[34] This quality also makes silver nanoparticles a relevant model system for other metallic nanoparticles, including copper, zinc, and iron, which have also been shown to oxidize and dissolve.[35–37]

Furthermore, both of us have extensive experience using silver nanoparticles, since they have been the focus of our research during our undergraduate careers. There are many already established protocols and best-practices for using the particles in the Wheeler Lab. The relevance of silver nanoparticles to our lab also enables us to build on established foundations of research

and support progress in the field. Thus, the particles are extremely relevant for the purpose of our study.

Human Serum Albumin

Human serum albumin is by far the most abundant blood protein and therefore makes up the majority of the protein corona in human systems.[38] We chose to work with only one protein to represent the protein corona in order to minimize the number of variables. Additionally, by using HSA we avoid biohazards associated with using human blood or human plasma. HSA was also an appealing choice because it is relatively inexpensive and can be purchased both glycated and non-glycated, minimizing the processing required prior to performing experiments.

HSA is also a suitable protein for our model because it is relatively well studied with regard to glycation. As stated in Section 1.2.7, it is known that glycation of HSA can occur at the *N*-terminus and any of the 59 lysine residues. This glycation occurs through the formation of a Schiff base, which can then form an Amadori product.[29]

It is known that patients with diabetes have 2 to 5-fold greater concentration of glycated HSA in their blood than a healthy person.[29] Understanding how glycation of HSA impacts the protein corona is therefore a solid foundation to understanding how glycation affects the protein corona more generally.

Liver hepatocellular carcinoma cells (HepG2)

Liver hepatocellular carcinoma cells (HepG2) are relevant for this study. They are widely used in nanoparticle toxicity studies, because the liver is relevant in nanoparticle toxicity. Nanoparticles have been found to accumulate in the liver, so the liver will experience higher concentrations of nanoparticles than other cell types, and therefore will be at greater risk of toxic effects of nanoparticle accumulation.[39]

1.5 Significance

As diabetes prevalence increases, the importance of considering the influence of glycation on the protein corona will also increase. Since diabetes is associated with higher rates of comorbidities, it is essential to contribute to research in this field. This work is also relevant because glycation is the most common post-translational modification.[31] Understanding the influence of glycation on the protein corona will be crucial in the development of nanomedicine for all patients.

1.6 Team and Management

The success of this research project relied on the collaborative and motivated nature of our team and the support of cross-disciplinary advisors. Collaboration between the Biochemistry and Bioengineering departments were essential in the design and execution of all elements of the project.

Dr. Prashanth Asuri served as our advisor through the Bioengineering Department at Santa Clara University. Dr. Korin Wheeler advised us on the biochemistry experiments. We met with both regularly to ensure that deadlines were met and project goals were being achieved. Support from graduate students in the Asuri Lab ensured that any issues with cell culture experiments were dealt with promptly. Bi-weekly meetings with Dr. Asuri were held to make certain that project process was being made and all thesis deadlines were met.

1.6.1 Budget

Funding for our project was provided by the Santa Clara University School of Engineering and the NIH.

1.6.2 Timeline

This senior thesis project was completed through the 2018-2019 academic year. A high-level Gantt Chart outlining the progress of the experiment is in the figure below (Figure 9). During fall quarter we completed all necessary safety training in order to take part in functional laboratory protocols for both the biochemistry and the bioengineering labs. By the end of the fall quarter we also studied and reviewed a significant portion of the applicable academic literature on our thesis topic and began training for lab-specific procedures, including cell culture. We additionally began performing some of the initial biochemistry experiments.

During winter quarter we primarily focused on the cell culture experiments, which required an unexpected amount of troubleshooting due to issues with cell growth. Once we identified and corrected the issue, we were able to complete most of the assays; we finished the rest in the spring. We also collected some initial biochemistry data with the help of other students in the Wheeler Lab. We also completed a rough draft of the thesis, without including results yet.

Throughout spring quarter we focused on wrapping up experiments, preparing for the senior design conference presentation, and editing the thesis for submission. We had a few biochemistry experiments to finish up. We also finished a few of the cell culture assays which we did not have time to complete in the winter. At the beginning of the quarter, we compiled our slides for the presentation, and practiced them repeatedly through the first half of the quarter leading up to the Senior Design Conference.

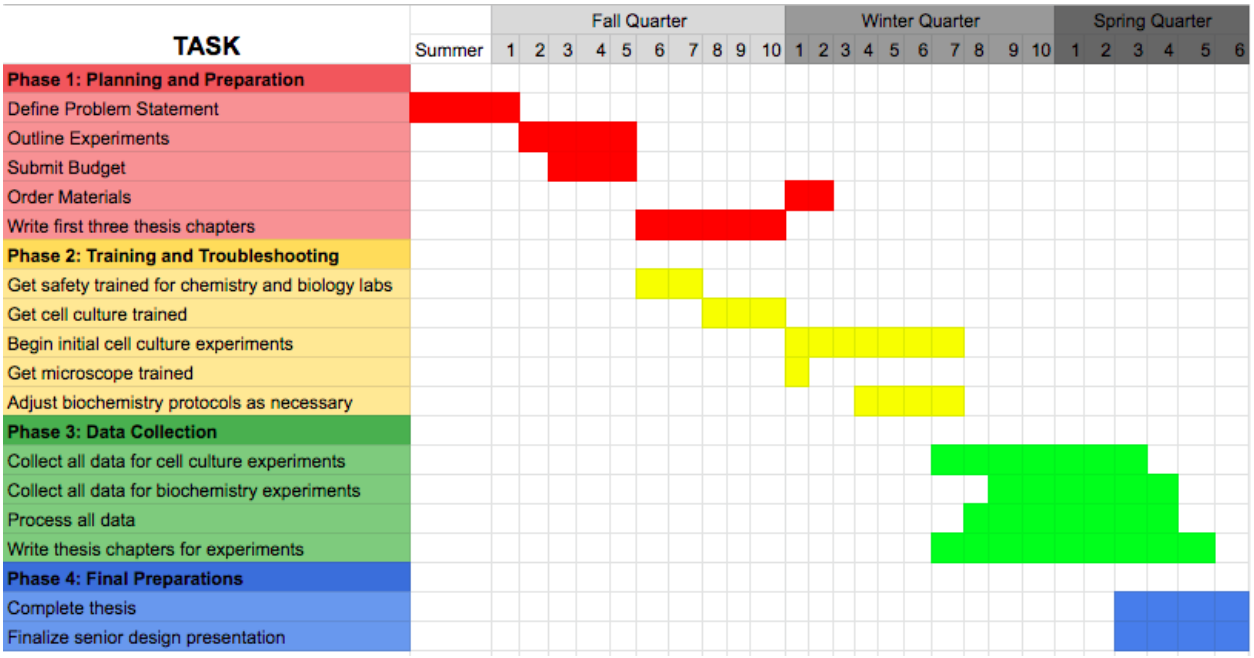

Figure 9. Gantt Chart showing the schedule of the Senior Design Project.

2. System Level Experimental Design

2.1 System Level Overview

The general approach for our project includes three subsystems, as described in the figure below. The first subsystem includes the system changes that we intend to make in order to study the effects of biological properties, which are dependent on the person, and nanoparticle properties, which can be controlled by scientists and engineers. These changes are the foundation for the project, and are the effects of these properties are explored in the other two subsystems. The second subsystems includes the biophysical experiments that were performed to characterize changes that occur to the nanoparticles and proteins when they interact with each other. The third subsystem includes cytotoxicity studies that were performed to evaluate how the nanoparticle-protein corona complexes interact with cells.

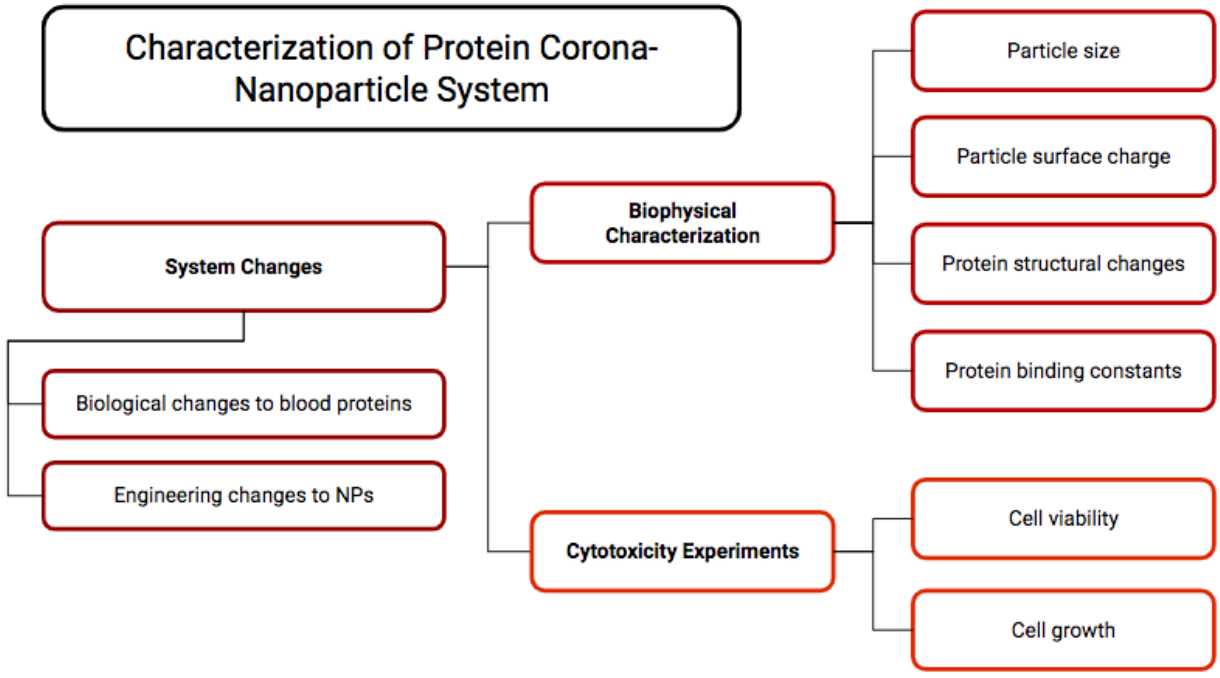

Figure 10. System overview.

Although the subsystems seem distinct, they are immediately relevant in biological systems. The two systems mimic the way that a nanoparticle would move through the human body if included in a medical device, summarized by Figure 11 below. The second subsystem (Biophysical Characterization) is concerned with the way that nanoparticles and blood proteins interact in the bloodstream, and how those interactions change the properties of NPs and the PC. The third subsystem, Cytotoxicity Experiments, concerns how the nanoparticle-protein corona complex interacts with cells upon traveling through the bloodstream.

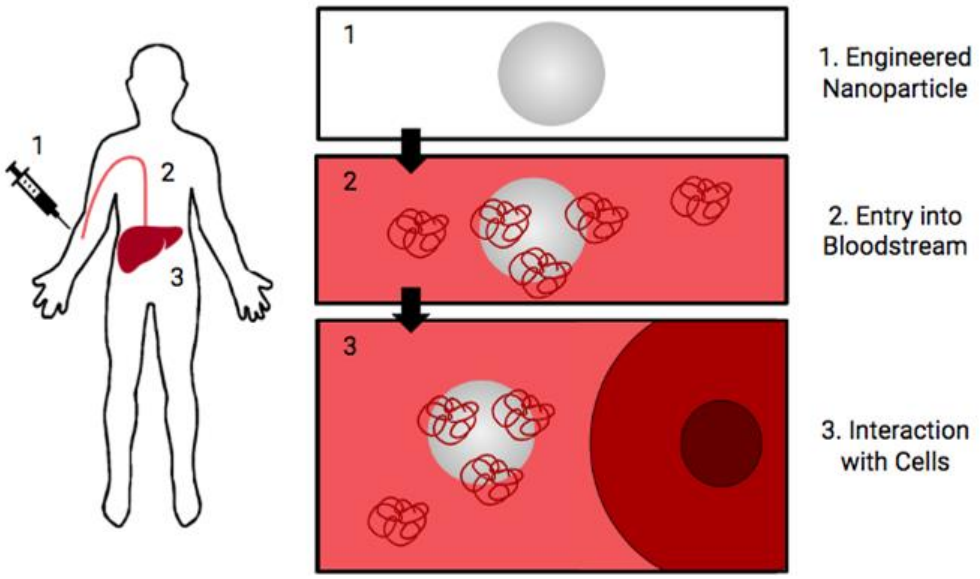

Figure 11. Design relevance in biological systems. Gray circle represents nanoparticle. Red clouds represent blood proteins. Large red circle with dark center represents human cells.

2.1.1 System Changes

The first branch of our experimental design involves altering the properties of the proteins and nanoparticles to determine the relative impact of each change on the nanoparticle-protein corona complex. For all experiments, we adhered to the following matrix (Figure 12) to determine the effects of both the engineered properties given to nanoparticles in the lab or the

innate biological characteristics of blood proteins, unique to every patient, that cannot be changed.

		Engineered properties	
		40 nm particles	80 nm particles
Biological properties	Glycated HSA		
	Unmodified HSA		

Figure 12. Experimental matrix.

For biological properties, we compared the impacts of a protein corona made of unmodified human serum albumin (HSA) or glycated HSA, which has sugar molecules randomly bound to the proteins, in order to study the effect that excess sugar in the blood has on the NP-PC complex. Albumin is by far the most abundant protein in the blood, constituting about half of blood serum. In the presence of glucose, proteins become glycated, meaning that sugar molecules randomly bind to the proteins. People with untreated or poorly controlled diabetes have consistently elevated blood glucose levels, causing their serum proteins to be glycated at a higher rate than people without diabetes. This is the basis for the hemoglobin A1c (HbA1c) test, a measure of a person's average blood glucose for 2 to 3 months, performed by measuring the percentage of hemoglobin molecules that have been glycated.[40] This glycation of blood proteins could affect how those proteins interact with nanoparticles. Thus, glycated HSA represents proteins that might be found in the bloodstream of a patient with diabetes, whereas unmodified HSA represents proteins that are likely to be found in the bloodstream of a healthy individual.

We hoped to elucidate the potential differences in NP-PC interactions and cytotoxicity by studying the impact of both glycated and unmodified HSA.

For the engineered properties of nanoparticles, we had a few options to study. After deciding that we would study silver nanoparticles, we could vary the nanoparticle size or surface chemistry. We chose to vary size because the unique properties of nanoparticles are derived from their size. For a baseline nanoparticle, we chose 40 nm citrate-coated silver nanospheres. For comparison, we also studied 80 nm citrate-coated silver nanospheres to observe the effect of size on nanoparticle fate and cellular toxicity.

2.1.2 Biophysical Characterization

In order to gain a baseline understanding of how citrate-coated silver nanoparticles and HSA interact with each other, we performed biophysical characterization experiments before moving into cell culture and cytotoxicity studies. As discussed in Section 1.2.4, nanoparticles and proteins undergo various changes when in contact with one another. Before looking at protein corona affected cell responses to nanoparticles, we wanted to understand how nanoparticles and proteins interact with one another. An understanding of how the size and surface charge of the nanoparticles change upon addition of proteins is key to understanding the basic patterns of their binding. Additionally, proteins can denature in the presence of nanoparticles, so we wanted to see how nanoparticles affect the folding or misfolding of the proteins. To gain insight into the kinetics of the particle-protein interactions, we also wanted to study the binding of proteins to the nanoparticles. By studying these nanoparticle-protein interactions, we hoped to gain insight into how changes in both the nanoparticle and the protein corona would impact cytotoxicity. For more details about these experiments, please refer to Chapter 4.

2.1.3 Cytotoxicity Studies

Cytotoxicity studies were performed to evaluate how changes in protein glycation and nanoparticle size would affect the toxicity of the nanoparticles to human liver cells. HepG2 cells, a commonly used cell line for looking at nanoparticle toxicity, were used in these studies. HepG2 is an immortalized cell line were derived from the human liver cancer. The cells therefore have good applicability in nanoparticle toxicity, as nanoparticles in the bloodstream will accumulate in the liver, thus exposing the cell type to higher concentrations of particle.

We plan to explore how the addition of nanoparticles with varied coronas influence the survival of HepG2 cells. To do this, we will perform an assay which quantifies cell viability, or the number of cells that are alive in a plate after exposure to nanoparticles. For more details about this experiment, please refer to Chapter 5.

2.2 System Level Constraints

There were several constraints that needed to be taken into account when designing, planning, and executing experiments. The constraints reflected relevant engineering constraints to be considered in any research project or product development process. These constraints required us to be flexible in our experimental approaches in order to accomplish the goals of the project within the required time frame for the Senior Design Project.

Cell Culture

The requirements of the cells were a major constraint when executing all cell culture experiments. HepG2 cells were chosen for the cell toxicity experiments because they are sensitive, and therefore would show changes in viability with the addition of nanoparticles.

Given their sensitivity, it was important to handle the cells with care when passaging and performing assays. We were careful when plating the cells and adding reagents to assays to avoid shearing cells or detaching them from the plate.

Time was a major constraint for all experiments because the Senior Design timeline requires that the project is completed in six months. Assays took about one week to complete, so we were mindful about reducing the total number of assays needed in order to optimize the time spent in lab. We also tried to be flexible in our scheduling of the time in lab, because we were constrained by the cell growth. HepG2 cells reached confluency after seven to ten days in the incubator, and needed to be at confluency before we were able to conduct the assays. Careful observation of the cells and planning were necessary in order to complete the cell culture experiments.

Biochemistry Experiments

Flexibility in timing was not as necessary for the biochemistry experiments because the materials were always ready to be used for experiments whenever lab partners had availability to be in lab. However, time was still a constraint for biochemistry experiments, because there were many experiments that needed to be performed. Four different characterization experiments needed to be performed in triplicate, on four different nanoparticle-protein combinations, plus control conditions for each experiment. Ultimately, there were over 100 individual experiments to be performed. We were very fortunate to have help completing the biochemistry experiments from non-Senior Design research students who were members of the Wheeler Lab. This enabled us to fully characterize the biochemical effects of a glycated protein corona on the engineered nanoparticles.

Nanoparticle cost

Money was also an important constraint in the design of both cell culture and biochemistry experiments. Silver nanoparticles are very costly, costing about $200 for a milliliter of particles. The particles, therefore, made up a large portion of the budget. Due to the high cost, we used the particles sparingly whenever possible. We ran several practice assays with acrylamide to get practice with the protocols before starting on nanoparticle assays. The extra time required to practice was worthwhile because it reduced the costs of the assays. We took care to minimize the number of assays needed, and were cautious to perform every step correctly to eliminate the need for further runs. Additionally, we used the lowest particle concentrations possible while still observing toxic effects.

2.3 Experimental Approach

We are one of the only Bioengineering Senior Design groups performing interdisciplinary research in two major areas: cell culture and biochemistry. This Senior Design Project is notable because it fills a gap in the current literature regarding the protein corona. Very little research has been published regarding the impact of post-translational modifications on nanoparticle-protein corona interactions, including glycation. We hoped to bridge this gap in understanding with this senior design project, and we believe that we have established a solid foundation upon which other researchers can work.

The experiments performed were based upon past experiments performed in Dr. Wheeler's biochemistry lab and Dr. Asuri's cell culture lab. Protocols for biophysical characterization experiments were obtained from Dr. Wheeler's lab work. Protocols for cell

culture experiments were obtained from Dr. Asuri's lab work. The protocols can be found in the appendix. Both sets of experiments were modified based on the current literature.

Furthermore, the work builds upon previous experiments performed in the Wheeler and Asuri Labs. The basis for our project was a previous collaboration between these two labs which looked at the protein corona formed by HSA on differently coated silver nanoparticles, both from the biophysical characterization and the cell culture approaches. This work served as a control against which we could compare our results for non-glycated HSA experiments. Our work then built upon this previous work by adding the same experiments with protein coronas formed from glycated HSA.

Our research represents the first steps toward assessing glycation effects on protein corona interaction with NPs and the resulting cell response. It provides the foundation for broader exploration of the impact of post-translational modifications on the protein corona. Our long-term goals are to contribute to the growing field of nanomedicine, and inform future research into the safety of nanoparticles used in the human body.

2.4 Engineering Standards and Realistic Constraints

The field of nanoparticles is in its nascent stages. As such, this research contributes to the first steps toward effective nanoparticle implementation for applications such as targeted drug delivery and biosensing. Our aim is to elucidate how a diabetes disease state may affect nanoparticle fate and toxicity in relevant applications. Later work may unlock the promising technology of nanomedicine and lead to better health. Thus, our project has significant contextual importance in terms of health and safety. Additionally, the design of our project takes into account more immediate economic and environmental concerns.

Promoting improvements in medical devices for the betterment of human health in a vulnerable population is a significant motivation behind our research. Our research into the protein corona aims to elucidate the impact of the protein corona on nanoparticle characteristics and on cellular toxicity. This is crucial when designing nanoparticles for medical use, because these particles will inevitably be coated in a protein corona, which may have distinctive characteristics due to disease state of the patient, or other factors such as patient age, sex, and habits. Diabetes is extremely prevalent in the US, so it is essential that research is done into the impact of this disease on the protein corona surrounding nanoparticles in order to ensure that nanomedicine is equally accessible to this population of patients. Furthermore, since diabetes has many associated comorbidities, patients with diabetes are more likely than the healthy population to require interventions which may benefit from the applications of nanoparticles. Increased research in the field of nanoparticles for medical use enables the field to continue moving forward to develop nanomedicine, which could revolutionize medicine. It could enable targeted drug delivery, reducing side effects from drugs and reducing the dose size, which could in turn reduce the price of drugs. Nanomedicine could be incorporated into medical devices, improving their sensitivity or reducing the chances that implants could be rejected. This could also make device-based interventions more efficient, cheap, and accessible to patients.

There are also immediate health and safety implications in performing the research for this project. Preserving our own health and safety while performing experiments in lab is paramount. We took a safety course and were tested on lab safety before we began to ensure a solid foundational understanding of safety expectations and protocols in the Chemistry and Bioengineering labs. We were properly trained in lab to perform experiments in a way that preserved the integrity of the experiments as well as our own health. Care was taken to ensure

proper labeling and disposal of nanomaterials and cells. We always wore gloves when handling nanomaterials and cells.

Our project considers economic factors as well, due to the limited budget available to us to complete the experiments. Experiments were designed to minimize the use of expensive reagents and chemicals as much as possible, and care was taken to reduce the number of times an experiment had to be performed by minimizing human error.

We were also mindful of environmental factors when designing the project experiments and methods. The biochemistry experiments employed "green chemistry" techniques, where small volumes of non-toxic reagents were used whenever possible. This has the dual effect of minimizing environmental factors as well as reducing cost of the experiment. In the cell culture experiments, biohazardous waste was properly disposed of and all cell culture plates and wells were bleached before being poured down the drain. In all experiments, waste that could pose a hazard to the environment was properly labeled and disposed of according to Bioengineering and Chemistry Department procedures, which follow EPA guidelines to preserve the environment.

2.4.1 Ethics

Ethical justification for the project

The project is justified because the research can have downstream influence on the development of nanomedicine and provide insight into the effect of diabetes on the fate and toxicity of nanoparticles for medical use. The common good approach to ethics provides justification for the project. The common good ethical approach states that the community is good and actions should contribute to the community. The approach considers the conditions that benefit all members of the community.[41] Performing research that increases the scientific knowledge positively influences the scientific community, which in turn positively influences

consumers who use products developed using scientific knowledge. Increased knowledge positively contributes to the welfare of everyone. The work we are doing does not stand alone. Rather, it joins similar work in the field to form a foundation of knowledge and insight about nanoparticle medicine in relation to the model species we are observing.

The project is also justified by the justice, or fairness, approach to ethics. The justice approach to ethics states that people should be treated equally unless unequal treatment is justified. The rights approach implies that those who are most negatively affected by something should also receive the greatest rewards that emerge from that thing.[41] Due to the comorbidities that arise with diabetes, people with diabetes are more likely to get other diseases that may one day benefit from nanomedicine, such as cardiovascular disease. People with diabetes are more likely to be negatively affected by cardiovascular disease than the general population, so this population should have an increased amount of research dedicated to them in finding treatments for their comorbidities. It is important that this particular population is studied in order to advance nanomedicine in a way that is inclusive to those who will benefit the most from it. Our senior design project includes people with diabetes in a field that often overlooks them, evidenced by the lack of published work about the effect of glycation on the protein corona and nanoparticles.

Ethics, the project, and what it means to be a good engineer

The Biomedical Engineering Society (BMES) has a published code of ethics for bioengineers in a variety of contexts.[42] Bioengineers work in a variety of areas, and there are different obligations for biological engineers based on their area of work. The ethical obligations for bioengineers engaged in research activities are as follows:

"Biomedical engineers involved in research shall:

1. Comply fully with legal, ethical, institutional, governmental, and other applicable research guidelines, respecting the rights of and exercising the responsibilities to colleagues, human and animal subjects, and the scientific and general public.
2. Publish and/or present properly credited results of research accurately and clearly."[42]

In order to adhere to the expectations of the first obligation, we have complied with Santa Clara University Departments of Biology and Bioengineering and Chemistry and Biochemistry policies throughout our work on the policy. Our mentors are both experienced researchers and have guided us to ensure that our experiments are meeting our goals, and complying with ethical standards for research. These policies are also compatible with larger governmental research guidelines. There are also guidelines for upholding research ethics generally, which we have also upheld where relevant.[43] We have striven to adhere to the commonly accepted behaviors of an ethical researcher. We have been honest in our communications with our research advisors, and in our presentation of data to audiences. We have not fabricated or falsified data, and have striven to perform all experiments in triplicate in order to provide appropriate error bars and show the relative replicability of our results. We have also been open in our communication with our research advisors, and open to criticism. We will be publishing this work for others to access. Our goal through publishing is to advance scientific knowledge in a poorly studied area; we are not aiming to advance our careers through publishing. We have been objective in our research. We have used common experiments and data-collection practices. We have no financial interests that influence the purpose of our research. We have aimed for integrity in our practices, and been consistent about the goals for our project. We have also been extremely careful in running experiments and collecting data with the project. We have taken careful notes and asked

questions when we were not sure. Thus, we have upheld the first obligation of bioengineers throughout the senior design process.

In order to address the second obligation, we are taking care to be responsible in the way that we present our work to others. The primary audience of our work is other scientists and engineers, who can use our work in context with a greater body of work in the field to inform future medical products. However, our work is also available to those who are not scientifically literate. Thus, we have a responsibility to ensure that the project is accessible to all of our audience members. Through clear documentation of our results, and a thoughtfully prepared thesis and conference presentation, we ensure that our work is accessible to a wide range of audiences.

Virtue ethics is a way of ethical thinking that goes beyond asking what not to do, as is typical in engineering ethics codes. Virtue ethics asks the question "is this action consistent with acting my best?"[41] In the context of senior design, acting our best has meant cultivating ethical habits of mind in relation to the challenges associated with the senior design project. These habits include techno-social sensitivity, commitment to the public good, respect for nature, and teamwork. Through promoting these habits, we will become better, more ethical engineers. This foundation of ethics in engineering is critical as we leave Santa Clara and enter graduate school or the workplace.

We have developed our techno-social sensitivity through work on this project. The motivation of the project recognizes the potential impact of nanomedicine. However, nanoparticles cannot be incorporated into medical devices, with potential positive impact, without understanding the toxicity of the particles upon their entry into the human body. Our work is foundational for the future development of the field. Thus, our project also incorporates

the public good. We are motivated by a desire to improve the inclusivity of research in the field to include those with diseases like diabetes.

We have developed respect for nature through this project. The experiments we have designed, especially the biochemistry experiments, have incorporated "green chemistry" concepts. We have tried to minimize the amount of buffer and materials used per experiment. Furthermore, we have disposed of all our toxic waste in accordance with school guidelines, to avoid the release of nanoparticles or other toxic chemicals into the environment. This approach is informed by a deep respect for nature, and an acknowledgement that the resources of the Earth are precious and must be protected.

We have also developed skills related to teamwork. Our project team is small, with only two members. We communicated clearly with one another to ensure that tasks were evenly distributed where appropriate, and also acknowledged the strengths and weaknesses of the partners. This ensures the quality of the project, while also respecting the time, skills, and efforts of all team members. We also learned to communicate clearly with our advisors, who are from two different disciplines and who have very different ways of approaching and conceptualizing the project. We took care to respect feedback from our mentors, even when there might be disagreement between the two. Throughout the process, we have learned more about what makes a good team, and what behaviors of good team members are. This is an experience and skill that will be invaluable outside of a senior design context, as we prepare to enter the workforce or higher education.

Engineers have high ethical standards that they must adhere to, which we have worked hard to uphold throughout the project. The senior design project also provides a way to foster ethical habits of mind, which inform high ethical standards throughout our lives.

Ethical pitfalls of the project

One of the primary concerns for many engineering projects is ensuring safety. Since our project is research-based, the risks associated with the project are entirely borne by us. The public will be indirectly affected by the results of the research, as our work may inform future developments in nanomedicine. Thus, many of the ethical concerns about risk are minimized. We both voluntarily accepted the risks associated with the research we were performing and were well-informed about potential sources of risk. These include: using toxic or irritating chemicals that should not be consumed, inhaled, or put onto the skin. We were safety trained prior to beginning research and understood the ways to ensure our personal safety and the safety of others by using the appropriate personal protective equipment (PPE) and disposing of waste properly.

There are few other ethical issues associated with the project. We are using HepG2 cells, which are immortal cells derived from liver cancer (hepatoblastoma) in a 15-year old white American male. It is widely used in research, and there are few ethical issues associated with its use. We do want to recognize that it was derived from human cells and are mindful of taking good care of the cells. We have tried to use the cells effectively and glean a significant amount of data from them in order to maximize their utility.

For initial experiments, we used human embryonic kidney (HEK-293) cells, which are also a widely used human immortal cell line. However, these cells were originally derived from a legally aborted fetus, so the ethical issues are a little unclear in comparison to the use of HepG2 cells.[44] However, since the cells have been used in many scientific experiments which have the potential to save many lives, the use of this cell line is acceptable using a utilitarian approach to ethics. Little is known about the identity of the parents or the motivation for the abortion. In the

absence of information, it can be difficult to make good ethical decisions. However, the fetus has already been aborted and nothing can be done to change that. By using the cells derived from the fetus, we can try to improve the common good and contribute to knowledge, using the cells to the best of our ability. We decided not to continue to with the HEK cells for our experiments because HepG2 cells were a better model system. It is also the ethical obligation of an engineer to choose appropriate models and ensure that our work is applicable to the real world.

3. Design Description

3.1 Design Overview

Based on the requirements of the system, as outlined in Chapter 2, we designed experiments to explore both the biophysical and cytotoxic elements of the project. The experiments are outlined in the figure below.

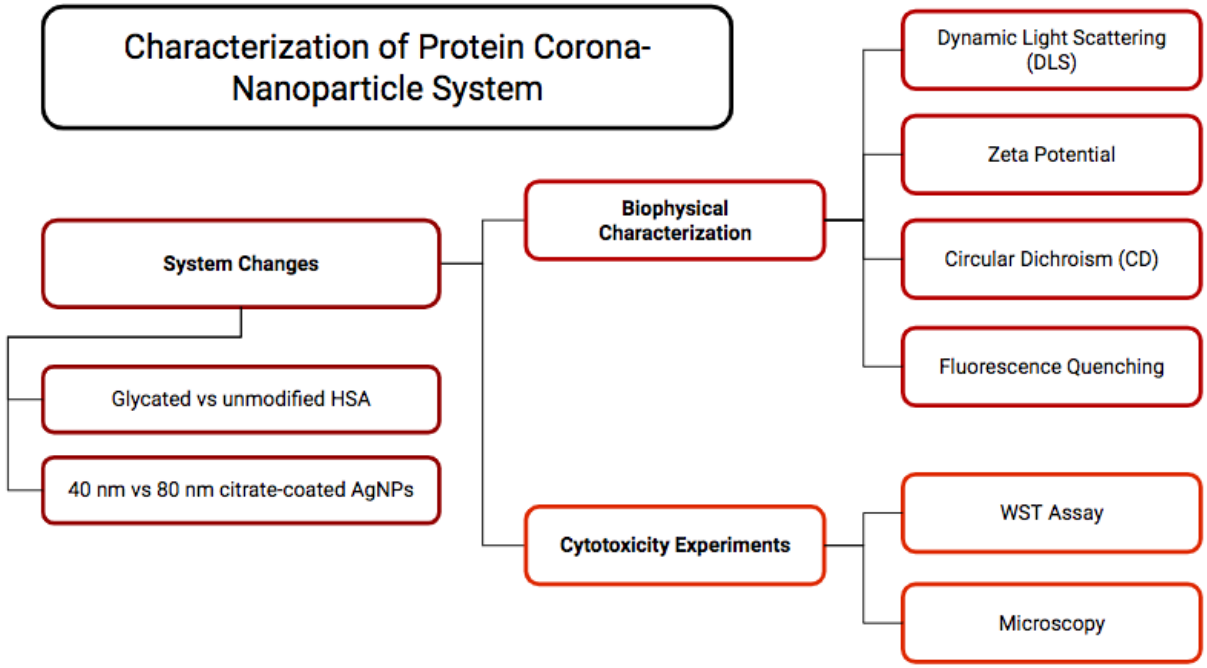

Figure 13. Outline of experiments performed.

3.1.1 Biophysical Characterization Experiments

In order to characterize the biophysical interactions of differently sized nanoparticles with glycated and unmodified HSA, dynamic light scattering (DLS), zeta potential, circular dichroism, and fluorimetry binding experiments were performed. DLS is used to measure the average diameter of nanoparticles and can be used to see how the HSA causes changes in nanoparticle size or agglomeration of nanoparticles. See Section 4.1 for more details on this

experiment. Zeta potential is used to measure the surface charge of nanoparticles. The citrate coating of our nanoparticles is negative, and binding of HSA can change this negative surface charge. See Section 4.2 for more details on this experiment. Circular dichroism is used to determine the secondary protein structure of proteins, such as random coils, α-helices, and β-sheets. The addition of nanoparticles often denatures proteins and shifts the balance of these secondary structures. See Section 4.3 for more details on this experiment. Because HSA has a tryptophan residue which is intrinsically fluorescent, fluorimetry experiments can be performed to determine how well and in what way HSA binds to nanoparticles. See Section 4.4 for more details on this experiment. All of these experiments will be performed with both 40 nm and 80 nm citrate-coated silver nanoparticles combined with both glycated and unmodified HSA. These experiments will elucidate the physical changes of both nanoparticles and proteins upon interaction.

3.1.2 Cytotoxicity Experiments

Colorimetric cell viability assays are a good way to explore the number of cells that survive exposure to nanoparticle-corona complexes. There are several commonly-used options for colorimetric cell viability assays, including MTT Tetrazolium, MTS Tetrazolium, WST, and Sulfohodamine B.[45] We chose to use a WST assay because it is a simple, water soluble assay, for which a protocol was already established in the lab for silver nanoparticles interacting the HepG2 cells. WST assays are widely used to measure the toxicity of chemicals to cells. The WST assay is a colorimetric assay that is used to count the number of metabolically active cells in a sample. It can therefore be used to assess cell viability and proliferation. By comparing the cell viability of HepG2 cells when exposed to different sizes of nanoparticles formed with protein coronas with glycated and non-glycated HSA, we were able to determine the effect of nanoparticle size

and protein glycation on the nanoparticle-protein corona complex cytotoxicity. We performed the assay after one and three days of exposure to the nanoparticle-corona complex. See Section 5.1 for more details about the experiment.

Information about cell growth is important to accompany the results from a WST assay because it provides context for the results at one and three days of exposure to the particle-corona complexes. We observed the percent confluency of control cells between day one and day three of the assays using a microscope. This helped us understand the normalized trends of the WST assay, by explaining possible differences in viability between day one and day three. See Section 5.2 for more details about the experiment.

3.2 Expected Results

The expected results are outlined below. Based on previous results from the research collaboration between the Wheeler and Asuri Labs, and from our literature search, we expected certain baseline results from our experiments between 40 nm citrate-coated silver nanoparticles and unmodified HSA. However, we were unsure of the impact that glycation may have, due to the lack of information in the literature about the impact of glycation on the PC. However, we can speculate about possible results based on based experiments, the literature, and what we know about glycated HSA.

3.2.1 Biophysical Characterization Expected Results

Dynamic Light Scattering

We expect that the citrate-coated nanoparticles may agglomerate with the addition of HSA to solution. This expectation is based upon previous experiments performed in the lab. The

larger 80 nm particles will likely be more stable and agglomerate less, because larger particles tend to remain more stable and monodisperse in solution compared to smaller particles. Furthermore, literature results on the changes in protein structure indicate that HSA does not change its structure significantly when it is introduced to nanoparticles, discussed in the circular dichroism section next. Agglomeration and crashing out of the particles tends to be accompanied by protein unfolding. Thus, since we don't expect protein unfolding, we do not expect significant agglomeration of the particles with addition of the proteins. For more details on this experiment, see Section 4.2.

Circular Dichroism

HSA secondary structure naturally consists of about 60% α-helices, 10% β-sheets, and 30% random coils. In the literature, albumin does not lose much of its structure when it interacts with gold or silver nanomaterials.[24,46,47] Thus, we do not expect significant changes to HSA secondary structure with the addition of either 40 or 80 nm NPs. Glycated HSA has a different structure than unmodified HSA, so it may be affected differently by the addition of NPs.[29] We are unaware of papers that discuss the impact of engineered nanomaterials on the structure of glycated albumin specifically. For more details on this experiment, see Section 4.3.

Zeta Potential

The surface charge of citrate-coated nanoparticles is negative at biological pH due to the carboxylate groups of citrate. We expect that the surface charge will become more positive upon addition of HSA because the protein will cover some of the negative surface of the particle. It is likely that unmodified HSA will make the surface charge more positively charged (or less negatively charged) than glycated HSA because unmodified HSA is more positively charged than glycated HSA. As discussed in Section 1.2.7, glucose binds to positively charged lysines on

the HSA surface.[29] Thus, glycated HSA will be less positively charged because the positively charged lysines have a post-translational modification. For more details on this experiment, see Section 4.4.

Fluorescence Spectroscopy

The literature shows varied results for binding constants of albumin to nanoparticles. The table below outlines the published results for binding constants of bovine serum albumin (BSA) to gold nanoparticles, a comparable model system. The table demonstrates the large variability in binding constants, with orders of magnitude of difference between various published sources for the same model system. Thus, based on the literature, we are unsure exactly what to expect from our results.

Table 1. Published binding constants of BSA to AuNPs.[48]

nanoparticle	shape	size (nm)	solvent	method	K_a (M^{-1})	author
citrate−GNP	sphere	18	water	fluorescence	2.34×10^{11}	Iosin et al. (2009)[37]
CTAB−GNP	rod	70 × 30	water	fluorescence	5.0×10^{4}	Iosin et al. (2009)[37]
Glut−GNP	sphere	40	PBS	fluorescence	3.16×10^{11}	Wangoo et al. (2008)[52]
citrate−GNP	sphere	20	water	CD	7.14×10^{8}	Truel et al. (2010)[53]
citrate−GNP	sphere	10	water	QCM	1.0×10^{6}	Brewer et al. (2005)[23]
citrate−GNP	sphere	15	PBS	fluorescence	3.0×10^{9}	Shang et al. (2007)[54]
citrate−GNP	sphere	51	HEPES	SCS	4.0×10^{3}	Dominguez-Medina et al. (2012)[55]

Previous fluorescence spectrometry experiments with unmodified HSA and 40 nm citrate-coated AgNPs performed in our lab show a K_d of just below $2 \times 10^{10} M^{-1}$. This is on the threshold of static and dynamic binding. We may expect similar results for our experiments. Glycation of the protein may decrease the strength of binding because the glycated protein is less positively charge than the unmodified protein, as discussed in the Zeta Potential expected results section above. Thus, it may bind more weakly to the surface of the NP than unmodified. For more details on this experiment, see Section 4.5.

3.2.2 Cytotoxicity Expected Results

We expect the citrate particles without a corona to have a slight cytotoxic effect on the cells. We are unsure what the impact of the protein corona will be on the toxicity of cells. There may be a decrease in toxicity, or the particles will remain cytotoxic.[49,50] For more details on our experimental plan, see Section 5.1.

4. Biophysical Characterization Experiments

4.1 Overview of Experiments

The purpose of the biochemistry experiments is to characterize the nanoparticle interaction with the protein corona. This involves characterizing change in NP properties upon the formation of the protein corona, as well as how the structure of the proteins in the corona changes as NPs are introduced into the system. In order to assess NP agglomeration upon addition of proteins, we performed dynamic light scattering experiments. To assess changes in surface charge of the particles, we performed zeta potential experiments. In order to determine changes in protein structure once proteins join the corona, we performed circular dichroism experiments. In order to quantify binding constants of the proteins on the NP, we performed fluorescence quenching experiments.

A visual overview of the biochemistry experiments is shown below (Figure 14). See Appendix A for an overview of materials and protocols used for all experiments.

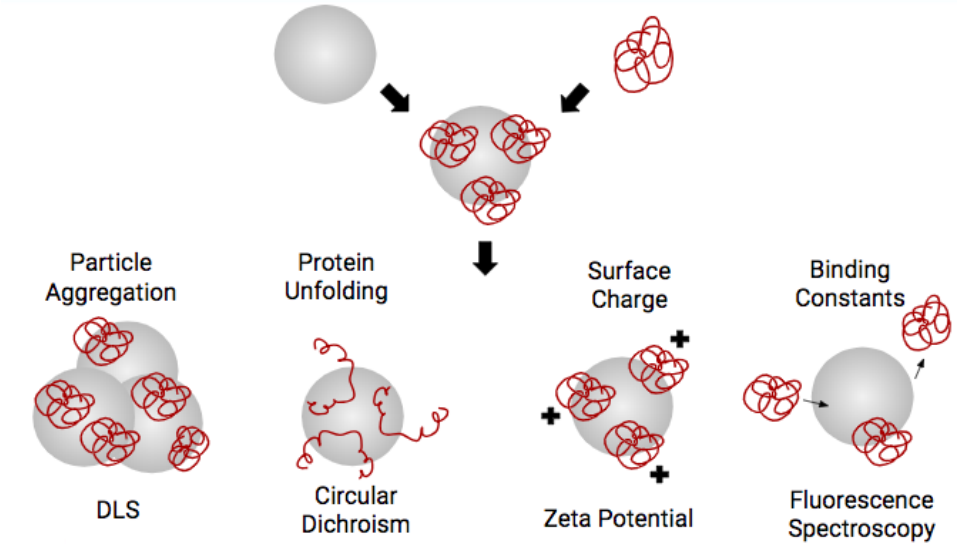

Figure 14. Schematic of biophysical characterization experiments.

4.2 Dynamic Light Scattering

4.2.1 Introduction

Dynamic light scattering (DLS) is a technique used to determine the size distribution of small molecules in solution. Nanoparticles are suspended in solution, and a beam of light is then cast through the solution. The light hits the nanoparticles and is scattered and detected by the instrument. The scattering patterns caused by the nanoparticles are analyzed by the instrument to give a distribution of the size of the nanoparticles.

By looking at the size distribution of nanoparticles before and after a given event, inferences can be made about the behavior of the nanoparticles during that event. For example, if the nanoparticles agglomerate upon addition of proteins, this will be reflected by an increased diameter as measured by DLS. DLS is therefore a powerful tool for analyzing the physical change in size that nanoparticles undergo as a result of exposure to proteins in solution.

4.2.2 Key Constraints

DLS posed several difficulties, primarily surrounding consistency of results. First, it is difficult to find the correct concentration of nanoparticles for the DLS instrument, while also not wasting the expensive nanoparticles. Additionally, even when using the same concentration, DLS results can be very inconsistent, so a large number of scans need to be taken to ensure accuracy. This posed logistical problems with both time and money being limited resources.

4.2.3 Design Description and Methods

Nanoparticles were first added to filtered nanopure water and measured alone. HSA was then added, mixed, and allowed to sit for ten minutes before being scanned. This was repeated for all nanoparticle and protein combinations, each of which were performed in triplicate.

For Z-average hydrodynamic measurements (ZetaPlus from Brookhaven Laboratories), the NP-protein complexes were made to be at a ratio of 2.9×10^4proteins per nanoparticle. To achieve this, 5 µg/mL glycated and unmodified HSA (from Sigma Aldrich) and 0.55 µg/mL 40 nm and 80 nm citrate coated NPs (from nanoComposix) were reacted in nanopure water filtered with a 0.02 µm inorganic membrane filter. Measurements were taken after 10 minutes incubation. See Appendix B for a detailed protocol.

4.2.4 Results and Discussion

DLS measurements had been completed in triplicate, and the diameter and polydispersity of one representative sample for each condition are shown in Table 2 below.

Table 2: Z-average hydrodynamic diameter of particles in solution. (Polydispersity).

	40 nm particles	**80 nm particles**
NP alone	44.18 nm (0.288)	86.46 nm (0.238)
Unmodified HSA	94.12 nm (0.170)	86.61 nm (0.217)
Glycated HSA	50.64 nm (0.091)	81.10 nm (0.227)

The 40 nm particles did not agglomerate upon addition of glycated HSA (50.64 nm), but did agglomerate in the presence of unmodified HSA (94.12 nm). This moderate amount of agglomeration indicates that several particles are agglomerating in the presence of the

unmodified HSA. However, this roughly doubling of size is an excessive amount of agglomeration. However, we did not notice any deposits at the bottom of the cuvette and thus did not observe particles crashing out of solution. For this reason, we believe that the 40 nm nanoparticle-protein system was stable in the presence of unmodified or glycated HSA.

The 80 nm particles did not agglomerate in the presence of unmodified HSA (86.61 nm) or glycated HSA (81.10 nm). Although there is a slight drop in the size of the nanoparticle upon addition of glycated HSA, we believe that this decrease does not represent an actually significant decrease in the size of the particle. Thus, the 80 nm nanoparticle-protein system was also stable in the presence of either unmodified or glycated HSA.

4.3 Circular Dichroism

4.3.1 Introduction

Circular dichroism (CD) is an absorption spectroscopy method that involves circularly polarized light. It absorbs right-handed and left-handed light in order to determine the structure of chiral molecules. We used CD to determine the secondary structure of proteins. A change in the spectrum measured by the CD instrument indicates a change in protein structure. It is possible to process the spectrum to determine how the secondary structure of the protein changes.

We use CD to determine how the protein structure changes as the NPs are added to a protein solution. As proteins in solution with the NPs form the PC, their structure may change. The citrate-coated NPs we are using are negatively charged, so the positively charged portions of HSA may deform in order to bind to the particle.

4.3.2 Key Constraints

The CD experiments are relatively straightforward. The major constraint was time; each scan takes about 30 minutes, and we performed over 24 scans.

4.3.3 Design Description and Methods

A CD scan was taken of glycated or unmodified HSA in nanopure water. 40 or 80 nm AgNP was added to the cuvette and another CD scan was taken to investigate the change in protein structure as a result of the addition of the NP. Each combination of HSA and particle was measured in triplicate.

To prepare the samples to be analyzed by CD, the NP-protein complexes were made to be at a ratio of 3.2×10^4proteins per nanoparticle. Protein dilutions were prepared at 0.0625 mg/mL HSA. This dilution was transferred into a quartz cylindrical cuvette with a pathlength of 1 mm and analyzed with an Olis Rapid-Scanning Monochromator. The wavelength range recorded was 185-260 nm, with the number of increments set to 150. Final concentration of protein was 70 µg/ml and final concentration of the nanoparticle was 7 µg/ml. The cuvette was washed with water and methanol in between scans. See Appendix C for a detailed protocol.

4.3.4 Results and Discussion

CD spectra indicate that protein structure does not change significantly upon addition of nanoparticles to glycated or unmodified HSA (Figure 15). This indicates that the proteins maintain their secondary structure with the addition of 40 and 80 nm nanoparticles. Protein unfolding is often accompanied by agglomeration of particles, which was not seen in the system according to DLS results. Thus, this data helps to confirm the data we saw with DLS. This data,

in combination with the DLS data, indicates that the system is stable; proteins are not unfolding and particles are not agglomeration to a significant degree.

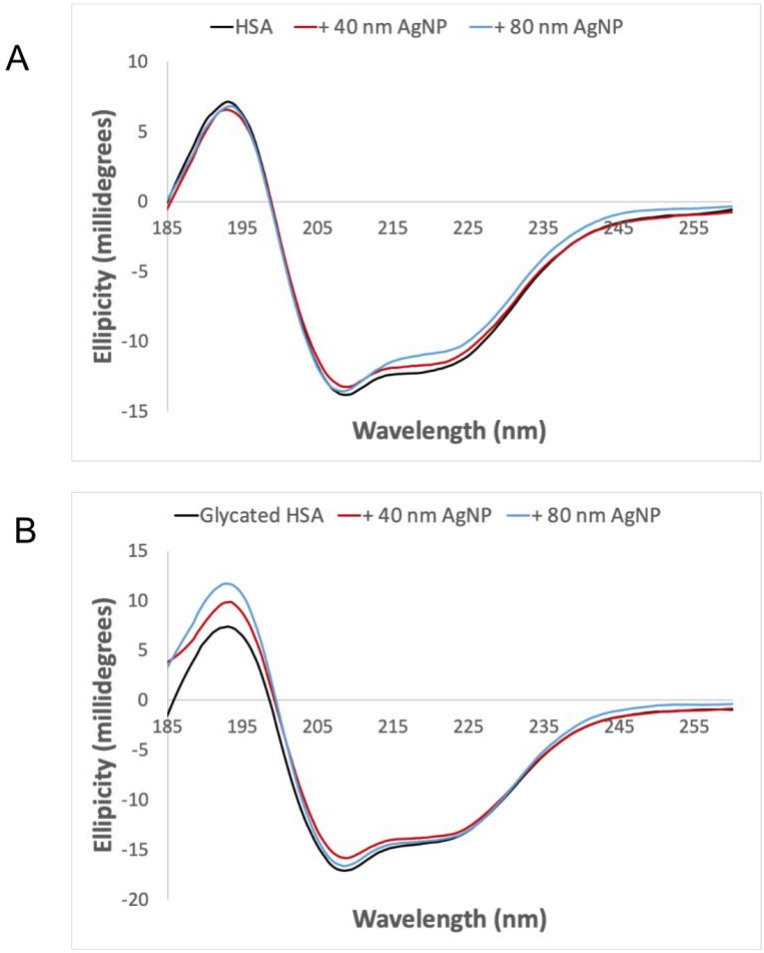

Figure 15. Circular Dichroism spectra. Representative spectra for CD experiments (performed in triplicate) characterizing unmodified HSA (A) and glycated HSA (B).

4.4 Zeta Potential

4.4.1 Introduction

Zeta potential is the surface electrical potential of particles. To measure this surface potential, nanoparticles are suspended in solution, and an electrode is placed into the solution. The electrode applies an electric field to the solution, causing charged particles to move toward

or away from the electrode. The velocity of the particles is proportional to their zeta potential. Thus, by measuring the velocity of the particles using optical techniques similar to those of DLS, the zeta potential of the nanoparticles can be determined by the instrument.

By observing the change in charge of the NPs upon addition of the proteins to solution, inferences can be made about the effect of the protein corona on NP surface charge. For example, the adsorption of proteins onto the surface of the nanoparticle will change the charge of the nanoparticle depending on the charge of the protein.

4.4.2 Key Constraints

Much like DLS, zeta potential experiments posed several difficulties, primarily surrounding consistency of results. First, it is difficult to find the correct concentration of nanoparticles for the instrument, while not wasting the expensive nanoparticles. Additionally, even when using the same concentration, zeta potential results can be very inconsistent, so a large number of scans need to be done to ensure accuracy. This posed logistical problems with both time and money being limited resources.

4.4.3 Design Description and Methods

Similar to DLS, nanoparticles were first added to filtered nanopure water and scanned alone. HSA was then added, mixed, and allowed to sit for ten minutes before being scanned. This was repeated for all nanoparticle and protein combinations, each of which were measured in triplicate.

For zeta potential measurements (ZetaPlus from Brookhaven Laboratories), the NP-protein complexes were made to be at a ratio of 4.0×10^3proteins per nanoparticle. To achieve this, 3.125 µg/mL HSA and 2.5 µg/mL NPs were reacted in nanopure water filtered with a 0.02

μm inorganic membrane filter. Measurements were taken after 10 minutes incubation. A Smoluchowski model was used to calculate zeta potential from electrophoretic mobility measurements. See Appendix D for a detailed protocol.

4.4.4 Results and Discussion

The average zeta potential measurements are shown below for all conditions.

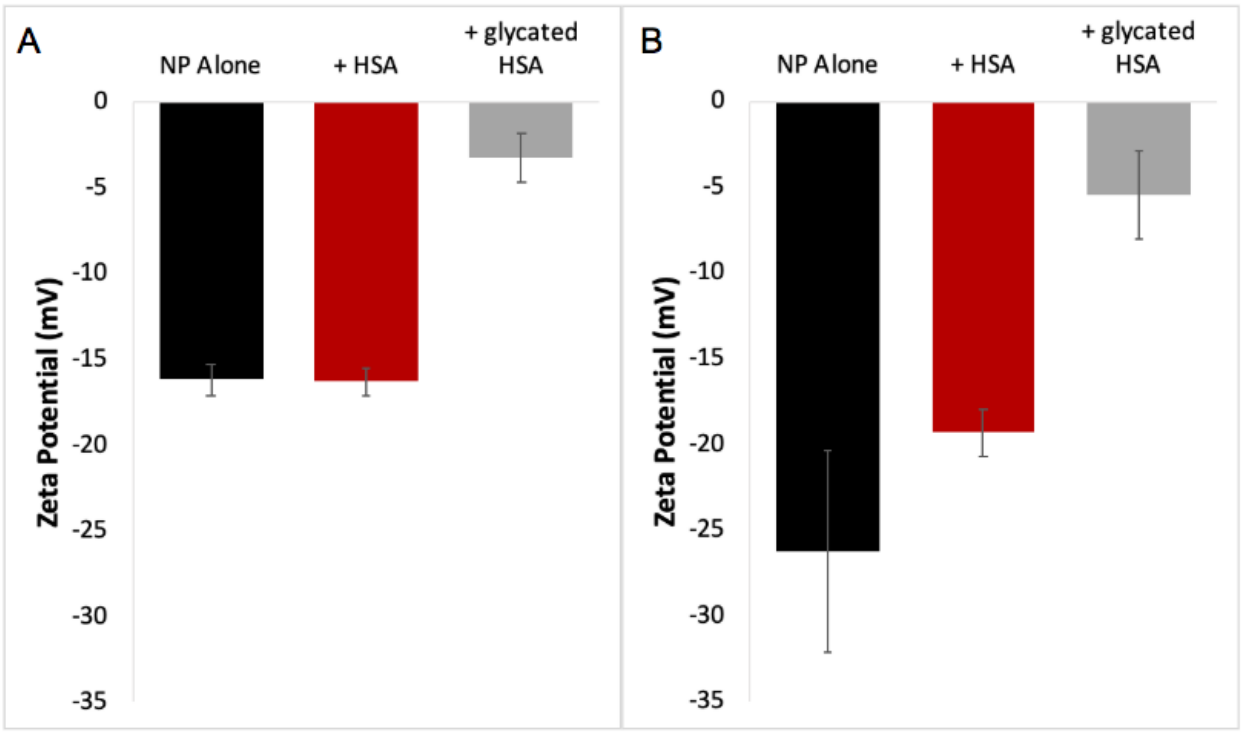

Figure 16: Average zeta potential measurement. (Error bars represent standard error of the mean). A. 40 nm nanoparticles. B. 80 nm particles.

Both the 40 nm and 80 nm particles are coated in citrate, a negatively charged ion (see 1.2.3). As expected, the particles are negatively charged in the absence of protein, due to this negatively charged surface coating.

The 40 nm particles begin with a surface charge of about -16.2 ± 1.0 mV. Upon addition of unmodified HSA, this stays constant at -16.3 ± 0.8 mV. However, upon addition of glycated HSA, the surface charge increases to about -3.3 mV.

The 80 nm particles begin with a surface charge of about -26.3 ± 5.9 mV. Upon addition of unmodified HSA, the surface charge increases to -19.3 ± 1.4 mV, and upon addition of glycated HSA, the surface charge increases to -5.4 ± 2.6 mV.

The changes in surface charge of the nanoparticle that we observed after the addition of protein indicates that the protein and nanoparticle are indeed interacting. When the protein interacts with the nanoparticle, it effectively shields some of the negatively charged citrate ions on the nanoparticle surface. However, although these zeta potential experiments do inform us that the nanoparticle and protein are actually interacting and possibly binding with each other, they cannot give us more specific insight into the nature or strength of this interaction. For this reason, fluorescence quenching binding affinity experiments were performed to give insight into the kinetics of nanoparticle and protein binding.

4.5 Fluorescence Quenching Binding Affinity

4.5.1 Introduction

HSA contains the amino acid tryptophan, which is intrinsically fluorescent. When HSA binds to the surface of an NP, the tryptophan fluorescence output changes, as shown in Figure 17. The changes in fluorescence can be graphed in a Stern Volmer plot or a Hill Plot. This analysis reveals the Hill Coefficient, which provides insight into the binding kinetics of the protein to the NP.

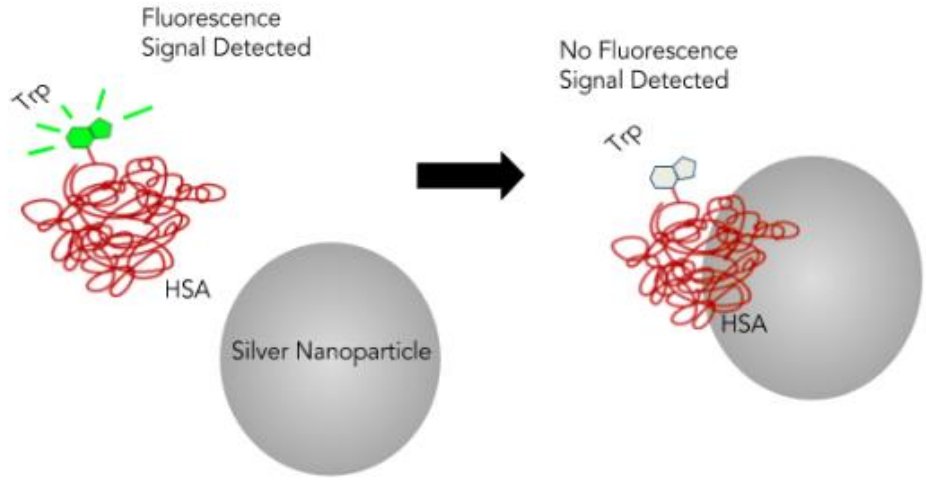

Figure 17. Changes in fluorescence due to binding of HSA to nanoparticle can provide insight into binding constants. Figure from Kira Fahy.

4.5.2 Key Constraints

The major constraint for this experiment was issues with the instrument. The lamp on the instrument was changed shortly after experiments began, which required redoing many of the experiments to adjust to the new lamp.

Repeated scans can photobleach the sample. Only one scan was taken upon each addition of NP.

4.5.3 Design Description and Methods

Glycated or unmodified HSA was added to a cuvette in 5 mM dibasic sodium phosphate buffer, pH 7.4. NPs were titrated into the solution; with each addition, a single fluorescence scan was taken. The change in fluorescence is measure and could be used to generate a binding curve for the proteins onto the NP.

Samples were prepared in a 5 mM dibasic sodium phosphate solution with 0.1 M HSA. 40 and 80 nm silver nanoparticles were titrated in to yield concentrations of NPs ranging from

2.66×10^{-12} M to1.26×10^{-11}M. The emission spectrum was measured on a Horiba Fluorolog 3 using an excitation wavelength of 280 nm and the fluorescence in CPS was read at 344 nm. The respective slit widths for excitation and emission were set at 2 nm and 12 nm. The temperature was adjusted to 20 degrees Celsius. The blank, with no added nanoparticle, was allowed to calibrate for five minutes before taking a reading, while each successive titration measurement had a temperature calibration time of two minutes. At each titration point, the sample was scanned only once to avoid the effects of photobleaching. The samples were titrated and thoroughly mixed in the Starna Cells sub-micro quartz cuvette, z = 15 mm. See Appendix E for a detailed protocol.

4.5.4 Results and Discussion

Sample fluorescence spectra show that there is a decrease in fluorescence upon addition of nanoparticles (see Figure 18), indicating that AgNPs can effectively quench the fluorescence of Human Serum Albumin.

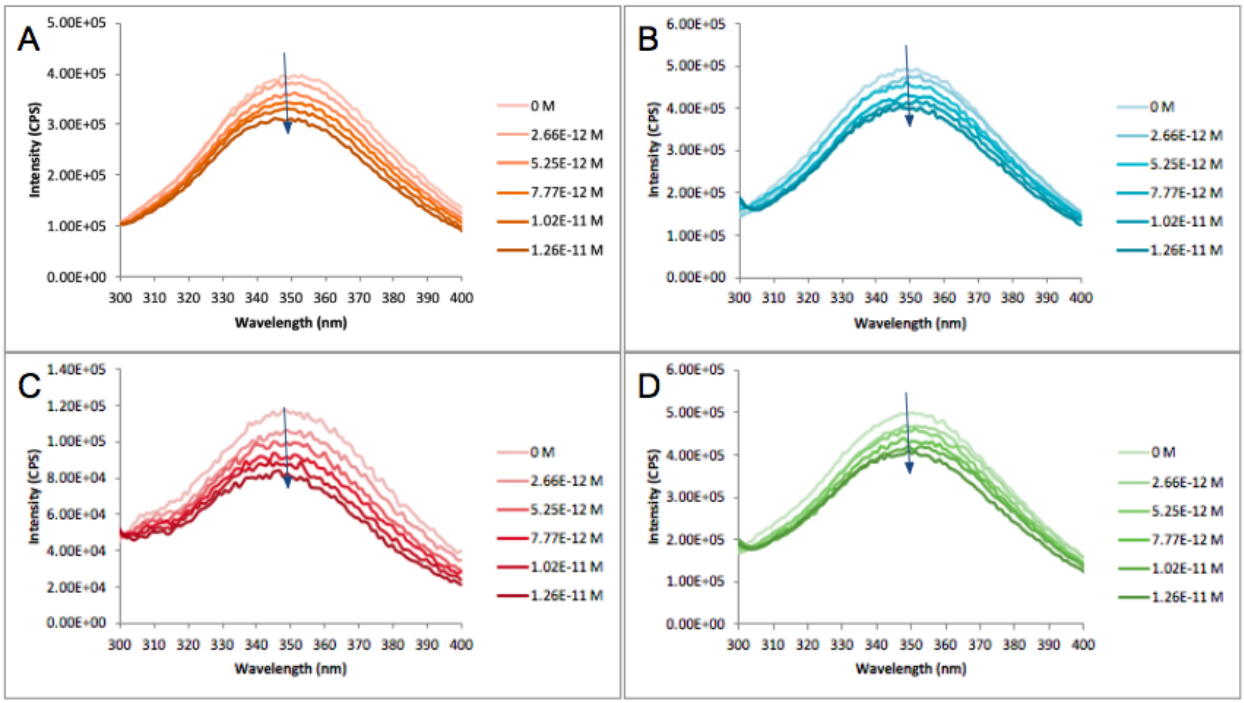

Figure 18. Addition of nanoparticle decreases intrinsic fluorescence of HSA. Sample plots from the following experiments (performed in triplicate): (A) HSA + 40 nm AgNP (B) HSA + 80 nm AgNPs (C) Glycated HSA + 40 nm AgNPs (D) Glycated HSA + 80 nm AgNPs.

The fluorescence quenching was observed through a Stern-Volmer plot (see Figure 19). The Stern-Volmer constant, K_d, for all combinations of nanoparticle and protein are below $2.0 \times 10^{10}\ M^{-1}$(see Table 3). This indicates that the quenching is collisional, or dynamic. The quenching of the fluorescent absorbance is due to transient collisions between the particles (quenching agents) and the intrinsically fluorescent proteins, rather than a stable bond between the two.[51]

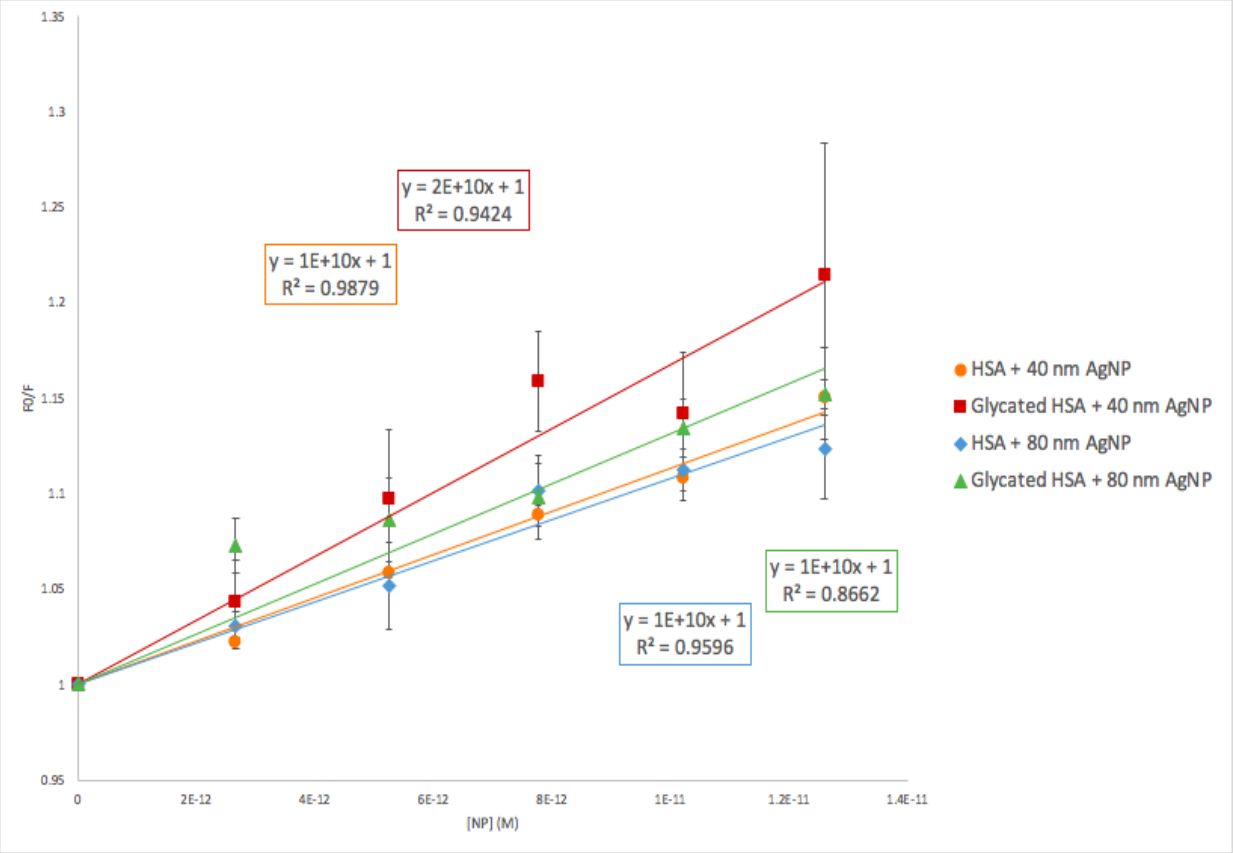

Figure 19. Stern-Volmer plot. Error bars represent standard deviation divided by the square root of the number of trials. Two outlier data points were removed due to an issue with the instrument identified during the experiment.

Table 3. K_d values derived from slopes on Stern-Volmer plot. K_d values are averages of three trials. Error is the standard deviation.

NP-protein combination	K_d (10^{10} M^{-1})
HSA + 40 nm AgNPs	1.13 ± 0.12
Glycated HSA + 40 nm AgNPs	1.87 ± 0.67
HSA + 80 nm AgNPs	1.08 ± 0.33
Glycated HSA + 80 nm AgNPs	1.31 ± 0.34

5. Cell Behavior Characterization Experiments

5.1 WST Assay

5.1.1 Introduction

The WST assay is a colorimetric assay that is used to count the number of metabolically active cells in a sample. It is commonly used to assess cell viability and proliferation. By comparing the cell viability of HepG2 cells when exposed to different sizes of nanoparticles formed with protein coronas with glycated and non-glycated HSA, we were able to determine the effect of nanoparticle size and PC glycation on the NP-PC complex cytotoxicity. We performed the assay on cells after one and three days of exposure to the NP or NP-PC complex.

A visual overview of the methods used in the experiments is below (Figure 20).

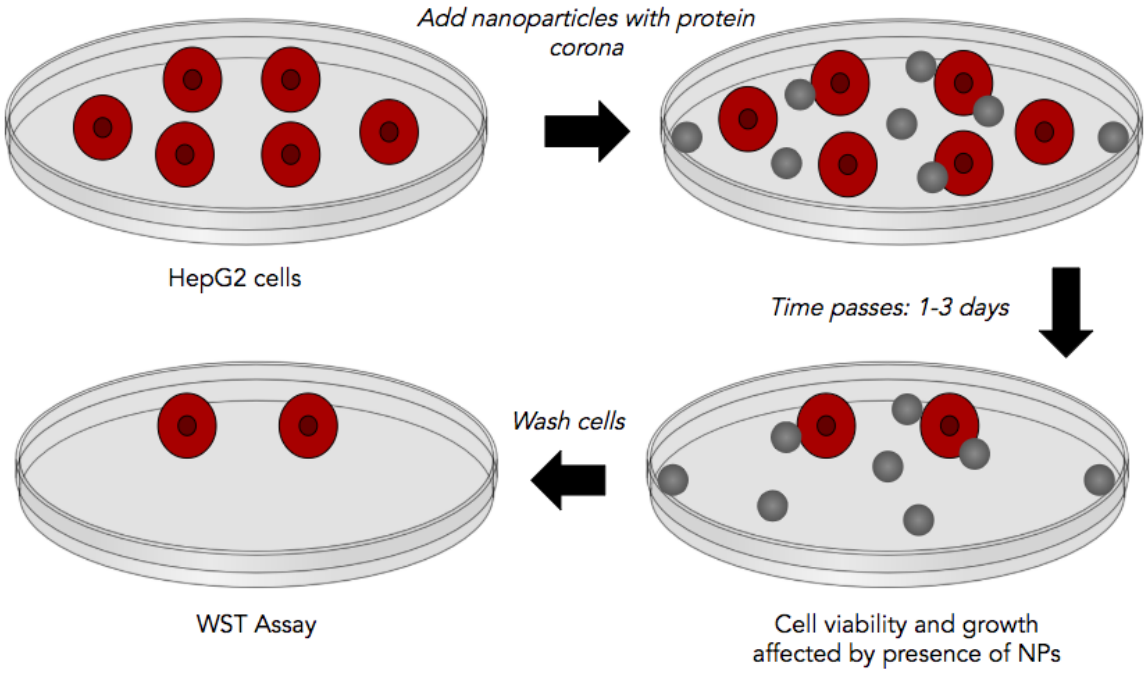

Figure 20. Schematic of WST assay.

5.1.2 Key Constraints

The major constraint associated with these experiments was time. The assays took 5 days to complete, and each experiment had to be performed at the same time each day. Given our busy schedules, it was difficult to find times that worked to complete a full assay. Furthermore, the cells grew extremely slowly. HepG2 cells typically take 7-10 days to become confluent, but we found that they generally took 10-14 days. With troubleshooting, we discovered that the issue was with the incubator we were using. Upon moving our cells to a different incubator, they grew at a significantly faster rate.

5.1.3 Design Description and Methods

HepG2 cells were maintained in DMEM supplemented with 10% fetal bovine serum, sodium pyruvate, MEM non-essential amino acids, and 1% penicillin–streptomycin at 37 °C in a 5% CO_2 humidified environment. The cells were passaged every 7-10 days using 0.25% trypsin/EDTA. For a detailed description of the passaging protocol, see Appendix F.

Cell viability when exposed to various nanoparticle preparations was measured using the formazan-based WST assay as per the manufacturer's instructions. Briefly, cells were seeded into 96-well flat-bottom plates at a cell density of 15,000 cells/well and allowed to proliferate for 48 hours before exposure to the nanoparticles. Cells were counted using an Orflo Moxi Z Mini Automated Cell Counter Kit. On day 0, the cells were exposed to 2 and 20 µg/mL of bare and HSA-coated nanoparticles (concentrations selected based on previous literature and experiments in our lab). Initial experiments indicated that higher concentrations of buffer did not influence cell viability, so we are certain that it is the nanoparticles which have a toxic effect on the cells. In order to investigate the impact of nanoparticles with the protein corona, nanoparticles were added in solution with glycated or unmodified HSA at a ratio of about 75 proteins to NPs, which

is sufficient to coat the outside of the nanoparticle in a single layer of protein. The nanoparticles were then added to the cells as in the bare particle experiments.

On days 1 and 3, the cells were washed with media to remove the nanoparticles. 10 μL of the WST solution for every 100 μL of culture media was added directly to the wells and the cells were incubated for 2 hours in a humidified incubator at 37 °C and 5% CO_2 and the absorbance was measured at 570 nm using a Tecan Infinite 200 PRO plate reader (Tecan, Switzerland). All experiments included control cells not exposed to the nanoparticles as well as control wells without cells and only the WST assay and the data is reported relative to the control conditions.

For a detailed protocol for the WST assay, please see Appendix G.

5.1.4 Results and Discussion

Results from the WST experiments are shown in the figure below (Figure 21). High concentrations of nanoparticles are toxic to the cells, indicated by extremely low cell viabilities at day 3 of exposure to nanoparticles or nanoparticles with the protein corona. Neither the size of the nanoparticles nor the presence or properties of protein corona does not have a significant impact on the toxicity of nanoparticles to cells at these concentrations.

At lower sub-lethal concentrations, the protein corona does appear to increase nanoparticle toxicity to cells, especially at day 3 of exposure. The cell viability for cells that have been exposed to nanoparticles with the protein corona is lower, regardless of nanoparticle size. Furthermore, it appears that the glycated HSA is more toxic to the cells than the unmodified HSA; however, more sensitive cell-based experiments (e.g. reactive oxygen species assay, lactate dehydrogenase assay) would be needed in order to confirm this result.

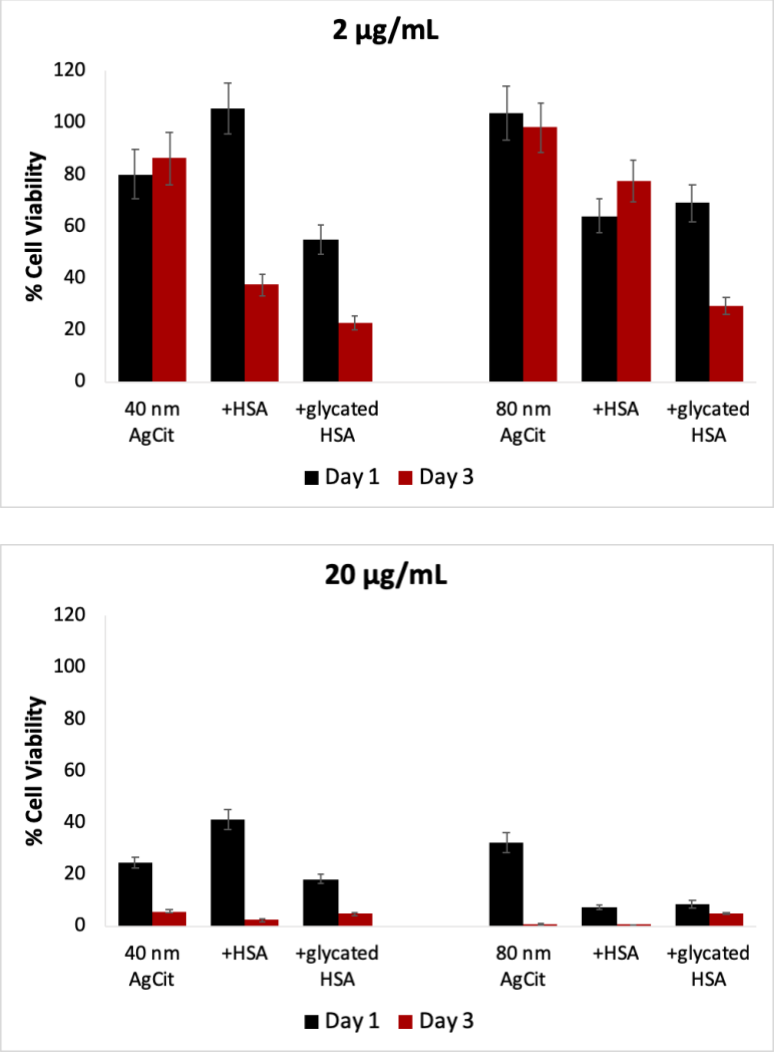

Figure 21. WST assay results at low and high concentrations of nanoparticle with protein corona.

6. Conclusions

6.1 Summary of Results and Connections

Biophysical characterization experiments indicated that the nanoparticle-protein corona system was largely stable. The proteins do not lose their structure upon interaction with nanoparticles and bind dynamically with the particles. The size of the nanoparticles and the glycation state of the proteins do not significantly change these results. The particles do not agglomerate with the addition of protein, and the surface charge of the naturally negatively charged particles becomes slightly more positively charged with the addition of proteins. Glycated HSA makes the nanoparticles more positively charged than unmodified HSA.

Cell toxicity experiments show that high concentrations of nanoparticles are toxic to cells. The presence of the protein corona does not have a significant impact on this toxicity. However, at sub-toxic concentrations the presence of the protein corona makes the nanoparticles more toxic to HepG2 cells. There is a slight increase in cytotoxicity for nanoparticles with a glycated HSA protein corona compared to nanoparticles with an unmodified HSA protein corona. However, this trend is not significant.

The reasons for the trends seen at low concentrations of nanoparticle in the cell behavior characterization experiments can perhaps be explained by the insights about interactions at the nanoscale provided by the biophysical characterization experiments. Thus, we connect the two arms of experiments. We suggest two theories about behavior at the nanoscale that may explain the toxicity trends.

Previous work in the lab has shown that the addition of unmodified HSA to 40 nm nanoparticles releases cationic silver into solution. The adsorption of proteins on the nanoparticle surface catalyzes the release of Ag^+ into solution (Figure 22). Ag^+ is known to be toxic to cells,

although the exact mechanism of toxicity is unknown.[52] Further experiments are required to investigate whether cationic silver is released for the other nanoparticle-protein corona combinations.

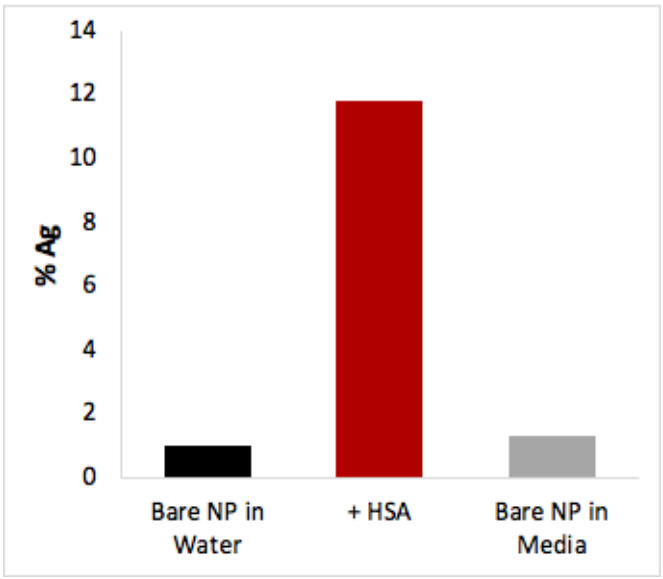

Figure 22. Ag^+ release measured by ICP-MS from 40 nm silver nanoparticle with addition of unmodified HSA. Data was previously collected in triplicate.

Another potential mechanism for increased nanoparticle toxicity with the presence of the protein corona is cell recognition of surface proteins. As seen in the Circular Dichroism data, the proteins retain their native secondary structure upon interaction with nanoparticles. Cells could recognize the proteins and be more likely to uptake the particles, thus leading to greater toxicity.

Although the impact of glycation of the protein corona on cell toxicity is not significant, the results seen in the cell behavior experiments is supported by biophysical characterization results. The Zeta Potential results showed that glycated HSA increases the surface charge of the particles, making the charge less negative. Cells are attracted to things that are positively charged, so the cells may interact with nanoparticles with a glycated protein corona more than nanoparticles with an unmodified HSA protein corona. This increased level of interaction may

correlate with increased toxicity. However, more sensitive cytotoxicity experiments are necessary to confirm this result.

6.2 Future Work

We completed all of the experiments that we initially planned to perform. We have a good understanding of the nanoparticle-protein corona system and how it impacts cell viability. However, future experiments would help provide more insight about potential modes of cell toxicity and greater understanding of the behavior of the system.

On the biophysical characterization side of the project, the experiment that would be most immediately beneficial to this work is a measurement of Ag^+ dissolution. As discussed in Section 6.1, the release of Ag^+ ions into solution has been implicated as one of the causes of nanoparticle toxicity to cells, and previous work in the Wheeler lab has shown that HSA can catalyze this dissolution. We therefore believe that the increased toxicity of the nanoparticle-protein corona system could be due to this protein-mediated dissolution of cationic silver. In order to further investigate this, we hope to perform inductively-coupled plasma mass spectrometry (ICP-MS) on all nanoparticle-protein conditions. This experiment would allow us to know if HSA catalyzes the release of cationic silver for both 40 and 80 nm particles, and if glycation increases the rate of this catalysis. By knowing this, we could gain more insight into the mechanism of nanoparticle toxicity to cells, and if this toxicity is due in large part to the dissolution of Ag^+.

On the cell toxicity side of the project, more sensitive cell assay could be performed, beyond the WST assay. The WST assay looks only at cell viability, so it can only give insight into whether the cells are dead or alive. Other cell assays would provide insight into cell

behavior at sub-lethal concentrations of nanoparticles and the mechanism of nanoparticle toxicity. Two such assays are reactive oxygen species and lactate dehydrogenase assays.

Reactive oxygen species (ROS) are molecules such as hydrogen peroxide that can cause damage to the cell by damaging nucleotides, proteins, and lipids. Production of ROS is necessary for cells that perform aerobic processes, but these ROS normally are produced and eliminated at a controlled rate. In situations of oxidative stress to the cells, ROS are created a much higher rate, and can cause damage to the cell.[53] By performing an ROS assay, we could determine if the cells are experiencing excessive oxidative stress due to the nanoparticles.

Lactate dehydrogenase (LDH) is an enzyme that catalyzes the conversion of lactate to pyruvate, coupled with the reduction of NAD^+ to NADH. When the cell membrane is damaged, LDH is released into solution and can be quantified with the LDH assay.[54] This assay would give insight into damage to the cell membrane, thus giving more information regarding the mechanism of nanoparticle toxicity.

6.3 Impact

This study has confirmed the findings of previous studies on the nanoparticle-protein corona complex. Although the field of nanomedicine has many exciting possible applications in targeted drug delivery, imaging, and other fields, those have not yet become reality. This is due in part to the unpredictability of how the nanoparticles will interact with cells once they have been coated in the protein corona. We have shown that at normally non-lethal concentrations of nanoparticles, the nanoparticles can actually become significantly more toxic to cells when coated in the protein corona. Additionally, changes to the protein corona may alter the toxicity of the nanoparticles. For this reason, it is imperative that nanoparticles be studied with the protein

corona in order to fully understand how the nanoparticles would interact with the body. Until this is done, nanoparticles cannot be safely implemented for biomedical applications.

7. Appendices

Appendix A. Materials information for all experiments.

Human Serum Albumin and Glycated Human Serum Albumin were ordered from Sigma Aldrich (A1653, A8301). Biopure 40 nm and 80 nm Ag nanospheres with citrate surface coatings were ordered from nanoComposix (AGCB40, AGCB80). All experiments were conducted using nanopure water.

Human hepatoma (HepG2) cells were obtained from ATCC (Manassas, VA); Dulbecco's modified Eagle medium (DMEM) from Mediatech (Manassas, VA); fetal bovine serum and penicillin—streptomycin from Invitrogen (Carlsbad, CA); sodium pyruvate and MEM non-essential amino acids from Life Technologies (Carlsbad, CA); and WST cell proliferation assay kit from Dojindo Molecular Technologies (Rockville, MD).

Appendix B. Protocol for DLS.

Protocol adapted from Wheeler lab standard protocols.

Safety Concern: Instrument is located in the Instrumental lab. It's imperative that you wear a lab coat and goggles when using this instrument.

Running Samples on the DLS

1. Open the BIC Particle Sizing Software (icon located on the desktop).
2. Wash out a polystyrene macro-cuvette with filtered DI water 3 times.
3. Fill with 2 – 3 mLs of DI water; use this as the blank. Always run a blank before running the actual sample. When running the blank, change the particle size option to <10 nm. Run the blank starting with step 5; these are the same steps when running the sample.
4. After running the blank put 1 – 3 mLs of sample into cuvette. If sample is less than 1.5 mLs, wrap a piece of scotch tape around the outside near the top of the cuvette; this holds the cuvette high enough so the laser shines through the sample rather than above it.
5. Open the sliding door on top of the instrument to load the sample.
6. Wipe outside of cuvette with kimwipe to remove fingerprints.
7. Remove the black circular cover and place the cuvette snugly in the hole. The laser should shine through the sample. Replace black cover if the cuvette completely fills the hole.
8. Close the sliding lid.
9. In the software, click on New, next to the New Measurements drop down menu. This will open a new window.
10. Click on SOP, and enter the title of your sample in the Sample ID space. Update the Group and Project ID as you need to. Enter your initials in the Notes.
11. Under Parameters, change the dust rejection option to fit with the size of NP that you are analyzing. Keep the set duration to 300 seconds. Leave the Temp at 25.0 deg C.
12. Under Sample Parameters, set the liquid medium to water.
13. Under Sample Parameters, set the Real Refractive Index of the Particles (AgNPs) to 1.333, and set the Imaginary Index to 0.000.
14. Select Uniform Spheres, not Thin Shells.
15. Click "OK" when parameters are set.
16. Once sample is loaded and the sliding door is back in place, click the "start" button on the bottom left. The instrument will take a few seconds to calibrate; do not bypass this step.
17. Numbers on the upper left of the window will change as the sample runs and measurements are taken.
18. If you want the experiment to stop at any time, click the "Stop" button on the top left of the screen (it's where the "start" button used to be) To continue an experiment after you stop it, click "continue" where the "stop" used to be.

19. To clear the experiment and start a whole new one, stop the experiment if it's running, then click "Clear".
20. Each run will automatically save onto the main page of the program. To access it right after you completed a run, simply click the refresh button and it will pop up at the top of the list.

Things to look for and keep in mind as the sample runs:

1. The "Count rate" and "Baseline Index" are in the upper left corner of the screen. You want the count rate to be relatively high, but not more than 600 kcps (kilocounts/sec).
2. The baseline index should be between 9.0-10.0/95.0% - 98.0%. The first number in this ratio indicates whether or not dust might be in your sample. A number below 8.8 or 9.0 indicates that dust might be present. The second number indicates how much of the data collected is being retained. If the numbers are too low or too high, the dust cutoff can be tweaked accordingly.

Appendix C. Protocol for Circular Dichroism.

Protocol from Kira Fahy, adapted from protocol written by Erik Berggren.

Circular Dichroism uses circularly polarized light to detect the chirality of species in a sample. We use it to determine the secondary structure of proteins.

How to turn on/off the instrument and computer (follow the numbers):

1. Turn on the nitrogen tank and the knobs leading to the instrument.
    a. Control the flow – the flow should be between 10-20 (aim for 15) across all three boards
2. Turn on the cooling boxes for the CD and the temperature controller
3. Turn on the power box (Bottom button). Press and hold the button to turn on the lamp. It is on when the green light turns on. DO NOT TURN ON THE LAMP IF THE COMPUTER IS ON.
4. Turn on the temperature controller.
5. Turn on the power cord next to computer power box. Wait a few seconds before turning on the computer.
6. Open Olis Program
7. To turn off instrument: Turn off everything in backwards order. When you no longer need the lamp on, turn off the lamp by pressing the power button (bottom). Keep the nitrogen and cooling box on for at least 5 minutes after the lamp is turned off. DO NOT FORGET TO TURN OFF THE NITROGEN TANK.
8. To turn off computer: Manually shutdown and then turn off the power cord.

How to set up the spectrum scan:

1. Open Olis Program on the desktop
2. Click on the "Data Collection" tab on the onscreen pop-up
3. Click on "DSM 1000"
4. In the "Live Display" Tab, you can set Wavelength, Integration Time, Number of Increments
    a. Wavelength
        i. For NCp7 and CP-CCHC: Far-UV from 190 nm – 290 nm
        ii. For azurin: Far-UV from 190 nm – 260 nm
        iii. For Atox1:
        iv. For HSA: 185 nm- 260 nm
    b. Integration Time:
        i. For HSA: 5 seconds
    c. Number of Increments:
        i. For HSA: 150

5. In the "Repeated Scans" tab, you can change the number of scans. If you do multiple scans, it may come out as a different file format than if you do one (it's easiest to do one).
6. Go back to "Live Display" and click on the Live Mode button. Check whether the voltages (red and blue numbers just below "Current Wavelength") are around 2
7. Start the scan by pressing "Collect Data"
8. Once the scan is finished (it usually takes around 20 minutes), save the scan with appropriate labeling.
9. When you want to do another scan, click on File>New Experiment and set up the new experiment in the same way. Conditions should remain the same in the software.

To save dataset:

1. Connect flashdrive to computer
2. Click anywhere on the graph
3. Right click, and click on "Save Dataset as ASCII"
4. Transfer the saved .asc file on the flashdrive, and remove safely (Do Not Pull Out of the Hard Drive Without Ejecting from the Computer software)
5. Connect to computer with Excel and Process data as XY Scatter with only the line present.

Data Processing in Origin

1. Enter Data in the appropriate columns of the Book# window
2. Leave Units and each column name in the comments sections (e.g. volume of titration)
3. How to Graph data:
    a. In the tab, click on "Plot"
    b. Click on "Line"
    c. Click on "Line" in the subset window
    d. "Plot Setup: Select Data to Create New Plot" Window pops up. Select the X and Y axes by checking the appropriate columns. For numerous data points, select "Y" for the remaining data columns.
4. How to process and smooth data:
    a. In the tab, click on "Analysis"
    b. Click on "Signal Processing"
    c. Click on "Smooth" in the subset window
    d. Click on "Open Dialog"
5. "Signal Processing:smooth" Window pops up.
    a. Select Input graph – which curve you want to be smoothed
        i. Selecting the sideways arrow key will take you to a window where you can click on "Select Columns" and you can choose the curve.
    b. Method: Savitzky-Golay

c. Points of Window: 15
d. Leave other conditions the same
e. Click “OK”
f. On the Legend featured on the graph, you can choose to deselect old curves so only the smoothed curve is present on the graph.

Appendix D. Protocol for Zeta Potential.

Protocol adapted from Wheeler lab standard protocols.

Safety Concern: Instrument is located in the Instrumental lab. It's imperative that you wear a lab coat and goggles when using this instrument.

Running Zeta Potential Samples

1. Open the BIC Particle Sizing Software (icon located on the desktop).
2. Wash out a polystyrene macro-cuvette with filtered nanopure water 3 times.
3. Put about 1.6 mL of sample into cuvette (usually about 5-10 uL of NPs with 1.6mL of filtered nanopure water works well, although you may have to increase the sample concentration slightly to make sure there is enough of a signal).
4. Open the sliding door on top of the instrument to load the sample.
5. Take an electrode from the drawer with the cuvettes and plug it into the internet-cable-like wire in the DLS instrument. Rinse it several times with DI water, then dry it with a Kimwipe.
6. Insert the electrode into the cuvette with the sample. If you have about 1.6 mL of sample, it should cover the metal parts of the electrode without spilling out of the cuvette. Look at the metal parts of the electrode and ensure that there are no air bubbles on the metal surface.
7. Place the cuvette and electrode into the instrument.
8. Close the sliding lid.
9. In the software, in the New Measurements drop down menu, select "ELS Zeta Potential Measurement", then click "New". This will open a new window.
10. Click on SOP, and enter the title of your sample in the Sample ID space. Update the Group and Project ID as you need to. Enter your initials in the Notes.
11. Under Parameters, run 5 cycles under "Manual" to check what kind of readings I'm getting. If the readings go well (not measuring 0 mV), run the actual data with it with "High Precision.".
12. Under Sample Parameters, the liquid medium should be set to water.
13. Under Sample Parameters, The Real Refractive Index of the Particles (AgNPs) is 1.333.
14. Under Sample Parameters, NP size can be inputted, although this is not necessary.
15. Click "OK" when parameters are set.
16. Once sample is loaded and the sliding door is back in place, click the "start" button on the bottom left. The instrument will take a few seconds to calibrate; do not bypass this step.
17. Numbers on the upper left of the window will actively change as the sample runs and measurements are taken.
18. Click the "Stop" button on the top left of the screen to stop the experiment at any time. To continue an experiment after you stop it, click "continue."

19. To clear the experiment and start a whole new one stop the experiment if it's running, then click "Clear".
20. Each run will automatically save onto the main page of the program. To access it right after you completed a run, simply click the refresh button and it will pop up at the top of the list.

Appendix E. Protocol for Fluorimetry.

Protocol from Kira Fahy.

Safety Concern**:** It's imperative that you wear a lab coat and goggles when using this instrument.

Running Samples

1. Follow the instructions that are taped to the instrument itself to turn on the fluorimeter. For best results, allow the lamp to warm up for about 30 minutes before taking data. (Turn on first thing in the morning and let it warm up while preparing the samples)
2. Turn on the monitor for the computer and click on fluorescence program located on the desktop (rainbow icon named FluorEsscence).
3. Click on the "M" located in the middle of the icon bar at the top (Red "M" in a yellow square with a blue border). Then click "Spectra" then "Emission" and then "Next." Then click on "Load".
4. Click on the saved method for this experiment called "Wheeler Fluorescence Quenching Expt with temp control_20180731".
5. Under "Excitation 1" the wavelength should be set at 280nm. Change the slit width to 2nm.
6. Under "Emission 1 [S1]" the start and end wavelengths should be 300 and 400nm. Adjust the slit width to 12nm.
7. On the left-hand side of the window, click on "Accessories." Click the tab that says, "Temperature Controller." The temperature should be set to 20 with a Tolerance of 1 degs. Under "EQ_Time (min)" change to 5 for the first run (the control with no nanoparticle) and back to 2 for each titration point after the control.
8. Load the cuvette into the chamber by lifting the large blue lid and then the smaller black lid. Find the "Q" on the cuvette and point that face toward the "Q" sticker inside the cuvette loading chamber. Place both lids back on and then press "Run" at the bottom of the screen.
9. After the first run, the program will have you title your project – once you name the project be sure to go under File > Save As and move your project file to a designated folder to keep things organized.

Experiment Setup

1. Fill an Eppendorf tube with about 100uL of 1 mg/ml stock HSA solution. This will be diluted 10x with buffer in the cuvette.
2. Using a larger Eppendorf tube, dilute 100mM stock Sodium Phosphate (Dibasic) buffer to a concentration of 5mM.
3. In another eppendorf tube, add 48 uL of Nanopure water and 2 uL of whatever NP you are testing. This will be what you use to titrate with.

4. The cuvette should be rinsed preferably overnight in a 20% nitric acid solution. Rinse with methanol and nanopure water and let dry completely before adding the solutions.
5. When the cuvette is dry, add 252uL of the 5mM buffer solution and 28uL of the 1mg/ml HSA. This gives a total volume of 280 uL, so the HSA is diluted 10x.
6. Wipe the outside of the cuvette with a Kimwipe and mix the solution by pipetting slowly up and down with a volume of about 100uL before loading it into the Fluorimeter.
7. Take only one scan at each titration point, as multiple scans can cause photobleaching of the sample.
8. After the control, add the nanoparticle solution in increments of 4uL until 20uL total have been added (the final volume will be 300uL). This will give six total data points for each trial. Leave the cuvette inside the machine, pipet in the 4uL of nanoparticle solution, and mix again with the pipet.
9. For the 4uL point, click the "Previous Experiment Setup" icon located to the right of the red "M". Change the temperature calibration time to 2 min and Run the sample. For the next four titration points (8uL, 12uL, 16uL, and 20uL), you only need to click the "Auto Run Previous Experiment" icon (looks like a play button) located to the right of the "Previous Experiment Setup" icon
10. After the sample has been scanned, click on the "Data" tab at the bottom of the window with the spectrum. Highlight the X and Y, and copy and paste to an excel sheet to compile all the data.
11. Run the experiment in biological triplicate. Between each trial, prepare a new sample after cleaning the cuvette. Let the cuvette sit in 20% Nitric Acid solution for about ten minutes and rinse with nanopure water and methanol.

µl NP added	**Total Volume in Cuvette**	**Concentration of NP in Cuvette (M)**
0	280	0
4	284	2.66×10^{-12}
8	288	5.25×10^{-12}
12	292	7.77×10^{-12}
16	296	1.02×10^{-11}
20	300	1.26×10^{-11}

Appendix F. Protocol for Passaging Cells.

Protocol from Eva Bouzos.

Cells should be passaged once they reach (or are near reaching) confluency.

1. Place cell culture media (DMEM +10% FBS +1% P/S) and 0.25% Trypsin-EDTA in the 37 ℃ water bath for 10 minutes. Once reagents are warm, use a paper towel to dry the tubes, spray them with ethanol and bring them into the hood.
2. Bring cell culture plate into the hood, aspirate media by tilting the plate away from you and touching the tip of the aspirating pipette to the surface of the media
3. Wash cells by adding 3 mL of media to the side of a 10 mm cell culture plate. Gently rock back and forth to wash all the cells. Aspirate media.
4. Detach the cells from the bottom of the plate by adding 3 mL of 0.25% Trypsin + EDTA directly to the cells. Place plate in incubator for 3 minutes. You will know that the cells have been exposed to trypsin for an ample amount of time when some, but not all of the cells have detached from the bottom of the plate.
5. Deactivate the trypsin by adding 6 mL of media to the plate. Tilt the plate towards you and pipette the media so that it runs down the plate and washes the cells off the bottom. Continue to gently pipette up and down until you no longer see the cells on the bottom of the plate. The bottom should no longer appear to be cloudy.
6. Transfer the cell suspension to a 15 mL centrifuge tube and centrifuge for 5 minutes at 1000-1500 rpm. If the pellet seems to be stringy after centrifugation, then the cells were exposed to trypsin for too long.
7. Aspirate the supernatant and then gently resuspend the pellet in 4 mL of media. The cells are well suspended when you no longer see clumps of cells in the media. It should look the same as media that does not have any cells in suspension.
8. If not counting cells:*
    a. Pipette 8 mL of media into a 10 mm plate
    b. Pipette desired amount of cell suspension to plate
        i. Amount determined by length of time you want between passages
        ii. 200 µL of cell suspension should enable passaging ~2-5 days (HEK)
9. Check cells under microscope. Spray plate with ethanol and place plate on the bottom shelf in the incubator.

*If counting cells and/or plating for an experiment:

a. Transfer 160 µL of cell suspension to micro centrifuge tube. Determine the cell concentration by inserting a cell count cassette into the Moxi automated cell counter. Pipette 75 µL of the cell suspension onto the cell count cassette and record the cell concentration. Repeat the process using the other side of the chip and take the average of the two cell concentrations

b. Calculate the amount of cell suspension needed to obtain *x* cells/well
c. Make the master mix by combining the amount of media needed to have 200 µL per well

Appendix G. Protocol for WST Assay.

Protocol adapted from Eva Bouzos.

The WST assay must be performed across six consecutive days. Protocols for each day are given below:

Preparation Day

1. Passage cells per protocol in Appendix F.
2. Using the cell concentration given by the Moxi cell counter, calculate the the appropriate dilution of cells in media. Each well must have 200 µL of cell/media solution, and there must be 15,000 cells per well.
    a. Plate 2 rows of control cells and 1 row for each concentration of nanoparticle to be tested. Plate 6 columns for each type of nanoparticle that you are testing.
3. Place plates in the incubator and allow cells to attach to the bottom of the plate. Allow 1 day for HEK cells, 2 days for HepG2 cells.

Day 0

1. Wash cells:
    a. Pipette media out of all wells.
    b. Add 50 µL of media to each well.
2. Make nanoparticle dilutions to achieve appropriate concentration of nanoparticles for each test condition. Dilute the nanoparticles in media so that the total volume for each well is 200 µL.
    a. If testing nanoparticle-protein corona condition, add protein to nanoparticles and allow to react for 10 minutes. Then add media.
3. Add nanoparticles:
    a. First, remove wash media that remains in wells.
    b. Then, add 200 µL of nanoparticle/media mix to conditions wells, and add 200 µL of media alone to control wells.
4. Place plate in incubator until day of WST assay.

Day 1

1. Assays for day 1 will be done in triplicate on 3 of the columns per nanoparticle type. Do not touch the other 3 columns for each nanoparticle type, as these will be used for day 3 assay.
2. Wash cells to remove nanoparticles from wells:
    a. Pipette media out of all wells.
    b. Add 50 µL of media to each well.
    c. Pipette media out of all wells.
    d. Add 50 µL of media to each well.

3. Make the WST solution:
    a. 110 µL of solution are needed per well.
    b. All day 1 wells should receive solution, as well as 1 additional row, which has no cells in it. This acts as a negative control.
    c. Calculate the amount of media needed (100 µL * number of wells) and WST reagent needed (10 µL * number of wells).
4. Add WST solution to wells:
    a. Pipette media out of wells.
    b. Add 110 µL of WST solution to each well.
5. Place the plate in the incubator for 2 hours.
6. After the plate has incubated for 2 hours, place the plate in the plate reader to gather data.
    a. Turn on lab computer and plate reader.
    b. Open folder on desktop called "Eva".
    c. Open "WST" script.
    d. Verify that the correct plate type is selected, and check the box indicating that "Plate cover is on".
    e. Select all wells that have WST solution in them which you would like analyzed.
    f. Save the script.
    g. Run the script.
    h. The script will automatically populate an Excel spreadsheet of data. You are interested in the "Mean" column. This is the data that is used for analysis.
7. Perform analysis in Excel.

Day 3

1. Repeat the protocol for Day 1 on the Day 3 wells.

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