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Targeted drug delivery: investigating protein corona behavior

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ABSTRACT

TARGETED DRUG DELIVERY: INVESTIGATING PROTEIN CORONA BEHAVIOR

by
Atharva Markale

Due to high specificity and less toxicity, nanoparticles have become promising for targeted drug delivery against cancer, although they face challenges when they enter the bloodstream, making their behavior unpredictable. The deposition of proteins on nanoparticles, the protein corona, changes their biosignature, affecting their circulation, drug release potency, targeting ability, and immunogenicity. The continuous exchange of protein layers on a nanoscale is complex to analyze.

Nanoparticle tracking analysis (NTA) is a method to characterize nanoparticles in blood plasma by combining principles of light scattering microscopy and Brownian motion. In this work, time-dependent experiments with plasma were conducted to analyze the effect of anticoagulants and types of animal species on protein corona formation. The anticoagulants were found to not affect protein corona formation, although they may be responsible for aggregation. The difference in protein composition between species affects corona formation. Increasing plasma concentration increased competitive binding, giving thicker protein coronas. It is observed that salt molarity affects protein corona formation and depends on plasma build up of species. The binding affinity of proteins decreased corona formation at higher speeds. Surface modification using PEG reduces protein corona formation and aggregation. Protein-based biointerfaces were added using physical and chemical adsorption methods, showing promising results for attaching albumin-based structures on nanoparticles for targeted drug delivery.

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by
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TARGETED DRUG DELIVERY: INVESTIGATING PROTEIN CORONA BEHAVIOR

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“To those who cheered or sledged because it motivates to chase harder!”

I would like to first pray to Ganpati Bappa. I would like to dedicate my work to my parents, Nitin &Geetanjali Markale; grandparents Vishnu &Mangala Markale. I would dedicate this work to my mentor, Dr. Kathleen McEnnis for guiding. I would dedicate this work to my friend Kaylie Green, for high score powerplay.

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CHAPTER 1

INTRODUCTION

1.1 Conventional Cancer Therapeutics

The World Health Organization terms a group of diseases with abnormal growth of cells or tissues which can spread to other parts of the body as cancer, a disease which accounted for 10 million deaths in 2020 [1]. There are various reasons, such as errors in cell division, inheritance of specific genes, or environmental factors that damage DNA, which causes abnormal growth of cells. These abnormal cells grow and multiply, forming clumps called tumors, which may be benign or malignant. Malignant tumors are termed cancer as they invade surrounding tissue and often metastasize across distant organs, disrupting the mechanism of tissues and, in turn, organs [2]. Cancer can develop in various tissues and each type of cancer has its peculiarities, risk factors, and treatment options. Conventionally, cancer treatment can include different methods, such as surgery, chemotherapy, and radiation therapy. The choice of treatment depends on the location of the cancer, grade of the cancer, whether it is curative or palliative, and various other factors.

The surgical option is a type of invasive therapy where the tumor is surgically removed from the patient's body. Less invasive methods such as laparoscopic interventions help in carrying out surgeries in the least invasive and painful manner. However, it is not preferred in the case of cancers that have metastasized across various vital organs or cancers that reoccur.

Radiation therapy uses ionizing radiation to kill cancer cells or cause mutations in cells ultimately resulting in cell death. The high energy radiation damages genetic make

up, hindering cancer cells growth and proliferation abilities. Normal cells that fall on the path of radiation also face damage, although they have faster regenerating and repairing abilities compared to cancer cells. Cancer cells lack this ability to repair fast, thus radiation stops their growth, preventing the further spread of tumors or resulting in cell death [3,4]. The goal of radiation therapy is to destroy or shrink tumors while minimizing damage to surrounding healthy tissues. With advances in radiation technology and imaging techniques, theranostics like 3D conformal radiotherapy (3DCRT), intensity modulated radiation therapy (IMRT), image-guided radiotherapy (IGRT), and stereotactic body radiation therapy (SBRT) have been used to treat various types of cancers. This therapy requires multiple sittings depending on the stage and type of cancer and is often prescribed with combinational therapy [4].

Chemotherapy is a widely used intervention; as its name suggests, it uses chemical agents, anticancer drugs, to kill cancer cells, inhibit cell proliferation, or reduce cancer metastasis. The molecules of anticancer drugs often target the DNA, RNA, or certain protein synthesis processes in tumorous cells, triggering apoptosis [5]. Some classes of anticancer drugs include alkylating agents, which alkylate DNA, RNA, or proteins; antimetabolites, which hinder the DNA and RNA synthesis processes; and anti-folates, which interfere with DNA replication and cell division processes [5]. Moreover there are antimicrotubular agents block molecules which are crucial for DNA synthesis stopping proliferation of the tumor. In combination with these anticancer drugs, antibiotics are also used against the cancer. Chemotherapy is an extensively prescribed therapy for cancer, particularly for patients in early stages to maximize their survival rates. The advantages of chemotherapy are that it has a wide number of drugs which increases number of combinations possible thereby having quick outcomes and

variability in the treatment of cancer. But by nature, chemo drugs are cytotoxic and may induce damage to healthy tissues too.

1.1.1 Disadvantages of Cancer Therapy and Need of Nanotherapy

Although these cancer therapeutics help in mitigating the disease and alleviating pain, they introduce several serious health hazards. Surgical interventions have their limitations depending on the age of the patient, location, and size of the tumor, and the reappearance and type of cancer. Radiation therapies use different types of radiation, which pose health hazards due to long-term effects, time of exposure, the risk to pregnancy and reproductive organs, and the risk of developing secondary cancer. Radiation therapy also causes damage to neighbor tissues, causing skin changes, fatigue, and damage to organs or structures near the treatment site.

Ideally, a chemotherapeutic treatment should deliver anticancer drugs to the specific site in a controlled manner and at optimal dosage, producing no harmful effects on non-cancerous cells. If the chemotherapy drugs are administered orally rather than via the bloodstream, they face metabolic and biological barriers before getting adsorbed and reaching the target. Thus, it forces intravenous drug delivery as the only option to administer antitumor drugs. Chemotherapy brings various side effects such as inducing systemic toxicity, non-specificity in targeting, lower biodistribution, immunosuppression, rapid clearance by the reticuloendothelial system, multidrug resistance, and lower tumor specificity and distribution [5,6]. Patients who have undergone chemotherapy have experienced nausea and vomiting, hypersensitivity, fatigue, neurotoxicity, sterility, infertility, and infusion reactions [5].

To mitigate these adverse effects, there is a need to design a drug delivery system with improved targeting and drug release abilities, increased tumor penetration, better

pharmacokinetics, and reduced systemic toxicity, specifically tailored for effectively targeting and eliminating cancer cells. Nanoparticles are small particles with dimensions on the nanoscale [7] with high surface area, and a high percentage of atoms on the surface having fewer neighboring atoms which gives rise to unique physicochemical properties [8, 9]. By using appropriate synthesis methods, the mechanical, thermal, electrical, optical, and magnetic properties of these nanoparticles can be controlled. This gives nanoparticles a wide range of applications in the pharmaceutical, biomedical, cosmetic, food and material industries.

Chemotherapy is a common method for treating cancer, but it affects both cancerous and healthy cells in the body. This creates a need for more precise and targeted therapies that can effectively combat cancer while minimizing harm to healthy cells. One promising approach is using nanoparticles to deliver drugs directly to cancer cells. By doing so, nanoparticles can help to reduce toxicity and increase efficacy. They can also enhance the accumulation of drugs in cancerous tissues while minimizing exposure to healthy tissues, resulting in fewer side effects for patients. Overall, this targeted approach can improve the quality of life and treatment outcomes for people with cancer.

1.1.2 Types of Nanoparticles

Since it is possible to engineer nanoparticles according to the application, they have shown promising results in invitro studies. When it comes to drug delivery applications nanoparticles are classified based on their nature: organic, inorganic or lipid based [10].

Organic nanoparticles include polymer nanoparticles, classified into polymer nanospheres, micelles, and dendrimers [10]. Polymer nanospheres are solid nanoparticles that contain drugs dispersed throughout a polymeric matrix. Polymer micelles are self-assembled structures with hydrophobic drug cores enveloped by a hydrophilic shell [10].

Dendrimers are highly branched macromolecules with precisely controlled structures suitable for drug encapsulation [10].

Inorganic nanoparticles can be categorized into silica, metallic, metal oxide, and quantum dots. Silicon dioxide-based silica nanoparticles are known for their biocompatibility and versatility [10]. Metallic nanoparticles, typically made of gold or silver, exhibit unique optical and catalytic properties, structural variability although have toxicity and solubility limitations [10]. Metal oxide nanoparticles, such as iron or titanium dioxide, are functional in imaging and drug delivery. Quantum dots are semiconductor nanoparticles with tunable optical properties suitable for diagnostics and imaging [10].

Lipid-based nanoparticles consist of liposomes, emulsions, and lipid nanoparticles and have properties like high bioavailability, payload flexibility, low encapsulation efficiency and formulation simplicity [10]. Liposomes are spherical vesicles formed by lipid bilayers that are versatile enough to encapsulate hydrophilic and hydrophobic drugs. Emulsions are nanoparticles formed by the dispersion of one liquid phase in another. Lipid nanoparticles include solid and nanostructured lipid carriers that offer controlled drug release [10].

In this paper we focus on polymeric nanospheres since we are using polystyrene nanoparticles in our experimentation. Polymeric nanoparticles are submicron colloidal particles suspended in immiscible medium classified as synthetic or natural depending on its source. Here we use polystyrene nanoparticles as a model system for studying protein corona formation. Polystyrene particles are commercially available, highly stable spherical particles, non-biodegradable and have the ability to load dyes thus acting as

ideal particles to study the interactions of proteins in plasma and nanoparticles in a real time microenvironment.

1.1.3 Disadvantages of Nanoparticles Based Therapy

Nanomedicine is a field of medicine that uses nanotechnology to diagnose, treat, and prevent diseases. Its benefits include delivering drugs directly to the disease site, bypassing biological barriers, enabling quicker and more precise diagnostics, and providing tailored treatments to each patient's specific requirements. Moreover, nanomedicine has the potential to address the issues of drug resistance, improve the efficacy of gene, cell, and RNA therapy, and outcomes in deadly diseases like cancer, tuberculosis, and HIV.

Precision drug delivery is the most significant advantage of nanoparticle-based drug delivery; it allows users to control release rates and target specificity by binding appropriate conjugates [11]. Their small size and high surface area increase bioavailability and solubility; thus, they become ideal candidates to cross the tightly bound endothelial junctions, showing enhanced permeability retention effects (EPR) [12]. Reduced dosage, controlled circulation time, and reduced systemic toxicity are advantages that make them ideal for designing cancer therapies.

Nanotherapy is a new field. Hence, it has disadvantages like variability in animal and human model studies, especially with the EPR effect, making it controversial to state it is a moonshot for cancers. Long-term toxicity and effects on vital organs still need to be adequately studied with less publicly available data. The cost of production is high since the synthesis and characterization of this nanoscale particle require high-powered, sophisticated instruments. Another issue nanoparticles face is regulatory approvals, which slow the pace of clinical studies and require lengthy approvals, which adds to the

cost of the product in the market. Although based on Class III device considerations, nanotherapy can be fast-tracked for approvals with preapproved drug combinations. There needs to be known appropriate molecular pathways to follow and alternative pathways to function along with properly established elimination mechanisms [13]. One such interaction of nanoparticles is with the protein microenvironment as soon as the nanoparticles enter the blood. The proteins envelop the particles, forming a layer that changes the biological characteristics of nanoparticles, which we will discuss in the next section. The shelf life of most nanoparticles is short and often requires special storage conditions.

1.1.4 Formation and Dynamics of Protein Corona

As soon as the nanoparticles are parentally administered, they come in contact with blood, a mixture of erythrocytes, electrolytes, and different proteins. When the nanoparticles enter this protein-rich medium, a protein layer gets deposited, enveloping the nanoparticle. This envelope of proteins over the nanoparticles is termed a protein corona [14]. The microenvironment in blood is dynamic, and due to free available protein and their high concentration in plasma, the protein corona becomes dynamic. It is often classified into soft and hard corona [15]. The soft corona is the outermost layer in which proteins having lower affinity are loosely bound, facilitating rapid exchange of proteins in and out of the protein corona [16]. The continuous exchange of proteins and unstable nature of soft corona makes it hard to isolate and characterize. Due to continuous exchange of proteins, there is a steric hindrance that comes into picture making protein-protein interaction more prevalent in deciding biological identity of the particle [16]. A hard corona is a tightly bound layer of proteins with a more stable protein complex and with less exchange of proteins. Since proteins with higher affinity make up the structure

of hard corona the biological identity is more dependent on protein nanoparticle interaction [16]. Protein corona formation is a complex process involving major interactions- 1) Between nanoparticle surface and protein 2) Between protein and protein 3) Between nanoparticle and medium [17]. These interactions are results of forces like van der Waals, electrostatic hydrogen bonds, hydrophobic interactions, π–π, Columbian and salt bridge forces. Protein-nanoparticle interactions are significantly influenced by the Van der Waals interaction. Larger nanoparticles show stronger van der Waal interactions, while proteins with flexible conformations can enhance contact and interaction strength [17]. Unfolded proteins usually bind more strongly than folded ones. Hydrogen bonding plays a crucial role in intermolecular interactions, with proteins offering a plethora of hydrogen donors and acceptors. In aqueous solutions, water molecules also act as hydrogen donors/acceptors, reducing contribution of H-bonding. Hydrophobic interactions arise from excluding ordered water molecules from nonpolar surface and tend to bind tightly to unfolded proteins due to exposed hydrophobic residues. protein-nanoparticle interactions are also influenced by π-π stacking interaction between aromatic rings, especially with sp2 carbon nanomaterials [17].

The protein corona is responsible for the biological identity of nanoparticles. The protein corona formation is an irreversible process resulting from complex intrinsic factors like nanoparticle size, surface area, morphology, material composition, shape, surface chemistry, and charge, which decides the fate of protein-nanoparticle interaction. Extrinsic factors include the ionic strength of the medium, protein concentration, pH, medium temperature, and composition.

Extensive studies have been conducted on the relationship between the size of nanoparticles and protein corona. It is seen that decreasing the size of the nanoparticle

increases the interaction with protein in a physiological medium, thereby increasing the size of the protein corona [14, 18]. The nanoparticle size affects factors like protein binding affinity and the curvature radius, which affects protein adsorption, leading to the varied composition of the protein corona [14]. Size and shape are other critical parameters that affect protein corona. There have been studies with nanorods, nanospheres, and nanotubes of different sizes, resulting in varied protein corona composition and formation. However, not much about biological identity and effects has been explored in the studies [19]. Another primarily studied parameter is the effect of charge on the nanoparticle surface. It is found that nanoparticles with a neutral charge of the surface have less protein corona formation [14]. A positively charged surface can trigger opsonization- the detection of surfaces by opsonins, which eliminates nanoparticles from the system and causes accumulation in vital organs like the liver [20]. Another critical factor that affects the nanomaterial and protein interaction is surface functionalization, which reduces protein absorption on the surface and gives stealth-like properties that help nanoparticles escape opsonin and immune checks. One such surface functionalization strategy is adding polyethylene glycol (PEG) coatings. Other parameters, like the hydrophobic or hydrophilic nature or the chemical nature of particles, influence protein corona formation.

Now let us overview the extrinsic factors dependent on the physiological medium responsible for protein corona formation. Protein corona is a time-dependent phenomenon. In previous work it was seen that the amount of protein in the corona did not significantly vary according to time. However, its composition varied, showing a continuous exchange of proteins in and out of the corona [21]. Temperature is another crucial aspect that may affect protein corona, although only a few studies have been

performed using blood or plasma. The ones carried out in protein solutions suggest that increased temperature increases protein adsorbed on the nanoparticle surface [22]. Protein concentration is another factor that plays a role in protein corona formation. As protein concentration increases, the protein corona's size increases. We have explored this idea in detail in upcoming chapters. Another factor is pH; proteins attach less to the surfaces of nanoparticles when the pH values differ significantly from the isoelectric point, forming less protein corona. The protein molecules on the surface repel other proteins due to strong electrostatic forces [23]. This fact is important when designing nanoparticle-based delivery for cancer because the pH of the tumor microenvironment is slightly acidic. One more factor is ionic strength, which affects protein corona; increasing ionic strength decreases negative surface potential, decreasing the absorption of proteins thus reducing protein formation [24].

Since blood plasma is a protein-rich medium, protein corona formation cannot be eliminated. There is little or no control over the environmental factors affecting protein corona. However, there is the possibility of controlling the intrinsic factors of nanoparticles while designing drug delivery applications. One approach to reduce protein corona and aggregation is adding coatings to particles.

1.1.5 Controlling Protein Corona for Drug Delivery

In the delivery of therapeutic payloads, manipulating the protein corona provides a nuanced approach to influence nanoparticle therapeutic agents' release kinetics and efficacy. The protein corona governs the destiny of nanoparticles and their internalization into cells. There are efforts to minimize immune recognition and clearance by enhancing the stealthiness of nanoparticles. This involves purposeful engineering of nanoparticle surfaces to exert control over the composition of the protein corona, thereby influencing

critical factors like biodistribution, cellular uptake, and therapeutic efficacy. Functionalizing techniques include engineering nanoparticle surfaces with PEG, protein interfaces, attaching ligands and zwitterions.

PEGylation: Once nanoparticles enter the bloodstream they get covered with proteins and the protein layer is responsible for circulation time, targeting and clearance time and mechanism. Opsonin like fibrinogen and gamma globulins increase macrophage detection and trigger faster phagocytosis [25, 26]. To avoid this polyethylene glycol (PEG) is used to increase the circulation time, solubilize hydrophilic drugs, provide stealth from macrophage detection – immunogenicity [27]. PEGylation discourages corona formation by obstructing protein-binding sites and creates a thermodynamic barrier to protein diffusion [28]. By controlling factors such as the molecular weight it is possible to optimize corona formation depending on the application. PEG also allows attachment of proteins, ligands, and antibodies to nanoparticles, thus it has a wide application in the pharmaceutical industry.

Zwitterion: When a molecule contains an equal number of positive and negatively charged functional groups, keeping the overall net charge of the molecule zero, it is zwitterion [29]. Zwitterionic materials possess antifouling properties because of their hydrophilic solid surface and high electrostatic interactions, which keep the net charge on the molecule neutral, reducing protein corona formation [28]. The high dipole movements due to equal solid charges on the surface produce high electrostatic interactions with water, forming hydration layers that keep protein molecules away from the surface of nanoparticles [30]. Zwitterionic nanoparticles are relatively new, and their potential in preclinical and clinical tests remains untapped.

Protein coatings: Protein coating is a process of attaching proteins to nanoparticles to modify their properties. It enhances biocompatibility, stability, and targeted delivery in biological systems, and can reduce immunogenic responses. These coatings are classified in two parts. The first part is coating nanoparticles with specific protein coatings such as albumin or immunoglobulin, depending on the application for drug delivery, and the second part is precoating nanoparticles with plasma. One of the most successful approaches is adding albumin coatings to nanoparticles, offering higher biodistribution, more bioavailability, lower toxicity, and immunogenicity [31]. Proteins also can target tumor sites actively or passively to receptors [32, 33]. Many of their drugs are studied in different phases of clinical trials. One such FDA-approved albumin-coated nanoparticle drug used in treating breast cancer is Nab-Paclitaxel [34].

Since immunoglobulins are major opsonins that help in the recognition and early clearance of nanoparticles in the system, a novel approach is utilized for preincubating nanoparticles with IgG-removed plasma. The nanoparticles were incubated in plasma without IgA to form a protein corona and then were exposed to the in vitro system.The research yielded that nanoparticle had less interaction with macrophages, and the particles exhibited stealth properties even after reintroduction in plasma [35]. Another proposed approach is precoating nanoparticles with patient plasma since protein corona buildup is patient-specific and can yield better results in increasing circulation time, preventing protein corona formation and having better-targeting capabilities with lower toxicity and higher bioavailability. These proposed mechanisms are limited to in vitro studies and lack evidence or preclinical data for successful application.

1.1.6 Objectives of Research

The research objective is to analyze the effect of varying the species of blood plasma on the protein corona. We also investigate if the type of anticoagulants affects protein corona formation. The experiments are done for 24 hours, taking readings at fixed intervals to look at protein corona formation over time in different dilutions of blood plasma. We also vary the speeds of the nanoparticles during measurement to find if the measurement speed affects the shear experienced by the particle and the protein layer developing over it. We also experimented with changing salt concentrations and how increasing concentration affects the formation of protein layers on nanoparticles. Towards the end, we develop biointerfaces to reduce protein corona formation by using PEGylation and coating with albumin, the most abundant protein found in plasma.

CHAPTER 2

NANO TRACKER ANALYZER NS 300

2.1 Characterization of Protein Corona

The protein corona is dynamic in nature and influences the biological identity of nanoparticles which is responsible for cellular uptake, immune recognition, clearance, and toxicity. Hence it is necessary to explore the structure and composition of protein deposition on nanoparticles. Characterization of the protein corona is either done using direct methods to count adsorbed protein on the nanoparticle surface or by indirect methods which use analytical techniques to estimate amount of protein and its composition [36]. To directly measure the protein corona structure or composition it is required to separate the nanoparticles in the physiological medium. This requires their removal from medium using separation techniques which often results in loss of the soft corona [37]. Direct structural analysis of the protein corona uses techniques like circular dichroism (CD), fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) and atomic force microscopy (AFM). Direct protein corona quantification techniques include using assays, mass spectroscopy (MS) and electrophoresis [36].

Indirect methods commonly used to measure size are dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), and diffuse correlation spectroscopy (DCS). Absorbance based techniques include surface plasmon resonance and ultraviolet visible spectroscopy (UV VIS). To quantify charge laser doppler anemometer (LDA) and tunable resistive pulse sensing (TRPS) is applied. For temperature-based measurement isothermal titration calorimetry (ITC) is applied, and for quantifying mass, quartz crystal

microbalance (QCM) is used [36]. For this thesis, we focus on indirect methods to measure protein corona size using nanoparticle tracking analysis. In the upcoming section we will focus on the working principle of NTA and its advantages over DLS.

2.1.1 NTA

Nanoparticle tracking analysis (NTA) is an analytical technique that helps uncover the complex behavior of nanoparticles with sizes between 10 – 1000 nm suspended in a liquid. The process starts by preparing and illuminating a liquid sample with a laser to induce light scattering. The microscope then focuses on the illuminated area, and a high-speed charged coupled camera captures the scattered light to record the Brownian motion of individual particles. Specialized software is used to analyze the video and track particle trajectories, which helps to determine particle size and concentration. The Stokes-Einstein equation is a critical component of NTA, which provides insights into particle size distribution by relating the diffusion coefficient of the nanoparticles to their size, temperature, and viscosity [38-43].

Strokes Einstein Equation: When any small particles are dispersed in a liquid, they move randomly in all directions. This is called diffusion, and it is measured by the diffusion coefficient. The particles move due to the energy transferred to them by surrounding water molecules. Although the particles move randomly, over time, their movements in any direction should balance out, resulting in almost zero net movement. However, when particles are observed over short time intervals, they move within specific volumes, and their movement can be recorded and measured as the mean square displacement, which is used to calculate the diffusion coefficient in different dimensions [42].

$$D = \frac{(x)^2}{2t} \qquad D = \frac{\overline{(x,\ y)^2}}{4t} \qquad D = \frac{\overline{(x,\ y,\ z)^2}}{6t} \qquad \text{…….(1)}$$

From the Stokes-Einstein relationship the particle diameter, d, can be calculated as function of the diffusion coefficient,D, at a temperature, T, and viscosity, η, of the liquid and Boltzmann's constant, k_B.

$$D = \frac{4k_B\,T}{3\pi\eta d} \qquad \text{……..(2)}$$

Since NTA looks at particles in two dimensions, x and y, we now solve the equation

$$d = \frac{4k_B\,T}{3\pi\eta t} \cdot \frac{4t}{(x,\ y)^2} = \frac{16k_B\,T}{3\pi\eta\overline{(x,\ y)^2}}$$

NTA simultaneously tracks several particles to determine their diameter. NTA operates in two modes, scattering mode and fluorescent mode with different filters supporting specific wavelengths. Here we use plasma which has a high amount of protein concentration which causes noise in scattering mode, not allowing the NTA to track particles. As we have fluorescently tagged particles, we switch to fluorescent mode in which NTA blocks all other light except the one it detects from fluorescently tagged objects. In this case we use yellow-green FluoSpheres with excitability at 505nm and emission at 515 nm and hence we work on laser with blue light with 488 nm wavelength and long pass filter of 500 nm. Every time we appropriately select the camera level and intensity by looking at the screen to optimize the quality of the image, which in turn is responsible for getting accurate sizes of particles.

CHAPTER 3

EFFECT OF TYPE OF ANTICOAGULANT AND PLASMA ON PROTEIN CORONA FORMATION

3.1 Methodology

Plasma Extraction: Bovine and goat blood with two variations of anticoagulants, Alsever's solution and sodium citrate, were purchased from Lampire Biological Laboratories. The obtained blood was centrifuged at 37^0C at 3000 rpm to separate out hematocrit from plasma. The suspended layer of plasma was removed and again the plasma was centrifuged. This process was repeated thrice to obtain clear yellow plasma solution. The obtained plasma solution with anticoagulants were stored at 4^0C for enhanced shelf life.

Protein Analysis: To measure the protein concentration in the plasma solutions we choose albumin as reference protein since albumin consists of more than 70% of proteins in plasma. Bovine albumin was used since plasma has maximum content of albumin, about 70%. 5 stock solutions were made with 1 to 5 molarity of albumin. A standard UV/VIS absorbance curve was plotted using these stock solutions. Plasma solutions with 10 µl, 100 µl, 500 µl, 1000 µl, and 1500 µl of plasma were made up to 2000 µl using saline solution. This step was done for both types of plasma with both anticoagulants. The absorbance values of the plasma samples were found, and the data was normalized using the known molarity absorbance values. This gave the albumin concentration in all the solutions allowing for comparisons across species and anticoagulant type to account for the differences in protein concentrations.

Viscosity measurements: A Ubbelohde viscometer was charged with the specified fluid using, ensuring the sample level was between the demarcated lines. Rubber tubing was connected to the top of the viscometer, and the viscometer was immersed in a 37^0C liquid bath. Gentle suction was initiated until the liquid ascended above the upper timing mark.The liquid was allowed to flow and the flow time for the liquid to pass the flow marks on the viscometer tube was recorded. This time was multiplied by viscometer constant and averaged over 10 readings to determine the viscosity of plasma solution [44].

24 hours experiments: The above solutions with different volumes of plasma were made for goat and bovine plasma with Alsever's solution and sodium citrate solution. 10 ul of 100x diluted fluorescent-labeled nanoparticles were incubated in plasma solutions, and NTA measurements were taken after equal intervals of time. The nanoparticle-embedded plasma solutions were kept at 37^0C in a incubator with a rotator with a speed of 25 rpm. The solutions were taken out of the incubator during the measurement and loaded into a syringe. The syringe was placed on a pump that loaded the colloidal solution in a microfluidic chip mounted inside the NTA for measurement of nanoparticle sizes. The NTA temperature was constant at 37^0C to mimic physiological temperature conditions. 10 videos, each lasting for 60 seconds, were captured one after another under a continuous flow rate at 50 AU.

Since plasma has proteins that create noise in the readings, we used fluorescently labeled nanoparticles with a laser of 488 nm wavelength and a long-pass filter of 500 nm. After each reading, the solution was taken off the syringe and inserted back into the stock under rotation at 37^0C. NTA uses the viscosity of water as the standard viscosity for particle size calculations. . Hence, we normalized the particle size according to the

measured viscosities of the plasma solutions. Since NTA measures diffussion coefficient and uses strokes einsteins equation to estimate size of nanoparticles. Since visosity and temperature are variables in strokes einstein equation we take in consideration their effects while determining nanoparticle size. As the viscosity of water, plasma and saline varies we multiply the NTA size by the ratio of the standard water viscosity from the NTA to the plasma solution-specific viscosity to calculate the actual size. To measure protein corona, we subtract the nanoparticle size in the plasma solution from the nanoparticle size in saline. Since the particles are spherical, the thickness is accounted twice for both hemispheres of the sphere, so we half this obtained thickness to get the real thickness of protein corona on nanoparticles.

The particle size data was used analyzed via statistical tests like ANOVA to determine statistical differences between groups.

3.1.2 Results and Discussion

Plasma with different volumes, such as 10 µl (0.5% v/v), 100 µl (5% v/v), 500 µl (25% v/v), 1000 µl (50% v/v), and 1500 µl (75% v/v), were taken and made up to 2000 µl using saline as a physiological medium. This process was carried out for goat and bovine plasma with sodium citrate and Alsever's solution as an anticoagulant. Increasing the plasma volume, irrespective of blood species type or anticoagulant type, was observed to increase the average size of the nanoparticles captured over 24 hours. The increase in plasma volume increased the concentration of protein content available in the solution in which nanoparticles were incubated. Increasing the protein content in the solution will increase the number of known protein molecules which will try to bind to the nanoparticle's surface. According to Langmuir's adsorption theory, several proteins in a solution influence the protein adsorption rate on the surface [16, 46]. Hence, more

available proteins will increase the adsorption rates, giving rise to thicker protein corona. Another way to explain this is according to binding site theory. The binding sites on the nanoparticle surface are constant and experience a continuous exchange of proteins. However, when there is higher protein availability after the formation of the hard corona in minutes, more proteins compete to stick to the surface, giving rise to a thicker protein corona over time, which increases the size of the protein corona formed.

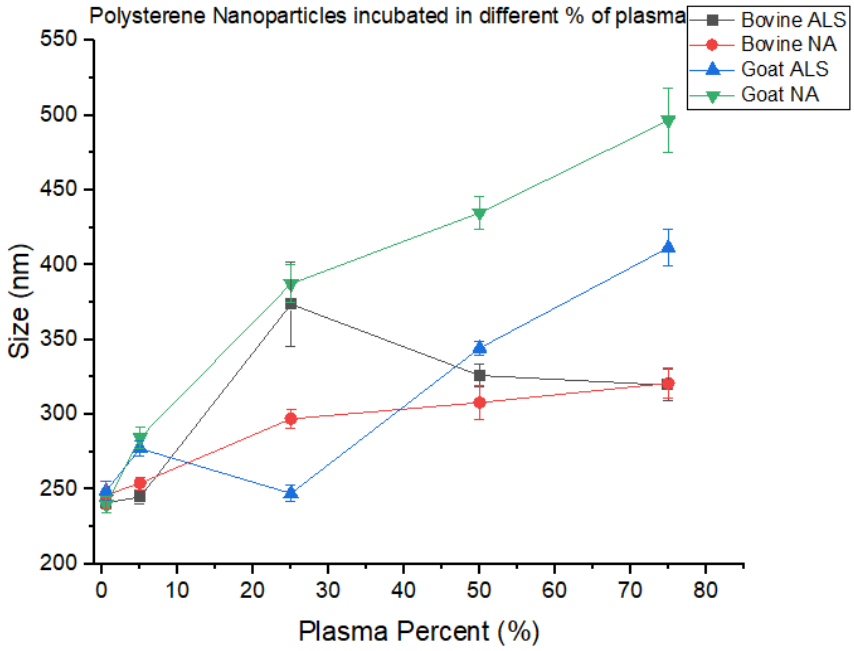


Figure 3.1 Size of polystyrene nanoparticles incubated in different types of plasma solutions at different % of plasma.

Appendix B shows graphs comparing nanoparticle size for all concentrations across both species and anticoagulants. When it comes to aggregation of the particles with components of the blood plasma, we qualitatively observed an increase aggregation with an increase in the concentration of protein content, however quantitative measurements of this multi-component aggregation are still ongoing. This increase in aggregation can be explained by increased protein-protein interaction, and higher collisions at higher concentrations will cause uneven folding or even denaturation of

proteins, giving rise to clumped protein sets that interact with nanoparticle surfaces and increase aggregation.

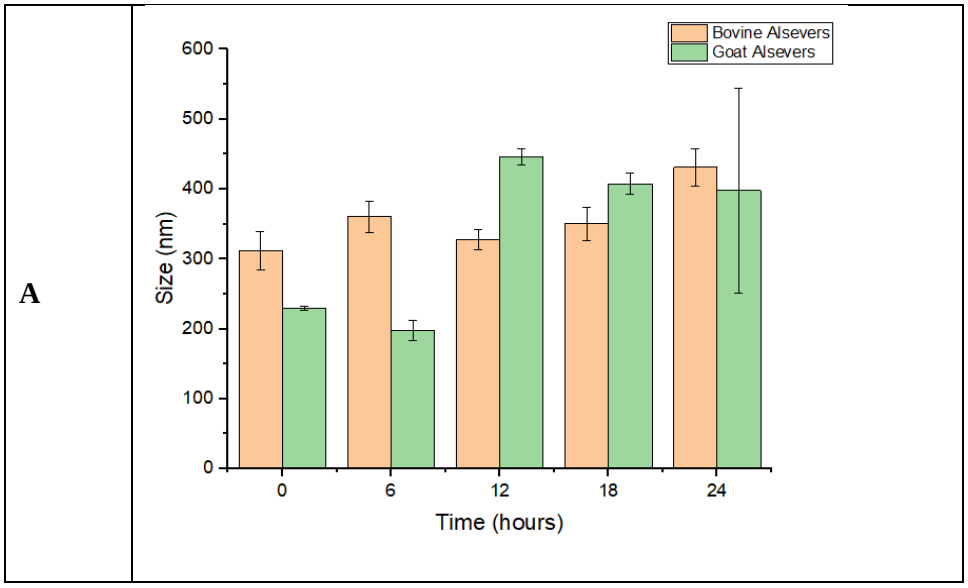
Similar studies were carried out previously on silica nanoparticles in a protein medium characterized by DLS, showing increased protein corona formation by increasing protein concentration [47]. The study showed that a higher protein corona gives rise to a thicker, harder corona. Another experimental study on liposomes by Digiacomo et al. pointed out that increasing protein availability in solution will increase protein corona formation and aggregation [48]. The zeta potential values showed a reduction in increasing protein concentration, pointing to the fact that there is an increase in the measured size of nanoparticles.

When average values of the amount of plasma vs size were graphed in Figure 3.1 it was visible that all the bovine plasma samples were similar and all the goat plasma samples were similar, however bovine plasma samples were different from the goat plasma samples. It was clear that anticoagulants have no significant effect on the plasma size. ANOVA test was performed between the groups of plasma comparing the variables of anticoagulant and species. A significant difference was found between average sizes of nanoparticles between goat and bovine plasma with the same anticoagulant. No significant difference was found in nanoparticle sizes between the the same species of plasma with either Alsever’s solution or sodium citrate solution. Anticoagulants' primary function is to stop blood clotting and preserve proteins in plasma. Since Alsever’s solution and sodium citrate plasma are made up of plasma-to-anticoagulent ratios of 1:1 and 9:1, respectively, there is a difference in protein content for the same amount of plasma volume. Hence, after normalizing the protein content axis, we see in Figure 3.1 that the nanoparticle size is larger in sodium citrate plasma solution irrespective of

species due to the higher plasma concentration. The dynamics of this phenomenon are discussed earlier for the protein corona vs plasma content experiment.

However, using Figure 3.1 when we compare the two anticoagulants with the same protein content, there are no significant differences in the pattern of increase or decrease in protein corona formation. The composition of the two salts is different. According to Lampire Biologics, the Alsever's solution is composed of 9.5 g/L sodium citrate, 21 g/L dextrose, 4.25 g/L sodium chloride, and 10 ml/L of 5.5% citric acid in deionized water. In contrast, sodium citrate is made from 38 g/L sodium citrate solution in deionized water. Ideally, the salt solutions are dissolved completely in whole blood, so even after the cells are removed, the salt is still evenly dissolved in the extracted plasma. Plasma is used instead of whole blood to avoid clogging the microfluidic channels of the NTA with the cells. As the hematocrit gets separated from plasma, the viscosities of different plasmas do not cause many differences; thus, no significant effect of anticoagulants is observed for protein corona formation on nanoparticles in plasma. Refering to figure 3.1 nanoparticle sizes in goat and bovine plasma showed significant differences compared to the same anticoagulant. Goat blood contains 7.5 g/dl of proteins compared to bovine blood, which has 6.6 g/dl [49]. Albumin, which is responsible for maintaining osmotic pressure and is involved in transportation, is the most abundant protein found in the blood. It is 3.4 g/dl in goat blood and 2.2 gm/dl in bovine blood. The globulins, which are responsible for immune function, are bulkier and of higher molecular weight than albumins. Goat blood contains 4.1 g/dl, and bovine blood contains 3.8 g/dl of globulin [49]. These numbers vary due to differences in genetic makeup, breeds, presence of disease, hydration state, and various other factors. However, we can safely use the range of values to derive general results. These differences in the

composition of proteins in different species cause differences in protein corona formation in goat and bovine plasma. The higher aggregation and protein corona formation rates observed in goat plasma are likely due to a larger amount of α1-globulins and α2-globulins, which increase immune activation. The increased protein content in goat blood increases protein-nanoparticle interactions, and a higher concentration of protein present in the plasma inhibits competitive binding, increasing protein layering. Muller and Simon, in their study, included sheep, mice, rabbits, and human plasma to study the effect of species on protein corona formation using DLS and SDS PAGE [50]. They observed differences in protein composition, especially immunoglobulins, across species, affecting aggregation, protein corona formation, and cellular uptake rates [50].The blood proteome was different in each species and each sample due to genetic variation, breed differences, presence of disease, genetic makeup, diet consumed, hydration state and several other reasons. These various factors are to be considered while translating research into clinical models as these variations cause changes in the expression of proteins in the blood, creating unique protein corona.



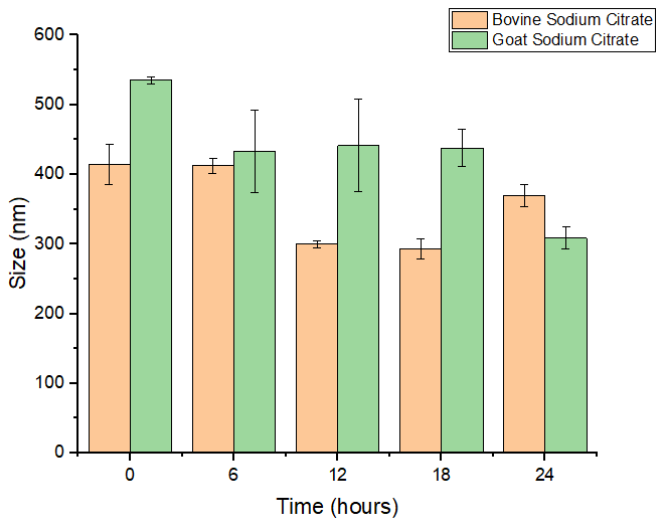
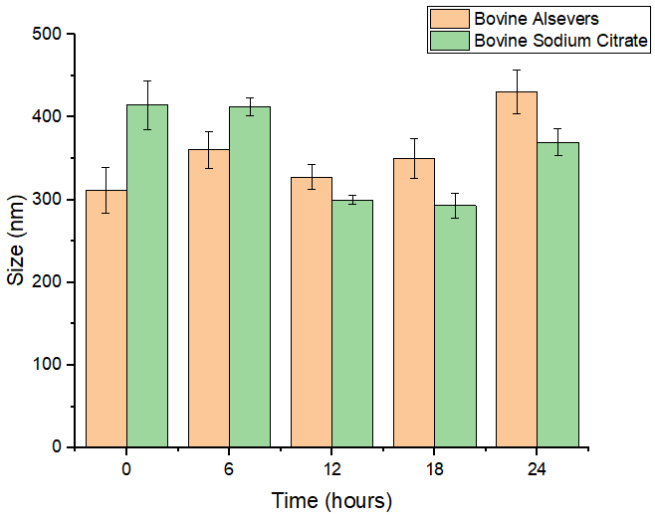
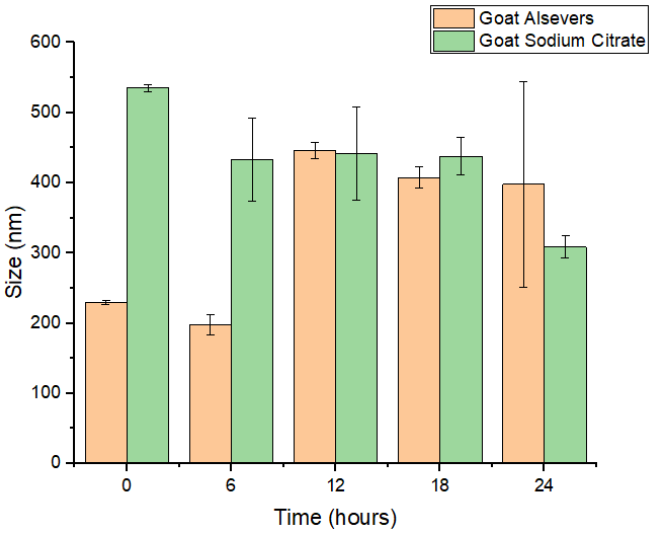
B	 Bovine Sodium Citrate Goat Sodium Citrate Size (nm) 0 100 200 300 400 500 600 0 6 12 18 24 Time (hours)
C	 Bovine Alsevers Bovine Sodium Citrate Size (nm) 0 100 200 300 400 500 0 6 12 18 24 Time (hours)
D	 Goat Alsevers Goat Sodium Citrate Size (nm) 0 100 200 300 400 500 600 0 6 12 18 24 Time (hours)

Figure 3.2 Size of polystyrene nanoparticles incubated in 100% plasma A: Comparison of species with the same anticoagulant (Alsever's solution), B: Comparison of species with the same anticoagulant (sodium citrate), C: Comparison of anticoagulants with the same species (Bovine), D: Comparison of anticoagulants with the same species (Goat)

CHAPTER 4

EFFECT OF INJECTION SPEED OF NANOPARTICLES ON PROTEIN CORONA FORMATION

4.1 Methodology

Following the experimental procedure of the 24 hours experiments presented in chapter 3 involving plasma dilutions with fluorescent particles, we ran a study to check if changing the speed of the syringe pump on the NTA changes the shear forces experienced by the nanoparticles and its effect on the size of the nanoparticles. All variations of plasma volumes (10 µl, 100 µl, 500 µl, 1000 µl, 1500 µl) were mixed with the appropriate volume of saline to make it up to 2000 µl and 20 µl of 100x diluted polystyrene nanoparticles were added to it. The syringe pump speeds were changed to 10, 20 and 30 AU and all other settings on the NTA were kept constant to find effect of speed on formation of protein corona.

4.1.2 Results and Discussion

In order to study the effect of speed on the injection of the nanoparticles on protein corona formation, the experimental setup was done as described in the methodology. This experiment was carried out for different volumes of plasma-to-saline combinations. Figure 4.1 represents results from the 500 µl plasma in 1500 µl saline solution, although this pattern of slopes is observed across different combinations as shown in Appendix C. It is observed that there is smaller protein corona formation at higher speeds and larger protein corona formation at lower speeds. At higher speeds, there is less time for protein-nanoparticle surface interaction time. Thus, it allows protein corona to develop fully, forming a small corona later. For lower speeds, the protein-to-nanoparticle surface

interaction is more significant, which allows the protein corona to mature, and then a thicker layer is developed.

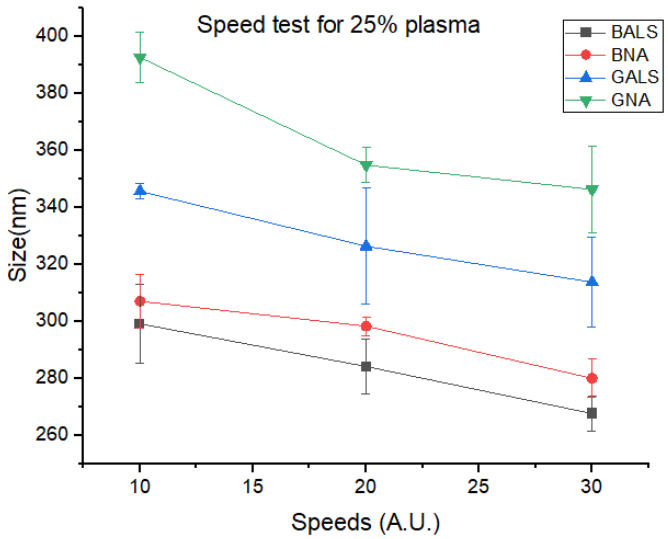


Figure 4.1 Size of polystyrene nanoparticles incubated in different types of 25% v/v plasma and measured using NTA at different syringe pump speeds. BALS- Bovine Alsevers, BNA- Bovine Sodium Citrate, GALS- Goat Alsevers, GNA- Goat Sodium Citrate.

Every particle moving in fluid experiences a drag force, which depends on several factors such as the speed of the moving particles, surface roughness, viscosity of the medium and sectional area of the body. At the nanoscale, the cross-sectional area is small and hence it can be ignored. Hence, the drag experienced is directly proportional to the velocity of the particles. The nanoparticles exist in colloidal solution, which makes them prone to Brownian motion. With external flow, the particles start flowing in a particular direction, although in a zigzag path. This movement causes particles to experience shear due to the relative motion between adjacent fluid layers in the plasma solution. The forces exert mechanical stress on the plasma proteins, potentially altering their conformation and exposing specific binding sites. Only proteins with high binding efficacy bind to the exposed site during these conditions. This selective adhesion caused by high-affinity binding sites is caused by conformational changes that take in proteins

due to shear forces. An increase in shear forces between nanoparticles and plasma proteins enhances their affinity-driven association, facilitating the exposure of specific protein binding sites and promoting interaction with complementary sites on the nanoparticle surface. Thus, at lower speeds, the lower and higher affinity proteins compete to bind, giving thicker layers of proteins on the surface of nanoparticles, whereas, at higher speeds, the shear alters the confirmation of proteins, allowing higher affinity proteins to bind the surface of nanoparticles.

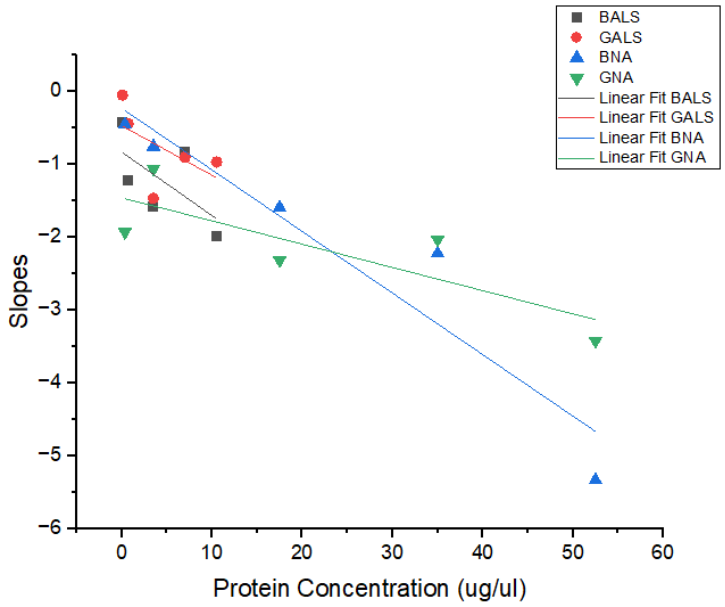


Figure 4.2 Linear fits for size of polystyrene nanoparticles incubated in different types of plasma measured by NTA at different syringe pump speeds. BALS- Bovine Alsevers, BNA- Bovine Sodium Citrate, GALS- Goat Alsevers, GNA- Goat Sodium Citrate.

Table 4.1 Statistical Values For Linear Fits For Slopes

Equation	**$y = a + b^2x$**			
Plot	**BALS**	**BNA**	**GALS**	**GNA**
Weight	No Weighting			
Intercept	-0.8344 ± 0.36	-0.23403 ± 0.34	-0.47178 ± 0.34	-1.46037 ± 0.37
Slope of Fig 4.2	-0.08716 ± 0.06	-0.0845 ± 0.016	-0.0679 ± 0.05	-0.03188 ± 0.01
Residue sum of squares	0.91751	1.48375	0.8106	0.93718

The pattern of a decrease in protein corona thickness with increased injection speed was continuously observed across all plasma values. Additionally, when the slopes

of speed tests conducted for all concentrations were plotted, they showed a decreasing trend with increased protein content. It can be hypothesized that there is a general trend of wearing off proteins on nanoparticles with increased protein concentration. At lower concentrations of proteins in solution, there is less competition on binding; thus, a hard corona is formed quickly. At higher concentrations, hard coronas take time to form due to the higher availability of proteins where proteins in abundance stick to the surface first, and then proteins with higher affinity get stuck on the surface.

Linear fits and R square tests were performed for further analysis. It was observed that the R-squared values for Alsever's solution and sodium citrate solutions were similar, irrespective of species. This shows that anticoagulants do not affect the wearing off of the proteins on the nanoparticles.

In the speed experiment significant size differences in protein coronas were observed across species, with goat plasma yielding a thicker protein corona than bovine plasma, attributed to the higher inherent protein concentration in goat plasma. These species-specific variations underscore the importance of tailoring nanoparticle formulations based on the specific plasma composition of the target species. The experiment also confirms the result of increase in particle size with increase in protein corona size when the data of the speed experiment is sampled according to volume of plasma at the same speed. Moreover, the choice of anticoagulant did not significantly affect particle size within the studied parameters, suggesting that anticoagulant selection may not be a critical factor influencing nanoparticle size dynamics. These findings in speed experiment reiterates the validity of results we obtain in the 24 hours experiment.

CHAPTER 5

ANALYZING SALT CONTENT ON PROTEIN CORONA FORMATION

5.1 Methodology

We follow the same procedure for loading nanoparticles and making the samples as stated in the 24 hours experiment section in chapter 3. Instead of NaCl, we use potassium chloride (KI) salt to study whether the increase in the molarity of salt solution changes protein corona formation. We made a 50% (v/v) solution of bovine Alsever's solution plasma for this test. The salt content is varied to make different salt solutions of 2%, 4%, 8%, 16%, 32% and 64%. Again, here we repeat the procedure of the 24-hour experiment by keeping 50 AU as the flow rate, 60-seconds capture rate with ten captures, and taking readings after each fixed time interval for 24 hours.

5.1.2 Results and Discussion

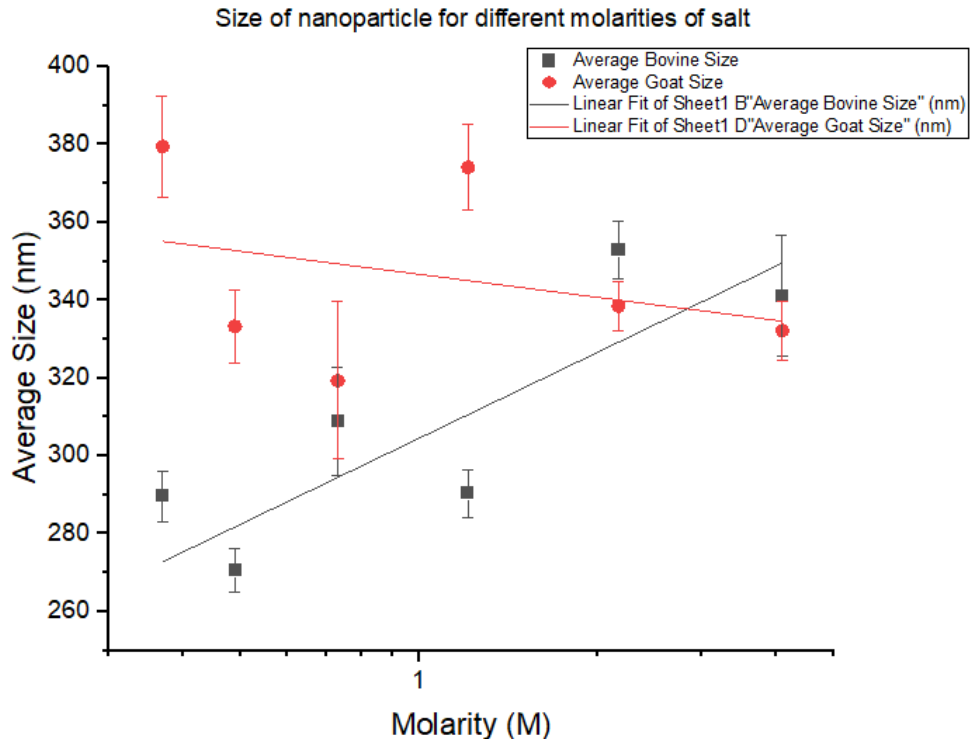


Figure 5.1 Size of polystyrene nanoparticles incubated in different types of plasma at different molarities of salt.

Different molarities of potassium iodide (KI) salt mixture was made and mixed with goat plasma and bovine plasma to study effect of molarity of salt on nanoparticle size and effect of salt on plasma species. Potassium iodide was chosen instead of sodium chloride because at higher molarities sodium chloride crystallizes. KI on other hand at higher molarities doesn't show this behavior. The average nanoparticle size suspended in bovine plasma showed a downtrend in protein corona size as the molarity increased and the solution neared its saturation point. On the other hand, an increase in size was observed for goat blood as its molarity increased. The variations in protein corona development at different salt concentrations in bovine plasma and goat blood can be attributed to several factors. Firstly, the distinct compositions of plasma proteins in these species play a pivotal role, as the protein corona forms by the adsorption of proteins onto nanoparticle surfaces upon interaction with biological fluids. Additionally, fluctuations in ionic strength, influenced by increasing molarity, may affect electrostatic interactions between nanoparticles and proteins differently in bovine and goat systems. The observed alterations in particle size approaching the saturation point could be linked to changes in available surface area for protein adsorption. At lower concentrations, a more extensive protein corona may form due to increased surface area, while near saturation, reduced general surface area may result in a smaller corona. Furthermore, species-specific interactions between proteins and nanoparticles and the solubility and aggregation tendencies under varying salt concentrations contribute to the distinct trends.

CHAPTER 6

BIOCONJUGATIONS TO CONTROL PROTEIN CORONA FORMATION

6.1 Methodology

In order to control protein corona formation and aggregation, altering the surface chemistry and adding coatings of specific materials, such as zwitterionic materials and PEGylation, are commonly used techniques. Here we study effect of PEGylation and adding protein coatings on nanoparticles. Since albumin is a widely available protein in blood, we constructed albumin coatings on carboxylated polystyrene nanoparticles. Physical and chemical adsorption techniques are used to add albumin coatings. For physical adsorption the polystyrene nanoparticles were incubated for 5 and 10 hours in 20%, 40% and 80% bovine serum albumin (BSA) solution. The albumin adsorbed particles were washed to remove excessively bound proteins. After this the nanoparticle size was measured in saline and plasma with same procedure discussed in chapter 3.

An EDC/NHS reaction is used on carboxylated (-COOH) polystyrene surfaces to activate carboxyl groups, allowing for efficient conjugation with molecules like PEG (here we use 10k PEG) and proteins(here albumin) containing primary amines (-NH2). The EDC NHS reaction involves the addition of 0.4 mg of EDC to 1 mL of MES buffer; however, due to the small quantity, 8 mg is measured and dissolved in 20 mL of MES buffer. Afterwards, 200 µL of the 2% stock particle solution is added to the 1 mL EDC solution, which is then vortexed to ensure proper mixing. The reaction is allowed to proceed for 10 minutes at room temperature on a rotator covered in foil. Subsequently, 4.4 mg of sulfur-NHS is added to the reaction, resulting in a final concentration of 5 mM. In the No-weigh format, 60 µL of MES is added to a tube to create a 920 mM solution,

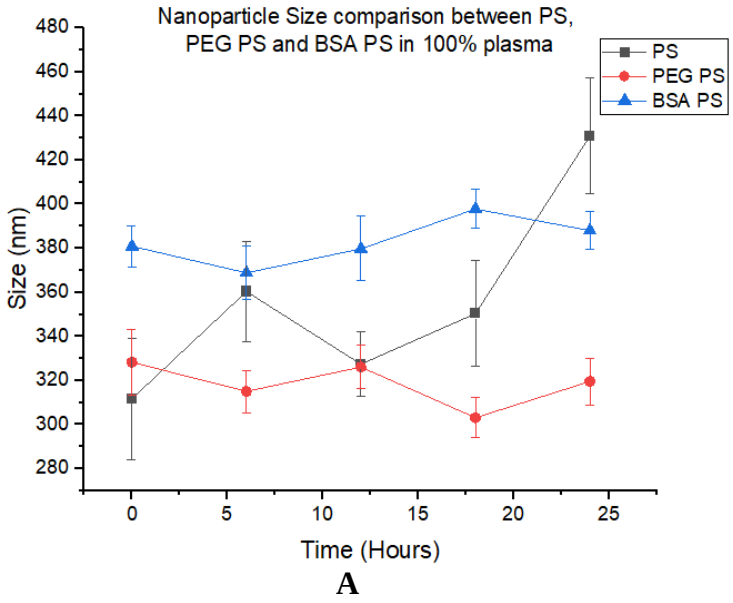
and then 88 µL of this solution is added to the 4 mL reaction to achieve a 5 mM concentration. After a brief vortex to mix the ingredients, the reaction continues at room temperature on a rotator covered in foil for 10-15 minutes. The supernatant is carefully removed after a centrifugation step at 15,000 rpm for 1 hour. The subsequent steps involve adding 1 mL PBS, tip sonication for particle dispersion, and adding either 15 mg of 10k linear PEG or 20% BSA.The solution is then thoroughly vortexed and allowed to react for 2 hours at room temperature, covered in foil, on a rotator. Following another centrifugation at 15,000 rpm for 1 hour, the supernatant is removed, and the particles are washed with saline. This washing process is repeated three times, and on the last wash, the solution is filled to 1 ml with saline. Finally, 400 µL of the reaction mixture is removed and combined with 600 µl of saline [45].

To measure protein corona formation for this experiment we followed the same experimental procedure as the 24 hours experiment mentioned in chapter 3.

6.1.2 Results and Discussion

The procedure of attaching PEG was carried out as described in the methodology section. Polyethylene glycol (PEG) have hydrophilic chains and are known for decreasing nanoparticle surface and protein interactions. PEG chain length, molecular weight [51] and density are three main variables deciding interaction between proteins and the nanoparticle surface. For measuring protein corona formed over PEG particles, a slightly modified version of the technique reported by Bannon. et al is used [45]. PEGylated particle size and bare polystyrene particle size are compared to get the PEG layer thickness. Chemical conjugation of PEG to polystyrene particles ensures stability of the coatings over time. Hence, now we compare this PEGylated size to particle size in plasma to get protein corona formation followingthe method described by Bannon. et al

[45]. Thus, now we can compare protein corona thicknesses rather than using overall nanoparticle size which also includes the PEG layer.



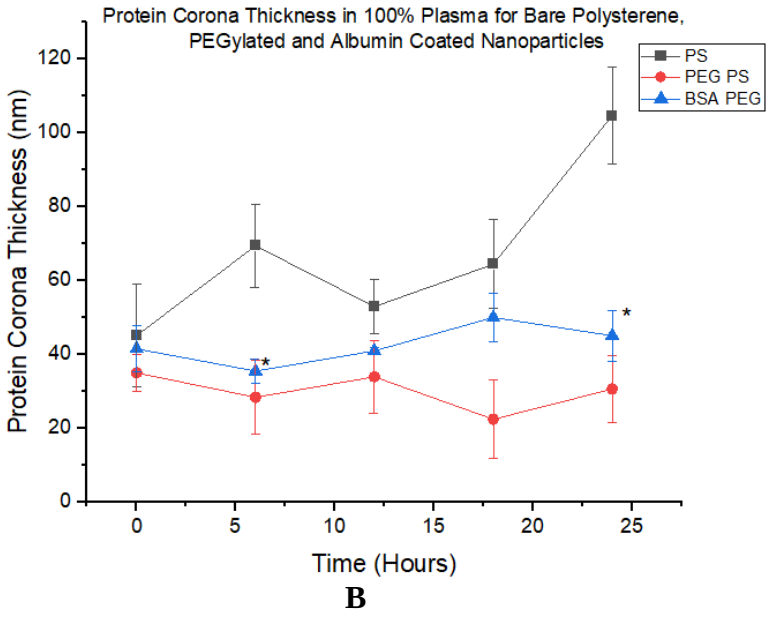


Figure 6.1 Comparison of protein corona thickness for bio-conjugated nanoparticles.

A: Comparison of particle size in 100% plasma for bare polystyrene, PEGylated and albumin coated nanoparticles. B : Comparison of protein corona thickness in 100% plasma for bare polystyrene, PEGylated and albumin coated nanoparticles.

We use PEG with a molecular weight of 10,000 g/mol (10k) to coat the particles. It was observed that PEGylated particles showed excellent stability over time, lower protein corona formation and aggregation over time. The mechanism behind the decreased protein corona in hydrophilic polymeric coatings like PEG is they promote the formation of hydration layers reducing direct contact with nanoparticle [52]. The hydrophilic chains extending from the nanoparticle surface reduce the bare surface area exposed, creating a physical barrier for proteins to bind to the surface and increase the hydrophilicity which repels proteins from interacting with the nanoparticles. The presece of the protein corona over PEG was characerized by Pelaz et al, which states proteins like serum albumin and fibrinogen penetrate between PEG chains giving rise to a thin protein corona. [52, 53]. PEG ligands have stealth-like properties, increase circulation time and reduce aggregation they have certain disadvantages like in vivo biodegradability, toxicity and accumulation in organs [52,54].

To overcome this issue we propose use of biointerfaces, protein-based coatings to optimize protein corona formation. Physical adabsorsorption of bovine serum albumin in solutions of 20%, 40% and 80% BSA was done with 5 hours and 10 hours incubation time. The particles were then washed in PBS at 2000 rpm to remove excessive bound proteins. These particles were tested in saline and bovine plasma solutions where they didn’t show any significant size differences than the bare particles at 0 and 6 hours. This shows that physical forces like Van der Waals forces are not strong enough to keep proteins bound on the nanoparticle surface.

Following the EDC-NHS reaction as stated in the methodology section, stable chemically conjugated albumin coated nanoparticles were synthesized and characterized for size measurements. For measuring size again, a modified procedure similar to Bannon et al. was followed as described in previous chapter to measure the thickness of the albumin coating and protein corona [45]. The results in the Figure 6.1 marked with asterik show that the protein corona formed on the nanoparticles is significantly lower than bare polystyrene particles, although slightly greater than PEG coatings. This result shows that cloaking nanoparticles with albumin initiates protein-protein interactions and optimizes protein corona formation. Albumin molecules stick to the surface of the polystyrene nanoparticles increasing their surface roughness adding hydration layers limiting the direct interaction between proteins and nanoparticle surface [55, 56]. However, we osberved that albumin interacts with other proteins giving rise to aggregation behavior. These results align with Peng et al. work where the demonstrated the albumin coatings significantly decrease protein corona formation compared to bare nanoparticles ad the protein corona grows over time on albumin coated particles.

CHAPTER 7

CONCLUSION AND FUTURE WORK

7.1 Conclusion

Protein corona formation is a dynamic process that changes nanoparticle's biological identity. Microenvironmental factors like pH, viscosity, salt molarity, type of species and temperature affect protein corona formation; conversely, nanoparticle surface roughness, density and shape also play an essential role in protein corona formation and aggregation behavior.

NTA NS 300 can track particles in plasma and present size distributions, combining principles of light-activated microscopy and Brownian motion. 24-hour experiments were performed at various % of plasma, in different types of plasma species and anticoagulants, and size profiling was done at equal intervals. Increasing % of plasma increased protein concentration, resulting in thicker protein corona formed around nanoparticles. This can be explained by Langmuir's adsorption theory and the binding site theory, where more available proteins lead to increased adsorption rates and thicker protein corona formation. Anticoagulants are responsible for preserving proteins and preventing blood clotting. Sodium citrate and Alsever's solution, commonly used anticoagulants to preserve plasma, showed no significant effect on protein corona formation. However, despite concentration and type of species, qualitative observations suggest that sodium citrate resulted in increased aggregation compared to Alsever's solution. Significant protein corona size differences were observed between goat and bovine plasma irrespective of type anticoagulants. Goat plasma contains more protein than bovine, resulting in higher protein corona formation and aggregation. The size of

nanoparticles in an individual sample's bloodstream is unique and influenced by various factors, such as hydration levels, protein density, and binding ability. Genetics, diseases, medication, pH levels, and temperature are some variables that can impact these factors, making it challenging to predict the precise behavior of nanoparticles.

Another experiment was conducted to check the effect of the speed of injection of nanoparticles on protein corona formation. Lower injection speeds resulted in increased protein corona thicknesses, whereas higher speeds resulted in lower protein corona thicknesses. This phenomenon can be explained by mechanical stress from the higher speeds that alter protein conformations, exposing specific binding sites. Conformational changes induced by shear forces drive this selective adhesion of proteins with high binding efficacy to exposed sites. Lower and higher affinity proteins compete to bind at lower speeds, resulting in thicker layers of proteins on the nanoparticle surface. At higher speeds, shear forces alter protein conformation, allowing proteins of higher affinity to bind to the nanoparticle surface. Another reason is the decreased time for protein-nanoparticle interactions at higher speeds only allows the highest affinity proteins to bind, and the rest get shed away due to the forces resulting in a thinner protein corona. This experiment was carried out for all variations of % of plasma and types of blood, and results aligned with the previous experiments' findings showing an increase in size with an increase in % plasma, anticoauglants do not affet protein corona formation and species varitation varies protein build up in turn affecting protein corona thhickness.

The size of nanoparticles in bovine plasma decreased as the molarity of the KI salt mixture increased and approached its saturation point. Conversely, in goat plasma, the size of nanoparticles increased as the molarity of the KI salt mixture increased and neared its saturation point. This difference is due to the variation in plasma protein buildup in

different species, which affects the adsorption of proteins on the surface of nanoparticles. Increasing the molarity of the salt mixture denatures protein structures, affecting their binding sites. Therefore, the distribution of individual proteins in a blood sample affects the formation of protein layers on the nanoparticles. Additionally, the ionic strength, which is influenced by an increase in molarity, may affect the electrostatic interactions between nanoparticles and proteins in bovine and goat systems.

Since blood comprises 57% blood plasma, the dynamic medium of protein exchange, it becomes impossible to avoid a protein corona entirely. However, protein corona formation can be optimized by changing the surface chemistry. One strategy is adding PEG coatings to improve circulation time and immunogenicity and reduce corona formation and aggregation. PEG adds hydration spheres and inhibits steric hindrance, reducing protein corona formation. Another way to reduce protein corona formation is adding biointerfaces such as protein coatings to the nanoparticles. Physical adsorption methods do not work since the Van der Waals and electrostatic interactions between the polystyrene nanoparticles and albumin molecules are weak. Hence, using EDC/NHS chemistry, albumin is covalently bonded to polystyrene nanoparticles. This results in stable protein coatings that increase the surface roughness and adds hydration layers, decreasing protein interactions, although may initiate aggregation behavior, which can be optimized using PEG coatings.

It is necessary to note that the results are highly specific and change with change in species due to variability in genetic makeup and extrinsic factors that affect blood, such as disease, hydration, diet and breed. These experiments were carried out in plasma and not whole blood; thus, factors, like the presence of red blood cells, will affect this phenomenon on a clinical scale. The protein corona formation is a complex process, and

in vitro results may vary from in vivo results, although these experiments will give a general trend of the behavior of nanoparticles in blood, which will help develop better drug delivery systems.

7.2 Future Work

Effect of Protein Corona on Drug delivery: Protein corona formation on nanoparticles adds a layer of molecules over the particle altering its surface chemistry, creating a shielding effect which may affect the drug release rates of particles. The hard corona has strongly bound proteins and are stable over time thus drug release rates through the hard corona will be different than the soft corona where proteins with lower affinity are bound and have weak interaction with nanoparticle surface. Thus the release rate will be different in the presence of a hard corona and soft corona. Since protein coronas evolve with time it is necessary to analyze drug release over time and compare the release rates with protein corona thickness to obtain the relationship between protein corona thickness and drug release.

This can be done by loading drugs into nanoparticles and incubating in plasma and saline similar to the 24 hour experiments were done. Every few hours the sample is taken from the release media and is tested with HPLC and cumulative release % is calculated using cumulative amount of drug released compared to initial amount of drug added. The release rate in saline and plasma can be compared to protein corona thickness data obtained from NTA. This process is to be repeated on PEG coated and BSA coated particles to analyze the effect of coatings on drug release along with protein corona formation on the drug delivery particles.

Cellular Uptake: Cellular uptake of nanoparticles typically occurs due to endocytosis, a process where external substances are brought inside cell using the cell membrane and

vesicles. Previous studies have observed that physicochemical properties of nanoparticles like shape, size, density, surface chemistry affect this process. Cancerous cells have increased ability of endocytosis; hence we propose to select MDA-MB-231, an aggressive human breast cancer cell line. These cell lines are incubated at appropriate conditions and density in multiwell cell plates. The cellsare incubated with bare nanoparticles or bare protein corona coated nanoparticles at the same timepoints we analyzed in the 24 hours experiment. Additionally, to study the effect of protein coatings, PEG and BSA coated nanoparticles with and without protein corona will also be incubated with the cells at the same time points. After incubating, the cells will be detached using trypsin and centrifuged to collect the pellet. This pellet will be washed in PBS thrice to remove excess nanoparticles. The cells are analyzed in flow cytometer to detect the fluorescent signal from the nanoparticles. The number of cells containing this fluorescent signal corresponds to the cellular uptake of nanoparticles. This experiment will differentiate the effectiveness of synthetic polymer-based PEG and protein based BSA coatings on cellular uptake, another critical step in drug delivery. It will also help in corelating the NTA size profiling data to cellular uptake, indicating how the protein corona thickness affects endocytosis.

APPENDIX A

VISCOMETRY

Table 1.1 Viscosity Chart for Different Volumes of Plasma in Saline at 37^0C

Plasma Volume (ul)	Saline + Goat Alsevers (m/s^2)	Saline + Goat Sodium Citrate (m/s^2)	Saline + Bovine Alsevers (m/s^2)	Saline + Bovine Sodium Citrate (m/s^2)
10	0.7271	0.7091	0.715	0.7210
50	0.7277	0.7192	0.7338	0.727
100	0.7304	0.7342	0.7399	0.7503
500	0.7697	0.7999	0.7644	0.805
1000	0.8336	0.9102	0.799	0.928
1500	0.8828	1.04	0.8366	1.014
2000	1.008	1.258	0.9586	1.191

Table 1.2 Viscosity Chart for Different Volumes of Plasma in Water at 37^0C

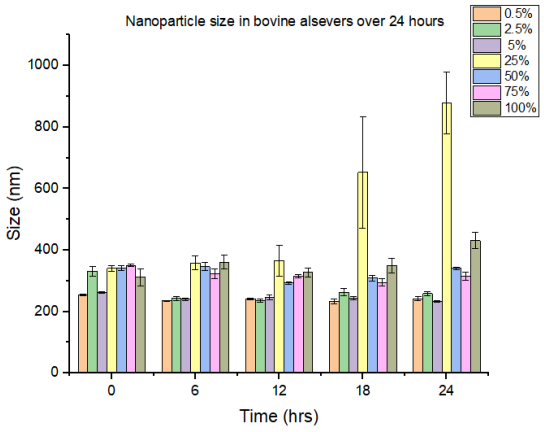
Plasma Volume (ul)	Water + Goat Alsevers (m/s^2)	Water + Goat Sodium Citrate (m/s^2)	Water + Bovine Alsevers (m/s^2)	Water + Bovine Sodium Citrate (m/s^2)
10	0.7194	0.7148	0.7274	0.7117
50	0.7403	0.721	0.7318	0.7156
100	0.7425	0.7334	0.7331	0.7386
500	0.7842	0.8066	0.7474	0.7905
1000	0.8462	0.8823	0.7767	0.88
1500	0.9521	1.028	0.8712	0.9822
2000	1.008	1.258	0.9586	1.191

The measured viscosity is kinematic viscosity, to convert into dynamic viscosity we multiply kinematic viscosity by density, here we consider the density to be 1.

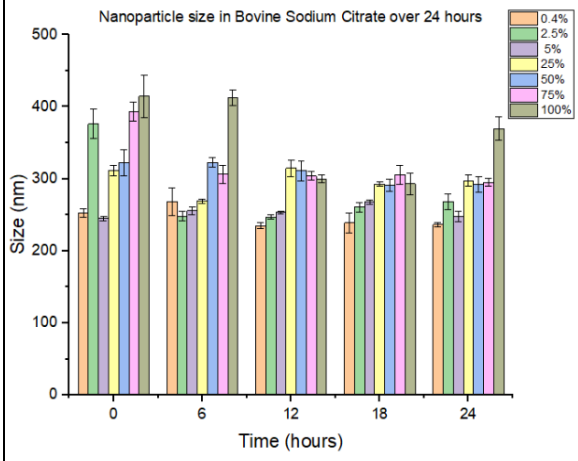
APPENDIX B

24 Hour Experiment

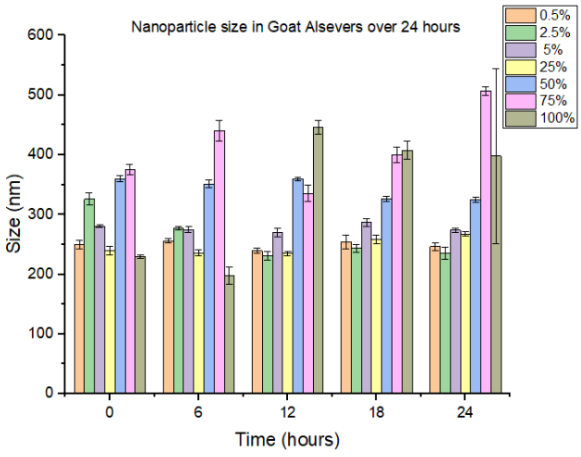
Table 2.1 Nanoparticle Size in 24 Hours Across Different Types of Plasma 37^0C



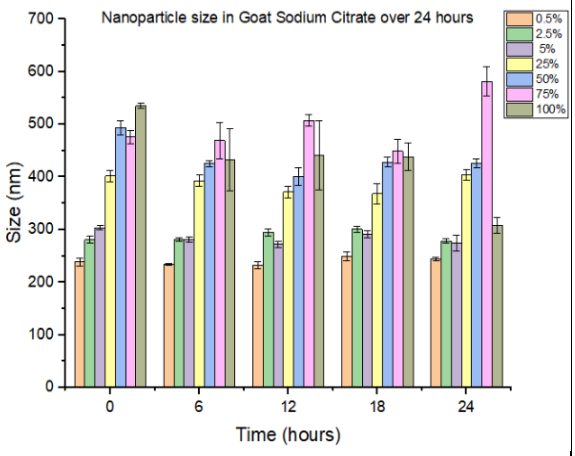
A. Nanoparticle size across 24 hours in bovine alsevers plasma at different plasma volumes.



B. Nanoparticle size across 24 hours in bovine sodium citrate plasma at different plasma volumes.

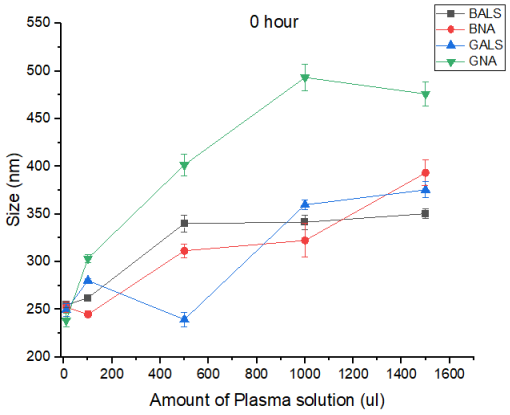


C. Nanoparticle size across 24 hours in goat alsevers plasma at different plasma volumes.

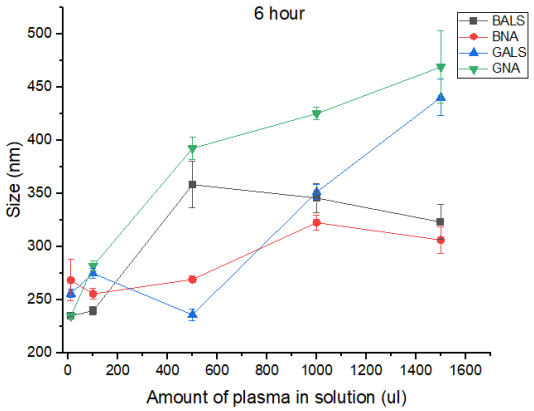


D. Nanoparticle size across 24 hours in goat sodium citrate plasma at different plasma volumes.

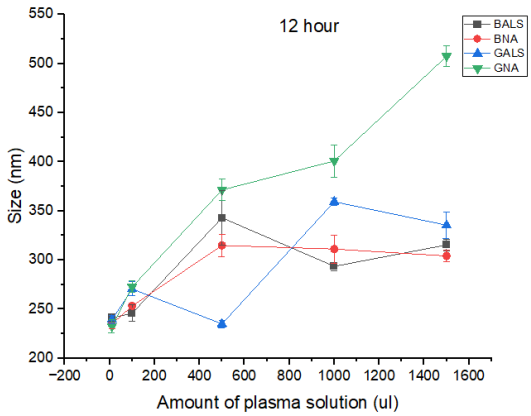
Table 2.2 Nanoparticle size comparison for Plasma content for each hour at 37^0C



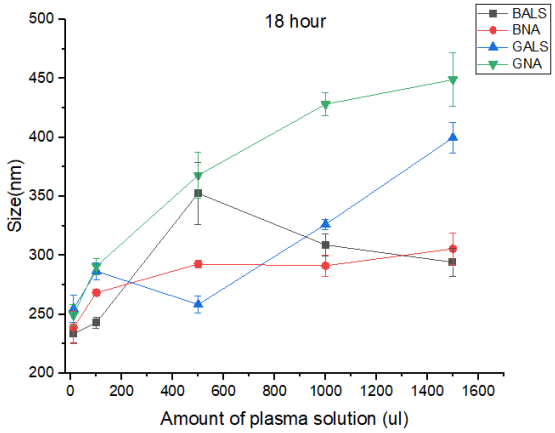
A. Size comparison for all types of plasma content for 0 hour



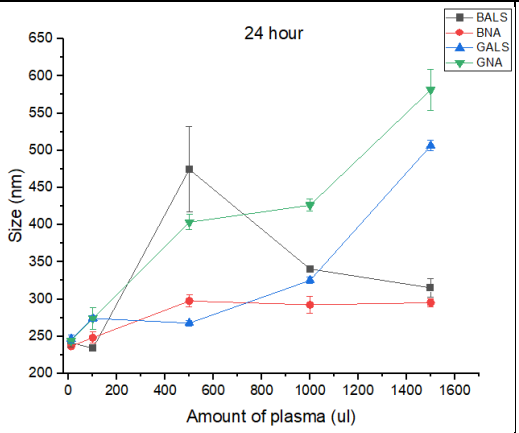
B. Size comparison for all types of plasma content for 6 hour



C. Size comparison for all types of plasma content for 12 hour



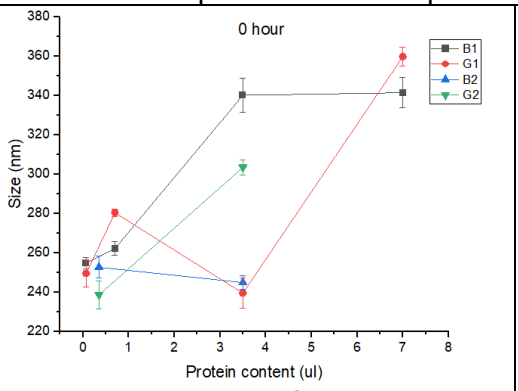
D. Size comparison for all types of plasma content for 18 hour



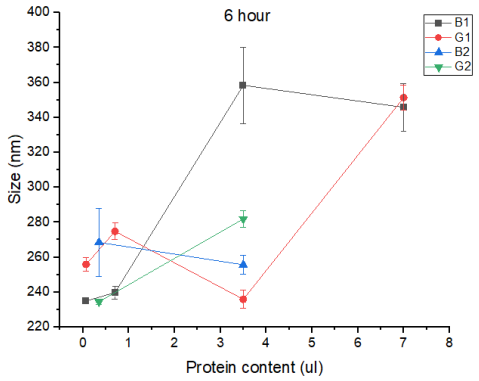
E. Size comparison for all types of plasma content for 24 hour

Nanoparticle size comparison based on protein content, Where B1 stands for bovine alsevers, B2 stands for Bovine Sodium citrate, G1 stands for goat alsevers and G2 stands for goat alsevers

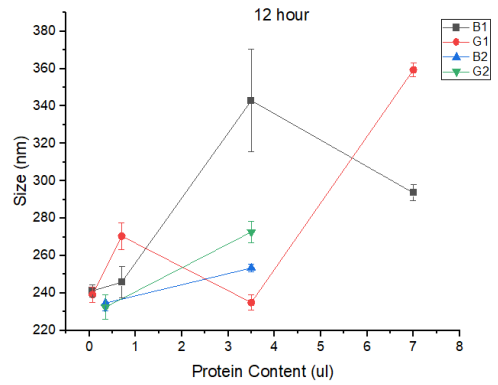
Table 2.3 Nanoparticle Size Comparison Based On Protein Content



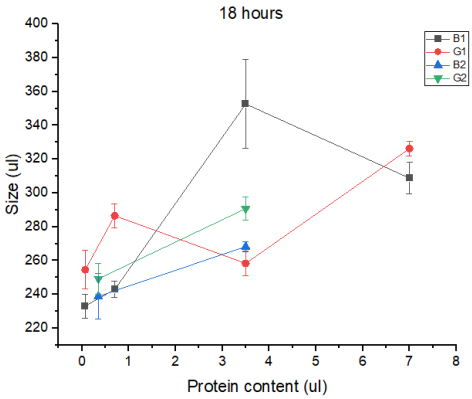
A. Size comparison at 0 hour



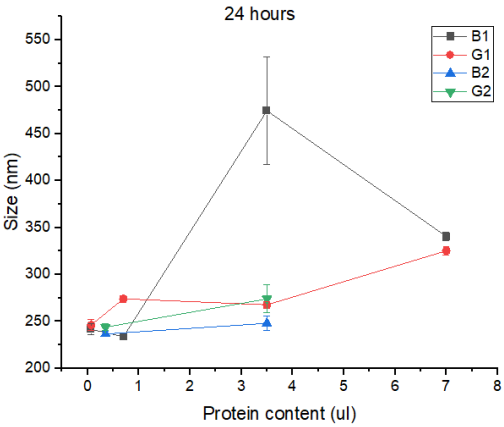
B. Size comparison at 6 hour



C. Size comparison at 12 hour



D. Size comparison at 18 hour

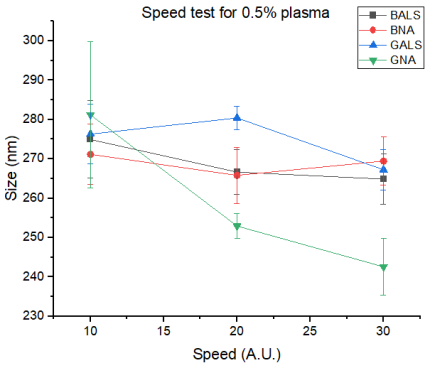


E. Size comparison at 24 hour

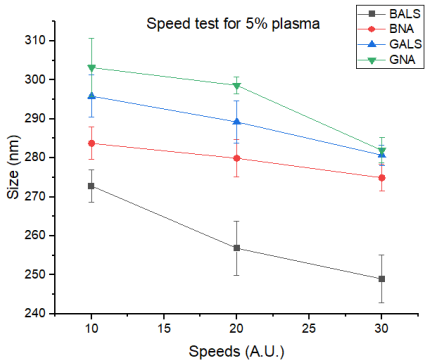
APPENDIX C

Speed Test

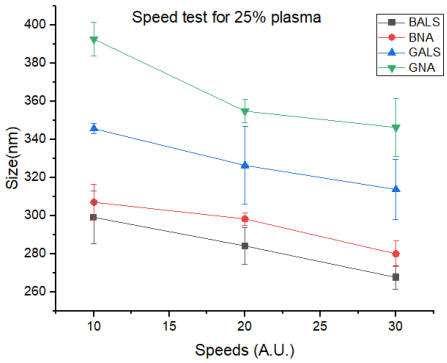
Table 3.1 Speed Tests at Different Volumes of Plasma



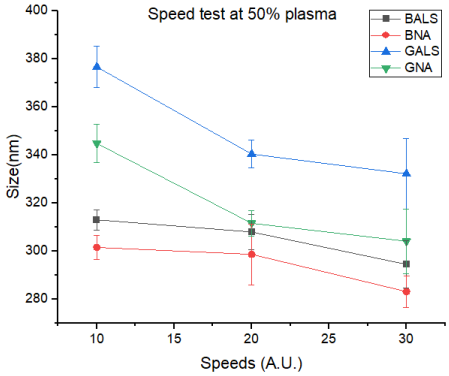
A. Speed test at 10, 20, 30 AU in 10 ul plasma



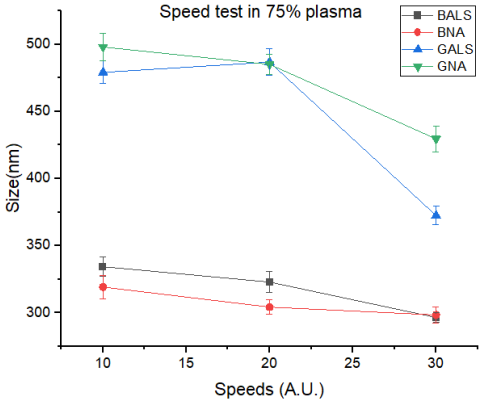
B. Speed test at 10, 20, 30 AU in 100 ul plasma



C. Speed test at 10, 20, 30 AU in 500 ul plasma



D. Speed test at 10, 20, 30 AU in 1000 ul plasma



E. Speed test at 10, 20, 30 AU in 1500 ul plasma

APPENDIX D

Salt Effect

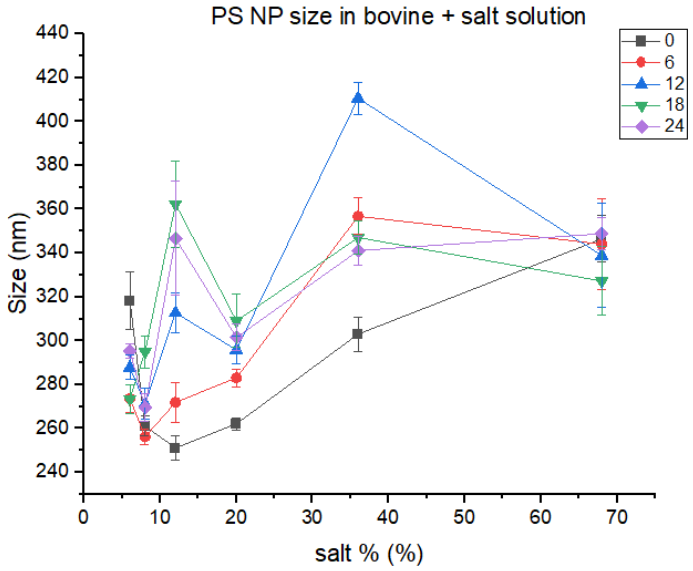


Figure 4.1 Size of Nanoparticles in 50% (v/v) bovine plasma at different concentration of salt.

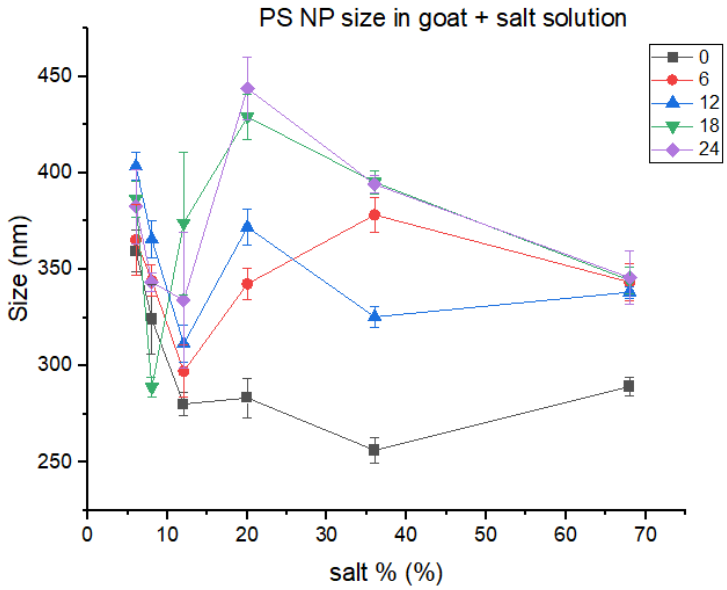


Figure 4.2 Size of Nanoparticles in 50% (v/v) goat plasma at different concentration of salt.

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