EFFICACY OF NANOPARTICLES IN ACHIEVING HEMOSTASIS

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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DEDICATION

To my loving mother, who has thoughtfully reviewed and painstakingly proofread this body of work.

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EFFICACY OF NANOPARTICLES IN ACHIEVING HEMOSTASIS

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ABSTRACT

Uncontrollable hemorrhage is significant cause of preventable death in the military and civilian setting. Hemostatic wound dressings have been created in an attempt to rectify this problem, but none currently on the market are highly effective at controlling hemorrhage resulting in a need for an effective hemostatic wound dressing. This study investigated the effects of gold, silver, and silica nanoparticles on blood coagulation time in order to determine if nanoparticle incorporation into a hemostatic wound dressing would effectively achieve hemostasis. Gold, silver, and silica nanoparticles were experimented with two different *ex vivo* studies to determine their effects on coagulation. A modified Lee White Method and a rotational viscometer were utilized to assess the nanoparticles ability to clot blood. Results obtained from the modified Lee White Method proved inconsistent and inconclusive demonstrating a need for improved testing methods. Results acquired from viscometer testing demonstrated that silica was effective in decreasing coagulation time indicating its potential use as a hemostatic agent and its prospective incorporation into a hemostatic wound dressing.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction to Hemostasis

1.1.1 Hemostasis and Coagulation

Hemostasis is the stoppage of bleeding of a hemorrhaging or injured blood vessel. It is a complicated process involving three main steps: blood vessel constriction, platelet plug formation, and coagulation. After injury occurs, blood vessels constrict limiting the amount of blood loss, platelets then adhere to subendothelial tissues and aggregate to form the primary hemostatic plug thus initiating the coagulation cascade (Colman et al. 2006; Riddel et al. 2007; Saito 1996). The coagulation cascade (Figure 1.1) can be broken down into two pathways: extrinsic and intrinsic, which ultimately come together and form a stable fibrin clot.

The extrinsic pathway is initiated when blood vessel injury occurs, exposing tissue factor (TF) to blood cells. TF combines with Factor VIIa creating the TF-VIIa complex, which then activates Factor X to Factor Xa and Factor IX to Factor IXa in the presence of Ca2+. Concurrently, the intrinsic pathway is initiated when Factor XII (Hageman factor) is activated to Factor XIIa by negatively charged surface contact, high molecular weight kininogen (HMWK), and kallikrein. Factor XIIa then activates Factor XI to Factor XIa, which in turn converts Factor IX to Factor IXa in the presence of Ca2+. Factor XIa combined with Factor VIIIa in the presence of Ca2+ forms a tenase complex activating Factor X to Factor Xa. The activation of Factor X to Factor Xa is where both

the extrinsic and intrinsic pathways coalesce into the common pathway (Mahdy and Webster 2004; Riddel et al. 2007).

After the activation of Factor X to Factor Xa to start the common pathway, Factor Xa combines with Factor Va, phospholipid, and Ca2+ to create prothrombinase. Prothrombinase converts prothrombin to thrombin. Thrombin then cleaves fibrinogen to fibrin and activates Factor XIII to Factor XIIIa, which is responsible for the cross-linking of the fibrin polymers to form a stable fibrin mesh thus achieving hemostasis (Mahdy and Webster 2004; Riddel et al. 2007).

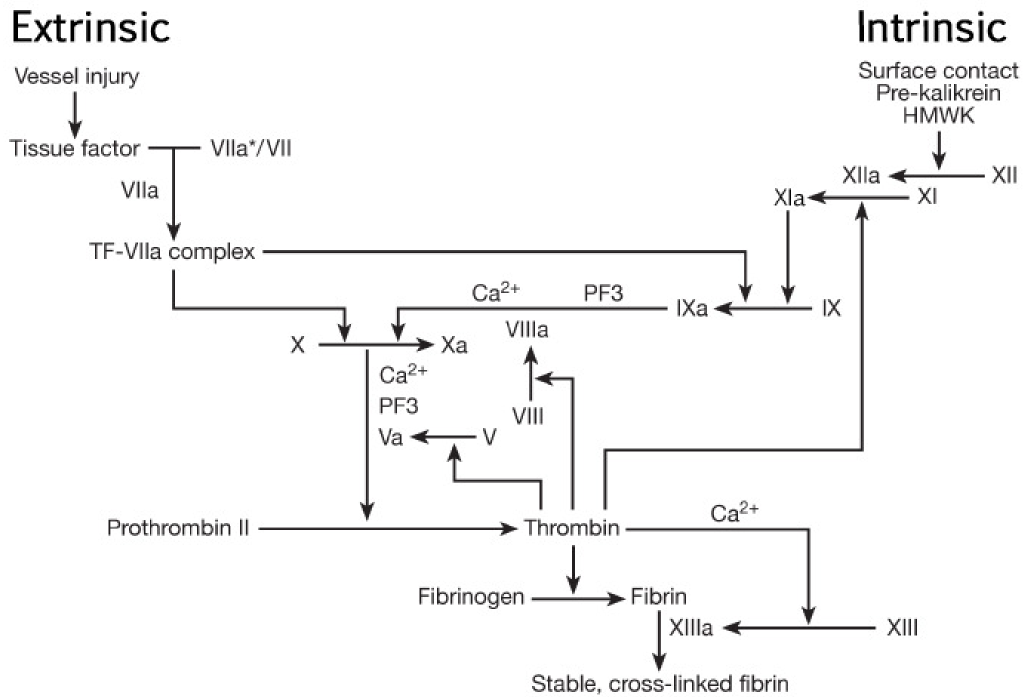

Figure 1.1: Coagulation Cascade (Modified from Mahdy and Webster 2004)

1.1.2 Importance of Hemostasis

Achieving adequate hemostasis becomes very important during various situations and under multiple conditions, such as: trauma, emergency medicine, surgery, and individuals with blood clotting disorders.

According to Wedmore et al. (2006) "uncontrollable hemorrhage accounts for almost 50% of combat fatalities and up to 80% of civilian trauma fatalities with the United States," and in other research conducted by Cox et al. (2009) up to 15% of deaths on the battlefield are preventable and 82% of those are from uncontrollable hemorrhage. Pusateri et al. (2006) also states that uncontrollable hemorrhage is the leading cause of death in military trauma and "the second leading cause of death in the civilian setting," thus, further solidifying the need for sufficient hemostasis in military trauma and emergency medicine situations.

In addition to trauma and emergency medicine, surgery also requires necessary hemostasis. Uncontrolled hemorrhaging during surgical procedures obstructs the view of the surgical field, increases the need for blood transfusions, can increase operating time, and increases the risks of post-operative complications (Villanueva 2008). In particular, cardiac surgery encompasses abundant blood loss due to the size of the surgical site, blood's contact with artificial surfaces, damage to coagulation factors and platelets, use of anticoagulants before surgery, and pre-existing platelet deficiencies in patients (Mahdy and Webster 2004; Skubas and Despotis 2001). In addition to cardiac surgery, vascular, neuro, and abdominal surgeries require sufficient monitoring of hemostasis and coagulation throughout (Grant 2007; Hirshberg and Walden 1997; Untch et al. 2007).

Besides surgery, blood clotting disorders such as: hemophilia A (Factor VIII deficiency), hemophilia B (Factor IX deficiency), and Von Willebrand disease further complicate the successfulness of hemostasis and coagulation under various circumstances. Hemophilia A and B are both inherited X-linked recessive disorders limited mostly to males caused by deficiencies in Factor VIII and Factor IX, respectively (Forbes 1996; Maclean and Makris 2005). Von Willebrand disease is characterized by a deficient or defective von Willebrand factor (vWF), which plays a role in platelet adhesion and aggregation and also binds to Factor VIII (Castman et al. 2005; Roberts and Ma 2006). Hemophilia and von Willebrand disease are considered the most common heritable bleeding disorders with an occurrence of von Willebrand between 1-4% (Forbes 1996; Nizzi et al. 2002). Furthermore, there are numerous other coagulation disorders ranging from inherited, acquired, and rare to multiple factor deficiencies, inhibitor defects, ones affecting adolescents, and others resulting from other diseases or surgery. With all of these variable circumstances requiring successful hemostasis and coagulation, there becomes a need for a supplement to the body's natural hemostatic response when that is not enough.

1.2 History of Hemostatic Wound Dressings

Traditional approaches to hemostasis have been to apply a tourniquet and pressure to the affected site where hemostasis is needed, but in more recent advances, hemostatic wound dressings have been created from various materials to supplement the body's own natural hemostatic process (Seyednejad et al. 2008). Table 1.1 details a variety of

hemostatic wound dressings, the materials they consist of, and the companies that produce them.

Table 1.1: Hemostatic Wound Dressings

Product	**Company**	**Material**
BioGlue®	CyroLife	Albumin, Glutaraldehyde
BloodSTOP®	Life Science Plus	Cellulose
Surgicel®	Ethicon	Cellulose
Celox™	Bio Stat, LLC	Chitosan
HemCon	HemCon Medical Technologies, Inc.	Chitosan
CoStasis®	Cohesion Technologies, Inc	Collagen
D-Stat	Vascular Solutions, Inc.	Collagen
TISSEEL	Baxter	Fibrin
Dry Fibrin Sealant Dressing (DFSD)	American Red Cross	Fibrinogen, Thrombin, Calcium, Factor XIII
FLOSEAL	Baxter	Gelatin, Thrombin
QuikClot® Combat Gauze	Z-Medica	Kaolin
Trauma Dex™	Medafor	Starch
QuikClot®	Z-Medica	Zeolite

With several materials available to produce hemostatic wound dressings, only two dressings have shown significant promise in achieving effective hemostasis, QuikClot® (Z-Medica) and the Dry Fibrin Sealant Dressing (DFSD) (American Red Cross).

QuikClot®: QuikClot® (Z-Medica) is a microporous aluminosilicate granular powder that facilitates coagulation by the absorption of water, therefore increasing the concentration of platelets and clotting factors (Seyednejad et al. 2008). It is individually packaged, inexpensive, easy to use, and has an expiration of 5 years (Cox et al. 2009;

Pusateri et al. 2006). In a study conducted by Cox et al. (2009), QuikClot® was shown to be successful at controlling bleeding in trauma patients, and in swine models, it has been shown to be effective in lethal Grade V liver injuries and mixed femoral arterial and venous injuries (Pusateri et al. 2006). However, there have been some significant problems associated with QuikClot®. Two relatively minor issues have surfaced while administering the product: the application to the wound site and the removal of the product. It was found that the granular powder was difficult to get directly into the wound and at times would blow away in the wind. Removal of the granules is achieved by surgical debridement and irrigation with a possibility of recurrent bleeding occurring (Cox et al. 2009). The major concern with QuikClot® is that upon application an exothermic reaction occurs with temperatures ranging from 42°C (107.6°F) to 140.4°C (284.7°F) in animal models and maximum temperatures between 42°C (107.6°F) to 44°C (111°F) *in vivo* creating mild to severe burns in and around the wound area (Alam et al. 2003,2004; Pusateri et al. 2004, 2006).

Dry Fibrin Sealant Dressing (DFSD): Dry Fibrin Sealant Dressing (DFSD) (American Red Cross) is composed of powdered human fibrinogen, thrombin, calcium, and Factor XIII incorporated onto a polypropylene backing providing key coagulation factors at a high concentration to the site of injury (Granville-Chapman et al. 2011). DFSD has proven to be effective in controlling bleeding in ballistic injuries, Grade V liver injuries, and aortic injuries in animal models (Holcomb et al. 1998, 1999; Sondeen et al. 2003). Despite DFSD's initial laboratory successes, it has still yet to be used in the field due to many reasons. DFSD is relatively expensive compared to other dressings on the market, costing around $1000 per 4x4 inch dressing, and the product itself is very brittle making

it difficult to conform to wounds. Most importantly, DFSD has not been FDA approved due to the human derived fibrinogen, which poses a risk of blood-borne pathogen transmission (Alam et al. 2005; Granville-Chapman et al. 2011; Sondeen et al. 2003).

Although QuikClot® and DFSD showed some success at achieving hemostasis in traumatic injuries, they failed to meet all the necessary criteria needed for an ideal hemostatic wound dressing. An ideal hemostatic wound dressing should be inexpensive, non-toxic, easily packaged with a long shelf-life, able to withstand various temperatures and environmental conditions, simple to apply requiring no mixing or special preparation, easily conformable to wounds yet durable, easily removable, and most importantly achieve hemostasis under various conditions. To date, there is no hemostatic wound dressing that meets the above aforementioned criteria.

1.3 Nanoparticles and their role in Hemostasis

1.3.1 Gold Nanoparticles

Currently, gold nanoparticles are being investigated for their use in targeted drug delivery systems, cancer treatments and therapy, and imaging technologies; nevertheless, limited research has been conducted with conflicting results on gold nanoparticles and their influence on coagulation (Ghosh et al. 2008; Jing-Liang and Min 2010; Lee and El-Sayed 2006; Pissuwan et al. 2011). It is not well-known whether or not gold nanoparticles support, hinder, or have no effect on coagulation. In research performed by Wiwanitkit et al. (2009) , gold nanoparticles were exposed to whole blood and platelet aggregation was evaluated. It was concluded that the gold nanoparticles did demonstrate platelet aggregation after being exposed with blood for 15 minutes, and the control

showed no platelet aggregation (Wiwanitkit et al. 2009). However, in a similar study performed on gold nanoparticles and their interaction with blood by Dobrovolskaia et al. (2009), results indicated that gold nanoparticles did not increase platelet aggregation or decrease coagulation time, but fibrinogen isoforms were found on the surface of the gold nanoparticles.

1.3.2 Silver Nanoparticles

Consistent with studies conducted on gold nanoparticles and coagulation, limited research performed on silver nanoparticles and their effect on blood coagulation have resulted in similar contradictory findings. In a recent study performed by Stevens et al. (2009), silicone catheters were coated with a silver nanoparticle emulsion; the blood interaction and antimicrobial ability of the silver nanoparticles were examined. It was found that silver nanoparticles increased platelet activation and adhesion and exhibited an antimicrobial effect by inhibiting bacterial growth having an antibiotic response on the silicone coated catheters (Stevens et al. 2009). However, in related research completed by Shrivastava et al. (2009), silver nanoparticles showed antiplatelet behaviors that inhibited platelet aggregation, secretion, and adhesion.

1.3.3 Silica Nanoparticles

In contrast to the contradictory evidence on the efficacy of silver and gold nanoparticles on blood coagulation, glass has a known blood clotting capability. Glass' negatively charged surface acts on the Hageman Factor (Factor XII) initiating the intrinsic pathway of the coagulation cascade (Margolis 1961; Rehmus et al. 1987). In a recent study performed by Dai et al. (2009), mesoporous silica spheres doped with

calcium (Ca^{2+}) and silver nanoparticles were characterized; their blood clotting ability and antimicrobial properties were evaluated. It was determined that the Ca^{2+} and silver nanoparticle doped silica spheres activated the intrinsic pathway of the coagulation cascade, induced platelet adhesion, and controlled hemorrhage in a femoral artery and liver injury animal model. The doped silica spheres also demonstrated antibacterial activity by a reduction of bacterial counts over time compared to the control.

CHAPTER 2

INTRODUCTION TO RESEARCH

2.1 Significance of Research

Uncontrolled hemorrhage is the leading cause of mortality in military trauma and the second leading cause of death in the civilian setting (Pusateri et al. 2006). Conventional approaches to controlling hemorrhage are to apply a tourniquet and pressure to the injured site, but those techniques do not always suffice to control bleeding. Thus, hemostatic wound dressings were created to supplement the body's own natural hemostatic response when that is not enough. As stated in Chapter 1, there are many hemostatic wound dressings available made from a variety of materials and are considered fairly successful under specific conditions, but none of them meet all the qualifications needed for an ideal hemostatic wound dressing. Those qualifications being inexpensive, non-toxic, easily packaged with a long shelf-life, able to withstand various temperatures and environmental conditions, simple application requiring no mixing or special preparation, easily conformable to wounds yet durable, easy removal, and most importantly achieve hemostasis under various conditions.

Recently, nanoparticles have been investigated for their use in a variety of technologies and treatments. Currently, gold nanoparticles are being used in targeted drug delivery systems, cancer treatments, and imaging technologies, and it has been established that silver nanoparticles possess antimicrobial properties (Clement and Jarrett 1994; Ghosh et al. 2008; Jamuna-Thevi et al. 2011; Jing-Liang and Min 2010; Lee and El-Sayed 2006; Lok et al. 2007; Pissuwan et al. 2011). However, very little conflicting

evidence exists on how gold and silver nanoparticles effect coagulation. Unlike gold and silver nanoparticles, glass has a known blood clotting affect by acting on Factor XII (the Hageman Factor), therefore initiating the intrinsic pathway of the coagulation cascade (Margolis 1961; Rehmus et al. 1987). Even though glass has a known blood clotting ability, there has been little research conducted on how silica nanoparticles would affect coagulation.

2.2 Research Objective

The overall research objective was to create an effective hemostatic wound dressing incorporated with nanomaterials capable of hemorrhage control under various circumstances. In doing so, certain tests needed to be established to address the nanoparticles ability to clot blood:

- An *ex vivo* coagulation test to determine gold, silver, and silica nanoparticles ability to clot blood
- Procedures and protocols for *ex vivo* testing of each type of nanoparticle
- Procedures and protocols for testing nanoparticles in whole blood and citrated blood

To create a simple basic *ex vivo* coagulation test, a modified Lee White Method was first used, in which nanoparticles impregnated in collagen gel were injected into blood collecting test tubes. Blood was drawn into those same tubes, and the tubes were put on a rotating test tube rack allowing them to slowly rotate back and forth for a determined amount of time. After rotating the tubes, they were allowed to sit for a

certain amount of time, and then the clotted blood and unclotted blood were separated and weighed (Ingram et al. 1953; Lee and White 1913; Tsang 1993).

After experimenting with the Modified Lee White Method and achieving inconsistent results, a viscometer was utilized to test the viscosity of blood over time. Nanoparticles were added to blood samples and the viscosity of the mixture was recorded over specific time intervals, in which the start of coagulation could be observed by increasing changes in viscosity of the solution.

In order to test individual types of nanoparticles during viscometer experimentation, individual procedures and protocols needed to be established based on the nanoparticles concentration, material state (i.e. dry, wet), and mass. Gold and silver nanoparticles testing methods were similar based on the fact that they are both water-based colloids, but parameters for silica nanoparticles were altered due to the difference in material state compared to gold and silver.

In addition to establishing methods for each individual type of nanoparticle, complementary procedures and protocols were needed for testing nanoparticles in whole blood and anticoagulated blood. This was accomplished by amending the parameters for whole blood testing and adding a reversing agent to undo the anticoagulant effect on the blood.

CHAPTER 3

EX VIVO STUDY I

3.1 Introduction

3.1.1 Modified Lee White Method

In attempting to create a fundamental ex vivo coagulation experiment in order to test the nanoparticles ability to clot blood, a modified version of the Lee White Method was developed. The original Lee White method employs the use of a blood sample inserted into a glass tube. The tube is then inverted every thirty seconds, and blood coagulation time is defined when the blood no longer flows freely in the tube (Lee and White 1913).

In the modified version of the Lee White Method, blood was collected in test tubes that contained collagen gel crosslinked with gold or silver nanoparticles. Collagen is a known hemostatic agent. After tissue injury, platelets adhere to collagen fibrils, degranulate, and then aggregate. Additionally, collagen rarely elicits serious adverse effects, and provides itself as a favorable scaffold for the gold and silver nanoparticles (Seyednejad et al. 2008). The tubes were then put on a rotating test tube rack, allowing the tubes to slowly rotate back and worth. After inverting the tubes for a pre-determined time, they were allowed to sit for a certain amount of time depending upon the test, and the clotted blood and unclotted blood were separated and weighed. The tube, which contained the largest blood clot with the least amount of unclotted blood after the test, was determined the most effective treatment.

3.2 Materials and Methods

3.2.1 Chemicals

Phosphate Buffered Saline (PBS) and 2-mercaptoethylamine (MEA) were obtained through Sigma Aldrich (Saint Louis, MO). Sodium Hydroxide (NaOH) was acquired through Fisher Scientific (Fair Lawn, NJ). Rat Tail Type I Collagen was purchased from BD Biosciences (Bedford, MA). Unconjugated 100nm (100AuNP) gold colloids and unconjugated 80nm silver colloids (80AgNP) were purchased from Ted Pella, Inc. (Redding, CA). Chemicals 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (NHS) were attained through Thermo Scientific (Rockford, IL).

3.2.2 Nanoparticle Functionalization and Crosslinking

Gold and silver nanoparticles were functionalized and crosslinked to collagen gel using a chemical crosslinking procedure (Appendix A). To functionalize the nanoparticles, 15µM MEA was used, which bound to the nanoparticles via a thiol group leaving a free amine group. After functionalizing the nanoparticles, 2mM EDC, 5mM NHS, 10X PBS, 1M NaOH, and distilled water were utilized to crosslink the free amine group on the functionalized nanoparticles to the carboxylic acid on collagen's C terminal end (Hermanson 1996). After functionalization and crosslinking, the collagen-nanoparticle samples were incubated at 37°C for 90 minutes to allow for gelling and polymerization. Once polymerized, gel samples were stored at room temperature. Each sample consists of a total volume of 0.25mL.

3.2.3 Ex vivo I studies

In an effort to determine the efficacy of the silver and gold nanoparticles ability to clot blood, a simple ex vivo test was created with varying parameters. Each test included the following 4 samples:

- Sample 1 = control, whole blood only
- Sample 2 = collagen gel
- Sample 3 = 100nm AuNP + collagen gel
- Sample 4 = 80nm AgNP + collagen gel

Test 1: Test 1 exposes previously polymerized gels to whole blood for a total of 10 minutes to determine the effects of the pre-polymerized gel and the nanoparticles ability to clot blood. Previously polymerized gels of 0.25mL in total volume were inserted into 6mL no additive blood collecting tubes. Blood was drawn into the tubes, and the tubes were placed on a rotating test tube rack. The four samples were allowed to slowly rotate at 180° for 5 minutes. After inverting the samples, they were permitted to rest for another 5 minutes. Once the samples had been rotated and rested, clotted blood was removed from each individual tube and placed onto separate petri dishes. The petri dishes with the clotted blood and the empty petri dishes were weighed.

Test 2: Test 2 exposes gels previously polymerized in tubes to whole blood for 10 minutes to determine the gel and nanoparticles effects on clotting blood. Unpolymerized gels of 0.25mL were inserted into 6mL no additive blood collecting tubes and placed in an incubator at 37°C for 90 minutes to allow for polymerization of the gels within in the tubes. After polymerization, the tubes were weighed, and blood was drawn into each tube. The tubes were placed on a rotating test tube rack, and the four samples

were allowed to slowly rotate at 180° for 10 minutes. After 10 minutes of rotation, unclotted blood from each sample was poured out into a petri dish, and the tubes and petri dishes were weighed.

Test 3: Test 3 examines the effect of unpolymerized gels and nanoparticles on whole blood for 10 minutes. Unpolymerized gels were inserted into 6mL no additive blood collecting tubes and weighed. Blood was drawn into the tubes, and the tubes were placed on a rotating test tube rack. The four samples were allowed to slowly rotate at 180° for 10 minutes. After 10 minutes of rotation, unclotted blood from each sample was poured out into a petri dish, and the tubes and petri dishes were weighed.

Test 4: Test 4 evaluates the effect of unpolymerized gels and nanoparticles on whole blood for 10 minutes with a filtering process. Unpolymerized gels were inserted into 6mL no additive blood collecting tubes and weighed. Blood was drawn into the tubes, and the tubes were placed on a rotating test tube rack. The four samples were allowed to slowly rotate at 180° for 10 minutes. After 10 minutes of rotation, unclotted blood from each sample was poured through a filter onto a petri dish. The petri dishes including filters and tubes were weighed.

Test 5: Test 5 assesses the effect of more fluid unpolymerized gels and nanoparticles on whole blood for 5 minutes with a filtering process. Unpolymerized gels were prepared with 178μL distilled water instead of 89μL and inserted into 6mL no additive blood collecting tubes and weighed. Blood was drawn into the tubes, and the tubes were placed on a rotating test tube rack. The four samples were allowed to slowly rotate at 180° for 5 minutes. After 5 minutes of rotation, unclotted blood from each sample was poured

through a filter onto a petri dish. The petri dishes including filters and tubes were weighed.

3.3 Results and Discussion

3.3.1 Ex Vivo Test 1

In test 1, blood was drawn into blood collecting tubes that consisted of four different samples: a control (whole blood), collagen gel, collagen gel crosslinked with 100nm gold nanoparticles, and collagen gel crosslinked with 80nm silver nanoparticles. The four samples were rotated for 5 minutes and then allowed to rest for 5 minutes. After resting, there was no unclotted free-flowing blood left in any tube. The clotted blood was removed from the tubes and weighed on a petri dish. The weight of the clotted blood was determined to be the difference between the weight of petri dish including the clotted blood and the initial weight of the empty petri dish (Table 3.1).

Table 3.1: *Ex Vivo* Test 1 Weights

	Before (g)	After (g)	Clotted Blood (g)
Sample 1 (Control)	15.68	19.18	3.50
Sample 2 (Collagen)	15.53	20.05	4.52
Sample 3 (AuNP)	15.62	18.71	3.09
Sample 4 (AgNP)	15.52	18.90	3.38

From results displayed in Table 3.1, sample 2 containing only collagen gel showed the largest weight of clotted blood after resting, while sample 3, which contained collagen gel crosslinked with gold nanoparticles, showed the least amount of clotted blood. Samples 1 and 4 were in between with the overall order of largest weight of clotted blood to least being: 2, 1, 4, and 3.

The results indicated that out of the four samples collagen gel was best at clotting blood, and that the addition of nanoparticles to the collagen gel did not add any extra benefit in the ability to clot blood. The results even suggest that that addition of gold nanoparticles had an adverse effect on clotting blood by the clotted weight of sample 3 being smaller than sample 1 the control. Although the results demonstrate and propose these findings, there are variabilities to account for in this experiment that could alter these conclusions: the amount of blood volume in each tube, the amount of gel inserted into each sample, and the amount of clotted blood removed from each tube.

3.3.2 Ex Vivo Test 2

In trying to lessen the degree of variability as in Test 1, Test 2 was modified by polymerizing the gels inside the blood collecting tubes. The four samples were rotated for 10 minutes with no resting afterwards, and the unclotted blood was poured out of each tube and weighed on a petri dish. The before and after weights of the tubes and petri dishes are listed in Table 3.2. The weight of clotted blood is considered the difference between the tubes after testing and before, and the weight of unclotted blood is the difference between the petri dishes after testing and before testing as shown in Table 3.3.

The sample that exhibited the best clotting ability was the sample with the highest percentage of clotted blood to total blood.

Table 3.2: *Ex Vivo* Test 2 Tube and Petri Dish Weights

	Tubes		**Petri Dish**	
	Before (g)	*After (g)*	*Before (g)*	*After (g)*
Sample 1 (Control)	7.35	8.01	15.63	20.37
Sample 2 (Collagen)	7.72	8.32	15.66	19.91
Sample 3 (AuNP)	7.72	8.22	15.65	18.50
Sample 4 (AgNP)	7.73	8.36	15.78	20.50

Table 3.3: *Ex Vivo* Test 2 Blood Weights

	Unclotted Blood (g)	**Clotted Blood (g)**	**Total Blood (g)**	**% (Clotted Blood/Total Blood)**
Sample 1 (Control)	4.74	0.66	5.40	12.22
Sample 2 (Collagen)	4.25	0.60	4.85	12.37
Sample 3 (AuNP)	2.85	0.50	3.35	14.93
Sample 4 (AgNP)	4.72	0.63	5.35	11.78

From the results presented in Table 3.3, sample 3 with gold nanoparticles showed the best clotting capability by exhibiting the highest percentage of clotted blood to total blood, and sample 4 with silver nanoparticles showed the lowest percentage. Samples 1 and 2 were in between with an overall order of highest to lowest percentage of 3, 2, 1, and 4. In showing contradictory results compared to Test 1, Test 2 was repeated (Test 2a) with results showing sample 1 with the highest percentage, sample 4 with the lowest percentage, and the overall order from highest to lowest percentage of 1,2,3, and 4 (Appendix B, Table B.1 and Table B.2). Results from both tests show no consistency.

3.3.3 Ex Vivo Test 3

In receiving inconsistent results from both Tests 1 and 2, Test 3 was adjusted from Test 2 by using unpolymerized gels compared to polymerized gels, therefore allowing for more interaction between the gels and the blood during mixing. The unpolymerized gels were inserted into blood collecting tubes, blood was drawn into the tubes, and the tubes were allowed to rotate for 10 minutes. After 10 minutes of rotation, unclotted blood from each sample was poured into a petri dish, and the tubes and petri dishes were weighed. The before and after weights of the tubes and petri dishes are shown in Table 3.4. The weight of clotted blood is the difference between the tubes after testing and before, and the weight of unclotted blood is the difference between the petri dishes after testing and before testing as shown in Table 3.5. The sample that exhibited the best clotting ability was the sample with the highest percentage of clotted blood to total blood.

Table 3.4: *Ex Vivo* Test 3 Tube and Petri Dish Weights

	Tubes		**Petri Dish**	
	Before (g)	*After (g)*	*Before (g)*	*After (g)*
Sample 1 (Control)	7.39	7.70	15.62	20.98
Sample 2 (Collagen)	7.61	8.15	15.52	20.68
Sample 3 (AuNP)	7.73	7.84	15.67	21.08
Sample 4 (AgNP)	7.65	8.30	15.53	19.80

Table 3.5: *Ex Vivo* Test 3 Blood Weights

	Unclotted Blood (g)	**Clotted Blood (g)**	**Total Blood (g)**	**% (Clotted Blood/Total Blood)**
Sample 1 (Control)	5.36	0.31	5.67	5.47
Sample 2 (Collagen)	5.16	0.54	5.7	9.47
Sample 3 (AuNP)	5.41	0.11	5.52	1.99
Sample 4 (AgNP)	4.27	0.65	4.92	13.21

From results shown in Table 3.5, sample 4 with silver nanoparticles exhibited the greatest clotting ability by having the highest percentage, and sample 3 with gold nanoparticles showed the lowest percentage. Samples 1 and 2 were in between with an overall order of highest to lowest percentage of 4, 2, 1, and 3. Furthermore, these results still show an inconsistency when compared to Tests 1 and 2 with possible variation due to

the loss of unpolymerized gel and blood clots when pouring out the unclotted blood into the petri dishes.

3.3.4 Ex Vivo Test 4

In trying to prevent the loss of unpolymerized gel and possible blood clots when measuring the unclotted blood as in Test 3, Test 4 was revised by an addition of a filter to prevent the unpolymerized gel and blood clots from passing out of the tubes onto the petri dishes. The unpolymerized gels were inserted into blood collecting tubes, blood was drawn into the tubes, and the tubes were allowed to rotate for 10 minutes. After 10 minutes of rotation, unclotted blood from each sample was poured through a filter onto a petri dish, and the tubes and petri dishes were weighed. The before and after weights of the tubes and petri dishes are displayed in Table 3.6. The weight of clotted blood is the difference between the tubes after testing and before, and the weight of unclotted blood is the difference between the petri dishes after testing and before testing (Table 3.7). The sample that exhibited the best clotting ability was the sample with the highest percentage of clotted blood to total blood.

Table 3.6: *Ex Vivo* Test 4 Tube and Petri Dish Weights

	Tubes		**Petri Dish**	
	Before (g)	*After (g)*	*Before (g)*	*After (g)*
Sample 1 (Control)	7.38	9.29	15.62	18.44
Sample 2 (Collagen)	7.77	8.01	15.73	20.17
Sample 3 (AuNP)	7.71	12.60	15.53	15.76
Sample 4 (AgNP)	7.77	9.22	15.63	19.47

Table 3.7: *Ex Vivo* Test 4 Blood Weights

	Unclotted Blood (g)	**Clotted Blood (g)**	**Total Blood (g)**	**% (Clotted Blood/Total Blood)**
Sample 1 (Control)	2.82	1.91	4.73	40.38
Sample 2 (Collagen)	4.44	0.24	4.68	5.13
Sample 3 (AuNP)	0.23	4.89	5.12	95.51
Sample 4 (AgNP)	3.84	1.45	5.29	27.41

From results presented in Table 3.7, sample 3 with gold nanoparticles demonstrated the greatest clotting ability by having the highest percentage, and sample 2 containing collagen gel with no nanoparticles exhibited the lowest percentage. Samples 1 and 4 were in between with an overall order of highest to lowest percentage of 3, 1, 4, and 2. Test 4 was repeated (Test 4a) with results showing sample 4 containing silver nanoparticles with the highest percentage, sample 1 with the lowest percentage, and the

overall order from highest to lowest percentage of 4, 2, 3 and 1 (Appendix B, Table B.3 and Table B.4). Results from both tests are not consistent and show no uniformity with previous tests.

3.3.5 Ex Vivo Test 5

In an attempt to obtain some consistency during experimentation, Test 5 was altered from Test 4 by doubling the amount of distilled water added in the nanoparticle crosslinking procedure (Appendix A) from 89μL to 178μL to allow for further interaction between the gels and blood during mixing. The unpolymerized gels were inserted into blood collecting tubes, blood was drawn into the tubes, and the tubes were allowed to rotate for 10 minutes. After 10 minutes of rotation, unclotted blood from each sample was poured through a filter onto a petri dish, and the tubes and petri dishes were weighed. The before and after weights of the tubes and petri dishes are displayed in Table 3.8. The weight of clotted blood is the difference between the tubes after testing and before, and the weight of unclotted blood is the difference between the petri dishes after testing and before testing (Table 3.9). The sample that exhibited the best clotting ability was the sample with the highest percentage of clotted blood to total blood.

Table 3.8: *Ex Vivo* Test 5 Tube and Petri Dish Weights

	Tubes		**Petri Dish**	
	Before (g)	*After (g)*	*Before (g)*	*After (g)*
Sample 1 (Control)	7.35	7.68	17.09	22.47
Sample 2 (Collagen)	7.64	7.51	17.00	22.41
Sample 3 (AuNP)	7.69	7.60	17.09	22.68
Sample 4 (AgNP)	7.80	7.83	17.13	22.29

Table 3.9: *Ex Vivo* Test 5 Blood Weights

	Unclotted Blood (g)	**Clotted Blood (g)**	**Total Blood (g)**	**% (Clotted Blood/Total Blood)**
Sample 1 (Control)	5.38	0.33	5.71	5.78
Sample 2 (Collagen)	5.41	-0.13	5.28	-2.46
Sample 3 (AuNP)	5.59	-0.09	5.50	-1.64
Sample 4 (AgNP)	5.16	0.03	5.19	0.58

Results presented in Table 3.9 show sample 1, the control, exhibiting the best clotting capability by having the highest percentage, and sample 2, collagen gel with no nanoparticles, displaying the lowest percentage. Sample 4 and 3 were in between with an overall order of highest to lowest percentage of 1, 4, 3, and 2.

Negative values were obtained for samples 2 and 3 suggesting the gels became too fluid upon adding the excess distilled water, therefore, allowing the gels to flow

through the filters negating the filtering process. Consequently, the gel weights were then added to the final petri dish weights resulting in an overall negative tube weight. These negative values indicate that the doubling the amount of distilled water produced gels that were too fluid yielding variable and inconsistent results.

3.4 Conclusion

Overall, ex vivo coagulation tests 1 through 5 showed inconsistent and contradictory results with gold and silver nanoparticles showing both a positive and negative effect on blood coagulation time indicating a lack of uniformity and reliability with the testing methods. The difficulty in the reproducibility of the test results precipitates the need for a change in coagulation testing methods.

CHAPTER 4

EX VIVO STUDY II

4.1 Introduction

4.1.1 Viscometer Coagulation Testing

Whole blood is considered a non-Newtonian fluid in that its viscosity changes over time when a shear stress is applied (Dee et al. 2002). From this information, a viscometer can be utilized to test the viscosity of whole blood over time with an increase in viscosity an indication of the onset time of coagulation. In previous studies, rotational viscometers have been employed to test the viscosity of whole blood over time, the effects of different variables on the viscosity of whole blood, and to determine the initiation time of coagulation (Antonova et al. 2008; Neslihan and Nurten 2010; Yamazoe et al. 2010). In this study, a rotational viscometer was utilized to test the effects of gold, silver, and silica nanoparticles on the onset time of coagulation for whole blood. Gold, silver, and silica nanoparticles were added to whole blood samples, a shear stress was applied to the samples using a rotational viscometer, and the viscosity of the samples were measured over time. The onset time of coagulation of the sample was determined by a significant increase in viscosity, and this was compared to a baseline viscometer test of whole blood only. The nanoparticle samples that exhibited an earlier onset coagulation time as compared to the baseline were considered to be a positive response in decreasing the time of blood coagulation.

4.2 Materials and Methods

4.2.1 Chemicals

Lyophilized Bovine Achilles Tendon Collagen, N,N-Dimethylmethanamide (DMF), Acetic Acid (AA), 2-mercaptoethylamine (MEA), Calcium Chloride Dihydrate ($CaCl_2$), and 15 nm Silicon Dioxide Nanopowder (SiNP) were obtained through Sigma Aldrich (Saint Louis, MO). Sodium Hydroxide (NaOH) was acquired through Fisher Scientific (Fair Lawn, NJ). Unconjugated 100nm (100AuNP) and 20nm (20AuNP) gold colloids and unconjugated 80nm silver colloids (80AgNP) were purchased from Ted Pella, Inc. (Redding, CA).

4.2.2 Viscometer Testing Set-Up

The viscometer testing set-up (Figure 4.1) included a Brookfield DV-II+ Pro Rotational Viscometer (Brookfield Engineering Laboratories Inc., Middleboro, MA) with a small sample adapter attachment (Figure 4.2), a Thelco Model 83 Water Bath (Precision Scientific Co., Chicago, IL) connected to the small sample adapter to maintain a temperature of 37°C ± 1°C within the small sample chamber, a StrykeFlow 2 disposable pump (Stryker Endoscopy, San Jose, CA) driven by a E3610A 30W DC Power Supply (Hewlett Packard, Rockville, MD) to circulate water through the small sample adapter chamber, and a laptop computer connected to the viscometer to record viscosity readings. The set parameters utilized for viscometer testing are shown in Table 4.1.

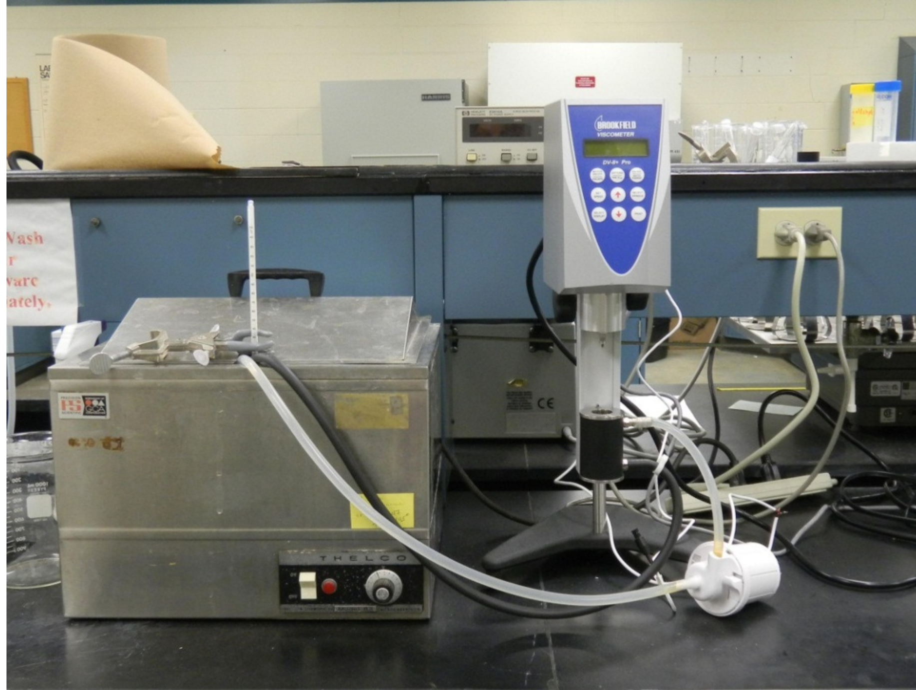

Figure 4.1: Viscometer Testing Set-Up

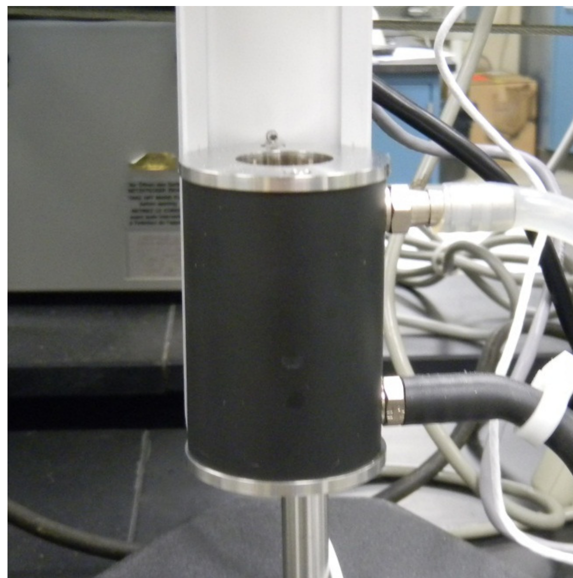

Figure 4.2: Small Sample Adapter

Table 4.1: Parameters for Viscometer Testing

Parameter	Setting
Spindle	#18
RPM	60
Temperature	37°C ± 1°C
Current	0.80A

4.2.3 Collagen Viscometer Testing

Lyophilized bovine collagen was tested in order to validate the use of a viscometer for blood coagulation testing due to collagen's known hemostatic abilities and for its potential use as a delivery agent for nanoparticles in a hemostatic wound dressing. *Whole Blood Testing:* A 0.25mL mixture consisting of 10mg/mL lyophilized bovine collagen and a 4:1 NaOH:DMF buffer solution (Appendix C) were added to 5mL of whole blood and placed into the small sample adapter of the viscometer. Viscosity readings were taken every 5 seconds and recorded.

4.2.4 Gold Nanoparticle Viscometer Testing

Unconjugated gold colloids consisting of 100nm and 20nm in size were tested in whole blood and citrated blood in order to determine their effects on coagulation.

4.2.4.1 Whole Blood Testing

Test 1: A 0.25mL aliquot of 100nm gold nanoparticles was centrifuged at 7000 RPM for 5 minutes. After centrifuging the nanoparticles, excess supernatant was removed from the top of the sample, leaving the nanoparticles sediment at the bottom. The

nanoparticles and a 0.25mL 4:1 NaOH:DMF solution were added separately to 5mL of whole blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded.

Test 2: A 0.25mL aliquot of 100nm gold nanoparticles was centrifuged at 7000 RPM for 5 minutes. After centrifuging the nanoparticles, excess supernatant was removed off the top and replaced with 50µL of $1x10^{-6}$M acetic acid. This acetic acid replacement procedure was repeated two more times for a total of 3 replacements with a final addition of 50µL of $1x10^{-6}$M acetic acid to the gold nanoparticles. This solution was then vortexed to ensure thorough mixing. The nanoparticle acetic acid solution and a 0.25mL 4:1 NaOH:DMF solution were added separately to 5mL of whole blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded. This same procedure was repeated for 20nm gold nanoparticles.

Test 3: A 0.25mL aliquot of 100nm gold nanoparticles was centrifuged at 7000 RPM for 5 minutes. After centrifuging the nanoparticles, excess supernatant was removed off the top and replaced with 50µL of $1x10^{-6}$M acetic acid. This acetic acid replacement procedure was repeated two more times for a total of 3 replacements with a final addition of 50µL of $1x10^{-6}$M acetic acid to the gold nanoparticles. To functionalize the nanoparticles, 5µL of a 10mg/ml MEA solution was added to the nanoparticle acetic acid solution and then vortexed to ensure thorough mixing. The functionalized nanoparticle acetic acid solution and a 0.25mL 4:1 NaOH:DMF solution were added separately to 5mL of whole blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded. This same procedure was repeated for 20nm gold nanoparticles.

4.2.4.2 Citrated Blood Testing

A 0.25mL aliquot of 20nm gold nanoparticles was centrifuged at 7000 RPM for 5 minutes. After centrifuging the nanoparticles, excess supernatant was removed off the top and replaced with 50µL of $1x10^{-6}$M acetic acid. This acetic acid replacement procedure was repeated two more times for a total of 3 replacements with a final addition of 50µL of $1x10^{-6}$M acetic acid to the gold nanoparticles. The nanoparticle acetic acid solution and 265µL of 0.02M $CaCl_2$ were added separately to 4.5mL of citrated blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded.

4.2.5 Silver Nanoparticle Viscometer Testing

Unconjugated silver colloids consisting of 80nm in size were tested in citrated blood in order to determine their ability to clot blood.

4.2.5.1 Citrated Blood Testing

A 0.25mL aliquot of 80nm silver nanoparticles was centrifuged at 7000 RPM for 5 minutes. After centrifuging the nanoparticles, excess supernatant was removed off the top and replaced with 50µL of $1x10^{-6}$M acetic acid. This acetic acid replacement procedure was repeated two more times for a total of 3 replacements with a final addition of 50µL of $1x10^{-6}$M acetic acid to the gold nanoparticles. The nanoparticle acetic acid solution and 265µL of 0.02M $CaCl_2$ were added separately to 4.5mL of citrated blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded.

4.2.6 Silica Nanoparticle Viscometer Testing

Silica nanoparticles consisting of 15nm in size were tested in citrated blood and whole blood in order to determine their effects on coagulation.

4.2.6.1 Citrated Blood Testing:

Test 1: An aliquot of 3.5mg of 15nm silica nanoparticles was added to 50µL of $1x10^{-6}$M acetic acid. The nanoparticle acetic acid mixture and 265µL of 0.02M $CaCl_2$ were added separately to 4.5mL of citrated blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded.

Test 2: Aliquots of 3.5mg and 7mg of 15nm silica nanoparticles and 265µL of 0.02M $CaCl_2$ were added separately to 4.5mL of citrated blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded.

Test 3: An aliquot of 14mg of 15nm silica nanoparticles was added to 265µL of 0.02M $CaCl_2$. This mixture was vortexed to ensure thorough mixing and then added to 4.5mL of citrated blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded.

4.2.6.2 Whole Blood Testing:

An aliquot of 14mg of 15nm silica nanoparticles was added to 5ml of whole blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded.

4.3 Results and Discussion

4.3.1 Collagen Viscometer Testing

A 10mg/mL mixture of collagen and a buffer solution consisting of NaOH and DMF was added to 5mL of whole blood, and viscosity readings were taken every 5 seconds and recorded. Results from Figure 4.3 show collagen having a significant impact on the onset time of coagulation by decreasing the time by about 6 minutes compared to the baseline, the buffer solution. In addition to the decreased coagulation time, collagen also exhibited a much higher viscosity after the onset of coagulation compared to the baseline. Repeated tests (Figure D.1) show similar results. The decreased coagulation time and the significantly increased viscosity after the onset of coagulation validated the use of a viscometer to test for differences in coagulation time with the addition of different nanoparticles.

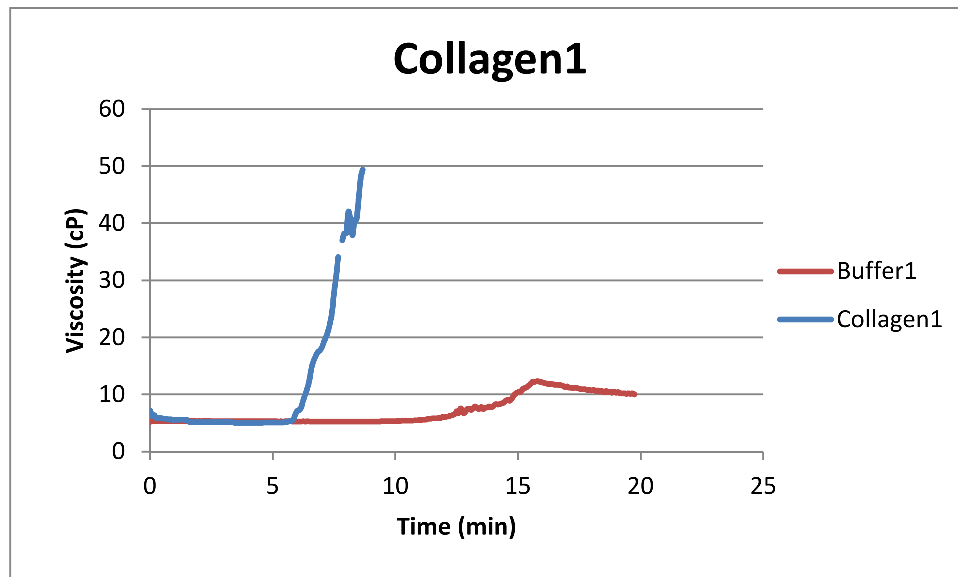

Figure 4.3: Collagen Whole Blood Test Rep 1

4.3.2 Gold Nanoparticle Viscometer Testing

4.3.2.1 Whole Blood

Test 1: In Test 1, 100nm gold nanoparticles and a 4:1 NaOH:DMF solution was added separately to 5mL of whole blood, and viscosity readings were taken every 5 seconds and recorded. Results shown in Figure 4.4 indicate that the addition of gold nanoparticles to the blood sample decreased the onset time of coagulation by about 3 minutes compared to the buffer solution alone with repeated tests (Figure D.2) displaying comparable results. However, this decrease in coagulation time is likely due to the water-based suspension that contained the gold nanoparticles. Presumably, the addition of the water from the gold nanoparticle suspension lysed the red blood cells in the sample presenting a "false" decreased onset time of coagulation. Further evidence from testing supports this explanation by physical observations of no visibly formed clots after the completion of testing with the addition of gold nanoparticles.

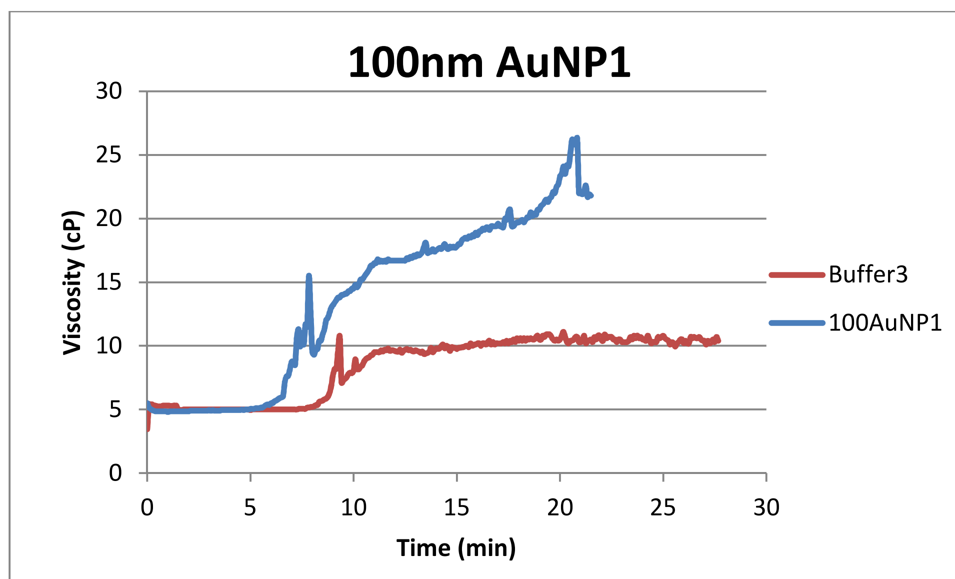

Figure 4.4: 100nm Gold Nanoparticle Whole Blood Test 1 Rep 1

Test 2: In Test 2, the procedure for the addition of gold nanoparticles to the blood sample was altered from Test 1 to try to prevent red blood cell lysis by the water-based suspension of the gold nanoparticles. A replacement procedure was performed 3 times with acetic acid on the water based suspension of the gold nanoparticles leaving a final volume of 50μL of gold nanoparticles suspended in acetic acid. The gold nanoparticle acetic acid solution and a 4:1 NaOH:DMF solution were added separately to 5mL of whole blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded. This procedure was performed with 100nm and 20nm gold nanoparticles. Results using 100nm gold nanoparticles are shown in Figures 4.5 and 4.6, and results using 20nm gold nanoparticles are shown in Figures 4.7 and 4.8.

Results presented in Figure 4.5 for 100nm gold nanoparticles illustrate that the addition of the gold nanoparticles decreased the onset time of coagulation by about 2

minutes compared to the baseline of acetic acid and NaOH:DMF. However, additional tests (Figure 4.6 and Figure D.3) demonstrate no difference between the addition of gold nanoparticles and the acetic acid NaOH:DMF solution suggesting that the gold nanoparticles have no effect on coagulation time. Supplemental testing supports this claim because a difference in coagulation time was unable to be replicated, and all other tests consistently display no difference in time between the gold nanoparticle addition and acetic acid NaOH:DMF solution.

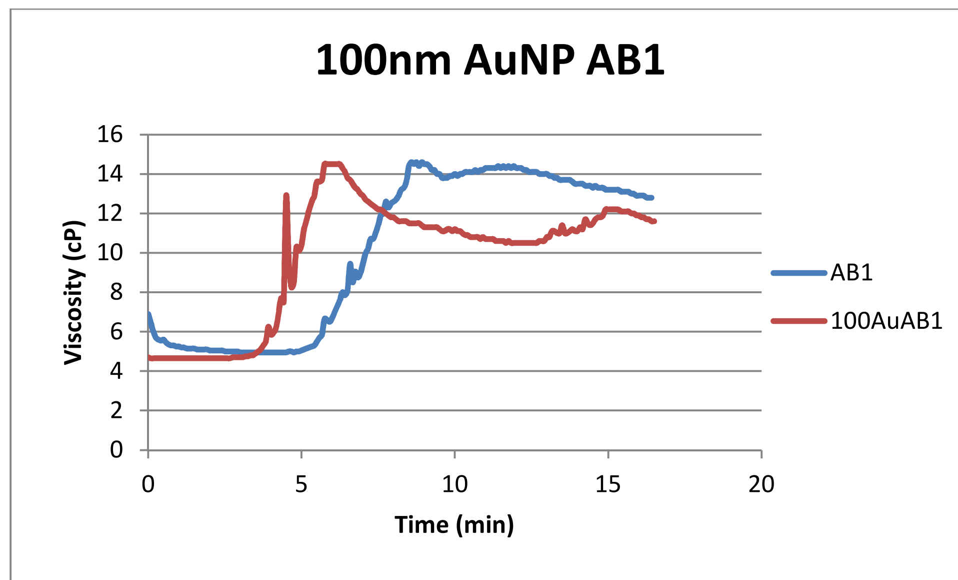

Figure 4.5: 100nm Gold Nanoparticle Whole Blood Test 2 Rep 1

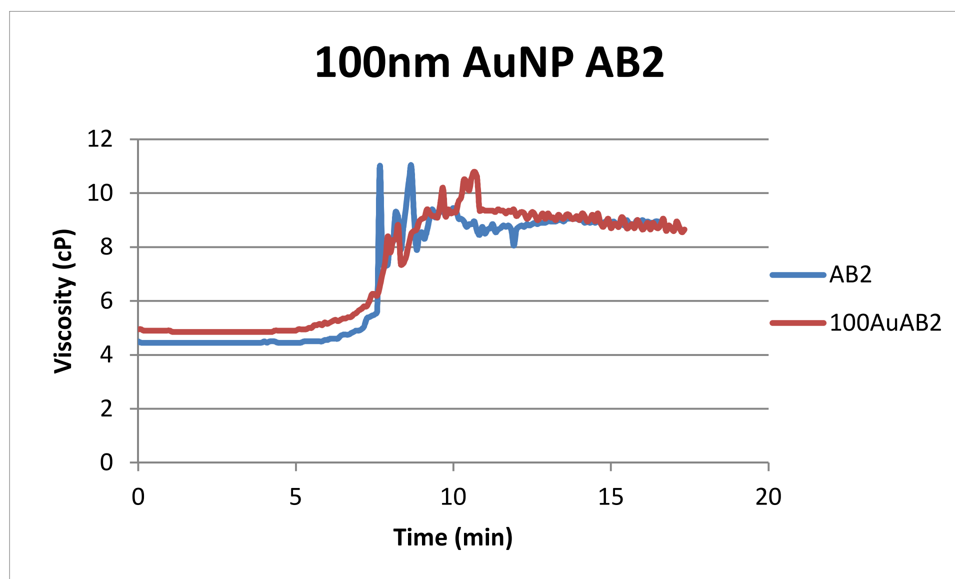

Figure 4.6: 100nm Gold Nanoparticle Whole Blood Test 2 Rep 2

Results from Figure 4.7 and 4.8 for 20nm gold nanoparticles display similar findings to that of the 100nm gold nanoparticles. The first repetition for 20nm gold nanoparticles (Figure 4.7) show that the addition of the gold nanoparticles decreased the onset time of coagulation by about 3 minutes compared to the baseline of acetic acid and NaOH:DMF, but in rep 2 (Figure 4.8) there is no significant difference in time between the addition of gold nanoparticles and the acetic acid NaOH:DMF solution. These similar findings indicate a corresponding conclusion that gold nanoparticles have no consistent effect on coagulation time possibly due to the gold nanoparticles themselves or the testing methods.

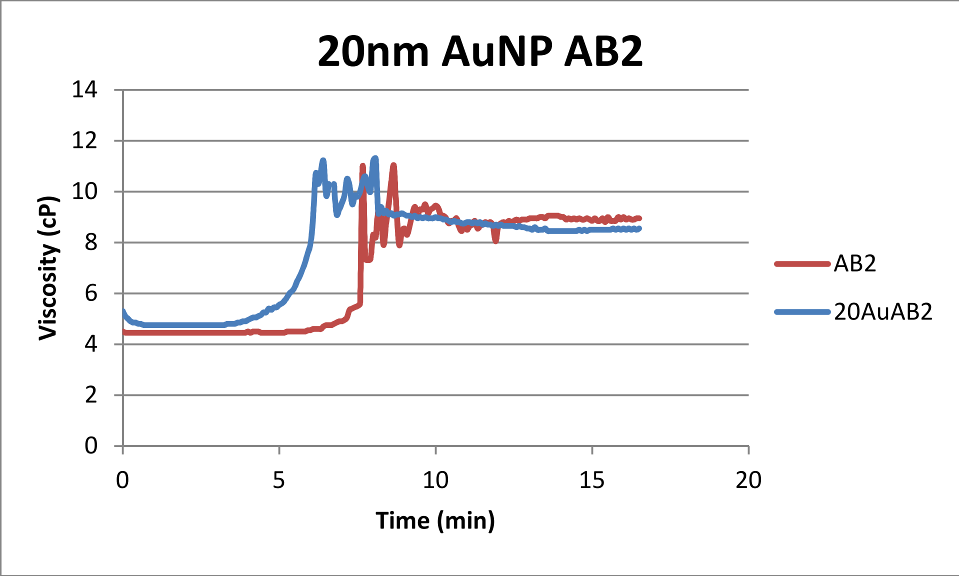

Figure 4.7: 20nm Gold Nanoparticle Whole Blood Test 2 Rep 1

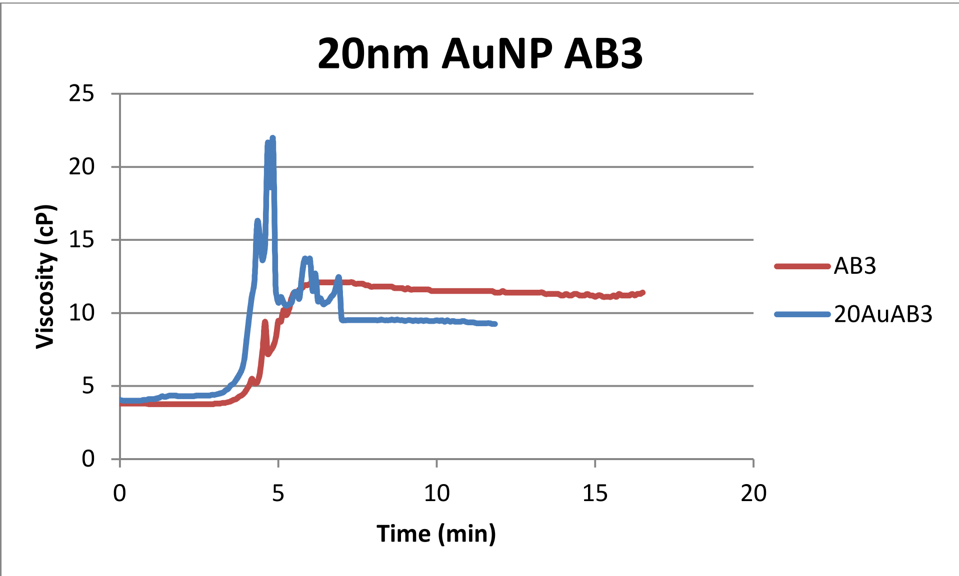

Figure 4.8: 20nm Gold Nanoparticle Whole Blood Test 2 Rep 2

Test 3: After receiving inconsistent results testing the gold nanoparticles themselves, Test 3 was modified by the addition of a functional group to the gold nanoparticles. The same acetic acid replacement procedure from Test 2 was repeated on the gold nanoparticles leaving a final volume of 50µL of gold nanoparticles suspended in acetic acid. Then, the nanoparticles were functionalized with 5µL of a 10mg/ml MEA solution adding a positive free amine group to the nanoparticles. The functionalized nanoparticle acetic acid solution and a 4:1 NaOH:DMF solution were added separately to 5mL of whole blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded. This procedure was performed on 100nm and 20nm gold nanoparticles.

From results displayed in Figure 4.9 for 100nm functionalized gold nanoparticles, the addition of functionalized gold nanoparticles did not alter the onset time of coagulation compared to the unfunctionalized gold nanoparticles and the baseline solution of acetic acid and NaOH:DMF. Repeated tests (Figure D.4) show comparable results indicating that the addition of the free amine group to the gold nanoparticles had no significant effect on coagulation time.

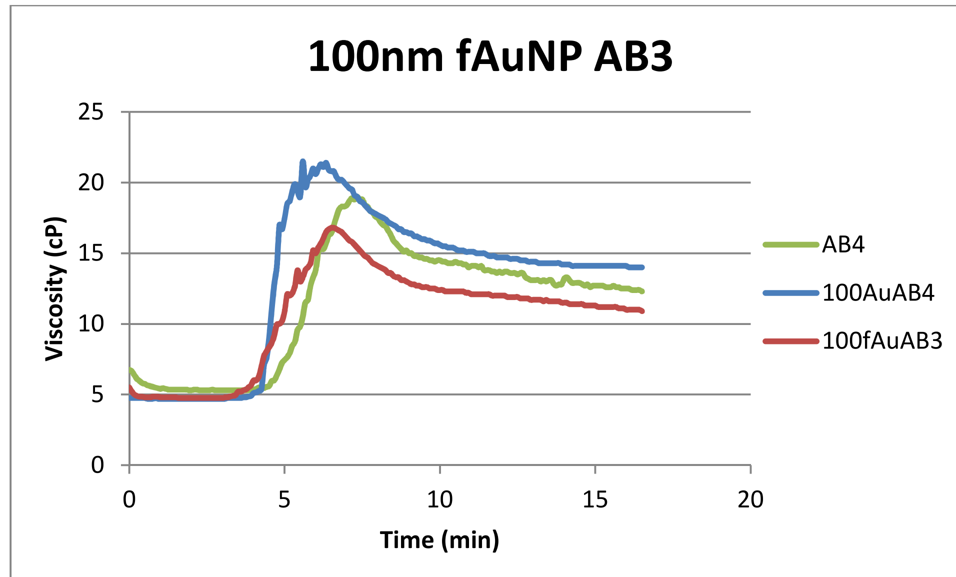

Figure 4.9: 100nm Functionalized Gold Nanoparticle Whole Blood Test 3 Rep 1

Results presented in Figures 4.10 and 4.11 show inconsistencies with respect to the onset time of coagulation time with the addition of 20nm functionalized gold nanoparticles. Figure 4.10 shows a decrease in the onset time of coagulation by almost 3 minutes compared to the baseline solution of acetic acid and NaOH:DMF but no difference between the functionalized and unfunctionalized gold nanoparticles. Alternatively, Figure 4.11 shows no difference between functionalized nanoparticles and the baseline solution of acetic acid and NaOH:DMF, but a decrease in coagulation time of about 3 minutes with the addition of unfunctionalized nanoparticles. Although the results are inconsistent with regards to the onset time of coagulation with the addition of functionalized gold nanoparticles, it can be concluded that the addition of the positive free amine group to the gold nanoparticles did not more effectively decrease the coagulation time compared to the unfunctionalized gold nanoparticles.

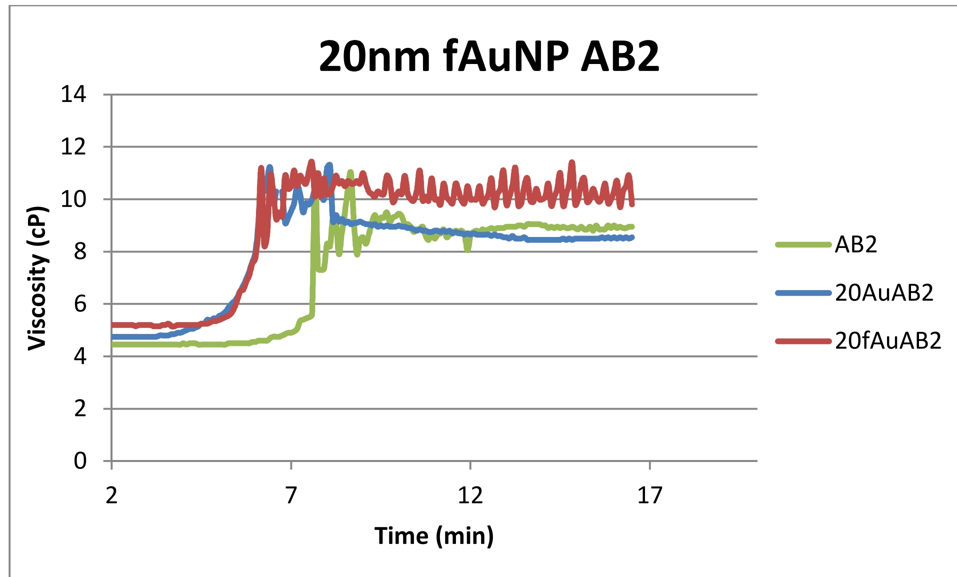

Figure 4.10: 20nm Functionalized Gold Nanoparticle Whole Blood Test 3 Rep 1

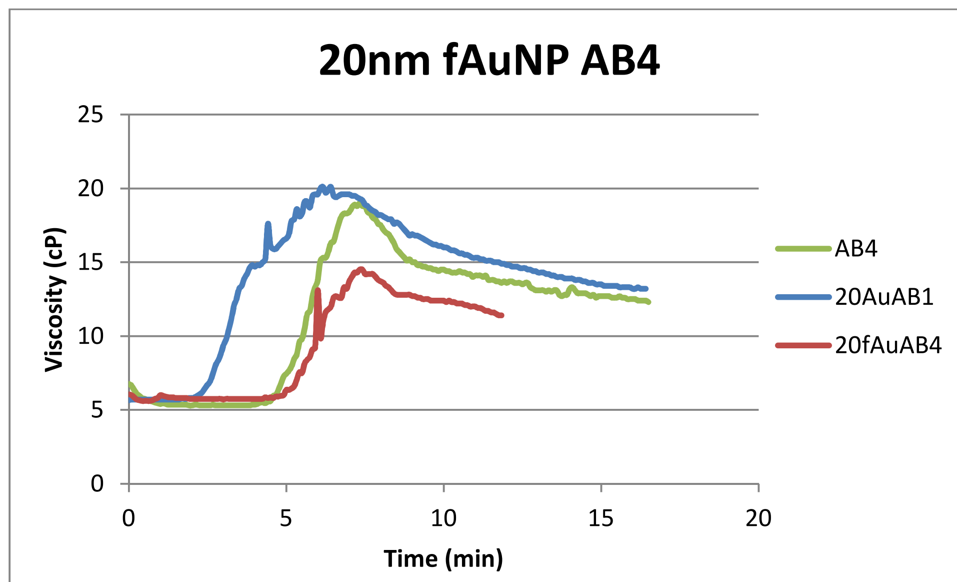

Figure 4.11: 20nm Functionalized Gold Nanoparticle Whole Blood Test 3 Rep 2

4.3.2.2 Citrated Blood

Gold nanoparticles were tested in citrated blood due to the ease of performing multiple tests with one venous blood draw. The acetic acid replacement procedure was performed on 20nm gold nanoparticles leaving a final volume of 50µL of gold nanoparticles suspended in acetic acid. The nanoparticle acetic acid solution and $CaCl_2$ were added separately to 4.5mL of citrated blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded.

Results presented in Figures 4.12 and 4.13 show inconsistencies with respect to the onset time of coagulation with the addition of 20nm functionalized gold nanoparticles. Figure 4.12 shows a decrease in the onset time of coagulation by almost 2 minutes with the addition of gold nanoparticles compared to the baseline solution of $CaCl_2$, but no significant difference between the gold nanoparticles and the acetic acid $CaCl_2$ solution. Alternatively, Figure 4.13 shows no sizable difference in coagulation times between the gold nanoparticles, acetic acid $CaCl_2$ solution, and the baseline solution of $CaCl_2$. These findings analogous to those seen in gold nanoparticle whole blood testing indicate a similar conclusion that the gold nanoparticles have no consistent effect on coagulation time conceivably due to the gold nanoparticles themselves or the testing methods.

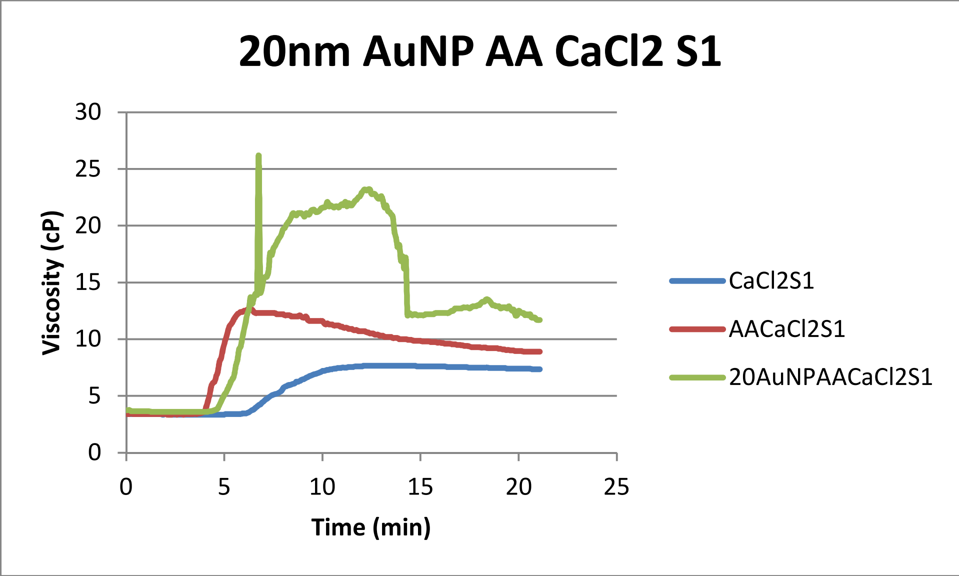

Figure 4.12: 20nm Gold Nanoparticle Citrated Blood Test Rep 1

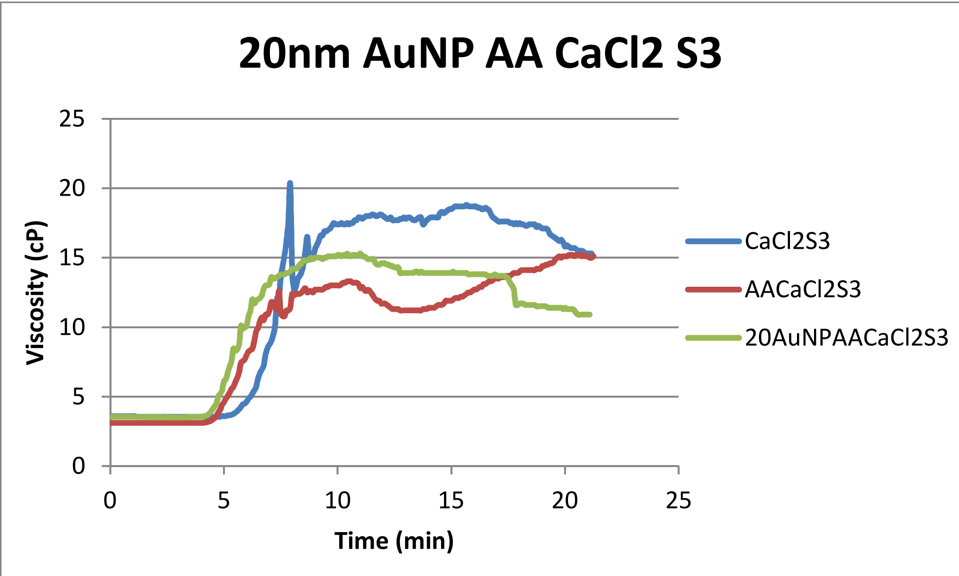

Figure 4.13: 20nm Gold Nanoparticle Citrate Blood Test Rep 2

4.3.3 Silver Nanoparticle Viscometer Testing

4.3.3.1 Citrated Blood

Silver nanoparticles consisting of 80nm in size were subjected to the acetic acid replacement procedure leaving a final volume of 50µL of silver nanoparticles suspended in acetic acid. The nanoparticle acetic acid solution and $CaCl_2$ were added separately to 4.5mL of citrated blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded.

Results displayed in Figure 4.14 shown a slight difference between the onset times of coagulation with the addition of silver nanoparticles decreasing the coagulation time by about 2 minutes compared to the baseline solution of $CaCl_2$. On the other hand, Figure 4.15 shows no difference between the coagulation times of the silver nanoparticles and the acetic acid $CaCl_2$ solution with an exception to the baseline solution of $CaCl_2$ in that it did not show any signs of coagulation by no increasing viscosity. The inconsistencies seen with the addition of silver nanoparticles are similar to those displayed in the gold nanoparticle testing as well leading to the same conclusion that both the silver and gold nanoparticles have no consistent effect on coagulation time presumably due to the nanoparticles themselves or the testing methods.

In addition to the inconsistencies regarding testing results, there were difficulties in initiating the clotting process by the addition of $CaCl_2$ to the citrated blood (Figure 4.15, Figure D.5, and Figure D.6). This difficulty was seen throughout all the nanoparticle citrated blood testing. A plausible explanation is that the concentration of

$CaCl_2$ that was added to the blood was too low to overcome the chelation of calcium ions in the blood by the citrate.

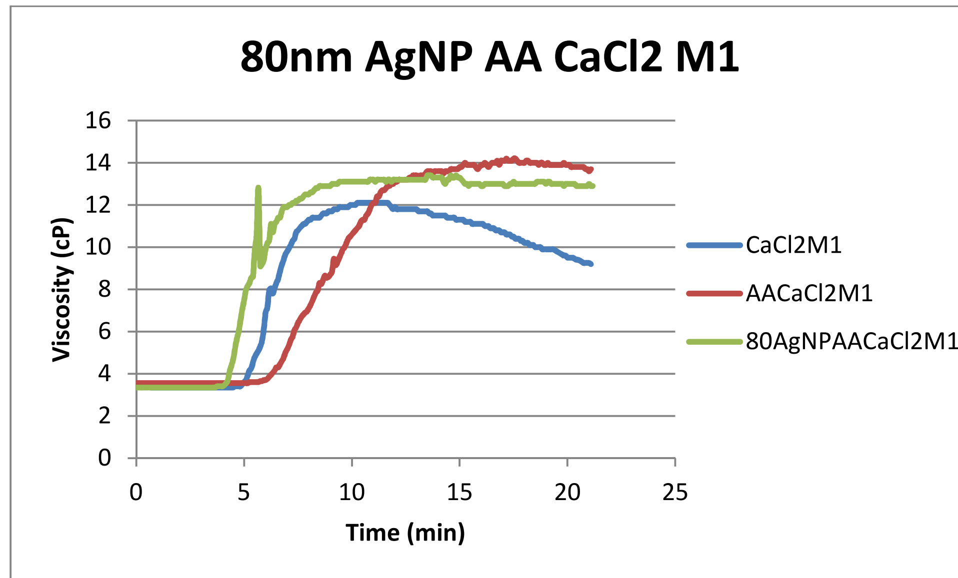

Figure 4.14: 80nm Silver Nanoparticle Citrated Blood Test Rep 1

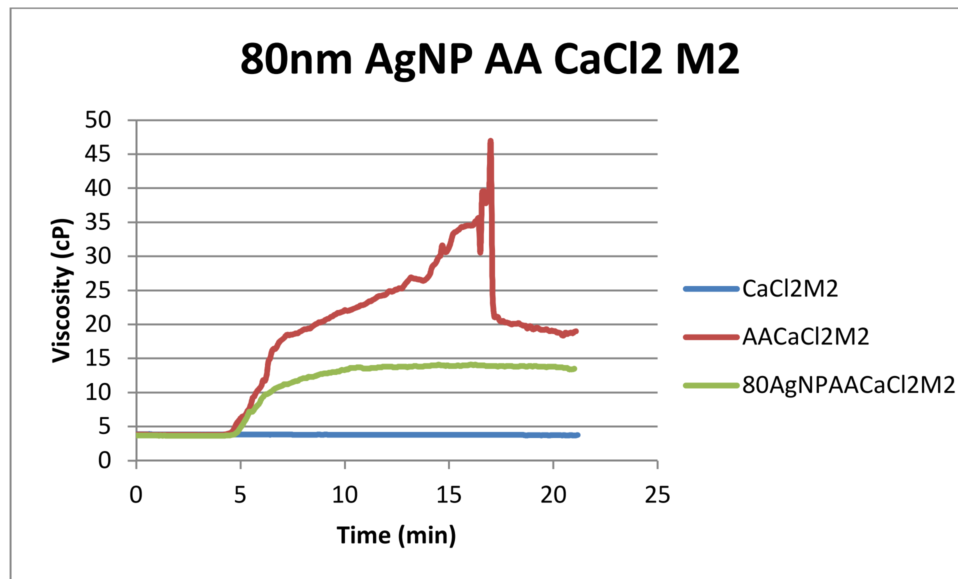

Figure 4.15: 80nm Silver Nanoparticle Citrated Blood Test Rep 2

4.3.4 Silica Nanoparticle Viscometer Testing

4.3.4.1 Citrated Blood

Test 1: An aliquot of 3.5mg of 15nm silica nanoparticles were added to acetic acid. The nanoparticle acetic acid mixture and $CaCl_2$ were added separately to 4.5mL of citrated blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded.

Results presented in Figure 4.16 show no significant differences in the onset times of coagulation for silica nanoparticles, acetic acid $CaCl_2$ solution, and the baseline solution of $CaCl_2$. The same result has occurred in multiple tests indicating a possible issue with the testing methods, specifically the addition of acetic acid to the nanoparticles. The acetic acid could be masking the effects of the nanoparticles, thus the start of coagulation is being caused by acetic acid and not the nanoparticles. This statement is supported by many test results showing no difference between the addition of nanoparticles and the acetic acid.

Although no differences were seen with respect to coagulation times, there was an increase in viscosity after the start of coagulation with the greatest increase seen by the addition of silica nanoparticles.

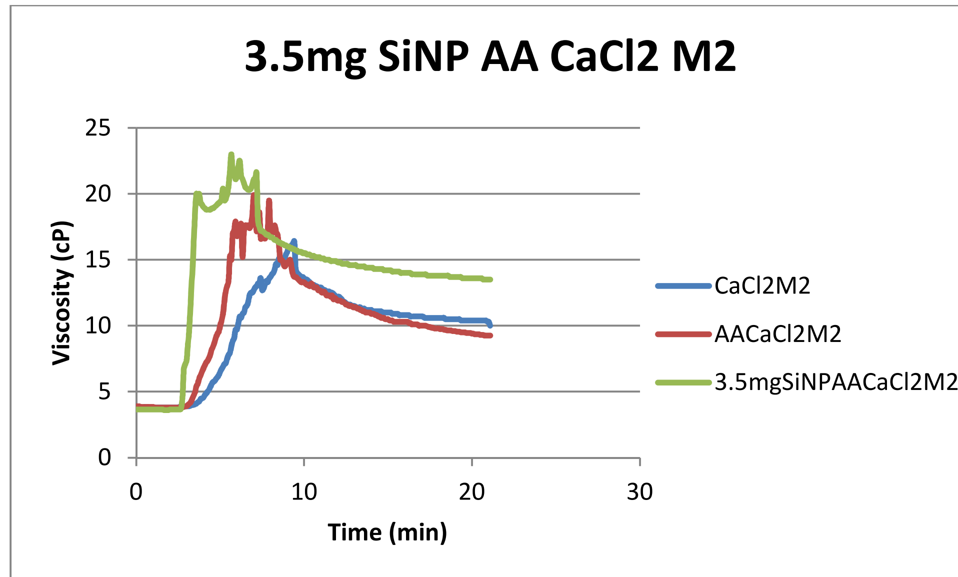

Figure 4.16: 3.5mg Silica Nanoparticle Citrated Blood Test 1

Test 2: In trying to improve the testing methods, Test 2 was altered from Test 1 by the removal of acetic acid. Aliquots of 3.5mg and 7mg of 15nm silica nanoparticles and $CaCl_2$ were added separately to 4.5mL of citrated blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded.

Results presented in Figure 4.17 for 3.5mg of silica nanoparticles show no difference in the onset times of coagulation between the silica nanoparticles and the baseline solution of $CaCl_2$, but an increase in viscosity after the start of coagulation can be seen with the silica nanoparticles having a greater viscosity than the baseline solution of $CaCl_2$.

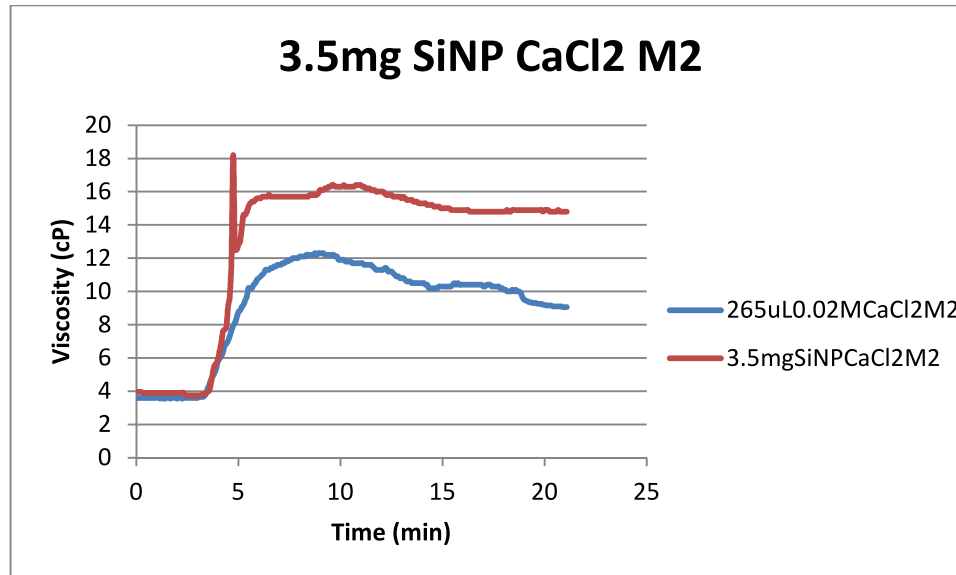

Figure 4.17: 3.5mg Silica Nanoparticle Citrated Blood Test 2

Results from Figure 4.18 for 7mg of silica nanoparticles display similar findings to that of the 3.5mg of silica nanoparticles. The results show no significant difference in the onset times of coagulation between the silica nanoparticles and the baseline solution of $CaCl_2$, and the silica nanoparticles possess a greater viscosity after the start of coagulation compared to the baseline. Even though there still is no difference in coagulation times between the silica nanoparticles and the baseline, the increased viscosities with the addition of the silica nanoparticles suggest a possible positive effect on coagulation by obtaining a more viscous blood sample with the addition of silica nanoparticles.

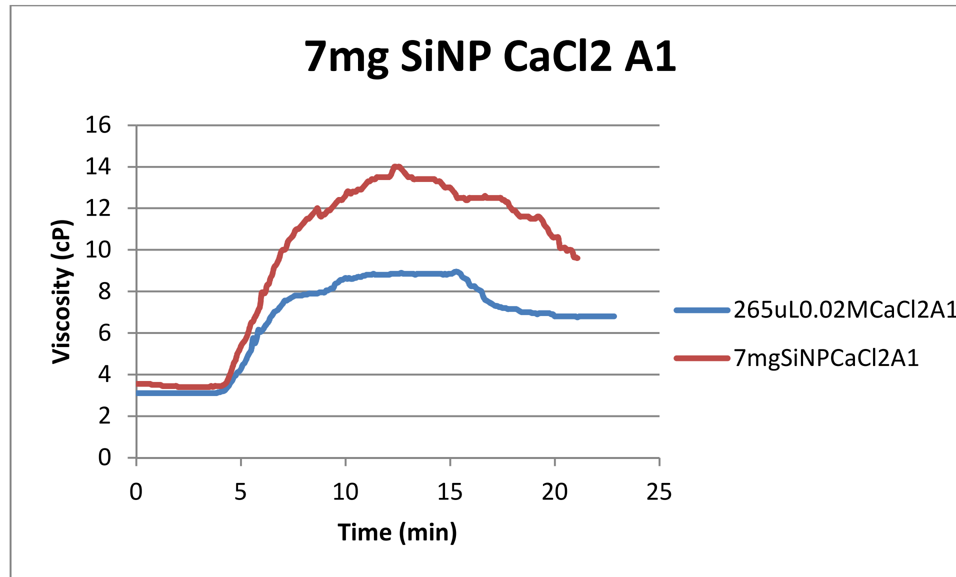

Figure 4.18: 7mg Silica Nanoparticle Citrated Blood Test 2

Test 3: In seeing an increased viscosity with the addition of 3.5mg and 7mg of silica nanoparticles, Test 3 was modified from Test 2 by increasing the amount of silica nanoparticles added to the citrated blood sample. An aliquot of 14mg of silica nanoparticles was added to $CaCl_2$. This mixture was vortexed to ensure thorough mixing and then added to 4.5mL of citrated blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded.

Results presented in Figure 4.19 show no difference in the onset times of coagulation between the silica nanoparticles and the baseline solution of $CaCl_2$, but there was a significant increase in viscosity after the start of coagulation with the addition of silica nanoparticles compared to the baseline. The blood sample became so viscous after the start of coagulation with the addition of the silica nanoparticles that the viscometer could not measure the viscosity of the sample at its current settings. Also, upon physical

examination after testing was completed, a large formed blood clot was observed indicating the silica nanoparticles positive effect on coagulation even though there was no detectable difference in coagulation times.

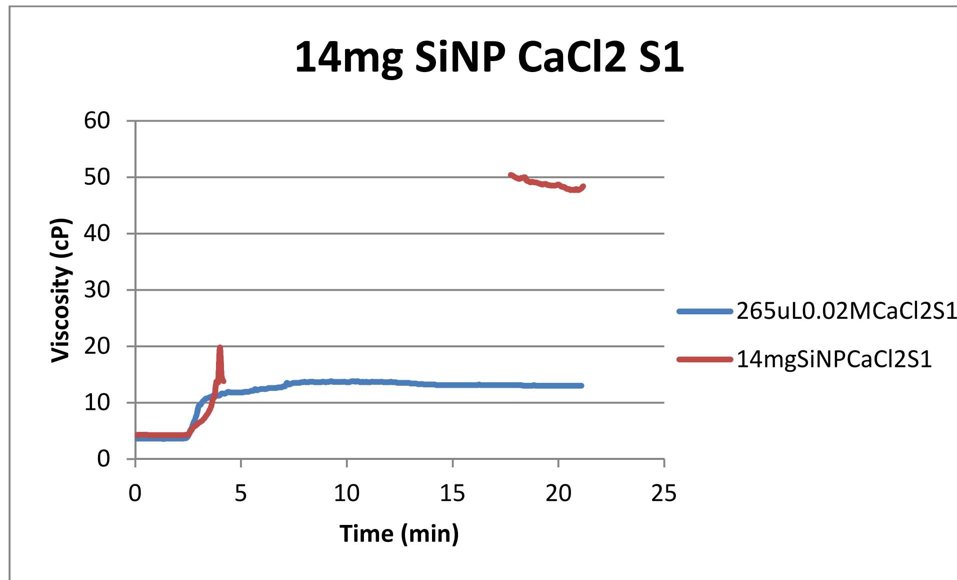

Figure 4. 19: 14mg Silica Nanoparticle Citrated Blood Test 3

4.3.4.2 Whole Blood

In attempting to detect measurable changes in the onset time of coagulation, 14mg of silica nanoparticles was added to 5ml of whole blood and placed in the small sample adapter. Viscosity readings were taken every 5 seconds and recorded.

Results displayed in Figure 4.20 show a decrease in the onset time of coagulation by about 2 minutes with the addition of silica nanoparticles compared to the baseline solution of $CaCl_2$ and a significant increase in viscosity after the start of coagulation with the addition of silica nanoparticles compared to the baseline. Repeated tests showed

similar results demonstrating silica nanoparticles effectiveness at initiating coagulation and decreasing the onset time of coagulation.

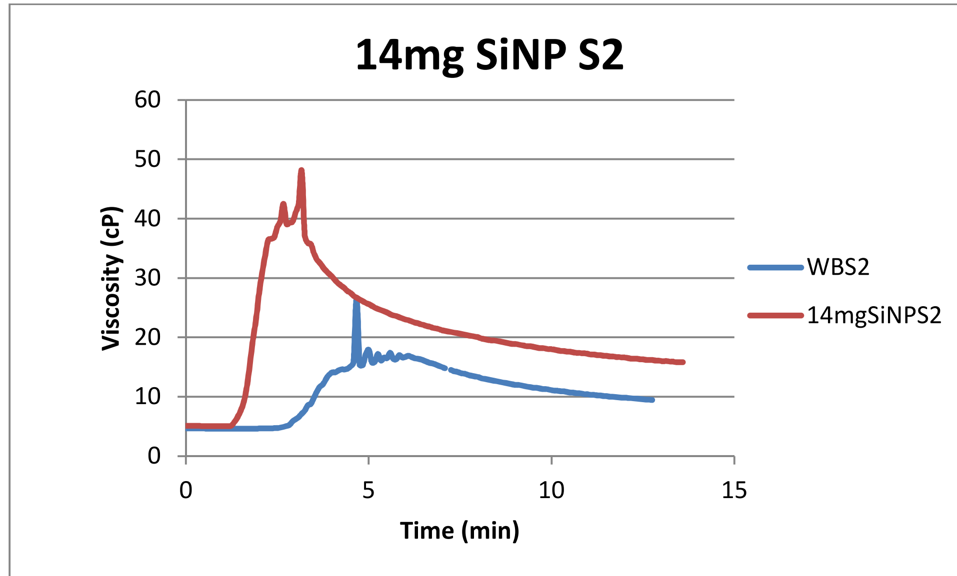

Figure 4.20: 14mg Silica Nanoparticle Whole Blood Test

4.4 Conclusion

A rotational viscometer was utilized to test the effects of collagen, gold nanoparticles, silver nanoparticles, and silica nanoparticles on blood coagulation time in whole blood and citrated blood.

Results from whole blood collagen testing show a significant decrease in the onset time of coagulation upon the addition of collagen validating the use of a rotational viscometer for testing differences in coagulation time with the addition of different nanoparticles.

Testing with 100nm and 20nm gold nanoparticles resulted in inconsistencies with respect to the onset times of coagulation with some tests displaying a decrease in coagulation times with the addition of nanoparticles while others exhibited no difference between the nanoparticles and the baseline solution. The inconsistencies were apparent in both whole blood testing and citrated blood testing.

Results for 80nm silver nanoparticle citrated blood testing also showed inconsistencies with respect to the onset times of coagulation with some tests displaying a decrease in coagulation times with the addition of nanoparticles whereas others exhibited no difference between the nanoparticles and the baseline solution.

Citrated blood testing with 15nm silica nanoparticles showed no difference in the onset time of coagulation compared to the baseline solution, but an increase in viscosity after the start of coagulation was observed with the silica nanoparticles. Moreover, whole blood testing with silica nanoparticles did show a consistent decrease in the onset time of coagulation and a greater increase in viscosity after the start of coagulation compared to the baseline solution.

Of all the nanoparticles tested, silica was the only nanoparticle that displayed a consistent decrease in the onset time of coagulation and a greater viscosity after the start of coagulation compared to the baseline solution demonstrating its potential use as hemostatic agent and its prospective incorporation into a hemostatic wound dressing.

CHAPTER 5

FUTURE WORK

5.1 Introduction

After testing gold, silver, and silica nanoparticles' effect on coagulation, it was determined that silica exhibited the greatest potential use as a hemostatic agent and its future incorporation into a hemostatic wound dressing.

5.2 Development of a Hemostatic Wound Dressing

In developing a hemostatic wound dressing with silica nanoparticle integration, methods and procedures need to be established in order to attach the nanoparticles to a dressing type of material. The attachment to the dressing material needs to be strong and durable, so the particles will not separate, yet flexible enough to allow for movements when applying the dressing to a wound. After attachment, characterization analysis will need to be performed to confirm that the silica nanoparticles successful attachment to the dressing.

Even though silver nanoparticles did not prove effective in clotting blood for both ex vivo studies, silver does contain antibacterial properties and would be an added benefit to a hemostatic wound dressing in preventing any possible infections in open wounds. Additional methods and procedures would need to be established for the attachment of the silver nanoparticles to the dressing in conjunction with the silica nanoparticles as well. After attachment is confirmed, bacterial studies would need to be

performed on the dressing in order to determine silver's ability to inhibit bacterial growth and adhesion.

5.3 Further Studies

After the development of the silica-silver nanoparticle hemostatic wound dressing, further studies need to be performed assessing the dressing's efficacy *in vivo*. Porcine animal studies where the dressing is tested under high pressure-high flow conditions are good measure of the dressing's ability to withstand significant amounts of blood volume within a small duration and successfully achieve hemostasis.

APPENDIX A
NANOPARTICLE FUNCTIONALIZATION AND CROSSLINKING PROCEDURE

A.1 Nanoparticle Functionalization:

1. Centrifuge 168µL of 100nm AuNP at 7000rpm for 5 minutes.
2. Remove 118µL of supernatant off the top leaving 50µL of AuNP left.
3. Add 1.14µL of a 10mg/mL solution of MEA to the 50µL of AuNP.
4. Vortex f-AuNP mixture.

A.2 Nanoparticle Crosslinking:

1. Mix 0.4mg EDC, 0.5mg NHS, 89µL distilled water, 50µL 10X PBS, and 5.75µL 1M NaOH and add to the f-AuNP solution.
2. Add 0.25mL rat tail collagen and pipette mix.
3. Incubate at 37°C for 90 minutes to allow for polymerization.
4. AuNP collagen gel can be stored at room temperature.

Each collagen nanoparticle sample comprises a total volume of 0.25mL.

*The procedure for 80nm AgNP is the same apart from the following exception: start with 855µL of 80nm AgNP and remove 805µL of supernatant after centrifuging.

A.3 Silver Nanoparticle Standardization

The amount of silver nanoparticles used in the functionalization procedure was standardized to equal amount of gold nanoparticles from the following calculations:

$$\frac{5.6x10^{9}\ particles\ of\ Au}{mL} \times 168\mu L = 9.408x10^{8}\ particles\ of\ Au$$

$$9.408x10^{8}\ particles\ of\ Au\ \times \frac{1\ mL}{1.1x10^{9} particles\ of\ Ag} = 8.55x10^{-4}\ L$$

$$= 855\mu L\ of\ AgNP$$

APPENDIX B
ADDITIONAL *EX VIVO* STUDY I TESTING RESULTS

Table B.1: *Ex Vivo* Test 2a Tube and Petri Dish Weights

	Tubes		**Petri Dish**	
	Before (g)	*After (g)*	*Before (g)*	*After (g)*
Sample 1 (Control)	7.37	7.80	15.64	20.82
Sample 2 (Collagen)	7.74	8.07	15.52	20.04
Sample 3 (AuNP)	7.67	7.96	15.64	20.76
Sample 4 (AgNP)	7.77	7.94	15.61	20.68

Table B.2: *Ex Vivo* Test 2a Blood Weights

	Unclotted Blood (g)	**Clotted Blood (g)**	**Total Blood (g)**	**% (Clotted Blood/Total Blood)**
Sample 1 (Control)	5.18	.43	5.61	7.66
Sample 2 (Collagen)	4.52	.33	4.85	6.80
Sample 3 (AuNP)	5.12	.29	5.41	5.36
Sample 4 (AgNP)	5.07	.17	5.24	3.24

Table B.3: *Ex Vivo* Test 4a Tube and Petri Dish Weights

	Tubes		Petri Dish	
	Before (g)	*After (g)*	*Before (g)*	*After (g)*
Sample 1 (Control)	7.35	7.73	15.66	20.86
Sample 2 (Collagen)	7.72	10.01	15.62	18.39
Sample 3 (AuNP)	7.62	8.97	15.56	19.38
Sample 4 (AgNP)	7.58	11.66	15.71	16.77

Table B.4: *Ex Vivo* Test 4a Blood Weights

	Unclotted Blood (g)	**Clotted Blood (g)**	**Total Blood (g)**	**% (Clotted Blood/Total Blood)**
Sample 1 (Control)	5.20	0.38	5.58	6.81
Sample 2 (Collagen)	2.77	2.29	5.06	45.26
Sample 3 (AuNP)	3.82	1.35	5.17	26.11
Sample 4 (AgNP)	1.06	4.08	5.14	79.38

APPENDIX C
EX VIVO STUDY II PROTOCOLS

C.1 Collagen and NaOH:DMF Buffer solution

1. Mix 0.05mL of DMF and 0.20mL of 0.5M NaOH, making a 0.25mL solution.

2. Add 2.5mg of lyophilized collagen to the solution.

Thus, making a 10mg/ml collagen and NaOH:DMF buffer solution.

APPENDIX D
ADDITIONAL *EX VIVO* STUDY II RESULTS

D.1 Collagen Whole Blood Viscometer Testing

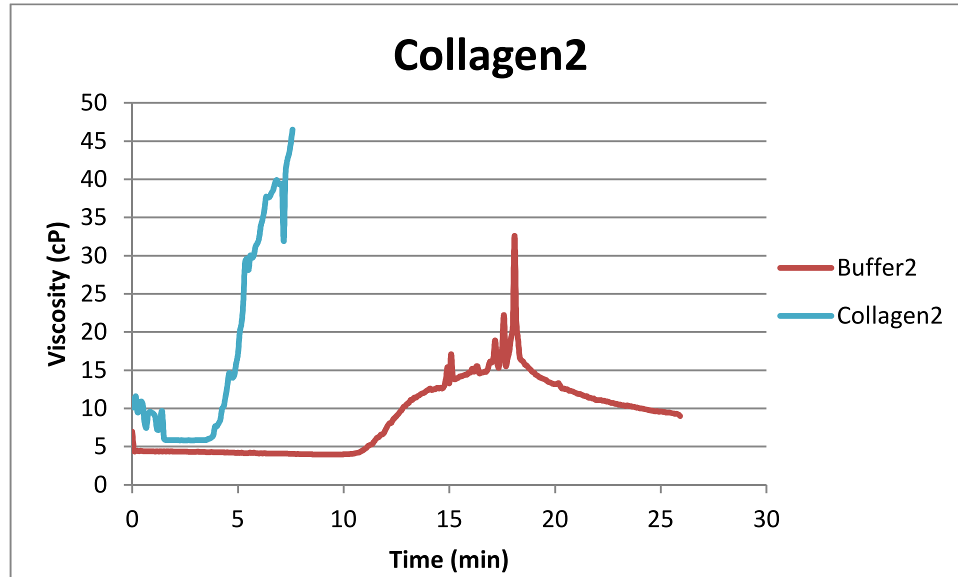

Figure D.1: Collagen Whole Blood Test Rep 2

D.2 Gold Nanoparticle Whole Blood Viscometer Testing

Test 1:

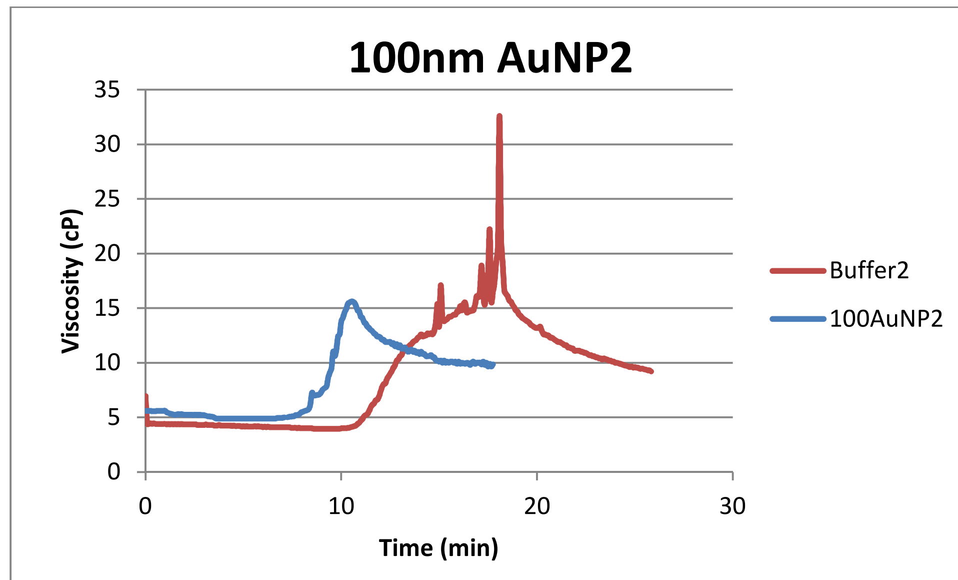

Figure D.2: 100nm Gold Nanoparticle Whole Blood Testing Rep 3

Test 2:

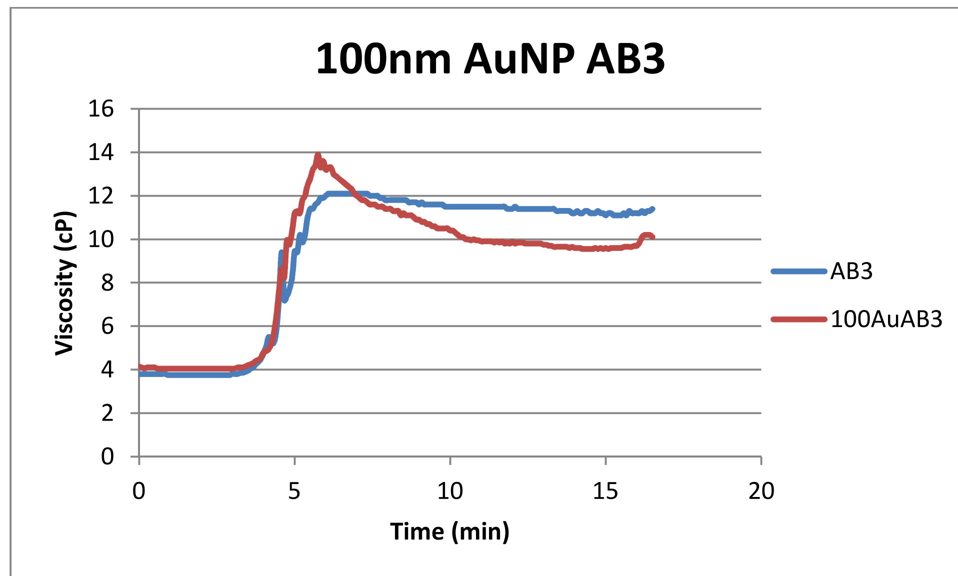

Figure D.3: 100nm Gold Nanoparticle Whole Blood Test 2 Rep 2

Test 3:

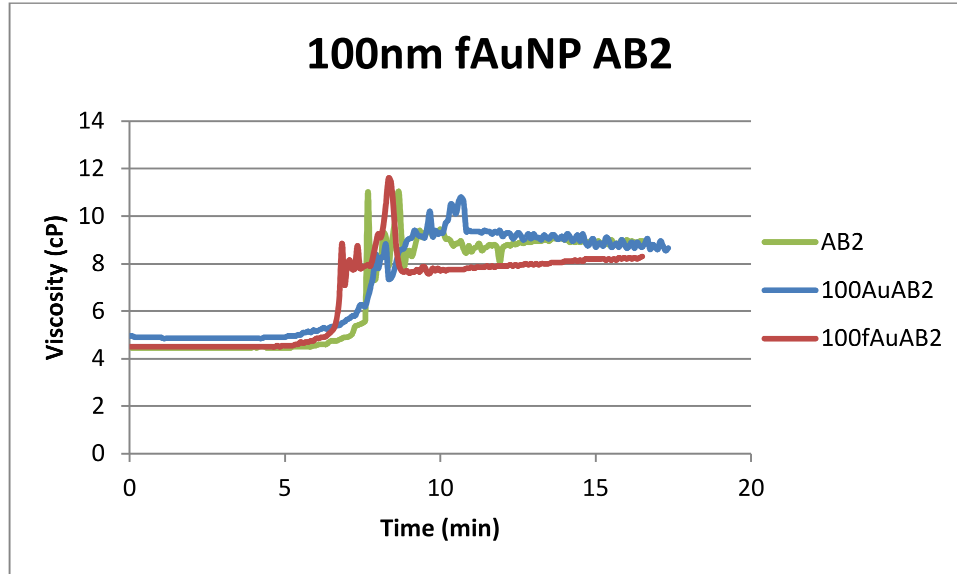

Figure D.4: 100nm Functionalized Gold Nanoparticle Whole Blood Test 3 Rep 2

D.3 Silver Nanoparticle Citrated Blood Viscometer Testing

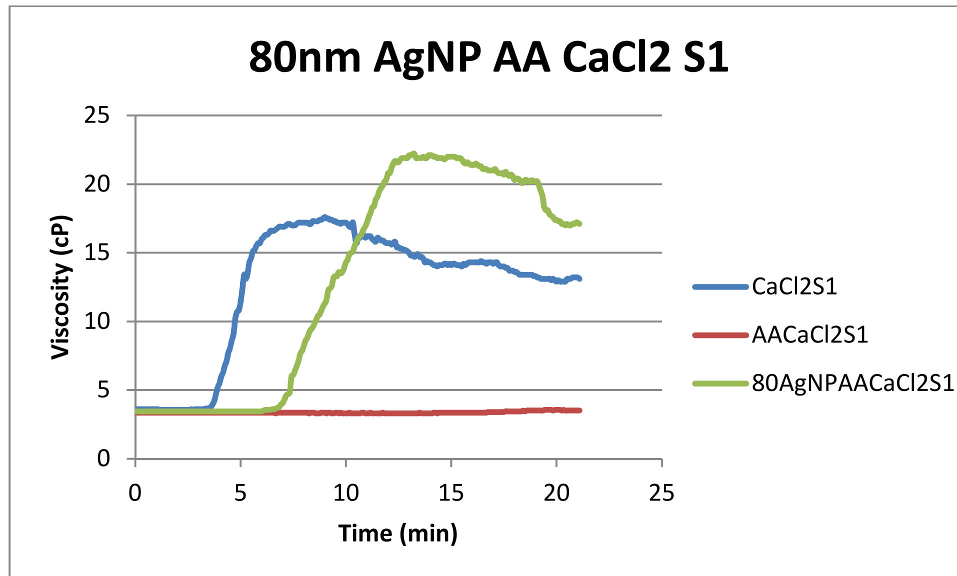

Figure D.5: 80nm Silver Nanoparticle Citrated Blood Test Rep 3

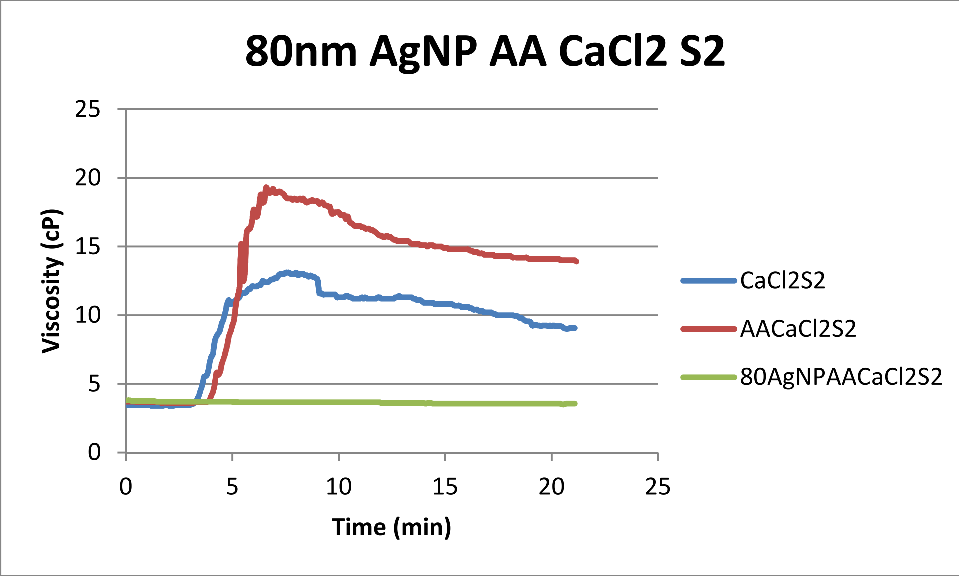

Figure D.6: 80nm Silver Nanoparticle Citrated Blood Test Rep 4

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