DISSERTATION

AN INVESTIGATION OF THE EFFECT OF SURFACE RELEASED NITRIC OXIDE ON FIBRINOGEN ADSORPTION

Submitted by

Sarah Marie Lantvit

Department of Chemistry

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Doctoral Committee:

Advisor: Melissa Reynolds

Thomas Borch
Ellen Fisher
Alan Kennan
Ketul Popat

ABSTRACT

AN INVESTIGATION OF THE EFFECT OF SURFACE RELEASED NITRIC OXIDE ON FIBRINOGEN ADSORPTION

The search for improved biomaterials is a continually ongoing effort to prevent the failure of medical devices due to blood clotting. Each group of researchers has their own set of methods to create the ideal material for biological systems. In the pursuit of materials to prevent blood clot formation, these attempts have been focused on alterations in surface properties, pre-adsorption of proteins, and release of drugs. In this work I took a high-throughput approach to the prevention of device failure by investigating a model material system. Starting with a nitric oxide (NO) releasing material, a sample preparation method was developed to ensure that surface properties could be compared to a non-NO releasing control. With this material, the effect of the NO release on fibrinogen adsorption to these surfaces could be isolated. Fibrinogen is instrumental in the formation of blood clots. Determining the effect that NO has on this protein will help determine why NO has been previously found to prevent clotting in blood-contacting systems. Once the model system was developed, further investigation into changes in the fibrinogen resulting from its interaction with the released NO could be undertaken. A full investigation was completed on control non-NO releasing, low NO flux, and high NO flux materials. A qualitative assessment of the fibrinogen adsorption shows that the high NO releasing material exhibits significantly higher fibrinogen adsorption compared to both the control and low NO flux materials. Quantitative assessment of fibrinogen adsorption was attempted through a variety of methods, which indicate that conformational changes are happening upon adsorption of fibrinogen to all materials. To this

end, FTIR spectra from the adsorbed fibrinogen and native fibrinogen were compared to elucidate changes in the protein's conformation. Control and low NO flux materials had too little protein to gain insight into these changes. For the high NO flux material, the fibrinogen had a significant decrease in α-helices and an increase in random chains compared to native fibrinogen. To begin understanding the effect that these changes will have on blood clot formation, these materials were further analyzed for platelet adhesion. A comparison of the control, low NO flux, and high NO flux materials with and without fibrinogen adsorbed to the material surface shows that the fibrinogen has a distinct effect on platelet adhesion and aggregation. The high NO flux materials exhibited less aggregation and full activation of platelets when fibrinogen was adsorbed prior to incubation with platelets than if fibrinogen was not present before incubation. Overall, the effect of NO on fibrinogen adsorption can be seen through these measurements. Nitric oxide release causes an increase in fibrinogen adsorption, as well as protein reorganization. Surprisingly, we see that this adsorbed fibrinogen actually improves the viability of platelets. Further study must be done using whole blood and *in vivo* measurements to fully understand what effect the adsorbed fibrinogen will have on the device. Despite this we can say that the adsorption of fibrinogen onto these NO releasing materials helps to improve the biocompatibility of this biomaterial due to its bulk adsorption and conformational changes.

ACKNOWLEDGEMENTS

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Finally, I would like to thank my family and friends for their support over the years. My parents have and always will be an inspiration to me. My husband deserves the greatest praise as he has been there to support and console me through all of the stages of graduate school.

This entire work is dedicated to my grandfather, Henry Duda, who passed right at the time I started graduate school. I know he has been here supporting and guiding me through this experience.

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CHAPTER I:

INTRODUCTION

I.1. Importance of Proteins in Blood Clot Formation

The use of blood-contacting medical devices increases annually.[1] Yearly, over half a million Americans alone have a stent implanted.[2] Still more undergo procedures for vascular graft implantation or dialysis using extracorporeal circuitry. The mortality rate for patients with either a coronary stent or bypass graft are at 8.0 and 7.6%, respectively.[3] For dialysis patients, this rate has been shown to be at 20% as of 2009.[4] While these rates have been dropping, they demonstrate the importance of continued improvement for blood-contacting medical devices.

The most common reason for blood-contacting device failure is thrombus formation, blood clots, and subsequent device occlusion. Despite the advances in medicine, millions of people still suffer from these complications annually.[5] For example, complications in extracorporeal circuitry use in dialysis results in an overall mortality rate of 39% (neonatal to adult) before discharge or transfer to another facility.[6] The majority of these complications arise from device failure due to clotting and sepsis. Several approaches have been adopted to overcome this challenge. As the scientific community looks toward developing enhanced biomaterials, a clearer understanding of blood clot formation is also required. A starting point is to compare the difference in blood clot formation on a blood vessel wall to a synthetic surface/implant. There are some major differences in which thrombus forms for synthetic materials when compared to the natural endothelium which are detailed in Figure I.1. In clot formation within the natural endothelium, the first step in clot formation is the activation and adherence of platelets to the site of injury (Figure I.1a). Subsequently, proteins begin to adhere. A specific example is fibrinogen, which will activate to

form fibrin, further adhering more platelets and proteins to the clot. Additionally, other cells will be attracted to the clot; if the system is working, clot formation will naturally halt after fixing the rupture in the blood vessel. For implanted materials this process has a key difference from the natural occurrence (Figure I.1b).[1,7,8] Immediately upon implantation of a device into the blood system, proteins adsorb to the surface of the material. In response to the adsorbed proteins, platelets begin to adhere and activate. The activated platelets then recruit more proteins and cells to form a clot. Because there is no signal to stop the clot formation, recruitment of cells and proteins will continue until the passageway is completely occluded and the device fails.[1]

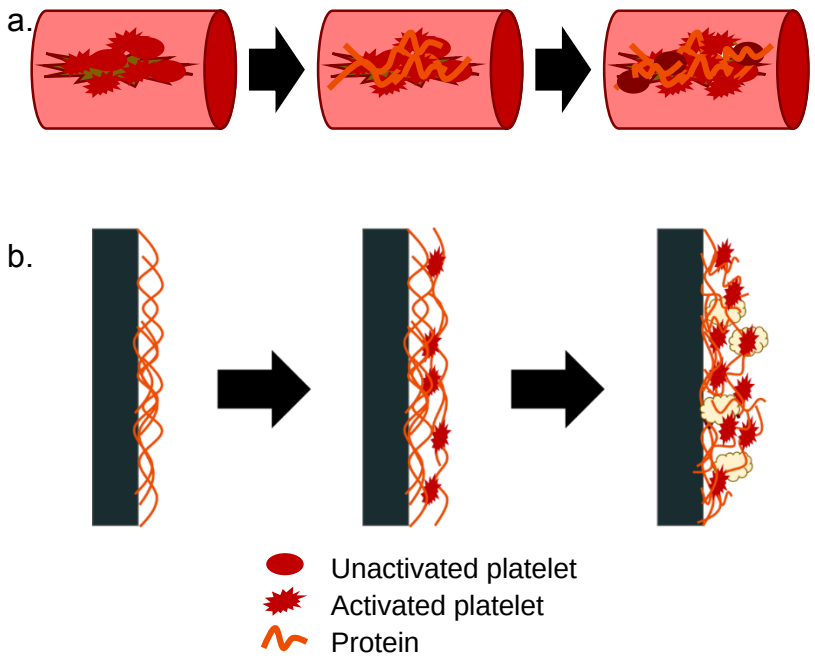

FIGURE I.1: Difference in the general steps of blood clot formation within the natural endothelium (a) and on a synthetic material (b) from initial response to final formation of a blood clot. Platelet adhesion is the first step in clot formation on the natural endothelium, while protein adsorption happens first on a synthetic material.

Protein adherence is a crucial step in the formation of blood clots on synthetic materials. Understanding the fundamental science behind clot formation on these devices will improve

materials in the future and reduce the complications associated with the failure of biomedical devices. Isolating which proteins adsorb onto the implant surface becomes a critical step as changing the amount, conformation, and activity of the adsorbed proteins affects the interactions of the protein layer with platelets and other cells. The ability to control unspecific protein adsorption improves implant compatibility in the body and reduce device failure due to clot formation.

Among all the proteins present in the blood stream, blood clotting proteins represent the major point of concern. The main players in blood clot formation are: fibrinogen, fibronectin, and vonWillebrand factor. These proteins are active in the formation of blood clots and interactions with platelets; therefore, they are potential risks for the failure of biomedical devices. By looking at the effect of the implantable material on the activity and conformation of these proteins, we can begin to understand the efficacy of the device to prevent blood clot formation during its use. As proteins are the body's first line of defense against synthetic implants, the focus of this work will be on protein adsorption, particularly fibrinogen, because it is the most concentrated protein in the blood stream. More specifically this work centers on the protein fibrinogen, the most concentrated of the clotting proteins in the blood stream.

I.2. Fibrinogen's Role in Blood

I.2.a. Structure

Fibrinogen is a 340 kDa protein found in the blood stream at a concentration of around 3 mg/mL.[9] Prior to the full crystal structure determination in 2009, only portions of fibrinogen had been investigated.[10] Fibrinogen has a distinct trinodular structure with two distal D domains and one central E domain (Figure I.2).[9] The E domain has a smaller αC domain positioned above it.

The relative charge of the protein differs slightly on different sides of the protein as seen in Figure I.2, represented by the yellow and purple circles. The αC domain is slightly positive and hydrophilic, whereas the D and E domains are slightly negative and hydrophobic in relation to the rest of the protein. This negatively charged region is much larger than the positively charged one. Binding through this region is less likely to be reversed. The D domains are connected to the rest of the protein through a coiled coil on each side comprised of Aα, Bβ, and γ chains. The αC domain is attached through an Aα chain on each side.

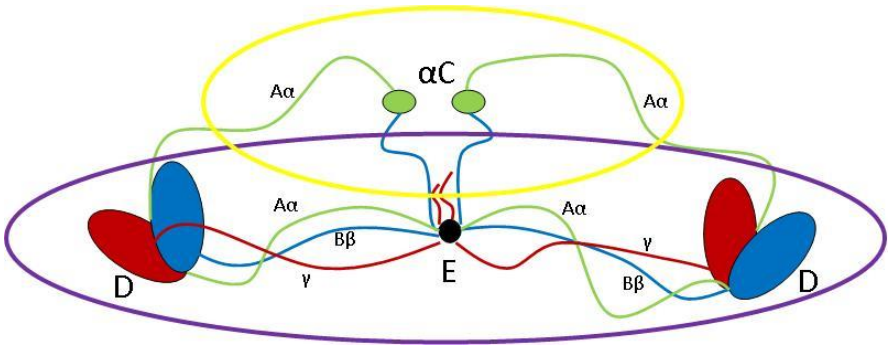

FIGURE I.2: Cartoon of fibrinogen with D, E, and αC domains and Aα, Bβ, and γ chains labeled. Purple circle indicates the area of the protein that is overall negatively charged and hydrophobic. Yellow circle indicates the area of the protein that is overall positively charged and hydrophilic.

The interaction of fibrinogen with platelets can be traced to three different motifs on the protein. The main motif for interaction with platelets is the RGD sequence; changes to this motif will lead to different interactions of the fibrinogen with platelets. The presence of RGD will allow for platelets to bind to the fibrinogen, however, lacking an additional amino acid such as S or F will not promote platelet aggregation.[11] Depending on the sequence, fibrinogen can adhere either only activated platelets or both activated and unstimulated ones. The availability of these motifs can be directly related to the ability of the fibrinogen molecules to interact with platelets in blood

clot formation. Overall fibrinogen is a flexible molecule and the interaction of its different chains and domains will be dependent on how it adsorbs to a material surface.

I.2.b. Binding

As described above, fibrinogen has various hydrophilic/hydrophobic and positive/negative domains within the structure of the molecule. The accessibility of these domains and specific binding residues after adsorption onto a material surface will dictate the binding abilities.

The binding of fibrinogen to a material surface is preferential compared to other proteins due to the size and concentration of the protein.[12] The larger the area of the protein available to interact with the surface, the less likely it will be that another protein will be able to displace it. In some cases, fibrinogen is replaced by other protein adsorption, but only if the other protein has a greater affinity for the surface properties of the material. The literature is still unclear; however, the prevailing thought is that fibrinogen binds due to charge and hydrophobic interactions with material surfaces. Because of fibrinogen's varied domain properties, the material surface properties will dictate which part of the fibrinogen will bind to it.

In blood clot formation, the main role of fibrinogen is to form fibrin which serves to bind the clot and hold it together. The formation of fibrin is triggered by the interaction of thrombin with adsorbed fibrinogen. Thrombin does not interact with free/native fibrinogen flowing through the blood, but it will trigger the formation of fibrin when fibrinogen is adsorbed. Thrombin will cleave the Aα chains from the central αC domain and subsequent knob-hole interactions with the strands from one fibrinogen molecule to the next will form the fibrin mesh.[13] Just as in the case with fibrinogen adsorption, the amount of fibrin formation has been shown to be affected by the charge and relative hydrophobicity of the material surface.[14]

I.2.c. Importance in Clot Formation

The coagulation cascade comprises many different proteolytic activities including, fibrin formation, and the interaction of twelve different clotting factors. However, when simplified, platelets and fibrinogen become the most important players in the activity of this pathway.[15] Platelet activation is instrumental in recruiting other cells and proteins to the site for clot formation. Fibrinogen adsorption becomes equally important in the formation of the mesh that holds the clot together and as a platelet activator and recruiter. Working together, these two players allow the coagulation cascade to proceed and create a blood clot. In the natural endothelium when all systems are working properly, platelets and fibrinogen work together to form a clot until the signal comes to end recruitment of new cells. The newly formed clot has then repaired any rupture in the vessel wall. In synthetic implants, there is no signal to stop clot formation and the coagulation cascade will continue until the area is fully occluded and the device fails.

Fibrinogen is the most concentrated blood clot forming protein present in the blood stream. It is instrumental in polymerizing to form fibrin which holds clots together. When in the natural lumen of blood vessels, fibrinogen is inactive in its native state flowing through the system. If a clot starts to form, the passing fibrinogen is attracted by the activated and adhered platelets to bind and produce fibrin through an interaction with thrombin. This will help hold in the blood clot and attract more platelets to the area. When an implanted device is inserted into the blood system, fibrinogen is one of the proteins that will be attracted to adsorb onto the material. Which proteins adsorb preferentially depends on the surface properties of the material (i.e. roughness, wettability, composition). The conformation and activity of these adsorbed proteins is unknown. It is these adsorbed proteins, fibrinogen included, that will interact then with platelets and begin blood clot formation.

I.3. Material Properties That Affect Protein Binding

The surface properties of a material affect the adsorption of proteins on its surface, particularly those of wettability,[12,16-19] roughness,[20] and composition.[21-23] Researchers are attempting to tailor the surface properties of materials to control protein adhesion and biofouling of the materials.[17,24,25] In addition, researchers are also introducing drug release from their materials to combat biofouling and control protein adsorption.[26-28] Changes in surface properties and the addition of drug release from the material will affect proteins in different ways. The study of these processes must isolate each in turn to determine which change (wettability, composition, drug elution, etc.) is causing the observed effect. There are many drugs being eluted from biomedical devices today for differing applications such as paclitaxel,[28] used in cancer treatments, pentoxifylline (PTX),[27] an anti-inflammatory, and sirolimus,[28] used to prevent rejection after kidney transplant. The focus of our group and this work, is on the release of nitric oxide (NO), a naturally occurring therapeutic for anti-inflammatory, anti-bacterial, vasodilation, etc. The main emphasis of this work is to control the surface properties and investigate the effects of surface released NO on fibrinogen adsorption, the first step in clot formation on synthetic implants.

I.3.a. Changes in surface properties and their effect on protein binding

The surface properties of a material are instrumental in determining which proteins will bind preferentially to a surface and how they will bind. Charge, wettability, roughness, and elemental composition are the four main players influencing protein adsorption. The charge on a material surface, whether it is positive or negative, will determine which amino acid residues on the protein will adsorb to the material surface.[9] For example, fibrinogen has both positively and negatively charged areas of its structure. If fibrinogen binds through the positively charged area,

it can be more easily removed from the material surface by other proteins, since it is a relatively small section of the protein that is involved in binding.[29] Proteins with larger positively charged sections will displace the fibrinogen from the material surface. In contrast, if the fibrinogen is bound by the (relatively large) negatively charged area of the protein, then it is likely to be irreversibly bound to the surface and other proteins will not be able to displace it. The same binding effects that surface charge has on proteins can be seen as true for surface wettability and hydrophobic or hydrophilic binding as well.

Many studies have investigated the effect wettability changes has on fibrin formation.[30-32] One study by the Schoenfisch group examined four different surfaces with varying surface functional groups to test the effect of changes in wettability and charge on fibrin proliferation.[14,30] Fibrin proliferation is important in thrombus formation as it binds the blood clot, holding it together. They studied surfaces modified with methyl-, hydroxyl-, amino-, and carboxyl- groups to serve as model hydrophobic, hydrophilic, positively charged, and negatively charged surfaces, respectively. The amount of fibrinogen adsorbed was comparable for hydrophobic, positively charged, and negatively charged surfaces, however, the hydrophilic surface had 50% less fibrinogen adsorbed to the material surface.[30] They found that the amount of fibrinogen adsorbed to the material surface does not dictate fibrin formation. However, the hydrophobic and positively charged surfaces exhibited far more fibrin than the hydrophilic and negatively charged materials. The observation that the amount of fibrinogen adsorbed on a surface does not dictate the activity of the fibrinogen, has also been reported by the Latour group and others.[33,34] This suggests that the surface properties of these materials dictate whether or not the fibrinogen will be highly prolific in fibrin formation or not. Control over the surface properties of the material of interest is essential to determine the effects on fibrinogen adsorption and thrombus formation. As shown by the effects

of the different surface properties in this study, it is instrumental that these properties be controlled to determine the effect of NO release on fibrinogen adsorption and activity.

Surface roughness is additionally important to protein adsorption to a surface with studies working toward specifically patterning surfaces to control biointeractions with the material surface. For example, work done by the Brennan group incorporates surface roughness to prevent biofouling.[35-37] In this study, they patterned a polydimethylsiloxane (PDMS) surface with ridges on the microscale, preventing fouling of the material by green algae. Further work on this material is aimed at preventing biofouling in the body. In contrast, a study by the Jandt group showed that the roughness of a material does have an effect on protein adsorption.[20] However, when the difference in roughness between two surfaces is within a 19 mm range (less than the size of the protein) there is no noticeable effect on protein interactions with the surface. This suggests that when roughness is on the nanometer scale, it can be eliminated as a contributing factor for protein adsorption when comparing materials. When the degree of roughness is on the order of the size of some proteins then the surface roughness will have an effect on adsorption.

We see that surface wettability and charge have a definite effect on the adsorption of fibrinogen to a material surface, as does the general surface composition. The functional groups on the surface of a material will change the surface wettability and the surface charge, for example, the inclusion of amine or carboxylic acid groups on the surface of the material will also have an effect on the charge and wettability. Additionally, it has been found that even for surfaces of comparable hydrophobicity, a slight change in the backbone of a polymer can change the interactions of a surface with bioagents, specifically, fibrinogen.[38] The importance of surface composition is directly related to the functional groups with which the proteins interact. Whether due to hydrophobic/hydrophilic interactions or charge interactions, there is also the possibility that

non-Van der Waals interactions and binding of the proteins could occur, should the appropriate changes to the material surface be made.

I.3.b. Drug release from materials and its effect on protein binding

In addition to surface properties, drug release has an effect on the adsorption and activity of proteins at a material's surface. There are a few different types of drugs that are currently being used in different medical applications that will interact with proteins. Notably, the elution of curcumin from coronary stents has been shown to reduce the amount of fibrinogen adsorbed to the device surface to prevent blood clot formation.[39] In contrast, a study by the Bartlett group provides evidence that nitric oxide release from a poly(vinyl chloride) based polymer may actually increase the amount of fibrinogen adsorbed to the material surface.[40] This type of NO releasing material also prevents blood clot formation on the device, which has been shown in multiple studies.[41-43] The current literature focuses only on NO's effect on platelet interactions, whereas my work focuses on the effect of NO on proteins involved in blood clot formation.

I.4. Methods of Blood Clot Prevention

I.4.a. Systemic drugs

Current methods of blood clot prevention include the administration of systemic anticoagulants. The most commonly prescribed anticoagulants are warfarin (Coumadin) and naturally derived heparin. These drugs will prevent blood clot formation by helping to thin the blood and interfering with the natural coagulation process. They are effective for preventing total occlusion of biomaterials and increasing the lifetime of implants. The main problem with the use of anticoagulants is that they act upon the entire circulatory system. This can lead to other

complications with the patient.[44-46] For example, if a patient experiences any rupturing of their blood vessels, the body's natural ability to form a clot and repair the damage is compromised. In this case the patients have a danger of bleeding out from the damaged blood vessel, whether this is an external or internal wound.

I.4.b. Modification of surface properties

An additional method of increased viability of biomaterials is the modification of their surface properties to reduce clot formation. As previously established, the surface properties of the material have a significant impact on its biocompatibility. To create materials with improved biocompatibility, much work is being done to modify surface properties. Many researchers are working to modify the currently existing materials to improve their function and lifetime. This is done through modification of the surface functional groups,[14,30] patterning textures,[36,37] and pre-adsorbing proteins[32,47,48] to the material surface. This allows the material to retain the bulk properties desired, but changes the surface chemistry and the interactions with the blood stream.

The effect of changing surface functional groups was previously mentioned in section I.3.a; here we present another method that few are pursuing, the modification of the surface texture. Varying the degree of surface roughness by more than 20 nm as well as patterning the material on the microscale affects which proteins that adsorb. By patterning the material's surface, the assertion is that the proteins that adsorb can be controlled and the material be made more biocompatible. This research is being pursued by the Brennan group, where they have already established a material that controls bioadhesion.[36,37] Using a specifically patterned polymeric material may be able to control the deposition of proteins, cells, platelets, etc. involved in the coagulation cascade to prevent and control clot formation.

Another major area of research in surface modification is that of pre-adsorbing proteins to the material surface. Proteins are the first to interact with an implant when placed into a biological system. The hope is to passivate the material surface by pre-adsorbing specific proteins before implantation. The main protein used by researchers is albumin, which is the most common protein in the blood stream, roughly 34 mg/ml in concentration. Additionally, albumin is not involved in the coagulation cascade. By pre-adsorbing albumin, the surfaces should retain their layer of protein and effectively eliminate blood clot formation by being covered with a protein that will not trigger blood clot formation.[49,50] Another candidate used for pre-adsorption trials is fibrinogen. Fibrinogen is an active clotting protein, however, the idea is that if there is already a passivating layer of fibrinogen on a material surface, other fibrinogen and clotting proteins will not adsorb after implantation and trigger blood clot formation. These methods have been shown to work reasonably well, but have yet to be utilized in a clinical setting to assess true viability of the material.

I.4.c. Drug release from materials

To localize the prevention of blood clotting to the device itself, one avenue of research is focused on the release of drugs from the material. Currently, a number of drugs are being used in different medical applications that will interact with proteins. First-generation, drug-eluting stents used aspirin and a platelet inhibitor like clopidogrel.[51] These prevented early stage thrombosis, but did not fully solve the complication of device failure. Sirolimus and paclitaxel eluting stents are currently the method most used to help prevent clot formation at the site of implant.[28] These have improved the lifetime of the stent, but are not a long-term solution. Usually the administration of a systemic anticoagulant is also required. One of the avenues of research in this field, and the one that our group focuses on is the inclusion of NO release into materials. The incorporation of NO

releasing donors into biomedical materials has been widely studied and can actually prevent the formation of blood clots *in vivo*.[42,52,53]

I.5. Nitric Oxide Properties and Benefits

Nitric oxide (NO) has long been thought of as a toxin. As a free radical, NO rapidly reacts with oxygen in the atmosphere to form NO_2 which is a toxin. Only just over twenty years ago was NO discovered to be a viable biological agent. Ignarro and others discovered that NO is the endothelium derived relaxing factor, and directly responsible for vasodilation.[54] NO is also found to be a neurotransmitter,[55] antibacterial agent,[56] promote wound healing,[57,58] and promote angiogenesis.[59] This led to an understanding of why nitroglycerine treatments worked on patients to thin the blood, it was due to a release of NO. Since this first realization, NO has been the subject of much research in the biomedical community.

I.5.a. NO donors

Because NO is a free radical, it will rapidly react with other moieties. This attribute led to research on how to store NO until it was needed in the system. Drago initially developed diazeniumdiolates in 1960s,[60,61] however, since NO was seen as a toxin, they were not actively pursued at that time. In 1993 the NO donors that we currently recognize as diazeniumdiolates were developed by Hrabie and Keefer.[62] Diazeniumdiolates are NO donors consisting of secondary amines, where the NO moiety is attached to the nitrogen on the amine site. The structure containing the secondary amine site will determine the stability of NO storage and the properties of the donor itself. In general, diazeniumdiolates are stable under highly basic conditions. Much of the work done with incorporating diazeniumdiolates into polymeric materials was initially done with the

donor (Z)-1-[N-Methyl-N-[6-(N-methylammoniohexyl)amino]]diazen-1-ium-1,2-diolate (MAHMA/NO) seen in Figure I.3b. While these materials released NO, they also released the NO donor.[63] The issue with NO donor leaching from the material is that after the NO is released from the donor, the NO can back react with the parent amine and form a nitrosamine. This byproduct is known to be cancer causing, thus the need to retain the NO donor in the polymeric material becomes important.

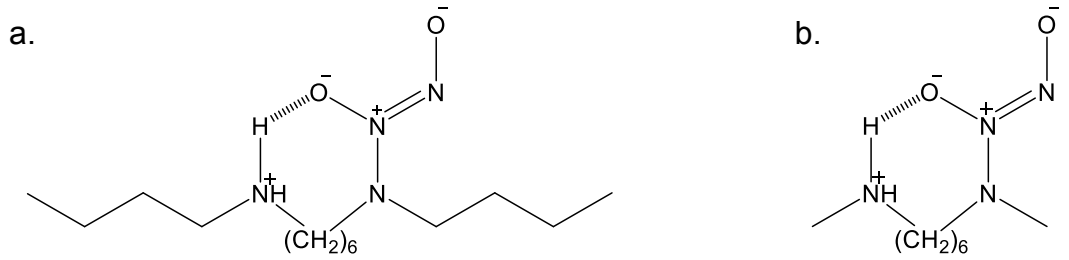

FIGURE I.3: Chemical structures of DBHD/N_2O_2 (a) and MAHMA/NO (b).

To this end, a more lipophilic NO donor was developed, diazeniumdiolated dibutylhexanediamine (DBHD/N_2O_2) (Figure I.3a).[64] This NO donor is thought to remain in the hydrophobic polymer system and allow for the release of NO without the leaching of the NO donor into the blood. The DBHD/N_2O_2 donor consists of a secondary amine modified with two NO moieties. It is stabilized by a second amine site separated by six methyl groups. On the end sides of the amine groups, there is a four carbon chain that is the reason why this donor is more lipophilic than MAHMA/NO, since MAHMA/NO has only one methyl group on each end of the molecule. Because release from these molecules happens at neutral and acidic pH, there is a need to control the pH to keep it from becoming basic when blended into a polymeric material. Potassium tetrakis(4-chlorophenyl) borate (KTpClPB) is used in a 1:1 ratio with the DBHD/N_2O_2 donor. This acts to control pH though ion exchange mechanisms with the donor molecule and water.[64] As the

NO donor releases NO, ammonium cations are formed on the molecule. Without the KTpClPB, ammonium hydroxide forms at these sites, however, when the salt is incorporated into the blended polymer, the borate ions can interact with the ammonium ions. At the same time, potassium and hydroxide ions can then diffuse from the polymer and into the aqueous media. This allows for a decrease in the production of ammonium hydroxide, thus keeping the pH regulated as NO is being released from the material.

I.5.b. Detection of nitric oxide release

The gold standard of NO detection is the use of chemiluminescence. This is done through the use of a nitric oxide analyzer (NOA) as detailed in Figure I.4.[65]

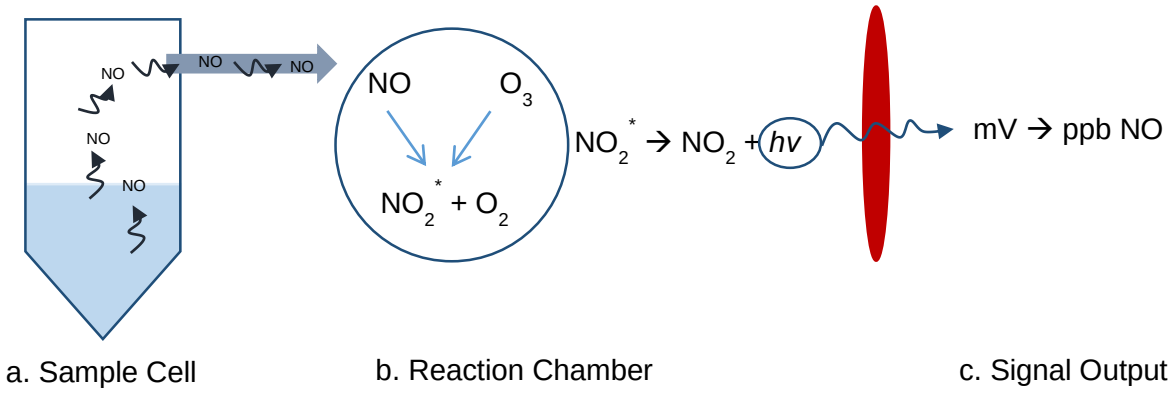

FIGURE I.4: Schematic of the detection method performed when using the NOA from sampling (a), chemiluminescence reaction (b), and final signal output (c).

We use a custom made sample cell set-up for our NOA to analyze our materials for NO release (Figure I.4a). This cell allows us to purge the testing area of all oxygen, which will interfere with the NO measurements. NO gas is transferred from the sample cell into the reaction chamber of the instrument. In the NOA's reaction chamber, NO is reacted with ozone to create excited state

NO_2 (Figure I.4b). When this relaxes, a photon of light is released and sent through a red filter on the instrument into the PMT for signal multiplication. The filter allows for only the light from the relaxation of excited state NO_2 to pass through to the PMT, eliminating any interference from other species that might interact with ozone from the reaction vessel. The signal output from the PMT is converted from voltage to ppb (Figure I.4c). To assess how many moles of NO are being released from our samples, the ppb is converted from a calibration constant. This constant is determined by detecting the amount of NO produces from a sodium nitrite solution and checked against an NO donor solution to ensure total recovery with error of 10% or less.

I.5.c. Benefits of NO release

The release of NO is found to have many beneficial properties.[59,66,67] In the body it is most well known as the endothelial derived relaxing factor. NO is naturally produced from interactions with nitric oxide synthase (NOS). There are three different types of NOSs: endothelial (eNOS), neural (nNOS), and induced (iNOS).[59,68,69] NO has been implicated as a vasodilator, antibacterial, antiplatelet, neurotransmitter, and wound healing agent, among other attributes.[70]

NO release has become a viable method of preventing biofouling of synthetic materials. Testing of materials *in vitro* shows that the incorporation of NO release will prevent the activation and adhesion of platelets to a material surface.[71,72] Further *in vivo* studies show that NO release not only mediates platelet adhesion and activation, but also prevents thrombus formation.[40,42,52,73] These studies utilized diazeniumdiolate NO donors blended into a polymer matrix to be used for different purposes such as vascular grafts and extracorporeal circuitry. Notably, a study performed by Fleser implanted DBHD/N_2O_2 doped vascular grafts into goats.[42] The NO releasing material remained mostly thrombus free. These grafts retained their viability for the entire study. However,

the control material needed to be removed prior to the end of the study as the thrombus formation within caused device failure. In addition to thrombus prevention, NO is also found to be prevalent in the mediation of bacteria. Schoenfish and coworkers, recently published a review, examining levels of NO needed to effectively eliminate the harmful bacteria, but allow for native and beneficial cells to remain alive, as well as promote anti-inflammatory, anti-cancer, and anti-bacterial properties of NO.[70,74] Although initial tests on NO for the mediation of blood clotting tested multiple release rates of NO, since the establishment of a threshold value associated with the elimination of serious thrombus conditions (>14 $\times 10^{-10}$ mol NO cm^{-2} min^{-1}),[40,52] only that level of NO has been tested.

In my study, the interest is in the effect of NO on the clotting protein fibrinogen. To establish how the release of NO affects the interactions of fibrinogen with these synthetic surfaces, different NO fluxes from the materials must be established. Thus we examined two release rates of NO, a high NO flux at the level therapeutically relevant for clot prevention, and a low NO flux just slightly below that of the high flux rate. Although these two fluxes are similar, when tested with fibrinogen (as outlined in Chapter III) there is a dramatic difference between the adsorption of fibrinogen to each surface.[75] Because we see this difference in fibrinogen adsorption with the high and low NO flux rates, it was determined that two rates would be sufficient. The two NO releasing composite materials allow for an establishment of any trends due to differing NO release rates. For this work we had two composite materials, one assay control, and an experimental control as needed for each assay.

I.6. Overall Goal of This Work

Delineating the factors that contribute to thrombus formation on implanted medical devices is of critical importance to the development of improved biomaterials. The biomaterial used herein will be one that releases NO. Since the first step in clot formation on synthetic materials is the deposition of proteins on the surface, adsorption of protein to NO releasing materials will be the focus of the current work. Until now, the effect of NO release on proteins has not been investigated. The general hypothesis of the work presented in this dissertation is depicted in Figure I.5.

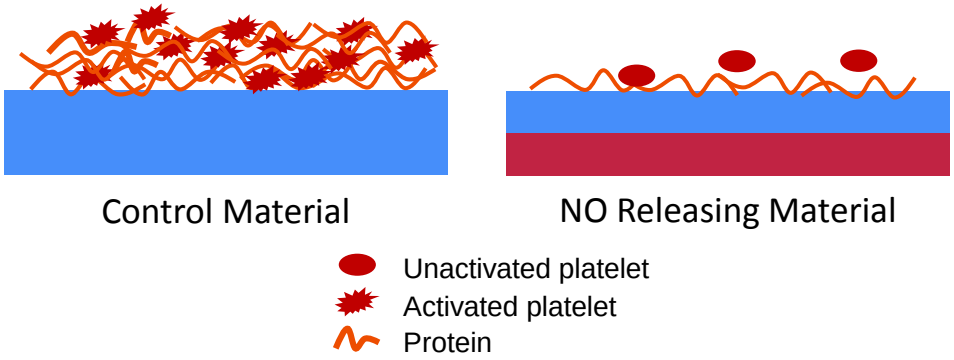

FIGURE I.5: Hypothesis of this dissertation. The control, non-NO releasing material (left) is theorized to have the greatest amount of fibrinogen adsorption to the material surface, as well as highly activating and adhering platelets. The NO-releasing material (right) will have the least amount of fibrinogen adsorbing the material surface, any platelets that do adhere will remain unactivated.

Fibrinogen is hypothesized to adsorb in higher concentrations to the control material than to an NO releasing material. We would assume that an NO releasing material of any NO release rate presented her will cause a decrease in fibrinogen adsorption. Due to its participation in the coagulation cascade, the adsorbed fibrinogen on the control material will likely cause increased platelet adhesion and activation compared to the NO releasing material.

It is well established that NO releasing materials will prevent the formation of thrombus on biomaterials.[63,76,77] To assess the effect of NO on biological processes, specifically fibrinogen adsorption, multiple NO release rates are explored. There are numerous reports of different

threshold values for how much NO is required to mediate thrombus formation on implanted devices. For the purposes of this study we use the threshold value of $>14 \times 10^{-10}$ mol NO cm^{-2} min^{-1} found by the Bartlett group.[40,73] Two levels of NO release and one control, non-NO releasing material were chosen. Two fluxes, a high and a low flux showed a significant difference in the amount of fibrinogen adsorbed to a material surface. Because they fall on and just below the optimal NO release properties of the polymeric material, the stark difference in fibrinogen adsorption shows that below the threshold NO dosage there is a different effect on fibrinogen adsorption. We demonstrate there is an increase in the adsorption of fibrinogen on these surfaces with a therapeutic NO release. Further investigation examines the conformation and ability for the adsorbed fibrinogen to interact with platelets. Herein is presented a picture of the effect of NO release on the primary clotting protein, fibrinogen.

Specific goals:

- Develop a material where surface properties (i.e. surface roughness, wettability, and composition) remain comparably similar across control and composite.
- Determine the relative amount of fibrinogen adsorbed to materials of varying NO release.
- Examine conformational changes in the protein upon adsorption to the surface of a model system.
- Assess the biocompatibility of the adsorbed fibrinogen through interactions with platelets.

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CHAPTER II:

CONTROLLING SURFACE PROPERTIES OF THE MATERIAL SYSTEM

II.1 Introduction

The surface properties of a material affect the subsequent interactions with biological components, including cells, proteins, and platelets.[1] To properly assess the interactions of a biomaterial with such species, the surface properties of the material must be determined and taken into account. Much work has been done toward the modification of material surface properties to induce certain interactions within biological systems. For example, work done by the Brennan group focuses on micropatterning polydimethylsiloxane (PDMS) surfaces to prevent biofouling.[2,3] This work revolves around the development of the Sharklet AF™ material, which is patterned with raised features to assess the effect of specifically patterned micrometer scale roughness on the prevention of fouling by green algae; however, they have recently begun to apply these materials to other biological systems. The Siedlecki group demonstrated that surface roughness and wettability impact fibrinogen adsorption, finding that on a more hydrophilic surface the adhesion of fibrinogen is generally stronger than that on hydrophilic materials, despite small changes in surface roughness.[4] In the same vein, work done by the Schoenfisch group correlated the surface wettability and charge to the amount of fibrin formation.[5] This work showed that the most fibrin was formed with hydrophobic and positively charged material surfaces; however, there was a greater amount of fibrinogen adsorbed to hydrophilic and negatively charged surfaces. These studies, among many others, show that the surface properties of materials have significant effects on the material's interaction with biological moieties, especially proteins.[6-12] To examine the effect of another aspect of a material on biological

interactions, the surface properties must be properly characterized to take into account whether they will be responsible for resulting interactions.

In the scope of this work, we explore the interactions of a model biomaterial surface with the protein fibrinogen. Fibrinogen is a prominent protein involved in the clotting process within the circulatory system; its use, therefore, will be representative of clotting proteins in general. Numerous material properties have been implicated in having an effect on fibrinogen adsorption, namely surface wettability,[4,8,9,12] roughness,[4,6,7] and composition.[5,11,13,14] The model biomaterial system under consideration for these studies is that of a nitric oxide (NO) releasing polymer. Previous studies have shown that these types of materials improve the biocompatibility of medical implants.[15-23] Notably, the inclusion of NO release from many classes of material has prevented gross thrombus formation for blood contacting medical devices *in vivo*.[15] The effect NO has on the coagulation cascade has long been attributed solely to its effect on mediating platelets.[15,18,24-26] NO is a well-established platelet mediator, thus helping to prevent adhesion and activation, which is the predominant cause of thrombus formation. However, the formation of blood clots on synthetic surfaces does not begin with platelet adhesion as it does in natural blood vessels. Instead, the first step in thrombus formation is the adhesion of protein.[1,27,28] The identity and conformation of proteins adsorbed to a material surface will have an effect on the adhesion likelihood of platelets and other clot contributors. Instead of focusing on NO primarily as an antiplatelet agent, it is necessary to also consider the effect of NO release on protein adhesion processes. Further, it is hypothesized that the protein conformation is important to fully understand why an NO releasing material provides a thrombus-free surface. A study by the Bartlett group established that, for a specific NO releasing surface, there was an increase in fibrinogen adsorption.[20] However, in this study there was no discussion of any changes in the

surface properties of the material. Because material properties other than NO release can attribute to biocompatibility differences, NO cannot be determined as the main cause of the phenomenon described in this study. To determine if this increase in fibrinogen adsorption indeed arises from NO release from the material, proper sample preparation methods must be developed to maintain comparable surface properties across all samples and controls. Until this is established, we cannot determine whether NO release is dependent on the increase in fibrinogen adsorption seen by the Bartlett group.

Considering the above issues, this work aimed to develop a sample preparation technique that would ensure that the surface properties of the material, aside from NO release, remained consistent across all samples and controls. The initial material investigated is based on literature formulations for NO releasing materials commonly used by the Bartlett group and others.[15,20,29,30] This two layered system is made with one base layer containing plasticized poly(vinyl chloride) (PVC) blended with the NO donor, diazeniumdiolated dibutylhexanediamine (DBHD/N_2O_2), with a single top coat containing only plasticized PVC. This specific material system was developed to help eliminate the leaching of the NO donor into the material, which was a concern with previous diazeniumdiolate donors.[31] The control is the same material without the NO donor to allow for comparison to the NO releasing material. From this initial material, multiple different formulations were tested to determine whether they would be suitable systems for isolating the effect of NO release on fibrinogen adsorption.

In addition to maintaining material surface properties across control and composite materials, the amount of NO release must maintain a specific surface flux. Through work done by the Bartlett and Meyerhoff groups, a surface flux that is found to be beneficial to preventing the formation of blood clots on materials is $>14.0 \times 10^{-10}$ mol NO cm^{-2} min^{-1} or greater.[20,22]

Thus, materials relevant for therapeutic application should maintain a relevant NO flux to assess the effect at that release rate.

Evaluation of the sample preparation methods described within this work is done by doping the active layer with DBHD/N_2O_2 donor or its parent amine, dibutylhexanediamine (DBHD). Both were used for these experiments since NO release from the donor results in the formation of the parent amine. If the parent amine can leach from the material during soaking or is detected initially on the surface, then this will happen over time as the NO is released from the donor molecule. All samples that made usable films were initially tested with water contact angle to assess viability. The reason this was used as the screening technique is the simplicity and low time and cost for each measurement. Materials that seemed promising were also tested using an NO analyzer (NOA) to determine the surface flux from the material. Only one sample preparation technique produced films that released NO at or above the goal surface flux and maintained comparable surface wettability to the control. This material is investigated more thoroughly in the next chapter.

II.2. Materials & Methods

II.2.a. Polymers

As described earlier, multiple polymer formulations were investigated toward an NO-releasing surface the exhibits no significant change in other surface properties. One formulation was prepared from high molecular weight poly(vinyl chloride) (PVC) from Aldrich (St. Louis, MO) and bis(2-ethylhexyl) sebacate (DOS) plasticizer from Acros Organics (New Jersey, USA). Tetrahydrofuran (THF) from Mallinckrodt (New Jersey, USA) was used as the solvent. Poly(lactic-*co*-glycolic-*co*-hydroxymethyl propionic acid) modified with cysteamine (PLGH-

cysteamine) was made in lab through a previously published method by Dr. Vinod Damodaran.[32] Dichloromethane (DCM) was obtained from EMD (Billerica, MA). Silicon rubber films were prepared using polydimethylsiloxane (PDMS), diaminoalkyltrimethoxylsilane (DACA-6) and toluene from Sigma (St. Louis, MO) according to a method previously published by the Meyerhoff group.[23] Tecoflex SG80-A was obtained from the Meyerhoff group.

II.2.b. NO releasing materials

NO, compressed was obtained from Matheson Trigas. For diazeniumdiolate donor preparation, *N,N'*-dibutyl-1,6-hexanediamine (DBHD) (Alfa Aesar, Ward Hill, MA) and acetonitrile (99.8%, Sigma) were purchased. Potassium tetrakis(4-chlorophenyl) borate (KTpClPB) was obtained from Ace (Korea). Nitrosating materials for PLGH-cysteamine, *tert*-butyl nitrite and methanol stored over molecular sieves to keep dry were procured from Aldrich and Fisher (Fair Lawn, NJ), respectively according to a previously published procedure.[32]

Glass slides (75×50 mm) from Corning (Corning, NY) and circular micro cover glass (12 mm diameter) from VWR (Radnor, PA) were purchased for contact angle sample preparation. Nylon and Millipore filters were used from general lab stock. Dulbecco's phosphate buffered saline (DPBS) was obtained from Hyclone® (Logan, Utah). All water used was purified using a Millipore Direct-Q 5 system (Billerica, MA) unless stated otherwise. All chemicals and materials were used as received.

III.2.c. Polymer Preparation

For plasticized PVC films, polymer formulations were created in three different ratios of PVC: DOS in THF. These ratios were 1 PVC: 2 DOS (1067 mg polymer and plasticizer per 10 mL), 2 PVC: 1 DOS (1062 mg per 10 mL), and 3 PVC: 1 DOS (1440 mg per 10 mL).

When used as a base coat, nitrosated PLGH-cysteamine was made by dissolving in DCM (50 mg per 150 µL). As a blended donor, a 94 wt% solution was made by blending nitrosated PLGH-cysteamine into the base coat formula (10 mg nitrosated PLGH-cysteamine per 100 µL base coat solution).

Silicon films were prepared by Jessica Joslin via preparation methods described by Zhang.[23] Briefly, PDMS was mixed with DACA-6 in toluene overnight, then allowed to mix with fumed silica. The resulting solution can be cured over two days under ambient moisture to initiate crosslinking of the polymer. The formulas used were 1 and 2 (0.03 wt% PDMS, DACA-6 and 0.2 wt% PDMS, DACA-6, respectively).

II.2.d. NO Releasing materials

Diazeniumdiolated dibutylhexanediamine (DBHD/N_2O_2) was synthesized by treating DBHD in acetonitrile with 80 psi NO on a custom-built high pressure reactor at room temperature for 24-48 h, as described previously.[33] This donor was blended in a 1:1 ratio with KTpClPB into polymer matrices. This was blended into the polymer base coat at either 3.5, 12.5, or 25 wt% of donor or parent amine based on the total PVC & DOS contained in the 2 PVC: 1 DOS solution.

PLGH-cysteamine was nitrosated due to exposure to *tert*-butyl nitrite in a 2 methanol: 1 DCM solution as previously described.[34] After reacting for 8 h, the solution was dried under vacuum and the isolated product was stored at -30 °C until ready for use.

Cured films of silicon rubber (SR) on glass slides were loaded with NO by placing these slides under NO at 80 psi at room temperature for ~24 h. As described previously by Zhang, this results in the diazeniumdiolated SR analogs. [23]

II.2.e. Sample Preparation

Glass rings of 24 mm diameter were secured to glass slides to make thin films for contact angle measurements. Aliquots of polymer solution were applied to the center of the glass rings as described for each film formulation below. Following an overnight curing period, the glass ring and edges of the polymer were removed for testing.

Films prepared for NO release measurements were made in a 96-well breakaway plate. Films were layered in the same manner as described below and allowed to cure overnight. The final samples were separated by well and an individual well was used for each NO measurement.

Circular glass coverslips with diameter 12 mm were used to make the SR films for both tests. SR-containing solutions were aliquoted onto glass slides contained within individual weigh boats. After a two day curing period, the top coat was added as described below and allowed to cure overnight. Slides were then removed from boats and tested as is.

II.2.f. Film Formulations

As described above, all samples were prepared in glass rings, 96-well plates, or on glass coverslips dependent upon the measurement and sample type. All formulations are displayed in

detail in Table II.1. Active layer base coats for all films drop coated into glass rings were in 398 µL aliquots, while the tops coats are 200 µL each unless otherwise noted. For formulation A, the preparation was based on the standard preparation generally performed in the literature. The base coat was blended with 25 wt% DBHD/N_2O_2 and a single top coat was applied after curing for one hour.

Films (n = 3) were created using a barrier layer between the active base coat and the top coat. The base coat contained 3.5 wt% DBHD to yield formulation B. The barrier layer was allowed to cure for 30 min before the top coat application. Formulations C and D were made with 25 wt% DBHD blended into the base coat. The base coat was allowed to cure for 30 min, and then a filter of consisting of either nylon (C) or Millipore materials (D) was gently pressed in before curing an additional 30 min. After this a top coat layer was applied.

To help contain the NO donor in the film to prevent leaching, two methods were employed using covalently attached NO donors. Nitrosated PLGH-cysteamine was used as a base coat layer for formulation E and allowed to cure 1.5 h before applying a top coat. Silicon rubber films for formulation F were prepared by applying an aliquot of the DACA-6 SR and allowing the film to cure for 3 days under ambient moisture. The SR films were cured onto circular glass cover slips, each base coat layer consisted of 100 µL of solution and each top coat being 50 µL. After curing, the top coats were applied allowing for 30 min curing time between coats. The reason for applying a plasticized PVC top coat to the SR films is to be able to compare to the plasticized PVC control films. These were allowed to cure overnight, and then loaded with NO as described above.

TABLE II.1: Sample formulations tested for development of material that retains comparable surface properties across control and composite.

Formulation	Polymers	Base Coats	Top Coats	Barrier Type	NO source
A: Literature Formulation	PVC/DOS	2 PVC: 1 DOS	1 coat: 1 PVC: 2 DOS	-	DBHD/N_2O_2
B: Tecoflex Barrier	PVC/DOS & Tecoflex SG80-A	2 PVC: 1 DOS	1 PVC: 2 DOS	2 Tecoflex SG80-A: 1 DOS	DBHD
C: Filter Barrier 1	PVC/DOS	2 PVC: 1 DOS	1 PVC: 2 DOS	Nylon Filter	DBHD
D: Filter Barrier 2	PVC/DOS	2 PVC: 1 DOS	1 PVC: 2 DOS	Millipore Filter	DBHD
E: PLGH Base Layer	PVC/DOS & PLGH	SNO-PLGH	1 PVC: 2 DOS	-	SNO-PLGH
F: SR Base Layer	PVC/DOS & Silicon Rubber	Silicon Rubber	3 coats: 1 PVC: 2 DOS	-	NO loaded Silicon Rubber
G: PLGH Blended	PVC/DOS & PLGH	2 PVC: 1 DOS & PLGH	3 coats: 1 PVC: 2 DOS	-	SNO-PLGH
H: SR Blended	Silicon Rubber	Silicon Rubber	3 coats: 1 PVC: 2 DOS	-	DBHD
I: Multi-Layer PVC	PVC/DOS	2 PVC: 1 DOS	9 coats: 2 PVC: 1 DOS & 1 PVC: 2 DOS	-	DBHD or DBHD/N_2O_2
J: Increased PVC	PVC/DOS	3 PVC: 1 DOS	3 coats: 2 PVC: 1 DOS & 3 PVC: 1 DOS	-	DBHD/N_2O_2
K: Control PVC	PVC/DOS	2 PVC: 1 DOS	1 PVC: 2 DOS	-	-

Another approach was to use blended materials like the literature formulation. The first materials tested were formulations G and H. For G, nitrosated PLGH-cysteamine was blended in 2 PVC: 1 DOS to make up the base coat to improve upon the mechanical properties of the native PLGH material. After curing for 1 h, three top coats were applied, curing for 30 min between

each coating. Formulation H consisted of 12.5 wt% DBHD blended into the SR solution. The film was allowed to cure for 3 days and then 3 top coats were applied, curing 30 min between coats.

To follow a similar approach to the literature formulation, two different layered formulations were attempted. Formulation I was made with 3.5 and 25 wt% DBHD as well as 25 wt% DBHD/N_2O_2 blended into the base coat. Top coats were applied in varying volumes, 395 µL for 2 PVC: 1 DOS, 200 µL for 1 PVC: 2 DOS, and 588 µL for 3 PVC: 1 DOS. The active base coat was allowed to cure for 1 h before applying top coats in the following order: (1) 2 PVC: 1 DOS, (2) 1 PVC: 2 DOS, (3) 1 PVC: 2 DOS, (4) 1 PVC: 2 DOS, (5) 2 PVC: 1 DOS, (6) 2 PVC: 1 DOS, (7) 2 PVC: 1 DOS, (8) 1 PVC: 2 DOS, (9) 1 PVC: 2 DOS. Each top coat was allowed to cure for 30 min between coatings. To minimize top coat layers, formulation J was made using 25 wt% DBHD/N_2O_2 blended into the base coat, which was allowed to cure for 1.5 h. Three top coat layers were applied in the order: (1) 2 PVC: 1 DOS, (2) 3 PVC: 1 DOS, (3) 2 PVC: 1 DOS, and were allowed to cure 30 min between coats.

Control films were made using PVC in a two layer format. Control formulation K was made in the same manner as formulation A without blending in any donor into the base coat. These films were used for material surface property comparison across all samples. Due to the prevalence of plasticized PVC used in biomedical devices (i.e. extracorporeal circuitry) and in the study performed by the Bartlett group, the used of PVC as the control material was necessary for drawing comparisons to previously published results.

II.2.g. Water Contact Angle

Samples were prepared to consider and eliminate leaching of the NO donor from the material matrix. Variable formulations were initially tested with static water contact angle (WCA) to evaluate surface wettability changes due to leaching of NO donor or parent amine. Films with the diazeniumdiolate donor were made with either donor or parent amine, where the parent amine formulation is a control representative of the film after the donor decomposes, releasing its payload of NO. Samples were tested either before or after soaking, in some promising cases where the contact angle was comparable to the control PVC film, both were tested. Soaking was performed for a total of 2.5 h, after which the soaked samples were pat dry using a Kimwipe.

Surface wettability was determined via water contact angle goniometry. Static water contact angles were achieved by depositing 2 μL of ultrapure water (>18 MΩ) onto the film's surface. All contact angles were determined by taking an image of the drop on the surface with subsequent analysis using the circle fitting parameter on the Drop Shape Analysis 1.50 software. Measurements were accomplished on a Krüss Drop Shape Analysis System DSA 10 (n=3 for preliminary studies; n=9 for model system).

II.2.h. NO release measurements

To further explore the films, NO release was measured from those whose contact angle was comparable to that of the control PVC material. Even if the material has a comparable hydrophobicity to the control, they must also be able to produce NO at a specific NO flux of $>14.0 \times 10^{-10}$ mol NO cm^{-2} min^{-1}, which has been shown to prevent thrombus formation on plasticized PVC materials.[20,22] NO release measurements were performed on these samples as

outlined in Chapter I.5. Samples in the 96-well breakaway set up were placed into a reaction cell and purged with nitrogen. An aliquot of 3.0 ± 0.2 mL DPBS was injected fill the well and surrounding cell. For glass coverslip samples, 3.0 ± 0.5 mL DPBS was added to the cell. The slide was pushed gently down with the injection needle to ensure that it was submerged in the solution. All samples were incubated via a water bath at 37 °C. Measurements were taken at 1 min intervals over a period of 1.5 h. Experiments were performed on a Sievers Nitric Oxide Analyzer (NOA) at cell pressure of 5.5 – 8.5 torr, supply pressure of 4.6 – 6.5 psi, and PMT cooler temperature of -12 °C. Total moles of NO released were calculated using a pre-determined calibration constant for each instrument. This was used to calculate the surface flux of the material using the surface area of the film, assuming a flat surface, and time of data collection to get a result in mol NO cm^{-2} min^{-1}.

II.3. Results

II.3.a. Control Films

PVC control (formulation K) exhibited a relatively hydrophobic contact angle at 95.2 ± 2.3° before soaking and 93.8 ± 3.7° after soaking in DPBS buffer for 2.5 h as seen in Table II.2. The films were visually smooth and uniform. As there was no NO donor added to the material for the control, NO release measurements were not performed on these materials.

II.3.b. Literature formulation

The blended NO donor material was significantly more hydrophilic than control PVC, with a contact angle of 86.6 ± 9.3° (Table II.2); as such, the film was not tested post soaking. The films were also visually smooth and uniform similar to the control PVC film (K). Although the

contact angle was not determined after soaking, NO release was evaluated for these films to serve as a comparison to other formulations. A total of 1.6 ± 0.5 $\times 10^{-7}$ mol NO was released by the film over the initial 1.5 h of contact with DPBS buffer. In contrast to the values reported previously 14.0 $\times 10^{-10}$ mol NO cm^{-2} min^{-1},[18,20] the average surface flux of these materials was 566 ± 162 $\times 10^{-10}$ mol NO cm^{-2} min^{-1}. These NO release values represent an n=3.

TABLE 2: Water contact angle determination for all sample preparation methods.

Formulation	Water Contact Angle (°)	
	Pre-Soak	Post-Soak
A: Literature Formulation	86.6 ± 9.3	-
B: Tecoflex Barrier	90.0 ± 3.7	-
C: Filter Barrier 1	91.9 ± 11.1	-
D: Filter Barrier 2	82.2 ± 2.4	-
E: PLGH Base Layer*	-	-
F: SR Base Layer	-	98.2 ± 4.7
G: PLGH Blended	83.6 ± 5.6	63.5 ± 19.2
H: SR Blended	-	73.9 ± 6.7
I: Multi-Layer PVC	92.0 ± 1.7	-
J: Increased PVC	-	92.4 ± 0.2
K: Control PVC	95.2 ± 2.3	93.8 ± 3.7

*Unable to determine WCA for formulation E due to sample issues.

II.3.c. Barrier layer

Tecoflex barrier films (formulation B) were visually rougher and significantly more hydrophilic than that of PVC: DOS alone (formulation K) prior to soaking, Table II.2 (90.0 ± 3.7° for B, compared to 95.2 ± 2.3° for K). Films were not tested after soaking due the already

increased hydrophilicity of the films. This change in surface wettability indicates that the films of formulation B with the added layer of Tecoflex are inappropriate for this study and thus were not tested further for NO release.

Films made with a layer created by a nylon filter (formulation C) were visually smooth despite areas where it was clear that there was no top coat present on the surface of the filter. As shown in Table II.2, the contact angle pre-soaking indicates a comparable hydrophobicity to that of PVC: DOS alone at 91.9 ± 11.1°; however, the spread of the angles from 83.2 – 104.4° indicated a non-homogeneous surface. The film with a nylon filter barrier was not tested for contact angle post soaking, nor NO release as the variability of the angles indicates a non-viable material.

Millipore filter films (formulation D) had a visual roughness to the surface, and exhibited a similar issue to the films using the nylon filter (formulation C) with uneven surface coating of the material where the top coat did not fully cover the entire filter. This surface was considerably more hydrophilic than that of PVC: DOS alone at 82.2 ± 2.4° for formulation D compared to 95.2 ± 2.3° for the control (formulation K) and so was not tested for contact angle post-soaking, nor tested for NO release.

II.3.d. Covalently Attached Donors

Films with nitrosated PLGH-cysteamine as a base coat (formulation E) and PVC as a top coat could not be tested for contact angle as the films became mangled in appearance after curing. Upon attempting to remove the films from the glass ring/slide set-up, the top coat peeled from the base. These films are not useful for this study because of these sample preparation issues and as such, were not tested for contact angle or NO release.

Silicon rubber NO loaded films (formulation F) were visually rougher than that of the PVC control, which was enhanced with the application of PVC: DOS top coats. The added top coats did not cover the entire surface of the SR. For the films that were coated with PVC: DOS prior to loading with NO, the film turned dark yellow post loading for 24 h with NO. Top coats stayed on the SR base layer through soaking, while only a few delaminated when attempting to transfer film to goniometer for measurement. Overall, the SR films exhibit a relative hydrophobicity comparable to that of PVC: DOS alone after soaking as seen in Table II.2 (98.2 ± 4.7° and 92.8 ± 3.7°, respectively).

For these formulation F films, NO release began prior to adding DBPS buffer as seen in Figure II.1 at the beginning of the NO release profile for this film (shown in blue). Once buffer was added, the release dropped and then rose again within ~10 min. The film then steadily continued to release NO over the 1.5 h with a gradual decrease in the surface flux. The total amount of NO released over 1.5 h was 1.37 $\times 10^{-8}$ mol NO with a surface flux ranging from 0.5-2.0 $\times 10^{-10}$ mol NO cm^{-2} min^{-1}. This flux is well within the range of the natural endothelium surface flux of 0.5-4.0 $\times 10^{-10}$ mol NO cm^{-2} min^{-1};[35] however, the steady decrease of NO release over the time period is not a desired characteristic of these films, as a steady state over which analysis can be made is preferable. Additionally, a flux above that of the natural endothelium is desired for synthetic materials with a plasticized PVC component (in this case, the top coat layers).

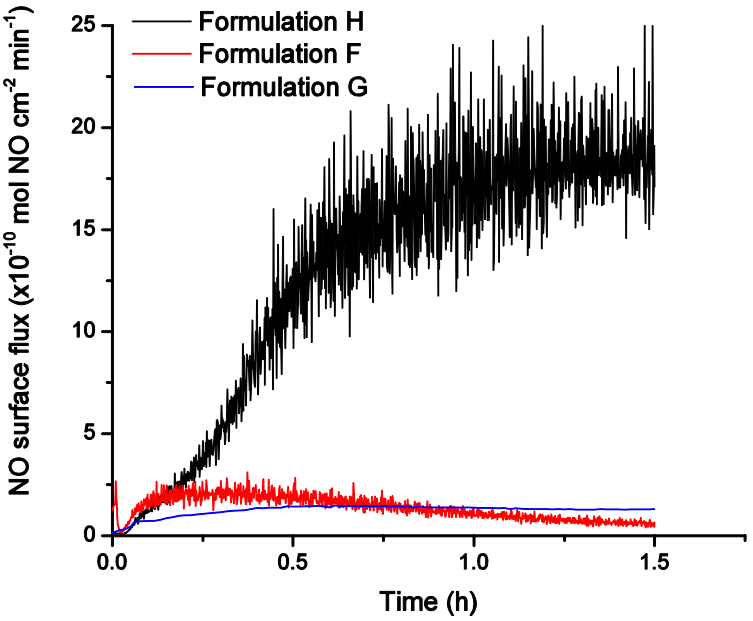

FIGURE II.1: Representative NO release measurements for covalently attached and blended sample methods. Surface flux was determined for films for which contact angles were obtained post-soaking.

II.3.e. Blended Materials

When PLGH-cysteamine was blended into PVC and used as a base coat (formulation G), the films were overall visually smooth in appearance except for ridges that appeared in the middle of the film and branched to the edges with patches of smooth film between the ridges. Contact angles from these films showed a significant increase in wettability as seen in Table II.2, especially after soaking (83.6 ± 5.6° and 63.5 ± 19.2° for formulation G compared to 95.2 ± 2.3° and 93.8 ± 3.7° for formulation K pre- and post-soak, respectively). This continued decrease in wettability after soaking supports the assertion that those films that exhibit increased hydrophilicity to the control PVC materials (formulation K) will continue to decrease in wettability upon soaking.

The films with nitrosated PLGH-cysteamine blended (formulation G) released 3.7 $\times 10^{-9}$ mol NO over a 1.5 h period at 37 °C as shown in Figure II.1. The NO reached a relatively steady state of release after 30 min. The average flux was around 1.3 $\times 10^{-10}$ mol NO cm^{-2} min^{-1}, which falls within the range of that released by the natural endothelium which is 0.5-4.0 $\times 10^{-10}$ mol NO cm^{-2} min^{-1}, but is not at the target surface flux level of >14.0 $\times 10^{-10}$ mol NO cm^{-2} min^{-1}. These films had minor mixing with the breakaway wells, which may hinder the release of NO from the material.

Silicon rubber blended films (formulation H) were more hydrophilic post-soaking than that of the control films (73.9 ± 6.7° and 93.8 ± 3.7° for SR blended films and control films, respectively). NO release from films steadily increased for ~30 min before reaching a relative steady state of release as shown in Figure II.1. Over 1.5 h, the DBHD/N_2O_2 blended SR films released a total of 1.3 $\times 10^{-7}$ mol NO with an average surface flux around 15.0 – 20.0 $\times 10^{-10}$ mol NO cm^{-2} min^{-1}. This flux is well above that of the natural endothelium, reaching the desired NO surface flux for these experiments, while this indicates a material useable for the model system, the hydrophilicity of the material eliminates formulation H as an option going forward.

II.3.f. Literature Based Blended Materials

Films of multi-layered top coats (formulation I) were visually thicker than that of the plasticized PVC control (formulation K). This is not necessarily an issue, as the films were overall visually smooth and uniform when plated on the glass slides. As measured by contact angle goniometry and shown in Table II.2, the pre-soaked exhibited comparable hydrophobicity to that of PVC: DOS alone before soaking (92.0 ± 1.7° and 95.2 ± 2.3°, for formulation I and K

respectively). These were not tested post soaking due to complications, described in the discussion, when preparing samples for NO release measurements.

As shown in Figure II.2, films released at a steady state of 0.4 $\times 10^{-10}$ mol NO cm^{-2} min^{-1} after ~6 min of data collection, falling below the release of the natural endothelium (0.5 – 4 $\times 10^{-10}$ mol NO cm^{-2} min^{-1}).[35] Over a 1.5 h time period, a total of 1.1 $\times 10^{-9}$ mol NO was released. At this release rate, these films with the nine top coat layers are not viable for use as a proposed model system. The addition of more NO donor may improve the NO surface flux, however, tests would have to be performed to ensure that the surface wettability has not changed due to the increase in blended donor.

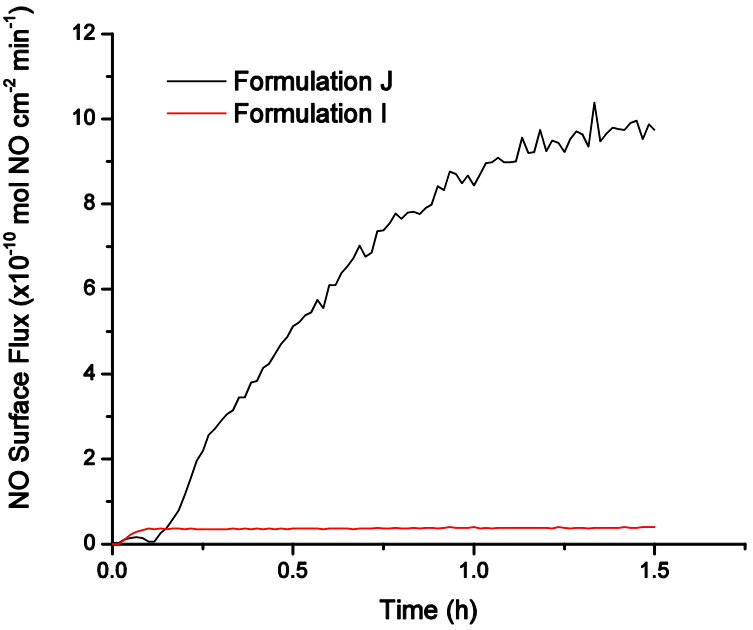

FIGURE II.2: NO release measurements of literature-based blended films. Multi-layered formulation I releases at a steady rate, yet does not release the target NO flux. Formulation J with increased PVC content has potential to release at the target level with continued time.

Formulation J films with the 3 PVC: 1 DOS used in the base coat layer are overall visually smooth and uniform, similar to the control PVC materials (formulation K). After

soaking for 1.5 h the films tested were of comparable hydrophobicity to that of PVC: DOS alone, exhibiting a WCA of 92.4 ± 0.2° for formulation J, compared to 93.8 ± 3.7° for the control PVC material. These films were not initially tested before soaking, the comparable wettability to the control found after soaking indicates that the films may also be comparable before soaking. Future testing of this material will include measurements of the contact angle both before and after soaking.

NO release was measured over 6 h for the films containing 3 PVC: 1 DOS. Over the initial 2 h of soaking in DPBS at 37 °C, there was a steady increase in NO release from the films after which they approached a steady state of release of NO during the duration of the collection time, as shown in Figure II.2. Over the initial 1.5 h, 2.1×10^{-8} mol NO was released by the film with an average surface flux of 6.2×10^{-10} mol NO cm^{-2} min^{-1}. Films made using formulation J are not currently a viable material for use as a model system as the NO surface flux remains below the target of 14.0×10^{-10} mol NO cm^{-2} min^{-1}. The addition of a higher weight percent of NO donor could increase this value, however, testing of the WCA to ensure that wettability is still maintained to be comparable to the plasticized PVC control will have to be completed.

II.4. Discussion

Of all of the sample preparation methods tested here, only one produced a material potentially viable for future studies. Formulation J, the film with increased PVC: DOS (3:1) in the base coat and three alternating top coat layers described in Table II.2, appeared to have the greatest promise of all the materials. To be a viable material, the sample had to exhibit a visual smoothness and comparable wettability to the control material, as well as be able to release NO at a surface flux of 14×10^{-10} mol NO cm^{-2} min^{-1} or greater. This surface flux should be relatively

stable for 1.5 h of release time to ensure no artifacts due to unstable NO release over the time period of which the protein will be adsorbing during the planned assay.

The literature formulation A, made similar to that used in previously published literature about NO's effect on biofouling with one active layer and a single 1 PVC: 2 DOS top coat (Table II.2), was found to not be a suitable material for isolation of NO release. Looking at the surface wettability of the commonly used sample preparation system, the material surface properties were not comparable to the control non-NO releasing plasticized PVC materials (formulation K). Thus, any tests run using this set up cannot claim NO release to be the sole contributor to the effects seen during in vivo and in vitro testing using blood and other biological agents since multiple surface properties are changing, not just NO release which is the parameter of interest. Formulation A was significantly more hydrophilic than the control film (formulation K) even prior to soaking the material. This lack of surface property control in the literature formation led to the search for determining a sample formulation whose surface properties were comparable to those of the control. In this way, NO's effects can been investigated using these materials.

To maintain comparison to previously published studies on NO's prevention of biofouling, the materials were to stay as close to the literature preparation as possible. Thus the first thought was to incorporate a filter or alternate polymer layer between the active layer and the top coat. The filter would allow for water or buffer to pass through the film and NO to pass out of the film, but keep the NO donor within the base layer. Both formulations C and D had a non-uniform surface, due to the polymer top coat failing to evenly coat the filter layer. This led to increased error in the contact angle determination, especially for the nylon filter in formulation D at 12.1% error on the measurement. At this time, the pore size of the filter layer was not considered, while a small enough pore size, might be able to prevent the leaching of the NO

donor from the material, that should not have a major effect on the coverage of the filter by the top coat layer. Since the top coat did not evenly cover the added filter, pore size was not considered and this method was abandoned. Additionally, using a filter layer for films made in a well plate for future protein testing presents the challenge of ensuring that the filter is cut so that it fully forms a barrier between the active layer and top coats, as well as lying flat in the well to not increase the roughness of the films by folding or bunching. Due to the complications found by using a filter layer as a barrier, a layer of Tecoflex polymer was considered between the active layer and top coats. Adding a layer of another type of polymer could also prevent leaching from the base layer by providing a barrier polymer layer on top of the donor blended base coat. However, formulation B did not work as well as planned and the surfaces were more hydrophilic than the control (formulation K) (90° and 95°, respectively). This increase in hydrophilicity may be due to mixing of the two polymers as the Tecoflex is soluble in the same solvent as the PVC materials. None of the barrier films ended up being viable methods of sample preparation for the isolation of NO release due to increased wettability compared to the control plasticized PVC and the lack of a visually smooth and uniform surface, particularly for the filter barrier films (formulations C and D).

All of these previous films used a blended NO donor, which inherently increases the chance of leaching from the material. As such, the next step was to use a covalently attached NO donor as the base coat. Formulation E used S-nitrosated PLGH-cysteamine as the base coat layer with PVC as the top coat. This was not a good combination as the PVC top coat warped the films so that films appropriate for contact angle measurements could not be made from this preparation method. The warping of the films and subsequent delamination of the top coat when attempting to remove the films from the glass slide likely results in the differences in the properties of the

different polymers. The delamination is likely due to the incompatibility of the two polymers with each other's solvents. The nitrosated PLGH is not soluble in THF which the plasticized PVC is blended into, thus the two films are not likely to bond to each other during curing, making delamination a possibility. The warping of the film may be due to the base coat of nitrosated PLGH not being fully cured when the top coat is applied, the introduction of the plasticized PVC and THF not being compatible with the nitrosated PLGH and causing an interruption in the polymerization, contributing to the warping of the films. The inability of formulation E to make visually smooth and uniform films eliminates it as a candidate for the model system needed here to isolate the effect of NO release.

Use of diazeniumdiolate-bound SR as a base layer, or formulation F, was another attempt to use a covalently attached donor. These films were coated with PVC as a top coat to compare to the plasticized PVC control materials. This comparison is necessary to both compare to previously published work on the interaction of fibrinogen with NO, and because the medical device that this model system should emulate is typically made with plasticized PVC (extracorporeal circuitry).[20] The top coats applied did not fully cover the entire SR film. These films become dark yellow after nitrosation, this is indicative of the formation of nitrosamines. This is not a desirable product to form for two reasons (1) the nitrosamine is highly stable and will not release NO under the conditions employed with this study; (2) nitrosamines are considered cancerous, any leaching of them from the material negates the formulation's ability to be used for biological applications. Additionally, the films had a visual roughness associated with them, although of comparable hydrophobicity to the control PVC films after soaking (98° and 94° for formulation F and K, respectively). It is possible that the inherent roughness of the films could produce a false reading of the relative hydrophobicity due to the interaction of the

water droplet with the material surface, however, the roughness was not measured for these films. Additionally, the SR films had a surface flux of NO ($0.5 – 2.0 \times 10^{-10}$ mol NO cm^{-2} min^{-1}) that is far below the necessary threshold of 14.0×10^{-10} mol NO cm^{-2} min^{-1} for our model system.[18,20] The surface flux is closer to that of the natural endothelium $0.5 – 4.0 \times 10^{-10}$ mol NO cm^{-2} min^{-1} rather than our target flux.[35] This may be a route to take when the amount of NO release needed is near that of the natural endothelium; however, for the purposes of this study we were aiming for a surface flux higher than the endothelium to prevent thrombus formation on plasticized PVC material surfaces, as such, this is not an appropriate material to move forward within the development of the model system for NO release isolation.

The lack of the covalently attached NO donor films to produce samples with properties required for these experiments led to using the same systems attempted for a covalently attached NO donor in the base coat, but blending the donor into the material. The first attempt was to blend the covalently attached nitrosated PLGH-cysteamine material into a PVC base coat, formulation G. This film was visually smooth in places, and ridged in others. The water contact angle revealed that the film was significantly more hydrophobic than the control PVC material with values at 84° and 95° for formulation G and K respectively. Although the film did not have an acceptable relative hydrophilicity compared to the control, it was still tested for NO release (Figure II.1). Its surface flux at 1.3×10^{-10} mol NO cm^{-2} min^{-1} is similar to that of the SR covalently attached film (formulation F) and not high enough to be considered for further study.

The SR films presented the possibility of a comparable hydrophobic surface to the control material, despite their innate roughness. Additionally, the SR films with covalently attached diazeniumdiolate moieties did not yield a high enough NO flux relevant to this study's biomedical applications. To address the possible use of these films with an increased NO release

capability, DBHD/N_2O_2 was blended into the material and top coated with PVC to make formulation H. This PVC top coat is again necessary to compare to the PVC control, which is representative of the material used to make the medical device of interest, extracorporeal circuitry. These blended films proved to be significantly more hydrophilic than the PVC control after soaking (74° for the blended SR films, compared to 94° for the control). Although formulation H is not viable due to this reason, the NO release from these films was at an acceptable level of 15 – 20 $\times 10^{-10}$ mol NO cm^{-2} min^{-1} as shown in Figure II.1.

Attempts to use covalently attached donors, barrier layers, and other polymers did not result in viable samples to be used as a model system for isolating the effects of NO release. Thus, studies returned to the investigation of PVC films and varying the number of top coats and PVC: DOS ratio of the layers. Both the multi-layered film (formulation I) and the film with increased PVC content in the base layer (formulation J) had comparable hydrophobicity to the control film as detailed in Table II.2 (92° and 95° for comparison of formulations I and K before soaking, 92° and 94° for formulations J and K after soaking). The NO surface flux from the formulation I films was 0.4 ± 0.2 $\times 10^{-10}$ mol NO cm^{-2} min^{-1} which is below the target NO flux of >14 $\times 10^{-10}$ mol NO cm^{-2} min^{-1}. Using this blended donor system, more NO donor could be added to the material in order to achieve the desired surface flux. The films for these measurements did not have a well-defined surface area as the polystyrene well blended in with the PVC: DOS films as shown in Figure II.3. When tested in a polypropylene well plate, there was no blending with the plate material. Still, the films exhibited a poorly defined surface. The films for formulation I involve nine top coat layers, and when they were plated in either the break-away wells or in a 96-well plate, the surface area of the material was not well defined (Figure II.3). The nine top coat layers were used because preliminary evaluation of the wettability of these PVC based materials

used varying numbers and PVC: DOS content of layers. When keeping the active layer at a 2 PVC: 1 DOS ratio, the nine layers of top coats were necessary to maintain comparable surface wettability with the control non-NO-releasing PVC material. This multi-layered method showed that enough PVC content could help achieve comparable surface properties between control and composite materials, but nine top coats was not the way to do this and so the next test was to add a greater ratio of PVC: DOS to the active layer.

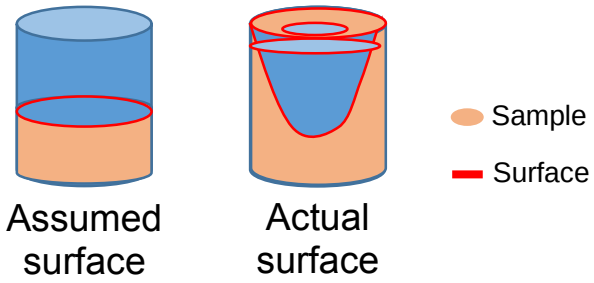

FIGURE II.3: Representation of the expected and actual surface of the multi-layered films used for formulation I. Surfaces were expected to be uniform with a defined surface area, however, the actual films dissolved the wells and became concave with no defined or uniform surface area. These films were made in the break-away wells for NOA experiments.

Formulation J has a base coat with a 3 PVC: 1 DOS ratio, which increases the PVC content compared to previous samples and the literature formulation which only had a 2 PVC: 1 DOS ratio. The increased PVC should help to contain the NO donor within the active layer.The plasticizer is added to increase the mobility of water through the polymer, as seen in previous formulations, this also increases the likelihood that the NO donor will also be more mobile and leach from the polymer material. By increasing the relative amount of PVC within the material, the relative amount of plasticizer decreases, hopefully decreasing the ability of the NO donor to leach from the material. This film has three top coats, two of 2 PVC: 1 DOS sandwiching a single top coat of 3 PVC: 1 DOS, none of which have greater DOS content than PVC. These

films also exhibited blending of the applied polymer solution with the breakaway well polymer; thus the surface area was not well-defined. When plated in a polypropylene well plate, blending of the film with the plate was eliminated, yielding visually uniform and smooth films. This film did not have the morphology issues associated with the multi-layered film (formulation I). Formulation J has comparable surface wettability to the control PVC films (formulation K) after soaking. Sample preparation in this way provides a promising solution to isolating the effect of NO. The NO surface flux was only at 6.2×10^{-10} mol NO cm^{-2} min^{-1}, which is below the target threshold value; however, blending more NO donor could result in NO release at the desired threshold value of $>14.0 \times 10^{-10}$ mol NO cm^{-2} min^{-1}. Understandably, this does mean that the films will have to be retested for surface wettability to ensure that the addition of more NO donor does not increase the hydrophilicity of the material. Overall, formulation J, the film with 3 PVC: 1 DOS in the active layer, is seen to be the most proming sample preparation technique for further study. The film is visually smooth and uniform, has comparable wetability to the control PVC material, and has the potential to release at the desired surface flux if more NO donor is blended into the active layer. Sample preparation using this technique should effectively create a surface where NO release is the only variable of change from the control material so that the effect of NO release on biological systems can be studied.

II.5 Conclusions

To investigate the effect of NO release form a synthetic material on a biological system, a material formulation was required that will maintain all other surface properties except for NO release to isolate NO as the main variable of study. This current work explored a variety of sample preparation options to control the surface properties of the material by keeping the composite surfaces comparable to the controls. The use of a barrier polymer or filter did not

produce a viable material to use for these experiments because the NO donor was still able to leach from the material causing increased hydrophilicity of the material in addition to the surface not being uniform. Additionally, the use of covalently attached NO donor systems and systems where the covalent system was blended within another system produced surfaces that did not solve the issue of keeping a comparable surface wettability to the control the incompatibility of the blended system with the PVC top coats and the lack of sufficient NO release. A multi-layered material appeared to be the solution, however, an impractical system due to film morphology issues and undefined surfaces. This led to the use of increased polymer in the layers of the material. The final sample preparation technique, formulation J, with the active layer containing 3 PVC: 1 DOS and three top coats of 2 PVC: 1 DOS, 3 PVC: 1 DOS, and 2 PVC: 1 DOS applied in that order, provided the best option for further study. This material was developed as the model system for determining the effect of NO on protein adsorption, as detailed in Chapter III of this dissertation.

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CHAPTER III:

DEVELOPMENT OF MODEL SYTEM AND INITIAL FIBRINOGEN ADSORPTION STUDIES[1]

III.1 Introduction

Implanted biomaterials are an integral part of various biomedical applications.[1] The incorporation of these materials that contact blood is usually complicated by foreign body reactions that are initiated by the coagulation cascade. The intrinsic coagulation cascade is marked by initial deposition of proteins on the material surface, which undergo conformational changes and trigger the activation and adhesion of platelets, finally resulting in gross thrombus formation.[2] Infection and inflammation then occur, preventing integration of the device within the body. To overcome these undesirable processes at the material-blood interface, a material can be functionalized with a therapeutic agent for controllable release to interact with and control various biological components.

One naturally-occurring therapeutic that has garnered significant attention due to its multiple biological functions is nitric oxide (NO). NO is involved in many signaling processes, namely vascular tonicity.[3-6] More specifically, NO is released from endothelial cells to result in vasodilation and platelet regulation. Since NO is naturally involved in regulating vascular processes, materials that release NO have gained interest as a way to prevent blood clot formation on implanted synthetic surfaces.[7-10] NO releasing materials have largely been designed by blending small molecule NO donors, such as *N*-diazeniumdiolates, within a hydrophobic polymer. Exposure

[1]*This work has been published previously in Journal of Biomedical Materials Part A with joint authors: Brittany J. Barrett and Melissa M. Reynolds and is used with permission. The main body of work was completed by this author with aid by Ms. Barrett. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 101A: 3201–3210, 2013.*

of the material to physiological conditions results in donor decomposition to yield NO. These *N*-diazeniumdiolate blended materials have certainly demonstrated excellent anti-platelet properties,[11-13] resulting in the minimization of thrombus formation,[8,14-16] and have further demonstrated anti-bacterial function.[17,18] The major biological agents involved in the formation of thrombus are platelets and fibrinogen (Figure III.1). Figure III.1 gives a simplified cartoon of the multi-step process of thrombus formation beginning with the deposition of proteins onto the material surface and culminating in the formation of a mature clot.[1,19-21] As this process begins with the initial deposition of proteins, it is critical to understand NO's role in this initial stage (Figure III.1a). Certainly NO is known to be involved in many physiological functions, but the full breadth of NO's involvement in biological systems is not yet fully known. Therefore, a greater understanding of NO's role in the entire coagulation process will enable better design of materials to eliminate clotting complications on device surfaces.

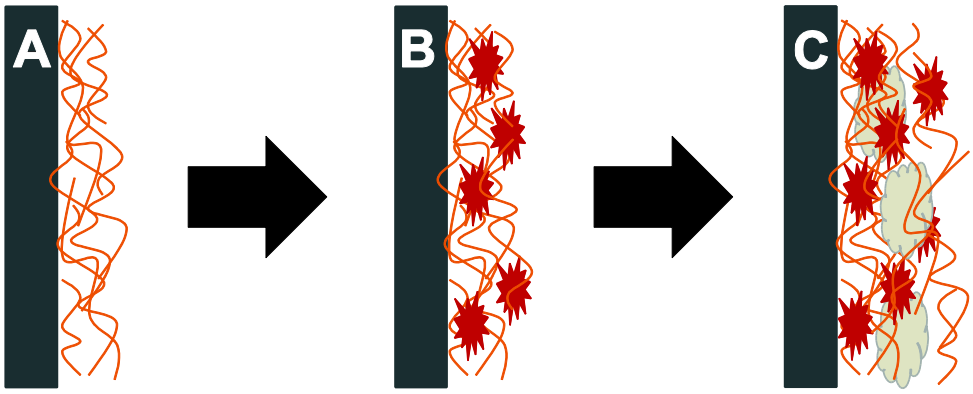

FIGURE III.1: Cartoon depicting the main steps of clot formation on surfaces. Synthetic devices adsorb proteins immediately upon implantation (A). Following, platelets adhere and activate (B). This ultimately leads to formation of a mature blood clot and device failure (C). In this work, we explore the influence of NO on fibrinogen binding to the material surface (A).

To date, no studies have directly determined the effect of NO on clotting proteins; however, one study examined the effect of an *N*-diazeniumdiolate blended poly(vinyl chloride) (PVC) coating on blood compatibility in a rabbit model.[22] This NO releasing coating demonstrated a

significant decrease in platelet adhesion and reduced thrombus formation compared to a PVC control film. Additionally, the adsorption of the clotting protein fibrinogen on the material surface was assessed through a fluorescence assay. A decrease in fibrinogen binding compared to the PVC control was observed, indicating that the NO releasing surface has an impact on protein adsorption. Further interest in the impact of NO materials on fibrinogen adsorption is established by Fleser et al. where they incorporated the NO-releasing PVC onto the interior of vascular grafts.[14] They saw a significant decrease in mature clot formation on the NO-releasing devices; however, there was also a thin layer of fibrin covering parts of the graft's surface. Fibrinogen is of the highest concentration of clotting proteins in the blood and its conversion to fibrin on a material surface marks a critical point in the coagulation cascade. Therefore, NO's ability to affect fibrinogen binding to a material surface is critical toward overall clot formation.

When considering the differences between the behavior of the NO releasing surface and the PVC control, the rabbit model experimental results were attributed to potential surface properties[22] whereas the vascular graft study attributed results solely to the release of NO.[14] In addition to the release of NO, the chemistry of the donor within the film during NO release may cause surface properties to differ from the expected (Figure III.2). It has long been established that the surface properties of a material largely influence its interaction with biological components. Fibrinogen adsorption and activation into fibrin is shown to be highly dependent on the surface properties of a material: composition,[23-26] wettability,[27-33] charge,[32-34] and roughness.[35,36] For this study we developed a sample preparation method to eliminate differences in these variables between control and NO-releasing surfaces (Figure III.2). By doing so, we can isolate, for the first time, the influence of NO on protein deposition on surfaces.

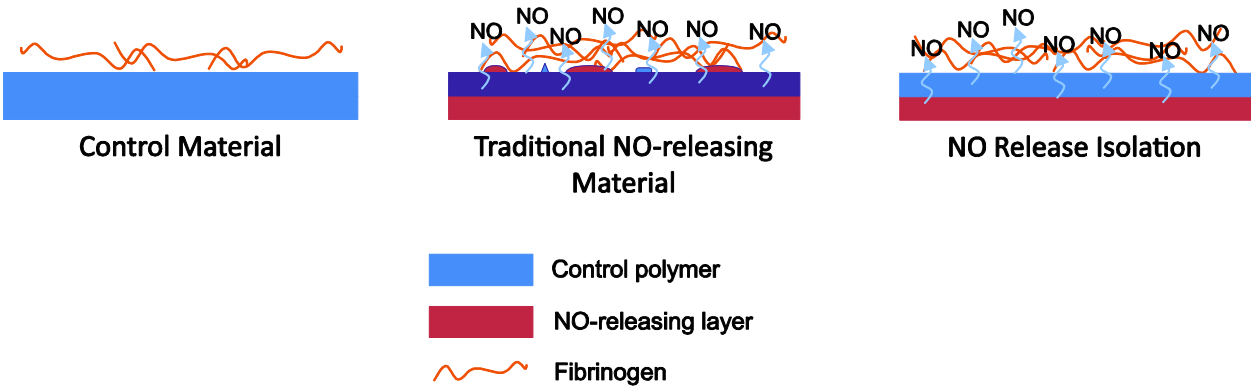

FIGURE III.2: To assess the effect NO has on fibrinogen adsorption, the surface properties must be normalized to the control. This cartoon illustrates the surface properties of the control and two possibilities of NO-releasing materials. The ideal material for assessing NO's effect is the one on the far right where the surface retains the properties of the control. Current NO releasing materials do not consider surface properties and may have changes in wettability, charge, roughness, and composition as shown by the purple region on the center material. Fibrinogen adsorption is seen to increase on NO-releasing materials; this increase is seen to be due to the release of NO because of the increase of adsorption on the NO release isolation material.

A model NO releasing plasticized PVC material system was developed with blended *N*-diazeniumdiolated dibutylhexanediamine (DBHD/N_2O_2) as the NO releasing species. This donor was incorporated to achieve an NO flux from the material >14 $\times 10^{-10}$ mol NO cm^{-2} min^{-1}, which is shown to preserve material function and limit coagulation.[37] Surface properties were established using contact angle goniometry, profilometry, and X-ray photoelectron spectroscopy. The release of NO from the material was measured using a nitric oxide analyzer (NOA), a chemiluminescence based detection method, and fibrinogen adsorption was established via fluorescent based ELISA. Taken together, we are able to see that NO release from the material surface influences fibrinogen deposition to a material that has been used to fabricate thrombus-free devices, an unforeseen result given the improved thromboresistivity of NO releasing surfaces. This result identifies another potential pathway for NO to influence clot formation on synthetic surfaces.

III.2. Materials & Methods

III.2.a. Materials

Polymer formulations were prepared from high molecular weight poly(vinyl) chloride (PVC) from Aldrich (St. Louis, MO) and bis(2-ethylhexyl) sebacate (DOS) from Acros Organics (New Jersey, USA). The additive, potassium tetrakis(4-chlorophenyl) borate (KTpClPB) was obtained from Ace (Korea). Tetrahydrofuran (THF) from Mallinckrodt (New Jersey, USA) was used as the solvent. Diazeniumdiolated dibutylhexanediamine (DBHD/N_2O_2) was synthesized by exposing *N,N'*-dibutyl-1,6-hexanediamine (DBHD) (Alfa Aesar, Ward Hill, MA) (99.8%, Sigma, St. Louis, MO) NO under 80 psi NO at room temperature for 48 h in acetonitrile as the solvent.[38] For amine detection 9-fluorenylmethyl chloroformate (FMOC-Cl) from Acros Organics (New Jersey, USA) and n-pentane (JT Baker, Pennsylvania, USA) were purchased.

For the fibrinogen binding assay, human plasma fibrinogen and Tween 20 were obtained from Calbiochem (La Jolla, CA) and the fluorescent conjugated goat IgG fraction to human fibrinogen was obtained from MP Biomedicals (Solon, OH). Dulbecco's phosphate buffered saline (DPBS) was obtained from Hyclone® (Logan, Utah). The Serotec Block Ace was purchased from AbD Serotec (Raleigh, NC). Black, round-bottom polypropylene, 96-well microtiter plates were obtained from Nalge Nunc International (Rochester, NY). Glass slides (75x50 mm) from Corning (NY) glass rings and polypropylene test tubes from lab stock. All water used in the experiment is deionized unless stated otherwise.

III.2.b. Film preparation

III.2.b.i. Polymer solution preparation

Two plasticized PVC formulations were made: 3 PVC: 1 DOS in THF (1080 mg PVC and 360 mg DOS per 10 mL) and 2 PVC: 1 DOS in THF (708 mg PVC and 354 mg DOS per 10 mL). A third solution was made with 1 wt% or 9.2 wt% DBHD/N_2O_2 blended in 1:1 ratio with KTpClPB into an aliquot of 3:1 polymer solution. This is diluted with THF in a 1:1 ratio of polymer solution to THF. Films were created in four layers: base layer and three top coats. The base layer consisted of either 3 PVC: 1 DOS (control film) 1 wt% or 9.2 wt% DBHD/N_2O_2 blended polymer (composite films). After curing for 1.5 h, a series of top coats in the order of 2:1, 3:1, 2:1 polymer solution were applied, each curing for 30 min between coats. The resulting film was allowed to cure at ambient conditions protected from the environment overnight.

III.2.c. Surface properties

Samples for surface property measurements were created in glass rings that had been secured to glass microscope slides. Films were tested both before and after a soaking step. For soaked films, samples were kept in the ring/slide set up and placed into a 37 °C DPBS bath after adding 2 mL of DPBS to the ring and allowed to soak for 2.5 h. Post soaking, films were pat dry with a Kimwipe. All films were prepared for surface analysis in the same manner.

III.2.c.i. Water contact angle

Static water contact angles were measured by depositing 2 µL of ultrapure water (>18 MΩ) onto the film's surface. All contact angles were determined by taking an image of the drop on the surface and analyzing with the circle fitting parameter on the Drop Shape Analysis 1.50 software.

Measurements were performed on a Krüss Drop Shape Analysis System DSA 10 (Krüss, Hamburg, Germany) (n=9).

III.2.c.ii. Optical profilometry

Roughness profiles were obtained by taking three measurements using the stitch function for an area of 1.28 mm × 0.960 mm for each film. Scans were taken using a -20× objective center read with 200 mm scans above and below center. Films were corrected for curvature by using a Fourier filter, period fit (5.0 mm). An additional reagent control containing KTpClPB and DBHD (1 wt% and 9.2 wt%) was run for this measurement. All measurements were performed and analyzed on a Zemetrics ZeScope Optical Profilometer and computer software (Zygo, Tucson, AZ) (n=9).

III.2.c.iii. X-ray photoelectron spectroscopy (XPS)

All elemental composition experiments were completed via X-ray photoelectron spectroscopy (XPS). XPS measurements were completed on a PHI-5800 spectrometer (Physical Electronics, Chanhassen, MN) using a monochromatic Al 7 mm filament ($h\nu = 187.85$ eV). Survey scans were taken over a range of 10-1100 eV for 5 min (21 scans total). Measurements were taken at a 45° take off angle defined at the angle between the surface and the analyzer. High resolution scans were taken for O 1s, C 1s, Cl 2p and N 1s with a pass energy of 23.50 eV at 0.1 eV per step. Two high resolution cycles were completed with two scans for O 1s and Cl 2p, one scan for C 1s and 5 scans for N 1s per cycle. All scans were taken utilizing a neutralizer set at 20.5 μA. Data was analyzed in Matlab (n=2).

III.2.d. Leaching solution study

Solution based UV-vis spectroscopy was performed on the collected soaking solutions of 24 wells with 100 µL DBPS placed in each well. Wells tested were blank plate, control film, 1wt% and 9.2 wt% composite films. Solutions were collected at 1 h into soaking, and then replaced with fresh 100 µL DPBS aliquots to soak for an additional 1.5 h to mimic the fibrinogen ELISA described in Section III.2.f. on fibrinogen adsorption. Collected solutions were tested for DBHD with a modified derivatization of the amine with 9-fluorenylmethyl chloroformate (FMOC-Cl) similar to that previously described for polyamines.[8,39] After reaction of the leachate with FMOC-Cl, excess FMOC-Cl was extracted with the addition of n-pentane.[40] The final solution was placed on an Evolution 300 UV−vis spectrophotometer (Thermo, USA) to detect absorbance at 265 nm. Measurements for KTpClPB were also performed on the UV-vis spectrometer to look for a peak at 235 nm.[41] For all measurements, sample absorbance was corrected by subtracting the absorbance of DPBS. Concentrations were determined via calculations from a calibration curve (concentrations from 0.05 – 10 µg/mL for both DBHD and KTpClPB). Comparisons to control and blank well plate were completed by subtracting their concentrations from those of the composite materials.

III.2.e. NO release study

Layered films were made as previously described in the bottom half of a polypropylene test tube to mimic conditions inside the fibrinogen assay well plate. To measure the release rate of the NO donor materials a single tube was placed into a custom-made reaction cell and purged with nitrogen. An aliquot of 0.6 mL DPBS was injected to fill the tube. All tests incubated via water bath at 37 °C. Measurements were taken at 1 min intervals over a period of 2.5 h. Experiments

were performed on a chemiluminescence NO analyzer (NOA) (Sievers 280i, Boulder, CO) at flow rate of 200 mL/min. Release of nitric oxide (NO) from the composite material was collected in ppb and converted to the reported moles of NO via a lab determined calibration constant. This was further converted to determine the flux of NO from the surface of the material by dividing by the surface area and time of collection (n=5).

III.2.f. Fibrinogen adsorption

Materials were prepared in a 96-well plate format, in sets of three columns each for control PVC, composite NO-releasing, and well plate control (Figure III.3). Fibrinogen adsorption to the surface was determined with a fluorescent based ELISA as described by Major et al. [22]

Briefly, 100 µL of DBPS was added to each well and allowed to incubate for 1 h at 37 °C. After which, the solution was removed and 100 µL of 3 mg/mL fibrinogen in DPBS solution was deposited into the well plate and allowed to incubate for 1.5 h. Post incubation, wells were washed eight times with wash buffer (1:10 Block Ace: water, 0.05% Tween 20). To block nonspecific binding, blocking buffer (1:4 Block Ace: water) was added in 100 µL aliquots and allowed to bind for 30 min. Wells were again washed three times with wash buffer and background fluorescence measurements taken. Goat IgG conjugated with fluorescein isothiocyanate (FITC) diluted 1:10 with dilutent (1:10 Block Ace: water) is allowed to adsorb in 100 µL aliquots for 1.5 h. Wells were washed three times with wash buffer and a final fluorescence reading is taken. All incubations done at physiological temperature (37 °C). Measurements taken at 485/20 nm (excitation) and 528/20 nm (emission) with a Synergy 2 microtiter plate reader (Biotek, Winooski, VA) (n=6).

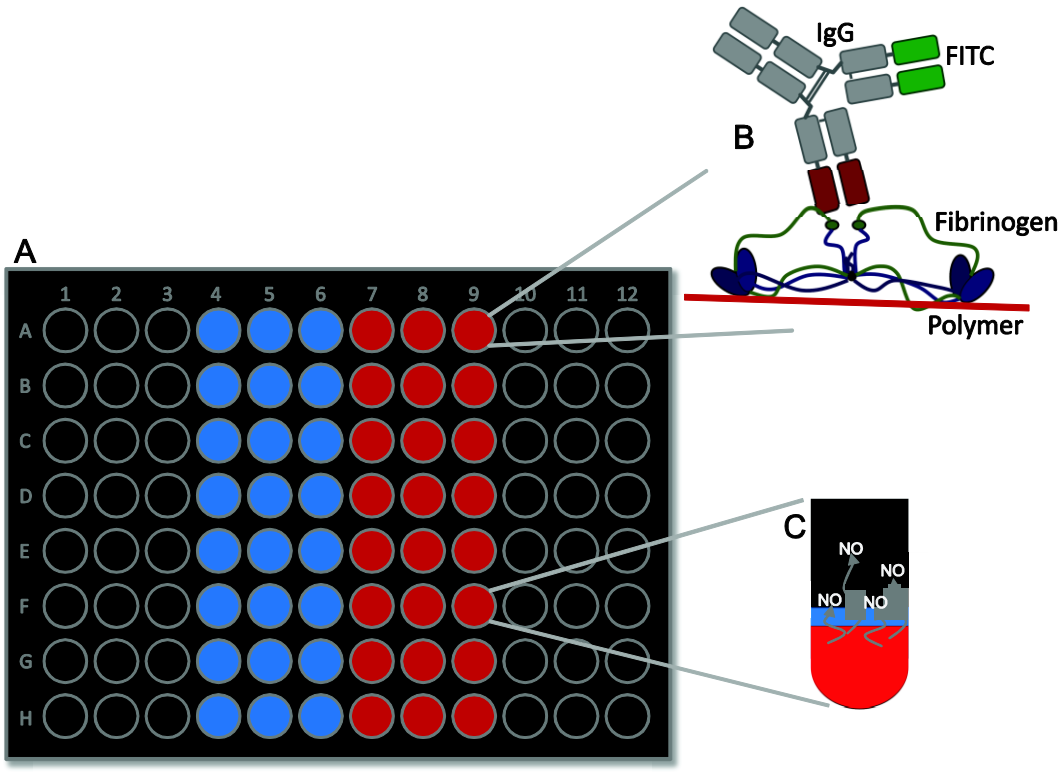

FIGURE III.3: Cartoon of fibrinogen ELISA. Shown is the well plate (A) with empty wells (black), control polymer (blue), NO-releasing polymer (red). The inset (B) gives an illustration of the adsorption of fibrinogen to the surface and the fluorescent tag used to detect the amount adsorbed. The layering within the NO-releasing wells (C) has the base layer containing the NO-releasing moiety and a top coat of control polymer which retains the surface properties of the control polymer alone.

III.3. Results

III.3.a. Surface properties

III.3.a.i. Water contact angle

The water contact angles measured for PVC control and DBHD/N_2O_2 containing composite films, both before and after soaking for 2.5 h, show a similar wettability for all films as shown in Table III.1 (range 86.0 – 92.0 °). Within error, the relative hydrophilicity of all films are the same. This indicates that soaking control and composite films does not change the relative hydrophilicity of the material during the time period of our model system (n=9).

TABLE III.1. Wettability and Roughness of Control and Composite Surfaces

Film	2.5 h DBPS Soak Status	Water Contact Angle (°)	Rq (nm)
1 wt% DBHD/N_2O_2	Before	87.4 ± 1.1	9.5 ± 8.5
	After	92.0 ± 2.8	7.7 ± 3.3
9.2 wt% DBHD/N_2O_2	Before	86.0 ± 0.7	9.5 ± 5.1
	After	88.6 ± 4.0	24.6 ± 17.1
Control PVC film	Before	88.4 ± 2.0	8.5 ± 3.6
	After	86.6 ± 2.3	26.0 ± 19.9
All values reported as mean ± standard deviation (n=9).			

III.3.a.ii. Optical profilometry

Composite and control films are found to have the same surface roughness both before and after soaking. The roughness is found to be on the nm scale, giving a roughness on the scale of the fibrinogen (5 × 5 × 45 nm).[42] The roughness, however, changes after soaking. For 1 and 9.2 wt% composite and control films the root mean square surface roughness (Rq) is 9.5 ± 8.5, 9.5 ± 5.1, and 8.5 ± 3.6 nm, respectively, before soaking in heated buffer. After soaking, the roughness changes to 7.7 ± 3.3, 24.6 ± 17.1 and 26.0 ± 19.9 nm for 1 and 9.2 wt% composite and control films, respectively. An additional control containing KTpClPB and either 1 wt% or 9.2 wt% DBHD was tested for roughness. This sample does not differ in roughness from the plasticized PVC control and composite films. There is a large spread in the measured values that does change after soaking (n=9). Our measured roughness range (7.7 – 26.0 nm) is similar to that previously reported by Cai et al. In the Cai work, this size difference in roughness has no effect on the amount of fibrinogen adsorbed.[43] As such, it is unlikely that roughness is a major contributor to fibrinogen binding in these studies.

III.3.a.iii. X-ray photoelectron spectroscopy

XPS was used to determine the presence of nitrogen on the surface of the films, also the similarity of the carbon, oxygen, and chlorine binding energies. The presence of nitrogen on control films would indicate a contaminant on the surface; however, the presence of nitrogen in the composite materials would indicate that the NO donor molecules are leaching to the surface of the material. We use nitrogen as an indicator due to its presence in DBHD/N_2O_2 and the lack of nitrogen in the PVC and DOS materials used for the top coats. During the survey scans of all materials both before and after soaking, there is no noticeable peak in the 396 – 409 eV binding energy range, indicating no nitrogen present on the surface of the material (Figure III.4).

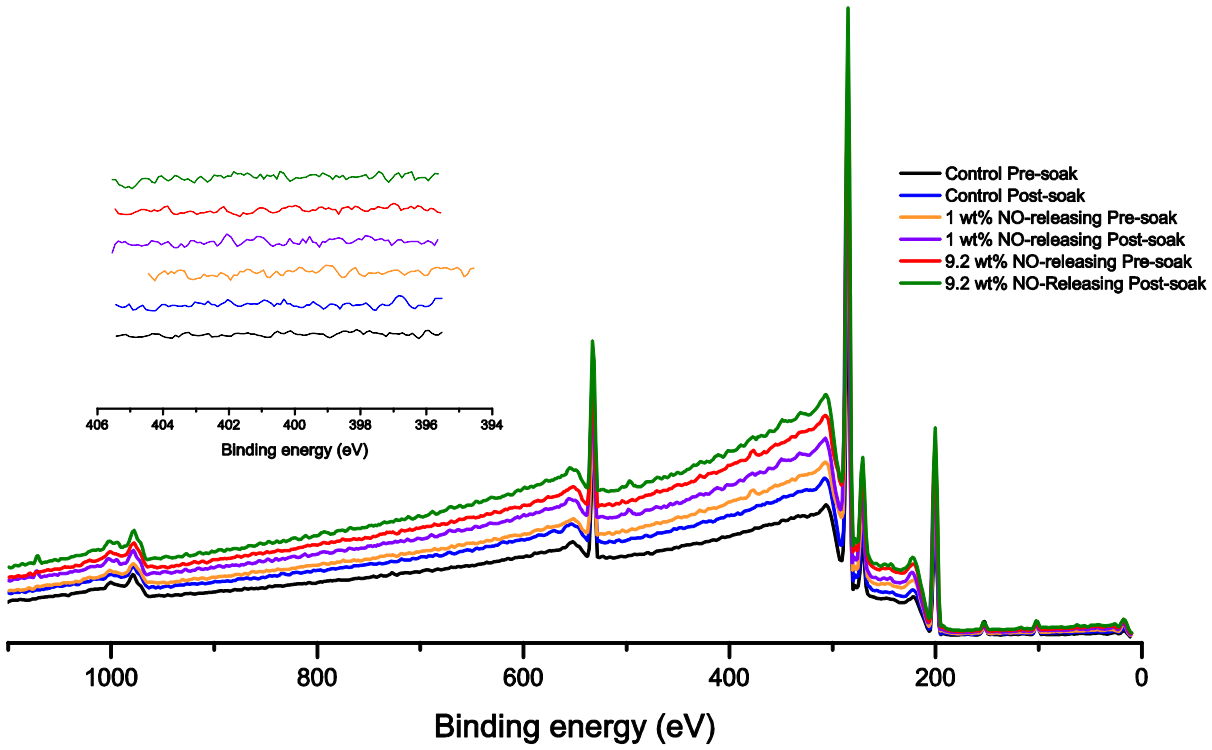

FIGURE III.4: Representative survey scans of material surfaces. Spectra overlay shows no change in surface composition for all samples. Inset is representative high resolution XPS scan for N1s peak. For all samples, peak to noise averages 0-3, indicating no peak for N1s.

Subsequent high resolution scans in the area confirm the absence of the N 1s peak (Figure III.4, inset). Because the only place nitrogen should be in the film is in the active layer of the composite material, the absence of the N 1s peak verifies that the active layer is not mixing with nor leaching to the surface of the material during the time period of interest. All other peaks (C 1s, O 1s, Cl 2p) remain comparable for both control and composite materials before and after soaking.

III.3.b. Leaching solution study

To further verify the absence of leaching of the added components, KTpClPB and DBHD/N_2O_2, solution based measurements were performed to determine whether they were leaching out of the material. Compared to blank plate and control material, no measurable diamine was detected in the leachate for 1 and 9.2 wt% composite materials. Less than 0.2 μg/mL of KTpClPB was found to leach from each composite well, compared to empty well plate and control material.

III.3.c. NO release

Figure III.5 shows 1.5 h of NO release after the film has been allowed to release for 1 h. After 1 h it was determined that the release of NO had achieved a relatively steady state flux of NO during the 1.5 h time period of interest. The importance of having a constant flux of NO for 1.5 h, is that the NO release during the fibrinogen adsorption step of the ELISA must be constant to determine the effect of a known amount of NO release on fibrinogen adsorption.

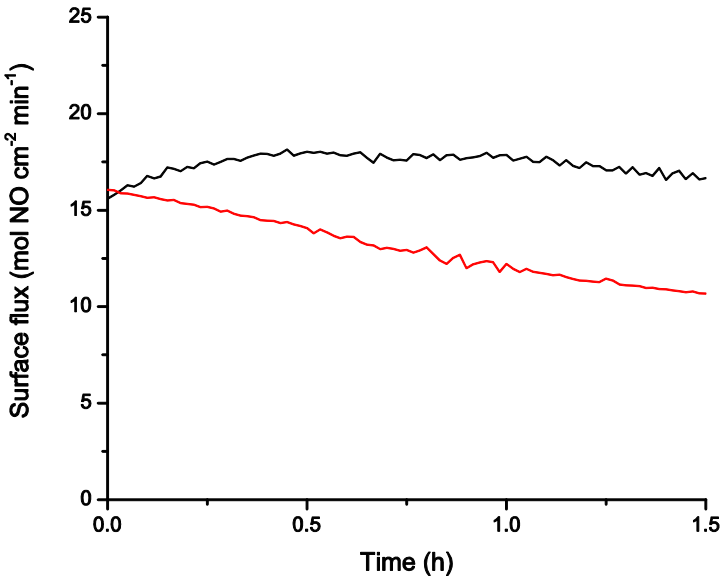

FIGURE III.5: Average surface flux of NO released from 1 wt% (red) and 9.2 wt% (black) composite materials. Shown is 1.5 h of measurement after film has been allowed to release for 1 h to achieve a stable release of NO. Spread in surface flux deviation over release period <5% (n=5).

The 1 wt% composite film releases a total of 7.63 ± 1.31 $\times 10^{-8}$ mol NO with an average flux of 13.0 ± 1.6 $\times 10^{-10}$ mol NO cm^{-2} min^{-1} during the 1.5 h period of interest. During this time period, a total of 1.07 ± 0.29 $\times 10^{-7}$ mol NO is released by the 9.2 wt% film at an average flux of 17.4 ± 0.5 $\times 10^{-10}$ mol NO cm^{-2} min^{-1} just above the level of NO flux shown to improve the biocompatibility of the material.[37] These releases are shown to be statistically different (95% CI), providing us with two different release rates of NO to monitor NO release from with the lower release rate being just under that previously shown to prevent thrombus formation on these materials.

III.3.d. Fibrinogen adsorption

The fluorescent based ELISA shows the amount of fibrinogen adsorbed to the surface of the polymer. All data are converted to % of 3 mg/mL fibrinogen control, run on the blank well plate, to facilitate comparison across assays. Results show a significant increase in the amount of fibrinogen adsorbed to the NO releasing surfaces compared to that of the polymer control (103 ± 31, 226 ± 99, and 2334 ± 496% of 3 mg/mL fibrinogen control for control, 1 wt%, and 9.2 wt% composite materials, respectively) (Figure III.6). There is a large difference also between the 1 wt% and 9.2 wt% composite materials showing that not only does NO release affect fibrinogen adsorption, but the amount of NO being released from the surface has an effect on the amount of fibrinogen adsorbed to the material's surface.

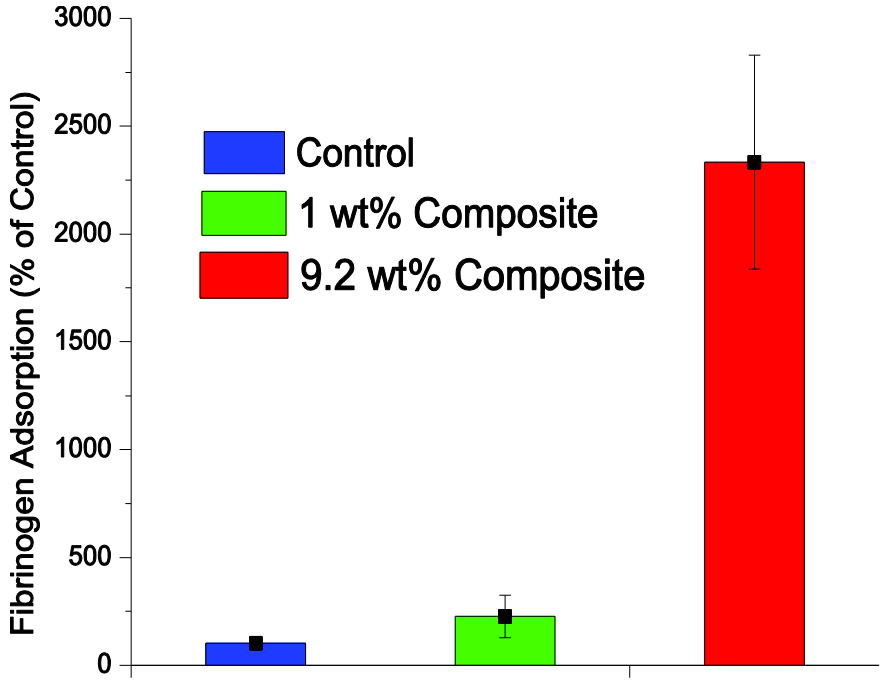

FIGURE III.6: Amount of fibrinogen adsorbed to the control, 1 wt%, and 9.2 wt% composite polymer surfaces. Values are calculated as % of 3mg/mL fibrinogen control and shown as mean ± standard deviation (n=16).

III.4. Discussion

With this study, we demonstrated that NO-releasing PVC materials exhibit an increase in the amount of fibrinogen adsorbed to the surface compared to a control PVC material. This provides valuable insight into the potential clotting pathway on synthetic materials. Previously, studies have shown that NO-releasing PVC materials provide a decrease in clot formation.[13-15,22,37,44] Work by the Bartlett group found that there was an increased amount of fibrinogen adsorbed to the NO-releasing material than the PVC alone.[22] They found that these materials maintained viability over the course of their study, implying that the increase in fibrinogen adsorption was not enough to interfere with the anti-platelet effects of the NO. They attribute the increase in fibrinogen adsorption to the hydrophobicity of the material and the charge added by the DBHD after NO release. Our materials eliminate the potential of the post-NO release DBHD influencing the surface properties of the material, and establish that the control and composite materials have the same degree of hydrophobicity. Because of this we are able to fully assess the reasons behind the increase of fibrinogen adsorption to the NO-releasing composite material.

We isolated the effect of NO release by controlling the surface properties of the PVC control and NO-releasing composite material. By modifying the sample preparation method of our samples we were able to achieve a normalization of surface properties across the samples and maintain a steady NO flux rate of $17.4 \pm 0.5 \times 10^{-10}$ mol NO cm^{-2} min^{-1}, this is within the flux established to promote viability of synthetic blood-contacting materials.[22,37] Additionally, we tested a lower flux rate of $13.0 \pm 1.6 \times 10^{-10}$ mol NO cm^{-2} min^{-1} to provide a comparison of fibrinogen adsorption at a flux of NO below that suggested to promote material viability. We measured the surface properties of both control and composite materials before and after soaking in DPBS at 37 °C for 2.5 h to mimic the conditions under which the fibrinogen adsorption assay

is performed. In addition to monitoring the surface properties we performed measurements on the soaking solutions for the films to determine whether the DBHD or KTpClPB were leaching. This set of tests ensured that the conditions under which fibrinogen adsorption is performed remain the same for both composite materials and the control.

Fibrinogen adsorption is influenced by the surface properties of a material, particularly, wettability, roughness, and composition. To isolate the effect of NO, these surface properties must remain the same on both control and composite materials. Surface wettability of control and composite materials ranged from 86.0 – 92.0 ° both before and after soaking the films. The closeness in the water contact angles indicates that the relative hydrophobicity of the materials did not change from control to composite, even after soaking the films. Wettability and adsorption can both be influenced by the roughness of the surface; to this end, we looked at the Rq of the material surfaces both before and after soaking in the nanometer range to ensure that any differences seen would be on the order of the size of fibrinogen (5 × 5 × 45 nm).[42] There is a change in roughness post soaking (9.5 ± 8.5 to 7.7 ± 3.3 nm, 9.5 ± 5.1 to 24.6 ± 17.1 nm, and 8.5 ± 3.6 to 26.0 ± 19.9 nm, for 1 wt%, 9.2 wt% composites and, control, respectively). Despite this change, the differences in roughness are within a range shown to have no noticeable difference on fibrinogen adsorption (change of 19 nm).[43] The spread in the measurements is large; this is especially true for the films after DPBS soaking. This large error and change in roughness post soaking is likely a result of the nature of polymeric material structures. A polymer is not a regularly ordered material, and thus different sections of the surface will be affected by the diffusion of buffer through the material in different ways causing the large deviation in roughness. Because the values for control and composite materials remain within a few nm of each other even after soaking (Table 1), the Rq values for the materials indicate that any changes are due to changes in the polymer itself during

the 2.5 h soak, so effects of changing surface roughness on fibrinogen adsorption will be the same for both materials. This indicates that the deviations are likely only from changes in the plasticized PVC and not the diffusion of any additives (e.g.: DBHD/N_2O_2 and KTpClPB) through the surface.

After ensuring that the wettability and roughness of the materials remain comparable across the samples, it was important to ensure that the surface composition does not change over the time period of fibrinogen adsorption during the assay. This was established by evaluating the elemental composition of the surface before and after soaking via XPS. Minor changes in polymer composition have been found to influence the amount of fibrinogen adsorbed to a surface, even a small change in the backbone of the polymer.[24] The changes exhibited by small changes in the polymer structure on fibrinogen adsorption indicates that if the NO donor or the DBHD after release of NO moves within the polymer to the surface of the material, it will influence the amount of fibrinogen adsorbed. DBHD differs from the plasticized PVC of the control by having nitrogen in it. By monitoring the XPS nitrogen peak we can determine whether the donor has leached to the surface (see Supporting Information, Figure SI1 from published paper). As seen in the Figure III.4 inset, the high resolution scans of the binding energies for N 1s were found to have no difference in the peak-to-noise ratio for all samples. Because the control polymer and top coats do not contain nitrogen, while the NO donor does, the absence of nitrogen on the surface of the material indicates that the donor molecules are remaining under the surface of the material. The overall binding environment on the surface remains the same for all composite and control samples (Figure III.4, inset). This confirms that the control and composite materials have comparable surface properties. The NO donor has remained in the active layer of the material and any changes to the surface can be attributed to changes in the plasticized PVC and remain the same for both control and composite materials. To further assess the retention of NO donor and salt within the film over the time period

of analysis, solution based UV-vis spectroscopy was performed on soaking solutions to see whether or not these molecules were present in the buffer post soaking. No detectable levels of diamine were found to leach from the composite materials compared to control and blank well plate. Presence of the diamine in the leaching solution, or nitrogen on the surface of the material would affect the amount of fibrinogen adsorbed to the surface of the material as previously established by the Schoenfisch group.[33] Through the UV-vis and XPS measurements we have established that amine interference is not a factor in the fibrinogen adsorption to our materials. The small amount of KTpClPB found to leach from the material (<0.2 μg/mL) is not going to have a major effect on fibrinogen adsorption. The monovalent cation K^+ will not have an effect on fibrinogen adsorption as only divalent cations have been found to have an effect.[45] These tests assure us that of the factors that affect fibrinogen adsorption to material surfaces (i.e. wettability, roughness, composition, drug release), the only one that will change from control to composite material during the time period of fibrinogen adsorption within the ELISA is the release of NO.

The release of NO from the composite material was determined via a direct chemiluminescence technique using an NOA. The NO release curves shown in Figure III.5 were obtained after the material has been allowed to release for 1 h. What we see is a steady release of NO over the course of the 1.5 h that only varies by 0.5 $\times 10^{-10}$ mol NO cm^{-2} min^{-1} for the entire time period for the 9.2 wt% composite material. This release ($17.4 \pm 0.5 \times 10^{-10}$ mol NO cm^{-2} min^{-1}) is significantly higher than that of the natural endothelium ($0.5 - 4 \times 10^{-10}$ mol NO cm^{-2} min^{-1})[46] and is found to be necessary for maintaining the viability of synthetic materials.[37] Additionally, we establish a second surface flux rate of NO release with a 1 wt% film ($13.0 \pm 1.6 \times 10^{-10}$ mol NO cm^{-2} min^{-1}) that retains a release varying by only 1.6 $\times 10^{-10}$ mol NO cm^{-2} min^{-1} over the 1.5 h fibrinogen adsorption period. This allows a comparison between two fluxes, one slightly above

the rate found to be necessary for synthetic materials to retain function (14×10^{-10} mol NO cm^{-2} min^{-1})[22] and one just below. By maintaining this flux rate over the course of fibrinogen adsorption during the ELISA, we are able to relate the rate of NO release on the adsorption of fibrinogen.

In the Bartlett group's study on the improved hemocompatibility of NO-releasing materials in a rabbit model, they observed an increased adsorption of fibrinogen to their materials, attributing this to potential changes in the surface of the material.[22] These were not tested, leaving a question as to whether it was the NO release or indeed a change in the surface properties of the NO-releasing material causing the increase in adsorption. The current study focuses on the possibility of NO increasing fibrinogen adsorption by normalizing the surface properties of the NO-releasing material to those of the control material. Previous studies, including the rabbit[22] and vascular graft[14] models, looking at the effect of NO release from PVC materials have not taken into account the effect of the material itself on the biological factor of interest.[13,15,37,44] We have established that the surface properties of wettability, roughness, and composition remain comparable for both control and composite materials and that there is no leaching of the diamine from the material, leaving NO release as the only changing variable. The NO release from the materials is at a level previously established to be effective at preventing aggregation and clot formation.[22,37]

We see in Figure III.6, that there is a significant increase in the amount of fibrinogen adsorbed to the NO-releasing material in comparison to the PVC control surface (103 ± 31, 226 ± 99, and 2334 ± 496 % of control for control, 1 wt%, and 9.2 wt% composite, respectively). This verifies that the increase seen in the work by the Bartlett group is likely influenced to the release of NO from the surface and not the surface properties as previously thought.[22] Fibrinogen is an active protein in the coagulation cascade, the increase of fibrinogen adsorption to a surface is currently viewed as leading to an increase in clot formation.[47,48] We find that there is an increase

in the amount of fibrinogen adsorbed to the NO releasing surface when compared to that of the control. This is in contrast to a study done by Charville et al. where they see that the adsorption of fibrinogen to control and NO releasing xerogels remains constant.[18] The primary reason for this difference is due to the differences in our systems. Charville and coworkers adsorb a solution of 20 μg/mL fibrinogen, while we adsorb a solution of 3000 μg/mL fibrinogen (a concentration at the level naturally found in blood). In addition, they tag 10% of the fibrinogen pre-adsorption and measure the concentration after eluting the proteins from the surface; we tag our fibrinogen after adsorption and read the fluorescence while the protein is still adsorbed to the surfaces. The increased amount of fibrinogen allowed to adsorb to our surfaces is the likeliest reason behind the differences in fibrinogen adsorbed to the surfaces. With more fibrinogen available to adsorb, the NO release has an effect on the and amount adsorbed, whereas the lower concentration used by Charville et al. in their study does not find a difference because at that low of a concentration of fibrinogen, there is not significant enough extra fibrinogen to adsorb to the surface and the amounts adsorbed to each surface end up being similar. The experiments presented in this paper now provide evidence that NO has an effect on increasing fibrinogen adsorption where this increase does not end in clot formation.

It is well established that NO-releasing materials are anti-thrombotic.[13-15,22,37,49] In addition, studies in mice have shown that the presence of fibrinogen is necessary to initiate the formation of a blood clot.[48] This leads one to presume that there would be a decrease in fibrinogen adsorption if the surface prevents thrombus formation. The significant increase in fibrinogen adsorption to the NO-releasing composite material (Figure III.6) indicates that there might be a change in the fibrinogen upon adsorption to the material surface. Since proteins adsorb to the surface immediately upon insertion,[20,50] the adsorbed fibrinogen, and not the material's surface, will affect

all subsequent biological moieties that pass the implanted device. Since it is well established that these surfaces will prevent clotting, there may be a change in the adsorbed fibrinogen that affects its interaction with other clotting factors such as platelets. Changes in the conformation and activity of fibrinogen on NO-releasing surfaces have not been determined to date. Fleser et al. found patches of fibrin on their NO-releasing grafts, however, there was no thrombus formation on these spots.[14] Based upon these findings, and the findings of the current work, it would seem that NO release allows fibrinogen to create an anti-thrombotic layer on the material surface, possibly altering its conformation such that it does not participate in coagulation. This presents a different way of looking at why NO incorporation into synthetic materials results in improved biocompatibility. Until now it has only been established that the beneficial effects of NO are due to its influence on platelets. The effect of NO seen on fibrinogen in this study offers, for the first time, a different potential pathway to improved biocompatibility of implantable materials.

III.5. Conclusions

We have shown that NO release from a polymeric material has a distinct effect on the adsorption of fibrinogen to the surface. The surface properties shown to have an effect on fibrinogen adsorption were normalized between the control PVC and composite NO-releasing films allowing for the isolation of NO release. NO increases the amount of fibrinogen adsorbed to a PVC-based synthetic material particularly at levels of NO flux that are seen to promote material viability. The increased fibrinogen adsorption indicates a potential change in the adsorbed fibrinogen. Fibrinogen is a major player in mature thrombus formation; proteins are the first to adsorb to synthetic materials. Interactions of other biological factors in blood are then with the adsorbed protein. These materials have previously been shown to prevent the formation of mature

blood clots, leading to a question about how the fibrinogen adsorbs to the material. Potential changes in the increased amount of fibrinogen adsorbed to the material surface may be the key to why NO release promotes the acceptance of synthetic devices into the body.

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CHAPTER IV:

DETERMINATION OF THE ADSORPTION ISOTHERM FOR FIBRINOGEN ON A MODEL SYSTEM

IV.1. Introduction

Fibrinogen adsorption to material surfaces is a common test of biocompatibility; however, the changes in the protein upon adsorption is not always well characterized. Currently, the main information on fibrinogen adsorption to material surfaces is that it preferentially adsorbs to hydrophobic materials compared to more hydrophilic materials.[1,2] Recent studies by the Schoenfisch group have looked into the effect of certain surface functional groups on fibrinogen adsorption and the proliferation of fibrin after thrombus activation. They found that the adsorption of fibrinogen was not significantly different for hydrophobic, negatively charged, and positively charged surfaces, however, the amount adsorbed to the hydrophilic surface was about half that adsorbed to the other surfaces.[3] This is consistent with previous findings on the preference of fibrinogen for a hydrophobic material. If the overall structure of fibrinogen is examined, we see that the greater portion of the protein is overall hydrophobic. By adsorbing through hydrophobic interactions, the bond will be stronger than that with the hydrophilic surfaces, as a greater area of the protein is able to interact with the surface in the hydrophobic case.

While fibrinogen adsorption to these model hydrophobic/hydrophilic surfaces has been well established,[4-8] it is additionally important to look at the adsorption of fibrinogen to other biomaterial surfaces. One major biomaterial class is those that release the biological agent nitric oxide (NO) from their surface to mimic conditions of the natural endothelium. Much work has

been done in making these materials and showing that they will prevent the formation of thrombus when used *in vivo*.[9-11] However, these materials had not been looked at for their effect on protein adsorption. Previous studies that combined fibrinogen and NO releasing materials adsorbed low concentrations of fibrinogen to assess bacteria viability with combined NO release, the effect of NO release on the fibrinogen was not considered.[12] The first study to suggest that fibrinogen may be influenced by NO release is that by the Bartlett group, which shows that there is an increase in fibrinogen binding to their NO releasing materials versus their control materials which did not contain NO.[13] To further the work done by their group, a recently published study by our group looked at the effect of NO on fibrinogen binding using a model system developed to maintain surface properties across control and composite materials; additionally varying the NO flux rate from the materials. We confirmed the results found by the Bartlett group showing that NO release does cause increased fibrinogen adsorption.[14] Additionally, this response is NO dose dependent and not a product of changes in surface properties of the materials. To further this work, an understanding of how the fibrinogen is adsorbed to the material is needed.

The adsorption of fibrinogen to the material surface is seen to be irreversible. To fully examine this phenomenon, a determination of the adsorption isotherm for the material must be performed. Generally, these isotherms are determined by measuring the adsorbent that is remaining in solution after a specific amount of time has passed.[15,16] This is used to gain information on the amount of adsorbent that has adsorbed to the adsorbate and from this an isotherm can be determined through known equations. The most commonly used isotherm is the Langmuir isotherm, proteins are generally assumed to follow this adsorption profile making it an ideal starting point for investigating fibrinogen adsorption to our model system.[16,17] This is the simplest determination and tells the relationship of the adsorbant and the adsorbate. To apply the

Langmuir isotherm, one must assume three things: (1) the adsorbate is strongly attracted to the surface, (2) the surface only has a specific amount of sites that the adsorbate can interact with, and (3) adsorption is only in a single layer. The isotherm is calculated using Equation IV.1:

Equation IV.1 $$[\Gamma] = [\Gamma_{max}] \frac{K_{ads} * [A]}{1 + K_{ads} * [A]}$$

Where Γ represents the amount adsorbed, Γ_{max} is the maximum amount adsorbed at a specific concentration, the Langmuir equilibrium constant, K_{ads}, and the amount of adsorbate in solution, [A]. By graphing the amount of protein in solution, [A], versus the amount adsorbed, [Γ], the Langmuir adsorption curve can be found. The adsorbate follows Langmuir adsorption if the curve increases, then plateaus. However, if the curve is a straight line the adsorbate displays hydrophobic partitioning for this surface. Langmuir adsorption indicates specific adsorption of the adsorbate to the adsorbant. Hydrophobic partitioning indicates that the adsorbate is not specifically adsorbed to the adsorbant; in this case the protein is in flux, both adsorbing and desorbing from the material surface. Knowing whether or not the protein adsorbs specifically gives insight into the potential for that protein to be displaced by others when in a mixture.

The issue with most adsorption studies on fibrinogen is that the fibrinogen is radiolabeled for detection after adsorption.[18,19] Although this is a standard method for performing this measurement it is not a viable method of detection for the present studies due to the use of radioactive species and lack of proper equipment with which to perform the work. There has been movement towards the use of fluorescent labeling for the detection of fibrinogen on material surfaces due to the complications associated with radiolabeling. In fact, the Schoenfish group has recently used a fluorescently tagged fibrinogen to determine the amount of fibrinogen adsorbed to a xerogel surface.[12] With this method, however, they measure the proteins after

eluting them from the material surface. While this can be a valuable method of detection, elution of the proteins from the material surface can denature the proteins. This presents a challenge to researchers, because denaturing causes structural changes in the protein allowing for different functional groups and amino acid residues to be available for interacting with the detection method. For example, if the protein restructures and the area in which an antibody binds is no longer available for interaction, then the use of that antibody will no longer give accurate results on the concentration of denatured protein. Additionally, one has to ensure that all of the protein elutes from the surface and depending on the amount of detergent used, not all of the protein may be removed for detection.

In this work, we utilize the model system from our previous work (Chapter III) to look at the effect of NO release on fibrinogen adsorption as a function of protein concentration. To measure protein adsorption, four different assays were attempted: bicinchoninic acid, and three different biotinylation assays. By varying the concentration of the fibrinogen, we can assess the adsorption of the protein on the material surface.

IV.2. Materials & Sample Preparation Methods

IV.2.a. Materials

Human plasma fibrinogen and biotin-*N*-hyrdoxysulfosuccinimide (biotin-NHS), water soluble were obtained from Calbiochem (La Jolla, CA. Dulbecco's phosphate buffered saline (DPBS) was obtained from Hyclone® (Logan, Utah). BCA protein assay kit and avidin-HABA were purchased from Pierce (Rockford, IL). Avidin-FITC, avidin-HRP, and amplex red were obtained from Invitrogen (Carlsbad, CA). Spin columns with 100 kDa MWCO were purchased from Fisher (Pittsburg, PA). For the making of the model system, poly(vinyl chloride) (PVC)

was obtained from from Aldrich (St. Louis, MO), bis(2-ethylhexyl) sebacate (DOS) from Acros Organics (New Jersey, USA), and tetrahydrofuran (THF) from Mallinckrodt (New Jersey, USA). The additive for composite films, potassium tetrakis(4-chlorophenyl) borate (KTpClPB) was obtained from Ace (Korea). For NO-release, diazeniumdiolated dibutylhexanediamine (DBHD/N_2O_2) was synthesized by exposing *N,N'*-dibutyl-1,6-hexanediamine (DBHD) (Alfa Aesar, Ward Hill, MA) (99.8%, Sigma, St. Louis, MO) NO under 80 psi NO at room temperature for 48 h in acetonitrile as the solvent.[20]

IV.2.b. Sample preparation

Samples were prepared in the same manner as previously reported.[14] In brief, a base coat of either control, low NO flux (1 wt% DBHD/N_2O_2), or high NO flux material (9.2 wt% DBHD/ N_2O_2) were applied to wells of a 96-well plate. After curing, three subsequent top coats of varying ratios of PVC: DOS were applied, curing between coats. After the final coat was applied, the films were left to cure overnight.

IV.3. Protein Assays

IV.3.a. Bicinchoninic acid assay

The bicinchoninic acid (BCA) assay is commonly used to determine whole protein concentration in solutions. It works through a Cu^{2+} reduction to Cu^{+} by interacting with thiol groups on the proteins. This reduced Cu^{+} then forms a complex with bicinchoninic acid (Figure IV.1) which is purple in color; the degree of color change can be monitored via absorbance measurements at a wavelength of 562 nm. Because this reaction happens with any protein with thiol containing amino acid groups, it is not selective for any specific protein. It is best used

when either a total amount of general protein is needed, or when only one protein is present in the solution. Since we are only testing one protein in solution, it is an ideal place to begin in making quantitative measurements of the fibrinogen adsorbed to my model materials. The previous assay used for determining fibrinogen adsorption, a fluorescent-based ELISA, was merely qualitative (Chapter III). While this is beneficial in determining relative amounts of adsorbed protein (measured in % of well plate control) across control and composite materials, actually determining adsorption isotherms for the system needs quantitative measurements. Using the BCA assay on this single protein system seems the ideal solution for quantification.

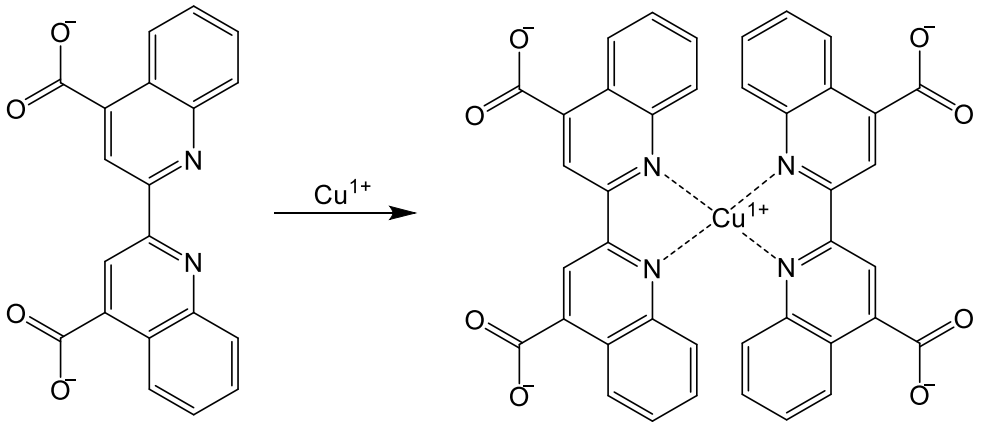


FIGURE IV.1: Reaction of bicinchoninic acid with cuprous ion. Left side shows bicinchoninc acid, the right side is the complex formed when reacting with the reduced copper.

There are two ways to perform this assay using my model system: (1) performing directly on the adsorbed protein, (2) using the protein solution left after adsorption. The issues with option (1) is that the assay is meant to be run on protein in solution, not adsorbed protein. The copper must be able to interact with the thiol groups on the protein, adsorbed protein could provide a challenge for this interaction. Despite this complication, I decided to see whether this assay could be adapted to work for adsorbed protein samples since copper is relatively small compared to the protein and should be able to interact with the entire molecule. To run the assay,

films were prepared in well plates as shown in Chapter III.2. Briefly, a four layer film was applied to the wells of a black, polypropylene 96-well plate. The first layer being the active layer containing either no nitric oxide (NO) donor for the control materials, or containing 1.0 or 9.2 wt% diazeniumdiolated dibutylhexanediamine (DBHD/NO) to make the low ($13.0 \pm 1.6 \times 10^{-10}$ mol NO cm^{-2} min^{-1}) and high ($17.4 \pm 0.5 \times 10^{-10}$ mol NO cm^{-2} min^{-1}) NO flux materials (Figure IV.2). These films were allowed to cure overnight to ensure that all of the solvent has left the film.

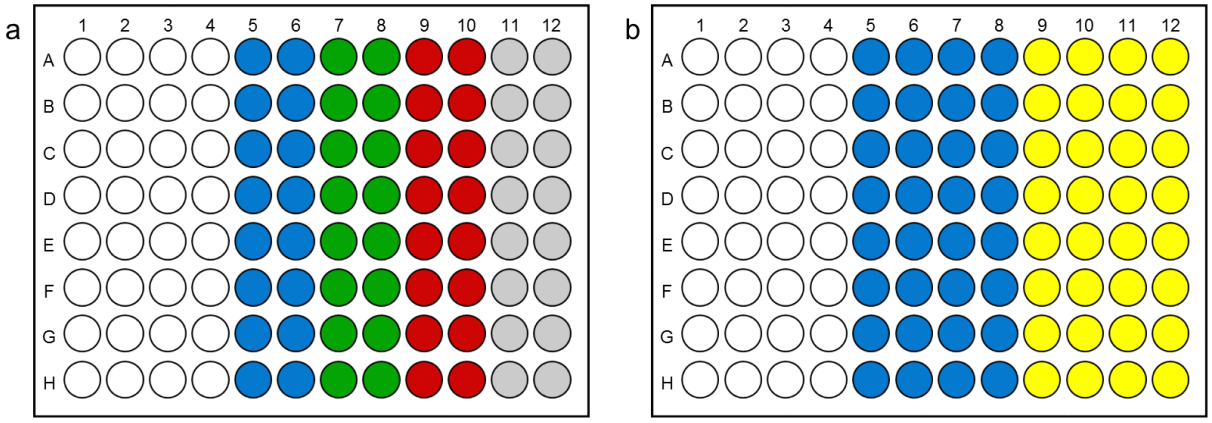


FIGURE IV.2: Well plate layout for BCA protein assay. Initial tests with 3 mg/mL fibrinogen run on all samples each time: control (blue), low NO flux (green), high NO flux (red), blank (grey). Tests on a range of concentrations from 3-0 mg/mL fibrinogen run on two films each time: control (blue), low or high NO flux (yellow) (n=3 for each concentration). Standards are plated in blank wells shown in white.

Initial testing to determine the viability of this method was performed using only the biologically relevant concentration of fibrinogen (3 mg/mL). Further testing to try and determine an adsorption isotherm was performed using a range of fibrinogen concentrations from 0 – 3 mg/mL. For each of these cases a stock 3 mg/mL solution was made by combining 0.75 mL of 44.4 mg/mL reconstituted fibrinogen into 11.1 mL Dulbecco's phosphate buffered saline (DPBS). Serial dilutions were made to get a range of concentrations between 0 and 3 mg/mL for isotherm calculations. As in Chapter III.2, samples were soaked for one hour prior to incubating

with fibrinogen to initiate NO release. Afterwards, fibrinogen was plated as shown in Figure IV.2 and allowed to incubate for 1.5 h. Post-incubation, remaining fibrinogen solution was removed and the plates were rinsed 8× with DPBS. Then the BCA assay was run on the samples. Standards of 0 – 3 mg/mL fibrinogen were plated 25 µL per well. To ensure the same volume for each sample, 25 µL 0.1% sodium dodecyl sulfate (SDS) was added to each well containing a sample coated with fibrinogen. The SDS was used to help the interaction of the adsorbed fibrinogen with the working reagent for the BCA assay. The working reagent comprises a mixture of cupric sulfate and bicinchoninic acid in a basic solution. SDS is commonly used to elute proteins from surfaces for further evaluation. Inclusion of this in the assay, will allow the adsorbed protein to partially elute from the material surface and interact with the working reagent despite whether or not protein is multi- or single layered on the surface. Elution of the proteins from the material surface does present possible complications due to the ability of detergents to denature proteins upon elution. SDS is compatible with this assay at up to 5%, so the 0.1% used here will not interfere with the assay.

After the inclusion of the samples or the SDS solution, 200 µL working reagent was added to each well, shaken in the plate reader for 30 s, and allowed to incubate for 30 min at 37 °C. After incubation, the plate is allowed to sit for 15 min to come to room temperature. At this time, 200 µL of solution from each well is transferred to a clean, dry, clear well plate for reading at 562 nm.

Initial results were promising for this method, however, the working range of the assay is 20 – 2000 µg/mL. After repeated iterations of this assay for the adsorbed fibrinogen, overall the results were not reproducible for this assay. The lower concentrations of protein allowed to adsorb to the surfaces did not adsorb enough protein to be within the lower range of the assay

itself. While the higher concentrations allowed to adsorb were within the range of the assay, the results were not reproducible across multiple trials nor within the same experiment. In the case of the control non-NO releasing film the response values were negative, indicating an issue with the assay itself, too little protein adsorption, or the protein conformation (discussed below). While the samples themselves do not contain any interfering species, perhaps even the inclusion of the SDS to elute protein from the surface did not allow for enough available protein for interaction with the working reagent. This does not allow for a full picture of the amount of fibrinogen adsorbed to the material surface to be obtained when the assay is performed. These results indicate that perhaps the BCA assay cannot be modified to accurately measure adsorbed fibrinogen, but only protein which is in solution. The changes in protein conformation upon adsorption may be due to the denaturing of the proteins upon adsorption or upon interaction with the SDS. Changes in protein conformation will affect the functional groups that are available for the copper to interact with, more or less being available after adsorption can give false results for this assay since the standards are assumed to be in native conformation while in solution. Changes in conformation due to adsorption are discussed in Chapter V.

Since the assay does not seem to be repeatable with adsorbed protein, option (2) was attempted to overcome this challenge. In this, I wanted to look at the fibrinogen remaining in solution after it has been allowed to adsorb to the material surface. To do this, the sample sizes were initially increased, using a 20 mL polytetrafluoroethylene (PTFE) scintillation vial for plating each film to mimic adsorption studies in the literature.[16,19] Following previous adsorption isotherm determinations, the vials were tested under static conditions and shaking conditions. Additionally, the fibrinogen was allowed to adsorb for either 1.5 h, or 24 h to ensure that the adsorption had been fully reached. These films were made in the same manner as those for the

well plate, however, since the size of the films increased the added volumes of protein added to each vial also increased by a factor determined by the change in diameter. Prior to adsorbing fibrinogen, DPBS was added to each vial and allowed to incubate for 1 h to initiate NO release. Fibrinogen solution in a rage from 0 – 3 mg/mL was added to each vial, including a vial with no polymer coating that had 3 mg/mL added to serve as an additional control. This was incubated for 1.5 h or 24 h at 37 °C depending on the test. Additionally, the vials were either gently shaken during this time period or left static. After the incubation period, vials were gently agitated, then a 25 µL aliquot of solution was removed. This aliquot was plated as in Figure IV.2 and the BCA assay was run. In the case of this assay, samples and standards were plated directly into a clean, clear 96-well plate. Working reagent was added, the plate shaken for 30 s, then the plate was allowed to incubate for 30 min at 37 °C. Post-incubation the plate was allowed to come to room temperature for 15 min, then read on the plate reader at 562 nm.

For these samples, initial test runs were done using the control polymer system for 1.5 and 24 h incubation times, as well as shaking versus static incubations. Figure IV.3 shows the results of this assay, changes in the reaction conditions (incubation time and static or shaken) had an effect on the individual results. Both the 24 h and 1.5 h samples tested under shaken conditions display similar trends in concentration of adsorbed fibrinogen. The values are the same within error for most data points. When a comparison of the samples was made for those incubated for 1.5 h under either shaken or static conditions, the trend for static conditions has a steeper slope, which is more noticeable as the stock concentration increases.

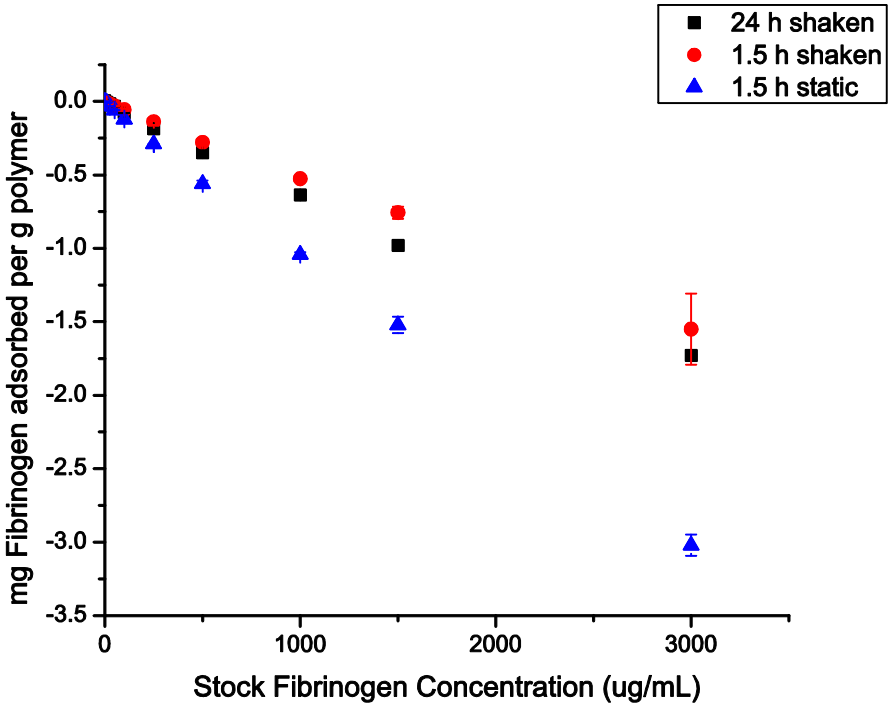


FIGURE IV.3: Comparison of BCA assay results for control film under different reaction conditions: 24 h shaken (blue), 1.5 h shaken (red), 1.5 h static (green). Incubation time has less effect on the values than shaken or static conditions does. Values represented in mean ± standard deviation (n=3 for all values).

The most noticeable aspect of the results in Figure IV.3 is that they are all negative. This is because for all reactions, the concentration of fibrinogen in solution after adsorption was larger than that before. Initially this looks like an error in measurement, however, when you being to look into the mechanism of this reaction, this response becomes clearer. The BCA assay works on the interaction of Cu^{2+} with thiol groups on the protein. When a protein is in its native form a certain number of thiol groups are available for interaction with the copper. If the protein becomes denatured or alters its conformation upon adsorption, then a different amount of thiol groups will be available for reaction with the copper. Based on the results in Figure IV.3, the length of time that a sample was adsorbed (1.5 or 24 h) does not seem to have an effect on the

structure of the proteins; however, whether the protein was shaken or under static conditions does have an effect on the denaturing or rearranging of the proteins conformation. In the second case, the protein was removed from the remaining solution after adsorption. It would be assumed that this protein was not denatured, however, there is the possibility that the protein interacts with the surface or proteins adsorbed to the surface. This interaction could potentially denature the protein leading to the increase in apparent solution concentration in the second situation after adsorption. Overall the BCA assay allows us to infer that the fibrinogen is undergoing a conformational change due to its interactions with the surface released NO. Further studies into the conformation of the adsorbed fibrinogen is investigated in Chapter V.

IV.3.b. Biotinylation

The BCA assay is good for testing total protein concentration of a system in solution, however, if more than one protein is present, another method is needed to distinguish between different protein types. The interaction of biotin and avidin has long been a detection method used to look at proteins in solution, or by performing a sandwich assay to look at protein on a material surface. The interaction between biotin and avidin is highly selective. Once bound, it can undergo even harsh reaction conditions and remain together. By attaching biotin to one protein in a system, the concentration of that protein can be determined despite being in a mixture. The biotin-avidin reaction was used to look at fibrinogen adsorption alone on a material surface as an initial step toward being able to investigate protein concentration within a mixed protein system. Three different detection methods were tested to determine the viability of the biotin-avidin reaction for use in fibrinogen detection on the model system surfaces.

For all biotin-avidin reactions two solutions were made: 3 mg/mL fibrinogen in DPBS, and 1 mg/mL biotin-NHS in water. Fibrinogen was biotinylated by adding biotin-NHS to the fibrinogen solution to make a solution with a 1:1 biotin: fibrinogen solution. This solution was incubated for 1 h at room temperature. Post incubation, the solution was added to spin columns in 4 mL aliquots. The tubes were spun at 3200 rpm for 1 h 15 min to remove unreacted biotin. The columns were rinsed 3×, centrifuging at 3200 rpm for 15, 20, and 15 min. Remaining solution was removed from the columns and DPBS was added to return the solution to the initial volume. During this, the well plate was incubated for 1 h at 37 °C with 100 uL DPBS per well. After incubation, the biotinylated fibrinogen was added to the wells (100 uL per well) and incubated for 1.5 h at 37 °C. Wells were washed for 8× with DBPS and a background fluorescence was read.

IV.3.b.i. Avidin bound FITC

For the first test, avidin bound to fluorescein isothiocyanate (avidn-FITC) was diluted to 0.586 mg/mL in DPBS to make a solution of 1:1 fibrinogen: avidin-FITC, assuming a maximum 3 mg/mL fibrinogen adsorbed to the material surface. Avidin-FITC solution was added in 25 µL aliquots to each well with adsorbed protein. The avidin was allowed to bind to the biotin for 45 min at room temperature. Avidin-FITC standards were made in DPBS over a 0-0.586 mg/mL concentration range. Post incubation, the well plate was washed 3× with DPBS. Standards were added to columns 1 – 3 in 25 µL solution per well. The plate was then read for final fluorescence measurements. All fluorescence measurements were taken at 485/20 nm (excitation) and 528/20 nm (emission) with a Synergy 2 microtiter plate reader (Biotek, Winooski, VA). To determine

the amount of fibrinogen adsorbed to the material surface final masses of adsorbed fibrinogen were normalized to the surface area of the film, the results of which can be seen in Figure IV.4.

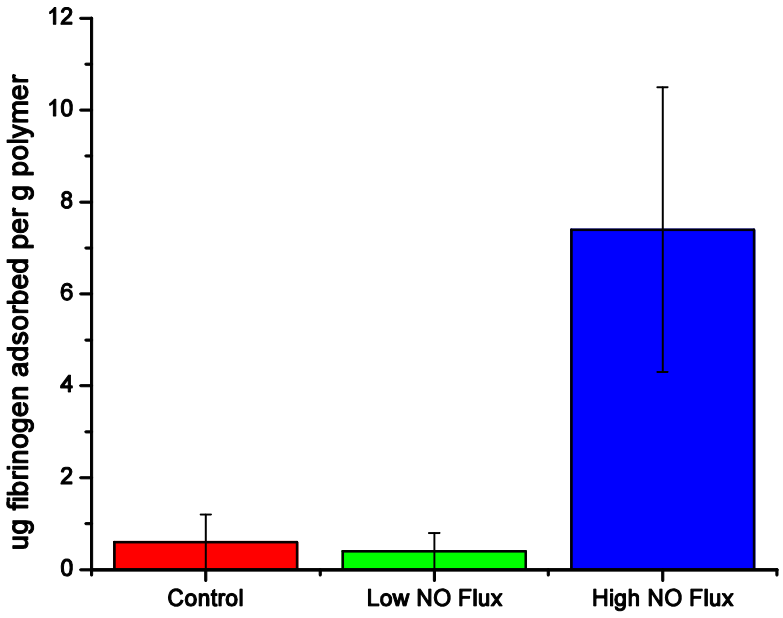


FIGURE IV.4: Fibrinogen adsorption as a function of polymer mass determined using avidin-FITC. Control (blue) and low NO flux (green) exhibit large error due to many responses being zero. Values represented as mean ± standard deviation (n=32).

The avidin bound FITC biotin assay produced repeatable results for the high NO flux materials. The limit of detection for this assay, however, was not sensitive enough for the control and low NO flux materials. As seen in Figure IV.4, the control and low NO flux samples did not produce a measurable fluorescence response with this method. As such, this assay can be used for higher concentrations of adsorbed fibrinogen, but is not sensitive enough for the lower concentrations. Despite this minor drawback, the method does confirm the results seen in Chapter III.3, that the adsorption of fibrinogen on the high NO flux materials was significantly

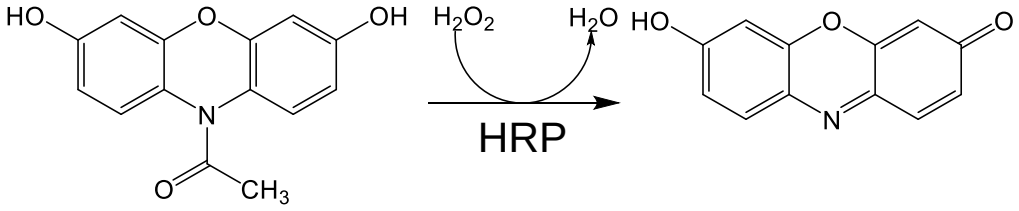
higher than that of the control and low NO flux materials. To increase the sensitivity of the biotin-avidin protein detection method, a second technique was attempted.

IV.3.b.ii. Horseradish peroxidase – amplex red reaction

The second biotin-avidin test attempted uses avidin bound to horseradish peroxidase (avidin-HRP), a method used to increase the sensitivity of most avidin-biotin reactions. In the presence of the covalently attached HRP, amplex red will interact with hydrogen peroxide (H_2O_2) in a 1:1 reaction to produce a fluorescent product, resorufin (Figure IV.5). All reactions in this method can be assumed to be 1:1 (i.e., biotinylated fibrinogen: avidin-HRP, amplex red: H_2O_2). A solution of avidin-HRP was made so that the concentration was 1:1 with 3 mg/mL fibrinogen in case all of the available fibrinogen adsorbed to the material surface. The avidin-HRP was added in 25 µL aliquots to the adsorbed fibrinogen and allowed to adsorb for 45 minutes. After rinsing 3× with DPBS, standards were added in 25 µL aliquots and 25 µL DPBS was added to the wells containing adsorbed protein. A solution containing amplex red and H_2O_2 was added to each well. The plates were allowed to incubate 30 min at room temperature, after which the fluorescence was measured at 530/25 nm (excitation) and 590/35 nm (emission).

This method provided a challenge in optimization for this model system. The standard assay protocol has a limit of detection of 1×10^{-5} U/mL HRP, or approximately $3.03 – 4.00 \times10^{-8}$ g/L HRP.[21] The higher end of the range was at 100 mU/mL or $3.03 – 4.00 \times10^{-4}$ g/L HRP. The major drawback of this assay is that, if maximum fibrinogen adsorption occurs to the material surfaces, the amount of HRP present will be 0.388 g/L HRP, assuming 1:1 interactions throughout the assay. This is a greater concentration than the high end of the assay range. The issue with going above the high end of the limit of detection is that excess HRP or H_2O_2 will

oxidize resorufin, the reaction product (Figure IV.5), to a non-fluorescent compound, resazurin. Despite this drawback, perhaps the assay could be used to look at the control and low NO flux materials, these materials have a much lower concentration of bound fibrinogen making them potentially within the working range of this assay. This would allow the avidin-HRP assay to be used in conjunction with the avidin-FITC assay to assess the full range of protein adsorption. Because the working range of this assay was determined with the standard protocol, I modified it in an attempt to increase the working range of the assay. To do this I decreased the amount of amplex red in the reaction to that which will interact in a 1:1 molar ratio with the bound HRP. To interact the amplex red in a 1:1 molar ratio with the potential amount of HRP present in the final wells, the ratio of H_2O_2 added to the wells based on the original protocol was well above the maximum level able to be detected in the assay (≥100 μM).



Amplex Red Resorufin

FIGURE IV.5: In the presence of HRP, Amplex red reacts with hydrogen peroxide to form the fluorescent molecule resorufin.

To help eliminate potential reductions in fluorescence by the inclusion of excess H_2O_2, different reaction conditions were employed to improve the sensitivity of the reaction. Ultimately, despite the modifications made to the assay, the response from the higher

concentrations of HRP used in this assay for the amount of fibrinogen bound does not make this a viable method for quantitative determination of adsorbed fibrinogen. If this method was to be explored further for this model system, a calibration curve made from a range of concentrations from the low end of the current calibration curve and lower (1.29×10^{-3} – 0 g/L HRP) might be beneficial to compare to results gained by running the assay on the control and low NO flux materials. The fluorescent response from these materials had only 12% and 11% error, respectively, associated with these measurements. It is possible that the amount of fibrinogen bound to these surfaces is lower than 10 µg/mL fibrinogen, which is a 1:1 molar ratio with the lowest end of the current calibration curve. With this refinement, the amount of fibrinogen adsorbed to all of the surfaces might be determined when used in conjunction with the avidin bound FITC assay. Despite the possibility of using two assays to determine quantitative fibrinogen adsorption, it is not an ideal situation due to the differences in incubation time and preparation, and so another biotinylation assay option was explored.

III.3.b.iii. Fluorescent avidin-HABA

To find an assay that will cover the entire range of adsorbed fibrinogen, a third method of biotin-avidin detection was attempted. The use of avidin bound with 4'-hydroxyazobenzene-2-carboxylic acid (HABA) has been commonly used as a method of detecting the degree of biotinylation of proteins. Usually, this is a colorimetric assay detected through absorbance measurements, however, to modify the assay for use with the adsorbed protein, a fluorescence based detection was used with a specialized avidin-HABA. When the HABA is displaced by biotin on the avidin, the avidin fluoresces. This way the reaction can be detected within the same well plate. With a traditional colorimetric version of this assay, the colored displaced HABA

would have to be removed from the well and placed into a clean, clear well plate for absorbance detection. For this reaction, a solution of avidin-HABA was made to react in a 1:1 manner with 3 mg/mL fibrinogen, assuming maximal adsorption. After the fibrinogen was allowed to adsorb to the material surface, unbound fibrinogen was rinsed off and 10 µL of buffer was added to each well. The stock solutions and standards were also plated with 10 µL aliquots and the avidin-HABA solution was added to each well with a standard or adsorbed fibrinogen. This is a rather fast reaction and after 5 min the fluorescence was detected using 485/20 nm (excitation) and 528/20 nm (emission).

This biotinylation assay provides a more direct approach than the previous assays and includes fewer steps. The displacement of HABA from avidin is the most common method of determining whether or not your materials have been biotinylated. Because of this, it provided a reasonable solution to having one assay to look at the adsorbed fibrinogen. Once the assay was run, the results for protein adsorption had a different trend than that seen with the fluorescent ELISA in Chapter III.3, the BCA assay, and the avidin-FITC assay. In this case, all of the surfaces had almost identical amounts of adsorbed fibrinogen, with the high NO flux material at the lowest relative concentration. This same trend was seen after multiple runs of the assay. In addition to the samples, a control aliquot of biotinylated fibrinogen was also tested. This resulted in a fibrinogen concentration lower than that of the adsorbed samples. What this currently indicates is that the fibrinogen might not be fully biotinylated, or that the concentration of fibrinogen in the biotinylated stock is below that able to be determined with this assay. The reason that there is a response within the low end of the calibration curve for the adsorbed fibrinogen is likely due to the fact that 100 µL of the fibrinogen solution are allowed to adsorb to the material surface prior to introduction of the avidin-HABA, whereas for the stock, only 10 µL

of the solution is allowed to interact with the avidin-HABA complex since this is done only during the final steps of the assay. Since all of the adsorbed fibrinogen samples give a fluorescence within the lowest standard and the blank, this is not the optimal assay for my system. If trying to detect either a highly biotinylated protein rather than only a 1:1 biotin: fibrinogen biotinylation, or trying to detect higher concentrations of the biotinylated fibrinogen, this assay could be beneficial. In this case, the limits of detection for the fluorescent HABA testing, was not sufficient for the detection of adsorbed fibrinogen.

The biotinylation of the fibrinogen should not have an effect on the adsorption of the fibrinogen, due to biotin being a much smaller molecule than the protein. The detection of the biotin, however, is achieved by using avidin, a much larger and bulkier molecule than the biotin. If the biotin is hidden within the adsorbed fibrinogen, then the avidin cannot reach it and bind. This will have an effect on the response of the assay, likely decreasing the response in most cases. Because we do not know how the protein is adsorbing to the material, the biotin could be in a position where the avidin cannot reach it.

Overall for the biotinylation assays, the avidin-FITC assay gives the most promising results for the high NO flux materials surface. If the avidin-HRP assay can be developed for lower protein concentrations, then it may be useful for determining the concentration of fibrinogen bound to the control and low NO flux materials. The challenge for all of these methods will be determining protein adhesion with varying amounts of fibrinogen, rather than the 3 mg/mL concentration. It will require the use of sensitive assays, like the avidin-HRP assay for the lower concentration range. However, as seen with attempting the avidin-HRP assay on these materials, the higher concentrations will need an assay like the avidin-FITC assay. This will take much trial and error to determine which assay is necessary for which concentrations of

fibrinogen allowed to adsorb to the material surfaces and a combination of multiple assays may have to be employed.

IV.4. Discussion

Determining quantitative amounts of bound fibrinogen on the surfaces of this model system will give a picture of what might be happening to the protein upon adsorption. Generally, the adsorption of proteins to surfaces is seen to be through mainly electrostatic interactions with the surface. The hydrophobic and hydrophilic parts of the protein preferentially binding to like surfaces and the negatively and positively charged areas of the protein interacting with materials though interactions with charged surfaces. In fibrinogen, half of the protein composes more negatively charged and hydrophobic amino acid side chains and the other half is more positively charged and hydrophilic amino acid side chains (Figure IV.6). Because the presence of hydrophobic amino acids is larger on one half of the protein, on a surface like PVC and our model system, the likely method of adsorption for the protein is through these hydrophobic interactions. An alternate way of thinking about this would be to look at the composition of the fibrinogen. Fibrinogen does include many amino acids, however, there are four that are more abundant than others: glutamate (Glu, 13.5%), aspartate (Asp, 12.2%), lysine (Lys, 7.8%), and arginine (Arg, 7.0%).[22] Interactions with the material surface may likely be with these amino acid groups. Both Glu and Asp are carboxy terminated, whereas Lys and Arg are amino terminated. Depending on the make-up of the material surface, these functional groups can help influence protein adsorption. In the case of the plasticized PVC films of the model system described in this work, it is unlikely that this will influence the fibrinogen adsorption. Most of the interactions with the fibrinogen and the model system surface are likely to be due to

hydrophobic interactions and any changes that might happen with the fibrinogen due to the localized NO release from the surface.

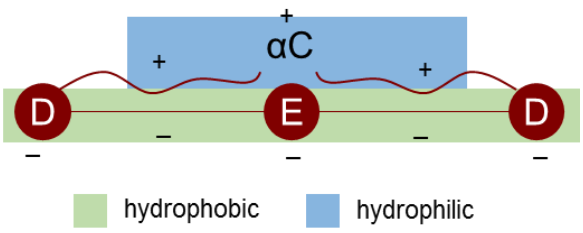


FIGURE IV.6: Simplified cartoon of fibrinogen displaying the general areas of the protein that contain overall more negatively charged and hydrophobic (green), as well as positively charged and hydrophilic (blue) amino acid groups.

The BCA and biotin assays do not appear to be the best approach to determine the amount of fibrinogen adsorbed to the material surface. Both types of assays have challenges that make them unsuitable for practical applications for this system. For the BCA assay, any difference in denaturation of the protein will have an effect on the response of the assay. This is not a direct assay since it deals with the interaction of available thiol groups with copper in the reaction solution. Any changes in the available thiol groups due to unfolding or denaturation of the protein will change the response of the BCA assay. Biotinylation assays rely on the interaction of biotin with avidin in order to obtain a response. While the biotin is a small enough molecule to be innocuous in the assay, avidin is a bulkier molecule that might not be able to infiltrate the adsorbed fibrinogen and interact with the biotin. Changes in protein conformation and the possibility that the fibrinogen adsorbs in multiple layers may cause the responses seen in the results for these assays. Because neither the rate of protein denaturation nor biotin interaction with the avidin can be measured during the run of the assay, an alternate method must be pursued.

All of the above methods rely on multiple steps. To help minimize the errors associated with these assays, turning to a simpler method should allow for quantitative measurements of fibrinogen adsorption. One method that we are currently pursuing in our lab is to directly tag the fibrinogen with FITC. After tagging is complete, the only steps remaining in the assay are to allow the fibrinogen to adsorb to the material surface and to obtain a final absorbance. Tagging the stock solution of fibrinogen with FITC allows both standards and adsorbed fibrinogen samples to be made from the same solution. Using the same batch of tagged fibrinogen for the entire assay ensures that it will not matter how much FITC has bound to the fibrinogen as all of the fibrinogen in a single batch should have the same relative amount of tagging. This method should provide concrete results on how much fibrinogen is adsorbed to the material surface, finally giving quantitative amounts of fibrinogen adsorbed to the material surface.

IV.5. Conclusions

Overall, we find that the results of our qualitative tests in Chapter III are generally seen to hold true with quantitative measures. The assays attempted, BCA and biotin-avidin, are inappropriate for determining quantitative measurements of fibrinogen adsorbed to our model surfaces. Potential conformational changes to the protein may be causing the BCA and biotin-avidin assays to be invalid, which are explored in Chapter V. Additionally, fibrinogen adsorbing in greater than a single layer may also be causing the results seen with these assays. Despite these challenges, what we know now is that the amount of fibrinogen on the control and low NO flux materials is significantly lower than that of the high NO flux materials. This poses a challenge when using most protein assays as it appears that the protein content of these materials is usually below the limits of detection. Ongoing work explores the possibility of tagging the

fibrinogen directly with FITC prior to adsorption. This should allow for the quantitative detection of fibrinogen on these model surfaces. Once quantitative information on fibrinogen adsorption can be obtained, a clearer picture of what is happening when the fibrinogen adsorbs can be seen.

These assays have shown that the adsorption of fibrinogen to the model system is not as straightforward as we thought. The conformation of the protein is likely changing as shown by the complications with running the BCA assay on the system. Additionally, the fibrinogen may not be adsorbed in a single layer, making the biotin-avidin assays invalid methods due to the inability of the bulky avidin to interact in a repeatable fashion with bound fibrinogen. Due to this, the Langmuir model is not appropriate for understanding fibrinogen adsorption to the model system's surfaces. These studies have led to the pursuit of understanding the conformational changes in the protein to determine what is happening to the protein when fibrinogen adsorbs to the control and NO releasing material surfaces.

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CHAPTER V:

CHANGES IN FIBRINOGEN CONFORMATION – ELUCIDATION OF SECONDARY STRUCTURE DIFFERENCES AFTER ADSORPTION

V.1. Introduction

The amount of protein bound to a surface does not necessarily dictate the interactions of a material with its biological surroundings. For example, in determining interactions with platelets and other biological entities it is the conformation of the adsorbed protein that is instrumental.[1-4] The majority of research surrounding protein adsorption is concerned with the amount of material attached to the surface. In the last ten years, researchers have begun to look for conformational changes of proteins upon adsorption. This research led to the understanding that not only does the amount of protein adsorbed matter, but also the manner in which the structure and conformation changes upon adsorption. Previously we have shown that there is an increased amount of fibrinogen adsorbed to our NO releasing material surfaces compared to control non-NO releasing materials.[5] Because fibrinogen is an integral part of the coagulation cascade, it would seem that an increase in fibrinogen adsorption should make the surfaces less biocompatible and prone to clotting. This is not the case. There have been many *in vivo* studies involving these same materials that show little to no thrombus formation on these NO releasing surfaces[6-8] suggesting that the fibrinogen must have a different conformation when exposed to NO releasing surfaces than on control surfaces.

Many theoretical studies have been performed on the adsorption mechanisms of fibrinogen to material surfaces.[9-12] Results offer two theories of the conformation in which fibrinogen adsorbs to a material surface: side-on and end-on (Figure V.1a and b).[10] Depending on which way the

fibrinogen adsorbs will also influence whether or not the adsorption is irreversible. Side-on adsorption is seen to be irreversible since it adsorbs lengthwise on the material surface. This allows for more of the functional groups to interact with the surface of the material. End-on, however, only has the protein interacting with one end on the material surface. As a result, the fibrinogen is not as strongly bound due to minimal surface contact. Additional findings by the Chen group were able to show that in addition to these basic adsorption patterns, the conformation of the protein in different bent positions can also be seen.[12] Not only does fibrinogen adsorb fully in a side-on manner, but whether the fibrinogen is in a bent or linear conformation can also be determined (Figure V.1 c).

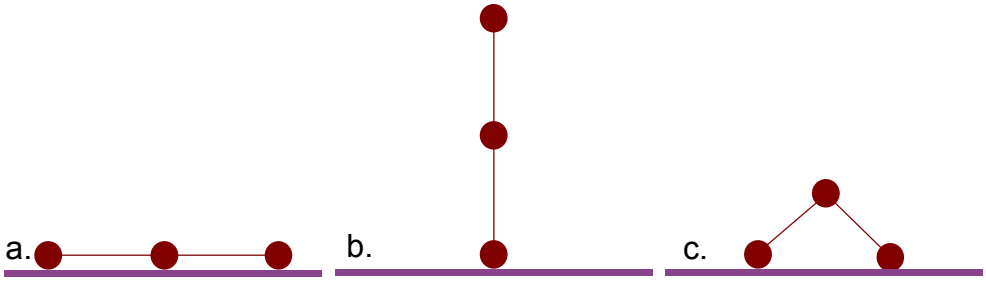

FIGURE V.1: Cartoons of possible conformations of adsorbed fibrinogen including: side-on (a), end-on (b), and bent (c) conformations.

Instrumentation methods commonly used to study the conformation of proteins include, NMR,[13] FTIR,[12,14-17] AFM,[11,16,18-20] and CD.[13,21-24] NMR is not optimized to look at adsorbed protein while still on the surface of the material. This technique is better suited to work in solution phase for these types of measurements. AFM is a powerful tool that can be used to reveal the morphology of the individual proteins on the surface. Indeed, fibrinogen is the preferred protein to use for AFM imaging of protein due to its well-defined tri-nodular shape, length, and relatively large size (340 kDa).[18] There have been many studies that have shown the adsorption of single fibrinogen molecules adsorbed to surfaces.[18,25,26] However, there is a potential complication with

this measurement technique. When a bulk amount of protein is adsorbed to the material surface, individual proteins are hard to distinguish using AFM. To counter these issues, parameters are usually modified to ensure optimal conditions for individual protein detection on material surfaces. While general conformation can be seen using AFM imaging, FTIR and CD are useful because both can give insight into the secondary structure of proteins. Changes in the structure of the protein, consequently will have an effect on the conformation. The benefit of knowing secondary structure in addition to conformation, is that conformational changes within the molecule that might not be readily apparent from AFM imaging, can be determined. For this we attempted the use of both FTIR and CD. The initial test was using CD due because experimental set-ups for these experiments had been optimized to be used under hydrated protein conditions.[4,27] This method allows for the determination of secondary structure such as, α-helices and β-sheets that can be compared across samples. Changes from native fibrinogen to that seen on the adsorbed materials can help give insight into how the protein is changing upon adsorption. The method utilized here is modified slightly from a system developed by the Latour group at Clemson University.[4] In this study, a custom made CD cuvette was constructed based on schematics previously published but modified to fit our CD system.

This specialized CD cuvette was used to look at the secondary structure of fibrinogen adsorbed to our model surfaces. The CD system is ideal for determining secondary structural changes, however, complications discussed herein caused us to use FTIR spectroscopy as an alternate method. For either method, any changes seen in the structure from native fibrinogen will give a picture of how the protein is unfolding upon adsorption to the material surface. Differences from control and NO releasing materials allow for an assessment of the effect NO has on the adsorbed fibrinogen. We found that the structure of the fibrinogen for high NO flux materials

exhibits differences after adsorption to the material surface, however, conformational changes for the control and low NO flux materials could not be determined due to low fibrinogen adsorption.

V.2. Materials & Methods

V.2.a. Materials

Human plasma fibrinogen was obtained from Calbiochem (La Jolla, CA). Dulbecco's phosphate buffered saline (DPBS) was obtained from Hyclone® (Logan, Utah). For model system preparation, poly(vinyl chloride) (PVC) was obtained from Aldrich (St. Louis, MO), bis(2-ethylhexyl) sebacate (DOS) from Acros Organics (New Jersey, USA), and tetrahydrofuran (THF) from Mallinckrodt (New Jersey, USA). For composite films, potassium tetrakis(4-chlorophenyl) borate (KTpClPB) was obtained from Ace (Korea). For NO-release, diazeniumdiolated dibutylhexanediamine (DBHD/N_2O_2) was synthesized by exposing *N,N'*-dibutyl-1,6-hexanediamine (DBHD) (Alfa Aesar, Ward Hill, MA) (99.8%, Sigma, St. Louis, MO) to NO under 80 psi at room temperature for 48 h in acetonitrile as the solvent.[28] For cuvette construction, quartz slides were obtained from Chemglass Life Sciences (Vineland, NJ) and Veroclear polymer from Stratasys (Eden Prairie, MN).

Polymer samples for control, low NO flux, and high NO flux were prepared in a similar manner as previously reported.[5] In brief, the low and high flux composite materials were prepared using a 1:1 ratio of KTpClPB to our NO donor, DBHD/N_2O_2. Then a base coat of either control, low flux, or high flux material were applied to a holder containing a quartz slide. After curing, three subsequent top coats of varying ratios of PVC: DOS were applied, curing between coats. After the final coat was applied, the films were left to cure overnight.

V.2.b. Atomic force microscopy

Samples were prepared by aliquoting polymer into glass rings affixed to glass slides in layers as described above. After allowing to cure overnight, films were soaked for 1 h in DPBS at 37 °C. Films were then incubated with either DPBS or 3 mg/mL fibrinogen in DPBS (in water or DPBS?) for 1.5 h. After soaking, films were rinsed once with DPBS and 10× with Millipore water (18.2 MΩ) before drying under nitrogen. The main difference in these films as opposed to the ones for ATR-FTIR is that they are affixed to a square glass slide with double sided tape (Scotch, Hutchinson, MN). This is to ensure that the sample does not move about on the sample stage during imaging. Images were taken at 1 μm × 1 μm scan size in tapping mode using a Digital Instruments Bioscope AFM (Bruker, Santa Barbara, CA).

V.2.c Circular dichroism

We constructed a custom CD cuvette based on the Latour group's previous studies[4] a schematic of the cuvette layout can be seen in Figure V.2. The main body of the cuvette was made using 3D printing. A model was sent to the 3D printing lab on Colorado State University campus. It was made using an Objet 30 Pro (Stratasys, Eden Prairie, MN) with their VeroClear polymer. Once the cuvette body was made specially cut quartz slides were glued to the ends of the cuvette to serve as the windows. Once the cuvette cured, it was tested for water fastness and found to hold water for over 3 h without leaking. Individual quartz slides were made to be skinnier than the size of the cuvette and fit well into the final product. Four slides of a single sample can be tested at a time to increase the signal from the adsorbed fibrinogen. Our cuvette was made to be slightly narrower than that of the Latour group to fit within our instrument parameters.

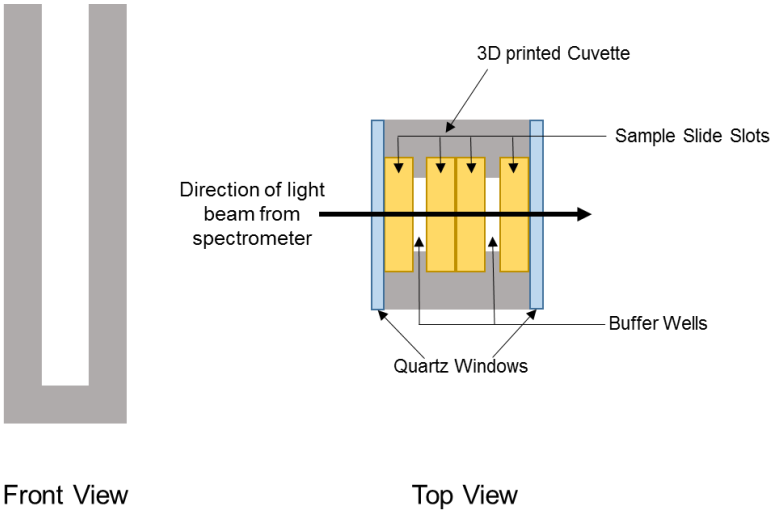

FIGURE V.2: Customized CD cuvette schematic. Adapted from a schematic made by the Latour Group from Clemson University. Slight changes made to width of cuvette to fit into standard sample holder of CD instrument, also cuvette main body made by using a 3D printer.[4]

V.2.d. ATR-FTIR

Samples were prepared in the same manner as those for AFM. Films were prepared both with adsorbed fibrinogen, and without. Films without fibrinogen were used as a control surface for each film and were soaked only in DPBS buffer. Post incubation, films were rinsed once with DPBS then 10× with Millipore filtered water (18.2 MΩ). Films were then removed from the glass rings and gently blown dry with nitrogen.

Amide I and II peaks were deconvoluted to determine the contribution of α-helices, β-sheets, β-turns, and random chains using the peak resolve function on the Omnic software. Peaks were found by using the find peaks option and applying a Gaussian/Lorentzian fit with a linear baseline. All peaks that were not centered on each of the four conformation factors and the amide II peak were deleted to eliminate peaks that should not be in the curve. Peaks were constrained to the wavenumber range associated with each factor.[14-16,29] Application of the peak fitting option with noise threshold of 10.0 absorbance units fit the peaks to the spectrum for the sample. The area

under each contributing peak was measured with Omnic software, these values were used to determine the relative contribution of each peak using ratios.

V.3. Results

V.3.a. Atomic force microscopy

Under AFM the individual conformations of adsorbed fibrinogen can be seen. Fibrinogen has a distinct tri-nodular structure that can be seen when adsorbed to a material surface. It is a 340 kDa protein with dimensions of 5 × 5 × 45 nm.[18] Previous work has shown that images at a 1 μm × 1 μm scan size are ideal to see this feature while displaying multiple fibrinogen.[30] Bending of the molecule and interactions between the molecules can be easily visualized. AFM analysis is less informative about protein layout when the protein is adsorbed in large quantities because individual molecules cannot be differentiated.

For all films, there is a distinct difference between those films without adsorbed fibrinogen, and those with (Figure V.3). When the films with fibrinogen were imaged, individual proteins were not apparent, indicating a bulk amount of fibrinogen adsorbed to the film surface. Despite not being able to distinguish between individual fibrinogen, the AFM images in Figure V.3 do show that the surfaces of all films are covered with protein. Fibrinogen coated films have an increased roughness compared to the same film without fibrinogen as shown by the scale bar in Figure V.3. The fibrinogen coated films have surface heights ranging from 0 to 10 nm, whereas the non-coated films only have heights that range from 0 to 5 nm. This increase in surface roughness indicates that there is fibrinogen on the material surface. Further, when the fibrinogen coated films were tested for roughness measurements using a Bruker AFM the high NO flux film had an average surface roughness (Sa) of 2.10 nm. The low NO flux and control films coated with

fibrinogen had average surface roughness of 0.665 and 0.651 nm respectively. The increase in surface roughness from the low NO flux and control materials to that of the high NO flux materials indicates that there may be a greater amount of fibrinogen coated onto the high NO flux surface. This result correlates well with the relative amounts of fibrinogen coverage found in Chapter III.

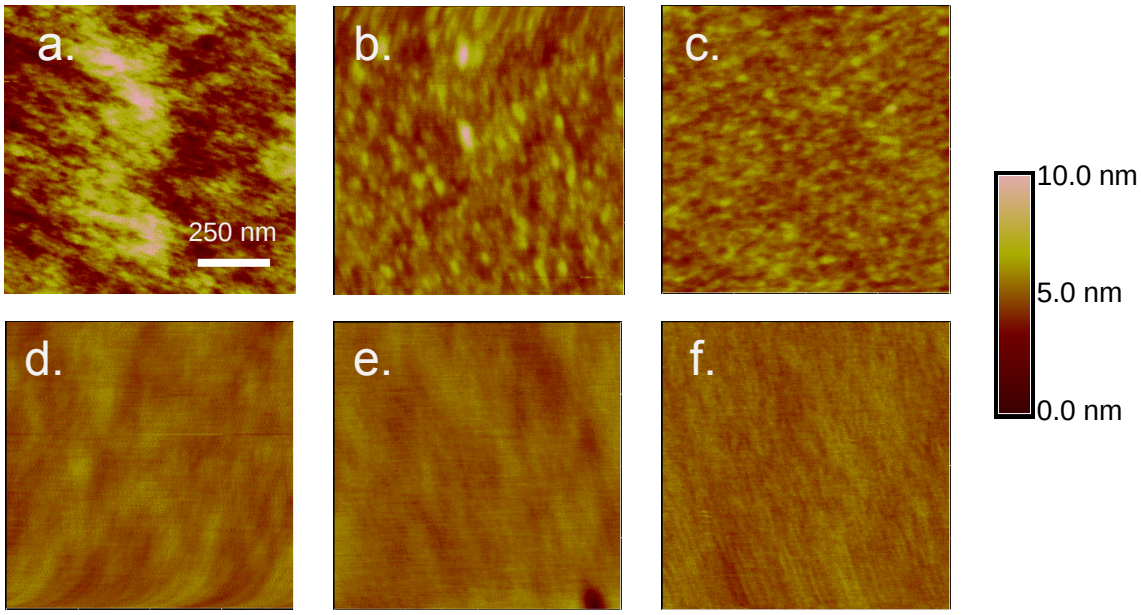

FIGURE V.3: AFM images of high NO flux (a,d), low NO flux (b,e), and control (c,f) surfaces with (top) and without (bottom) fibrinogen adsorption. Scale bar shows relative heights of the image.

Imaging of these films with AFM was difficult due to sample drift. This can be seen in the images of Figure V.3 where the image seems to have a bit of curvature to it. This drift artifact is primarily due to the sample being mounted using double sided tape, as well as the sample continuously drying during measurements. The samples were soaked prior to AFM imaging and as the polymer dries out, the film adjusts causing the artifacts seen in the images. Since these artifacts are found for all samples, it is not seen as an interference. The comparisons made here between the different films can still be considered accurate.

V.3.b. Circular dichroism

Generally, CD is the optimal method for determining the secondary structure of adsorbed proteins.[4,31,32] But for this system, it is not viable. The custom made cuvette worked well on its own, however, once slides with high NO flux films were added to the cell, the diode crashed out, making scans impossible to complete. To ensure that the cuvette was not the issue, the slides were tested in a general use CD cuvette. Again the diode crashes out within the range of detection needed to look at protein secondary structure, 190-200 nm.[4] The reason that my system is not compatible with CD experiments is because of the composition of the film. The plasticized PVC films are blended with DBHD/N_2O_2 and a KTpClPB salt. The primary issue is with the salt content. Too much salt, even high concentrations of phosphate buffered saline solution, can crash out the CD diode. Looking at the structure of KTpClPB shows why this salt in particular is going to interfere with CD measurements. Figure V.4 shows the phenyl groups on the salt, these aromatic groups are known to interfere with CD spectroscopy. With the high NO flux material, the donor and salt are at such high concentrations that the diode cannot cope with it.

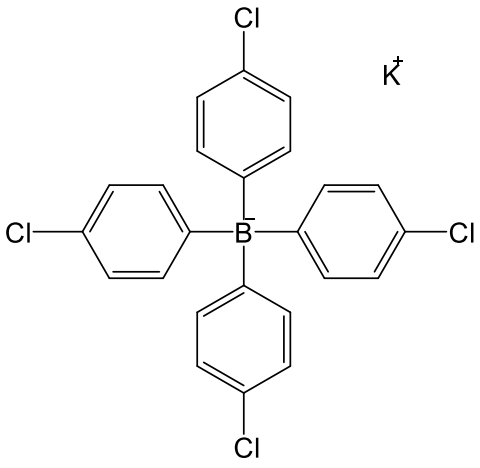

FIGURE V.4: Molecular structure of KTpClPB. Shows the aromatic rings that are part of the structure and known to interfere in CD measurements.

Although CD spectroscopy is not going to be a good method to use for determining secondary structure of adsorbed fibrinogen on my model system, there is the potential for using ATR-FTIR. With FTIR, the response from the proteins can be separated from that of the film itself.

V.3.c. ATR-FTIR

Control, low NO flux, and high NO flux materials were tested both with and without fibrinogen using the attenuated total reflection (ATR) crystal on a Thermo FTIR system. Samples were compared to determine the presence of amide I and II peaks for the presence of fibrinogen on the material surface. In the initial IR spectra, the control and low NO flux films did not differ greatly from that of the polymer alone post-soak (Figure V.5).

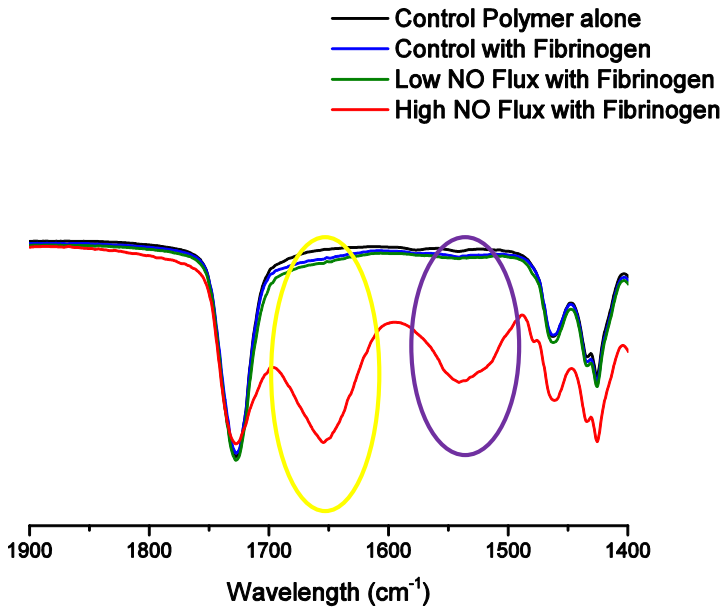

FIGURE V.5: IR spectra of polymer with and without fibrinogen adsorption control (black without and blue with), low NO flux (green), high NO flux (red). Films without fibrinogen adsorption are shown with dashed lines, whereas those with fibrinogen adsorption are solid. The amide I and II peaks are highlighted in yellow and purple, respectively.

There is a slight growth in the spectrum in the 1700 – 1600 cm^{-1} range. This indicates that there is protein present on the surface, however, it is not concentrated enough to resolve the amide bands from the spectrum. The high NO flux material had prominent amide I and II peaks that appeared for the fibrinogen coated material compared to the film without protein. To be able to resolve the amide peaks from all spectrum, the non-fibrinogen coated films were used as a baseline before measuring the spectrum of the fibrinogen coated materials.

The amide I band is found from 1700 – 1600 cm^{-1} and the amide II is found from 1600 – 1500 cm^{-1} as shown in Figure V.5. In general the amide I band is used for deconvolution since it is more sensitive to conformational changes. Using the amide I peak the contributions of α-helices, β-sheets, β-turns, and random chains were determined for each film as shown in Figure V.6.

Knowing which conformation factors are present in the sample is necessary because we will compare it to the fibrinogen in its native form. This comparison will allow for conformational changes to be observed. Native fibrinogen was measured by reconstituting fibrinogen in deuterated water. This solution was applied to the ATR crystal and measured using blank deuterated water as a background. The amide I and II peaks were isolated from this spectrum and the same peak fitting was applied to the native fibrinogen as it was to the fibrinogen adsorbed to the polymer surface (Figure V.6). All peaks were shifted to a lower frequency for the native fibrinogen compared to the adsorbed fibrinogen spectra as seen in Figure V.6, this is likely due to the interactions of the protein with the deuterated solvent. At this point, the relative peak heights of each aspect of fibrinogen's conformation could be compared across samples.

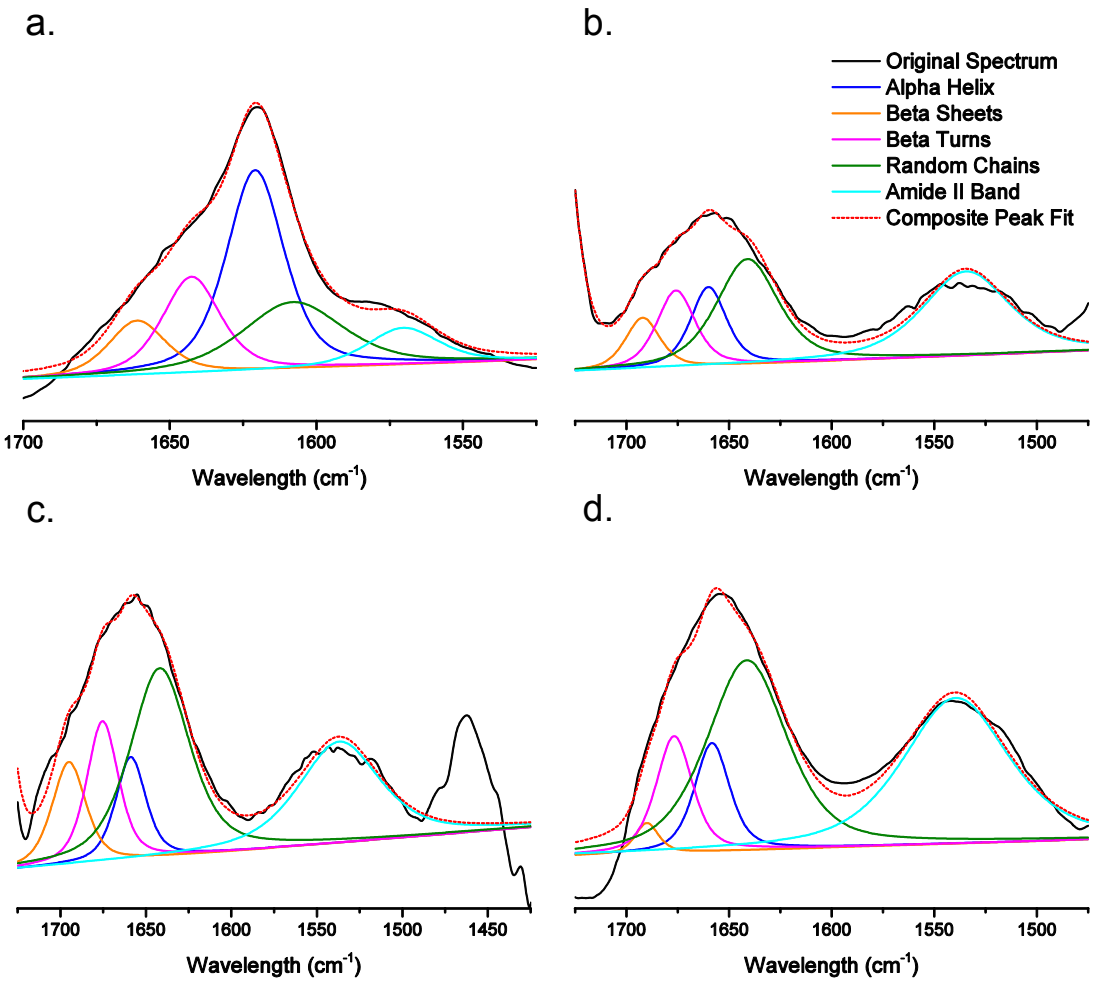

FIGURE V.6: Comparison of native (a) and adsorbed fibrinogen on control (b), low NO flux (c), and high NO flux (d) materials. Adsorbed fibrinogen is on the high NO flux material. Peak deconvolution performed using Thermo Omnic software.

We wanted to compare the peak deconvolution for both native and adsorbed fibrinogen to elucidate any changes in secondary structure. The first notable difference between the amide I peak deconvolution of all spectra is the difference in the α-helix peak. In native fibrinogen this peak is the most dominant feature of the fibrinogen. This makes sense, as fibrinogen is composed of two sets of coiled coils. When the fibrinogen is adsorbed to the surface of both the control and NO-releasing materials, the relative amount of α-helix contribution to the amide I peak is decreased due to rearrangement of the protein. For adsorbed fibrinogen, the random chains make up the

largest contributing factor to the fibrinogen conformation as shown in Figure V.6 of the deconvolution of the amide I peaks for each case. The area under the curve for each structural feature found under the amide I peak was determined and used to calculate the relative contribution of each feature (Table V.1). Calculation of the ratio of α-helix to random chain contribution in Table V.1 shows that the random chain contribution increases significantly upon adsorption. For native fibrinogen the ratio is 1.77, however, this drops to 0.27, 0.29, and 0.45 for the high NO flux, low NO flux, and control surfaces respectively. This is a 75% decrease in α-helix contribution upon adsorption to the control material; the high and low NO flux materials have an 85 and 84% decrease in alpha helix contribution compared to native fibrinogen. This indicates that the release of NO contributes to further unfolding of the protein beyond what the polymer itself initiates.

TABLE V.1: Summary of area under the curve for each structural feature found under the amide I peak during deconvolution

Sample	α-helix	β-sheet	β-turn	Random Chain	α-helix: Random Chain
Native Fibrinogen	0.712	0.189	0.340	.402	1.77
Control	0.058	0.036	0.060	0.129	0.45
Low NO Flux	0.036	0.040	0.052	0.125	0.29
High NO Flux	0.897	0.169	0.984	3.374	0.27

All values are unitless. Area is used only for internal sample comparison, only ratio values are appropriate for comparison across all samples. Control, Low NO Flux, and High NO flux measurements represent adsorbed fibrinogen while Native Fibrinogen is unbound fibrinogen.

Overall, the adsorption of protein to a material surface causes a change in protein structure regardless of whether or not NO is being released. Interestingly, the low and high NO flux materials have a greater decrease in α-helix contribution than the control material. This indicates that NO release is having an effect on protein conformation different from that of the control

material. As this testing was only performed on one set of samples, further testing is needed to determine if these trends remain true for these films.

These changes in the deconvolution of the amide I peak shows that there is a rearrangement of the fibrinogen molecule when it adsorbs to a surface releasing NO, regardless of whether it is a therapeutic amount of NO (>14 $\times 10^{-10}$ mol NO cm^{-2} min^{-1}) or not. The decrease in the α-helices and increase in random chains indicates that the protein unfolds upon adsorption. Since plasticized PVC materials releasing NO have been proven to prevent blood clot formation *in vivo*, this conformational change may indicate that the protein is changing in such a way that it promotes surface passivation. At this time, we can only speculate about the composition of the random chains of fibrinogen exhibited in the adsorbed case. Presumably these chains are not the ones that will interact with platelets, causing them to activate and adhere due to the anti-thrombogenic properties of NO releasing materials in the literature.

Alternately, we could make the conjecture that the unfolding of the fibrinogen and increase in random chains due to interaction with released NO could be detrimental to clot formation. With this model system, the surface properties were kept comparable across all of the samples. In the literature, the NO releasing materials were assumed to have similar surface properties, however, this was never tested. In work done by the Bartlett group, the surface properties were assumed to be more hydrophilic with NO release than the control material due to the presence of the NO donor. This alteration in surface properties of the material could induce other changes in the fibrinogen conformation that differ from those on the control material regardless of the presence of NO release. In this case, the material may be less susceptible to inducing blood clot formation due to the interactions of the fibrinogen with both NO and changes in their material's surface properties. Testing my model systems using an *in vivo* system is beyond the scope of this project. Future work

could use this system to run tests on whole blood and *in vivo* to determine whether or not the materials that have isolated NO release from any other surface property changes still have the same ability to prevent blood clotting.

V.4. Discussion

The adsorption of fibrinogen to a material surface results in a change to its conformation. When the conformation of the protein changes from its native state, then the interactions it can have with other proteins and cells will also be affected. In this study we looked at the effect of NO release on the conformation of fibrinogen, due to its importance in the coagulation cascade. Fibrinogen adsorption to other materials has been well documented and methods are in place to look at changes in conformation from its native configuration.[4,18,33] Common methods include CD, ATR-FTIR, and AFM. These methods were used here to attempt to determine how fibrinogen changes upon adsorption to a material that is releasing NO.

Analysis of the films with AFM spectroscopy were unable to distinguish between individual proteins. Part of the reason for this is that the polymer films are not completely flat surfaces, most of the studies looking at fibrinogen adsorption under AFM have the protein adsorbed to a flat surface, such as mica.[18,34,35] This is unfortunate as we cannot see in what way the fibrinogen is adsorbed onto the material surface (i.e. side-on, end-on, bent). Despite this complication, we can say that there is protein on the material surface. Comparisons between the films with and without fibrinogen shows a change from a relatively smooth surface to one that is coated in something. In this case, that something is fibrinogen. What we are seeing with all films is the adsorption of bulk fibrinogen on the material surface. In order to actually look at the

conformational changes that may be happening with the adsorbed fibrinogen, further studies had to be completed. For that we looked to CD and FTIR spectroscopy.

Experiments involving CD to determine conformational changes are very common in the field. The challenge with CD spectroscopy is that the plane of polarized light has to be able to pass through the film. If the film is too opaque, this becomes an issue. Additionally, the presence of aromatic rings and salts will interfere with the signal. This interference is due to the strong interaction of the polarized light with both salts and the aromatic rings. When CD was attempted on the model system used for this work, the diode crashed out almost immediately upon beginning the measurement. This is likely due to the presence of the KTpClPB salt within the films. The NO releasing films have a 1:1 ratio of this salt to the NO donor and as such, the high NO flux material has a large amount of this salt blended within it. Looking at the salt, we can already see that it will have an interference due to the aromatic rings on the borate. That the salt is also of a high concentration is an additional contributing factor to the diode crashing out. This method was not appropriate for determining changes in protein conformation.

Due to the inability of CD to be used for detection of fibrinogen conformational changes, ATR-FTIR was used. FTIR is another method that is commonly found in the literature to look at protein conformation. Most of these methods are used to look at proteins in solution, however, in the case of FTIR use of the ATR accessory allows films with adsorbed protein to be easily examined. FTIR is not a surface sensitive method. In order to isolate the signal from the protein on the surface from that of the film, the film was examined without fibrinogen and compared to a spectrum obtained with fibrinogen on the surface. The films were all relatively uniform in their structure; and post fibrinogen adsorption, there was no noticeable change in the most of the spectrum. The main change seen was in the range of 1700-1400 cm^{-1} where the amide I and II

peaks appear. The amide I peak is the most affected by changes in protein conformation, so this was the peak used to deconvolute the data and determine which structural features were contributing most to what was measured.

The control and low NO flux materials only showed significant amide I and II peaks when the polymer itself was taken as the background before scanning the fibrinogen coated film. When polymer is not used as the background, the control and low NO flux materials only show a slight increase in the area of the amide peaks compared to the polymer alone (Figure V.5). In contrast, the high NO flux material showed a strong amide I and II peaks whether or not the polymer was used as a background. This indicates there is a large amount of fibrinogen adsorbed to the high NO flux material surface compared to the control and low NO flux materials, confirming what has been seen in Chapters III and IV, as well as the results for AFM in this chapter. Previously we attempted to determine the Langmuir adsorption isotherm for the fibrinogen on these model materials in Chapter IV. The intensity of the amide I and II peaks in the IR show that there may be more than a monolayer of fibrinogen adsorbed to the material surface, confirming that Langmuir isotherms are inappropriate for this system. Using the spectra where the polymer is taken as the background, the contributions of α-helices, β-sheets, β-turns, and random chains to the amide I peak can be determined. With this, we were able to examine the changes in protein conformation and begin to understand what is happening with the protein when introduced to NO release from a material surface. The deconvolution of the amide I peak showed a decrease in the contribution of α-helices to the conformation of protein when adsorbed to a material surface. Additionally, an increase in the contribution of random chains to the structure of the protein was seen for all adsorbed protein systems compared to the native fibrinogen. This indicates unfolding of the protein upon adsorption to the material surface. Since the protein appears to adsorb in greater than a

monolayer, the unfolding of the fibrinogen may also be due to interaction with other adsorbed fibrinogen molecules. Native fibrinogen in the blood will not adhere to nor activate platelets as it passes through the system. Fibrinogen adsorbed to a material surface in the presence of NO is altered in conformation from its native state. This could produce fibrinogen that will interact with and activate platelets, causing the formation of a mature blood clot.

Previous studies on an NO releasing plasticized PVC system show that the inclusion of NO release prevents occlusion of the device by blood clot formation. The main difference between the systems used for these previous tests and those used here is that the surface properties of the material are similar to a non-NO releasing control. Previous studies did not look into any changes in surface properties. We showed in Chapter II.3 that there is an increase in surface wettability for materials prepared in the same manner as those in the literature. Surface wettability is well known to have an effect on the adsorption of proteins. The difference in surface properties from our films to those in the literature may lead to a difference in the ability of the material to prevent blood clot formation. In both previous studies by our group (shown in Chapter III)[5] and in previous work done by the Bartlett group[36] there was an increase in fibrinogen adsorption to these NO releasing materials compared to the non-NO releasing control material. The similarities here could indicate that the conformational changes seen here are due to the NO interactions with the fibrinogen and not with surface interactions due to increased hydrophilicity of the Bartlett group's system. Further studies must be done on our model systems to determine the viability of the material to prevent blood clot formation *in vivo*. As current work focuses mainly on the interaction of fibrinogen with NO, *in vivo* measurements are beyond the scope of this dissertation.

V.5. Conclusions

The studies performed here confirm previous findings that the high NO flux system has significantly more fibrinogen adsorbed to the material surface compared to the control material and the low NO flux system. Additionally, the fibrinogen undergoes a conformational change upon adsorption to all surfaces compared to native fibrinogen. For the first time we see that fibrinogen adsorbed to NO releasing materials have increased structural unfolding compared to the control non-NO releasing material. The increase in the contribution of the random chains to the amide I peak for NO releasing materials along with the increased biocompatibility of these materials in literature indicates that this unfolding likely improves the function of these materials. Conformational changes in the adsorbed fibrinogen on NO releasing materials may lead to the formation of a passivating layer upon the material's surface, which helps prevent the formation of blood clots. Ultimately, whether these changes in conformation have an effect on the prevention of blood clot formation on this model system is unclear. Further studies must be completed on whole blood and *in vivo*. To begin to get at the viability of these materials, *in vitro* platelet studies were completed on the system to look at the effect that the adsorbed fibrinogen has on platelet adhesion. These studies are presented in Chapter VI. We have shown in this chapter that NO release results in conformational changes to fibrinogen upon adsorption that differs from a control non-NO releasing material and native fibrinogen in solution.

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CHAPTER VI:

PLATELET ACTIVITY ON FIBRINOGEN ADSORBED MATERIALS

VI.1. Introduction

Blood clot formation during injury and on a synthetic surface occurs via two different pathways. In either case platelets play an important role in this process. In the presence of the natural endothelium, platelets are the first responders to damage. They will form a plug at the site of injury and recruit other cells and proteins to heal the area. When a synthetic material is introduced into a biological system, the difference in clot formation is that the first responders to foreign objects are proteins. After the initial adsorption of proteins to the material, platelets and other cells are recruited to form a clot, eventually leading to device failure. The prevention of device failure is currently a major area of research. The method used here to combat this is to include the release of nitric oxide (NO) from the material used to make the device. This is method rests on the fact that NO is produced by the natural endothelium. By including NO release into biomedical devices, it should more closely mimic the natural endothelium and help prevent device failure resulting from blood clots. In fact, *in vivo* studies have shown that the incorporation of NO release has prevented significant blood clot formation in vascular grafts[1] and extracorporeal circuitry[2] compared to control materials.

In the numerous studies on NO's mediation of blood clot formation, the focus has been on NO's effect on platelets. NO has been well established as a platelet mediator, helping to prevent both activation and adhesion. The issue with only looking at platelets is that proteins have the first interactions with implanted materials, not platelets. We have previously established that the clotting protein fibrinogen adsorbs in greater concentration to NO releasing materials than it does

to control materials.[3] Additionally, we see that the fibrinogen adsorbed to the high NO flux material changes in conformation from its native state in Chapter V. How these conformational changes influence the end result of blood clot prevention is yet unclear. Continued studies on these fibrinogen coated, NO releasing materials look into the effect of the system on platelet adhesion and activation. By combining studies on fibrinogen conformation and platelet activation and adhesion, we now know that separately NO has an effect on both fibrinogen and platelets. The question now is, what does the pre-adsorbed fibrinogen have on platelets? There have been many studies that examine platelet activity in the presence of bound fibrinogen.[4-8] The main difference in previous literature and the studies described herein, is the inclusion of NO release from the material surface.

In this study, platelet adhesion and activation is examined for model NO releasing materials in the presence and absence of pre-adsorbed fibrinogen. Comparing platelet activity between both sets of samples will allow us to understand what results we are seeing from the NO, and which are arising from the pre-adsorbed fibrinogen. We see that the fibrinogen coated NO-releasing materials have less surface coverage than those without fibrinogen for both the low and high NO flux systems. Further the low and high NO flux materials exhibits greater unactivated platelets and less fully activated platelets overall. These results indicate that the fibrinogen conformational changes found in the NO flux systems may have an effect on platelet activation and adhesion. The interaction of NO with fibrinogen may actually be a large contributing factor in the improved platelet viability of these materials.

VI.2. Materials & Methods

VI.2.a. Materials

Polymer formulations were prepared from high molecular weight poly(vinyl) chloride (PVC) from Aldrich (St. Louis, MO) and bis(2-ethylhexyl) sebacate (DOS) from Acros Organics (New Jersey, USA). Potassium tetrakis(4-chlorophenyl) borate (KTpClPB) was obtained from Ace (Korea). Tetrahydrofuran (THF) from Mallinckrodt (New Jersey, USA) was used as the solvent. Diazeniumdiolated dibutylhexanediamine (DBHD/N_2O_2) was synthesized by exposing *N,N'*-dibutyl-1,6-hexanediamine (DBHD) (Alfa Aesar, Ward Hill, MA) (99.8%, Sigma, St. Louis, MO) NO under 80 psi NO at room temperature for 48 h in acetonitrile as the solvent.[9]

For fibrinogen binding, human plasma fibrinogen was obtained from Calbiochem (La Jolla, CA) and Dulbecco's phosphate buffered saline (DPBS) was obtained from Hyclone® (Logan, Utah). Glass slides (75x50 mm) from Corning (NY) glass rings from lab stock. All water used in the experiment is deionized unless stated otherwise.

VI.2.b. Sample preparation

Samples are prepared in a manner described in Chapter III.2 with a few changes made to the sample preparation method, as detailed below. Blood was acquired from healthy individuals through venipuncture and drawn into standard 10 mL vacuum tubes coated with EDTA. Dulbecco's phosphate buffered saline (DPBS) and fibrinogen are the same as in Chapter III.2. Glass cover slips and 24-well plates were obtained from VWR (Radnor, PA) and Corning (Tewksbury, MA), respectively. Glass coverslips were coated by Surfatek LLC with a specialized metallic coating. Glutaraldehyde, sucrose, sodium calcodylate, ethanol, and hexamethlydisilizane were obtained from Sigma Aldrich. Calcien-AM was obtained from Invitrogen (Eugene, OR).

The platelet assay was modified off of a previously established method from the Popat group.[10,11] Coverslips were placed in the center of a glass ring affixed to a glass slide. Samples are prepared by drop coating layers of polymer on top of specially coated coverslips to make our model system. To ensure that the samples could be removed from the set-up the first coating was of plasticized polymer without NO donor blended into the solution. After this coating cured for 1.5 h layers were applied starting with a base coat of either 0, 1.0, or 9.2 wt% DBHD/N_2O_2 to create the control, low NO flux, and high NO flux materials. A series of three top coats were applied to the system as described in Chapter III.2. After curing overnight, the samples were transferred to two 24 well plates, one plate for testing with fibrinogen and one for testing without. Samples include, control coated coverslip, control PVC, and low and high NO releasing composite materials.

VI.2.c. Platelet assay

An aliquot of DPBS was added to all samples, 500 µL per well and plates were incubated for 1 h at 37 °C. After this initial incubation, the DPBS was aspirated off. In one plate, 500 µL of DPBS was added again to each well, in the second plate, 500 µL of 3 mg/mL fibrinogen in DPBS was added to each well. Both plates were incubated for 1.5 h at 37 °C. Post incubation, solution was aspirated out of all wells and samples were washed 4× with DPBS.

During the incubations, blood was collected from a healthy donor. This blood was centrifuged at 300 g for 15 min to separate the plasma from the rest of the blood. The plasma was then gently removed from the vials and pooled together. Pooled plasma was added to all sample wells in 500 µL aliquots. This was allowed to interact with the surfaces for 2 h at room temperature. Post-incubation, the plasma was aspirated from the wells and the samples were washed 2× with

DPBS. At this time half of the samples were prepared for fluorescence measuring of platelet adhesion and half were prepared for SEM imaging for platelet activation.

VI.2.d. Fluorescence microscopy

Platelet adhesion studies were completed by staining the cells with calcein-AM. Samples were incubated with 2 µM calcien-AM in DPBS solution (500 µL per well) for 30 min at room temperature. During this time samples were kept from light. The solution was then aspirated out and samples were rinsed; they were kept wet by adding DPBS after rinsing until measurement. Samples were immediately imaged using a fluorescent microscope with a high efficiency filter set 62 HE BP 474/28 (green) from Ziess (Jena, Germany). Images were taken at 10 and 20× magnification (n=3 for all samples).

VI.2.e. Scanning electron microscopy

Platelet activation was determined by imaging using scanning electron microscopy (SEM). Platelets were dehydrated and fixed onto the sample surfaces. Fixing the platelets is done by soaking the films first in primary fixidant (9% glutaraldehyde, 0.1 M sodium calcodylate, 0.1 M sucrose) for 45 min. They were then soaked in secondary fixidant (primary fixidant minus the glutaraldhyde) for 45 min. To dehydrate the platelets, they were placed in a series of increasing ethanol concentration solutions (35, 50, 70, 100% ethanol) for 10 min each. After the last soaking in 100% ethanol solution, they were further dried by incubating in hexamethyldisilizane for 10 min. After the final soak, films were removed from all solutions and allowed to air dry before storing in a clean well plate until imaged on a JEOL JSM-6500F SEM (JEOL USA, Peabody, MA). Samples were sputter coated with a ~10 nm layer of gold prior to imaging. SEM images

were taken at 15 kV and 100, 1000, and 5000× magnification. The images were used to determine the percentage of unactivated, semi-activated, and fully activated platelets based on their morphology. Unactivated platelets are normal with a compact central body. Semi-activated platelets exhibit short dendrimers and fully activated are platelets with many long dendrimers.

VI.2.f. Data analysis

Fluorescence and SEM images were analyzed using ImageJ software (NIH, Bethesda, MD). The analyze particles parameter was used to determine platelet coverage with the fluorescence images. Platelets were counted and grouped by unactivated, short dendritic, and long dendritic using the cell counter plug-in available for the program. Standard error of the mean (sem) used for all error reporting (n=3 for all samples). Statistics were run by Jim zumBrunnen in the Colorado State University Statistics Lab.

VI.3. Results

VI.3.a. Platelet coverage

Platelet coverage was determined through detection using fluorescence microscopy. Images were taken at 10 and 20× magnification. The 10× magnification images were used to determine the overall platelet coverage of the materials. Percent coverage was calculated for each sample and situation and shown in Figure VI.1 (n=3). Overall the platelet coverage was found to be the same across all samples. There are no statistical differences in the data, however, general trends did appear. The inclusion of fibrinogen caused a general increase in platelet coverage on the non-NO releasing control materials (0.60 ± 0.23% and 0.39 ± 0.10% coverage with and without fibrinogen adsorption, respectively). The NO releasing materials show an opposite trend to the

control material; fibrinogen adsorption on these surfaces causes a decrease in platelet coverage. This trend is evident in the low NO flux material with platelet coverage for the pre-adsorbed fibrinogen material exhibiting less platelet coverage than that without fibrinogen (0.17 ± 0.06% v 0.43 ± 0.22%, respectively). The increase in NO release from the high NO flux materials did not appear to have a different effect on platelet coverage compared to the low NO flux materials. The general trend for platelet coverage on NO materials continues with the high NO flux materials. With pre-adsorbed fibrinogen, there is a general decrease in platelet coverage compared to when no fibrinogen has been adsorbed at 0.08 ± 0.02% and 0.25 ± 0.20% coverage for with and without adsorbed fibrinogen. While the trends indicate a difference in coverage, currently the platelet coverage is statistically the same across all materials.

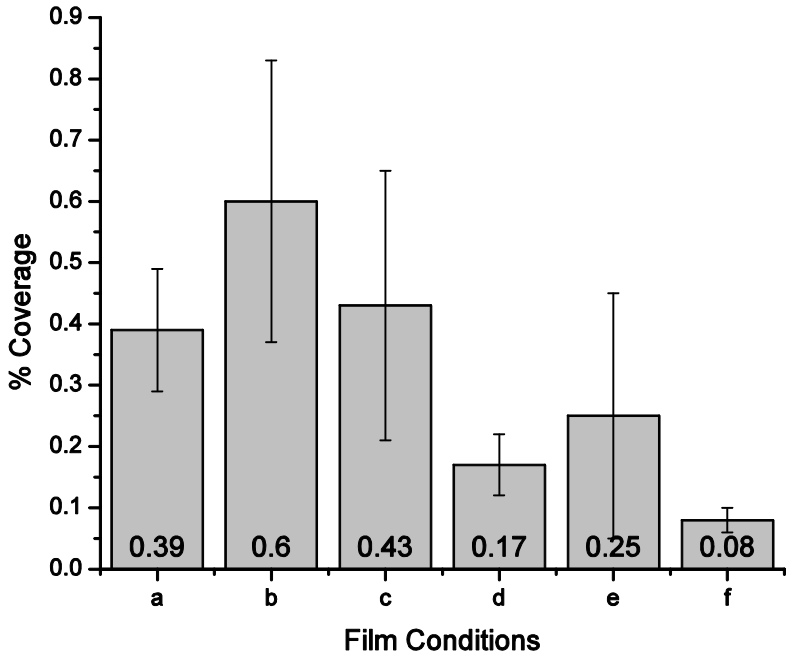

FIGURE VI.1: Platelet coverage of samples with and without fibrinogen pre-adsorbed onto the material surface (control (a), control with fibrinogen (b), low NO flux (c), low NO flux with fibrinogen (d), high NO flux (e), high NO flux with fibrinogen (f). Values represent mean ± sem (n=3).

VI.3.b. Platelet activation

Platelet activation was assessed by imaging the fixed platelets using SEM. Images were taken at 100, 1000, and 5000× magnification. Overall coverage of the samples were seen in the 100× magnification images while the 1000× images were used for counting the platelets and determining the degree of activation (n=3). Platelets were assessed as either unactivated, short dendritic, or long dendritic as seen in Figure VI.2.

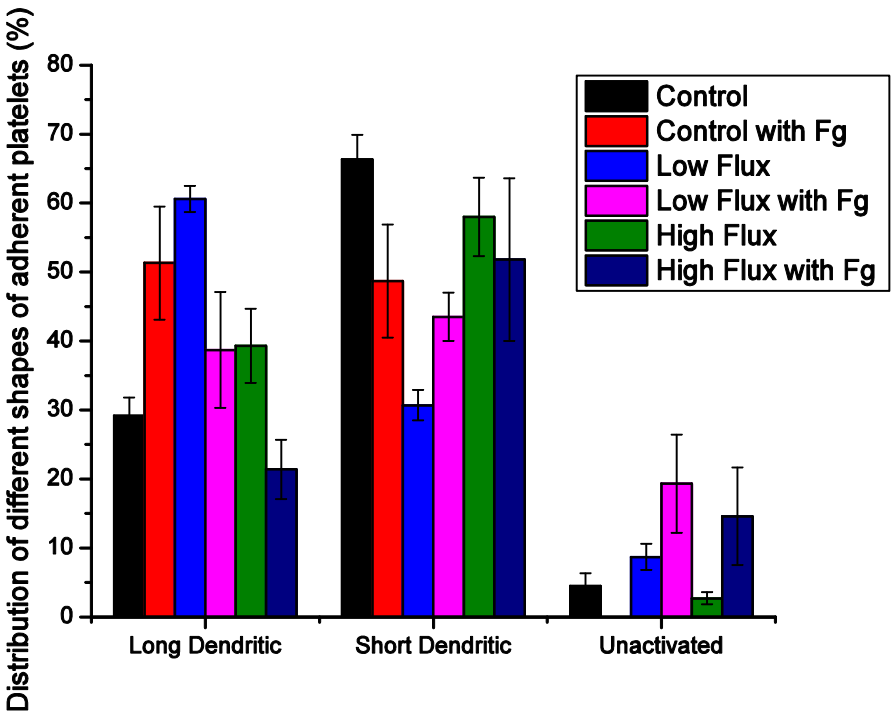

FIGURE VI.2: Distribution of adherent platelets to all samples with and without fibrinogen adhesion prior to platelet incubation. Values shown are mean ± sem (n=3).

Overall, the degree of activation of the platelets was not found to be statistically different for any material with or without fibrinogen adsorption. Again, trends appear in the data, however, more repetition is necessary to determine significance. The inclusion of adsorbed fibrinogen shows a general trend in the percentage of long dendritic and unactivated platelets with and without NO release. The control, non-NO releasing material has more long dendritic platelets and less

unactivated platelets with fibrinogen pre-adsorbed (51.3 ± 8.2% long dendritic, 0.0 ± 0.0% unactivated), compared to without fibrinogen (29.2 ± 2.6% long dendritic, 4.5 ± 1.8% unactivated). The opposite is true for the NO releasing materials. Low NO flux materials show a decrease in long dendritic platelets on films with pre-adsorbed fibrinogen (38.7 ± 8.4% with and 60.6 ± 1.9% without fibrinogen). The high NO flux material with pre-adsorbed fibrinogen has the least amount of long dendritic platelets compared to all other surfaces at 21.4 ± 4.3%. The films with the highest amount of unactivated platelets are the low and high flux NO materials with pre-adsorbed fibrinogen (19.3 ± 7.1 and 14.6 ± 7.1%, respectively). Whereas the low NO flux material with fibrinogen and the high NO flux material without fibrinogen had less unactivated platelets (8.7 ± 1.9% and 2.7 ± 0.9%, respectively). Fibrinogen adsorption and NO release appears to have an effect on the activation of platelets based on the trends in the data, however, this current data shows no statistical differences.

VI.4. Discussion

Assessment of the platelet coverage and degree of activation was completed on three films, control, low NO flux, and high NO flux. All films were tested with and without fibrinogen adsorption prior to incubation with platelets. Films containing NO donor blended into the base layer released the same amount of NO during platelet incubation whether it was pre-incubated in fibrinogen or DPBS. In this way everything except for the adsorption of fibrinogen was maintained on all sets of samples. The current data shows that fibrinogen adsorption and NO release has no significant effect on platelet adhesion and aggregation because there is no statistical difference in the data across all samples. Despite this, general trends appeared in the data indicating that increased repetition of the experiments may lead to differences in the data. Overall, the general

trend of the data indicates that the high NO flux material with pre-adsorbed fibrinogen has the least amount of platelet adhesion and activation indicating potential improved biocompatibility over the control material. This study shows that fibrinogen adsorbed in the presence of NO release may improve the biocompatibility of plasticized PVC materials.

The control materials had a different trend compared to the NO releasing materials with and without pre-adsorbed fibrinogen. In the case of the control film with fibrinogen adsorbed to the material surface, there was an increase in platelet coverage compared to only incubating in DPBS prior to introducing platelets (0.60 ± 0.23 and 0.39 ± 0.10% with and without fibrinogen, respectively). Further, there were more fully activated platelets on the material with fibrinogen, as indicated by the increase in the amount of long dendrites found on the material surface from only 29 ± 3% without pre-adsorbed fibrinogen to 51 ± 8% with. This increase in platelet activation and coverage indicates potential changes in conformation of the fibrinogen upon adsorption. Indeed, in Chapter V conformational studies via ATR-FTIR indicate unfolding of the protein upon adsorption to the control material. Should these trends continue with repetition of the experiments, this would indicate that fibrinogen adsorbed onto plasticized PVC alters its conformation in such a way that platelets are induced to adhere and activate. This will eventually lead to the formation of a blood clot when introduced into the circulatory system. We can see the degree of activation in Figure VI.3. Visually, the control film with pre-adsorbed fibrinogen has the worst biocompatibility seen with the tested films. There were no unactivated platelets found on the fibrinogen coated control material surface. Both control films with and without pre-adsorbed fibrinogen are shown to have fully activated platelets in Figure VI.3. Although there are less platelets shown on the control material without fibrinogen, both of the platelets are fully activated, similar to those of the control material with pre-adsorbed fibrinogen. This data indicates that the

control material, particularly with pre-adsorbed fibrinogen, is not very biocompatible compared to the other films tested. Since fibrinogen is naturally present in the blood stream, one would assume

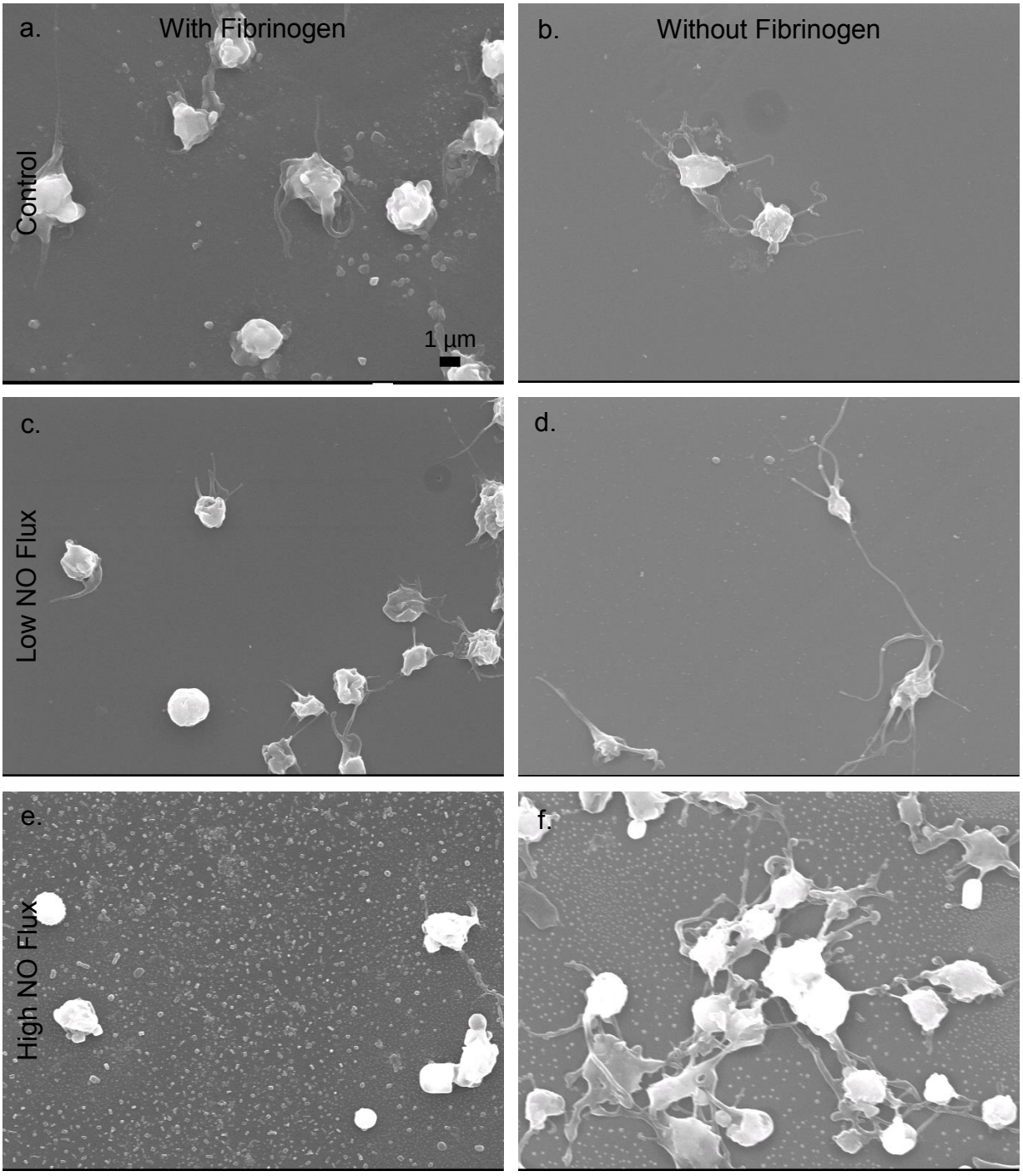

FIGURE VI.3: SEM images of platelet activation for control (a, b), low NO flux (c, d), and high NO flux (e, f) materials with (left) and without (right) pre-adsorbed fibrinogen. Images are taken at 5000× magnification to show degree of activation.

that the control with adsorbed fibrinogen is more comparable to what would happen in the body. The trends of this study confirms previous studies where the control polymer exhibits significantly more blood clot formation than the NO releasing system because the control with fibrinogen has greater overall platelet activation compared to the same films with NO release.[1,2,12-14] Although these results only focus on the interaction of these materials with platelets, the films exhibit significant platelet activation. This supports previous studies *in vivo* which have seen significant blood clot formation on these surfaces. Since platelet adhesion and activation is increased on the control material with fibrinogen adsorbed to the surface, we can infer that the protein conformation changes upon adsorption to promote platelet activation and aggregation.

Both the low and high NO flux materials exhibit similar trends in regards to platelet adhesion and activation. These trends are opposite to those found for the control material, indicating that NO release has an effect on the system and can be seen in Figure VI.4. The low NO flux materials exhibit a decrease in platelet coverage with pre-adsorbed fibrinogen (0.43 ± 0.22% coverage without fibrinogen versus 0.17 ± 0.6% coverage with, respectively). The same is true for the high NO flux material; platelet coverage with pre-adsorbed fibrinogen is 0.08 ± 0.02% while the same material without fibrinogen has 0.25 ± 0.20% coverage. Overall, values for platelet coverage is not statistically different across all samples, however, these trends do stand out amongst the data. This indicates that on NO releasing materials, fibrinogen adsorption may decrease the amount of platelet coverage. Additionally, the data suggests that the high NO flux material with pre-adsorbed fibrinogen may have the least amount of platelet coverage of all materials. This would indicate that the high NO flux system with fibrinogen adsorption may present the best solution to improved biocompatibility. This trend is visually seen in Figure VI.4, however, further examination and repetition is necessary to confirm this trend.

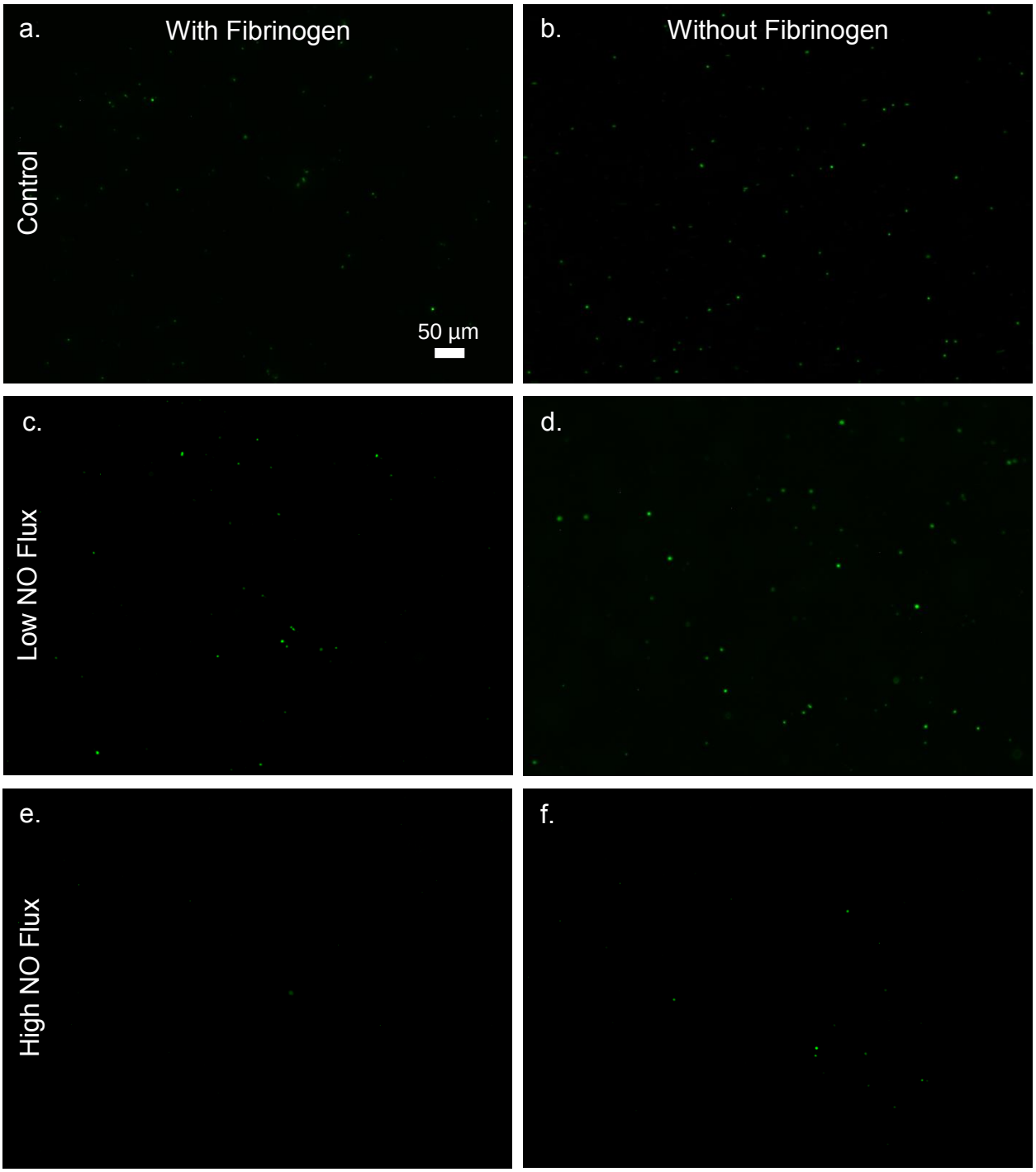

FIGURE VI.4: Fluorescent microscope images of overall platelet coverage of control (a, b), low NO flux (c, d), and high NO flux (e, f) materials with (left) and without (right) pre-adsorbed fibrinogen. Images taken at 10× magnification.

Platelet activation on both low and high NO flux materials remains statistically the same to both each other and the control, non-NO releasing material. Despite this, the trends in the data

suggest that the presence of NO and fibrinogen may produce an improved biomaterial. The amount of fully activated platelets for both low and high NO flux materials decreases for films with pre-adsorbed fibrinogen as seen in Figure VI.2 (61 ± 2% to 39 ± 8% for low NO flux materials and 39 ± 5% to 21 ± 4% for high NO flux materials). Further, the high and low NO flux materials had the greatest amount of unactivated platelets when fibrinogen was pre-adsorbed compared to all materials tested. The trend for unactivated platelets on the NO releasing materials is opposite the trend seen for coverage and the amount of fully activated platelets. This increase in unactivated platelets indicates that the combination of NO release and fibrinogen adsorption may be important in the prevention of platelet activation for improved biocompatibility of the materials. The decrease in activated platelets and increase in the amount of inactivated platelets can be seen in Figure VI.3. The high NO flux material has the greatest contrast visually when comparing the SEM images from pre-adsorbed fibrinogen materials to those without fibrinogen. Figure VI.3f shows large amounts of adhesion, activation, and aggregation of platelets on the high NO flux material without fibrinogen. When compared to the SEM images for the high NO flux system with pre-adsorbed fibrinogen (Figure VI.3e), the system without fibrinogen is clearly less biocompatible. This difference is not as obvious for the SEM images for the low NO flux material, however, the low NO flux system without fibrinogen exhibits longer dendrites and more fully activated platelets than the system with pre-adsorbed fibrinogen (Figure VI.3c and d). While the SEM images show significant differences in the platelet activation across all samples, analysis of the data shows that none of the differences are significant. Further repetition is needed to confirm these trends.

The trends found in this data indicate that the release of NO from a plasticized PVC material improves its biocompatibility when fibrinogen is pre-adsorbed. Overall, platelet adhesion and activation is decreased when both fibrinogen is adsorbed and NO is being released. Since there

is no statistical difference between any of the data points for either adhesion or activation, we can only make conjectures about what the trends in the data mean for our system. Interestingly, there does not seem to be a difference in platelet adhesion and activation for the different NO fluxes. Both the high and low NO flux materials show the same trend toward improved biocompatibility in the presence of pre-adsorbed fibrinogen compared to both the same materials without fibrinogen and both control, non-NO releasing materials. Conformational changes seen in Chapter V indicate that fibrinogen unfolds as it adsorbed to the plasticized PVC regardless of whether or not it releases NO. There is a slight difference in the unfolding of fibrinogen with NO release compared to without. This difference in conformation may be the reason why the trends for NO releasing materials show improved biocompatibility with adsorbed fibrinogen. The data found in these studies indicate that the amount of fibrinogen adsorbed onto the surface of the material may not matter as much as the conformation in which it is adsorbed. Previous studies also found that it was the unfolding of the protein causing the interactions with platelets and not the amount of protein.[15-18] There are specific amino acid sequences on the fibrinogen that interact with platelets.[19] When these sites are not available to the platelets, they are less likely to adhere and activate. The fibrinogen adsorbed to the NO releasing materials appears to have conformational changes that inhibit platelet adhesion and activation compared to that of the same materials without fibrinogen and both non-NO releasing control materials. Although these trends are readily apparent in the data, at this time there is no statistical difference in the platelet activation and adhesion to the materials regardless of NO release or fibrinogen adsorption. Currently, the results seen here indicate that neither NO release or fibrinogen adsorption have an effect on platelet adhesion or activation. This is in contrast to previous findings for NO release where they found that NO release from polymeric materials actually decreased the amount of platelet adhesion and activation.[13,20-24]

None of these previous studies took into account the changes in surface properties that the inclusion of NO donors has on the material. The materials used for this dissertation have controlled the surface properties and ensured that they remain comparable for all control and composite materials. It is likely that the changes in surface properties for the previous studies had an influence on the results they saw, particularly for studies performed *in vivo* and with pre-adsorbed protein.

VI.5. Conclusions

The studies presented here bring us to a better understanding of the interaction between fibrinogen, NO, and platelets. Overall, the data indicates that there is no change in platelet adhesion and activation regardless of NO release or fibrinogen adsorption. There is currently no statistical evidence to suggest whether or not the adsorption of fibrinogen in the presence of surface released NO will prevent platelet activation and adhesion. However, what we do see is that there are trends in the data that indicate the possibility of NO release and fibrinogen adsorption to have an effect on platelets. Further study and repetition of the experiments presented here are needed to confirm these trends. The control, non-NO releasing material exhibits an increase in platelet coverage and percentage of fully activated platelets when there is pre-adsorbed fibrinogen compared to without. There is also a decrease in unactivated platelets for the control material, the fibrinogen coated system was found to have no unactivated platelets. The NO releasing materials, regardless of flux had a reverse trend with a decrease in platelet adhesion and fully activated platelets, as well as an increase in the amount of unactivated platelets when fibrinogen is pre-adsorbed. These trends indicate, for the first time, that it may be the combined presence of both NO release and fibrinogen adsorption that have an impact on platelet adhesion and activation.

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CHAPTER VII:

CONCLUSIONS AND FUTURE DIRECTIONS

VII.a. Experimental Findings

Nitric oxide (NO) is a versatile molecule involved in many biological pathways.[1-4] Since Ignarro determined that NO was the endothelium derived relaxing factor that is the cause for vasodilation, it has been the focus of much research.[5-11] Previous literature focuses on the ability of NO to prevent platelet adhesion and activation. However, as I have established throughout this dissertation, there are more than just platelets involved in blood clot formation on synthetic materials.[12-14] In this work, I explored the interactions of NO with fibrinogen, a prominent clotting protein. At the onset of my studies I hypothesized that the NO releasing material would have the least amount of fibrinogen bound to the surface and also activate the least amount of platelets (Chapter I, Figure I.5). To test this hypothesis, I developed a model system to be able to test fibrinogen adsorption, conformation, and effect on platelets.

My model system was developed utilizing the NO donor diazeniumdiolated dibutylhexanediamine (DBHD/N_2O_2) in blended films that release NO at two different NO fluxes 13.0 $\times 10^{-10}$ mol NO cm^{-2} min^{-1}as a low NO flux and 17.4 $\times 10^{-10}$ mol NO cm^{-2} min^{-1} for the high NO flux (Chapter III). These NO releasing films were prepared such that they maintain comparable surface properties to the control, non-NO releasing material. This model system was then utilized for the determination of fibrinogen adsorption to the material surface, analysis of the changes in conformation upon adsorption, and the effect of the adsorbed fibrinogen on platelets. As a result, this study has broadened our knowledge of the effect of surface released NO on fibrinogen adsorption.

These studies have shown that, contrary to my initial hypothesis, NO release from a material surface increases the amount of fibrinogen adsorbed to the material surface. This adsorbed fibrinogen is altered in conformation from its native state over 1.5 h of adsorption time due to NO release. The adsorption of fibrinogen onto NO releasing materials may improve the biocompatibility of the system. The release of NO in the presence of pre-adsorbed fibrinogen may likely cause less platelet coverage than the control non-NO releasing film based on the trends in the data. Further, the amount of fully activated, long dendritic platelets appears to decrease with fibrinogen adsorption on NO releasing materials compared to the same NO flux without fibrinogen adsorbed to the material surface. This study shows that NO releasing materials exhibit potential improved biocompatibility when fibrinogen is adsorbed to the material surface compared to the same NO flux without fibrinogen, with regards to platelet adhesion and activation. To further understand the biocompatibility of this system, these tests must be repeated to determine whether or not the trends seen in this dissertation continue to hold true. Additionally, whole blood and *in vivo* testing must be performed using these NO releasing materials to explore the full biocompatibility of the system.

Previous literature has determined that NO releasing materials will prevent mature blood clot formation *in vivo*.[13,15,16] These materials, however, have not been assessed for surface property changes. It is well documented that changes in surface properties of materials will have a distinct effect on the adsorption of proteins.[17,18] The materials used for exploration within this dissertation finally eliminate the contributions of surface properties when investigating the effect of NO on protein adsorption and biocompatibility. I found that the release of NO from these model materials will alter the conformation of fibrinogen upon adsorption and this adsorbed fibrinogen may improve the reaction of the material to platelets compared to a material without fibrinogen

adsorbed. Trends in the data exhibit a decrease in major platelet aggregation to samples with the same rate of NO release when fibrinogen is allowed to adsorb prior to incubation with platelets than when there is no fibrinogen adsorbed. Further testing on these materials should repeat these experiments, then examine the effect that NO has on other proteins in the blood stream, primarily albumin and other clotting proteins. These studies will help investigate what effect NO has on individual aspects of blood clot formation.

In addition to examining the effect of NO on different proteins, these materials must also be tested for biocompatibility under whole blood conditions. Because the surface properties of my model system remain comparable across both NO releasing materials and non-NO releasing materials, the biocompatibility of these NO releasing materials may be altered. The high NO flux material in my model system does release $>14 \times 10^{-10}$ mol NO cm^{-2} min^{-1}, which is seen to be the amount of NO release needed to produce therapeutic effects.[16] The question remaining then is whether or not changes in surface properties for the materials tested in previous studies had a major effect on whether their NO releasing materials exhibited improved biocompatibility to the controls. To answer this, my model system will need to be examined thoroughly in not only how fibrinogen is affected by NO release from the material, but also how the material responds during *in vivo* testing similar to that done on previous systems.

VII.2. Future Directions

VII.2.b. Biocompatibility testing with proteins

We have established that NO is having an effect on fibrinogen such that there is a large relative amount adsorbed to the surface. The conformational changes in the fibrinogen cause less activation in platelets on a high NO flux material surface compared to the same surface without

fibrinogen. This decrease in platelet activation indicates that the fibrinogen conformation is such that it improves the biocompatibility of the material compared to control and currently used materials. Looking at the fibrinogen adsorption alone is a good starting point, considering that fibrinogen is a major protein involved in the formation of blood clots. The next steps for determining the biocompatibility of these material surfaces would be to examine competitive binding of different proteins on the surface in addition to fibrinogen. Proteins such as vonWillebrand factor, and fibronectin are also involved in blood clot formation.[19] While they are not found in as great of concentration in the blood stream as fibrinogen, understanding the effect NO has on these proteins will further our understanding of the coagulation process for these NO releasing biomaterials. Further, the adsorption of albumin to these material surfaces will be instrumental in biocompatibility testing. Albumin is the present in the highest concentration in the blood stream (34 mg/mL), while it is not involved in blood clot formation, the adsorption of this protein could provide improved performance of biomaterials.

These proteins need to be tested both individually and in a competitive fashion with fibrinogen and the other proteins on the model system. By testing the proteins individually, the same tests that have been run here with fibrinogen can be tested with each protein. Doing this can help us understand the effect NO has on multiple different proteins present in the blood stream. To fully assess blood compatibility of the NO releasing materials, these proteins must also be tested together for competitive adsorption. There is a challenge to making these measurements. We have established in Chapter IV that the total protein assay, the BCA assay, is not a practical assay to use when there are potential changes in protein structure. Further, this assay is not selective for the type of protein and will give you the total protein concentration adsorbed to the material surface. Ideally, one would like to be able to test all of the proteins in one mixture and be able to tag for

specific proteins to assess the overall coverage of that protein. By tagging a different protein each time, the competitive adsorption of each protein can be determined. We have established in Chapter IV that using a tagging method such as biotin with the intent to detect the protein using the interaction of biotin with avidin is not a good choice when looking at these adsorbed protein systems. Depending on the conformation of the adsorbed protein, and potential adsorption in layers, the avidin may be too large of a molecule to get between the protein layers and within the changes in conformation to actually interact with the biotin. To overcome these challenges, the possibility of labeling each protein individually with FITC, then mixing it in with the other proteins prior to allowing the protein mixture to adsorb to the material surface should be explored. The viability of this method on a single protein solution is currently being explored within our group on fibrinogen.

These methods are for measuring a quantitative amount of protein adsorbed to the material surface, if one only cares about qualitative assessments of the protein there are other options to pursue. The important piece of these experiments would be to understand which proteins preferentially adsorb to NO releasing materials using the model system explored in Chapter III. Specifically, we are interested in which proteins preferentially adsorb to the high NO flux material, as this is the material that appears to be the most promising based on both the studies performed here and previous literature. Because of this, using an ELISA with antibodies specific for each protein may give a good picture of which proteins are preferentially adsorbed to the NO releasing surface. In Chapter III, I performed a fluorescent based ELISA to determine relative adsorption of fibrinogen on the material surface. By pairing fibrinogen with either vonWillebrand factor, fibronectin, or albumin alone or by pairing it with a mixture of all of the proteins, competitive binding with fibrinogen can be established. This assay is selective for fibrinogen alone.

Preliminary studies on a mix of fibrinogen and albumin, shows the selectivity of this assay for fibrinogen. When the assay was tested with albumin alone, the fluorescent response of the assay was not above background. Later a mix of both albumin and fibrinogen was allowed to adsorb to the material. There was a definite fluorescent response from this assay, indicating that fibrinogen is adsorbing to the material surface (Figure VII.1). By finding commercially available antibody tags that are specific for each protein, this assay can be run to detect each protein individually using multiple assays. If different proteins can be labeled with antibodies that are tagged with molecules fluorescing at different wavelengths, one assay may be able to give the overall adsorption of each protein in solution.

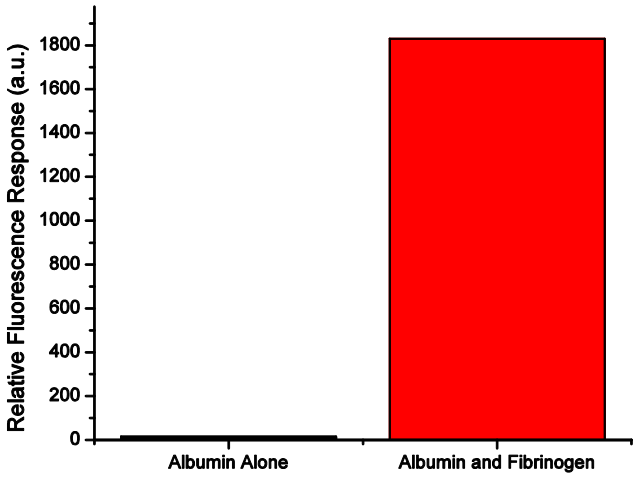

FIGURE VII.1: Fluorescent response of the ELISA run with albumin alone (left) and a mix of albumin and fibrinogen (right).

VII.2.b. Biocompatibility testing with whole blood

Testing individual proteins is an important first step in determining biocompatibility of NO releasing materials. As previously stressed, the first step in blood clot formation on synthetic materials is the adsorption of proteins to the material surface. Investigating the effect NO has on these proteins is an integral first step in determining why NO prevents blood clot formation on

blood contacting medical devices. We have already established that NO release from out model system has an effect on adsorbed fibrinogen, particularly on the amount adsorbed, conformation, and effect on platelet adhesion and activation. The studies described in the previous section will help determine the effect that NO has on how much protein is adsorbed for other clotting proteins (vonWillebrand factor and fibronectin) as well as on albumin, which is not involved in the coagulation cascade. To further these studies, the adsorbed protein systems must be tested further.

The first step would be to run platelet assays on these proteins, just as I ran on the fibrinogen coated materials. These experiments must be run on materials where individually adsorbed proteins or a mixture of all of the proteins has been allowed to adsorb prior to platelet incubation. Examining the amount of platelets adsorbed to these surfaces and the degree of platelet activation will give a broader picture of the biocompatibility of these model materials.

After the assessment of these materials with platelets alone, these systems must be tested under whole blood conditions. These tests should be performed both with and without pre-adsorbing the protein to the material surface. Pre-adsorption of proteins must be completed to assess its effect in a whole blood situation, despite the fact that these proteins will be present in the blood. It is also possible that our materials perform better when protein is pre-adsorbed to the material surface. Current literature suggests that adsorbing protein onto the surface of a biomaterial prior to insertion into the blood stream, may create a passivation layer that will prevent the fouling of the device due to blood clot formation.[20-22] Testing of the pre-adsorbed proteins will give insight into whether or not this is happening with our NO releasing materials. While we see certain results under our current research conditions, it may be that proteins feel a different effect from the NO release in whole blood than proteins in a buffer solution.

Blood has many components that can scavenge NO, particularly hemoglobin.[4,23] This scavenging could lead to a reduced amount of NO reaching the proteins and produce different effects. One way to test for this would be to utilize antibodies specific for different proteins and tag for those proteins after whole blood has been allowed to interact with the material. The challenge of this experiment would be the possibility of the antibody not being able to find the protein. After exposure to whole blood, the materials will likely be coated with more than just proteins, cells, platelets, and other biological components can also adsorb to the material. If this is a layered effect, protein close to the surface might be blocked from interaction with antibodies for tagging purposes. This is an issue we have already seen in our attempts to obtain a quantitative measure of fibrinogen adsorption to our model system. With more components available to adsorb to the material under whole blood conditions, this may become an even bigger challenge to overcome.

Overall, interactions of the materials with whole blood will be able to be imaged using scanning electron microscopy (SEM) to determine differences in coverage. Platelets and red blood cells are easily imaged using this technique. Thus, the activation and aggregation of platelets and their effect on the red blood cells can be imaged. Additionally, fluorescent confocal microscopy can be utilized to measure the thickness of any aggregation on the material surface. Another way to examine the thickness of biofilm coverage would be to apply the coatings of my model system onto a quartz crystal microbalance (QCM). This method is highly sensitive to changes in mass of the system. Films can be measured before and after incubation with whole blood to determine the mass of biological components on the surface of the material. Use of QCM may also be of use in determining thickness of adsorbed protein layers in the preliminary studies on protein solutions. The usage of SEM to look at aggregation and the determination of the thickness of these aggregates

using confocal microscopy or QCM, will give an overall picture of whether or not the NO releasing materials I developed as a model system will provide the improved biocompatibility seen with NO releasing materials in previous studies. Comparison between the model system with either pre-adsorbed fibrinogen alone or whole blood alone will indicate whether or not the proteins will adsorb similarly in both buffer systems and potential *in vivo* tests. This will also show whether the surface adsorbed protein provide a passivation layer when adsorbed to a surface in the presence of NO release.

VII.2.c. Biocompatibility testing under flow conditions

All of these assays discussed to this point have been run only under static conditions. We know based on previous research done in collaboration with the Neeves group at Colorado School of Mines that there is a gradient of NO release under flow conditions and this has an effect on the biocompatibility of the material.[24] The materials tested were diazeniumdiolated dibutylhexanediame (DBHD/N_2O_2) blended base coat with a single top coat layer applied on top. Despite a slight difference in sample preparation compared to the model system used in this work, the models used here can apply to any NO releasing polymer system. NO releasing layers were coated onto a glass slide and patterned with collagen prior to introduction of blood into the flow assay using a microfluidic device. Transport of the NO through the microchannel was determined computationally using a commercial finite element method software (COMSOL) by our collaborators at Mines and diagrammed in Figure VII.2. A model system to measure NO transport into the channel, at the wall, and through the platelet rich layer (PRL) was developed such that NO was released at a constant flux from the wall into a fluid where it can react with both hemoglobin and oxygen.[24] What we see is that the wall shear rate will actually vary the apparent concentration

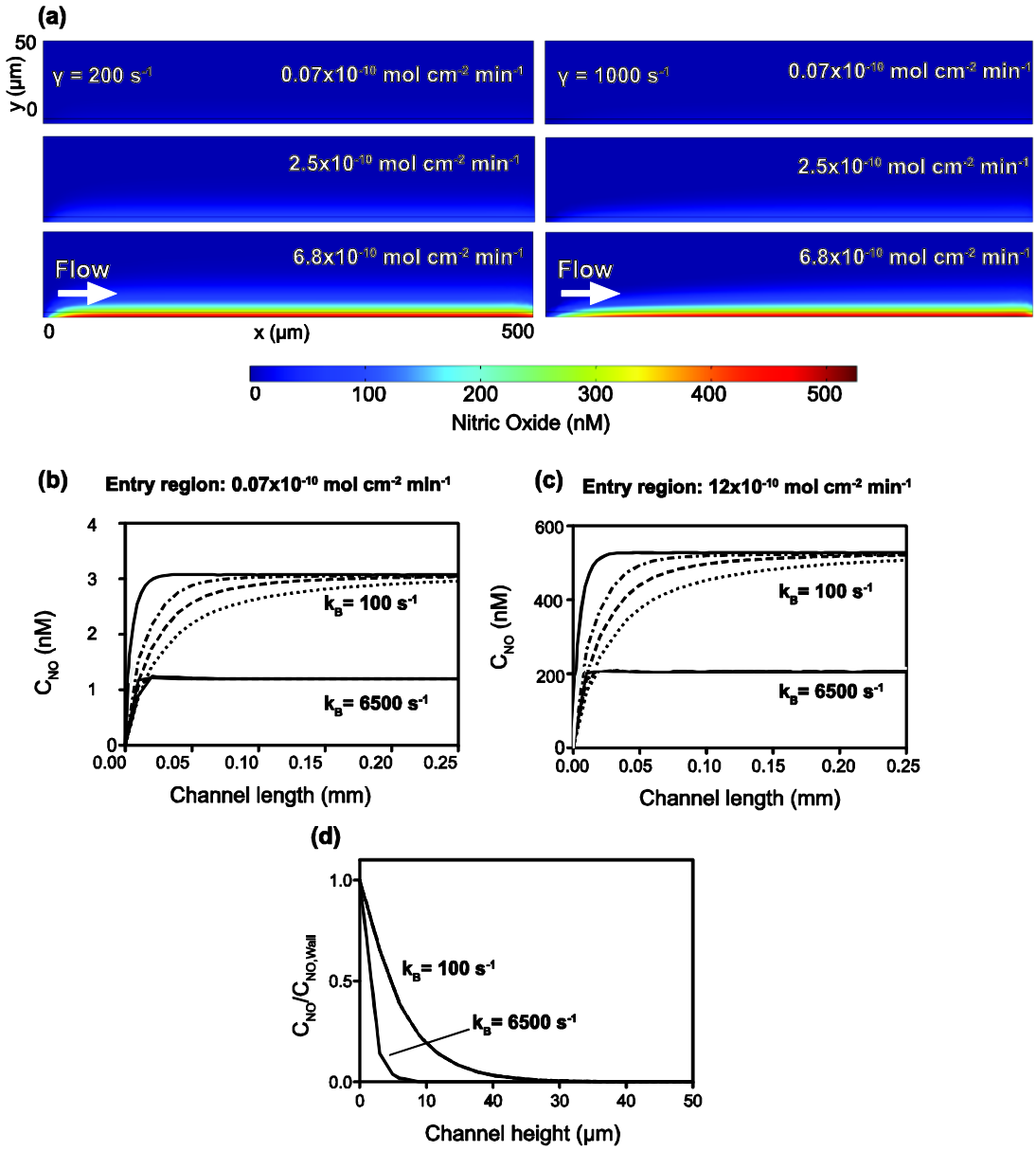

FIGURE VII.2: (a) NO distribution within the entry region of the channel as function of NO flux at shear rates of 200 and 1000 s^{-1} at kB = 100 s^{-1}. NO wall concentrations in the entry region for fluxes of 0.07 $\times 10^{-10}$ mol cm^{-2} min^{-1} (b) and 12 $\times 10^{-10}$ mol cm^{-2} min^{-1} (c), at shear rates of 200 s^{-1} (dotted dashed line), 500 s^{-1} (dashed line) and 1000 s^{-1} (dotted line). The solid line indicates static conditions. (d) Normalized NO concentration into the lumen of the channel (perpendicular to flow) at x = 5.5 mm as a function first order rate constant for elimination in the blood (kB). The profile in the height direction is effectively independent of shear rate. (Springer and Annals of Biomedical Engineering, 41, 2013, 2197, Transport Limitations of Nitric Oxide Inhibition of Platelet Aggregation under Flow, J. L. Sylman, S. M. Lantvit, M.C. VeDepo, M. M. Reynolds, K. B. Neeves, Figure 5, © 2013 Biomedical Engineering Society, with kind permission from Springer Science and Business Media.)

of NO seen over the length of the channel. As the solution continues to flow down the channel, the shear rate has less of an effect on the transport of NO (Figure VII.2b-c). For the k_B = 100 s^{-1} conditions, the wall shear rate had a greater effect on the NO concentration within the entry region, once past this, the NO concentration was independent of shear rate. Conditions with k_B = 6500 s^{-1} had no effect on the NO concentration whether under static conditions or different wall shear rates. Where k_B represents NO elimination by hemoglobin in red blood cells. Further, penetration into the channel was seen to be greater when k_B = 100 s^{-1} than k_B = 6500 s^{-1} as seen in Table VII.1 where the Peclet (Pe) number varies with different shear rates indicating that convection dominates over diffusion outside the momentum boundary layer. Pe is the relative rate of convection to the rate of diffusion within the system.

These studies show that the flow rate of the system will have an effect on the distribution of NO throughout a channel or vessel. This flow of NO is also seen to have an effect on platelet aggregation. In this same study done in conjunction with the Neeves group, whole blood was run over the NO releasing material. This material was patterned with collagen to induce platelet aggregation and blood clot formation. What was seen is, as the NO flux from the material increases, the size of the platelet aggregates decrease (Figure VII.3a-c). This indicates that the flow of NO, computationally mapped above, is interacting with the whole blood system and having an effect on platelets. As the shear rate increases, the size of aggregates continues to decrease (Figure VII.3a-c). Additionally, the thickness of these aggregates significantly decreases as the NO flux of the material increases regardless of the wall shear rate (Figure VII.3d).

TABLE VII.1. Experimental conditions and computational results from whole blood flow assays over NO releasing polymer films.

Wall shear rate (s^{-1})	Flux ($\times 10^{-10}$ mol cm^{-2} min^{-1})	Pe*	Effective penetration depth, λ (μm) k_B = 100 s^{-1}**	Effective penetration depth, λ (μm) k_B = 6500 s^{-1}**	Avg NO conc in PRL (nM) k_B = 100 s^{-1}***	Avg NO conc in PRL (nM) k_B = 6500 s^{-1}***
200	0.07	19	0	0	3	1
200	0.33	19	1.5	1.5	12	3
200	0.4	19	2.7	1.8	14	4
200	2.5	19	13	4.8	90	24
200	6.8	19	19	6.8	240	65
200	12	19	22	8.4	430	110
500	0.07	48	0	0	2.5	0.67
500	0.33	48	1.5	1.5	12	3
500	0.4	48	2.7	1.8	14	4
500	2.5	48	13	4.8	90	24
500	6.8	48	19	6.8	240	65
500	12	48	22	8.4	430	110
1000	0.07	1000	0	0	2.5	0.67
1000	0.33	1000	1.5	1.5	12	3
1000	0.4	1000	2.7	1.8	14	4
1000	2.5	1000	13	4.8	90	24
1000	6.8	1000	19	6.8	240	65
1000	12	1000	22	8.4	430	110

The NO flux from polymer films was measured experimentally. The effective penetration depth (λ) and the average NO concentration in PRL were calculated for simulations.

* The Pe number is calculated from Suppl. Eq. 14

** The effective penetration depth was found at x = 5.5 mm and was determined to be the depth in which concentration was greater than or equal to 12 and 3 nM for a k_B of 100 and 6500 s^{-1}, respectively. If the concentration of NO was less than these values, it was considered a subthreshold concentration to induce inhibition and is denoted by zero in the table.

*** The average concentration of NO in the PRL was determined over the range of x = 5-10 mm and y = 1-3 μm.

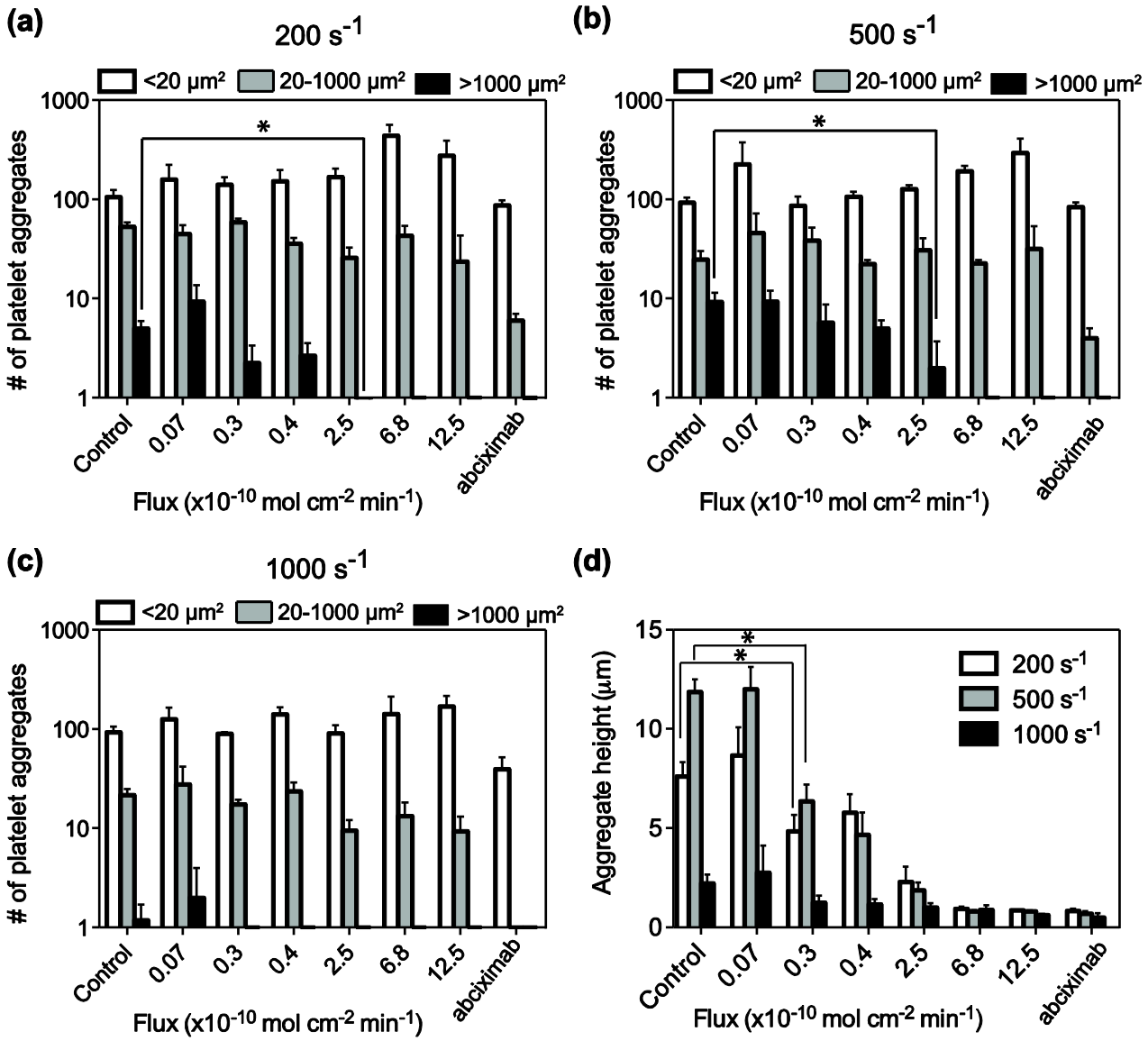

FIGURE VII.3: Platelet aggregates decrease in size with increasing NO flux. Platelet aggregate areas are categorized by size; <20 µm^2, 20–1000 and >1000 µm^2 for each NO flux at (a) 200, (b) 500 and (c) 1000 s^{-1}. (d) Platelet aggregate heights are shown at the same conditions as aggregate areas (n = 3–4 donors for all conditions). All conditions are compared to abciximab, representative of maximal platelet aggregation inhibition. Error bars indicate standard error of the mean across donors at each condition. Significant differences ($p < 0.05$) are denoted by asterisk. (Springer and Annals of Biomedical Engineering, 41, 2013, 2200, Transport Limitations of Nitric Oxide Inhibition of Platelet Aggregation under Flow, J. L. Sylman, S. M. Lantvit, M.C. VeDepo, M. M. Reynolds, K. B. Neeves, Figure 4, © 2013 Biomedical Engineering Society, with kind permission from Springer Science and Business Media.)

These studies show that not only does NO release have an effect on the ability of platelets to aggregate, but the shear rate of the solution will also play a role in this. We see in Figure VII.3a-c that the relative sizes of platelet aggregates varies dependent on the flow rate for different fluxes of NO. For instance, at a flux of 6.8 $\times 10^{-10}$ mol NO cm^{-2} min^{-1} the number of platelet aggregates that are <20 μm^2 decreases as the shear rate is increased from 200 s^{-1} to 1000 s^{-1}. Further, the shear rate can also be seen to have an effect on the height of the aggregates (Figure VII.3d). This effect is greater at lower fluxes of NO, however, there are still slight differences to be seen at the higher NO fluxes despite all shear rates having much shorter aggregates at these fluxes.

These same parameters can be applied to the model system that we explored in this dissertation. By using this established experimental set-up for microfluidic testing, films can be prepared in the manner that we have prepared them for my model system and tested for similar clotting properties. This set-up is specially designed to induce clot formation by patterning the material with collagen prior to flowing blood over the material. Because of this, the testing method that the Neeves group has developed is an excellent way to test the biocompatibility of a material before *in vivo* testing. In this case, if the NO releasing materials in my model system reduces clot formation under the conditions of this test, then we know that this is a viable system for preventing blood clot formation.

Ultimately these materials will be needed to be tested *in vivo*. Static testing can only be so helpful since blood is constantly in flow within the body. Using the flow system developed by the Neeves group is a good first step towards *in vivo* testing as it is under flow conditions. Altering the flow rate can give information on what might happen in the body during periods where blood flow may be changing it its rate. An additional step before *in vivo* testing would be the use of a flow loop (Figure VII.4). My model system is primarily plasticized poly(vinyl chloride) (PVC)

with an NO donor blended in the base layer. PVC is commonly used in the production of extracorporeal circuitry (ECC) systems. A flow loop set-up in a laboratory is similar to the use of ECC in that the blood is pumped around in a circuit using a peristaltic pump to mimic the pumping of blood done in the body. As the blood flows throughout the system, it can go into either a control, non-NO releasing material or tube coated in the same manner as my model system that releases NO. After running this system, the degree of clot formation within the tubes can be assessed. This can be done with an optical microscope or by fixing the clot onto the tubing and imaging with SEM.

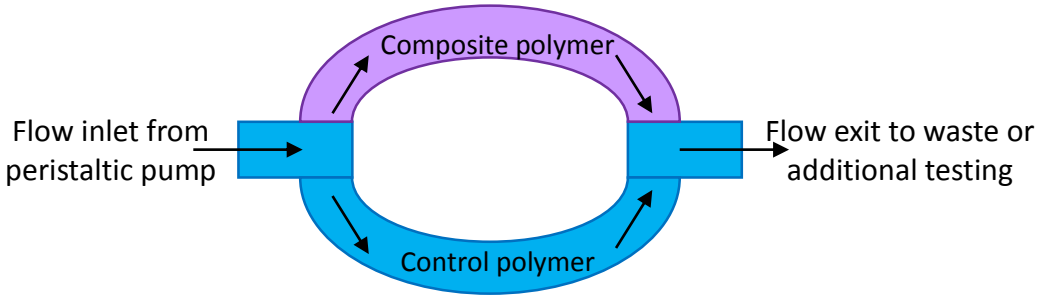

FIGURE VII.4: Model of flow loop set-up for testing whole blood compatibility with our material system. The composite and control polymers are tested at the same time with the flow of protein, plasma, or whole blood solution flowing continuously through the loop.

Once these preliminary tests have been completed, the coating of medical devices such as ECC systems in the same manner as my model system should be completed. These devices can then be tested *in vivo*. The original literature study that led me to question the effect of NO on fibrinogen and develop this model system also ran *in vivo* testing on their coated ECC system using a rabbit model.[16] It is reasonable to refer back to this study completed by the Bartlett group to examine their *in vivo* techniques. Testing materials prepared in the same manner as my model system using their methods will give a good experimental basis for comparing the results with my

materials to those previously found with a system that did not maintain the material's surface properties. This will allow the actual biocompatibility of my model system to be fully assessed.

VII.3 Final Statements

Over the course of this dissertation, I have shown that surface released NO has an effect on fibrinogen as it adsorbs to a material surface. Initially I hypothesized that NO releasing materials would have overall less fibrinogen coverage and less platelet activation and adhesion than the control material (Chapter I, Figure I.5). I was able to develop a model system where the effect of NO was isolated by having a system where surface wettability, roughness, and composition is comparable across all control and composite materials. Interestingly, when this model system was tested for fibrinogen adsorption and it was seen that a significantly greater amount of fibrinogen was adsorbed to the surface of the high NO flux material compared to the control and low NO flux materials (Figure VII.5).[25] The manner in which the fibrinogen adsorbed to the material surface made quantitative measurements of this protein presents an ongoing challenge for this research. I have determined that the amount of fibrinogen adsorbed onto the high NO flux materials is enough that there is more than a monolayer present. We can tell that such a high amount of protein adsorbs based on the intensity of the amide I and II peaks in the FTIR spectra. While we can see that protein is adsorbed to the control and low NO flux material, it is not enough to be able to determine structural features from the spectra without subtracting the polymer itself. FTIR data comparing the amide I and II peaks of fibrinogen adsorbed to any material and native fibrinogen shows distinct changes in conformation. Further, both high and low NO flux materials displayed a difference in conformation from the control, non-NO releasing material. This indicates that the NO release may induce different conformation upon adsorption

than what happens on the control material. Contrary to initial my initial hypothesis, all materials exhibited statistically the same amount of platelet coverage and activation as seen in Figure VII.5. Trends in the data suggest that the combination of NO release and pre-adsorbed fibrinogen may be more biocompatible as they trended toward less platelet coverage and activation. Overall, the results point toward NO release having a significant effect on the amount of fibrinogen adsorbed to the material surface. Additionally, the conformation of the fibrinogen changes upon adsorption to all materials with slight differences in the presence of NO. Platelet adhesion and activation to these fibrinogen coated surfaces does not differ, indicating that any changes in fibrinogen's conformation due to NO release will not have an effect on platelets. Adsorbed fibrinogen may not be the reason that NO releasing materials have previously been found to prevent blood clot formation *in vivo*. Repetition of the conformation and platelet studies are necessary to confirm these findings.

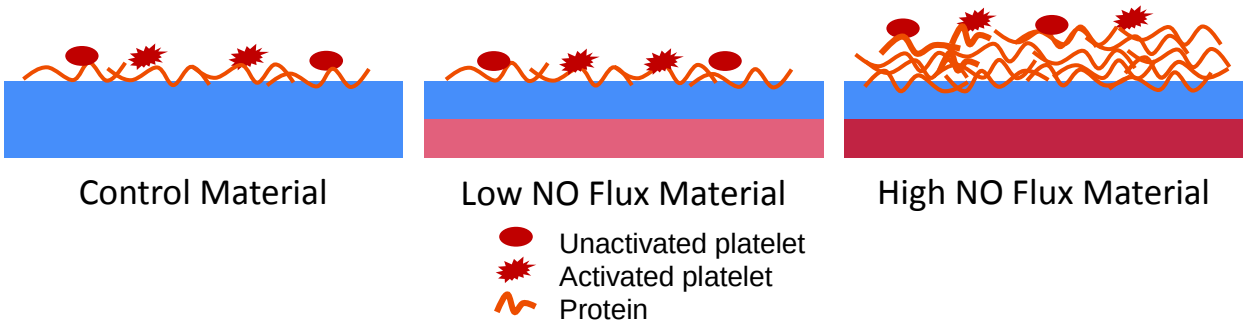

FIGURE VII.5: General findings of this dissertation. The high NO flux material (right) displayed the greatest amount of fibrinogen adsorption across all samples. Another NO releasing material, the low NO flux system (middle), showed significantly less fibrinogen adsorption which was closer to the amount adsorbed to the control material (left). Interestingly all materials exhibited statistically the same amount of platelet adhesion and activation despite differences in amount of fibrinogen and slight conformational changes found using FTIR.

To further the studies performed for this dissertation, these films must be studied to determine the effect NO may have on other proteins, specifically vonWillebrand factor,

fibronectin, and albumin. These proteins must be tested alone and in conjunction with each other and fibrinogen to determine competitive protein adsorption. Once the effect of NO on these proteins has been established, testing must be performed using whole blood in both static and flow conditions. Two flow conditions present valid methods of looking at this material, the collagen patterned microfluidic device designed by the Neeves group, and a blood flow loop. These studies will provide a good basis for assessing the biocompatibility of the system before moving to *in vivo* testing. In the end, to ensure that the materials I developed for my model system perform similarly to previous NO releasing material systems, they must be tested *in vivo*.

Testing my model system must be done in steps. To really understand why NO release prevents blood clot formation when added to polymeric materials, each step of the coagulation cascade must be investigated. My initial testing presented in this dissertation for NO's effect on fibrinogen is a fundamental starting point. Protein is the first to adsorb to synthetic devices when they are placed into biological systems. Fibrinogen, being a primary protein involved in blood clot formation is a good starting point in assessing the effect NO has on each step of the clotting process. Use of my model system eliminates all other variables except for NO release, providing a platform for determining localized NO release's effect on the stages of blood clot formation. Once each step is analyzed a better picture as to why NO release makes materials more biocompatible can be realized.

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