INFLUENCE OF SURFACE COMPOSITION AND WETTABILITY UPON
CONTACT ACTIVATION OF BLOOD PLASMA

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by

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ABSTRACT

Coagulation resulting from contact activation due to blood-material interactions remains a challenge in the use of blood-contacting medical devices. Our group is investigating the protein-surface interactions influencing material-induced blood plasma coagulation with the goal of contributing towards the rational development of biomaterials with improved hemocompatibility. Traditional biochemical theory of contact activation imparts specific activating abilities to negatively-charged hydrophilic surface. However, recent studies have indicated a need to revise this view as activation of blood factor XII (FXII), a contact activation protein, is not specific to negatively-charged surfaces. The central hypothesis of this dissertation proposes that all material surfaces are potential activators of the intrinsic coagulation cascade, but protein adsorption moderates the molecular interactions of contact activation. Measurements of FXII activation in buffer solutions in the presence of mixed thiol self-assembled monolayers suggest a minimum of FXII activation for surfaces with wettabilities nearing 45 dyne/cm. From direct measurement of kallikrein activation at test surfaces we observed that contact activation reactions involving FXII, high-molecular weight kininogen, and prekallikrein are not specific to negatively-charged hydrophilic surfaces, lending further support for the role of protein adsorption-competition at procoagulant surfaces. Preliminary investigations into contact activation of blood plasma coagulation patterned thiol-modified surfaces suggest spatial chemical heterogeneity influences blood plasma coagulation. In summary, the studies described in this dissertation underscore the need to view all materials as potential activators of the intrinsic coagulation cascade with protein adsorption moderating interactions of contact activation proteins.
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Chapter 1
Introduction

The use of blood-contacting medical devices is prevalent in the clinical setting, with uses ranging from routine blood collection to more invasive procedures, such as percutaneous coronary interventions and cardiopulmonary bypass. Thrombosis due to interaction of blood with blood-contacting materials remains a significant risk, necessitating the use of anticoagulants which have their own associated risk of uncontrolled bleeding (Rose, Gelijns et al. 2001; Copeland, Smith et al. 2004; Pae, Connell et al. 2007). Approximately 84% of 660,000 hospitalized patients who underwent coronary angioplasty in 2003 received a stent (Thom 2006) and in the year 2000 it was estimated that about 1000 left-ventricular assist devices (LVAD) were implanted in patients in the United States with thousands more that may have benefited from LVAD use (Lysaght and O'Loughlin 2000). As more blood-contacting medical devices are being increasingly looked at to serve long-term purposes, e.g. ventricular assist devices for bridge-to-recovery or bridge to transplant applications (Ueno, Bergin et al. 2000; Hunt, Abraham et al. 2005; Birks, Tansley et al. 2006; Topkara, Dang et al. 2006), biomaterial thrombogenic potential is increasingly important (Miller, Pagani et al. 2007; Nielsen, Kirklin et al. 2008; Achneck, Sileshi et al. 2010). In this section the mechanisms of thrombosis will be briefly discussed, with emphasis on the intrinsic coagulation cascade. Protein adsorption will also be discussed, as it contributes significantly to the interaction of the proteins of the intrinsic coagulation cascade with a biomaterial surface.
1.1 Hemostasis Overview

There are five important components of the vasculature’s ability to balance procoagulant and anticoagulant processes in promoting hemostasis: the vascular endothelium, platelets, blood coagulation factors, blood coagulation inhibitors, and fibrinolysis. In response to injury to the vasculature platelets, the cellular component of hemostasis, serve to form mechanical barriers preventing further loss of blood from the vessel. The protein factors of the blood coagulation cascade serve to amplify the signal of the injury culminating in the production of thrombin, and consequently, the formation of fibrin from plasma fibrinogen. A fibrin mesh is created which ensnares and interacts with platelet aggregates, thus strengthening the mechanical plug, the thrombus. Platelets and the blood coagulation cascade work synergistically, as each serves to activate the other; the membranes of activated platelets being cofactors for tenase and prothrombinase complexes of blood coagulation (Zwaal, Comfurius et al. 1998), and thrombin being a potent activator of platelets. To limit the effect of thrombin to the site of injury in vivo, important coagulation factor inhibitors exist, including tissue factor pathway inhibitor (tFPI), protein C, and protein S. Fibrinolysis is important in limiting thrombus formation by preventing excessive fibrin formation (Bachmann 2001), allowing subsequent repair mechanisms to proceed. In fibrinolysis, the plasma protein plasminogen is activated, forming the serine protease plasmin which proceeds to degrade the fibrin mesh.
1.1.1 Blood Coagulation

The blood coagulation cascades consist of a series of enzyme activation steps which culminate in the production of fibrin. Traditionally, the cascades have been grouped into the extrinsic and intrinsic pathways, which both lead into the common pathway. However, the extrinsic and intrinsic pathways are not completely independent and there are a variety of feedback interactions. Figure 1-1 shows a simplified diagram of the cascades.

As shown in Figure 1-1, either the extrinsic or intrinsic pathway can activate factor X (FX). FXa then activates prothrombin to produce thrombin. Thrombin cleaves fibrinogen into monomers of fibrin which polymerize into fibrin strands. The zymogen factor XIII (FXIII) is activated by thrombin and promotes covalent crosslinking of fibrin strands. The extrinsic pathway is the principle pathway initiated in vivo, as with vascular trauma (Colman, Clowes et al. 2001). Tissue factor (TF) is an intrinsic membrane protein of sub-endothelial cells. When blood plasma comes in contact with injured vessel endothelium, exposed TF binds with plasma coagulation factor VII (FVII) and forms a TF-FVIIa complex. This complex can activate factor XI (FXI) and factor X (FX), thus leading to the common pathway.
1.1.2 Contact Activation

The intrinsic pathway results in the activation of factor IX (FIX) which in turn activates FX, thus entering the common pathway. The intrinsic pathway is initiated when the blood plasma comes in contact with a non-physiologic surface, mediating the activation of FXII, hence “contact activation.” FXIIa then activates FXI (Bouma and Griffin 1977) which activates FIX. Prekallikrein (PK) and high-molecular weight kininogen (HK) are also important actors in contact activation. FXI and PK each circulate in plasma as a complex with HK. It is believed that HK protects these proteins from C1H-inhibitor and other plasma...
inhibitors (Schapira, Scott et al. 1981; Scott, Schapira et al. 1982), and has other attributes which will be discussed shortly.

The most common widespread view of contact activation imparts FXII and HK activating abilities specific to anionic surfaces, such as glass, kaolin, dextran sulfate, etc (Colman and Schmaier 1997). Binding of FXII to anionic surfaces via “anionic binding domains” rich in positive-charged amino acids induces activation of FXII to FXIIa, often termed “autoactivation”. FXIIa consists of two chains, a heavy and light chain, connected via a disulfide bond. The light chain contains a serine protease containing the typical aspartate-histidine-serine catalytic triad, typical of chymotrypsin-like proteases (Cool, Edgell et al. 1985; Hedstrom 2002). HK also possesses anionic binding domains (Kunapuli, DeLa Cadena et al. 1993) which are believed to help bring PK and FXI into an orientation favorable to activation (Griffin and Cochrane 1976; Meier, Thompson et al. 1977; Wiggins, Bouma et al. 1977). Surface-generated FXIIa activates PK complexed with HK at the surface (Griffin 1978) and the resulting kallikrein can activate FXII (Silverberg, Dunn et al. 1980) such that there is reciprocal-activation. However, it has been recently observed that Kal-mediated FXII activation (reciprocal activation) is the principal pathway for FXII activation, producing 75% of the FXIIa yield within the intrinsic pathway and is proportional to the initiating amount of FXIIa produced through autoactivation (Chatterjee, Guo et al. 2009). Further studies indicate that the role of the surface in contact activation may be confined to the autoactivation of FXII (Chatterjee, Vogler et al. 2006). Notably, in Chatterjee et al (Chatterjee, Thornton et al. 2009), chromogenic assays of FXIIa-mediated PK hydrolysis indicate that an anionic, hydrophilic surface is not a necessary cofactor for PK hydrolysis and that prekallikrein hydrolysis is not localized to an activation complex on the
procoagulant surface. This study also demonstrated that protein-adsorption competition is an important facet of blood plasma coagulation as the introduction of a competitive protein, bovine serum albumin, to octadecyltrichlorosilane-treated glass vials containing FXII and PK in PBS resulted in a rapid increase in Kal production, likely due to the displacement of adsorbed zyomogens, or their activated enzymes, from the hydrophobic surface. However, the role of HK was not addressed in Chatterjee et al and is a focus of study in Chapter 3.

1.1.3 Fibrinolysis

Thrombosis and coagulation are major risks long associated with the use of blood-contacting biomaterials. However, despite the prevalent use of these materials, a comprehensive and reliable model describing the role of the material surface in blood plasma coagulation has yet to be established even as much investigation is focused upon the intrinsic pathway. However, fibrinolysis is also an important part of hemostasis as it serves to limit thrombus formation by preventing excessive fibrin formation through the action of plasmin. Plasmin is a serine protease resulting from the activation of plasminogen, which is synthesized in the liver and is present in the bloodstream at a concentration of approximately 2.4 µM (Robbins and Summaria 1976). Plasmin is capable of hydrolyzing fibrinogen, non-crosslinked fibrin, crosslinked fibrin (Figure 1-2), as well as other plasma proteins. During fibrinolysis fibrin degradation products are produced, including a crosslinked derivative known as D-Dimer that can be distinguished from fibrinogen degradation products (Francis and Marder 2001). The presence or absence of this unique crosslinked derivative can have
important clinical implications in the evaluation of thrombotic risk or monitoring the effectiveness of anticoagulant therapy.

It is well known that plasminogen has a strong affinity for lysine residues (Deutsch and Mertz 1970; Bachmann 2001) and that the ε-amino group of terminal lysyl residues in fibrin is important in their interaction (Christensen 1984; Warkentin, Johansen et al. 1998). This property of plasminogen has been utilized in the development of biomaterial surfaces with fibrinolytic properties (Woodhouse and Brash 1994; Fowers and Kopecek 1997; McClung, Clapper et al. 2000; McClung, Clapper et al. 2003; McClung, Babcock et al. 2007) so that nascent clots might be lyzed before they become large enough to become pathologic. FXIIa is capable of activating plasminogen (Schousboe 1997; Schousboe, Feddersen et al. 1999), as well as the physiologic activators tissue-type and urokinase type plasminogen activator (t-PA and u-PA, respectively), making this a logical approach for creating biomaterials with better blood compatibility, although the practicality of such an approach is still in question.
1.2 Relevance of protein adsorption to contact activation

The response elicited when a biological fluid, such as blood plasma, is brought into contact with a material is influenced by the properties of the vicinal water. Although protein adsorption may often be the response of interest, there are preceding interactions with the surface, such as surface hydration, electrostatic double-layer formation, or hydrophobic interactions (Andrade and Hlady 1986; Vogler 1999). Protein adsorption to solid surfaces is an important concern for many applications, with blood-contacting biomaterials being no exception. As mentioned previously, the current widespread view of contact activation imparts functionality to FXII and HK that is specific to anionic-hydrophilic surfaces. However, less attention has been given to other factors involved in protein adsorption and contact activation; such as the roles of the solvent and adsorption competition, and their
possible effects on contact activation (Vogler 2001; Zhuo, Siedlecki et al. 2007; Vogler and Siedlecki 2009).

The investigation of protein adsorption has borne many models but few overarching principles, and as such a comprehensive review is not practical here. For the purpose of this dissertation, protein adsorption will be viewed from a Guggenheim perspective (Guggenheim 1967; Butt, Graf et al. 2006), from which a three-dimensional interphase is envisioned between the biomaterial solid phase and the bulk solvent, which in most, if not all, biological systems is water. This is in contrast to the Langmuirian model of protein adsorption in which the adsorption of molecules to a surface occurs at specific sites, forming a monolayer. These sites are limited in number and selective. In multiple-protein solutions, the bioselectivity of these specific sites influences the monolayer composition during adsorption. Furthermore, the composition of the monolayer may change over time, i.e. the “Vroman effect”, due to this competitive adsorption of proteins for the finite availability of adsorption sites at the surface (Slack and Horbett 1995). This is not to be confused with an earlier interpretation of the Vroman effect, in which fibrinogen adsorbed to a negatively-charged surface such as glass or kaolin is displaced or undergoes a “conversion” through some action of HK (Vroman, Adams et al. 1980; Scott, Silver et al. 1984). Indeed, several studies have shown that plasma proteins other than fibrinogen show peak adsorption behavior from multiple-protein mixtures (Brash and Tenhove 1984; Breemhaar, Brinkman et al. 1984; Horbett and Schway 1988).

Recent developments concerning protein adsorption utilizing the Guggenheim perspective have yielded important insights into contact activation. Krishnan et al (Krishnan, Siedlecki et al. 2003) proposes that protein molecules adsorb to the interphase according to a homology of molecular size of the protein, such that smaller proteins would fill a single layer
while larger proteins would form multiple layers. It was observed that plasma proteins with molecular weights spanning 10-1000 kDa, including those proteins involved in contact activation, exhibit similar trends in protein adsorption, thus giving credence to the concept that dehydration of the vicinal surface water is the driving force for protein adsorption (Krishnan, Liu et al. 2005). Solvent-solvent interactions also have a role in selective protein adsorption which is largely influenced by the size of the protein. The interphase between a hydrophobic surface and bulk solvent (water) has a limited capacity for proteins. As a result, smaller proteins are present in larger numbers compared to larger proteins, since fewer larger proteins are needed to obtain the same interfacial concentration (weight per volume) as the smaller proteins (Noh and Vogler 2007). It has also been shown the FXII autoactivation occurs at nearly equal efficiency upon contact with either hydrophobic or anionic-hydrophilic surfaces in neat buffer solutions (Zhuo, Siedlecki et al. 2006). However, FXIIa yield, arising from FXII interaction with hydrophilic glass beads, was observed to be greater in buffer solution containing plasma proteins unrelated to contact activation (i.e. IgG, IgM, albumin) than in neat buffer solutions. Conversely, FXIIa yield, arising from FXII interaction with hydrophobic silanized-glass beads, was observed to be lower in buffer solution containing plasma proteins unrelated to contact activation than in neat buffer solutions (Zhuo, Siedlecki et al. 2007). Thus the presence of these proteins leads to a diminution of FXII activation at hydrophobic surfaces, giving an apparent specificity of FXII activation to anionic, hydrophilic surfaces. As the interphase would have a limited capacity to accommodate proteins, there would be competitive adsorption (Noh and Vogler 2007). The selective pressure exerted by this competitive adsorption is consistent with the observations made by Vogler and coworkers (Krishnan, Siedlecki et al. 2004; Krishnan, Sturgeon et al. 2004) who
propose that protein concentration measured in weight per unit volume is the significant energetic driver of adsorption. It is reasoned that the reduced efficiency of FXII activation at hydrophobic surfaces is due to adsorption competition between FXII and other plasma proteins (Zhuo, Siedlecki et al. 2006; Zhuo, Siedlecki et al. 2007; Vogler and Siedlecki 2009).

1.3 Thiol Self-Assembled Monolayers and Surface Wettability

In order to directly ascertain the effect of wettability on the adsorption or activity of the various components of blood plasma it is desirable to utilize a series of materials spanning a range of wettability from hydrophobic to hydrophilic. Self-assembled monolayers (SAMs) utilizing alkanethiols adsorbed to smooth gold surfaces are very practical in this purpose, as various functional groups can be utilized and surfaces of mixed hydrophobic and hydrophilic character can be created. Surfaces with mixed hydrophilic and hydrophobic character have recently been used in the study of complement deposition (Hirata, Hioko et al. 2003), human serum albumin adsorption and competition with fibrinogen (Martins, Ratner et al. 2003), as well as fibrinogen adsorption and platelet adhesion and activation (Rodrigues, Goncalves et al. 2006). Mixed thiol SAMs also are prominent in the study of contact activation in Chapter 2 of this dissertation.

In the surface dose-response relationships of biomaterials, four factors are prominent: surface area, surface energy, surface chemistry, and surface roughness (Vogler 2001). Mixed thiol SAMs allow for the convenient study of the first two, as the surface energy of a two-component mixed thiol monolayer can be varied by adjusting the proportion of the thiol components present on the surface without introducing any new chemically different
functional groups. In Martins et al (Martins, Ratner et al. 2003) and Rodriguez et al (Rodrigues, Goncalves et al. 2006), studies of human serum albumin and human fibrinogen adsorption competition onto mixed hydroxyl-/methyl-terminated thiol SAMs indicates that surfaces with mid-range wettability exhibit increased albumin adsorption and decreased fibrinogen adsorption. Regarding contact activation, and specifically Factor XII, Golas et al (Golas, Parhi et al. 2010) investigated contact activation of factor XII in phosphate-buffered saline using silane-modified glass beads as the procoagulant. The results of the study suggest a range of minimal activation of the intrinsic coagulation pathway falling within 20-40 dyne/cm surface energy. However, as the procoagulant used were silane-modified beads, different functionalities were introduced when producing procoagulants with differing wettabilities (i.e. aminopropyltrietoxysilane versus vinyltriethoxysilane). Chapter 2 investigates whether these trends persist when using mixed thiol self-assembled monolayers, as their use allows for the surface energy of a two-component mixed thiol monolayer to be varied by adjusting the proportion of the thiol components present on the surface without introducing any new chemically different functional groups, thus limiting the array of chemistries present over the range of surface energy investigated.

1.4 Central Hypothesis

The current widespread view of contact activation imparts coagulation factor XII (FXII) activating abilities specific to anionic hydrophilic surfaces. Literature suggests that an activation complex forms on anionic hydrophilic surfaces, where FXII is activated to FXIIa which then activates prekallikrein to kallikrein and coagulation factor XI (FXI) to FXIa. However, previous studies observed that FXII activation is not specific to anionic
hydrophilic surfaces and PK hydrolysis does not require a surface-located activation complex (Zhuo, Siedlecki et al. 2006; Zhuo, Siedlecki et al. 2007; Chatterjee, Thornton et al. 2009).

As mentioned previously, surface chemistry and surface energy are important factors in the surface area dose-response relationship of blood-contacting biomaterials. It is clear that the anionic-hydrophilic surface chemistry is insufficient to usefully describe contact activation, especially as it seems to be at odds with the general principle that proteins generally adsorb more efficiently to hydrophobic surfaces than hydrophilic surfaces (Brash and Horbett 1995; Noh and Vogler 2006). In Xu and Siedlecki (Xu and Siedlecki 2007) a step-wise transition is demonstrated between protein adsorbant and protein non-adsorbant materials in the range of 60-65° contact angles for bovine serum albumin, fibrinogen and human FXII. This step is consistent with other studies (Berg, Eriksson et al. 1994; Vogler 1999; Sethuraman, Han et al. 2004) suggesting that efforts to create fully wettable surfaces to prevent protein adsorption may be unnecessary. However, as was demonstrated in Golas et al (Golas, Parhi et al. 2010) fully-wettable surfaces may entail adverse effects on biocompatibility in terms of FXII activation. Thus there is a need to further explore the role of the surface in contact activation in order to develop and refine a new paradigm which considers the role of protein adsorption and surface wettability, providing new insights into developing surfaces with improved hemocompatibility. This leads to the central hypothesis of this work which is:

*All material surfaces are potential activators of the intrinsic coagulation cascade, but protein adsorption moderates the molecular interactions of contact activation.*
1.5 Significance

The current view of contact activation is limited in its usefulness as a model to create biomaterials with better biocompatibility in regard to preventing the initiation of blood coagulation. In effect, the only guideline proffered is to avoid the use of anionic-hydrophilic materials. However, the use of hydrophobic materials does not eliminate the initiation of blood coagulation nor thrombus formation, as evidenced by the need for anticoagulation when using such blood-contacting materials in vivo. By investigating the role of the surface and protein adsorption, it is hoped that better models of biomaterial induced coagulation may be constructed which may then help in the development of blood-contacting biomaterials and applications, such as biosensors, artificial organ replacement, dialysis, etc.
1.6 Citations


Chapter 2

Contact activation of Hageman Factor XII and blood plasma coagulation by mixed thiol self-assembled monolayers

Abstract

A minimum for FXII activation in neat buffer solutions is seen for mixed thiol-adsorbed SAMs having water adhesion tension, $\tau = 45-50$ dyne/cm. One- and two-component thiol-modified surfaces were prepared and utilized as the procoagulant material for in vitro coagulation assays as well as FXII activation in neat buffer solution. Mixed carboxyl/methyl-, hydroxyl/methyl-, and amine/methyl-thiol modified surfaces were prepared by immersing gold-coated coverslips in 1mM of thiol in ethanol solutions for 24 hours. XPS was utilized to verify the sample surface’s thiol composition and contact angles measured to determine the sample surface’s wettability. These samples were then used in in vitro coagulation assays using a 50% mixture of recalcified plasma in phosphate buffered saline. Alternatively, the samples were placed into purified FXII solutions for 30 minutes to assess FXII activation in neat buffer solution. While plasma coagulation studies support a role for anionic surfaces in contact activation, the results suggest FXII activation in neat buffer solution is related to surface wettability, with a minimum observed at mid-range wettabilities, and no statistically distinguishable differences in FXII activation seen between pure carboxyl- and pure hydroxyl-terminated surfaces.
Contact activation of blood plasma coagulation by mixed thiol self-assembled monolayers

2.1. Introduction

It is well known in hematology laboratories that blood and blood plasma coagulates (clots) in hydrophobic plastic test tubes more slowly than in hydrophilic glass test tubes. This common knowledge, together with the uncommon observation that blood of a particular patient, John Hageman, did not clot quickly in glass tubes, led Oscar Ratnoff to discover the contact activation system of blood plasma coagulation. Intense research following Ratnoff’s discoveries, continuing to this day, led to elaboration of the biochemistry of the plasma coagulation cascade and formulation of a biochemical mechanism for contact activation. This mechanism proposed that the zymogen Hageman factor (FXII) binds to anionic hydrophilic surfaces through chemically-specific interactions that somehow cleave a disulfide bridge in FXII, creating the active protease αFXIIa. FXIIa subsequently potentiates the series of zymogen-enzyme conversions that comprise the plasma coagulation cascade. Thus it was explained that hydrophobic plastic tubes bearing no surface-resident anionic functionalities do not efficiently activate FXII, causing and blood and blood plasma to clot more slowly than blood or blood plasma contained in hydrophilic glass tubes with surface-resident anionic groups (see ref. (Vogler and Siedlecki 2009) and the many citations therein for a recent review that supports this section).

Subsequent work in our laboratories developed mathematical models of plasma coagulation that could be statistically fit to experimental data measuring decrease in plasma coagulation time (CT) as a function of activator surface area (so-called surface area titrations). A variable parameter of these models quantified the “catalytic potential” of
activator materials (a.k.a. procoagulants) (Vogler, Graper et al. 1995; Vogler, Graper et al. 1995; Zhuo, Miller et al. 2005; Guo, Bussard et al. 2006). Using oxidized polymers and silanized glass particles spanning a range of surface energies (water wettability), it was shown that catalytic potential followed a systematic trend with surface energy, low for poorly-water-wettable (hydrophobic) materials and rising sharply with increasing hydrophilicity. These findings were in complete accord with the idea that FXII bound to hydrophilic anionic surfaces through chemically-specific interactions. Similar data obtained using glass discs bearing silane self-assembled monolayers (SAMs) generally followed this trend but with some interesting departures (Vogler, Graper et al. 1995). Notably, carboxyl-terminated SAMs were much more activating than anticipated on a surface energy basis and cationic ammonium-terminated SAMs less activating than anticipated on a surface energy basis. The technical difficulty of producing activators with different well-defined chemistry prevented a thorough study of surface-chemical specificity in contact activation of blood plasma. The matter of chemical specificity was effectively dropped from our hands until activation experiments performed in neat buffer solutions of FXII showed that FXII activation was not, in fact, chemically-specific for anionic hydrophilic surfaces (Zhuo, Siedlecki et al. 2006).

Indeed, it has been shown that FXII activation in neat buffer solution exhibits a parabolic response to activator surface energy, high at both hydrophobic and hydrophilic extremes of water wettability and falling through a minimum over a range of intermediate surface energy (Golas, Parhi et al. 2010). Stated more exactly, the yield of activated fragments with procoagulant properties in plasma exhibits a parabolic response to activator surface energy. This important refinement reflects a growing suspicion that different FXII
activated fragments, possibly many different activated fragments, are produced by contact with an activator surface, only some of which may have procoagulant properties in plasma (Golas, Parhi et al. 2010). This growing suspicion again raises the question of surface-chemical specificity in contact activation: do different activator surface chemistries with the same nominal surface energy produce different yield of activated fragments with procoagulant properties in plasma?

This chapter investigates this question by measuring activation of both blood plasma and FXII in neat buffer solution by activator surfaces bearing well-defined surface chemistry. Activator surfaces were prepared using thiol adsorption onto gold substrata, creating SAMs with mixed surface chemistry by co-adsorption of two different thiols with different terminal functional groups over a broad range of binary-pair compositions. Measurements reveal a heretofore unobserved chemical specificity in surface activation of FXII which may lead to new surface engineering routes to hemocompatible biomaterials.

2.2. Materials and Methods

This work measured activation of FXII and blood plasma by SAM surfaces supported on a gold-coated rectangular glass coverslips. Single coverslips were immersed in solutions contained in a plastic UV-Vis spectrophotometer cuvette. A drawback of this approach compared to previous studies using either silanized glass particles or silane-SAM glass coverslips (see Section 2.1) was that activator surface area was comparatively low. However, the advantage of this system was the control over surface chemistry afforded by the thiol-on-gold system and the ability to engineer surfaces with a controlled gradient of surface chemistries using mixed thiols (Ulman 1996; Hirata, Hioko et al. 2003; Martins,
Ratner et al. 2003; Rodrigues, Goncalves et al. 2006). The following sections are organized to detail methods of surface engineering and measurement of calibration curves that quantified coagulation time results in terms of FXIIa yield. Section 3 characterizes activator surfaces and quantifies limits of detection.

2.2.1. Gold Substrates and Monolayer Formation

A 10 nm titanium adhesion layer and 200 nm gold layer were sputter coated (Thin Films, Inc., NJ) onto cleaned borosilicate glass coverslips measuring 10.5mm x 22mm. These substrates were used in the preparation of all thiol self-assembled monolayers. Dodecanethiol (Sigma-Aldrich), 11-mercaptoundecanoic acid (Sigma-Aldrich), and 11-mercaptoundecanol (Sigma-Aldrich) were used as received. 1 mM thiol solutions in anhydrous ethanol were used with mixtures made by varying the ratios of the single-component solutions. Gold substrates were cleaned extensively before monolayer formation. First, the gold substrates were immersed in gold cleaning solution (Sigma-Aldrich) for 3 minutes followed by 3 rinses using deionized water (Millipore, 18.2 mΩ) and 3 rinses using ethanol. Next the samples were air-plasma cleaned, 15 minutes per side (100 W, Harrick) followed by 3 rinses with ethanol. The samples were allowed to sit in ethanol for at least 1 hour to allow more than sufficient time for the gold surface to become reduced (Ron and Rubinstein 1994; Ron, Matlis et al. 1998). Finally the samples were immersed in a 0.125% (v/v) of butyltrichlorosilane (Gelest) in chloroform (Sigma-Aldrich) for 15 minutes, and then rinsed with chloroform and subsequently ethanol. This step was included to block any exposed glass due to insufficient gold coating of the coverslip edges or inadvertent scratching from handling the samples.
Prepped substrates were immersed in 1 mM thiol solutions at room temperature for 20 hours, protected from light. Stock solutions of 1 mM 11-mercaptoundecanoic acid and 11-amino-1-undecanethiol were prepared as noted above with the addition of trifluoroacetic acid or triethylamine, respectively, to 2.5% (v/v) (Wang, Chen et al. 2005). After incubation, the samples were rinsed with ethanol, with the exception of samples exposed to the 11-mercaptoundecanoic acid or 11-amino-1-undecanethiol solutions, for which a solution of 0.1 M NaOH or 0.1 M HCl, respectively, in deionized water was used in lieu of ethanol in an intermediate rinse. All samples were dried under N₂ and stored under vacuum, protected from light, for subsequent use.

2.2.2. SAM Characterization

Static, horizontal contact angles were measured via the sessile drop method with deionized water using a Kruss goniometer (KRÜSS Gmbh Contact Measuring System G10, Hamburg, Germany). The Contact angles of 5 separate 20µL drops were measured and averaged.

XPS analysis of the thiol-adsorbed samples was performed using a Kratos Axis Ultra XPS (Kratos Analytical Inc., Chestnut Ridge, NY) with aluminum Kα as the radiation source. Survey spectra and high resolution C(1s), O(1s), S(2p) and Au(4f) spectra were taken. Spectra were analyzed using the software program Casa XPS.

A Nanoscope IIIa Multimode atomic force microscope (Digital Instruments, Inc., Santa Barbara, CA) utilizing a silicon nitride tip was used to obtain height and friction images of the mixed SAMs in an effort to detect single-component domains.
2.2.3. FXIIa Plasma Coagulation Assay

Human platelet poor plasma (PPP), less than two days outdated, was obtained from M.S. Hershey Medical Center Blood bank. Multiple outdated lots were pooled and aliquoted in 15 mL polypropylene tubes and frozen at -20ºC until use. FXIIa was obtained from Enzyme Research Laboratories (South Bend, IN). Plasma coagulation time (CT) was used to quantify FXIIa activity in solution using FXIIa titration calibration curves relating [FXIIa], in PEU/mL, to CT. FXIIa titration curves were generated by mixing 0.5 mL of plasma thawed to room temperature with 0.4 mL PBS containing increasing amounts of FXIIa in polystyrene semi-micro cuvettes (Brand, VWR). The contents are recalcified with 0.1 mL of 0.1 M CaCl₂ and mixed on a hematology mixer (Labquake, Barnstead/Thermolyne) at 8 rpm. The coagulation time recorded was the time from recalcification to coagulation, as seen from a change in the liquid state of the mixture to a gelatinous state with the presence of fibrils. The specific methods for performing the coagulation-time assay used herein has been described in further detail previously (Vogler, Graper et al. 1995; Zhuo, Miller et al. 2005).

2.2.4 Substrate-induced contact activation in platelet poor plasma

A single, thiol-modified substrate was placed in a 2.5 mL polystyrene semi-micro cuvette with 0.5 mL of plasma thawed to room temperature and 0.4 mL PBS. The nominal surface area of the substrate was estimated to be 472 mm², as calculated from the manufacturer supplied dimensions of the coverslip. The contents were recalcified with 0.1 mL of 0.1 M CaCl₂ and mixed on a hematology mixer at 8 rpm. The coagulation time recorded was the time from recalcification to coagulation, as seen from a change in the liquid state of the mixture to a gelatinous state.
2.2.5 FXII activation in neat buffer solutions

FXII activation in the presence of thiol-modified surfaces was measured by recording the coagulation time of PPP upon addition of an aliquot of the test solution and referencing the corresponding FXIIa titration calibration curve. A single, thiol-modified substrate was placed in a 2.5 mL polystyrene semi-micro cuvette. A test solution of 30 μg/mL FXII in PBS was added to each cuvette and the cuvette was gently rocked for 30 minutes, after which 100 μL aliquots were taken to measure FXII activation. The FXIIa assay was performed by equilibrating 500 μl of thawed PPP in a semi-micro cuvette, mixed with the 100 μL aliquot of test solution obtained following the 30 minute activation period and diluting with sufficient additional PBS to bring the total volume to 900 ml. Coagulation was induced by recalcification with 100 ml of 0.1 M CaCl₂ and tube contents were mixed on a slowly-turning hematology mixer. The coagulation time was recorded and used to calculate the equivalent FXIIa activity by referencing back to the FXIIa-titration curve described in section 2.3. Two separate lots of FXII were used in this work, Enzyme Research Laboratories (ERL), South Bend, IN and Haemotologic Technologies, Inc. (HTI), Essex Junction, VT, as well as two separate lots of pooled plasma. A FXIIa-titration calibration curve was generated for each lot of pooled plasma as described in section 2.3. Aliquots of each lot of FXII (3 μg aliquots which corresponds to how much FXII would be expected in 100 μL of test solution) were tested for the presence of FXIIa activity. Both lots had low nascent FXIIa activity with the lot from HTI having a [FXIIa]eq of 1.79 ± 1.40 x 10⁻⁴ PEU/mL and the lot from ERL having a [FXIIa]eq of 3.95 ± 2.24 x 10⁻⁴ PEU/mL.

Statistical analyses were performed by parametric ANOVA (Holm-Sidak’s test) using SigmaPlot software. Means of experimentally determined [FXIIa]eq for each surface type
were compared pair-wise and the differences were considered statistically significant for p-values less than the critical p-value as determined by the Holm-Sidak test ($\alpha=0.05$).

### 2.3. Results and Discussion

This section characterizes mixed-thiol-SAM activator surfaces and compares catalytic potential of these surfaces to activate plasma coagulation, as measured by reduction in observed coagulation time. SAM surface energy was taken to be the significant surface property causing activation (Vogler and Siedlecki 2009) and coagulation time was therefore scaled against SAM water wettability measured as water adhesion tension $\tau = \gamma_v \cos \theta_a$, where $\gamma_v = 71.97$ mJ/m$^2$ is the interfacial tension of water at 20°C and $\theta_a$ is the observed advancing contact angle of water on the SAM surface. As an alternative scaling, coagulation time was converted to the equivalent amount of FXIIa produced inferred from a FXIIa titration curve.

#### 2.3.1. Surface Characterization

##### 2.3.1.1. XPS analysis

Survey spectra for thiol-adsorbed samples revealed the presence of Au, C, O, and S. No Si or Ti peaks were observed nor peaks indicating other extraneous elements. Small oxygen peaks were detected for methyl-terminated SAMs but this is consistent with findings from other groups (Bain, Eval et al. 1989; Castner, Hinds et al. 1996; Martins, Fonseca et al. 2003). High resolution S2p peaks were as expected, fitted well by a doublet with peaks
centered at 162.2 and 163.5 eV with a 2:1 peak-area ratio (Castner, Hinds et al. 1996), indicating bound thiol species. Figure 2-1 shows an example of such a peak. No peaks were detected in with the range of 164-169 eV indicating the absence of oxidized sulfur species or unbound thiols.

![Figure 2-1: High resolution S2p peaks showing peaks at 162.2eV and 163.5eV, indicating the presence of bound thiols.](image)

Single-component SAMs exhibited peaks consistent with the adsorbed thiol. High resolution C1s spectra for methyl-terminated SAMs show peaks centered at 284.6eV. Hydroxyl- and carboxyl-terminated SAMs have additional peaks at 286.7 eV and 289.5 eV, respectively, corresponding to the C-O bond and carbonyl carbon. Amine-terminated SAMS showed peaks at approximately 405eV as expected. Figure 2-2 shows examples of the characteristic high resolution scans for the one-component thiol SAMs.
Figure 2-2: (a) C1s peak of carboxyl terminated surface; (b) C1s peak of hydroxyl-terminated surface; (c) C1s peak of methyl-terminated surface; (d) N1s peak of amine-terminated surface.
2.3.1.2. Contact Angle Goniometry

Figures 2-3(a,b,c) relate the advancing contact angles to the solution composition. For the mixed hydroxyl-/methyl-terminated SAMs it is expected that the methyl-terminated thiol will be preferentially adsorbed as it is less soluble in ethanol than the hydroxyl-terminated thiol (Bain, Evall et al. 1989). This effect is not quite as stark in the mixed carboxyl-/methyl-terminated thiol series. For the mixed amine-/methyl-terminated SAMs, a dramatic increase in contact angle isn’t noted until the solution fraction percentage of 11-amino-1-undecanethiol is 50% or less.

![Graph showing contact angle versus 11-MU-OH solution fraction](image)

**Figure 2-3 (a)** Contact angles of mixed hydroxyl-/methyl-terminated thiol-modified substrates corresponding to the solution percentage of 1 mM 11-mercaptoundecanol (11-MU-OH) in ethanol, i.e. a 70% solution contains 7 parts 1 mM 11-mercaptoundecanol in ethanol to 3 parts 1 mM dodecanethiol in ethanol (n=3).
Figure 2-3 (b) Contact angles of mixed carboxyl-/methyl-terminated thiol-modified substrates corresponding to the solution percentage of 1 mM 11-mercaptoundecanoic acid in ethanol, i.e. a 70% solution contains 7 parts 1 mM 11-mercaptoundecanoic acid (11-MUA) in ethanol to 3 parts 1 mM dodecanethiol in ethanol (n=3).
Figure 2-3 (c) Contact angles of mixed amine-/methyl-terminated thiol-modified substrates corresponding to the solution percentage of 1 mM 11-amino-1-undecanethiol in ethanol, i.e. a 70% solution contains 7 parts 1 mM 11-amino-1-undecanethiol in ethanol to 3 parts 1 mM dodecanethiol in ethanol (n=3).

2.3.1.3. Atomic Force Microscopy

Height images of cleaned, bare gold surfaces were as expected, with features between 1-2 nm in size and typical of the grain structure of gold sputter-coated surfaces evident. Extensive friction force AFM images of these mixed thiol-adsorbed surfaces did not detect any discernible domains that would be indicative of phase separation. Figure 2-4 depicts typical height images and friction force images of a mixed thiol-adsorbed surface, as well as a typical height image of the bare gold surface.
The thiols used in constructing these mixed surfaces were similar in chain length, differing only in terminal functional group, thus no topographical thiol-related features were expected. However, if there had been marked phase separation it would likely have been detected using
friction force microscopy. In a previous study, 400 nm islands of an amine-terminated silane dispersed in a butyltrichlorosilane background on glass substrates were easily visualized by these same types of friction mode atomic force microscopy images (Miller, Guo et al. 2006). While these islands are detectable using friction mode, findings from other investigators indicate that phase separated domains in mixed thiol-adsorbed SAMs of comparable chain length are less than or equal to 15 nm$^2$ and undetected using friction force microscopy (Kakiuchi, Iida et al. 2001; Brewer and Leggett 2004). Thus, while no gross domains were seen in the images, the presence of smaller domains less than 15 nm$^2$ cannot be completely ruled out. Figure 2-5 depicts two examples of patterned two-component thiol surfaces imaged using friction force microscopy. Note that the friction force trace and retrace images are inverses of each other, as would be expected, and that the height image might not necessarily show the pattern. For these patterned surfaces, thiols with different chain lengths were used and may be detected topographically. However, as the difference in chain length is on the order of 1-2 nm, stamping and imaging conditions greatly influence the likelihood of seeing the pattern topography.
Figure 2-5: From left to right – height, friction mode retrace, and friction mode trace AFM images of patterned thiol surfaces. Top – Bare gold surface was stamped with a post pattern PDMS stamp inked with 16-mercaptopoundecanoic acid and then backfilled with octanethiol. The oval pattern corresponds to the stamped 16-mercaptopoundecanoic acid. Pattern was imaged using a hydrophobic, octadecyltrichlorosilane modified probe. Bottom – Bare gold surface was stamped with a linear pattern PDMS stamp inked with octadecanethiol and backfilled with 11-mercaptopoundecanoic acid. The light colored bands in the friction retrace image (middle) correspond to the stamped octadecanethiol. Pattern was imaged using a hydrophilic, plasma cleaned probe.
2.3.2. FXIIa Plasma Coagulation Assay

Figure 2-6a,b show the FXIIa titration curves for the two lots of human platelet-poor plasma used in this study. Figure 2-6a corresponds to the plasma lot used in substrate-induced coagulation studies discussed in Section 2.3.3 and substrate-induced FXII activation in neat-buffer solution experiments using FXII obtained from Enzyme Research Laboratories. Figure 2-6b corresponds to the plasma lot used for substrate-induced FXII activation in neat-buffer solution experiments using FXII obtained from Haemotologic Technologies, Inc.

Table 2-1 lists the coagulation times (mean ± SD; n=3) for the controls in which no exogenous FXIIa were added. Also listed is a practical quantification limit set at 10 times the standard deviation of the control coagulation times subtracted from the mean coagulation time of the controls and the corresponding [FXIIa]eq. Alpha or beta errors (probability of a false positive or false negative, respectively) are extremely unlikely for a sample with a coagulation time greater than this practical limit.
Figure 2-6: FXIIa titration curves for the two lots of plasma showing coagulation times versus concentrations of exogenous FXIIa. The smooth curves result from a least squares fitting of a mathematical model developed in Guo et al (Guo, Bussard et al. 2006). (a) FXIIa titration curve for the lot of plasma used in substrate-induced coagulation studies discussed in section 2.3.3 and substrate-induced FXII activation in neat-buffer solution experiments using FXII obtained from Enzyme Research Laboratories. (b) FXIIa titration curve for the lot of plasma used for substrate-induced FXII activation in neat-buffer solution experiments using FXII obtained from Haemotologic Technologies, Inc. The shaded areas represent exogenous concentrations of FXIIa, and their corresponding coagulation times with respect to the modeled curve, which fall outside of the practical limit of quantification for this assay.
Table 2-1: Practical limits of quantification for the two lots of plasma used in this study as discussed in Section 2.3.3. Control Coagulation Time – the mean coagulation time (± standard deviation) of the controls to which no exogenous FXIIa was added; Practical Quantification limit – the mean Control Coagulation Time minus 10 times the standard deviation; [FXIIa]eq – The equivalent of exogenous FXIIa needed to elicit the Practical Quantification Limit coagulation time.

<table>
<thead>
<tr>
<th>Plasma Lot</th>
<th>Control Coagulation Time (minutes, mean ± SD, n=3)</th>
<th>Practical Quantification Limit (minutes)</th>
<th>[FXIIa]eq (PEU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>58.20 ± 1.64</td>
<td>41.80</td>
<td>3.59 x 10^-4</td>
</tr>
<tr>
<td>B</td>
<td>56.87 ± 0.99</td>
<td>46.97</td>
<td>3.50 x 10^-4</td>
</tr>
</tbody>
</table>

2.3.3. Coagulation Assays

Thiol-modified substrates were utilized in plasma coagulation assays as detailed in Section 2.2.4. Figures 2-7(a,b,c) depict the coagulation times for mixed carboxyl/methyl-, mixed hydroxyl/methyl-, and mixed amine/methyl-terminated thiol surfaces while Figures 2-8(a,b,c) shows the equivalent amount of FXIIa activity [FXII]eq, as estimated from a FXIIa titration calibration curve. Two differences between the mixed carboxyl- and mixed hydroxyl terminated surfaces are noted. First, the transition between a pure methyl-terminated surface to a pure carboxyl-terminated surface has different effects on coagulation time and [FXII]eq as compared to the hydroxyl-terminated series. The data shows a decrease in [FXII]eq and increase in coagulation time as the methyl-terminated content in the carboxyl-terminated series increases. However, the same trend is not seen in the hydroxyl/methyl-terminated series, as there is a decrease in [FXII]eq and increase in coagulation times to near baseline levels for surfaces with water adhesion tension approximating 45 dyne/cm. Given that the [FXII]eq for this surface is even less than that of either a pure hydroxyl or methyl surface it cannot be considered that it is simply an effect due to additive effects of the separate areas of each chemical component. The spatial distribution of the methyl- and
hydroxyl-terminated thiols and the resulting wettability has effects upon the interaction of the surface and FXII which must be addressed.

A second important difference is the near 40-fold increase in the magnitude of $[\text{FXII}]_{\text{eq}}$ produced when comparing pure carboxyl-terminated surfaces to pure-hydroxyl terminated surfaces in plasma. Although, both surface types are hydrophilic, in platelet-poor plasma the carboxyl-terminated surface appears to be a much stronger activator. This appears in line with the widespread view that anionic-hydrophilic surfaces are strong activators of contact activation. However, what is not obvious from the traditional view of coagulation is why the hydroxyl-terminated surface is also capable of being an activating surface to an even greater extent than a protein-adsorbent methyl-terminated surface. This observation, along with previous results, suggests that the problem is much more complex than simply activation by anionic surfaces, and must include factors such as surface wettability and protein adsorption properties of materials.

Figure 2-7c shows coagulation times for mixed amine-/methyl-terminated thiol surfaces that appear similar to the hydroxyl surface, where materials having mid-range wettability seem to have little to no coagulation activity in plasma. These materials are statistically indistinguishable from the baseline coagulation times, and in fact for samples with adhesion tension nearing 0 dyne/cm, the coagulation time appears slightly greater than baseline. As seen in Figure 2-8c, this increased coagulation time would represent a “negative” value for $[\text{FXIIa}]_{\text{eq}}$, and while a negative value for $[\text{FXIIa}]_{\text{eq}}$ is not possible, it may in fact be indicative of activation of an inhibitory pathway. However, we currently do not have sufficient data to speculate on which pathway is being activated.
Figure 2-7 (a) Coagulation time (CT) of carboxyl-/methyl-terminated thiol-modified substrates. (CT ± sd, n=3). The dashed and dotted lines indicate the mean baseline coagulation time +/- standard deviation (n > 3) in which no thiol-modified substrate has been added to the sample cuvette.

Figure 2-7 (b) Coagulation time (CT) of hydroxyl-/methyl-terminated thiol-modified substrates (mean ± SD, n=3). The dashed and dotted lines indicate the mean baseline coagulation time +/- standard deviation (n > 3) in which no thiol-modified substrate has been added to the sample cuvette.
Figure 2-7 (c) Coagulation time (CT) of amine-/methyl-terminated thiol-modified substrates (mean ± SD, n=3). The dashed and dotted lines indicate the mean baseline coagulation time +/- standard deviation (n > 3) in which no thiol-modified substrate has been added to the sample cuvette.
Figure 2-8 (a) [FXIIa]eq, calculated from coagulation times, of carboxyl-/methyl-terminated thiol-modified substrates.

Figure 2-8 (b) [FXIIa]eq, calculated from coagulation times, of hydroxyl-/methyl-terminated thiol-modified substrates (mean ± SE, n=3).
Figure 2-8 (c) [FXIIa]eq, calculated from coagulation times, of amine-/methyl-terminated thiol-modified substrates (mean ± SE, n=3).
2.3.4. FXII activation in neat buffer solution

Figures 2-9 and 2-10 summarize the results for FXII activation experiments in neat buffer solution. Data shown in Figure 2-9 was obtained using FXII supplied from Enzyme Research Laboratories while data shown in Figure 2-10 was obtained using FXII supplied by Haematologic Technologies, Inc. and a second, separate lot of pooled PPP. Note that the [FXIIa]eq produced by the control blank for data in Figure 2-9 is nearly 50% greater than that for the data in Figure 2-10. This is explained by the manufacturer reported activity determined via clotting assay. The activity of the FXII used for data in Figure 2-9 was approximately 44% greater than that used for the data shown in Figure 2-10 (40.31 PEU/mg and 28 PEU/mg for the ERL and HTI supplied FXII, respectively).

In each of these data sets, there is a decreased level of FXII activation for surfaces with wettibilities nearing 45 dyne/cm that is statistically indistinguishable from the control blank of the set. It is worth noting that the polystyrene control blank has a measured contact angle of approximately 73°±3 and the total area of the cuvette exposed to the test solution is approximately 1.77 times the sample surface area.

Furthermore, in both data sets the one-component hydroxyl-, carboxyl-, and methyl-terminated surfaces had increased levels of FXII activation as compared to the 45 dyne/cm surface types, suggesting a possible minimum for FXII activation at mid-range wettabilities, and consistent with the results previously published by Golas et al. (Golas, Parhi et al. 2010).

An important difference between the work of Golas et al. and this study is the means of surface preparation; in the previous work an array of different silane chemistries was used to create surfaces with varying wettability versus a limited number of thiol chemistries mixed in different ratios in this study. In this study, it is shown that surfaces bearing a limited number
of terminal groups (carboxyl, hydroxyl, amine) all exhibit minimal FXII autoactivation levels regardless of chemistry as wettability nears 45 dyne/cm, thus supporting the idea that FXII autoactivation is minimized for surfaces with mid range wettability. Increased autoactivation is seen near both the hydrophobic and hydrophilic extremes regardless of surface chemistry presented.

It is important to emphasize that the experimentally determined values for [FXIIa]eq in these FXII activation studies in both plasma and neat buffer solution are not an actual measured value of FXIIa produced. The [FXIIa]eq value is determined using a FXIIa titration calibration curve relating known quantities of commercially purchased FXIIa, applying vendor supplied values of activity in PEU/mL, to the coagulation time resulting from addition of these activated products to plasma. Comparison of experimental values to the calibration curve yields an equivalent amount of purified FXIIa that would need to be added to the sample plasma aliquot to elicit the same measured coagulation time. The literature shows that FXII autoactivation results in a number of fragments with amidolytic activity, with further evidence indicating that different fragments have varying procoagulant activity (Revak, Cochrane et al. 1978; Dunn, Silverberg et al. 1982; Vanderkamp and Vanoeveren 1994). With this consideration the [FXIIa]eq determined by FXII activation in neat buffer studies may represent a compilation of different FXIIa fragments with associated procoagulant activities. Furthermore, the presence and actions of other plasma proteins must be considered when interpreting values of [FXIIa]eq determined from the plasma coagulation studies in this paper.
Figure 2-9: (top) FXII (ERL) activation (mean ± SD, n ≥ 5) in neat buffer solution. Surface types are listed along with their contact angle ± SD. * denotes statistical significance as compared to OH mix (51° ± 1), NH₂ mix (51° ± 1), and the control blank and determined via the Holm-Sidak method with α = 0.05. ‡ denotes statistical significance as compared to OH mix (51° ± 1) only. (bottom) FXII (ERL) activation (mean ± SD, n ≥ 5) in neat buffer solution plotted against water adhesion tension. The average of FXII activation for the blank (no additional surface area added) has been subtracted from the sample FXII activation levels.
Figure 2-10: (top) FXII (HTI) activation (mean ± SD, n≥5) in neat buffer solution. Surface types are listed along with their contact angle ± SD. * denotes statistical significance as compared to OH mix (52°±1) and the control blank and determined via the Holm-Sidak method with α=0.05. (bottom) FXII (HTI) activation (mean ± SD, n≥5) in neat buffer solution plotted against water adhesion tension. The average of FXII activation for the blank (no additional surface area added) has been subtracted from the sample FXII activation levels.
The FXII activation studies performed in neat buffer solution clearly show that FXII can be activated in the presence of both neutral and charged hydrophilic surfaces as well as hydrophobic surfaces. However, the differences between neat buffer and plasma activation highlight the role of adsorption-competition. From the neat buffer studies, the pure carboxyl-terminated surface produced a statistically significant greater [FXIIa]eq than both the purely methyl-terminated surface and the control “blank” (Figure 2-10), while the pure methyl-terminated surface produced a statistically significant greater [FXIIa]eq than the control blank. Summarizing the [FXIIa]eq produced:

\[ \text{COOH (16^\circ \pm 2)} > \text{CH}_3 > (102^\circ \pm 1) > \text{COOH/CH}_3 (52^\circ \pm 1). \]

However, the plasma coagulation studies for the mixed carboxyl series (see Figures 2-7(a) and 2-8(a)) do not indicate a minimum [FXIIa]eq near these mid-level water adhesion tension values around \( \sim 45 \text{ dyne/cm} \). In fact, it’s until samples have water contact angles greater than 60° that coagulation times begin to notably increase, and at pure methyl-terminated samples a minimum [FXIIa]eq is measured. Thus, although both the pure methyl-terminated and pure carboxyl-terminated surfaces produced [FXIIa]eq above control in buffer, the hydrophobic surface appears to be a highly inefficient activator of blood coagulation in plasma.

Although the carboxyl mixture series studies seem to lend further support to the adsorption dilution effect in blood coagulation, it does not address why the midrange wettability (45 dyne/cm, 52°±1 contact angle) mixed carboxyl-samples appear to be efficient activators when neat-buffer studies indicate that less [FXIIa]eq is produced than their pure
methyl- or carboxyl-terminated counterparts. It may be helpful to look at the hydroxyl mixture series studies to discern a possible explanation. From the neat buffer studies, a similar trend to the carboxyl series is observed. The pure hydroxyl-terminated surface produced a statistically significant greater [FXIIa]eq than both the purely methyl-terminated surface and the control blank, while again the pure methyl-terminated surface produced a statistically significant greater [FXIIa]eq than the control blank. To summarize, in terms of measured [FXIIa]eq:

$$\text{OH (28º±1, Figure 2-8; 28º±0, Figure 2-9)} > \text{CH3 (101º±1; 102º±1)} > \text{OH/CH3 (51º±1; 52º±1)}.$$

The plasma coagulation studies for the hydroxyl series show a much different trend than did the carboxyl series. Within the hydroxyl series, a maximum coagulation time, statistically indistinguishable from the “baseline” coagulation time of the control blank, is noted near 45 dyne/cm, while coagulation times for pure hydroxyl-terminated samples are only slightly lower than those of the pure methyl-terminated samples. Furthermore, as noted in section 2.3.3, the [FXIIa]eq corresponding to these coagulation times are nearly 40x less than those in the carboxyl mixture series, even though these two materials produce similar amounts of [FXIIa]eq in buffer. A possible explanation for the similarities seen in neat-buffer studies and the differences between the coagulation studies for the hydroxyl and carboxyl subsets is the presence of other proteins and pathways in plasma, including PK and HK. Previous studies have shown that an anionic surface is not necessary for FXII activation or FXIIa mediated hydrolysis of prekallikrein (Zhuo, Siedlecki et al. 2006; Zhuo, Siedlecki et al. 2007; Chatterjee, Thornton et al. 2009). This investigation provides further support to the
notion that an anionic surface is not necessary for FXII autoactivation, but does not address
the role that surfaces may play in other alternative activation and inhibition pathways.

2.4. Conclusion

Previous investigations into contact activation and surfaces of varying wettability
utilized silane-modified glass as model surfaces (Miller, Guo et al. 2006; Zhuo, Siedlecki et
al. 2006; Zhuo, Siedlecki et al. 2007). While a multitude of surfaces can be created via silane
SAMs, surfaces with well-defined nanoscale chemical heterogeneity have proven difficult to
develop. Thiol-adsorbed SAMs offer a practical means to create a set of surface chemistries
that differ only in the proportion of one terminal functional group, i.e. carboxyl, hydroxyl,
amine, to another, i.e. methyl. In this manner, a range of surface wettability can be explored
and the FXII activation results can be viewed with respect to the wettability, without having
to consider the possibility that different functional groups may somehow be influencing the
results. As such, this study extends our previous work (Golas, Parhi et al. 2010)
demonstrating that surfaces with midrange wettability (20-40 dyne/cm) exhibit minimal
activation of FXII. While plasma coagulation studies would appear to support a role for
anionic surfaces in contact activation, the results suggest FXII activation in neat buffer
solution is a function of surface wettability, with a minimum at mid-range wettability.
Taken together, these results demonstrate that the composition of the solution and the surface
properties of the material both influence contact activation, as well as demonstrating that
activation of FXII cannot be viewed as being specific to anionic surfaces as believed.
2.5. Citations


Chapter 3

High-molecular weight kininogen and the moderation of prekallikrein-factor XII interactions in surface activation of coagulation

Abstract

A chromogenic assay was used to measure kallikrein activity generated by FXIIa-mediated conversion of prekallikrein to kallikrein in the presence of high molecular weight kininogen (HK). Cleaned glass and octadecyltrichlorosilane-treated glass were used as the test hydrophilic and hydrophobic surfaces, respectively. The results presented in this chapter (1) suggest HK increases the generation of kallikrein activity when FXIIa is present; (2) suggest that an anionic hydrophilic surface is not a necessary cofactor for FXIIa-mediated hydrolysis of prekallikrein in the presence of HK; (3) suggest that prekallikrein hydrolysis in the presence of HK does not occur directly on the activating surface; (4) suggest that the ability of HK to increase the generation of kallikrein activity can compensate for the loss of activity that may be associated with adsorption to a hydrophobic surface.
Chapter 3

High-molecular weight kininogen and the moderation of prekallikrein-factor XII interactions in surface activation of coagulation

3.1 Introduction

The use of blood-contacting medical devices is prevalent in the clinical setting, with uses ranging from routine blood collection to more invasive procedures, such as percutaneous coronary interventions, and very invasive procedures such as cardiopulmonary bypass. Thrombosis due to interaction of blood with blood-contacting materials remains a significant risk, necessitating the use of anticoagulants which have their own associated risk of uncontrolled bleeding (Rose, Gelijns et al. 2001; Copeland, Smith et al. 2004; Pae, Connell et al. 2007). As more blood-contacting medical devices are being increasingly looked at to serve long-term purposes, e.g. ventricular assist devices for bridge-to-recovery or bridge to transplant applications (Ueno, Bergin et al. 2000; Hunt, Abraham et al. 2005; Birks, Tansley et al. 2006; Topkara, Dang et al. 2006), their thrombogenic potential is increasingly important (Miller, Pagani et al. 2007; Nielsen, Kirklin et al. 2008; Achneck, Sileshi et al. 2010).

The intrinsic pathway is initiated when the blood plasma comes in contact with a non-physiologic surface, mediating the activation of FXII, hence “contact activation.” FXIIa then activates FXI (Bouma and Griffin 1977) which activates FIX. Prekallikrein (PK) and high-molecular weight kininogen (HK) are also important participants in contact activation. FXI and PK each circulate in plasma as a complex with HK (Mandle, Colman et al. 1976). It is believed that HK protects these proteins from C1H-inhibitor and other plasma inhibitors
(Schapira, Scott et al. 1981; Scott, Schapira et al. 1982), and has other attributes which will be discussed shortly.

The most widespread model of contact activation imparts FXII and HK activating abilities specific to anionic surfaces, such as glass, kaolin, dextran sulfate, etc (Colman and Schmaier 1997). Binding of FXII to anionic surfaces via “anionic binding domains” rich in positive-charged amino acids induces activation of FXII to FXIIa, often termed “autoactivation”. HK also possesses anionic binding domains (Kunapuli, DeLa Cadena et al. 1993) which are believed to help bring PK and FXI into an orientation favorable to activation (Griffin and Cochrane 1976; Meier, Thompson et al. 1977; Wiggins, Bouma et al. 1977). Surface-generated FXIIa activates PK complexed with HK at the surface (Griffin 1978) and the resulting kallikrein can activate FXII (Silverberg, Dunn et al. 1980) such that there is reciprocal-activation. However, it has been recently observed that Kal-mediated FXII activation (reciprocal activation) is the principal pathway for FXII activation, producing 75% of the FXIIa yield within the intrinsic pathway and is proportional to the initiating amount of FXIIa produced through autoactivation (Chatterjee, Guo et al. 2009). Further studies indicate that the role of the surface in contact activation may be confined to the autoactivation of FXII (Chatterjee, Vogler et al. 2006). Notably, in Chatterjee et al (Chatterjee, Thornton et al. 2009), chromogenic assays of FXIIa-mediated PK hydrolysis indicate that an anionic, hydrophilic surface is not a necessary cofactor for PK hydrolysis and that prekallikrein hydrolysis is not localized to an activation complex on the procoagulant surface. This study also demonstrated that protein-adsorption competition is an important facet of blood plasma coagulation as the introduction of a competitive protein, bovine serum albumin, to octadecyltrichlorosilane-treated glass vials containing FXII and PK in PBS resulted in a rapid
increase in Kal production, likely due to the displacement of adsorbed zymogens, or their activated enzymes, from the hydrophobic surface.

However, PK is complexed with HK as it circulates in human blood plasma and is an important cofactor in the intrinsic coagulation cascade. It has been demonstrated that using a monoclonal antibody to block the prekallikrein and FXI binding sites of HK inhibits the HK-dependent coagulation activity in normal plasma activated by dextran sulfate (Schmaier, Schutsky et al. 1987; Reddigari and Kaplan 1989). It has been put forth that cleavage of HK enhances interaction with anionic surfaces and its coagulant activity (Scott, Silver et al. 1984), though it is unclear whether it is the cleaved product or the action of cleaving that enhances coagulant activity. Both FXIIa and kallikrein cleave HK in similar manners (Wiggins 1983; Colman and Schmaier 1997). The presence of HK was not addressed in Chatterjee et al (Chatterjee, Thornton et al. 2009). This chapter serves to expand upon the work presented in Chatterjee et al and elucidate the effect of HK on the moderation of prekallikrein-factor XII interactions in surface activation of coagulation by protein-adsorption competition.

3.2. Materials and Methods

3.2.1. Zymogens and Proteins

Human prekallikrein, human kallikrein, human FXIIa, human HK, and corn trypsin inhibitor were obtained from Enzyme Research Lab (South Bend, IN). FXII was obtained
from Haemotologic Technologies, Inc. (Essex Junction, VT) and mouse IgG (Fab)\textsubscript{2} fragment from Jackson ImmunoResearch Laboratories (West Grove, PA). Human serum albumin, >99% and essentially fatty acid free, was obtained through Sigma-Aldrich.

3.2.2 Model Surface Preparation

Hydrophobic and hydrophilic surfaces were prepared as detailed previously (Guo, Bussard et al. 2006; Chatterjee, Thornton et al. 2009) and briefly described here. Borosilicate glass vials (5 mL, 12x75 mm; Kimble Glass, Inc., Vineland, NJ) were immersed sequentially in a solvent series rinse consisting of ethanol, acetone, and chloroform. The glass vials were then dried and plasma treated in a glow-discharge chamber for 30 minutes at 100 W. Plasma cleaned glass vials were used as the model hydrophilic surface. Hydrophobic vials were prepared by silanization of the plasma-cleaned vials with n-octadecyltrichlorosilane (OTS) obtained from Gelest Inc. (Morrisville, PA). The plasma-cleaned vials were filled with 5% OTS in chloroform (volume/volume), capped, and let sit for 60 minutes. Treated vials were rinsed twice with chloroform and annealed overnight at 70°C in a vacuum oven. Water wettability of the model surfaces was estimated by measuring the water contact angles of identically treated borosilicate glass coverslips. The contact angles were measured using the sessile drop method with 18 M\text{\textOmega} water (Millipore Siplicity 185 system) and a Kruss Goniometer.
3.2.3. Kallikrein Generation Assay

Each hydrophilic or hydrophobic surface-modified glass vial contained 1 mL protein solution in phosphate buffered saline (PBS). Generally, the protein solution contained 20 μg/mL PK, which is approximately 50% of the normal human concentration, and FXIIa at 0.05 μg/mL or FXII as noted in the text or figures. The concentrations of PK, FXIIa, and FXII chosen were consistent with those used in Chatterjee et al (Chatterjee, Thornton et al. 2009). HK, when present was added at a 1:1 molar concentration to PK. Prior to addition to the assay, HK and PK were incubated together for 30 minutes at room temperature to allow more than sufficient time for them to complex (Mandle, Colman et al. 1976). In instances which HK was not added, the PK was allowed to sit at room temperature for 30 minutes as well, so as not to introduce an additional variable to the study. Mouse IgG F(ab)2 fragment was used as a dummy protein to serve as a control for the addition of HK. Once FXIIa or FXII was added, the last agent added to the protein solution, the surface-modified vials were capped with parafilm and set rotating on a hematology mixer at 8 rpm. To assay Kall generation, 15 μL aliquots were removed from the vials at 0, 2, 4, 8, 12, 16, 22, and 30 minute time points. At 30 minutes the assay was stopped. The 15 μL was transferred to a 90-well plate for chromogenic determination of the corresponding Kall concentration, which will be described shortly. For human serum albumin (HSA) displacement studies, a 38 μL aliquot of an HSA solution in PBS was added to the vials at the 10.5 minute time point yielding an HSA concentration of 5 mg/mL.
3.2.4. Chromogenic Assay

The chromogenic substrate Pefachrome-PK® (Centerchem, Inc., Norwalk, CT) was used to determine the concentration of kallikrein at each defined timepoint. Stock vials containing 25mg of the substrate were refrigerated at 4°C and reconstituted to 4 mM in 18MΩ Millipore water just prior to using and used within two days of being reconstituted. 15 μL aliquots of the protein solution from the kallikrein generation assay were placed in wells of a 90-well plate containing 200 μL tris-buffered saline, 25 μL of the 4 mM Pefachrome-PK® solution, and corn trypsin inhibitor at 25 times the molar of FXIIa or FXII used. The corn trypsin inhibitor was added because it is a FXII inhibitor (Hojima, Tankersley et al. 1980; Rand, Lock et al. 1996) and would inhibit any additional FXIIa activation of prekallikrein during the chromogenic assay. The chromogenic assay was allowed to react for 5 minutes at 37°C and then stopped with the addition of 40 μL 10% acetic acid (Mallinckrodt Baker, Paris, KY). The absorbance (ΔA405nm) was measured at 405nm using a Molecular Devices spectrometer. Control experiments performed previously (Chatterjee, Thornton et al. 2009) did not indicate any measurable effects by corn trypsin inhibitor, FXIIa, or HSA on the reaction of kallikrein with the substrate in this assay.

3.2.5. Statistical Analysis

Statistical analyses were performed by parametric ANOVA (Tukey’s test) using SigmaPlot (Systat Software, Inc.) when the assumption of equal variances was valid. Otherwise, Dunn’s non-parametric ANOVA was used to compare groups. Groups were compared pairwise. Means of measured ΔA405nm of the test aliquots inputted into the
chromogenic assay at a given time were compared pair-wise and the differences were considered statistically significant for p< 0.05.

3.3. Results and Discussion

3.3.1. Kallikrein generation in solutions of PK, HK, and FXIIa

Using the kallikrein-specific chromogenic substrate, the timecourse of FXIIa-mediated hydrolysis of PK in PBS in hydrophobic- or hydrophilic modified glass vials was investigated. Contact angles of representative glass and OTS-coated witness samples are given below in Table 3-1.

Table 3-1: Contact angles of sample surfaces.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Contact Angle (sessile drop method, mean±sd, n=3)</th>
</tr>
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<tbody>
<tr>
<td>OTS</td>
<td>108°±1</td>
</tr>
<tr>
<td>Glass</td>
<td>7°±1</td>
</tr>
</tbody>
</table>

This set of experiments shows the effect of the addition of HK without any confounding effects from FXII activation. The protein solution contained 20 μg/mL PK and FXIIa at 0.05 μg/mL. HK, when present, was added at a 1:1 molar concentration to PK. Prior to addition to the assay, HK and PK were incubated together for 30 minutes at room temperature to allow more than sufficient time for them to complex (Mandle, Colman et al. 1976). In instances which HK was not added, the PK was allowed to sit at room temperature for 30
minutes as well, so as not to introduce an additional variable to the study. Mouse IgG F(ab)2 fragment was used as a dummy protein to serve as a control for the addition of HK. Figure 3-1 summarizes the results (mean ± SD) of four tested conditions: hydrophilic vials with or without HK added, and hydrophobic vials with or without HK added. Figures 3-2(a-d) break out the results of the trials into subgroups consisting of trials using hydrophilic versus hydrophobic vials (3-2a and 3-2b), or trials with HK added versus without HK added (3-2c and 3-2d).

**Figure 3-1:** Summary of results for kallikrein generation in solutions of PK and FXIIa, with and without HK. Hydrophobic- or hydrophilic-modified glass vials contained 20 μg/mL PK and FXIIa at 0.05 μg/mL. HK, when present, was added at a 1:1 molar concentration to PK. Open squares – hydrophilic vials with HK; Closed squares – hydrophobic vials with HK; open diamonds – hydrophilic vials without HK; closed diamonds – hydrophobic vials without HK. The right axis displays the measured absorbance readings while the left axis corresponds to equivalent kallikrein (Kal) concentrations determined by a standard curve prepared at 405 nm. (n≥3)
As Figure 3-1 shows, hydrophilic or hydrophobic vials containing PK, FXII, and HK showed increased chromogenic substrate hydrolysis compared to hydrophobic or hydrophilic vials not containing HK, as reflected by the increased $\Delta A_{405nm}$. As fixed-point chromogenic assays were used in this study, it cannot be determined from these trials whether the increased activity towards the chromogenic substrate is due to increased kallikrein generation or an acceleration of kallikrein’s hydrolysis of the chromogen via the presence of HK. Breaking down the summary into subgroups, Figure 3-2a shows kallikrein generation of solutions of PK and FXIIa without HK in hydrophobic or hydrophilic vials.

![Figure 3-2a: Summary of results for kallikrein generation in solutions of PK and FXIIa without HK. Hydrophobic- or hydrophilic-modified glass vials contained 20 $\mu$g/mL PK and FXIIa at 0.05 $\mu$g/mL. Open diamonds – hydrophilic vials without HK; closed diamonds – hydrophobic vials without HK. The right axis displays the measured absorbance readings while the left axis corresponds to equivalent kallikrein (Kal) concentrations determined by a standard curve prepared at 405 nm. The symbol # denotes statistical significance with respect to the hydrophobic vials. (n≥3)
In the hydrophobic vials there was an increase in kallikrein activity before reaching a steady state yield after approximately 12 minutes. Kallikrein activity was significantly higher in the hydrophilic vials for t > 12 min (p < 0.05). These results are similar to those of Chatterjee et al. in which lower kallikrein generation in hydrophobic vials was attributed to protein adsorption to the walls of the vessel, attenuating interactions between the proteins in solution. However, there is a difference in timescale as the results here display a longer time elapsed before a steady yield is obtained and before statistically significant differences are observed, i.e. kallikrein activity was significantly higher in the hydrophilic vials for t > 12 min (p < 0.05) in this study versus t > 4 min (p < 0.05) in Chatterjee et al. This may be due to differences in the activity of the enzymes used for each study.

Figures 3-2b and 3-2c display kallikrein activity of solutions of PK and FXIIa in hydrophilic vials and hydrophobic vials, respectively. In each graph three conditions are shown: PK and FXIIa solutions with HK included, mouse IgG fragment included, or nothing else included.
Figure 3-2b: Summary of results for kallikrein activity in hydrophilic glass vials of solutions of PK and FXIIa with either HK, mouse IgG fragment, or nothing added. Hydrophilic-modified glass vials contained 20 μg/mL PK and FXIIa at 0.05 μg/mL. HK or IgG, when present, was added at a 1:1 molar concentration to PK. Open squares – hydrophilic vials with HK; open triangles – hydrophilic vials with IgG; open diamonds – hydrophilic vials without HK or IgG. The right axis displays the measured absorbance readings while the left axis corresponds to equivalent kallikrein (Kal) concentrations determined by a standard curve prepared at 405 nm. The symbols # and * denote statistical significance with respect to the vials with IgG or vials without IgG or HK, respectively. (n ≥ 3)

As Figure 3-2b shows, there is a significant increase in kallikrein activity when HK is present in hydrophilic glass vials as compared to when HK is not present, regardless of whether the dummy IgG protein was included. This result is consistent with the numerous studies linking HK with negatively charged surfaces (Meier, Thompson et al. 1977; Thompson, Mandle et al. 1977; Scott, Silver et al. 1984) as an increase in kallikrein activity was observed, presumably through the increased action of FXII on prekallikrein. However, when the effect of the addition of HK in the presence of a hydrophobic surface (Figure 3-2c) is investigated, the historically assumed specificity of contact activation to negatively-charged surfaces again breaks down.
Figure 3-2c: Summary of results for kallikrein activity in hydrophobic glass vials of solutions of PK and FXIIa with either HK, mouse IgG fragment, or nothing added. Hydrophobic-modified glass vials contained 20 μg/mL PK and FXIIa at 0.05 μg/mL. HK or IgG, when present, was added at a 1:1 molar concentration to PK. Closed squares – hydrophobic vials with HK; closed triangles – hydrophobic vials with IgG; closed diamonds – hydrophobic vials without HK or IgG. The right axis displays the measured absorbance readings while the left axis corresponds to equivalent kallikrein (Kal) concentrations determined by a standard curve prepared at 405 nm. The symbols # and * denote statistical significance with respect to the vials with IgG or vials without IgG or HK, respectively. (n≥3)

As shown in Figure 3-2c there is an increase in kallikrein activity in the presence of HK as compared to the trials in hydrophobic vials without HK or with IgG fragment added as a dummy protein. Although the level of kallikrein activity isn’t as high as that from the trials of hydrophilic vials with HK (Figure 3-2d), such a level wouldn’t be expected due to the effect of protein adsorption to the walls of the hydrophobic vials. Furthermore, the difference of the means between the two groups at each time point kallikrein in Figure 3-2d is not great enough for statistical significance. With FXIIa available, it appears HK is able to enhance the generation of kallikrein activity in the presence of a hydrophilic or hydrophobic surface.
**Figure 3-2d**: Summary of results for kallikrein generation in solutions of PK and FXIIa with HK. Hydrophobic- or hydrophilic-modified glass vials contained 20 μg/mL PK and FXIIa at 0.05 μg/mL. *Open squares* – hydrophilic vials with HK; *closed squares* – hydrophobic vials with HK. The right axis displays the measured absorbance readings while the left axis corresponds to equivalent kallikrein (Kal) concentrations determined by a standard curve prepared at 405 nm. (n≥3)

In a separate control experiment, OTS-coated vials were blocked by overnight incubation with HSA in PBS followed by several rinses with PBS. This blocking was done in order to minimize any FXIIa adsorption to the OTS surface that might be occurring. As shown in Figure 3-3, kallikrein activity in OTS vials blocked with HSA was no different than that in hydrophilic glass vials. Note that Figure 3-3 also contains the results of control experiments utilizing FXII and discussed in the next section.

In summary, the presence of HK had an effect of increased kallikrein activity generation in hydrophobic as well as hydrophilic vials, thus suggesting that a negatively-charged surface is not necessary for FXIIa-mediated hydrolysis of prekallikrein with or without the presence of HK.
Figure 3-3: Control experiment results. *Open squares* – hydrophilic glass vials with 20 μg/mL PK, HK at 1:1 molar ratio to PK, and 0.05 μg/mL FXIIa; *closed squares* – hydrophobic OTS-coated vials preblocked with HSA containing 20 μg/mL PK, HK at 1:1 molar ratio to PK, and 0.05 μg/mL FXIIa; *closed triangles* – hydrophobic OTS-coated vials containing 20 μg/mL PK, HK at 1:1 molar ratio to PK, and 0.05 μg/mL FXII; *closed diamonds* – hydrophobic OTS-coated vials preblocked with HSA containing 20 μg/mL PK, HK at 1:1 molar ratio to PK, and 0.05 μg/mL FXII

### 3.3.2. Kallikrein generation in solutions of PK, HK, and FXII

After investigating the effects of the addition of HK on kallikrein activity in the presence of hydrophilic or hydrophobic surfaces, the kallikrein chromogenic assay was used to investigate kallikrein activity resulting from prekallikrein hydrolysis mediated through the interaction of FXII with the model hydrophilic or hydrophobic surfaces. As in Chatterjee et al. (Chatterjee, Thornton et al. 2009), the goal of these studies was to investigate the role of
adsorption to the surface on contact activation and reciprocal activation amplification step of FXII and prekallikrein interactions. Results summarized in Figure 3-4 show there was very little kallikrein activity generated in hydrophobic vials containing 0.05 μg/mL FXII, 20μg/mL prekallikrein. This was true whether the hydrophobic vials contained eqmolar quantities, relative to the molar prekallikrein concentration, of HK, IgG fragment dummy protein, or neither. No statistically significant differences were observed between these three conditions in hydrophobic vials. There were significantly higher amounts of kallikrein activity observed at glass surfaces. Similarly to Chatterjee et al., there was an initial lag period followed by a consistent rise in kallikrein activity. At time points after four minutes, statistically significant amounts of kallikrein activity were observed relative to those generated by hydrophobic surfaces. This was generally expected as FXIIa molecules generated via autoactivation at hydrophobic surfaces would adsorb to the surface, becoming unable to participate in further reactions with kallikrein. Thus, subsequent reciprocal-activation reactions would be attenuated. A statistically significant increase in kallikrein activity in hydrophilic vials containing HK relative to hydrophilic vials with and without the dummy IgG fragment was only observed at the 30 minute time point. However, over the 30 minute time span, the level of kallikrein activity in hydrophilic vials containing HK seems to begin to diverge from the level of kallikrein activity in hydrophilic vials without HK.

The results of control experiments, shown in Figure 3-3 along with control experiments discussed in the preceding section, show that little kallikrein activity was generated when 0.05 μg/mL FXII was added to hydrophobic OTS-coated vials pre-blocked with HSA containing 20 μg/mL prekallikrein with HK added at a 1:1 molar ratio of prekallikrein. As Figure 3-3 also shows that kallikrein activity is generated in hydrophobic
OTS-coated vials pre-blocked with HSA and 0.05 μg/mL FXIIa added (in contrast to FXII) this confirms that generation of kallikrein activity in the presence of HK requires FXIIa. This was expected as HSA-blocking of the OTS surface should minimize autoactivation of FXII and subsequent reciprocal activation processes. This result is also consistent with previous studies demonstrating less efficient coagulation induced by surfaces pre-blocked with bovine serum albumin (Chatterjee, Vogler et al. 2006; Chatterjee, Thornton et al. 2009).

**Figure 3-4:** Summary of results for kallikrein generation in solutions of PK and FXIIa, with and without HK. Hydrophobic- or hydrophilic-modified glass vials contained 20 μg/mL PK and FXII at 0.05 μg/mL. HK, when present, was added at a 1:1 molar concentration to PK. **Open squares** – hydrophilic vials with HK; **open diamonds** – hydrophilic vials without HK; **open triangles** – hydrophilic vials with IgG fragment; **closed squares** – hydrophobic vials with HK; **closed diamonds** – hydrophobic vials without HK; **closed triangles** – hydrophobic vials with IgG fragment. The right axis displays the measured absorbance readings while the left axis corresponds to equivalent kallikrein (Kal) concentrations determined by a standard curve prepared at 405 nm. (n=3)
3.3.3. HSA displacement of FXII activation products at OTS surfaces

The results discussed in the preceding sections suggest protein adsorption to the hydrophobic OTS-coated surface attenuates FXIIa-mediated prekallikrein hydrolysis in the presence of HK, just as similar experiments in Chatterjee et al suggested such adsorption prevents the interactions needed for reciprocal-activation. However, with the addition of HK to the system, attenuation of kallikrein activity generation in OTS-coated vials containing 0.05 μg/mL FXIIa was not as severe (Figures 3-2c, 3-2d) as it was in such experiments in Chatterjee et al without HK. Vogler and coworkers have proposed modeling protein adsorption to hydrophobic surfaces as the partitioning of proteins from bulk solution into an interphase, where the protein concentration scales with the bulk-solution concentration up to a saturating maximum (Krishnan, Liu et al. 2005; Noh and Vogler 2006). It would be expected that some fraction of FXIIa formed at the surface would partition back into the bulk solution, which could be amplified by HK to potentiate the response. In order to test the hypothesis that displaced protein moieties would be available for interactions leading to increased kallikrein activity generation, HSA was used at high concentrations relative to FXII, prekallikrein, and HK to displace adsorbed enzymes or zymogens from the hydrophobic surface.

Little kallikrein activity was seen when HSA at 5 mg/mL was added, after 10.5 minutes, to hydrophobic OTS-coated vials containing solutions of PK, HK at 1:1 molar ratio to PK, and FXII at 0.05 μg/mL. Although slightly increased when compared to controls where only an equivalent amount of PBS was added, this increase was not statistically significant. The concentration of FXII used for these experiments was then increased 10-fold to 0.5 μg/mL. Using this FXII concentration greater amounts of kallikrein activity were
observed as compared to the 0.05 μg/mL FXII trials. However, upon addition of HSA at 10.5 minutes, a rapid increase in kallikrein activity was not observed as compared to the negative controls in which an equivalent volume of PBS was added (Figure 3-5). This is in contrast to Chatterjee et al in which addition of BSA to the test vials resulted in rapid kallikrein activity generation presumably through displacement of the adsorbed proteins.

Figure 3-5: Changes in kallikrein activity generation in solutions of 20 μg/mL PK, HK added at 1:1 molar ratio to PK, and either 0.05 μg/mL or 0.5 μg/mL FXII in OTS-coated vials. Closed circles – OTS vials containing 0.5 μg/mL FXII with HSA added to 5 mg/mL at 10.5 minutes. Closed squares- OTS vials containing 0.5 μg/mL FXII with PBS added at 10.5 minutes. Closed triangles - OTS vials containing 0.05 μg/mL FXII with HSA added to 5 mg/mL at 10.5 minutes. Closed triangles - OTS vials containing 0.5 μg/mL FXII with PBS added at 10.5 minutes. (n=3)

Control experiments in which hydrophilic glass vials were used containing solutions of at PK at 20 μg/mL, HK at 1:1 molar ratio to PK, FXII at 0.05 μg/mL and the addition of HSA to 5 mg/mL at 10.5 minutes also showed no statistically significant difference in kallikrein activity generation at each time point (Figure 3-6).
Figure 3-6: Changes in kallikrein activity generation in solutions of 20 μg/mL PK, HK added at 1:1 molar ratio to PK, and either 0.05 μg/mL or 0.5 μg/mL FXII in hydrophilic glass vials. Open circles - glass vials containing 0.5 μg/mL FXII with HSA added to 5 mg/mL at 10.5 minutes. Open squares - glass vials containing 0.5 μg/mL FXII with PBS added at 10.5 minutes. (n=3)

The results of these studies suggest that the ability of HK to increase the generation of kallikrein activity can compensate for the loss of activity that may be associated with adsorption to a hydrophobic surface. Presumably, the zymogen or zymogen activation products released into bulk-solution after addition of the HSA was not enough to induce an increase in kallikrein activity as compared to the PBS controls.

3.4. Conclusion

A chromogenic assay was used to measure kallikrein activity generated through FXIIa-mediated conversion of prekallikrein to kallikrein in the presence of high molecular weight kininogen (HK). The results presented in this chapter (1) indicate the presence of HK
increases the generation of kallikrein activity when FXIIa is present; (2) indicate that an anionic hydrophilic surface is not a necessary cofactor for FXIIa-mediated hydrolysis of prekallikrein in the presence of HK; (3) suggest that prekallikrein hydrolysis in the presence of HK does not occur directly on the activating surface; (4) suggest that the ability of HK to increase the generation of kallikrein activity can compensate for the loss of activity that may be associated with adsorption to a hydrophobic surface.
3.5. Citations


Chapter 4

Investigation into the role of fibrin(ogen)olysis in blood plasma coagulation induced by mixed thiol self-assembled monolayers

Abstract

In Chapter 2, a small but statistically significant increase was observed in blood plasma coagulation times of mixed amine-/methyl-terminated thiol surfaces with contact angles of approximately 90°, as compared to other homogeneous amine- or methyl-terminated surfaces. The investigations outlined in this chapter were intended to determine if fibrinolysis or fibrinogenolysis may be responsible for this increase. D-Dimer assays, fibrinogen degradation product agglutination assays, and plasmin activity chromogenic assays were employed to determine whether any increase in fibrinolysis, fibrinogenolysis, or plasmin activity was observed in plasma coagulation assays of thiol chemisorbed surfaces. D-Dimer assays revealed only a transient spike in D-Dimer levels for all sample types tested, possibly due to increased epitope availability prior to the fibrin polymers forming an insoluble clot. Fibrinogen degradation products were not elevated, and chromogenic assays also revealed no increases in plasmin activity. This suggests fibrinolysis, fibrinogenolysis, and plasmin activity were not factors in the increased coagulation times previously observed.
Chapter 4

Investigation into the role of fibrin(ogen)olysis in blood plasma coagulation by mixed thiol self-assembled monolayers

4.1 Introduction

Thrombosis and coagulation are major risks long associated with the use of blood-contacting biomaterials. However, despite the prevalent use of these materials, a comprehensive and reliable model describing the role of the material surface in blood plasma coagulation has yet to be established. Indeed, many investigations focus upon the intrinsic pathway, just as Chapter 2 of this dissertation investigated the contact activation of blood plasma coagulation by mixed thiol self-assembled monolayers. However, fibrinolysis is also an important part of hemostasis and an interesting result described in Chapter 2 motivated this investigation into the possibility that the material surface influences fibrinolysis to such an extent that in vitro coagulation assays of such materials show extended coagulation times.

As described in Chapter 2, plasma coagulation times for mixed amine-/methyl-terminated thiol surfaces yielded interesting results for samples with wetting tension nearing 0 dyne/cm in that the blood plasma coagulation times were statistically significant increased as compared to coagulation times of other homogeneous amine- or methyl-terminated surfaces. Neat buffer FXII activation studies for these surfaces indicate that they are indeed capable of producing FXII autoactivation products at levels similar to those of homogeneous amine- or methyl-terminated surfaces. Previously, in Miller et al. (Miller, Guo et al. 2006), it was shown that a silane-based mixed film comprised of approximately 400 nm diameter islands of 3-aminopropyltriethoxysilane (APTES) backfilled with butyltricholorsilane (BTS) was an inefficient activator of the intrinsic coagulation cascade as compared to the
hydrophilic and hydrophobic control surfaces (plasma-cleaned glass beads and BTS- or octadecyltriethoxysilane-modified glass beads, respectively). Miller et al. suggests that this nanoscale chemical distribution may decrease contact activation. However, the mixed amine-/methyl-terminated thiol surfaces that yielded increased coagulation times did not possess a nanoscale chemical heterogeneity, thus prompting possible other explanations for the observed results.

Fibrinolysis serves to limit thrombus formation by preventing excessive fibrin formation through the action of plasmin. Plasmin is a serine protease resulting from the activation of plasminogen, which is synthesized in the liver and is present in the bloodstream at a concentration of approximately 2.4 μM (Robbins and Summaria 1976). Plasmin is capable of hydrolyzing fibrinogen, non-crosslinked fibrin, crosslinked fibrin, as well as other plasma proteins. It is well know that plasminogen has a strong affinity for lysine residues (Deutsch and Mertz 1970; Bachmann 2001) and that the ε-amino group of terminal lysyl residues in fibrin is important in their interaction (Christensen 1984; Warkentin, Johansen et al. 1998). This property of plasminogen has been utilized in the development of biomaterial surfaces with fibrinolytic properties (Woodhouse and Brash 1994; Fowers and Kopecek 1997; McClung, Clapper et al. 2000; McClung, Clapper et al. 2003; McClung, Babcock et al. 2007) so that nascent clots might be lysed before they become large enough to become pathologic. FXIIa is capable of activating plasminogen (Schousboe 1997; Schousboe, Feddersen et al. 1999), as well as the physiologic activators tissue-type and urokinase type plasminogen activator (t-PA and u-PA, respectively). Kallikrein itself is also able to activate u-PA (Ichinose, Fujikawa et al. 1986), lending thought to plasminogen-sequestering surfaces as a reasonable approach for creating biomaterials with better blood compatibility. As the
results described in Chapter 2 indicate, the mixed amine-/methyl-terminated thiol surfaces with contact angles of approximately 90° are capable of producing FXII activating products at levels similar to the hydrophobic methyl surfaces. It is hypothesized that these surfaces may influence the extent of fibrinolysis and/or fibrinogenolysis, thus increasing the observed coagulation time.

The objective of this study was to investigate whether there was any increase in fibrinolysis or fibrinogenolysis prior to coagulation of blood plasma in the presence of test surfaces. Furthermore, plasmin activity in the presence of test surfaces was assessed via chromogenic assay. Results do not indicate the presence of increased fibrinolysis or fibrinogenolysis, suggesting that some other factor or pathway may be responsible for the increased coagulation times of mixed amine-/methyl-terminated thiol surfaces with hydrophobic contact angles of approximately 90°.

4.2 Materials and Methods

4.2.1 Preparation of pooled human platelet poor plasma

Human platelet poor plasma (PPP), less than two days outdated, was obtained from M.S. Hershey Medical Center Blood bank. Multiple outdated lots were pooled and aliquoted in 15 mL polypropylene tubes and frozen at -20°C until use.
4.2.2 Quantification of plasminogen activity in pooled human plasma

The plasminogen activity of the pooled human PPP was verified using a commercially available Biophen Plasminogin kit purchased through Aniara (Mason, Ohio). The kit is comprised of a chromogenic assay for the quantitative measurement of plasminogen activity in human plasma. In a 90-well microplate well 50 μL of the test plasma (diluted 1:30 in phosphate buffered saline) was placed along with 50 μL of streptokinase solution provided with the kit and reconstituted with 2.5 mL of distilled water and preincubated at 37°C. This was left to incubate at 37°C for three minutes, after which 50 μL of chromogenic substrate was introduced and allowed to incubate for three minutes. The reaction was then stopped by adding 50 μL 10% chilled acetic acid. The absorbance was measured using a spectrophotometer set at 405nm and the results compared to a calibration curve, generated using a dilution series prepared from a normal plasma control provided in the kit. The plasminogen activity of the test sample was calculated from this curve. Three different vials of the pooled human PPP were assayed, with four samples taken per vial.

4.2.3 Surface Preparation

A 10 nm titanium adhesion layer and 200 nm gold layer were sputter coated (Thin Films, Inc., NJ) onto cleaned borosilicate glass coverslips measuring 10.5mm x 22mm. These substrates were used in the preparation of all thiol self-assembled monolayers. Dodecanethiol (Sigma-Aldrich), 11-mercaptoundecanoic acid (Sigma-Aldrich), 11-mercaptopoundecanol (Sigma-Aldrich), and 11-amino-1-undecanethiol (Asemblon) were used as received. 1 mM thiol solutions in anhydrous ethanol were used with mixtures made by
varying the ratios of the single-component solutions. Gold substrates were cleaned extensively before monolayer formation. First, the gold substrates were immersed in gold cleaning solution (Sigma-Aldrich) for 3 minutes followed by 3 rinses using deionized water (Millipore, 18.2 mΩ) and 3 rinses using ethanol. Next the samples were air-plasma cleaned, 15 minutes per side (100 W, Harrick) followed by 3 rinses with ethanol. The samples were allowed to sit in ethanol for at least 1 hour to allow sufficient time for the gold surface to become reduced (Ron and Rubinstein 1994; Ron, Matlis et al. 1998). Finally, the samples were immersed in a 0.125% (v/v) of butyltrichlorosilane (Gelest) in chloroform (Sigma-Aldrich) for 15 minutes, and then rinsed with chloroform and subsequently ethanol. This step was included to block any exposed glass due to insufficient gold coating of the coverslip edges or inadvertent scratching from handling the samples.

The cleaned, silane-treated samples were immersed in 1 mM thiol solutions at room temperature for 20 hours, protected from light. Stock solutions of 1 mM 11-mercaptoundecanoic acid and 11-amino-1-undecanethiol were prepared as noted above with the addition of trifluoroacetic acid or triethylamine, respectively, to 2.5% (v/v) (Wang, Chen et al. 2005). After incubation, the samples were rinsed with ethanol, with the exception of samples exposed to the 11-mercaptoundecanoic acid or 11-amino-1-undecanethiol solutions, for which a solution of 0.1 M NaOH or 0.1 M HCl, respectively, in deionized water was used in lieu of ethanol in an intermediate rinse. All samples were dried under N₂ and stored under vacuum, protected from light, for subsequent use.

Static, horizontal contact angles were measured via the sessile drop method with deionized water using a Kruss goniometer (KRÜSS Gmbh Contact Measuring System G10,
Hamburg, Germany). The Contact angles of 5 separate 20 μL drops were measured and averaged.

### 4.2.4 D-Dimer quantification

A commercially available ELISA kit (Aniara; Mason, Ohio) was utilized to assay the D-Dimer content of human plasma in the presence of model test surfaces. A single, thiol-modified substrate was placed in a 2.5 mL polystyrene semi-micro cuvette with 0.5 mL of plasma thawed to room temperature and 0.4 mL PBS. The nominal surface area of the substrate was estimated to be 472 mm², as calculated from the manufacturer supplied dimensions of the coverslip. The contents were recalcified with 0.1 mL of 0.1 M CaCl₂ and mixed on a hematology mixer at 8 rpm. The coagulation time recorded was the time from recalcification to coagulation, as seen from a change in the liquid state of the mixture to a gelatinous state. At various times prior to and sometimes after coagulation, a 16 μL aliquot of the 50% plasma solution were removed from the cuvette and placed into a well of the ELISA microplate containing 184 μL sample diluent giving a 12.5 dilution factor. This was allowed to incubate for one hour at room temperature, after which the well is washed five times using 300 μL quantities of the wash solution. 200 μL of the peroxidase-coupled anti-D-Dimer monoclonal antibody conjugate solution was added to the well and allowed to incubate for one hour at room temperature. Again the well is washed using the wash solution as before. After this washing, 200 μL of the peroxidase substrate (tetramethylbenzidine with hydrogen peroxide) was added to the well and allowed to incubate for five minutes at room temperature. The reaction was then quenched by the addition of 50 μL 0.45M sulfuric acid.
The absorbance was then measured at 450 nm, and the resulting D-Dimer concentration was calculated by referencing a calibration curve constructed using the kit-supplied standard 200 ng/mL D-Dimer calibrator. The baseline D-Dimer concentration of the human plasma was determined by assaying the 50% plasma solution just after recalcification.

4.2.5 Fibrinogen Degradation Product (FDP) agglutination assay

The commercially available Thrombo-Wellcotest kit (Remel, Lenexa, KS) was used in the semi-quantitative testing for the presence of fibrinogen degradation products in 50% human plasma in contact with mixed thiol self-assembled monolayers. In principle, the assay involves the addition of an antibody adsorbed latex particle in glycine buffer suspension to a sample of interest. The antibodies are specific to fibrinogen degradation products, including fragments X, Y, D, and E, as well as fibrin monomer. If the sample contains fibrinogen degradation products at a concentration of 2 μg/mL or greater, the latex particles will agglutinate and be visible to the naked eye.

A single, thiol-modified substrate was placed in a 2.5 mL polystyrene semi-micro cuvette with 0.5 mL of plasma thawed to room temperature and 0.4 mL PBS. The contents were recalcified with 0.1 mL of 0.1 M CaCl₂ and mixed on a hematology mixer at 8 rpm. After 30 minutes, the 1 mL 50% human plasma mixture was transferred to a kit-supplied sample collection tube containing soya bean trypsin inhibitor and Bothrops atrox venom. After the plasma had clotted, the clot was transferred to a 15-mL polypropylene centrifuge tube and centrifuged for 10 minutes at 1000 rcf in order to separate serum from the bulk of the clot. A dilution series consisting of serum diluted with glycine saline buffer was created. One drop of each serum dilution (100% serum, 10%, 5%, 2.5%, 0%) was transferred to a
separate position on a glass slide. One drop of the latex suspension, was added to each of the serum dilutions and briefly stirred. The glass slide is then gently rocked for exactly two minutes. A positive result is indicated when macroscopic agglutination is seen.

4.2.6 Chromogenic assay of plasmin activity

A chromogenic assay was employed to investigate whether plasmin activity could be detected in human plasma in the presence of mixed amine-/methyl-terminated thiol self assembled monolayers. A single, thiol-modified substrate was placed in a 2.5 mL polystyrene semi-micro cuvette with 0.5 mL of plasma thawed to room temperature and 0.4 mL PBS. The contents were recalcified with 0.1 mL of 0.1 M CaCl\(_2\) and mixed on a hematology mixer at 8 rpm. Starting at 5 minutes after recalcification , and every 10 minutes thereafter until coagulation, a 25 \(\mu\)L aliquot was taken from the sample cuvette and placed into the well of a 90-well microplate containing 235 \(\mu\)L tris-buffered saline and 20 \(\mu\)L of 5 mM Pefachrome PL (Centerchem, Norwalk, CT). This mixture was allowed to incubate for five minutes at 37°C until the reaction is arrested by the addition of 40 \(\mu\)L 10% acetic acid. The absorbance is then read at 405 nm.

4.3 Results and Discussion

4.3.1 Quantification of plasminogen activity in pooled human plasma

Results indicate that the pooled human plasma used in these studies have a plasminogen activity of 101.8% ± 5.7% (n=3) as compared to the normal control plasma
(Biophen Normal Control Plasma, Aniara; Mason, Ohio). The normal control plasma itself had a plasminogen activity of 97% as established against the NIBSC International Standard (National Institute for Biological Standards and Control, United Kingdom). Thus, the pooled human plasma used in the following studies can be considered to have a normal plasminogen activity.

4.3.2 D-Dimer quantification

Five different surfaces were chosen for this investigation: methyl-terminated, carboxyl-terminated, amine-terminated, and mixed amine-methyl terminated thiol chemisorbed surfaces. Their corresponding contact angles can be seen in Table 4-1.

Table 4-1: Contact angles of thiol self-assembled monolayers measured via the sessile drop method. For the mixed amine-/methyl-terminated surfaces, the ratio of the 1mM methyl and amine thiol solutions used to create the surface is given (methyl:amine).

<table>
<thead>
<tr>
<th>Thiol-chemisorbed surface</th>
<th>Sessile Drop Contact Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxyl-terminated, -S(CH)₁₁COOH</td>
<td>18°±2</td>
</tr>
<tr>
<td>Methyl-terminated, -S(CH)₁₁CH₃</td>
<td>103°±1</td>
</tr>
<tr>
<td>Amine-terminated, -S(CH)₁₁NH₂</td>
<td>35°±2</td>
</tr>
<tr>
<td>Mixed amine-methyl (10:10)</td>
<td>51°±1</td>
</tr>
<tr>
<td>Mixed amine-methyl (4:16)</td>
<td>89°±2</td>
</tr>
</tbody>
</table>
Figure 4-1: D-Dimer concentration measured via ELSIA versus time from coagulation. Dashed lines indicate the upper and lower bounds for the baseline D-Dimer concentration range (avg±SD).

Figure 4-1 illustrates the results of the D-Dimer ELISA experiments. When the absorbance is plotted against the time from coagulation, a spike in D-Dimer levels is clearly noted, but after coagulation and soon after the D-Dimer levels plummet. It is also seen that the D-Dimer levels peak at roughly the same range for the various thiol-chemisorbed surfaces. It is likely that this spike results from the availability of the D-Dimer epitope as fibrin polymerization begins, but before the soluble fibrin polymers are taken up in the formation of an insoluble clot (Adam, Key et al. 2009). The D-Dimer assay is intended to measure the D-Dimer fragment via a monoclonal antibody specific to epitopes in the fibrin D-Dimer region. However, the assumption that the D-Dimer specifically results from the degradation of
crosslinked fibrin has been challenged (Eisenberg, Jaffe et al. 1990; Lawler, Bovill et al. 1990; Bauer and Weitz 2001). Soluble fibrin polymers containing the D-Dimer region can also be recognized (Francis, Marder et al. 1980; Francis, Marder et al. 1980).

The coagulation times for the samples used for the D-Dimer ELISA are depicted in Figure 4-2 and Figure 4-3. Although, the coagulation times for the mixed amine-/methyl-terminated thiol surfaces with contact angles of 89°±2 did not have coagulation times at or above the baseline coagulation times, they are still significantly elevated (p ≤ 0.001) when compared to the methyl-terminated surface coagulation times. Results of statistical analyses are depicted in Figure 4-3

**Figure 4-2:** Coagulation times of thiol chemisorbed surfaces employed for the D-Dimer ELISA.
Figure 4-3: Coagulation times of mixed amine-/methyl-terminated thiol chemisorbed surfaces plotted against water adhesion tension (n ≥ 12). # - Indicates statistical significance determined via ANOVA, Holm-Sidak method, p ≤ 0.001. The dashed line and grey box indicate the average and standard deviation, respectively, for the coagulation time for the control blank.

4.3.3 Fibrinogen Degradation Product (FDP) agglutination assay

The results of the semi-quantitative FDP agglutination assay give no indication that fibrinogen degradation is occurring in the coagulation assay, with or without a sample surface presence, as increased levels of FDP relative to the control plasma were not observed. Results are tallied in Table 4-2. The control plasma consisted of 0.5 mL of pooled plasma thawed to room temperature with 0.4 mL PBS and 0.1 mL of 0.1 M CaCl₂ added, giving a 50% dilution. Immediately after recalcification the plasma was assayed. The resting FDP level in normal subjects is estimated at 4.9±2.8 μg/mL (Das, Allan et al. 1967) and the result of agglutination assays of the control plasma was consistent with this estimate.
The results of the agglutination assay are semi-quantitative, given the nature of the assay, results are tallied according to the concentration range corresponding to the dilution for which agglutination was seen. Table 4-2: FDP agglutination assay results. Given the semi-quantitative nature of the assay, results are tallied according to the concentration range corresponding to the dilution for which agglutination was seen.

<table>
<thead>
<tr>
<th>Sample</th>
<th>0-2 μg/mL FDP</th>
<th>2-20 μg/mL FDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH3 (101°±1)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>NH2 (44°±1)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>NH2 mix (86°±2)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Blank</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Control Plasma</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Of course, as the results of the agglutination assay are only semi-quantitative, there may still be differences in FDP levels between the different sample types. However, as no stark differences were discerned and that results for each sample, including the control, varied between 0-2 and 2-20 μg/mL, it is unlikely that FDP levels for the samples assayed would be substantially higher than those of the control plasma.

4.3.4 Chromogenic assay of plasmin activity

No differences in plasmin activity were observed between the various surfaces tested with negligible plasmin activity seen in all cases. Results are summarized in Figures 4-4a,b. Samples of heat treated plasma (50% in phosphate buffered saline) were tested for plasmin activity as a control with an average absorbance reading of 0.148±0.047 (n=2). This low level reading is likely due to the yellow hue of the plasma itself as the heat treatment would have inactivated any nascent proteolytic activity.
Figure 4-4a,b: Absorbance readings of chromogenic assays performed on 25 μL sample aliquots taken from the cuvette containing the sample surface and recalcified 50% plasma in PBS. Aliquots were placed into the well of a 90-well microplate containing 235 μL tris-buffered saline and 20 μL of 5 mM Pefachrome PL. This mixture was allowed to incubate for five minutes at 37°C until the reaction is arrested by the addition of 40 μL 10% acetic acid. The absorbance is then read at 405 nm.
Plasmin is rapidly inactivated by its inhibitor, \( \alpha_2 \)-antiplasmin, so it is possible that plasmin activity would not be seen via chromogenic assay. However, plasmin exists in plasma at a concentration roughly double of \( \alpha_2 \)-antiplasmin concentration (Bachmann 2001). Also, as the D-Dimer assay revealed only a transient spike in D-Dimer levels, likely due to increased availability prior to the fibrin polymers forming an insoluble clot, and fibrinogen degradation products were not elevated, any plasmin activity was likely limited and transient.

4.4 Summary

In Chapter 2, a small but noticeable increase in blood plasma coagulation times of mixed amine-/methyl-terminated thiol surfaces with hydrophobic contact angles of approximately 90°, as compared to other mixed amine-/methyl-terminated surfaces. The investigations outlined in this chapter were intended to determine if fibrinolysis or fibrinogenolysis may be responsible for this increase. A battery of assays (D-Dimer, FDP, plasmin activity) suggest that the increased coagulation times of mixed amine-/methyl-terminated thiol surfaces with water adhesion tension approximating 0 dyne/cm are likely not due to an increase in fibrinolysis or fibrinogenolysis through the action of plasmin. Although previous studies of these mixed surfaces indicate levels of FXII activation in neat buffer solution are consistent with those of pure methyl surfaces, the trend of increased coagulation times persists, with statistical significance shown in this chapter (Figure 4-3. Additionally, in Miller et al. the water adhesion tensions of the mixed amine/methyl surfaces and the BTS coated surfaces both approximated 0 dyne/cm, thus the increased coagulation times do not seem to be a consequence of macroscale wettability. However, this does not
rule out possible effects of localized wettability on the nanoscale. The response elicited when a biological fluid, such as blood plasma, is brought into contact with a material is influenced by the properties of the vicinal water. Initial events may include surface hydration, electrostatic double-layer formation or hydrophobic interactions, followed by protein adsorption, then cellular adsorption, adhesion, or activation. It is generally known that hydrophobic surfaces adsorb more protein than hydrophilic surfaces but conformation or orientation of the adsorbed protein at the interface also influences the biologic response to the biomaterial surface.

Broadly speaking, there are five important components of the vasculature’s ability to balance procoagulant and anticoagulant processes in promoting hemostasis: the vascular endothelium, platelets, blood coagulation factors, blood coagulation inhibitors, and fibrinolysis. As vascular endothelium was not a factor in the coagulation assays of the mixed amine-/methyl-surfaces, it is safe to rule it out as a possible actor resulting in the increased coagulation times seen for the 0 dyne/cm amine-/methyl terminated surfaces. Furthermore, as the results of this study suggest, fibrinolysis and fibrinogenolysis may be viewed as unlikely causes. As platelet-poor plasma was used in these experiments, the interaction of platelets in this setting is also unlikely. This now leaves blood coagulation factors and blood coagulation inhibitors. Localized water wettability may be affecting protein adsorption, conformation, and/or orientation at the surface which would consequently affect the biologic response of the blood coagulation factors and inhibitors contained in human blood plasma.
4.5 Conclusion

Results of D-Dimer assays, fibrinogen-degradation product agglutination assays, and plasmin chromogenic assays suggest that fibrinolysis, fibrinogenolysis, or plasmin activity are not responsible for the trends seen in the coagulation times of mixed methyl-/amine-terminated thiol surfaces. This suggests that some other factor or pathway may be responsible for the increased coagulation times of mixed amine-/methyl-terminated thiol surfaces with hydrophobic contact angles of approximately 90°.
4.6 Citations


Chapter 5

Preliminary investigation into the contact activation of blood plasma coagulation in response to surfaces with nanometer-scale chemical patterning

Abstract

This chapter concerns the preliminary investigation into the effect of nanometer-scale patterning on the contact activation of blood coagulation of thiol self-assembled monolayers. Carboxyl-terminated and methyl-terminated thiols were chosen as the components of the microcontact printed surfaces as one-component carboxyl-terminated and methyl-terminated thiol chemisorbed surfaces represent two extremes in terms of the response of contact activation to those surfaces. Microcontact printing using contact inked PDMS stamps formed from silicon masters were used to pattern gold surfaces. Preliminary results indicate nanometer-scale patterning of surfaces does lead to statistically-significant increased coagulation times, relative to surfaces with equivalent, albeit macro-scale, areas of carboxyl-and methyl-terminated thiol chemisorption. These increased coagulation times suggest that the patterning of the surface may affect inducement of the intrinsic coagulation cascade and that it is not simply a matter of the cumulative carboxyl-terminated thiol backfilled area.
5.1. Introduction

The use of blood-contacting medical devices is prevalent in the clinical setting, with uses ranging from routine blood collection to more invasive procedures, such as percutaneous coronary interventions, and very invasive procedures such as cardiopulmonary bypass. Thrombosis due to interaction of blood with blood-contacting materials remains a significant risk, necessitating the use of anticoagulants which have their own associated risk of uncontrolled bleeding (Rose, Gelijns et al. 2001; Copeland, Smith et al. 2004; Pae, Connell et al. 2007). The response elicited when a biological fluid, such as blood plasma, is brought into contact with a material is influenced by the properties of the vicinal water (see Chapter 1 for further discussion), with protein adsorption often being the response of interest. Water-wettability of surfaces has been shown to have considerable influence on contact activation of blood plasma coagulation (Vogler, Graper et al. 1995; Zhuo, Siedlecki et al. 2006; Zhuo, Siedlecki et al. 2007; Vogler and Siedlecki 2009). For further discussion on contact activation and the importance of protein adsorption, please refer to sections 1.1.2, 1.2, and the references included therein. Furthermore, Chapter 2 of this dissertation discussed the contact activation of blood plasma coagulation by mixed thiol self-assembled monolayers. The mixed thiol self-assembled monolayers in that study were formed by the chemisorption of two different thiol moieties from solution and did not contain any well-defined patterning, or any significant domain separation. However, nanometer-scale patterning or domain formation is of interest as it has been put forth that the microphase
separation in polyurethane materials, a material widely used in biomedical applications (Lamba, Woodhouse et al. 1998), contributes to its hemocompatibility (Bernacca, Gulbransen et al. 1998; Xue and Greisler 2003). Indeed, studies indicate microphase separation of the hydrophilic hard segment and hydrophobic soft segment mediate the local surface environment and influence protein and cellular interactions at the surface (Xu, Runt et al. 2010; Xu and Siedlecki 2010). The influence of surface domains on human blood plasma coagulation was the motivation for a study by Miller et al. (Miller, Guo et al. 2006) which investigated silane-based mixed films on glass substrates having nanoscale chemical heterogeneity formed by the stepwise adsorption of an amine-terminated silane, 3-aminopropyltriethoxysilane (APS), and a methyl-terminated silane, n-butytrichlorosilane (BTS). Their results showed that their mixed films with nanometer-scale chemical heterogeneity were inefficient at activating the intrinsic coagulation of human blood plasma when compared to their one-component control surfaces.

This chapter concerns the preliminary investigation into the effect of nanometer-scale patterning on the contact activation of blood coagulation of thiol self-assembled monolayers. Carboxyl-terminated and methyl-terminated thiols were chosen as the components of the microcontact printed surfaces as one-component carboxyl-terminated and methyl-terminated thiol chemisorbed surfaces represent two extremes in terms of the response of contact activation to those surfaces. Microcontact printing using PDMS stamps formed from silicon masters were used to pattern gold surfaces. Preliminary results suggest nanometer-scale patterning does increase the coagulation time of the surface, relative to a surfaces with equivalent, macro-scale, areas of carboxyl-, and methyl-terminated thiol chemisorptions.
Further testing is needed to confirm these preliminary results as well as an exploration of different patterned surfaces.

5.2. Materials and Methods

5.2.1. PDMS stamp fabrication

Nanotextured silicon substrates consisting of a linear pattern with manufacturer reported dimensions of an 833 nm period, 416 nm wide and 250 nm deep grooves (Figure 5-1), were used in preparing polydimethylsiloxane (PDMS) stamps for microcontact printing. In practice, actually dimensions of the silicon master varied substantially from manufacture reported, as shown in Figure 5-1. The commercially supplied silicon substrate (Lightsmyth, Eugene, OR) was first fluorinated using tridecafluoro-1,1,2,2-tetrahydrooctyl(dimethylchlorosilane (Gelest). The silicon master was cleaned using a solvent series rinse consisting of ethanol, acetone, then chloroform, and then dried under nitrogen. The master was immersed for two hours in a 1% solution (v/v) of the fluorinated silane in chloroform. The master was subsequently rinsed with chloroform, sonicated in chloroform for one hour, then rinsed once more with chloroform before finally dried under nitrogen. The PDMS stamp was formed by covering the silicon master, placed in a Teflon dish, with a 10:1 mixture of SYLGARD 184 and its curing agent. The PDMS covered master was left to stand for at least one hour at room temperature under vacuum until any air bubbles were removed from the mixture. After air bubbles were removed, final curing was performed at 70°C under vacuum for at least four hours. Flat-surfaced inking pads were
fabricated using nearly identical conditions with the substitution of a smooth silicon master in place of the patterned master.

![AFM image of a silicon master used for PDMS stamp preparation. Tapping mode in air was used. The trenches (dark bands) measured approximately 200 nm deep and 260 nm wide at the bottom. This width is likely underestimated due to the geometry of the probe used. The probe, being conical, would not be able to reach the very edges of the bottom of the trench. The period, consisting of one ridge (light band) and adjacent trench was approximately 748nm.](image)

**Figure 5-1:** AFM image of a silicon master used for PDMS stamp preparation. Tapping mode in air was used. The trenches (dark bands) measured approximately 200 nm deep and 260 nm wide at the bottom. This width is likely underestimated due to the geometry of the probe used. The probe, being conical, would not be able to reach the very edges of the bottom of the trench. The period, consisting of one ridge (light band) and adjacent trench was approximately 748nm.

### 5.2.2. Formation of patterned surfaces

The contact-inking (Libioulle, Bietsch et al. 1999; Fujihira, Furugori et al. 2001) method of microcontact printing was used to create the patterns on the gold-coated coverslips. A 10 nm titanium adhesion layer and 200 nm gold layer were sputter coated (Thin Films, Inc., NJ) onto cleaned borosilicate glass coverslips measuring 10.5mm x 22mm. Gold substrates were cleaned extensively before monolayer formation. The gold and titanium was removed from the edges of the coverslips using a Dremel diamond wheel point. This was done by hand, as the coverslips were too delicate for the use the Dremel power tool. After
the edges were stripped, the coverslips were briefly rinsed using a solvent series, and subsequently cleaned. First, the gold substrates were immersed in gold cleaning solution (Sigma-Aldrich) for 3 minutes followed by 3 rinses using deionized water (Millipore, 18.2 mΩ) and 3 rinses using ethanol. Next the samples were air-plasma cleaned, 15 minutes per side (100 W, Harrick) followed by 3 rinses with ethanol. The samples were allowed to sit in ethanol for at least 1 hour to allow more than sufficient time for the gold surface to become reduced (Ron and Rubinstein 1994; Ron, Matlis et al. 1998). Finally the samples were immersed in a 0.125% (v/v) of butyltrichlorosilane (Gelest) in chloroform (Sigma-Aldrich) for 15 minutes, and then rinsed with chloroform and subsequently ethanol. This step was included to block any exposed glass due to insufficient gold coating, or purposeful gold removal, of the coverslip edges and any inadvertent scratching from handling the samples.

The flat PDMS inking pad, used to transfer the octadecanethiol to the PDMS stamp, was immersed in a octadecanethiol solution (0.3-1.0 mM as noted later in the text) in ethanol overnight. The inking pad was then removed from the thiol solution and had any excess solution removed by a dry nitrogen stream. The patterned PDMS stamps were then placed on the thiol-impregnated inker pads for one minute. The stamps were then brought into contact with the cleaned gold surfaces for 30 seconds under a 100 g weight, and the substrates then immersed in a 1 mM solution of 11-mercaptoundecanoic acid in ethanol for approximately 15 minutes.

Static, horizontal contact angles were measured via the sessile drop method with deionized water using a Kruss goniometer (KRÜSS Gmbh Contact Measuring System G10, Hamburg, Germany). The contact angles of 3 separate 20 μL drops were measured and averaged.
5.2.3. Atomic Force Microscopy of patterned surfaces

A Nanoscope IIIa Multimode atomic force microscope (Digital Instruments, Inc., Santa Barbara, CA) utilizing plasma-cleaned silicon nitride tip was used to obtain height and friction images of the mixed SAMs in an effort to detect single-component domains. Contact mode in air was used to acquire height and friction images for the sample surfaces while tapping mode in air was used to obtain height images of PDMS stamps.

5.2.4 Substrate-induced contact activation in platelet poor plasma

A single, thiol-modified substrate was placed in a 2.5 mL polystyrene semi-micro cuvette with 0.5 mL of plasma thawed to room temperature and 0.4 mL PBS. The nominal surface area of the substrate was estimated to be 472 mm$^2$, as calculated from the manufacturer supplied dimensions of the coverslip. The contents were recalcified with 0.1 mL of 0.1 M CaCl$_2$ and mixed on a hematology mixer at 8 rpm. The coagulation time recorded was the time from recalcification to coagulation, as seen from a change in the liquid state of the mixture to a gelatinous state. Statistical analyses were performed by parametric ANOVA (Holm-Sidak’s test) using SigmaPlot software. Means of experimentally determined coagulation times for each surface type were compared pair-wise and the differences were considered statistically significant for p-values less than the critical p-value as determined by the Holm-Sidak test ($\alpha=0.05$).
5.3. Results and Discussion

5.3.1. Stamp fabrication and microcontact printing of surfaces

Figure 5-2 shows a height image, from the top-down perspective, of a PDMS stamp fabricated from the silicon master. The PDMS stamp was imaged after being used for microcontact printing, thus the two artifacts noted on the surface were likely picked up during use or though exposure to the ambient environment.

![Figure 5-2: Top-down image of PDMS stamp; Tapping mode AFM in air was used.](image)

In general, microcontact printing of the gold-sputter-coated surfaces was difficult to accomplish in a reproducible and uniform fashion. Octadecanethiol was used as the thiol inking solution for two reasons. First, no success was achieved in using carboxyl-terminated thiols as the thiol in the inking solution. This is understandable as sufficient time is needed
to form a well-ordered surface. Generally, thiol chemisorptions to the surface occurs quickly, ranging from milliseconds to minutes, but more time is needed for the reorganization to occur that leads to a densely packed and well-ordered surface (Love, Estroff et al. 2005). In stamping applications, too much contact time with the stamp can lead to diffusion of the thiol past its intended areas of deposition, while too little time leads to an incompletely covered and disordered surface, detracting from the goal of achieving a hydrophilic pattern in a hydrophobic background. A technique called microcontact displacement printing was attempted in order to try and utilize carboxyl-terminated thiols as the inking solution. This is discussed in section 5.3.3. Second, octadecanethiol was used instead of a shorter chain methyl-terminated thiol as shorter chain thiols have faster lateral diffusion rates than longer chain thiols (Stranick, Parikh et al. 1994; Delamarche, Geissler et al. 2002). Lateral diffusion of the thiol molecules across the surface is a chief concern in producing acceptable patterns. Therefore time, distance, and concentration are important parameters of the printing process. As the stamp’s dimensions were obtained from the silicon master, the parameter of distance was set, leaving time of stamping and concentration of the thiol inking solution as parameters to vary.

A set of thiol-patterned surfaces were fabricated using a flat PDMS inking pad immersed in 0.3 mM octadecanethiol solution in ethanol overnight. The inking pad was then removed from the thiol solution and had any excess solution removed by a dry nitrogen stream for approximately 10 seconds. The patterned PDMS stamps were then placed on the thiol-impregnated inker pads for one minute. The stamps were then brought into contact with the cleaned gold surfaces for 30 seconds, and then immersed in a 1 mM solution of 11-
mercaptoundecanoic acid in ethanol for approximately 15 minutes. Figure 5-3 shows a diagram of the stamping process.

**Figure 5-3:** Preparation of patterned samples using the contact inking method. Light grey shading represents octadecanethiol. Dark grey shading represents 11-mercaptoundecanoic acid. The ridges of the PDMS stamp are approximately 500 nm wide.
Three patterned samples were imaged using atomic force microscopy. Three areas per sample were imaged. Contact mode in air using plasma cleaned tips was used to acquire height and friction images for the sample surfaces. All areas imaged showed patterning. Representative images of the surfaces are shown in Figure 5-4 below.

**Figure 5-4:** From left to right — height, friction mode retrace, and friction mode trace AFM images of patterned thiol surfaces. Bare gold surface was stamped with a linear pattern PDMS stamp inked with octadecanethiol and backfilled with 11-mercaptoundecanoic acid. The light colored bands in the friction retrace image (middle) correspond to the stamped octadecanethiol. Pattern was imaged using a hydrophilic, plasma cleaned probe.

The wider bands in the friction trace and retrace images correspond to the octadecanethiol stamped areas, while the thinner bands correspond to the 11-mercaptoundecanoic acid backfilled areas. The cumulative area of 11-mercaptoundecanoic acid backfilled areas was estimated to be 40% by comparing band widths. Contact angles of the three patterned samples imaged, as well as the octadecanethiol and 11-mercaptoundecanoic acid controls are shown in Table 5-1.
Table 5-1: Contact angles of sample surfaces (mean ± SD, n=3) measured using the sessile-drop method.

<table>
<thead>
<tr>
<th>Sample (% 11-mercaptoundecanoic acid backfilled)</th>
<th>Contact Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patterned (40%)</td>
<td>73°±4</td>
</tr>
<tr>
<td>Octadecanethiol stamped control (0%)</td>
<td>96°±0</td>
</tr>
<tr>
<td>11-mercaptoundecanoic acid backfilled control (100%)</td>
<td>24°±0</td>
</tr>
</tbody>
</table>

The average contact angle measured for the patterned samples is consistent with that predicted by the Cassie equation, which predicts the effective contact angle for a composite surface (Cassie and Baxter 1944). The equation is listed below:

$$\cos \theta_c = f_1 \cos \theta_1 + f_2 \cos \theta_2$$  \hspace{0.5cm} (1)

wherein $f_1$ and $f_2$ are the area fractions of the composite surface with contact angles $\theta_1$ and $\theta_2$ respective. $\theta_c$ is then the effective contact angle. Using 0.4 for $f_1$, 0.6 for $f_2$ and the contact angles given in Table 5-1 for $\theta_1$ and $\theta_2$, $\theta_c$ is predicted to be 72°, which is statistically identical to the measured value.

5.3.2. Plasma coagulation assay of patterned surfaces

The patterned substrates were used in plasma coagulation assays as detailed in section 5.2.4. A series of control samples were prepared using flat, octadecanethiol inked PDMS stamps to approximate a range of cumulative 11-mercaptoundecanoic acid backfilled areas, in which the 11-mercaptoundecanoic acid backfilled area is contiguous. This is in contrast to the patterned substrates in which the cumulative 11-mercaptoundecanoic acid backfilled area
is arrayed in alternating bands. By comparing the coagulation times of the patterned substrates to those in the control series of an approximate cumulative 11-mercaptoundecanoic acid backfilled area, we strive to elucidate whether the nanometer-scale pattern affects the coagulation times such that it is not simply the result of the cumulative area the 11-mercaptoundecanoic acid backfilled sections. Figure 5-5 plots the coagulation times of the three patterned substrates tested and the flat-stamped controls against their cumulative 11-mercaptoundecanoic acid backfilled area.

Figure 5-5: Coagulation times of the three patterned substrates tested and the flat-stamped controls against their cumulative 11-mercaptoundecanoic acid backfilled area. The “Cumulative Carboxyl Surface Area” represents the amount of area of the 462 mm² substrate chemi-sorbed with 11-mercaptoundecanoic acid. The black curve represents a mathematical model taken from previously published work that was fit to the control substrates. The blue curves represent the bounds of the 95% confidence interval while the red curves represent the bounds of the 95% prediction interval.
A mathematical model (Eq. 2) taken from previously published studies (Vogler, Graper et al. 1995; Zhuo, Miller et al. 2005; Miller, Guo et al. 2006) was fit to the control series. This was done in order to then construct confidence and prediction bands so that coagulation times of the patterned surfaces could be better compared to the controls.

\[
CT = -\left(\frac{1}{k_p}\right)\ln\left[\frac{k^{\text{AT}}_{\text{act}}A + e^{-k_pCT_0}}{k^{\text{AT}}_{\text{act}}A + 1}\right]
\]  (2)

The model compares surface-induced coagulation to a negative control. In previous studies, this negative control was the time to coagulation in vessels without any additional procoagulant added, represented as \(CT_0\) in Eq. 2. Here, the negative control is the time to coagulation in a semi-microcuvette with the octadecanethiol flat-stamped hydrophobic control. This was deemed more appropriate, for as the cumulative 11-mercaptoundecanoic acid backfilled areas of the substrates in the control series decreases, there remains a substantial cumulative octadecanethiol stamped area with its associated contribution to surface-induced coagulation. Only the cumulative 11-mercaptoundecanoic acid backfilled areas (represented as “A” in Eq. 2) were considered in comparing amount of surface area present to coagulation time, as these surfaces are much more efficient activators of coagulation as compared to methyl surfaces. For instance, a substrate with a cumulative 11-mercaptoundecanoic acid backfilled area of 50% was considered to be contributing 231 \(\text{mm}^2\) (0.5 x 462 \(\text{mm}^2\)) to surface-induced coagulation.

As Figure 5-5 on the previous page shows, the patterned substrates had coagulation times well outside the bounds of the prediction interval, which are pictured as the red outermost curves on the graph. The mean coagulation time of the patterned substrates was also compared to the means of the control series coagulation times. As shown in Figure 5-6,
the patterned substrates had a mean coagulation time that was increased relative to the control series. Parametric ANOVA analysis using SigmaPlot revealed this increase to be statistically significant, $p \leq 0.001$. 

Figure 5-6: Coagulation time means of patterned and control series surfaces (100%, 50%, 25%, 12.5%, and 6.25% cumulative 11-mercaptoundecanoic acid backfilled area). Each substrate presented nominally 462 mm$^2$ surface area, not including the edges. For example, a substrate with 50% cumulative 11-mercaptoundecanoic acid backfilled area would then have 231 mm$^2$ with 11-mercaptoundecanoic acid chemisorbed and 231 mm$^2$ with octadecanethiol chemisorbed. Error bars represent 1 SD. * denotes statistical significance, $p \leq 0.001$ as determined by parametric ANOVA. (n=3)

5.3.3. Microcontact displacement printing

As previously explained, microcontact printing with a methyl-terminated thiol followed by backfilling with a carboxyl-terminated thiol was problematic in that the edges of the gold-coated coverslips were coated with a carboxyl-terminated thiol. This area, although only a few square millimeters, is enough to cause a dramatically decreased coagulation time
when the sample is used in the coagulation assay. Microcontact printing with a carboxyl-terminated thiol followed by backfilling with a methyl-terminated thiol was unsuccessful as surfaces with uniform patterning could not be produced. The technique of microcontact displacement printing (Dameron, Charles et al. 2005; Dameron, Hampton et al. 2005) was attempted in order to use a carboxyl-terminated thiol inking solution and a methyl-terminated thiol backfilling solution. In brief, microcontact displacement printing is different from microcontact printing in that the gold surface is first coated with 1-adamantanethiol. This thiol can be displaced by alkanethiols either in solution or through contact printing. However, the presence of the 1-adamantanethiol on the gold surface hinders lateral diffusion of alkanethiols, an attribute which may be useful in microcontact printing applications.

1-adamantanethiol coated gold-surfaces were prepared by overnight immersion in 10 mM 1-adamantanethiol in ethanol. Subsequently, the adamantathiol coated surfaces were stamped with PDMS stamps inked with 25 mM 11-mercaptoundecanoic acid for 3 minutes. The surfaces were then immersed in 1 mM octanethiol in ethanol solution for 20 minutes. The stamped sample is then imaged using contact mode AFM with a plasma-cleaned SiN tip under air. Figure 5-7 shows the results of this process.

Figure 5-7: Height (left), friction retrace (middle), and friction trace (right) AFM images of microcontact displacement printed surface.
A very faint pattern can be seen in the friction trace and retrace images, but the lack of contrast indicates an incomplete displacement of the adamantanethiol with the 11-mercaptoundecanoic acid. Contact angle measurements of control samples that had been stamped using a flat, non-patterned PDMS pad support this assertion as well as they were not even in the hydrophilic range (66°±2). As such, these patterns were unsuitable for application in blood plasma coagulation studies. It was then thought instead to remove the gold and titanium from the edges of the coverslips, exposing the glass to OTS-silanization, in order to eliminate edge effects from the 11-mercaptoundecanoic acid backfilling step.

5.4. Conclusion and Future Direction

As shown in section 5.3.2, the patterned surfaces exhibited elevated coagulation times as compared to control samples with similar, or even greater, cumulative 11-mercaptoundecanoic acid backfilled areas. This suggests that the patterning of the surface may affect inducement of the intrinsic coagulation cascade and that it is not simply a matter of the cumulative carboxyl-terminated thiol backfilled area.

As this was a preliminary investigation there are further questions for future studies involving the plasma coagulation response to patterned surfaces. First, what affect would a pattern consisting of islands versus bands have on activation of the intrinsic coagulation cascade? The banded patterning of the substrates in this study had nanometer-scale widths, while their lengths were centimeter-scale. It should be investigated whether nanometer-scale islands would have a different affect on the activation of the intrinsic coagulation cascade. Second, how does the distribution of the pattern affect activation of the intrinsic coagulation
cascade? Patterns bearing the same cumulative backfilling area but differing in the size of their features should be utilized in plasma coagulation assays to discern how large the features can be and still affect activation of the intrinsic coagulation cascade. Presumably, there would be a limit at which substrates with larger features would behave in the plasma coagulation assay similarly to the controls having only two, contiguous hydrophobic and hydrophilic areas.

This study suggests that nanometer-scale surface patterning decreases activation of blood plasma coagulation. Further investigations into this may provide a means for the rational development of patterned surfaces with improved hemocompatibility.
5.5. Citations


Chapter 6

Summary

Coagulation and thrombosis resulting from blood-material interactions remains a challenge in the use of blood-contacting cardiovascular materials, necessitating the use of anticoagulants which have their own associated risk of uncontrolled bleeding. Developing new materials with improved hemocompatibility will entail a better understanding of the role of the surface in influencing the biological response resulting from blood-material interaction. The formation of a thrombus on the surface of a blood-contacting material in vivo involves activation of the intrinsic coagulation cascade, also termed contact activation, and the adhesion and activation of platelets. As both processes are by themselves very complex, the work discussed in this dissertation has focused on the role of the surface in the intrinsic coagulation cascade.

The common widespread view of contact activation imparts activating abilities specific to anionic, hydrophilic surfaces whereby contact activation proteins in blood plasma, such as FXII and high-molecular weight kininogen, bind to anionic surfaces via anionic binding domains rich in positive-charged amino acids (see 1.1.2 for further discussion and references). This view is limited in its usefulness as a model to develop biomaterials with better hemocompatibility as its only guideline is to avoid the use of anionic, hydrophilic materials. However, the use of hydrophobic materials does not eliminate the initiation of blood coagulation nor thrombus formation as evidenced by the need for anticoagulation when using such blood-contacting materials in vivo. Furthermore, previous studies have demonstrated the importance of protein adsorption at the biomaterial surface to contact activation leading to an apparent specificity to anionic surfaces (see 1.2). The central
hypothesis of this work posits that all material surfaces are potential activators of the intrinsic coagulation cascade, but protein adsorption moderates the molecular interactions of contact activation.

Mixed carboxyl/methyl-, hydroxyl/methyl-, and amine/methyl-thiol modified surfaces were prepared and utilized as the procoagulant material for in vitro coagulation assays as well as FXII activation in neat buffer solution (2.1). A minimum for FXII activation in neat buffer solutions is seen for mixed thiol-adsorbed SAMs having water adhesion tension, \( \tau = 45\text{-}50 \text{ dyne/cm} \) (2.3.3). While plasma coagulation studies (2.3.2) appear to support a role for anionic surfaces in contact activation, the results suggest FXII activation in neat buffer solution is related to surface wettability, with a minimum observed at mid-range wettabilities. These results demonstrated that the composition of the solution, i.e. blood plasma versus buffer, and the surface properties of the material both influence contact activation, as well as demonstrate that activation of FXII cannot be viewed as being specific to anionic surfaces.

Previous investigations observed that kallikrein mediated reciprocal-activation of FXII was the principal pathway of FXII activation at test surfaces and that FXII-mediated hydrolysis of prekallikrein does not appear to occur at by the assembly of those molecules at the material surface (see 1.1.2 and references therein). In Chapter 3, these studies were extended by using a chromogenic assay to measure kallikrein activity generated through FXIIa-mediated conversion of prekallikrein to kallikrein in the presence of high molecular weight kininogen (HK). Cleaned glass and octadecyltrichlorosilane-treated glass were used as the test hydrophilic and hydrophobic surfaces, respectively. The results observed suggest that HK increases the generation of kallikrein activity when FXIIa is available in the presence of either a hydrophilic or hydrophobic surface (3.3.1); an anionic hydrophilic
surface is not a necessary cofactor for FXIIa-mediated hydrolysis of prekallikrein in the presence of HK (3.3.1-3); prekallikrein hydrolysis in the presence of HK does not occur directly on the activating surface (3.3.1); and the ability of HK to increase the generation of kallikrein activity can compensate for the loss of activity that may be associated with adsorption to a hydrophobic surface (3.3.3). This investigation, as the others that preceded it, demonstrated that contact activation is not specific to anionic surfaces and supports that the apparent specificity to anionic hydrophilic surfaces is instead a reduced efficiency of contact activation at hydrophobic surfaces due to adsorption competition between FXII and other plasma proteins (see 1.2 and reference therein).

As described in Chapter 2, plasma coagulation times for mixed amine-/methyl-terminated thiol surfaces yielded interesting results for samples with wetting tension nearing 0 dyne/cm in that the some coagulation times appeared greater than baseline, although statistical significance was not achieved (2.3.2). Neat buffer FXII activation studies for these surfaces indicate that they are indeed capable of producing FXII autoactivation products (2.3.1), suggesting that another actor or pathway may be involved. Since it has been observed that plasminogen has a strong affinity for lysine residues and that the ε-amino group of the terminal lysyl residues in fibrin is important in their interaction (see 4.1 and references therein), it was hypothesized that the mixed amine-/methyl- surfaces may influence the extent of fibrinolysis and/or fibrinogenolysis. However, results of D-dimer assays (4.3.2), fibrinogen-degradation product agglutination assays (4.3.3), and plasmin chromogenic/fluorgenic assays (4.3.4) suggest that fibrinolysis or fibrinogenolysis are not responsible for the trends observed in the coagulation times of mixed amine-/methyl-terminated thiol surfaces. It was also shown that the coagulation times for mixed amine-
methyl-terminated thiol surfaces with wetting tension nearing 0 dyne/cm were significantly elevated, \( p \leq 0.001 \), as compared to one-component methyl- or amine- terminated surfaces (Figure 4-3). This suggests that some other phenomena may be causing the decrease in contact activation, possibly associated with protein adsorption and interactions at the chemically heterogeneous surface.

The effect of nanometer-scale patterned chemically heterogeneous surfaces was addressed in Chapter 5. Carboxyl-terminated and methyl-terminated thiols were chosen as the components of the microcontact printed surfaces as one-component carboxyl-terminated and methyl-terminated thiol chemisorbed surfaces represent two extremes in terms of the response of contact activation to those surfaces. Microcontact printing using contact inked PDMS stamps formed from silicon masters were used to pattern gold surfaces. Preliminary results indicate nanometer-scale patterning does increase the coagulation time of the surface, relative to a surfaces with equivalent, macro-scale areas of carboxyl-, and methyl-terminated thiol chemisorptions (5.3.2). This suggests that the patterning of the surface may affect inducement of the intrinsic coagulation cascade and that it is not simply a matter of the cumulative carboxyl-terminated thiol backfilled area.

In summary, the studies described in this dissertation demonstrate a need for further investigation into the roles of cofactors (i.e. HK) into the mechanisms of contact activation as well as the effects of surface chemical heterogeneity. For instance, FXI also circulates in human blood plasma complexed with HK (Thompson, Mandle et al. 1977) and both FXIIa and kallikrein cleave HK in similar manners (Wiggins 1983; Colman and Schmaier 1997). As was demonstrated in Chapter 3, an anionic hydrophilic surface is not a necessary cofactor for FXIIa-mediated hydrolysis of prekallikrein in the presence of HK (3.3.1-3) and
prekallikrein hydrolysis in the presence of HK does not occur directly on the activating surface. This brings us to the next logical step of investigation, i.e. the effect of adsorption-competition upon FXIa generation in a model system consisting of FXII, prekallikrein, FXI, and HK.

Further investigations into the effects nanometer-scale patterned chemically heterogeneous surfaces on contact activation should focus on aspects such as pattern geometry, pattern size, and chemical functionality. It is likely that varying these aspects would have significant ramifications on protein interactions at the surface, affecting contact activation.

It is the goal of the investigations in this dissertation to contribute to the understanding of the role of the surface in contact activation and provide direction towards future investigations. Increased understanding of the role of the surface in contact activation is essential for the development biomaterials with improved hemocompatibility.
Citations


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