AN ABSTRACT OF THE THESIS OF

Omkar Joshi for the degree of Master of Science in Chemical Engineering presented on May 27, 2004.
Title: Fibrinogen Adsorption at Heparinized Silica Interfaces: History Dependence and Protein Concentration Effects

Abstract approved:

Redacted for privacy

Dr. Joseph McGuire

Adipic dihydrazide-modified heparin was prepared and subsequently immobilized on surface-activated silica wafers. X-ray photoelectron spectroscopy (XPS) was used to quantify the elemental composition of the silica surface at each stage of activation and results showed that successful immobilization had taken place. Contact angle measurements were made to verify the high hydrophilicity of the heparinized and unheparinized silica surfaces. In situ ellipsometry was used to study protein concentration effects on adsorption kinetics exhibited by human plasma fibrinogen on unheparinized and heparinized silica. The adsorbed amount of fibrinogen increased with concentration, on each type of surface. At all concentrations, fibrinogen adsorbed in diminished amounts and at a slower rate on heparinized as compared to unheparinized silica. Buffer elution experiments showed that fibrinogen was less tightly bound on heparinized silica. To examine the history dependence of
adsorption, fibrinogen was added for a second time, after rinsing. The
difference in adsorption rates between the first and second adsorption cycle
evaluated at identical mass density, indicated that post-adsorptive
molecular rearrangements had taken place. These rearrangements
presumably involved aggregation of adsorbed fibrinogen, in this way
increasing unoccupied area at the interface. Fibrinogen was postulated to
be more mobile on heparinized silica.
Fibrinogen Adsorption at Heparinized Silica Interfaces: History Dependence and Protein Concentration Effects

by

Omkar Joshi

A THESIS

submitted to

Oregon State University

in partial fulfillment of the requirements for the degree of

Master of Science

Presented May 27, 2004
Commencement June 2005
I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Omkar Joshi, Author
ACKNOWLEDGEMENTS

I express my sincere gratitude to Dr. Joe McGuire for his enormous support and encouragement through these last two years. Because of the incredible research environment that he provided, I can wholeheartedly say that I worked “with” Dr. McGuire, not just “under” Dr. McGuire. I thank Dr. Michelle Bothwell and Dr. Karyn Bird for being available for guidance every time I asked for help. I thank Dr. Mike Schimerlik and Dr. Mike Penner for kindly serving on my committee. I must mention Dr Woo-Kul Lee and Phyllis Buchholz for assisting with the experiments, at various stages.

I had many good times (and some “bad” moments, which were usually laughed away later on!) with my dear colleague, Hyo Jin Lee. I thank her for being such an admirable lab-mate and co-worker. I will cherish our unique friendship for a long time to come.

I had a great time at Oregon State University. I sincerely thank my lab-mates, my friends in Chemical Engineering and my friends from the Indian community in Corvallis, for making that happen. Finally, I thank my family for their love and support, which was undiminished even though I was half way across the world from them.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2. LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td>2.1 Protein adsorption and biocompatibility</td>
<td>4</td>
</tr>
<tr>
<td>2.2 Recent work on protein adsorption</td>
<td>6</td>
</tr>
<tr>
<td>2.3 Heparin: Role as an anticoagulant and effectiveness of surface heparinization</td>
<td>8</td>
</tr>
<tr>
<td>2.4 Fibrinogen: Properties and role in biomaterial compatibility</td>
<td>13</td>
</tr>
<tr>
<td>2.5 Concentration effects in protein adsorption</td>
<td>16</td>
</tr>
<tr>
<td>3. MATERIALS AND METHODS</td>
<td>21</td>
</tr>
<tr>
<td>3.1 Adipic dihydrazide modification of heparin</td>
<td>21</td>
</tr>
<tr>
<td>3.2 Surface activation of silica wafers</td>
<td>23</td>
</tr>
<tr>
<td>3.3 ADH-heparin attachment</td>
<td>25</td>
</tr>
<tr>
<td>3.4 Surface characterization</td>
<td>26</td>
</tr>
<tr>
<td>3.5 Evaluation of adsorption kinetics</td>
<td>28</td>
</tr>
<tr>
<td>4. RESULTS AND DISCUSSION</td>
<td>32</td>
</tr>
<tr>
<td>4.1 Surface characterization</td>
<td>32</td>
</tr>
<tr>
<td>4.2 Evaluation of adsorption kinetics</td>
<td>35</td>
</tr>
<tr>
<td>5. CONCLUSIONS</td>
<td>65</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>66</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>72</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Structure of major and minor disaccharide sequence in heparin</td>
<td>9</td>
</tr>
<tr>
<td>2.2</td>
<td>Schematic of fibrinogen structure</td>
<td>14</td>
</tr>
<tr>
<td>3.1</td>
<td>Heparin immobilization on silica wafers</td>
<td>31</td>
</tr>
<tr>
<td>4.1</td>
<td>Elemental composition (%) on the surfaces at various stages of activation</td>
<td>34</td>
</tr>
<tr>
<td>4.2</td>
<td>Adsorption kinetics on unheparinized silica, fibrinogen concentration 0.05 mg/mL</td>
<td>37</td>
</tr>
<tr>
<td>4.3</td>
<td>Adsorption kinetics on unheparinized silica, fibrinogen concentration 0.1 mg/mL</td>
<td>38</td>
</tr>
<tr>
<td>4.4</td>
<td>Adsorption kinetics on unheparinized silica, fibrinogen concentration 0.5 mg/mL</td>
<td>39</td>
</tr>
<tr>
<td>4.5</td>
<td>Adsorption kinetics on heparinized silica, fibrinogen concentration 0.05 mg/mL</td>
<td>40</td>
</tr>
<tr>
<td>4.6</td>
<td>Adsorption kinetics on heparinized silica, fibrinogen concentration 0.1 mg/mL</td>
<td>41</td>
</tr>
<tr>
<td>4.7</td>
<td>Adsorption kinetics on heparinized silica, fibrinogen concentration 0.5 mg/mL</td>
<td>42</td>
</tr>
<tr>
<td>4.8</td>
<td>Adsorption kinetics at 0.05 mg/mL fibrinogen concentration. Comparison between heparinized and unheparinized silica</td>
<td>45</td>
</tr>
<tr>
<td>4.9</td>
<td>Adsorption kinetics at 0.1 mg/mL fibrinogen concentration. Comparison between heparinized and unheparinized silica</td>
<td>46</td>
</tr>
<tr>
<td>4.10</td>
<td>Adsorption kinetics at 0.5 mg/mL fibrinogen concentration. Comparison between heparinized and unheparinized silica</td>
<td>47</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES (CONTINUED)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.11</td>
<td>( \Gamma_{ad} ) values at various fibrinogen concentrations</td>
<td>49</td>
</tr>
<tr>
<td>4.12</td>
<td>Adsorption kinetics on unheparinized silica at various fibrinogen concentrations</td>
<td>51</td>
</tr>
<tr>
<td>4.13</td>
<td>Adsorption kinetics on heparinized silica at various fibrinogen concentrations</td>
<td>52</td>
</tr>
<tr>
<td>4.14</td>
<td>History dependence: unheparinized silica, 0.05 mg/mL fibrinogen concentration</td>
<td>56</td>
</tr>
<tr>
<td>4.15</td>
<td>History dependence: unheparinized silica, 0.1 mg/mL fibrinogen concentration</td>
<td>57</td>
</tr>
<tr>
<td>4.16</td>
<td>History dependence: unheparinized silica, 0.5 mg/mL fibrinogen concentration</td>
<td>58</td>
</tr>
<tr>
<td>4.17</td>
<td>History dependence: heparinized silica, 0.05 mg/mL fibrinogen concentration</td>
<td>59</td>
</tr>
<tr>
<td>4.18</td>
<td>History dependence: heparinized silica, 0.1 mg/mL fibrinogen concentration</td>
<td>60</td>
</tr>
<tr>
<td>4.19</td>
<td>History dependence: heparinized silica, 0.5 mg/mL fibrinogen concentration</td>
<td>61</td>
</tr>
<tr>
<td>4.20</td>
<td>Comparison of experimental data with diffusion controlled model at fibrinogen concentration 0.05 mg/mL</td>
<td>63</td>
</tr>
<tr>
<td>4.21</td>
<td>Comparison of experimental data with diffusion controlled model at fibrinogen concentration 0.1 mg/mL</td>
<td>63</td>
</tr>
<tr>
<td>4.22</td>
<td>Comparison of experimental data with diffusion controlled model at fibrinogen concentration 0.5 mg/mL</td>
<td>64</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>Contact angles for silica wafers at various stages of activation</td>
<td>33</td>
</tr>
<tr>
<td>4.2</td>
<td>Elemental composition (%) on the surfaces at various stages of activation</td>
<td>34</td>
</tr>
<tr>
<td>4.3</td>
<td>Adsorbed mass at various stages</td>
<td>36</td>
</tr>
<tr>
<td>4.4</td>
<td>Fraction of fibrinogen which is rinsable</td>
<td>44</td>
</tr>
</tbody>
</table>
# LIST OF APPENDICES

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Least squares minimization procedure to fit adsorption data.</td>
<td>73</td>
</tr>
<tr>
<td>B</td>
<td>Immobilized heparin detection using the toluidine blue assay.</td>
<td>77</td>
</tr>
<tr>
<td>C</td>
<td>NMR analysis of adipic dihydrazide-modified heparin.</td>
<td>79</td>
</tr>
</tbody>
</table>
**LIST OF APPENDIX FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.1</td>
<td>NMR of native heparin, molecular weight 3,500</td>
<td>80</td>
</tr>
<tr>
<td>C.2</td>
<td>NMR of native heparin, molecular weight 3,500 Expansion of Figure C.1 in the area of 3.0 – 5.6 ppm</td>
<td>81</td>
</tr>
<tr>
<td>C.3</td>
<td>NMR of adipic dihydrazide-modified heparin</td>
<td>82</td>
</tr>
<tr>
<td>C.4</td>
<td>NMR of adipic dihydrazide-modified heparin Expansion of Figure C.3 in the area of 1.0 – 5.0 ppm</td>
<td>83</td>
</tr>
</tbody>
</table>
# LIST OF APPENDIX TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.1</td>
<td>Values of parameters used for data fitting.</td>
<td>74</td>
</tr>
<tr>
<td>B.1</td>
<td>Heparin amount calculated by the toluidine blue assay.</td>
<td>78</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Biomaterials engineering is a rapidly progressing field. Recent advances in this field have led to a number of successful technologies in medicine and surgery. At the same time, despite several years of research, we are not close to obtaining a truly non-thrombogenic material. Noted researcher Buddy Ratner has referred to this problem as the "blood compatibility catastrophe" (Ratner, 1993).

The clinical success of a biomaterial largely depends on how well its surface promotes or discourages biological responses, including adsorption of macromolecules and adhesion of cells, platelets, and bacteria to the surface. Millions undergo surgery each year and many of them suffer from post-surgical complications caused by adverse biological response to the implanted biomaterial. Thrombosis at the site of an implant is a very common occurrence. Long-term anticoagulant therapy generally includes coumarin or related compounds. The major drawback of this treatment is that patients have to endure the toxic effects of these compounds. Short-term anticoagulant therapy involves systemically injecting heparin, either in the unfractionated or low molecular weight form.
This approach leads to some problems since the entire blood stream of the patient is “anticoagulated”.

A possible alternative is heparin immobilization on the biomaterial, so that the anticoagulant effect will be localized at the site of implant. Various heparinized materials are already available commercially, e.g., stents, catheters and extra-corporeal circuits. Even though these materials are widely used, the mechanism which makes them more favorable biomaterials is not well-understood.

Protein adsorption is one of the first events that takes place when a biomaterial contacts blood. The success of a biomaterial is, to a large extent, governed by its protein adsorption characteristics. Several factors, such as the rate of adsorption, strength of binding and conformational state of adsorbed proteins, contribute to the biocompatibility of the material.

The objective of this research was to synthesize and characterize heparinized silica surfaces and study the adsorption behavior of the plasma protein fibrinogen on them. A comparison with a model hydrophilic surface, untreated silica, was made to examine the effects of surface heparinization. Protein adsorption is widely accepted to be a concentration-dependent phenomenon. Three different fibrinogen concentrations were studied to examine the effect of concentration on adsorption. In addition,
the interfacial arrangement of adsorbed fibrinogen was evaluated by applying a relatively new theoretical approach, relating to the history dependence of protein adsorption.
2.1 Protein adsorption and biocompatibility

Proteins are known to influence a wide variety of biological processes that occur at interfaces (Horbett and Brash, 1986). Some practical examples of the relevance of protein adsorption are contact lens fouling, foaming of protein solutions and fouling of equipment in the food processing industry. Horbett and Brash noted that any process involving an interface which is in contact with a protein solution is likely to be influenced by protein adsorption to the interface.

Horbett (1982) presented a broad review on the general aspects of protein adsorption. Proteins are surface active and are known to preferentially locate at interfaces because of their polymeric structure and amphoteric nature. Multiple contacts with the surface may form due to the large size of protein molecules, leading to strong binding. Various modes of binding with a wide array of surfaces are possible due to the presence of polar, charged and nonpolar amino acid side chains in proteins. Proteins are also known to undergo conformational changes upon adsorption, which are attributed to the tendency for nonpolar residues to be internalized.
Protein adsorption is especially relevant in the case of implanted biomaterials. Many of the biological processes involved require the interaction of cells with the biomaterial (Horbett, 1982). The biomaterial may not elicit any active cellular response, but may become reactive by promoting the adsorption of specific proteins at the interface. Thus the adsorbed proteins can be thought to be “mediating” cellular response.

A “blood compatible” surface may be thought of as a special case of the general term “biocompatible” surface. Labarre (2001) defines a blood compatible surface as a surface which is able to keep under control coagulation and inflammation processes at its interface with normal blood, in given hemodynamic conditions.

The first event that occurs when a biomaterial contacts blood, is the adsorption of proteins at the interface (Young, 1982). This starts the “coagulation cascade” in the vicinity of the biomaterial (Basmadjian, 1997). Adsorbed proteins may desorb or may be displaced by other proteins, called the Vroman effect. The coagulation cascade results in the formation of thrombin, which further accelerates coagulation reactions and results in the conversion of fibrinogen into strands of fibrin.

Basmadjian et al (1997) presented a review of mathematical models of the coagulation process and concluded that it is not possible to produce
inert biomaterials which do not lead to any clot formation. They suggested that producing materials which are actively antithrombogenic, e.g. heparinized surfaces, might be a more successful approach.

2.2 Recent work on protein adsorption

Tangpasuthadol et al (2003) studied the adsorption of lysozyme and bovine serum albumin (BSA) at the surface of chemically modified chitosan films, using the bicinchoninic acid (BCA) assay. The chitosan surface became more hydrophobic when a stearoyl group was attached, and more hydrophilic when reacted with acid anhydrides. Increased adsorption of both proteins was observed on the films reacted with the stearoyl group, which suggested that the hydrophobic surface enhanced protein adsorption. The hydrophilic surface reduced the adsorption of BSA but increased the adsorption of lysozyme. The authors postulated that at pH 7.4, the carboxylic acid groups present on the surface of films reacted with acid anhydrides and were converted to negatively charged carboxylate ions. Since BSA is a carboxylic acid-rich protein, charge-charge repulsion may explain reduced adsorption. On the other hand, lysozyme is positively charged at pH 7.4, so hydrogen bonding and charge-charge attraction may be responsible for increased adsorption.
Sariri (2002) treated contact lenses made of hydroxyethyl methacrylate (HEMA) with the natural phospholipid lecithin. The amount of lysozyme, albumin and insulin adsorbed onto treated and untreated lenses was compared using UV absorption spectroscopy. The treatment significantly reduced the adsorption of all proteins. Adsorption was further reduced as the lecithin film thickness was increased. Amounts adsorbed on negatively charged untreated HEMA surfaces were in the descending order of lysozyme, insulin and albumin. Isoelectric points show that lysozyme has a net positive charge, insulin is almost uncharged and albumin is negatively charged at neutral pH. The authors concluded that the charge of a protein determines its adsorption onto charged surfaces.

Brusatori et al (2003) studied the adsorption of proteins under an applied electric field using optical waveguide lightmode spectroscopy in an effort to direct the structure and formation rate of the adsorbed protein layer. The rate and extent of albumin and cytochrome c adsorption was significantly increased by the applied voltage. In the case of negatively charged albumin, the transport-limited and initial surface-limited adsorption rates were greatly increased with voltage but were nearly independent of voltage for positively charged cytochrome c. Adsorption rates onto a surface of higher coverage were greatly increased for both proteins. The authors attributed this to more oriented adsorbed molecules and formation of a multilayer.
Tanaka et al (2002) compared the adsorption behavior of albumin and fibrinogen on poly(2-methoxyethylacrylate) (PMEA), poly(2-hydroxyethylmethacrylate) (PHEMA) and polypropylene (PP) surfaces using the dynamic quartz crystal microbalance (QCM) method. The authors observed that the detachment rate constant of PMEA was higher than those for PHEMA and PP. The degree of the conformational changes of the proteins studied by circular dichroism were in the following order PP > PHEMA > PMEA, which the authors believed was related to the detachment rate constant. The results suggested a weaker interaction between the proteins and PMEA as compared to the other polymers.

2.3 Heparin: Role as an anticoagulant and effectiveness of surface heparinization

Heparin belongs to a family of biological macromolecules called glycosaminoglycans (GAGs). It was discovered in 1916 by Jay Maclean at John Hopkins University. Other commonly known GAGs are hyaluronic acid (HA), heparan sulfate (HS), chondrotin sulfate. GAG’s are unbranched, polydisperse, acidic polysaccharides, often covalently linked to a protein core to form proteoglycans (PGs). GAGs extend from a protein core in a brush-like structure. The core protein size ranges from 10 kDa to greater than 500 kDa, and the number of attached GAG chains varies from 1 to greater than 100 (Islam, 2003)
Heparin is a polydisperse, highly sulfated, linear polysaccharide made up of repeating 1-4 linked uronic acid and glucosamine residues. Due to the extremely complex nature of heparin, its precise structure remains unknown (Islam, 2003; Capila, 2002).

![Structure of major and minor disaccharide sequence in heparin](image)

**Figure 2.1** Structure of major and minor disaccharide sequence in heparin

Heparin is the most commonly used clinical anticoagulant. The antithrombotic action of heparin is due mainly to its antithrombin III (ATIII)-mediated anticoagulant activity. ATIII forms tight, irreversible complexes with its target enzymes such as thrombin, factor Xa, etc. Heparin accelerates this normally slow process by a factor of 2000. Antithrombotic activity of heparin may also be linked to some other factors. Because of its high content of sulfo and carbonyl groups, heparin has the highest negative charge density of all biological macromolecules and, hence, it increases the electronegative potential of the vessel wall. Also, it may increase the release of anticoagulant active HS and tissue factor pathway inhibitor (Islam, 2003; Capila, 2002).
Low molecular weight (LMW) heparins are prepared by the controlled chemical and enzymatic depolymerization of heparin. The use of LMW heparin has recently exceeded that of heparin. This can be attributed to the fact that LMW heparins have more predictable pharmacological action, sustained activity, better bioavailability and a better therapeutic index (Capila, 2002).

Heparin has to be administered intravenously, since it has a low bioavailability when given subcutaneously and is inactive when given orally. This approach has its own disadvantages, primarily that the heparin will be present in the entire bloodstream rather than just at the site of implant, where thrombosis is expected to occur. This leads to the approach of heparin immobilization on biomaterials. In one of the earliest studies of heparinized surfaces, Gott (1963) reported that heparin-coated graphite rings remained free from blood clots for at least 14 days. Since then, heparinized surfaces have attracted a lot of scientific and commercial interest.

Chanard et al (2003) found that for patients undergoing hemodialysis, heparinized membranes allow for reduction in systemic heparin doses. They noted that surface-attached heparin retains, to an extent, its anticoagulant property. The biological activity of heparin is reduced when bound to a surface as compared to heparin in solution. This
could be due to a variety of factors: chemical modification of the ATIII binding site during coupling, reduced flexibility of the immobilized heparin chain, decreased accessibility of heparin for components involved in the coagulation cascade, steric hindrance caused by deposited proteins (van Delden, 1996)

Heparin immobilization on a surface can be achieved physically, ionically or covalently. Covalent attachment is stronger than the others (van Delden, 1996). Marconi et al (1997) evaluated the biological activity of heparinized ethylene-vinyl alcohol copolymers using APTT measurements. They found that samples heparinized covalently were more active than samples heparinized ionically. Nadkarni et al (1994) described covalent coupling at the aldehyde group on the reducing end of heparin on amine-functionalized matrices and surfaces containing hydrazido groups. They stated that covalent immobilization through the reducing end of heparin mimics the orientation of the naturally occurring proteoglycan, reduces steric interference and exposes all of heparin's binding sites.

Nemets and Sevastianov (1991) studied the influence of the method of heparin immobilization on the protein adsorption and platelet activation. Direct immobilization of heparin leads to a reduced probability of interaction between ATIII and corresponding binding sites on heparin. This
steric hindrance is considerably reduced when heparin is immobilized using a spacer arm, leading to increased anticoagulant activity.

Number of studies have shown that heparinized surfaces exhibit a diminished thrombogenic response (Blezer, 1997; Svenmarker, 1997; Johnell, 2002), but other researchers report inconclusive findings. Also, the exact mechanism of heparin's biological activity at interfaces is still not well understood.

Using an ELISA analysis, Weber et al (2002) found that there is less adsorption of complement protein C3, fibrinogen and fibronectin on heparinized surfaces as compared to uncoated PVC surfaces. Adsorption of high molecular weight kininogen, however, was increased. Adsorption to heparinized surfaces for the former group of proteins is slower, suggesting that there is a different adsorption mechanism at work at the two surfaces. Immunoblot patterns showed some diffuse bands for fibronectin and fibrinogen eluates from unheparinized surfaces, but were not observed for heparinized surfaces. They concluded that some of the improved hemocompatibility of heparinized materials may be attributed to the difference in non-specific degradation of adsorbed proteins.

employing enzyme immunoassays and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. They found less platelet adhesion on the heparinized surface as compared to the uncoated polypropylene surface, independent of the amount of heparin administered systemically. Also, they found that the amount and composition of the adsorbed protein was not altered between the heparinized and uncoated surface. In particular, they found no change in adsorption pattern for fibrinogen.

2.4 Fibrinogen: Properties and role in biomaterial compatibility

Fibrinogen (molecular weight 340,000 Da) is a dominant plasma protein. It occurs in plasma at concentrations of 3-4 mg/mL (Nygren, 1992). It is a part of the coagulation cascade. The coagulation cascade results in the production of thrombin, which results in the conversion of fibrinogen into fibrin, and in turn leads to clot formation.

Also, the cell-surface receptor glycoprotein IIb-IIIa can bind on fibrinogen, which permits platelet aggregation (Phillips, 1988)

A fibrinogen molecule consists of three distinct polypeptide chains and each molecule contains a pair of each chain (McKee, 1966; Hemmerle, 1999). The three globules or chains are connected by two regions formed
of supercoiled alpha helixes. The two terminal globules, called D fragments, are composed of two compactly folded chains (β and γ). Each terminal region also contains an α chain that is hydrophilic and floats freely in solution. The central globule is called the E fragment.

The following is a schematic presentation of the fibrinogen molecule (Hemmerle, 1999)

![Schematic of fibrinogen structure](image)

**Figure 2.2** Schematic of fibrinogen structure

Vroman et al (1980) have reported that normal intact plasma deposits fibrinogen on most surfaces studied within the first few seconds of contact. Thus, the study of fibrinogen adsorption on biomaterials becomes crucial.

Fibrinogen adsorption at a solid-liquid interface is an important phenomenon to be studied for several reasons (Schmitt, 1983): it is abundant in plasma, it adsorbs preferentially as compared to other proteins, and it is believed to play an important role in platelet adhesion.
Brash (1983) and Schaaf (1992) have found that only a small percentage of adsorbed fibrinogen molecules desorb on rinsing with pure buffer. There is also only a small turnover between fibrinogen molecules as studied by a radiolabeling technique. The authors concluded that fibrinogen, largely, is irreversibly adsorbed.

Retzinger et al. (1994) studied fibrinogen adsorption on microscopic polystyrene-divinylbenzene beads. They theorized that fibrinogen exists in two adsorbed states or conformations on the surface, the desorbable and the nondesorbable species. The nondesorbable species dominates if there is large area available for each fibrinogen molecule. They report that this species is very stable at interfaces and is a denatured form of fibrinogen, not functional in fibrin gelation. The denatured form is not found in solution, hence it can be concluded that it is a consequence of adsorption.

Wertz et al (2002) put forth a slightly different model. They state that adsorbed fibrinogen can exist in many possible conformations and/or orientations and not just two states as reported by Retzinger et al. The number and nature of states could depend on the adsorption history and the surface chemistry. Initially, fibrinogen is thought to adsorb non-specifically on both hydrophobic and hydrophilic surfaces, which results in a random
mixture of end-on and side-on adsorbed molecules. After this, fibrinogen increases its number of segment-surface contacts, in other words, its "footprint". This is similar to denaturation (unfolding) on a hydrophobic surface and reorientation (rolling-over) on a hydrophilic surface. Thus the reversible and irreversibly bound populations may consist of molecules with many different footprint sizes.

2.5 Concentration effects in protein adsorption

Greater quantities of proteins adsorb from solutions of higher bulk concentrations. Chan and Brash (1981) found that initially, adsorbed amounts on a glass surface increased steeply with increase in bulk fibrinogen concentration and a plateau is reached at about 0.25 mg/mL.

The Langmuir adsorption isotherm cannot explain the increase in protein adsorption with increasing protein solution concentration since it assumes equilibrium between adsorption and desorption. Soderquist and Walton (1980) have described a model for the adsorption process which involves three stages: a brief initial period of reversible adsorption, a second stage where slow conformation change occurs and adsorption is essentially irreversible followed by slow desorption of denatured material. In the case of irreversible adsorption, adsorption is completed when all accessible adsorption sites are occupied. Thus, the amount of protein
adsorbed would be expected to be constant, depending on the number of adsorption sites and independent of the solution concentration (Brynda, 1986). Hence, the reason for concentration dependence on protein adsorption still remains unclear.

Rapoza and Horbett (1990) studied the effect of adsorption conditions, such as protein concentration and adsorption time, on the elutability of baboon fibrinogen and albumin from polyethylene and polystyrene. They observed that elutability of the fibrinogen adsorbed increased with an increase in fibrinogen solution concentration. In particular, elutability increased from 30% at 0.001 mg/mL to 75% at 1 mg/mL fibrinogen concentration on polyethylene. They also observed that proteins adsorbed from low bulk concentrations exhibited lower elutability levels at high surfactant concentrations and required higher surfactant concentrations to effect elution as compared to proteins adsorbed from high bulk concentrations. They concluded that proteins adsorbed from low bulk concentrations show tighter binding. The authors attributed this phenomenon to molecular spreading, in which adsorption from a dilute solution results in more initial contacts per adsorbed molecule.

Schmitt et al (1983) studied fibrinogen adsorption on various surfaces using radiolabeling. For high concentrations (> 0.5 mg/mL), adsorption values were in the range expected for a close-packed monolayer.
Adsorption was also surface specific in this region. For low concentrations, adsorption data was found to fit a power law equation and was observed to be largely independent of surface.

Lee (2002) studied the adsorption kinetics and dodecyltrimethylammonium bromide-mediated elution of the wild type and two structural stability mutants of bacteriophage T4 lysozyme at silica surfaces, using *in situ* ellipsometry. Experiments were performed at different solution concentrations, ranging from 0.01 to 1 mg/mL. Plateau values of adsorbed mass were found to increase with increasing solution concentration. Comparison of the data to an adsorption mechanism consisting of three adsorption states, which differ by binding strengths, showed that the fraction of adsorbed molecules present in the most tightly bound state decreased with increasing solution concentration. The index of binding strength decreased with increasing concentration. This was in agreement with the observation that at a less crowded interface, a protein will more readily alter conformation upon adsorption, allowing more noncovalent bonds to be established. The interfacial region is likely to be less crowded when adsorption occurs from solutions of low concentration, resulting in greater resistance to elution.

Elofsson et al (1997) studied the adsorption of β-lactoglobulin A and B onto hydrophilic and methylated silica surfaces by *in situ*
ellipsometry. The adsorption isotherms were observed to have two distinct levels of adsorbed amounts. The authors suggested that the two levels indicated different modes of binding at high and low protein concentrations. They attributed the observation to the preferential adsorption of β-lactoglobulin dimers at increasing concentrations. It was observed that the initial adsorption rates, at a concentration where mainly monomers were present, were very fast. This suggested an initial adsorption of monomers, which at higher concentrations were exchanged for dimers.

Singla (1996) studied the adsorption kinetics exhibited by the wild type and two synthetic stability mutants of T4 lysozyme at hydrophilic and hydrophobic silica surfaces. Each protein was adsorbed to the hydrophilic surface, first from a solution of concentration 0.01 mg/mL, followed by rinsing with buffer, a second addition of protein at 0.1 mg/mL, rinsing with buffer again, and finally, addition of protein at 1.0 mg/mL. The results were compared with single-step adsorption of protein at 1.0 mg/mL. Mutant I3W has been reported to be most resistant to elution and is believed to adsorb in a state characterized by higher binding strength. At low protein concentration, adsorbed molecules would adopt this state more readily. This was consistent with the results for I3W adsorption, in which similar plateau values were observed for multi-step and single-step adsorption. For the wild type and mutant I3C, which do not undergo post-adsorptive structural change to the same extent as I3W, the plateau for the
multi-step adsorption experiment was lower than that for the single-step adsorption experiment.
CHAPTER 3
MATERIALS AND METHODS

3.1 Adipic dihydrazide modification of heparin

The procedure for adipic dihydrazide (ADH) modification of heparin was adapted from procedures published for the ADH modification of hyaluronic acid (HA) by Pouyani and Prestwich (1994), Pouyani, Harbison and Prestwich (1994), Luo et al (2000) and Vercruysse et al (1997). The authors wanted to devise a chemical methodology for the modification of hyaluronic acid (HA) which would allow coupling under mild, aqueous conditions, preserve the molecular size distribution of HA used and produce materials that could be characterized spectroscopically. The same methodology is expected to work for heparin because heparin has a similar glycosaminoglycan structure as HA.

Two hundred (200) mg of low molecular weight heparin (Celsus Laboratories, lot number FH-19002) was dissolved in 50 mL of distilled deionized water (DDW) i.e. the concentration of heparin solution was 4 mg/mL. To this mixture, 3.5 g of ADH (Avocado Research Chemicals Ltd) was added. The pH of the reaction mixture was adjusted to 4.75 using 0.1 N HCl and the mixture was kept stirred for at least 30 minutes. Three hundred eighty two (382) mg of 1-[3-(dimethylamino)propyl]-3-
ethylcarbodiimide hydrochloride (EDCI, Aldrich) was added next and the pH of the reaction mixture was maintained at 4.75 by periodically adding 0.1 N HCl. The pH of the reaction mixture was then adjusted to 7.0 using a very small amount of 1 N NaOH. Spectra/Por Dialysis tubing with a molecular weight cutoff of 3,500 (Spectrum Laboratories Inc, CA) was soaked in DDW at room temperature for 3-4 hours. The final reaction mixture was transferred to the pretreated dialysis tubing and dialyzed for 3 days against 100 mM sodium chloride (Mallinkrodt), followed by dialysis against DDW for 1 day in a 2 L vessel, contents of which were kept stirred. The solution was then centrifuged at 30,000 rpm (Allegra 6R, Beckman, Palo Alto, CA) for 15 minutes and the supernatant was frozen at -80 °C (VC1340-AIX, Revco Scientific, Asheville, NC) at an angle of about 45°, before lyophilizing. Lyophilization (Bench Top 5SL, Vir Tis Company Inc., Gardiner, NY) was carried out for at least 48 hours. The lyophilized heparin was stored in the refrigerator until further use.

Nuclear magnetic resonance (NMR) analysis of modified heparin was performed to verify successful modification and to quantify percentage of modified heparin (Appendix C).
3.2 Surface activation of silica wafers

3.2.1 Cleaning

Silicon (Si) wafers (crystal grade CZ, type N, Boron doped, orientation 1-0-0, thickness 525 ± 18 μm, resistivity 0.01-0.02 ohm-cm) were purchased from WaferNet, Inc (San Jose, CA). Using a tungsten pen, wafers were cut into approximately 1x3 cm wafers. These wafers were rinsed in 25 mL acetone.

To prepare the hydrophilic silica surface, which is free of organic residues and dust, the procedure described by McGuire et al (1995) was used. Each wafer was placed in a test tube and a 10 mL mixture of NH₄OH : H₂O₂ : H₂O (1:1:5 volume ratio) was added. They were then heated at 80 °C for 10 minutes in a constant temperature water bath (Blue M, NC). Wafers were then rinsed in 20 mL of distilled deionized water (DDW). The DDW was made using a Barnstead MegaPure MP-3A system (Barnstead International, Dubuque, Iowa). Next, a 10 mL mixture of HCl : H₂O₂ : H₂O (1:1:5 volume ratio) was added and wafers heated at 80 °C for 10 minutes. Wafers were then rinsed in 20 mL DDW and dried with nitrogen. Wafers were stored in 50% ethanol solution to maintain hydrophilicity, until further use.
3.2.2 Amination

Next, the silica wafers were silanized using 3-aminopropyl triethoxy silane (3APTS, Aldrich) using the procedure described by Buechi and Bachi (1979) and the SPI Supplies website. Hydrophilic silica wafers, cleaned according to the procedure described previously, were immersed in acetone for at least 2 hours, to ensure that the wafers were clean to the degree necessary. A 2% (v/v) solution of 3APTS in acetone was prepared and was preheated to 50 °C inside an incubator (VWR). Silica wafers were placed in test tubes and the test tubes were completely filled with 2% 3APTS solution. The tubes were stored for 24 hours at 50 °C inside the incubator. After 24 hours, the wafers were dipped in acetone for 1 minute to remove the 3APTS. Wafers were then dipped in DDW for 1 minute. Wafers were dipped in acetone for 1 minute, this time with agitation. After this, wafers were dipped in DDW for 1 minute, with agitation. Wafers were then dried with nitrogen.

3.2.3 Carboxylation

Mason et al (2000) observed that there is a greater amount of HA attachment to aminated samples if the aminated sample has been treated with succinic anhydride. They hypothesized that this was because succinic
anhydride produces an extended arm to which the HA can become covalently linked.

Aminated silica wafers were exposed to 10 mM (1.0007 g/L) succinic anhydride (Aldrich) in dry N-N-dimethyl formamide (EM Science) solution for 10 hours at room temperature. The wafers were then washed for 30 minutes in 10 mL DDW.

3.3 ADH-heparin attachment

The procedure as described by Mason et al (2000) was used, modified for heparin. The carboxylated silica wafers were placed in a 20 mL solution each of 1.2 mg/mL of ADH heparin, 50 mM (12.285 g/L) bis-tris hydrochloride (Sigma) and 1 mM (0.192 g/L) EDCI for 24 hours. The reaction was performed at 4 °C at 90 rpm in an Aros 160 Adjustable Reciprocating Orbital shaker (Thermolyne), to prevent bacterial growth. To remove physically adsorbed heparin, the silica wafers were then washed with DDW at a low flow rate at 4 °C.

A schematic of heparin attachment to silica is shown in Figure 3.1 at the end of the chapter.
3.4 Surface Characterization

3.4.1 Contact angle measurements

When a liquid phase contacts both a second fluid phase and a solid surface, a net, characteristic orientation of the liquid-fluid interface with respect to the solid surface occurs. This orientation is reflected in the "contact angle". Contact angle measurements can give information regarding the hydrophilic/hydrophobic character of the solid surface.

Contact angle measurements were made using a FTA 32 Video (First Ten Angstroms) instrument. A 1-3 μL drop of DDW was placed on the surface of silica wafers at various stages of activation and on silica wafers with heparin attached, and contact angles were noted.

3.4.2 X-ray Photoelectron Spectroscopy (XPS)

The XPS technique (or more particularly, Electron Spectroscopy for Chemical Analysis, ESCA) is based on electrical and magnetic analysis at high resolution of the electrons which are emitted from a substance on irradiation with X-rays. Core electrons that are ejected in this process have energy that is very specific for the element from which the atom originates.
All elements can be studied even if the element occurs together with other elements and is present in small amounts (Siegbahn, 1967).

The XPS technique was used to investigate the elemental composition of the heparinized surface. This would determine the efficacy of heparin immobilization.

The XPS experiments were carried out using a Kratos HSI instrument. Monochromatized Al-Kα radiation was used with a 200 W power source. Charge neutralization was done with a low energy electron flood gun. The surface composition was analyzed from survey scan data acquired at 80 eV pass energy. High resolution data was acquired by 20 eV pass energy.

The XPS measurements were taken for unheparinized silica, silica with the linkers attached (i.e. after carboxylation), and the heparinized silica. Also, in order to quantify the amount of heparin immobilized, the toluidine blue assay was performed on unheparinized, “linkers only” and heparinized silica (Appendix B).
3.5 Evaluation of adsorption kinetics

3.5.1 Buffers

Sodium phosphate buffer (0.01M), pH 7, was used in all experiments. Sodium phosphate dibasic heptahydrate, 1.08 g/L (Mallinckrodt) and 0.53 g/L sodium phosphate monobasic monohydrate (Fisher Biotech) were used to prepare the buffer solutions and the above salts were dissolved in DDW. The buffer solutions used for ellipsometry were filtered using a 0.45 μm syringe filter.

3.5.2 Fibrinogen

Fibrinogen (F4883, Lot 029K7602) was purchased from Sigma and used without any further purification.

Fibrinogen was dissolved in a phosphate buffer solution, which had been pre-warmed, by layering the protein on top of the solution. This solution was then placed in a 37 °C incubator, while it was being slowly stirred, for 4 hours. The fibrinogen solution was filtered through an Acrodisc 0.45 μm Supor membrane syringe filter (Pall Corporation, MI), just before it was used. For the second cycle of protein addition, fibrinogen solutions remained inside the incubator at 37 °C with stirring.
For the purpose of the one-film-model ellipsometry program, the values of the ratio between molar mass and molar refractivity (M/A) and partial specific volume of fibrinogen were taken as 4.246 g/mL and 0.719 mL/g respectively (Wahlgreen, 1993).

### 3.5.3 Ellipsometry

An ellipsometer measures optical changes in the state of polarization upon the reflection of chromatic, collimated and polarized light, in terms of two components $\Psi$ and $\Delta$. The effect of reflection is characterized by the angle $\Delta$, defined as change in phase, and the angle $\Psi$, the arctangent of the factor by which the amplitude ratio changes, between two component plane waves into which the electric field oscillation is resolved (Archer, 1968). Ellipsometry allows for adsorption experiments to be studied in situ and in real time, so that we can precisely study adsorption kinetics (Elwing, 1998).

An automatic ellipsometer (Model L-104 SA, Gaertner Scientific Corp., Chicago, IL) was used. The angle of reflection was the same as the angle of incidence, 70°. The light source was a 1 mW He-Ne laser with a wavelength of 6328 Å.
Each silica sample was suspended in a fused quartz trapezoid cuvette and 4.5 mL of 0.01 M phosphate buffer, pH 7, was injected. Bare surface values of $\Psi$ and $\Delta$ were measured for 30 minutes. One half mL of fibrinogen solution at various concentrations was then added, while being stirred. Adsorption was monitored for 30 minutes. The silica wafer was then rinsed for 5 minutes with 0.01 M phosphate buffer, pH 7, at a flow rate of 31.6 mL/min. Twenty-five minutes later, 0.5 mL of the fibrinogen solution at the same concentration used previously was added again and adsorption was monitored for 30 minutes. Values of $\Psi$ and $\Delta$ were recorded every 15 seconds.

A one-film-model ellipsometry program (Krisdhasima, 1992) was used to determine the adsorbed mass of the protein film on the silica surface, based on the values of $\Psi$ and $\Delta$ recorded. Three fibrinogen concentrations were studied, 0.05, 0.1 and 0.5 mg/mL and data were collected for unheparinized and heparinized silica. Each experiment was performed at least twice and the adsorbed mass obtained for duplicate experiments was within $\pm$ 0.05 $\mu$g/cm$^2$. 
Figure 3.1 Heparin immobilization on silica wafers
CHAPTER 4
RESULTS AND DISCUSSION

4.1 Surface characterization

4.1.1 Contact angle measurements

Table 4.1 shows the values of contact angles on silica wafers at various stages of activation. When a drop of DDW was placed on unheparinized silica, the drop had no characteristic shape and spread rapidly on the surface. Based on this, the contact angle of unheparinized silica was interpreted to be 0°. The attachment of amino and carboxyl groups on the “linkers only” silica made the surface hydrophobic, as evidenced by the contact angle of 44.46°. Heparin is extremely hydrophilic. Heparin attachment, therefore, reduced the contact angle. The value recorded was 16.54°. The contact angle recorded for heparinized silica is higher than that for unheparinized silica, which is contrary to that expected. Some “hydrophobic pockets” may remain after heparin immobilization, thus explaining the slightly higher contact angle on the heparinized silica.
**Table 4.1** Contact angles for silica wafers at various stages of activation

<table>
<thead>
<tr>
<th>Surface</th>
<th>Contact Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unheparinized</td>
<td>0</td>
</tr>
<tr>
<td>Linkers only</td>
<td>44.46 ± 1.33</td>
</tr>
<tr>
<td>Heparinized</td>
<td>16.54 ± 0.96</td>
</tr>
</tbody>
</table>

**4.1.2 X-ray photoelectron spectroscopy analysis**

Table 4.2 shows atomic percentages of elements detected on unheparinized silica, silica with all linkers and heparinized silica. Figure 4.1 is a graphical view of the elemental surface composition detailed in Table 4.2. The amount of silicon decreased proceeding from unheparinized to heparinized silica. The decrease in the amount of silicon gives an estimate of layer thickness. Assuming a uniform layer thickness, the lesser the amount of silicon detected, thicker the layer on top of the substrate. The trends in oxygen, nitrogen and carbon also indicate the progressive attachment of organic groups on the surface. Sulfur is detected only on the heparinized silica; since heparin is the only component containing sulfur. All these data indicate that surface heparinization was successful. The carbon detected on the unheparinized silica was from the contaminants in air and typical for this type of experiment (Golledge, personal...
communication). The trace amount of copper detected on the unheparinized silica also was thought to be contamination.

Table 4.2 Elemental composition (%) on the surfaces at various stages of activation

<table>
<thead>
<tr>
<th>Sample</th>
<th>C</th>
<th>O</th>
<th>N</th>
<th>S</th>
<th>Si</th>
<th>Na</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>unheparinized</td>
<td>19.9</td>
<td>27.9</td>
<td>1.90</td>
<td>nd</td>
<td>50.1</td>
<td>tr</td>
<td>0.20</td>
</tr>
<tr>
<td>linkers only</td>
<td>38.3</td>
<td>24.8</td>
<td>4.8</td>
<td>nd</td>
<td>32.1</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>heparinized</td>
<td>60.3</td>
<td>22.1</td>
<td>6.2</td>
<td>0.90</td>
<td>10.5</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd: not detected, tr: trace

Figure 4.1 Elemental composition (%) on the surfaces at various stages of activation
4.2 Evaluation of adsorption kinetics

4.2.1 Adsorption kinetic data

Representative plots of the adsorption kinetic data, obtained for fibrinogen at concentrations of 0.05, 0.1 and 0.5 mg/mL on unheparinized and heparinized silica are shown in Figures 4.2 to 4.7. Best fit curves describing the data were generated (Appendix A). Values of adsorbed mass after 30 minutes ($\Gamma_{ad1}$), after 60 minutes ($\Gamma_{rinse}$) and at 90 minutes ($\Gamma_{ad2}$) based on the best fit curves are reported in Table 4.3. These values correspond to the values of adsorbed mass at the end of the first adsorption cycle, at the end of rinsing and at the end of the second adsorption cycle, respectively. The entries in Table 4.3 are best discussed with reference to comparison of adsorption behavior on heparinized and unheparinized surfaces (Section 4.2.2), and the effects of concentration on adsorption (Section 4.2.3). The second adsorption step will be discussed with reference to a history dependent kinetic model (Section 4.2.4)
Table 4.3 Adsorbed mass at various stages

<table>
<thead>
<tr>
<th>Surface</th>
<th>Fibrinogen concentration (mg/mL)</th>
<th>$\Gamma_{ad1}$ (µg/cm²)</th>
<th>$\Gamma_{rinse}$ (µg/cm²)</th>
<th>$\Gamma_{ad2}$ (µg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>unheparinized silica</td>
<td>0.05</td>
<td>0.260</td>
<td>0.233</td>
<td>0.292</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.318</td>
<td>0.284</td>
<td>0.337</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.354</td>
<td>0.272</td>
<td>0.373</td>
</tr>
<tr>
<td>heparinized silica</td>
<td>0.05</td>
<td>0.109</td>
<td>0.072</td>
<td>0.161</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.166</td>
<td>0.097</td>
<td>0.206</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.219</td>
<td>0.134</td>
<td>0.208</td>
</tr>
</tbody>
</table>
Figure 4.2  Adsorption kinetics on unheparinized silica, fibrinogen concentration 0.05 mg/mL
Figure 4.3  Adsorption kinetics on unheparinized silica, fibrinogen concentration 0.1 mg/mL
Figure 4.4 Adsorption kinetics on unheparinized silica, fibrinogen concentration 0.5 mg/mL
Figure 4.5  Adsorption kinetics on heparinized silica, fibrinogen concentration 0.05 mg/mL
Figure 4.6  Adsorption kinetics on heparinized silica, fibrinogen concentration 0.1 mg/mL.
Figure 4.7  Adsorption kinetics on heparinized silica, fibrinogen concentration 0.5 mg/mL
4.2.2 Comparison between unheparinized and heparinized silica surfaces

Figures 4.8 to 4.10 provide a comparison of the adsorption kinetics of fibrinogen on heparinized and unheparinized silica at the three different fibrinogen concentrations (0.05, 0.1, and 0.5 mg/mL). The amount of fibrinogen adsorbed on the heparinized silica was less than that on unheparinized silica. The $\Gamma_{ad1}$ values presented in Table 4.3 quantify this observation, which is apparent at each concentration. The initial rate of adsorption for the first cycle of protein addition was much lower for heparinized silica as compared to unheparinized silica at each concentration. It is a common observation that proteins adsorb in lesser amounts on hydrophilic surfaces relative to hydrophobic surfaces (Sagvolden et al, 1998). Heparin is a very hydrophilic biopolymer. Heparin immobilization, therefore, is expected to render the surface extremely hydrophilic. Unheparinized silica is also hydrophilic. The difference in amounts of fibrinogen adsorbed may be attributed to the presence of a three-dimensional hydrophilic environment on the heparinized silica compared to the “flat” hydrophilic surface on the unheparinized silica.
Table 4.4  Fraction of fibrinogen adsorbed which is rinsable

<table>
<thead>
<tr>
<th>Fibrinogen concentration (mg/mL)</th>
<th>Fraction rinsable ( \left( \frac{\Gamma_{ad1} - \Gamma_{rinse}}{\Gamma_{ad1}} \right) )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>unheparinized</td>
</tr>
<tr>
<td>0.05</td>
<td>0.107</td>
</tr>
<tr>
<td>0.1</td>
<td>0.109</td>
</tr>
<tr>
<td>0.5</td>
<td>0.231</td>
</tr>
</tbody>
</table>

Values in Table 4.4 indicate that more desorption occurred on heparinized as compared to unheparinized silica, at all concentrations. This meant that fibrinogen molecules were less tightly bound to heparinized as compared to unheparinized silica. Prior studies state that fibrinogen undergoes a conformational change after adsorption to a surface (Retzinger et al, 1994). The fibrinogen molecules that have undergone conformational change are thought to be more tightly bound, so rinsability can be used to estimate the degree of conformational change taking place (Bohnert, 1986). Based on results presented here, it was interpreted that fibrinogen underwent less conformational change on heparinized surfaces.
Figure 4.8  Adsorption kinetics at 0.05 mg/mL fibrinogen concentration
Comparison between heparinized and unheparinized silica
Figure 4.9 Adsorption kinetics at 0.1 mg/mL fibrinogen concentration
Comparison between heparinized and unheparinized silica
Figure 4.10  Adsorption kinetics at 0.5 mg/mL fibrinogen concentration
Comparison between heparinized and unheparinized silica
4.2.3 Adsorption kinetics: concentration effects

Figure 4.11 shows the $\Gamma_{ad1}$ values at each fibrinogen concentration studied, for both heparinized and unheparinized surfaces. The $\Gamma_{ad1}$ values increased steeply going from fibrinogen concentration of 0.05 mg/mL to 0.1 mg/mL. The $\Gamma_{ad1}$ values increased less steeply going from fibrinogen concentration of 0.1 mg/mL to 0.5 mg/mL. This observation suggests that the surfaces were approaching their respective saturation levels as the concentration increased. With increasing fibrinogen concentration, less free space is available for adsorption. This phenomenon adequately explains the saturation trend in the case of unheparinized silica, but not in the case of heparinized silica since less fibrinogen is adsorbed on heparinized silica. As the number of fibrinogen molecules adsorbed on the surface increases, the energy barrier for further adsorption increases. Due to the high negative charge density on the heparinized silica, the activation energy associated with adsorption may reach some critical value at a lower adsorbed mass. This may possibly explain the saturation trend for heparinized silica.

Figures 4.12 and 4.13 show comparisons of adsorption kinetics at the three fibrinogen concentrations studied, on unheparinized and heparinized silica, respectively. On unheparinized silica, the first cycle adsorption rate increased in proceeding from a fibrinogen concentration of 0.05 mg/mL to 0.1 mg/mL, but remained substantially similar from
0.1 mg/mL to 0.5 mg/mL. This supports the theory that the surface was approaching saturation with fibrinogen at higher concentrations.

Figure 4.11  $\Gamma_{adl}$ values at various fibrinogen concentrations

Table 4.4 displays the fraction of adsorbed molecules that are "rinsable", i.e. their adsorption is reversible by buffer elution. For unheparinized silica, the fraction rinsable increased as concentration increased. At lower concentrations, more space will be available at the interface, so molecules will tend to unfold more, resulting in more noncovalent bonds. At high concentrations, the interface will be crowded with molecules so the protein will not have enough space to unfold and will elute in larger amounts.
There was no clear trend in rinsability with change in concentration on the heparinized silica. Clearly, fibrinogen molecules elute in larger amounts from heparinized silica as compared to unheparinized silica, although fibrinogen adsorbs in lesser amounts. This observation is consistent with fibrinogen being less tightly bound to the heparinized silica.
Figure 4.12  Adsorption kinetics on unheparinized silica at various fibrinogen concentrations
Figure 4.13  Adsorption kinetics on heparinized silica at various fibrinogen concentrations
4.2.4 Analysis based on a history dependent kinetic model

Proteins exhibit long relaxation times or irreversible transitions when adsorbed at an interface (Tie et al, 2002). Adsorbed protein layers are essentially not at equilibrium at each stage of formation, and the dependence on formation history persists for a very long time. Calonder et al (2001) introduced the rate of adsorption as a measure of the history dependence. When adsorption is not mass transfer controlled, the rate of adsorption depends on the interaction between the adsorbing molecules and molecules previously adsorbed. The interaction in turn depends on the structure of the interfacial layer. The rate of adsorption, therefore, can be a measure of interfacial structure and, correspondingly, of history dependence.

Tie et al (2002) studied the adsorption of fibronectin, cytochrome c and lysozyme on SiTiO₂ using optical waveguide lightmode spectroscopy (OWLS) in multi-step mode, where an adsorbing surface is alternately exposed to a protein solution and a solution free of protein. The adsorption rate during the second step exceeded that observed during the initial step. The authors isolated the intrinsic adsorption rate (a kinetic constant depending solely on the adsorbent-adsorbate system), the interfacial cavity function (defined as the probability of an incoming protein molecule landing in a cavity on the surface free from other protein) and the intrinsic
desorption rate from the OWLS kinetic data. The authors reported that the significantly greater rate observed during the second adsorption step is due to an increase in the cavity function, which itself is a parameter reflecting the adsorbed layer structure. The authors postulated that the difference in rates was evidence of structural rearrangement; in other words formation of clusters of adsorbed molecules. For a given mass density, a surface filled with aggregates would present more open space than one with a random distribution of molecules.

The nature of adsorption experiments performed here, protein adsorption followed by desorption followed by protein addition, allows one to look at protein films of the same mass density (and presumably at the same thermodynamic state), differing only by their formation histories. If the adsorbed protein films are at equilibrium, the same (adsorbed mass-dependent) rates of adsorption would be expected during the two cycles of protein adsorption, since at equilibrium the proteins would have identical structural characteristics.

Figures 4.14 to 4.19 show adsorption kinetics for the first adsorption cycle on both heparinized and unheparinized silica surfaces, at each fibrinogen concentration. The data for the first ten minutes of the second adsorption cycle has been overlaid on these plots, shifted back in time to the point in the first adsorption cycle with the same initial mass
density. In the case of unheparinized silica, higher adsorption rates were observed for the second cycle as compared to the first, at fibrinogen concentrations of 0.1 and 0.5 mg/mL. This may be attributed to structural rearrangements, or the formation of clusters which clears space for subsequently adsorbing molecules. The adsorption rates for the two protein adsorption cycles were not significantly different on unheparinized silica at 0.05 mg/mL fibrinogen. It was thought that the effect of rearrangement was less pronounced for adsorption from low bulk concentration solutions, since there would be areas unoccupied by adsorbed molecules.

On heparinized silica, the adsorption rate of the second cycle was observed to be more than that of the first cycle, at all concentrations. This effect increased as the fibrinogen concentration increased. The difference in adsorption rates between the two adsorption cycles was observed even at the lowest fibrinogen concentration (0.05 mg/mL). At the lowest concentration, no difference in adsorption rate between the two adsorption cycles was apparent with unheparinized silica. We have noted that fibrinogen is less tightly bound to heparinized silica. The weaker binding is consistent with fibrinogen molecules having greater mobility on the heparinized surface, facilitating molecular rearrangement. The argument for greater mobility on the heparinized surface is further supported by the steeper difference in the adsorption rates between cycles on heparinized, as compared to unheparinized silica, at each concentration.
Figure 4.14  History dependence: unheparinized silica, 0.05 mg/mL fibrinogen concentration
Figure 4.15  History dependence: Unheparinized silica, 0.1 mg/mL fibrinogen concentration
Figure 4.16  History dependence: Unheparinized silica, 0.5 mg/mL fibrinogen concentration
Figure 4.17  History dependence: Heparinized silica, 0.05 mg/mL fibrinogen concentration
Figure 4.18  History dependence: heparinized silica, 0.1 mg/mL fibrinogen concentration
Figure 4.19  History dependence: heparinized silica, 0.5 mg/mL fibrinogen concentration
4.2.5 Mass transfer considerations

Before any kinetic analysis can be performed on adsorption data, it is necessary to evaluate whether mass transfer effects need to be considered. The adsorbed mass of a protein can be determined if the adsorption is diffusion controlled, using an approach described by McGuire (2002). In particular,

\[ \Gamma = 2C_{\text{bulk}} \left( \frac{Dt}{\pi} \right)^{1/2} \]

where

- \( \Gamma \): adsorbed mass per square area
- \( C_{\text{bulk}} \): bulk solution protein concentration
- \( D \): diffusion coefficient of protein
- \( t \): time

The diffusion coefficient of fibrinogen as reported by Gorman (1971) is \( 54.6 \times 10^{-7} \text{ cm}^2/\text{min} \). Plots of predicted adsorbed mass, using the diffusion controlled model, were prepared for each protein concentration and compared with the experimental data (Figures 4.20 to Figure 4.22). Only the first six minutes of each experiment were studied, since mass transfer would only be an important consideration at short time durations. The diffusion controlled model predicts much higher values of adsorbed mass as compared to the experimental values. Also, the initial slope of the diffusion controlled model is much higher than experimentally observed. In
particular, even though the data for the first few minutes of each experiment are somewhat scattered, it is reasonable to conclude that adsorption is not in the mass transfer regime and limited by kinetics, at all concentrations.

Figure 4.20 Comparison of experimental data with diffusion controlled model at fibrinogen concentration 0.05 mg/mL

Figure 4.21 Comparison of experimental data with diffusion controlled model at fibrinogen concentration 0.1 mg/mL
Figure 4.22 Comparison of experimental data with diffusion controlled model at fibrinogen concentration 0.5 mg/mL
CHAPTER 5
CONCLUSIONS

Successful heparin immobilization on silica was achieved. Less fibrinogen adsorbed and the rate of adsorption was lower on heparinized as compared to unheparinized silica. Adsorption was concentration dependent, on both surfaces, i.e., more fibrinogen adsorbed with increasing fibrinogen concentration. Buffer elutability results indicated that more fibrinogen was eluted from heparinized silica, which is interpreted as fibrinogen being weakly bound to the heparinized silica and possibly having undergone less conformational change. Second cycle adsorption rates were greater than the first cycle adsorption rates, which is attributed to post-adsorptive molecular rearrangement. Fibrinogen was postulated to be more mobile on heparinized, as compared to unheparinized silica.

Based on protein adsorption characteristics, heparinized silica appears to be a more favorable biomaterial as compared to unheparinized silica.


Johnell, M., G. Elgue, R. Larsson, A. Larsson, S. Thelin, and A. Siegbahn. 2002. Coagulation, fibrinolysis, and cell activation in patients and shep mediastinal blood during coronary artery bypass grafting with


APPENDICES
APPENDIX A

Least squares minimization procedure to fit adsorption data

Best fit curves for the adsorption data were generated to get values of adsorbed mass at particular times during the experiment and to assist visual comparison of data. The model chosen to fit the data was as follows.

\[ \Gamma = A + Be^{-k_1t} + Ce^{-k_2t} \]

where \( \Gamma \): adsorbed mass (\( \mu g/cm^2 \))

\( t \): time (min)

\( A, B, C, k_1, k_2 \): parameters which can be varied

The adsorption data was divided into three regions, the first adsorption cycle, desorption, and the second adsorption cycle, each consisting of 30 minutes. The model shown above was used in each case to fit the experimental data. For the purpose of data fitting, the desorption stage and the second adsorption cycle were considered to begin at zero time. The optimum values for the five parameters were estimated using the MATLAB function ‘fminsearch’ to minimize the sum of least square errors. The values obtained for the parameters for each stage are displayed below.
Table A.1 Values of parameters used for data fitting

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Stage</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>k₁</th>
<th>k₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>unheparinized</td>
<td>First ads</td>
<td>0.2920</td>
<td>-0.0366</td>
<td>-0.1981</td>
<td>0.2557</td>
<td>0.0613</td>
</tr>
<tr>
<td>0.05 mg/mL fibrinogen</td>
<td>Desorption</td>
<td>0.1153</td>
<td>0.0225</td>
<td>0.1227</td>
<td>0.1124</td>
<td>0.0017</td>
</tr>
<tr>
<td>unheparinized</td>
<td>Second ads</td>
<td>0.1109</td>
<td>-0.1494</td>
<td>0.2737</td>
<td>0.0371</td>
<td>0.0057</td>
</tr>
<tr>
<td>0.1 mg/mL fibrinogen</td>
<td>First ads</td>
<td>0.3655</td>
<td>-0.0968</td>
<td>-0.6315</td>
<td>0.0239</td>
<td>0.6642</td>
</tr>
<tr>
<td>Desorption</td>
<td>0.2575</td>
<td>-0.0066</td>
<td>0.0726</td>
<td>0.0699</td>
<td>0.0331</td>
<td></td>
</tr>
<tr>
<td>Second ads</td>
<td>0.0220</td>
<td>-0.0589</td>
<td>0.3149</td>
<td>0.2022</td>
<td>-2.8625</td>
<td></td>
</tr>
<tr>
<td>* 10⁻⁵</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>unheparinized</td>
<td>First ads</td>
<td>0.2201</td>
<td>0.1152</td>
<td>-0.3352</td>
<td>-0.0051</td>
<td>0.2568</td>
</tr>
<tr>
<td>0.5 mg/mL fibrinogen</td>
<td>Desorption</td>
<td>0.2089</td>
<td>-0.1042</td>
<td>0.2505</td>
<td>0.0101</td>
<td>0.0193</td>
</tr>
<tr>
<td></td>
<td>Second ads</td>
<td>2.9813</td>
<td>-2.6196</td>
<td>-0.0760</td>
<td>1.3696 * 10⁻⁴</td>
<td>0.3217</td>
</tr>
<tr>
<td>heparinized</td>
<td>First ads</td>
<td>0.1096</td>
<td>-0.6114</td>
<td>0.5896</td>
<td>0.2166</td>
<td>0.2712</td>
</tr>
<tr>
<td>0.05 mg/mL fibrinogen</td>
<td>Desorption</td>
<td>0.6807</td>
<td>-0.6010</td>
<td>0.1020</td>
<td>-4.087 * 10⁻⁴</td>
<td>0.4795</td>
</tr>
<tr>
<td></td>
<td>Second ads</td>
<td>0.3543</td>
<td>-0.2047</td>
<td>-0.0853</td>
<td>0.0019</td>
<td>0.7552</td>
</tr>
<tr>
<td>heparinized</td>
<td>First ads</td>
<td>0.1946</td>
<td>-0.1810</td>
<td>0.0037</td>
<td>0.0609</td>
<td>0.0607</td>
</tr>
<tr>
<td>0.1 mg/mL fibrinogen</td>
<td>Desorption</td>
<td>0.0471</td>
<td>0.0929</td>
<td>0.0827</td>
<td>0.0209</td>
<td>0.8336</td>
</tr>
<tr>
<td></td>
<td>Second ads</td>
<td>0.2132</td>
<td>-0.0293</td>
<td>-0.0529</td>
<td>0.8736</td>
<td>0.0645</td>
</tr>
<tr>
<td>heparinized</td>
<td>First ads</td>
<td>0.2325</td>
<td>-0.0264</td>
<td>-0.0897</td>
<td>0.1297</td>
<td>0.0646</td>
</tr>
<tr>
<td>0.5 mg/mL fibrinogen</td>
<td>Desorption</td>
<td>0.1272</td>
<td>0.0511</td>
<td>0.0412</td>
<td>0.0880</td>
<td>0.0880</td>
</tr>
<tr>
<td></td>
<td>Second ads</td>
<td>0.2080</td>
<td>-0.4980</td>
<td>0.3647</td>
<td>1.1198</td>
<td>0.9614</td>
</tr>
</tbody>
</table>
The MATLAB program used for the least squares minimization procedure:

```matlab
%program to fit ellipsometry data using the model
%A + B exp(-k1*t) + C exp(-k2*t)
format compact;
clear all;

%reading experimental data cycle from Excel file
edatal = xlsread('hep_30_0.05_first_ads.xls');
t = edatal(:,1);
ads = edatal(:,2);

%input starting values for parameters
disp(' ');  disp(['Model : A + B exp(-k1 * t) + C exp(-k2 * t)']);
disp(' ');  A = input('Starting estimate of A ');
B = input('Starting estimate of B ');
C = input('Starting estimate of C ');
k1 = input('Starting estimate of k1 ');
k2 = input('Starting estimate of k2 ');

%using fminsearch to get values of the five parameters
[search,sigma] = fminsearch('fitdata fhl ',[A,B,C,k1,k2],[],t,ads);

disp(' ');  disp('estimated values of parameters are');
A_est = search(1)
B_est = search(2)
C_est = search(3)
k1_est = search(4)
k2_est = search(5)

ads_est = A_est + B_est *exp(-k1_est.*t) + C_est.*exp(-k2_est.*t);

figure;
plot(t,ads_est,'r-',t,ads,'k.);
title('Fitted plot');
xlabel('Time (mm)');
ylabel('adsorbed mass microgm/sq cm');
```
%function fitdata_fn1 to evaluate the values of parameters

function sigma1 = fitdata_fn1(invalues,t,ads);
A_in = invalues(1);
B_in = invalues(2);
C_in = invalues(3);
k1_in = invalues(4);
k2_in = invalues(5);

ads_cal = A_in + B_in.*exp(k1_in.*t) + C_in.*exp(-k2_in.*t);
ads_diff = (ads_cal-ads).^2;

sigma1 = sum(ads_diff);
APPENDIX B

Immobilized heparin detection using the toluidine blue assay

The procedure for the detection and possibly quantification of covalently immobilized heparin was adapted from Smith (1980). Upon binding to the polyanionic substrate heparin, the metachromatic dye toluidine blue undergoes color change. The decrease in absorbance can be related to the heparin content based on standardization curves.

A 0.2% NaCl solution was prepared by dissolving 0.002 g NaCl per mL of DDW. Twenty-five mg toluidine blue (SPI Chem) was dissolved in 500 mL 0.01 N HCl containing 0.2% NaCl. To generate a standard curve 10 mg of adipic dihydrazide-modified heparin was dissolved in 100 mL 0.2% NaCl solution.

Toluidine blue solution (2.5mL) was pipetted into test tubes (8 standards, 2 controls, 2 heparinized silica samples and 1 blank control). Heparin standards measuring 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, and 0.5 mL were pipetted in tubes 1-8. The heparin amounts ranged from 10 µg to 50 µg. The unheparinized and the “linkers only” controls were placed tubes 9 and 10. The two heparinized samples were placed in tubes 11 and 12. Tube 13 was the blank control; it did not contain either the heparin standards or the silica pieces. The total volume of each tube was made up to be 5 mL by adding 0.2% NaCl. Each test tube was vortexed for 30 seconds. Hexane
(5 ml) was then added to each test tube. Each test tube was agitated again for 30 seconds. A 0.6 mL sample of the aqueous layer of each tube was taken and diluted with 3 mL absolute ethanol. Absorbance was read at 631 nm.

Results of some selected experimental runs are presented here. The toluidine blue assay provided a value for the amount of heparin, which was converted into surface concentration by dividing by the area of the silica piece studied.

Table B.1 Heparin amount calculated by the toluidine blue assay

<table>
<thead>
<tr>
<th>Experiment date</th>
<th>Heparin amount (µg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>03-15-2004</td>
<td>9.47</td>
</tr>
<tr>
<td>03-16-2004</td>
<td>3.41</td>
</tr>
<tr>
<td>03-26-2004 (1)</td>
<td>0.48</td>
</tr>
<tr>
<td>03-26-2004 (2)</td>
<td>1.5</td>
</tr>
<tr>
<td>03-26-2004 (3)</td>
<td>5.56</td>
</tr>
</tbody>
</table>

The heparin amounts calculated showed a lot of variation. Also, the lower end of these values is close to the non-specific amounts detected on unheparinized and “linkers only” silica controls. It was concluded that the results confirmed heparin attachment, but the assay was not sensitive or accurate enough to quantify the amount of heparin attached.
NMR analysis of adipic dihydrazide-modified heparin

Nuclear Magnetic Resonance (NMR) analysis of adipic dihydrazide-modified heparin and native heparin was performed to verify successful modification and to quantify percentage of heparin modified. Analysis was performed at the NMR facility in the Department of Chemistry at Oregon State University. However, the experiments were not further pursued because of lack of expertise in the technique and in the interpretation of data. Some representative data obtained by using deuterium oxide (Cambridge Isotope Laboratories, Inc) are presented here. However the reader is cautioned not to assume these data to be accurate.
Figure C.1 NMR of native heparin, molecular weight 3,500
Figure C.2 NMR of native heparin, molecular weight 3,500.
Expansion of Figure C.1 in the area of 3.0 – 5.6 ppm.
Figure C.3 NMR of adipic dihydrazide-modified heparin
Figure C.4 NMR of adipic dihydrazide-modified heparin
Expansion of Fig C.3 in the area of 1.0 - 5.0 ppm.