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Heparin was modified with adipic dihydrazide and covalently linked to surface-activated silica. Contact angle measurements were made to determine changes in surface at various stages of the derivatization. X-ray photoelectron spectroscopy was used to analyze the elemental composition of the surface at each step of immobilizing heparin as well and showed that the heparinization was successful. Adsorption time effects on human plasma fibrinogen adsorption at each type of surface were studied by *in situ* ellipsometry. Less fibrinogen adsorption and a slower adsorption rate were observed for heparinized silica relative to unheparinized silica in all cases. The history dependence of adsorption was examined by recording the sequential adsorption of fibrinogen. Differences between the rates of the first and the second adsorption step recorded at identical initial values of adsorbed mass indicated structural rearrangement of the molecules on

the surface. Increased adsorption time in the first adsorption step led to more structural rearrangements, with the effect of history dependence observed to be more pronounced on the heparinized silica. It is believed that these rearrangements involve fibrinogen aggregate formation at the interface, presumably facilitated on heparinized silica by enhanced molecular mobility.

Fibrinogen Adsorption at Heparinized Silica Interfaces: History Dependence and Adsorption Time Effects

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF TABLES

LIST OF APPENDICES

LIST OF APPENDICES FIGURES

FIBRINOGEN ADSORPTION AT HEPARINIZED SILICA INTERFACES: HISTORY DEPENDENCE AND ADSORPTION TIME EFFECTS

CHAPTER 1

INTRODUCTION

Interactions between proteins and surfaces are nearly instantaneous and cause profound alterations in the physical-chemical and biological properties of the interface (Horbett and Brash, 1995). Protein behavior at interfaces is relevant to many bioprocesses, including therapeutic protein separation and purification, the biocompatibility of biomaterials, solid-phase immunoassays, and biotechnology in general. The mechanisms that control these biointerfacial interactions have contributed to the subject of much research, but they are only partly understood and studies on protein behavior at interfaces remain active.

The clinical success of a biomaterial depends on how well its surface promotes or discourages biological responses. Interfacial phenomena have been considered crucial in biomedical devices where the biomaterials are in contact with human tissue (Wojeciechowski and Brash, 1996). While the mechanical requirements of such implants are fairly well characterized, the biocompatibility and long-term interaction of materials with the physiological environment are still not fully understood.

Thrombosis on surfaces is one of the major problems encountered with implants (Reynolds et al., 1993). Some of the major events that occur during thrombosis are protein adsorption, platelet attachment, and thrombus growth. Vascular occlusion and downstream organ damage can result by the growth and subsequent embolization of blood aggregates induced by thrombosis.

The conventional treatment and prevention of thrombosis is by use of anticoagulants like heparin. But there are serious side effects leading to hemorrhage and long term use is not recommended for children or pregnant women. So localizing the anticoagulant activity of the heparin to the specific site of thrombosis, as opposed to potentially life-long systemic administration, is highly desirable.

A number of case studies suggest that heparinized biomaterial surfaces show a diminished thrombogenic response, but results have not been uniformly favorable (Mahoney and Lemole, 1999; Olsson et al., 2000; Svenmarker et al., 2001; Johnell et al., 2002). Heparin is reported to lose part of its anticoagulant activity due to the surface binding procedure (Nemets and Sevatianov, 1991). In any event, the spatial and structural character of heparin at interfaces is not well controlled and the interactions between surface-bound heparin and plasma proteins are not well understood.

Our objective was to immobilize heparin to a surface, characterize the surface, and evaluate its interaction with fibrinogen, which is abundant

in plasma and plays a central role in thrombosis. A recent advance in the study of biopolymer adsorption at interfaces that emphasizes the history dependence of protein adsorption was applied to identify specific factors governing fibrinogen adsorption. The effect of adsorption time on interfacial behavior, including its effect on history dependence, was also examined.

CHAPTER 2

LITERATURE REVIEW

2.1 Protein adsorption and biocompatibility

2.1.1 Biocompatibility

Foreign materials react to blood in various ways, for instance: (1) initiating inflammation, (2) activating the intrinsic coagulation system and then promoting platelet deposition, or (3) activating complement pathways (Baier, 1978). Protein adsorption is one of the first events that occur when a synthetic material is in contact with blood. Studies have shown that the properties of the surface influence the composition of the adsorbed protein and the composition of the protein film has an effect on the thrombotic response (Young et al., 1982; Horbett, 1982). So the clinical success of a biomaterial depends on how well its surface promotes or discourages these biological responses.

Sagvolden et al. (1998) using atomic force microscopy (AFM) showed that proteins adsorb better on hydrophobic than hydrophilic surfaces. They found that the adhesion forces on the surfaces depended on different properties. On hydrophobic surfaces, the binding strength depended on the structural rigidity of the protein, while on hydrophilic

surfaces, protein and surface charge had more influence. But Rapoza and Horbett (1990), who studied postadsorptive transitions in fibrinogen on polymers, found that fibrinogen adsorbed more on rigid polymers regardless of whether the polymer was hydrophilic or hydrophobic even though the binding strength was much lower on hydrophilic polymers.

The adsorption of plasma proteins is complex due to the enormous amount of surface active proteins interacting with each other. It involves competitive adsorption, where the composition of the adsorbed layer keeps changing over time (Vroman et al., 1980). Biocompatibility is directly related to identifying the proteins that adsorb to the blood-contacting surface. Fibrinogen is one of the proteins known to promote platelet adhesion (Ginsberg et al., 1988). Slack and Horbett (1988) have studied fibrinogen adsorption from plasma and binary protein solutions using radiolabeling of baboon fibrinogen. Fibrinogen adsorption to the surface reached its maximum more quickly as the plasma concentration increased, but fibrinogen adsorbed more from slightly diluted plasma. This study showed that fibrinogen binds quickly but weakly from plasma and is therefore replaced by other proteins that have a stronger affinity for the surface. To get a better understanding of protein adsorption from mixed protein systems, sequential adsorption of human serum albumin (HSA), immunoglobulin G (IgG), and fibrinogen at a hydrophobic surface was done with ellipsometry and total internal reflection fluorescence spectroscopy (TIRF) (Malmsten et al., 1997).The sequential adsorption of

fibrinogen and IgG seem to drop dramatically when HSA was preadsorbed suggesting that HSA can be used as a blocking agent on biomaterials.

Protein adsorption can become irreversible by changes of conformation and specific binding. But the degree of conformational change depended on the specific protein and the surface. The conformation state and type of the adsorbed protein were reported to influence platelet reactivity initiating the events leading to thrombosis (Barbucci and Magnani, 1994). The correlation between adsorbed protein conformation and biocompatibility provided information on developing nonthrombotic materials and in part controlled triggering of the non-specific self-protection mechanisms of blood (Morrisey, 1977).

Hydrophilic silicon oxide was modified by chemically anchoring an organic monolayer bearing terminal phosphorylcholine (PC) groups (Lu et al., 2001). The authors characterized the structure of the PC layers and their effectiveness in inhibiting nonspecific protein deposition. Neutron measurements showed that PC monolayer coatings were as effective as the PC polymer coatings in reducing protein adsorption in vitro. The monolayer coating may have advantages when dealing with modification of dialysis membranes and nanofiltration membranes where the size of membrane pores is crucial to regulation of permeate flux. Inglis et al. (2001) demonstrated how a biocompatible polymer can be easily patterned upon another polymer substrate using the soft lithographic technique. It would create a surface with two spatially different properties, which resists

protein adsorption. This technique would allow a cost effective method of microfabrication, avoiding the more difficult use of gold, self assembly, or self-assembled monolayers as protein resistant surfaces.

2.1.2 Time effects in protein adsorption

History dependence caused by conformation changes of adsorbed protein is considered important in determining the behavior of the surface that the protein is bound to. Lenk et al. (1991) using an attenuated total reflection (ATR) infrared technique studied the time-dependent transitions of adsorbed fibrinogen on two types of surfaces and found that less fibrinogen was removed from the surface as the residence time on the surface increased. So over time, fibrinogen underwent structural changes and bound more tightly to the surface. Through comparison of short-term with long-term adsorption by infrared spectra, loss of helical and random structure with an increase in β-structures with time gave proof of structural change.

Wertz and Santore (1999) studied the kinetic behavior of albumin and fibrinogen adsorption and relaxation onto C16 self-assembled monolayers by using total internal reflectance fluorescence (TIRF). Albumin was adsorbed to the surface and after different relaxation periods of the albumin, fibrinogen was adsorbed subsequently. Prior to fibrinogen adsorption, short albumin exposures were ineffective in preventing

fibrinogen adsorption. But at longer incubation times of albumin, the amount of fibrinogen adhesion was subsequently reduced. As albumin adsorbs and relaxes on the surface, the area available for fibrinogen decreases.

Competitive adsorption behavior exhibited by the wild-type T4 lysozyme and two of its structural stability variants was studied by ^{125}I radioisotope labeling (Lee et al., 2004). When exchange between adsorbed protein and dissolved protein occur, the more stable variants are removed from the surface by less stable variants. Thus, less stable proteins exhibited an advantage in competitive adsorption over the more stable proteins.

Schaff et al. (1992) simplified the protein adsorption to three steps:

(1) transport of the molecules from the bulk to the surface by diffusion or diffusion/convection;

(2) adsorption of dissolved macromolecules interacting with the solid surface;

(3) conformational changes of the molecules adsorbed on the surface.

They found that knowledge of time scale of each step was needed to understand the different mechanisms controlling the process.

The technique of scanning angle reflectometry (SAR) was used to observe fibrinogen adsorption on silica surfaces. The fibrinogen molecules adsorbed "side-on" where the axis is oriented parallel or adsorbed "end-on" where the axis is oriented perpendicular to the interface. Two characteristic

time scales were found through this study. The time to form a layer covering the surface "side-on" takes about 5 minutes, while it takes about 1 hour for the fibrinogen to go through conformational changes adsorbing "end-on" and tightening to an irreversible bond. But the scanning procedure was not automated, so it could not give real time results.

Van Tassel et al. (2001) quantitatively analyzed history dependent adsorption using optical waveguide lightmode spectroscopy (OWLS). A multiple step adsorption process was used to compare same composition systems of differing formation histories. The protein that is adsorbed on the surface undergoes conformational changes and aggregates, as well. A different structure would form if the same protein was adsorbed subsequently. The rate of adsorption depends on the formation history of the adsorbed layer because not only does it depend on the bulk concentration and the amount of protein already adsorbed, but also the structure already attached to the surface. The rate of adsorption increased when going from the initial step to the next step and the increase was more significant when the initial adsorption was extended for a considerable amount of time.

Tiberg et al (2001) used neutron reflectometry to investigate the time-dependent β-casein adsorption at the silica-aqueous solution interface. They have reported that adsorption of β-casein to silica is slow compared to a hydrophobic surface. They have suggested that more time allowed extensive molecular rearrangements in the interfacial regions. During this

time, a large excess of protein is built up forming an asymmetric surface bound bilayer, in contrast to the monolayer structure formed at hydrophobic surfaces.

2.2 Fibrinogen and fibrinogen adsorption

2.2.1 The physical properties and role of fibrinogen

Fibrinogen is an adhesive protein that promotes adhesion and aggregation of platelets, and has a central role in thrombosis and haemostasis (Ginsberg et al., 1988; Zucker and Vroman, 1969; McManama et al., 1986). Clotting occurs when fibrinogen is broken down by an enzyme called thrombin into short fragments of fibrin (Mosesson, 1998).

Fibrinogen is a 340,000 Da molecule, 47.5 nm in length, composed of two identical halves bound together through a network of disulfide bonds, each half containing three different peptide chains described as Aα, Bβ, and γ (Henschen et al., 1983, Doolittle, 1983). Hall and Slayter (1959) first introduced the trinodular model of fibrinogen by using electron microscopy. It has three domains connected by two regions. The two outer D domains consist of β and γ compactly folded chains while a hydrophilic α chain flows freely in the solution and the N-terminal regions of all the six chains are folded into a central E domain.

2.2.2 Fibrinogen adsorption

Fibrinogen adsorption has been studied extensively due to its important role in blood coagulation and its ability to promote platelet adhesion (Zucker and Vroman, 1969; Ginsberg et al., 1988; Phillips et al. 1988). Others have found that bound fibrinogen is not the main reason for platelet activation, but rather that the conformation of fibrinogen adsorbed on surfaces plays a more important role (Lindon et al., 1986; Pekala et al., 1986). The conformational changes of fibrinogen due to adsorption and desorption on TiO_2 were studied using differential scanning calorimetry (DSC), circular dichroism (CD), and fluorescence spectroscopy. The decrease of the α-helix content was observed (Yongli et al., 1999).

The adsorption of fibrinogen to hydrophilic and hydrophobic quartz surfaces was measured and showed different conformations suggesting that the surface energy affects the conformation of adsorbed fibrinogen (Nygren and Stenberg, 1988). AFM measurements showed that fibrinogen tends to aggregate and appear globular on hydrophobic surfaces, while on hydrophilic surfaces it adsorbs as isolated single molecules keeping its trinodular structure (Marchin and Berrie, 2003).

Several studies were done on elutability to gain a better look at molecular properties affecting fibrinogen adsorption on surfaces (Bohnert and Horbett, 1986; Rapoza and Horbett, 1990; Wahlgren et al., 1993). Rapoza and Horbett (1990) studied the effects of concentration and

adsorption time on the elutability of fibrinogen using surfactant solutions of varying structures and concentrations. They found that fibrinogen tends to bind tighter when adsorbed from low bulk concentration solution and when surface residence time increased. The degree of elutability was also dependent on the surface on which fibrinogen was adsorbed. Wahlgren et al. (1993) examined the elutability of fibrinogen by cationic and anionic surfactants on hydrophobic and hydrophilic surfaces. Less fibrinogen was adsorbed for all the surfactants used on the hydrophobic surface but only the addition of anionic surfactant led to a significant decrease in the amount adsorbed on the hydrophilic surface.

2.3 Heparin and heparinized surfaces

2.3.1 Heparin

Heparin is a glycosaminoglycan, which is a family of polysaccharides with alternating uronic acid and glycosamine residues. Its molecular size ranges from 3000 to 40,000 Da with an average molecular size of about 15,000 Da (Tyan et al., 2002). Because it is highly sulfated, heparin has the highest negative charge density of biological macromolecules (Capila and Linhardt, 2002).

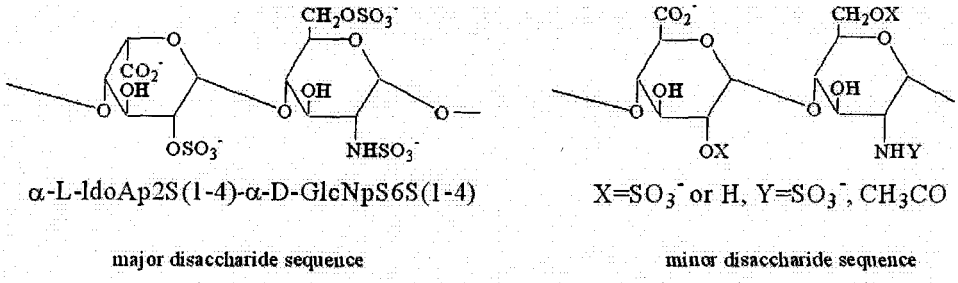


Figure 2.1 Structures of heparin

Heparin is known for its anticoagulant property. It is also known to show multiple activities, such as being antiatherosclerotic and antiinfective, inhibiting complement activation, acting as an anti-inflammatory agent, and having numerous roles in angiogenesis and cancer (Islam and Linhardt, 2003). Although it is the most commonly used clinical anticoagulant, the exact structure is still not known due to its complex nature.

Rosenberg and Damus (1973) suggested one of the first analysis of heparin interacting with anti-thrombinIII (AT-III) and through years of research found that a specific sequence in heparin binds to AT-III causing a conformation change in AT-III, which accelerates the inhibition of blood coagulating factors (Lindahl et al, 1983). It not only binds to AT-III, but several other proteins that are responsible for thrombosis (Engelberg, 1996).

Heparin is usually injected intravenously but has serious side effects that can lead to bleeding. These complications and the knowledge of the coagulation cascade have led to the development of low-molecular

weight heparins (LMWH) which bind less to the other heparin-binding proteins (Lane et al., 1986).

2.3.2 Heparinized surfaces

The anticoagulant property of heparin has made it favored as a coating for blood-contacting biomaterials. Gott et al. (1963) were some of the first to investigate the possibilities of heparin on surfaces as anticoagulant coatings. When heparin is immobilized, adsorption of blood proteins and adhesion and activation of platelets are reduced on the surface of the material (Barbucci and Magnani, 1994; Niimi et al., 1999). AT-III binding and cleavage of plasma proteins are primarily responsible for antithrombotic effects of immobilized heparin (Weber et al., 2002). But some results show otherwise and the exact mechanism of heparin activity on surfaces is still not well understood (Blezer et al., 1997; Olsson et al., 2000). Anticoagulant activity of heparin is reduced when immobilized because of competition for binding sites of other heparin binding proteins with AT-III and the non-specific binding of plasma proteins, which hinders the binding of AT-III (Van Delden et al., 1996).

Heparin is immobilized through different methods. It can be bonded ionically to the surface, but because of the weak binding strength, heparin can be leached from the surface to the blood (Engbers and Feijen, 1991; Schmitt et al., 1983). For long-term usage, it is appropriate to

covalently bond heparin to the surface. Covalent bonding of heparin through its reducing end provides all of heparin's binding sites, reduces steric repulsion, and mimics the arrangement of the naturally occurring proteoglycan (Nadkarni et al., 1994). Also, hydrophilic spacers are used so that heparin has higher mobility and is more bioactive, which would enhance its anticoagulant activity (Marconi et al., 1997). The longer the spacers are, the more bioactive it became, but Park et al. (1988) found there was no chain length effect on platelet adhesion.

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of modified heparin

3.1.1 Heparin modification

Heparin modification was based on methods to modify hyaluronic acid (HA), which is another glycosaminoglycan described by Pouyani and Prestwich (1994), Pouyani et al. (1994), Vercruysse et al. (1997), and Luo et al. (2000). The chemical modification targets the carboxyl groups of heparin through carboiimide compounds. The use of adipic dihydrazide (ADH, Avocado Research Chemicals Ltd, Lot F6744B) provides multiple pendant hydrazide groups for further coupling and crosslinking in mild and aqueous conditions.

Two hundred mg of low molecular weight heparin sodium (heparin, Celsus Laboratories, Inc., Cincinnati OH, Lot FH-19002) was dissolved in 50 mL of water such that the concentration of the heparin solution was 4 mg/ml. To this mixture, 3.5 g of adipic dihydrazide (ADH, Avocado Research Chemicals Ltd, Lot F6744B) was added. The pH of the reaction was then adjusted to 4.75 using 0.1 N HCl (Fischer Scientific, Fair Lawn, NJ) and maintained for 30 minutes. Next, 382 mg of 1-ethyl-3-[3-

(dimethyl-amino)propyl]carboiimide (EDCI, Aldrich Chemical Co., Inc. Milwaukee, WI) was added. The pH of the mixture was maintained at 4.75 for 2 hours or until no further increase in pH was observed at room temperature using 0.1 N HCl. The reaction was stopped by raising the pH to 7.0 by adding a small amount of 1 N NaOH (Fischer Scientific, Fair Lawn, NJ).

Dialysis tubing (MW 3,500, Spectrum Laboratories, Inc., Rancho Dominguez CA) was soaked in deionized distilled water (DDW, Barnstead Mega-PureR, Dubuque, IA) at room temperature for 3-4 hours and then rinsed. The reaction was transferred to the prewashed dialysis tubing filling it up to 1/3 to 1/2 of the volume. It was dialyzed in a 2 L vessel containing NaCl (Mallinckrodt Inc., Paris, KY) solution with a concentration of 5.84 g/L. The NaCl solution was changed every 2 hours for the first 6 hours and every day subsequently for 3 days. On the fourth day, NaCl solution was discarded and changed to DDW.

The dialyzed mixture was centrifuged at 21 °C, 3000 rpm (Allegra 6R Centrifuge, Beckman, Palo Alto, CA) for 15 minutes. The pellet was discarded while the supernatant was frozen in a -80 °C freezer (UC1340-A14, Revco Scientific, Asheville, NC) for 2 hours at an angle of 45°. The frozen mixture was lyophilized (Benchtop 5SL, VirTis Company, Inc., Gardiner, NY) for at least 48 hours.

A nuclear magnetic resonance spectroscopy (NMR) analysis is used to gain information about the chemical composition of a substance. It was

used to identify ADH heparin and quantify the percentage of modification (APPENDIX B).

3.2 Surface preparation

3.2.1 Unheparinized surface

The procedure to obtain hydrophilic surfaces has been previously described by McGuire et al. (1995). Since silica has a hydrophilic character, this method is used as a cleaning procedure to get rid of the oil and dust from the surface. Silicon (Si) wafers (hyperpure, type N, boron doped, plane 1-0-0) were purchased from Wafernet (San Jose, CA). They were cut into 1 cm X 3 cm plates using a tungsten pen. Each Si plate was rinsed with 25 mL acetone and then placed in a 10 mL mixture of NH_4OH: H_2O_2: H_2O (1:1:5, volume ratio). Then they were heated for 10 minutes at 80 °C in a water bath. After rinsing with 20 mL of DDW, each Si plate was placed into a mixture of HCl: H_2O_2: H_2O (1:1:5, volume ratio) and they were heated for 10 minutes at an 80 °C water bath. After rinsing with 20 mL of DDW, the plate were stored in 20 mL of 50 % ethanol to maintain their hydrophilic properties.

3.2.2 Aminated surface

The following treatment was slightly modified from the method described by Buechi and Bachi (1979) to aminate silica using 3-aminopropyltriethoxy-silane (3-APTS, Aldrich Chemical Co. Inc., Milwaukee, WI). The surfaces were aminated to enhance attachment of the heparin. To ensure their cleanliness, the hydrophilic Si plates were immersed in anhydrous acetone (Mallinckrodt Inc., Paris, KY) for 2 hours. Each Si plate was placed into a test tube filled with 2 % (volume/volume) solution of 3-APTS in acetone. Then they were kept at 50 °C for 24 hours. After 24 hours, the plates were dipped in anhydrous acetone for 1 minute followed by DDW for 1 minute and then dipped in anhydrous acetone for 1 minute with agitation followed by DDW for 1 minute with agitation. Each plate was then dried with nitrogen.

3.2.3 Carboxylated surface

The following method was based on the procedure described by Mason et al. (2000) to attach HA to polymeric biomaterials. ADH-heparin can be attached to aminated surfaces through several weak bonds, but succinic anhydride (Aldrich Chemical Co. Inc., Milwaukee, WI) produces an extended arm to which ADH-heparin can become covalently linked, which helps maintain its bioactivity.

The aminated surfaces were treated with 10 mM (1.0007 g/L) succinic anhydride in dry N, N-Dimethylformamide (DMF, EM Science, Gibbstown, NJ) solution. After 10 hours, surfaces were washed for 30 min in 10 mL DDW.

3.3 Immobilization of heparin

After carboxylation, each surface was placed in a solution containing 1.2 mg/ml of ADH-heparin, 50 mM (12.285 g/L) Bis-tris hydrochloride (Sigma-Aldrich Inc., St. Louis, MO) and 1mM (0.192 g/L) EDCI for 24 hours. They were placed on a shaker (AROS 160, Barnstead/Thermolyne, Dubuque, Iowa) at 90 rpm at 6 °C to prevent bacterial growth. After 24 hours, the surfaces were washed in DDW overnight at a low flow rate to remove physically adsorbed heparin. A schematic of the chemistry involved is presented at the end of this chapter (Fig.3.1).

. The toluidine blue assay is used to quantify polyanionic substrates. It was used to detect and quantify covalently immobilized heparin (APPENDIX C).

3.4 Surface analysis

3.4.1. Contact angle

Contact angles are measurements used to characterize the solid surface by calculating the solid-vapor-liquid tension. The tensions between solid and liquid, solid and vapor and liquid and vapor are the three forces involved when a drop is formed on a surface. Contact angle can be defined as the angle in the liquid phase at the three-phase line of contact. It is used to obtain information on the hydrophilicity or the hydrophobicity of the surface.

Contact angles were measured with the First Ten Angstroms 32 Video (FTA 32, First Ten Angstroms, Portsmouth, VA). The contact angles of the Si plates of each step were measured by dropping 1 μL of DDW on the surface and then getting an image of it. The measurements based on the drop shape analysis were made using the image.

3.4.2 X-ray Photoelectron Spectroscopy (XPS)

X-ray Photoelectron Spectroscopy (XPS), also known as Electron Spectroscopy for Chemical Analysis (ESCA), is an extremely sensitive surface analysis technique. X-ray is used to excite the surface by emitting photoelectrons. The high resolution energy analysis of these photoelectrons

gives information of the elemental and chemical bonding of a surface. But it is a bit difficult to get a quantitative analysis and is usually used as a qualitative tool.

XPS was used to get a qualitatively evaluate the immobilization of heparin. The unheparinized surface and the carboxylated surface, which is the surface with all the linkers, were measured and compared with the heparin attached surface.

Experiments using XPS were done on a Kratos HSI instrument. Monochromatized Al-Kα radiation was used with a source power of 200W. A low energy flood gun was used to neutralize the charge. The composition was analyzed from survey scan data acquired at 80 eV pass energy. High resolution data was done by 20 eV pass energy.

3.5 Adsorption kinetics

3.5.1 Buffers

The buffer used for the experiments is a 0.01 M sodium phosphate buffer, pH 7. Sodium phosphate dibasic heptahydrate (1.08 g/L, Mallinckrodt Inc., Paris, KY).and 0.53 g/L of sodium phosphate monobasic monohydrate (FischerBiotech, Fair Lawn, NJ) were mixed together in DDW. The buffer was filtered with a 0.45 μm syringe filter (Acrodisc® Syringe Filters, Pall Corporation, Ann Arbor, MI).

3.5.2 Fibrinogen

Fibrinogen (F-4883, Sigma-Aldrich CO., St. Louis, MO) was used without further purification. It was dissolved by layering it on the top of the pre-warmed phosphate buffer. The solution was placed in an incubator set at 37 °C while being slowly stirred. Fibrinogen was stirred for 4 hours and then was filtered with a 0.45 μm syringe filter prior to the ellipsometry experiment. The fibrinogen solution was kept in the incubator while being stirred until ready for use.

3.5.3 Ellipsometry

Ellipsometry is the measurement of the effect of reflection on the state of polarization of light. The state of polarization is defined by the phase and amplitude relationships between the two component plane waves into which the electric field oscillation is resolved (Archer, 1968). The effect of reflection is characterized by the angle Δ, which is the change in phase, and the angle ψ, which is the arctangent of the factor by which the amplitude ratio changes. Ellipsometry is used to determine the thickness and refractive index of thin films on reflective surfaces, such as metal and ceramic surfaces. In situ ellipsometry is ideal for monitoring the growth of

films, especially determination of real time protein adsorption kinetics (Elwing, 1998).

A Si plate was suspended on a mount and was slowly lowered to a fused quartz trapezoid cuvette which had 4.5 mL of 0.01M phosphate buffer, pH 7. The bare surface was measured for 30 minutes and then 0.5 mL of 0.1 mg/mL fibrinogen was added while being stirred. Different adsorption times were applied and then the surface was rinsed with clean buffer for 5 minutes. After 25 minutes, 0.5 mL of fibrinogen at the same concentration was added for a second cycle and monitored again. The Δ and ψ was measured every 15 seconds.

Following the data collection, a one-film-model ellipsometry program (Krisdhasima, 1992) was used to calculate the amount of protein adsorbed over time. To obtain the adsorbed amount of fibrinogen, the value of the ratio of the molecular weight to molar refractivity (M/A) used was 4.246 g/ mL and the partial specific volume used was 0.719 mL/g (Wahlgreen, 1993).

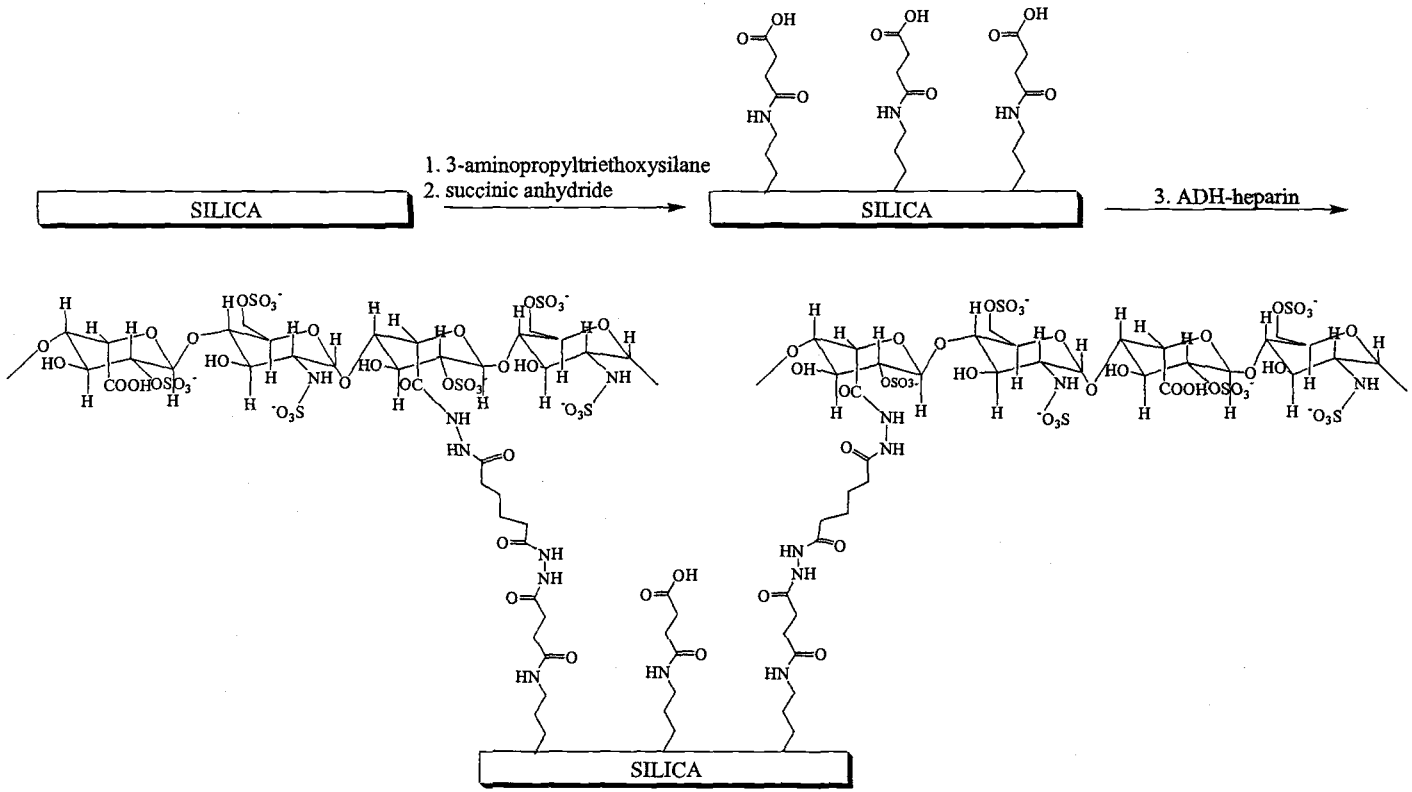


Figure 3.1 Heparin attachments to surface activated silica

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Surface Analysis

4.1.1 Contact Angle Measurement

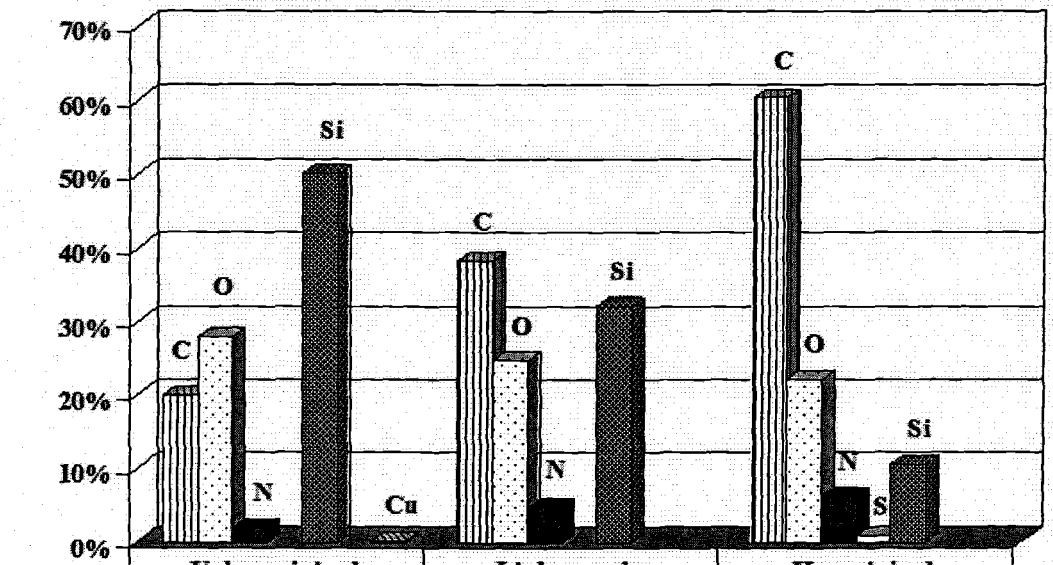
Contact angles were measured at each of the steps in immobilizing heparin. Measurements were repeated at least 6 times and the averages and the standard deviations were tabulated in Table 4.1. The DDW droplet on the unheparinized silica had no particular shape making it difficult to analyze the shape of the drop. Silica with only the linkers present showed an increase in the contact angle values indicating a hydrophobic surface. Since heparin is very hydrophilic, the heparinized silica gave a lower contact angle value than the silica with only the linkers. But contrary to expectations, it was higher than the unheparinized silica. This is possibly due to sites where heparin was not attached and only the linker was present.

Table 4.1 Values of contact angle of each step of heparinization

Surface	Contact angle (degree)
Unheparinized	0
Linkers only	44.46 ± 1.33
Heparinized	16.54 ± 0.96

4.1.2 X-ray Photoelectron Spectroscopy (XPS) Analysis

Each step of immobilization was analyzed by XPS for surface characterization. Fig. 4.1 shows the elemental atomic percent values of the unheparinized silica, silica with only linkers and heparinized silica. The amount of silicon measured decreased in the proceeds of heparinizing the silica surface indicating that other elemental components were being attached. Linker attachment was confirmed with the increase of carbon and nitrogen. The presence of sulfur on the heparinized silica is entirely consistent with heparin being immobilized on the surface, since heparin is the only component used that has sulfur. Carbon detected on the unheparinized silica was thought to be from the atmosphere and traces of copper and sodium from contamination (Golledge, personal communication).



Atomic Percentage	Unheparinized	Linkers only	Heparinized
C	19.90%	38.30%	60.30%
O	27.90%	24.80%	22.10%
N	1.90%	4.80%	6.20%
S	nd	nd	0.90%
Si	50.10%	32.10%	10.50%
Na	tr	nd	nd
Cu	0.20%	nd	nd

Figure 4.1 XPS analysis of each step for heparinizing silica

4.2 Adsorption Kinetics

4.2.1 Adsorption Data

Representative plots for the adsorption kinetics of fibrinogen on unheparinized and heparinized surfaces are shown in Figs 4.2 to 4.7. Fibrinogen was allowed to adsorb from a 0.1 mg/mL solution for 15, 30 or 60 min, then desorbed for 30 min, and then introduced again at the same concentration for the same period of time as the first adsorption step (i.e.15, 30 or 60 min).

Less fibrinogen adsorbed to heparinized surfaces for all time periods. For the 60-min adsorption period, adsorption of fibrinogen on heparinized silica measured 0.206 $\mu g/cm^2$ .The value of fibrinogen adsorption on unheparinized silica was found to be 0.377 $\mu g/cm^2$, which agrees well with previously reported values (Wahlgren et al, 1993).

Adsorption of fibrinogen increased over time for both surfaces. A plateau value was reached for the 30 and 60-min adsorption tests, while the amount of absorbed fibrinogen did not attain a plateau over the 15-min adsorption period in the first adsorption. It is reasonable to assume that the interface had not yet been "saturated" with fibrinogen molecules at the end of 15 min.

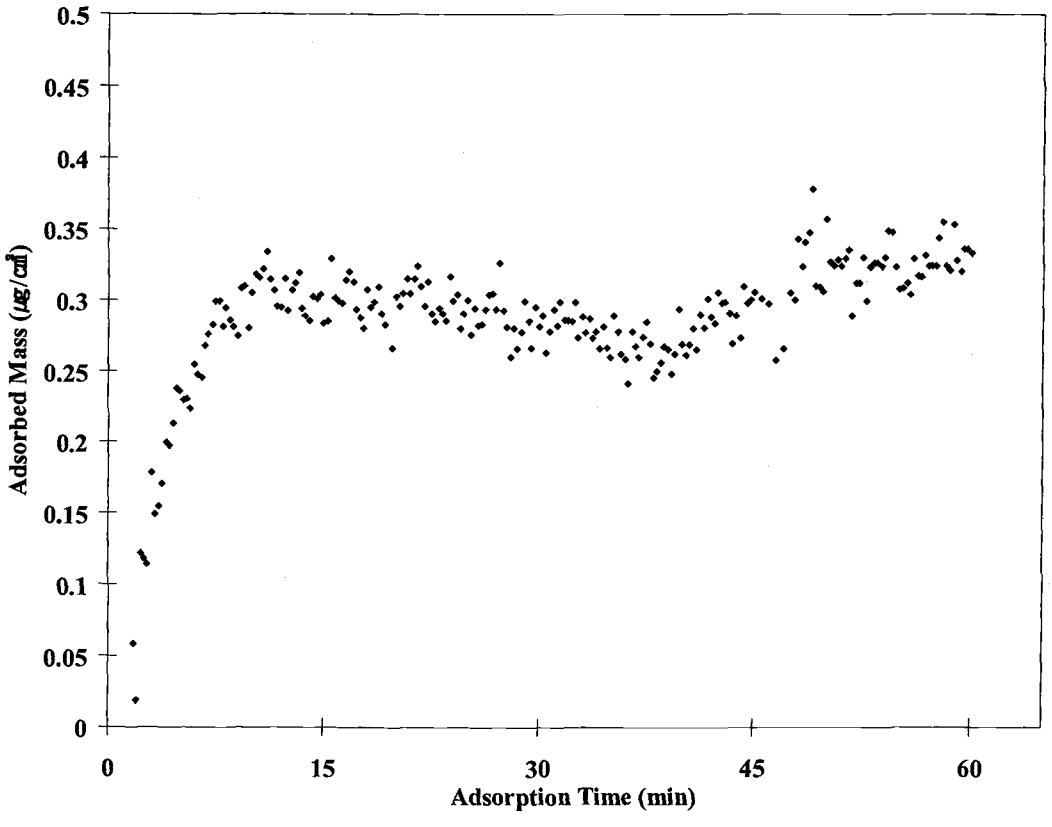


Figure 4.2 Adsorption of 0.1 mg/ml fibrinogen for 15 min on unheparinized silica

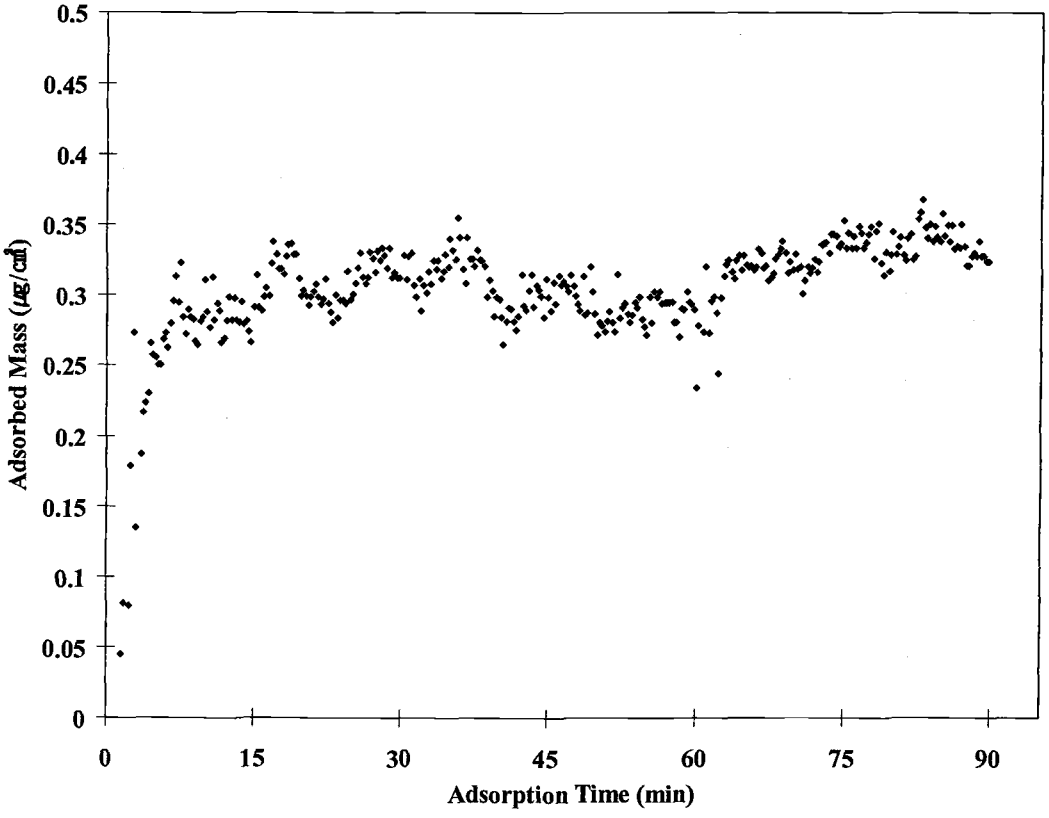


Figure 4.3 Adsorption of 0.1 mg/ml fibrinogen for 30 min on unheparinized silica

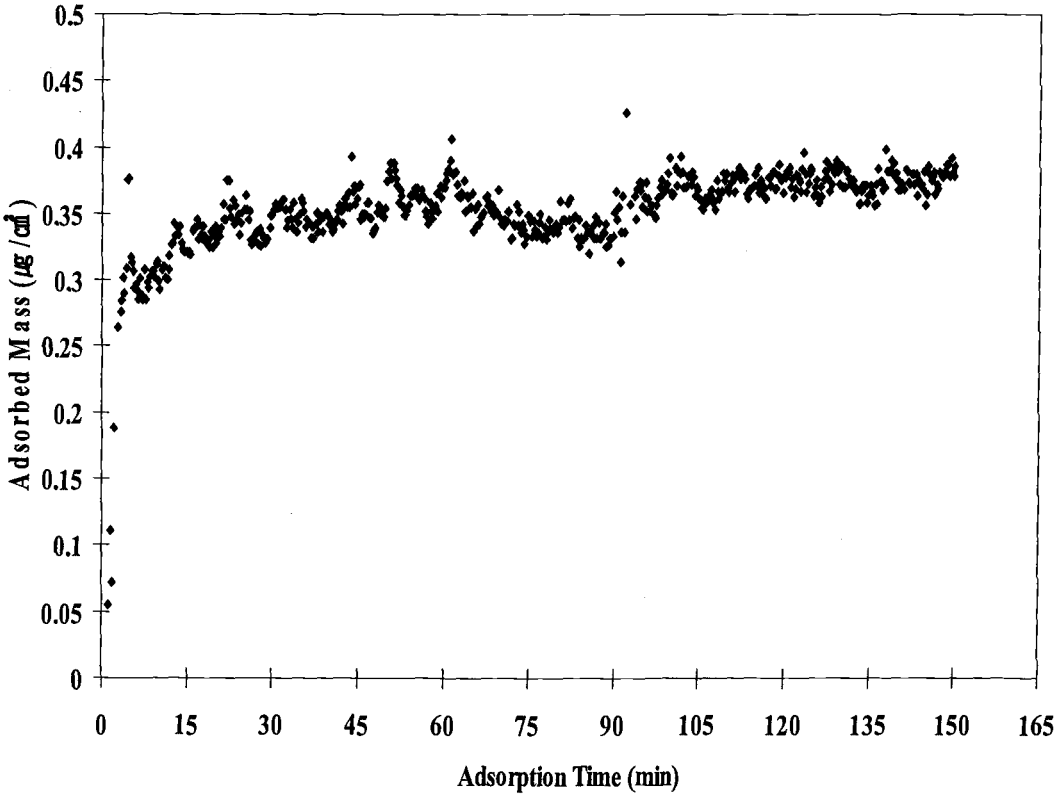


Figure 4.4 Adsorption of 0.1 mg/ml fibrinogen for 60 min on unheparinized silica

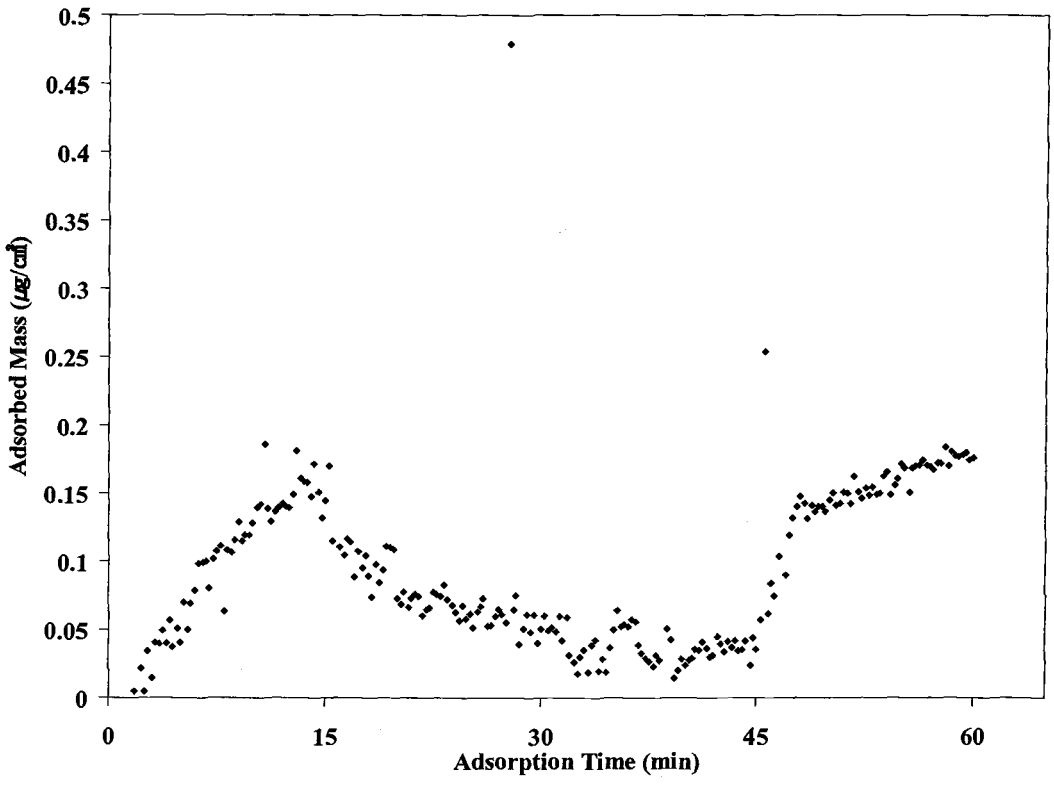


Figure 4.5 Adsorption of 0.1 mg/ml fibrinogen for 15 min on heparinized silica

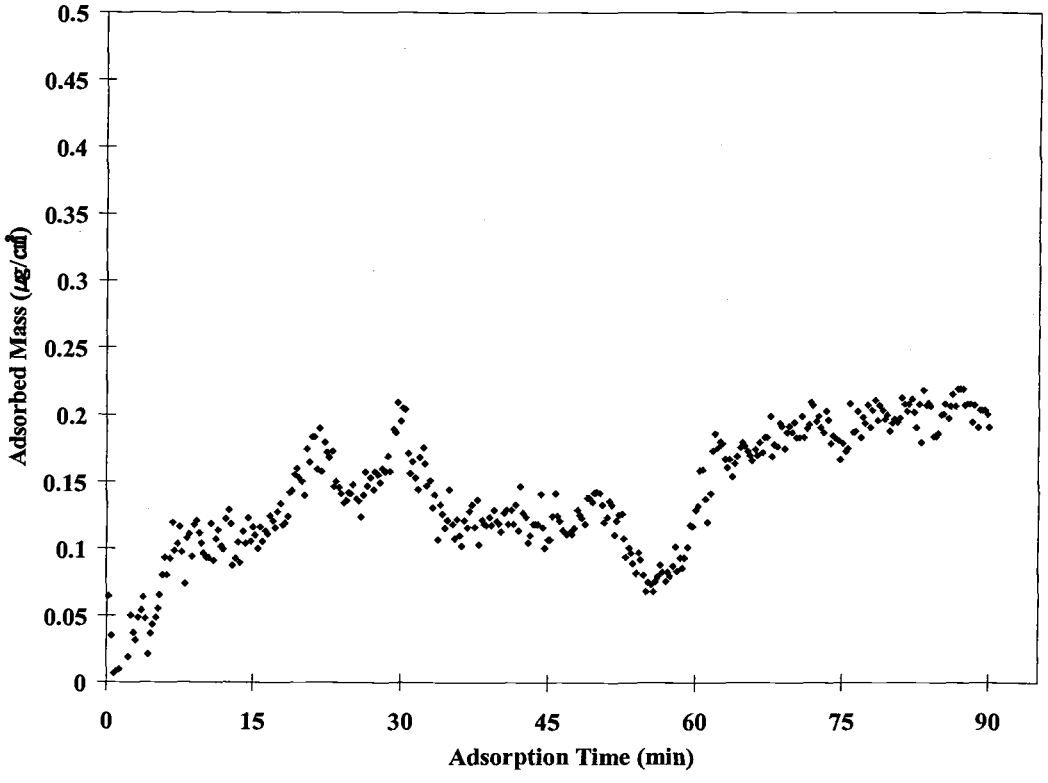


Figure 4.6 Adsorption of 0.1 mg/ml fibrinogen for 30 min on heparinized silica

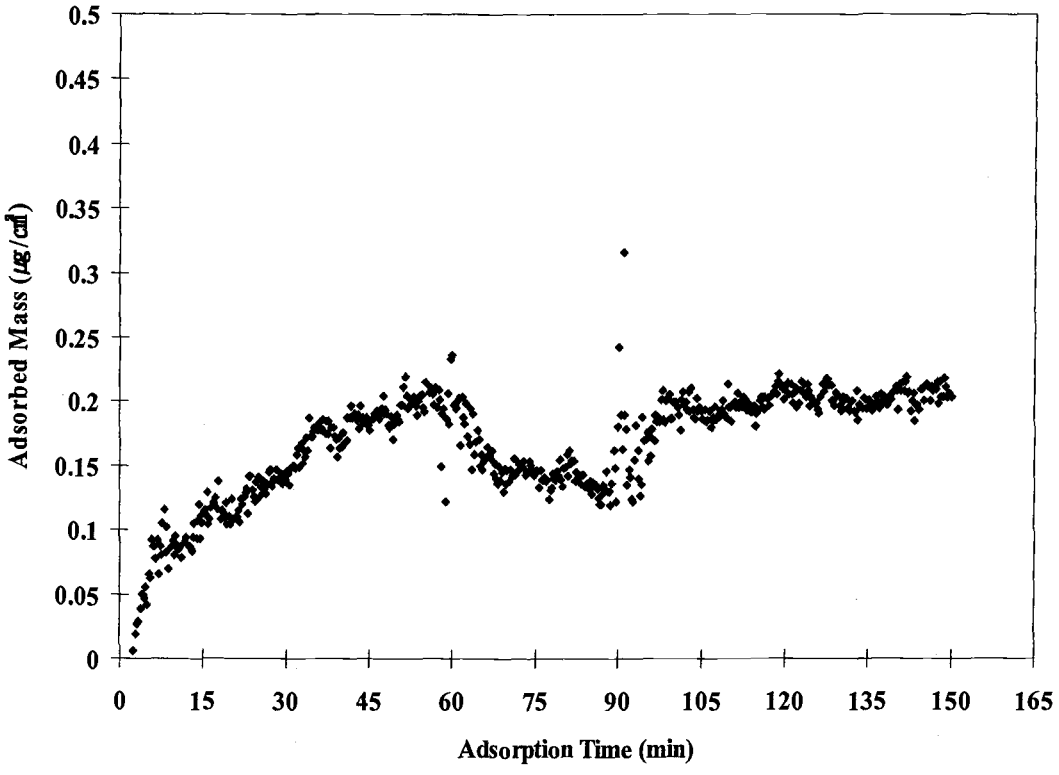


Figure 4.7 Adsorption of 0.1 mg/ml fibrinogen for 60 min on heparinized silica

4.2.2 Analysis of Time Effects on fibrinogen adsorption

Protein behavior is often characterized by "history dependence" owing to the slow-relaxation of non-equilibrium structures at the interface (Calonder et al, 2001). Optical waveguide lightmode spectroscopy (OWLS) in "multi-step"mode, where an adsorbant surface is alternatively exposed to a protein solution and one free of protein, was used to compare adsorption rates on interfacial layers of identical composition but of differing formation histories. The adsorption kinetics consist of the intrinsic adsorption rate (a kinetic constant depending solely on protein and adsorbent surface properties), the interfacial cavity function (a parameter describing the state of the adsorbed layer), and the intrinsic desorption rates of molecules in different adsorbed states. In the absence of transport limitations (i.e. when adsorption is not diffusion controlled), the rate of adsorption depends not only on bulk concentration and the amount already adsorbed, but also on the structure that is already formed on the surface. A schematic of this concept is shown in Fig 4.8. In Fig. 4.8, the adsorbed mass is the same for both cases, but the adsorbed layer structures are different due to different formation histories. A greater adsorption rate would be expected for the case illustrated in Fig.4.8 (b) since there would be a higher probability of an incoming macromolecule landing in a "cavity" on a macromolecule-free surface. This "cavity" could be attributed to clustering of adsorbed molecules.

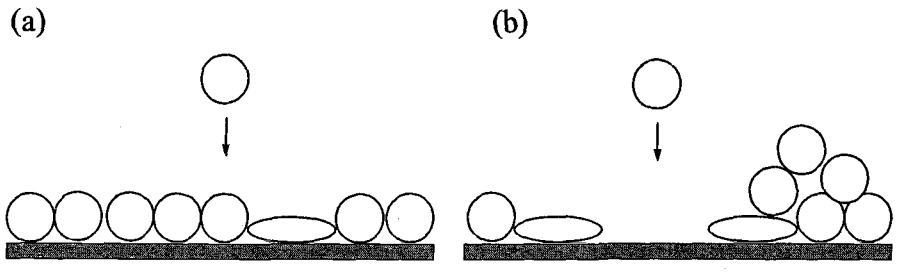


Figure 4.8 Macromolecule adsorption to a surface at which molecules of the same type have already adsorbed

Thus, adsorption rate data provides important information relevant to adsorbed layer structure.

Three-step experiments in which the adsorbed amount was measured continuously with alternating cycles of protein adsorption and rinsing was used to compare adsorbed layers of the same mass but of different formation histories. At equilibrium adsorbed protein layers of identical density and temperature have the same structural properties; the rates of subsequent adsorption would be expected to be the same. However, as seen in the results from Figs 4.2 to 4.7, the rate of the second adsorption step was different from the first adsorption step. The difference in the adsorption rates indicates different structures of the adsorbed layer.

Figs 4.9 to 4.14 show the data of Figs 4.2 to 4.7 with best fit curves to assist in the quantitative comparison of kinetic behavior (APPENDIX A). The second adsorption step is shifted in time to better compare the first and second step adsorption kinetics for unheparinized and heparinized surfaces.

On the unheparinized silica, the slope of the second adsorption step was greater than that recorded for the first adsorption step. With reference to Figs 4.9 to 4.14, the initial kinetics where the mass density is the same for the two adsorption steps were compared. The difference between the initial kinetics increased as the adsorption time of the first step increased. Presumably, a sufficient amount of time is needed for the aggregation and rearrangement of adsorbed molecules on the surface. That is, a longer adsorption time allows molecules to aggregate and, therefore, present more open space.

For heparinized silica, the second adsorption slope was greater than the first adsorption cycle, and was enhanced compared to the unheparinized silica. This enhanced adsorption slope may be attributed to fibrinogen being less tightly bound on heparinized silica, enhancing its mobility. Greater mobility would allow more clustering among the adsorbed molecules.

Values from the best fit curves of the adsorbed fibrinogen in the first adsorption cycle (Γ_{ads1}), rinsing cycle (Γ_{rinse}), and the second adsorption cycle (Γ_{ads2}) are reported in Table 4.2. Each value was recorded at the end of each cycle.

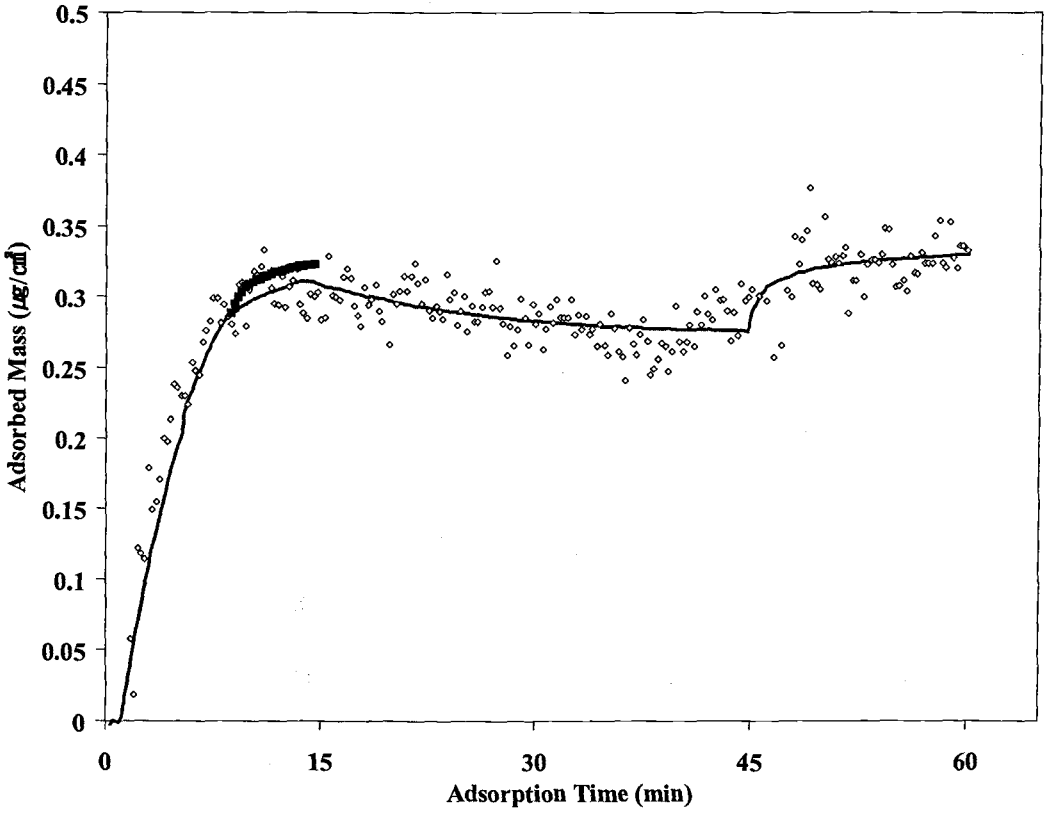


Figure 4.9 Adsorption of fibrinogen at 0.1 mg/ml on unheparinized silica for 15 min showing history dependence ◇ -raw data, — -fitted data, ▬ -compared data

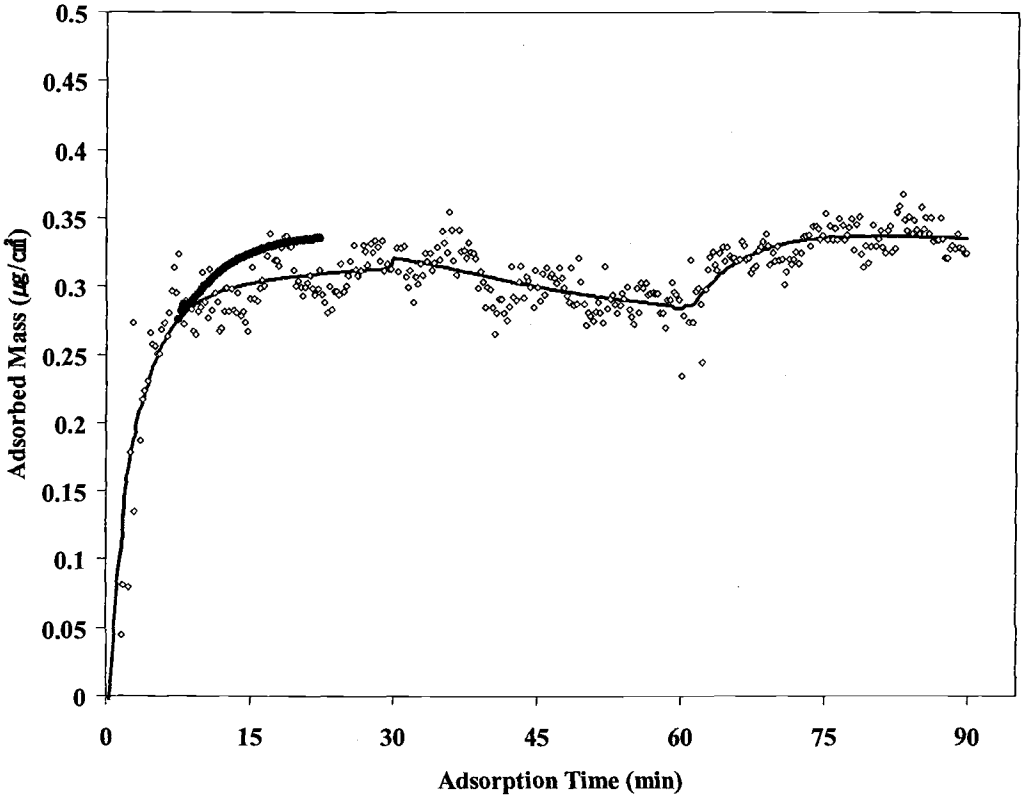


Figure 4.10 Adsorption of fibrinogen at 0.1 mg/ml on unheparinized silica for 30 min showing history dependence ◇ -raw data, — -fitted data, ▬ -compared data

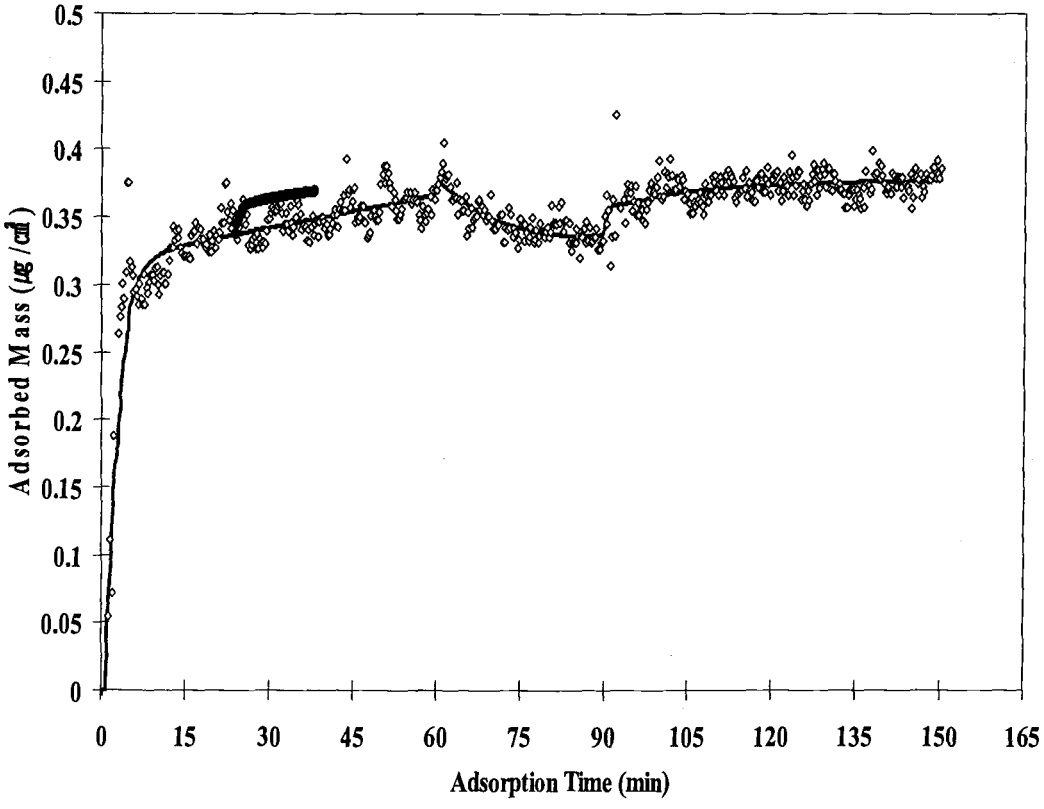


Figure 4.11 Adsorption of fibrinogen at 0.1 mg/ml on unheparinized silica for 60 min showing history dependence ◇ -raw data, — -fitted data, ■ -compared data

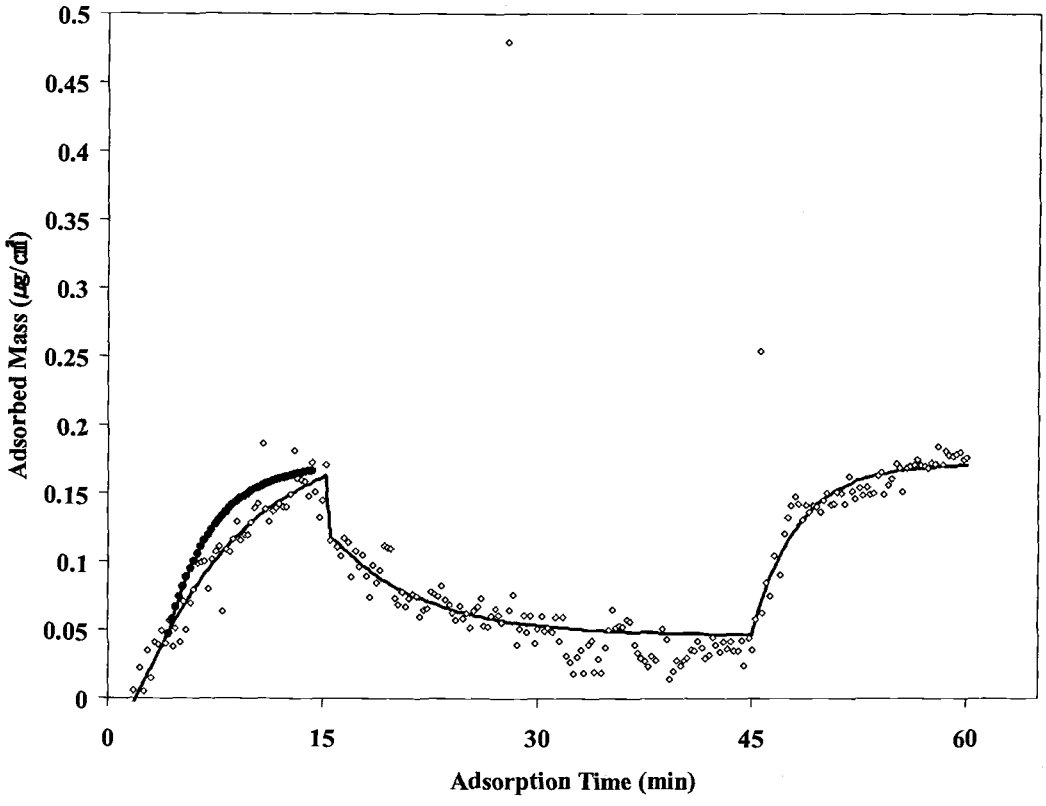


Figure 4.12 Adsorption of fibrinogen at 0.1 mg/ml on heparinized silica for 15 min showing history dependence ◇ -raw data, — -fitted data, ■ -compared data

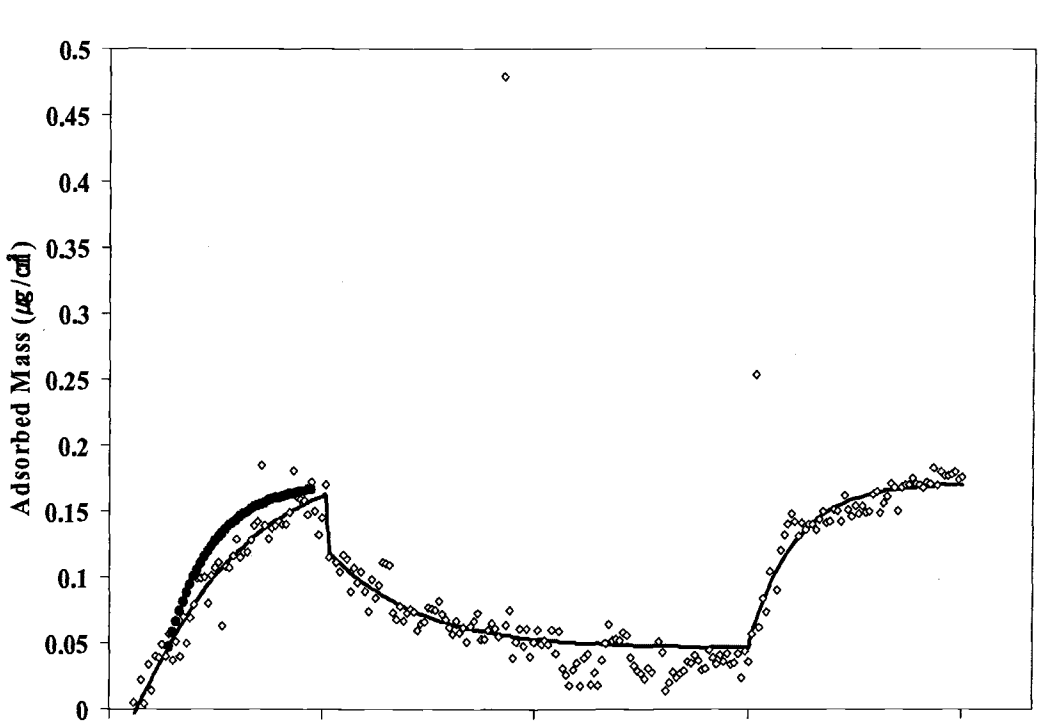


Figure 4.13 Adsorption of fibrinogen at 0.1 mg/ml on heparinized silica for 30 min showing history dependence ◇ -raw data, — -fitted data, ■ -compared data

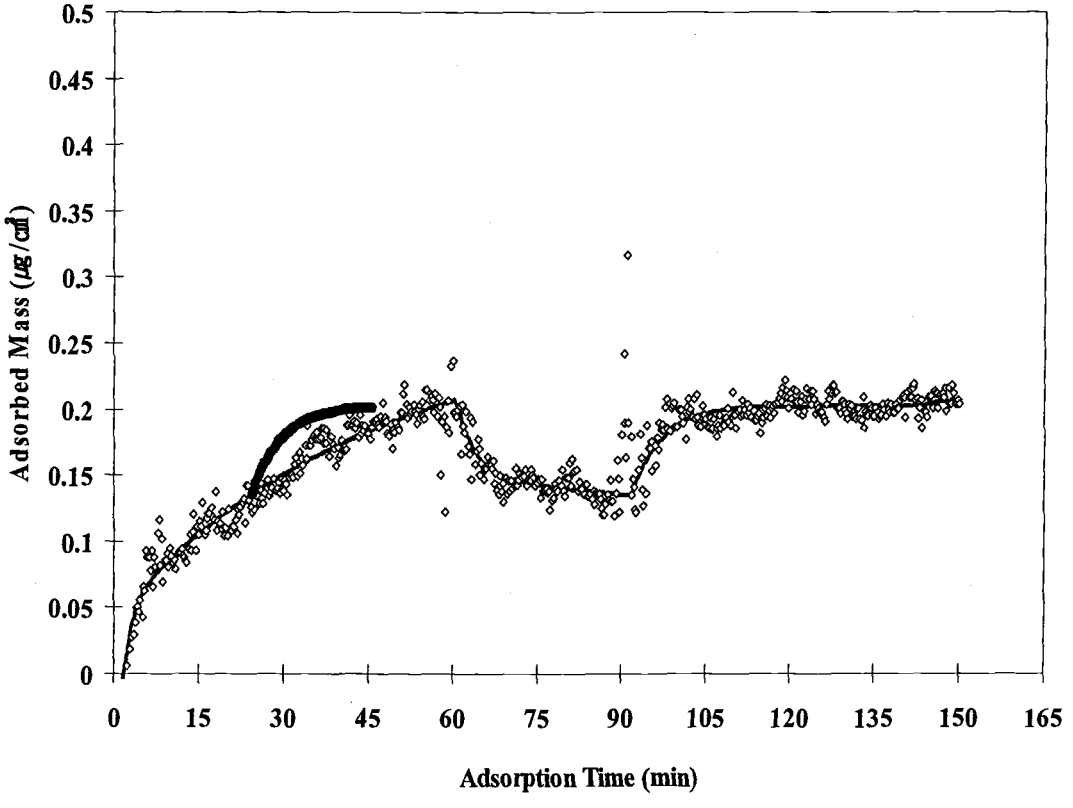


Figure 4.14 Adsorption of fibrinogen at 0.1 mg/ml on heparinized silica for 60 min showing history dependence ◇ -raw data, — -fitted data, ■ -compared data

More fibrinogen adsorbed as adsorption time increased. Formation of the fibrinogen film was apparently complete only after an adequate amount of time had passed. But on the heparinized silica, no plateau could be seen on the first adsorption step suggesting the fibrinogen film had not fully developed. This implies that the heparin on the silica is hindering fibrinogen adsorption.

Table 4.2 Values of the adsorbed amount of fibrinogen for each step

Ads Time (min)	Unheparinized			Heparinized		
	Γ_{ads1} ($\mu g/cm^2$)	Γ_{rinse} ($\mu g/cm^2$)	Γ_{ads2} ($\mu g/cm^2$)	Γ_{ads1} ($\mu g/cm^2$)	Γ_{rinse} ($\mu g/cm^2$)	Γ_{ads2} ($\mu g/cm^2$)
15	0.311	0.276	0.330	0.132	0.024	0.178
30	0.318	0.284	0.337	0.166	0.097	0.206
60	0.377	0.337	0.377	0.206	0.135	0.206

4.2.3 Surface Comparison

Comparisons of the adsorption kinetics of fibrinogen at 0.1 mg/ml on the unheparinized and heparinized silica for the adsorption times of 15, 30 and 60 min are shown in Figs 4.15 to 4.17. Proteins tend to adsorb less on hydrophilic surfaces. Less fibrinogen adsorbed to the heparinized silica than on the unheparinized silica. But from our contact angle data,

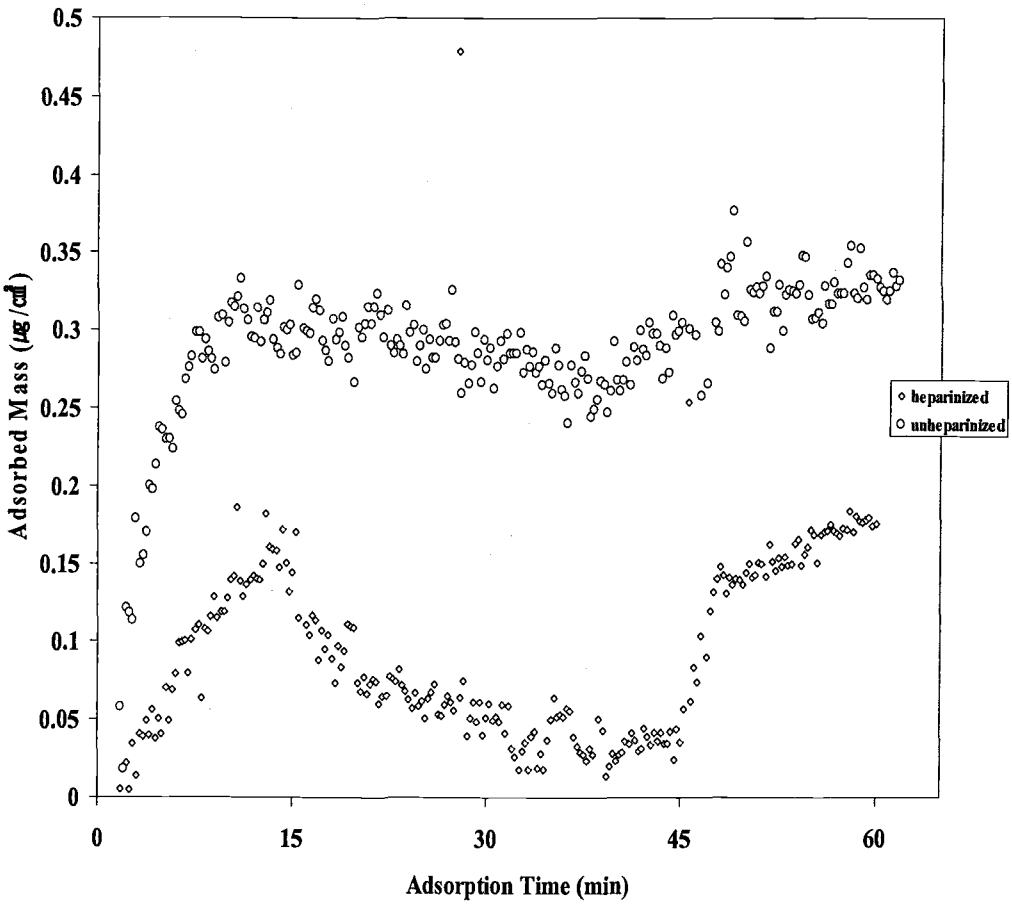


Figure 4.15 Comparison of fibrinogen adsorption at 0.1 mg/mL on heparinized and unheparinized silica for 15 min

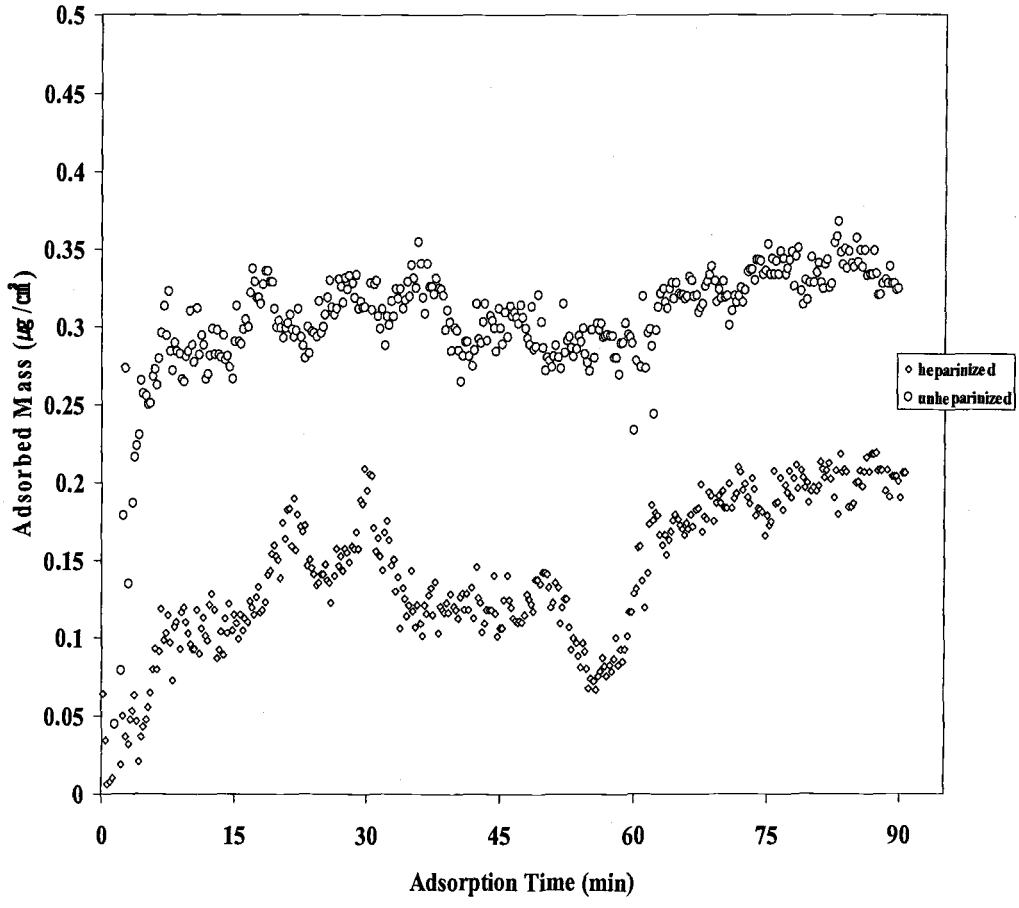


Figure 4.16 Comparison of fibrinogen adsorption at 0.1 mg/mL on heparinized and unheparinized silica for 30 min

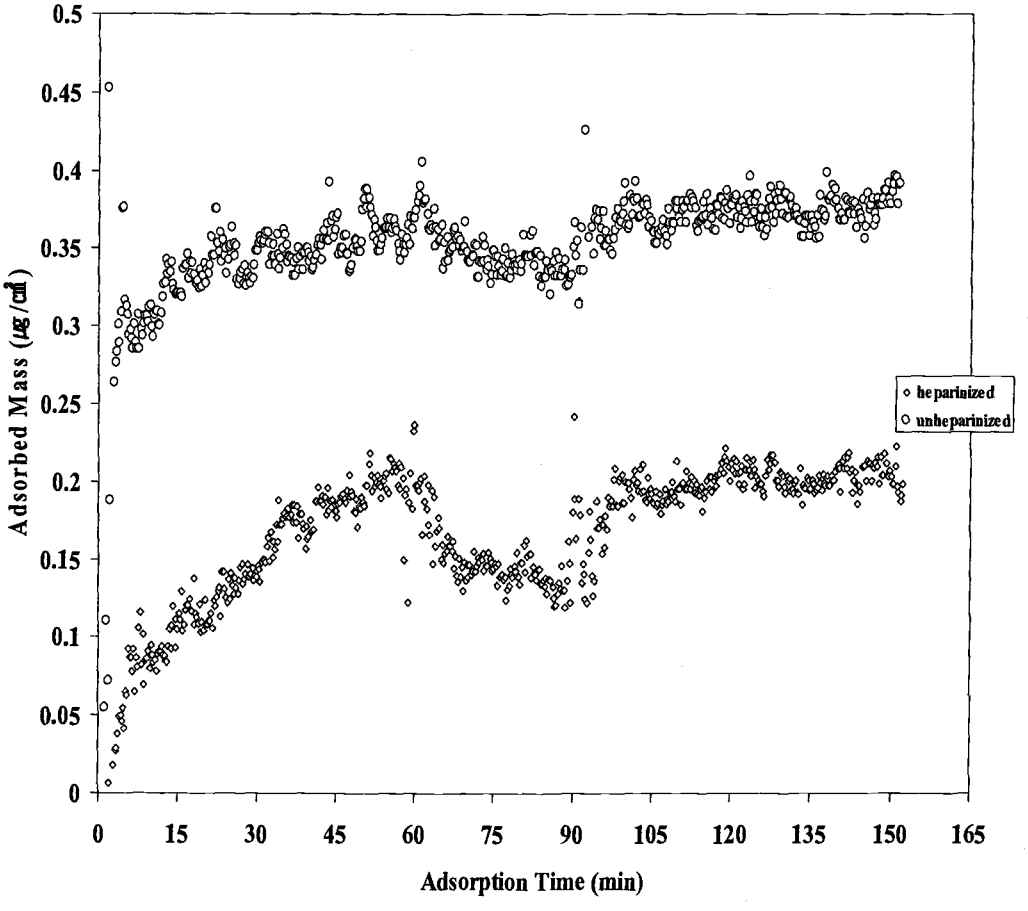


Figure 4.17 Comparison of fibrinogen adsorption at 0.1 mg/mL on heparinized and unheparinized silica for 60 min

unheparinized silica was observed to be more hydrophilic. Apparently the immobilization of heparin has a steric repulsion effect on the fibrinogen causing less adsorption at the surface. The rate of adsorption on heparinized silica is less than on the unheparinized silica as well suggesting that the immobilized heparin on the silica hinders the adsorption of fibrinogen. Table 4.3 shows the fraction of fibrinogen eluted. More of the fibrinogen was elutable from the heparinized surface than from the unheparinized surface. These values could be used as an estimate of how tightly fibrinogen is bound to the surface (Bohnert and Horbett, 1986). Thus, it is reasonable to assume that the adsorbed fibrinogen is not as tightly bound to the heparinized silica as it is to the unheparinized silica.

Table 4.3 Fraction of fibrinogen rinsable from unheparinized and heparinized surfaces

Adsorption Time (min)	Fraction Rinsable $\left(\frac{\Gamma_{ads1} - \Gamma_{rinse}}{\Gamma_{ads1}}\right)$	
	Unheparinized	Heparinized
15	0.112	0.821
30	0.109	0.418
60	0.106	0.344

4.3 Mass transfer

Transport-limited adsorption (i.e. diffusion controlled) was considered where there is no significant energy barrier to adsorption at the interface. The adsorption process may be diffusion controlled and can be tested using the approach described by McGuire (2002).

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

where

Γ : adsorbed mass per square area

C_{bulk} : bulk concentration of protein solution

D : diffusion coefficient of protein

t : time of adsorption

The diffusion coefficient for fibrinogen was reported to be 0.91 × 10^{-7} cm^2/sec by Gorman et al. (1971). Fig 4.18 is a plot of the amount of fibrinogen adsorbed, which was calculated assuming diffusion control, against time. These calculated data compared with experimental data of fibrinogen adsorbed to heparinized and unheparinized silica. Since the initial adsorption data was nearly the same for each time period, the first 15 minutes were studied for each surface. The diffusion control rate is much

faster than the experimental data rates. If the adsorption studies were diffusion controlled, we would expect more fibrinogen to adsorb to the surface. Based on this, it can be concluded that the adsorption studies on the heparinized and unheparinized silica were not diffusion controlled.

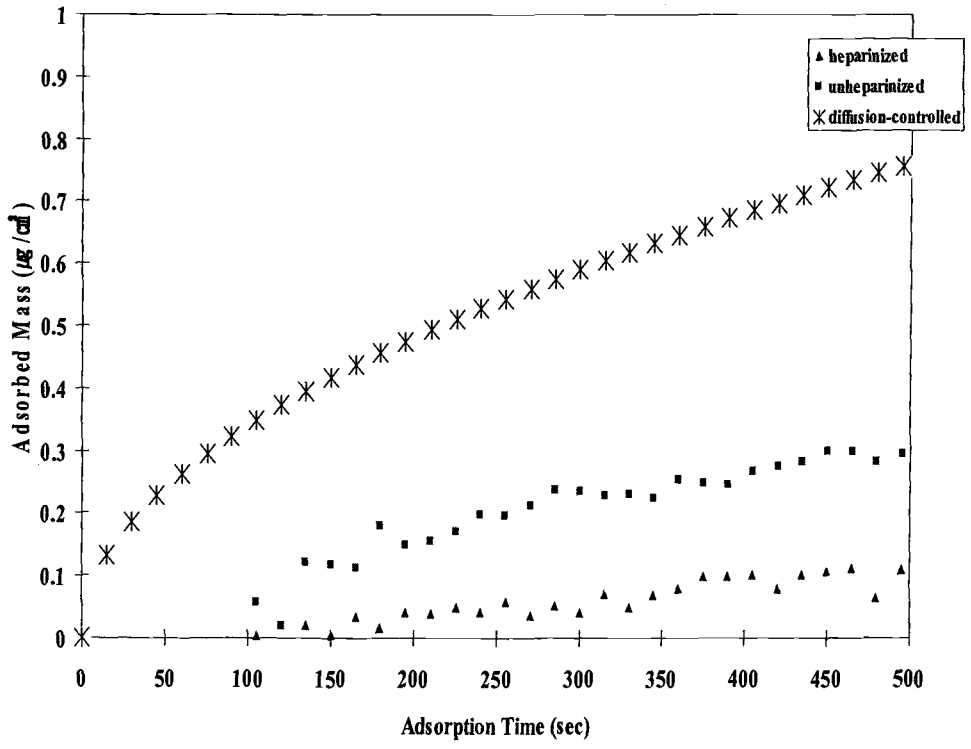


Figure 4.18 Comparison of diffusion controlled data and experimental data at fibrinogen concentration at 0.1 mg/mL

CHAPTER 5

CONCLUSIONS

Heparin was chemically modified and immobilized to surface activated silica. Contact angle measurements and XPS analysis verified successful heparin immobilization. Fibrinogen adsorption on unheparinized and heparinized silica was measured using ellipsometry. The adsorption data was analyzed with respect to a history dependent kinetic model. The results indicated a greater rate for the second adsorption step. Based on this, it may be concluded that longer adsorption times lead to more structural transitions. The effect of history dependence was more pronounced on the heparinized silica, which may be attributed to the weaker binding of fibrinogen, thus enhancing its mobility. This may be due to the immobilized heparin sterically hindering the fibrinogen. It can be concluded that immobilized heparin might enhance the biocompatibility of the surface.

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APPENDICES

APPENDIX A

Fitting data by least squares

Least squares estimation is used to calculate the values of the coefficients in a model from the experimental data. It is applied by minimizing the sum of the squares of the error between the predicted and the experimental values of the dependent variable for each data point. Kinetic data were fit to the following equation:

$$\Gamma = A + Be^{-k_1 t} + Ce^{-k_2 t}$$

where Γ is the adsorbed mass of protein ($\mu g/cm^2$) at time t (min), and A,B,C, k_1,k_2 are coefficients related to the final value of adsorbed mass. The data was divided into three parts, first adsorption step, rinsing step, and the second adsorption step. Each section was fitted with the equation above and was assumed to start from zero time. The function 'fminsearch' in MATLAB was used to generate the best fit curves. The MATLAB program used is shown in the following pages.

Program to fit ellipsometry data using the model

```
%A + B exp(-k1*t) + Cexp(-k2*t)
format compact;
clear all;

%reading experimental data cycle from Excel file
edata1 = xlsread('15_third_hep.xls');
t = edata1(:,1);
ads = edata1(:,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
options=optimset;
options=optimset(options,'MaxFunEvals',100000,'MaxIter',100000);
[search,sigma] = fminsearch('fitdata_fn1',[A,B,C,k1,k2],options,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 (min)');
ylabel('adsorbed mass microgram/sq cm');
```

```
%function c_mid_1st_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

NMR analysis of modified heparin

Nuclear Magnetic Resonance (NMR) was used to identify adipic dihydrazide-modified heparin and quantify the percentage of modification. The NMR analysis was performed at the facility in the Chemistry department at Oregon State University. Due to lack of expertise in interpreting the NMR spectra, the experiments were not pursued further. Some representative spectra were obtained by using deuterium oxide (Cambridge Isotope Laboratories, Andover MA). The reader should not assume these data to be accurate.

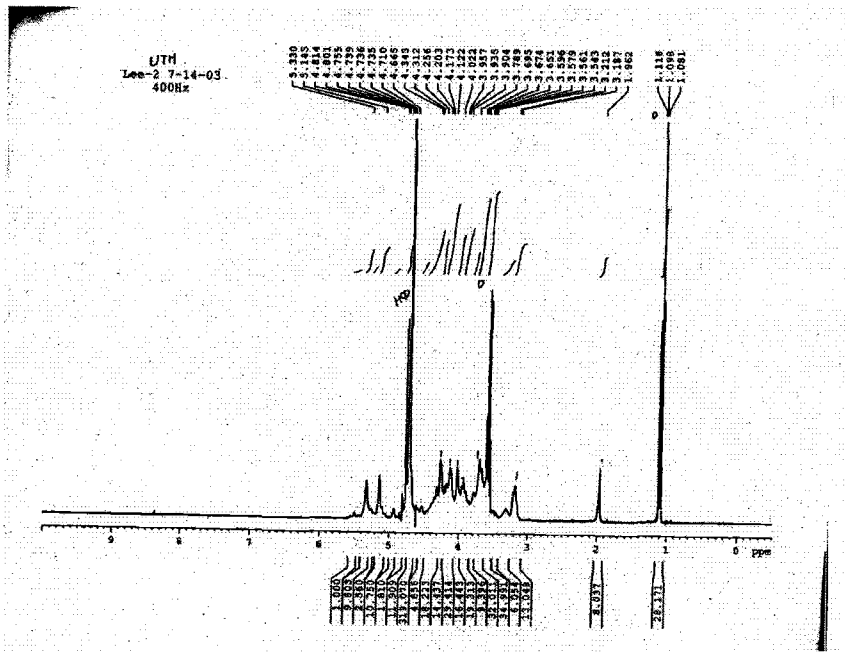

Figure B.1 NMR spectra of unmodified heparin

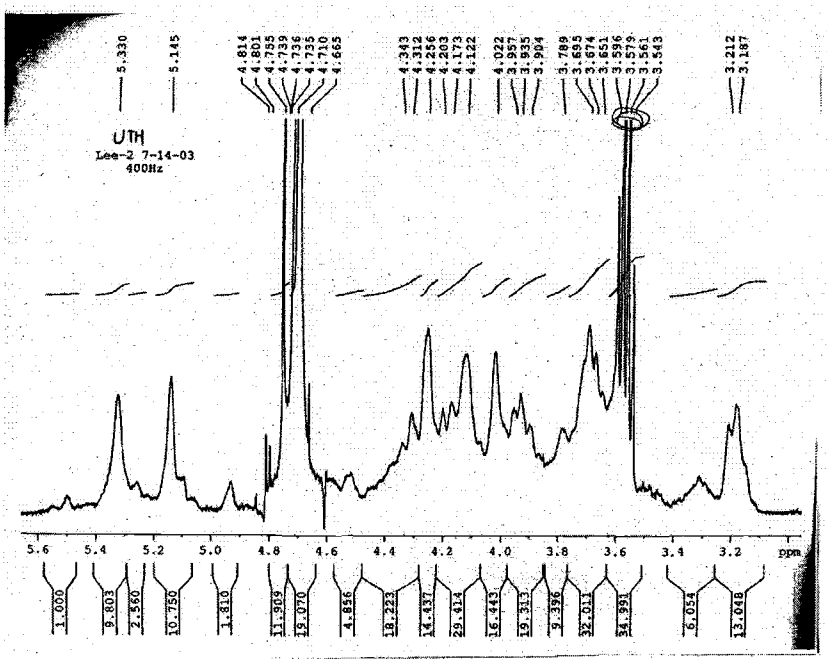

Figure B.2 Expansion of the NMR spectra of unmodified heparin between 3.0 and 5.6 ppm

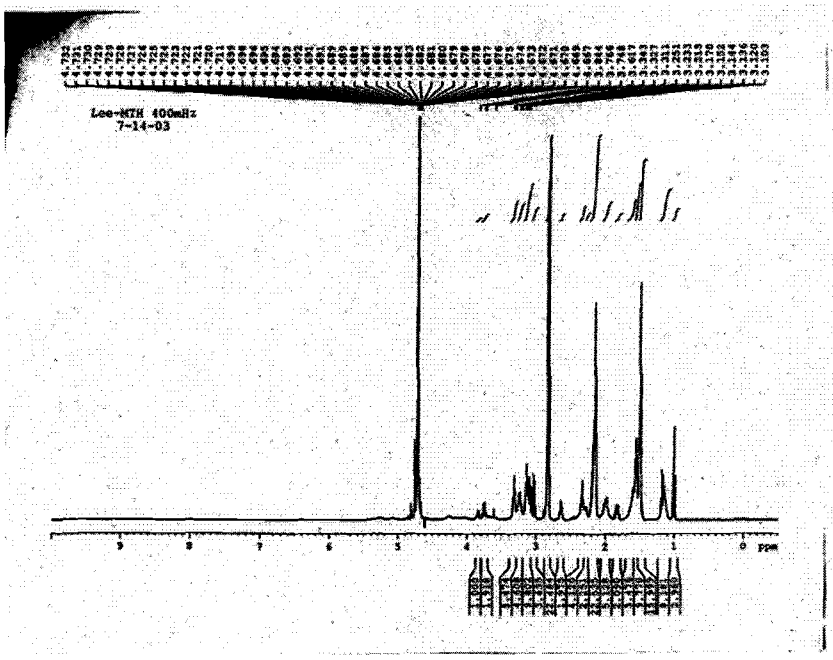

Figure B.3 NMR spectra of modified heparin

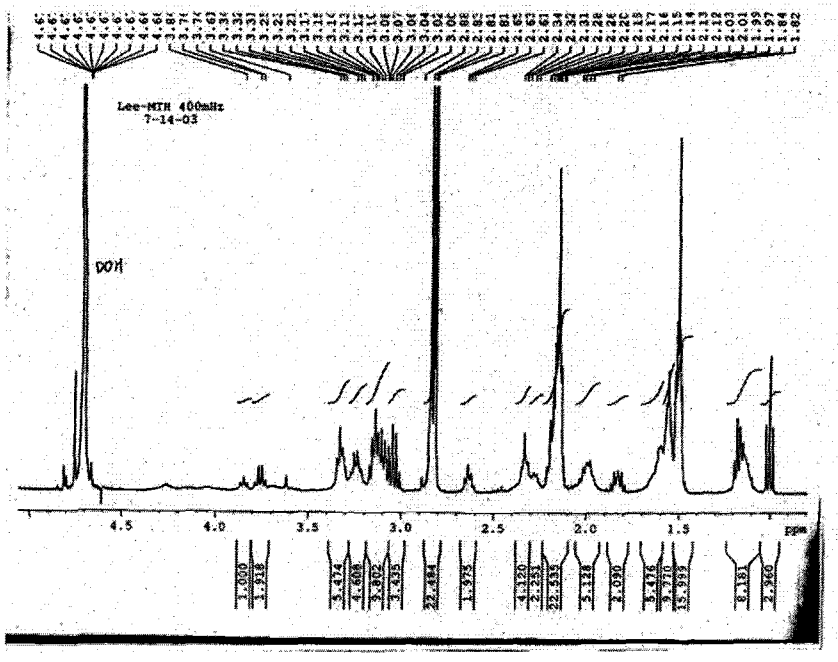

Figure B.4 Expansion of the NMR spectra of modified heparin between1.0 and 5.0 ppm

APPENDIX C

Toluidine Blue Assay: Detection of immobilized heparin

The toluidine blue assay was based on a method to detect and quantify covalently immobilized heparin described by Smith et al. (1980). Toluidine blue which is a metachromatic dye changes color after binding to a polyanionic substrate. Decrease in absorbance would indicate more binding of the substrate.

Twenty five mg of toluidine blue (SPI Chem) was dissolved in 500 mL 0.01 N HCl containing 0.2% NaCl. A standardization curve was generated with 10 mg of adipic dihydrazide-modified heparin dissolved in 100 mL 0.2% NaCl solution. Two and one half mL of toluidine blue solution were pipetted into test tubes. Heparin amounts ranged from 10 μg to 50 μg for the standard curve.

The samples consisted of unheparinized, linkers only, heparinized silica plates. Unheparinized and linkers only silica plates were used as controls to compare with heparinized silica. Each piece was cut into approximately $2cm^2$. They were put into 2.5 mL of toluidine blue solution, as done with the standards. The blank control, which did not contain either the heparin standards or the silica pieces, was analyzed as well. The total volume for the standards, controls, and the samples were made up to be 5 mL by adding 0.2 % NaCl. Then each test tube was agitated using a

vortex mixer for 30 sec. Five mL of hexane was added and then agitated again for 30 sec. Six tenth of a mL of the aqueous layer, which is the bottom layer, was sampled and diluted with 3 mL 100% ethanol. Absorbance was read at 631nm.

Results of the experiments are recorded in this table below. The amount of heparin detected was divided by the area of the silica piece.

Table C.1 Heparin amount detected by toluidine blue assay

Experiment Date	**Immobilized heparin ($\mu g/cm^2$)**
March 15, 2004	9.47
March 16, 2004	3.41
March 26, 2004 (1)	0.48
March 26, 2004 (2)	1.5
March 26, 2004 (3)	5.56

The heparin amount detected by the toluidine assay varied from 0.5 to10 $\mu g/cm^2$. The controls, which are unheparinized and linkers only silica plates, gave similar values of as those obtained at the lower end of the amount of heparin detected. It was concluded that heparin attachment was detected but this assay was not sensitive enough to give an accurate assessment of the heparin immobilized. For future work, it is suggested

that the range of heparin standards be from 0 μg to 10 μg. This might help in reducing the variation of heparin being detected.