Synthesis and Anticoagulant Function of Heparin Containing Block Copolymers on Polystyrene Microspheres

by
Allyson Kaye Fry

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

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Allyson Kaye Fry for the degree of Master of Science in Chemical Engineering
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Abstract approved:

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Joseph McGuire

Contact of blood with the surfaces of synthetic materials is associated with spontaneous protein adsorption, initiating platelet aggregation, the coagulation cascade, and the eventual development of a stable clot. Current therapy to inhibit implant-induced thrombosis is life-long administration of systemic anticoagulants. An alternative to the systemic administration of anticoagulant drugs is to attach a functional anticoagulant agent to the implant surface, thus imparting site-specific activity. Unfractionated heparin (UFH) and an end-aminated form of UFH (HepNH2) were reacted with 2-iminothiolane (2-IT), producing free thiol groups at the sites of internal and terminal amines. Thiolation was quantified using Ellman's assay and o-phthalaldehyde. Thiolated heparin retained anticoagulant activity as shown by the activated partial thromboplastin time (APTT) and anti-Factor Xa (anti-FXa) assays. Surface immobilized, pyridyl disulfide-activated polyethylene oxide chains were used as tethers to attach heparin "end-on" to 1.15 μm polystyrene microspheres. Spectroscopic monitoring of the progress of the reaction indicated that similar amounts of UFH and HepNH2 were attached to the microspheres. The APTT assay showed no anticoagulant activity on heparinized microspheres, due either to the presence of an insufficient amount of immobilized heparin, or to steric constraints

inhibiting the formation of a functional heparin-antihrombin complex. However, immobilized heparin did retain anti-FXa activity, with significantly greater activity being recorded at surfaces treated with thiolated HepNH2 than those treated with thiolated UFH.

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APPROVED:

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Chair of the School of Chemical, Biological, and Environmental Engineering

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Dean of the Graduate School

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.

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Allyson Kaye Fry, Author

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CHAPTER 1
INTRODUCTION

Biomedical materials are used in a variety of devices from contact lenses and catheters to synthetic joints and organs. These materials have obvious benefits for patients and are major contributors in the advance of modern medicine. However, materials used for blood contact in particular are not without complications and require improvement before they can be deemed fully compatible with the human body.

Contact of blood with synthetic material surfaces is associated with spontaneous protein adsorption, initiating platelet aggregation, the coagulation cascade, and the eventual development of a stable clot. Thrombus formation on implanted materials is a leading complication resulting in removal of millions of catheters, stents, and other biomedical products each year (Mao et al, 2004, Ratner, 2007). Current therapy to inhibit thrombosis is life-long administration of systemic anticoagulants, such as coumarin derivatives (e.g. warfarin) or intravenous heparin injections. These therapies can result in severe side effects, especially in the case of injury or surgery, since their pharmacological effects are distributed throughout the body and are not limited to the location of greatest concern, the site of the implant.

An alternative to the systemic administration of anticoagulant drugs is to attach an anticoagulant agent to the implant surface, thus imparting site-specific activity. Heparinized coatings have been used in coronary stents, catheters, and extracorporeal devices for some time, but their effectiveness is debatable. Several factors contribute to this outcome. Binding methods that do not consider the orientation and structure of heparin required for functional interaction with the surrounding environment lead to suboptimal performance. Also, due to the highly heterogeneous chemical composition of heparin, improved characterization and control of chemical modification and binding procedures are needed in order to ensure retention of function upon immobilization.

The purpose of this study was to synthesize a thiolated heparin that could be attached "end-on" to surface immobilized pyridyl disulfide-activated polyethylene oxide (PEO) chains, and evaluate retention of anticoagulant activity in the immobilized form. Two types of heparin were chemically modified to include thiol groups at the sites of primary amines: unfractionated heparin (UFH) and an end-aminated form of UFH (HepNH2). The reaction was designed such that the amine group located at the end of the HepNH2 chain would be modified to a greater extent than internal amines. Heparin composition and activity was monitored before and after thiolation. Thiolated heparin was then covalently linked to polystyrene microspheres coated with pendant, end-activated PEO chains, thereby producing a heparinized surface. Anticoagulant activity was determined before and after surface attachment.

CHAPTER 2
LITERATURE REVIEW

2.1Heparin structure and function

Heparin is the most widely used anticoagulant today. It is a glycosaminoglycan composed of alternating 1→4 linked iduronic acid and glucosamine monosaccharide subunits. Heparin is very highly sulfated (Fig. 1), making it the highest negatively charged non-synthetic molecule. It is synthesized as a proteoglycan with many polysaccharide chains extending outward from a protein backbone. The chemical makeup is highly heterogenous, varying especially among species and tissues. The molecular weight for unfractionated heparin (UFH) varies from 4-40 kDa and has an average of 2.7 sulfate groups per disaccharide (Islam and Linhardt, 2003, Capila and Linhardt, 2002).

Heparin's anticoagulant activity is due to an interaction with antithrombin (AT) that is mediated by a specific pentasaccharide sequence (Fig. 2). Antithrombin is a serine protease inhibitor (serpin) that acts on many members of the protein coagulation cascade (Fig. 3). This cascade is a series of enzymatic reactions that concludes in the conversion of fibrinogen to fibrin, which can then cross-link and form a stable clot. It is initiated by contact with negatively charged surfaces such as the extracellular matrix (intrinsic pathway) or by cellular injury (extrinsic pathway). These pathways converge into a common pathway at the activation of Factor X (FXa). The cascade actually is quite complex, composed of feedback inhibition and activation, cofactors, and complex binding interactions, and would be described more completely as a meshwork. Antithrombin is most commonly known for inhibiting thrombin; however, it also has the ability to inhibit FVa, FIXa, FXa, FXIa, and FXIIa, as well as other proteins in the coagulation cascade. For thrombin inhibition to take place, the heparin chain must be long enough to complex with both AT and thrombin. For inhibition of other factors, binding with AT only is sufficient. Therefore, the pharmacological effect of heparin varies by chain length (Walker and Royston, 2002).

It is interesting to note that heparin's primary pharmaceutical use is most likely not its primary role in the body. In the body heparin is synthesized and stored in mast cells and released from the cell during certain types of immune responses. Mast cells are found in close proximity to blood vessels in areas that are exposed to the external environment, such as the intestine and respiratory tract. In fact, bovine lung and porcine intestinal mucosa are the primary sources of heparin for pharmaceutical and laboratory purposes. It is suspected that the primary physiological role is that of an immune and complement regulator since it cannot directly inhibit clot formation and is present in species lacking coagulation systems (Walker and Royston, 2002). A closely related molecule, heparan sulfate (HS), is found abundantly in the extracellular matrix and as components of endothelial cell membranes. While HS actually does play the role of an anticoagulant, it has a substantially decreased activity when compared to heparin (Bourin et al, 1993).

The proteoglycan form of heparin is processed for pharmaceutical use by cleaving away the protein, leaving only the unfractionated polysaccharide chain. Three enzymes can act on heparin: heparinase, heparin lyase, and heparatinase (Ampofo et al, 1991). Partial depolymerization can also occur by oxidation or reduction of glycosidic bonds, or by treating with hydrochloric acid, which degrades the chain into smaller fragments making low molecular weight heparin (LMWH). Because of its smaller size of 3-12 kDa, LMWH has a reduced affinity for nonspecific interactions with positively charged species, allowing it to remain active and in circulation for longer. Low molecular weight heparin can cross membranes with greater ease and has a longer plasma half-life, leading to a greater availability in the body for a pharmacological effect, but it is less likely to contain the pentasaccharide. Even shorter varieties can be synthesized that are composed of only the pentasaccharide sequence (fondaparinux), which has even higher bioavailability and very predictable pharmacokinetics. Varieties shorter than 18 monosaccharides affect coagulation primarily by inhibition of FXa (Dinwoodey and Ansell 2006, Hirsh et al 1998).

Differential centrifugation and chromatography methods have shown that around a third of heparin molecules bind to AT while the other two thirds do not. In fact, only 30% of heparin molecules contain the AT pentasaccharide. A sample of heparin with a specific activity of 155 U/mg, is actually a combination of high affinity heparin (348-388 U/mg) and low affinity heparin (19-52 U/mg). High affinity fractions can be isolated to produce heparin with higher anticoagulant activity heparin (Petitou et al, 2003).

2.2 Protein adsorption and triblock copolymers

Blood clotting is only one step in hemostasis. When injury occurs to a vessel, the surrounding musculature is triggered immediately to constrict, limiting blood flow to the area to decrease blood loss. The second step is activation of platelets by binding to collagen, the major component of the extracellular matrix, a process that is facilitated by von Willebrand factor. With the aid of fibrinogen, platelets aggregate and form a plug over the site of injury. The final step is the process of coagulation described previously, which forms a stronger patch, allowing the body time to permanently repair the damage (Minors, 2007).

Platelet aggregation and coagulation can also be activated whenever a foreign material is in contact with blood. Upon insertion into the blood stream, any material will instantaneously adsorb a non-specific layer of proteins onto the surface 1-10 nm in thickness. Proteins are naturally surface active agents that will undergo conformational unfolding and spread along the surface to decrease the interfacial energy. It is this configuration change that causes failure of an implant by activating coagulation, the immune system, and platelets, and creating a site for clot formation and adherence of infectious bacteria (Castner and Ratner, 2002).

One strategy to increase hemocompatibility is to chemically alter the surface of the implant to prevent this initial protein adsorption. Triblock copolymers (Pluronic®) are a class of surfactants composed of a core hydrophobic polypropylene oxide (PPO) chain flanked by hydrophilic polyethylene oxide (PEO) chains (Fig. 4). The length of the polymer chains can be varied, altering the characteristics and capabilities of the

construct. Li et al (1994) found that the polymers, when adsorbed onto polystyrene surfaces, displayed PEO chain mobility that was directly dependent on length. The higher mobility of the chains, the greater the ability to sterically repel proteins from adsorbing to the surface. Pluronic® F108 has been shown to have the greatest protein repulsion capability of its class due to its long, highly mobile PEO chains composed of 141 subunits. It also has the greatest surface adherence to common hydrophobic biomaterials due to the length of the PPO chain, 44 subunits, which allows the copolymer to make strong hydrophobic associations (Li et al, 1996). In this study, we use 1.15 μm polystyrene (PS) microspheres as the hydrophobic surface for triblock adsorption.

In this study we use a modified form of F108 called end-group activated Pluronic® (EGAP) that has pyridyl disulfide (PDS) groups at the free ends of the PEO chains. Modifying a surface with an anticoagulant theoretically could prevent clot formation at the interface without producing an unnecessary systemic effect. Positioning heparin at the end of the PEO chains offers the heparin pentasaccharide greater access to the surrounding environment than if bound directly to a surface. Triblocks, therefore, provide a tether arm that decreases steric interference while increasing molecular interactions and, possibly, the activity of the molecule being tethered.

EGAP offers an additional opportunity to increase the biocompatibility of a surface. The terminal disulfide chemistry provides a relatively simple method to covalently link molecules with a specific function to the surface (Fig. 5). Thiolated heparin covalently binds to triblock copolymers by displacement of PDS groups. The method chosen in this study, discussed later, allows internal and terminal thiolation, and thus two different orientations of surface attachment (Fig. 6).

Neff et al (1997 and 1999) covalently linked cell adhesion proteins to EGAP in order to control cell attachment to a polystyrene surface. Andersson et al (2005) used EGAP to immobilize factor H to the surface to reduce complement activation, and reported that even though an EGAP coated surface did not activate coagulation, the coating increased complement activation. Covalently-linked factor H did not detach

from the copolymer, and bound in an active conformation that could decrease complement activation.

2.3 Immobilized heparin

Goddard and Hotchkiss (2007) offer a comprehensive review of modern polymer surface conjugation techniques and preparations, as well as common analysis methods. Over a dozen distinctly different heparin immobilization methods can be found in the literature. They include nonspecific, ionic, and covalent binding techniques, forming heparinized surfaces with a wide repertoire of orientations, capabilities, and characteristics.

Nonspecific heparin coatings can be achieved by covalently binding heparin to a large protein such as albumin or fibrinogen. The protein then preferentially adsorbs onto the surface, forcing heparin to be located there as well. These types of coatings are designed to be simple, non-time consuming methods for binding or biochemical interaction studies. Very little control over the surface orientation exists, and stability with time is not a concern (Mahoney et al, 2004). Heparin can also be embedded in a polymer for a time-release effect (Chung et al, 2006).

Nguyen et al (2003) bound heparin to the surface of ionically associated polyethyleneimine coated membranes, which gave the membrane a high positive charge. The negatively charged heparin would then be ionically attracted to the highly positively charged surface. Although standard clotting time assays showed that the surface had no anticoagulant activity, anion-cation staining procedures showed that there was, in fact, heparin bound to the surface. Though ionic coatings are appealing, given the simplicity of the method and charged nature of heparin, such procedures are rarely found in the literature, because they are consistently ineffective.

Covalent attachment of heparin to a surface is performed by modification at hydroxyl, amine, or carboxyl groups within the polysaccharide. The modification is performed to create functional groups that are reactive with that of the surface, usually a polymer or silica.

Marconi et al (1997) used adipoil chloride and hexamethylene diisocyanate to couple hydroxyl groups within heparin to hydroxyl groups of an ethylene-vinyl alcohol copolymer. They reported that surfaces containing lower amounts of heparin were found to have more anticoagulant activity, and attributed this to lower steric hindrance. A decrease in steric hindrance increasing the activity of the surface is supported by the evidence that activity is increased by placing a spacer arm between the surface and heparin. Formamide can also be used to covalently link heparin through hydroxyl groups. (Byun et al, 1996, Nadkarni et al, 1997).

Numerous studies used *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC, WSC, or EDAC) to link carboxyl groups of heparin with aminated surfaces (Che et al, 2005, Lin et al, 2004, Wissink et al, 2001, Wang et al, 2005) or conversely, to react amine groups of heparin with carboxylated surfaces (Kung et al, 2006). Reaction of EDC with a carboxyl group forms an unstable intermediate that is readily hydrolyzed. The efficiency of EDC modification is often increased with the use of N-hydroxysuccinimide (NHS) or N-hydroxysulfosuccinimide (Sulfo-NHS), which reacts with the unstable EDC intermediate to form a semi-stable amine reactive ester. Each reported successful immobilization using dye techniques or x-ray photoelectron spectroscopy (XPS). The heparinized surfaces affected the coagulation cascade by increasing clotting times, while also decreasing platelet and protein adhesion to the surface.

Wissink et al (2001) used EDC and NHS to crosslink collagen to a polymer surface, afterward using the various molar ratios of EDC to carboxylated heparin to covalently link heparin to the collagen matrix. Though results from thrombin inhibition assays report significant anticoagulant activity of all surfaces, significantly greater activity was reported for surfaces prepared with a lower EDC to heparin ratio. Since heparin contains a low amount of amine groups, reaction with EDC can theoretically link heparin molecules to each other and not just to the surface, forming a meshwork. Heparin binding sites then become less accessible to the surrounding environment.

Joshi et al (2006) used adipic dihydrazide (ADH) to covalently link the carboxylate groups of heparin to carboxylated silica surfaces. Though the anticoagulant activity of ADH reacted heparin at the surface was never determined, anti-FXa assays performed on ADH modified heparin in solution showed decreased activity compared to unmodified heparin, presumably because of covalent links formed between heparin by ADH. Crosslinking of heparin limits the activity of the surface by decreasing accessibility. Accessibility is also a concern anytime heparin is anchored by internal functional groups. Therefore, many coating techniques have been designed to mimic HS endothelial orientation by employing end-point attachment chemistries.

Biotin is bound to streptavidin into a complex by one of the strongest noncovalent associations known, making the association, for practical purposes, irreversible. This chemistry has been exploited for many applications, including immunoassays and chromatography. Nardella et al (2004) used biotinylated heparin for end-point attachment to streptavidin coated microplates. Tseng et al (2006) used the biotin-streptavidin complex to end-point attach heparin to membrane-mimetic thin films subjected to physiologically relevant flow regimes. The coated surfaces were not only stable, but could functionally interact with the environment even under shear stress.

Another method used to end-point attach heparin is reductive amination (Bjorklund et al, 1997, Denizli, 1999). This technique requires that a terminal reducing sugar be available for reaction. Since heparin is synthesized on a protein backbone, pharmaceutical processing sometimes leaves fragments of the amino acid linkage to the end of the polysaccharide chain. Partial depolymerization with strong acid can be use to expose the reducing end, which can then be reacted with sodium cyanoborohydride ($NaCNBH_3$) to create a terminal amine group (Larm et al, 1983). Since most internal amine groups are sulfated or acetylated at the amine position (Comper, 1981, Toida et al, 1997, Ampofo et al, 1991), the unique terminal chemistry can be used to covalently bind heparin to a surface.

Nadkarni et al (1994) created three different functional groups on the reducing end of heparin, an amine, a hydrazide, and an ester, to end-point attach heparin to

sepharose columns. Protamine was used as a probe to assess binding site availability. It was reported that columns with end-point attached heparin displayed an increased amount of binding sites compared to commercial sepharose columns. Commercial columns bind heparin non-directionally, most likely by internal functional groups and/or multiple linkages between heparin and the column. Also, end-modification of heparin that introduced a spacer arm bound relatively more protamine than heparin attached to the sepharose column with shorter or no spacer arm, presumably from decreased steric inhibition.

Osmond et al (2002) used a BIAcore surface plasmon resonance biosensor to analyze the difference in protein-heparin interactions between heparin anchored through internal or terminal functional groups. Heparin was biotinylated via carboxylate groups, internal amines, and at reductively aminated termini. To measure binding of proteins, avidin, lactoferrin, antithrombin, and thrombin were injected over streptavidin coated sensor chips after exposure to biotinylated heparin. The surface with the highest binding affinity was that with heparin attached by the reducing end, followed by internal amines. Heparin attached by carboxylated groups had the lowest affinity. Though the difference in modification chemistry would have some influence on results, this study is very supportive of the theory that orientation and accessibility plays a critical role in the effectiveness and activity of heparinized surfaces.

2.4 Heparinized surfaces in clinical practice

Wendel and Ziemer (1999) review heparin coatings used in extracorporeal devices, those used to circulate blood outside of the body during bypass surgeries. The authors note that since the circulating blood is in contact with around 3 m^2, it is extremely important for the surface to be hemocompatible to prevent massive activation of coagulation, platelets, and immune activity. At the time of publication, four heparinized coatings were approved for clinical use; one ionic, two covalent, and one covalently bound to non-specifically adsorbed peptides.

The Corline Heparin Surface® (CHS) by Corline is prepared by exposing the surface to an unpublished pretreatment and a polymeric amine, after which heparin is

end-point attached with a disulfide bond by reacting with the cross-linker *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP). This results in around 70 nmol of end-point disulfide-bound heparin for each amine polymer bound to the surface. The method was created in cooperation with Rolf Larsson, a Swedish scientist and pioneer in heparinized coatings. Stents coated in this way had significantly less platelet and coagulation activation when compared to uncoated stents (Christensen et al, 2001). However, a 1999 clinical study by Haude et al (2003) found that use of CHS coated stents in small coronary arteries (2.0 to 2.6 mm) showed no significant difference in clinical performance from bare stents. Polydimethylsiloxane (PDMS) channels prepared with CHS significantly increased hemocompatibility and resulted in a surface with hydrophilic properties (Thorslund et al, 2005). Johnell et al (2005) found that a single layer of CHS does not cover the surface completely and that a double layer is needed to create a uniform surface. Kristensen et al (2006) exposed a CHS surface to citrated plasma at continuous high shear rates for three weeks. By measuring the chemical composition with x-ray photoelectron spectroscopy (XPS) before and after exposure it was determined that the surface ability to bind heparin did not decrease after the three week exposure to plasma.

The Carmeda Bioactive Surface® (CBAS) was developed by Swedish scientist Olle Larm, and is now a product of Medtronic. The method begins by partially degrading heparin, exposing reducing ends. The surface is prepared by exposure of heparin to polyethyleneimine. Partially degraded heparin is then covalently bound to the surface by reductive amination. Larm et al (1983) determined only the CBAS, when compared to other ionic and covalent heparinized surfaces, stably bound heparin, completely inhibited thrombin activity, and prevented blood clotting when exposed to whole blood for 30 min. Sanchez et al (1995) used CBAS to determine that UFH bound to antithrombin prevented surface activation of FXII but an identical surface prepared with LMWH could not. Sanchez et al (1996) determined that coagulation inhibition of the heparinized surface is directly related to the density of AT binding sites. Inhibition decreased with increased density with no inhibition on surfaces expressing greater than 4 pmol/cm^2 specific AT binding sites. Heparin

surfaces without AT binding sites, like any other negatively charged surface, promote coagulation. It is suggested that AT catalysis, therefore, overcomes the charged nature of heparin. Videm et al (2004) determined that CBAS also inhibits neutrophil adhesion and proliferation.

Cornelius et al (2003) exposed both the CBAS and CHS to human plasma, and determined that both surfaces adsorbed large amounts of AT with less contact activation and almost no activation of FXII when compared to controls. In comparison to each other, CBAS adsorbs four times as much AT than CHS. Though this indicates that CHS is a less active surface, studies have shown that it is still effective in clinical practice.

2.5 2-Iminothiolane

2-Iminothiolane (Traut's reagent, 2-IT) is a cyclic thioimidate that reacts with primary amines to introduce thiol groups (Fig. 7). The reactivity of Traut's reagent is pH dependent, with a reactive range from pH 7-10. Other amine reactive compounds that introduce thiol groups (N-Succinimidyl-S-acetylthioacetate (SATA) or *N* -Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP)) have a reactive range of pH 7-9. Alkaline conditions are more favorable for amine modifications since they are protonated and unreactive at neutral and acidic conditions. We chose 2-IT as a crosslinker based on the pKa values of the amine groups with the intention of modifying end-amines preferentially over those located internally (Traut et al, 1973).

2-Iminothiolane was originally used to create disulfides in proteins to aid in ribosome structure and interaction determination (Traut et al, 1973, Jue et al, 1978). 2-Iminothiolane has also been used to modifiy polysaccharides. The degree of reaction is dependent on both concentration and pH of solution. The half life at pH 10 is 210 h, making it a suitable chemical for long alkaline incubations. The maximum thiol quantity is reached between one and two hours at room temperature. The decrease in thiol groups present after this time is presumably due to formation of disulfide bonds. It is also known that 2-IT is capable of reacting with hydroxyl and thiol groups, but the reaction time is 100 times slower than the reaction with amines.

Therefore, side reactions can be considered negligible unless reacting overnight or longer (Bernkop-Schnurck et al, 2003, Alagon and King, 1980, Tarentino et al, 1993).

CHAPTER 3
MATERIALS AND METHODS

Unfractionated heparin sodium (UFH) and unfractionated heparin sodium amine (HepNH2), both from porcine intestinal mucosa, were obtained from Celsus Laboratories (Cincinnati, OH). Polystyrene (PS) microspheres with a diameter of 1.15 μm were obtained from Seradyn (Indianapolis, IN). Cysteine-HCl and 5,5'-Dithio-*bis*-(2-nitrobenzoic acid) (DTNB, Ellman's Reagent) are products of Pierce (Rockford, IL). 2-Iminothiolane (2-IT, Traut's reagent), glucosamine 6-sulphate, and o-phthalaldehyde (OPA) were obtained from Sigma-Aldrich (St. Louis, MO). Automated titration equipment with pH probe, drop counter, and software were purchased from MeasureNet Technology (Cincinnati, OH). Univettes, KC1Δ coagulometer, and APTT reagents were obtained from Trinity Biotech (Berkeley Heights, NJ). The Chromogenix COATEST Heparin test kit for the anti-FXa assay was obtained from Diapharma Group (West Chester, OH). Microcon centrifugal filters with molecular weight cut-off of 3000 Da were obtained from Millipore (Billerica, MA). Dithiothreitol (DTT) was obtained from VWR (West Chester, PA). Flat bottom black 96 well polystyrene plates were products of Whatman (Clifton, NJ). All water is distilled-deionized water (DDW) purified by a Barnstead MP-3A system (Dubuque, IA). Pluronic® F108 (triblock copolymer) was a gift from BASF (Florham Park, NJ). End-group activated Pluronic® (EGAP) was a gift from Allvivo Vascular (Lake Forest, CA). Human plasma was harvested from human donors in accordance with Oregon State University Institutional Review Board Guidance.

3.1 Amine characterization

Titrations were performed to determine the pKa of the amine groups present in UFH and HepNH2, and used to determine the optimum pH for the modification of heparin by 2-iminothiolane. Each of the two heparins was dissolved separately in water to a concentration of 1 mg/mL. Two burettes, one filled with 0.1M NaOH, the other with 0.1M HCl, were used to dispense acid or base into a continuously stirred

heparin solution. The MeasureNet automated titration system with pH probe and drop counter was used to record volume and pH data.

3.2 Amine quantification

Amine quantification was carried out with the use of OPA (Parsons et al, 1984). Amine content was determined at pH 10 in 400 mM sodium borate buffer. A buffered OPA solution was created by combining 1 mL of 10 mg/mL OPA in ethanol with 50 μL 2-mercaptoethanol and 20 mL buffer and stored at 4°C for up to two weeks. To perform the assay, 100 μL buffered OPA solution was mixed with 100 μL of analyte dissolved in water. The fluorescence of the solution was recorded at excitation and emission wavelengths of 355 nm and 460 nm, respectively, until maximum intensity was reached. As a standard for heparin, glucosamine 6-sulphate was used for calibration.

3.3 Triblock coating of microspheres

Triblock copolymer coating of PS microspheres was performed according to Li et al (1994). Briefly, polystyrene microspheres were warmed to room temperature and mixed gently. In each of ten 1.5 mL centrifuge tubes, 100 μL of 10% solids stock microspheres and 900 μL water was combined, vortexed, and centrifuged at 13.4 RCF for four minutes. The supernatant was discarded and the wash cycle was repeated twice more using 1 mL water each. A solution of 0.4% EGAP in water was added in 1 mL aliquots to five of the tubes containing washed microsphere pellets. To each of the other five, 1 mL of a 0.4% F108 solution was added. All tubes were incubated by rotating at room temperature for 24 h. After incubation coated microspheres were stored at 4°C for up to two weeks.

3.4 Determination of coating efficiency

The content of triblock adsorbed to the microsphere surface was determined by cleaving the end groups with DTT. Coated microspheres in solution were centrifuged at 13.4 RCF to create a pellet. The supernatant was discarded and replaced with 50

mM DTT in 150mM phosphate buffer at a pH of 7.3 (Neff et al, 1997). The DTT solution was allowed to react 15 min, centrifuged, the supernatant collected, and the absorbance measured at 343 nm. The extinction coefficient ε_{343}=8060 $M^{-1}cm^{-1}$ of PDS in solution was used to determine the concentration of PDS in the supernatant. This concentration was used to calculate the coverage and compared to the theoretical maximum (Li et al, 1994).

3.5 Thiolation of heparin

Heparin was modified to contain thiol groups by reacting with 2-iminothiolane (Fig. 7). Unfractionated and HepNH2 were dissolved in water to a stock concentration of 15 mg/mL. A fresh solution of 2-IT was made by dissolving 10 mg in 1 mL 20 mM sodium borate buffer with 1 mM EDTA at pH 10. In separate vials, 75 µL stock heparin solution was combined with 270 µL concentrated 2-IT and the volume brought up to 1.5 mL with buffer. Water was used in place of heparin solutions as a control solution. Samples were incubated at room temperature on a 600 rpm vortex mixer for 2 h and used immediately.

3.6 Purification of thiolated heparin

Thiolated heparin was purified to remove unreacted 2-IT. Two centrifugal filters were filled to a capacity of 500 µL with each heparin solution. Samples were centrifuged 90 min at 13.4 RCF to a stop volume of 10 µL. Filters were then inverted in new microcentrifuge tubes and centrifuged for three minutes at 0.1 RCF to recover the purified, thiolated heparin. Samples were brought up to 500 µL in 20 mM phosphate buffered saline containing 1mM EDTA and 150 mM NaCl at pH 7.5 for addition to microspheres. Two centrifugal filters for each heparin solution and control blank were filled to a capacity of 250 µL and centrifuged 50 min to a stop volume of 10 µL. One set was brought up to 100 µL in 100 mM phosphate buffer with 1 mM EDTA at pH 8.0 for thiol content determination, while the other set was brought up to 100 µL in water for amine quantification and bioactivity measurements.

3.7 Determination of thiol content

Thiol content of 2-IT reacted heparins was determined using the Ellman assay (Ellman, 1959). Buffer used was 100 mM phosphate buffer with 1 mM EDTA at pH 8.0. Cysteine-HCl was dissolved in buffer to a stock concentration of 10 mM. Standards were made by diluting the stock solution to a range of 0.25-2.0 mM in buffer. In separate microcentrifuge tubes, 90 μL standard or sample were combined with 893 μL buffer and 18 μL of a 4 mg/mL of Ellman's reagent in buffer, freshly made. Samples were mixed by vortexing, reacted for 15 min at room temperature, and the absorbance determined at 412 nm.

3.8 Heparinized microspheres

Heparinized surfaces were produced by reacting thiolated heparin with triblock coated microspheres. Thiolated heparin displaces PDS of EGAP PEO chains (Fig. 5) to attach to the surface via thiol groups located internally or terminally (Fig. 6). Six microcentrifuge tubes, half containing 500 μL EGAP coated microspheres and the other half containing 500 μL F108 coated microspheres were centrifuged at 13.4 RCF. The supernatant was discarded and replaced with 1 mL of water and vortexed to remove any unbound triblock copolymer. The wash cycle was repeated for a total of three times. After final removal of the wash water, 500 μL centrifuged, purified sample or blank buffer were added to an aliquot of each type of pelleted microsphere. The pellet was dispersed by pipette mixing and vortexing before incubating at 4°C for 24 h by end-over-end rotation at 8 rpm.

3.9 Determination of heparinization

After coupling thiolated heparin to coated microspheres overnight, microsphere solutions were pelleted at 13.4 RCF for 4 min. The supernatant was collected and filtered through 0.45 um syringe filters to remove any residual microspheres. The absorbance of each sample was measured at 343 nm and quantified as described in DTT cleavage (section 3.4). For bioactivity measurements, the pellet

was dispersed in either 50 μL water for the APTT or 320 μL COATEST buffer for the anti-FXa assay.

3.10 Plasma collection

Plasma was collected to test the anticoagulant activity of the materials. Human blood was harvested using 3.8% sodium citrate to inhibit clotting during storage. The whole blood was then centrifuged at 4000 RCF at 4°C for 15 min to separate cells and platelets from plasma. Plasma was collected, separated into 600 μL aliquots, and stored at -80°C until needed.

3.11 Activated Partial Thromboplastin Time (APTT)

Anticoagulant activity was determined using the APTT assay. Plasma was thawed from -80°C at 37°C. APTT reagents (phospholipids and calcium chloride) were warmed to 37°C in stationary univettes in a coagulometer. Once reagents reached 37°C, 100 μL plasma and 100 μL phospholipids were combined in the continuously spinning univette in a KC1Δ coagulometer and allowed to incubate for 5 min. After incubation, 100 μL $CaCl_2$ was added. Clotting time was determined as the time from addition of $CaCl_2$ to the point when the stainless steel ball in the univette became embedded in the gelatinous clot and did not move freely. Assay of samples was performed by replacing 10 μL of pure plasma with the sample of interest.

3.12 Anti-FXa assay

The COATEST anti-FXa assay was used to determine anti-FXa activity. Stock solutions of 0.1 IU/mL heparin were made in 0.9% saline. Dilutions were made in 50 mM COATEST tris buffer at pH 8.4 to final concentrations of 0.1, 0.3, 0.5, and 0.7 U/mL plasma and total volume of 400 μL, and added to 100 μL AT and 100 μL plasma. A blank was prepared using saline in place of heparin solutions. The assay was performed on the coagulometer at 37°C. In univettes, 125 μL of a sample was added and incubated 4 min. At the end of the incubation, 62.5 μL room temperature FXa was mixed into the heparin solutions. After a 30 sec. incubation, 125 μL S2222

chromogenic reagent at 37°C was added to heparin solutions, pipette mixed, and incubated for 3 min. Directly after this 187.5 µL 20% acetic acid was added to all solutions in order to stop the reaction. An additional 187.5 µL of water was added to the blank. The absorbance was measured at 405 nm. Samples containing microspheres were prepared by suspending a centrifuged pellet in 275 µL buffer, 40 µL of AT, and 40 µL plasma to give a final volume of 380 µL. The assay was then performed as described, in triplicate.

3.13 Statistical analysis

The paired t-test is used to analyze the difference in data sets that are dependent or matched. The paired t-test was used to analyze the difference in thiol content found using the Ellman assay. The null hypothesis is that the mean difference between paired sets is zero. It is assumed that the differences are normally distributed. The paired t-test is more powerful than the Student's t-test when the difference between data sets is small compared to the difference within each data set. To perform the test, the standard deviation of the observed differences is calculated by

$$s_d = \sqrt{\left[\sum_{i=1}^{n} d_i^2 - \left(\sum_{i=1}^{n} d_i\right)^2 n\right] / (n-1)}$$

where d_i is the difference between the i^{th} matched pair and n is the number of matched pairs. The test statistic t is then calculated by

$$t = \frac{\bar{d}}{s_d / \sqrt{n}}$$

where $\bar{d}$ is the mean of the differences between the matched pairs. If $t > t_{n-1,\ 1-\alpha/2}$ or $t < t_{n-1,\ 1-\alpha/2}$ then the null hypothesis is rejected. The p-value is twice the area to the left of t under a t_{n-1} distribution.

Satterthwaite's Method is used to analyze the mean values between two independent data sets with unequal variances. It was used to analyze results from PDS displacement, amine content, and the anti-FXa assay. The null hypothesis is that the mean values of the two data sets are equal. The test statistic *t* is calculated by

$$t = \frac{\overline{x}_1 - \overline{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

where $\overline{x}_1$, s_1^2, and n_1 are the mean, variance, and sample size of the first data set and $\overline{x}_2$, s_2^2, and n_2 are the mean, variance, and sample size of the second data set. The degree of freedom, *d*f, is equal to n_1-*1* for all instances in this study. If $t > t_{df,\ 1-\alpha/2}$ then the null hypothesis is rejected. The p-value is twice the area to the right of *t* under a t_{df} distribution (Rosner, 2000).

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Amine characterization

The pKa quantifies the ability of a molecule to lose or gain a proton. The buffering region is traditionally the range ±1 pH unit from the pKa. The pKa can therefore be found experimentally by acid base titrations. Titration of UFH revealed one buffering region at an alkaline conditions, with a pKa of roughly 12 (Fig. 8). Titration of HepNH2, however, reveals two distinct buffering regions, each having a distinctly different pKa. The first buffering region is centered at pH 12, the same value as UFH. This pKa, presumably, can be attributed to amine groups contained internally since these are the only basic groups that both heparins have in common. The second buffering region is centered at roughly pH 9 and is representative of end-chain amine groups.

Amine groups will become unprotonated and increasingly more reactive as the pH increases above the pKa. Preferential reaction can occur by choosing reaction conditions sufficiently basic with respect to terminal amines, causing them to be unprotonated and reactive, yet sufficiently acidic with respect to internal amines, causing them to be protonated and unreactive. This ideal range exists from pH 9-12. Previous work has been performed to covalently couple UFH to a surface using SATA and other similar thiol producing crosslinkers, without success. The reactive range of these compounds (pH 7-9) is not conducive to preferential thiolation. Instead we chose 2-IT with reactivity up to pH 10 for thiolation of heparin.

4.2 Amine quantification

Amine content was determined using fluorescence detection with OPA. The limit of detection was 1 μM using glucosamine 6-sulfate as a standard. Amine content per molecule of heparin was determined before and after thiolation (Fig. 9). Unfractionated heparin was found to have less than one unsubstituted amine group per

molecule of heparin, while the average amine content of HepNH2 was almost 2.5 groups per molecule of heparin.

Since heparin is such a highly heterogeneous molecule, unsubstituted amine content varies significantly among species and tissues. Comper (1981) states that up to 3.2% of glucosamine monosaccharides can contain unsubstituted amines, Toida et al (1997) states that 2.82% of glucosamine in HS from porcine intestinal mucosa is unsubstituted. However, a study by Ampofo et al (1991) does not consider unsubstituted amines in the possible disaccharide compositions since they are found in less than 1% of the population. Using an average molecular weight of heparin of 12500 Da (manufacturer value) and an average disaccharide size of 580 Da (Pavlov et al, 2003), we found that unsubstituted amines are present in 3.2% of glucosamine units. Thus, amine content of UFH used in this study coincides quite well with values described in the literature.

The amine content of HepNH2 was found to be almost two groups per molecule more than UFH. Aminated heparin was produced by reductive amination of UFH, a process which is supposed to specifically target the reducing end of heparin. However, our results show that amine groups were not only created at the reducing end of the polysaccharide, but internally as well. Manufacturer data declares that HepNH2 contains 1.5 amine groups per molecule. Most amine assays are reactive toward primary amines only (e.g. ninhydrin, dabsyl or dansyl chloride, 2,4,6-Trinitrobenzene sulfonic acid (TNBSA)). If the amine assay is performed at a pH below the pKa of the functional group, amine groups can be protonated and unreactive, resulting in detected amounts less than what is actually present in the molecule. It is reasonable to believe that amine content, as determined by the manufacturer, varied from the amine content found in this study because the manufacturer assayed for amine content in neutral or acidic conditions.

4.3 Triblock coating of microspheres

Neff et al (1997) determined the maximum surface density of triblock copolymers to be 3.3 mg/m^2, consistent with a “footprint” of 7.4 nm^2 per molecule.

The EGAP used was 66.72% derivatized (i.e., 66.72% of PEO chains contain PDS groups), which gives a maximum of 7.872 nmol of EGAP groups for each 500 μL aliquot of coated microspheres (302 nmol/m^2). Using an absorbance reading from DTT cleaved PDS groups, we determined that an aliquot of microspheres contains 6.055 nmol (232 nmol/ m^2) of EGAP groups (data not shown). Though this is less than the absolute maximum, the triblock copolymer coating is sufficient for creating a heparinized surface. The difference can be attributed to loss of microspheres during washing, spontaneous loss of PDS groups during storage before the test took place, and the possibility of less than maximum surface coating.

4.4 Thiolation of heparin

Fluorescence detection shows a decrease in amine content after thiolation of heparin (Fig. 9). The decrease between UFH and UFH-SH and between HepNH2 and HepNH2-SH was determined to be statistically significant ($p < 0.001$ and $p < 0.01$, respectively). The change represents a decrease of 53% of total amine content for UFH and 85% for HepNH2 (Table 1). Therefore, end-amine groups were not exclusively thiolated, but they were modified to a higher degree than internal amine groups, as was intended.

Total thiol content was determined using Ellman's assay (Fig. 10). Thiol content for UFH-SH and NH2-SH was determined to be significantly greater than the blank ($p < 0.001$), as reported in Table 2. The difference between HepNH2-SH and UFH-SH was also found to be significantly different ($p < 0.05$). Thiol groups detected on HepNH2-SH using the Ellman assay are approximately equal to the decrease in amine content detected with OPA. The degree of thiolation for UFH-SH, however, is much greater than the decrease in amine content. This can be partially attributed to cross reactivity with hydroxyl groups (Alagon and King, 1980) and insufficient removal of unreacted 2-IT.

Activity was tested before and after thiolation using APTT. HepNH2 has decreased activity when compared to an equimolar concentration of UFH (Fig. 11). This is as expected since the activity determined by the manufacturer is 175 U/mg for

UFH and 107 U/mg for HepNH2. Therefore, reductively aminating heparin resulted in a loss of activity. Using APTT we can see that thiolation also results in a loss of activity (Fig. 12). For UFH, the loss in activity increases with increased concentration. For HepNH2, the difference in activity cannot be detected until very high concentrations. The decrease in activity could be due in part to disulfide formation and cross-linking of polysaccharide chains, as this would decrease accessibility of the binding sites and inhibit heparin-AT-thrombin complex formation.

4.5 Heparinized microspheres

Quantification of displaced PDS groups after incubation of microspheres with thiolated heparin was used to quantify heparin attached to the microsphere surface. There was no significant difference between surface-bound UFH and surface-bound HepNH2. Though there is a wide variation between samples, it is important to note that the displaced PDS quantity never exceeds that of the theoretical maximum of 7.872 nmol determined by Neff et al (1997). The variation arises from the variation in thiolation. Testing for surface coverage using DTT before heparin incubation would allow determination of a coupling efficiency, but was not performed in this study.

The APTT assay revealed no significant change in clotting time for heparinized microspheres, thus no anticoagulant activity was detected (Table 3). The APTT is the clinical standard for testing the concentration of heparin in blood. It is a relatively crude test of the intrinsic clotting system that is not sensitive to any single factor, but measures the final length of time it takes to form a clot. Any lengthening of time with respect to a control is primarily brought about through the inhibition of thrombin, and to a lesser extent FXa. The assay is insensitive to LMWH and UFH heparin concentrations below 0.1 U/mL (Hirsh et al, 1998). The absence of significant results could therefore be due to low levels of anticoagulant activity, not necessarily the absence of activity. However, there is no evidence recorded here that suggests immobilized heparin retained the ability to associate with both AT and thrombin.

Surface activity was also determined using the anti-FXa assay (Fig. 14). This assay specifically measures the formation of heparin-AT-FXa complexes which lies at

the intersection of the extrinsic and intrinsic clotting pathways. Excess FXa that is not complexed with Heparin-AT cleaves the substrate releasing a chromophore into solution that is detected at 405 nm. The signal of the chromophore is indirectly proportional to heparin concentration. Heparin standards were used to create an exponential calibration curve. As shown in Fig. 14, heparin was detected on the surface of EGAP coated microspheres only. The thiolated UFH and HepNH2-SH content of EGAP coated microspheres was significantly greater than the content of an equivalent volume of F108 coated microspheres ($p < 0.05$). The surface content of heparin on HepNH-SH treated microspheres was significantly greater than that on UFH-SH treated microspheres ($p < 0.01$), even though it was shown that similar amounts of heparin were apparantly attached to the surface. The increased activity is presumably due to a greater retention of heparin function resulting from end-on attachment to the triblock coated microspheres.

Activity values of 175 U/mg for UFH and 107 U/mg for HepNH2 given by the manufacturer were verified using the anti-FXa assay. Using these values we can relate the anti-FXa activities of the microspheres to actual amounts (grams and moles) of heparin that was apparently present on the surface (Table 4). It is important to note that this is not the actual amount of heparin present at the surface. Molar amounts of HepNH2-SH are much lower than EGAP coating results from DTT cleavage, 4.78 pmol versus 4.56 nmol, respectively. It is possible that the actual amount of heparin attached to the surface is not detected by the anti-FXa assay because it is not free to interact with the environment. It is also possible that the same molar amount of UFH-SH is also linked to the polystyrene microspheres, but because of the “side-on” orientation of the molecule it has a significantly decreased activity. Since HepNH2-SH attaches with the bulk of the molecule apparently located away from the surface, the activity is not inhibited as much by the covalent linkage.

CHAPTER 5
CONCLUSION

Heparin was successfully thiolated using 2-IT. Thiolation occurred to a higher degree in HepNH2 than UFH, as was intended by reacting at a high pH. Comparision of Ellman assay and OPA results suggest that 2-IT reacts not only with amine groups of heparin, but presumably with hydroxyl groups as well. Unfractionated heparin and HepNH2 displaced similar amounts of PDS groups upon reaction with end-activated the triblock copolymers. Anticoagulant activity on microspheres was not detected by the APTT, due to steric interference with the ability to properly bind AT and form a functional heparin-AT-thrombin association and/or a low surface content of heparin. Heparin was detected on the surface using the anti-FXa assay. Higher anti-FXa activity was seen from microspheres treated with thiolated HepNH2 relative to those treated with thiolated UFH.

This study provides evidence that end-point attachment of heparin to a surface is advantageous over attaching the molecule by a position internally. The combination of specifically oriented heparin with triblock polymers creates a surface that is not only resistant to thrombus formation, but also might retain the ability to inhibit protein adsorption. Though this study did not include measures of stability and performance of the material over time, these topics ought to contribute to the subject of future research.

CHAPTER 6
LIST OF FIGURES

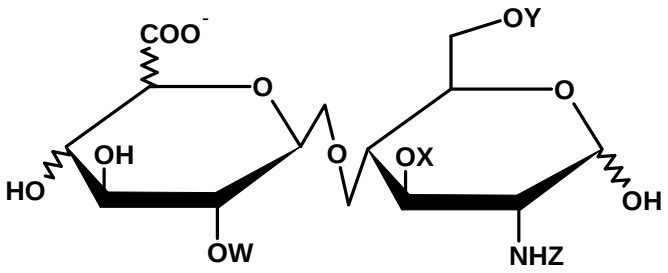

Figure 1.
Disaccharide sequence of heparin. Iduronic acid (left) is most commonly sulfated at the C2 position (W = SO_3^-), while glucosamine (right) is unsubstituted at C3 (X = H) and sulfated at C6 (Y = SO_3^-) and the amine position at C2 (Z = SO_3^-). (Adapted from Islam and Linhardt, 2003)

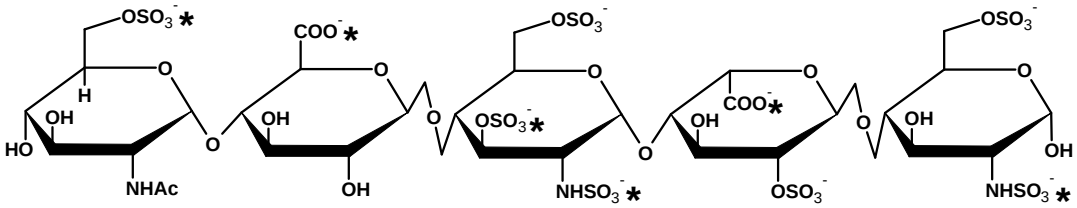

Figure 2.
Pentasaccharide sequence of heparin that constitutes the site of binding to AT. Functional groups highlighted by (*) are required to induce the conformational change in AT that leads to increased thrombin inhibition. (Adapted from Petitou et al, 2003)

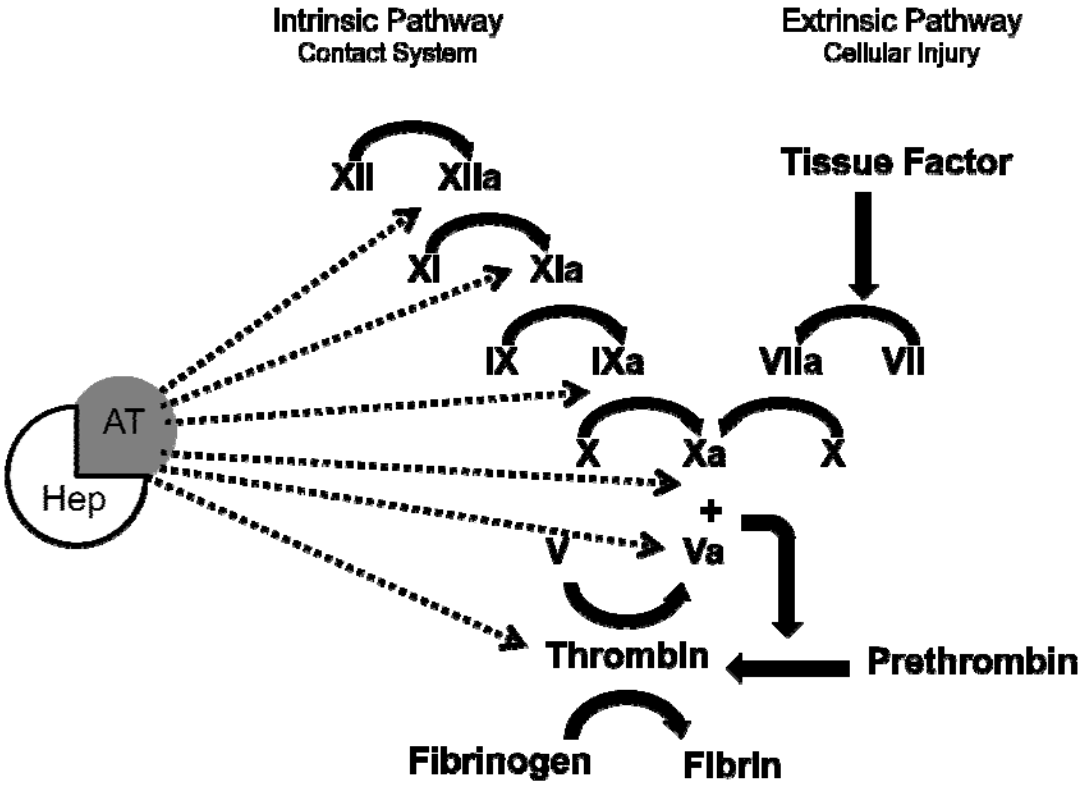

Figure 3.
Enzymatic conversion (solid arrows) of proteins within the coagulation cascade is inhibited by the heparin-antithrombin complex (dotted arrows). The primary activity of AT is toward FXa and thrombin. (Adapted from al-Lamee and Taktak, 1998)

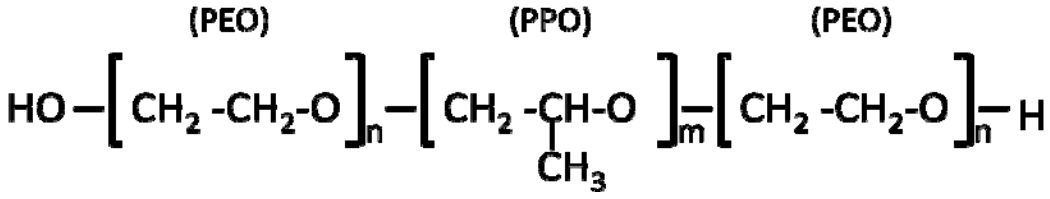

Figure 4.
Chemical structure of triblock copolymers where *n* and *m* represent number of monomers. For F108 *n* = 141 and *m* = 44.

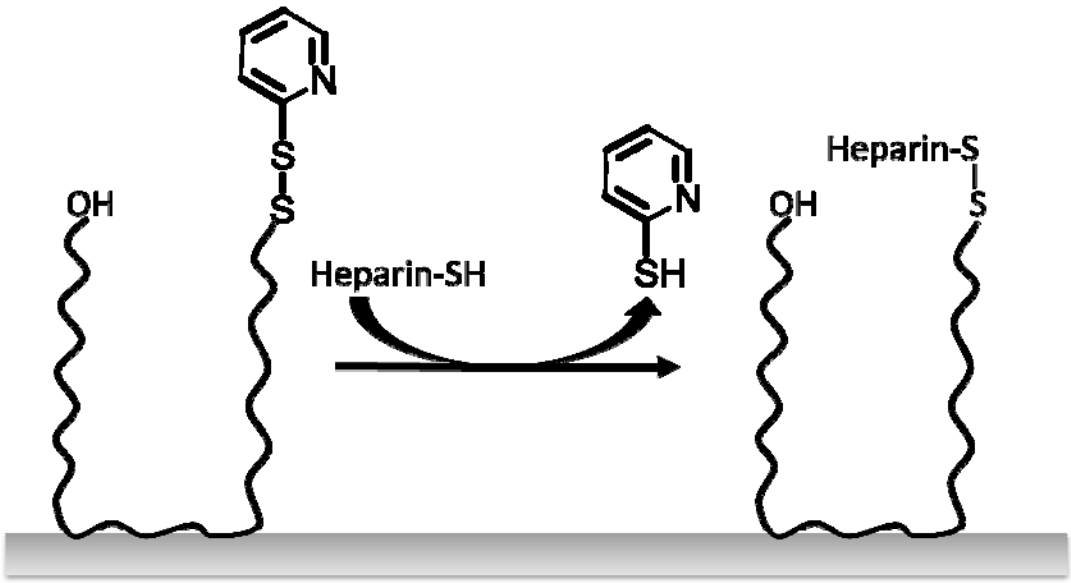

Figure 5.
Subsitution of Pluronic® triblock end-groups groups with thiolated heparin.

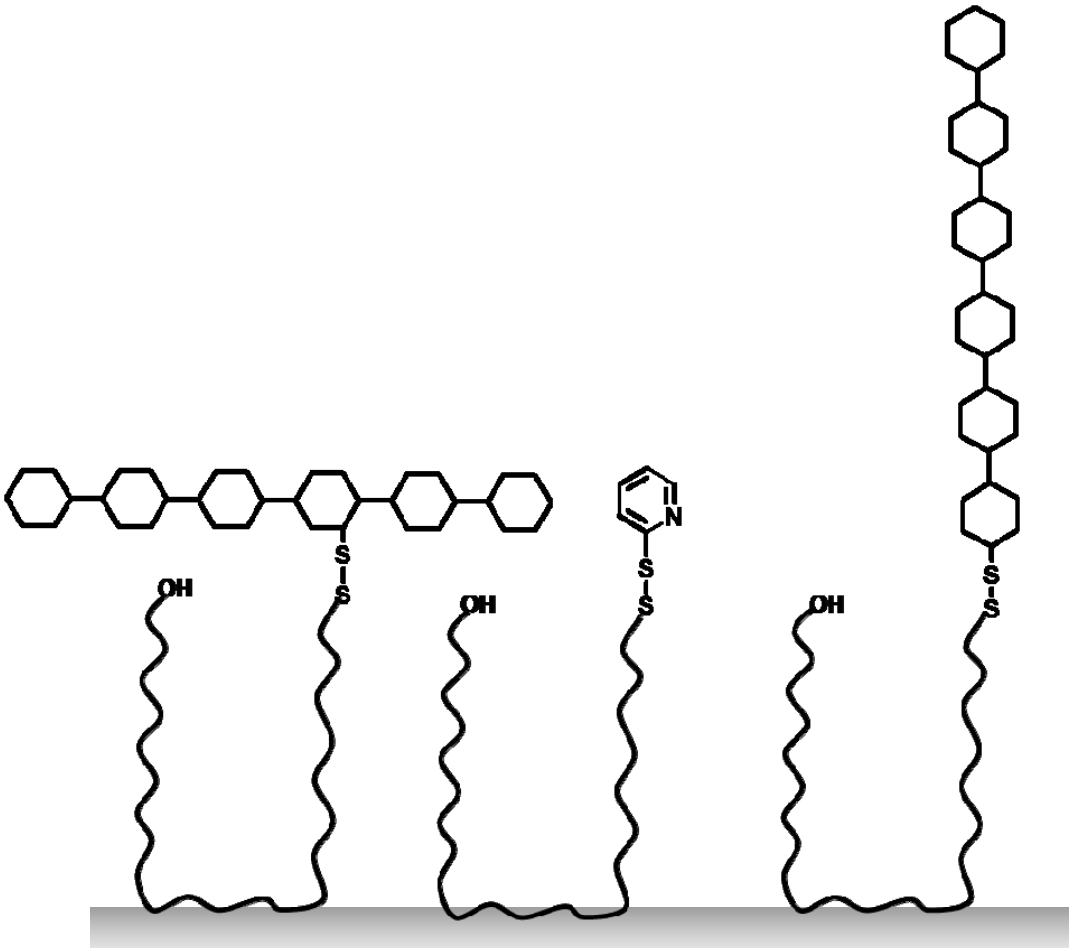

Figure 6.
Heparinized triblock coated surface. Heparin can covalently couple to PDS-activated triblocks via thiol groups located internally (left), or terminally (right).

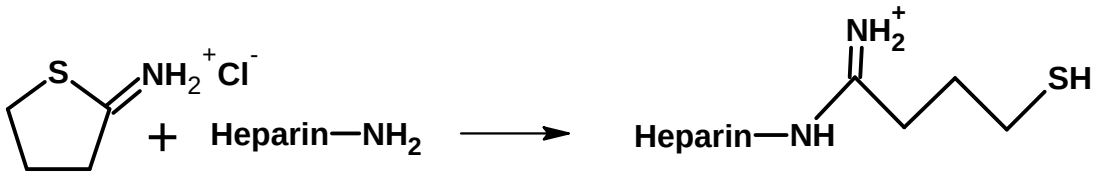

Figure 7.
Reaction scheme of 2-IT with the end amine of HepNH2. The modification can also take place with internal amines of HepNH2, as well as UFH.

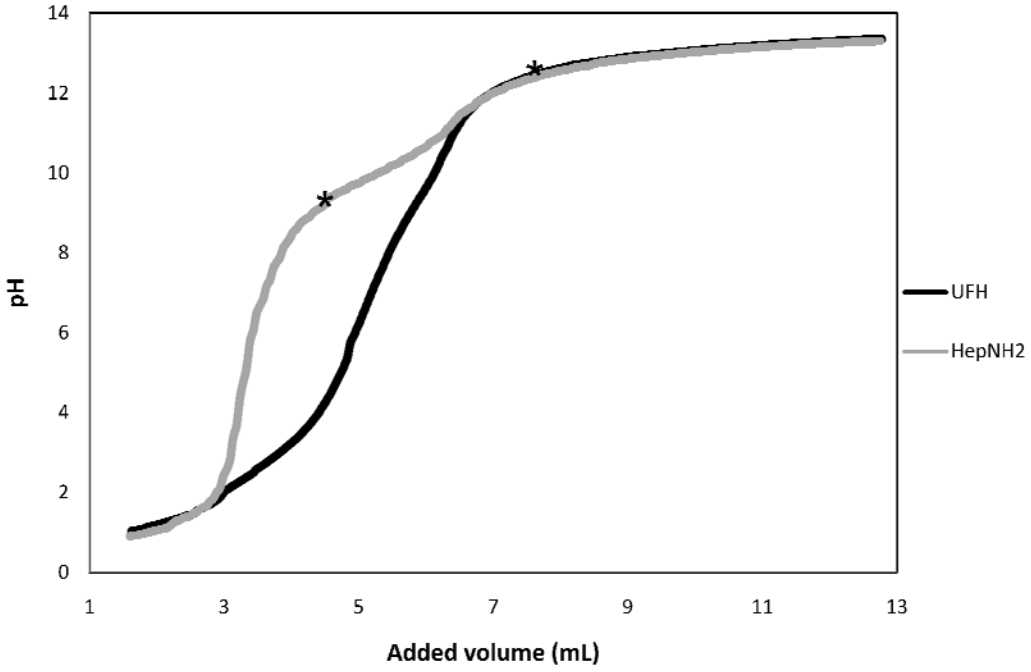

Figure 8.
Titration of UFH and HepNH2 reveals two chemically distinct amine groups, (denoted by *), those found internally and those present at the end of the chain by chemical modification. Each is characterized by a different pKa (internal pKa ≈ 12, terminal pKa ≈ 9).

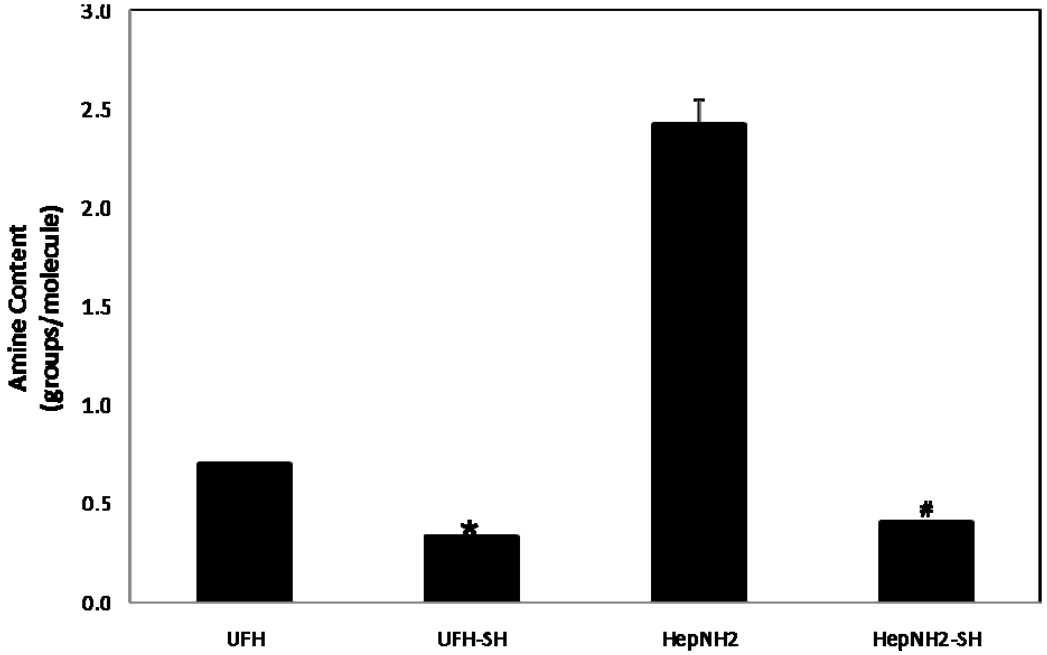

Figure 9.
Amine content of UFH and HepNH2 before and after thiolation. Amine groups were detected by reacting with OPA (n=3). UFH-SH and HepNH2-SH amine content is significantly different from UFH and HepNH2 (* = $p < 0.001$ and # = $p < 0.01$).

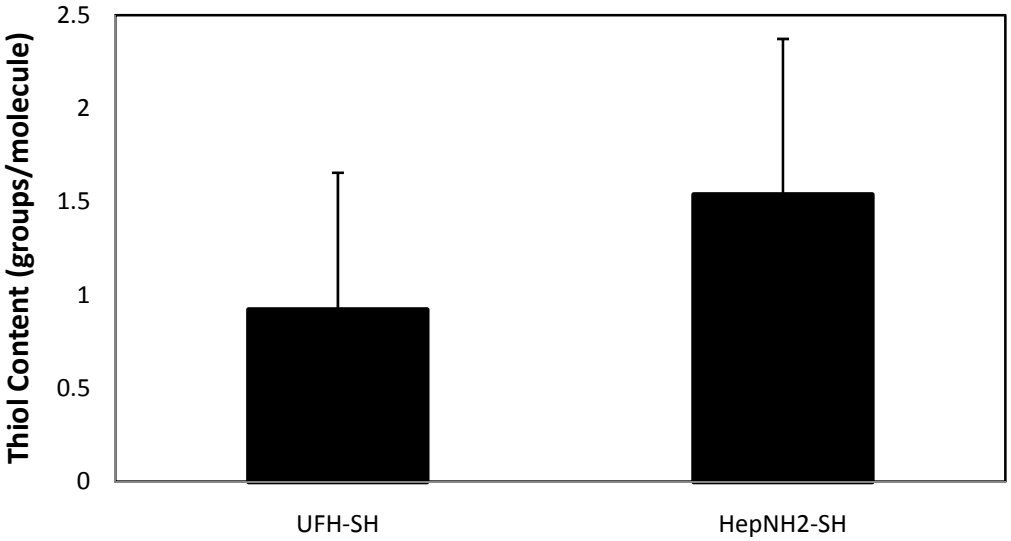

Figure 10.
Thiol content of heparin treated with 2-IT as determined by Ellman's assay (n=13). UFH-SH and HepNH2-SH thiol content are significantly different from blank values ($p < 0.001$) and significantly different from each other ($p < 0.05$).

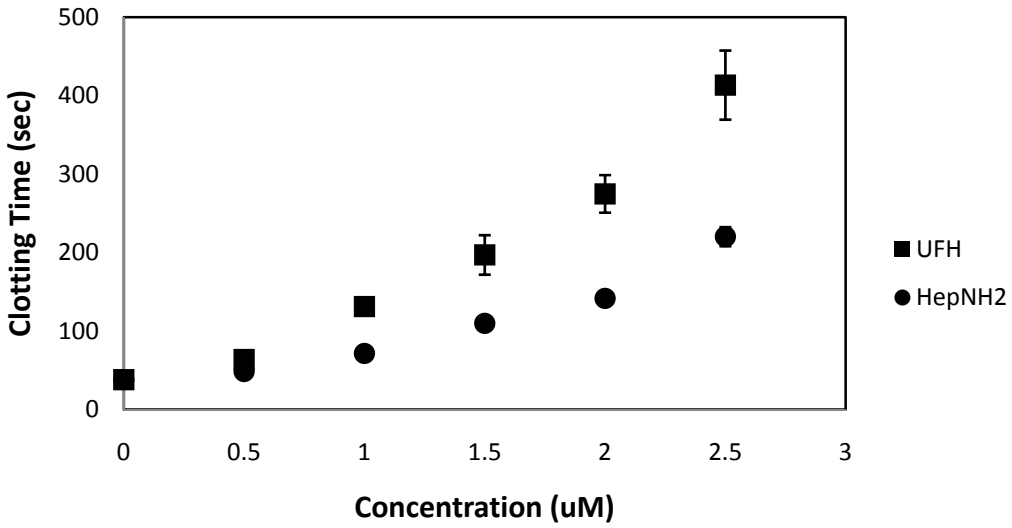

Figure 11.
Anticoagulant activity of UFH and HepNH2 as determined by APTT (n=3).

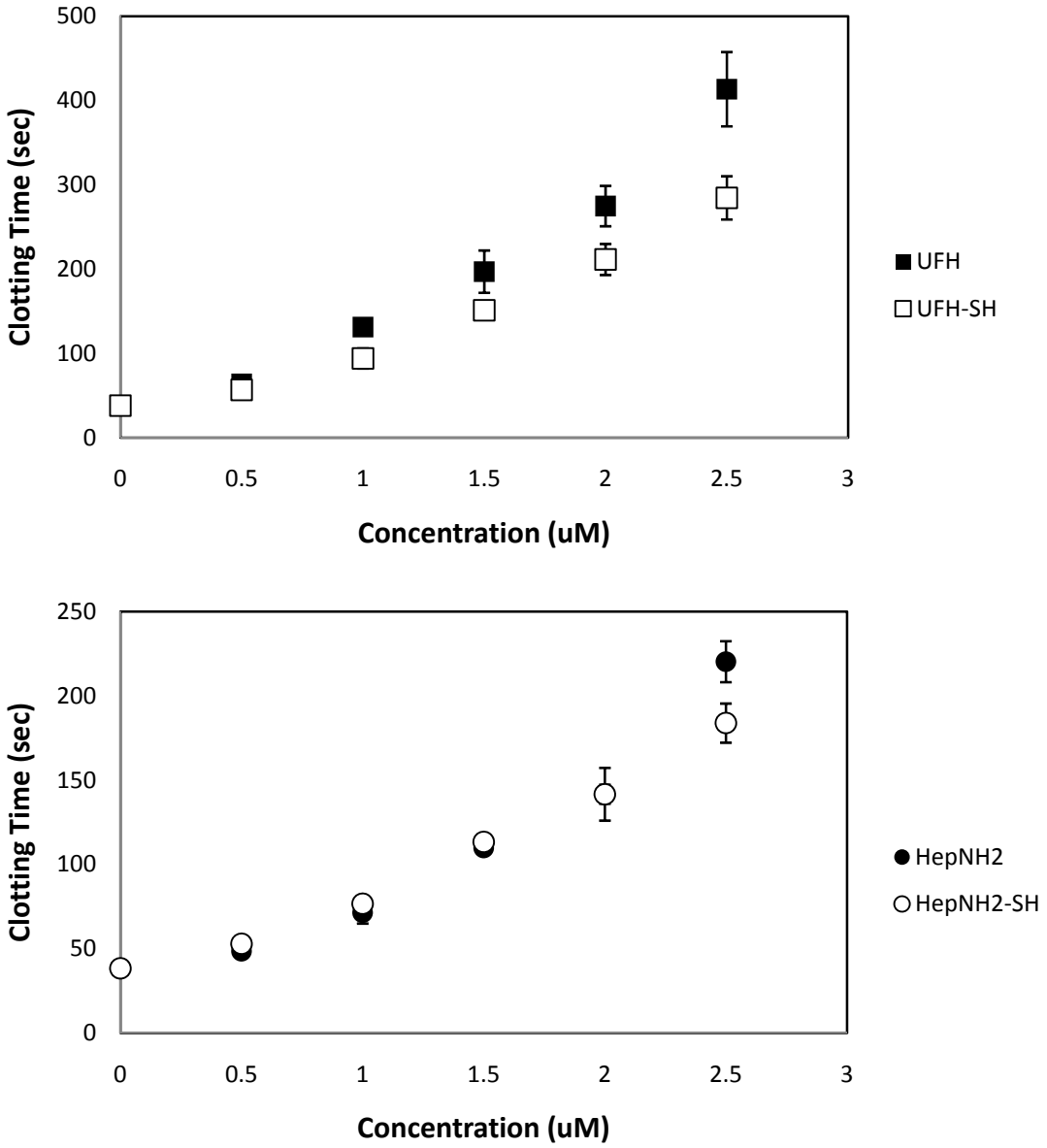

Figure 12.
Anticoagulant activity of UFH (top) and HepNH2 (bottom) before and after reaction with 2-IT. Black markers are before thiolation, white markers are after thiolation (n=3).

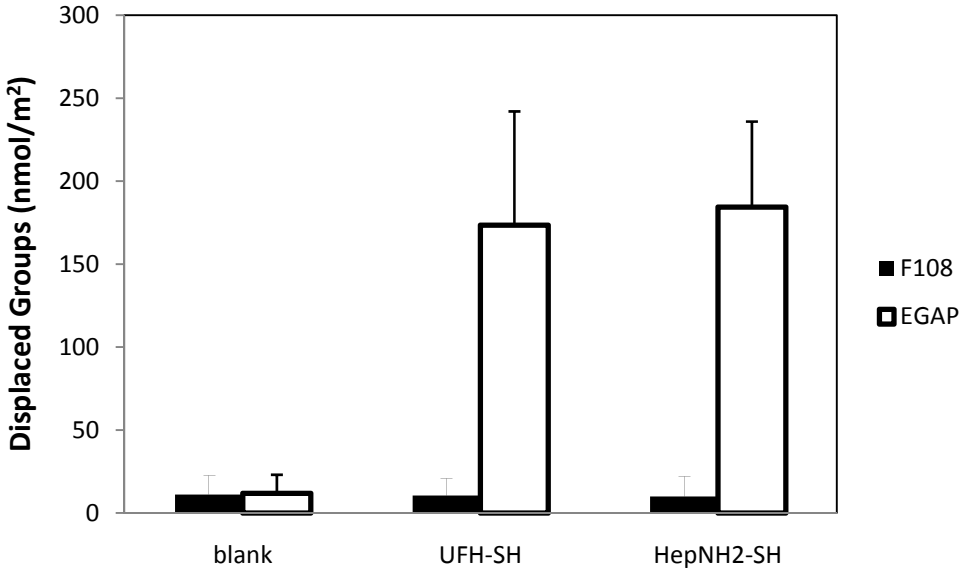

Figure 13.
Quantification of pyridyl 2-thione release from triblock coated microspheres upon introduction of thiolated heparin as determine by absorbance at 343 nm (n=12).

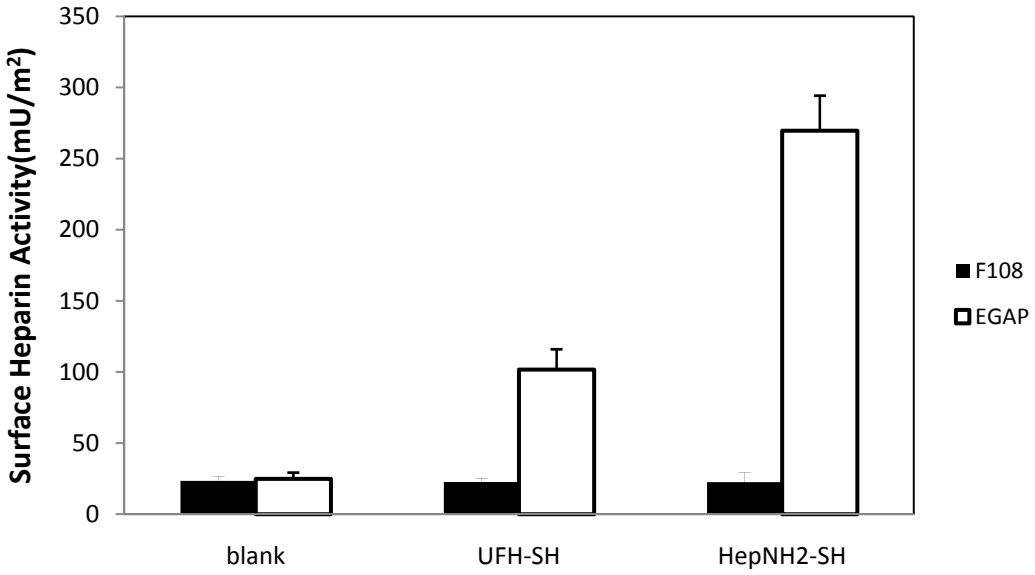

Figure 14.
Surface heparin activity of triblock-coated, heparinized PS microspheres as determined by the anti-FXa assay. Heparin was detected only on the surface of EGAP coated microspheres (n=3). UFH-SH and HepNH2-SH activity on EGAP coated microspheres is significantly different compared to equivalent F108 coated microspheres ($p < 0.05$).

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Table 1.
Amine content of UFH and HepNH2 before and after thiolation (n=3).

	average groups/molecule	standard deviation	p-value	thiolation efficiency
UFH	0.70	0.0062	p > 0.001	53%
UFH-SH	0.33	0.0016		
HepNH2	2.7	0.29	p > 0.01	85%
HepNH2-SH	0.40	0.0087		

Table 2.
Thiolation results from Ellman assay (n=13).

	average groups/molecule	standard deviation
blank	0.2538	0.1110
UFH	0.4213	0.1872
NH2	0.5336	0.1852

	p-value
blank vs UFH	$p < 0.001$
blank vs HepNH2	$p < 0.001$
UFH vs HepNH2	$p < 0.05$

Table 3.
Clotting time for heparinized microspheres using APTT (n=3).

	treatment	average sec	standard deviation
Control		38.53	0.90
F108	**blank**	37.40	2.91
	UFH-SH	43.13	0.67
	HepNH2-SH	43.80	0.10
EGAP	**blank**	39.43	2.41
	UFH-SH	39.73	1.55
	HepNH2-SH	41.77	3.21

Table 4.
Results for anti-FXa assay of heparinized microspheres (n=3). Activity is detected in mU, which can be converted to μgram and nmol detected on the surface. Activity of UFH-SH and HepNH2-SH on EGAP coated microspheres was significantly different from equivalent F108 coated microspheres.

	mU	ngram	pmol	p-value
UFH-SH	2.007	11.47	0.917	$p < 0.05$
HepNH2-SH	6.391	59.73	4.778	$p < 0.05$

CHAPTER 8
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