2005-06-08

Autologous Fibrinogen Purification and Concentration For Use in Fibrin Sealant

Steven M. Alston
Brigham Young University - Provo

AUTOLOGOUS FIBRINOGEN PURIFICATION AND CONCENTRATION FOR USE IN FIBRIN SEALANT

by

Steven M. Alston, Jr.

A dissertation submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

Department of Chemical Engineering

Brigham Young University

August 2005

BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

of a dissertation submitted by

Steven M. Alston, Jr.

This dissertation has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

_____	_____
Date	Kenneth A. Solen, Chair
_____	_____
Date	S. Fazal Mohammad
_____	_____
Date	William G. Pitt
_____	_____
Date	John N. Harb
_____	_____
Date	Larry L. Baxter

BRIGHAM YOUNG UNIVERSITY

As chair of the candidate's graduate committee, I have read the dissertation of Steven M. Alston, Jr. in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill the university's requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

_____ _____

Date

Kenneth A. Solen
Chair, Graduate Committee

Accepted for the Department

_____

W. Vince Wilding
Department Chair

Accepted for the College

_____

Alan R. Parkinson
Dean, Ira A. Fulton College of Engineering and Technology

ABSTRACT

AUTOLOGOUS FIBRINOGEN PURIFICATION AND CONCENTRATION FOR USE IN FIBRIN SEALANT

Steven M. Alston, Jr.

Department of Chemical Engineering

Doctor of Philosophy

Fibrinogen concentrates are used widely as a sealant during and after surgery to reduce blood loss. Commercially available fibrin sealants are made from pooled human blood, which carries the risk of blood-borne diseases, and are expensive. These concerns have brought to focus the need for autologous fibrinogen concentrates. This need has been addressed by utilizing a unique approach in which fibrinogen is precipitated from plasma with protamine.

The physical properties of fibrin sealant prepared from fibrinogen precipitated with protamine were evaluated. The optimal precipitation conditions included a plasma protamine concentration of 10 mg/mL at room temperature. Under these conditions 96% ± 4% of the fibrinogen present in the plasma was precipitated and 98% ± 0.9% of the

precipitated fibrinogen was clottable. In addition, it was shown that almost 50% of the factor XIII in the plasma was also precipitated along with the fibrinogen.

The tensile and adhesion strengths and kinetics of fibrin sealant prepared from protamine-fibrinogen concentrate were evaluated. Tensile strength and adhesion strength both increased with increasing fibrinogen concentration. Addition of calcium chloride significantly increased the tensile and adhesion strengths. The addition of aprotinin and ε-aminocaproic acid (used to inhibit natural fibrinolysis) to the fibrinogen concentrate was shown to have no effect on the mechanical properties of the sealant. Kinetic experiments showed that the clotting time decreased as the thrombin and fibrinogen concentrations were increased.

A rat model with controlled renal incisions was employed to evaluate the hemostatic efficacy of the fibrin sealant made from the protamine-fibrinogen concentrate. The fibrin sealant significantly reduced the blood loss and bleeding time when compared with controls (no sealant, plasma, and a commercial product). The sealant also significantly reduced blood loss and bleeding time in rats that were anticoagulated with heparin.

A mathematical model based on tensile strength and adhesion strength was developed to predict the bleeding time in the animal wound. Model predictions showed that the ability of the fibrin sealant to reduce bleeding time, and therefore blood loss, was limited by the adhesion strength.

ACKNOWLEDGEMENTS

Completing this project would not have been possible without the help and support of many people. I would like to briefly acknowledge their contributions.

Dr. Solen has been a mentor and advisor throughout this project. He has encouraged me to take ownership of the project by allowing me the freedom to make important decisions regarding the direction the project should take. I appreciate the many helpful ideas and solutions he discussed with me and then let me make the final decision.

Working with Dr. Mohammad was a valuable part of my graduate education. I appreciate the many critiques of my experimental techniques and conclusions drawn from those experiments. These experiences helped to further my ability to think critically and logically.

Dr. Sukavaneshvar was another important part in completing this project. I appreciate his many suggestions and critiques of my work. He helped me to realize the ultimate goal of the project.

I appreciate the help given by Dr. Woolley in obtaining the required drugs for the animal experiments. The animal work would not have gone as smooth as it did were it not for his assistance.

I would like to thank the many undergraduate research assistants that helped with the research. Eddie Bywater and Adam Broderick provided a lot of help with the in-vitro

tests. I appreciate the time volunteered by John Ashton to help with the animal experiments.

Last, but certainly not least, I wish to thank my loving wife Jackee. She has supported me every step of the way through this project. Without her love and support this project would not have been possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

1.1 Reducing Blood Loss During Surgery

A common method for reducing blood loss during/after surgery is to mimic the last step of the physiological coagulation mechanism through the use of fibrin sealant (also called fibrin glue). The sealant, formed by mixing a concentrated solution of fibrinogen with thrombin and Ca^{2+} to produce fibrin, is applied to bleeding wounds and suture lines to stop bleeding within minutes [1].

1.2 Preparation of Fibrinogen Concentrate

Conventionally, fibrinogen concentrates are either prepared from a cryoprecipitated concentrate of fibrinogen from blood banks or an autologous plasma (i.e. from the patient's own blood). However, the blood-bank derived cryoprecipitates are extracted from homologous (i.e. from other humans) blood and therefore carry the risk of transmitting blood-borne pathogens (i.e. hepatitis, HIV). The risk is eliminated by deriving fibrinogen from autologous plasma, but the relatively low concentration of fibrinogen in plasma (3 mg/mL) results in a weak sealant [2].

Commercial fibrinogen preparations are available but their use is limited due to high cost. The Food and Drug Administration recently (1998) approved the use of

Tisseel VH (Baxter Healthcare Corp.,Westlake Village, CA) for use in the United States. In this product, the fibrinogen is isolated from pooled human blood, but efforts have been made to reduce the risk of HIV and other viral contaminants by heat inactivation or solvent/detergent extraction. However, it is well recognized that the risk of viral contamination cannot be fully eliminated from any preparation obtained from blood collected from multiple donors. Further, the preparation of fibrinogen concentrates is a complex, time-consuming process. More recently, methods have been developed to prepare concentrated fibrinogen from autologous blood in the operating room, but the methods require complex procedures and equipment that would be difficult to extend to emergency situations.

1.3 Goals of this Dissertation

The specific goals of this dissertation were to: 1) develop a quick, inexpensive process to harvest concentrated fibrinogen from autologous patient blood for use as a fibrin sealant; 2) evaluate the in-vitro mechanical properties of the fibrinogen concentrate; and 3) evaluate the efficacy of the fibrinogen concentrate in an in-vivo animal wound model.

CHAPTER 2: BACKGROUND

2.1 Blood and Coagulation

The blood volume of an average adult is about 5 liters, consisting of formed elements (~40 vol%) and plasma (~60 vol%) [3]. The formed elements in blood include erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets). Blood plasma is a dilute (0.15 N) electrolyte solution containing inorganic ions, numerous proteins, and other molecules [4].

The body has inherent mechanisms to limit blood loss as a lack of blood flow rapidly leads to cell, tissue, and organ death. Hemostasis is the arrest of bleeding, and coagulation plays an important role in hemostasis by helping to minimize blood loss from the body [3]. Coagulation occurs when appropriate stimuli activate a group of procoagulant plasma proteins, which activate each other in an enzymatic cascade to eventually form a viscous gel (Figure 2.1). The intrinsic pathway (activated by components wholly contained within the vasculature [3]) and/or the extrinsic pathway (activated by tissue injury) lead to activation of the common pathway and the formation of a stabilized fibrin clot.

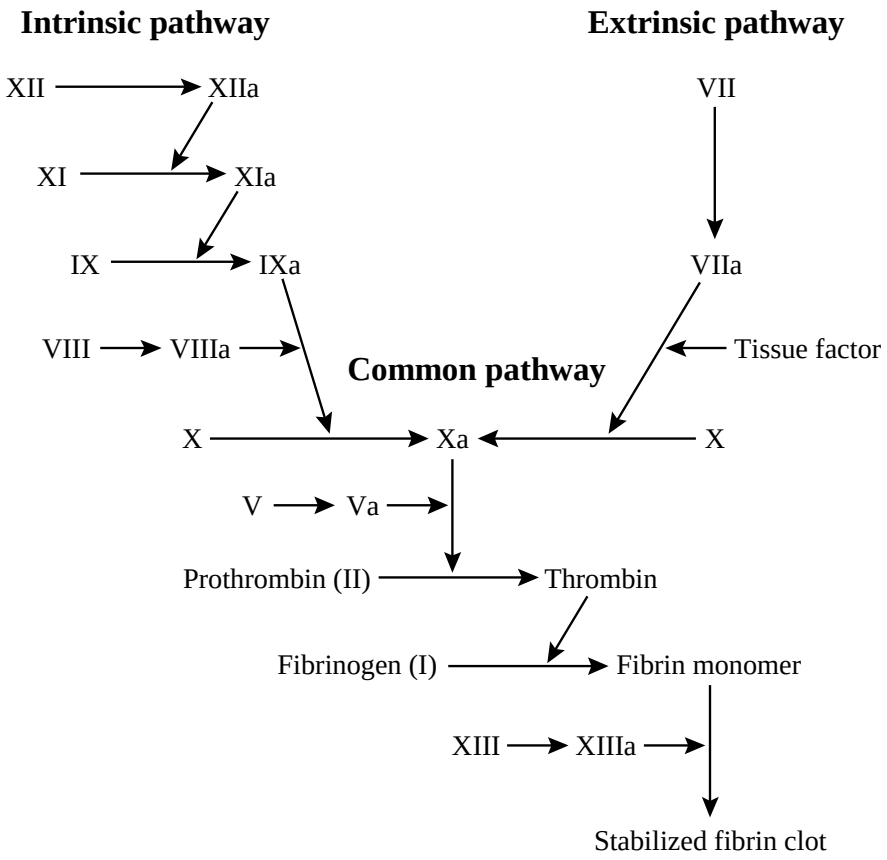

Figure 2.1: A simplified version of the coagulation cascade.

2.2 The Role of Fibrinogen

Fibrinogen is a soluble blood protein that comprises 3 mg/mL of plasma [5]. It is converted into an insoluble fibrin network in the presence of thrombin as part of the intrinsic and extrinsic coagulation cascades. Thrombin cleaves fibrinopeptides A and B from the fibrinogen molecule to form fibrin monomer, which polymerizes to form a weak fibrin clot. Factor XIII, activated by thrombin and Ca^{2+}, catalyzes crosslinking of the fibrin molecules, resulting in a stable clot [6].

2.3 Fibrin as a Tissue Sealant

While the first documented use of fibrin as a tissue adhesive dates back to 1909, Cronkite and associates are credited as being the first to combine fibrinogen and thrombin to form an adhesive gel for attaching skin grafts during World War II [7]. Matras [8] is recognized for having used a concentrated fibrinogen solution to form a viscous gel for peripheral nerve repair, thus bringing fibrin glue on the stage as an effective tissue sealant. These early forms of fibrin sealant lacked adequate adhesive strength due to their low fibrinogen concentrations. It wasn't until the 1970s that plasma fractionation methods emerged allowing the production of more concentrated protein solutions [9]. However, the Food and Drug Administration (FDA) prohibited the use of commercial fibrin sealants in 1978 because of the high risk of hepatitis transmission [2]. During this time fibrin sealants continued to be used in Europe, and today several commercial preparations are available. The FDA recently (1998) approved the use of Tisseel VH (Baxter-Immuno, Vienna, Austria) for commercial use in the United States. Other commercial sealants are currently in clinical trails for FDA approval.

Considering the critical role that fibrin plays in hemostasis, and possibly in wound repair, it has been recognized for decades as an attractive primary ingredient upon which to base the development of an effective tissue sealant. The major limitations in developing a fibrin-based sealant that can be widely utilized have been the risk of blood-borne infections, and the costs associated with harvesting concentrated protein solutions. Despite these limitations, fibrin sealant has been used successfully in almost every field of surgery including cardiothoracic [10], neurologic [11], ophthalmologic [12],

orthopedic [13, 14], vascular [15], and other fields [16, 17]. Other potential applications contemplated for utilizing fibrin-based sealant include delivery of antibiotics at the wound site to ward infection [18], delivery of growth factors or other additives to improve wound healing [19], and delivery of chemotherapeutic agents to treat malignancies [20].

2.4 Composition of Fibrin Sealant

The main components in a fibrin sealant are fibrinogen and thrombin, although other constituents such as factor XIII and antifibrinolytic agents may also be present [21]. The performance of a fibrin sealant is determined by the individual components present in the final product.

Fibrinogen is the main structural component in fibrin sealant and can range in concentration from 3 mg/mL to 130 mg/mL [21]. It has been shown that the mechanical strength and clotting time of fibrin sealant are directly related to the fibrinogen concentration [9, 22]. Increasing the fibrinogen concentration increases the tensile and adhesion strengths and decreases the clotting time of fibrin sealant. It is desirable to have a fibrinogen concentration of 60 mg/mL or greater in order to provide complete hemostasis [23].

The second major component of fibrin sealant is thrombin, which is present at a concentration of 4-1000 U/mL [21, 24]. The main function of thrombin is to convert fibrinogen into fibrin. Because of this, the clotting time is determined by the concentrations of both fibrinogen and thrombin. Products that have a low thrombin

concentration will clot at a much slower rate than those with high concentrations of thrombin [21].

Factor XIII is activated by thrombin, in the presence of calcium, and is responsible for cross-linking fibrin. The concentration of factor XIII in fibrin sealants ranges from 0 to 80 U/mL [25]. The presence of factor XIII has been shown to significantly increase the tensile and adhesion strengths of fibrin sealant and improve the in-vivo hemostatic efficacy [26-28]. The cross-linking of fibrin stabilizes the clot and makes it less susceptible to fibrinolytic breakdown. Factor XIII has also been shown to crosslink adhesive proteins, such as fibronectin [29], thrombospondin [30], and von Willebrand factor [31] to fibrin, increasing the adhesion strength.

Some fibrin sealants may have a fibrinolytic inhibitor, such as aprotinin or ε-aminocaproic acid, added to them to increase clot stability [32]. It has been shown that clots formed from fibrin sealant containing aprotinin or ε-aminocaproic acid exhibit decreased lysis [33]. However, it has also been shown that fibrinolytic inhibitors are not necessary in fibrin sealant and may negatively affect tissue remodeling [34].

2.5 Isolation of Fibrinogen

Since the main component of fibrin sealant is fibrinogen, many different methods have been developed to harvest fibrinogen for this purpose.

2.5.1 Cryoprecipitation

The ‘gold standard’ for isolating fibrinogen from human blood is cryoprecipitation, which utilizes the low fibrinogen solubility at cold temperatures. Plasma is frozen at –80 ^{o}C for at least 12 hours, thawed for several hours at 4 ^{o}C, followed by centrifugation. The supernatant is then decanted leaving a yellowish precipitate of fibrinogen, which is then reconstituted in a small amount of supernatant plasma. Fibrinogen concentrations obtained from cryoprecipitation range from 20-40 mg/mL [35-37]. Because cryoprecipitation requires no chemical manipulation, this approach has been used widely in blood banking to provide a crude clotting factor concentrate to manage hemostatically deficient patients. While cryoprecipitation steps are utilized for large-scale preparation of fibrinogen from pooled blood, this approach is not practical for the harvesting of fibrinogen from small volumes of blood on short notice. Cryoprecipitate also carries the potential risk of virus transmission because it is prepared from pooled donor blood.

2.5.2 Ultrafiltration

Ultrafiltration combines the use of pressure and semi-permeable membranes to separate molecular species by size, shape, and/or charge. This technique has been used to concentrate plasma and has been suggested for use in preparing fibrin sealant. Interpore Cross, Inc. (Irvine, CA) has developed a plasma concentrator based on ultrafiltration [38], in which platelet-rich plasma is delivered to an ultrafiltration chamber with a molecular weight cutoff of 30,000 Daltons. The concentrated plasma is collected out of the bottom of the chamber and can be used to prepare fibrin sealant. Harvesting fibrinogen by this

approach yields a final fibrinogen concentration of approximately 6 mg/mL [39]. In addition, a significant portion of the fibrinogen is denatured in the process, rendering only 70% of the collected protein clottable [39].

2.5.3 Centrifugation

The Cell Saver is a centrifugal blood concentrator that separates and concentrates red blood cells, platelets, and plasma proteins by removing plasma from whole blood [40-42]. The physical separation of blood components is based on the differences in density and particle size [43]. Fibrinogen harvested from the cell saver has been investigated for use in fibrin sealant [44]. The efficacy of fibrin sealant made from concentrated fibrinogen collected from the cell saver was compared to the efficacy of a commercial fibrin sealant. The commercial fibrin sealant performed significantly better than the cell saver fibrin sealant due to the significantly higher fibrinogen concentration in the commercial sealant.

2.5.4 Chemical Precipitation

Methods have also been proposed to precipitate fibrinogen using chemical agents such as ethanol, polyethylene glycol (PEG), or ammonium sulfate [45-47]. Chemical precipitation methods present many advantages over cryoprecipitation. First, the entire preparation requires only 90 minutes, eliminating the need for pre-surgical patients to donate blood 1-2 days prior to surgery. The fibrinogen yield with chemical precipitation is greater than cryoprecipitation, thereby reducing the amount of plasma required. These factors make it possible to use autologous patient blood for preparing the fibrinogen. The

use of autologous blood eliminates the risk of blood-borne virus transmission. The concentration of fibrinogen prepared from chemical precipitation ranges from 30 to 50 mg/mL [2].

However, chemical precipitation with these agents does have some disadvantages. For example, ethanol precipitation leaves elevated levels of ethanol in the fibrinogen concentrate that can result in premature clotting of the fibrinogen [2]. Factor XIII, which is necessary for stabilizing the clot, has reduced activity in the presence of ethanol, resulting in reduced clot tensile strength [48]. Ammonium sulfate precipitates 55% of the fibrinogen and a large amount of albumin that can interfere with clotting [49]. PEG precipitation requires that prothrombin be absorbed with $BaSO_4$ and $MgSO_4$ before addition of PEG to precipitate fibrinogen [50]. In addition, PEG may modify the fibrinogen and yield it unclottable [51].

Protamine has also been shown to precipitate fibrinogen [52-54]. Protamine is a small (MW 5 kDa), positively charged protein harvested from fish sperm. It is routinely used to restore clotting function in patients that have been anticoagulated with heparin. Protamine has been shown to precipitate up to 100% of the fibrinogen in the plasma [54]. The precipitation step is rapid, and recoveries have been reported of up to 100% of the fibrinogen [54].

The Vivostat system is a medical device that utilizes chemical precipitation to prepare patient-derived fibrinogen concentrate [55]. 120 mL of the patient's blood is placed into the processor, and the automated processing is started to obtain platelet-poor plasma. The plasma is reacted with biotin-batroxobin for 10 minutes at 37 ^{o}C which results in the formation of an acid soluble fibrin I polymer. The polymer is removed with

centrifugation and dissolved in a sodium acetate buffer (pH 4). The solution is filtered to isolate fibrin I and is loaded into a syringe. Another syringe is prepared containing a carbonate/bicarbonate buffer (pH 10). The two syringes are applied simultaneously resulting in the formation of a fibrin clot. The Vivostat system takes approximately 30 minutes to process the patient's blood and to concentrate fibrin I.

2.5.5 Chromatographic Methods

Fibrinogen has been shown to bind to several different immobilized ligands via affinity and ion exchange chromatography. All of these approaches require plasma to be passed through a column containing the ligand bound to a matrix. The fibrinogen in the plasma binds to the ligand and is then eluted from the gel [5, 56]. These approaches have been employed to study molecular interactions of fibrinogen with other blood components as well as the function of fibrinogen, however, these approaches have not been proven conducive for preparation of fibrinogen concentrate.

2.5.5.1 Fibrin Monomer Matrix

The high affinity of fibrinogen for fibrin monomer has been employed for purifying fibrinogen [57-59]. Fibrin monomer immobilized on a chromatography gel matrix can be utilized to bind fibrinogen in plasma. The fibrinogen can then be harvested from the fibrin monomer.

Experiments showed that 90% of the applied fibrinogen bound to the fibrin monomer gel matrix. 60 to 95% of the bound fibrinogen was eluted with a buffer of 0.05

M Tris-H_3PO_4, 2.0 M NaBr, and 0.005 M EDTA at pH 5.3. Clotting assays showed that 85 to 100% of the recovered fibrinogen was clottable.

Fibrinogen is adsorbed from plasma onto immobilized fibrin monomer with high specificity. The eluted fibrinogen appears not to be altered as shown from the clottability. However, fibrinogen is denatured in media with a pH lower than 5.5 [60]. In order to recover functional fibrinogen, it is necessary to keep the contact time with the buffer as short as possible. This technique has also been shown to remove fibrin degradation products (FDP) from plasma [57].

2.5.5.2 Heparin Matrix

Heparin immobilized on Sepharose was shown to bind fibrinogen, and binding assays showed that approximately 30 minutes were required to reach a maximal binding of ^{125}I-labeled fibrinogen [61]. Labeled fibrinogen binding was completely inhibited in the presence of NaCl at concentrations of 0.5 M and greater. At a concentration of 50 mg/mL free heparin, the binding was completely inhibited. More than 80% of the bound labeled fibrinogen was displaced in the presence of 200-fold excess of unlabeled fibrinogen.

Heparin immobilized on Sepharose is also used to remove and purify other blood proteins. Currently this technique is used commercially to produce concentrates of anti-thrombin III, an important coagulation inhibitor[62]. Anti-thrombin III is easily removed from heparin-Sepharose by gradient elution with NaCl [5].

2.5.5.3 Protamine Matrix

Fibrinogen was also shown to bind to protamine immobilized on Sepharose beads (protamine-Sepharose) [63, 64]. The fibrinogen formed a non-specific ionic bond with the immobilized protamine. Binding experiments in columns showed that 100 % of the applied fibrinogen adsorbed to the protamine-Sepharose within 10 minutes. Elution of 65-80% of the bound fibrinogen was achieved with a buffer of 0.05 M tris/HCl, 0.005 M Na_2EDTA, and 0.005 M epsilon-amino caproic acid (εACA) at a pH of 4.5. Clotting assays further showed that approximately 6.5 mg of pH 4.5-elutable fibrinogen adsorbed to 1 mL of protamine-Sepharose.

In the work reported here, preliminary attempts were made to purify and concentrate fibrinogen using protamine-Sepharose chromatography. Approximately 90% of the fibrinogen applied to a column packed with protamine-Sepharose bound to the gel. However, since 45% of the bound fibrinogen could be eluted from the gel, this approach was not used.

2.6 Fibrin Sealant Preparations

2.6.1 Commercial Sealants

The only FDA-approved, commercially-available fibrin sealant in the United States is Tisseel VH. The Tisseel VH kit is supplied with four separate vials containing freeze-dried human fibrinogen (75-115 mg/mL), freeze-dried human thrombin (500 IU/mL), bovine aprotinin solution (3000 KIU/mL), and calcium chloride solution (40

mmol/L) [23]. Also included with the kit are two identical syringes and a Duploject applicator (Figure 2.2).

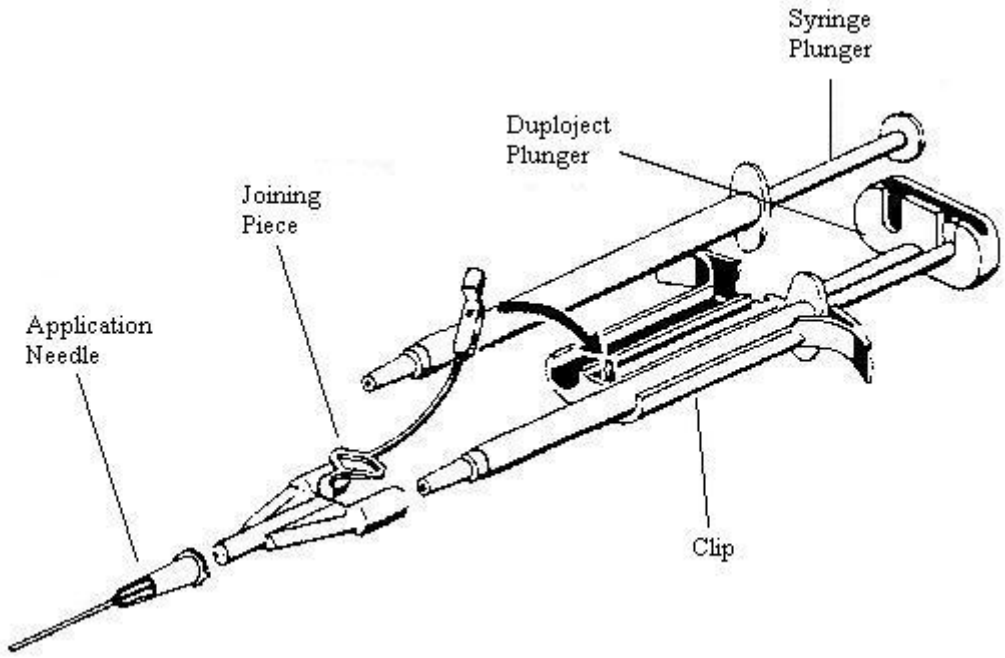

Figure 2.2: Duploject applicator[65].

The fibrinogen is reconstituted with the aprotinin solution while the thrombin is reconstituted with the calcium chloride. The fibrinogen/aprotinin is loaded into one syringe and the thrombin/calcium chloride into the other. The syringes are loaded into the Duploject applicator that has a common plunger to insure that the two components are delivered with equal volume. The solutions then travel through a common joining piece where they are mixed uniformly before being expelled through a blunt needle onto the wound site [23].

Commercial preparations have been used in Europe for over 20 years and many are available for use. Table 2.1 outlines some of the available sealants and their compositions [1, 24, 25].

Table 2.1: Composition of commercially-available fibrin sealants in Europe.

	Fibrinogen (mg/mL)	Thrombin (U/mL)	Factor XIII (U/mL)	Fibrinolytic Inhibitor
Beriplast (Aventis Behring)	65-115	400-600	40-80	Aprotinin (3000 KIU/mL)
Biocol (LFB)	127	558	11	Aprotinin (3000 KIU/mL)
Bolheal (Kaketsuken)	80	250	57	Aprotinin (1000 KIU/mL)
Hemaseel APR (Haemacure)	75-115	500	None	Aprotinin (3000 KIU/mL)
Quixil (Omrix)	60-100	1000	None	Tranexamic acid (95 mg/mL)
Tissucol (Baxter-Immuno)	70-110	500	10-50	Aprotinin (3000 KIU/mL)
VIGuard (Vitex)	50-95	200	3-5	None

Use of commercial fibrin sealant is often prohibited by cost and availability. The cost of Tisseel is $132 per mL of fibrinogen concentrate [23]. If a significant volume of sealant is needed, the use of Tisseel can quickly become cost prohibitive. Commercial sealants are available only if stocked in plentiful quantities, which occasionally may not be the case. The relevance of the latter requirement is illustrated in a recent medical log in which a surgeon related that “Fibrin glue was applied (home-made, since the only Tisseel carried at big hospital (sic) was in the 1cc amounts).” [66].

2.6.2 Home-Made Sealants

The alternative to using a commercial fibrin sealant is to use a home-made fibrin sealant. There are multiple recipes for preparing home-made fibrin sealant, all of which

consist of mixing plasma or cryoprecipitate with bovine thrombin [23, 67-69]. The thrombin component is mixed with calcium chloride and loaded into a syringe. The fibrinogen component is loaded into a second syringe. In most applications the contents of the two syringes are applied simultaneously [69]. In other methods, the two syringes are attached to a multiple lumen catheter and the two syringes are squirted simultaneously, which mixes the two solutions and dispenses the sealant onto the wound site [67]. Because home-made sealants are made from plasma or cryoprecipitate, they have much lower fibrinogen concentrations than found in commercial sealants.

2.6.3 Risk of Virus Transmission

The safety of commercial fibrin sealants is ensured by four complementary approaches: (1) careful selection of blood donors; (2) extensive testing of donated blood for viral markers; (3) rigorous removal of infectious human viruses by a range of protein purification methods; and (4) the use of virus-inactivation methods during the manufacturing process [70]. Virus inactivation methods used are solvent/detergent treatment and vapor heat treatment [1, 70]. These techniques are effective against viruses with a lipid envelope, such as HIV, hepatitis B and C, but are less effective against viruses without a lipid envelope, such as hepatitis A and parvovirus B19 [71]. Tisseel VH has been used in over 8 million procedures worldwide without any confirmed cases of HIV; hepatitis A, B, or C; or Creutzfeldt-Jakob Disease to date [72]. There have been reported cases of parvovirus B19 transmission from commercial sealants [73, 74], which has been remedied by implementing more rigorous testing of donated blood.

When cost or availability prohibits the use of commercial sealants, home-made sealants are used. Blood donated at a blood bank undergoes less rigorous testing for viral markers than plasma used in commercial fibrin sealants. Furthermore, blood-bank blood does not undergo protein purification and virus-inactivation methods used in commercial fibrin sealants and is associated with a higher risk of viral transmission [70].

2.7 In-Vitro Evaluations

2.7.1 Tensile Strength

The clinical performance of a fibrin sealant is influenced by its physical properties such as tensile strength and adhesion strength [75]. Many factors including fibrinogen concentration, calcium concentration, the presence of other plasma proteins, ionic strength, and temperature affect the rate and extent of fibrin polymerization and, ultimately, the mechanical properties [25]. The optimization of these properties is important to both the manufacturers and surgeons.

Tensile strength is mainly a function of fibrinogen concentration and is frequently used as an indicator of quality [76]. A number of different models have been proposed to evaluate the tensile strength of fibrin sealant.

Rubenstein [77] reported forming cylindrical clots in siliconized glass tubes for testing tensile strength. The clots were cured for 30 minutes and removed from the tubes. The two ends of the cylindrical clots were placed in clamps, and a cup was attached to the

bottom clamp. Weights were added to the cup until the clot ruptured. The tensile strength was calculated from the amount of weight required to rupture the clot.

Nowotny, *et al*. [78] and Velada *et al*. [79] molded clots for tensile strength testing. Clots were formed by injecting fibrinogen and thrombin into a mold. The clots were removed from the mold and placed in an Instron machine. The tensile strength was determined by pulling apart the clots and measuring the force required to rupture the clot.

2.7.2 Adhesion Strength

Adhesion strength is also a function of fibrinogen concentration and is a good indicator of how a fibrin sealant will perform in sealing a wound. Many different methods have been proposed for testing the adhesion strength of a fibrin sealant. Some of these methods consist of using silastic [76] or other artifical materials [46]. These models have little clinical relevance as they do not measure the adhesion of fibrin sealant to tissue.

Silver, *et al*. [47] have measured the adhesion strength of fibrin sealant using collagen. Collagen films were made and cut into two equal halves. The two halves were overlapped by 1 cm and fibrin sealant was applied between the overlapping pieces. The films were cut into 1 cm wide strips and put into an Instron machine. Adhesion strength was calculated as the strength required to cause the overlapping joints to fail.

Dickneite, *et al*.[25], Kjaergard, *et al*.[75], and Laitakari, *et al*. [80] have all reported measuring the adhesion strength with animal or human tissue. Fresh rat skin, porcine skin, human dura, or human vascular tissue were affixed to small blocks with sutures or pins. Fibrin sealant was applied between two sample blocks and allowed to

cure. The adhesion strength was measured as the maximum force required to pull apart the sample blocks.

2.8 Animal Model Evaluation

Although the in-vitro mechanical properties of a fibrin sealant are good indicators of quality and performance, a better indicator is how well the sealant stops bleeding in an in-vivo animal wound model. Many different types of animal models have been employed to evaluate fibrin sealant.

A kidney excision model has been reported for evaluating fibrin sealant in rats and rabbits [25-27, 81]. The animal was anesthetized and the abdomen was opened and the renal artery was clamped off to stop blood flow to the kidney. A piece of the kidney, generally 20%, was excised and any blood loss from the wound was absorbed with gauze. Fibrin sealant was applied to the wound area and allowed to cure for a predetermined amount of time. The artery clamps were removed and blood flow to the organ was restored. The fibrin sealant performance was evaluated by blood loss which was measured by soaking up blood with pieces of pre-weighed gauze. This model does not evaluate how well a fibrin sealant performs when applied to a bleeding wound, because blood flow to the kidney was stopped before the sealant was applied.

An aorta/artery bleeding model has been used to evaluate the efficacy of fibrin sealant in rabbits and dogs [82-84]. The animal was anesthetized and the abdomen was opened. The aorta or other artery was clamped proximally and distally to occlude blood flow and was severed in the middle. The vessel was sutured back together with sutures

placed 0.3 cm apart. Fibrin sealant was applied and allowed to cure for a predetermined amount of time. Blood flow was restored by removing the clamps and blood loss was measured by soaking up blood with pieces of pre-weighed gauze. As in the kidney excision model, this model provides a good evaluation of how much pressure a fibrin sealant can withstand, but does not address how well a fibrin sealant performs when applied to a bleeding wound.

A liver incision model has been proposed as an evaluation of fibrin sealant performance in rats [34, 44]. The animal was anesthetized and the abdomen was opened, exposing the liver. A 1 to 3 cm long incision was made in the liver, and fibrin sealant was applied. Blood loss was measured by soaking up blood with pieces of pre-weighed gauze. This model provides a good evaluation of how well a fibrin sealant performs when applied to a bleeding wound. Because the average blood pressures are much lower in the kidney[3], this model is not a good measure of how much pressure a fibrin sealant can withstand. Extending this model to the kidney would evaluate how well a fibrin sealant performs when applied to a higher pressure bleeding wound.

2.9 What is Missing?

Fibrin sealants have been shown to be very effective in reducing blood loss during and after surgery. Purifying and concentrating fibrinogen remains the limiting step in the production of fibrin sealant. Conventional methods currently used, such as cryoprecipitation and chemical precipitation, present many drawbacks such as long processing time, large blood volume, and the use of chemicals that can be toxic to the

body. Commercial sealants are available, but carry the burden of high cost and availability. Home-made sealants suffer from a higher risk of virus transmission and low fibrinogen concentrations.

From the discussion it is obvious that a method to rapidly and inexpensively purify and concentrate autologous fibrinogen is needed. Protamine has been shown to precipitate up to 100 % of fibrinogen from plasma. Because of this, protamine was considered an excellent candidate for use in preparing autologous fibrinogen. This dissertation reports analysis of fibrinogen concentrate prepared by protamine precipitation based on in-vitro mechanical properties and hemostatic efficacy in an animal model.

CHAPTER 3: OBJECTIVES

The overall goal of the research was to evaluate fibrinogen concentrate prepared from protamine precipitation for use in a fibrin sealant. This overall goal was divided into the following 5 objectives:

1. Develop a method to rapidly harvest and concentrate fibrinogen with protamine and evaluate the concentrate for fibrinogen and factor XIII content and functionality.
2. Develop in-vitro models to evaluate the tensile strength and adhesion strength of a fibrin sealant. Evaluate the quality of the precipitated fibrinogen by evaluating the tensile strength and adhesion strength using these models.
3. Evaluate the kinetic behavior of the precipitated fibrinogen.
4. Determine the hemostatic efficacy of the protamine-fibrinogen concentrate in an animal model.
5. Mathematically model the performance of the fibrin sealant in an animal model to optimize the fibrin sealant operating conditions.

CHAPTER 4: PREPARATION AND EVALUATION OF FIBRINOGEN CONCENTRATE

4.1 Experimental Methods

Blood was collected from healthy adult human donors by venapuncture into sodium citrate (Sigma Chemical Co., St. Louis, MO; final concentration 0.38 g/100mL) and centrifuged for 30 minutes at 1200 *g* to obtain platelet-poor plasma (PPP). The plasma was used immediately or stored at -80 ^{o}C.

Fibrinogen was precipitated from pooled human plasma (minimum of eight donors) by addition of protamine sulfate (Sigma Chemical Co., stock concentration 40 mg/mL). Following the addition of protamine, the plasma was centrifuged at 1000 *g* for 5 min to sediment the precipitate. The plasma was then decanted, and the remaining precipitate was dissolved in the same volume (as the initial plasma) of 0.2 M sodium citrate (37^{o}C, pH 7.4). Fibrinogen concentrations in the starting plasma and concentrate were evaluated with an enzyme-linked immunosorbent assay (ELISA) (AssayPro LLC, Brooklyn, NY). Samples were added to wells pre-coated with an antibody specific for fibrinogen and competed with a biotinylated fibrinogen for antibody binding sites. The sample fibrinogen was sandwiched between the capture antibody and a streptavidin-peroxidase conjugate. All unbound material was washed away and a peroxidase enzyme

substrate was added resulting in color change. The color development was stopped and the intensity of the color was measured with a Dynex MRX microplate reader (Dynex Technologies, Chantilly, Virginia). Sample fibrinogen concentrations were calculated from a standard curve. The percentage of fibrinogen harvested was taken to be the ratio of fibrinogen in the concentrate to the fibrinogen in the initial plasma sample.

4.2 Effect of Protamine Concentration

The final concentrations of protamine in the plasma were varied from 9 mg/mL to 12 mg/mL as guided by the literature [53, 54] to obtain the optimal yield of fibrinogen. At a plasma protamine concentration of 10 mg/mL, 96% ± 4% (n=4) of the fibrinogen in the plasma was found in the concentrate (Figure 4.1). The molar concentration of protamine in the plasma was 2000 μM compared to the molar concentration of fibrinogen of 8.8 μM. Plasma concentrations of protamine below 10 mg/mL did not precipitate all of the fibrinogen in the plasma. Plasma concentrations above 10 mg/mL resulted in a precipitate comprised of very tiny aggregates that would not completely dissolve back into solution.

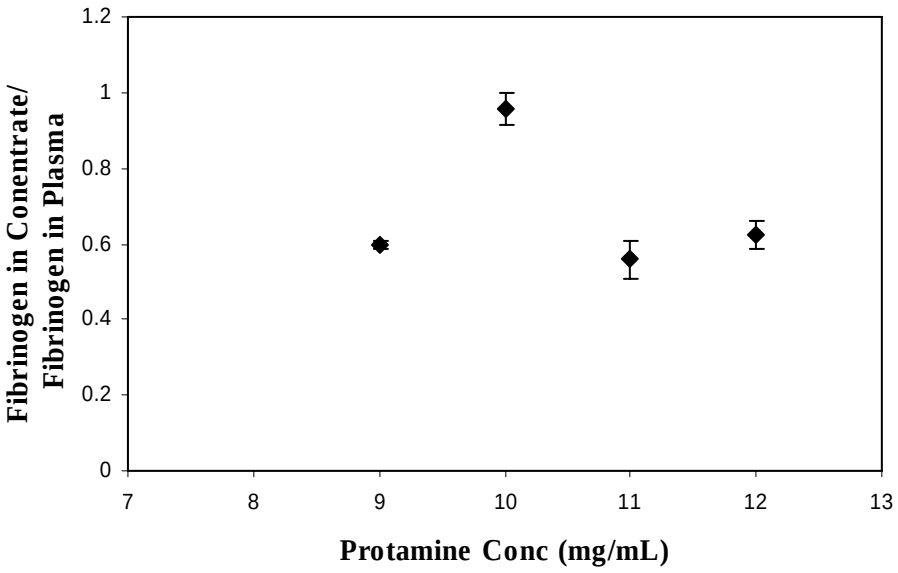

Figure 4.1: The amount of fibrinogen precipitated as a function of the final protamine concentration in the plasma (n=4). The results are displayed as the ratio of fibrinogen in the concentrate to fibrinogen in the original plasma. At a protamine concentration of 10 mg/mL ~96% of the fibrinogen in the original plasma precipitated. Data are shown as mean values ± SD.

4.3 Effect of Temperature

The temperature-dependent nature of the fibrinogen precipitation was investigated by bringing the plasma protamine concentration to 10 mg/mL in plasma samples at 37, 22, 15, and 7 ^{o}C. At a plasma temperature of 22 ^{o}C (room temperature), 96% ± 4% (n=4) of the fibrinogen was found in the precipitate (Figure 4.2). Plasma temperatures lower than room temperature did not increase the yield of fibrinogen in the concentrate. At a plasma temperature of 37 ^{o}C (body temperature), 75% ± 6% of the fibrinogen in the plasma was found in the concentrate.

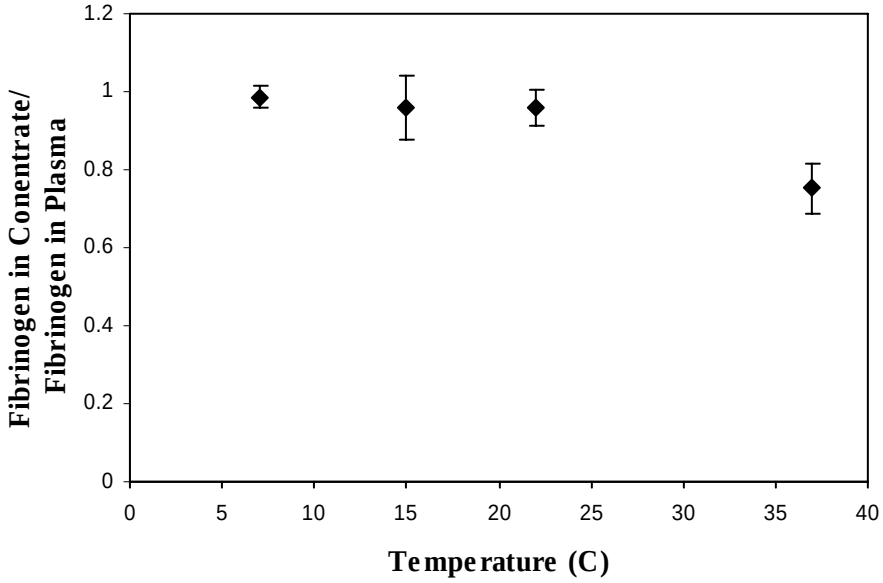

Figure 4.2: The amount of fibrinogen precipitated as a function of temperature (n=4). The results are displayed as the ratio of fibrinogen in the concentrate to fibrinogen in the original plasma. At temperatures of 22°C and below, ~96% of the fibrinogen in the original plasma was precipitated. This decreased to ~75% when the temperature was raised to 37°C. Data are shown as mean values ± SD.

4.4 Evaluation of Factor XIII

The presence of factor XIII has been shown to significantly improve the in-vitro and in-vivo performance of a fibrin sealant [21, 26-28]. Earlier publications have indicated that protamine may bind to factor XIII [63, 64], suggesting that factor XIII may also be harvested by the protamine-fibrinogen precipitation method investigated here.

The fibrinogen concentrate was evaluated for factor XIII content. Protamine was added to room temperature plasma to a concentration of 10 mg/mL. The amount of factor XIII in the initial plasma and in the fibrinogen concentrate was determined with ELISA. Samples were added to wells pre-coated with an antibody specific for factor XIII. The sample factor XIII was sandwiched between the capture antibody and a

biotinylated polyclonal antibody specific for factor XIII, which was recognized by a streptavidin-peroxidase conjugate. All unbound material was washed away and a peroxidase enzyme substrate was added resulting in color change. The color development was stopped and the intensity of the color was measured with a Dynex MRX microplate reader. Sample factor XIII concentrations were calculated from a standard curve. At these conditions, 47% ± 0.6% (n=4) of the factor XIII in the initial plasma was found in the concentrate.

4.5 Clottability of Precipitated Proteins

To confirm the clottability of the recovered fibrinogen, 1 mL of fibrinogen concentrate was mixed with 100 μL of bovine thrombin (Vital Products, Inc, Boynton Beach, FL, 500 Units/mL) and allowed to clot. The clot was centrifuged for 2 min at 3500 *g* and the supernatant was drawn off. The amount of fibrinogen in the clot supernatant and the amount of fibrinogen in the concentrate were measured with ELISA. The fraction of clottable fibrinogen was taken to be the ratio of the difference between the amount of fibrinogen in the concentrate and clot supernatant to the amount of fibrinogen in the concentrate. The clottablility of the fibrinogen was found to be 98% ± 0.9% (n=4).

Factor XIII requires Ca^{2+} as a cofactor in order to participate in clotting [3]. To evaluate the clottability of the harvested factor XIII, the above process was repeated with the addition of calcium chloride (Spectrum Quality Products, Inc., Gardena, CA). The amount of fibrinogen and factor XIII in the clot supernatant and the amount of fibrinogen

and factor XIII in the concentrate were measured with ELISA. No change in the clottability of the fibrinogen was observed when calcium chloride was added to the concentrate. 30% ± 1% of the precipitated factor XIII participates in clotting (n=4).

4.6 Effect of Heparin

Fibrin sealants are routinely used in surgery that requires the patient to be anticoagulated with heparin. The amounts and clottability of fibrinogen and factor XIII harvested from plasma that was anticoagulated with heparin were measured. Blood was drawn into porcine heparin (ESi Pharmaceuticals, Cherry Hill, NJ; final concentration 2 U/mL) and centrifuged for 30 minutes at 1200 *g* to obtain PPP. Protamine was added to a known amount of plasma to bring the plasma concentrations to 10, 11, and 12 mg/mL. Fibrinogen concentrate was prepared as previously described. The amounts of fibrinogen and factor XIII in the concentrate were measured with ELISA.

The maximum yield of fibrinogen occurred at a protamine concentration of 11 mg/mL in plasma in contrast to 10 mg/mL seen when no heparin was present, precipitating 95% ± 1% (n=4) of the fibrinogen in the plasma. At this protamine concentration, 31% ± 3% (n=4) of the factor XIII in the plasma was found in the concentrate. The observed increase in protamine concentration needed to maximize the yield of harvested fibrinogen was expected because protamine binds to heparin as well as to fibrinogen. Protamine is used to bind to and neutralize the anticoagulating effect of heparin in patients following cardiopulmonary bypass surgery [85]. 1 mg of protamine will neutralize 100 U of heparin. The increase in protamine concentration is required to

bind all of the heparin along with binding the fibrinogen. There were no observed changes in protein clottability when the heparin was present.

CHAPTER 5: IN-VITRO MECHANICAL PROPERTIES

The clinical performance of a fibrin sealant is influenced by its physical properties such as tensile strength and adhesion strength [75]. Many factors including fibrinogen concentration, calcium concentration, the presence of other plasma proteins, ionic strength, and temperature affect the rate and extent of fibrin polymerization and, ultimately, the mechanical properties [25]. This chapter will detail the development of a model to evaluate the tensile strength and a model to evaluate the adhesion strength and the optimization of these properties.

5.1 Tensile Strength

5.1.1 Experimental Method

Fibrin clots for tensile strength testing were cast in a mold as shown in Figure 5.1. Two separate dies made of plexiglass, held together with screws, formed the mold. Removable plexiglass holders were put into both ends of the mold and sealed with O-rings. Stiff sponges were used to provide an anchor for the clot to form around and were tucked into the holders and held in place with bolts. The sample diameter was 2 mm in the center and 6.5 mm at the ends, the length was 31 mm, and the mold had a total volume of 1.5 mL. The samples were prepared by continuously emptying a syringe of

fibrinogen and a syringe of thrombin into a common duct where they were mixed. The mixture then entered the mold through the sponges until it discharged from the opposite end. Care was taken to ensure that air bubbles weren't formed in the clot as this would weaken the sample. The plexiglass dies were removed after the sample had cured for 30 minutes, and the sample and holders were transferred to an Instron Model 1120 Universal Testing Instrument (max load 500 g). Stress-strain was recorded while the sample was strained at 100 mm/min until it ruptured. Tensile strength was taken as the maximum stress sustained.

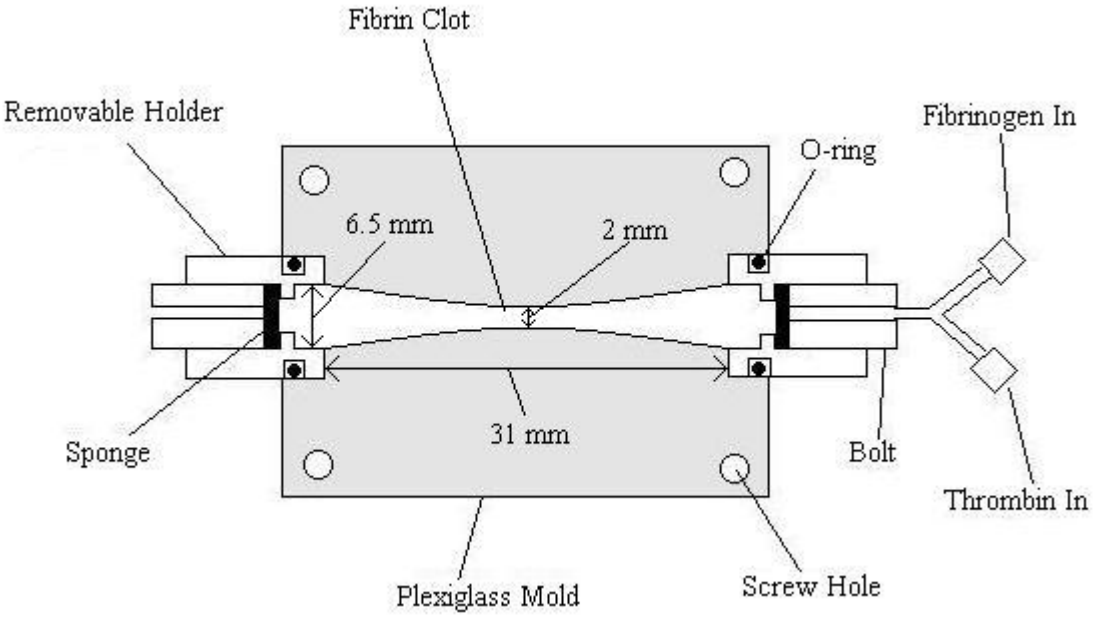

Figure 5.1: Mold for casting clots to test for tensile strength. Fibrinogen and thrombin were injected into the mold as shown. The plexiglass mold was removed for tensile testing.

5.1.2 Effect of Thrombin

The effect of the amount of thrombin on tensile strength was determined by holding the fibrinogen concentration constant and changing the amount of thrombin used.

Samples were prepared from a 15 mg/mL fibrinogen concentrate and 75, 100, or 125 U of bovine thrombin (final concentrations 45, 60, 75 U/mL clot). Clots were cast into the molds and cured for 30 min. No significant differences (p=0.51) were observed between the three different amounts of thrombin (Figure 5.2). The rest of the tensile strength experiments were performed with a final thrombin concentration of 60 U/mL clot.

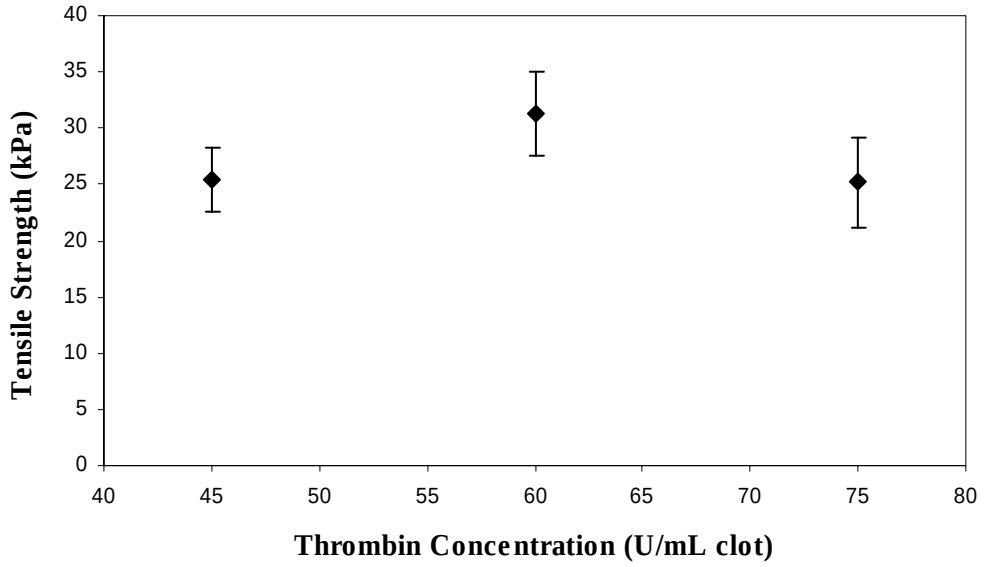

Figure 5.2: Tensile strength as a function of final thrombin concentration in the clot (n=4). Fibrinogen concentration was 15 mg/mL. No significant differences (p=0.51) in tensile strength were observed at the different thrombin concentrations. Data are shown as mean values ± SD.

5.1.3 Effect of Fibrinogen Concentration

To evaluate the effect of fibrinogen concentration on tensile strength, samples were prepared with fibrinogen concentrations of 15, 30, 45, and 60 mg/mL. Controls of pooled plasma (3 mg/mL), pure fibrinogen (15 mg/mL), and Tisseel (average concentration 95 mg/mL) were used. Clots were cast in the molds and cured for 30 min.

Tensile strength was found to increase approximately linearly with increasing fibrinogen concentration as shown in Figure 5.3.

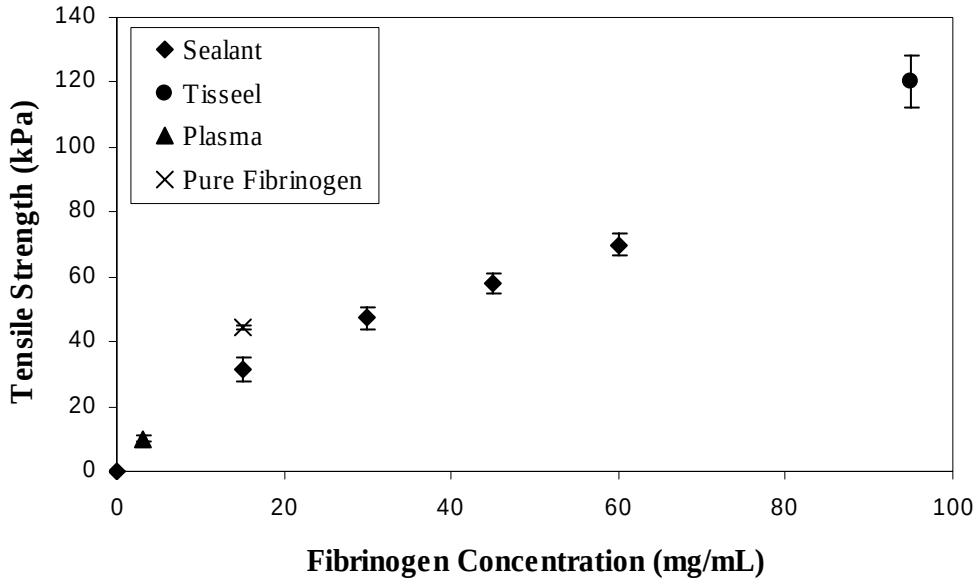

Figure 5.3: Tensile strength as a function of fibrinogen concentration (n=4). Controls of plasma, pure fibrinogen, and Tisseel were used. The tensile strength increased linearly with fibrinogen concentration. The tensile strength of plasma and Tisseel lie near the curve. The tensile strength of pure fibrinogen was significantly greater than that of the protamine-fibrinogen ($p<0.05$). Data are shown as mean values ± SD.

The tensile strength of the samples formed with plasma and Tisseel fall near the curve with the samples formed with the protamine-fibrinogen concentrates. The tensile strength of the 15 mg/mL pure fibrinogen sample was significantly higher than that of the 15 mg/mL protamine-fibrinogen sample ($p<0.05$). The major difference between these two concentrate preparations is the precipitation with protamine.

To verify that the addition of protamine adversely affected the tensile strength, samples were prepared from a 15 mg/mL pure fibrinogen concentrate and a 15 mg/mL

fibrinogen concentrate prepared by protamine precipitation of pure fibrinogen (Figure 5.4). The fibrinogen concentration of each sample was measured to ensure that the fibrinogen concentrations were the same. The tensile strength of the precipitated pure fibrinogen was significantly lower than the pure fibrinogen ($p<0.05$). This was due to the presence of protamine in the concentrate.

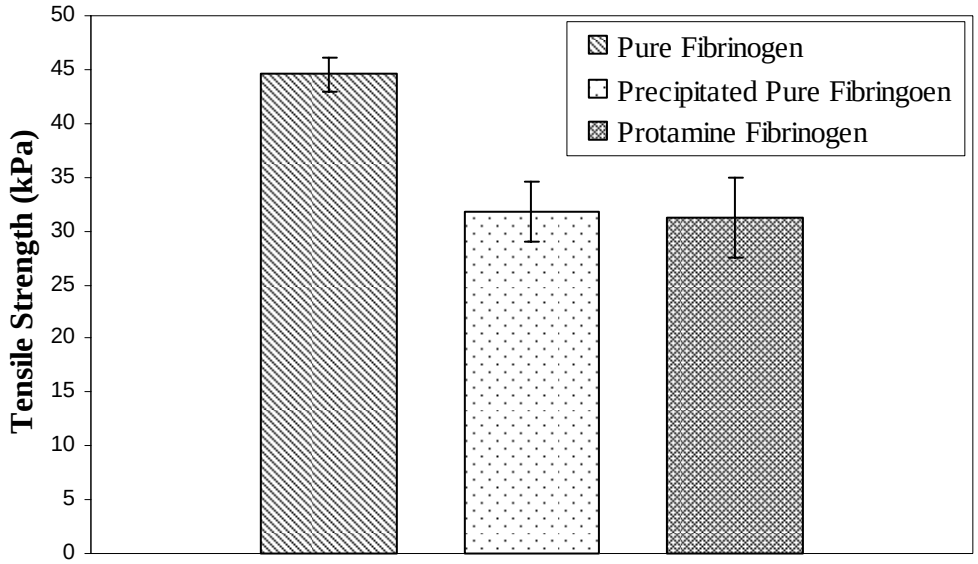

Figure 5.4: Tensile strength of 15 mg/mL fibrinogen concentrates prepared from pure fibrinogen, precipitated pure fibrinogen, and protamine-fibrinogen (n=4). Precipitation of the pure fibrinogen concentrate significantly lowered the tensile strength ($p<0.05$). Data are shown as mean values ± SD.

5.1.4 Effect of Calcium

The effect of calcium on clot tensile strength was investigated by adding calcium chloride to a 15 mg/mL solution of protamine-fibrinogen. Final concentrations of calcium chloride in the fibrinogen concentrate were 0, 0.0036, 0.0089, 0.018, 0.035, 0.05, 0.075, and 0.1 M. Clots were cast in the molds and cured for 30 min. The concentration

of calcium chloride in the concentrate yielding the highest tensile strength was in the range of 0.0089 to 0.05 M (Figure 5.5).

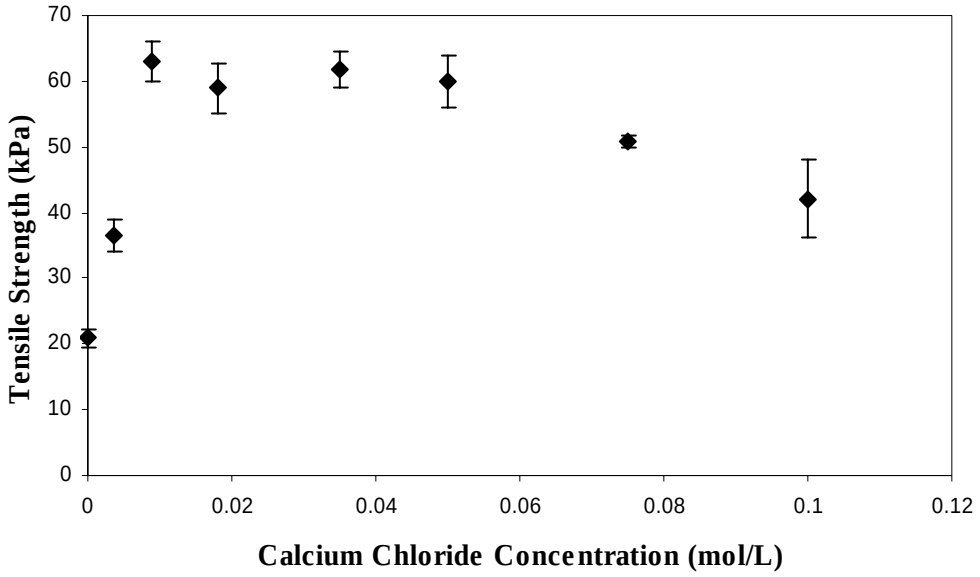

Figure 5.5: Tensile strength as a function of calcium chloride concentration (n=4). A fibrinogen concentration of 15 mg/mL was used. The tensile strength reached a maximum in the range of 0.0089 M to 0.05 M calcium chloride. Above 0.05 M calcium chloride, the tensile strength decreased. Data are shown as mean values ± SD.

The observed increase in clot tensile strength when calcium chloride was added to the fibrinogen concentrate was probably due to the calcium-dependent effect of factor XIII. The concentration of factor XIII in the 15 mg/mL protamine-fibrinogen concentrate was approximately 20 μg/mL. It has been shown that factor XIII requires calcium as a cofactor to crosslink fibrin [6] and increases tensile strength [26-28]. There was no calcium present in the fibrinogen concentrate before adding calcium chloride, so the cofactor required for factor XIII to function was not available. After calcium chloride was added, factor XIII had the required cofactor and could function properly.

Calcium chloride concentrations below 0.0089 M were insufficient to produce the maximum tensile strength. At these concentrations, most of the calcium was probably bound to the free sodium citrate in the protamine-fibrinogen concentrate. Once the threshold of 0.0089 M was reached, enough calcium was present to bind all of the free sodium citrate with excess to provide a cofactor for factor XIII. At calcium chloride concentrations above 0.05 M, enough free calcium was probably present in the protamine-fibrinogen concentrate to displace sodium citrate from protamine, resulting in partial precipitation of the fibrinogen and reduction in the tensile strength.

To verify that the increase in tensile strength when calcium chloride was added to the fibrinogen concentrate was due to factor XIII, the tensile strength of samples prepared from pure fibrinogen with and without factor XIII and calcium was measured. Samples were prepared from a 15 mg/mL pure fibrinogen concentrate with nothing added, 0.0089 M calcium chloride, 10 μg/mL factor XIII, and 10 μg/mL factor XIII with 0.0089 M calcium chloride (Figure 5.6). The increase in tensile strength to approximately 90 kPa when factor XIII and calcium were added together (Figure 5.6) more than explains the effect of calcium summarized in Figure 5.5.

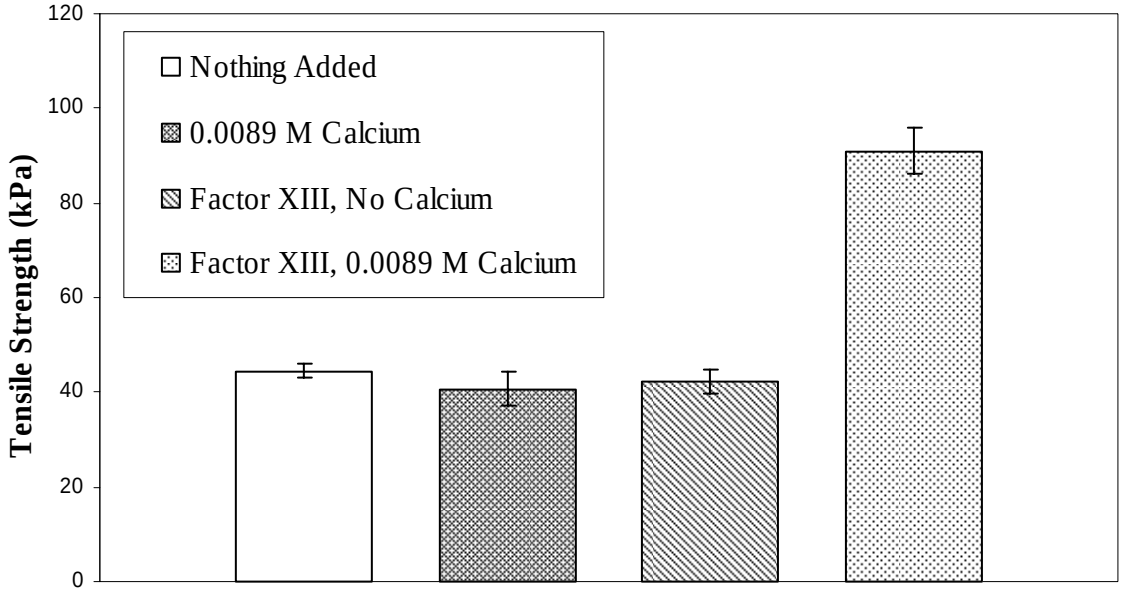

Figure 5.6: Tensile strength as a function of factor XIII content and calcium content (n=4). Fibrinogen concentration of 15 mg/mL was used. 0.0089M Calcium and 10 μg/mL factor XIII alone did not cause an increase in tensile strength. When calcium and factor XIII were added together, the tensile strength increased significantly over the other samples ($p<0.05$). Data are shown as mean values ± SD.

The combined effect of fibrinogen concentration and calcium on tensile strength was evaluated by preparing samples with fibrinogen concentrations of 15, 30, 45, and 60 mg/mL and a calcium chloride concentration of 0.0089 M. Controls of pooled plasma (3 mg/mL), pure fibrinogen (15 mg/mL), and Tisseel (average concentration 95 mg/mL) were used (Figure 5.7).

At each fibrinogen concentration the addition of calcium chloride to the protamine-fibrinogen concentrate significantly increased the tensile strength compared with the protamine-fibrinogen concentrate without calcium ($p<0.05$). The tensile strength of protamine-fibrinogen with calcium was greater than that of Tisseel even with one-half of the fibrinogen concentration. This was probably because Tisseel does not contain any factor XIII. No change in tensile strength was observed when calcium chloride was

added to pure fibrinogen, which was expected because pure fibrinogen contained no factor XIII.

No change in tensile strength was also observed when calcium chloride was added to citrated plasma. This may have been because free calcium was already present in the citrated plasma even when no calcium chloride was added. This would have led to factor XIII activity in both cases of no calcium chloride and with calcium chloride. This observation may also have been due to the low factor XIII concentration in plasma. The plasma concentration of factor XIII is 10 μg/mL [3] while the factor XIII concentration in 15, 30, 45, and 60 mg/mL protamine-fibrinogen concentrates was approximately 20, 50, 70, and 95 μg/mL

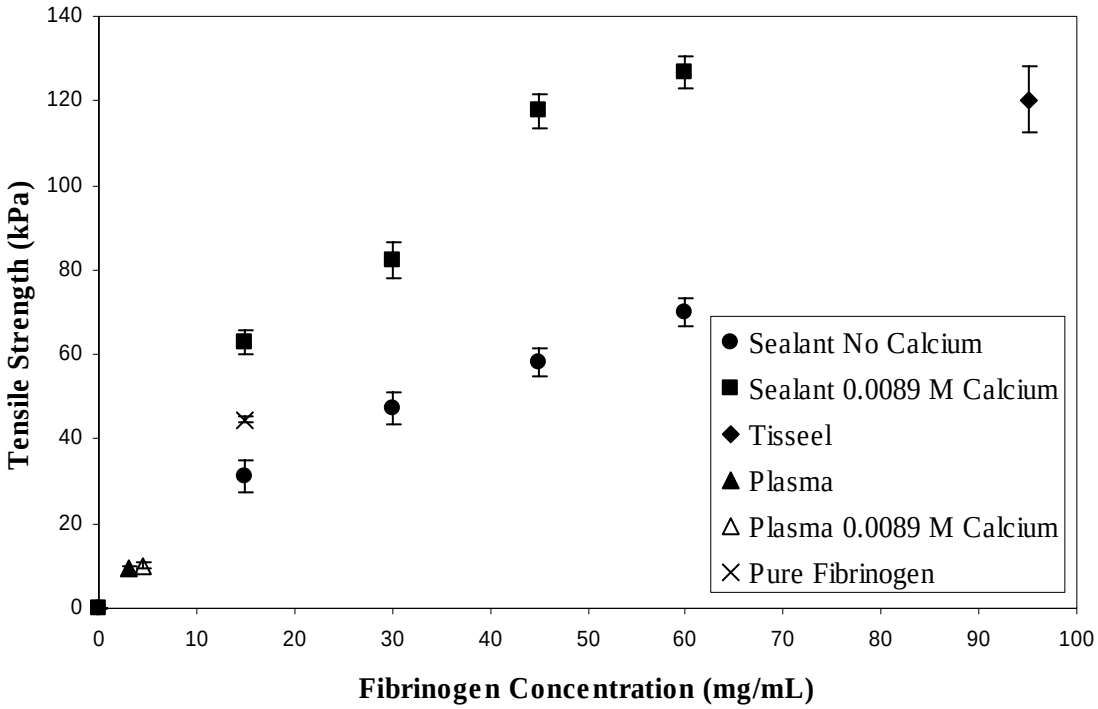

Figure 5.7: Tensile strength as a function of fibrinogen concentration with (0.0089 M) and without calcium chloride (n=4). Controls of plasma, plasma with 0.0089 M calcium, pure fibrinogen, and Tisseel were used. Adding calcium significantly increased the tensile strength as compared to pure fibrinogen, Tisseel, and fibrinogen without calcium ($p<0.05$). Data are shown as mean values ± SD.

5.1.5 Effect of Cure Time

The effect of cure time on the tensile strength was evaluated by casting clots in the molds and allowing them to cure for 1, 5, 10, 15, 30, and 60 minutes. Samples were prepared from a 15 mg/mL fibrinogen concentrate with 0.0089 M calcium chloride and without calcium chloride.

Clots prepared from fibrinogen to which calcium was added reached a maximum tensile strength in 1 minute (Figure 5.8). It is possible that the cure time required for clots with calcium is less than 1 minute, however the shortest cure time that could be measured was 1 minute. Clots prepared without calcium chloride reached a maximum tensile strength in about 5 minutes.

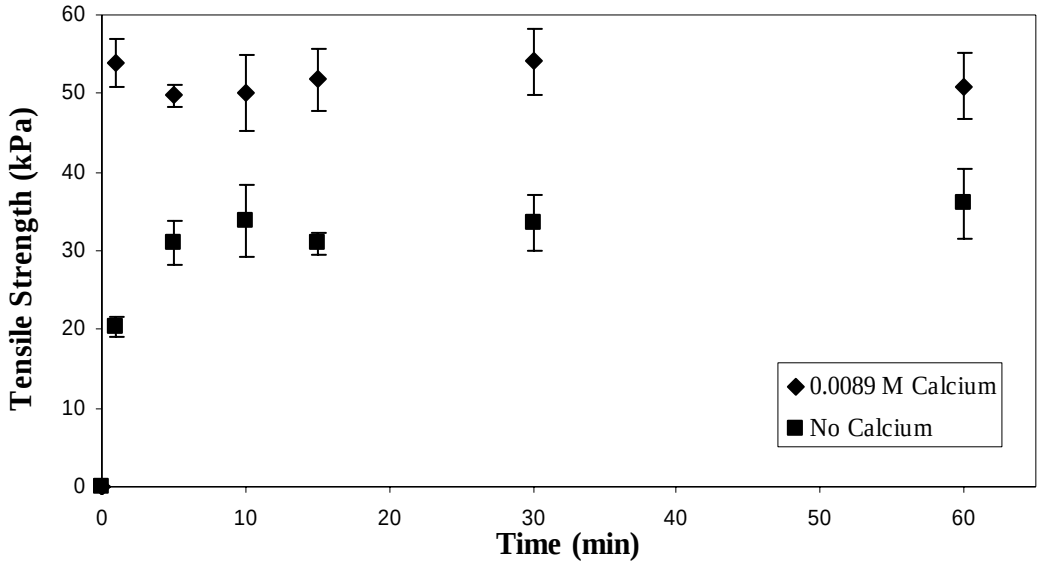

Figure 5.8: Tensile strength as a function of cure time with (0.0089 M) and without calcium chloride (n=4). Fibrinogen concentration was 15 mg/mL. Sealant was kept at 37°C and samples were cured at 22°C. Samples containing calcium chloride reached a maximum tensile strength in 1 minute or less. Samples without calcium chloride required 5 minutes to reach the maximum strength. Data are shown as mean values ± SD.

5.1.6 Effect of Aprotinin and ε-Aminocaproic Acid

The effect of the presence of fibrinolytic inhibitors on tensile strength was investigated. Samples were prepared from a 15 mg/mL fibrinogen concentrate with 0.0089 M calcium chloride and without calcium chloride. Aprotinin (Trasylol Injection, Bayer Corp., West Haven, CT) was added to the fibrinogen concentrate to bring the total concentration to 3000 KIU (Kallikrein Inhibitor Units)/mL. ε-Aminocaproic (Sigma Chemical Co.) was added to the fibrinogen concentrate separate from aprtotinin to bring the total concentration to 10 mg/mL. There were no significant changes in tensile strength upon addition of the antifibrinolytic agents (Figure 5.9).

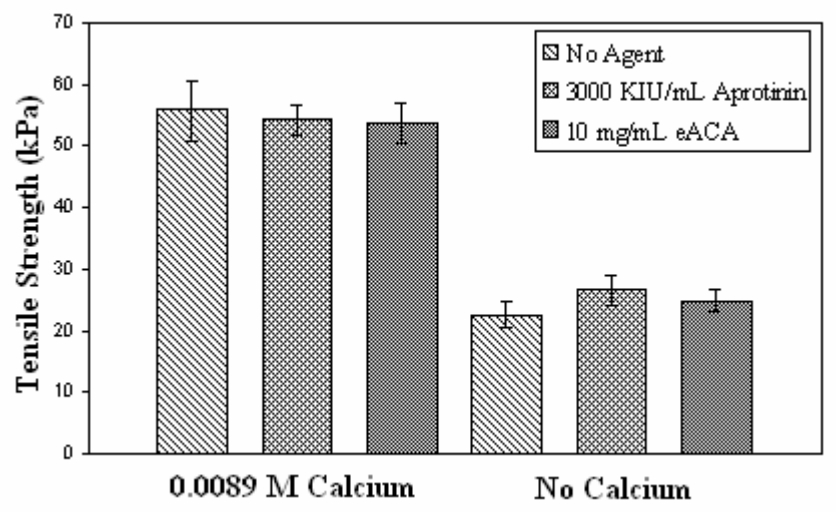

Figure 5.9: Tensile strength of samples prepared with antifibrinolytic agents with (0.0089M) and without calcium chloride (n=4). Fibrinogen concentration of 15 mg/mL was used. Antifibrinolytic agents used were aprotinin and epsilon-aminocaproic acid (eACA). No significant differences in tensile strength were observed when the antifibrinolytic agenets were added. Data are shown as mean values ± SD.

5.2 Adhesion Strength

5.2.1 Experimental Method

Samples for adhesion strength testing were prepared by sealing together two pieces of vascular tissue and then pulling them apart, simulating how a sealant would perform when applied to cut tissue. Bovine aorta was obtained from a local abattoir and was prepared in four steps as shown in Figure 5.10. First, a lengthwise incision was made through the aorta wall and the aorta was laid flat. The aorta was then cut into smaller strips. Each strip was approximately 3 cm long and 1 cm wide. Because clots would not adhere to the endothelial lining of the intima, it was necessary to split each strip down the length of the wall. Each strip was cut down the length of the wall between the adventitia and intima, yielding a strip with media on one side and intima on the other and a strip with media on one side and adventitia on the other. Sealant was applied (0.1 mL), covering an approximate area of $1cm^2$, between the medial linings of the aorta strips and a 100 g weight was placed on top of the overlapping joint. The exact area of the overlapping joint was carefully measured with a digital caliper. The samples were allowed to cure for 30 minutes and then placed in an Instron Model 1120 Universal Testing Instrument (max load 500 g). Stress-strain was recorded while the sample was strained at 100 mm/min until the overlapping joint failed. Adhesion strength was taken as the maximum stress sustained.

The adhesion model provided a good simulation of sealant being applied to cut tissue. The model did not provide a good simulation of fibrin sealant being applied to sutured vascular tissue or skin.

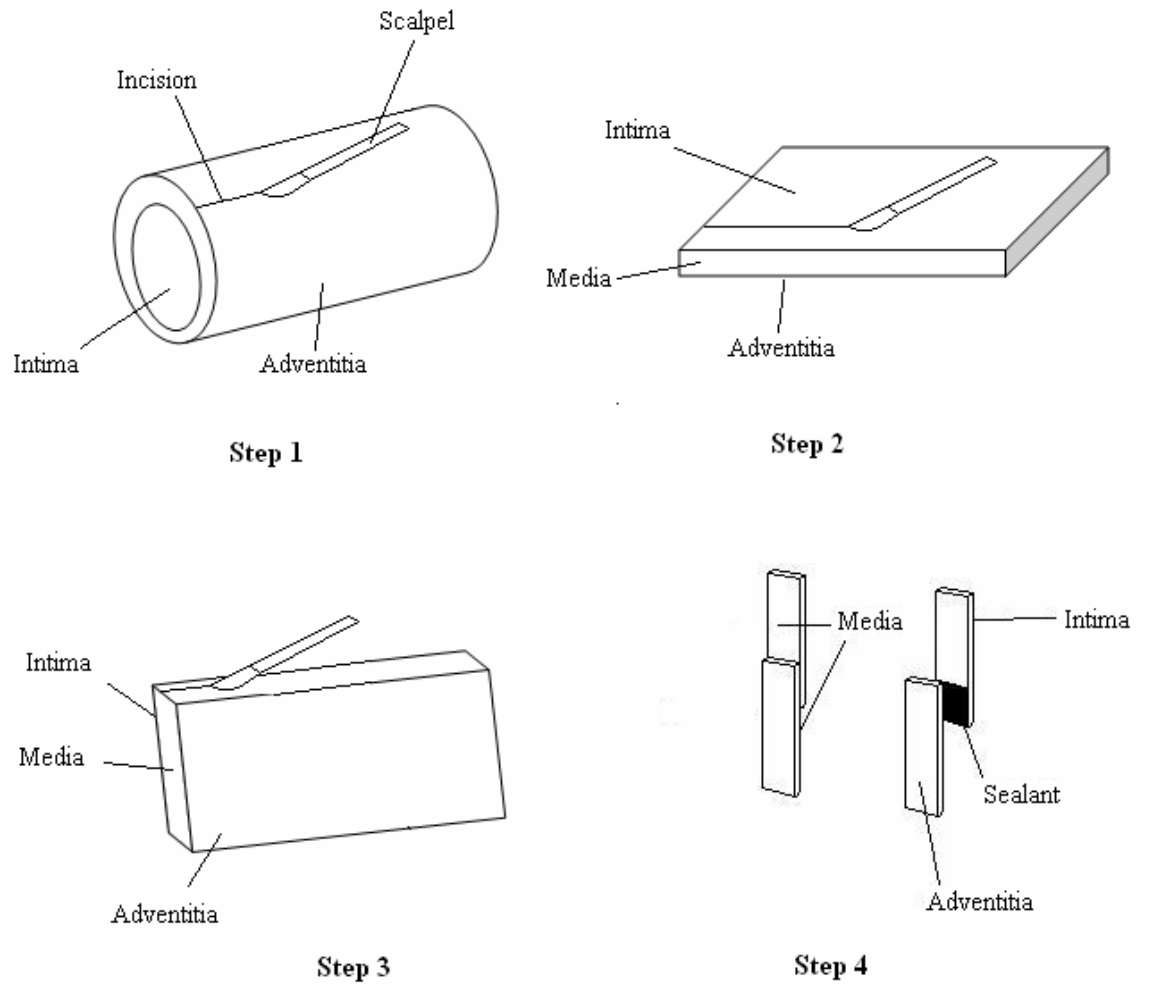

Figure 5.10: Steps in preparing samples for adhesion strength testing. Step 1) the aorta was sliced lengthwise and laid open. Step 2) the aorta was cut into strips. Step 3) each strip was split down the media to give two strips of aorta. Step 4) sealant was applied to the media and the strips were overlapped.

5.2.2 Effect of Thrombin

The effect of the amount of thrombin on adhesion strength was determined by keeping the fibrinogen concentration constant and changing the amount of thrombin used. Sealant was prepared as previously described (see Chapter 4). Samples were prepared from a 15 mg/mL fibrinogen concentrate and 75, 100, or 125 U of bovine thrombin (final concentrations 45, 60, 75 U/mL clot). No significant differences ($p=0.74$) were observed

between the adhesion strengths from the three different amounts of thrombin (Figure 5.11). The rest of the adhesion strength experiments were performed with a final thrombin concentration of 60 U/ml clot.

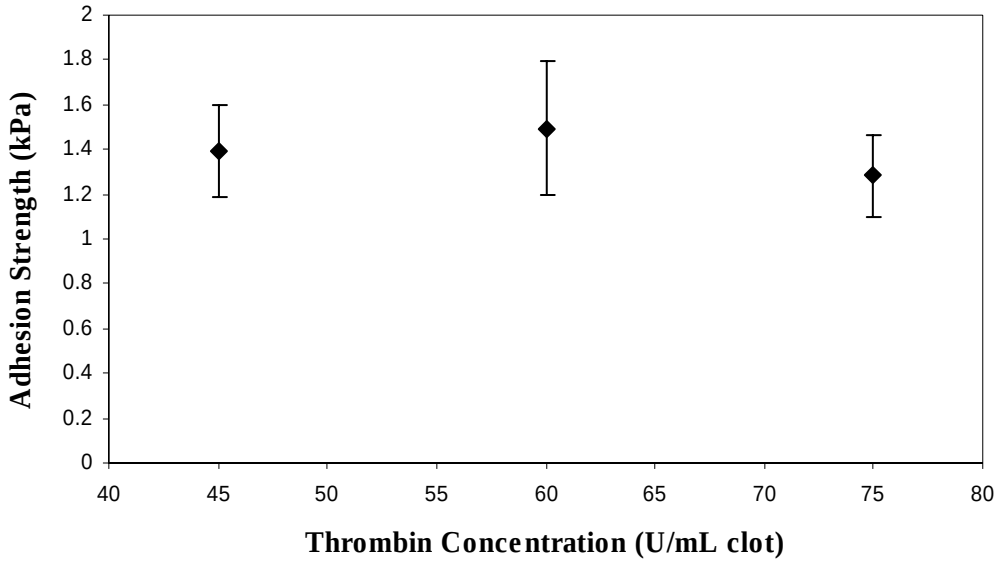

Figure 5.11: Adhesion strength as a function of final thrombin concentration in the clot (n=8). Fibrinogen concentration was 15 mg/mL. No significant differences (p=0.74) were observed at the different thrombin concentrations. Data are shown as mean values ± SD.

5.2.3 Effect of Fibrinogen Concentration

To evaluate the effect of fibrinogen concentration on adhesion strength, samples were prepared with fibrinogen concentrations of 15, 30, 45, and 60 mg/mL. Controls of pooled plasma (3 mg/mL), pure fibrinogen (15 mg/mL), and Tisseel (average concentration 95 mg/mL) were used. Adhesion strength was found to increase approximately linearly with increasing fibrinogen concentration (Figure 5.12).

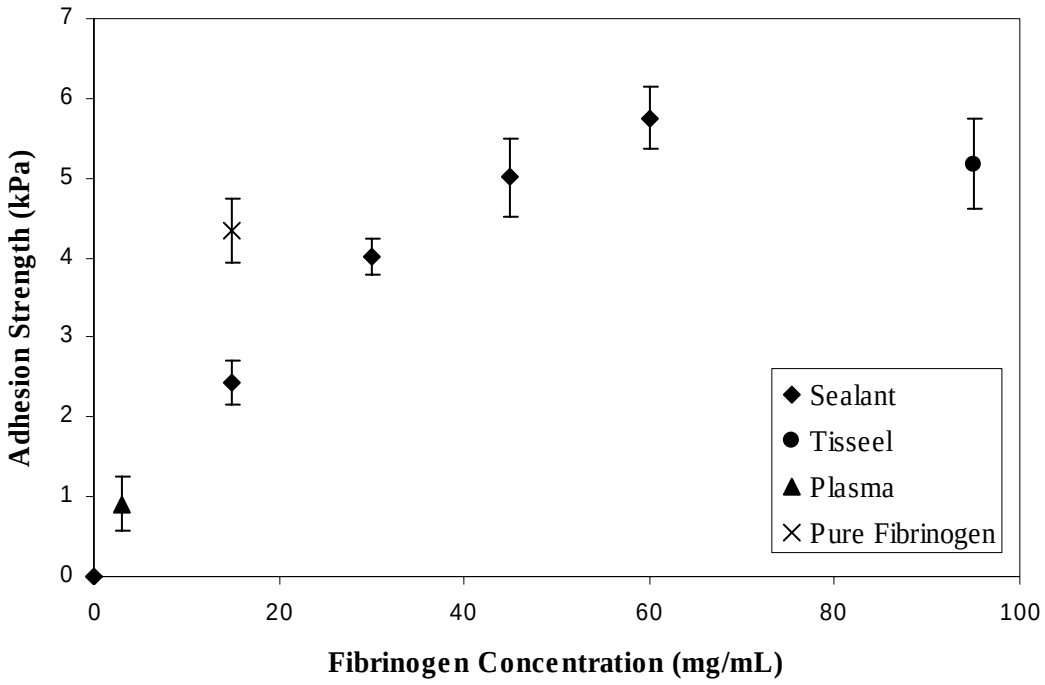

Figure 5.12: Adhesion strength as a function of fibrinogen concentration (n=8). Controls of plasma, pure fibrinogen, and Tisseel were used. The adhesion strength increased linearly with fibrinogen concentration. The adhesion strength of plasma falls near the curve. The adhesion strength of Tisseel fell below the curve. The adhesion strength of pure fibrinogen was significantly larger than the protamine-fibrinogen ($p<0.05$). Data are shown as mean values ± SD.

The adhesion strength of the sample formed with plasma falls near the curve with the samples formed from protamine-fibrinogen concentrate. The adhesion strength of Tisseel is much lower than what is suggested by its fibrinogen concentration. This observation may be evidence that protamine not only precipitates fibrinogen and factor XIII, but also precipitates adhesive proteins as well. The concentrations of these adhesive proteins may be higher in the protamine-fibrinogen concentrate than in Tisseel. The tensile strength of the 15 mg/mL pure fibrinogen sample is significantly greater than the 15 mg/mL protamine-fibrinogen sample ($p<0.05$). The presence of protamine in the

fibrinogen concentrate lowers the adhesion strength of a clot formed as compared to a clot formed with concentrated pure fibrinogen.

5.2.4 Effect of Calcium

The effect of calcium on adhesion strength was investigated by adding calcium chloride to a 15 mg/mL solution of protamine-fibrinogen. Final concentrations of calcium chloride in the fibrinogen concentrate were 0, 0.0018, 0.0036, 0.0089, 0.018, 0.035, 0.05, 0.075, and 0.1 M. The concentration of calcium chloride in the concentrate yielding the highest adhesion strength was in the range of 0.0036 to 0.1 M (Figure 5.13).

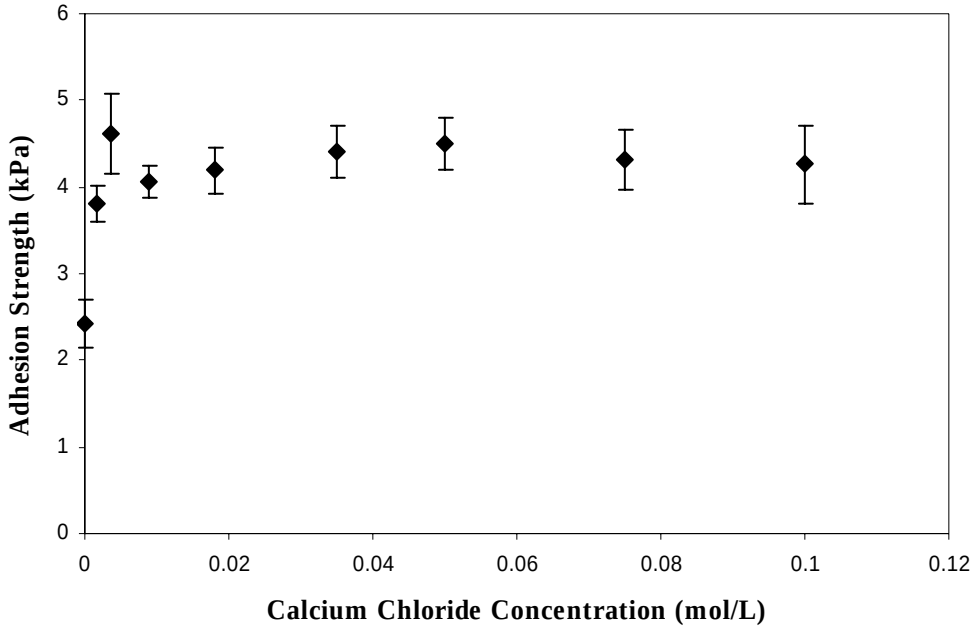

Figure 5.13: Adhesion strength as a function of calcium chloride concentration (n=8). A fibrinogen concentration of 15 mg/mL was used. The adhesion strength reached a maximum in the range of 0.0036 M to 0.1 M calcium chloride. Data are shown as mean values ± SD.

The observed increase in adhesion strength when calcium chloride was added to the fibrinogen concentrate was due to the calcium-dependent effect of factor XIII. It has been shown that factor XIII requires calcium as a cofactor to crosslink fibrin and adhesive proteins and increase adhesion strength [29-31]. There was no calcium present in the fibrinogen concentrate before adding calcium chloride, so the cofactor required for factor XIII to function was not available. After calcium chloride was added, factor XIII had the required cofactor and could function properly.

Calcium chloride concentrations below 0.0036 M were insufficient to produce the maximum adhesion strength. Unlike tensile strength, the maximum adhesion strength remained constant over the range of calcium chloride concentrations once the threshold of 0.00036 M was reached. Tensile strength is a strong function of fibrinogen concentration, while adhesion strength is a function of not only fibrinogen, but adhesive proteins as well. Calcium chloride concentrations above 0.05 M may precipitate enough fibrinogen to affect the tensile strength, but may not be enough to affect the adhesion strength due to the significant influence of adhesive proteins.

The combined effect of fibrinogen concentration and factor XIII on adhesion strength was evaluated by preparing samples with fibrinogen concentrations of 15, 30, 45, and 60 mg/mL and calcium chloride concentration of 0.0089 M. Controls of pooled plasma (3 mg/mL), pure fibrinogen (15 mg/mL), and Tisseel (average concentration 95 mg/mL) were used (Figure 5.14).

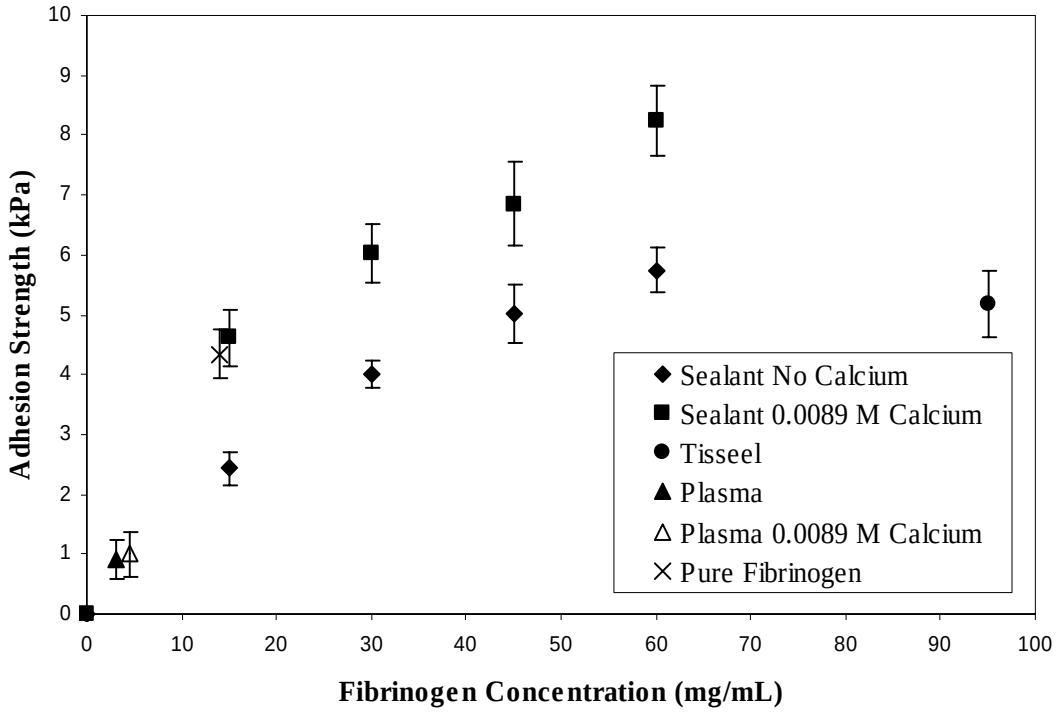

Figure 5.14: Adhesion strength as a function of fibrinogen concentration with (0.0089 M) and without calcium chloride (n=8). Controls of plasma, pure fibrinogen, and Tisseel were used. Adding calcium significantly increased the adhesion strength as compared to Tisseel and fibrinogen without calcium ($p<0.05$). No significant difference was observed for pure fibrinogen. Data are shown as mean values ± SD.

At each fibrinogen concentration the addition of calcium chloride to the protamine-fibrinogen concentrate significantly increased the adhesion strength compared with the protamine-fibrinogen concentrate without calcium ($p<0.05$). No change in adhesion strength was observed when calcium chloride was added to pure fibrinogen, probably because there was no factor XIII present in the pure fibrinogen concentrate. No change in adhesion strength was also observed when calcium chloride was added to citrated plasma. This may have been because free calcium was already present in the citrated plasma even when no calcium chloride was added. This would have led to factor

XIII activity in both cases of no calcium chloride and with calcium chloride. This observation may also have been due to the low factor XIII concentration in plasma. The adhesion strength of Tisseel was found to be significantly less than that of clots formed with 30, 45 and 60 mg/mL protamine-fibrinogen concentrates with 0.0089 M calcium chloride ($p<0.05$).

5.2.5 Effect of Cure Time

The effect of cure time on adhesion strength was evaluated by preparing samples and allowing them to cure for 1, 5, 10, 15, 30, and 60 minutes. Samples were prepared from a 15 mg/mL fibrinogen concentrate with 0.0089 M calcium chloride and without calcium chloride (Figure 5.15). Clots prepared from fibrinogen to which calcium was added reached a maximum adhesion strength in 15 minutes. Clots prepared without calcium chloride reached a maximum adhesion strength in about 5 minutes.

The time required to reach the maximum tensile strength is much lower than the time required to reach the maximum adhesion strength. This provides evidence that the kinetics of fibrin polymerization, which the cure time for tensile strength is dependent on, are much faster than the kinetics of bond formation between the fibrin network and the adhesive proteins.

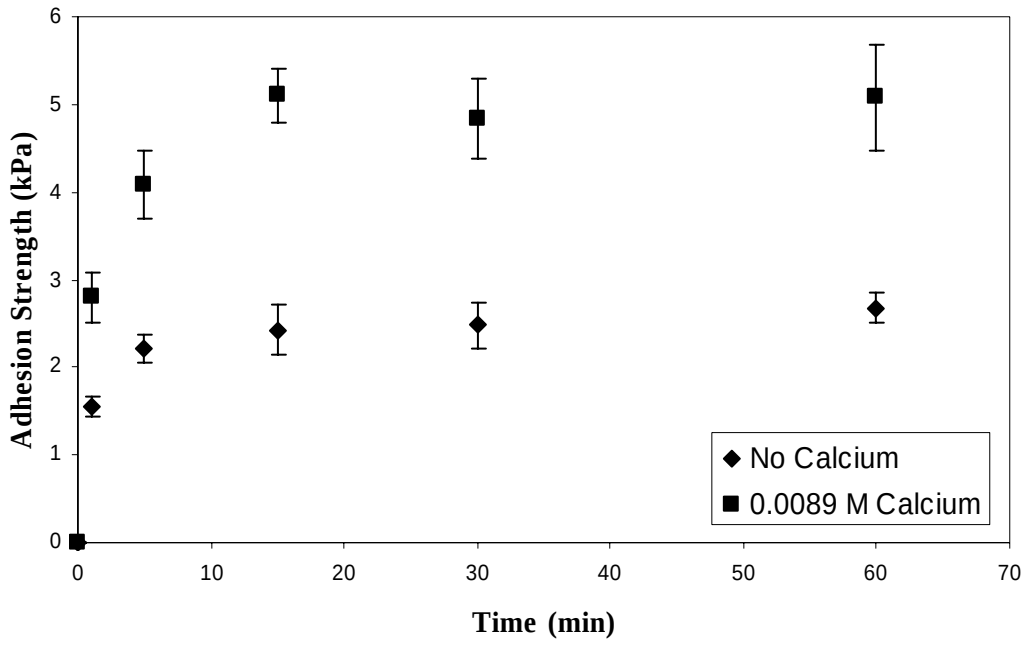

Figure 5.15: Adhesion strength as a function of cure time with (0.0089 M) and without calcium chloride (n=8). Fibrinogen concentration was 15 mg/mL. Sealant was kept at 37°C and samples were cured at 22°C. Samples containing calcium chloride reached a maximum tensile strength at 5 minutes. Samples without calcium chloride required 5 minutes to reach the maximum strength. Data are shown as mean values ± SD.

5.2.6 Effect of Aprotinin and ε-Aminocaproic Acid

The effect of the presence of fibrinolytic inhibitors on adhesion strength was investigated. Samples were prepared from a 15 mg/mL fibrinogen concentrate with 0.0089 M calcium chloride and without calcium chloride. Aprotinin was added to the fibrinogen concentrate to bring the total concentration to 3000 KIU/mL. ε-Aminocaproic was added to the fibrinogen concentrate separate from aprotinin to bring the total concentration to 10 mg/mL. There were no significant changes in adhesion strength upon addition of the antifibrinolytic agents (Figure 5.16).

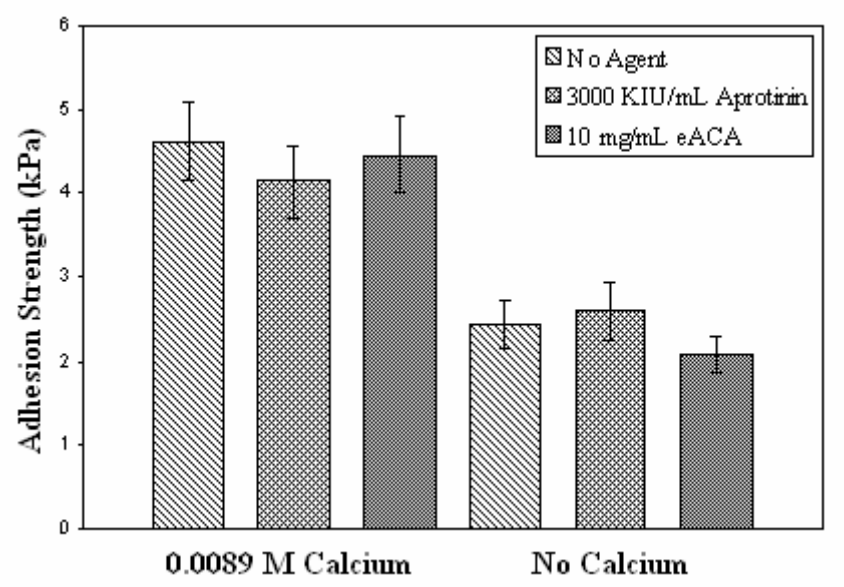

Figure 5.16: Adhesion strength of samples prepared with antifibrinolytic agents with (0.0089M) and without calcium chloride (n=8). Fibrinogen concentration of 15 mg/mL was used. Antifibrinolytic agents used were aprotinin and epsilon-aminocaproic acid (eACA). No significant differences in Adhesion strength were observed when the antifibrinolytic agents were added. Data are shown as mean values ± SD.

5.3 Donor Variation

A major criticism of conventional autologous fibrin sealants is the poor reproducibility of their mechanical properties and clinical performance [79]. Plasma fibrinogen levels vary considerably between individuals, being affected by age, sex, race, alcohol intake, and smoking, as well as certain disease states. Fibrinogen concentrations of 2-6 mg/mL are typical of patients undergoing surgery [86-89]. These variations in fibrinogen concentration are manifested as variations in concentration of fibrin sealants prepared from autologous donations, which leads to poor reproducibility [49, 90].

The effect of donor variation on the mechanical properties of the protamine-precipitated fibrinogen concentrate was evaluated. Blood was drawn into sodium citrate

(final concentration 3.8 gm/100 mL) from 10 donors, 5 male and 5 female. The fibrinogen concentration in the plasma was measured with the Clauss method [91]. Diluted fibrinogen was clotted in the presence of excess thrombin in a CoaData 2000 Fibrintimer (Labor GmbH, Hamburg, Germany). The clotting time was recorded and the fibrinogen concentration was calculated from a standard curve. The fibrinogen was precipitated with protamine as described and concentrated to 15 mg/mL without the addition of calcium. The adhesion and tensile strengths were measured as previously described and were compared to fibrinogen concentrate from pooled donor plasma (Figure 5.17).

The adhesion and tensile strengths of the individual donors were not significantly different from the pooled plasma values. One donor had a high plasma fibrinogen concentration (4 mg/mL), reflected in the increased adhesion and tensile strengths over the other samples. Because the mechanical properties are dependent on the fibrinogen concentration, an autologous sealant made from a patient with an abnormally low fibrinogen concentration (<2 mg/mL) will have mechanical properties significantly lower than the pooled plasma values.

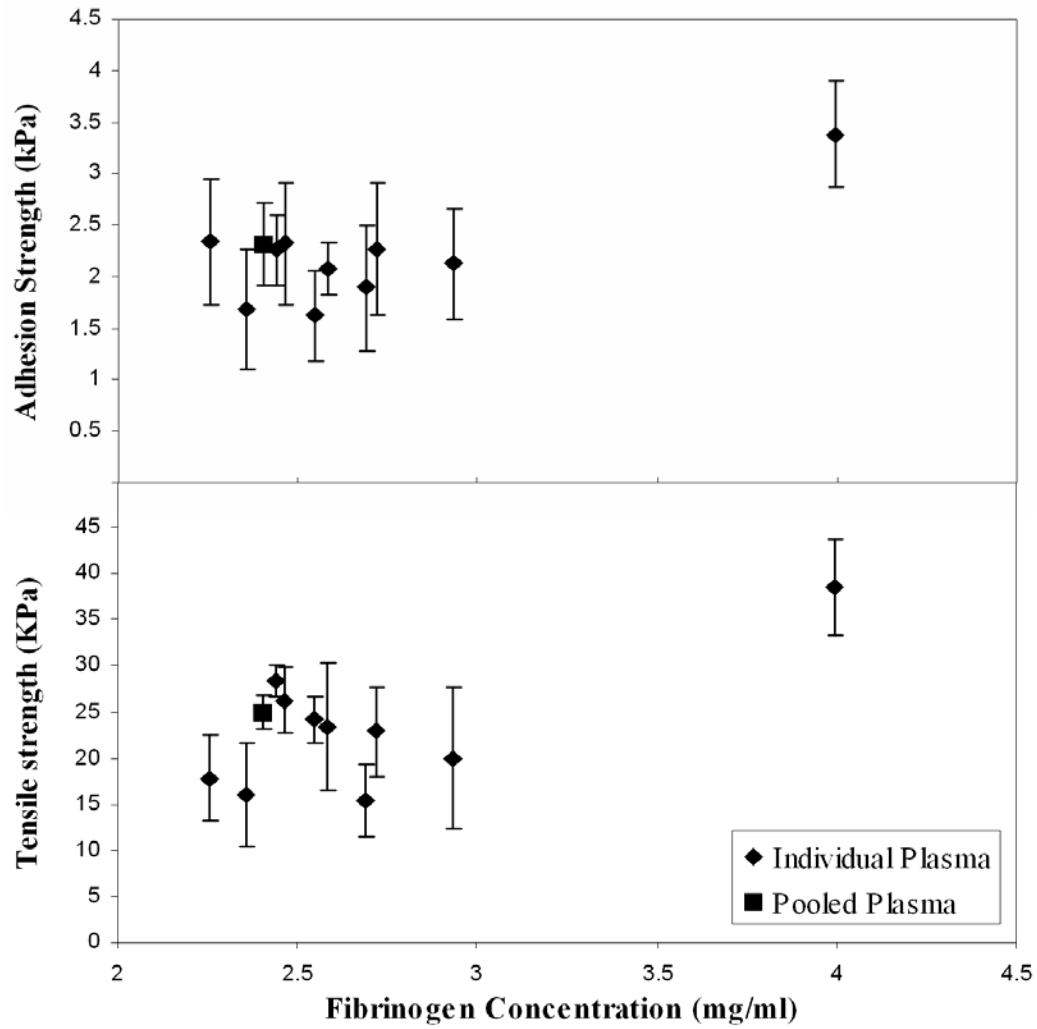

Figure 5.17: Tensile strength (n=4) and adhesion strength (n=8) as a function of individual fibrinogen concentration. Fibrinogen concentration of 15 mg/mL without calcium was used. The tensile and adhesion strengths of the donors were not different from the pooled plasma (except for the outlier). Data are shown as mean values ± SD.

CHAPTER 6: KINETICS

While tensile strength and adhesion strength are good indicators of how well a fibrin sealant will perform, the most important parameter for hemostasis is the speed of clotting, which is a function of thrombin concentration and fibrinogen concentration [24].

6.1 Experimental Methods

The kinetics of the thrombin-fibrinogen interaction in the fibrin sealant were determined by measuring the clotting curve [22, 92, 93]. The clotting curve was measured as an increase in absorbance at 420 nm as a function of time using a Beckman Coulter DU 640 spectrophotometer (Beckman Coulter, Inc, Fullerton, CA). 0.5 mL of fibrinogen concentrate was put into a cuvette and placed in the spectrophotometer. 50 μL of bovine thrombin was injected into the cuvette to start the reaction and the absorbance was monitored continuously up to 180 seconds. To confirm that the thrombin and fibrinogen were completely mixed, samples were also prepared by injecting the sealant into a cuvette using a Duploject. No differences between the clotting curves for the two methods of sample preparation were observed. It was assumed that both techniques provided complete mixing.

A sample clotting curve is shown in Figure 6.1. Initially there is a time interval during which the optical density remains close to zero. This time interval is associated with the enzymatic events involved before clot formation [94]. The optical density then increases in a sigmoid shape. As fibrin monomers polymerize, the optical density increases with time [94]. The slope at the inflection is extrapolated to zero to give the predicted time required for the thrombin to convert the fibrinogen into fibrin monomer, or the clotting time. The slope at the inflection represents the rate of the hypothetical polymerization reactions involved in clot formation [92].

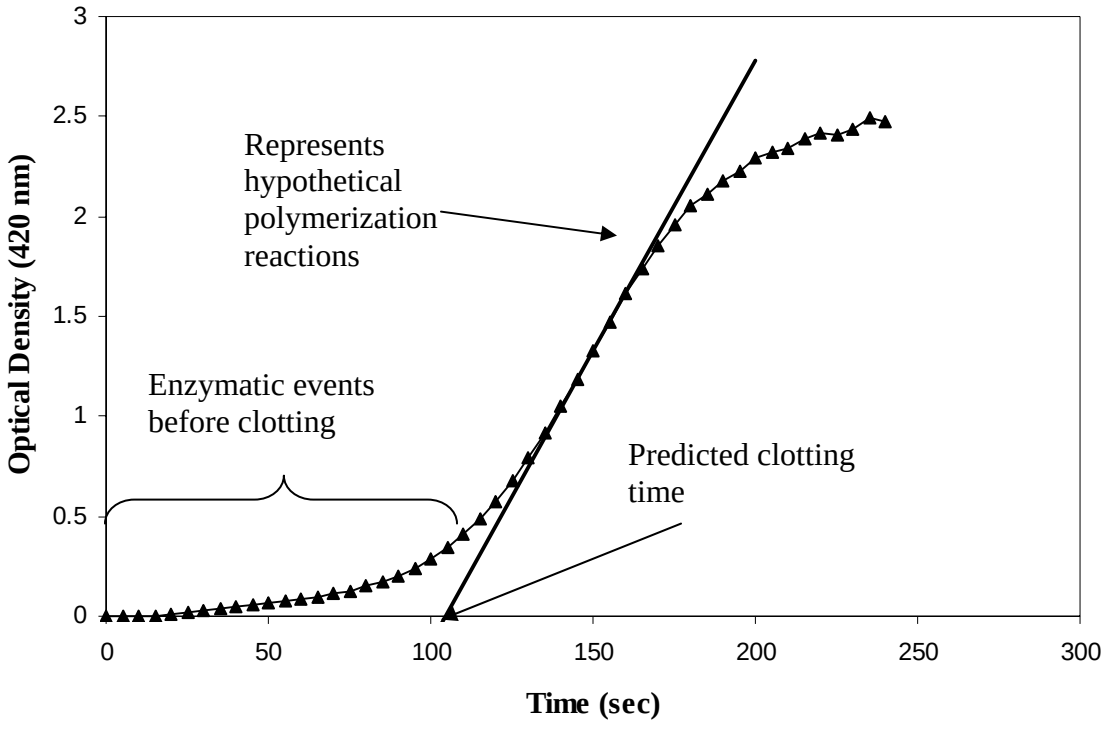

Figure 6.1: A sample clotting curve. The clotting time was calculated by extrapolating the slope at the inflection to an optical density of zero. The clotting time represents the enzymatic cleavage of fibrinogen to fibrin by thrombin. The slope through the inflection represents the rate of the polymerization steps in clot formation.

6.2 Effect of Thrombin Concentration

The effect of thrombin concentration on clotting time was determined by measuring the clotting curves at thrombin concentrations of 10, 25, 50, 100, 200, 300, 400, and 500 U/mL and a fibrinogen concentration of 15 mg/mL with no calcium. The clotting time decreased as the thrombin concentration increased (Figure 6.2). At a thrombin concentration of 10 U/mL, the time required for the fibrinogen to be converted to fibrin was 57.6 seconds. When the thrombin concentration was increased to 500 U/mL the clotting time decreased to 2.5 seconds.

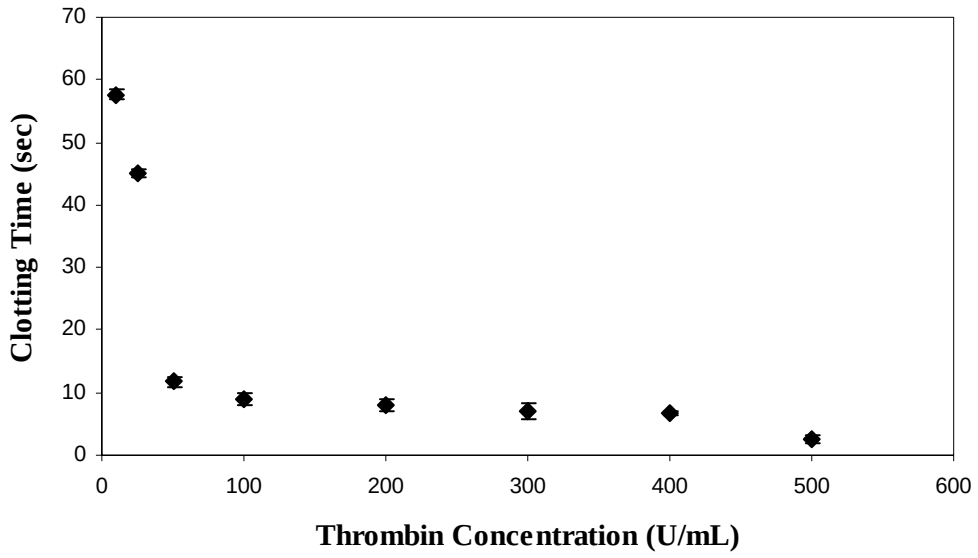

Figure 6.2: Clotting time as a function of thrombin concentration (n=2). Fibrinogen concentration of 15 mg/mL was used. The clotting time decreased as the thrombin concentration increased. Data are shown as mean values ± SD.

6.3 Effect of Fibrinogen Concentration

The effect of fibrinogen concentration on clotting time was determined by measuring the clotting curves at fibrinogen concentrations of 30, 15, 9, 6, 3.75, 2, and 1 mg/mL and a thrombin concentration of 100 U/mL with no calcium. The clotting time decreased as the fibrinogen concentration increased (Figure 6.3). The clotting time at a fibrinogen concentration of 1 mg/mL was 28.1 seconds. When the fibrinogen concentration was increased to 6 mg/mL the clotting time decreased to 8.4 seconds. At fibrinogen concentrations above 6 mg/mL no change in the clotting time was observed with increasing fibrinogen concentration. The enzymatic reaction at this point was no longer limited by the amount of fibrinogen, but by the amount of thrombin. As shown previously, increasing the thrombin concentration to 500 U/mL decreased the clotting time of the 15 mg/mL fibrinogen to 2.5 seconds.

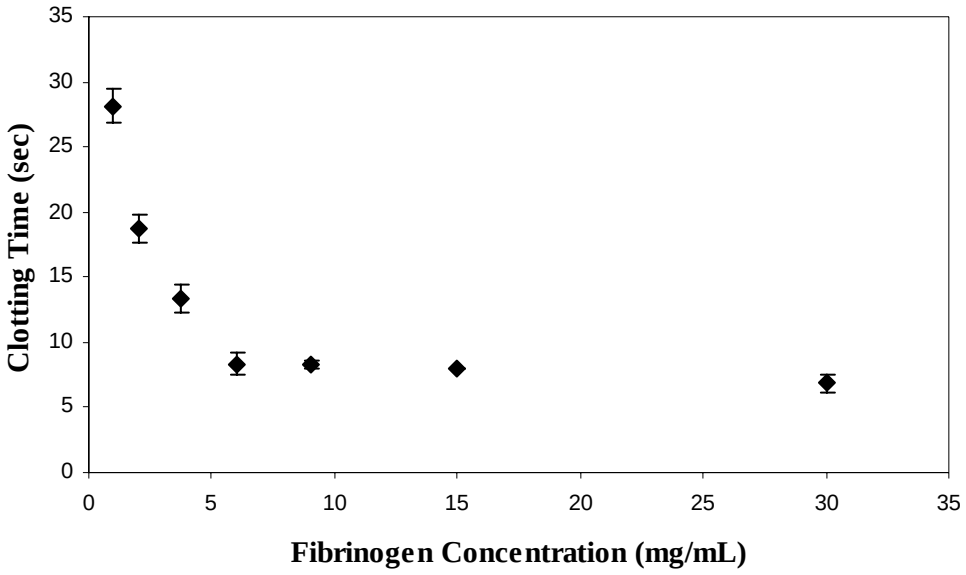

Figure 6.3: Clotting time as a function of fibrinogen concentration (n=2). Thrombin concentration of 100 U/mL was used. The clotting time decreased as fibrinogen concentration increased. Data are shown as mean values ± SD.

6.4 Kinetic Parameters

The enzymatic reaction between thrombin and fibrinogen can be described with Michaelis-Menton enzyme kinetics [95]. The fibrinogen concentration and thrombin concentration data were fit with the Mechaelis-Menton equation (Equation 6.1):

$$-r_A = \frac{k_{cat} C_E C_A}{K_M + C_A} \tag{6.1}$$

where C_E is the enzyme concentration, C_A is the substrate concentration, K_M is the apparent equilibrium constant for the dissociation of the enzyme-substrate complex, and k_{cat} is the rate constant for the formation of product from the enzyme-substrate complex. Values for K_M and k_{cat} are reported in Table 6.1 using thrombin as the enzyme and fibrinogen as the substrate for clots prepared from protamine-fibrinogen concentrate and

previously published values for clots prepared from pure fibrinogen. The fit constants agree favorably with previously published values showing that protamine does not adversely affect the kinetics of clotting.

Table 6.1: Kinetic constants for the Michaelis-Menton equation.

	This work	Mihalyi [96]	Martinelli & Scheraga [97]
K_M ($x10^5$) (mol/L)	1.8 ± 0.4	0.6	1.13
k_{cat} (min^{-1})	0.45 ± 0.1	0.101	0.033

6.5 Operating Conditions

Clotting times were measured for sealants prepared with fibrinogen concentrations of 15, 30, and 45 mg/mL with and without calcium and a thrombin concentration of 500 U/ml (Figure 6.4).

The addition of calcium to the fibrin sealant did not change the kinetic behavior at the different fibrinogen concentrations. The addition of calcium to the fibrin sealant decreased the clotting time on average 0.18 seconds. The addition of calcium did change the mechanical properties of the sealant, as has been shown, and should be included in the final preparation. The clots formed from the sealant polymerized very rapidly as well. All preparations were completely polymerized at 20 seconds.

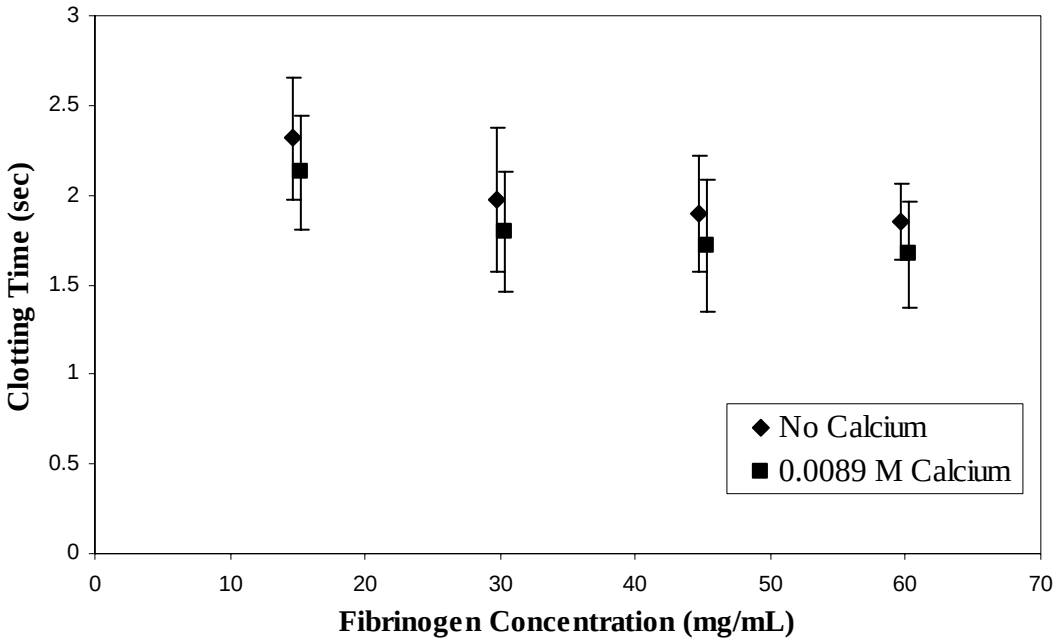

Figure 6.4: Clotting time for fibrinogen concentrations of 15, 30, and 45 mg/mL with (0.0089 M) calcium and without (n=2). Thrombin concentration of 500 U/mL was used. The mean clotting times for all of the preparations were 2.5 seconds or less. Adding the calcium reduced the clotting times on average by 0.18 seconds. Samples were completely polymerized within 15 seconds. Data are shown as mean values ± SD.

6.6 Commercial Product

The clotting curve for Tisseel was measured and compared to fibrin sealant with a fibrinogen concentration of 95 mg/mL (average fibrinogen concentration in Tisseel) and a thrombin concentration of 500 U/mL (Figure 6.5). Samples were prepared by injecting the sealant into a cuvette using a Duploject.

The shapes of the clotting curves for Tisseel and the sealant prepared from 95 mg/mL protamine-fibrinogen were the same. Both sealants clotted so rapidly that the clotting time could not be measured. The polymerization of both sealants was also very rapid.

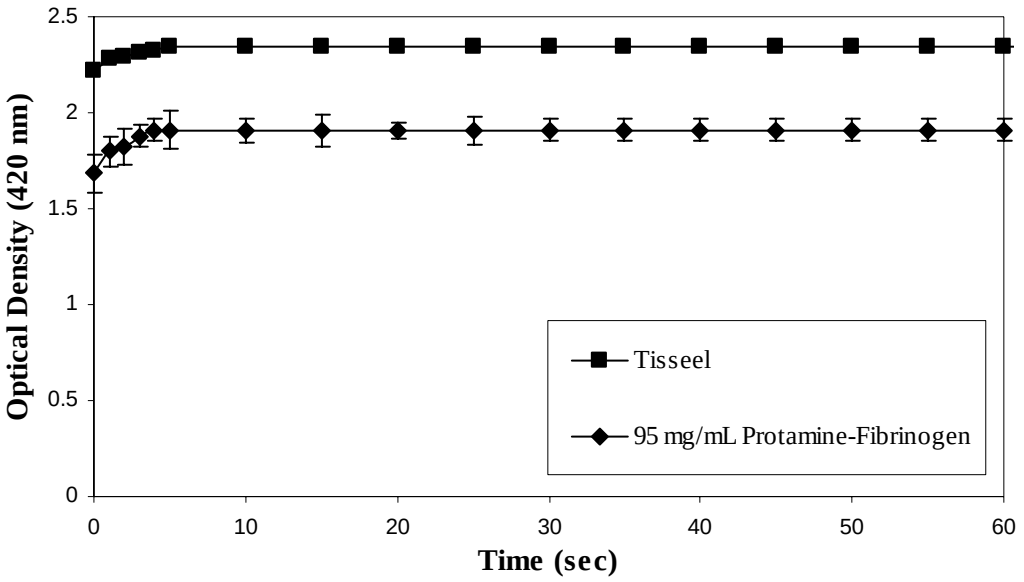

Figure 6.5: Absorbance at 420 nm as a function of time for a 95 mg/mL fibrinogen concentrate (n=2) and Tisseel (n=1). The sealant prepared from the protamine-fibrinogen concentrate and Tisseel exhibit similar kinetics. Data are shown as mean values ± SD.

CHAPTER 7: ANIMAL MODEL

7.1 Experimental Methods

Adult male Wistar rats (*Rattus rattus*) (250-400 gm) were anesthetized by an intraperitoneal injection (0.1 mL/100 g) of a cocktail of xylazine (10 mg/kg) (Butler Co., Columbus, OH) and ketamine (90 mg/kg) (Fort Dodge Labs, Fort Dodge, IA). Breathing rate and depth and response to a toe or tail pinch were monitored throughout the procedure to ensure proper anesthesia.

Laparotomy was performed to open the abdominal cavity and expose the kidneys. The abdominal cavity was dried with gauze to minimize accumulated body fluids immediately before making a 1.00 cm long, 0.5 cm deep incision in the kidneys using a scalpel cutting guide. The scalpel cutting guide provided a way to make a repeatable incision of 1.00 ± 0.05 cm long and 0.5 ± 0.01 cm deep. The wound was then allowed to bleed for 5 seconds, wiped clean, and 0.3 ml of fibrin sealant was applied to the wound with a Duploject. This procedure was repeated in the opposite kidney with a control sealant. The order in which the sealant and controls were applied was randomized as was the kidney used.

Blood loss was measured by absorbing blood around the wound site, without touching the wound or sealant, with pre-weighed gauze and weighing the gauze after the

blood was absorbed. The gauze was stored in small sealed bags between blood absorption and weighing to minimize evaporation. This was repeated until blood no longer stained a clean piece of gauze. Bleeding time was measured by starting a timer when the fibrin sealant was applied and stopping the timer when blood no longer stained a clean piece of gauze. Blood samples were collected for hematology and aggregometry analysis at the end of the experiments via cardiac puncture while the animal was under anesthesia.

The animals were euthanized at the end of each experiment due to the severe damage done to the kidneys. Humane euthanasia was enacted by cardiac injection of pentobarbital (200 mg/kg) (Fort Dodge Animal Health, Fort Dodge, IA) while the animal was still under anesthesia. Death was determined by respiratory and cardiac arrest.

7.2 Experimental Results

7.2.1 Hematology and Aggregometry

Complete blood counts were performed at the Utah Artificial Heart Institute on blood samples collected from the animals. The results shown in Table 7.1 are consistent with published data for Wistar rats [98].

Aggregometry was performed to verify that the anesthetics were not adversely affecting platelet functionality. 500 μL of PRP were aggregated with 50 μL of adenosine diphosphate (Sigma Chemical Co.; stock concentration 1 mM). Aggregation was measured in a Chronolog 560-ca Dual Sample Aggregometer (Chronolog Corp.,

Havertown, PA). Maximum platelet aggregation was 65% ± 21%, which agrees with the published value of 68% ± 30% [98].

Table 7.1: Hematology of Wistar rats. Data are shown as mean values ± SD.

	This Work	Lewis, *et al*.
Red Blood Cells ($x10^6/\mu L$)	6.6 ± 0.5	5.9 ± 0.4
White Blood Cells ($1/\mu L$)	5.1 ± 2.4	5.4 ± 1.9
Hematocrit (%)	41.0 ± 3.0	---
Hemoglobin (g/dL)	14.2 ± 0.8	13.0 ± 0.7
Platelets ($x10^3/\mu L$)	800 ± 155	1109 ± 310

7.2.2 Sealant Performance

Blood loss and bleeding time were evaluated for sealants prepared from 15, 30, 45, and 60 mg/mL fibrinogen concentrates with 0.0089 M calcium chloride and without calcium chloride. Control sealants used were pooled plasma, 50 U thrombin in 0.2 mL sodium citrate, and Tisseel. A control where nothing was applied to the wound was also used.

All of the fibrin sealant preparations significantly reduced the blood loss and bleeding time over the controls ($p<0.05$) (Figure 7.1). For sealant preparations with added calcium, no significant differences were observed between the different fibrinogen concentrations. The only significant difference between sealants with and without calcium was at a fibrinogen concentration of 15 mg/mL.

Blood loss and bleeding time for Tisseel were significantly greater than the results for 30, 45, and 60 mg/mL fibrinogen concentrates with calcium chloride and 60 mg/mL

fibrinogen concentrate without calcium chloride ($p<0.05$). This observation is attributed to the low adhesion strength of Tisseel.

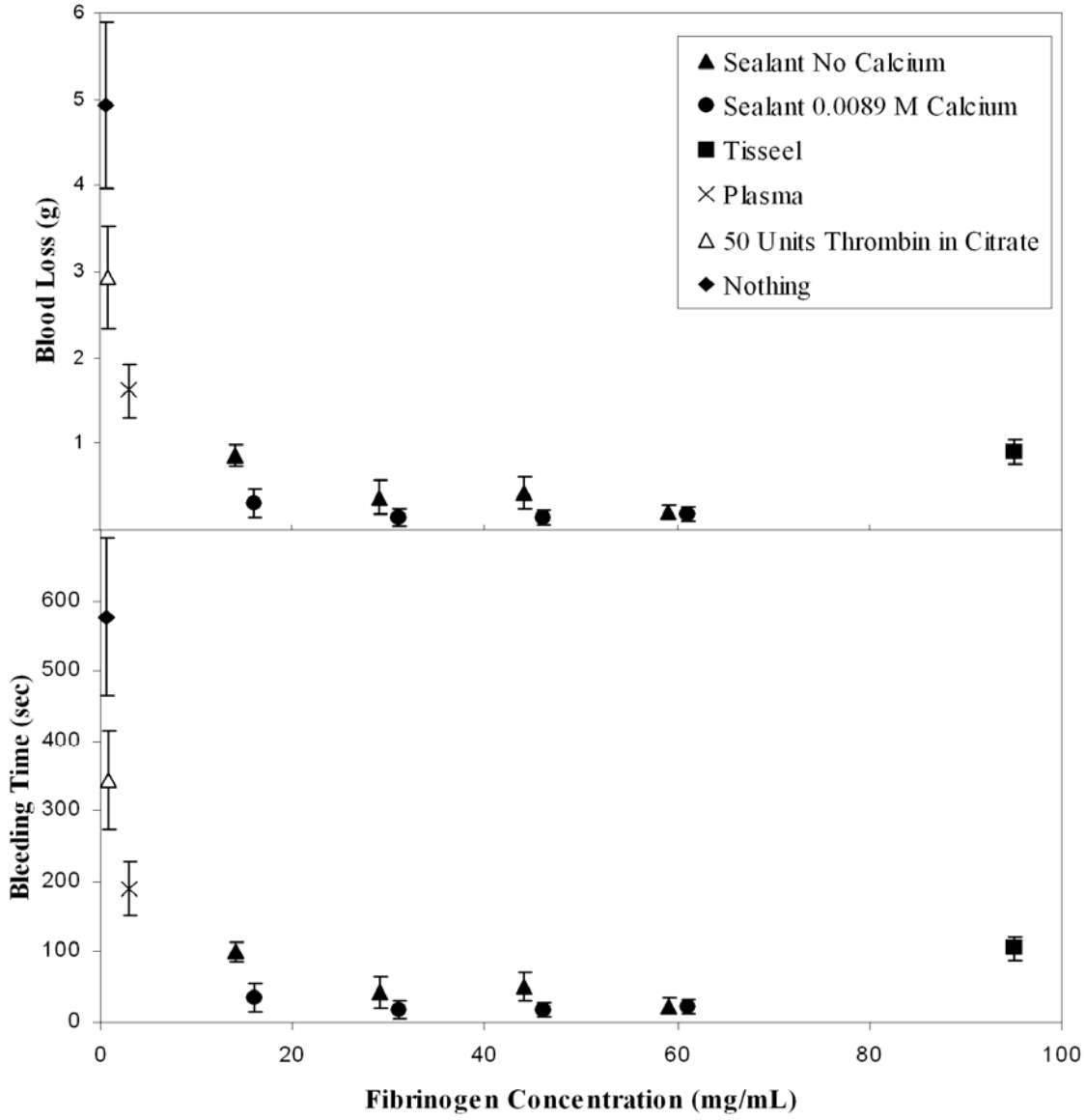

Figure 7.1: Bleeding time and blood loss as a function of fibrinogen concentration (n=5). Controls used were nothing, plasma, thrombin, and Tisseel. All sealant preparations significantly decreased the blood loss and bleeding time over the controls of plasma, thrombin, and nothing ($p<0.05$). 30, 45, and 60 mg/mL fibrinogen concentrates with calcium and 60 mg/mL fibrinogen concentrate without calcium significantly lowered blood loss and bleeding time over Tisseel ($p<0.05$). Data are shown as mean values ± SD.

7.2.3 Anticoagulated Rats

Fibrin sealants are often used in patients undergoing surgery that requires anticoagulation (e.g. open-heart surgery requiring cardiopulmonary bypass). Heparin is the most widely used anticoagulant in such surgery, and a heparin concentration of 2-3 U/mL in the blood provides adequate anticoagulation for that application [85].

The performance of fibrin sealant made from protamine-fibrinogen concentrate was evaluated by measuring blood loss and bleeding time in heparinized rats. The surgical procedure already described was followed. A bolus injection of heparin (100 U/mL) was given in the vena cava following laparotomy to bring the concentration in the blood to 2-3 U/mL. Sealant was prepared from 30 and 60 mg/mL fibrinogen concentrates with a calcium chloride concentration of 0.0089 M. A control sealant of pooled plasma and a control where nothing was applied to the wound were used.

Both fibrin sealant preparations significantly lowered the blood loss and bleeding time over the controls ($p<0.05$) (Figure 7.2). The blood loss and bleeding time at both fibrinogen concentrations were significantly greater in the heparinized rat than in the non-heparinized rat ($p<0.05$).

The increase in blood loss and clotting time in the heparinized rat compared with the non-heparinized rat is evidence that the animal's native coagulation system contributes in reducing blood loss and bleeding time. Application of the fibrin sealant in a non-heparinized rat initiates clotting of the animal's blood at the wound site. The fibrin sealant and clotting of the native blood combine to help reduce the blood loss and bleeding time. Normal clotting function in a heparinized rat is inhibited and doesn't aid in stopping bleeding, as observed by an increase in blood loss and clotting time.

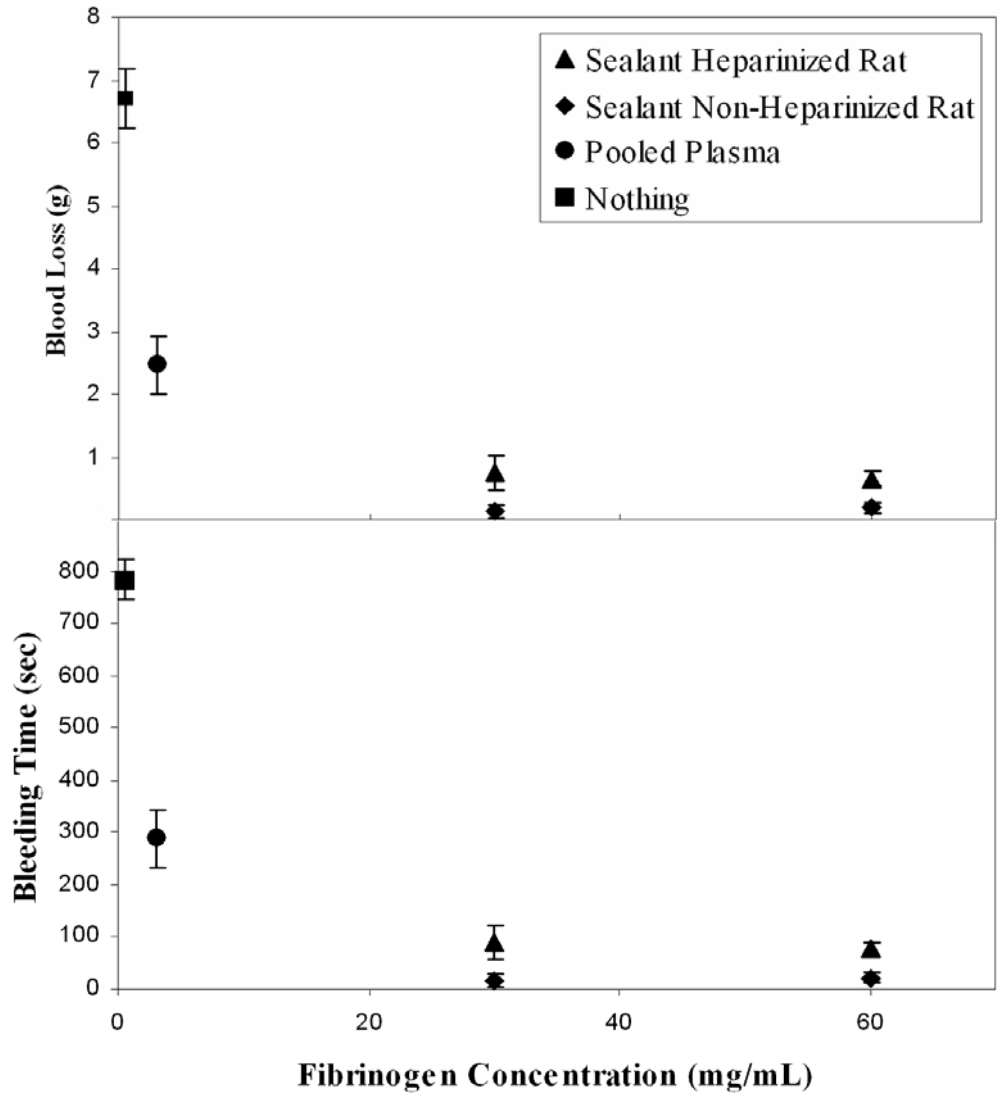

Figure 7.2: Blood loss and time to stop bleeding in heparinized rats (n=5). Sealants prepared from 30 and 60 mg/mL fibrinogen concentrated with 0.0089 M calcium were used. Controls of nothing and plasma were used. The sealants significantly lowered blood loss and bleeding time in the heparinized rats over the controls ($p<0.05$). Blood loss and bleeding time were greater in the heparinized rat than in the non-heparinized rat. Data are shown as mean values ± SD.

7.3 Mathematical Model of Animal Wound

The rat kidney wound was modeled mathematically in order to determine the influence of the individual mechanical properties on the efficacy of the sealant. A force balance was performed on the incision and the sealant applied to it (Figure 7.3).

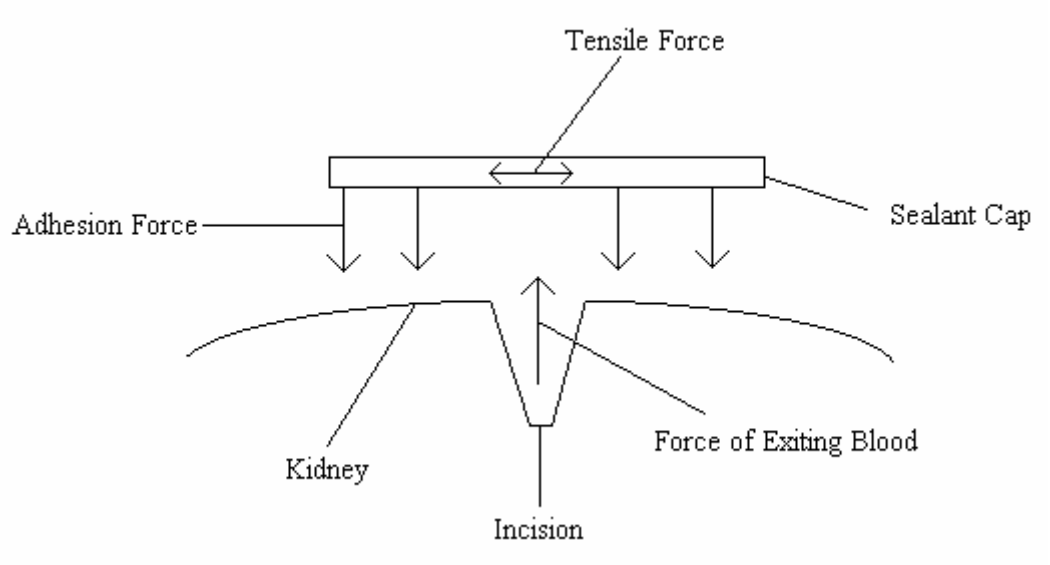

Figure 7.3: Force balance on kidney incision in the animal model.

In order for the fibrin sealant to stop the wound from bleeding, the adhesive force and tensile force of the sealant must be greater than the force exerted by the blood exiting the wound as shown in Equations 7.1 and 7.2:

$$F_B \leq F_T \tag{7.1}$$

$$F_B \leq F_A \tag{7.2}$$

where F_B is the force of the blood exiting the wound, F_T is the tensile force of the sealant, and F_A is the adhesion force. These forces were calculated as shown in Equations 7.3, 7.4, and 7.5:

$$F_B = A_I P_B \tag{7.3}$$

$$F_T = \sigma A_I \tag{7.4}$$

$$F_A = \tau (A_S - A_I) \tag{7.5}$$

where A_I is the cross-sectional area of the incision, P_B is the blood pressure in the kidney, σ is the tensile strength (see Chapter 5), τ is the adhesion strength (see Chapter 5), and A_S is the total surface area of the sealant cap. If one of the mechanical forces of the sealant is less than force of the blood, then the sealant will not stop the wound from bleeding.

7.3.1 Model Development

The force of the blood exiting the wound was calculated as the blood pressure in the kidney multiplied by the cross-sectional area of the incision. The mean arterial pressure in a male Wistar rat, which is assumed to be the same in the renal artery, is approximately 100 mmHg [99]. Ketamine/xylazine anesthetic used in rats has been shown to lower the mean arterial pressure by 10 mmHg [100, 101]. Hence, the mean pressure in the renal artery is estimated to be 90 mmHg. The pressure in the interlobar, arcuate, and interlobular arteries is the same as in the renal artery [3]. Based on the size of the incision made relative to the size of the kidney, it was assumed that the interlobar, arcuate, and interlobular arteries were cut, and the pressure of the blood exiting the wound was assumed to be 90 mmHg. The incision was l cm long and 1 mm wide at the surface of the kidney yielding a cross-sectional area of 10 mm^2 and a force of blood exiting the wound of 0.12 N.

The force that sealant can hold is a combination of the tensile force and the adhesive force. The sealant has to have a strong enough tensile force to prevent the blood

from rupturing the clot and a strong enough adhesive force to adhere to the kidney and anchor the clot. The tensile and adhesion forces were calculated from the time-dependent curves shown previously (see Chapter 5). The adhesive force was calculated from the adhesive strength and the surface area of the kidney covered by the applied sealant. The sealant was assumed to form a circular disc with a diameter of 1.1 cm and surface area of 0.95 cm^2. A lower surface area than was actually measured was assumed to account for the formation of blood leak channels under the sealant. The cross-sectional area of the incision was subtracted from the surface area of the sealant disc to give the total surface area of the sealant contacting the kidney tissue. The tensile force was calculated from the cross-sectional area of the incision and the tensile strength.

7.3.2 Results

The bleeding time was predicted for the conditions described using fibrin sealant with fibrinogen concentrations of 15, 30, 45, and 60 mg/mL, thrombin concentration of 500 U/mL, and with and without calcium chloride. The predicted bleeding time was calculated as the time required for the tensile and adhesion forces to reach the same force exerted by the blood coming out of the wound. The greater of the two times for the tensile and adhesion force development determined the bleeding time. The calculated bleeding times were compared to the experimental values (Figure 7.4). For the sealant containing calcium, the predicted values matched the experimental values within an average of 6 seconds with a maximum difference of 10 seconds. The predicted values for sealant without calcium matched the experimental values within an average of 22 seconds with a maximum difference of 50 seconds.

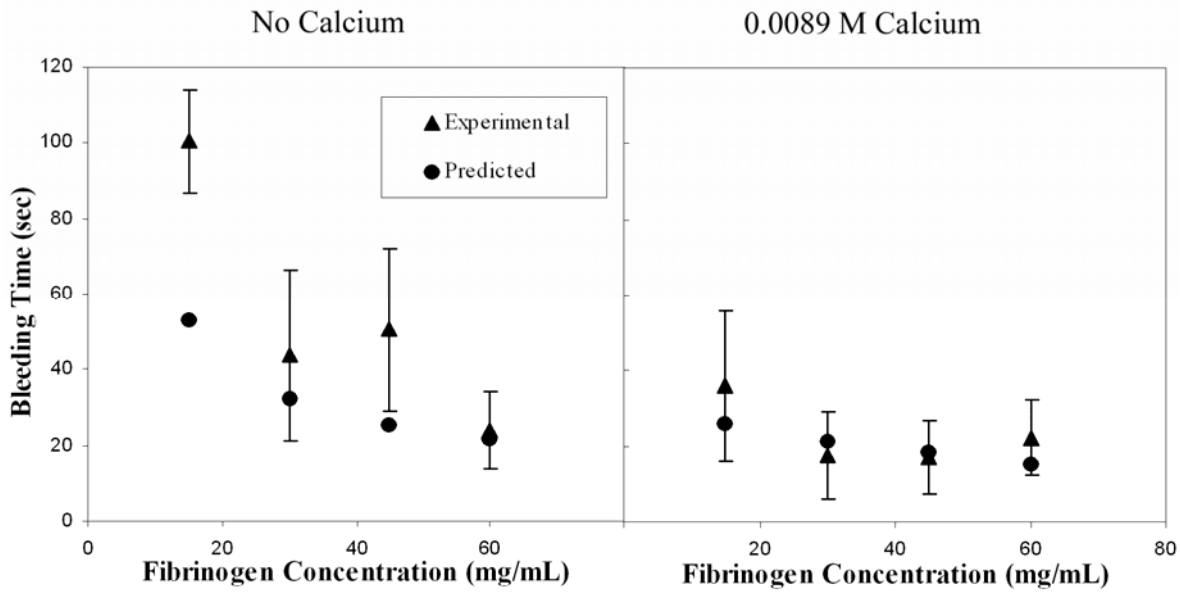

Figure 7.4: Predicted and experimental (n=5) bleeding times. The predicted bleeding times for sealants with calcium match the experimental results within an average of 6 seconds. The predicted bleeding times for sealants without calcium match the experimental results within an average of 22 seconds. Experimental results are shown as mean values ± SD.

The bleeding time calculated from the adhesion force at each sealant formulation was much greater than the bleeding time calculated from the tensile force (Figure 7.5). The time required for the tensile strength to reach its maximum strength is not only much shorter than the time required for the adhesion strength, but the magnitude of the maximum tensile strength is also much higher than adhesion strength (see Chapter 5). These factors contribute to the differences in the predicted bleeding times calculated for adhesion and tensile. As was shown in the animal model, the presence of calcium reduces the bleeding time.

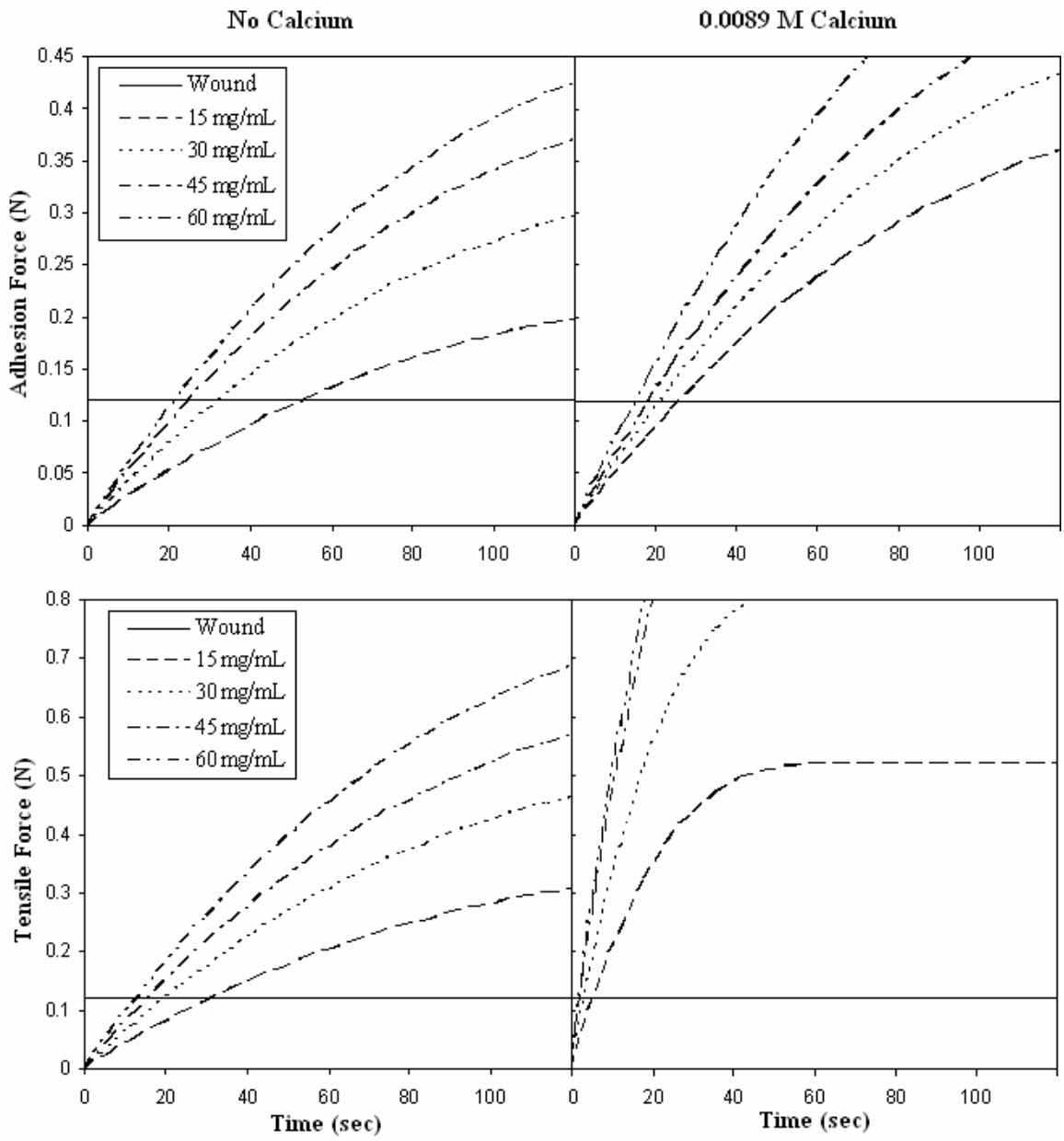

Figure 7.5: Time curves of adhesion force and tensile force as a function of time compared to the force exerted by the blood exiting the wound. Sealants preparations with 15, 30, 45, and 60 mg/mL fibrinogen concentrations with (0.0089 M) and without calcium chloride were modeled. The bleeding time for all sealant preparations was limited by the adhesion force.

The effect of changing the force of the exiting blood on bleeding time was investigated by predicting the bleeding time for wounds with blood pressures in the range

of 0 to 100 mmHg. Calculations were made for a sealant with a fibrinogen concentration of 15 mg/mL with and without calcium (Figure 7.6).

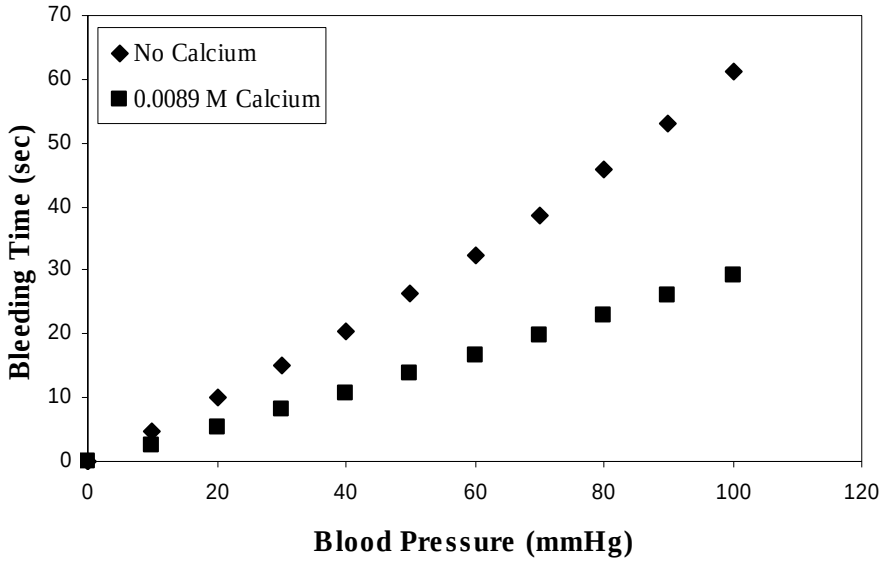

Figure 7.6: Predicted bleeding time as a function of blood pressure in the wound. A sealant prepared from 15 mg/mL fibrinogen concentrate with (0.0089 M) and without calcium chloride was modeled. The bleeding time decreased as the pressure in the wound decreased. The sealant with calcium had lower bleeding times at all pressures than the sealant without calcium.

The predicted bleeding times in sealants with and without calcium decreased as the blood pressure in the wound decreased. At lower blood pressures, the force exerted by the exiting blood was lower, which resulted in a lower adhesion force required to stop bleeding. The sealant can stop a low-blood-pressure wound from bleeding faster than it can stop a high-blood-pressure wound.

The effect of changing the surface area of adhesion on the predicted bleeding time was investigated. Bleeding times were predicted for surface areas of 0.1, 0.5, 1, and 2

cm^2 and a blood pressure of 90 mmHg. Calculations were made for a sealant with a fibrinogen concentration of 15 mg/mL with and without calcium (Figure 7.7).

The bleeding time increased as the adhesion surface area decreased. The surface area required to stop bleeding was 0.5 cm^2 or greater in both sealant preparations. A surface area of 0.1 cm^2 was too low for both preparations of fibrin sealant to stop the wound from bleeding.

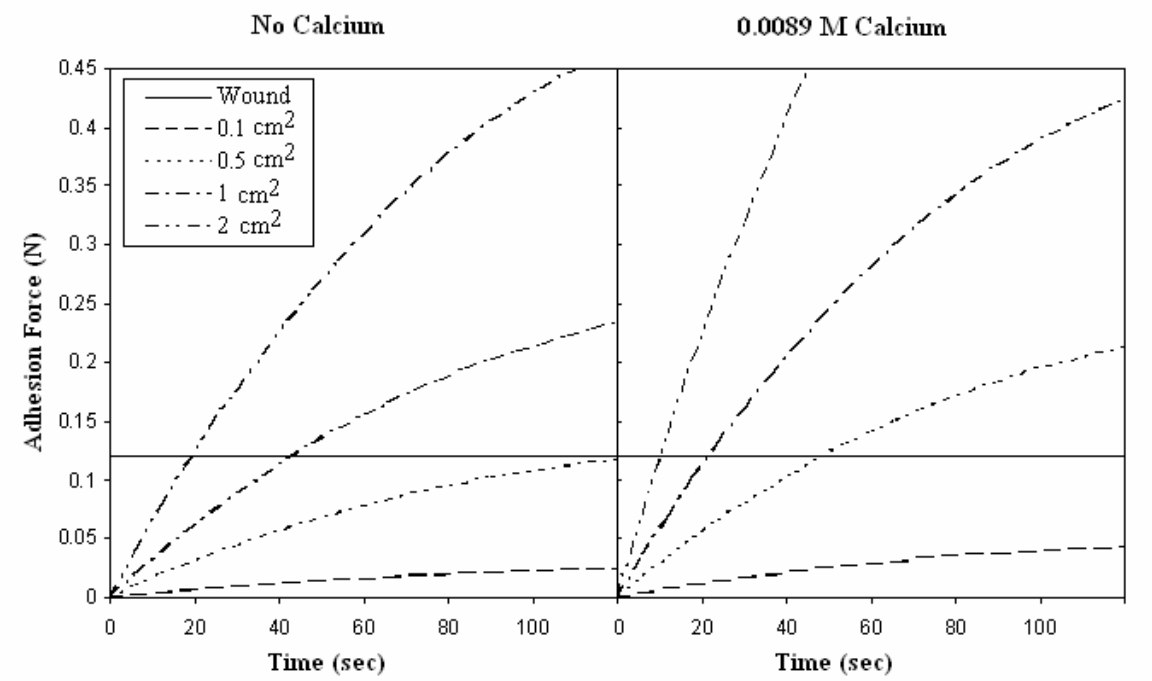

Figure 7.7: Adhesion force as a function of time at different adhesion surface areas. Surface areas of 0.1, 0.5, 1, and 2 cm^2 were predicted. Predictions were made for a 15 mg/mL fibrinogen concentration with (0.0089 M) and without calcium chloride.

The ability of a fibrin sealant to stop a wound from bleeding is limited by the adhesion strength of the sealant as shown with the mathematical model. It has been shown that the adhesion strength increased with fibrinogen concentration and addition of calcium. When sealing a high-pressure wound, a high fibrinogen concentration with

added calcium should be used to reduce the bleeding time and blood loss. If the sealant is to be used for sealing a low-pressure wound, a low fibrinogen concentration with added calcium will be adequate in reducing the bleeding time and blood loss. It should also be noted that enough fibrin sealant must be applied to cover the wound and surrounding tissue in order to provide enough surface area for the sealant to adhere to the tissue.

CHAPTER 8: SUMMARY AND CONCLUSIONS

This dissertation reports the in-vitro and animal experimental evaluation of a rapid and inexpensive method to produce autologous fibrinogen concentrate for use in fibrin sealant. Following are the new contributions to the field.

8.1 Novel Use of Protamine to Harvest Fibrinogen

For the first time, fibrinogen was harvested by precipitation with protamine sulfate and concentrated for use in a fibrin sealant. At the optimal conditions, 96% ± 4% of the fibrinogen present in the plasma was precipitated with protamine, and 98% ± 0.9% of the precipitated fibrinogen was clottable. In addition, it was shown that almost 50% of the factor XIII in the plasma was precipitated along with the fibrinogen.

8.2 More Consistent Methods to Test Tensile and Adhesion Strength

In-vitro models to evaluate tensile strength and adhesion strength were designed and fabricated and these models provided more control than previously-published models. This was accomplished by using precise molds with controlled location of failure and repeatable tissue preparation which lowered the variability. Tensile strength

and adhesion strength both increased with increasing fibrinogen concentration. Addition of calcium chloride to the concentrate significantly increased the tensile and adhesion strengths. When calcium was added to the concentrate, clots reached their maximum tensile strength in less than a minute and reached their maximum adhesion strength in 5 minutes. The maximum tensile and adhesion strengths in the presence of calcium were significantly greater than the tensile and adhesion strengths of Tisseel. The addition of antifibrinolytic agents to the fibrinogen concentrate was shown to have no effect on the mechanical properties of the sealant.

8.3 Kinetic Study

Kinetic experiments showed that the clotting time decreased with increased thrombin concentration and fibrinogen concentration and that protamine did not adversely affect the kinetics. Michaelis-Menton constants were calculated from the fibrinogen and thrombin concentration data and agreed favorably with previously published constants for pure fibrinogen and thrombin, showing that the protamine did not affect the clotting kinetics. The fibrinogen concentrate was completely clotted and polymerized in less than 20 seconds.

8.4 Relevant Animal Model

A rat model was developed to simulate a bleeding wound to evaluate the hemostatic efficacy of a fibrin sealant made from the protamine-fibrinogen concentrate

and the model provided more control (lower variability) than previously-published models. This was accomplished by having a consistent, repeatable incision and a better representation (actively-bleeding wound) of how the sealant would be used in surgery. The fibrin sealant significantly reduced the blood loss and bleeding time when compared to no sealant, plasma, and Tisseel. The sealant also significantly reduced blood loss and bleeding time in rats that were anticoagulated with heparin.

8.5 Mathematical Model

For the first time, a mathematical model based on tensile strength and adhesion strength was developed to predict the bleeding time in the animal wound. Model predictions showed that the ability of the fibrin sealant to reduce bleeding, and therefore blood loss, was limited by the adhesion strength. The model also showed that in order to minimize the bleed time and blood loss, a high fibrinogen concentration (60 mg/mL) with calcium should be used.

From the research conducted for this dissertation, it may be concluded that autologous fibrinogen can be rapidly and inexpensively harvested with protamine sulfate. The fibrinogen concentrate can be used in all arenas of surgery to decrease bleeding and decrease healing time. These findings have considerable implications in trauma situations where the need for fibrin sealant can be high and time is short.

CHAPTER 9: FUTURE DIRECTIONS

The research conducted for this dissertation suggests some potential areas of future research. This chapter briefly outlines some of these possibilities.

9.1 Autologous Thrombin

In order for a fibrin sealant to be truly autologous, both the fibrinogen and thrombin must be of an autologous source. Currently the thrombin component of autologous fibrin sealant is almost always obtained from a commercially-available bovine source. However, patient immune reaction to the bovine thrombin has been reported [102-105]. It is estimated that 30% of all patients treated with bovine thrombin develop antibodies to their native coagulation factors, which can lead to bleeding complications. In addition, the possibility of viral contamination in bovine thrombin can not be ruled out, particularly in light of recent outbreaks of mad cow disease. Commercial preparations of human thrombin are becoming more widely used but carry the risk of virus transmission.

A rapid and inexpensive method to harvest and concentrate autologous thrombin would reduce patient exposure to bovine thrombin and any associated coagulopathies. Autologous thrombin could be combined with the autologous fibrinogen described in this dissertation to give a truly autologous fibrin sealant.

9.2 Whole Blood Precipitation

The method described in this dissertation to harvest fibrinogen requires plasma to be separated from whole blood. This can be accomplished with centrifugation, plasmapheresis, or filtration. Directly precipitating the fibrinogen from whole blood would eliminate this step and reduce the processing time for harvesting autologous fibrinogen. Preliminary attempts were undertaken to precipitate fibrinogen from whole blood with little success. When protamine was added to whole blood, aggregates comprised of red blood cells, protamine, and fibrinogen formed, which broke up the network of fibrinogen precipitate. This resulted in harvesting <20 % of the fibrinogen in the plasma.

It has been shown that protamine binds to red blood cells [106-108]. The aggregates formed when protamine was added to whole blood were probably due to protamine binding red blood cells and fibrinogen together. If the binding sites on the red blood cells were to be masked with a chemical agent that does not interact with fibrinogen, it may be possible to precipitate fibrinogen from whole blood.

REFERENCES

1. Jackson, M.R. Fibrin sealants in surgical practice: An overview. *Am J Surg* 2001; 182(2 Suppl):1S-7S.
2. Gammon, R.R., N. Avery, and P.D. Mintz. Fibrin sealant: an evaluation of methods of production and the role of the blood bank. *J Long Term Eff Med Implants* 1998; 8(2):103-16.
3. Guyton, A.C. and J.E. Hall, *Textbook of Medical Physiology*. 9th ed. 1996, Philadelphia: W. B. Saunders Company.
4. Cooney, D.O., *Biomedical Engineering Principles: An Introduction to Fluid, Heat, and Mass Transport Processes*. 1976, New York: Marcel Dekker, Inc.
5. Andersson, L.-O. Purification and studies of components of the haemostatic system by affinity chromatography. *J Chromatogr* 1981; 215:153-164.
6. Spotnitz, W.D. Fibrin sealant in the United States: clinical use at the University of Virginia. *Thromb Haemost* 1995; 74(1):482-5.
7. Cronkite, E.P., E.L. Lozner, and J. Deaver. Use of thrombin and fibrinogen in skin grafting. *JAMA* 1944; 124:976-978.
8. Matras, H., et al. [Suture-free interfascicular nerve transplantation in animal experiments]. *Wien Med Wochenschr* 1972; 122(37):517-23.
9. Mintz, P.D., et al. Fibrin sealant: clinical use and the development of the University of Virginia Tissue Adhesive Center. *Ann Clin Lab Sci* 2001; 31(1):108-18.
10. Wolner, E. Fibrin gluing in cardiovascular surgery. *Thorac Cardiovasc Surg* 1982; 30(4):236-7.
11. Shaffrey, C.I., et al. Neurosurgical applications of fibrin glue: augmentation of dural closure in 134 patients. *Neurosurgery* 1990; 26(2):207-10.
12. Lagoutte, F.M., L. Gauthier, and P.R. Comte. A fibrin sealant for perforated and preperforated corneal ulcers. *Br J Ophthalmol* 1989; 73(9):757-61.
13. Shah, M.A., A.M. Ebert, and W.E. Sanders. Fibrin glue fixation of a digital osteochondral fracture: case report and review of the literature. *J Hand Surg [Am]* 2002; 27(3):464-9.
14. Athanasiou, K.A., et al. Basic science of articular cartilage repair. *Clin Sports Med* 2001; 20(2):223-47.
15. Drake, D.B. and R.E. Ferguson, Jr. Fibrin sealants in microvascular surgery: current status. *J Long Term Eff Med Implants* 2001; 11(1-2):65-72.
16. Loose, H.W. and P.J. Haslam. The management of peripheral arterial aneurysms using percutaneous injection of fibrin adhesive. *Br J Radiol* 1998; 71(852):1255-9.

17. Houston, K.A. and O.D. Rotstein. Fibrin sealant in high-risk colonic anastomoses. *Arch Surg* 1988; 123(2):230-4.
18. Singh, M.P., et al., *Sustained release of antibiotics from fibrin sealant*, in *Surgical Adhesive Sealants: Current Technology and Applications*, D.H. Sierra and R. Saltz, Editors. 1996, Technomic: Lancaster.
19. Currie, L.J., J.R. Sharpe, and R. Martin. The use of fibrin glue in skin grafts and tissue-engineered skin replacements: a review. *Plast Reconstr Surg* 2001; 108(6):1713-26.
20. MacPhee, M.J., et al., *Fibrin sealant: a versatile delivery vehicle of drugs biologics*, in *Surgical Adhesive Sealants: Current Technology and Applications*, D.H. Sierra and R. Saltz, Editors. 1996, Technomic: Lancaster.
21. Wozniak, G. Fibrin sealants in supporting surgical techniques: the importance of individual components. *Cardiovasc Surg* 2003; 11(S1):17-21.
22. Marx, G. and A. Blankenfeld. Kinetic and mechanical parameters of pure and cryoprecipitate fibrin. *Blood Coagul Fibrinolysis* 1993; 4(1):73-8.
23. MacGillivray, T.E. Fibrin sealants and glues. *J Card Surg* 2003; 18:480-485.
24. Martinowitz, U. and W.D. Spotnitz. Fibrin tissue adhesives. *Thromb Haemost* 1997; 78(1):661-6.
25. Dickneite, G., et al. A comparison of fibrin sealants in relation to their in vitro and in vivo properties. *Thromb Res* 2003; 112(1-2):73-82.
26. Dickneite, G., et al. The importance of factor XIII as a component of fibrin sealants. *J Surg Res* 2002; 107:186-195.
27. Marx, G. and X. Mou. Characterizing fibrin glue perforance as modulated by heparin, aprotinin, and factor XIII. *J Lab Clin Med* 2002; 140:152-160.
28. Phillips, M., G. Dickneite, and H. Metzner. Fibrin sealants in supporting surgical techniques: strength in factor XIII. *Cardiovasc Surg* 2003; 11(S1):13-16.
29. Hansen, M.S. Fibronectin and coagulation factor XIII increases blood platelet adhesion to fibrin. *Thromb Res* 1984; 34:551-556.
30. Bale, M.D. and D.F. Mosher. Thrombospondin is a substrate for blood coagulation factor XIIIa. *Biochemistry* 1986; 25:5667-5673.
31. Hada, M., et al. Covalent crosslinking of von Willebrand factor to fibrin. *Blood* 1986; 68:95-101.
32. Vinazzer, H. Fibrin sealing: physiologic and biochemical background. *Facial Plastic Surgery* 1985; 2(4):291-295.
33. Pipan, C.M., et al. Effects of antifibrinolytic agents on the life span of fibrin sealant. *J Surg Res* 1992; 53:402-407.
34. Krishnan, L.K., et al. Fibrinolysis inhibitors adversely affect remodeling of tissues sealed with fibrin glue. *Biomaterials* 2003; 24:321-327.
35. Dresdale, A., et al. Preparation of fibrin glue from single-donor fresh-frozen plasma. *Surgery* 1985; 97:750-754.
36. Mosesson, M.W. Fibrin polymerization and its regulatory role in hemostasis. *J Lab Clin Med* 1990; 116:8-17.
37. Spotnitz, W.D., et al. Fibrin glue from stored human plasma: an inexpensive and efficient method for local blood bank preparation. *Am Surg* 1987; 53:460-464.
38. Hood, A., *Device and method for concentrating plasma*. January 29, 2002, Interpore Orthopedics, Inc.: USA.

39. Kevy, S.V. and M.S. Jacobson. Comparison of methods for point of care preparation of autologous platelet gel. *J Extra Corpor Technol* 2004; 36(1):28-35.
40. O'Neill, E.M., et al. Autologous platelet-rich plasma isolated using the Haemonetics Cell Saver 5 and Haemonetics MCS+ for the preparation of platelet gel. *Vox Sang* 2001; 81(3):172-5.
41. Nakamura, Y., et al. Comparative study of cell saver and ultrafiltration nontransfusion in cardiac surgery. *Ann Thorac Surg* 1990; 49(6):973-8.
42. Johnson, H.D., et al. Comparative analysis of recovery of cardiopulmonary bypass residual blood: cell saver vs. hemoconcentrator. *J Extra Corpor Technol* 1994; 26(4):194-9.
43. Koster, A., et al. Autologous plasma and platelet sequestration at the beginning of cardiopulmonary bypass: a pilot investigation in five patients undergoing extended vascular surgery in deep hypothermia. *Asaio J* 2002; 48(1):106-9.
44. Sirieix, D., et al. Comparative study of different biological glues in an experimental model of surgical bleeding in anesthetized rats: platelet-rich and -poor plasma-based glue with and without aprotinin versus commercial fibrinogen-based glue. *Ann Vasc Surg* 1998; 12(4):311-6.
45. Vila, V., et al. A rapid method for isolation of fibrinogen from human plasma by precipitation with polyethylene glycol 6,000. *Thromb Res* 1985; 39:651-656.
46. Yoshida, H., K. Hirozane, and A. Kamiya. Comparative study of autologous fibrin glues prepared by cryo-centrifugation, cryo-filtration, and ethanol precipitation methods. *Biol Pharm Bull* 1999; 22(11):1222-5.
47. Silver, F.H., M.C. Wang, and G.D. Pins. Preparation of fibrin glue: a study of chemical and physical methods. *J Appl Biomater* 1995; 6(3):175-83.
48. Sierra, D.H. Fibrin sealant adhesive systems: a review of their chemistry, material properties and clinical applications. *J Biomat Appl* 1993; 7:309-352.
49. Park, M.S. and C.I. Cha. Biochemical aspects of autologous fibrin glue derived from ammonium sulfate precipitation. *Laryngoscope* 1993; 103(2):193-6.
50. Brennan, M. Fibrin glue. *Blood Rev* 1991; 5(4):240-4.
51. Barker, T.H., et al. Modification of fibrinogen with poly(ethylene glycol) and its effects on fibrin clot characteristics. *J Biomed Mater Res* 2001; 56(4):529-35.
52. Ferguson, J.H. The action of heparin, serum albumin (crystalline), and salmine on blood-clotting mechanisms. *Am J Physiol* 1940; 130:759-70.
53. Mylon, E., M.C. Winternitz, and G.J. Suto-Nagy. The determination of fibrinogen with protamine. *J Biol Chem* 1942; 143:21-27.
54. Portmann, A.F. and W.D. Holden. Protamine (salmine) sulphate, heparin, and blood coagulation. *J Clin Invest* 1949; 28:1451-1458.
55. Kjaergard, H.K., et al. The Vivostat system for the automated preparation of autologous fibrin sealant. 1997; 2:204-206.
56. Burnouf, T. and M. Radosevich. Affinity chromatography in the industrial purification of plasma proteins for therapeutic use. *J Biochem Biophys Methods* 2001; 49(1-3):575-86.
57. Heene, D.L., et al. Separation of fibrinogen degradation products (FDP) by means of adsorption to insolubilized fibrinmonomer (FM-ag). *Thromb Haemost* 1979; 41:677-686.

58. Heene, D.L. and F.R. Matthias. Adsorption of fibrinogen derivatives on insolubilized fibrinogen and fibrinmonomer. *Thromb Res* 1973; 2:137-154.
59. Matthias, F.R., G. Hocke, and H.G. Lasch. Isolation of fibrinogen from plasma by affinity chromatography on insolubilized fibrinogen (FG-ag) and insolubilized fibrinmonomer (FM-ag). *Thromb Res* 1975; 7:861-870.
60. Furlan, M., *Structure and function of normal fibrinogen*, in *Current Problems in Clinical Biochemistry 14*, E.A. Beck and M. Furlan, Editors. 1984, Hans Huber Publishers: Berne.
61. Mohri, H. and T. Ohkubo. Fibrinogen binds to heparin: the relationship of the binding of other adhesive proteins to heparin. *Arch Biochem Biophys* 1993; 303(1):27-31.
62. Farooqui, A.A. and L.A. Horrocks. Heparin-Sepharose affinity chromatography. *Adv Chromatogr* 1984; 23:127-148.
63. Dempfle, C.E. and D.L. Heene. Purification of human plasma fibrinogen by chromatography on protamine-agarose. *Thromb Res* 1987; 46:19-27.
64. Dempfle, C.E. and D.L. Heene. Isolation and purification of fibrinogen/fibrin degradation products by chromatography on protamine-agarose. *Thromb Res* 1987; 48:223-232.
65. Tisseel Package Insert, Baxter Healthcare Corporation.
66. Unkown. A chance to cut is a chance to cure. *Meblog Links* 2004. http://www.cut-to-cure.blogspot.com/2004_03_01_cut-to-cure_archive.html.
67. Cohn, S.M., et al. Recipe for poor man's fibrin glue. *J Trauma* 1998; 44(5):907.
68. Fattahi, T., M. Mohan, and G.T. Caldwell. Clinical applications of fibrin sealants. *J Oral Maxillofac Surg* 2004; 62(2):218-24.
69. Toma, A.G., E.W. Fisher, and A.D. Cheesman. Autologous fibrin glue in the repair of dural defects in craniofacial resections. *J Laryngol Otol* 1992; 106(4):356-7.
70. Joch, C. The safety of fibrin sealants. *Cardiovasc Surg* 2003; 11(Suppl 1):23-8.
71. McCullough, J. Progress toward a pathogen-free blood supply. *Clin Infect Dis* 2003; 37(1):88-95. Epub 2003 Jun 25.
72. Data on file, Baxter Healthcare Corporation.
73. Hino, M., et al. Transmission of symptomatic parvovirus B19 infection by fibrin sealant used during surgery. *Br J Haematol* 2000; 108(1):194-5.
74. Kawamura, M., et al. Frequency of transmission of human parvovirus B19 infection by fibrin sealant used during thoracic surgery. *Ann Thorac Surg* 2002; 73(4):1098-100.
75. Kjaergard, H.K., et al. Development of a model for measurement of adhesion strength of fibrin sealant to human tissue. *Eur Surg Res* 1999; 31:491-496.
76. Siedentop, K.H., J.J. Park, and B. Sanchez. An autologous fibrin tissue adhesive with greater bonding power. *Arch Otolaryngol Head Neck Surg* 1995; 121(769-772).
77. Rubenstein, E. Studies on clot tensile strength. *Thromb Diath Haemorrh* 1967; 17(3-4):552-560.
78. Nowotny, R., et al. Preparation of fibrin clot samples for tensile stress-strain testing. *Biomaterials* 1981; 2:55-56.

79. Velada, J.L., et al. Reproducibility of the mechanical properties of Vivostat system patient-derived fibrin sealant. *Biomaterials* 2002; 23(10):2249-54.
80. Laitakari, K. and J. Luotonen. Autologous and homologous fibrinogen sealants: adhesive strength. *Laryngoscope* 1989; 99:974-976.
81. Tuthill, D.D., et al. Assessment of topical hemostats in a renal hemorrhage model in heparinized rats. *J Surg Res* 2001; 95:126-132.
82. Basu, S., et al. Comparative study of biological glues: cryoprecipitate glue, two-component fibrin sealant, and "French" glue. *Ann Thorac Surg* 1995; 60(5):1255-62.
83. Kheirabadi, B.S., et al. Comparative study of the efficacy of the common topical hemostatic agents with fibrin sealant in a rabbit aortic anastomosis model. *J Surg Res* 2002; 106(99-107).
84. Schelling, G., et al. The effectiveness of a fibrinogen-thrombin-collagen-based hemstatic agent in an experimental arterial bleeding model. *Ann Surg* 1987; 205(4):432-435.
85. Hardman, J. and L. Limbird, eds. *The Pharmacological Basis of Therapuetics*. 10th ed. 2001, McGraw-Hill: New York.
86. Balleisen, L., et al. Epidemiological study on factor VII, factor VIII and fibrinogen in an industrial population: 1. Baseline data on the relation to age, gender, body-weight, smoking, alcohol, pill-using, and menopause. *Thromb Haemost* 1985; 54:475-479.
87. Lee, A.J., et al. Fibrinogen in relation to personal history of prelevant hypertension, diabetes, stroke, intermittent claudication, coronary heart disease and family history: the scottish heart health study. *Br Heart J* 1993; 69(338-342).
88. Lip, G.Y.H. Fibrinogen and cardiovascular disorders. *Q J Med* 1995; 88:155-165.
89. Tarallo, P., et al. Reference limits of plasma fibrinogen. *Eur J Clin Chem Clin Biochem* 1992; 30:745-751.
90. Casali, B., et al. Fibrin glue from single-donation autologous plasmapheresis. *Transfusion* 1992; 32:641-643.
91. Clauss, A. Gerinungs physiologische schnell methods zur bestimmung des fibrinogens. *Acta Haematol* 1957; 17:237.
92. De Christofaro, R. and E. Di Cera. Phenomenological analysis of the clotting curve. *J Prot Chem* 1991; 10(5):455-468.
93. Wolfe, J.K. and D.F. Waugh. Relations between enzymatic and association reactions in the development of bovine fibrin clot structure. *Arch Biochem Biophys* 1981; 211(1):125-142.
94. Hantgan, R.R. and J. Hermans. Assembly of fibrin. A light scattering study. *J Biol Chem* 1979; 254(22):11272-11281.
95. Nelson, D. and M. Cox, *Lehninger Principles of Biochemistry*. 3rd ed. 2000, New York: Worth Publishers.
96. Mihalyi, E. Changes in pH associated with clotting of fibrinogen. Kinetic studies of the pH shift and correlation of the pH change with the release of fibrinopeptides and the ensuing polymerization. *Biochemistry* 1991; 30:4753-4762.
97. Martinelli, R.A. and H.A. Scheraga. Steady-state kinetic study of the bovine thrombin-fibrinogen interaction. *Biochemistry* 1980; 19:2343-2350.

98. Lewis, J.H., et al. Comparative hematology and coagulation studies on rodentia (rats). *Comp Biochem Physiol* 1985; 82A(1):211-215.
99. Porter, J. Personal Communication.
100. Sumitra, M., et al. Cardiorespiratory effects of diazepam-ketamine, xylazine-ketamine and thiopentone anesthesia in male Wistar rats--a comparative analysis. *Life Sci* 2004; 75(15):1887-1896.
101. Wixson, S., et al. The effects of pentobarbital, fentanyl-droperidol, ketamine-xylazine and ketamine-diazepam on core and surface body temperature regulation in adult male rats. *Lab Anim Sci* 1987; 37(6):743-749.
102. Carroll, J.F., et al. Immunologic assessment of patients treated with bovine fibrin as a hemostatic agent. *Thromb Haemost* 1996; 76(6):925-31.
103. Israels, S.J. and M.T. Leaker. Acquired inhibitors to factors V and X after exposure to topical thrombin: interference with monitoring of low molecular weight heparin and warfarin. *J Pediatr* 1997; 131(3):480-3.
104. Rapaport, S.I., et al. Clinical significance of antibodies to bovine and human thrombin and factor V after surgical use of bovine thrombin. *Am J Clin Pathol* 1992; 97(1):84-91.
105. Streiff, M.B. and P.M. Ness. Acquired FV inhibitors: a needless iatrogenic complication of bovine thrombin exposure. *Transfusion* 2002; 42:18-26.
106. Antohi, S. and V. Brumfeld. Polycation-cell surface interactions and plasma membrane compartments in mammals. Interference of oligocation with polycationic condensation. *Z Naturforsch* 1984; 39(7-8):767-775.
107. Barfod, N.M. and B. Larsen. Polymerization of protamine sulphate by carbodiimide and interaction of isolated protamine polymers with human red blood cells. *Biochimica et Biophys Acta* 1976; 427(1):197-207.
108. Remmel, R.P., A.K. Copa, and D.M. Angaran. The effects of hemodilution, pH, and protamine on lidocaine plasma protein binding and red blood-cell uptake in vitro. *Pharm Res* 1991; 8(1):127-130.