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THE *in vitro* STABILITY OF CROSS-LINKED FIBRIN AND FIBRIN/HYALURONAN-COATED POLYURETHANES IN THE PRESENCE OF PLASMIN

By

Christine Adelle Bense, B.A.Sc.

A Thesis
Submitted in Conformity with the Requirements for the Degree
Master of Applied Science and Engineering

Graduate Department of Chemical Engineering
University of Toronto
1998

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The goal of this research was to evaluate the \textit{in vitro} stability of thin fibrin coatings on polymeric materials in the presence of plasmin. The effects of cross-linking and the presence of hyaluronan in the fibrin matrix on the relative stability of the coatings were investigated. Degradation assays indicated that cross-linking significantly enhanced the stability of fibrin coatings on both Tegaderm\textsuperscript{®} and PCL/HDI/Phe; however, the persistence of the coating on the woven Corethane\textsuperscript{TM} was not as influenced by cross-linking of the thin fibrin layer. The presence of HA within the fibrin matrix destabilized the coatings on both Tegaderm\textsuperscript{®} and PCL/HDI/Phe, but enhanced the stability of the fibrin layer on Corethane\textsuperscript{TM}. The chromogenic substrate assay data showed cross-linking did not affect the specific plasmin activity on the coatings; therefore, the increased stability resulting from cross-linking was not likely achieved through a reduction of fibrinolysis. The presence of HA within the fibrin matrix decreased the plasmin activity on all coated samples, most probably by interfering with the ternary complex between fibrin, plasminogen and tPA that is responsible for accelerating the rate of plasminogen activation. Therefore, although HA decreased the fibrinolysis of the cross-linked fibrin coatings, it mechanically weakened the fibrin coatings, making them more susceptible to removal from their respective substrates during the degradation experiments. A thin, cross-linked, fibrin-coated...
polyurethane provides a theoretically attractive biomaterial for use in a wound dressing application and should be subject to ongoing research.
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1.0 Introduction

A biomaterial is "a nonviable material used in a medical device, intended to interact with a biological system" (Williams, 1987). Biomaterials are synthesized in a variety of shapes and forms, including composites, coated systems (Ratner and Hoffman, 1996) and devices such as vascular prostheses, substitute heart valves, stents, catheters and contact lenses. The biocompatibility of devices which interact with the human body is often a measure of performance or success of the device at a specific task (Ratner and Hoffman, 1996). Biocompatibility is determined by the physical and chemical make-up of the material itself and is defined as "the ability of a biomaterial to perform with an appropriate host response in a specific application" (Williams, 1987). Although the biocompatibility of devices is application-specific, biomaterial design is often approached from a general standpoint. Research is frequently driven by the desire to develop a practical material which may find use in multiple applications.

Wound dressings are biomaterials which have received much attention in recent years. They have several important functions including the prevention of protein and fluid losses, protection of the wound against bacterial invasion and promotion of the healing process. Many attempts have been made to fulfill these requirements; however, success has not yet been met. Existing materials are not completely effective in assisting the healing of large, deep, or chronic wounds; however, composites containing both biological and artificial components have shown improved wound healing (Cooper and Spielvogel, 1994; Quinn et al, 1985). For this and other important reasons, it is believed that a bilayer skin replacement based on a fibrin-coated polyurethane may provide a potentially successfully alternative to existing wound dressings.
Much research has focused on fibrin as a haemostatic barrier and a wound healing agent (Dinges et al, 1986; Redl and Schlag, 1986) and the stability of fibrin clots in the presence of plasmin has been extensively studied (Norrmn et al, 1985; Azoury et al, 1989; Urano et al, 1989; Kudinov et al, 1990; Fredenburgh et al, 1992; Reilly and Hutzelmann, 1992). The protein and platelet interactions with cross-linked fibrin-coated surfaces that were developed using the protocol described in this work have also been examined (Skarja et al, In Press). However, the stability of the fibrin coating on materials, particularly polyurethanes, has not yet been investigated. Since fibrin is readily attacked and digested by the enzyme plasmin in vivo and the coating may need to be stable on the surface of a wound dressing long enough to modulate cellular infiltration, determining the parameters which impact the resistance of the coatings to fibrinolysis was deemed essential.

In this investigation, fibrin was coated onto three different polyurethane materials using a technology previously developed to investigate “mature thrombus” formation and for cardiovascular applications. The factor XIIIa cross-linked fibrin coating protocol was modified and the coating stability was investigated with a view to its use in a bilaminar wound dressing. In chronic wounds, a thin fibrin coating may provide a wound dressing with superior healing ability. This notion stemmed directly from the high degree of success attained by fibrin sealant in surgery as both a barrier against blood loss and a wound healing agent. Therefore, the fibrin coating was evaluated here as a potential dermal substitute in a bilayer skin replacement.

1.1 Adult Tissue Repair

When the skin undergoes trauma, the process of wound repair is initiated. Although small acute wounds often heal quickly, chronic wounds persist for extended periods of time and can cause significant adverse health affects. Normal adult tissue repair involves four stages: haemostasis, inflammation, cellular proliferation and tissue
remodeling as illustrated in Figure 1-1. The nonhealing of chronic wounds is believed to result when one or more of these processes is impaired (Lawrence, 1992).

1.1.1 Haemostasis

Haemostasis is the body's natural and immediate response to tissue and blood vessel damage. It is a complex process which involves two main processes: coagulation and fibrinolysis.

Figure 1-1: Time course of wound healing. The deposition of fibrin marks the beginning phase of the healing process. Fibrin provides a provisional matrix for the adhesion and migration of cells during the proliferative phase. Adapted from Mast (1992).
1.1.1.1 Coagulation

The coagulation pathway is a natural defense system that works to maintain the integrity of the circulatory system following blood vessel injury (Furie and Furie, 1988). The coagulation cascade is a system of enzymatically catalyzed reactions that results in the formation of an insoluble clot when thrombin converts soluble fibrinogen to insoluble fibrin. The coagulation pathway is shown in Figure 1-2.

Fibrinogen is a structural protein which is found in relatively high concentrations in blood (3-4 mg/mL).

Figure 1-2: The coagulation pathway. Both intrinsic and extrinsic pathways converge to a common pathway which involves the generation of thrombin and the subsequent formation of insoluble, cross-linked fibrin. Adapted from Ratner and Hoffman (1996).
It is a large trinodal protein comprised of three pairs of disulfide-bonded polypeptide chains. The α, β and γ chains possess molecular weights of 67 kDa, 56 kDa and 47 kDa, respectively. The amino terminals of the chains form the central E domain of the fibrinogen molecule (Doolittle, 1978) and the carboxy-terminals of the β and γ chains fold to form distinct globular D domains. Figure 1-2 provides a representation of this structure.

![Diagram of fibrinogen structure](image)

**Figure 1-3:** Structure of fibrinogen. The carboxy and amino terminals of the α, β and γ chains illustrated in A) comprise the D and E domains, respectively as shown in B). Dashed lines represent the paired disulfide bonds that stabilize the fibrinogen molecule. Adapted from Dang (1989).

A three-dimensional illustration of the fibrinogen molecule is shown in Figure 1-4. As this figure indicates, the carboxy-terminals of the α chains actually fold back away from the D domains and form a folded domain above the central E domain. A more detailed presentation of the fibrinogen molecule is illustrated in Figure 1-5.

Fibrinogen is cleaved by thrombin, a trypsin-like serine protease that is also responsible for platelet activation and the stimulation of endothelial cells. Thrombin
binds to the central E domain of the fibrinogen molecule, cleaving the protein. Upon cleavage of fibrinogen, two moles each of fibrinopeptides FPA and FPB are liberated and fibrin I and fibrin II monomers are formed. The release of FPA and FPB exposes polymerization sites at the E and D domains of the monomers. Therefore, when the E and D domains of adjacent monomers interact with each other, polymerization occurs and fibrin bundles are formed (Doolittle, 1978; Nossel, 1981; Weisel et al, 1985; Dang and Bell, 1989).

Figure 1-4: Three-dimensional structure of fibrinogen. The dimeric central E domain of the molecule which is comprised of the amino terminals of the α, β and γ chains is represented by two spheres. The hydrophilic carboxy-terminal ends of the α chains depart and fold back away from the globular D domains to form a folded domain above the central E domain of the molecule. Each D domain is shown as two spheres, representing the folded ends of the β and γ chains. Structural data was obtained from electron microscopy and x-ray diffraction studies (Hantgan et al, 1987).

Fibrin bundles are further stabilized by factor XIII which renders clots insoluble by covalently cross-linking the fibrin polymers. Factor XIII is inactive until it is cleaved by thrombin at its amino terminus, at which point, it undergoes calcium-induced
conformational changes. Once activated, it catalyzes the intermolecular covalent cross-linking of fibrin by covalently linking glutamine and lysine residues on adjacent fibrin molecules forming cross-linked species including γ-γ dimers and α-polymers. The formation of these fragments is illustrated in Figure 1-6 and marks the formation of an insoluble barrier against blood loss (Greenberg, 1993; Achyuthan et al, 1994).

Figure 1-5: Schematic diagram of fibrinogen adapted from Hantgan et al (1987). The seven pairs of disulfide bonds that link the six polypeptide chains of the molecule are represented by straight lines. Points marked T and P indicate thrombin and plasmin cleavage sites. The cross-linking sites on the lysine and arginine residues of the carboxy-terminal ends are marked by XL.

There are four to six γ-glutamyllysyl cross-links per monomer of fibrin, of which, two sets form between γ chains and the remainder between α chains. In vivo, γ-γ dimers form within 2-5 minutes; however, α-polymers are more complex and require up to 24 hours to form completely (McDonagh et al, 1987). In fact, each α polymer consists of at least five or six adjacent α chains. Once the covalent isopeptide bonds are formed and fibrin has been converted from a soluble to an insoluble form, the fibrin
possesses increased mechanical stability and resistance to plasmin digestion. The stabilized fibrin bundles then act as a basis for continued thrombus formation.

**Figure 1-6:** Covalent stabilization of fibrin via factor XIIIa. Glutamine and lysine residues on adjacent soluble fibrin molecules are covalently linked by the enzyme factor XIIIa, forming insoluble, cross-linked fibrin. The resulting ε(γ-glutamyl)lysyl bonds form the basis of several cross-linked fragments including γ-γ dimers and α-polymers. Adapted from (McDonagh et al, 1987).

1.1.1.2 Fibrinolysis

The fibrinolytic system maintains vascular function by degrading fibrin, the end product of coagulation (Fears, 1989). In doing this, it improves blood flow and facilitates the healing process after injury and inflammation (Ratner and Hoffman, 1996). In this system of enzymatically catalyzed reactions, plasminogen is converted to plasmin which, in turn, cleaves fibrin polymers as illustrated in Figure 1-7.
Figure 1-7: Simplified schematic representation of the fibrinolytic pathway. Tissue-type plasminogen activator is shown in this illustration since it was used to cleave plasminogen in this investigation; however, urokinase-like plasminogen activator (uPA) also exists. Plasminogen activator inhibitors are not shown. Adapted from Francis and Marder (1987).

Plasminogen is the primary proenzyme of the fibrinolytic system. It is a protein composed of a single polypeptide chain whose normal plasma concentration is 2.2 μmol/L (Mayer, 1990). As shown in Figure 1-8, the plasminogen molecule contains kringie structures that contain lysine binding sites (LBS). Plasminogen binds to fibrin via the strong LBS in kringie I and is activated to plasmin by plasminogen activators, such as tissue-type plasminogen activator (tPA) (Ratner and Hoffman, 1996). At the fibrin surface, plasminogen activation occurs at an accelerated rate compared to in plasma due to the ternary complex that forms between fibrin and both plasminogen and tPA (Wiman and Hamsten, 1990). The rate constants \( k_{cat}/K_M \) for enzyme-catalyzed reactions during activation of plasminogen by tPA increase from \(< 5.6 \times 10^3 \text{ M}^{-1} \times \text{sec}^{-1}\) to \(1.7 \times 10^6 \text{ M}^{-1} \times \text{sec}^{-1}\) in the presence of fibrin.
Plasmin is a two-chain protein linked by two disulfide bonds. It cleaves lysyl and arginyl bonds in fibrin, releasing fibrin-fibrinogen degradation products (FDP) into the circulating blood (Ratner and Hoffman, 1996). The cleavage of non cross-linked fibrin is identical to that of fibrinogen in kinetics and results in the liberation of derivatives with the same structure (Francis and Marder, 1987). However, the cleavage of cross-linked fibrin produces D dimers and other FDPs (Siebenlist and Mosesson, 1994) which are illustrated in Figure 1-9. The cross-link bonds in stabilized fibrin are responsible for the generation of these specific high molecular weight degradation fragments.

![Figure 1-8: Schematic representation of plasminogen (Lijnen and Collen, 1982). Kringles are labeled I-V.](image)

More extensive degradation of cross-linked fibrin via plasmin proteolysis results in the liberation of smaller fragments including individual $\gamma$-$\gamma$ dimers and $\alpha$-polymers. In fact, these FDPs can be used to identify stabilized fibrin via application of SDS-
PAGE since distinct bands representing γ–γ dimers and α-polymers appear at 100 kDa and 200 kDa, respectively (Francis et al, 1979; Serrano et al, 1993; Mosesson, 1996). In this case, the degradation products are reduced gel products (RGPs).

![Diagram](image)

**Figure 1-9:** Plasmin degradation of cross-linked fibrin. A series of noncovalently bound complexes results from the degradation of a two-stranded protofibril of fibrin. The α-chain extensions are not shown in this illustration for convenience. Adapted from (Francis and Marder, 1987).

Once haemostasis is initiated, fibrin is laid down and platelets adhere to the wound site via integrins. These platelets become activated, secrete their granule contents and aggregate forming a clot. One of the most important components of platelet granules is platelet-derived growth factor (PDGF). Platelet derived growth factor is known to be chemotactic for fibroblasts (Kirsner and Eaglstein, 1993). Cytokines contained in platelet granules are partially responsible for the initiation of the inflammatory response.
1.1.2 Inflammation

Inflammation occurs in response to tissue injury (Wahll, 1992; Ratner and Hoffman, 1996). This reaction of vascularized living tissue acts to protect tissue from the adverse impacts of an injurious agent. Inflammation also initiates the wound healing process and is critical to the formation and growth of fibrous tissue. The inflammatory response is a series of complex reactions involving various types of cells. It is dominated by the influx of blood-borne cells and the subsequent release of their cytokines (Clark and Henson, 1988).

Acute inflammation, the initial response, is marked by a process called exudation which involves the movement of fluid and leukocytes from the vascular system into the surrounding tissue (Anderson, 1993). These leukocytes migrate to the injury site where they accumulate and become activated. As a result, these cells phagocytose microorganisms and foreign materials. In addition, activated monocytes and platelets release cytokines which upregulate the cell adhesion molecules on macrophages, fibroblasts and epithelial cells, causing these cells to accumulate at the wound site. Macrophages contain various cytokines which induce fibroblast and keratinocyte infiltration and proliferation (Clark and Henson, 1988). The persistence of these cells is characteristic of chronic inflammation; however, if the inflammatory stimulus is eliminated, the healing response begins (Anderson, 1993; Ratner and Hoffman, 1996).

1.1.3 Cellular Proliferation

The next stage in wound healing is cellular proliferation. Here, fibroblasts and epithelial cells are activated to migrate from adjacent tissue into the wound site (Anderson, 1993; Martin, 1997). The cells then proliferate and secrete collagen, elastin and proteoglycans which constitute the main components of the extracellular matrix. Cell migration and proliferation are stimulated by the interaction of regulatory factors
produced by both platelets and mononuclear phagocytes (Morgan, 1992). These factors include platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and tumor necrosis factor (TNF). Fibroblasts and epithelial cells form granulation tissue to reconstruct the damaged area. When the wound space is filled with new tissue, angiogenesis occurs, the wound matrix becomes less cellular, and collagen within the wounded region is remodelled to increase the tensile strength of the newly-formed tissue.

1.1.4 Tissue Remodeling

Reepithelialization of the wound is followed by contraction of underlying connective tissue which shrinks to bring the wound margins together (Martin, 1997). It is generally believed that once the fibrin clot has been completely invaded and replaced by activated fibroblasts, some of the fibroblasts are converted to myofibroblasts that express α-smooth muscle actin and resemble smooth muscle cells. These cells are capable of generating strong contractile forces (Martin, 1997). Depending on the size of the original wound, varying degrees of contraction and scarring occur as the collagen is cross-linked and the wound reaches the final stages of healing.

1.2 Role of Fibrin in Tissue Repair

Fibrin is critical to wound healing, not only because it facilitates cellular infiltration, but because it acts as a haemostatic barrier to form temporary wound closure (Schlag et al, 1986). Early wound repair involves the local deposition of a provisional matrix comprised of fibrin which facilitates the adhesion and migration of fibroblasts and keratinocytes into the injured region (Schlag et al, 1986; Kirsner and Eaglestein, 1993; Brittberg et al, 1997; Martin, 1997). This provisional matrix provides scaffolding for the orderly and controlled restructuring of the tissue. In its absence, fibroblastic tissues lay down collagen in a random fashion, resulting in a scar (Ratner and Hoffman,
In addition, studies have shown that adhesion proteins such as fibronectin, laminin, thrombospondin and vitronectin contain domains specific for binding to fibrin (Rudolph, 1992). Therefore, fibrin plays a structural role in tissue injury and remodeling, it stimulates the formation of granulation tissue and it may promote reepithelialization which is of particular concern in chronic skin wounds (Falanga, 1988).

1.3 Fibrin Glue

Fibrin’s critical role in the wound healing process has resulted in its extensive use as a glue or sealant in surgery. In the early 1900’s, fibrin powder and fibrin patches were used to control bleeding from damaged organs. In the 1940’s, fibrin solutions containing thrombin were used to seal nerve anastomoses and fix skin grafts. By 1975, the two-component fibrin sealant, Tisseel®, was developed (Redl and Schlag, 1986; Schlag et al, 1986). Tisseel® contains highly concentrated fibrinogen, factor XIII, thrombin, calcium chloride, and aprotinin solution. Compared to plasma, fibrin adhesive contains a thirty-fold higher concentration of fibrinogen and a fifty-fold higher concentration of factor XIII.

The technique of fibrin sealing, which involves the conversion of fibrinogen to fibrin, imitates the final stages of coagulation. Fibrin adhesives are used primarily to prevent blood loss following surgery, but have also been shown to improve the natural repair of musculoskeletal tissues (Brittberg et al, 1997). When applied to skin grafts, fibrin sealant mechanically fixes the graft, promotes neovascularization, decreases the loss of fluid from the wound and promotes reepithelialization (Dinges et al, 1986). Studies have shown that sealed skin grafts increase the rate of granulation tissue formation and fiber proliferation (Schlag and Redl, 1989), but reduce the total amount of granulation tissue which forms (Dinges et al, 1986).
Although fibrin glue is most frequently used on its own to reduce blood loss in surgical procedures, it can also be used in conjunction with biomaterials. TachoComb™, for example, is a commercially prepared wound dressing composed of collagen fleece coated with fibrin sealant which has shown superior haemostasis. In addition, fibrin adhesives have potential as delivery vehicles for drugs and other biological materials.

The main problem associated with the use of fibrin sealant initially involved the transmission of hepatitis through virally-contaminated blood used in fibrinogen preparation. The FDA revoked the license for fibrinogen concentrates in the United States in 1978; however, careful donor selection and heat treatment have since resolved this issue. Another problem associated with the use of fibrin sealant was revealed by studies that suggested that fibrin glue may not positively influence impaired wound healing (Dinges et al, 1986). More recent work has shown that large quantities of fibrin may have an inhibitory effect on cell migration and may even cause delayed wound healing (Schlag et al, 1986; Brittberg et al, 1997). Therefore, large quantities of fibrin may promote an exaggerated inflammatory response which may lead to delayed tissue repair.

However, other work using smaller quantities of fibrin sealant has shown that thin film application of fibrin to wound dressings may have a positive influence on tissue repair. Fibrin sealant can be applied in a number of ways, including various spray applications. The spray catheter is connected to a pressurized gas source which allows coating of extensive surfaces with a small amount of sealant. A thin film spray application may be optimum for the sealant to promote wound healing (Redl and Schlag, 1986).

Evidently, fibrin sealant provides a superior barrier against blood loss, and when applied as a thin film, may have the ability to promote the wound healing process. The application of a thin, cross-linked fibrin surface coating as described in this work is
similar to this fibrin glue technology. Although fibrin glue is used to repair damaged tissue during surgery, theoretically it can be transferred to a wound dressing application and attain the same degree of success if applied as a thin layer.

1.4 Hyaluronan

Hyaluronan (sodium hyaluronate, hyaluronic acid, HA) is a naturally occurring high molecular weight, linear polysaccharide which is a major constituent of the extracellular matrix. In fact, HA makes up approximately 3% (dry basis) of the human body (Sedlak, 1995). It is composed of N-acetyl-D-glucosamine and D-glucuronic acid and has the ability to bind up to 400 times its weight with water. The structural formula of HA is illustrated in Figure 1-10.

![Figure 1-10: Structural formula of hyaluronan. HA is a simple glycosaminoglycan that consists of up to several thousand sugar residues in a straight chain. Note the absence of sulfate groups. There is increasing evidence that HA plays an important role in cell migration during development and wound repair. Adapted from Goa and Benfield (1994).](image)
Studies have shown that wound sites are normally initially depleted of HA; however, the body responds by producing more HA receptors to facilitate its accumulation (Sedlak, 1995). Therefore, the local concentration of HA increases at the wound site during tissue repair as it transports growth factors to the lesion (Goa and Benfield, 1994; McCourt et al, 1994). High concentrations of large molecular weight forms of HA appear to promote the recruitment of stimulated macrophages and neutrophils which are necessary for reducing inflammation and for wound healing (Laurent and Fraser, 1986; West and Kumar 1989). The local concentration and molecular weight of HA also has been shown to influence angiogenesis (West and Kumar, 1989). In addition, HA has a high affinity for fibrin, with which it forms a supporting extracellular matrix that modulates fibroblast proliferation and granular tissue formation (Fournier and Doillon, 1994; Goa and Benfield, 1994). In fact, HA is considered a provisional matrix component (Svee et al, 1996).

In fetal wounds, a prolonged HA-rich environment facilitates cell mobility, cell proliferation, and regeneration which ultimately leads to rapid and scarless healing (Adzick and Longaker, 1992). This mechanism is absent in adult wounds where HA is initially deposited at the wound site via the fibrin clot. At the wound site, hyaluronidase is produced and HA is removed. Sulfated glycosaminoglycans are then deposited and collagen is laid down in a scar pattern. It has been postulated that an HA-rich extracellular matrix (ECM) may provide a permissive environment for the orderly deposition of collagen by acting as a carrier for biologically active proteins (Adzick and Longaker, 1992). The same work has shown that membranes treated with HA close faster than untreated controls and show much less scar tissue. Finally, hyaluronan may define the space in which cells proliferate and grow (Wight et al, 1991). Therefore, HA appears to play an essential role in the wound healing process, and theoretically is a desirable additive for a wound dressing.
1.5 Potential Use

A fibrin-coated polyurethane is an example of a novel biomaterial which may find use in multiple applications. In particular, a fibrin coated surface may provide a potential alternative to existing wound dressing materials that are not completely effective in assisting the healing of large, deep or chronic wounds.

1.5.1 Wound Dressings

The primary function of wound dressings is to provide a healing environment to a damaged region of the skin. A successful wound dressing must prevent protein and fluid losses, protect the wound against bacterial invasion and aid the healing process. Wetting, conforming and adhering to the wound surface are critical properties of any dressing. Dressings also must be permeable to moisture in order to prevent the accumulation of fluid and the swelling of the wound bed. In addition, dressings should act to reduce pain, which they often do by limiting inflammation and by protecting exposed nerve endings. Dressings should create an environment that promotes the formation of granulation tissue, appropriate wound contraction and epithelialization (Quinn et al, 1985). Existing materials meet only some of these requirements.

1.5.1.1 Existing Wound Dressings

As shown in Figure 1-11, wound dressings fall into three categories: conventional, synthetic and biological. The most notable advantage of conventional dressings, which include cotton wool and gauze, is their adsorptive capacity (Quinn et al, 1985). These dressings are also cost efficient and easy to store. Synthetic dressings include films, gels, foams and composites. In general, these dressings provide an adequate and conformable barrier for the wound. However, films often fail at preventing infection, gels lack durability and foams and sponges allow ingrowth of epithelium which leads to secondary trauma when the dressing is removed. Despite
these disadvantages, various combinations of synthetic dressings are thought to possess much promise as successful alternatives and are currently being studied.

![Figure 1-11: Options available for the treatment of wounds. Synthetic composites comprised of various combinations of synthetic dressings also exist. Adapted from (Quinn et al, 1985).](image)

The third category of wound dressings, biological dressings, approach ideal coverage (Parente, 1997). Evidently, the best cover for a wound is skin itself; however, both autografts and allografts are limited by supply (Herndon, 1997). Porcine xenografts are limited by problems in rejection, infection and cost (Quinn et al, 1985; Wang et al, 1997). Dressings seeded with epithelial cells have received much attention recently, but despite their success as skin replacements, they are limited by the time required to culture the cells.
Some of the most successful dressings to date include biological derivatives combined with synthetic materials. Studies have shown that bilaminate wound dressings are particularly effective because they mimic the morphologic characteristics of skin (Pritt, 1997). An artificial skin composed of a ‘Silastic’ epidermis and a porous bovine collagen-shark cartilage chondroitin-6-sulfate dermis has been developed (Yannas et al, 1981). This bilayer dressing has attained a high degree of success, but is very expensive to produce and can induce infection. Other biosynthetic dressings including ‘Biobrane’, ‘Dermodress’ and ‘Integra’, which are collagen-based bilaminete skin substitutes, have paved the way for more recent developments in this continually advancing field (Pritt, 1997). Each biosynthetic wound dressing is associated with several distinct advantages associated with their ability to achieve rapid wound closure and cosmetically acceptable results, but each is also ultimately limited in some way. Therefore, there is a demand for the development of novel dressings that surpass those that are currently in practice in these situations.

1.6.1.2 A Novel Wound Dressing Material

A fibrin-coated polyurethane is an example of a bilaminate biosynthetic wound dressing. The physical properties of a polyurethane support its use as a conformable, but durable wound dressing material. The polyurethanes used here were occlusive materials, meaning they were impervious to microbes but were permeable to water vapour and oxygen. Occlusive dressings induce exudation and stimulate the development of granulation tissue which facilitates successful and often complete tissue repair (Eaglestein, 1991). The formation of granulation tissue is also stimulated by fibrin which may promote reepithelialization in chronic wounds (Falanga, 1988). The physical structure of a fibrin coating would likely contribute to the adhesive properties of the wound dressing, an essential property of any successful skin graft. In light of these factors, it is believed that an occlusive bilayer wound dressing based on a
polyurethane coated with a thin layer of cross-linked fibrin may provide enhanced healing of large, deep, or chronic wounds.
2.0 STATEMENT OF OBJECTIVES AND CONTRIBUTIONS

The main objective of this work was to investigate the hypothesis that covalently cross-linking a fibrin layer coated onto a material would increase the fibrin layer’s stability in the presence of plasmin. It was also hypothesized that by incorporating hyaluronan (HA) into the cross-linked fibrin coating, its stability would be further enhanced. The stabilities of non cross-linked, cross-linked fibrin and cross-linked fibrin/HA coatings on varying substrates were studied.

The contributions of this work were as follows:

1) Fundamental information about the relative stabilities of various fibrin coatings on different materials produced using the methods of Rubens et al (Rubens et al, 1992; Skarja et al, 1998). We anticipated that the covalent stabilization of the fibrin coating on a polyurethane would enhance its stability in the presence of plasmin. We also thought that the incorporation of hyaluronan into the fibrin matrix would further enhance the stability of the cross-linked fibrin coatings.

2) Data linking the extent of cross-linking of the fibrin coating to its resulting stability in the presence of plasmin. The degree of cross-linking of the fibrin layer was determined using SDS-PAGE.

3) A method for incorporating hyaluronan (HA) into the cross-linked fibrin coating. Prior to this study, the protocol for the preparation of the non cross-linked and cross-linked fibrin coatings existed; however, the addition of HA to this protocol had to be developed.
4) Information about the specific enzymatic activity of plasmin on fibrin-coated polyurethanes. Results from this study revealed the effect of cross-linking and hyaluronan on the fibrinolysis of the fibrin coatings.

2.1 DISCUSSION

A biomaterial with a thin fibrin coating may provide a successful alternative to conventional wound dressings. Such a fibrin layer could provide the dressing with adhesivity to the wound bed and a provisional matrix for the adhesion and migration of cells. Both have shown to favorably affect tissue repair. However, it may be possible to further enhance the healing ability of the fibrin coating by impregnating the coating with additives, such as hyaluronan (HA), that contribute to the healing process.

HA has been shown to closely interact with fibrin. Therefore, when HA is incorporated into a cross-linked fibrin matrix, HA will likely bind to fibrin and occupy some of the void space between the fibres in the fibrin matrix. HA may disrupt the ternary complex that forms between fibrin, tPA and plasminogen. This complex promotes an accelerated rate of fibrinolysis. In the event that either tPA or plasminogen is prevented from binding to the fibrin matrix because binding sites are occupied or sheltered by the intertwined HA matrix, the overall rate of plasmin digestion of the fibrin coating should decrease and the coating should be more stable. However, HA also has a high affinity for water. Therefore, it is also possible that the swollen fibrin/HA matrix may have more available sites for the adhesion of both plasmin and tPA, thus enhancing the effect of the ternary complex and accelerating the overall degradation of the fibrin coating. The specific interaction of HA with fibrin is not well understood and the effect of HA on the in vitro stability of a cross-linked fibrin coating has not been previously studied. It was hypothesized that HA would increase the stability of the cross-linked fibrin coating.
It was also hypothesized that cross-linking a thin fibrin coating using factor XIII would increase the stability of the coating on a material. Cross-linked fibrin possesses covalent isopeptide bonds between glutamyl and lysyl residues on adjacent fibrin molecules. These bonds do not exist in non cross-linked fibrin. Since more energy is required to equally digest cross-linked fibrin than non cross-linked fibrin, it was predicted that cross-linking a thin fibrin coating would increase its stability in the presence of plasmin.
CHAPTER THREE
FIBRIN COATING PREPARATION AND CHARACTERIZATION

3.0 INTRODUCTION

The application of a thin fibrin surface coating is one method by which the biocompatibility of artificial materials may be enhanced (Rubens et al, 1995). It is generally believed that in order for biomaterials to attain biocompatibility, their surfaces must interact with tissues and blood without inducing uncontrolled thrombus formation and inflammation (Beumer et al, 1994).

The factor XIIIa cross-linked fibrin coating was originally designed to mimic a mature thrombus in order to achieve a potentially non-thrombogenic material suitable for cardiovascular applications (Rubens et al, 1992). Studies involving the platelet reactivity and the protein adsorptivity of both the thermally denatured fibrinogen layer and the thin cross-linked fibrin layer have been performed (Skarja et al, 1998; Rubens et al, 1992; Rubens et al, 1995). Fibrin’s ability to adhere to a wound and its structural role in tissue repair also have been extensively studied (Schlag et al, 1986; Kirsner and Eaglestein, 1993; Fournier and Doillon, 1994; Martin, 1997; Britberg et al, 1997). In this work, the original coating was modified for potential use as a wound dressing.

The protocol used has been shown to achieve thin and uniform surface coverage (Rubens et al, 1992; Skarja et al, 1998). The fibrin layer studied was approximately 50 μm in thickness, as determined using SEM. Studies involving fibrin glue have shown thin film application may be required for fibrin to promote wound healing (Redl and Schlag, 1986). The stability of the thin fibrin layer coated onto a substrate may influence healing when applied as a wound dressing; however, few investigations have examined this relationship. Therefore, investigating the properties that affect the stability of the coating was deemed the next logical step in the development of a biomaterial using this technology.
The system under investigation in this study was comprised of a polyurethane, onto which a bilayer fibrin coating was attached (Rubens et al, 1992; Skarja et al, 1998). Initially, a layer of thermally denatured fibrinogen (TDF) was covalently bound to the substrate. A thin layer of fibrin was then polymerized onto the TDF surface as illustrated in Figure 3-1. In this investigation, non cross-linked fibrin, factor XIIIa cross-linked fibrin and cross-linked fibrin/HA layers were coated on three different polymeric materials: Corethane™, Tegaderm®, and an experimental polyurethane (PCL/HDI/Phe: a polyurethane composed of a polycaprolactone (PCL) soft segment, hexamethylene diisocyanate (HDI), and a phenylalanine (Phe)-based chain extender (Skarja and Woodhouse, In Press)). These three polyurethanes have varying surface morphologies and chemical characteristics. As occlusive dressings, the Tegaderm® and the PCL/HDI/Phe polyurethanes induce exudation and stimulate the development of granulation tissue (Eagelstein et al, 1991). This often results in the complete healing of the wounded region. The desire to investigate the stability of fibrin coatings on a woven substrate led to the inclusion of Corethane™ in this study. In addition, use of this substrate permitted more detailed SEM information because of the fibrin layer formed.

![Figure 3-1: The fibrin coating. In this investigation, non cross-linked fibrin, factor XIIIa cross-linked fibrin and factor XIIIa cross-linked fibrin/ha layers were prepared on three different polyurethane substrates. The stable thermally denatured fibrinogen (TDF) surface (Voegel et al, 1987) functioned to anchor the thin fibrin layer to the underlying material.](image-url)
This comparative study focused on determining the relative stabilities of non cross-linked fibrin, cross-linked fibrin and cross-linked fibrin/HA coatings on three different polyurethanes in the presence of plasmin. Fibrin coatings were prepared in the presence or absence of both factor XIII and calcium ions during the synthesis stage in order to prepare both non cross-linked and cross-linked layers. Cross-linked coatings were prepared in both the presence and absence of hyaluronan (HA). All coatings were constructed on three different polyurethanes: Corethane™, Tegaderm®, and PCL/HDI/Phe.

The substrates used in the study were initially characterized using scanning electron microscopy (SEM) and contact angle analysis. The fibrin coatings were then characterized using SDS-PAGE and SEM.

3.1 EXPERIMENTAL METHODS
3.1.1 Polyurethane Surfaces

Three polyurethanes were used as substrate for the fibrin coatings. Corethane™ (Pfizer) is a commercially available vascular graft material which is also used to coat metal stents. It is a polycarbonate polyurethane whose woven morphology produces a large available surface area. Tegaderm® (3M) is an occlusive wound dressing material with an acrylic adhesive backing which possesses relatively flat and uniform surface characteristics. Like both Corethane™ and Tegaderm®, PCL/HDI/Phe is a polyurethane; however, its surface appears qualitatively slightly less textured than Tegaderm® and contains small diameter pits caused by the casting technique used for its synthesis. In addition, PCL/HDI/Phe (1:2:1 reaction stoichiometry) is an experimental biodegradable polyurethane which was designed as a potential wound dressing material (Skarja and Woodhouse, In Press). Corethane™ and Tegaderm® are non-degradable.
3.1.2 Fibrinogen

The plasminogen-free fibrinogen (Calbiochem) used in this study was derived from human plasma. Prior to use, the fibrinogen was dissolved in distilled deionized water and was then dialyzed against tris buffered saline (TBS) (0.05 M tris (ICN), 0.1 M NaCl (ACP), pH 7.4). The buffer was changed at 1 and 5 hours before the protein was left to dialyze overnight. The concentration of the fibrinogen was determined spectrophotometrically at 280 nm.

Human injectible $^{125}$I-labelled fibrinogen was obtained from both McMaster University Medical Centre and from Dr. J.L. Brash from the Department of Chemical Engineering at McMaster University. The protein was prepared in tris buffer, pH 7.4 and iodinated using the Iodine Monochloride method. Free iodide was removed by extensive dialysis against tris buffer. The labeled protein had a specific activity of approximately 70 $\mu$Ci/mg.

3.1.3 Hyaluronan

Pharmaceutical grade hyaluronan was obtained from Hyal Pharmaceutical Corporation. The molecular weight of the HA used was 500-800 kDa.

3.1.4 Coating Procedure

3.1.4.1 Cross-Linked Fibrin Coating

The protocol for the construction of the factor XIIIa cross-linked fibrin coating was adapted from Rubens et al and Skarja et al (Rubens et al, 1992; Skarja et al, 1998). The base material (either Corethane™, Tegaderm® or PCL/HDIPhe) was punched into small circular disks using a metal punch. The disks were rinsed with methanol (ACP Chemicals Inc.) and soaked in tris buffered saline (TBS) (0.05 M tris (ICN), 0.1 M NaCl (ACP), pH 7.4) for 1 hour at room temperature. The samples were then immersed in 1.0 mg/mL fibrinogen (Calbiochem) in TBS for 2 hours at room temperature, after which, they were incubated at 70°C for 15 min. After incubation, the samples were
rinsed three times in TBS, marking the final step in the preparation of the thermally
denatured fibrinogen (TDF) layer.

To polymerize a thin fibrin layer onto the TDF surface, the samples were then
incubated in 1 μg/mL human thrombin (Sigma) in hypotonic TBS (0.025 M tris, 0.05
M NaCl, pH 7.4) for 5 minutes at room temperature. Following three consecutive rinses
in hypotonic TBS, the samples were immersed for 5 minutes in a 0.5 mg/mL fibrinogen
solution containing 0.1 μg/mL factor XIII and a calcium-containing buffer (0.05 M tris,
0.10 M NaCl, 2mM CaCl₂ (BDH), pH 7.4). The samples were then rinsed three times
with hypotonic TBS and were soaked in 10 μmol/L D-phe-pro-arg chloromethyl ketone
(PPACK) (Calbiochem) in TBS for 5 minutes to inactivate any residual thrombin.
Following three final rinses in TBS, the samples were stored overnight in TBS at 4°C.
Previous investigations have shown that this method produces a thin fibrin layer on the
material (Rubens et al, 1992; Skarja et al, 1998).

3.1.4.2 Cross-Linked Fibrin/Hyaluronan Coating Preparation

The cross-linked fibrin/HA coatings were prepared using the same protocol as
for the cross-linked fibrin coatings; however, 6 μg/mL hyaluronan in TBS was added to
the cross-linking stage of the protocol, thus incorporating the HA into the fibrin coating.

3.1.4.3 Non Cross-Linked Fibrin Coating Preparation

The non cross-linked fibrin coatings were prepared using the same protocol as
for the cross-linked coatings; however, factor XIII was not added and the calcium-
containing buffer was replaced by hypotonic TBS.
3.1.5 Contact Angle Analysis

Water contact angles were measured using the sessile drop technique. The advancing and receding contact angles were measured by goniometer (rame-hart) and the contact angle hysteresis associated with each substrate was determined.

3.1.6 Fibrin Coating Characterization: SDS-PAGE

To confirm that the fibrin coatings were successfully cross-linked via their exposure to the cross-linking agent, factor XIII, the eluants from both cross-linked and non cross-linked coatings were run on SDS-PAGE.

Initially, several fibrin-coated samples were incubated in a 37°C water bath in a solution containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 62.5 mM tris-HCl (pH 6.8), 0.05% (w/v) bromophenol blue and distilled deionized water. The eluant was then removed, boiled for 5 minutes and run in a polyacrylamide gel.

3.1.7 Fibrin Coating Characterization: Scanning Electron Microscopy

To determine the characteristics of the base materials used and of the fibrin coating on each of the substrates, the surfaces of both coated and uncoated samples were examined using SEM. The extent of surface coverage achieved by the coating procedure employed was also determined using the same microscopic technique.

Both coated and uncoated samples of each base material were fixed in a 4% formaldehyde solution. The samples were then dried using a critical point dryer, gold-coated, and examined under a scanning electron microscope.

3.2 RESULTS AND DISCUSSION
3.2.1 Fibrin Coating Characterization: SDS-PAGE

The eluants from both cross-linked and non cross-linked coatings were run on a polyacrylamide gel and are shown in Figure 3-2 in lanes 1 and 2, respectively. Three
distinct bands at approximately 65 kDa, 55 kDa and 50 kDa in both lanes 1 and 2 represent the α, β and γ chains of fibrin, respectively (Fischer, 1990; Rubens et al, 1995; Serrano et al, 1993; Mosesson et al, 1996). The additional faint band in lane 2 at approximately 100 kDa represents γ–γ dimers (Fischer, 1990; Rubens et al, 1995; Serrano et al, 1993; Mosesson et al, 1996). This additional band, which is particularly bold in lane 2, is evidence of intermolecular covalent cross-linking since γ–γ dimers are specific products of cross-linked fibrin. The ε-amino(γ-glutamyl) lysine isopeptide bonds formed between adjacent γ chains of fibrin as a result of the cross-linking action of plasma transglutaminase (factor XIIIa) in the presence of Ca²⁺ within minutes after cross-linking was initiated. Although this band also existed for the non cross-linked sample as well, it was very faint. Trace amounts of factor XIII present in the fibrinogen used for the construction of both the non cross-linked and factor XIIIa cross-linked fibrin coatings were likely responsible for the cross-linking indicated by this faint band since factor XIII-free fibrinogen was not used in this study.

An additional band at approximately 200 kDa was detected in lane 2, demonstrating the existence of αγ–γ dyads within the digested cross-linked fibrin sample (Shainoff et al, 1991). These are unique hybrid products of cross-linked fibrin which result from co-cross-linking between γ–γ dimers and α chains of fibrin at one fourth the rate of γ–γ dimer formation. This additional band was very faint, suggesting that these products were few in number. Evidently, the duration of the cross-linking step employed and the concentration of the factor XIII used were sufficient to enable simple cross-linking to occur within the fibrin polymer. Under these conditions, however, more extensive cross-linking marked by γ multimerization was not evident.
Figure 3-2: 2% SDS-PAGE gel confirming factor XIIIa activity. Lanes 1 and 2 contained eluant from non cross-linked and factor XIIIa cross-linked fibrin coatings, respectively. The dark bands at approximately 65, 55 and 50 kDa in both lanes 1 and 2 represent the α, β and γ chains of fibrin, respectively. The additional bold band at approximately 100 kDa represents γ-γ dimers, unique products of cross-linked fibrin.

3.2.2 Fibrin Coating Characterization: Scanning Electron Microscopy

Both uncoated and fibrin-coated samples of Corethane™, Tegaderm® and PCL/HDI/Phe were submitted for microscopic analysis. Figures 3-3A and 3-3B show samples of uncoated Corethane™ at 50X and 1000X magnification, respectively. The porous woven morphology of this polyurethane is responsible for its large available surface area. Cross-linked fibrin-coated Corethane™ is shown in Figure 3-3C. Here, a relatively thin, but uniform, layer of fibrin covers the material’s available surface area.
At this magnification, the woven structure of the Corethane™ can still be observed underneath the translucent fibrin layer. The region in the upper right corner of the micrograph shows a portion of an adjacent uncoated sample.

A cross-linked fibrin-coated sample of Tegaderm® is shown in Figure 3-4C. The fibrin layer appears dense and opaque compared to the coated Corethane™ sample in Figure 3-3C; however, due to the nature of the Tegaderm® surface which is shown in Figures 3-4A and 3-4B, it was difficult to obtain a true sense of the opacity of the coating. The fibrin layer appears to completely cover the substrate’s surface, with the exception of a few proportionally small regions which signify defects. These exposed areas may have been a result of handling and manipulation of the samples during their preparation for microscopic analysis; however, a definite cause was not determined.

Figures 3-5A and 3-5B show samples of uncoated PCL/HDI/Phe at 50X and 1000X magnification, respectively. The dense cross-linked fibrin layer in Figure 3-5C appears similar to the fibrin coating on the Tegaderm® sample; however, the coating here is less uniform and appears to have peeled away from the surface in more areas, leaving the underlying thermally denatured fibrinogen layer exposed. Both materials are polyurethanes; however, unlike PCL/HDI/Phe, Tegaderm® has an acrylic adhesive backing on one side and appears slightly more textured than the former. These differences may have influenced the amount of fibrin which originally polymerized onto the Tegaderm® surface and contributed to the enhanced persistence of the fibrin coating through the SEM preparation. Again, it is unknown whether the defects in the fibrin coating were artifacts of SEM preparation or if they were present prior to microscopic analysis; however, the exposed regions were proportionally small.

The surface characteristics of both the Tegaderm® and the PCL/HDI/Phe made it difficult to detect differences between fibrin and fibrin/HA coatings. Therefore, these coatings were constructed on Corethane™, whose porous structure made it possible to observe the effect of adding HA to the fibrin coating.
Figure 3-3A: Scanning electron micrograph showing uncoated sample of Corethane™ at 50X magnification. Note the large available surface area due to the woven morphology of this polycarbonate polyurethane.

Figure 3-3B: Scanning electron micrograph showing uncoated Corethane™ at 1000X magnification.
Figure 3-3C: Scanning electron micrograph illustrating fibrin-coated Corethane™ at 50X magnification. The fibrin coating was prepared using 0.5 mg/mL fibrinogen and 1 μg/mL human thrombin.
Figure 3-4A: Scanning electron micrograph showing uncoated sample of Tegaderm at 50X magnification. This commercially available wound dressing material possesses a mildly textured surface that may be a result of the casting technique used during processing.

Figure 3-4B: Scanning electron micrograph showing uncoated Tegaderm at 1000X magnification.
Figure 3-4C: Scanning electron micrograph illustrating fibrin-coated Tegaderm at 50X magnification. The fibrin coating was prepared using 0.5 mg/mL fibrinogen and 1 μg/mL human thrombin.
Figure 3-5A: Scanning electron micrograph showing uncoated sample of PCL/HDI/Phe at 50X magnification. This novel biodegradable polyurethane possesses a relatively smooth surface with small diameter pores distributed throughout.

Figure 3-5B: Scanning electron micrograph showing uncoated PCL/HDI/Phe at 1000X magnification.
Figure 3-5C: Scanning electron micrograph illustrating fibrin-coated PCL/HDI/Phe at 50X magnification. The fibrin coating was prepared using 0.5 mg/mL fibrinogen and 1 μg/mL human thrombin.
Cross-linked fibrin coatings are shown at 500X magnification and 2000X magnification in Figures 3-6A and 3-6C, respectively. Similar coatings containing HA are illustrated in Figures 3-6B and 3-6D. Evidently, the fibrin/HA coatings achieve more complete and uniform surface coverage than the fibrin coatings. The fibrin/HA coatings also appear to form more dense and compact coatings than those shown in Figures 3-6A and 3-6C. Studies have shown that HA increases the rate of fibrin polymerization over 500% (Weigel et al, 1989), which explains this observation. With respect to biomaterial design and the application of a surface coating to an artificial substrate, complete and uniform surface coverage is desired.

The scanning electron micrographs were used to determine the surface characteristics of both the uncoated, fibrin-coated and fibrin/HA-coated samples of each substrate. In all cases, the fibrin coating resembled a dense network which covered most of the substrate’s exposed surface. It was interesting to note that the extent of void surface coverage was consistent with the observed surface smoothness of the substrates. This observation suggested that surface roughness may have influenced the adhesion of the fibrin coating to the underlying material. Although the TDF surface was essentially irreversibly bound to the substrate via protein denaturation (Rubens et al, 1993), surface roughness is related to a material’s surface area which influenced the amount of fibrinogen that initially adhered to the substrate. Another observation worth noting is that the fibrin coating appeared densely-packed and thick on both the Tegaderm® and the PCL/HDIPhe; however, upon closer examination, the fibrin coating actually maintained the same porous matrix structure that was seen on the Corethane™ sample. An enhanced magnification image of the fibrin coating on Tegaderm® is shown in Figure 3-7. This illustration clearly shows the fibrin meshwork that polymerized to the thermally denatured fibrinogen surface. The porous nature of the fibrin coating may provide a “provisional matrix” for the adhesion to and migration of cells into the coating during the healing process (Schlag et al, 1986).
Figure 3-6A: Scanning electron micrograph showing fibrin-coated sample of Corethane™ at 500X magnification.

Figure 3-6B: Fibrin/HA-coated sample of Corethane™ at 500X magnification. Note what appears to be a more uniform surface coverage attained by this coating compared to that in Figure 3-6A.
Figure 3-6C: Scanning electron micrograph showing fibrin-coated sample of Corethane™ at 2000X magnification.

Figure 3-6D: Fibrin/HA-coated sample of Corethane™ at 2000X magnification. Again, note the enhanced surface coverage attained by this coating compared to that in Figure 3-6A.
Figure 3-7: Enhanced magnification of Figure 3-4C illustrating fibrin-coated Tegaderm at 5000X magnification. This coating shows the true porous matrix structure of the fibrin coating which, at 50X magnification, appears densely-packed and smooth.
Although the effect of surface morphology on the stability of the fibrin coating was not the main focus of this work, contact angle analysis of the Tegaderm® and PCL/HD/Ph surfaces was used to determine the contact angle hysteresis of both materials. Hysteresis can be used as an indicator of surface roughness and also can be used to indicate the relative hydrophobicity and hydrophilicity of materials. As shown in Table 3-1, the contact angle hysteresis for the PCL/HD/Ph was greater than that for the Tegaderm® samples. However, Tegaderm® was the more textured of the two materials according to the surface characterization that was performed via SEM. Therefore, differences in surface roughness were not solely responsible for the measured differences in hysteresis. It is possible that differences in the scale of roughness of the two substrates influenced the measured hysteresis; however it may have also resulted from one of several conditions.

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<td>PCL/HD/Ph</td>
<td>82.2 ± 2.0</td>
<td>47.0 ± 2.2</td>
</tr>
</tbody>
</table>

Table 3-1: Contact angle analysis. All values represent the mean of 10 measurements ± standard deviation. Both the adhesive and non adhesive sides of the Tegaderm® were analyzed.

These conditions include surface chemical heterogeneity, the absorption of a thin film of liquid to the test surface which altered the surface dynamics, surface entropy, or surface reorientation or deformation (Wu, 1982; Woods, 1990). Other researchers have shown
that the surface of a segmented polyurethane tends to reorient in response to an aqueous environment (Andrade et al, 1985). This phenomenon may provide a possible explanation for the contact angle hysteresis measured in this study.

The contact angle data also indicated that the advancing angle of the PCL/HDI/Phe was greater than that for the Tegaderm®. The magnitude of the advancing angle is reflective of the surface energy of the material being tested. Repulsion of the water droplet from the surface occurs in response to a difference in surface energies. Since water is a high energy liquid and like attracts like, large advancing angles are indicative of a low energy surface, or a hydrophobic surface. Therefore, the results suggested that Tegaderm® had a slightly higher surface energy than the PCL/HDI/Phe. The adhesive side of the Tegaderm®, however, maintained a surface energy comparable to that of the novel biodegradable polyurethane. Evidently, the acrylic adhesive backing enhanced the hydrophobicity of the wound dressing.
CHAPTER FOUR
STABILITY STUDIES IN THE PRESENCE OF PLASMIN
AND HYALURONAN

4.0 INTRODUCTION

Cellular infiltration into the wounded region requires that the fibrin coating remain available on the polyurethane surface; therefore, assessing the stability of the fibrin coatings in the presence of plasmin was of primary concern in this work. Although a typical dressing is applied for a period of two weeks, the length of time over which the fibrin layer should remain intact on the surface is not known. In vivo, cellular infiltration and proliferation begins approximately three days after wounding; however, the presence of a provisional matrix in excess of three days may be beneficial for the wound. Studies that extend beyond the scope of this work must be performed in order to determine how long the fibrin coating should remain stable on the material for the dressing to attain success in vivo.

The stability of the cross-linked fibrin coating on a material has not been studied to date. The degradation assays were not performed using physiological concentrations of the required components because of constraints imposed by the in vitro nature of the study. Therefore, the measured stabilities were not applicable to an in vivo situation. However, the relative stabilities of the coatings in the presence of plasmin were evaluated. In addition, the total plasmin activity on the coatings was determined and was used to investigate fibrinolysis of the fibrin coatings on the materials.

4.1 EXPERIMENTAL METHODS

4.1.1 Plasmin Degradation of Fibrin Coatings

Fibrin-coated samples were prepared using the protocol previously described in section 3.1.4; however, a 0.5 mg/mL $^{125}$I-labelled fibrinogen solution was used to polymerize the fibrin layer to the TDF surface. The samples that had been left overnight
at 4°C were counted using a gamma counter and were then placed in individual wells of a 96-well tissue culture plate (Nunclon) in TBS at 37°C. Then, 0.1 mg/mL human plasminogen (Calbiochem) was added to each well. After a 10 minute adsorption period, 0.1 µg/mL tissue-type plasminogen activator (tPA) (J.L. Weitz) was added to each well, except those containing the control samples. After a one hour incubation period, the samples were removed from the wells and rinsed 3 times in fresh TBS. Again, the samples were placed in individual counting vials with TBS and the radioactivity of the samples was quantified.

4.1.2 Chromogenic Substrate Assay for Plasmin Activity

The specific enzymatic activities of non cross-linked fibrin, cross-linked fibrin and cross-linked fibrin/hyaluronan coatings was determined using the following protocol. Fibrin-coated samples that had been left overnight at 4°C were placed in individual wells of a 96-well tissue culture plate (Nunclon) containing 0.1 mg/mL human plasminogen in TBS at 37°C. After a 10 minute adsorption period, 0.1 µg/mL tPA and 0.32 mmol/L D-val-leu-lys p-Nitroanilide (S2251) (Sigma) were added to each well. Again, tPA was not added to those wells containing the control samples. The 96-well tissue culture plate was placed in a plate reader at 37°C (Spectra Thermo) and the optical density (OD) of the samples was read at 25 second intervals for one hour at 405 nm.

Direct determination of the sample ODs was possible for assays involving fibrin-coated Tegaderm® because the material's translucence made spectrophotometric analysis of material-containing wells possible. Corethane™ and PCL/HDl/Phe, on the other hand, are both opaque substrates. For this reason, a kinetic assay using the acid-stop method was performed for these base materials.
The acid-stop method involved adding glacial acetic acid (ACP) to each well to arrest the cleavage of the chromogenic substrate. Immediately following the addition of tPA and S2251 to the appropriate wells, acid was added to triplicate wells at 10 minute intervals for 1 hour at 37°C, beginning at t=0. The absorbance of the entire plate was measured upon completion of the assay.

4.2 RESULTS AND DISCUSSION
4.2.1 Plasmin Degradation of Fibrin Coatings

A gamma counter was used to measure the amount of radiolabelled fibrinogen on both the controls and the samples before and after plasmin digestion of the fibrin-coated materials was performed. Initial counts of the radiolabelled samples indicated that, on each of the substrates used, the cross-linked fibrin coatings were comprised of double the fibrinogen constituting the non cross-linked coatings. The covalent stabilization of the fibrin samples evidently provided a greater resistance to removal from each substrate as a result of the protocol employed. The initial counts, which are shown in Table 4-1, also revealed that approximately 170 times more fibrinogen adhered to the Corethane™ than both the Tegaderm® and the PCL/HDI/Phe which maintained comparable amounts of fibrinogen following the initial coating preparation. The available surface area of Corethane™ could not be accurately estimated due to its complex structure; therefore, the amount of fibrinogen per unit area which adsorbed to the material was not determined. However, its highly woven structure was likely responsible for the relatively large quantity of fibrinogen which adhered to its surface. Not only was there a larger surface area for the adsorption of fibrinogen onto this material, but its porous structure provided reservoirs for the absorption of protein during the coating procedure.
<table>
<thead>
<tr>
<th>Corethane™</th>
<th>Cross-Linked</th>
<th>1680</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non Cross-Linked</td>
<td>820</td>
</tr>
<tr>
<td>Tegaderm®</td>
<td>Cross-Linked</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Non Cross-Linked</td>
<td>3</td>
</tr>
<tr>
<td>PCL/HDI/Phe</td>
<td>Cross-Linked</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Non Cross-Linked</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 4-1: Amount of $^{125}$I-Fg per sample (n=3). Initial counts of each sample were used to determine the amount of $^{125}$I-labelled fibrinogen which adhered to each sample via the coating procedure employed.

Table 4-2 illustrates the results that were obtained from the degradation assays performed for each of the coatings. These results, which are expressed as percent degradation ± the corresponding 95% confidence interval, are represented graphically in Figures 4-1 and 4-2. Cross-linking the fibrin coatings consistently increased their stabilities in the presence of plasmin; however, this difference was not statistically significant for the coated Corethane™ samples. The stabilities of the fibrin coatings on both the Tegaderm® and the PCL/HDI/Phe may have been significantly influenced by cross-linking of the thin fibrin layer since the persistence of the coatings on these substrates depended on the interaction of the fibrin layer with the underlying thermally denatured fibrinogen layer. Cross-linking of the fibrin layer to itself and to the thermally denatured fibrinogen layer increased the stability of the coatings on these substrates. In other words, the fibrin coating on the Corethane™ may have achieved a great deal of its stability by winding around the fibrils in the substrate and by absorbing into pores in the material and becoming entrapped. Because its stability depended greatly on the substrate’s morphology, whether or not the fibrin layer was cross-linked...
did not influence its resulting stability to a significant degree. The morphologies of the flat wound dressing materials did not likely confer much stability to their fibrin coatings; therefore, the covalent stabilization of the fibrin layer via factor XIII likely influenced the resulting stability of the coatings to a greater extent than those on the Corethane™ samples.

Although cross-linking the fibrin layers did increase their stabilities on the substrates, the enhancement was not as significant as was originally expected. The susceptibility of fibrin clots to fibrinolysis has been extensively studied, and evidence both supporting and rejecting the increased stability of cross-linked fibrin clots over non cross-linked clots exists. This discrepancy has led to the notion that the extent of cross-linking within the fibrin matrix is related to the fibrinolytic resistance of the fibrin polymer (Eaglestein, 1991).

<table>
<thead>
<tr>
<th></th>
<th>Non Cross-Linked Fibrin (%)Degradation ± 95% C.I.</th>
<th>Cross-Linked Fibrin (%)Degradation ± 95% C.I.</th>
<th>Cross-Linked Fibrin/HA (%)Degradation ± 95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corethane™</td>
<td>73.0 ± 5.0 (n = 21)</td>
<td>69.6 ± 3.2 (n = 30)</td>
<td>52.6 ± 4.1 (n = 24)</td>
</tr>
<tr>
<td>Tegaderm®</td>
<td>56.4 ± 3.0 (n = 27)</td>
<td>38.5 ± 3.4 (n = 27)</td>
<td>45.2 ± 2.6 (n = 48)</td>
</tr>
<tr>
<td>PCL/HDI/Phe</td>
<td>53.6 ± 7.7 (n = 15)</td>
<td>45.5 ± 7.3 (n = 15)</td>
<td>52.3 ± 3.8 (n = 15)</td>
</tr>
</tbody>
</table>

Table 4-2: Summary of degradation assay results. All results are presented as % degradation ± 95% confidence interval. Following a ten minute plasminogen adsorption period to each of the coatings, the fibrin layers were incubated with tPA in tris buffer for one hour at 37°C.
The SDS-PAGE results obtained here gave evidence of \(\gamma-\gamma\) dimers and few higher molecular weight fibrin degradation products. Studies have shown that the stability of fibrin clots is not significantly affected by the formation of either \(\gamma-\gamma\) dimers or \(\alpha\) polymers, but is enhanced only with the formation of \(\gamma\) multimers within the fibrin matrix (Falanga, 1988). These \(\gamma\) multimers are more complex than \(\alpha\) polymers and are formed only after a much longer incubation period approaching 100 hours and extending into days (Siebenlist and Mosesson, 1994). In this investigation, the modest difference between the plasmin-induced degradation of cross-linked and non cross-linked fibrin coatings was likely reflective of the short cross-linking step employed and the moderate degree of \(\gamma\) cross-linking which was subsequently reached.

![Figure 4-1: Graphical representation of degradation assay results for cross-linked and non cross-linked samples. All coated polyurethanes were exposed to a one hour incubation period with 0.1 mg/mL plasminogen and 0.1 \(\mu\)g/mL tPA at 37°C. Error bars represent 95% confidence intervals.](image)
The degradation assay results also showed that the fibrin-coated Corethane™ was less resistant to plasmin digestion than both the fibrin-coated samples of Tegaderm® and PCL/HDIPhe. The large available surface area of the Corethane™ resulted in a fibrin-coated surface which also had a large exposed surface area, and in turn, had more available tPA-binding sites than either of the coated flat wound dressing materials. Studies have shown that the adsorption of more tPA ultimately leads to faster fibrinolytic rates (Gabriel, 1992). It is also likely that plasminogen and tPA were able to penetrate into the fibrin layer, given the porous structure of the Corethane™. Consequently, the coatings on this substrate may have been degraded from within. Therefore, the inferior level of stability attained by fibrin-coated Corethane™ compared
to coated samples of both Tegaderm® and PCL/HDI/Phe was reflective of the coating’s enhanced susceptibility to plasmin attack. It should be noted that there was no statistically significant difference between the stabilities of the coated Tegaderm® and PCL/HDI/Phe samples. Therefore, the degradation assay results exposed a potential relationship between the substrate morphology and the resulting stability of the fibrin coatings.

The degradation assay results also showed that HA destabilized the fibrin coatings on the flat wound dressing materials. HA may have interfered with the polymerization of the cross-linked fibrin layer onto the thermally denatured fibrinogen layer. Since the fibrin coatings on both the PCL/HDI/Phe and Tegaderm® relied on the TDF layer for anchorage to the substrates, disruption of the polymerization step may have destabilized the coatings on these materials. It is also possible that the stability of the coatings may have been decreased in response to swelling. Studies have shown that HA has a high affinity for fibrin, with which it binds and forms a matrix structure (Weigel et al, 1989). However, HA also has a strong association with water and upon contact with water, HA swells and forms a gel. It is likely that when the HA-containing fibrin coatings were incubated with plasminogen and tPA in TBS for a period for one hour at 37°C, the HA within the coatings swelled, causing the fibrin matrix to swell. Therefore, incorporation of the glycosaminoglycan into the cross-linked fibrin layers likely exerted forces on the fibrin layers that may have caused the coatings to come loose from both the PCL/HDI/Phe and Tegaderm® samples.

Although hyaluronan destabilized the coatings on both the flat wound dressing materials, the incorporation of HA into the fibrin layer enhanced the stability of the cross-linked fibrin-coated Corethane™ samples. Unlike the PCL/HDI/Phe and Tegaderm®, the morphology of the Corethane™ made it possible for the fibrin layer to wind around the fibrils in the substrate and absorb into the pores of the woven material. Therefore, the persistence of the coating on this substrate was not solely dependent on
its interaction with the TDF layer. Although HA may have interfered with the polymerization step, this interference did not affect the stability of the coating on the Corethane™. Rather, the polymerization of the fibrin entrapped on and in the substrate may have been enhanced by the presence of HA in the coating. Studies have shown that HA increases the rate of fibrin polymerization over 500% (Weigel, 1989). Because the morphology of the substrate was likely responsible for maintaining fibrin on the surface, the presence of HA in the fibrin matrix likely contributed to the stability of the fibrin layer by making it physically more difficult for fibrin to be removed from the surface.

4.2.2 Chromogenic Substrate Assay for Plasmin Activity

A plate reader was used to measure the absorbance of the 96-well tissue culture plates in which the synthetic chromogenic substrate assays for plasmin were performed. Both controls and samples were performed in triplicate. The true sample ODs and their corresponding times were used to construct the kinetic curve of OD versus time, as shown in Figure 4-3. Linear regression analysis was performed on the linear portion of the graph and the slope of the regression line corresponding to the Michaelis-Menton parameter, $V_{max}$, was determined. This parameter was then used to calculate the enzymatic activity of the plasmin in each well using the following formula:

$$\text{Activity} = \frac{0.01 \times V \times V_{max}}{50 \times S}$$

where: $V$ = the total volume of each well (280 μL), $V_{max}$ = the maximum rate = the slope of the kinetic curve, $S$ = the amount of enzyme per well (pmol), and where 50 mOD/min = 0.01 CU/mL (Schlag and Redl, 1986).

The coated materials were assayed in the wells containing the plasminogen solution; therefore, both bound and unbound activities were accounted for in the assays.
Studies have shown that, the catalytic activity of plasmin is enhanced in the presence of fibrin (Gabriel, 1992; Mayer, 1990) and the activation of plasminogen is increased one thousand times that of unbound plasmin when fibrin-bound (Haber, 1989; Hoyalertz, 1982; Norrman et al, 1985; Weitz, 1990). Consequently, the protocol used here assayed both but it is likely that a significant proportion of the fibrinolysis can be attributed to the bound plasminogen.

![Image](Figure 4-3: Typical kinetic curve showing the characteristic lag phase, followed by the linear portion of the graph from which the plasmin activity was determined.)

A graphical representation of the chromogenic substrate assay results which implied a correlation between the substrate used and the resulting stability of the fibrin coating is shown in Figures 4-4 and 4-5. There was no statistically significant difference between the activities associated with the Tegaderm® and PCL/HD/Phc samples. This result was consistent with the degradation assay results for these two samples which demonstrated that the stabilities of the coatings on each of these two
materials were not statistically different. However, the enzymatic activity of the plasmin assayed with fibrin-coated Corethane™ samples was significantly greater than that associated with the former two substrates as the data in Table 4-3 shows. This result was also in accordance with the degradation assay results which revealed the lower level of stability attained by the coated Corethane™ samples compared to coated samples of both Tegaderm® and PCL/HDl/Phe. As mentioned earlier, the large available surface area of the fibrin-coated Corethane™ translated to a fibrin-coated surface with more available tPA-binding sites than either of the flat wound dressing materials. Since the adsorption of more tPA ultimately leads to faster fibrinolytic rates (Gabriel, 1992), the plasmin activity on the fibrin-coated Corethane™ samples exceeded that on either of the other flat polyurethanes.

![Graphical representation of chromogenic substrate assay results for cross-linked and non cross-linked samples.](image)

**Figure 4-4:** Graphical representation of chromogenic substrate assay results for cross-linked and non cross-linked samples. All assays were performed in triplicate. Error bars represent standard errors of the means.
<table>
<thead>
<tr>
<th></th>
<th>Specific Enzymatic Activity (CU/µg Plg)</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corethane&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>Non Cross-Linked Fibrin 10.31</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>Cross-Linked Fibrin 11.80</td>
<td>2.68</td>
</tr>
<tr>
<td></td>
<td>Cross-Linked Fibrin/HA 9.35</td>
<td>0.97</td>
</tr>
<tr>
<td>Tegaderm&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Non Cross-Linked Fibrin 5.78</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>Cross-Linked Fibrin 7.76</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td>Cross-Linked Fibrin/HA 5.97</td>
<td>0.94</td>
</tr>
<tr>
<td>PCL/HDI/Phe</td>
<td>Non Cross-Linked Fibrin 7.84</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Cross-Linked Fibrin 7.56</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>Cross-Linked Fibrin/HA 6.32</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Table 4-3: Tabulated chromogenic substrate assay results. The enzymatic activity of plasmin on each of the coatings was determined using both the direct kinetic method and the acid-stop method. All assays were performed in triplicate. The 95% confidence intervals were used to determine the statistical significance of the results.

The chromogenic substrate assay results also showed that there was no statistical difference between the activity of the plasmin assayed with the non cross-linked samples and that with the factor XIIIa cross-linked samples. The cross-linked fibrin layers required cleavage in more places than the non cross-linked coatings in order to achieve the same amount of plasmin digestion under the same set of experimental conditions because of the presence of the covalent cross-links within the fibrin matrix. Therefore, this result was expected since cross-linking does not affect plasmin activity but, as the degradation assay results indicated, it does affect the resulting stability of the fibrin coating.
The chromogenic substrate assay results also showed that the incorporation of HA into the fibrin matrix decreased the specific plasmin activity on the substrates used in this study. This result was consistent with work performed by Fournier and Doillon (1987). HA likely interfered with the formation of the ternary complex that existed between fibrin, plasminogen and tissue type plasminogen activator. Since the activation of plasminogen is increased one thousand fold when fibrin-bound, less plasminogen was likely converted to active plasmin, which resulted in a decreased plasmin activity assayed using the chromogenic substrate ELISA assay. It is also possible that, by binding to the fibrin matrix, HA decreased the number of available plasmin digestion sites by occupying or interfering with the lysyl and arginyl residues on fibrin that are attacked by plasmin. HA could have also interfered with the activation of plasminogen,
a possibility originally proposed by Weigel et al (1989). The specific means of interaction between fibrin and hyaluronan is not well understood at this time; therefore, the cause for decreased fibrinolysis due to the presence of HA in the fibrin coating could not be definitively determined. Although these results indicated that HA decreased the plasmin activity on the coatings, the degradation results showed that the presence of HA in the fibrin matrix did not consistently enhance the stability of the cross-linked coatings.

To recap, the degradation results showed that HA destabilized the coatings on both the flat wound dressing materials. Since HA decreased the plasmin activity on these two substrates, the decreased stability of the fibrin/HA coating was likely due to physical weakening of the coating due to the presence of HA within the fibrin matrix, and not to an increased fibrinolytic rate. The presence of HA in the fibrin matrix made the coatings more susceptible to removal from their substrates. Therefore, the effects of swelling caused by HA and/or interference with the polymerization of the fibrin layer onto the TDF layer overcame any stabilizing effects due to decreased fibrinolysis.

The degradation results also showed that HA enhanced the stability of cross-linked fibrin-coated Corethane™. As previously mentioned, this enhanced stability was likely due to the fact that Corethane™'s morphology made it possible for fibrin to become trapped in the substrate where the rate of fibrin polymerization was increased by HA. It was likely more difficult for HA to alter the physical integrity of the coatings on this substrate because of the material's morphology and its effect on stabilizing the fibrin layer. Therefore, HA may have enhanced the stability of the coatings on this substrate by increasing the rate of fibrin polymerization, by interfering with the removal of “loose” fibrin from the substrate and by decreasing the fibrinolysis of the coatings.

Finally, it should be noted that under the conditions employed here, the percent degradation of the fibrin coated materials exposed to plasmin for a period of one hour was determined. However, since a complete set of physiological experimental
conditions were not employed, it was not possible to obtain a rough estimate of the specific time frame within which the coated materials would likely maintain a stable surface layer \textit{in vivo}. A tPA concentration equaling 25 times greater than that typically found in plasma was chosen for use in this study. In order to attain a measurable degree of fibrinolysis within the two hour time period during which tPA remains active \textit{in vitro}, this high concentration was required. Slower fibrinolytic rates and increased fibrin stability would be anticipated under physiological conditions.
CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.0 SUMMARY

In this research, the effects of cross-linking and the presence of hyaluronan in the fibrin matrix on the in vitro stability of fibrin coatings on three different polyurethane substrates in the presence of plasmin were investigated. The substrates and coatings were initially characterized and compared using SEM, SDS-PAGE and water contact angle analysis. Degradation assays and chromogenic substrate assays were then performed to determine the stability of the coatings. The findings from this work suggest that fibrin coatings are worthy of continued investigation. A cross-linked fibrin/HA coating may provide a wound dressing with enhanced healing ability.

5.1 CONCLUSIONS

(1) The effect of cross-linking on the stability of fibrin coatings was determined. The degradation assay results indicated that, despite a definite trend of enhanced stability in response to cross-linking, there was no statistically significant difference between the stabilities of non cross-linked and factor XIIIa cross-linked fibrin coatings on Corethane™ in the presence of plasmin. The enhanced resistance of the cross-linked fibrin coatings on Tegaderm® and PCL/HDI/Phe to removal from the surface may have been reflective of the dependence of the thin fibrin layer on the TDF layer for anchorage to the substrates. Cross-linking the fibrin layer to itself and to the TDF surface had a significant impact on the stability of the coatings on the flat wound dressing materials. The morphology of the Corethane™, on the other hand, had a significant impact on the persistence of the fibrin layer on the surface; therefore, whether or not the fibrin layer was cross-linked had little influence on the resulting stability of the fibrin layer.
The chromogenic substrate assay results showed that cross-linking did not significantly affect the specific plasmin activity on any of the fibrin-coated materials. The increased stability of the cross-linked fibrin coatings was likely due to the fact that the presence of the covalent cross-links within the fibrin matrix required that the coatings be cleaved in more places in order to achieve the same amount of degradation as the non cross-linked coatings under the same set of experimental conditions. Therefore, cross-linking the fibrin coatings did not affect the fibrinolysis of the coatings, but did enhance their stability.

(2) The effect of the substrate used on the resulting stability of the fibrin coatings in the presence of plasmin was determined.

Degradation results showed that the fibrin coatings on the Corethane™ samples were significantly less stable than on both Tegaderm® and PCL/HDI/Phe in the presence of plasmin. The large available surface area of Corethane™ resulted in a fibrin-coated surface which also had a large exposed surface area, and in turn, had more available tPA-binding sites than either of the coated flat wound dressing materials. Studies have shown that the adsorption of more tPA ultimately leads to faster fibrinolytic rates (Gabriel et al, 1992) which explains this decreased stability.

(3) The effect of hyaluronan on the in vitro stability of cross-linked fibrin coatings was determined.

The degradation assay results indicated that the presence of HA in the cross-linked fibrin coating destabilized the coatings on the flat wound dressing materials but enhanced the stability of the cross-linked fibrin coatings on Corethane™. Despite the fact that HA has a high affinity for fibrin (Weigel et al, 1989), it also has a strong association with water with which it swells and forms a gel. It is likely that the HA within the coatings swelled, causing the fibrin matrix to swell. This may have
physically weakened the coatings on the Tegaderm® and PCL/HDIPhe, making them more susceptible to removal from the substrate via sample manipulation involved in the degradation experiments. The destabilization of the coatings may have also resulted from interference of HA with the polymerization of the fibrin layer onto the thermally denatured fibrinogen layer. With respect to the flat substrates, the coatings relied solely on their interaction with the TDF layer for anchorage to the substrates; therefore, any interference with this attachment would explain destabilization of the fibrin layers. Unlike the flat materials, Corethane™'s morphology likely had a significant influence on the resulting stability of the fibrin coating it supported. During the fibrin polymerization step, fibrin may have absorbed into the pores of the woven substrate and become entangled around the fibrils of the substrate. Even though HA may have interfered with the attachment of fibrin onto the TDF surface, it may have significantly increased the rate of polymerization of the fibrin that was trapped on and in the material. The presence of HA in the fibrin coating may have also made it more difficult for “loose” fibrin to be physically removed from the substrate, enhancing its persistence on the surface.

The chromogenic substrate assay results indicated that the presence of HA in the fibrin coating resulted in a decreased plasmin activity on each sample, regardless of the substrate used. The HA may have interfered with the penetration of the plasminogen and tPA into the fibrin matrix, disrupting the ternary complex that forms between these components and fibrin. HA has a high affinity for fibrin; therefore, it is also possible that HA not only filled the void space within the fibrin matrix, but bound to the matrix and occupied or interfered with potential sites for plasmin digestion. The interaction of HA with the lysyl and arginyl residues on fibrin likely explained the decreased plasmin activity associated with HA-containing fibrin coatings, although this decreased rate of fibrinolysis was not sufficient to enhance the overall stability of the fibrin coatings.
Therefore, although HA had differing effects on the stabilities of thin cross-linked fibrin coatings in the presence of plasmin, the effects were not controlled by alterations of the fibrinolytic process. Rather, HA physically weakened the coatings by interfering with the polymerization of fibrin onto the thermally denatured fibrinogen surface and/or by causing the fibrin coatings to swell. The effect of the physical weakening of the cross-linked fibrin coatings appears to have exceeded the enhanced resistance conferred to the coatings by decreasing plasmin activity on the surface. The morphology of Corethane™ was likely responsible for enabling its fibrin coating to withstand destabilization via mechanically weakening. The combination of the substrate’s ability to absorb and trap fibrin and the ability of HA to both increase the rate of fibrin polymerization and decrease plasmin activity on the coating’s surface were likely responsible for the increased persistence of the thin fibrin layer that was observed.

5.2 FUTURE WORK

Future work should focus on investigating the stability of more extensively cross-linked fibrin coatings in the presence of plasmin. \( \gamma \) multimerization within the fibrin coating may be attained by increasing the duration of the cross-linking step or by increasing the factor XIIIa levels employed. The stability of cross-linked fibrin/HA coatings in the presence of a plasmin-free environment should also be studied. In doing this, the mechanical stability of the cross-linked coatings may be assessed and may be isolated from the fibrinolytic resistance of the fibrin coatings. The effects of mechanical stability and fibrinolysis on the overall stability of the fibrin coatings may be more clearly defined. Finally, the effect of the substrate morphology on the resulting fibrinolytic resistance of the fibrin coatings should be further studied, with particular emphasis on an assessment of the activity of plasmin bound to the surface of the fibrin coating.
References


