FGF-2 DELIVERY FROM HEPARINIZED PDMS AND COLLAGEN MATERIALS

BASIC FIBROBLAST GROWTH FACTOR (FGF-2) DELIVERY FROM HEPARIN MODIFIED SURFACES FOR ARTIFICIAL CORNEA APPLICATIONS

By

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Abstract

Device anchoring of artificial cornea implants, through tissue integration of stromal tissue, is necessary to ensure long-term success. In this work, the delivery of basic fibroblast growth factor (FGF-2), a key modulator in corneal wound healing, via heparin modified materials was investigated as a means of sustained, soluble growth factor delivery for stimulation of device anchorage. Two materials types, commonly used for ophthalmic applications and currently under investigation for use in artificial cornea applications, were utilized. Poly (dimethyl siloxane) (PDMS) is currently under investigation as the base material for keratoprosthetic devices; dendrimer crosslinked collagen has been examined as the basis for use as a tissue engineered corneal equivalent.

PDMS surfaces were modified directly or indirectly, through a poly (ethylene oxide) (PEO) spacer, to contain functionalized reactive NSC groups capable of binding heparin and FGF-2 Surface modifications were characterized with attenuated total reflection Fourier transform infrared spectrophotometer (ATR-FTIR), X-ray photoelectron spectroscopy (XPS) and water contact angles. Heparin coverage was assessed with metachromatic and bioactivity assays.

Heparinized collagen gels were crosslinked with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and polypropyleneimine octaamine G2 dendrimers. Gel integrity was assessed with water uptake, differential scanning calorimetry, and heparin and dendrimer stability.

Both materials were exposed to radiolabelled FGF-2 and growth factor immobilization and delivery were quantified. Heparinized PDMS surfaces were capable

of binding on average 100 ng/cm^2 of FGF-2, while heparinized collagen gels had higher FGF-2 immobilization, 300 ng, likely attributed to their higher heparin densities and the fact that the bulk gel rather than the surface only was modified. Delivery of FGF-2 from the heparinized materials revealed a first order release profile, with an initial burst of FGF-2, followed by gradual growth factor release. Release rates, over a 2 week period, reached 6.5% and 50%, for 1 day and 3 day FGF-2 exposed heparinized PDMS modified surfaces, while heparinized dendrimer crosslinked collagen gels released 40%.

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Lastly, I'd like to dedicate this thesis to the spirit of my mother. Although I was too young to know her as a researcher, her desire to continue her education and impact her research community will always be an inspiration to me.

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Nomenclature

AT-III	Antithrombin III
ATR-FTIR	Attenuated total reflection Fourier transform infrared spectrophotometer
BMP	Bone morphogenic protein
BL	Bowman's layer
COLL, C-	Collagen
diNHS	2-N'N-hydroxysuccinimide
DC1107	Polymethylhydrogen siloxane fluid $(MeHSiO)_n$
DM	Descemet's membrane
DSC	Differential scanning calorimetry
ECM	Extracellular matrix
EDC	1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride
EDTA	Ethylenediamine tetraacetic acid
EFW	Endotoxin Free Water
EGF	Epidermal growth factor
FGF-2	Basic fibroblast growth factor
FGFR	Fibroblast growth factor receptor
G2	Generation two
G-CSF	Granulocyte colony stimulating factor
HCl	Hydrochloric acid
HEP	Heparin
HGF	Hepatocyte growth factor

HSPs	Heparan sulphate proteoglycans
IGF	Insulin-like growth factor
KGF	Keratocyte growth factor
IL	Interleukin
LOEX	Laboratoire d'Organogenese Experimentale
LMWH	Low molecular weight heparin
MCP-1	Monocyte chemotactic protein-1
MMPs	Matrix metalloproteinases
NaCl	Sodium chloride
NGF	Nerve growth factor
NHS	N-hydroxysuccinimide
NSC	N-succinimidyl carbonate
PAMAM	Polyamido amine
PEG	Poly ethylene glycol
PEO	Poly (ethylene oxide)
PDMS	Poly (dimethyl siloxane)
PDGF	Platelet derived growth factor
PNIPAAM	Poly(N-isopropyl acrylamide)
PPI	Polypropyleneimine
ePTFE	Polytetrafluoroethylene
pHEMA	Poly(2-hydroxyethyl methacrylate)
PMMA	Poly(methyl methacrylate)

1. INTRODUCTION

Cornea diseases or corneal trauma are a major cause of blindness, second only to cataracts (Whitcher, Srinivasan, and Upadhyay. 2001). The most successful treatment is an allograft corneal transplant, which has a reported 80% success rate for the first 2 years (Duan, Klenkler, and Sheardown. 2006). Despite the high success rates, long-term graft rejection still occurs, with a failure rate of 65% after 5 years, a number which is further complicated by poor patient pre-operative conditions (Corvol, Malemud, and Sokoloff. 1972; Griffith et al 2003). Furthermore, the National Coalition for Vision Health documented 2,602 cornea transplants performed across Canada in 2001; however, at this time there were 3,269 Canadians who were still awaiting transplantation (National Coalition for Vision Health. 2005). Given the aging population and the increasing popularity of laser *in situ* keratomileusis (LASIK), which renders corneas inappropriate for transplantation, donor demand is estimated to continue to surpass eye bank supplies.

An alternative to cornea transplantations, usually used in the cases where host tissue is too diseased for allografts or where allograft surgery is prone to high rates of failure, are the use of artificial cornea replacements in the form of keratoprostheses or tissue engineered corneal equivalents (Duan, Klenkler, and Sheardown. 2006). A suitable artificial cornea replacement must be non toxic, interact well with host tissue and have similar physical characteristics to the natural cornea, including transparency, appropriate refractive index, as well as adequate tensile strength, and high permeability to oxygen and cell nutrients (Chirila et al. 1998; Duan, Klenkler, and Sheardown. 2006; Griffith et al. 2003).

Furthermore, for long term success, the artificial cornea should integrate seamlessly with the host tissue. As shown in Figure 1-1, successful host integration potentially requires interactions between the device and all three cornea layers, the epithelium, stroma, and endothelium (Duan, Klenkler, and Sheardown. 2006).

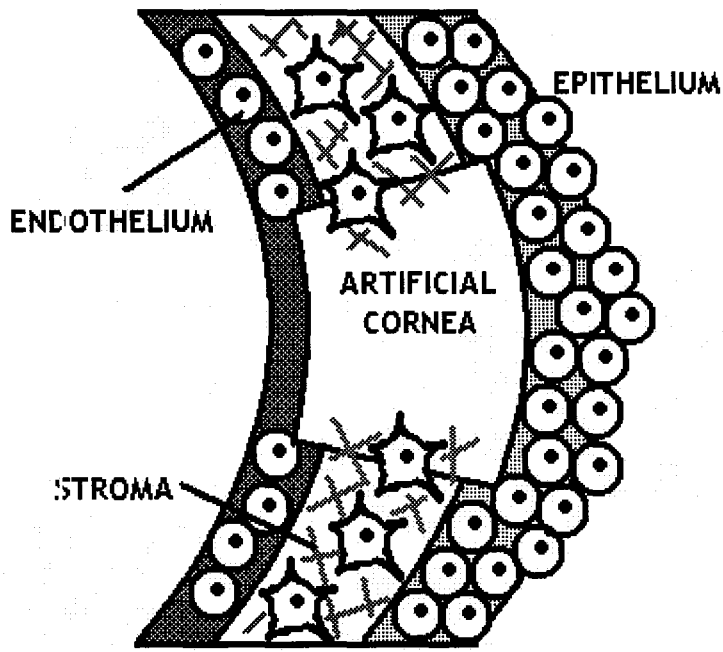


Figure 1-1. Requirements for an artificial cornea. An artificial corneal implant requires integration with all three corneal cell types through epithelial coverage, stromal ingrowth, endothelial compatibility and the regeneration of nerve cells.

Specifically, the device must support epithelial overgrowth on the anterior surface for a wettable and self renewing layer but avoid epithelial downgrowth and subsequent device extrusion (Carlsson et al. 2003). Stromal integration involves fibroblast infiltration and extracellular matrix production to avoid device exposure and tissue necrosis. Finally endothelial cell compatibility and support of nerve regeneration is necessary for corneal health. Many of the interactions between these cells and the natural

corneal extracellular matrix are mediated by growth factors, many of which are sequestered by heparan sulphate proteoglycans through highly specific heparin binding sites for delivery in soluble form.

Therefore, in the current work, heparin modification of artificial cornea materials was examined as a method of facilitating the delivery of growth factors to promote corneal cell interactions with these materials. Specifically, the potential of the materials for delivering the stromal stimulating growth factor basic fibroblast growth factor (FGF-2) for promoting cellular interactions was examined.

2. BACKGROUND

2.1 THE CORNEA

The cornea is a tough, elastic and transparent tissue at the front of the eye that serves as the eye's primary refractive element and protects the internal ocular structures against pathological agents (Chirila et al. 1998; Krachmer, Mannis, and Holland. 2005). The cornea is heavily innervated with sensory nerve fibres, avascular and thus immune privileged. As illustrated in Figure 2-1, the cornea consists of five layers: the epithelium; the Bowman's layer, (BL); the stroma; the Descemet's membrane (DM); and the endothelium.

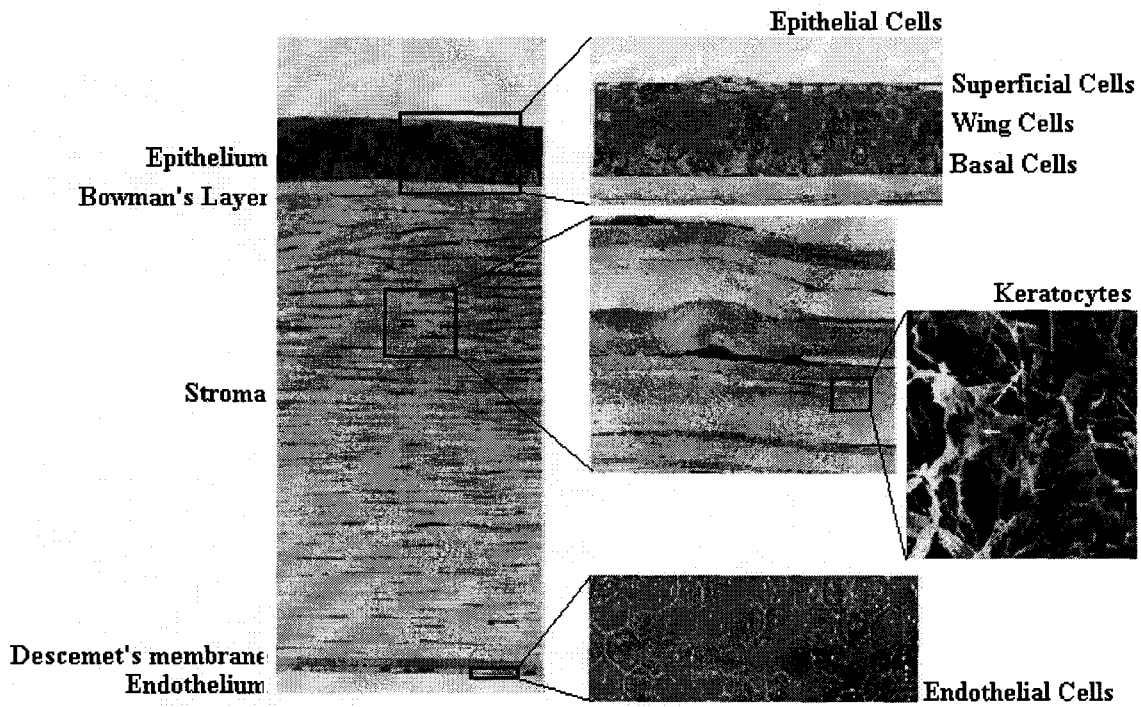


Figure 2-1. Histology of the cornea, illustrating the epithelium and its main cellular layers, the Bowman's layer, the dense lamellar collagen structure and interconnecting keratocytes of the stroma, the Descemet's membrane and the hexagonal cells of the endothelium. Permission pending (Beuerman and Pedroza. 1996; Ojeda, Ventosa, and Piedra. 2001).

The epithelium consists of three phenotypes of non-keratinized, stratified squamous epithelial cells, arranged in 5 to 6 cellular layers. Cells are connected through tight intercellular junctions, creating a semi-permeable membrane for hydration, and a highly protective barrier against environmental threats. The first few layers consist of superficial epithelial cells that stabilize the tear film and prevent dehydration of the cornea. Beneath this are the epithelial wing cells, with a posterior layer of basal epithelial cells that rest on a basement membrane of collagen type IV and laminin, adjacent to Bowman's layer (BL). BL is comprised of Type I and III collagen fibrils as well as proteoglycans, such as the heparin sulphate proteoglycan, perlecan (Krachmer, Mannis, and Holland. 2005; Zieske. 2001).

The stroma, representing 90% of the total cornea thickness, is a highly structured layer of keratocytes, nerve fibres, and extracellular matrix (ECM) components. This layer provides the cornea with its strength, stability and transparency (Chirila et al. 1998; Fini. 1999; Krachmer, Mannis, and Holland. 2005). Keratocytes are scarce, spindle-shaped cells, connected to one another and the ECM via long and narrow gap junctions. They produce ECM constituents to maintain cornea strength and function, contain actin filaments, which allow the cells to contract and perhaps ultimately influence cornea shape, express corneal crystallin proteins responsible for transparency through destructive interference of scattering light, and display integrins to relay ECM information. These cells are greatly influenced by the ECM and by any injury to the stromal layer, whereby they can become activated fibroblasts and myofibroblasts (Fini. 1999).

The ECM of the stromal layer is comprised of collagen, mainly type I with minute amounts of types III, V and VI, water, and glycosaminoglycans (Chirila et al. 1998; Meek and Boote. 2004). The collagen is arranged into thin, uniformly parallel fibrils, layered orthogonally to provide strength and transparency, by scattering light via destructive interference (Chirila et al. 1998; Fini. 1999). Keratocytes also produce matrix metalloproteinases (MMPs) to degrade collagen and restructure the ECM; however this is tightly regulated by cytokines and the turnover of collagen in the ECM is quite slow in a healthy cornea, averaging a 2-3 year time span (Krachmer, Mannis, and Holland. 2005). The glycosaminoglycans within the stroma, with the exception of hyaluronan, bind to core proteins to form proteoglycans (Krachmer, Mannis, and Holland. 2005). For instance, glycosaminoglycans containing chondroitin or dermatan sulphate combine with decorin, while those containing keratin sulphates can bind to lumican, keratocan or mimecan (Krachmer, Mannis, and Holland. 2005; Zieske. 2001). Through cell and ECM interactions, proteoglycans also influence collagen fibril formation and thus stroma transparency (Krachmer, Mannis, and Holland. 2005; Michelacci. 2003; Rada, Cornuet, and Hassell. 1993). Proteoglycans have the potential to retain water and together with the endothelium, they maintain the homeostatic hydration of the stroma (Krachmer, Mannis, and Holland. 2005).

The stroma lies on the highly elastic and strong DM, which contains type IV collagen fibrils, as well as laminin, and fibronectin (Chirila et al. 1998; Krachmer, Mannis, and Holland. 2005). The posterior side of the DM is adjacent to the endothelium, which consists of a single layer of hexagonal endothelial cells. The

endothelium is responsible for hydrating the stroma through its ion transport with the eye's aqueous humour.

2.2 STROMAL WOUND HEALING

Wound healing occurs to restore tissue structure and function after extensive trauma. The wound healing response differs according to the size and depth of injury, which in turn influences the healing rate (months versus years), and scar tissue formation (Zieske. 2001). It is mediated by cell migration, proliferation, adhesion, and differentiation, all orchestrated by various signaling proteins, such as growth factors. Growth factors are polypeptides that stimulate or inhibit a variety of cellular activities, and affect their own cellular regulation through the up- or down-regulation of receptors and cellular gene expression (Lee. 2000). They typically remain inactive or as partially inactive precursors, usually in the ECM, only to be activated during wound healing via proteolytic cleavage and subsequently binding to cellular receptors (Lee. 2000).

Stromal wound healing is a complex cascade of events orchestrated by cellular interactions, apoptosis, proliferation and migration, matrix production and remodelling and eventual wound closure. A generalized corneal wound healing cascade is summarized in Figure 2-2 (Klenkler and Sheardown. 2004; Wilson et al. 2001).

A wound that penetrates the epithelium and BL and goes into the stroma severs intercellular communications and damages cells and the surrounding ECM (Wilson, Lui, and Mohan. 1999; Wilson, Netto, and Ambrósio. 2003). Stroma wound healing begins with keratocyte apoptosis which creates an acellular void that is believed to stimulate subsequent wound healing responses (Zieske. 2001). The keratocyte apoptosis is a

consequence of released signalling proteins from damaged epithelial cells, including interleukin (IL)-1 ligands (IL-1α & IL-1β) and soluble Fas ligands (Mohan et al. 1997; Wilson et al. 1996). Released signalling proteins diffuse into the stromal layer, stimulating keratocytes to produce Fas, which in turn binds to the Fas receptors on keratocytes to induce apoptosis (Ahmadi and Jakobiec. 2002). However, other mechanisms may be present, as Fas knockout mice with induced corneal injury also exhibited keratocyte apoptosis, and tumor necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β) also induced keratocyte apoptosis *in vitro* (Mohan et al. 1997; Wilson, Lui, and Mohan. 1999). Also, there is the hypothesis that intercellular connections between keratocytes may transmit an apoptosis signal (Wilson, Netto, and Ambrósio. 2003). Despite the mystery of keratocyte apoptosis, it is believed to initiate the next stages of wound healing (Krachmer, Mannis, and Holland. 2005; Zieske. 2001).

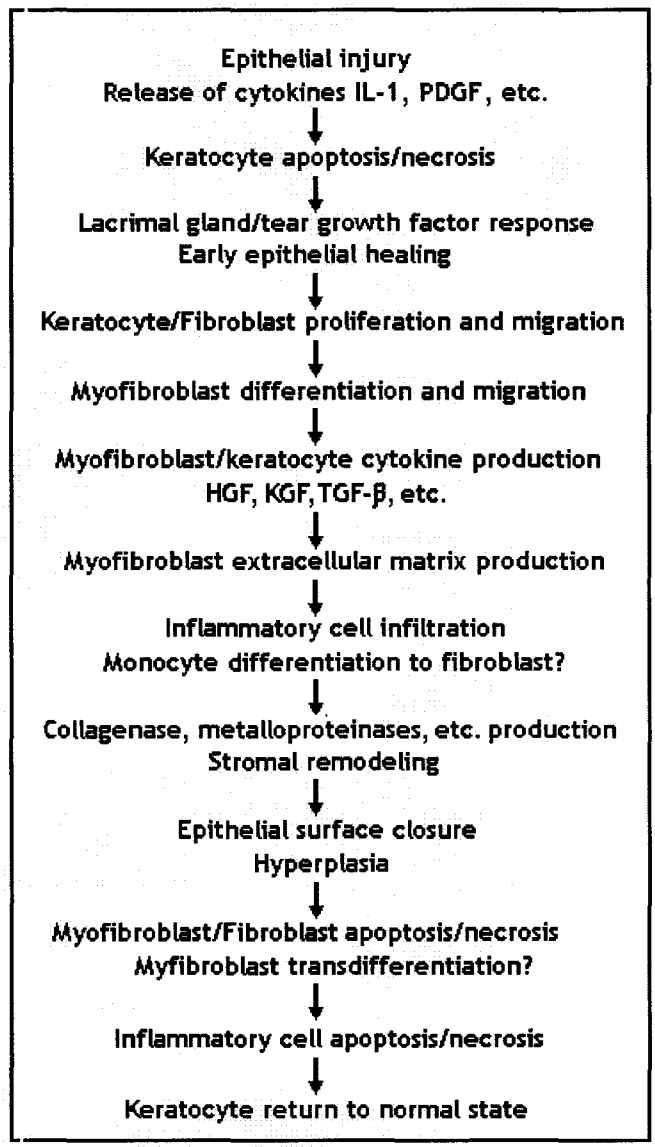


Figure 2-2. The corneal wound healing cascade. Awaiting permission (Klenkler and Sheardown. 2004; Wilson et al. 2001).

Keratocyte apoptosis is followed by the transformation of surrounding keratocytes into a fibroblast phenotype, evidenced by an increase in size, and altered organelle population, morphology and protein expression (Fini. 1999). They cease to express corneal crystallin proteins but gain a fibronectin receptor. As they proliferate and migrate

into the wound, they deposit various ECM components atypical of the healthy stroma, forming a provisional matrix. It has been demonstrated that the composition of the provisional matrix stimulates fibroblast migration and may facilitate wound repopulation during wound healing (Andresen et al. 2000; Zieske. 2001). This provisional matrix consists of various ECM components, such as collagen IV, collagen VII, laminin-1, fibronectin and tenascin (Garana et al. 1992; Latvala et al. 1995; Zieske. 2001). Collagen types I and III are also secreted but have not been shown to influence cell migration and proliferation in the provisional matrix (Andresen et al. 2000; Zieske. 2001). Proteoglycan production is altered as keratin sulpate proteoglycans are down-regulated, while decorin sulphate proteoglycans are generated (Hassell et al. 1983). This atypical ECM, together with the absence of crystallin, results in the formation of an opaque scar tissue (Fini. 1999).

Fibroblast phenotypic transformation follows, whereby activated fibroblasts are transformed into myofibroblasts (Fini. 1999). This transformation is attributable to platelet derived growth factor (PDGF) and transforming growth factor beta (TGF-β) (Jester et al. 1996; Jester et al. 2002). The myofibroblast phenotype is characterized by α-smooth muscle actin expression, and increased appearance of intracellular actin stress fibres and cell focal adhesions (Jester et al. 1994; Jester, Rodrigues, and Herman. 1987). Myofibroblasts contract the wound by binding intracellular stress fibers and α-actin to the transmembrane protein, $\alpha_1\beta_2$-integrin, which in turn has a high affinity for binding extracellular fibronectin, pulling the wound together in a "shoe-string" manner (Jester, Petroll, and Cavanagh. 1999).

Myfibroblast mediated wound contraction is followed by ECM remodelling, whereby the synthesized provisional matrix is remodelled by surrounding cells, to reform tissue similar to an unwounded cornea (Fini and Stramer. 2005; Fini. 1999). Remodelling is greatly influenced by matrix metaproteinases (MMP) that remodel the arrangement of collagen fibrils. MMP-1, or collagenase, degrades type 1 collagen by attacking its triple helix; MMP-3, or stomelysin-1, degrades proteoglycans; gelatinase A, or MMP-2, degrades fibronectin, collagen, and basement membrane components; while MMP-9, or gelatinase B, denatures collagen, basement membrane components and elastin, and may also influence cell migration into the ECM.

Finally, myofibroblasts either transform back to a quiescent keratocyte phenotype, or like some inflammatory cells, undergo apoptosis or necrosis, to restore corneal homeostasis (Fini. 1999; Wilson et al. 2001). As well epithelial cells repopulate the anterior surface.

It should be noted that stromal wound healing irregularities often occur after severe injury whereby the stromal collagen fibrils are not as homogeneous as before, thus obstructing cornea clarity (Krachmer, Mannis, and Holland. 2005). With the replacement of normal corneal tissue with scar tissue, the original strength of the cornea does not return, resulting in an average loss of tensile strength of 70% (Krachmer, Mannis, and Holland. 2005). In some instances, the resulting keratocyte phenotype is irregular, or fibroblasts and myofibroblasts are still present, which interrupts light scattering (Fini and Stramer. 2005; Krachmer, Mannis, and Holland. 2005). Also, the keratocyte population may be too dense for optimal transparency and newly infiltrating nerve fibers may lack

normal sensitivity (Krachmer, Mannis, and Holland. 2005). There are also the imbalances associated with abnormal or prolonged signaling protein expression. For instance, over-expression of certain growth factors can lead to epithelial hyperplasia and subsequent vision loss (Klenkler and Sheardown. 2004). However, certain growth factors, specifically PDGF and fibroblast growth factor-2 have a beneficial role in enhancing the wound healing process (Fini and Stramer. 2005).

2.2.1 Growth Factors in Corneal Wound Healing

Throughout the corneal wound healing process, various growth factors are released by the lacrimal gland, corneal cells or infiltrating inflammatory cells that regulate wound healing, specifically cell proliferation and migration, as illustrated in Figure 2-3 (Imanishi et al. 2000; Klenkler and Sheardown. 2004).

Upon stromal injury and disruption of BL, signalling proteins from the lacrimal gland, tear fluid, and corneal epithelial layer are able to diffuse into the stroma and influence wound healing, whereas in a healthy cornea, these signalling proteins would not normally be present (Fini and Stramer. 2005). Inflammatory cells, such as monocytes, macrophages, granulocytes and T-cells, arrive to the wound site from limbal blood vessels or the tear film (Wilson et al. 2001). Their summoning is believed to be brought on by injured epithelial cells that release interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α). This further activates keratocytes to produce inflammatory chemotatic agents, such as monocyte chemotactic protein-1 (MCP-1) and granulocyte colony stimulating factor (G-CSF) (Hong et al. 2001; Tran et al. 1996). These inflammatory cells are believed to remove cell debris from keratocytes that undergo apoptosis (Wilson

et al. 2001). It has also been hypothesized that monocytes and macrophages may undergo phenotypic transformation into fibroblasts, similar to what occurs in bone resorption where osteoblasts are derived from macrophages and monocytes. However; there is no current research which supports this theory (Wilson et al. 2001).

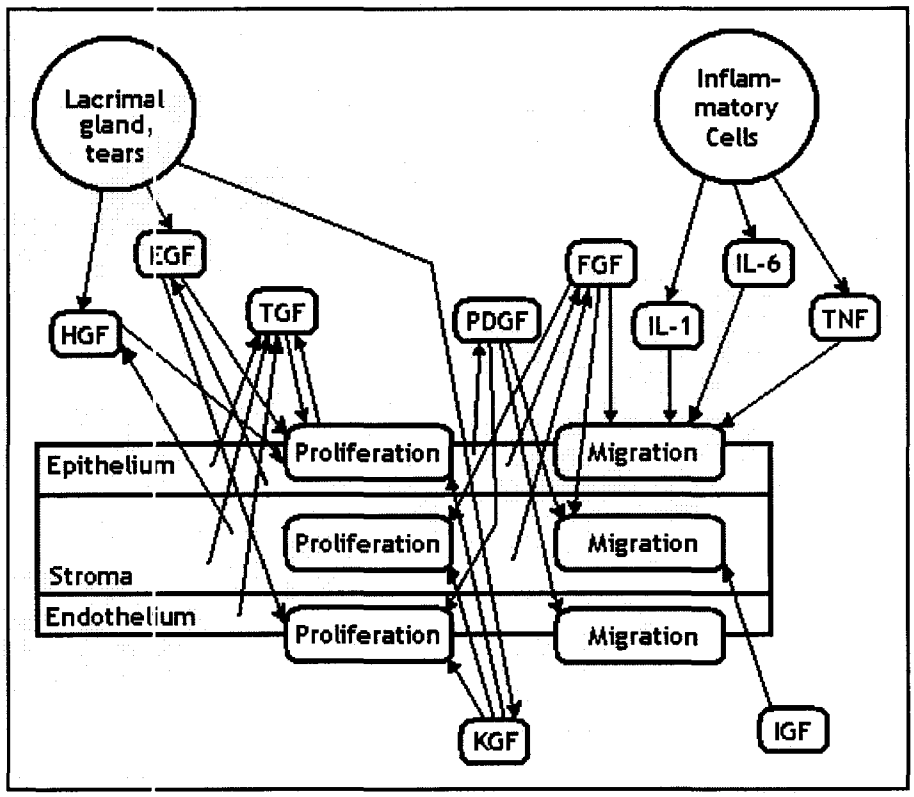


Figure 2-3. Signaling proteins involved in corneal wound healing. Awaiting permission (Imanishi et al. 2000; Klenkler and Sheardown. 2004).

As mentioned earlier, keratocyte apoptosis is stimulated by IL-1 and TNF-α, that is released from damaged epithelial cells and from the tears. IL-1 also activates keratocytes to secrete keratocyte growth factor (KGF) and hepatocyte growth factor (HGF), which further influence wound healing (Weng et al. 1997). KGF and HGF, also secreted from the lacrimal gland (Li et al. 1996; Wilson, Liang, and Kim. 1999),

stimulate epithelial and endothelial proliferation and migration and keratocyte proliferation, but fail to stimulate fibroblasts (Carrington and Boulton. 2005; Wilson et al. 1993). Besides IL-1, cytokines IL-6 and TNF stimulates epithelial cell migration in the presence of fibronectin (Wang et al. 1994). Endothelial cells can also express IL-1 and its receptor (Hoppenreijs et al. 1996).

PDGF released from the damaged BL influences fibroblast migration, and stimulates the further secretion of HGF and KGF (Li and Tseng. 1997; Seppa et al. 1982). PDGF also stimulates epithelial cell migration, but only in the presence of fibronectin; this most likely occurs once the provisional matrix is laid (Kamiyama et al. 1998). Endothelial cells express PDGF, which also induces endothelial cell proliferation and migration (Iguchi et al. 1995; Imanishi et al. 2000; Kamiyama et al. 1995).

Epidermal growth factor (EGF), from the lacrimal gland (Wilson, Liang, and Kim. 1999), epithelium and stroma (Wilson et al. 1999), induces epithelial and endothelial cell proliferation. Keratocytes exposed to EGF have demonstrated weak proliferation through low affinity EGF membrane receptors (Imanishi et al. 2000; Joyce et al. 1995; Wilson et al. 1994). EGF stimulated epithelial migration has been observed, and is enhanced in the presence of fibronectin (Nishida et al. 1992; Sheardown et al. 1997).

Fibroblast growth factor (FGF-2) and its receptors are expressed by all three corneal cell types as well as by the lacrimal gland (Hoppenreijs et al. 1996). FGF-2 can influence the migration and proliferation of epithelial cells (Grant et al. 1992),

keratocytes (Andresen, Ledet, and Ehlers. 1997) and endothelial cells (Gospodarowicz, Mescher, and Birdwell. 1977; Hoppenreijs et al. 1994; Hoppenreijs et al. 1996).

Activated TGF-β, produced by all corneal cell types (Ahmadi and Jakobiec. 2002) and the lacrimal gland (Yoshino et al. 1996), acts as an inhibitor for HGF, KGF and EGF expression, and is thought to control excess epithelial cell proliferation (Imanishi et al. 2000). As well, TGF-β, released from epithelial cells, acts as a chemotactic agent for corneal fibroblasts, and stimulates alpha smooth muscle actin expression in keratocytes, *in vitro* (Ahmadi and Jakobiec. 2002). However, TGF-β expression is inhibited by FGF-2, which may counter balance the effects of TGF-β during corneal wound healing (Song et al. 2002).

Other growth factors are also active in the wounded cornea. Insulin-like growth factor (IGF), isolated in the epithelium and stroma (Saghizadeh et al. 2001), has been shown to stimulate endothelial cell proliferation and migration (Choi et al. 1995; Hoppenreijs et al. 1996), and keratocyte migration (Andresen, Ledet, and Ehlers. 1997). Nerve growth factor (NGF) has been detected in the all layers of the cornea and can stimulate epithelial and stromal proliferation, and may be linked to fibroblast differentiation into myofibroblasts (Lambiase et al. 1998; Micera et al. 2006; You, Kruse, and Volcker. 2000). Also present in the cornea are bone morphogenic protein (BMP), which has been shown to induce fibroblast migration, and vascular endothelial growth factor (VEGF), whc may play a role in corneal neovascularization (Kim et al. 1999; Saghizadeh et al. 2001; van Setten. 1997).

During corneal wound healing, growth factors also regulate provisional matrix remodelling, through stromal synthesis of matrix metalloproteinases (MMP) and collagen (Fini and Stramer. 2005). IL-1 and TNF have been shown to up-regulate collagenases (MMP-1, and -13) and stromelysins (MMP-3, -10, and -11) (Li de et al. 2003), while TGF-β1 can stimulate them in addition to gelatinase (MMP-9) (Kim et al. 2004). PDGF influences MMP-1 and -9 expression (Ahmadi and Jakobiec. 2002), and there is evidence that FGF-2 stimulates MMP-1 production by stromal cells (Strissel, Rinehart, and Fini. 1997). Collagen production is mediated by IL-6, which both increases collagen production and inhibits MMP-2 production (Malecaze et al. 1997), and TGF-β which decreases collagenase production (Strissel, Rinehart, and Fini. 1995).

2.3 BASIC FIBROBLAST GROWTH FACTOR (FGF-2)

Basic fibroblast growth factor (FGF-2), is a 18 kDa protein consisting of 146 amino acids arranged into 12 anti-parallel β-sheets, further folded into a trigonal pyramidal structure (Nugent and Iozzo. 2000; Okada-Ban, Thiery, and Jouanneau. 2000). It contains 4 cysteines and many basic residues, giving it an isoelectric point of 9.6. Figure 2-4 illustrates a stereo view of FGF-2 (Faham, Linhardt, and Rees. 1998).

FGF-2 is found in the normal ECM (Aktas and Kayton. 2000), in many basement membranes (Folkman et al. 1988), and in many tissues and cells, including the retina (Ohsato et al. 1997), aqueous humour (Tripathi, Borisuth, and Tripathi. 1992), cornea epithelium (Wilson, Lloyd, and He. 1992; van Setten, Fagerholm, and Cuevas-Sanchez. 1995), neural tissues, pituitary (Marin and Boya. 1995), placenta (Florkiewicz and Sommer. 1989), and smooth muscle and endothelial cells (Cordon-Cardo et al. 1990).

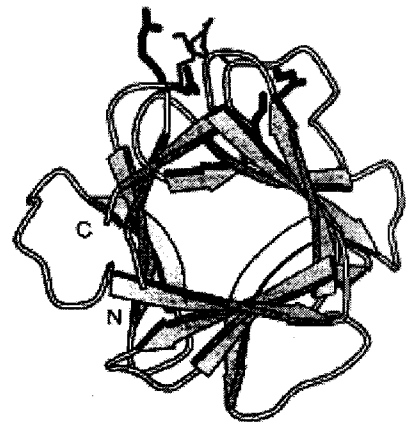


Figure 2-4. A stereo view of the FGF-2 molecule. Awaiting permission (Mohammadi, Olsen, and Ibrahimi. 2005).

Within the cell, the 18kDa FGF-2 is localized in the cytoplasm, while larger isoforms of FGF-2 (22, 22.5 and 24 kDa) have been detected in the cell nucleus. However, their cellular role is yet unknown (Powers, McLeskey, and Wellstein. 2000; Renko et al. 1990).

The ECM acts as a reservoir for FGF-2, as a means of regulating its bioavailability (Flaumenhaft et al. 1989; Folkman et al. 1988), and to protect against acid and heat denaturation (Gospodarowicz and Cheng. 1986), and proteolytic cleavage (Saksela et al. 1988; Sommer and Rifkin. 1989). To regulate FGF-2 bioavailability, its release from the ECM by controlled polysaccharide degradation, either by enzymatic cleavage, with proteases or heparanases, or by binding to a carrier protein, FGF binding protein, which can transport FGF-2 to cell receptors (Powers, McLeskey, and Wellstein. 2000). Despite the large amount of FGF-2 in the ECM reservoir (10-500 ng/mL), its release is highly regulated by extracellular matrix proteoglycans, as there is a low molar concentration required to activate its receptors (Powers, McLeskey, and Wellstein. 2000).

2.3.1 Cell Secretion

FGF-2 is secreted by many cell types, including fibroblasts (Akimoto et al. 1999; Aktas and Kayton. 2000) and macrophage cells (Akimoto et al. 1999; Sunderkotter et al. 1991). In contrast other synthesized proteins, which are secreted via the endoplasmic reticulum and Golgi pathway, FGF-2, lacking a consensus signal peptide (Mignatti, Morimoto, and Rifkin. 1992), is secreted by a different, as yet unknown mechanism. It has been suggested that secretion is possibly a passive process, through apoptosis, or physical injury, as *in vitro* induced cellular injury has stimulated vascular smooth muscle cells (Crowley et al. 1995) and endothelial cells (Gajdusek and Carbon. 1989; McNeil et al. 1989) to release FGF-2. Other studies with FGF-1, which also lacks a signal sequence for secretion, suggest that high temperatures (42°C, 2 hr) can induce release (Jackson et al. 1992). However, under these conditions, FGF-1 had lost its bioactivity, mostly likely due to thermally induced protein denaturation. The other hypothesis involves non-lethal membrane disruptions, such as exocytosis, or vesicles, whereby FGF-2 is translocated across the cell plasma membrane (Schafer et al. 2004). However, earlier immunofluorescent localization of FGF-2 failed to locate it in secretory vesicles (Renko et al. 1990), and to date, the secretion process remains unresolved.

2.3.2 Biological Functions

FGF-2 is pleiotropic, via intracrine, autocrine and paracrine mechanisms. It stimulates proliferation in many cells phenotypes including fibroblasts (Mignatti, Morimoto, and Rifkin. 1991; Shipley et al. 1989), endothelial cells (Sa and Fox. 1994), keratinocytes (O'Keefe, Chiu, and Payne. 1988; Shipley et al. 1989), chrondrocytes

(Corvol, Malemud, and Sokoloff. 1972; Kato et al. 1983) and smooth muscle cells (Skaletz-Rorowski et al. 1996). *In vitro* studies with endothelial cells indicate a chemotactic and mitogenic role to FGF-2 (Galzie, Kinsella, and Smith. 1997). FGF-2 can also stimulate the migration of other cells, such as circulating macrophages (Sunderkotter et al. 1994), to the area, which may in turn stimulate the release of additional FGF-2 or other growth factors, to influence other cellular responses (DeBlois, Cote, and Doillon. 1994; Pieper et al. 2002). Release of FGF-2 in low concentration (nanogram and pictogram) amounts, has been shown to stimulate local FGF-2 expression (Edelman et al. 1991; Pieper et al. 2002).

FGF-2 is a mediator of angiogenesis, neovascularization, wound healing (Tsuboi and Rifkin. 1990), embryonic development (Cohn et al. 1995; Kimelman et al. 1988), neuronal development, vascular tone (Zhou et al. 1998) and regeneration of bone, cartilage (Cuevas, Burgos, and Baird. 1988), nerve (Ortega et al. 1998) and retinal tissues (Ozaki et al. 1998). A few biological functions of FGF-2 are summarized in Table 2-1.

A lack of or inappropriate expression of FGF-2 can result in various pathological conditions, including tumour growth (Bikfalvi et al. 1997). For example, theoretical strategies for cancer treatment have included the suppression of angiogenesis to tumour cells through inhibition of FGF-2 signaling, which has been shown to inhibit pancreatic cancer *in vitro* and *in vivo* (Wagner et al. 1998). FGF-2 is down regulated by interferons α and β in human carcinomas and systemic administration of interferon α inhibits FGF-2 expression (Okada-Ban, Thiery, and Jouanneau. 2000). A few pathological implications associated with irregular FGF-2 expression are summarized in Table 2-2.

Table 2-1. Biological roles of FGF-2.

Biological System	Biological Role	Reference
Angiogenesis and Neovessel formation	Endothelial cell and smooth muscle cell manipulation	(Cox and Poole. 2000; Flamme and Risau. 1992; Gajdusek, Luo, and Mayberg. 1993; Schwartz and Liaw. 1993; Yoshida, Anand-Apte, and Zetter. 1996)
	Regulates expression of other biological molecules	(Klein et al. 1993)
	Vessel formation and hypotensive influence	(Cuevas et al. 1991; Dono et al. 1998; Ingber. 1991)
	Wound healing, cardial infarction healing & atherosclerosis Healing	(Harada et al. 1994; Parish et al. 1995; Six et al. 2004; Villaschi and Nicosia. 1993; Yanagisawa-Miwa et al. 1992)
Lung	Development and branching	(Brettell and McGowan. 1994)
	Wound healing	(Goldsmith, Gammon, and Garver. 1991; Henke et al. 1991)
Hematopoietic system	Influences myelopoiesis	(Wilson et al. 1991)
	Modulation of megakaryocytopoiesis	(Avraham et al. 1994)
	Influences hematopoietic cell types	(Allouche. 1995; Brunner et al. 1993; Oliver et al. 1990)
Nervous system	Development	(Dono. 2003; Tsai and Kim. 2005)
	Cell influence (neurons, oligodendrocytes & astrolial cells)	(Enokido et al. 1992; Gomez-Pinilla, Vu, and Cotman. 1995; Grinspan et al. 1993; Miyagawa, Saito, and Nishiyama. 1993; Vescovi et al. 1993)
	Wound healing and regeneration	(Gomez-Pinilla, Lee, and Cotman. 1992; Logan and Berry. 1993)

Table 2-1. Biological roles of FGF-2 (con't).

Biological System	Biological Role	Reference
Reproductive system	Regulates cell function (Leydig cells) and stimulates cell expression (germ cells)	(Han et al. 1993; Murono et al. 1993)
Skin	Melanocyte proliferation and differentiation	(Halaban et al. 1992)
	Wound healing (keratinocytes)	(Kurita et al. 1992; Stenberg et al. 1991)
	Induces endothelium and neovessels	(Pierce et al. 1992)
Eye	Development	(Esteve and Bovolenta. 2006; Le and Musil. 2001)
	Proliferation; migration and differentiation of lens cells	(McAvoy and Chamberlain. 1989; Schulz et al. 1993)
	Retinal regeneration and healing	(Faktorovich et al. 1992; Goureau et al. 1993; LaVail et al. 1992; Park and Hollenberg. 1993; Schuschereba et al. 1994)
	Corneal wound healing	(Andresen, Ledet, and Ehlers. 1997; Gospodarowicz, Mescher, and Birdwell. 1977; Grant et al. 1992; Hoppenreijs et al. 1994; Hoppenreijs et al. 1996)
Mesoderm	Mesoderm induction; development and maintenance	(Cornell and Kimelman. 1994; Gillespie et al. 1992; Isaacs, Tannahill, and Slack. 1992)
Muscle and skeleton	Skeletal muscle growth and differentiation	(Kudla et al. 1995; Templeton and Hauschka. 1992)
	Limb development	(Savage et al. 1993)
	Osteoblast proliferation and differentiation	(Globus, Patterson-Buckendahl, and Gospodarowicz. 1988)
	Induces expression of other biological molecules	(Noda and Vogel. 1989)
	Wound healing and bone growth regeneration	(Coffin et al. 1995; Eppley et al. 1991)
Digestive system	Intestinal epithelial proliferation	(Dignass, Tsunekawa, and Podolsky. 1994)
	Wound healing (ulcers & mucosa)	(Folkman et al. 1991; Szabo et al. 1994)

Table 2-2. Pathological implications of FGF-2.

System	Biological Role	Reference
Angiogenesis	Tumour formation	(Kuwabara et al. 1995; Li et al. 1994; Nguyen et al. 1994)
Nervous system	Neurodegenerative diseases and tumours	(Stopa et al. 1990; Tooyama et al. 1993a; Tooyama et al. 1993b; Zagzag et al. 1990)
Muscle and skeleton	Muscular disorders	(D'Amore et al. 1994)
Digestive system	Cancer	(Iida et al. 1994)

FGF-2 cell signalling involves its association with cell surface fibroblast growth factor receptors (FGFRs), of which there are two forms, FGFR-1 and FGFR-2, capable of equal FGF-2 interaction (Quarto and Amalric. 1994). FGFRs are transmembrane proteins with an extracellular immunoglobulin domain, a single-membrane-spanning domain and an intracellular tyrosine kinase domain, of which the there are many sub-variants differing in the extracellular domain. FGF-2 mainly binds to FGFRs with high affinity, but there is also a FGFR low affinity binding site capable of heparin binding (D'Amore. 1990; Venkataraman et al. 1999). The number of FGFRs per cell varies based on cell type and FGF concentration, mainly ranging from 10^3-10^4 receptors/cell (Conrad. 1998). A second receptor lies on cell surfaces, comprised of heparin sulphate proteoglycans, which has low affinity binding for FGF-2, and will be discussed in a subsequent section.

2.3.3 FGF-2 – FGFR Interactions

To activate FGFR, FGF-2 must dimerize the receptor to activate the tyrosine kinase, which results in autophosphorylation of the receptor tyrosine residues for subsequent stimulation of cellular signal transduction pathways (Coughlin et al. 1988;

Dailey et al. 2005). FGF-2 activates a number of cellular responses by phosphorylating intracellular proteins in a series of cascade reactions, leading to nuclear translocation where transcription factors are phosphorylated and activated.

Atypical to many growth factors, FGF-2 does not degrade in the lysosome after interacting with its receptors but rather truncates into various polypeptides (Galzie, Kinsella, and Smith. 1997). These polypeptides are translocated into the cell cytoplasm and into the nucleus, and are further believed to stimulate growth signals (Hawker and Granger. 1992; Olsnes, Klingenberg, and Wiedlocha. 2003; Wesche et al. 2006). This unique translocation to the nucleus is due to interactions between FGF-2 and heparan sulphate proteoglycans.

2.4 HEPARAN SULPHATE PROTEOGLYCANS

Heparan sulphate proteoglycans (HSPs) are sulphated glycosaminoglycans, heterogeneous in structure, produced by many cell types (Gallagher, Lyon, and Steward. 1986). They consist of a protein core with carbohydrate side chains. The main protein cores are syndecan, glypican, perlecan, CD44 and betaglycan (Galzie, Kinsella, and Smith. 1997).

HSPs are found in the ECM, in many basement membranes, and on cell membranes, as low affinity binding sites. They aid with cell adhesion, growth factor receptor activation and FGF-2 signalling (Dowd, Cooney, and Nugent. 1999; Hagen, Michael, and Butkowski. 1993; Kwan et al. 2001; Nugent and Iozzo. 2000). HSPs can bind cells and to ECM components, namely collagen, fibronectin and laminin, through specific electrostatic interactions (Gallagher, Lyon, and Steward. 1986; Koda, Rapraeger,

and Bernfield. 1985). HSP-ECM complexes have increased stability with high molecular weight glycosaminoglycans, possibly due to increased HSP protein interactions (Salchert et al. 2004). HSPs also have binding sites for many growth factors, including FGF-2, VEGF, TGF-β, NGF, and PDGF (Zamora et al. 2002). HSP-FGF-2 binding have been shown to increase its thermal stability (Gospodarowicz and Cheng. 1986) and protect it from tryptic and chymotryptic digestion (Sommer and Rifkin. 1989) as well as plasmin induced proteolytic degradation (Saksela et al. 1988). Proteases including heparanase and plasmin, can cleave HSP bonds in the ECM and release FGF-2 for cellular stimulation, or can cleave HSP side chains still bonded to FGF-2 for increased biostability and bioactivity (Bashkin et al. 1989; Saksela and Rifkin. 1990).

2.4.1 HSP – FGF-2 Interactions

HSP and FGF-2 interactions are polyionic, whereby the positively charged site on FGF-2 ionically binds to the acidic segments of HSPs, with low affinity (Tabata. 2000). Binding *in vitro* has been shown to be dependant on FGF-2 concentration and exposure time (Moscatelli. 1992). An *in vitro* cell culture exposed to FGF-2 for a short time will give a prolonged cellular response after the FGF-2 removed, due to FGF-2 bound to HSP cell receptors (Galzie, Kinsella, and Smith. 1997).

Of recent interest is the idea that membrane HSP congregate towards lipid rich areas and the influence of environment on FGF-2 binding (Chu, Buczek-Thomas, and Nugent. 2004). The results indicated that disruption of the lipid raft hindered FGF-2 binding and increased its dissociation from the cell surface. It was further suggested that these lipid clusters may aid the autophosphorylation of dimer membrane receptors.

2.4.2 HSP – FGF-2 Internalization

There is evidence of internalized HSP-FGF-2 complexes, which may explain the high levels of FGF-2 within the cell cytoplasm and its translocation to the nucleus (Amalric et al. 1994; Hsia, Richardson, and Nugent. 2003). Upon internalization of HSPs, there are two possible pathways: HSP degradation; and cleavage of HSPs into smaller, but long lasting, oligo- or polysaccharides that can bind to intracellular FGF-2, providing it with increased resistance against cellular catabolic reactions (Conrad. 1998; Reiland and Rapraeger. 1993; Sperde and Nugent. 1998). HSP is believed to accelerate FGF-2 nuclear localization and regulate nuclear delivery (Sperde and Nugent. 1998).

2.4.3 HSP – FGF-2 – FGFR Interactions

Interactions between HSPs, FGF-2 and FGFR have also been observed and there are many proposed models (Figure 2-5) (Powers, McLeskey, and Wellstein. 2000). The significance of these interactions correlates to the stabilizing effect of HSP on the FGF-2 and FGFR complex, creating high affinity binding (Forsten-Williams, Chua, and Nugent. 2005). There is also evidence that the binding of FGF-2 to HSP induces a conformal change in the FGF-2 molecule (Figure 2-6(a) and (b)), which may present it to the FGFR in a way that favours its integration (Powers, McLeskey, and Wellstein. 2000; Prestrelski, Fox, and Arakawa. 1992).

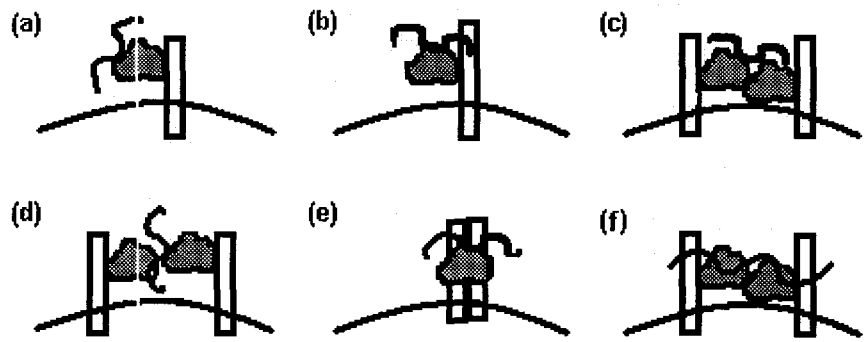


Figure 6. A few proposed models for HSP – FGF-2 – FGFR interactions. (a) HSP may initiate a conformal change in FGF-2 for favoured FGFR binding. (b) HSP may facilitate the interaction between FGF-2 and FGFR by interacting with both molecules. (c) HSP may allow the formation of FGF-2 dimers which then bind to two FGFRs. (d) HSP may facilitate the dimerization of FGFR-FGF-2 complexes. (e) HSP may facilitate the bivalent interaction between one FGF-2 and two FGFRs. Awaiting permission (Mohammadi, Olsen, and Ibrahimi. 2005).

One interaction model depicts HSP binding to two FGF-2 molecules thereby creating a dimeric molecule that easily dimerizes two FGFRs (Figure (6c)). A second model has HSP interacting with one FGF-2 and one FGFR creating a dimer between the protein and receptor, capable of interacting with another FGFR-FGF-2 complex (Figure 6(d)). There is also the theory that the two binding sites on FGF-2 for FGFR, in the presence of HSP, are capable of bridging two FGFRs (Figure 6(e)). Another possibility is that two FGF-2 molecules bind individually to 2 FGFRs, with one HSP binding across them (Figure 6(f)).

A relationship between HSP and FGFR in terms of regulating cellular signalling has also been demonstrated; it has been shown that reducing HSPs cell surface concentrations can lead to up-regulation of FGFR expression, and *vice versa* (Padera et al. 1999).

The internalization of the FGF-2 molecule occurs by direct binding either to the FGFR, to cell membrane bound HSPs, or to both (Ornitz et al. 1992; Schlessinger. 2000; Yayon et al. 1991). It is this mechanism, and the presence of HSPs, that determines the cellular response (Quarto and Amalric. 1994; Reiland and Rapraeger. 1993). This was recently seen in retinal pigment epithelial cells, whereby FGFR-1 and FGFR-2 induced different signalling pathways (Rosenthal et al. 2005). Chinese hamster ovary cells deficient in HSPs induced a different FGF-2-FGFR-1 signalling pathway compared to cultures where heparan sulphate was added (Lundin et al. 2003). This work suggested that FGF-2 alone will bind to FGFR-1 with low affinity and in the presence of HSP binds with high affinity. Further studies with smooth muscle cells indicate that FGF-2 induced proliferation is independent of FGFR but dependent on HSP mechanisms (Natke et al. 1999).

2.4.4 FGF-2 – HSP Interactions in the Wounded Cornea

FGF-2 has been localized in the cornea, conjuctiva, retina, aqueous humour and limbus (Reid. 1994). FGF-2 binds to the heparan sulfate proteoglycans in the basement membrane of the murine cornea, and to the basement membrane and Descemet's membrane of the bovine cornea (Reid. 1994).

In wound healing, platelets, neutrophils and lymphoma cells can release heparanase with can degrade ECM-HSP bonds in Descement's membrane, releasing FGF-2 for cell usage (Ishai-Michaeli, Eldor, and Vlodavsky. 1990). Other inflammatory cells can release corneal endothelium modulation factor, which in turn can stimulate FGF-2 synthesis (Kay, Gu, and Smith. 1994). FGF-2 can then induce wound healing by

stimulating epithelial healing, endothelial proliferation, keratocyte migration and keratocyte expression of an HSP, keratin sulphate proteoglycan (Hoppenreijs et al. 1994; Krachmer, Mannis, and Holland. 2005; Rieck et al. 1994; Zieske. 2001). While HSP is not normally expressed in a healthy stroma, upon injury, corneal stormal cells begin to express HSPs, and this is believed to greatly influence the wound healing response through increased FGF-2 bioavailability and subsequent fibroblast differentiation (Hsia, Richardson, and Nugent. 2003; Rapraeger, Krufka, and Olwin. 1991).

Also of interest is the characterization of the response of cornea stromal fibroblasts to low density populations (from 1000 to 35000 cells/cm^2) in which cells demonstrated increased FGF-2 binding (Richardson, Trinkaus-Randall, and Nugent. 1999). This up-regulation could be interpreted as a means of augmenting stromal proliferation and migration during wound healing. The nuclear localization and cell proliferation of the cells was also increased in the presence of FGF-2 and HSPs, when cultured on fibronectin, compared to those seeded on collagen type I (Hsia, Richardson, and Nugent. 2003). This implies that the wound healing response of fibrin matrix production may enhance fibroblast proliferation. Also of importance are the HSP side chains that, upon translocation, are cleaved from the protein backbone, but remain bound to FGF-2 for its transportation to the cell nucleus.

2.5 HEPARIN

Heparin is a glycosaminoglycan of varying length, with alternating units of glucosamine and iduronic acid that is commonly secreted by mast cells (Faham, Linhardt, and Rees. 1998; Galzie, Kinsella, and Smith. 1997). It is structurally very similar to the

side chains of cell membrane heparan sulphate proteoglycans except that it is highly sulphated, as seen in Figure 2-6.

Heparin interacts with a variety of proteins, such as antithrombin and annexin V, and is currently used as an anticoagulant and as a coating to create more blood compatible anticoagulant surfaces (Brash. 2000; Hinrichs et al. 1997b; Mulloy and Linhardt. 2001; Zhou and Meyerhoff. 2005).

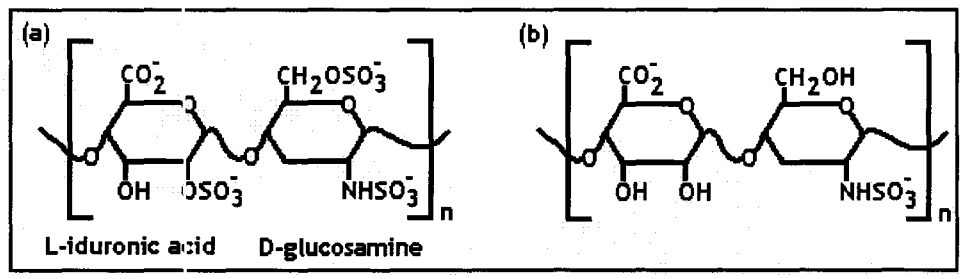


Figure 2-6. Molecular repeating units of heparin (a) and heparan sulphate (b), illustrating the higher degree of sulphination on heparin molecules. Awaiting permission (Faham, Linhardt, and Rees. 1998; Mulloy and Linhardt. 2001).

2.5.1 Heparin Interactions with FGF-2 and FGFR

Heparin also has the potential to interact with growth factor molecules, including FGF-2, to which it binds with high affinity (Faham, Linhardt, and Rees. 1998). The binding affinity of FGF-2 to heparin increases with increasing chain length, and it is also possible to bind more than one FGF molecule to a heparin chain; either in a *cis-* or *trans-* formation or in a way that complements both.

Heparin has been shown to form tertiary structures with FGF and FGFR, although there is some debate about the minimum length required for this interaction (hexasaccharide versus octasaccharide) (Pellegrini. 2001; Schlessinger. 2000). Current models mirror previously mentioned HSP-FGF-2-FGFR interactions.

One thing to note is the effect of heparin dose and application to cells. Low heparin concentrations (< 1 μg/mL), in the presence of FGF-2, were found to stimulate fibroblast proliferation while high concentrations (> 10 μg/mL) hindered it (Fannon, Forsten, and Nugent. 2000; Skaletz-Rorowski et al. 1996; Yamashita et al. 1992). This was further investigated with corneal stroma cells, where proliferation was hindered at heparin concentrations ranging from 200-5000 μg/mL (Denk and Knorr. 1999).

2.6 ARTIFICIAL CORNEA

Second only to cataracts, cornea disease, trauma, scarring or ulceration is a major cause of vision loss (Whitcher, Srinivasan, and Upadhyay. 2001). Currently, the most accepted and successful treatment for cornea blind patients is corneal allograft surgery whereby donor tissue is implanted into the host. However there are risks of disease transmission and an increasing shortage of donor tissue highly due to the aging population and the increasing popularity of laser *in situ* keratomileusis (LASIK) surgery for refractive cornea errors, which damages the tissue for donation (Carlsson et al. 2003; Chirila et al. 1998; Duan, Klenkler, and Sheardown. 2006; Griffith et al. 2003). Thus there is a need for alternative substitutes, namely keratoprostheses and tissue engineered corneal equivalents (Carlsson et al. 2003; Duan, Klenkler, and Sheardown. 2006).

The ideal artificial cornea must be non toxic, transparent with a suitable refractive index, have comparable textile strength to the native cornea, interact well with the corneal cellular layers and nerve network, promote cellular communication, and be able to provide the semi-permeable membrane characteristics of the native cornea for oxygen

and nutrient diffusion (Chirila et al. 1998; Duan, Klenkler, and Sheardown. 2006; Griffith et al. 2003).

2.7 KERATOPROSTHESES

There are two main keratoprosthesis designs: the optical stem with skirt implant, also referred to as the collar button design, and the core and skirt model (Khan, Dudenhoefer, and Dohlman. 2001). Both are illustrated in Figure 2-7. A typical collar button design has a transparent optical core joined to 2 plates, one anterior and one posterior, which sandwich the cornea and anchor the device. The core and skirt keratoprosthesis consists of a transparent, flexible material of suitable optical refraction, surrounded by a skirt to anchor or suture the device, to the cornea's stromal intralamellar region.

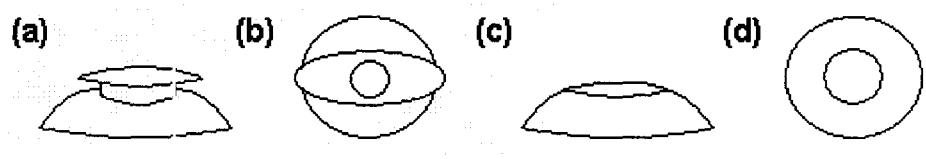


Figure 2-7. The two most common keratoprosthesis designs (Khan, Dudenhoefer, and Dohlman. 2001). The button and collar model from the side (a) and from the anterior surface (b). The core and skirt model side profile (a) and viewed from above (b).

The optic core must be transparent with similar optical properties to the eye and the skirt of the device must be strong enough to withstand placement and suturing. The optic stem should be as short as possible to avoid anterior chamber penetration (Hicks et al. 2000). Keratoprosthesis with porous skirts are being explored, as they potentially allow ingrowth of host tissue and permit adequate oxygen and nutrient permeability to

integrated cells (Carlsson et al. 2003). The pore should be on the micron range, usually between 10 – 30 μm, and interconnected to ensure cell-cell interactions and ECM deposition (Griffith et al. 2003). The anterior surface of the device should encourage epithelialization. However, epithelial downgrowth which commonly leads to device extrusion, should be avoided. The posterior surface of the device should inhibit cellular integration to maintain device clarity (Duan, Klenkler, and Sheardown. 2006). The two most successful prototype devices include the collar button and core and skirt designs.

2.7.1 Collar Button Keratoprostheses

The Dohlman-Doane keratoprosthesis device, composed of a poly(methyl methacrylate (PMMA) collar-button design, over a clinical seven year study, resulted in many corneal complications, including extrusion, inflammation, glaucoma, retroprosthetic membrane, retinal detachment and hindered vision (Yaghouti et al. 2001). However, many of these negative outcomes were attributed to patient preoperative conditions, with only non-cicatrizing conditions achieving amicable results. Future work with this device focuses on improving device design, surgical procedures, and aligning the appropriate postoperative procedures (anti-inflammatory drugs, antibiotics, contact lens bandages, shunts, etc.) with preoperative diagnosis (corneal burns, auto-immune diseases, glaucoma, etc.) (Aquavella et al. 2005; Subhransu et al. 2002).

PMMA had also been combined with other polymers in an effort to exploit the biointegration or strength of these other polymers. The Seoul-type keratoprosthesis, has a stem made of surface treated PMMA with a flange of fluorinated silicone, a porous poly(urethane) or polypropylene skirt, for fibroblast infiltration, and a polypropylene

haptic, to interact with the sclera, providing the keratoprosthesis double fixed anchoring and increased mechanical stability (Kim et al. 2002b). Despite promising preliminary animal studies indicating corneal neovascularization, fibroblast integration, collagen deposition, no extrusion with mild tissue necrosis, and decreased inflammation (Kim et al. 2002a), human trials were disappointing (Kim et al. 2002b). Implantation led to skirt exposure, irregardless of skirt material, retroprosthetic membrane formation, retinal detachment, glaucoma and endophthalmitis, perhaps due to preoperative circumstances.

2.7.2 Core and Skirt Keratoprostheses

The AlphaCor™ keratoprosthesis, previously known as the Chirila KPro, is composed of poly(2-hydroxyethyl methacrylate) (pHEMA), a flexible, hydrophilic, permeable polymer, that through curing manipulation with water content, can have an interconnecting, non-porous and transparent centre and a porous and opaque skirt (Hicks et al. 2000). Corneal anchorage through stroma fibroblast integration in animal studies was demonstrated with this device; however delayed healing, and inflammation did occur. Although preliminary human clinical trials with the device have been promising, with 80% device retention after one year, complications typical of other keratoprosthetic devices, including tissue melting, retroprosthetic membrane formation, optic damage and poor biointegration (Hicks et al. 2003) were observed. In some patients, pHEMA calcification occurred, which is thought to be due to polymer degradation or protein adsorption. Epithelialization of the device's anterior surface did not occur and was deemed unnecessary by the research group, as extrusion was not evident (Hicks et al. 2000); however, it can be argued that an absence of an anterior epithelial layer may

increase the chance for bacterial infection and may affect the quality of the tear film (Duan, Klenkler, and Sheardown. 2006). Other implantation techniques and device designs are being investigated to minimize clinical complications, and improve the mechanical strength of the polymer, through the use of various crosslinking agents. As well, different surface modification techniques have been used to increase hydrophobicity and decrease the risk of bacterial adhesion.

Porous polytetrafluoroethylene (ePTFE) has been explored as a haptic support, in the BIOKOP prostheses, of which there are two prototypes (I and II), type I with a PMMA optic core, and type II with one of silicone. BIOKOP I implanted in rabbits demonstrated keratocyte migration and collagen production (Legeais et al. 1994). However, implantation into humans was not as successful, with glaucoma, necrosis, extrusions, endophothalmitis, retroprosthetic membrane formation and lens dislocation (Legeais et al. 1995). It was determined that a PMMA optic core was inappropriate for this keratoprosthesis. Further design lead to BIOKOP II with an optic core based on poly (dimethyl siloxane) (PDMS) with a polyvinylpyrrolidone coating (Legeais and Renard. 1998). The PDMS and ePTFE were crosslinked at the optic-skirt interface to overcome the issue of compromised strength typically seen at composite keratoprosthetic interfaces (Duan, Klenkler, and Sheardown. 2006). Short-term clinical trials showed improved results over the previous BIOKOP type with only a few retroprosthetic membranes and extractions due to buccal muscal instability as epithelial surface coverage was not evident (Legeais and Renard. 1998). However, a recent long-term clinical study of this keratoprosthesis in humans, many with preoperative complications, resulted in skirt

exposure, retroprosthetic membranes, endophthalmitis and device extrusion (Alio et al. 2004).

2.7.3 Complications and Future Directions

Synthetic keratoprostheses are advantageous as they could provide off the shelf availability, improved optical properties and reduce the risk of disease transmission associated with allografts (Duan, Klenkler, and Sheardown. 2006; Griffith et al. 2003). Many do not interact well with host tissue resulting in device extrusion, tissue rejection (stromal melting and epithelial thinning), aqueous humour leakage, infection, retroprosthetic membrane formation, retinal detachment and glaucoma, mainly attributable to device rigidity, poor permeability of nutrients and oxygen (Duan, Klenkler, and Sheardown. 2006; Griffith et al. 2003; Llloyd, Faragher, and Denyer. 2001). Composite keratoprostheses generally have compromised mechanical strength at the core-skirt interface (Duan, Klenkler, and Sheardown. 2006). Some keratoprosthesis demonstrate moderate stromal anchoring; but issues remain with biointegration, primarily with the epithelium, and inflammatory reactions, which may be attributable to exposure of the device to the tear layer (Duan, Klenkler, and Sheardown. 2006; Griffith et al. 2003).

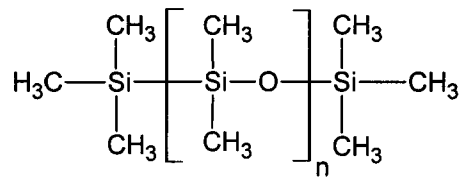
Further modification of devices with extracellular matrix proteins, namely collagen, fibronectin and laminin, or with oligopeptides, including cell adhesion peptides RGDS, YIGSR, PHSRN and PDSGR has shown increased epithelialization of a keratoprosthetic anterior surface *in vitro* (Duan, Klenkler, and Sheardown. 2006; Griffith et al. 2003). Growth factors, specifically epidermal growth factor (EGF) and

transforming growth factor beta (TGF-β) have been tethered to polymers to manipulate epithelialization. Success was observed with the former material; the latter was hypothesized to stimulate stromal migration but was found to be inhibitory in tethered form (Duan, Klenkler, and Sheardown. 2006).

Ideally a keratoprosthesis, and the implantation surgery and preoperative techniques, would promote a response similar to that seen in wound healing, and not induce excessive macrophage activation or foreign body inflammation (Hicks et al. 2000).

2.8 POLY (DIMETHYL SILOXANE)

Poly (dimethyl siloxane) (PDMS) elastomer, shown in Figure 2-8, is prepared by combining a silicon molecule with alkene groups to a hydrolyzed silicon molecule in the presence a catalyst, usually platinum (Brook. 2000).



PDMS

Figure 2-8. The poly (dimethyl siloxane) elastomer chemical composition comprises of a silicone core and branched methyl groups (Maciejewski et al. 2006).

With its transparency, high gas permeability, low toxicity, relative mechanical strength, and thermal and oxidative stability, PDMS has been explored for biomedical applications (Chen et al. 2005; Liu and Sheardown. 2005; Maciejewski et al. 2006) and has been widely used as an ophthalmic biomaterial. However, given the hydrophobic

methyl groups attached to the silicon core, PDMS is susceptible to non-specific protein absorption (Bartzoka, McDermott, and Brook. 1999), which is undesirable for many biological applications. For example, in blood contacting applications, protein adsorption may lead to coagulation, complement activation, thrombosis and haemolytic damage (Brash. 2000). While contact lenses based on silicone rubber have recently seen great success, they are prone to tear protein and lipid adsorption leading to lens fouling and patient discomfort (Lloyd, Faragher, and Denyer. 2001). In artificial cornea, PDMS has shown low epithelial cell adhesion in vitro (Aucoin et al. 2002). Therefore, to improve these properties, various modification techniques have been used.

PDMS modified via microwave frequency plasma polymerization with allyl alcohol to create reactive hydroxyl groups, which, after a series of subsequent reactions, allowed for synergistic peptide immobilization of laminin and fibronectin derivatives, namely YIGSR, RGDS, PDSGR and PHSRN, and improved corneal epithelial cell attachment *in vitro* (Aucoin et al. 2002). This technique was also applied to PDMS surfaces for the tethering of a bifunctional poly (ethylene glycol) spacer for immobilization of transforming growth factor beta-2 (TGF-β2) to impede corneal epithelial cell adhesion (Merrett et al. 2003). While, as noted above, the *in vitro* results were somewhat unexpected, the potential for modification of PDMS in this manner was clear.

Alternatively, modification with poly (ethylene oxide) (PEO), a highly hydrophilic, non-toxic and non-immunogenic polymer, to minimize hydrophobicity as well as related protein adsorption (Chen et al. 2005b; Kingshott, Thissen, and Griesser.

2002) and cell adhesion (Chen et al. 2006; Llanos and Sefton. 1993; Shen et al. 2002) has been investigated. Block copolymers of PDMS, PEO and heparin, developed in 1988 for anti-thrombogenic applications, demonstrated a heterogeneous microphase-separated structure that reorganized the immobilized heparin to its surface upon hydration, with the heparin molecule keeping its biological activity. Biologically these materials showed low levels of platelet activation, (Grainger et al. 1990a; Grainger et al. 1990b; Grainger, Kim, and Feijen. 1988).

2.8.1 Polysiloxane Keratoprosthesis

Polysiloxanes were successfully tested for biocompatibility in animal eyes by Lieb *et. al.* in 1959, and later unsuccessfully as canine corneal intralamellar disks by Bowen *et al.* in the 1960s (Chirila et al. 1998). Some success was achieved later by Dohlman *et al.* to implant silicone into the corneal stroma of edema patients, which later evolved to an artificial corneal endothelium (Brown and Dohlman. 1965; Dohlman and Dube. 1968). Silicone keratoprosthesis subsequently appeared in the 1970s by Ruedemann *et al.* in the shape of a saucer or satellite, with a supporting perforated plate or a Dacron® cloth skirt flange (Chirila et al. 1998; Reudemann. 1974). Human implantation of the device over a 10 year period was promising given its infancy, with short-term success in some patients; however long-term success was not achieved with many complications arising.

Recently the use of silicone rubber in keratoprosthesis applications has been reprised (Lee et al. 1996). In an effort to create a more corneal cell compatible surface, a homobifunctional pHEMA film was deposited on the silicone surface, via plasma

induced graft polymerization. Epithelial coverage was observed *in vitro* and *in vivo* although there was evidence of epithelial downgrowth and device clarity was compromised with certain pHEMA concentrations. To prevent epithelial downgrowth, a heterobifunctional silicone surface was created with pHEMA or collagen on the anterior surface for epithelial growth, and either 2-methacryloyloxethyl phosphorylcholine or bisamino polyethylene oxide on the posterior side for epithelial inhibition (Chang, Lee, and Hsiue. 1998). *In vitro* and *in vivo* results with the collagen surface in particular were encouraging, with only slight complications (opacity and neovascularization). However, there have been no recent results with this system.

The Aachen keratoprosthesis, first mentioned in 2000, is entirely composed of transparent silicone rubber, with a aspheric optical centre, a haptic suturing section and protruding arms for scleral suturing later to be covered by the conjunctiva (Langefeld et al. 2000). Designed primarily for short applications during vitrectomy, *in vivo* implantation resulted in edema in some cases but generally the eyes maintained retinal attachment. While promising, there were issues with host compatibility, including cell integration, which must be addressed.

Plasma polymerization of PDMS with allyl amine and subsequent functionalization of PEO with tethered epidermal growth factor (EGF), for the use as the anterior surface of a keratroprosthesis, was found to significantly improve corneal epithelization *in vitro* (Klenkler and Sheardown. 2006; Klenkler et al. 2005).

More recently PDMS and PEO were combined during the room temperature vulcanization process to form bulk polymers with reduced protein adsorption (Chen et al.

2005; Chen, Brook, and Sheardown. 2004). Using a novel method, PEO modified PDMS surfaces have been generated by first creating a reactive Si-H layer through acid catalyzed equilibration with a polymethylhydrogen siloxane fluid $(MeHSiO)_n$, DC1107, and subsequent hydrosilylation with allyl terminated PEO (Figure 2-9) (Chen et al. 2005b).

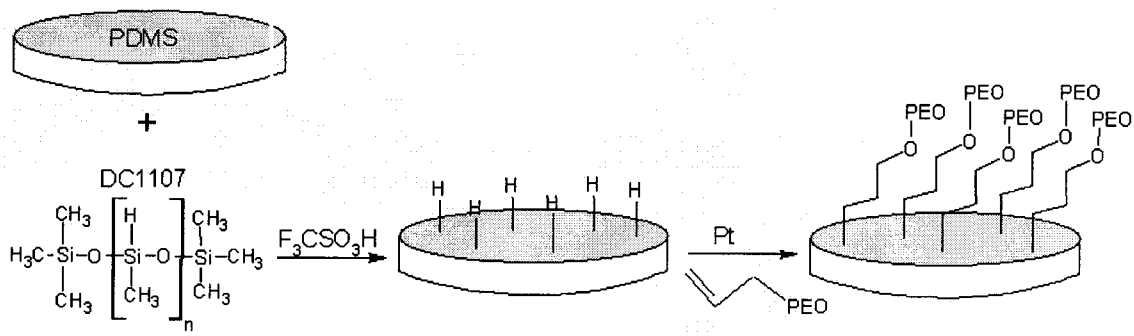


Figure 2-9. Direct modification of PDMS with DC1107, in the presence of triflic acid (F_3CSO_3H), forms surface Si-H functionalization, which can further react, in the presence of platinum, via hydrosilylation, with allyl terminated PEO (Chen et al. 2005b).

By using a heterobifunctional PEO molecule, various biological components, including cell adhesion molecules, growth factors, and heparin could be immobilized on the PDMS substrate, resulting in high biological specificity (Chen et al. 2005a; Chen et al. 2006).

The high hydrophobicity of PDMS would be expected to impact its performance *in vivo*, as it lacks the appropriate glucose permeability necessary for cell survival (Liu and Sheardown. 2005). Therefore, a composite interpenetrating network of PDMS and poly(N-isopropyl acrylamide) (PNIPAAM) has been created. The IPN demonstrated

appropriate oxygen and glucose permeability, improved hydrophilicity, and greater mechanical strength than control PDMS homopolymers.

2.9 COLLAGEN

Collagen is one of the most abundant proteins in the body, mediating cell adhesion through interactions with over 50 molecules, namely glycosaminoglycans, proteins and growth factors (Friess. 1998; Salchert et al. 2004). It consists of at least 1000 amino acids, arranged into three polypeptide chains, stabilized by the steric repulsion of proline and hydroxyproline residues' pyrrolidine rings, which fold into a triple helix configuration via hydrogen bonding. Every third residue is glycine, which gives collagen its tight helix formation and tertiary structure. There is a high percentage of proline, compared to other proteins, as well as rare amino acids, such as hydroxyproline and hydroxylysine, the latter of which allows sugar attachment to the collagen molecule (Friess. 1998). At either end of the collagen structure, there exists amino acids not in the helical formation, termed telopeptides, which are involved in fibril crosslinking.

2.9.1 Natural Collagen Crosslinking

In vivo collagen is crosslinked via intra- and intermolecular bonds. Intramolecular thermal crosslinking is initially regulated by lysyl oxidase (also called protein lysine 6-oxidase) where, in telopeptide regions, lysine and hydroxylysine residues are selectively converted to allysine and hydroxyallysine (Figure 2-10(a)) (Friess. 1998). Allysine and hydroxyallysine are very reactive aldehydes that can form crosslinks, via

aldol condensation (Figure 2-10(b)), between two α-chains in the telopeptide regions of the same collagen molecule. Aldol condensation initiates with the first aldehyde, in the presence of a base, becoming an enolate ion that can react with the carbonyl group of the second aldehyde molecule (Friess. 1998; Solomons. 1996).

Intermolecular crosslinking, between the helical region of a quarterly staggered collagen molecule and the telopeptide region of an adjacent collagen molecule, occurs between aldehyde residues and ε-amino groups from either lysine or hydroxylysine via aldimine formation (Figure 2-10(c)) (Friess. 1998; Solomons. 1996). Further reactions occur to form polyfunctional crosslinks with histidine, lysine or hydroxylysine through additional condensations (Friess. 1998). Fibril formation is believed to be driven by hydrophobic and electrostatic interactions (Rosenblatt, Devereux, and Wallace. 1993; Salchert et al. 2004; Wallace. 1990). Extraction of collagen involves breaking these natural crosslinks resulting in a material that, for use in biomaterials and tissue engineering applications, requires external crosslinking. However, thermally crosslinked collagen, in which the collagen fibrils reassociate under elevated temperature conditions, is generally very weak (Duan and Sheardown. 2006b) and upon *in vivo* implantation, it has a high enzymatic turnover rate (Olde Damink et al. 1996). Thus techniques are needed to increase the mechanical and biological stability of crosslinked collagen gels, while remaining biocompatible. Various crosslinking agents are being explored to enhance collagen gel matrices, such as carbodiimide, gluteraldehyde, hexamethylene diisocyanate and acyl azide.

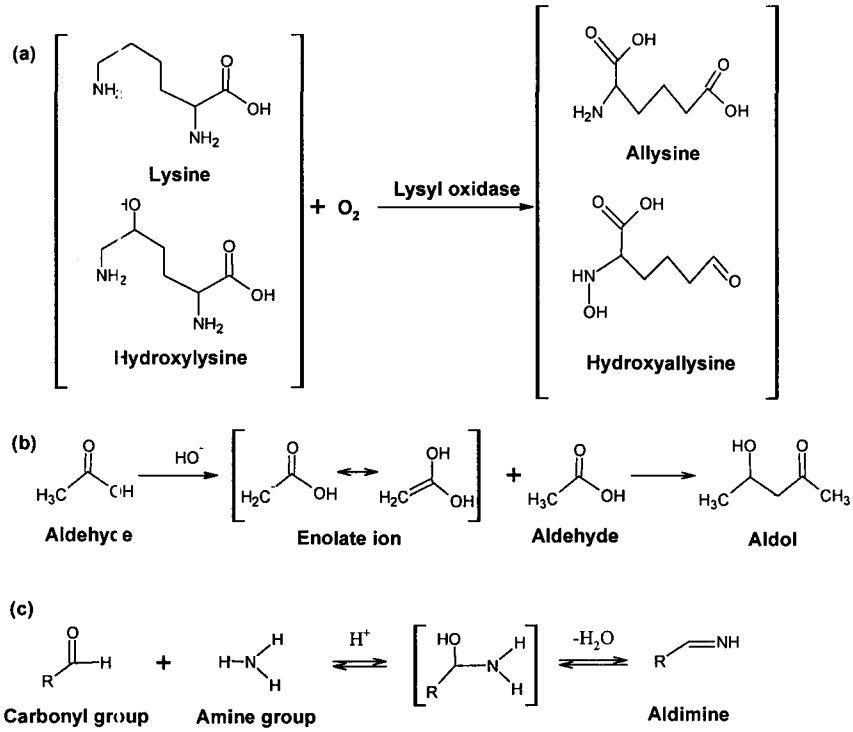


Figure 2-10. Intramolecular and intermolecular collagen crosslinking mechanisms (Friess. 1998; Solomons. 1996). Intramolecular crosslinking begins with an enzymatic reaction (a), whereby lysine and hydroxylysine are converted to the aldehydes allysine and hydroxyallysine. Next two aldehydes of the same collagen molecule undergo aldol condensation (b), whereby the first aldehyde is converted to an enolate ion that can react with the carbonyl group of the second aldehyde molecule. Intermolecular crosslinking occurs when aldehyde residues combine with amino groups on lysine or hydroxylysine to form aldimine crosslinks (c).

2.9.2 Chemical Crosslinking of Collagen with Carbodiimide

Chemical crosslinking collagen with carbodiimides, such as 1-ethyl-3-(3dimethyl aminopropyl) carbodiimide (EDC), induces isopeptide bonds. More specifically, the EDC reacts with the carboxyl group of glutamic acid or aspartic acid on the collagen chain (Figure 2-11) (Grabarek and Gergely. 1990; Wissink, M. J. B. et al. 2001), forming an unstable and highly reactive O-acylisourea ester, which can react with an amine group on lysine, creating a stable amide bond. However, the intermediate O-acylisourea ester is

highly susceptible to hydrolysis, which can lead to the regeneration of the carboxylate molecule and no subsequent crosslinking (Grabarek and Gergely. 1990; Olde Damink et al. 1996). To improve EDC mediated reactions, N-hydroxysuccinimide (NHS) is added, as it can react with the O-acylisourea ester, creating a semi-stable amine reactive succinimidyl ester. This NHS-ester has a slower hydrolytic rate and can further be modified to create the stable amide bond (Grabarek and Gergely. 1990).

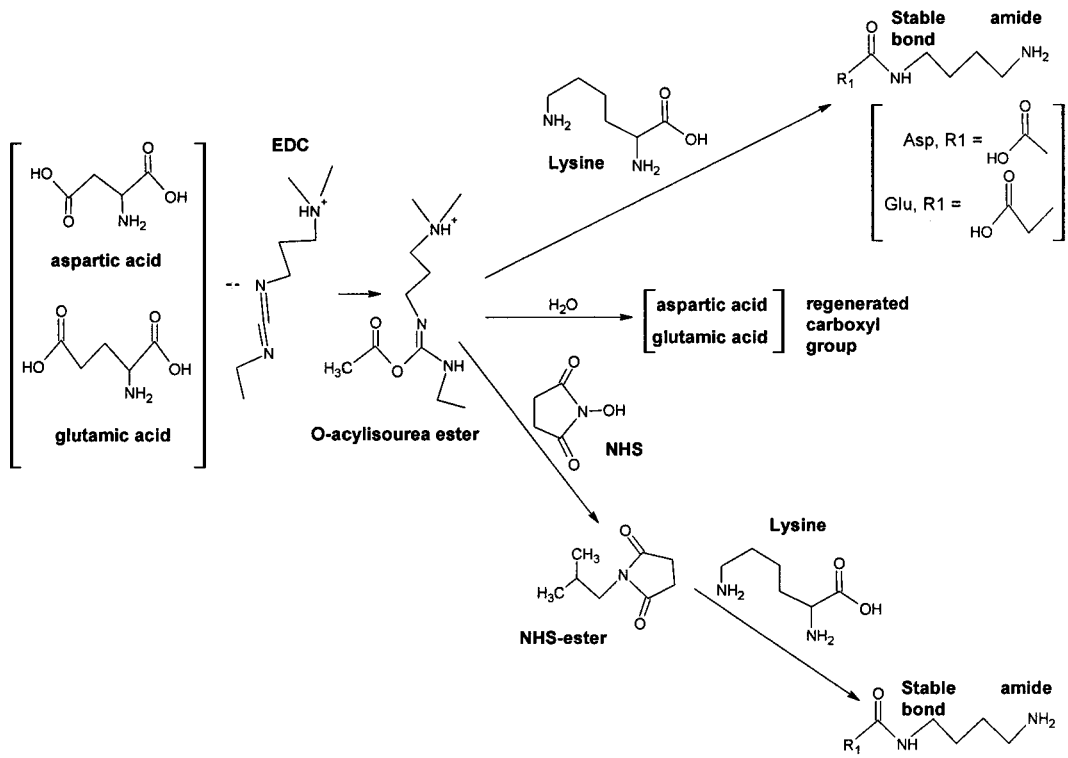


Figure 2-11. Reaction mechanism for chemically crosslinking collagen via EDC and NHS (Grabarek and Gergely. 1990; Wissink, M. J. B. et al. 2001). The carboxylate molecule reacts with EDC to form O-acylisourea ester (a), which can further react with an amine to create a stable amide bond between the carboxyl and amine molecules (b). To prevent hydrolysis of the O-acylisourea ester intermediate (c), NHS is added to create a more stable NHS-ester (d) that can further react to form the amide complex (e).

Carbodiimide crosslinking is "zero-length", as direct covalent crosslinks between collagen are formed (Grabarek and Gergely. 1990). EDC/NHS therefore merely acts as a crosslinking catalyst that is washed away after crosslinking occurs. This zero-length crosslinking is limited by the number of amine groups available within the collagen molecule, typically 30 per 1000 (or with incomplete amine hydrolysis, 75 per 1000), compared to the 120 per 1000 carboxylic acid groups (Duan and Sheardown. 2005). It is also likely that not all carboxylic acid groups are activated by EDC, further decreasing crosslinking density. As such, collagen matrices fabricated in this manner often lack the desired strength required for tissue engineering constructs. Collagen crosslinked via EDC/NHS has however been shown to be non-toxic *in vitro* and *in vivo* (Wissink, M. J. B. et al. 2001).

2.10 DENDRIMERS

Dendrimers are multivalent, globular molecules whose core is linked to many branches, usually fabricated with reactive groups able to covalently attach molecules for drug delivery (Boas and Heegaard. 2004; Gillies and Frechet. 2005). Molecular weight can also be manipulated by adding additional cascaded branches, or generations (Boas and Heegaard. 2004). Two of the more common dendrimers are polyamido amine (PAMAM) and polypropyleneimine (PPI) dendrimers. The latter has a 1,4-diaminobutane core and reactive amine branches, as shown in Figure 2-12 (Gillies and Frechet. 2005).

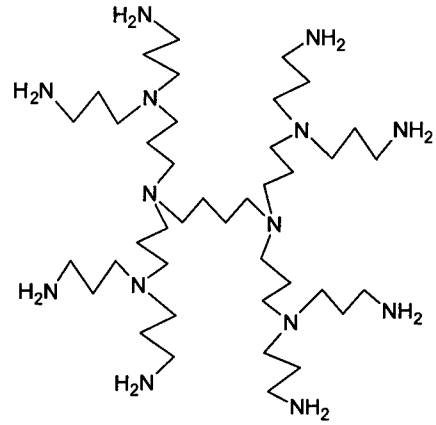


Figure 2-12. Generation two (G2) polypropylene imine dendrimer, with a 1,4 diaminobutane core and amine branches (Gillies and Frechet. 2005).

Of concern is the cationic nature of PAMAM and PPI animo-terminated branches, as *in vitro* studies have shown cellular toxicity through binding of the dendrimer to the cellular membrane, resulting in membrane damage and eventual lysis. However, lower generation dendrimers, and those surface modified with poly (ethylene glycol) (PEG), or complexed with oglionucleotides have been shown to have reduced cytotoxicity (Boas and Heegaard. 2004). Conversely, *in vivo* studies, with injected cationic PAMAM dendrimers, failed to show significant toxicity or immunogenicity in mice, although there is the risk of liver accumulation (Boas and Heegaard. 2004; Gillies and Frechet. 2005). Currently various dendrimers are being exploited for drug delivery of anticancer drugs, gene delivery through DNA coupling, although targeting, systemic half-life and toxicity remain ongoing issues (Gillies and Frechet. 2005).

2.11 TISSUE ENGINEERED CORNEAL IMPLANTS

Tissue-engineered cornea equivalents consist of cell based repair strategies, either using immortalized cells lines of primary/low passage limbal of central cornea cells, that

also incorporate extra cellular matrix components, particularly collagen as over 85% of the cornea is Type I collagen (Duan, Klenkler, and Sheardown. 2006). Several groups have developed collagen based materials for use as tissue engineered corneal matrices.

2.11.1 Collagen Based Corneal Implants

A 3-D collagen thermogel, has been developed by the Laboratoire d'Organogenese Experimentale (LOEX) group, using a sandwich approach (Carlsson et al. 2003; Duan, Klenkler, and Sheardown. 2006). First, corneal fibroblasts are cultured to produce a collagenous extracellular matrix. These sheets are subsequently layered into a 3-D matrix, after which epithelial cells are seeded on top to create an environment similar to the natural cornea (Duan, Klenkler, and Sheardown. 2006). There are issues with the matrix strength, but filter paper anchorage rings have been explored for matrix stability (Duan, Klenkler, and Sheardown. 2006).

In another model, a lyophilized, dehydrothermally crosslinked collagen sponge matrix, with controlled porosity, and strength, has shown to support epithelial, keratocyte and endothelial cell migration and proliferation and extracellular matrix production *in vitro* (Orwin and Hubel. 2000). Characterization of the sponge matrices revealed a stronger modulus when seeded with keratocytes than collagen gels, although it remained lower than the native cornea. Transparency was adequate with keratocyte seeded sponges and was further enhanced with the addition of proteoglycans (Orwin, Borene, and Hubel. 2003). There was also evidence of sponge contraction, caused by fibroblast differentiation into myofibroblasts and subsequent matrix remodelling. Water permeability studies indicated that permeability fluctuated with time and the structure

was not on par with the permeability of the native cornea (Borene, Barocas, and Hubel. 2004).

Collagen containing chondroitin-6-sulfate crosslinked with glutaraldehyde resulted in gels with increased transparency and strength, compared to thermally crosslinked collagen (Doillon et al. 2003). *In vitro*, in the presence of protease inhibitors and ascorbic acid to hinder matrix degradation and promote collagen synthesis, the surfaces were compatible with immortalized corneal cell lines and promoted keratocyte ion channel development (Griffith et al. 1999). However, there are concerns over the biostability and cytotoxicity of glutaraldehyde crosslinked collagen (Duan and Sheardown. 2005). Further experimentation led to the development of a collagen crosslinked with a N-isopropylacrylamide, acrylic acid and acryloxysuccinamide copolymer that had superior transparency, tensile strength and reactive functional groups for the integration of additional molecules, such as extracellular matrix components and growth factors (Li et al. 2005). *In vivo*, these surfaces promoted epithelial overgrowth, stromal ingrowth, and nerve regeneration without excessive inflammation (Li et al. 2003).

2.11.2 Collagen Crosslinked with Dendrimers

As an alternative method of generating corneal tissue engineering scaffolds, the amine groups of collagen were amplified using polypropyleneimine octaamine dendrimers with EDC/NHS for collagen crosslinking (Duan and Sheardown. 2005). Compared with thermal and chemical crosslinkers including EDC/NHS and glutaraldehyde, dendrimer crosslinked collagen showed high mechanical strength, good

optical clarity, biological stability and high crosslinking density (Duan and Sheardown. 2005; Duan and Sheardown. 2006b) and the potential to add further biological functionalization through free reactive groups on the dendrimer (Duan et al. 2006a (Ahead of print)).

Preliminary cytotoxicity of dendrimer crosslinked collagen was evaluated *in vitro* with corneal epithelial cells, to which positive biocompatibility results were obtained, yet there exists a need for extensive cellular studies to assess protein production and mitochondrial activity (Duan and Sheardown. 2006b).

2.12 GROWTH FACTOR DELIVERY SYSTEMS

Drug delivery systems for bioactive factors, such as growth factors and cytokines, have been widely explored for the targeting of tissue restoration, maintenance, structure and function, through an artificial environment to stimulate desired cellular activities (Lutolf and Hubbell. 2005; Saltzman and Olbricht. 2002). Of concern are the administration route, drug release profile, delivery method, targeting and fabrication of the delivery vehicle (Sinha and Trehan. 2003). Also, the biophysical, biochemical, and physiological characteristics of the growth factor as well as its molecular size, biological half-life, and the dose requirements need to be considered in the design of an effective delivery system. The amount of growth factor released is crucial. There must be an adequate amount present to stimulate the desired physiological response of the cells; however, it should not be present in an amount such that it stimulates receptor down regulation, whereby cells decrease the number growth factor receptors, diluting the desired biological response (Nimni. 1997). Maintenance of growth factor biological

activity during manufacturing and release is also crucial (Tabata. 2003). Degraded protein may cause immunogenic responses that could compromise the integrity of the delivery system, potentially altering the release profile and bioactivity (Sinha and Trehan. 2003).

2.12.1 Insoluble Growth Factor Delivery

Typically the time needed to generate a cell response is longer than the half life of the growth factor (Saltzman and Baldwin. 1998; Lee. 2000). Therefore, parenteral delivery, via intravenous of intramuscular injection, of growth factors is not an effective delivery method as growth factors rapidly disperse from the injection site and are prone to proteolysis (Tabata. 2003). For example, rapid degradation and loss of bioactivity of FGF-2 has been noted when it has been injected or ingested (Saltzman and Baldwin. 1998; Lee. 2000). To maintain growth factor concentrations in the therapeutic range, high doses and frequent injections are necessary, which can increase side effects and perhaps lead to undesirable cellular responses (Saltzman and Baldwin. 1998). Oral delivery of proteins is hindered by the intestinal lumen, a poorly permeable interface through which the drug must pass to be effective, and by the many proteases and peptidases that line its structure, which can denature the protein (Sinha and Trehan. 2003). Another risk with systematic delivery systems lies with the pluripotency of growth factors, as it may be desired to influence only one population of cells and not another (Saltzman and Baldwin. 1998).

Therefore, localized controlled delivery systems are widely investigated for growth factor delivery, whereby the drug is incorporated by some means into a polymer

or hydrogel carrier (Babensee, McIntire, and Mikos. 2000; Saltzman and Baldwin. 1998). The drug release usually occurs via diffusion, although it can be affected by material characteristics (degradable or non-degradable) and shape (reservoir or matrix), and the amount of drug loaded. The most common systems include reservoir devices, poly-dispersed matrices, microspheres and hydrogels as shown in Figure 2-13.

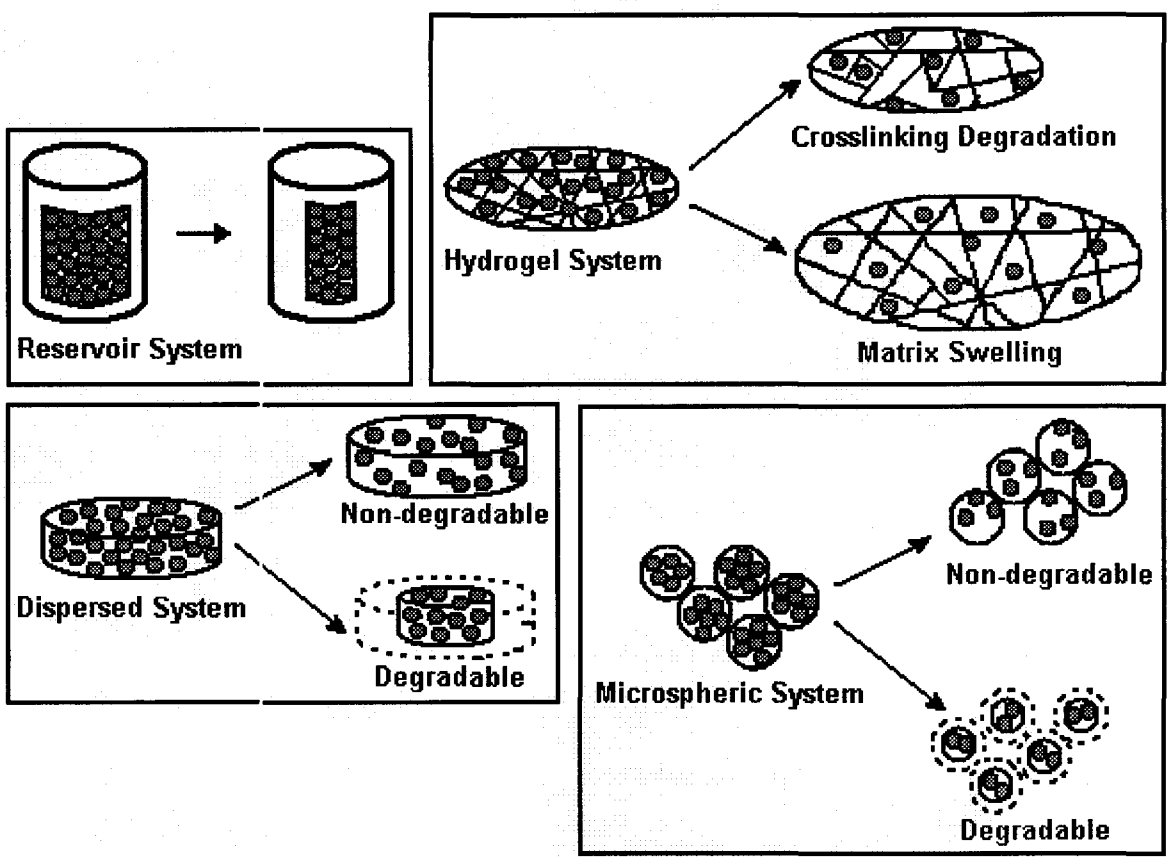


Figure 2-13. Localized and controlled delivery systems for protein delivery (Saltzman and Baldwin. 1998). Reservoir systems have the growth factor contained in its core, whereby the release is controlled by diffusion. Degradable or non-degradable dispersed systems have the growth factor suspended throughout the matrix. Hydrogel systems hold the growth factor within its crosslinks, which can degrade, releasing the growth factor, or the matrix can swell allowing for drug diffusion. Microspheric systems encapsulate the growth factor within multiple non-degradable or degradable microspheres that release the growth factor.

Reservoir systems have the growth factor surrounded by a polymer coating; however the large size of growth factors greatly reduces their diffusion from the material and there are incidences of dose-dumping, whereby damage to the polymer coating is followed by massive protein release (Saltzman and Baldwin. 1998). Alternatively, polymer matrices containing dispersed growth factor have favourable release, as they are capable of slowly delivering macromolecules. Hydrogel delivery systems have also been widely examined for protein release, although the kinetics of release from hydrogels are not optimal. Microspheres encapsulating growth factors protect the protein from degradation and rapid clearance *in vivo* (Sinha and Trehan. 2003). If degradable, the microspheres do not require removal; however given their small size and dispersement, premature termination of the treatment is a common challenge as they are difficult to locate and remove (Saltzman and Baldwin. 1998).

2.12.1.1 Insoluble FGF-2 Delivery

Although insoluble delivery of pharmaceutical drugs is promising, FGF-2 delivery with this method has encountered much difficulty. Table 2-3 lists polymer and hydrogel systems that have been used to deliver FGF-2 and the associated drawbacks of those systems.

Table 2-3. Examples of insoluble FGF-2 delivery systems.

Polymer delivery system	FGF-2 Release rate	Drawbacks	Reference
Ethylene-vinyl acetate copolymer matrix and tubes	37% over 2 weeks	- Loss of FGF-2 activity	(Edelman et al. 1991)
Acidic and basic gelatin hydrogels	- Acidic gels: 30% in 1 day; - Basic gels: <90% in 1 day	- Rapid release via diffusion and degradation	(Tabata and Ikada. 1999; Tabata, Nagano, and Ikada. 1999)
Dextran-epichlorohydrin hydrogels	90% in 26 days	- Non-degradable; thus 2nd surgery required or dextranase administration to avoid fibrin formation	(Dogan, Gumusderelioglu, and Aksoz. 2005)
Nitrocinnamate-derived polyethylene glycol hydrogel system	50% to 70% over 5 days	- Loss of FGF-2 activity (without heparin)	(Andreopoulos and Persaud. 2006)

Release from many of these systems is diffusion controlled, which may result in rapid release kinetics in the absence of an alternative controlling technique (Tabata. 2000). Ideally it would be desirable to deliver the protein at a rate that is proportional to the rate of site elimination (Saltzman and Baldwin. 1998). Unbound FGF-2 has a short half life, as it is prone to rapid enzymatic degradation (Saksela et al. 1988), resulting in a circulation clearance time of only 1.5 minutes when administered intravenously in rats (Whalen, Shing, and Folkman. 1989). Therefore it is desired to deliver the growth factor using an alternative method, either through tethering or via soluble delivery systems.

2.12.2 Immobilized Growth Factor Delivery

Growth factors can be covalently conjugated to carrier polymers or proteins for sustained localized delivery (Saltzman and Baldwin. 1998). The conformation of the

attached growth factor is of critical importance, to maintain its biological activity, but resist receptor internalization for sustained signaling. As well, alteration or denaturation of the protein may lead to inflammation (Brevig et al. 2005).

Growth factors immobilized to surfaces have shown to increase cell stimulation and surface receptor population, compared to free or adsorbed growth factors *in vitro* (DeLong, Moon, and West. 2005; Gomez et al. 2006 (in press); Nimni. 1997). A variety of growth factors including EGF (Klenkler and Sheardown. 2006; Klenkler et al. 2005) and TGF-β (Merrett et al. 2003) have been covalently attached, including FGF-2, whereby it was complexed to biotin and tethered to biotinylated collagen via avidin-biotin interactions (Stompro, Hansbrough, and Boyce. 1989).

Typically immobilized growth factor systems are used to create biologically active surfaces for cellular stimulation and adhesion. Conjugated growth factors in some cases have demonstrated increased stability and failed to aggravate immunogenic responses (Saltzman and Baldwin. 1998). It may be difficult to utilize this delivery method for cellular responses that require internalization of the growth factor, if it remains tethered to the surface. However, removed from the delivery vehicle, the growth factor is still susceptible to enzymatic degradation and rapid clearance.

2.12.3 Soluble Growth Factor Delivery

Stabilizing agents are frequently added to stabilize the protein, protecting against proteolytic cleavage or maintaining bioactivity, either during fabrication, storage or delivery. These stabilizing agents can consist of other proteins, sugars, polyols, amino acids, chelating agents or inorganic salts (Sinha and Trehan. 2003). Additionally,

stabilizing agents can also be used to decrease the protein release rate (Saltzman and Baldwin. 1998).

2.12.3.1 Soluble FGF-2 Delivery with Heparin

Binding of FGF-2 to heparin allows FGF-2 to be delivered in soluble form without degradation, as heparin has been shown to stabilize acidic and basic fibroblast growth factor over a high temperature range (Sinha and Trehan. 2003). For instance, when FGF-2 and heparin have been combined in an intravenous solution administered continuously to rats, growth factor circulation time increased 3 fold, and systemic concentrations were maintained at higher levels than when FGF-2 was delivered alone (Whalen, Shing, and Folkman. 1989). FGF-2 delivery systems with heparin have shown to prolong growth factor release (Sakiyama-Elbert and Hubbell. 2000), and prolonged storage and encapsulation of FGF-2 has been achieved through its binding to heparin-sepharose beads (Lee. 2000). Also, heparin added to the release medium has been shown to increase FGF-2 release (Liu et al. 2002), presumably aiding in the translocation of FGF-2 from the delivery system. Thus, FGF-2 delivery with heparin is amicable for sustained FGF-2 stability and bioactivity; a few research examples are highlighted in Table 2-4.

Table 2-4. Soluble delivery of FGF-2.

Delivery system	FGF-2 Release rate	Experimental Findings	Reference
Alginate gels with heparin-sepharose beads	25% in 2 weeks	- Heparinase increased FGF-2 release to 85% in 2 weeks - Heparin allowed for retained FGF-2 bioactivity (87%) - High ionic solutions strip FGF-2 from heparin (all released in 8 hours)	(Edelman et al. 1991)
Fibrin and fibrin-collagen matrices with heparin	<80% in 48 hours	- *In vitro*: Increased cell DNA replication - *In vivo*: Increased cell growth compared to unheparinized matrices and locally administered heparin; evidence of slight inflammation	(DeBlois, Cote, and Doillon. 1994)
Fibrin matrices with heparin	None shown	- *In vitro*: neurite length was enhanced with 1 and 5 μg/mL of FGF-2 - Suggested cell receptor saturation at 10 μg/mL - Neurite stimulation did not differ between bound and unbound FGF-2 - Sustained FGF-2 delivery achieved with heparin binding	(Sakiyama-Elbert and Hubbell. 2000)
Hyaluronate-heparin conjugate gels	50% in 2 weeks	- *In vitro*: enhanced fibroblast stimulation - Release decreased in sucrose buffer but increased with ionic strength - FGF-2 release enhanced when heparin - Evidence of heparin-FGF-2 complexes being released	(Liu et al. 2002)
Benzyl-bis (dimethylsilylmethyl) oxycarbamoyl-heparin surfaces	None shown	- *In vitro*: enhanced endothelial cell proliferation & capillary tube formation - *In vivo* (rats): increased density of granulation tissue - Fibronectin and fibrinogen reduced FGF binding	(Zamora et al. 2002)
Collagen-heparin matrices, crosslinked with carbodiimide	53% in 4 weeks	- Heparinized collagen bound more FGF-2 than pure collagen matrices - *In vivo* (rats): evidence of increased angiogenesis and tissue regeneration - Reduced inflammation with heparinized matrices	(Pieper et al. 2002)
Poly ethylene glycol (PEG) diacrylate hydrogel	None shown	- *In vitro*: enhanced smooth muscle cell proliferation and migration - PEG conjugation unaffected FGF-2 activity, contrary to other work	(DeLong, Moon, and West. 2005)

Table 2-4. Soluble delivery of FGF-2 (con't).

Delivery system	FGF-2 Release rate	Experimental Findings	Reference
Heparinized collagen matrices	65% in 28 days	- FGF-2 binding decreased with increasing ionic strength - Surfaces without FGF-2 stimulated endothelial cell proliferation	(Wissink et al. 2001; Wissink, M. J. B. et al. 2001)
Chitosan heparin hydrogel	30% in 1 day	*In vitro*: enhanced human umbilical vascular endothelial cell growth *In vivo* (mice): induced prolonged neovascularization	(Ishihara et al. 2003)
Heparinized polystyrene bound to collagen membranes	None shown	*In vitro*: enhanced fibroblast and endothelial cell proliferation	(Ishihara et al. 2001)
Chitosan heparin hydrogel	20% in 1 day	- *In vitro*: human umbilical vascular endothelial cell stimulation, further enhanced with gel degradation - *In vivo* (mice and rats): enhanced vascularization	(Fujita et al. 2004)
Heparinized poly (lactide glycolide) nanospheres in fibrin gel	60% in 28 days	- FGF-2 release increased with increased fibrinogen loading (85%) - *In vitro*: increased human umbilical vascular endothelial cell proliferation over 2 weeks - *In vivo* (mice): evidence of neovascularization	(Jeon et al. 2006)

3. SCOPE OF PROJECT

For the success of an artificial cornea, material modification is required to enhance tissue integration including anterior epithelialization, stromal ingrowth and anchoring and nerve regeneration. Modification techniques include creating an environment that stimulates cell adhesion by introducing cell adhesion molecules and tethering growth factors for cell migration and proliferation. Previous studies have investigated epithelial coverage and downgrowth inhibition with epidermal growth factor (EGF) and transforming growth factor beta (TGF-β), respectively. Stromal anchoring has been improved by creating porous matrices that allow fibroblast migration. However, direct stimulation of the stroma with growth factors has not been addressed.

Basic fibroblast growth factor (FGF-2) has been shown to be a key modulator of stromal wound healing, through its effects on proliferation and migration of various cell types. In the cornea, FGF-2 is expressed in the epithelial basement membrane. Upon damage, it is released from the extracellular matrix and has been shown to stimulate fibroblast proliferation and migration. As well, it may be involved in nerve regeneration.

Many growth delivery systems lack the ability to protect growth factors from proteolytic cleavage and degradation for improved bioavailability and long-term healing. Growth factor storage and protection *in vivo* is mediated by association with extracellular matrix glycosaminoglycans, such as heparan sulphate proteoglycans (HSPs). HSPs have been shown to improve FGF-2 stability by protecting it from proteolytic cleavage, and heat damage, while during wound healing, HSPs release FGF-2 for cellular stimulation, and have aided in its translocation for cellular signaling.

In this study we investigate the heparin modification of two materials, intended for use in keratoprostheses and tissue engineered corneal equivalents as a stromal stimulant for device anchorage through fibroblast integration. Through the immobilization of heparin and uptake and release of basic fibroblast growth factor (FGF-2), it is hypothesized that the materials will better mimic the soluble delivery of the growth factor that is often seen in wound healing.

The first material selected was poly (dimethyl siloxane) (PDMS), which has been proposed by the Sheardown group as a potential substrate material for an artificial cornea. While material modifications will be needed to improve glucose and low molecular weight permeability, proof of concept was examined with PDMS only. The modified material was characterized by attenuated total reflection Fourier transform infrared spectrophotometry (ATR-FTIR), X-ray photoelectron spectroscopy (XPS), and water contact angles. The amount of immobilized heparin and its biological activity were quantified.

The second material selected was dendrimer crosslinked collagen, under investigation as a corneal tissue engineering scaffold. Heparin incorporation into the collagen scaffolds was characterized by water uptake, and differential scanning calorimetry (DSC), while matrix stability was assessed using colorimetric assays.

Both materials were exposed to radiolabeled FGF-2. The amount of immobilized growth factor was quantified. Release under physiological conditions was assessed to assess the potential of these materials for growth factor delivery.

4. MATERIALS AND METHODS

4.1 MATERIALS

Sylgard 184 silicone elastomer kit and polymethylhydrogen siloxane fluid (DC 1107) [$(MeHSiO)_n$, 30 centistokes] were from Dow Corning (Mississauga, ON). The α-allyl-ω-N-succinimidyl carbonate-poly (ethylene oxide) (allyl-PEO-NSC), average molecular weight of 550, was purchased from JuTian Chemical Co. (Nanjing, China), while the monoallyl-hydroxyl-terminated poly (ethylene oxide) (allyl-PEO-OH) was purchased from Clariant (Canada) Inc., (Markham, ON). 2-N'N-hydroxysuccinimide (diNHS) was purchased from Sigma-Aldrich (Oakville, ON).

Pepsin dissolved bovine cornium Type I collagen (with less than 20% type III collagen) was a gift from Inamed Corporation, (Santa Barbara, USA). Second generation (G2) polypropyleneimine octaamine (PPI) dendrimer was purchased from SyMO-Chem (Eindhoven, The Netherlands). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (Oakville, ON).

Broad spectrum heparin was purchased from Sigma-Aldrich (Oakville, ON), while low molecular weight heparin (LMWH) was manufactured by Calbiochem, EMD Biosciences Inc (San Diego, USA). Basic fibroblast growth factor (FGF-2) was purchased from R & D Systems (Minneapolis, USA).

Additional reagent information is located in Appendix A, as is equipment information.

4.2 POLY (DIMETHYL SILOXANE) PREPARATION

Poly (dimethyl siloxane) (PDMS) surfaces were fabricated according to manufacturer's instructions. Briefly, Sylgard 184 silicone elastomer base and its curing agent were well mixed in a 10:1 w/w ratio in a 10 mm Petri dish (4 g base: 0.4 g curing agent). The resulting film was degassed under vacuum for 1 hour, and then allowed to cure at room temperature for 5 days. The film was punched into 0.625 cm (¼") discs, with an approximate 0.5 mm thickness, and washed with anhydrous methanol prior to use.

Figure 4-1 illustrates PDMS fabrication and subsequent PDMS surface modification reactions.

4.2.1 PDMS Modification

PDMS surfaces were first modified to obtain a surface oriented functionalized reactive Si-H group, based on the methods described by Chen *et al.* (Chen et al. 2005a; Chen et al. 2006). Thirty PDMS discs were placed in a 10 dram glass vial and immersed in a mixture of 5 mL anhydrous methanol, 3 mL polymethylhydrogen siloxane fluid (DC 1107), and 50 μL of triflic acid [$CFSO_3H$]. The vial was shaken vigorously for 30 minutes at room temperature. Discs were then washed with anhydrous methanol, to remove the triflic acid, followed by washing with dried n-hexane, to remove any residual DC 1107. Each Si-H modified disc, PDMS-SiH, was dried under N_2 and placed under high vacuum for at least 8 hrs. Two methods were subsequently used to tether N-succinimidyl carbonate (NSC) terminal poly (ethylene oxide) (PEO) groups to the

PDMS-SiH functionalized surface: a direct grafting method and an indirect grafting method.

4.2.2 Direct Grafting Method

Twenty PDMS-SiH functionalized surfaces were placed in a 10 dram glass vial with 3 mL of molecular sieve dried 2-methoxyethyl ether, 0.6 g allyl-PEO-NSC and 20 μL of Karstedt's platinum catalyst $[(Pt)_2(H_2C{=}CH\text{-}SiMe_2OSiMe_2CH{=}CH_2)_3]$. The vial was purged with N_2, tightly sealed, placed in a 50°C oil bath and stirred for 15-20 hrs. The resulting surfaces, PDMS-PEO-NSC, were washed with dried acetone, dried with N_2 and placed in a desiccator for 12 or more hrs. Control hydroxyl-terminated surfaces (PDMS-PEO-OH) were prepared by submersing the PDMS-PEO-NSC surfaces in potassium phosphate buffer (pH 8.0) for 3 days, then washing with Milli-Q water and wicking dry.

4.2.3 Indirect Grafting Method

In a 10 dram glass vial, 10 PDMS-SiH surfaces were immersed in 1 mL 2-methoxyethyl ether and 1 mL allyl-PEO-OH and catalyzed with 15 μL Karstedt's platinum. The vial was gently shaken on an orbital shaker for 2 hrs, after which surfaces (PDMS-PEO-OH) were rinsed with acetone, dried with N_2 and desiccated for 10 hrs.

Afterwards, in a 10 dram glass vial, 0.6 g diNHS was dissolved in 1 mL anhydrous acetonitrile and 100 μL triethylamine (TEA) for 10 minutes. Ten PDMS-PEO-OH surfaces were added, purged with N_2 and gently shaken on an orbital shaker for

6 hrs. Then surfaces were rinsed with anhydrous acetonitrile and 2-methoxyethyl ether, dried with N_2 and desiccated for at least 12 hrs.

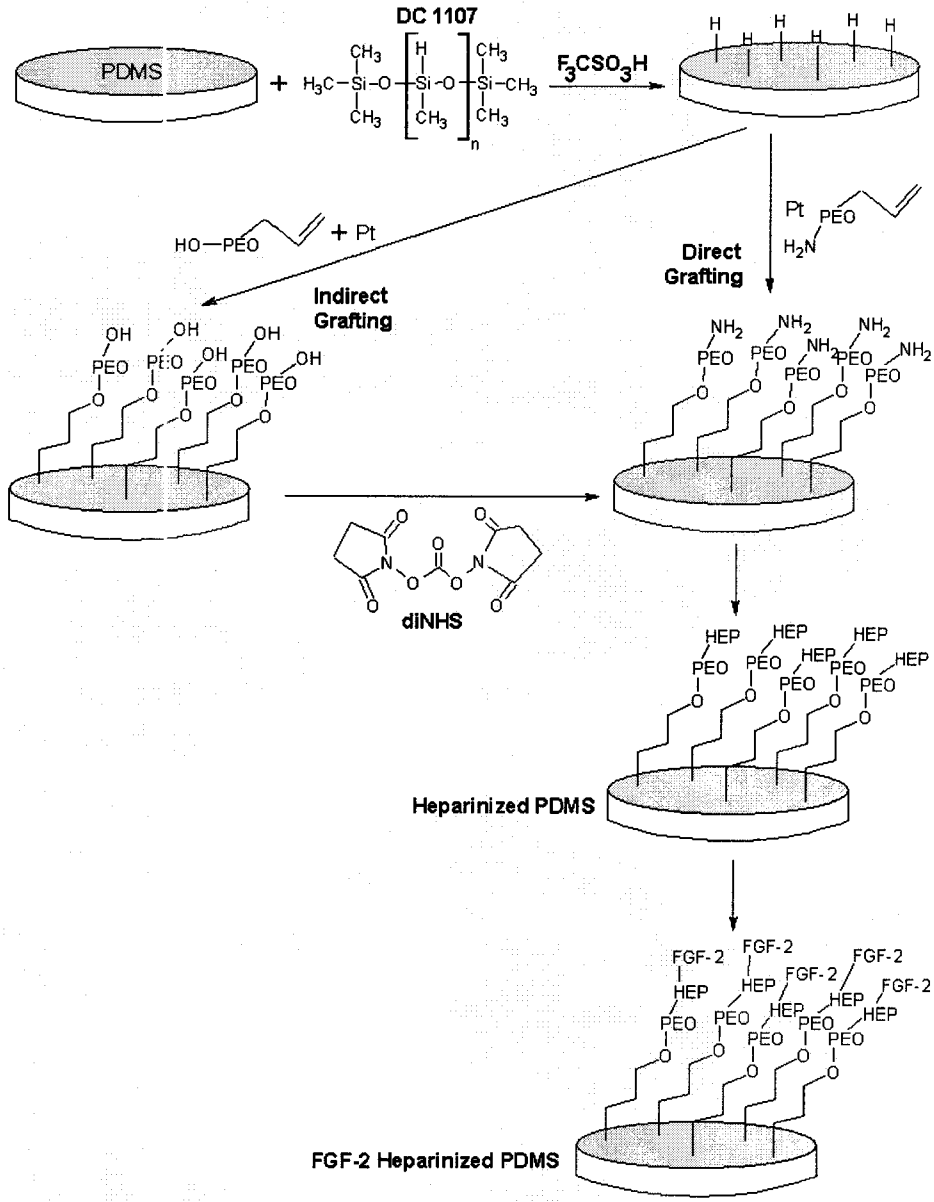


Figure 4-1. PDMS Modification. Direct and indirect PDMS modification begins with SiH functionalization of the PDMS surface, following exposure of PDMS to DC1107 and triflic acid. Direct grafting modifies PDMS-SiH surface with allyl-PEO-NSC, while indirect grafting consists of allyl-PEO-OH and subsequent diNHS modification. PDMS-PEO-NSC surfaces are then exposed to heparin to create heparinized surfaces, followed by FGF-2 exposure.

4.2.4 Heparinization of PDMS Surfaces

To create heparinized surfaces, PDMS-PEO-NSC surfaces, generated using both direct and indirect methods, were immersed in 5 mL of a 10 mg/mL heparin solution in phosphate buffered saline (PBS, pH 8.0) for at least 6 hrs. Surfaces were then removed, rinsed with PBS, and wicked dry. Surfaces were also modified with low molecular weight heparin (LMWH) in a similar manner.

4.3 PDMS SURFACE CHARACTERIZATION

Surface characterization was performed on PDMS based surfaces via attenuated total reflection fourier transform infrared spectrophotometer (FTIR-ATR), X-ray photoelectron spectroscopy (XPS), and water contact angles. Heparinization of the surfaces was quantified via colorimetry and biological activity assays.

4.3.1 Attenuated Total Reflection Fourier Transform Infrared Spectrophotometer (ATR-FTIR)

Attenuated total reflection Fourier transform infrared spectrophotometry (ATR-FTIR) provides transmittance spectra correlating to atomic and molecular unit vibrations resulting from exposure to infrared radiation and total internal reflection (Merrett et al. 2002; Ratner et al. 1996). Given two materials with different indices of refraction, the infrared beam is restricted to analyzing the surface of a sample, with a penetration depth usually on the order of 1-5 μm (Merrett et al. 2002; Ratner et al. 1996). A Bio Rad FTS-40 FTIR equipped with a DTGS detector and a vertical ATR module, with a zinc selenide crystal, was used to scan PDMS, PDMS reaction intermediates and the final PDMS-PEO-Heparin surfaces to verify surface reactions.

4.3.2 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS), also referred to as electron spectroscopy for chemical analysis (ESCA), is utilized for elemental surface quantification (Ratner et al. 1996). Infrared X-rays are sent to the surface, causing the emission of photoelectrons, whose energy is quantified and translated to binding energy. Binding energies (E_B), which are characteristic to each element and its environment, are quantified by the following equation:

$$E_B = h\upsilon + E_K + \Phi \qquad \text{(Eq.1)}$$

Where: $h\upsilon$ = photon energy (energy from the X-ray)

E_K = kinetic energy (of emitted electron)

Φ = work function

This method can identify all elements, except hydrogen and helium, in quantities greater than 0.1 atomic percent. The depth of analysis can range from 10-250 Å, with a spacial resolution between 10-150 μm.

XPS was performed at Surface Interface Ontario, University of Toronto, using a Leybold (Specs) MAX 200, and peaks were analyzed with the corresponding software. A sampling depth of 10 nm was performed for low resolution and high resolution spectra, at take off angles of 20° and 90°.

4.3.3 Water Contact Angles

Water contact angles are used to estimate surface energy through the liquid wetting of a surface and the surface tension that results (Ratner et al. 1996). The energy of the surface (γ_{sv}) can be related to the drop's liquid-vapour surface tension (γ_{lv}) and the

interfacial tension between the solid and the drop (γ_{sl}), combined with the contact angle (θ). This is illustrated in Figure 4-2 and in the following equation:

$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos \theta \qquad \text{(Eq.2)}$$

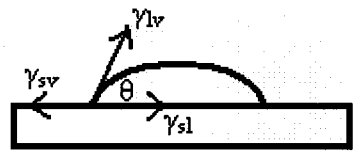


Figure 4-2. Contact angle measurements using the sessile drop method.

In the current work, contact angles were used to monitor surface wettability changes with PDMS surface modification. Advancing and receding sessile drop contact angles were measured on a bench top goniometer (NRL C.A. Goniometer, Ramé-hart, USA). Milli-Q water was used with a drop volume of 20 µL. Contact angles were performed in triplicate on each side of the surface.

4.3.4 Heparin Density

Heparin density on the PDMS-PEO-Heparin surfaces was quantified via a slightly adapted version of the toluidine blue assay (Hinrichs et al. 1997a). Briefly, surfaces were exposed to a 0.5% toluidine blue solution (with 0.2% sodium chloride (NaCl) and 0.01 N hydrochloric acid (HCl)) overnight. Subsequently, surfaces were rinsed 3 times in Milli-Q water, dabbed dry and placed in 700 µL of 4:1 ethanol: 0.1 N sodium hydroxide (NaOH) solution overnight. Surfaces were subsequently removed from the solution and the absorbance of the ethanol solution read at 540 nm. Absorbance values were standardized against standard heparin solutions. Standards were created by exposing known concentrations of dissolved heparin in PBS (pH 7.4) to 0.5% toluidine blue

solution (0.2% NaCl and 0.01 N HCl) overnight, after which a precipitate forms. Standards were then centrifuged (Allegra X-12R Centrifuge, Beckman Coulter) at 1000g for 10 min and the supernatant carefully removed. The pellet was resuspended in a 4:1 ethanol: 0.1 N NaOH solution, diluted to appropriate concentrations, and read at 540 nm.

4.3.5 Heparin Activity

As an alternative means to quantify heparin immobilization, its activity was assessed by exploiting its involvement with antithrombin III and factor Xa (Ingber. 1991; Teien and Lie. 1977), with an anti-Factor Xa assay kit (Stachrom® Heparin, Diagnostica Stago, France) according to the manufacturer's instructions. Briefly, surfaces were exposed to 100 µL purified bovine antithrombin III (AT-III) and either 800 µL for unfractionated heparin, or 1mL for LMWH, 0.1N tris (hydroxymethyl) methylamine and ethylenediamine tetraacetic acid (TRIS-EDTA) buffer (pH 8.4) for 2 min at 37°C. Then, surfaces and 200 µL of the AT-III solution were transferred to a new test tube, and 200 µL purified bovine factor Xa was added and the samples were incubated for 30 seconds at 37°C. Next, 200 µL of the chromogenic substrate (CBS 31.39) was added, the samples incubated for an additional 30 sec at 37°C and the reaction terminated with the addition of 200 µL of glacial acetic acid. Surfaces were removed and the solutions read at 540 nm. Calibration curves were created with known concentrations of aqueous unfractionated and low molecular weight heparin solutions.

4.4 COLLAGEN GEL PREPARATION

Collagen gels were fabricated based a method adapted from the work of Duan and Sheardown (Duan and Sheardown. 2005). Heparin modified collagen gels were prepared by first dissolving 1 mL collagen (66 mg/mL) in 1.0 N hydrochloric acid to give a 4% solution, followed by the addition of heparin solution (30 mg/ml). Since, heparin is only stable under highly acidic conditions for up to 1 hour (Jandik et al. 1996); crosslinking was initiated as quickly as possible. Crosslinking was achieved thermally or via two chemically methods. The first chemical crosslinking was achieved with a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysuccinimide (EDC/NHS) mixture, and second the chemical crosslinking solution contained EDC/NHS components combined with a second generation polypropyleneimine octaamine G2 dendrimer which has been shown to result in highly crosslinked, stable collagen gels. Where appropriate, 1.0 N NaOH was added to adjust the pH as the EDC/NHS reaction has been shown to be optimized at a pH of 5.5 (Olde Damink et al. 1996). Prior to crosslinking, endotoxin free water (EFW) was added so that the collagen mixture had a final collagen concentration of 30 mg/mL. The mixture was pressed between glass plates and left overnight to ensure complete crosslinking. Thermally gelled collagen controls were prepared without the addition of the EDC/NHS by incubation of the mixture overnight in a 37°C oven. The solution pH in this case was raised to 7.4. Control collagen gels containing no heparin were also fabricated. The resultant gels were punched into 0.625 cm (¼") surfaces. These were stored at room temperature, but

surfaces were reconstituted in Milli-Q water prior to experimentation. Collagen gel recipes are listed in Table 4-1.

Table 4-1. Collagen Surface Preparation Instructions. Volumes are in microliters (μL), unless otherwise stated.

Surface	Abbr.	Water	1.0 N HCl	Heparin	Amount of crosslinker	Crosslinking solution	1.0 N NaOH	pH
Collagen	COLL	990	110	-	-	-	100	7
Collagen-Heparin	C-H		100	450	-	-		7
Collagen with EDC	C-EDC	780	120	-	200	EDC*	100	5.5
Collagen with EDC, and heparin	C-H-EDC	380	100	450	200	EDC*	60	5.5
Collagen with EDC, and dendrimer	C-G2	900	100	-	200	G2[a]	-	5.5
Collagen with EDC, dendrimer and heparin	C-H-G2	450	100	450	200	G2[a]	-	5.5
Collagen with EDC, 100% more dendrimer	C-20%G2	700	100	-	200	20%G2[b]	-	5.5
Collagen with EDC, 100% more dendrimer, with heparin	C-H-20%G2	200	100	450	200	20%G2[b]	-	5.5
Collagen with 100% more EDC and dendrimer	C-2G2	900	100	-	400	2G2[t]	-	5.5
Collagen with 100% more EDC and dendrimer with heparin	C-H-2G2	450	100	450	400	2G2[t]	-	5.5

*1mL of EDC crosslinker solution contains 75 mg EDC, and 45 mg NHS.
[a]1mL of G2 crosslinker solution contains 75 mg EDC, 45 mg NHS, and 38 mg G2.
[b]1mL of 20% G2 crosslinker solution contains 75 mg EDC, 45 mg NHS, and 38 mg G2.
[t]1mL of 2G2 crosslinker solution contains 150 mg EDC, 90 mg NHS, and 76 mg G2.

4.5 BULK COLLAGEN GEL CHARACTERIZATION

Collagen gels were characterized mainly for bulk properties with water uptake, differential scanning colorimetry (DSC). Heparin and dendrimer stability within the collagen matrix was assessed with release studies analyzed with metachromatic assays.

4.5.1 Water uptake

Water content of the collagen gels was measured to characterize the collagen crosslinking. Collagen gels were freeze dried in a Modulyo D model freeze dryer (Thermo-Savant) and weighed to determine the dry weight (W_d). Afterwards, gels were reswollen in Milli-Q water for 1 hour, wicked dry to remove assess water and weighed again (W_w). The water uptake was calculated according to the following formula:

$$Water Uptake(\%) = \frac{W_d - W_w}{W_w} * 100\% \qquad \text{(Eq.3)}$$

4.5.2 Differential Scanning Calorimetry (DSC)

Differentical scanning calorimetry (DSC) is traditionally used to study thermally induced transition changes (McElhaney. 1982). A reference material and the sample are heated at identical rates so their temperature difference remains constant at zero, creating a steady baseline of heat supplied as a function of temperature. Upon heating, if a thermally induced change occurs to the sample, the temperature difference between the reference and sample deviates from zero. In an effort to rectify the temperature difference, the DSC instrument supplies more or less heat to the sample, disrupting the baseline. A negative deflection from the baseline constitutes an exothermic reaction,

with the magnitude of deviation dependant on the magnitude of differential heating rate, and denotes the sample's denaturation temperature.

Denaturation temperatures for collagen modified surfaces were determined via differential scanning calorimetry using a TA instruments DSC 2910. Collagen gels were immersed in endotoxin free water (EFW) for 2 hrs, dabbed dry, and transferred into aluminum hermetic pans. They were exposed to a heating rate of 2 °C/min over a temperature range of 15°C to 100°C. Peaks deviating from the baseline were monitored and analyzed with accompanying DSC software. A hermetic pan filled with endotoxin free water was used as a reference.

4.5.3 Heparin Stability

Release of free heparin from the collagen gels was examined in order to quantify the total amount of heparin immobilized within the gels. Hydrated collagen gels were immersed in PBS buffer (pH 7.4) and shaken in a waterbath, at 37°C. The PBS was replaced hourly for the first 8 hrs, then twice daily for 2 days, then once on the fourth day and finally once on the seventh day. Samples were stored at 4°C prior to analysis for heparin concentration via the toluidine blue assay (Kang et al. 1996; MacIntosh. 1941; Smith, Mallia, and Hermanson. 1980).

4.5.4 Toluidine Blue Assay

In a 5 mL test tube, typically 1.5 mL of sample or standard heparin solutions (0-2 μg/mL heparin in 0.2% NaCl) and 0.15 mL of toluidine blue solution (0.005% toluidine blue dye with 0.2% NaCl and 0.01 N HCl) were combined. The test tubes were vortexed

for 30 seconds and 1 mL n-hexane added, then shaken by hand for an additional 30 seconds. The solution was allowed to phase separate, after which the aqueous layer removed and analyzed at 630 nm. Sample absorbance values were standardized against known standard heparin solutions and heparinized gels were standardized against their non-heparin gel counterparts.

4.5.5 Dendrimer Stability

Dendrimer reactivity with and release from the collagen matrix was assessed by analyzing release samples with a Coomasie blue assay (Bradford. 1976; Sapan, Lundblad, and Price. 1999). Although this assay is primarily used for protein quantification, through protein-dye interactions, its use was extended here, as a crude means of detecting changes in pH. Alkaline substances have been shown to interfere with the dye absorbance, through a conversion from the cationic to the neutral to the anionic form of the dye, causing an absorbance shift at 595 nm (Stoscheck. 1990). Heparin was not shown to interfere with the dye, consistent with Bradford's observations of non-interfering carbohydrates (Bradford. 1976).

A release study was performed similar to the heparin stability release, whereby hydrated collagen surfaces were immersed in PBS (pH 7.4), shaken at 37°C and sampled periodically. 200 µL of the release sample, in triplicate, was pipetted into a 96-well plate and mixed with 100 µL of Coomassie blue solution (50.1 mg Coomassie blue dye powder dissolved in 25 mL 95% ethanol and 50 mL 85% O-phosphoric acid and 500 mL Milli-Q water). After 5 minutes, the samples were read at 595 nm and subsequent absorbencies were compared against standard curves of known dendrimer concentrations in PBS.

4.6 FGF-2 RADIOLABELING, IMMOBILIZATION AND RELEASE

Both PDMS and collagen based surfaces, with and without heparin, were exposed to radiolabeled FGF-2 to assess growth factor immobilization and assess growth factor release.

4.6.1 ^{125}I-FGF-2 Radiolabeling Procedure

FGF-2, reconstituted in tris (hydroxymethyl) methylamine (TRIS) buffer (pH 7.4), was radiolabeled via the IODOGEN method (Klenkler et al. 2005). The growth factor solution (100 μL) was combined with 5 μL of ^{125}I (500 μCu / 5 μL) in an Iodogen coated vial and stirred for 15 minutes at room temperature. Free iodine was removed via dialysis (MWCO 3500) with TRIS buffer over a 24 hour period. The final concentration of the solution was determined spectrophotometrically from known unlabeled FGF-2 concentrations.

Free unbound isotope was measured by trichloroacetic acid (TCA) precipitation. In duplicate, 1 mL of 0.1% bovine serum albumin (BSA), 2 μL ^{125}I-FGF-2 and 0.5 mL TCA (1.4%) were combined in an eppendorf tube, allowed to react for 10 minutes and centrifuged for 2 minutes. The eppendorf supernatant, 0.5 mL, was combined with 0.5 mL TRIS buffer and its activity read in a gamma counter (Wallac Wizard 3" 1480 Automatic Gamma Counter, Perkin Elmer Life Sciences). Two background vials and 2 control solutions, containing 1 mL TRIS buffer and 2 μL ^{125}I-FGF-2, were also counted. The amount of free isotope was calculated via:

$$\%FreeIodogen = 3*100*\frac{SampleAverage}{ControlAverage} \qquad \text{(Eq.4)}$$

4.6.2 ^{125}I-FGF-2 Immobilization

PDMS modified surfaces were exposed to 750 μL of ^{125}I-FGF-2 solution for 24 hrs and 3 days. Collagen surfaces were soaked in Milli-Q water for 24 hours, freeze dried and exposed to 750 μL of ^{125}I-FGF-2 solution for 4 days. After exposure times, the soak solution and surfaces were dabbed dry and read for activity. To determine FGF-2 adsorption, surfaces were rinsed three times in TRIS (pH 7.4) for 1 min, dabbed dry and read for activity. Rinsing solutions were also read.

4.6.3 ^{125}I-FGF-2 Release Studies

Radioactive surfaces were placed into 1 mL of fresh TRIS buffer, in a 37°C shaking waterbath. Initially, release samples were taken every 30 minutes for the first 2 hrs, then periodically to obtain a gradual FGF-2 release profile. Surface and sample radioactivity were measured for activity and translated to known concentrations of ^{125}I labeled FGF-2.

5. PAPER 1

Heparin Modified Poly (Dimethyl Siloxane) Surfaces for the Delivery of Basic Fibroblast Growth Factor

The following paper describes the research performed on PDMS modified surfaces, intended for keratoprosthetic applications, for soluble FGF-2 delivery through heparin immobilization. This research expands on previous work by Chen *et al.* whereby PDMS elastomer surfaces were directly modified with N-succinimidyl carbonate (NSC) terminated poly (ethylene oxide) (PEO) spacers for heparin immobilization (Chen et al. 2005a; Chen et al. 2005b). The paper also examines a new more robust method of generating the reactive surfaces and compares heparinization and release results obtained using these two methods. In the previous work, the intended application was as a blood contacting material. The current application is related to 1) growth factor delivery and 2) biomaterials for keratoprosthetic applications.

Although the exploitation of heparin immobilization for blood contacting materials has been widely examined (Brash. 2000; Jee et al. 2004; Klement et al. 2002); the use of heparinized materials as growth factor delivery vehicles for angiogenic and wound healing applications has also been studied (DeLong, Moon, and West. 2005; Ishihara et al. 2003; Liu et al. 2002). Many of those delivery systems are hydrogel based and lack long-term stability. The significance of heparinization stems from the inability to delivery growth factors, without carrier molecules, for extended time periods, as they

are rapidly degraded upon parental administration (Lee. 2000; Tabata. 2003) and are highly unstable in solution (Edelman et al. 1991). However, the development of heparinized surfaces for delivery and ultimately for inducing subsequent cellular events associated with the materials have not been studied. This work provides evidence that heparinized PDMS materials are capable of delivering growth factor over sustained time periods.

Heparin Modified Poly (Dimethyl Siloxane) Surfaces for the Delivery of Basic Fibroblast Growth Factor

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Abstract

Tissue integration between an artificial corneal implant and the host eye, necessary for device anchoring is of critical importance in ensuring the long-term success of the implant. Initiation of wound healing responses, leading to stromal integration, has been widely shown to facilitate device anchoring.

FGF-2 was immobilized to a heparin modified poly (dimethyl siloxane) (PDMS) surface through a poly (ethylene oxide) (PEO) spacer, as a means of delivering the growth factor in a soluble form to initiate a wound healing response. Two similar chemical methods were examined for tethering the reactive PEO spacer to the PDMS. In the first method, an α-allyl-ω-N-succinimidyl carbonate-poly(ethylene oxide) (allyl-PEO-NSC) spacer was attached which could be directly functionalized with heparin. The second method involved tethering an α-allyl-hydroxyl-poly(ethylene oxide) (allyl-PEO-OH) which was subsequently functionalized with the reactive N-succinimidyl carbonate (NSC) group for heparin attachment.

Both PEO modification methods were successful, although PEO surface coverage was not quantifiable. Results suggested that direct immobilization gave inconsistent heparin surface coverage while the indirect method generally resulted in successful and consistent heparinization. Both modification methods resulted in surfaces that were capable of binding on average 100 ng/cm^2 of FGF-2. Delivery of FGF-2 from the directly and indirectly modified surfaces revealed a first order release profile, with an initial burst of FGF-2 followed by gradual release. Over 2 weeks, approximately 6.5% of

the total bound growth factor was released from surfaces exposed to FGF-2 for one day, while 3 day exposure resulted in a release of 50%.

Keywords: FGF-2; keratoprosthesis; corneal stroma; PEO; PDMS; surface modification

5.1 INTRODUCTION

Second only to cataracts in numbers of patients affected, diseases of the cornea are a major cause of blindness. The most popular and successful treatment by corneal transplantation is limited in some instances by graft rejection and failure, donor tissue shortages and the risk of disease transmission (Chirila et al. 1998; Whitcher, Srinivasan, and Upadhyay. 2001). In certain cases, the only alternative is a keratoprosthesis.

The success of this type of implant has been shown to depend on compatibility with host corneal tissues (Duan, Klenkler, and Sheardown. 2006), specifically the maintenance of a corneal epithelial cell layer on the surface, for promoting tear interactions and preventing bacterial infection, and the ingrowth of corneal stromal cells for device anchorage (Griffith et al. 2003). Epithelial coverage *in vitro* has been manipulated by the creation of hydrophilic surfaces (Legeais and Renard. 1998) and the tethering of adhesion peptides (Aucoin et al. 2002) and growth factors (Klenkler et al. 2005; Merrett et al. 2003). Stromal integration has been improved with the use of porous skirts allowing anchorage through fibroblast migration and extracellular matrix production (Hicks et al. 2000; Kim et al. 2002; Legeais et al. 1994); however tissue necrosis and device extrusion remain prevalent over long-term implantations.

As a means of enticing stromal integration, extracellular matrix components are being researched *in vitro* for stromal integration; they include hydoxyapatite (Mehta et al. 2005), fibronectin (Langefeld et al. 1999), and various growth factors (Andresen et al. 2000; Andresen, Leclet, and Ehlers. 1997). Growth factors are the primary mediators of wound healing in the eye, thus it is reasonable to hypothesize that the delivery of

appropriate growth factors may stimulate the cellular healing responses capable of initiating cellular integration into the keratoprosthesis (Imanishi et al. 2000). Basic fibroblast growth factor (FGF-2), an 18kDa polypeptide, found in the cornea basement membranes (Folkman et al. 1988) and epithelium (Wilson, Lloyd, and He. 1992; van Setten, Fagerholm, and Cuevas-Sanchez. 1995), has been shown to stimulate all corneal cell types, including fibroblast proliferation and migration (Shipley et al. 1989).

Although, direct topical application of FGF-2 to wounded corneas has been shown to increase healing rates (Rieck et al. 1994), therapies are hindered by the short half-life of FGF-2 (Whalen, Shing, and Folkman. 1989). Thus a soluble delivery method is desirable for sustained long-term release. *In vivo*, extracellular matrix glycosaminoglycans, heparan sulphate proteoglycans (HSPs), are upregulated in stromal wound healing (Hsia, Richardson, and Nugent. 2003), and bind to FGF-2 released from damaged epithelial cells, protecting it from proteolytic degradation (Saksela et al. 1988), as well as serving as a delivery reservoir for FGF-2. Therefore it is reasonable to hypothesize that heparinization of surfaces may be useful for controlling the release of FGF-2.

In this work, poly (dimethyl siloxane) (PDMS) was modified with a bifunctional poly (ethylene oxide) (PEO) spacer that was subsequently heparinized for immobilization and subsequent release of FGF-2. Release of the growth factor as a function of heparin density and other factors were examined.

5.2 MATERIALS AND METHODS

5.2.1 Materials

PDMS components and polymethylhydrogen siloxane fluid (DC 1107) [$(MeHSiO)_n$, 30 centistokes] were purchased from Dow Corning (Mississauga, ON). α-allyl-ω-N-succinimidyl carbonate-poly (ethylene oxide) (allyl-PEO-NSC, MW 500) was purchased from JuTian Chemical Co. (Nanjing, China), while the monoallyl-hydroxyl-terminated poly (ethylene oxide) (allyl-PEO-OH, MW 550) was purchased from Clariant (Canada) Inc., (Markham, ON). Triflic acid, Karstedt's platinum catalyst, unfractionated heparin and N-N'disuccinimidyl carbonate (diNHS) were purchased from Sigma-Aldrich (Oakville, ON), while low-molecule weight heparin (LMWH) was obtained from Calbiochem (San Diego, USA). FGF-2 was purchased from R & D Systems (Minneapolis, MN).

5.2.2 PDMS Surface Preparation

PDMS surfaces were prepared according to manufacturers instructions. Briefly, the elastomer base and curing agent were combined in a 10:1 (w/w) ratio, placed under vacuum for approximately 1 hour, and then allowed to cure at room temperature for at least 2 days. The film, with a thickness of approximately 0.5 mm, was punched into 0.625 cm (¼") discs and washed with anhydrous methanol prior to use. Surfaces were then modified to incorporate a reactive Si-H group based on the methods described by Chen *et al.* (Chen et al. 2005a; Chen et al. 2006). Briefly thirty PDMS discs were placed in a 10 dram glass vial, immersed in a mixture of 5 mL anhydrous methanol, 3 mL DC 1107 ($(MeHSiO)_n$), and 50 μL triflic acid ($CFSO_3H$), and shaken vigorously for 30

minutes at room temperature. Discs were then washed with anhydrous methanol and dried n-hexane, subsequently dried with N_2 and placed under high vacuum for 8 hours to remove residual solvents.

Two PEO spacers were immobilized to PDMS surfaces, allyl-PEO-NSC (MW 500) (direct modification), and allyl-PEO-OH (indirect modification). The PDMS-PEO-NSC surfaces were ready for subsequent heparinization while the PDMS-PEO-OH surfaces were subsequently modified with diNHS to incorporate the functional group for biological functionalization.

Direct grafting was achieved by placing 20 PDMS-SiH functionalized surfaces with 3 mL dried 2-methoxyethyl ether, 0.6 g allyl-PEO-NSC and 20 μL of Karstedt's platinum catalyst $[(Pt)_2(H_2C{=}CH\text{-}SiMe_2OSiMe_2CH{=}CH_2)_3]$. The mixture was purged with N_2, tightly sealed, placed in a 50°C oil bath and stirred for 15-20 hours. The resulting surfaces, PDMS-PEO-NSC, were washed with dried acetone, dried with N_2 and placed in a desiccator for at least 12 hours. Control hydroxyl-terminated surfaces (PDMS-PEO-OH, direct (D)) were prepared by submersing the PDMS-PEO-NSC surfaces in potassium phosphate buffer (pH 8.0) for 3 days, washed with Milli-Q water and wicked dry.

Indirect grafting was achieved by immersing 10 PDMS-SiH surfaces in 1 mL 2-methoxyethyl ether, 1 mL allyl-PEO-OH and 15 μL Karstedt's platinum catalyst. The vial was gently shaken on an orbital shaker for 2 hrs, after which surfaces (PDMS-PEO-OH, indirect (InD)) were rinsed with acetone, dried with N_2 and desiccated for 10 hrs. To these surfaces was added a pre-dissolved mixture of 0.6 g diNHS in 1 mL anhydrous

acetonitrile and 100 µL triethylamine (TEA). The mixture was purged with N_2 and gently shaken on an orbital shaker for 6 hrs. Surfaces were then rinsed with anhydrous acetonitrile and 2-methoxyethyl ether, dried with N_2 and desiccated for at least 12 hrs

The mechanisms are illustrated in Figure 5-1.

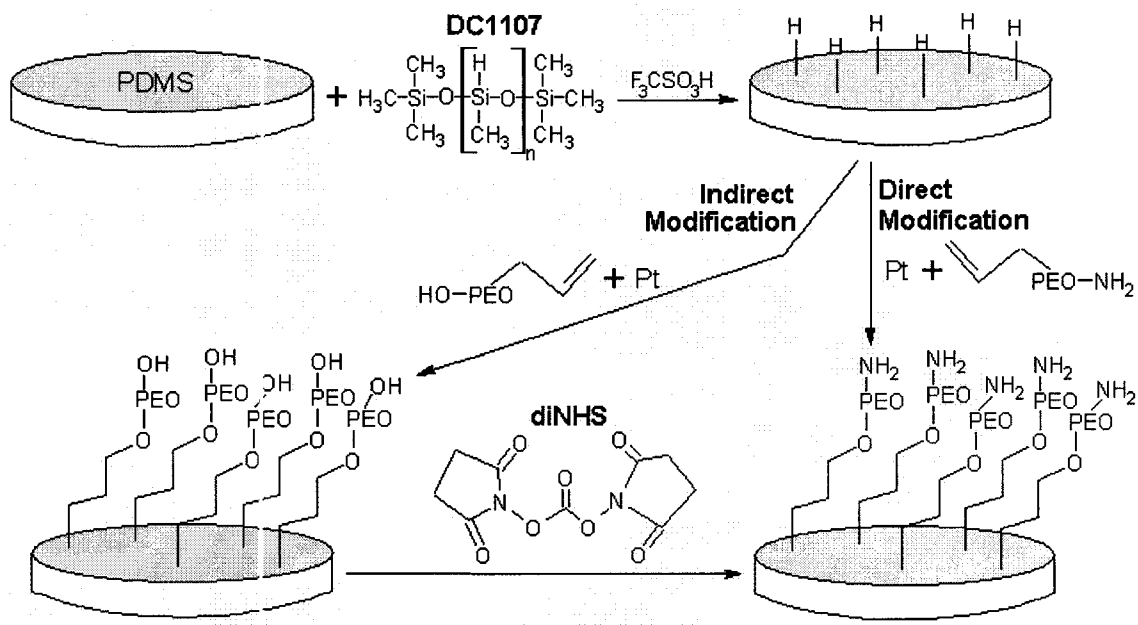

Figure 5-1. Direct and indirect PDMS modification. PDMS Si-H functionalization initiates with DC1107 and triflic acid (F_3CSO_3H). Subsequent PEO reactions were direct with the hydrosylation of allyl-PEO-NSC, in the presence of platinum (Pt), or indirect with an allyl-PEO-OH hydrosylation followed by NSC functionalization with diNHS.

Heparinization of directly and indirectly modified PDMS surfaces was achieved through exposure of 30 surfaces to 5 mL of a 10 mg/mL heparin solution in phosphate buffered saline (PBS, pH 8.0) for at least 6 hrs. Surfaces were then removed, rinsed with PBS, and wicked dry. Indirectly modified surfaces were also exposed to LMWH in a similar manner.

5.2.3 Attenuated Total Reflection Fourier Transform Infrared Spectrophotometer

A Bio Rad FTS-40 Fourier transform infrared spectrophotometer (FTIR) equipped with a DTGS detector and a vertical attenuated total reflection (ATR) module, with a zinc selenide crystal, was used to scan PDMS, PDMS reaction intermediates and the final PDMS-PEO-Heparin surfaces to verify surface reactions.

5.2.4 X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) was performed at Surface Interface Ontario, University of Toronto, using a Leybold (Specs) MAX 200. Peaks were analyzed with the corresponding software. Low resolution and high resolution spectra were collected at take off angles of 20^{o} and 90^{o}.

5.2.5 Water Contact Angles

Advancing and receding sessile drop contact angles were measured on a bench top goniometer (NRL C.A. Goniometer, Ramé-hart, USA). Milli-Q water was used with a drop volume of 20 μL. Contact angles were performed in triplicate on each side of the surface.

5.2.6 Heparin Density

Heparin density was quantified via a slightly adapted version of the toluidine blue assay (Hinrichs et al. 1997; MacIntosh. 1941). Briefly, surfaces were exposed to a 0.5% toluidine blue solution (with 0.2% sodium chloride (NaCl) and 0.01 N hydrochloric acid (HCl)) overnight. Subsequently, surfaces were rinsed 3 times in Milli-Q water, dabbed dry and placed in 700 μL of 4:1 ethanol: 0.1 N NaOH solution overnight. The surfaces

were then removed from the solution and the absorbance of the ethanol solution read at 540 nm. Absorbance values were standardized against standard heparin solutions, created by exposing known concentrations of dissolved heparin in PBS (pH 7.4) to 0.5% toluidine blue solution (0.2% NaCl and 0.01 N HCl) overnight. Standards were then centrifuged at 1000g for 10 min and the supernatant carefully removed. The pellet was resuspended in a 4:1 (v/v) ethanol: 0.1 N sodium hydroxide (NaOH) solution and read at 540 nm.

5.2.7 Heparin Activity

As an alternative means to quantify heparin immobilization, its activity was assessed by exploiting its involvement with antithrombin III and factor Xa (Ingber. 1991; Teien and Lie. 1977) and compared to the activity of known heparin concentrations. Heparin activity on heparinized PDMS surfaces was quantified using an anti-Factor Xa assay kit (Stachrom® Heparin, Diagnostica Stago, France) according to the manufacturer's instructions. Briefly, surfaces were exposed to 100 µL purified bovine antithrombin III (AT-III) and either 800 µL for unfractionated heparin, or 1 mL for LMWH, in 0.1 N tris (hydroxymethyl) methylamine-ethylenediamine tetraacetic acid (TRIS EDTA) buffer (pH 8.4) for 2 min at 37°C. Then, the surfaces and 200 µL of the AT-III solution were transferred to a new test tube, and 200 µL purified bovine factor Xa was added and the samples were incubated for 30 sec at 37°C. Next, 200 µL of the chromogenic substrate (CBS 31.39) was added, the samples incubated for an additional 30 sec at 37°C and the reaction terminated with 200 µL glacial acetic acid. Surfaces were removed and the solutions read in a UV spectrophotometer at 540 nm. Calibration curves

were created with known concentrations of aqueous unfractionated and low molecular weight heparin.

5.2.8 ^{125}I Labelled FGF-2 Immobilization

FGF-2, reconstituted in tris (hydroxymethyl) methylamine (TRIS) buffer (pH 7.4), was radiolabelled via the IODO-GEN method (Pierce, USA). Briefly, 100 μL FGF-2 was exposed to 5 μL of ^{125}I (500 μCu / 5 μL) in an Iodogen coated vial and stirred for 15 minutes at room temperature. Free iodide was removed by overnight dialysis against TRIS buffer. Free unbound isotope was measured by trichloroacetic acid (TCA) precipitation with bovine serum albumin (BSA).

The ^{125}I labelled FGF-2 was then reacted with the various heparinized surfaces described above, for 24 hrs or 3 days. To assess FGF-2 adsorption, surfaces were rinsed in fresh 1mL TRIS for 1 min, three times.

5.2.9 ^{125}I Labelled FGF-2 Release Studies

Radioactive FGF-2 containing surfaces were placed into fresh TRIS buffer, in a 37°C shaking waterbath. Release samples were taken at regular intervals in order to capture the FGF-2 release profile. Initial surface radioactivity and the radioactivity of the samples were measured with a gamma counter (Wallac Wizard 3" 1480 Automatic Gamma Counter, Perkin Elmer Life Sciences) and translated to FGF-2 release profiles.

5.2.10 Statistical Analysis

Data, in triplicate, was averaged and variability assessed with standard deviations, unless otherwise stated. Statistical significance was determined with a single factor Anova test, with $p < 0.05$.

5.3 RESULTS AND DISCUSSION

5.3.1 PDMS Surface Preparation

PDMS surfaces were prepared by siloxane vulcanization, whereby dimethylsilicone elastomer is crosslinked with hydromethylsilicone in the presence of platinum. Further modification, under acidic conditions with triflic acid and DC 1107, resulted in functionalization of the PDMS surface with reactive Si-H groups.

Next, PEO, either premodified with NSC groups or –OH termination for subsequent NSC functionalization, was grafted. The former method, termed direct grafting, yielded PEO modified surfaces with reactive functional groups for subsequent modification (PDMS-PEO-NSC). Surfaces that stuck together or to the surface of the vial were discarded to ensure that only high quality surfaces were subsequently functionalized and tested. Surfaces generally remained clear throughout the reaction process, although surfaces reacting for more than 24 hrs were stained black, presumably due to platinum poisoning.

Indirect grafting first involved the tethering of allyl-PEO-OH, in the presence of platinum, to create hydroxyl-terminated PDMS surfaces, PDMS-PEO-OH. Despite the short reaction time for PDMS-PEO-OH fabrication, many surfaces were damaged and discoloured due to platinum poisoning; decreasing the amount of platinum added did not

seem to alter this result. Doubling the solvent and PEO amounts did improve the yield of clear surfaces. As with the previous reaction, damaged or translucent surfaces were discarded and not used for subsequent reactions. In the second stage of preparation of the indirect grafting surfaces, the PDMS-PEO-OH was reacted with diNHS, through nucleophilic substitution, to yield the reactive NSC surface. To facilitate the dissolving of diNHS, triethylamine (TEA) was added to acetonitrile. As TEA is a nucleophile, it has the potential to compete for PDMS-PEO-OH, but given the small amount added, it is assumed that its interference would be minimal.

The resulting PDMS-PEO-NSC surfaces had a slight gold tint presumably due to the presence of an impurity in the diNHS, as reaction with a higher purity was found to decrease the tinting. In the future it would be desirable to react with even higher purity diNHS to eliminate tinting altogether, particularly for use in cornea applications.

Heparinization with unfractionated heparin (HEP) was achieved by exposing the directly and indirectly modified PDMS-PEO-NSC surfaces to heparin solutions. To assess steric hindrance of the heparin binding, low molecular weight heparin (LMWH) was also exposed to surfaces fabricated indirectly. These surfaces were then exposed to concentrations of FGF-2 to generate PDMS-PEO-HEP-FGF-2 (direct and indirect) and PDMS-PEO-LMWH-FGF-2 (indirect) surfaces. No visible surface abnormalities were observed in either of those processes.

5.3.2 ATR FTIR

ATR FTIR spectra for the various PDMS surfaces modified by the direct grafting method are shown in Figure 5-2; spectra for the indirect grafted surfaces are shown in Figure 5-3.

Unmodified PDMS has a characteristic methyl peak at ~3000 cm^{-1}, while Si-H functionalization results in the appearance of a peak at ~2166 cm^{-1} due to the Si-H stretch, shown in both Figures. Subsequent reactions of the PDMS-Si-H surface, whether reacted by the indirect or direct method, results in the disappearance of this Si-H stretch. With the addition of PEO to the surface, a peak appears at 2870 cm^{-1} characteristic of the CH_2 stretch of PEO, while peaks 1455 cm^{-1}, 1349 cm^{-1}, 1288 cm^{-1} represent the CH_2 scissoring of PEO, the antisymmetric OCH_2-CH_2 stretch and symmetric OCH_2-CH_2, respectively. The addition of reactive NSC groups to the surface is evident by fingerprint peaks at 1600cm^{-1} and 1200cm^{-1}. Direct grafting of allyl-PEO-NSC to the surface gave peaks corresponding to PEO tethering, along with the reactive NSC group, while the indirect grafting of allyl-PEO-OH followed by the diNHS reaction gave a similar result. There were no differences between direct and indirect methods overall, except that it was very difficult to create directly modified surfaces with predominant PEO and NSC peaks, presumably due to factors such as the sampling depth of the ATR-FTIR technique. It is possible that longer reaction times may have favoured increased generation of reactive groups but platinum poisoning of these surfaces had to be considered.

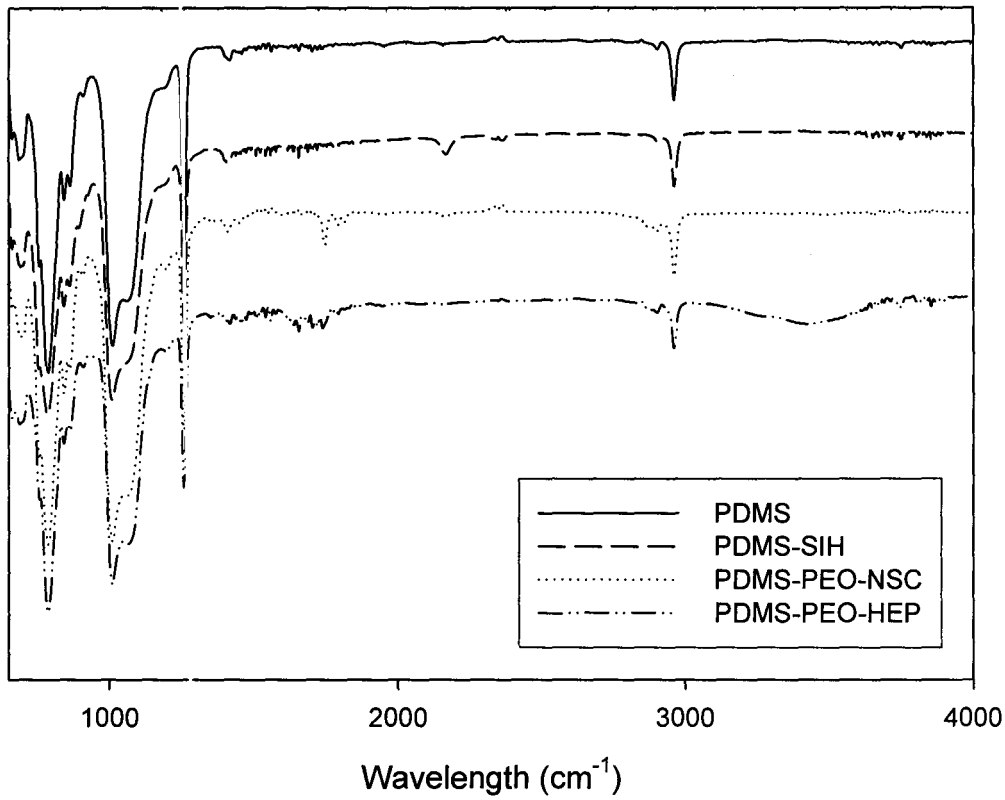

Figure 5-2. ATR-FTIR spectra for directly modified PDMS. PDMS surfaces show a peak at 3000 cm^{-1}, characteristic of its methyl groups. Si-H functionalization is evident by the appearance of a peak at 2166 cm^{-1}. NSC functionalization is characterized by a peak appearance at 1600 cm^{-1} and 1200 cm^{-1} and the disappearance of the Si-H peak. Heparinized surfaces have a broad peak at 3300 cm^{-1} and sharp peaks at 1250 cm^{-1} and 1620 cm^{-1}.

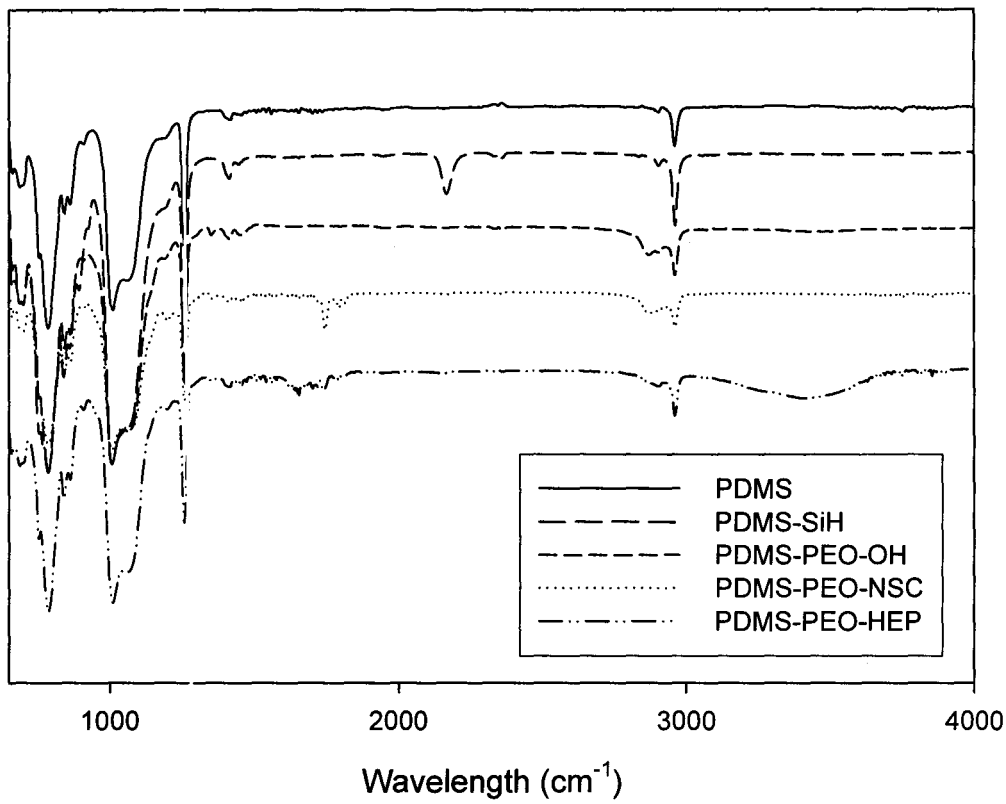

Figure 5-3. ATR-FTIR spectra for indirectly modified PDMS. PDMS surfaces have a peak at 3000 cm^{-1}, representative of its methyl groups. The addition of Si-H functionalization is evident by the appearance of a peak at 2166 cm^{-1}. PEO grafting to the surface is noted by the disappearance of the Si-H peak and mainly by the appearance of a peak at 2870 cm^{-1}. NSC functionalization is characterized by a peak appearance at 1600 cm^{-1} and 1200 cm^{-1}. A broad peak at 3300 cm^{-1} and sharp peaks at 1250 cm^{-1} and 1620 cm^{-1} are representative of heparinized PDMS-PEO surfaces.

FTIR, shown in Figure 5-4, was performed to verify modification with unfractionated heparin and LMWH. Both had sharp peaks at 1250cm^{-1} and 1620cm^{-1}, characteristic of sulphonated and amino side groups, and a broad peak between 3300cm^{-1} and 3500cm^{-1}, representative of the hydroxyl groups. Similar FTIR spectra have been presented elsewhere for heparin modified surfaces (Kreitz, Domm, and Mathiowitz. 1997).

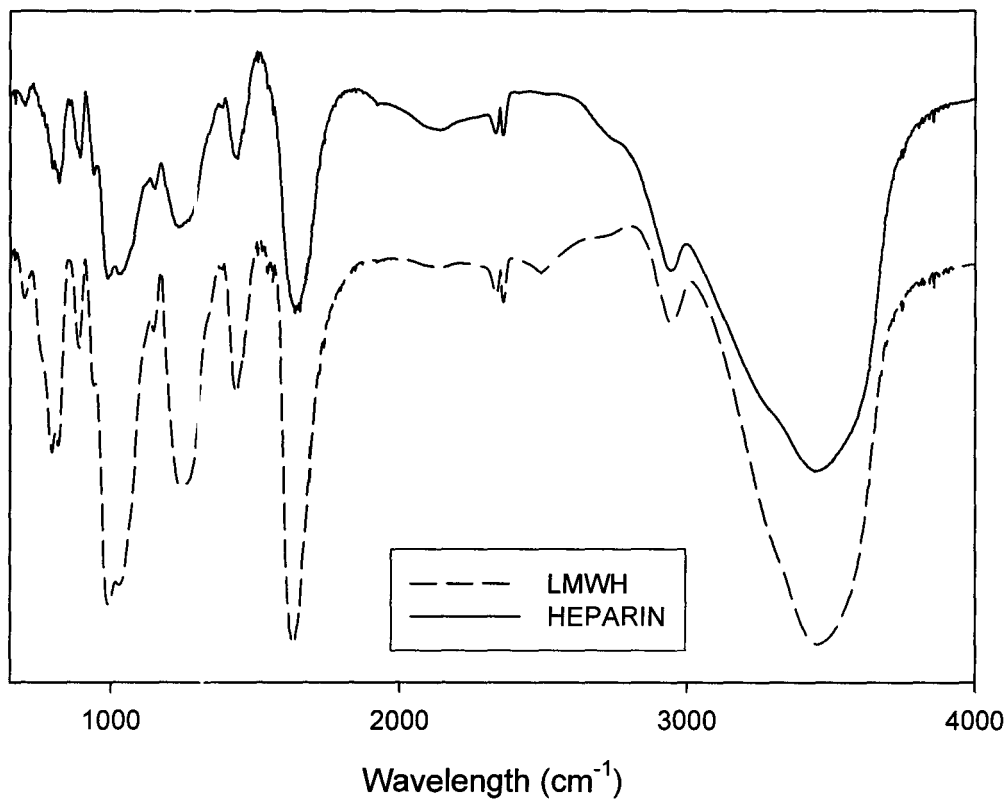

Figure 5-4. FTIR spectra of unfractionated and LMWH heparin, demonstrating the broad for hydroxyl groups peak at 3300 cm^{-1} and sharper peaks at 1250 cm^{-1} and 1620 cm^{-1}, representing its sulphonated and amino side chains.

5.3.3 X-ray photoelectron spectroscopy

XPS analysis of directly and indirectly modified PDMS surfaces, at take off angles of 20° and 90°, are summarized in Table 5-1. High resolution C1s spectra of PDMS and its modified counterparts are shown in Figure 5-5.

Unmodified PDMS, showed expected amounts of carbon, oxygen and silicone (Merrett et al. 2003) and further showed a high resolution carbon peak at 284.4 eV, indicative of C-H bonds of the PDMS methyl carbon groups.

In both grafting methods, PEO grafting correlated to an increase in C1s and O1s, and a decrease in Si2p, as expected. PEO high resolution spectra showed two peaks, the first indicative of the unmodified PDMS carbon molecules, while the peak at 286.4 eV represents the C-O bonds of PEO, providing evidence of PEO grafting. This peak is also representative of the percent of PEO on the surface (Merrett et al. 2002), which supports higher PEO coverage via indirect grafting compared to direct grafting methods. Also of interest are the inconsistencies seen with PEO grafting, when comparing two heparinized surfaces fabricated via indirect grafting.

The elemental heparin ratio is estimated to be C:O:N:S = 12:14:2:1 (Chen et al. 2005a). Therefore an increase in C1s, O1s and C:O ratio, as well as the appearance of S2p would be expected following heparinization of the surface. Although there was no great increase in C1s, heparinized surfaces did show an increase in O1s, indicative of the hydroxyl functional groups of heparin. Sulphur, S2p, was also evident, but in much smaller amounts than would be expected. Only 0.1 atom% was observed for direct and indirect heparinization with 0.2 atom% present for indirect modification with LMWH.

Previous studies of immobilized heparin on poly (urethane) based surfaces with carboxyl or amine groups resulted in sulphur percentages of 0.1 and 0.5, respectively (Kang et al. 1996), while Chen *et al.*'s heparinized PDMS-PEO surfaces, fabricated via direct grafting, showed 0.4% S2p (Chen et al. 2005a). The C:O ratio did increase with direct grafting methods, providing evidence of surface immobilized heparin on the surface; this was not as prevalent with indirect grafting, likely attributable to poor PEO grafting and subsequent heparinization. Not surprisingly, as with other studies, high resolution spectra failed to conclusively demonstrate heparin modification (Chen et al. 2005a; Sefton et al. 2001), presumably due to the presence of similar functional groups on the unmodified surfaces. Therefore, XPS analysis of the materials failed to conclusively demonstrated heparinization. However, the high vacuum environment and highly surface active nature of the PDMS substrate materials may have been a contributing factor.

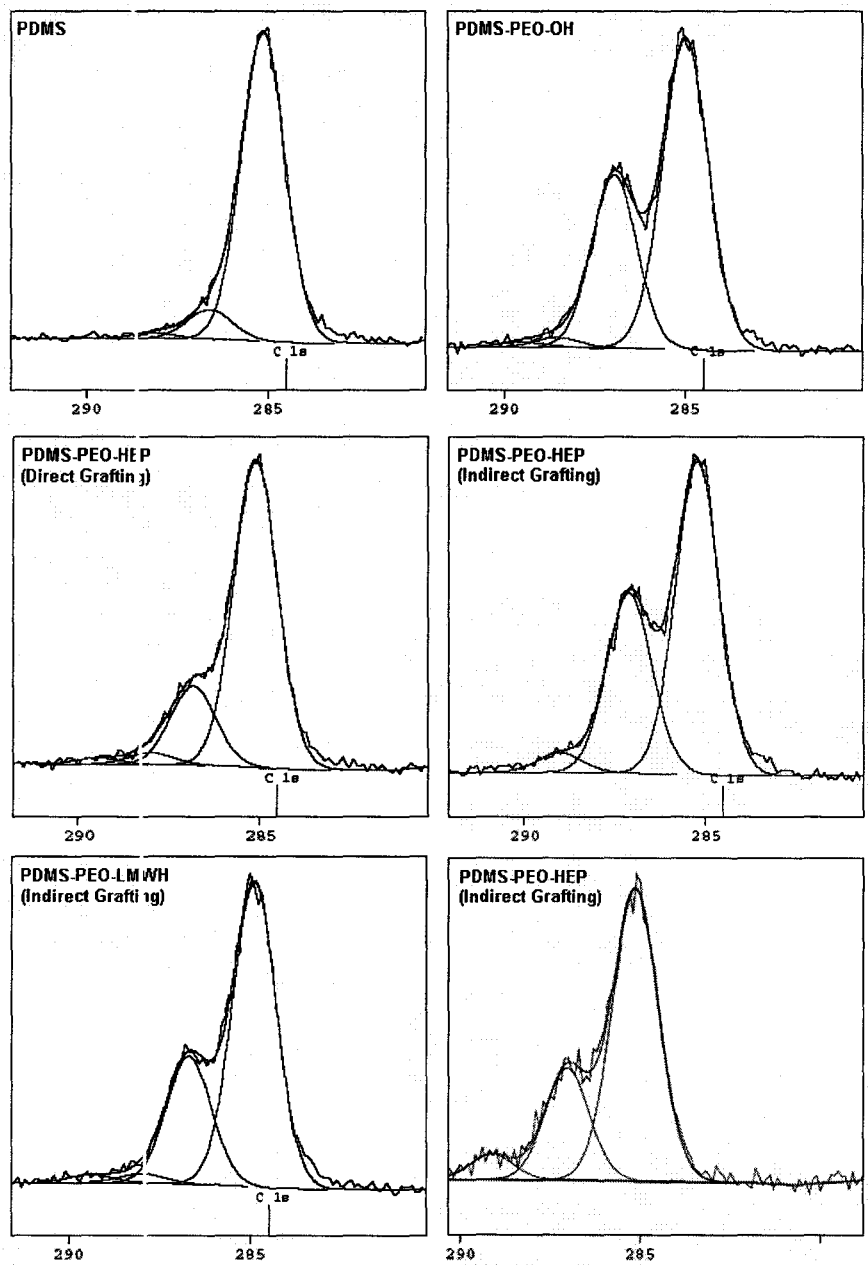

Figure 5-5. XPS high resolution C1s spectra, at a take off angle 90°, for PDMS and subsequent modified surfaces. Distinct peaks are visible representing PEO grafting, however, heparin binding was not as clearly indicated. A peak at 284.4 eV represent the C-H bonds of PDMS, while a peak 286.4 eV represents the C-O bonds of attached PEO.

Table 5-1. Summary of XPS results for unmodified PDMS and directly and indirectly modified PDMS surfaces.

Modification Method	Surface	Take off Angle (°)	Atom %								
			C 1s					O 1s	C:O ratio	Si 2p	S 2p
			Total	285	286	288	289				
Unmodified	PDMS	20	48.7	62.2	7.6	3.0	2.5	25.9	1.88 : 1	25.4	0.0
		90	46.9	65.4	13.0	9.2	0.0	26.6	1.79 : 1	26.5	0.0
Direct Grafting	PDMS-PEO-OH	20	51.2	39.5	9.0	2.7	0.0	25.4	2.01 : 1	23.4	0.0
		90	52.7	36.3	14.0	2.4	0.0	25.3	2.08 : 1	21.6	0.0
	PDMS-PEO-HEP	20	52.7	58.9	11.5	3.2	3.3	24.5	2.15 : 1	22.7	0.1
		90	52.8	56.6	16.8	2.7	1.2	26.5	1.99 : 1	20.6	0.1
Indirect Grafting	PDMS-PEO-OH	20	51.7	76.7	15.3	3.2	4.8	24.5	2.11 : 1	23.7	0.0
		90	50.7	74.6	22.2	1.7	1.6	26.6	1.91 : 1	22.7	0.0
	PDMS-PEO-HEP	20	48.9	77.5	15.0	3.8	3.8	26.1	1.87 : 1	25.1	0.0
		90	48.8	64.6	15.0	18.9	2.1	29.3	1.67 : 1	21.8	0.0
	PDMS-PEO-LMWH	20	51.0	79.2	12.7	4.0	4.2	24.3	2.10 : 1	24.6	0.1
		90	51.8	70.5	25.4	1.8	2.3	25.5	2.00 : 1	22.4	0.2

5.3.4 Water Contact Angles

Advancing and receding contact angles were measuring for unmodified and directly and indirectly modified PDMS surfaces and summarized in Figure 5-6.

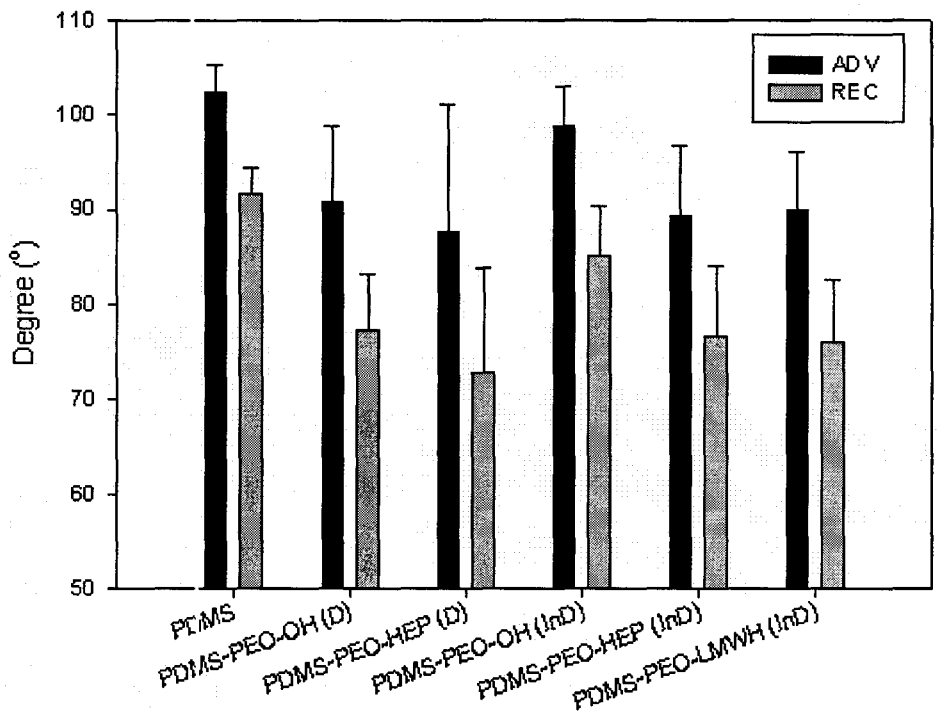

Figure 5-6. Advancing (adv) and receding (rec) contact angles for modified PDMS surfaces. Unmodified PDMS had a high contact angle, indicating surface hydrophobicity. PDMS surfaces modified with PEO had a lower contact angle, thus increased hydrophilicity, with heparinized surfaces demonstrating more hydrophilicity.

Unmodified PDMS surfaces had a high advancing contact angle of 102 ± 2.8° and a receding angle of contact angle of 91.5 ± 2.7°, characteristic of its hydrophobic and relatively uniform surface. Following direct and indirect modification to create PDMS-PEO-OH surfaces, both the advancing ($p = 0.002$ and 0.049, respectively) and receding angles ($p = 0.000055$ and 0.005, respectively) decreased significantly, indicative of a more hydrophilic surface, thus supporting PEO tethering. The contact angles were lower for indirectly

modified surfaces, compared to directly modified surfaces. Heparinized surfaces modified directly did not differ from their PEO controls, while indirectly heparinized surfaces did differ. There was no significant difference between LMWH and unfractionated heparin.

Compared with previous work (Chen et al. 2005a; Chen et al. 2005b), the contact angles observed in the current study were considerably higher, along with the variability. This could be due to a lower level of surface grafting. As well, upon exposure to air, the PDMS-PEO elastomer reorganizes, exposing the hydrophobic PDMS methyl groups to the surface, to decrease surface energy, which in turn could increase hydrophobicity (Chen et al. 2005a). However, in water, the elastomer can reorient to have the hydrophilic PEO to the surface. Therefore it would be prudent to perform these experiments with hydrated surfaces, use the captive bubble water technique or measure contact angles in a humid environment.

Finally, using a goniometer to determine contact angles depends greatly on individual technique; as such the variability seen here could also be due to novice experimenter skills.

5.3.5 Heparin Density

Heparinized surfaces were exposed to toluidine blue solution to determine and visualize the surface coverage of heparin. Results, shown in Figure 5-7 demonstrate that toluidine blue dye forms a complex with the heparin molecule, a reaction hypothesized to be through hydrophobic and ionic interactions (Moxon and Slifkin. 1973) and this reaction can also occur with immobilized heparin (Smith, Mallia, and Hermanson. 1980). As expected, unmodified PDMS and modified PDMS-PEO-OH surfaces did not react with the dye, nor was there evidence of dye adsorption. Heparinized PDMS surfaces exposed to toluidine

blue however became very dark blue, suggesting that a reasonable heparin layer had been formed despite the negative XPS results.

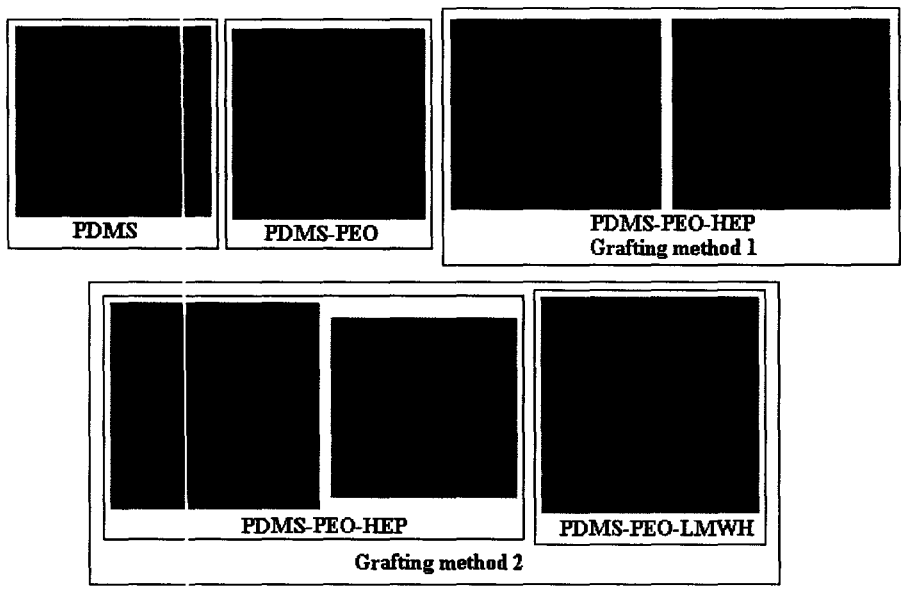

Figure 5-7. PDMS surfaces exposed to toluidine blue dye to illustrate surface coverage of heparin. Control surfaces, PDMS and PDMS-PEO-OH, did not bind toluidine blue, while heparinized surfaces turned blue. Direct grafting has inconsistent heparin coverage, while direct grafting shows more uniform binding.

Interestingly, directly modified surfaces showed incomplete dye binding, and thus uneven heparin coverage. Instances of incomplete coverage correlated well with ATR-FTIR results with weak NSC and PEO peaks. Uniform heparin coverage was achieved with indirectly modified surfaces; however there were batches that showed no staining. Again, these results were consistent with ATR-FTIR results.

Ethanol solutions severed the heparin-dye complex (Hinrichs et al. 1997), allowing toluidine blue to be retrieved from heparinized surfaces, quantified and related back to heparin concentration. Heparin densities determined using this method are summarized in Table 5-2.

As PDMS and PDMS-PEO-OH surfaces did not bind toluidine blue, their calculated heparin density was assumed to be background interference and the average was subtracted from the results for the heparinized surfaces. On average, directly grafted surfaces had a relatively low heparin density at 0.34 $\mu g/cm^2$, and these surfaces showed high variability. The highest densities obtained with the direct method, roughly 1.2 $\mu g/cm^2$, were similar to what was obtained with the indirect method. While there was some variability between indirectly modified batches, it was slightly lower than values corresponding to the directly modified surfaces. There was generally low variability within batches, regardless of modification method.

Table 5-2. Heparin Density (via extracted toluidine blue dye) of modified PDMS surfaces.

Modification Method	Surface	n	Heparin Density ($\mu g/cm^2$)		
			Average	Max.	Min.
Direct grafting	PDMS-PEO-HEP	2	0.339	1.254	0.000
Indirect grafting	PDMS-PEO-OH	5	0.012	0.092	0.000
	PDMS-PEO-HEP	7	0.919	2.171	0.224
	PDMS-PEO-LMWH	5	1.077	1.736	0.157

Immobilized heparin has been quantified elsewhere. Polysulphone surfaces had reported heparin densities of 1.38$\mu g/cm^2$ (Xu et al. 2001), while heparinized polyurethane surfaces had densities of 1.4 to 2$\mu g/cm^2$ (Kang et al. 1996). Earlier work of fabricating heparinized PDMS-PEO surfaces via direct PEO grafting resulted in surfaces with heparin densities of 0.68$\mu g/cm^2$ (Chen et al. 2005a). In the current work, similar heparin densities

were obtained. However further optimization of grafting methods is needed to decrease batch to batch variation.

5.3.6 Heparin Activity

Heparin activity was determined through an anti-Factor Xa assay kit, to ensure appropriate heparin configuration for further FGF-2 conjugation, and as an alternative means to quantify heparin density through antithrombogenic and concentration correlations. Heparin activity is routinely used to determine heparin concentration in plasma (Fitch et al. 1999), despite a disproportional relationship between surface heparin density and anticoagulant activity (Lindhout et al. 1995). Heparin activity is greatly influenced by heparin heterogeneity, specifically the occurrence of a high-affinity pentasaccharide sequence, and chain length (Lin, Sinha, and Betz. 2001), whereby smaller heparin molecules (>18 saccharides) are incapable of binding to antithrombin III and thrombin simultaneously (Hirsh and Raschke. 2004).

Only surfaces that demonstrated heparin immobilization with toluidine blue were assessed for heparin activity. The results are summarized in Table 5-3.

Table 5-3. Heparin density (via Xa activity) of modified PDMS surfaces.

Modification Method	Surface	n	Heparin density (μg/cm^2)		
			Average	Max	Min
Direct grafting	PDMS-PEO-Hep	4	1.104	1.633	0.432
Indirect grafting	PDMS-PEO-Hep	8	1.243	1.604	0.881
	PDMS-PEO-LMWH	4	0.566	0.879	0.205

Heparin densities, found via heparin activity assay, were similar between PDMS modification methods, at ~1.1μg/cm^2, although there exists batch to batch variation, similar to what was seen with toluidine blue quantification. The large variations within directly modified batches could be attributed to improper mixing and larger batch sizes. Insufficient mixing and diNHS chemical impurities could result in indirect grafting variation. As well, platinum poisoning could unknowingly damage the surface rendering it unsuitable for modification.

Not unexpectedly, heparin densities between the two types of heparin were not comparable in this analysis as they were when dyed with toluidine blue. With LMWH a heparin density of 0.566μg/cm^2, half the value of unfractionated heparinized surfaces, was observed. This is attributable to the presence or lack of presence of the high-affinity pentasaccharide sequence that interacts with antithrombin III. LMWH, a result of depolymerized heparin chains, will have a large percentage of chains that are too short for inhibition and will contain fewer pentasaccharide sequences, rendering it less effective for antithrombin III binding (Hirsh and Raschke. 2004). It is estimated that 25 to 50% of LMWH species are incapable of antithrombin binding. Thus it could be expected to see a similar loss in activity, as was evident here, through the difference in heparin densities between unfractionated and LMWH modified surfaces.

5.3.7 ^{125}I Labelled FGF-2 Immobilization

Heparinized surfaces were exposed to ^{125}I labelled FGF-2 to assess the amount of bound FGF-2 to the surface. Figure 5-8 illustrates FGF-2 binding over 24 hours and 3 days, and the amount of growth factor that remained after rinsing with TRIS buffer.

PDMS and, somewhat surprisingly, PEO surfaces seemed to adsorb FGF-2 to a high degree. While high levels of adsorption on PDMS surfaces are not unexpected due to their hydrophobic nature, the tethering of PEO using this method has been previously demonstrated to significantly decrease levels of adsorbed protein. It is believed that two factors are likely contributing to this effect. XPS and other results seem to demonstrate that, although not directly quantifiable, the PEO density of these surfaces is potentially lower on these materials than on materials previously synthesized. As well, it is possible that FGF-2, a smaller protein than was used in previous studies may be able to interact with and adsorb to the surfaces.

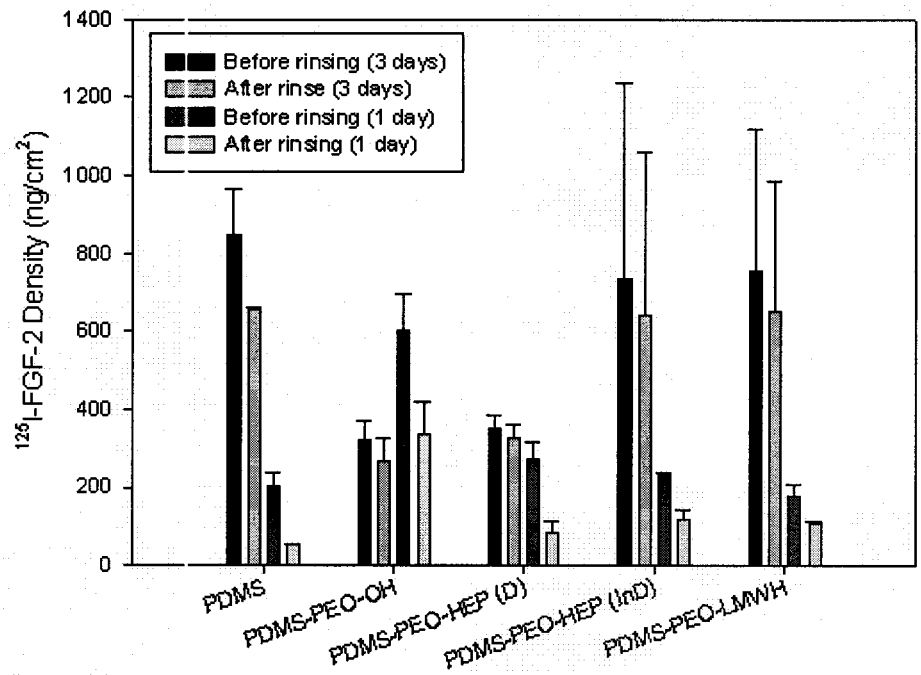

Figure 5-8. FGF-2 immobilization on modified PDMS surfaces after a soaking in ^{125}I-FGF-2 for 1 and 3 days, and after 3 consecutive one minute rinses in TRIS buffer. Values are averages of 2 surfaces, with standard error bars.

There exists a large difference in the amount of FGF-2 taken up by the directly modified surfaces compared with the indirectly modified surfaces, although this may have

resulted from batch to batch variation. However, the amount of FGF-2 taken up by the indirectly grafted surfaces was relatively unaffected by the use of broad spectrum heparin compared with LMWH, which is unexpected, as the binding affinity of FGF-2 to heparin increases with increasing molecular chain length (Faham, Linhardt, and Rees. 1998). The variability seen on the PDMS-PEO-HEP and PDMS-PEO-LMWH surfaces is attributed to the high variability of PEO bound to the surface and its subsequent reaction to heparin, evident by the variable heparin densities discussed earlier. There were significant ($p = 0.005$) increases in the amount of growth factor associated with the surfaces after 3 days of exposure compared with one day of exposure suggesting that longer time periods were necessary to obtain the highest potential for FGF-2 release.

5.3.8 ^{125}I Labelled FGF-2 Release Studies

Release of the radiolabeled FGF-2 into TRIS buffer (pH 7.4) at 37°C are shown in Figure 5-9, for the first 38 days of release from the surfaces soaked for 24 hours, and in Figure 5-10, for surfaces soaked for 3 days over the first 29 days of release. As expected, release of FGF-2 for all PDMS surfaces began with an initial burst of FGF-2 followed by first order gradual release. Large error and improper percentage release values can be attributed to batch variation and their averaging.

Heparinized surfaces exposed to ^{125}I-FGF-2 for 1 day released, over 2 weeks, approximately 6.5% of originally immobilized FGF-2, while 3 day exposure studies lead to a release rate of 50%. It is unexpected that the release rates differ so much, perhaps related to surface exposure times and FGF-2 adsorption. There was also evidence of high free iodine for surfaces soaked for 24 hrs, thus those results may not truely represent FGF-2 release.

Indirectly modified PDMS-PEO-HEP had a slower release rate indicative of surface heparinization and subsequent FGF-2 immobilization. Surprising is the high release rate of PDMS-PEO-OH surfaces, however this could be explained by incomplete surface coverage and FGF-2 adsorption eluted to earlier with the FGF-2 immobilization studies. Differences between unfractionated heparin and its low molecular weight counterpart could be attributable to chain length, which may influence storage capabilities of FGF-2. The late burst of PDMS-PEO-LMWH in Figure 10 is unexpected and likely due to experimental error.

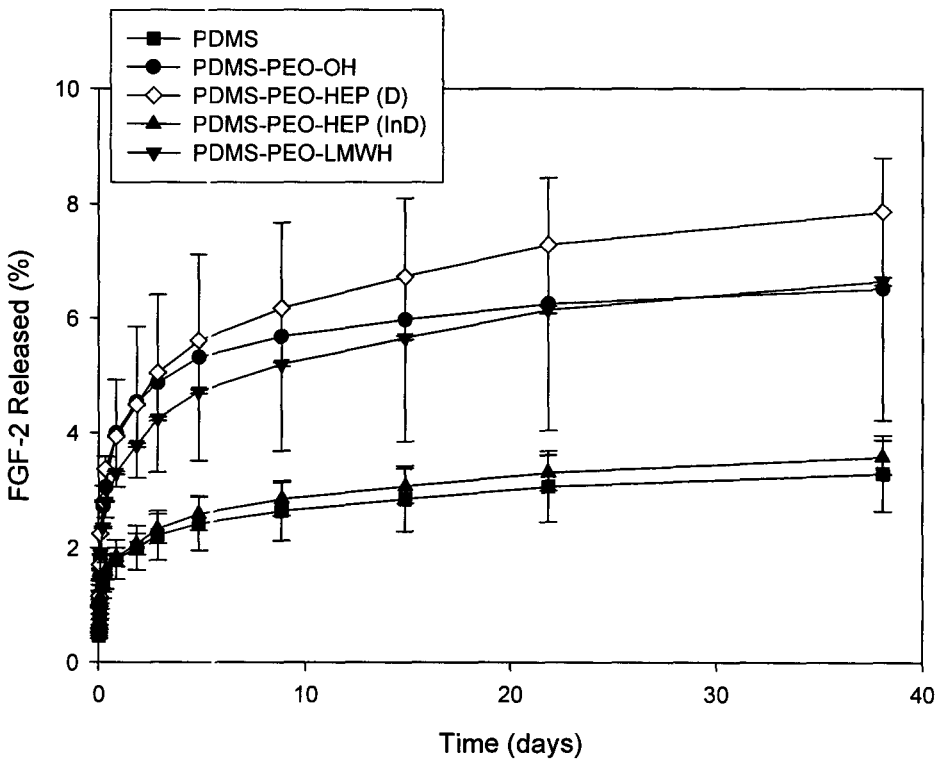

Figure 5-9. Cummulative ^{125}I-FGF-2 release from PDMS modified surfaces after soaking for 24 hr. Values are averaged with standard error bars.

Clearly these materials are able to release the growth factor over a period suitable for stimulating wound healing, suggesting that heparin modified materials may be useful for growth factor delivery in artificial cornea applications. Similar results have been observed in

other studies using alginate gels with heparin-sepharose beads (Edelman et al. 1991), hyaluronate-heparin gels (Liu et al. 2002), and heparinized poly (lactide glycolide) nanospheres in fibrin matrices (Jeon et al. 2006) which showed FGF-2 release rates of 25%, 50% and 30%, respectively, over a 2 week period.

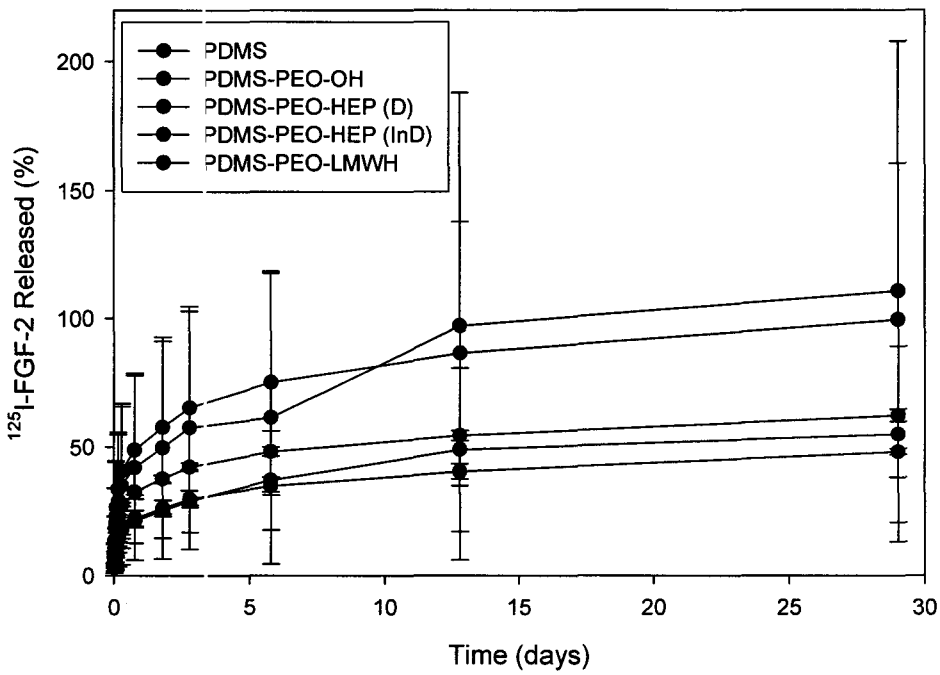

Figure 5-10. Percent release FGF-2 release from PDMS modified surfaces after 3 days of soaking. Values are averaged with standard error bars.

Of extreme importance is the maintenance of FGF-2 activity during release. Although *in vitro* cell stimulation of released FGF-2 was not performed, there is evidence of FGF-2 instability when released without heparin. For instance, ethylene-vinyl acetate copolymer matrices were capable of delivering 37% of FGF-2 over 2 weeks. However all bioactivity was lost, evident from the lack of stimulation of fibroblasts (Edelman et al. 1991). Thus PDMS and PDMS-PEO-OH surfaces that adsorbed FGF-2 are likely to have adsorbed and released denatured protein, incapable of stimulating the desired wound healing response.

FGF-2 activity was shown to be maintained when released with heparin-sepharose beads and stored FGF-2 solutions could be stabilized with the addition of heparin (Edelman et al. 1991). Thus the soluble FGF-2 release with heparin seen here is more likely to maintain FGF-2 activity. Studies of the efficacy of these released growth factors are ongoing. Interestingly, heparin added to release media has been found to increase release (Liu et al. 2002) and is capable of stabilizing FGF-2 further. It would be noteworthy to conduct release studies of that nature, to better predict *in vivo* release, as there is increased glycosaminoglycan production during corneal wound healing (Hassell et al. 1983) and the presence of free heparin is likely.

5.4 CONCLUSIONS

PDMS-PEO-heparin surfaces were fabricated using two methods, termed direct and indirect. Although both methodologies were straight forward and resulted in PDMS-PEO-Heparin surfaces, the direct modification method showed batch variation and highly reactive surfaces were difficult to reproduce. Indirect modification resulted in frequent surface damage and platinum poisoning but is generally thought to be more promising and overall simpler to perform. With uniform PEO coverage, heparin densities on the order of 1 $\mu g/cm^2$ were obtained; much of the heparin on these surfaces was active. High levels of FGF-2 could be immobilized for subsequent release. Heparinized PDMS modified surfaces soaked for 3 days released 50% (over 2 weeks), of initially immobilized FGF-2, indicating that slow and gradual release was possible. These materials have potential for the delivery of FGF-2 in corneal wound healing and artificial cornea applications.

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6. PAPER 2

Dendrimer Crosslinked Collagen Matrices Modified with Heparin for the Delivery of Basic Fibroblast Growth Factor

This paper describes the fabrication of polypropyleneimine octaamine generation 2 (G2) dendrimer crosslinked gels, expanding on previous work by Duan and Sheardown through the integration of heparin for soluble FGF-2 delivery for applications of tissue engineered corneal equivalents (TECEs).

Heparinized growth factor delivery systems have gained critical importance, as FGF-2 delivery without carrier molecules results in rapid protein release and premature denaturation of the growth factor (Edelman et al. 1991). Previously fabricated heparinized collagen based growth factor delivery systems were intended as degradable hydrogels for angiogenesis and wound healing (DeBlois, Cote, and Doillon. 1994; Pieper et al. 2002; Sakiyama-Elbert and Hubbell. 2000; Wissink et al. 2001; Wissink, M. J. B. et al. 2001) and lack the mechanical stability required for TECEs. The collagen gels with polypropyleneimine octaamine G2 dendrimer integration achieved by Duan and Sheardown demonstrate increased stability, when compared to traditional chemical crosslinking techniques (Duan and Sheardown. 2005; Duan and Sheardown. 2006b).

In this work, free amine groups on the dendrimers were exploited to permit the incorporation of FGF-2 and to generate biologically active collagen matrices through the

incorporation of heparin. Characterization of these surfaces indicates similar integrity to unheparinized surfaces although optimization of reaction kinetics may improve the results.

Heparinized dendrimer crosslinked collagen, exposed to FGF-2, demonstrated high growth factor immobilization and prolonged growth factor release. However release kinetics from unheparinized materials were not significantly different. As well, analysis of FGF-2 bioactivity is pending.

Dendrimer Crosslinked Collagen Matrices Modified with Heparin for the Delivery of Basic Fibroblast Growth Factor

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Abstract

A tissue engineered corneal equivalent must be transparent and durable, with properties comparable tc the refractive index and tensile strength of a natural cornea. Tissue integration between the tissue engineered corneal equivalent and the host eye is of critical importance to ensure long-term implant success. As in wound healing, it is hypothesized that corneal tissue integration can be achieved by the stimulation of fibroblast migration through growth factor stimulation. Therefore it is necessary to delivery appropriate growth factors to the cells in an active form. Here we investigate heparinized chemically crosslinked collagen gels and their ability to deliver basic fibroblast growth factor (FGF-2).

Collagen gels crosslinked with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) chemistry, and varying amounts of polypropyleneimine octaamine generation 2 (G2) dendimer were synthesized with immobilized heparin.

Swelling studies indicated that thermal gel stability was compromised by heparin addition, where as there was insignificant difference between chemically crosslinked gels and their heparinized counterparts. Differential scanning calorimetry indicated much higher denaturation temperatures for dendrimer crosslinked gels (96°C), which was slightly lowered with the addition of heparin (89°C). Interestingly, dendrimer crosslinked gels with or without heparin gave multiple denaturation peaks, as did heparinized EDC gels, thought to be the result of the heterogeneous crosslinking possible between collagen, dendrimer and heparin. Heparin leaching was observed with uncrosslinked heparin, decreasing with increasing chemical crosslinking. Finally, release of FGF-2 from collagen gels gave a first

order release profile, with an initial burst, followed by a prolonged gradual release. Heparinized dendrimer crosslinked gels released approximately 40% over 2 weeks.

Keywords: collagen; crosslinking; dendrimers; EDC; FGF-2; corneal stroma; tissue engineered corneal equivalents

6.1 INTRODUCTION

The cornea, a tough, transparent tissue located at the front of the eye, protects the optical elements from ultraviolet light and environmental hazards, as well as performing more than 80% of the refractive function of the eye. Corneal and ocular trauma account for 2 million new cases of corneal blindness annually; making cornea blindness the second leading cause of blindness worldwide after cataracts (Whitcher, Srinivasan, and Upadhyay. 2001). Allograft surgery, the most widely used treatment, has an initial success rate of 80%. However there are instances of graft rejection and failure in severe cases (Chirila et al. 1998). Furthermore, with increasing demand for corneal donor tissue, almost doubling in the past decade, an increasing shortage of tissue (National Coalition for Vision Health. 2005), and the chance of allotypic disease transmission, a reasonable synthetic alternative is desirable. Unfortunately, an acceptable artificial cornea implant that interacts well with host tissue is not yet available (Carlsson et al. 2003).

Tissue-engineered cornea equivalents (TECE) are artificial corneal implants based on cellular repair strategies, whereby extracellular matrix components are combined with immortalized corneal cells lines to increase host integration (Duan, Klenkler, and Sheardown. 2006). Much interest has surrounded collagen based TECE (Doillon et al. 2003; Germain et al. 1999; Duan and Sheardown. 2005; Orwin, Borene, and Hubel. 2003) since the corneal structure is predominantly type I collagen (Meek and Boote. 2004). However, pure collagen thermal gels are limited by weak intrafibrillar interactions, opacity, and are prone to rapid enzymatic degradation *in vivo* (Olde Damink et al. 1996), rendering them unsuitable for most TECE applications. An alternative is to introduce chemical crosslinkers which can either

facilitate covalent crosslinking of collagen fibrils or act as bridges between collagen fibrils and increase collagen gel strength (Rault et al. 1996; Wallace and Rosenblatt. 2003).

Crosslinking with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) catalyzes "zero-length" collagen crosslinking (Grabarek and Gergely. 1990), and achieves increased crosslinking density (Olde Damink et al. 1996), but is limited by the number of amine groups in the collagen molecule (Duan and Sheardown. 2005; Liu et al. 2006a; Liu et al. 2006b). Duan and Sheardown hypothesized that increasing the number of amine groups may increase the degree of crosslinking and utilized multifunctional amine terminated dendrimers as crosslinking agents (Duan and Sheardown. 2005), resulting in materials with mechanical properties suitable for suturing and which showed good *in vitro* compatibility (Duan and Sheardown. 2006b). Furthermore, they demonstrated that the incorporation of biological functional groups, through free amine groups present on the dendrimer, was possible (Duan et al. 2006a (Ahead of print)).

To enhance the cytocompatibility and host integration with a TECE, growth factor delivery may be desired as growth factors are active in corneal wound healing by stimulating cell migration (Imanishi et al. 2000). Basic fibroblast growth factor (FGF-2) has been shown to increase healing (Rieck et al. 1994) and influence fibroblast migration in the stroma (Shipley et al. 1989). However given its short half life *in vivo* (Whalen, Shing, and Folkman. 1989), appropriate delivery methods are required. Heparin, a glycosaminoglycan, capable of stabilizing FGF-2 (Sommer and Rifkin. 1989) and maintaining its bioactivity (Gospodarowicz and Cheng. 1986) in a manner similar to *in vivo* extracellular heparan sulphate proteoglycans (Saksela et al. 1988), has been utilized to facilitate the delivery of FGF-2 in a soluble form (Edelman et al. 1991; Fujita et al. 2004; Ishihara et al. 2001;

Ishihara et al. 2003; Jeon et al. 2006; Liu et al. 2002; Sakiyama-Elbert and Hubbell. 2000; Zamora et al. 2002). In this work, we investigated EDC and G2 dendrimer crosslinked collagen gels with immobilized heparin for the delivery of FGF-2.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Concentrated collagen suspensions (98 mg/mL) of pepsin digested bovine cornium purified type I collagen, with less then 20% type III collagen, was a generous gift from Inamed Corporation (California, USA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and unfractionated heparin were purchased from Sigma-Aldrich (Oakville, ON). Polypropyleneimine octaamine generation two (G2) dendrimer was purchased from SyMO-Chem (Eindhoven, The Netherlands). FGF-2 was purchased from R & D Systems (Minneapolis, USA).

6.2.2 Collagen Gel Fabrication

Collagen gels were fabricated based a method adapted from the work of Duan and Sheardown (Duan and Sheardown. 2005). Collagen was diluted to 6% (v/v) with phosphate buffered saline (PBS, pH 7.4), and 1 mL was acidified with 1.0 N hydrochloric acid (HCl) prior to heparin treatment and crosslinking. Various heparin containing collagen gels fabricated.

Thermally crosslinking heparin-collagen (C-H) gels were prepared by adding 450 μL heparin solution (30 mg/mL), adjusting the pH to 7.4 with 1.0 N sodium hydroxide (NaOH), diluting the suspension with endotoxin free water (EFW) for a final collagen concentration of 30 mg/mL, and incubating overnight at 37°C.

Carbodiimide crosslinked heparin collagen (C-H-EDC) gels were prepared with the addition of 200 μL of a 1 mL EDC/NHS solution of 75 mg EDC and 45 mg NHS (molar ratio of EDC:NHS:COOH = 5:5:1). Dendrimer heparin collagen gels were crosslinked with the EDC/NHS solution combined with a second generation polypropyleneimine octaamine dendrimer (G2). Three dendrimer gels were created with different crosslinking solutions containing different EDC:NHS:dendrimer weight ratios. The first, C-H-G2, had 200 μL of a (2.27:1.36:1) crosslinking solution of EDC:NHS:G2 (w/w/w); the second, C-H-20%G2, contained a higher molar amount of the crosslinking solution (1.98:1.19:1) (w/w/w) crosslinking solution; while the highest dendrimer concentration gel, C-H-2G2, had a (0.99:0.59:1) (w/w/w) crosslinking solution. In all cases, the amount of heparin in the systems remained constant.

The chemical crosslinking solutions were added to the collagen suspension after the addition of heparin. Where appropriate, 1.0 N NaOH was added to adjust the pH to 5.5, to stabilize the EDC/NHS reaction (Olde Damink et al. 1996). As with the thermally crosslinked collagen gels, the final collagen concentrations of the chemically crosslinked gels were 30 mg/mL. Collagen gels without heparin were fabricated as controls. All suspensions were pressed between glass plates and left overnight to ensure complete crosslinking, and then punched into 0.625 cm (¼") diameter surfaces. Gels were dried and stored at room temperature until needed. Surfaces were reconstituted in Milli-Q water prior to experimentation.

6.2.3 Water uptake

Water content of the collagen gels was measured to characterize the collagen crosslinking. The dry weight (W_d) of the gels was determined following freeze drying. Following reswelling in Milli-Q water for 1 hour, the gels were wicked dry to remove surface moisture and the wet weights (W_w) were determined. The water uptake was calculated according to the following formula:

$$WaterUptake(\%) = \frac{W_d - W_w}{W_w} * 100\% \qquad \text{(Eq.1)}$$

6.2.4 Differential Scanning Calorimetry

Denaturation temperatures for collagen materials were determined by differential scanning calorimetry (DSC) using a TA instruments DSC 2910. Collagen gels were immersed in EFW for 2 hrs, dabbed dry, and transferred into aluminum hermetic pans. They were exposed to a heating rate of 2°C/min over a temperature range of 15°C to 100°C. Peaks deviating from the baseline were monitored and analyzed for denaturation temperature with the accompanying DSC software. A hermetic pan filled with EFW was used as a reference.

6.2.5 Heparin Stability

Release of free heparin from the collagen gels was examined in order to assess the stability of the immobilized heparin. Hydrated collagen gels were immersed in PBS (pH 7.4) and shaken in a waterbath, at 37°C. Samples were taken hourly for the first 8 hours, then twice daily for 2 days, then once on the fourth day and finally once on the seventh day. Samples were stored at 4°C prior to analysis for heparin concentration using the toluidine blue assay (MacIntosh. 1941; Smith, Mallia, and Hermanson. 1980).

6.2.6 Toluidine Blue Assay

In a 5 mL test tube, 1.5 mL of sample or standard heparin solution (0-2 μg/mL heparin in 0.2% sodium chloride (NaCl)) and 0.15 mL of toluidine blue solution (0.005% toluidine blue dye with 0.2% NaCl and 0.01 N HCl) were combined. The test tubes were vortexed for 30 seconds and 1 mL n-hexane added. The mixture was then shaken for an additional 30 seconds. The solution was allowed to phase separate, after which the aqueous layer was removed and analyzed at 630 nm. Sample absorbance values were standardized against known standard heparin solutions and heparinized gels were standardized against their non-heparin gel counterparts.

6.2.7 Dendrimer Stability

Dendrimer reactivity with and release from the collagen matrix was assessed by analyzing release samples with a Coomasie blue assay (Bradford. 1976; Sapan, Lundblad, and Price. 1999). Although this assay is primarily used for protein quantification, through protein-dye interactions, its use was extended here, as a crude means of detecting changes in pH. Alkaline substances have been shown to interfere with the dye absorbance, through a conversion from the cationic to the neutral to the anionic form of the dye, causing an absorbance shift at 595nm (Stoscheck. 1990). Heparin was not shown to interfere with the dye, consistent with Bradford's observations of non-interfering carbohydrates (Bradford. 1976).

A release study was performed similar to the heparin stability release, whereby hydrated collagen surfaces were immersed in PBS (pH 7.4), shaken at 37°C and sampled periodically. 200 μL of the release sample, in triplicate, was pipetted into a 96-well plate and mixed with 100 μL of Coomassie blue solution (50.1 mg Coomassie blue dye powder

dissolved in 25 mL 95% ethanol and 50 mL 85% O-phosphoric acid and 500 mL Milli-Q water). After 5 minutes, the samples were read at 595 nm and subsequent absorbencies were compared against standard curves of known dendrimer concentrations in PBS.

6.2.8 ^{125}I Labelled FGF-2 Immobilization

FGF-2 was reconstituted in tris (hydroxymethyl) methylamine (TRIS) buffer (pH 7.4), and radiolabelled via the IODOGEN method (Pierce, USA) (Klenkler et al. 2005). Growth factor (100 µL) was combined with 5 µL of ^{125}I (500 µCu / 5 µL) in an Iodogen coated vial and stirred for 15 minutes at room temperature. The solution was subsequently dialyzed 3 times with TRIS buffer over a 24 hour period. Free unbound isotope amount was determined by trichloroacetic acid (TCA) precipitation. Collagen gels were soaked in 5 mL EFW for 24 hrs to remove any unbound heparin and dendrimer, freeze dried, and exposed to ^{125}I labelled FGF-2 to assess FGF-2 immobilization through radioactivity, read on a gamma counter (Wallac Wizard 3" 1480 Automatic Gamma Counter, Perkin Elmer Life Sciences). To determine FGF-2 adsorption, surfaces were rinsed three times in TRIS for 1 min, dabbed dry and read for activity.

6.2.9 ^{125}I Labelled FGF-2 Release Studies

Radioactive gels were placed into 1 mL fresh TRIS buffer, in a 37°C shaking waterbath. Release samples were obtained at regular intervals in order to determine the FGF-2 release profile. Surface and sample radioactivity were measured with a gamma counter (Wallac Wizard 3" 1480 Automatic Gamma Counter, Perkin Elmer Life Sciences) and translated to known concentrations of ^{125}I labelled FGF-2.

6.2.10 Statistical Analysis

Data is presented as mean values with variability expressed as standard deviations, unless otherwise stated. Statistical significance was determined with a single factor Anova test, with $p < 0.05$.

6.3 RESULTS AND DISCUSSION

6.3.1 Collagen Gel Fabrication

Thermally and chemically crosslinked collagen gels containing heparin were prepared, as were control gels containing no heparin. Two chemical crosslinking solutions were used, the first containing EDC/NHS, while the second solution was variations of EDC and NHS combined with dendrimer. Thermally crosslinked collagen gels required a pH of 7.4 to gel, while EDC/NHS crosslinking has been reported to be optimal at pH 5.5, as this enables carboxylic acid groups to be functionalized efficiently with EDC (Olde Damink et al. 1996).

The appearance and relative strength of collagen gels are presented in Table 6-1. Thermally crosslinking collagen gels with or without heparin were opaque in appearance. It has been observed by McPherson *et al.* that opacity increases with increasing low levels of heparin (5-20 μg/mg collagen) but a saturation point is achieved (>160 μg heparin/mg collagen) with no further opacity effects (McPherson et al. 1988). The transparency of pure collagen gels tended to improve with the addition of chemical crosslinkers but the addition of heparin led to more opaque gels. Heparin influences collagen fibrillogenesis, whereby increasing heparin concentrations disrupts fibril assembly and increases collagen fibril diameter resulting in greater opacity (McPherson et al. 1988). In this work, the concentration of heparin added to the gels was quite high at 204 μg heparin/mg collagen. Thus the

occurrence of opacity is not unexpected. For TECE applications, optimization of the heparin concentration is required for transparent implants.

Table 6-1. Macroscopic Properties of Collagen Gels

Surface	Appearance	Relative Mechanical Strength
COLL	Translucent	Poor
C-H	Translucent	Poor
C-EDC	Transparent	Fair
C-H-EDC	Translucent	Fair
C-G2	Transparent	Good
C-H-G2	Translucent	Good
C-20%G2	Transparent	Good
C-H-20%G2	Translucent	Good
C-2G2	Transparent	Good
C-H-2G2	Translucent	Good

The relative mechanical strength can be attributed to interactions between carboxyl acid and amine groups either present on the collagen molecule itself, or between collagen and the introduced chemical crosslinkers. Thermally gelled collagen (COLL) matrix formation is mediated by fibril hydrophobic and electrostatic forces, whose interactions are stabilized at higher temperatures (30-37°C) (Rosenblatt, Devereux, and Wallace. 1993; Wallace. 1990). At room temperature, pure collagen gels become more fluid, resulting in the more delicate surface observed here. Heparinized collagen thermal gels (C-H) had a slight increase in relative strength, as heparin influences the generation of larger diameter fibrils, which may affect gel stability (McPherson et al. 1988).

Relative mechanical strength of collagen gels was greatly improved with the addition of chemical crosslinkers as expected. The crosslinking of collagen facilitated by the EDC/NHS mixture (C-EDC) allows for "zero-length" direct covalent crosslinks between collagen fibrils (Grabarek and Gergely. 1990). This occurs through the formation of stable

amide bonds through carboxylic acid side groups, specifically glutamic and aspartic acid, binding to lysine groups (Olde Damink et al. 1996). Crosslinking collagen with G2 dendrimer (C-G2), further increased relative mechanical strength, as the limited amine groups available for crosslinking are amplified by the presence of the G2 dendrimer (Duan and Sheardown. 2005). No difference in relative mechanical strength was observed with increasing crosslinking amounts of C-20%G2 and C-2G2. There was no noticeable difference in strength between heparinized chemically crosslinked collagen matrices and their control counterparts. However, the addition of heparin to the collagen solution prior to crosslinker addition could result in the activation of heparin carboxylic acid groups, rendering them capable of amide bonding to the collagen fibrils or to the G2 dendrimer.

6.3.2 Water uptake

Water uptake, shown in Figure 6-1, was determined from dry and wet weights of freeze dried and re-hydrated collagen surfaces, respectively. Swelling can be loosely correlated to gel crosslinking as electrostatic and hydrophobic forces affect porosity and fiber rigidity, both of which influence water uptake (Rosenblatt, Devereux, and Wallace. 1993; Wallace. 1990). Thermally crosslinked collagen gels had unusually moderate swelling at only 86%, whereas previous work has demonstrated higher values near 100% (Duan and Sheardown. 2005). This difference could be due to dehydration methods, whereby collagen was freeze-dried prior to reswelling, since the surface appeared discoloured and of irregular shape (compared to other more disc like surfaces), which was not recovered upon reswelling. Also, the soaking time of 1 hr may not have been enough for these surfaces to adequately rehydrate.

Water uptake increased significantly ($p = 0.0001$) in thermal gels to 97% with the addition of heparin, which can be attributed to the increased porosity of a less uniform cohesive matrix fabricated when heparin interferes with collagen fibril formation (Guidry and Grinnell. 1987). There is a high affinity receptor for heparin on the collagen molecule (Keller, Keller, and Kuhn. 1986) and heparin can interact with positively charges groups on collagen, but heparin binding does not do so uniformly *in vitro*, which can create a poorly crosslinked collagen matrix.

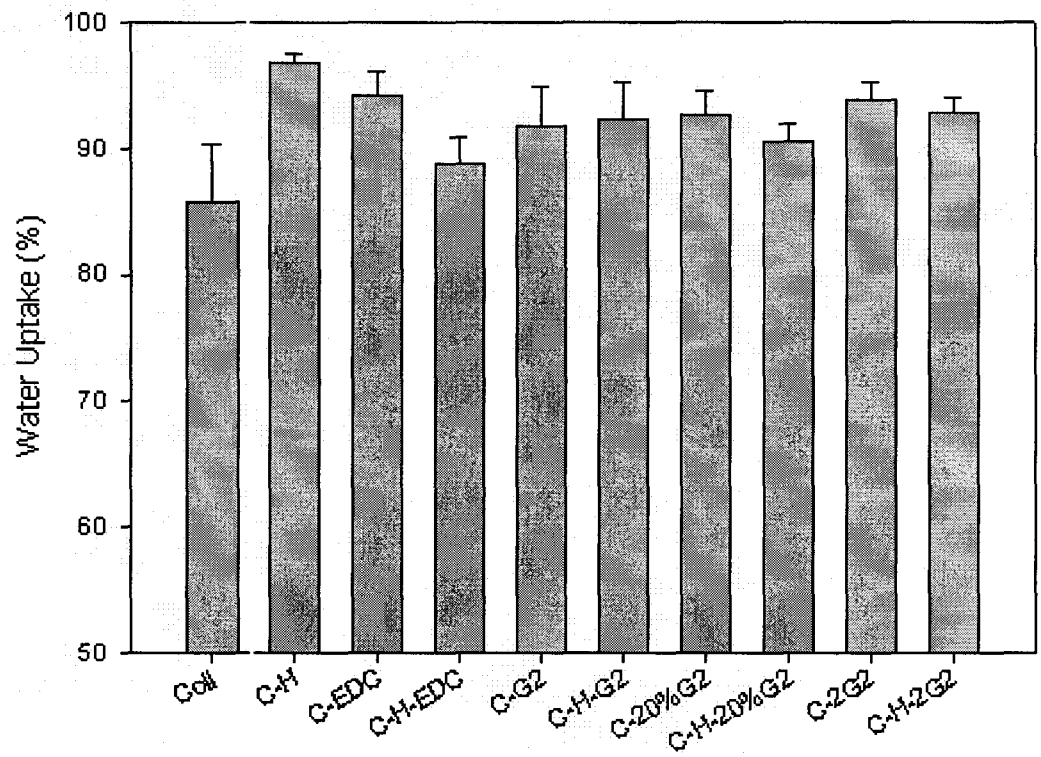

Figure 6-1. Solvent uptake of collagen modified materials. Thermally crosslinked gels had surprisingly low swelling, while heparinized thermal gels had higher swelling, indicative of a weaker matrix. Chemical crosslinking with EDC/NHS and its heparinized counterpart has increased crosslinking respectively, similar to dendrimer crosslinked gels crosslinked with or without heparin.

The use of a chemical crosslinker did not significantly decrease the water uptake of these gels. However, in most cases, the addition of heparin either the swelling, suggesting that heparin was also participating in the crosslinking reaction, and did not affect the water uptake. Consistent heparin incorporation may be possible by activating with EDC/NHS prior to combining with the collagen solution. Significantly ($p = 0.03$) lower water uptake was observed with the addition of heparin to the EDC/NHS crosslinked gels (C-EDC versus C-H-EDC). Heparinized G2 dendrimer crosslinked collagen gels had swelling capabilities similar to their unheparinized counterparts, indicating similar crosslinking. In these gels, heparin, through EDC/NHS activation, would be able to bind to collagen and dendrimer, creating a highly complex but stable mesh network.

Water uptake trends observed by Duan and Sheardown for unheparinized collagen surfaces showed swelling increasing with decreasing crosslinking, with crosslinking in the order of Coll < C-EDC < C-G2 (Duan and Sheardown. 2005). The discrepancy seen here may be due to the small size of the surfaces weighed; it may be worthwhile to reexamine water uptake using larger surfaces and soaking the materials for longer time periods.

6.3.3 Differential Scanning Colorimetry

Differential scanning calorimetry (DSC) has been routinely used to assess crosslinking stability of collagen materials (Figueiro et al. 2006; Lee et al. 1995). Hydrated collagen surfaces were exposed to a temperature range of 5 to 100°C at a heating rate of 2°C/min and the corresponding denaturation temperatures for collagen gels are shown in Figure 6-2.

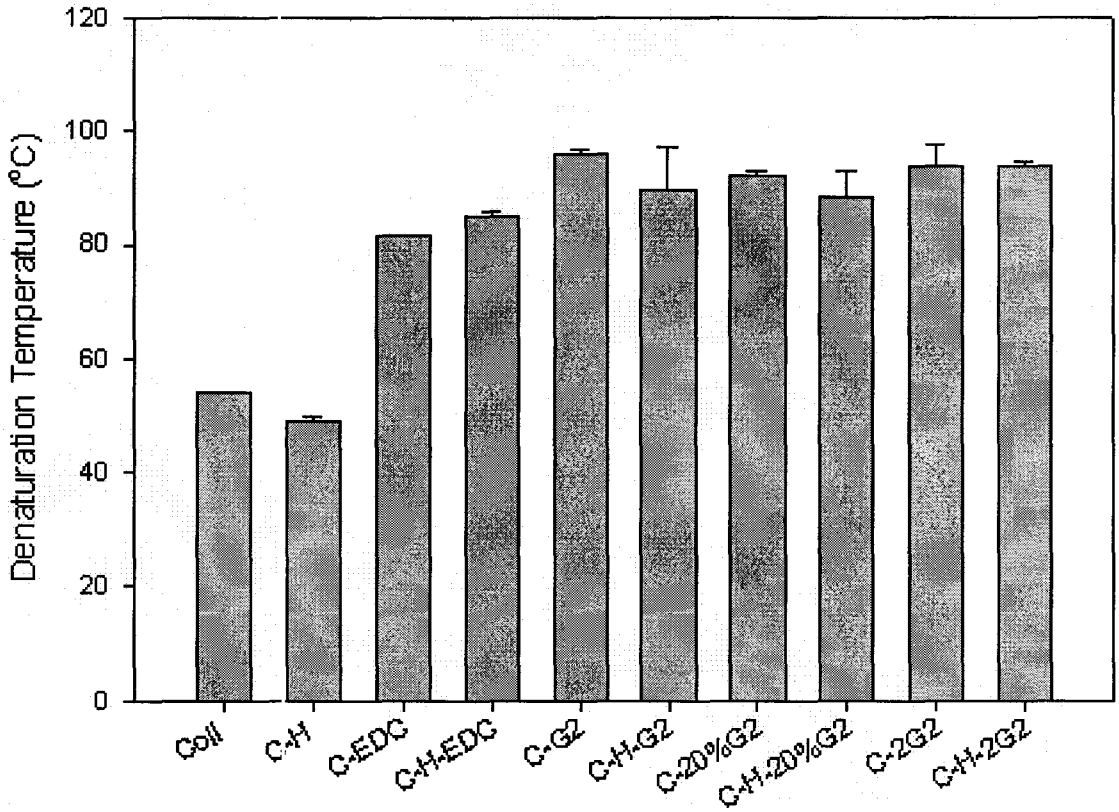

Figure 6-2. Denaturation temperatures of collagen surfaces. Thermally crosslinked gels had lower denaturation temperatures, while chemically crosslinked gels demonstrated higher stability through higher denaturation temperatures. Values are the mean with standard error.

Multiple denaturation temperatures have been observed for fibrillar collagen, those below 50°C and those slightly above 50°C, representing non-fibrillar and fibrillar components, respectively (Wallace et al. 1986). In this work, the collagen denaturation temperature was calculated as the average temperature needed to denature both fibrillar and non-fibrillar components. Thermally crosslinked collagen gels had a denaturation temperature of 53°C, which decreased significantly ($p = 0.02$) to 49°C with heparinization, providing further evidence of heparin fibril interference towards gel stability.

EDC/NHS crosslinked collagen gels with and without heparin had higher denaturation temperatures, 81°C and 84°C, respectively. With the addition of G2 dendrimer crosslinker, the denaturation temperature of the gels increased further to near 90°C, indicating increased crosslinking and stability. Differences were insignificant ($p > 0.05$) between collagen gels and their heparinized counterparts. Previously observed denaturation temperatures of unheparinized thermally and chemically crosslinked collagen gels were comparable to the values seen here (Duan and Sheardown. 2005; Wissink, M. J. B. et al. 2001).

Interestingly, many collagen crosslinked gels had numerous denaturation temperatures; these are summarized in Table 6-2. Referred to earlier, collagen has two denaturation temperatures due to non-fibrillar and fibrillar components, and these can be attributed to the lower denaturation temperatures seen here. Moderate temperatures, similar in value to C-EDC's denaturation temperature could represent denaturation of chemically crosslinked collagen fibrils. Heparin integration via EDC/NHS activation resulted in direct heparin-collagen binding, whose denaturation may be represented at 73°C, as seen as a denaturation peak in C-H-EDC gels.

Table 6-2. Denaturation temperatures for collagen gels.

Surface	Denaturation Temperature (°C)			
COLL	53.68			
C-H	49.85			
C-EDC	58.26	81.54		
C-H-EDC	59.3	73.405	84.63	
C-G2	63.12	73.41	89.20	97.11
C-H-G2	61.46	70.67	88.83	97.79
C-20%G2	61.43	76.605	83.262	93.32
C-H-20%G2	61.26	76.03	82.755	91.72
C-2G2	52.44	66.14	82.4	94.98
C-H-2G2	56.36	66.505	82.83	93.45

The lower denaturation temperatures in dendrimer crosslinked collagen could represent collagen fibrils, and their binding through EDC/NHS. There was evidence of multiple high temperature denaturation peaks, averaged in the data shown here, that could represent complex and heterogeneous dendrimer interactions with collagen, whereby dendrimer can bind completely to collagen, or partially through its 8 reactive amine groups. Further peaks were noted with heparinized dendrimer gels, likely attributable to various heparin interactions within the collagen and dendrimer matrix. To verify the integrity of denaturation temperatures, DSC was performed again on the surfaces, as collagen degradation is an irreversible process. Denaturation peaks noted in the first scan were not present in the second scan.

6.3.4 Heparin Release From and Stability within the Gels

Heparin stability within the heparinized collagen gels was assessed by incubating surfaces under physiological conditions and periodically quantifying heparin concentrations of withdrawn samples via a modified version of the toluidine blue assay. A calibration curve was created from known heparin concentrations in PBS, while heparinized gels were normalized against their unheparinized counterparts. Heparin release profiles are shown in Figure 6-3.

Initially all heparin gels were fabricated to contain 119.2 μg. Heparin released with decreasing collagen crosslinking, which thermally gelled heparinized collagen (C-H) releasing 35% of heparin, followed by 24% heparin released from EDC/NHS crosslinked gels (C-H-EDC), and 18%, 16% and 6% heparin released from G2 dendrimer gels, C-H-G2,

C-H-20%G2, and C-H-2G2, respectively. The data indicate that activation of heparin by EDC/NHS allows for heparin binding to the collagen fibrils or G2 dendrimer, and that increasing EDC/NHS and G2 dendrimer amounts allows for greater heparin integration. The variability in the amount of heparin released observed with heparinized chemically crosslinked gels is likely due to the possible heterogeneity of collagen binding.

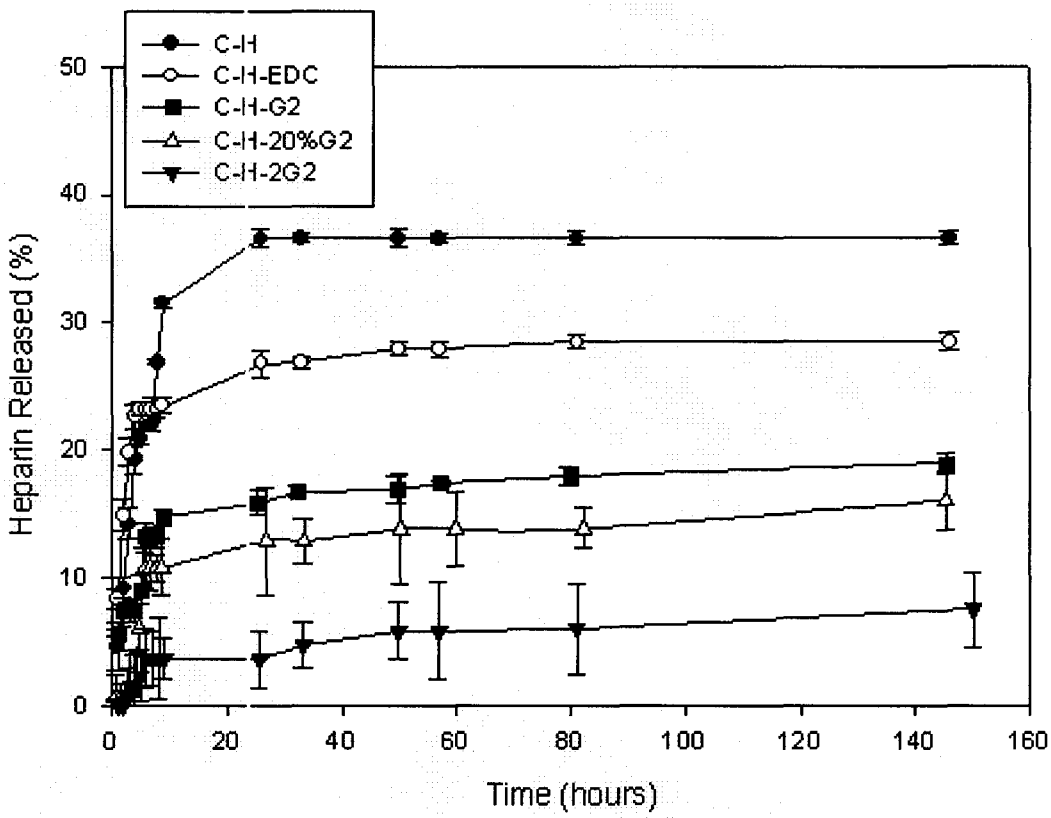

Figure 6-3. Heparin release profiles of collagen surfaces. Higher degrees of crosslinking were able to immobilize more heparin, with thermal gel releasing the most heparin (36%).

It would be interesting to manipulate the amount of EDC/NHS and dendrimer required for controlled heparin immobilization and delivery, as the addition of heparin to release mediums has increased FGF-2 release and enhanced growth factor stability once released (Edelman et al. 1991). In fact, it may be beneficial to allow the release of heparin-

FGF-2 complexes early in wound healing applications to ensure cellular activation if extracellular matrix proteoglycan concentrations are low.

The amount of immobilized heparin could also potentially be increased by adding heparin to the EDC/NHS mixture, allowing for full carboxyl activation and greater probability of reaction with collagen. Furthermore, pre-reaction with the G2 dendrimer, as in previous work, may also be desirable as it would provide dendrimer heparin complexes; however optimization would be required to ensure maximum crosslinking without compromising stability.

6.3.5 G2 Dendrimer Stability

There is controversial data surrounding dendrimer toxicity, whereby cationic dendrimers induce toxicity, through cell membrane lysis and accumulate in the liver (Boas and Heegaard. 2004; Gillies and Frechet. 2005). Dendrimer immobilization will also result in stronger collagen matrices for the desired TECE applications. For these reasons, dendrimer stability was assessed by monitoring changes in pH with the Coomassie blue assay, as pH adjusts the metachromatic absorbance of the dye (Stoscheck. 1990). To eliminate side effects of collagen degradation over the release time, collagen gels without dendrimer were also monitored. Results are presented in Figure 6-4.

The collagen gel, its heparinized counterpart, and C-H-EDC did not appear to interfere with the coomassie assay. Dendrimer crosslinked collagen, C-G2, C-20%G2 and C-2G2 did release < 10% of their dendrimer, which released within the first 2-3 hours. Approximately 20% of the original dendrimer amount was released in a similarly rapid fashion from C-H-G2 and C-H-20%G2 gels. Both of these gels had equal amounts of

EDC/NHS, which may not be adequate for both heparin and collagen activation for complete dendrimer incorporation. This is further supported by the C-H-2G2 gel, which released less than 5%, as the gel was activated with a larger amount of EDC/NHS. Extensive rinsing of the dendrimer crosslinked gels would eliminate the risk of leaching dendrimer during further experimentation.

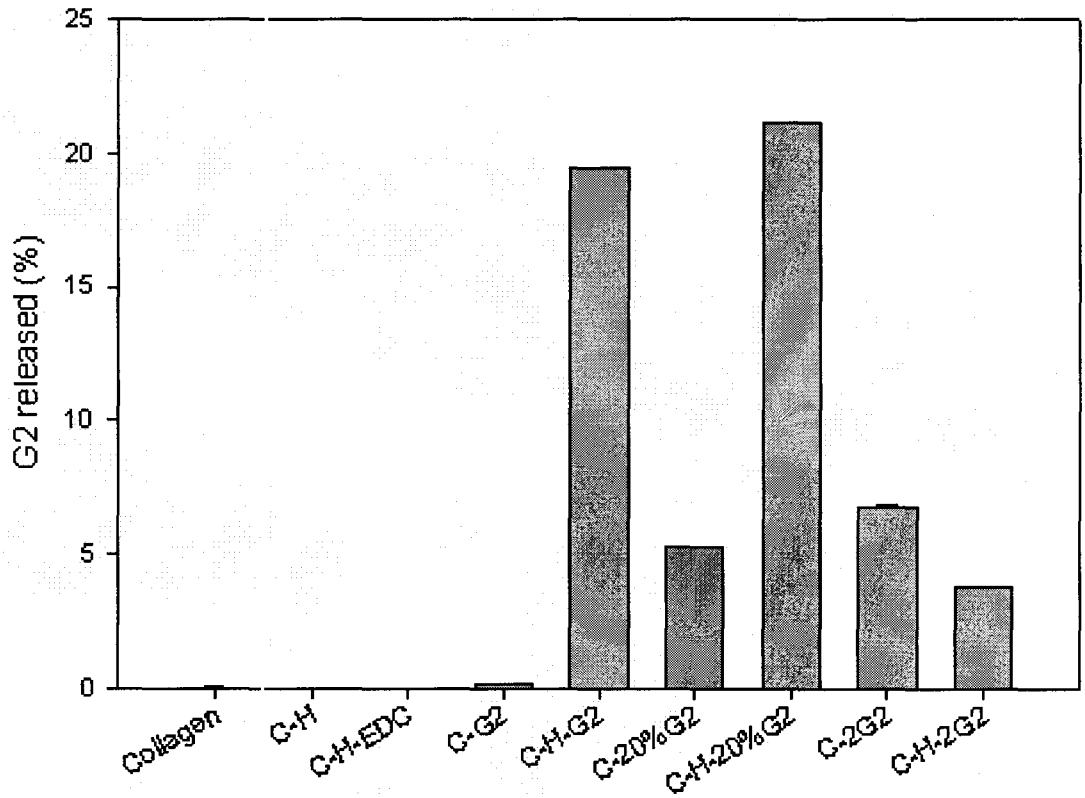

Figure 6-4. G2 dendrimer stability within collagen gels. All heparinized chemically crosslinked gels released dendrimer (20%), while their unheparinized counterparts released much less (<10%). However, C-H-G2 had increased dendrimer stability, perhaps attributable to an increased concentration of EDC/NHS, which likely accommodates chemical activation of both collagen and heparin.

6.3.6 ^{125}I Labelled FGF-2 Immobilization

Collagen gels were exposed to ^{125}I labelled FGF-2 to assess the amount of bound FGF-2 to the material. Collagen surfaces were soaked in Milli-Q water for 24 hours prior to

^{125}I-FGF-2 exposure to remove unbound heparin and dendrimer. Figure 6-5 illustrates FGF-2 binding after 4 days of soaking in ^{125}I labelled FGF-2 solution and the amount of growth factor that remained after rinsing three times with TRIS buffer.

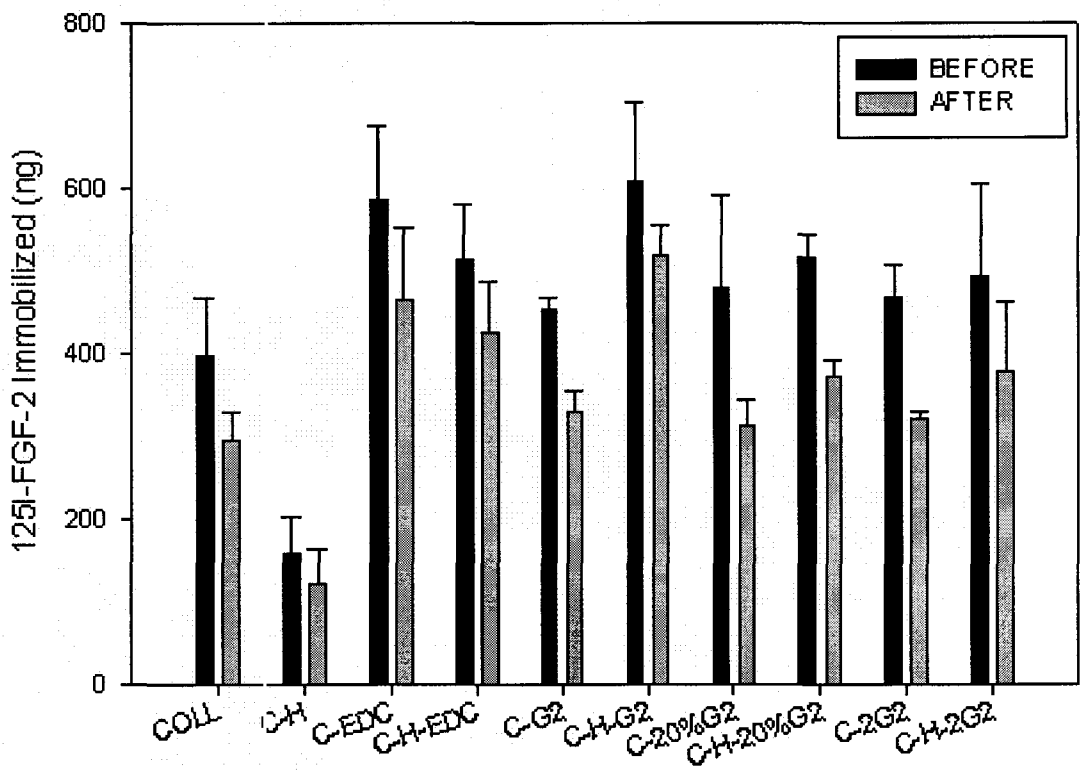

Figure 6-5. Immobilized FGF-2 to collagen surfaces before and after rinsing with TRIS buffer. Thermally crosslinked gels, with and without heparin, had lower FGF-2 immobilization, likely attributable to matrix porosity and instability. Chemically crosslinked gels had comparable immobilization.

On average, an approximate loss of 25% ^{125}I-FGF-2 from all surfaces was observed after rinsing with TRIS buffer, and retention did not differ greatly between surfaces ($p = 0.21$).

Collagen thermal gels immobilized ~ 300 ng of FGF-2 after rinsing, which can be attributed not only to absorption into the dried gel, but also to the growth factor binding domains on the collagen molecule (Kanematsu et al. 2004a). C-H gels immobilized less

FGF-2 ($p = 0.005$) likely due to the gel's loose fibril arrangement and poor heparin retention. EDC/NHS crosslinked gels, with ($p = 0.036$) or without heparin ($p = 0.002$), resulted in higher FGF-2 immobilization. Dendrimer crosslinked gels did not differ significantly from COLL or EDC/NHS crosslinked gels, presumably due to the increase in heparin binding and a decrease in collagen domains for FGF-2 binding.

6.3.7 ^{125}I Labelled FGF-2 Release Studies

Release of the ^{125}I-FGF-2 from collagen based gels, into TRIS buffer at 37°C, is shown in Figures 6-6 and 6-7 for the first 26 days of release. As expected, release of FGF-2 for all collagen gels began with an initial burst of FGF-2 followed by first order release.

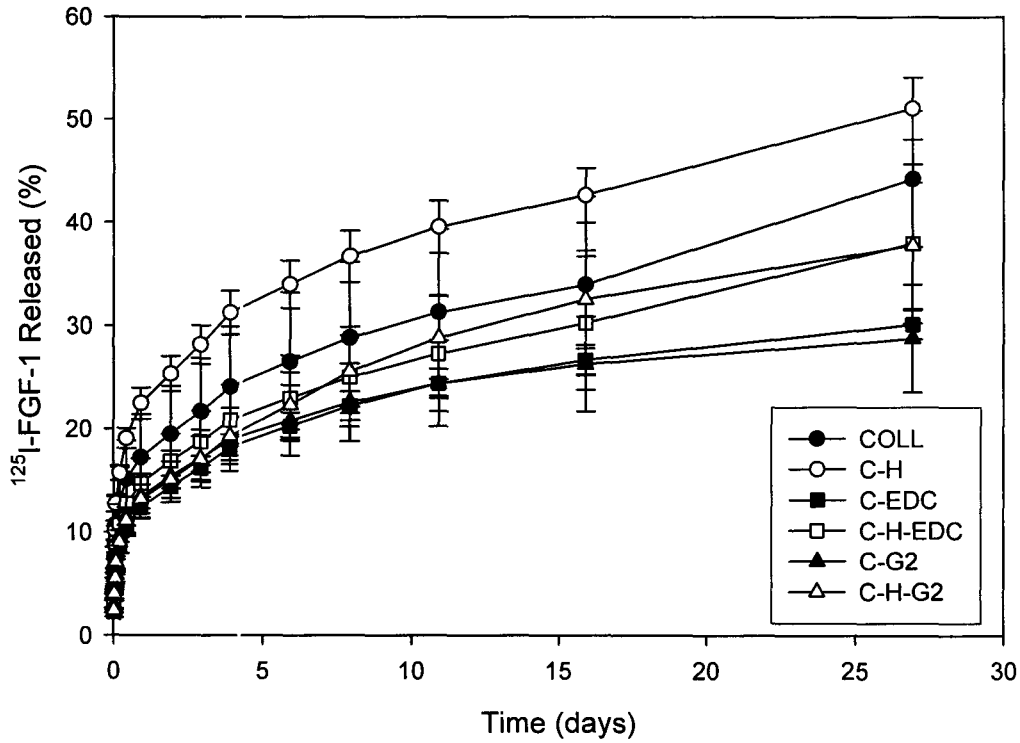

Figure 6-6. FGF-2 released from collagen surfaces, with or without heparin, thermally and chemically crosslinked with EDC/NHS and G2.

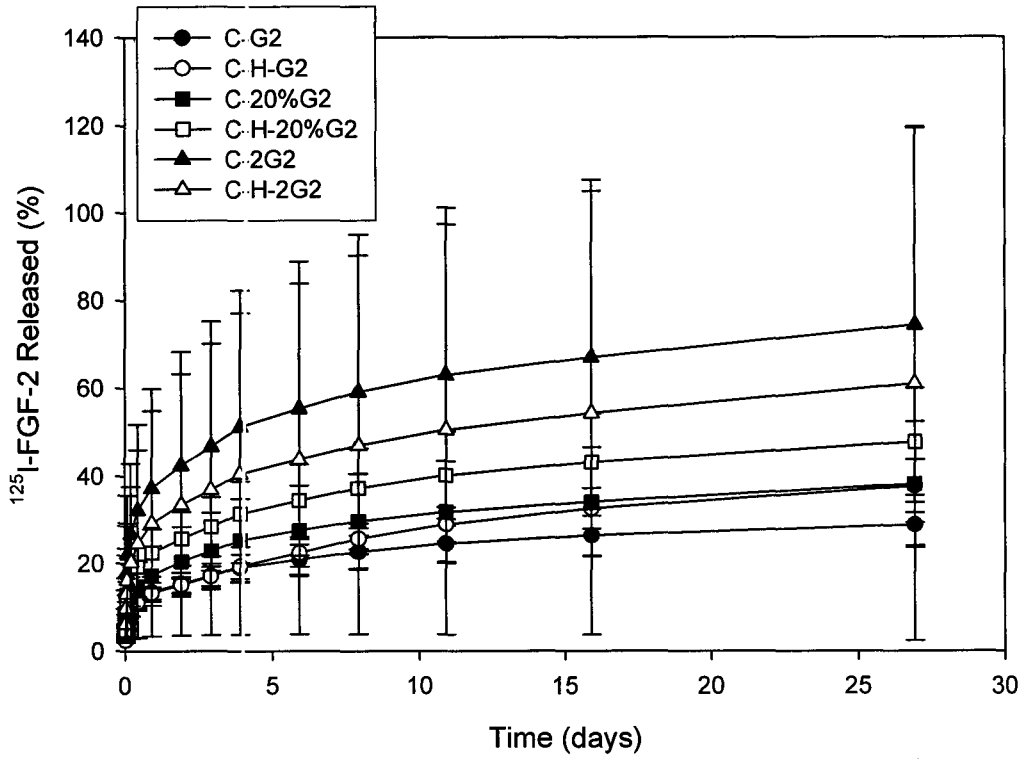

Figure 6-7. FGF-2 released from G2 dendrimer crosslinked collagen surfaces, with or without heparin, crosslinked with various amounts of crosslinking solutions.

Over a 2 week period, 20-60% of the incorporated FGF-2 was released from the gels, with higher percentages being released from the dendrimer gels with high initial loadings. The large error bars seen with dendrimer crosslinked gels may be attributable to differences in FGF-2 retention, perhaps influenced by crosslinking variability and heparin availability. It is unknown what release rates will be necessary to stimulate corneal stromal cells. However, given that the relative loading differed by a factor of more than two, it is reasonable to hypothesize that different release rates could be obtained. Future work will focus on the effect of the released growth factor on corneal stromal cells. Furthermore, despite the promising release of FGF-2 from collagen gels, either thermally or chemically crosslinked, there is evidence of growth factor instability when released without heparin (Edelman et al.

1991). Thus there is a high probability that FGF-2 incorporated in the matrix and later released may be rapidly denatured, and studies of this nature are ongoing. Heparin added to the release medium has also shown to influence release rates (Liu et al. 2002), and since glycosaminoglycan production is augmented during wound healing (Hassell et al. 1983), that would also be worth investigating.

Collagen matrices crosslinked with gluteraldehyde demonstrated sustained FGF-2 release, but this was also affected by matrix degradation (Kanematsu et al. 2004b). Other collagen delivery systems for other heparin-binding growth factors, TGF-β (Schroeder-Tefft, Bentz, and Estridge. 1997) and VEGF (Steffens et al. 2004), have also shown promise for tissue engineering.

Heparinized collagen crosslinked with EDC/NHS has been developed by Wissink *et al.* for vascular graft applications (Wissink et al. 2001). They observed FGF-2 immobilization of 22% of the original FGF-2 solution and released 40% FGF-2 in 28 days. Research by Pieper *et al.* demonstrated increased angiogenesis with FGF-2 incorporated into EDC/NHS crosslinked collagen gels (Pieper et al. 2002).

6.4 CONCLUSIONS

Heparinized collagen surfaces were gelled thermally and chemically through EDC/NHS activation and dendrimer crosslinking. Heparinized thermal gels demonstrated a loose, unstable matrix, indicated by the high swelling, low denaturation temperature, increased heparin instability and decreased FGF-2 immobilization.

Combination of heparin with EDC/NHS allowed for its covalent immobilization within the collagen matrix. Surfaces had increased stability, compared to thermal gels, with

moderate swelling, improved crosslinking, evident from the high denaturation temperature of 84°C, and only had 25% unbound heparin.

Further addition of G2 dendrimers to the EDC/NHS crosslinked gels resulted in materials with very moderate swelling and high denaturation temperatures. These materials allowed for increased heparin retention, provided the amount of crosslinker was adequate to activate both heparin and collagen fibrils for dendrimer incorporation, as was the case with C-H-2G2.

FGF-2 immobilization was comparable with all chemically crosslinked gels, and release of FGF-2 was in a first order profile resulting in approximately 20-60% released over 2 weeks, depending on crosslinking densities. Thus, these surfaces would be adequate for wound healing applications, through their FGF-2 delivery. However for the application of TECEs, more optimization of heparin integration and matrix stability are required.

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7. CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Two materials types, commonly used for ophthalmic applications and currently under investigation for use in artificial cornea applications, were investigated; PDMS based materials for a keratoprosthetic devices and dendrimer crosslinked collagen gels for use as a tissue engineered corneal equivalent.

The two methodologies, direct and indirect modification, were successful for PDMS-PEO-heparin fabrication. However, direct modification method showed batch variation and highly reactive surfaces were difficult to reproduce, while indirect modification resulted in frequent surface damage and platinum poisoning but is generally thought to be more promising and overall simpler to perform. Heparin densities on the order of 1 $\mu g/cm^2$ were obtained, with uniform PEO coverage, and surface bound heparin was active. High levels of FGF-2 could be immobilized for subsequent release. Heparinized PDMS modified surfaces soaked for 1 or 3 days released 7.5% (over 20 days) and 50% (over 5 days), respectively, of initially immobilized FGF-2, indicating that slow and gradual release was possible.

Heparinized collagen crosslinked with EDC/NHS and G2 dendrimer were created. Surfaces demonstrated moderate swelling and high denaturation temperatures. These materials allowed for increased heparin retention, provided the amount of crosslinker was adequate to activate both heparin and collagen fibrils for dendrimer incorporation. FGF-2 immobilization was successful with release reaching 30% over 11 days.

Thus these materials have potential for the delivery of FGF-2 in corneal wound healing and artificial cornea applications.

7.2 Recommendations

Large variability was seen on the PDMS-PEO-HEP and PDMS-PEO-LMWH surfaces, attributable to the high variability of PEO bound to the surface and its subsequent reaction to heparin. Thus further optimization of these reaction mechanisms are required to decrease surface damage, decrease variability, and ensure consistent heparinization and subsequent FGF-2 delivery.

Heparinized collagen gels crosslinked with dendrimer require optimization of reaction kinetics, while considering heparin integration, and subsequent transparency, and overall matrix stability Further work in this area should involve investigating heparin activation with EDC/NHS prior to combining with the collagen solution. Furthermore, pre-reaction with the G2 dendrimer as in previous work may also be desirable as it would provide dendrimer heparin complexes; however optimization would be required to ensure maximum crosslinking without compromising stability.

Finally, maintenance of FGF-2 activity during release is of crucial importance. Although *in vitro* cell stimulation of released FGF-2 was not performed, there is evidence of FGF-2 instability when released without heparin (Edelman et al. 1991). Non-heparinized surfaces that adsorbed or bind FGF-2 are likely to have adsorbed and released denatured protein, incapable of stimulating the desired wound healing response. Thus the soluble FGF-2 release with heparin seen here is more likely to maintain FGF-2 activity. It is recommended that *in vitro* studies with fibroblast cells be performed to assess FGF-2 activity and the efficacy of these delivery systems.

Also of interest is the presence of heparin in release medium and its ability to increase release and stabilizing FGF-2 further (Liu et al. 2002). It would be noteworthy to conduct

release studies of that nature, to better predict *in vivo* release, as there is increased glycosaminoglycan production during corneal wound healing (Hassell et al. 1983) and the presence of free heparin is likely. In the case of heparinized collagen matrices, it may be beneficial to allow an initial release of heparin to facilitate FGF-2 stability and translocation.

8. APPENDIX A.

Reagents

Chemical	Abbreviation	Supplier	Product ID
Acetic acid, glacial		Anachemia, Kirkland Lake, ON	00565-320
Acetone		EMD Biosciences, California, USA	AX0120-2
Acetonitrile, anhydrous		Sigma-Aldrich*	271004
Albumin, bovine serum	BSA	Sigma-Aldrich*	A4503
α-allyl-ω-N-succinimidyl carbonate-poly(ethylene oxide)	Allyl-PEO-NSC	JuTian Chemical Co., Nanjing, China	MW 500
α-allyl-hydroxyl-poly(ethylene oxide)	Allyl-PEO-OH	Clariant (Canada) Inc, Markham, ON	MW 550
Basic fibroblast growth factor, human recombinant	FGF-2	Invitrogen, Burlington, ON	13256029
Basic fibroblast growth factor, human recombinant	FGF-2	R and D Systems, Minnesota, USA	233-FB-001MG/CF
Collagen, Type I		Inamed Corporation, California, USA	
Coomassie Blue Dye (G250)		Fluka, division of Sigma-Aldrich*	27815
Endotoxin free water	EFW	Sigma-Aldrich*	W3500
Ethanol, 95%	EtOH	Commercial Alcohols Inc, Brampton ON	
Heparin sodium salt	HEP	Sigma-Aldrich*	H4784
Heparin, low molecular weight	LMWH	Calbiochem, division of EMD Biosciences, California, USA	375097
n-Hexane		EMD Biosciences, San Diego, CA, USA	HX0293-1
Hydrochloric acid (1.0N)	HCl	Sigma-Aldrich*	H9892
Methanol, Anhydrous		Sigma-Aldrich*	322415
2-Methoxyethyl ether		Fluka, Sigma-Aldrich*	32210
Milk Protein, skim (dried)		Carnation, Nestle Canada Inc, North York, ON	
Molecular Sieves type 4A		Caledon Laboratory Chemicals, Georgetown, ON	5114-8

*Sigma-Aldrich, Oakville, ON.

Reagents (con't)

Chemical	Abbreviation	Supplier	Product ID
N-(3-dimethylaminopropyl-N'-ethylcarbodiimide hydrochloride	EDC	Sigma-Aldrich*	E6383
N-Hydroxysuccinimide	NHS	Sigma-Aldrich*	130672
N-N'disiccinimidyl carbonate	diNHS	Sigma-Aldrich*	225827
Platinum Catalyst, Karstedt's	Pt	Sigma-Aldrich*	479519
o-Phosphoric acid, 85%		Fisher Scientific, Ottawa, ON	A144
Polymethylhydrogen siloxane fluid [$(MeHSiO)_n$]	DC 1107	Dow Corning, Mississauga, ON	
PPI-Dendrimer (DAB-Am-8)	G2	SyMO-Chem, Eindhoven, The Netherlands	
Sodium Chloride	NaCl	Caledon Laboratory Chemicals, Georgetown, ON	7560-1
Sodium Hydroxide (1.0N)	NaOH	Sigma-Aldrich*	S2770
Stachrom® Heparin, anti-Factor Xa assay kit		Diagnostica Stago, France	00906
Sylgard 184 elastomer kit		Dow Corning, Mississauga, ON	
Triethylamine	TEA	Anachemia, Kirkland Lake, ON	94300-320
Toluidine Blue dye		Sigma-Aldrich*	T3260
Trichloroacetic acid	TCA	Fisher Scientific, Ottawa, ON	A322
Triflic Acid ($CFSO_3H$)		Sigma-Aldrich*	347817

*Sigma-Aldrich, Oakville, ON.

Buffer Solutions

Buffer	Components	Supplier
Phosphate buffer solution (PBS)	Disodium hydrogen phosphate	EM SX0720-1
	Sodium dihydrogen phosphate	EM SX0710-1
	Sodium chloride	EM SX0420-1
TRIS buffer solution	TRIS (hydroxymethyl aminomethane)	EM TX1529-1
Potassium hydrogen phosphate (KH2PO4) buffer	Potassium phophate monobasic	EMD PX1565-1
	Sodium hydroxide	EM SX0590-1

Laboratory Equipment

Equipment	Manufacturer
Attenuated total reflection Fourier Transform Infrared Spectrophotometer (FTIR-ATR)	BioRad (FTS-40; ZnSe crystal)
Differential scanning colorimetry (DSC)	TA instruments DSC 2910
Gamma Counter	
Goniometer	NRL C.A. Goniometer
Modulyo D Freeze Dryer	Thermo Savant
Microplate Reader	BioRad (Model 550)
Oil bath with stirrer and electronic temperature controller	IKA (RCT basic) and IKA (ETC-D4 fuzzy)
Vacuum Pumps	Thermo Savant VLP200; BOC Edwards RV8
Vortex	Fisher Scientific

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