DENDRIMER CROSSLINKED COLLAGEN GELS MODIFIED WITH EXTRACELLULAR MATRIX COMPONENTS

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By

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ABSTRACT

Collagen crosslinking with a polypropyleneimine octaamine dendrimers, via carbodiimide chemistry, was further exploited to demonstrate the ability of this technology for various tissue engineering strategies, including tissue engineered corneal equivalents (TECE) and blood-contacting biomaterials. In addition, modification with extracellular matrix components and other biomimetic molecules may enhance tissue-host interactions for greater *in vivo* compatibility.

First, the efficacy of the dendrimer crosslinking technology was further validated with commercially available collagen-based materials, from bovine or human sources (Chapter 4: Paper 1), as determined via transmittance, water uptake, differential scanning calorimetry, collagenase stability and *in vitro* cell compatibility. Despite gel formation, the matrix integrity was compromised with collagen-based materials manufactured under acidic conditions and purified via freeze-drying.

To continue the theme of dendrimer crosslinked collagen gels as TECE materials, growth factor incorporation was investigated with epidermal growth factor (EGF) and heparin-binding EGF (HB-EGF), as a method for improving device epithelialization and subsequent host integration. However, given the short half lives of these growth factors, an effective growth factor delivery system is necessary to protect growth factor bioactivity. As heparan sulphate proteoglycans sequester and release heparin-binding growth factors *in vivo*, the use of heparinized dendrimer crosslinked collagen (CHG) gels for HB-EGF delivery would provide prolonged, controlled delivery, while maintaining growth factor effectiveness (Chapter 5: Paper 2). HB-EGF release was prolonged and capable of inducing human cornea epithelial cell (HCEC) proliferation. Thus, HB-EGF delivery from CHG gels could aid in TECE device retention through enhanced device-host integration via epithelialization.

Alternatively, tethering EGF or HB-EGF to dendrimer crosslinked collagen (CG) gels could also supply growth factor stimulation in a manner that maintains bioactivity, while stimulating growth factor receptors continually with minute concentrations

(Chapter 6: Paper 3). Growth factor uptake and bioactivity was assessed with radiolabeled growth factor and through *in vitro* epithelial cell culture, respectively. Surface-modification of CG gels with growth factors demonstrated greater bioactivity, compared to growth factor bulk-modification of CG gels.

Finally, dendrimer crosslinked collagen gels, with pre-activated heparin (PH gels) were investigated as a tissue engineered blood-contacting biomaterial (Chapter 7: Paper 4), as we hypothesized that biomaterial induced coagulation is not only influenced by an anticoagulant surface, but also by the underlying material and that improved blood-biomaterial interactions may be achieved by utilizing a natural polymer that emulates biomimetic properties. Pre-activation of heparin was utilized to increase heparin gel content, while antithrombotic properties were evaluated via antithrombin and fibrinogen adsorption and plasma recalcification times. PH gels had increased heparinization, but extensive crosslinking compromised antithrombin-heparin interactions, compared to CHG gels. CHG gels demonstrated improved antithrombotic properties and further evaluation of these gels for blood-contacting applications is warranted.

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LIST OF ABBREVIATIONS AND SYMBOLS

AT: antithrombin
C: collagen
CG: dendrimer crosslinked collagen gel
CHG: heparinized dendrimer crosslinked collagen gel
COLL: collagen thermal gel
COOH: carboxylic acid group
ECM: extracellular matrix
EDC: N-(3-dimethylaminopropyl)-N-ethylcarbodiimide
EFW: endotoxin free water
EGF: epidermal growth factor
Fg: fibrinogen
FGF-2: basic fibroblast growth factor
G2: Polypropyleneimine octaamine generation two dendrimer
GF: growth factor
HB-EGF: heparin-binding epidermal growth factor
HC: human collagen
HCEC: human cornea epithelial cells
HCl: hydrochloric acid
hECM: human extracellular matrix
NaOH: sodium hydroxide
NH_2: amine group
NHS: N-hydroxysuccinimide
PBS: phosphate buffer saline
PC: Purecol®
PH: pre-activated heparinized dendrimer crosslinked collagen
PPI: polypropyleneimine
PPP: platelet poor plasma
RGD: Arginine-Glycine-Aspartic Acid
RGDS: Arginine-Glycine-Aspartic Acid-Serine
TECE: tissue engineering corneal equivalent
THM: time to half maximum
W_d: dry weight
W_w: wet weight
YIGSR: Tyrosine-Isoleucine-Glycine-Serine-Arginine

1 INTRODUCTION

Collagen is a natural polymer utilized as a tissue engineering substrate for cornea and blood vessel applications (Malafaya PB *et al*. 2007; Cen L *et al*. 2008). Extraction and purification of collagen results in reduced mechanical strength, and upon *in vivo* implantation, high degradation rates (Friess W. 1998). Thus techniques are needed to increase the mechanical and biological stability of collagen gels, while maintaining *in vivo* compatibility.

Collagen crosslinked, via carbodiimide chemistry, with generation two polypropyleneimine octaamine dendrimers was developed previously as an alternative method of generating corneal tissue engineering scaffolds (Duan X and Sheardown H. 2005). The amine group amplification from the dendrimers resulted in gels with high mechanical strength, good optical clarity, biological stability and high crosslinking density, compared to collagen crosslinked thermally or with other chemical crosslinkers (Duan X and Sheardown H. 2005; Duan X and Sheardown H. 2006). Furthermore, compatibility with corneal epithelial cells was enhanced with biological functionalization of cell adhesion peptides (Duan X and Sheardown H. 2007; Duan X *et al*. 2007), while heparinization of dendrimer crosslinked collagen gels led to retention and sustained delivery of basic fibroblast growth factor (Princz MA and Sheardown H. 2008).

Herein, we continue the work with dendrimer crosslinking by demonstrating the ability of this technique to crosslink other collagen-based materials, which may be used in various tissue engineering applications [Chapter 4: Paper 1]. Further evaluation of the potential of these gels was performed using a novel growth factor system, whereby heparinized dendrimer crosslinked collagen gels were utilized for delivery and release of heparin-binding epidermal growth factor (HB-EGF) for increasing human cornea epithelial cell (HCEC) proliferation, to ultimately aid in artificial cornea retention through enhanced device-host integration [Chapter 5: Paper 2]. Different methods of incorporating growth factors into dendrimer crosslinked collagen gels were examined. Specifically, covalent attachment of HB-EGF and epidermal growth factor (EGF) was achieved and the effect on human corneal epithelial cell (HCEC) proliferation was

examined [Chapter 6: Paper 3]. Finally, since heparin is an effective anticoagulant, the dendrimer crosslinked collagen gels were fabricated with heparin pre-activated with carbodiimide, for increased heparin retention [Chapter 7: Paper 4], to decrease the thrombotic nature of collagen and improve blood compatibility (Wissink MJB *et al*. 2001).

2 BACKGROUND

2.1 Collagen Type I

As one of the most abundant proteins in the body, collagen provides biological and structural support in the soft and hard connective tissues of skin, tendons, cornea, bones, cartilage, blood vessels and ligaments (Malafaya PB *et al*. 2007; Cen L *et al*. 2008). Of the 28 different types of collagen, collagen type I mediates cell adhesion via interactions with over 50 molecules including glycosaminoglycans, proteins and growth factors (DiLullo GA *et al*. 2002), and is found in the skin, tendon, bone, cornea, dentin, fibrocartilage, large vessels, intestine, and uterus (Friess W. 1998).

Type I collagen, a fibrillar collagen, is comprised of three polypeptide chains organized with a characteristic glycine-X-Y amino acid sequence (Figure 2-1), where X and Y positions are usually held by proline and hydroxyproline, respectively (Prockop DJ and Kavirikko KI. 1995).

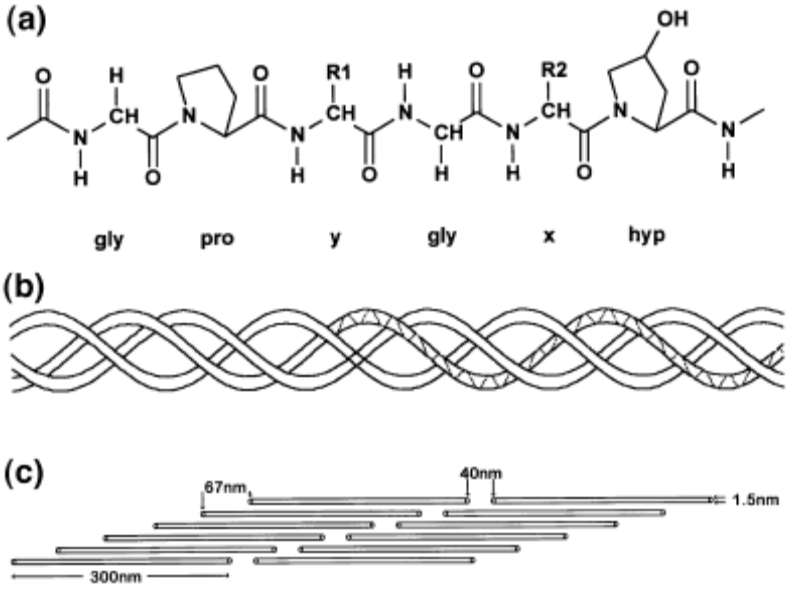

Figure 2-1. Chemical structure of type I collagen shown as (a) an amino acid sequence, (b) triple helix formation, and (c) fibril assembly (Friess W. 1998; Reproduced with permission).

Glycine facilitates the formation of a unique triple-helical structure that is stabilized by hydrogen bonds and electrostatic interactions, and following extracellular secretion

and proteomic cleavage, it self-assembles into staggered fibrils in the extracellular matrix (ECM). The ECM is a fibrillar network of proteins, proteoglycans and glycosaminoglycans, providing structural support and environmental cues that influences cellular behaviour (Taipale J and Keski-Oja J. 1997).

Collagen extraction and isolation procedures, via enzymatic digestion, cleave natural fibril crosslinks and remove telopeptide regions, resulting in a mildly immunogenic collagenous material that does not mimic its previously observed strength, and, for use in biomaterial and tissue engineering applications, it may collapse or degrade too quickly upon *in vivo* implantation (Friess W. 1998). Thus, prior to use as a tissue engineering scaffold, chemical crosslinking techniques are needed to increase the mechanical and biological stability of collagen gels, while maintaining *in vivo* compatibility.

2.2 Chemical Collagen Crosslinking

Formaldehyde, glutaraldehyde, polyepoxy, hexamethylenediisocyanate, carbodiimide and acyl azide are among the various collagen crosslinking methods that have been used to increase matrix strength (Khor E. 1997; Friess W. 1998). Formaldehyde [HCHO] reacted with lysine or hydroxylysine, with tyrosine, glutamine or asparagine, resulting in the formation of C-O-C bonds, but has demonstrated toxicity with collagen-based constructs (Ruderman RJ *et al*. 1973; Uludag H *et al*. 1999; Friess W. 1998; Madhaven K *et al*. 2010). Glutaraldehyde [HCO-$(CH_2)_n$-HCO] crosslinking formed bonds between lysine or hydroxylysine groups (Cheung DT and Nimni ME. 1982; Weadock K *et al*. 1983; Friess W *et al*. 1996). However, glutaraldehyde crosslinks were heterogeneous, brittle and cytotoxic (Cooke A *et al*. 1983; Weadock K *et al*. 1983; Huang-Lee LLH *et al*. 1990). Hexamethylene diisocyanate [OCN-$(CH_2)_6$-NCO] activated collagen lysine groups for amide bond formation (van Gulik *et al*. 1989; Khor E. 1997), while collagen carboxylic acid groups were modified to contain reactive acyl azide end groups [CON_3] that could bind amine groups (Petite H *et al*. 1990). However, collagen matrices crosslinked with hexamethylenediisocyanate, acyl azide, or glutaraldehyde, were found to be cytotoxic following implantation in rats, while collagen crosslinked with carbodiimide demonstrated decreased toxicity and degradation for enhanced tissue regeneration (van

Wachem PB *et al*. 1994). Finally, collagen crosslinking of lysine or hydroxylysine residues with a poly(epoxy) compound, 1,4-butanediol diglycidyl ether, demonstrated good biocompatibility in rats (Zeeman R *et al*. 1999; van Wachem *et al*. 1999).

2.2.1 Carbodiimide Collagen Crosslinking

Collagen crosslinking with 1-ethyl-3-(3dimethyl aminopropyl) carbodiimide (EDC) forms zero-length crosslinks within the collagen matrix (Grabarek Z and Gergely J. 1990; Olde Damink LHH *et al*. 1996). Specifically, EDC activates the carboxyl group of glutamic acid or aspartic acid on the collagen chain, forming a highly reactive O-acylisourea ester, and when stabilized with N-hydroxysuccinimide (NHS), the intermediate ester can react with an amine group on lysine, creating a stable amide bond. EDC/NHS activation of collagen carboxylic acid groups, and the subsequent amine reaction, is shown in Figure 2-2.

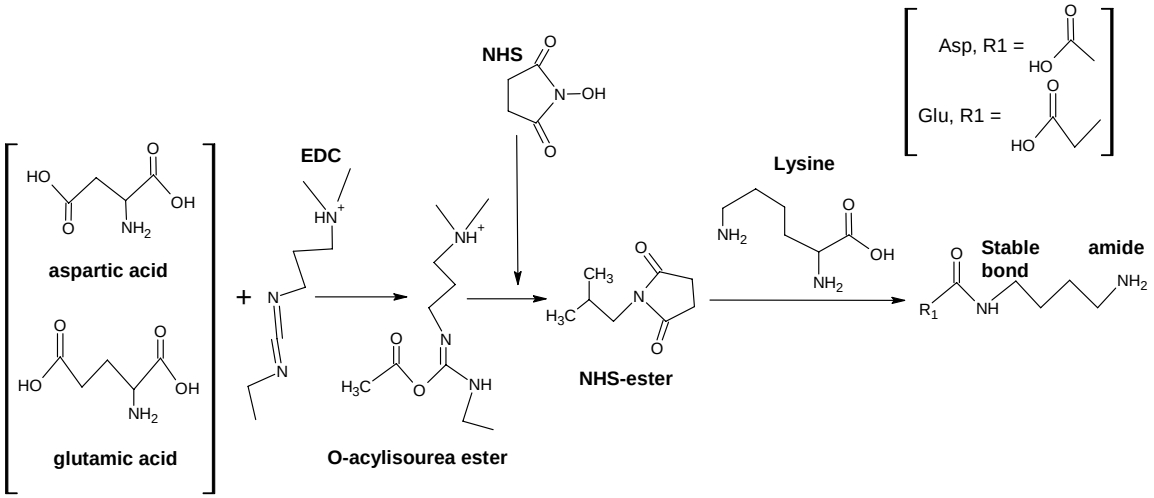

Figure 2-2. Reaction mechanism for EDC/NHS chemical activation of collagen carboxylic acid groups (Grabarek Z and Gergely J. 1990; Olde Damink LLH *et al*. 1996; Reproduced with permission). The carboxylate molecules of collagen, aspartic and glutamic acid, react with EDC to form an O-acylisourea ester, which following NHS addition, transforms into a more stable NHS-ester that can further react to form the amide complex.

Collagen EDC/NHS crosslinking is limited by the number of amine groups available within the collagen molecule, typically 30 per 1000, compared to the 120 per 1000 carboxylic acid groups (Olde Damink LHH *et al*. 1996; Duan X and Sheardown H. 2005).

It is also likely that not all carboxylic acid groups are activated by EDC, further decreasing crosslinking density. Regardless, collagen matrices fabricated in this manner have increased strength, compared to thermal gels, and have been shown to be non-toxic *in vitro* and *in vivo* (Wissink MJB *et al*. 2001).

2.2.2 Dendrimer Collagen Crosslinking

An alternative method of increasing collagen crosslinking density was developed by the Sheardown lab, whereby collagen suspensions were crosslinked, via EDC/NHS chemistry, with polypropyleneimine octaamine generation two dendrimers (Duan X and Sheardown H. 2005). The dendrimer utilized, shown in Figure 2-3, contains a 1,4 diaminobutane core and eight amine branches (Gillies ER and Frechet JMJ. 2005; Omidi Y *et al*. 2005).

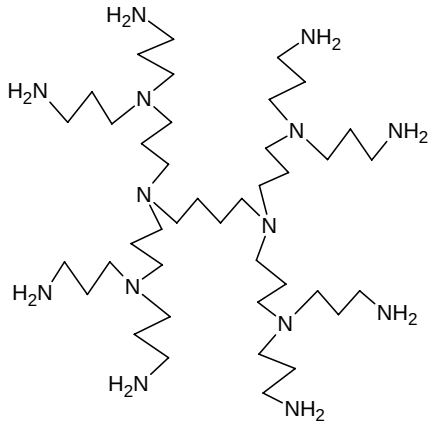

Figure 2-3. Generation two polypropyleneimine octaamine dendrimer, with a 1,4 diaminobutane core and eight amine branches (Duan X and Sheardown H. 2006; Reproduced with permission).

The amplification of amine groups within the EDC/NHS crosslinking of collagen resulted in gels with decreased water uptake, increased denaturation temperatures and increased collagenase stability, suggesting a stronger network compared to thermal and EDC/NHS crosslinked collagen gels (Duan X and Sheardown H. 2005). Additional gel characterization revealed increased transmittance, mechanical strength, and glucose permeability, which supported cornea epithelialization *in vitro* (Duan X and Sheardown H. 2006). Cornea cell compatibility of dendrimer crosslinked collagen gels was further

enhanced with biological functionalization using laminin-based cell adhesion peptides (Duan X *et al*. 2007; Duan X and Sheardown H. 2007), and heparin for the delivery of basic fibroblast growth factor (Princz MA and Sheardown H. 2008).

2.3 Heparin/Heparan Sulphate Proteoglycans

Heparin is a linear, heterogeneous glycosaminoglycan of varied length with alternating units of glucosamine and iduronic acid, commonly secreted *in vivo* by mast cells (Li JP and Vlodavsky I. 2009). Heparin is structurally very similar to the side chains of cell membrane heparan sulphate proteoglycans (HSPG), except that it is highly sulphated, as seen in Figure 2-4 (Mulloy B and Linhardt RJ. 2001).

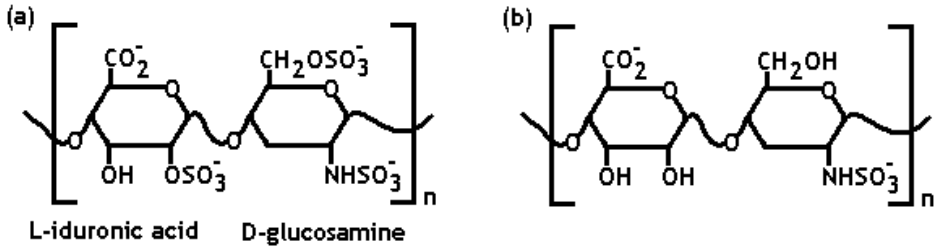

Figure 2-4. Molecular repeating units of (a) heparin and (b) heparan sulphate, illustrating the higher degree of sulphation on heparin molecules (Mulloy B and Linhardt RJ. 2001; Reproduced with permission).

Both HSPG and heparin influence development, wound healing, coagulation and inflammation *in vivo*, via binding or sequestering of various proteins (Mulloy B and Linhardt RJ. 2001; Alexopoulou AN *et al*. 2007; Li J and Vlodavsky I. 2010). Development and wound healing are mediated via HSPG/heparin interactions with various growth factors, including basic fibroblast growth factor (FGF-2) (Faham S *et al*. 1998), heparin-binding epidermal growth factor (Thompson SA *et al*. 1994), transforming growth factor beta (McCaffrey TA *et al*. 1992), vascular endothelial growth factor (Vaisman N *et al*. 1990), nerve growth factor (Lee MK and Lander AD. 1991) and platelet derived growth factor (Shing Y *et al*. 1984).

Coagulation (see Sections 2.10 & 2.11) is affected by the binding of HSPG/heparin with antithrombin (AT), and their inhibition of circulatory plasma proteins, including thrombin and factor Xa (Rosenberg RD and Damus PS. 1973; Petitou M *et al*. 2003).

Clinically, unfractionated heparin (UFH) and low molecular weight heparin (LMWH) are administered systemically for prevention and maintenance of venous and arterial thrombosis (Weitz DS and Weitz JI. 2010). UHF is a heterogeneous mixture of heparin moieties, of 10-50 saccharide units, with a molecular weight averaging 15 kDa, while LMWH is comprised of 6 to 30 saccharides, averaging 4.5 kDa in molecular weight (Day JRS *et al*. 2004). Binding of heparin to AT causes a conformal change in the molecule, resulting in increased AT anticoagulant activity (Krishnaswamy A et a. 2010). Roughly 30% of UHF and 15-25% of LMWH contain a pentassacharide sequence able to bind antithrombin and inactivate factor Xa, while a saccharide sequence of 18 is necessary for tertiary binding of heparin, antithrombin and thrombin (Weitz JI. 1997).

Inflammation is influenced by HSPG of blood vessel endothelial cells, which can facilitate leukocyte adhesion, activation and subsequent translocation from circulation (Tanaka Y *et al*. 1993; Selvan RS *et al*. 1996; Parish CR. 2006). Inflammatory cell manipulation is mediated by heparin-binding chemokines, including interleukin (IL)-1, IL-2, IL-6 (Ramsden L and Rider CC. 1992; Najjam S *et al*. 1998), IL-8 (Webb LM *et al*. 1993; Spillmann D *et al*. 1998), RANTES (Oravecz T *et al*. 1997), IL-10 (Salek-Ardakani S *et al*. 2000), and microphage inflammatory protein-1α (Stringer SE *et al*. 2002), in addition to cell adhesion molecules, such as leukocyte(L)-selectin (Norgard-Sumnicht K and Varki A. 1995) and platelet(P)-selectin (Koenig A *et al*. 1998). Following chemokine stimulation during inflammation, HSPG expression is upregulated and specific HSPG side chain motifs are expressed, which may further affect the inflammatory response by targeting certain chemokines and cells (Parish CR. 2006). However, the administration of heparin reduces inflammation by competitively binding L- and P-selectin (Nelson RM *et al*. 1993) and human platelet GMP-140 (Skinner MP *et al*. 1989), isolating them from endothelial HSPG and avoiding cell adhesion to blood vessel walls (Li J and Vlodavski I. 2010). Consequently, the clinical use of anti-inflammatory heparin therapies may not be viable due to increased bleeding risks (Li J and Vlodavski I. 2010).

HSPG/heparin-protein interactions are governed by ionic interactions between positive groups on the protein and the negatively charged sulpho and carboxyl groups on

the heparin molecule (Joung YK *et al*. 2008). Protein immobilization to HSPG/heparin can increase protein stability, protect proteins from proteolytic degradation, as is the case with heparin/HSPG binding to FGF-2 (Gospodarowicz D and Cheng J. 1986; Saksela O *et al*. 1988). Furthermore, heparin/HSPG can stabilize and enhance FGF-2-receptor dimerization and signalling (Yayon A *et al*. 1991; Forsten-Williams K *et al*. 2005), however this phenomenon has not been confirmed with all other heparin-binding proteins (Mulloy B and Rider CC. 2006).

2.3.1 Heparin & Collagen Interactions

Type I collagen contains high and low affinity binding sites capable of heparin binding (Keller KM *et al*. 1986). *In vitro*, heparin was found to influence collagen gel transparency by disrupting collagen fibril assembly and increasing collagen fibril diameter, in a dose-dependant manner whereby collagen gel opacity increased from 5-20 μg heparin per mg collagen, reaching saturation point at 160 μg heparin per mg collagen (McPherson JM *et al*. 1988). This trend was also observed with heparinized dendrimer crosslinked collagen gels fabricated with 0.2-204 μg heparin per mg collagen (Princz MA and Sheardown H. 2008).

2.4 The Cornea

The cornea, a transparent, avascular tissue at the front of the eye, serves as the eye's primary refractive element and protective pathological barrier (DelMonte DW and Kim T. 2011). The cornea consists of five layers: the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium (Beuerman RW and Pedroza L. 1996; Ojeda JL *et al*. 2001).

2.4.1 The Corneal Epithelium

The corneal epithelium is comprised of three phenotypes of non-keratinized, stratified squamous epithelial cells, arranged in 5 to 6 cellular layers (Beuerman RW and Pedroza L. 1996; DelMonte DW and Kim T. 2011). The superficial epithelial cells are connected via tight intracellular junctions, which stabilize the tear film preventing corneal dehydration, and protect posterior ocular tissues by maintaining an impenetrable barrier. The middle epithelial wing cells are the transitional layer between the superficial and

basal epithelial cells, consisting of 2 to 3 cellular layers. The basal layer consists of one single layer of round cells, capable of mitosis, which facilitate cornea cell regeneration, with each newly formed basal cellular layer pushing towards the corneal anterior, while flattening, for weekly superficial cornea cell replacement.

2.4.2 Corneal Wound Healing

Corneal wound healing, to restore tissue structure and function after injury, is a complex series of events governed by cellular interactions, apoptosis, proliferation and migration, and the production of various ECM components and cytokines (Wilson SE *et al*. 2001; Zieske JD. 2001). Epithelial injury leads to anterior keratocyte apoptosis (Wilson SE *et al*. 1996), followed by the transformation of surviving, surrounding keratocytes into a fibroblast phenotype (Fini ME. 1999). As they proliferate and migrate into the wound, they deposit a provisional matrix, consisting of proteins and proteoglycans atypical of a healthy cornea, resulting in the formation of opaque scar tissue (Fini. 1999; Zieske JD. 2001). Next, fibroblasts transform into myofibroblasts that contract the wound closed (Jester JV *et al*. 1999), followed by ECM remodeling into a normal, unwounded cornea tissue (Fini ME. 1999; Fini ME and Stramer BM. 2005). Myofibroblasts either transform back to a quiescent keratocyte phenotype, or undergo apoptosis or necrosis, to restore corneal homeostasis (Fini ME. 1999; Wilson SE *et al*. 2001). While the stromal layer heals, epithelial cells migrate and proliferate to repair the anterior corneal surface.

Throughout the corneal wound healing process, various growth factors are released by the lacrimal gland, remaining corneal cells or infiltrating inflammatory cells (Li Q *et al*. 1996; Mohan RR *et al*. 1997; Wilson SE *et al*. 1999), which regulate corneal tissue regeneration via cell proliferation, migration and apoptosis (Imanishi J *et al*. 2000; Klenkler BJ and Sheardown H. 2004).

2.5 Growth Factors

Growth factors are polypeptides, usually sequestered in the extracellular matrix (ECM), that regulate a variety of cellular functions, including ECM deposition and

degradation (Schultz GS and Wysocki A. 2008), in addition to cytokine and cell receptor regulation via cellular gene expression (Lee SJ. 2000).

2.5.1 Epidermal Growth Factor (EGF)

Epidermal growth factor (EGF), shown in Figure 2-5, consists of an acidic single chain 53 amino acid sequence, influences epithelial cell cytoskeletal actin arrangement, migration and proliferation in autocrine, paracrine and possibly juxtacrine mechanisms, in addition to initiating the secretion of certain extracellular matrix components, including fibronectin and hyaluronic acid (Carpenter G and Cohen S. 1990; Boonstra J *et al*. 1995; Schneider MR and Wolf E. 2009).

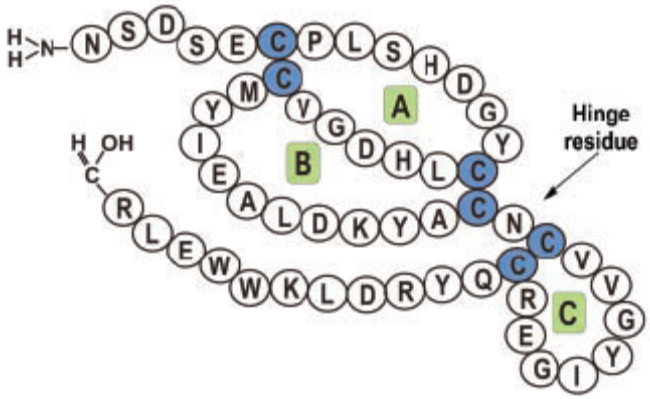

Figure 2-5. Epidermal growth factor (EGF) amino acid sequence, illustrating cysteine residues (C, blue) involved in the formation of disulphide bonds (block A, B & C) (Schneider MR and Wolf E. 2009; Reproduced with permission).

EGF is synthesized as a membrane bound protein, proEGF, over 1200 amino acids long of 140-170 kDa (Bell GI *et al*. 1986; Massague 1993). Proteolytic cleavage releases soluble EGF (Mroczkowski B *et al*. 1988). Characteristic of all EGF-family growth factors, EGF contains six cysteine residues within the sequence $CX_7CX_{4–5}CX_{10–13}CXCX_8GXRC$ (C, cysteine; G, glycine; R, arginine; X, any amino acid), facilitating the formation of three disulphide bonds between C1–C3, C2–C4 and C5–C6, which are necessary for EGF binding to its receptor (Dreux AC *et al.* 2006).

The epidermal growth factor receptor (EGFR) is a 170 kDa tyrosine kinase cell receptor, containing extracellular, transmembrane and intracellular domains, with the

latter domain containing multiple phosphorylation sites (Singh AB and Harris RC. 2005). There are 4 EGFR phenotypes capable of homo- or heterodimerization upon ligand binding, resulting in endocrine, autocrine, paracrine and juxtacrine activation of cellular pathways for cell survival, proliferation, and migration (Dreux AC *et al*. 2006). All EGFR phenotypes are found within in the corneal epithelium, and EGFR signaling is greatly involved in corneal homeostasis, while EGFR ligands are active in corneal wound healing (Ahmadi AJ and Jakobiec FA. 2002; Yu FX *et al*. 2010). EGF binds the EGRF receptor ErbB1 at both low and high affinity binding sites (Carpenter G *et al*. 1978; Prywes R *et al*. 1986; Massague J and Pandiella A. 1992).

EGF is found within numerous tissues including the submaxillary gland, lacrimal gland, kidney, thyroid gland and pancreas (Imanishi J *et al.* 2000). Within the eye, EGF is expressed by epithelial, endothelial and keratocyte cells (Wilson SE *et al.* 1994b; Ahmadi AJ and Jakobiec FA. 2002). Following corneal injury, EGF messenger RNA is increased in the lacrimal gland, resulting in augmented EGF concentrations in tears (Wilson SE *et al.* 2001; Sheardown H and Cheng YL. 1996). EGF released from the lacrimal gland, epithelium and stroma can induce epithelial cell proliferation, influencing corneal wound healing (Wilson, Liang, and Kim. 1999; Wilson SE *et al*. 1999).

2.5.2 EGF & Corneal Epithelial Cells

EGF stimulates corneal epithelial cell proliferation and migration, which can be enhanced in the presence of fibronectin, transforming growth factor-beta and reactive oxygen species (Wang X *et al*. 1994; Wilson SE *et al*. 1994a; Wilson SE *et al*. 1994b; Nishida T *et al*. 1992; Sheardown H *et al*. 1997; Nakamura M and Nishida T. 1999). *In vitro* EGF has been shown to stimulate cornea epithelial cell proliferation in a dose-dependant manner from 0.1 to 10 ng/mL (Hongo M *et al*. 1992; Wilson SE *et al*. 1994a; Imanishi J *et al*. 2000), while higher EGF concentrations did not appear to enhance proliferation (Hongo M *et al*. 1992). Epithelial cell migration was affected at concentrations of 10-50 ng/mL (Grant MB *et al*. 1992; Wilson SE *et al*. 1994a). Topical administration of EGF accelerated epithelial wound closure, resulting in decreased healing times (Boisjoly HM *et al*. 1993; Sheardown H *et al*. 1993; Baldwin HC and

Marshall J. 2002; Yu FX *et al*. 2010), while continuous delivery of EGF for 8 hours from polyacrylic acid polymer significantly augmented corneal wound healing in rabbits (Sheardown H *et al*. 1997).

2.5.3 Heparin-binding Epidermal Growth Factor (HB-EGF)

Heparin-binding epidermal growth factor (HB-EGF) is a 14-22 kDa protein capable of binding heparan sulphate proteoglycans and heparin (Nishi E and Klagsbrun M. 2004). Initially synthesized as a 208 amino acid transmembrane protein, shown in Figure 2-6, the HB-EGF precursor interacts with epidermal growth factor receptors, heparan sulphate proteoglycans, membrane protein CD9, diphtheria toxin, and integrin α3β1 as a juxtacrine growth factor (Raab G and Klagsbrun M. 1997; Iwamoto R and Mekada E. 2000).

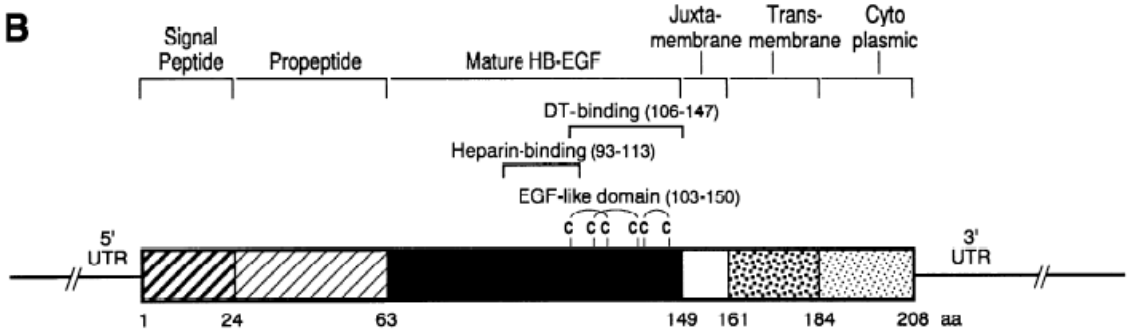

Figure 2-6. HB-EGF domain structure, illustrating mature HB-EGF, the EGF-like domain sequence and the heparin-binding domain (Raab G and Klagsbrun M. 1997; Reproduced with permission).

Following proteolytic ectodomain shedding, the soluble, mature form of HB-EGF contains an EGF-like domain (amino acids 103-150) and heparin-binding motif (amino acids 93-113), which facilitates EGFR and heparan sulphate proteoglycan binding, initiating cell signaling cascades in autocrine and paracrine mechanisms (Fang L *et al*. 2001; Nishi E and Klagsbrun M. 2004). HB-EGF can bind EGFR phenotypes ErbB1 (Higashiyama S *et al*. 1992) and ErbB4 (Elenius K *et al*. 1997). In addition, the cytoplasmic end of proHB-EGF, following mature HB-EGF shedding, can translocate to the cell nucleus and initiate gene replication (Nanba D and Higashiyama S. 2004).

HB-EGF has been implicated in heart and lung development, wound healing, smooth muscle cell hyperplasia, brain injury and cancer, via its influence on enhanced proliferation of epithelial cells, fibroblasts and smooth muscle cells, but not endothelial cells (Davis-Fleischer KM and Besner GE. 1998; Nishi E and Klagsbrun. 2004).

2.5.4 HB-EGF & Corneal Epithelial Cells

In vitro, HB-EGF augmented human corneal epithelial cell proliferation and migration (Wilson SE *et al*. 1994a), while wounded rabbit epithelial cells had accelerated healing following HB-EGF stimulation (Block ER *et al*. 2004). Migration of injured human cornea epithelial cells *in vitro* was hindered by HB-EGF inhibitors, and rescued following HB-EGF addition (Xu KP *et al*. 2004a; Xu KP *et al*. 2004b; Yin J and Yu FS. 2008). More recently, keratinocyte-specific HB-EGF deficient mice with corneal wounds demonstrated delayed wound healing, determined to be a result of decreased cell migration and attachment, but not proliferation, to which the authors suggest may be due to the absence of proHB-EGF (Yoshioka R *et al*. 2010). Furthermore, enhanced wound healing of corneal epithelial cells was observed following administration of HB-EGF, compared to EGF, following 2 minutes of growth factor exposure (Tolino MA *et al*. 2011).

2.6 Growth Factor Delivery

Growth factor delivery has been investigated for manipulating tissue structure, restoration, and regeneration by creating an artificial environment capable of stimulating desired cellular activities (Lutolf MP and Hubbell JA. 2005; Saltzman WM and Olbricht WL. 2002). Delivered growth factor concentrations must be able to stimulate the desired response of target cells or tissue, while avoiding growth factor cell receptor down-regulation, which would hinder growth factor effectiveness (Nimni ME. 1997).

Systemic growth factor administration, either intravenously or orally, is not an effective growth factor delivery method, given the high dosages and frequent injections that would be necessary to maintain a therapeutic window, and the risk of increased, undesirable side effects with growth factor pluripotency. (Baldwin SP and Saltzman WM.

1998; Lee SJ. 2000; Tabata Y. 2003). Furthermore, growth factors are prone to rapid proteolytic cleavage and thus have short circulatory half-lives.

As such, localized, controlled and long-term growth factor delivery systems are desirable (Babensee JE *et al*. 2000; Baldwin SP and Saltzman WM. 1998). The delivery mechanism, release profile and target tissue, in relation to growth factor characteristics of charge, molecular size, biological half-life and required dosage must be considered in engineering an appropriate delivery system (Sinha VR and Trehan A. 2003). Of equal importance is the preservation of growth factor biological stability during device manufacturing, installation and usage, and following release, as denatured growth factors may cause adverse immunogenic responses, compromising the delivery system integrity and potentially altering cellular responses (Tabata Y. 2003; Sinha VR and Trehan A. 2003).

Current soluble growth factor delivery methods include encapsulation or entrapment of the protein from reservoir devices, microspheres and hydrogels, and tethering proteins directly to a substrate (Baldwin SP and Saltzman W. 1998). Many delivery systems are based on natural polymers, including albumin, alginate, chitosan, collagen, dextran, fibrin, gelatin, heparin, and hyaluronic acid (Uebersax L *et al.* 2009), as growth factor activity is tightly regulated *in vivo* through extracellular matrix interactions (Taipale J and Keski-Oja J. 1997).

2.6.1 Collagen-based Soluble Growth Factor Delivery Systems

Collagen-based delivery systems were utilized to deliver bone morphogenetic protein (Takaoka K *et al*. 1991), platelet derived growth factor-BB and insulin-like growth factor-1 (Wells MR *et al*. 1997) for tissue regeneration in rats. Type I collagen matrices delivered basic fibroblast growth factor, heparin-binding epidermal growth factor, hepatocyte growth factor, insulin like growth factor-1, platelet derived growth factor-BB, and vascular endothelial growth factor (Kanematsu A *et al*. 2004), while elsewhere collagen type I was able to bind and maintain bioactivity of basic fibroblast growth factor (Kanematsu A *et al*. 2004) and hepatocyte growth factor (Marui A *et al*. 2005). To increase scaffold strength, collagen was combined with hydroxyapatite, for the delivery

of transforming growth factor-beta1 (Sun JS *et al*. 2003), bone morphogenetic protein (Itoh S *et al*. 2001) and nerve growth factor (Letic-Gavrilovic A *et al*. 2003).

2.6.2 Heparinized Soluble Growth Factor Delivery Systems

To further maintain growth factor bioactivity and influence release rates, stabilizing agents are frequently added, either during fabrication, storage or delivery, (Sinha VR and Trehan A. 2003; Baldwin SP and Saltzman WM. 1998). One such stabilizing agent utilized in protein delivery is heparin, given the known binding interactions between some growth factors and heparin/heparan sulphate (Mulloy B and Rider CC. 2006; Joung YK *et al*. 2008). Thus, heparinized delivery systems that bind, sequester, and deliver growth factors in a prolonged and controlled manner are acceptable (Silva AKA *et al*. 2009).

Heparinized growth factor delivery systems have been used extensively with basic fibroblast growth factor, transforming growth factor beta, vascular endothelial growth factor, nerve growth factor and platelet derived growth factor (Joung YK *et al*. 2008; Zhang S and Uladag H. 2009). Heparin has been conjugated to numerous synthetic or natural scaffolds, and/or microspheres, including but not limited to poly(lactic-co-glycolic acid), poly(ethylene glycol), fibrin, alginate, and collagen (Silva AKA *et al*. 2009).

2.6.3 Heparin-Collagen Soluble Growth Factor Delivery Systems

Collagen, heparin and transforming growth factor beta mixtures were utilized for connective tissue regeneration *in vivo* (Schroeder-Tefft JA *et al*. 1997). Matrices of heparin and collagen, crosslinked via carbodiimide chemistry, demonstrated enhanced growth factor bioactivity *in vivo*, when utilized for the delivery of basic fibroblast growth factor (Wissink MJB *et al*. 2000; van Wachem PB *et al*. 2001; Pieper JS *et al*. 2002), vascular endothelial growth factor (Steffens GC *et al*. 2004; Yao C *et al*. 2006), platelet derived growth factor (Sun B *et al*. 2009), stromal cell-derived factor 1 alpha (Bladergroen BA *et al*. 2009), and co-delivery of basic fibroblast growth factor and vascular endothelial growth factor (Nillesen STM *et al*. 2007). Similarly, vascular endothelial growth factor delivered from a heparin-collagen coating increased cell proliferation *in vitro* (Wolf-Brandstetter C *et al*. 2006). Heparinized dendrimer

crosslinked collagen gels delivered basic fibroblast growth factor, releasing 40% of the growth factor over 2 weeks (Princz MA and Sheardown H. 2008), while collagen-hydroxyapatite heparinized matrices, crosslinked with EDC/NHS, delivered bone morphogenic protein-2 for one week (Teixeira S *et al*. 2010).

2.7 Growth Factor Immobilization

Growth factor immobilization may be advantageous in controlling protein concentration via prevention of diffusion to surrounding tissues, cell uptake and degradation, and providing prolonged activation of cell signaling (Kuhl PR and Griffith-Cima LG. 1996). Covalent protein attachment to various substrates has been achieved through step-wise, bulk or micropatterning techniques, whereby growth factors are bound directly or via spacer chains to the substrate, or modified via protein-engineering to control substrate attachment (Ito Y. 2008).

Tethered growth factors have demonstrated increased biological stability and availability *in vitro*, compared to soluble or physically adsorbed protein (Baldwin SP and Saltzman WM. 1998; Kobsa S and Saltzman WM. 2008). Growth factor biological activity following covalent attachment relies on the protein conformation, as the growth factor must maintain receptor activity. However, the observed cellular response of immobilized growth factors could differ from cell exposure to soluble growth factor, as there would be continual receptor stimulation and no internalization of the protein (Masters KS. 2008). Transforming growth factor beta-2 (Bentz H *et al*. 1998; Mann BK *et al*. 2001; Merrett K *et al*. 2003), basic fibroblast growth factor (Stompro BE *et al*. 1989; DeLong SA *et al*. 2005; Nur-E-Kamal A *et al*. 2008), nerve growth factor (Gomez N and Schmidt CE. 2006), platelet-derived growth factor-BB (Saik JE *et al*. 2001), vascular endothelial growth factor (Shen YH *et al*. 2008; Leslie-Barbick JE *et al*. 2011a) and a vascular endothelial growth factor mimetic peptide (Leslie-Barbick JE *et al*. 2011b) are among the growth factors tethered either directly or via poly(ethylene) glycol spacers to various synthetic or natural polymers.

2.7.1 Immobilized EGF

EGF has a plasma half life of only 2.52 minutes; hence immobilization may prolong its bioactivity (Baldwin SP and Satlzman MV. 1998). EGF immobilization via star poly(ethylene oxide) to aminosilane-modified glass demonstrated growth factor activity through rat hepatocyte proliferation and ECM production, in a similar fashion to soluble EGF (Kuhl PR and Griffith-Cima LG. 1996). Poly(dimethyl siloxane) was modified with poly(ethylene) glycol (PEG) spacers for EGF attachment and demonstrated bioactivity with human cornea epithelial cells proliferation and adhesion (Klenkler BJ *et al*. 2005; Klenkler BJ and Sheardown H. 2006; Klenkler BJ *et al*. 2010). Amine-terminated PEG was combined with polycaprolactone for EGF tethering, and supported human primary keratinocyte growth and stimulated wound healing of diabetic ulcer *in vivo* (Choi JS *et al*. 2008).

Other spacer tethering mechanisms include EGF bound to a biotin-thiazolidine ring capable of binding streptavidin (Wade JD *et al*. 2002), to an amine-reactive ester and phenyl azide heterobifunctional crosslinker on polystyrene (Stefonek-Puccinelli TJ and Masters KS. 2009), to amine-modified polycaprolactone, and to gelatin nanofibrillar scaffolds via suberic acid bis(N-hydroxyl-succinimide ester) (Tigli RS *et al*. 2011).

Although the use of spacer molecules can achieve specific protein orientation and improve the stability and availability of the growth factor to its receptor through reduced steric hindrance, it can also result in heterogeneous surface coverage and non-specific adsorption of the growth factor (Liberelle B *et al*. 2010). Furthermore PEGylated growth factors may not elicit the desired cellular response, as EGF covalently attached to PEG maintained proliferative activity, but required the cell adhesion peptide sequence, Arginine-Glycine-Aspartic acid-Serine (RGDS), to enhance migration in one study (Gobin AS and West JL. 2003). Also, soluble EGF stimulated keratinocyte proliferation to a greater extent than tethered EGF, while the opposite was observed with keratinoctye migration (Stefonek-Puccinelli TJ and Masters KS. 2009).

2.8 Blood Coagulation

When a tissue is injured, the biological response of acute inflammation, granular tissue and scar formation, and tissue regeneration, begins with the affected area being flooded with blood, and the initiation of the coagulation cascade (Ratner BD and Bryant SJ. 2004). Blood coagulation is orchestrated by numerous serum proteins, also known as coagulation factors, which undergo a cascade of proteolytic reactions resulting in fibrin clot formation, as illustrated in Figure 2-7 (Hanson SR and Harker LA. 1996; Gorbet MB and Sefton MV. 2004). Coagulation is initiated by the activation of either the intrinsic or extrinsic coagulation pathway.

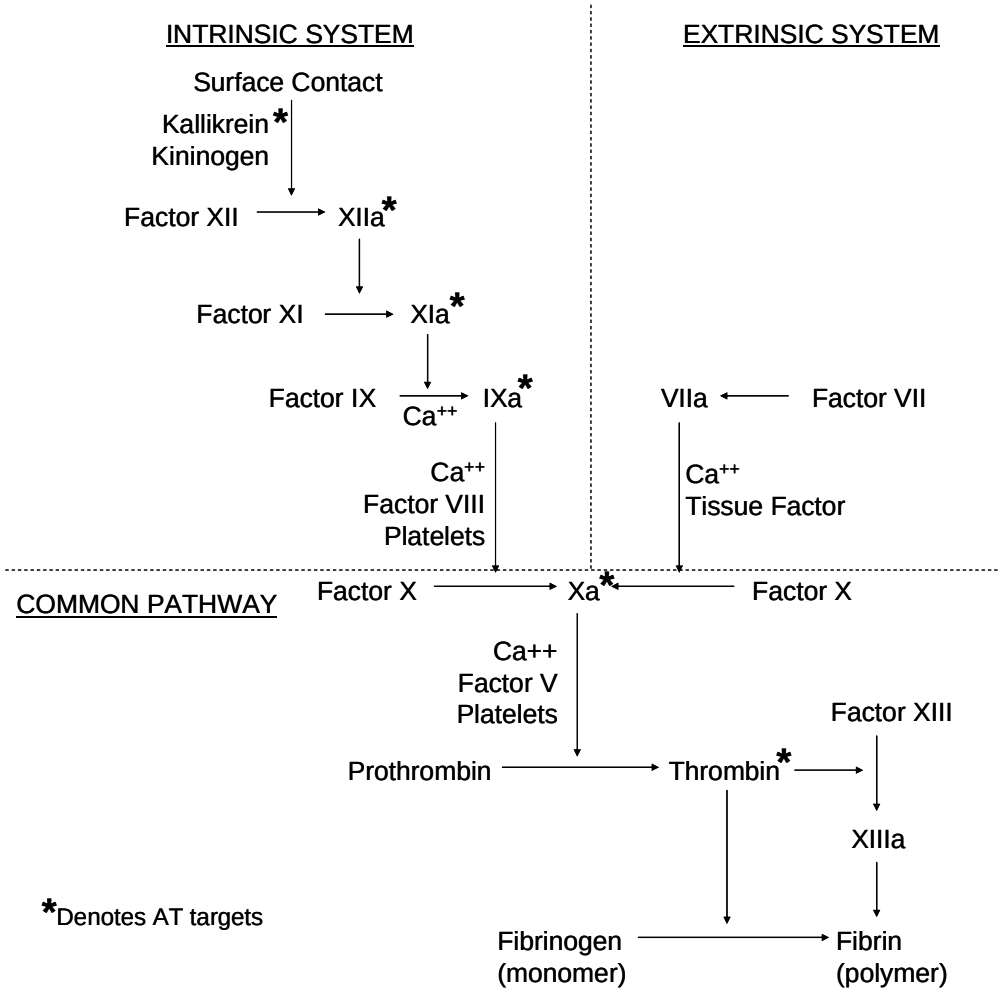

Figure 2-7. The coagulation cascade, illustrating AT targets (*) (Adapted from Hanson SR and Harker LA. 1996; Krishnaswamy A *et al.* 2010; Reproduced with permission).

Initiation of the intrinsic pathway is via contact activation, or adsorption, of factor XII to a surface, usually negatively charged, which facilitates its activation into factor XIIa (Colman RW. 1993). Factor XIIa converts prokallikrein to kallikrein, and together with high molecular weight kininogen (HMWK), activates factor XI (Hanson SR and Harker LA. 1996; Gorbet MB and Sefton MV. 2004). The extrinsic pathway is initiated via activation of factor VII (FVII), following its conjugation to tissue factor (TF), which is expressed on damaged endothelial cells or activated monocytes/macrophages/platelets at the site of vascular injury (Colman RW. 1993; Markiewski MM *et al*. 2007). The TF-FVII complex can be activated by thrombin, factor IXa, factor Xa and other TF-VIIa complexes (Gorbet MB and Sefton MV. 2004). The intrinsic and extrinsic pathways merge to a common pathway with the activation of factor X, whereby factor Xa converts prothrombin to thrombin, while thrombin facilitates the transformation of fibrinogen to fibrin, resulting in fibrin clot formation, which is further polymerized by factor XIII (Hanson SR and Harker LA. 1996).

2.9 Anticoagulation Mechanisms

In undamaged vascular tissue, coagulation is regulated by an intact endothelium; circulating plasma protease inhibitors, such as antithrombin, protein C, and protein S; the fibrinolytic system of plasminogen conversion to plasmin, which dissolves fibrin clots; and systemic clearance or dilution of activated plasma proteins via the liver or as a consequence of blood flow (Colman RW. 1993).

2.9.1 Antithrombin (AT)

The plasma protease inhibitor, antithrombin (AT) is a 58 kDa molecule, comprised of 432 amino acids, circulating in plasma at a concentration of 150 μg/mL, with a 3 day half-life (Quinsey NS *et al*. 2004; Rau JC *et al*. 2007). It is a major regulator of the coagulation cascade through inhibition of thrombin, factor IXa, factor Xa, factor XIIa and kallikrein, as illustrated in Figure 2-7 (Krishnaswamy A *et al*. 2010).

The antithrombotic activity of AT is increased upon binding to heparin, via a specific pentasaccharide sequence, found within 1/3 of unfractionated heparin formulations. This induces a molecular conformational change in AT and enhances its affinity for proteases

(Olson ST *et al*. 2010). AT activity is further increased by longer heparin chains, longer than 18 monosaccharide units, which facilitate binding with thrombin, factor Xa or factor IXa (Huntington JA. 2011). Following heparin induced AT-thrombin binding, heparin can be released and facilitate further AT-thrombin inhibition (Day JRS *et al*. 2004).

2.10 Blood-Material Interactions

Upon implantation of any foreign material into vascularized tissue, proteins non-specifically adsorb onto the substrate, typically leading to adhesion and activation of platelets and leukocytes, coagulation and complement activation (Sefton MV *et al*. 2000). Thrombus formation following material implantation can initiate either the intrinsic or extrinsic coagulation pathways, via protein adsorption, and/or monocytes/macrophage or damaged endothelial cell expression of TF (Gorbet MB and Sefton MV. 2004). Protein adsorption is rapid, with much research focused on fibrinogen adsorption (Courtney JM *et al*. 2004).

2.10.1 Fibrinogen (Fg)

Fibrinogen (Fg), a 340 kDa plasma protein, is regarded as a crucial protein in blood coagulation and blood-material interactions given its dual roles in fibrin clot formation and platelet adhesion via the glycoprotein receptor GPIIb-IIIa (Bennett JS. 2001; Geer CB *et al*. 2007). Fg adsorption and fibrin polymerization onto blood-contacting biomaterials differs according to surface charge and hydrophobicity. Despite similar Fg adsorption amounts on hydrophobic and negatively charged surfaces, the clot structures have been shown to differ according to the fibrin layers and corresponding crosslinking network (Evans-Nguyen K and Schoenfisch MH. 2005). These differences in clot formation may be due to protein orientation upon adsorption, as Fg adsorption amounts did not correlated to platelet adhesion (Wu Y *et al*. 2005). In addition, adsorbed Fg may be replaced by HMWK over time, and initiate the intrinsic coagulation cascade (Gorbet MB and Sefton MV. 2004).

2.11 Tissue Engineering

The multidisciplinary field of tissue engineering aims to restore, maintain, improve, or replace damaged tissue via direct cell transplant therapies, tissue-inducing scaffolds, or

cell containing scaffolds (Langer R and Vacanti JP. 2003). Current tissue engineering therapies rely on manufacturing compatible 3-dimensional scaffold biomaterials, whether synthetic or natural, with appropriate biomechanics for sustained cellularization at the implantation site. The scaffold must control cell behaviour, promote low immunogenic responses and increase vascularization, if necessary, to promote *de novo* tissue regeneration (Langer R. 2000). Specifically, biomaterial modification techniques include surface or bulk manipulation to influence porosity, roughness, mechanical strength, degradation, and cell-scaffold interactions via incorporation or delivery of growth factors or cell adhesion peptides (Lavik E and Langer R. 2004).

Natural biomaterials, typically comprised of protein, polysaccharide or polyhydroxyalkanoates origins, are deemed advantageous for their cell compatibility, low cost and availability and ability to mimic cell microenvironments, despite batch-to-batch variation and weak mechanical properties (Malafaya PB *et al*. 2007). However, enhanced isolation, purification and crosslinking techniques to control and predict biomaterial properties may improve those disadvantages. Collagen has been utilized in numerous tissue engineering applications, including skin or dermal dressings, bone, cartilage, nerve, bladder, renal, and cornea (Yang C *et al*. 2004; Malafaya PB *et al*. 2007; Cen L *et al*. 2008; Fagerholm P *et al*. 2009).

2.12 Tissue Engineered Corneal Equivalents (TECE)

A major cause of vision loss is corneal blindness, as a result of corneal disease, trauma, scarring or ulceration (Whitcher JP *et al*. 2001). Corneal allograft surgery, whereby donor tissue is implanted into the host, is met with risks of disease transmission and an increasing shortage of donor tissue; thus, there is a need for artificial corneal substitutes (Carlsson DJ *et al*. 2003; Chirila TV *et al*. 1998; Duan D *et al*. 2006; Griffith M *et al*. 2002).

Tissue-engineered cornea equivalents (TECE) are artificial corneal implants based on cellular repair strategies, whereby corneal cells are grown and encouraged to produce their own extracellular matrix, or scaffolds are fabricated with extracellular matrix components and combined with corneal cells (Duan D *et al*. 2006; Shah A *et al*. 2008).

The ideal artificial corneal substitute must be comparable to the native cornea in transparency and strength, be non-toxic, interact seamlessly with the host's remaining corneal tissue to promote cellular communication, and provide a semi-permeable membrane for oxygen and nutrient diffusion (Chirila TV *et al*. 1998; Griffith M *et al*. 2006; Duan D *et al*. 2006). Much interest has surrounded collagen-based TECE, since the corneal extracellular matrix contains predominantly type I collagen (Griffith M *et al*. 2002; Meek KM and Boote C. 2004).

2.12.1 Collagen-Based TECE

Lyophilized, dehydrothermally crosslinked collagen sponges, fabricated from insoluble bovine collagen, supported human primary corneal cell growth *in vitro* (Orwin EJ and Hubel A. 2000), and demonstrated increased strength and transparency, when fabricated with chondroitin sulphate proteoglycans (Orwin EJ *et al*. 2003), higher insoluble collagen concentrations (Crabb RAB *et al*. 2006), or soluble collagen with UV crosslinking (Crabb RAB and Hubel A. 2008). However, further optimization of strength and transparency is necessary to better mimic the native human cornea (Ruberti JW and Zieske JD. 2008).

The Laboratoire d'Organogenese Experimentale (LOEX) 3-D collagen-based TECE was created by a self-assembly approach, whereby sheets of human primary corneal epithelial, fibroblast and endothelial cells were layered *in vitro* (Germain L *et al*. 2000; Proulx S *et al*. 2010). Corneal constructs demonstrated histological and immunofluorescent staining similar to native human corneas, despite a thinner stromal layer (Proulx S *et al*. 2010). Minor opacity of the construct was observed, deemed to be from an unorganized stromal collagenous matrix. Whether the construct mimics corneal strength remains to be verified, and filter paper anchorage rings were necessary to facilitate device handling (Duan D *et al*. 2006; Proulx S *et al*. 2010).

Phospholipase A2 was utilized to decellularize porcine corneal tissue, which did not alter collagen fibrils and only 80% of proteoglycans, and were compatible with cornea cells *in vitro*, and rabbit corneas *in vivo* (Wu *et al*. 2009). Scaffolds were later lyophilized

to manipulated pore size and permeability for enhanced keratocyte infiltration (Xiao J *et al*. 2011).

Griffith *et al*. have developed a series of collagen-based TECE. Collagen, containing chondroitin sulphate, was crosslinked with glutaraldehyde for increased transparency and strength, compared to thermally crosslinked collagen, and was compatible with immortilized cornea cells *in vitro* (Doillon CJ *et al*. 2003), and was tolerated following preliminary implantation into canine eyes (Bentley E *et al*. 2010). However, the biostability and cytotoxicity of glutaraldehyde may be problematic (Cooke A *et al*. 1983; Weadock K *et al.* 1983; Huang-Lee LLH *et al.* 1990; Duan X and Sheardown H. 2005). Collagen crosslinked with an N-isopropyl acrylamide copolymer exhibited adequate clarity, the ability to incorporate cell adhesion peptides, and good *in vivo* porcine and dog compatibility, but did not have mechanical properties comparable to the native cornea (Li F *et al*. 2003; Li F *et al*. 2005; Bentley E *et al*. 2010). Porcine collagen (Liu Y *et al*. 2006a; Liu Y *et al*. 2006b; Lagali NS *et al*. 2007; McLaughlin CR *et al*. 2008) and recombinant human collagen, crosslinked with EDC/NHS, demonstrated good optical clarity, nutrient diffusion, and tissue regeneration following animal implantation (Liu W *et al*. 2008; Merrett K *et al*. 2008). To increase device strength, human collagen-based TECE were also prepared with chitosan and poly(ethylene) glycol (Rafat M *et al*. 2008), a glycopolymer (Merrett K *et al*. 2009; Deng C *et al*. 2010), and phosphorylcholine (Liu W *et al*. 2009; McLaughlin CR *et al*. 2010; Hackett JM *et al*. 2011), and all demonstrated good *in vivo* compatibility. Human implantation of human recombinant collagen type III crosslinked with EDC/NHS, following anterior lamellar keratoplasty in 10 patients, demonstrated improved visual acuity, ocular surface quality and corneal sensitivity after 7 months (Fagerholm P *et al*. 2009) and corneal epithelialization and innervation after 2 years (Fagerholm P *et al*. 2011), further validating the usage of collagen-based acellular TECE. However, surgical suturing methods deemed necessary to secure the TECE of inadequate ocular strength, hindered optical clarity.

2.12.2 Dendrimer Crosslinked Collagen TECE

Collagen gels were crosslinked with amine-terminated polypropyleneimine octaamine generation two dendrimers as a TECE. The amplification of amine groups resulted in collagen-based gels with improved crosslinking density, mechanical strength, good optical clarity, and biological stability, compared to thermally, EDC/NHS and glutaraldehyde crosslinked collagen gels, and demonstrated corneal epithelial cell compatibility *in vitro* (Duan X and Sheardown H. 2005; Duan X and Sheardown H. 2006). Following gel functionalization with laminin-based adhesion peptides, the dendrimer crosslinked collagen-based gels supported epithelial cell stratification, adhesion and proliferation and neurite extension from dorsal root ganglia (Duan X and Sheardown H. 2007; Duan X *et al*. 2007). Further gel development included the incorporation of heparin for basic fibroblast growth factor uptake and delivery, ultimately to enhance stromal integration following TECE implantation (Princz MA and Sheardown H. 2008); although *in vitro* research was not conducted with heparinized gels to verify cell compatibility. In addition, as dendrimer crosslinked collagen-based gels have not been examined *in vivo*, corneal tissue and inflammatory response to these materials is unknown, as is whether they will preserve corneal architecture and function.

2.13 Tissue-Engineered Blood-Contacting Biomaterials

The success of tissue-engineered blood-contacting biomaterials, such as tissue engineered blood vessels or related devices, relies on blood compatibility and accordance with the coagulation cascade. To prevent activation of the coagulation cascade upon biomaterial implantation, substrates have been modified with antithrombic agents or non-fouling peptides to prevent non-specific protein adsorption, encourage the adsorption of proteins that favour a non-thrombogenic response, activate plasma inhibitors of the coagulation pathways, or initiate the fibrinolytic system for thrombi dissolution (Courtney JM and Forbes CD. 1994; Courtney JM *et al*. 1995; Brash JL. 2000). Furthermore, some tissue engineered blood-contacting biomaterials utilize biomimetic strategies, whereby extracellular matrix or cellular components are incorporated to emulate native tissue blood vessels (Ratner BD. 2004). Biomimetic strategies include mimicking the

endothelium through incorporation of lipid and cell membrane proteins, including glycosaminoglycans or promoting endothelialization with extracellular matrix components or cell adhesion peptides, including collagen (Tanzi MC. 2005; Jordan SW and Chaikof EL. 2007).

2.13.1 Collagen-Based Blood-Contacting Biomaterials

Despite having prothrombotic properties (Parsons TJ *et al*. 1983; Heemskerk JWM *et al*. 2005), collagen has been utilized in blood-contacting biomaterial applications. Nanofibres of collagen and poly(lactic acid)-co-poly(epsilon-caprolactone), intended for vascular tissue engineering, were fabricated to mimic an extracellular matrix architecture and support endothelial cell adhesion and antithrombotic phenotype (He W *et al*. 2005). Elsewhere, collagen and chitosan nanofibrous scaffolds demonstrated increased strength and endothelial and smooth muscle cell compatibility (Chen ZG *et al*. 2010). Collagen-based tissue engineering constructs seeded with human umbilical vein endothelial cells demonstrated reduced platelet adhesion and activation, compared to pure collagen constructs, although leukocyte activation and plasma recalcification times were not affected (McGuigan AP and Sefton MV. 2008). Thus despite cell compatibility and promotion of endothelial antithrombotic properties, thrombosis still requires maintenance.

2.13.2 Heparinized Blood-Contacting Biomaterials

Heparin has been widely used as a released or tethered anticoagulant to create more blood compatible antithrombotic surfaces (Brash JL. 2000; Mulloy B and Linhardt RJ. 2001; Zhou Z and Meyerhoff ME. 2005). Heparin conjugation methods are appealing to ensure long-term thrombolysis, given the rapid circulatory half-life of heparin, and to avoid adverse side effects (Weitz DS and Weitz JI. 2010). As summarized in Table 2-1, heparin has been directly adsorbed, tethered, or released from various substrates. In addition, to reduced thrombosis, heparinized surfaces were combined with a non-fouling aspect following immobilization to poly(ethylene) glycol (PEG) or PEO spacers.

Table 2-1. Heparinized Blood-Contacting Biomaterials.

Heparinized Blood-Contacting Biomaterials		
Incorporation Method	**Base Material**	**Reference(s)**
Bound and released from	Cellulose	(Hinrichs WLJ *et al.* 1997)
	Chitosan-poly(ethylene-vinyl)-acetate	(Vasudev SC *et al.* 1997)
Released from	Poly(N-isopropylacrylamide) and poly(etherurethane)-urea block copolymer	(Gutowska A *et al.* 1995)
Tethered to	Poly(ethylene oxide)	(Tay SW *et al.* 1989)
	Poly(vinyl acetate)	(Smith BAH and Sefton MV. 1992); (Smith BAH and Sefton MV. 1993)
	Poly(ethylene oxide)-Poly(dimethylsiloxane) block copolymers	(Grainger DW *et al.* 1990)
	Poly(vinylsiloxane)-poly(ethyleneimine)	(Blezer R *et al.* 1998)
	Polypropylene	(Tyan YC *et al.* 2002)
	Glass	(Keuren JFW *et al.* 2003)
	Polyethylene	(Cornelius RM *et al.* 2003)
	Poly(lactic acid)	(Jee KS *et al.* 2004)
	Poly(dimethylsiloxane)	(Olander B *et al.* 2003); (Thorslund S *et al.* 2005)
Tethered via PEG or PEO	Poly(ethylene)	(Yuan S *et al.* 1993)
	Polystyrene	(Byun Y *et al.* 1996)
	Poly(ethylene-terephthalate)	(Kim YJ *et al.* 2000)
	Poly(dimethylsiloxane)	(Chen H *et al.* 2005)
	Poly(vinyl chloride) and Polyurethane	(Wan M *et al.* 2004); (Zhou Z and Meyerhoff ME. 2005); (Wu B *et al.* 2007)
	Stainless steel	(Chuang TW *et al.* 2008)

Heparin has also been conjugated to other proteins for increased potency. Heparin-albumin conjugates, for reduced cell adhesion, were adsorbed to glass, poly(vinyl chloride) coated glass, cellulose acetate coated glass (Hennink WE *et al*. 1984), polystyrene (Hennink WE and Feijen J. 1984), and polyurethane (Engbers GHM *et al*.

1997), and immobilized onto polystyrene (van Delden CJ *et al*. 1997; Bos GW *et al*. 1999). Heparin-AT conjugates, capable of thrombin interaction, have been administered systemically to rabbits (Chan A *et al*. 1997), tethered to PU (Klement P *et al*. 2002; Du YJ *et al*. 2007), and immobilized to gold via PEO spacers (Sask KN *et al*. 2010; Sask KN *et al*. 2011).

Despite various heparin conjugation and delivery methods with promising *in vitro* antithrombotic characteristics, coagulation improvements were not clinically observed with heparin-coated stents (Haude M *et al*. 2003; Jordan SW and Chaikof EL. 2007). Perhaps the coagulation is not only related to the surface characteristics, but also to the bulk; hence utilization of a biomimetic base material may further improve antithrombotic properties.

2.13.3 Collagen-Heparin Blood-Contacting Biomaterials

Collagen has been combined with heparin for decreased thrombogenesis (Keuren JFW *et al*. 2004) and platelet activation (Kauhanen P *et al*. 2000), even though collagen demonstrates prothrombogenic properties, through platelet binding and activation (Parsons TJ *et al*. 1983; Heemskerk JWM *et al*. 2005),

Collagen reacted with heparin, EDC and NHS, demonstrated decreased contact activation and thrombin inhibition, decreased platelet adhesion, spreading and aggregation, compared to thermal collagen gels (Wissink MJB *et al*. 2001). Collagen bound to periodate oxidized heparin exhibited AT binding, decreased thrombin generation, and moderately reduced platelet activation, despite platelet adhesion (Keuren JFW *et al*. 2004). Polyelectrolyte layer-by-layer deposition of heparin onto collagen had prolonged clotting times, decreased platelet adhesion and activation, and facilitated endothelialization (Chen J *et al*. 2010; Lin Q *et al*. 2011). Finally, the antithrombotic properties of fibroin and collagen scaffolds were enhanced through heparin incorporation, evident via cell capability, and delayed clotting times due to heparin release (Lu Q *et al*. 2007).

2.14 Background References

Ahmadi AJ and Jakobiec FA. Corneal wound healing: cytokines and extracellular matrix proteins. International Ophthalmology Clinics 2002; 42: 13-22.

Alexopoulou AN, Multhaupt HAB and Couchman JR. Syndecans in wound healing, inflammation, and vascular biology. The International Journal of Biochemistry & Cell Biology 2007; 39: 505-528.

Babensee JE, McIntire LV and Mikos AG. Growth factor delivery for tissue engineering. Pharmaceutical Research 2000; 17: 497-504.

Baldwin HC and Marshall J. Growth factors in corneal wound healing following refractive surgery: A review. Acta Ophthalmologica Scandinavica 2002; 80: 238-247.

Baldwin SP and Saltzman WM. Materials for protein delivery in tissue engineering. Advance Drug Delivery Reviews 1998; 33: 71.

Bell GI, Fong NM, Stempien MM, Wormsted MA, Caput D, Ku L, Urdea MS, Rall LB and Sanchez-Pescador R. Human epidermal growth factor precursor: cDNA sequence, expression, *in vitro* and gene organization. Nucleic Acid Research 1986; 14: 8427-8446.

Bennett JS. Platelet-fibrinogen interactions. Annals New York Academy of Sciences 2001; 936: 340-354.

Bentley E, Murphy CJ, Li F, Carlsson DJ and Griffith M. Biosynthetic corneal substitute implantation in dogs. Cornea 2010; 29: 910-916.

Bentz H, Schroeder JA and Estridge TD. Improved local delivery of TGF-beta2 by binding to injectable fibrillar collagen via difunctional polyethylene glycol. Journal of Biomedical Materials Research 1998; 39: 539-548.

Beuerman RW and Pedroza L. Ultrastructure of the human cornea. Microscopy Research and Technique 1996; 33: 320-335.

Bladergroen BA, Siebum B, Siebers-Vermeulen KG, Van Kuppevelt TH, Poot AA, Feijen J, Figdor CG and Torensma R. *In vivo* recruitment of hematopoietic cells using stromal cell-derived factor 1 alpha-loaded heparinized three-dimensional collagen scaffolds. Tissue Engineering Part A 2009; 15A: 1591-1599.

Blezer R, Cahalan L, Cahalan PT and Lindhout T. Heparin coating of tantalum coronary stents reduces surface thrombin generation but not factor IXa generation. Blood coagulation and fibrinolysis 1998; 9: 435-440.

Block ER, Matela AR, SundarRaj N, Iszkula ER and Klarlund JK. Wounding induces motility in sheets of corneal epithelial cells through loss of spatial constraints: Role of heparin-binding epidermal growth factor-like growth factor signaling. The Journal of Biological Chemistry 2004; 279: 24307-24312.

Boisjoly HM, Laplante C, Bernatchez SF, Salesse C, Giasson M and Joly MC. Effects of EGF, IL-1 and their combination on *in vitro* corneal epithelial wound closure and cell chemotaxis. Experimental Eye Research 1993; 57: 293-300.

Boonstra J, Rijken P, Humbel B, Cremers F, Verkleij A, and van Bergen en Henegouwen P. The epidermal growth factor. Cell Biology International 1995; 19: 413-430.

Bos GW, Scharenborg NM, Poot AA, Engbers GHM, Beugeling T, van Aken WG and Feijen J. Blood compatibility of surfaces with immobilized albumin-heparin conjugate and effect of endothelial cell seeding on platelent adhesion. Journal of Biomedical Materials Research 1999; 47: 279-291.

Brash JL. Exploiting the current paradigm of blood-material interactions for the rational design of blood-compatible materials. Journal of Biomaterials Science, Polymer Edition 2000; 11: 1135-1146.

Byun Y, Jacobs HA, Fiejen J and Kim SW. Effect of fibrinogen on the binding of antithrombin III to immobilized heparin. Journal of Biomedical Materials Research 1996; 30: 95-100

Carlsson DJ, Li F, Shimmura S and Griffith M. Bioengineered corneas: how close are we? Current Opinion in Ophthalmology 2003; 14: 192-197.

Carpenter G and Cohen S. Epidermal growth factor. The Journal of Biological Chemistry 1990; 265: 7709-7712.

Carpenter G, King L Jr and Cohen S. Epidermal growth factor stimulates phosphorylation in membrane preparations *in vitro*. Nature 1978; 276: 409-410.

Cen L, Liu W, Cui L, Zhang W and Cao Y. Collagen tissue engineering: development of novel biomaterials and applications. Pediatric Research 2008; 63: 492-496.

Chan A, Berry L, O'Brodovich H, Klement P, Mitchell L, Baranowski B, Monagle P, and Andrew M. Covalent anti-thrombin-heparin complexes with high anticoagulant activity. The Journal of Biological Chemistry 1997; 272: 22111-22117.

Chen H, Chen Y, Sheardown H and Brook MA. Immobilization of heparin on a silicone surface through a heterobifuncitonal PEG spacer. Biomaterials 2005; 26: 7418-7424.

Chen J, Chen C, Chen Z, Chen J, Li Q and Huang N. Collagen/heparin coating on titanium surface improves the biocompatibility of titanium applied as a blood-contacting biomaterial. Journal of Biomedical Materials Research Part A 2010; 95: 341-349.

Chen ZG, Wang PW, Wei B, Mo XM and Cui FZ. Electrospun collagen-chitosan nanofiber: a biomimetic extracellular matrix for endothelial cell and smooth muscle cell. Acta Biomaterialia 2010; 6: 372-382.

Cheung DT and Nimni ME. Mechanism of crosslinking of proteins by glutaraldehyde II. Reaction with monomeric and polymeric collagen. Connective Tissue Research 1982; 10: 201-216.

Chirila TV, Hicks CR, Dalton PD, Vijayasekaran S, Lou X, Hong Y, Clayton AB, Ziegelaar BW, Fitton JH, Platten S, Crawford GJ and Constable IJ. Artificial Cornea. Progress in Polymer Science 1998; 23: 447-472.

Choi JS, Leong KW and Yoo HS. *In vivo* wound healing of diabetic ulcers using electrospun nanofibres immobilized with human epidermal growth factor (EGF). Biomaterials 2008; 29: 587-596.

Chuang TW, Lin DT and Lin FH. Immobilization of NaIO4-treated heparin on PEG-modified 316L SS surface for high anti-thrombin-III binding. Journal of Biomedical Materials Research 2008; 86A; 648-661.

Colman RW. Mechanisms of thrombus formation and dissolution. Cardiovascular Pathology 1993; 2: 23S-31S.

Cooke A, Oliver RF and Edward M. An *in vitro* cytotoxicity study of aldehyde-treated pig dermal collagen. British Journal of Experimental Pathology 1983; 64: 172-176.

Cornelius RM, Sanchez J, Olsson P and Brash JL. Interactions of antithrombin and proteins in the plasma contact activation system with immobilized functional heparin. Journal of Biomedical Materials Research 2003; 67A: 475-483.

Courtney JM and Forbes CD. Thrombosis on foreign surfaces. British Medical Bulletin 1994; 50: 966-981.

Courtney JM, Lamba NMK, Gaylor JDS, Ryan CJ and Lowe GDO. Blood-contacting biomaterials: bioengineering viewpoints. Artificial Organs 1995; 19: 852-856.

Courtney JM, Lamba NMK, Sundaram S and Forbes CD. Biomaterials for blood-contacting applications. Biomaterials 2004; 15: 737-744.

Crabb RAB, Chau EP, Evans MC, Barocas VH and Hubel A. Biomechanical and microstructural characteristics of a collagen film-based corneal stromal equivalent. Tissue Engineering 2006; 12: 1565-1575.

Crabb RAB and Hubel A. Influence of matrix processing on the optical and biomechanical properties of a corneal stromal equivalent. Tissue Engineering Part A 2008; 14: 173-182.

Davis-Fleischer KM and Besner GE. Structure and function of heparin-binding EGF-like growth factor (HB-EGF). Frontiers in Bioscience 1998; 3: d288-299.

Day JRS, Landis RC and Taylor KM. Heparin is much more than just an anticoagulant. Journal of Cardiothoracic and Vascular Anesthesia 2004; 18: 93-100.

DelMonte DW and Kim T. Anatomy and physiology of the cornea. Journal of Cataract and Refractive Surgery 2011; 37: 588-598.

DeLong SA, Moon JJ and West JL. Covalently immobilized gradients of bFGF on hydrogel scaffolds for directed cell migration. Biomaterials. 2005; 26: 3227-3234.

Deng C, Li F, Hackett JM, Chaudhry SH, Toll FN, Toye B, Hodge W and Griffith M. Collagen and glycopolymer based hydrogel for potential corneal application. Acta Biomaterialia 2010; 6: 187-194.

DiLullo GA, Sweeney SM, Korkko J, Ala-Kokko L and San Antonio JD. Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. The Journal of Biological Chemistry 2002; 277: 4223-4231.

Doillon CJ, Watsky MA, Hakim M, Wang J, Munger R, Laycock N, Osborne R and Griffith M. A collagen-based scaffold for a tissue engineered human cornea: physical and physiological properties. The International Journal of Artificial Organs 2003; 26: 764-773.

Dreux AC, Lamb DJ, Modjtahedi H and Ferns GAA. The epidermal growth factor receptor and their family of ligands: Their putative role in atherogenesis. Artherosclerosis 2006; 186: 38-53.

Du YJ, Brash JL, McClung G, Berry LR, Klement P and Chan AK. Protein adsorption on polyurethane catheters modified with a novel antithrombin-heparin covalent complex. Journal Biomedical Materials Research, Part A 2007; 80A: 216-225.

Duan D, Klenkler BJ and Sheardown H. Progress in the development of a corneal replacement: keratoprostheses and tissue-engineered corneas. Expert Reviews of Medical Devices 2006; 3: 59-72.

Duan X and Sheardown H. Crosslinking of collagen with dendrimers. Journal of Biomedical Materials Research 2005; 75A: 510-518.

Duan X and Sheardown H. Dendrimer crosslinked collagen as a corneal tissue engineering scaffold: Mechanical properties and corneal epithelial cell interactions. Biomaterials 2006; 27: 4608-4617.

Duan X and Sheardown H. Incorporation of cell-adhesion peptides into collagen scaffolds promotes corneal epithelial stratification. Journal of Biomaterials Science, Polymer Edition 2007; 18: 701-711.

Duan X, McLaughlin C, Griffith M and Sheardown H. Biofunctionalization of collagen for improved biological response: Scaffolds for corneal tissue engineering. Biomaterials 2007; 28: 78-88.

Elenius K, Paul S, Allison G, Sun J and Klagsbrun M. Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation. The EMBO Journal 1997; 16: 1268-1278.

Engbers GHM, Dost L, Hennink WE, Aarts PAMM, Sixma JJ and Feijen J. An *in vitro* study of the adhesion of blood platelets onto vascular catheters: Part I. Journal of Biomedical Materials Research 1997; 21: 613-627.

Evans-Nguyen K and Schoenfisch MH. Fibrin proliferation at model surfaces: influence of surface properties. Langmuir 2005; 21: 1691-1694.

Fagerholm P, Lagali NS, Carlsson DJ, Merret K and Griffith M. Corneal regeneration following implantation of a biomimetic tissue-engineered substitute. Clinical and Translational Science Journal 2009; 2: 162-164.

Fagerholm P, Lagali NS, Merrett K, Jackson WB, Munger R, Liu Y, Polarek JW, Soderqvist M and Griffith M. A biosynthetic alternative to human donor tissue for inducing corneal regeneration: 24-month follow-up of a phase 1 clinical study. Science Translational Medicine 2011; 2: 1-8.

Faham S, Linhardt RJ and Rees DC. Diversity does make a difference: fibroblast growth factor-heparin interactions. Current Opinion in Structural Biology 1998; 8: 578-586.

Fang L, Li G, Liu G, Lee SW and Aaronson SA. p53 induction of heparin-binding EGF-like growth factor counteracts p53 growth suppression through activation of MAPK and PI3K/Akt signaling cascades. The EMBO Journal 2001; 20: 1931-1939.

Fini ME. Keratocyte and fibroblast phenotypes in the repairing cornea. Progress in Retinal and Eye Research 1999; 18: 529-551.

Fini ME and Stramer BM. How the cornea heals: Cornea-specific repair mechanisms affecting surgical outcomes. Cornea 2005; 24: S2-S11.

Forsten-Williams K, Chua CC, and Nugent MA. The Kinetics of FGF-2 Binding to Heparan Sulfate Proteoglycans and MAP Kinase Signaling. Journal of Theoretical Biology 2005; 233: 483-499.

Friess W. Collagen: biomaterial for drug delivery. European Journal of Pharmaceuticals and Biopharmaceuticals 1998; 45: 113-136.

Geer CB, Rus IA, Lord ST and Schoenfisch MH. Surface-dependent fibrinopeptide A accessibility to thrombin. Acta Biomaterialia 2007; 3: 663-668.

Germain L, Carrier P, Auger FA, Salesse C and Guerin SL. Can we produce a human corneal equivalent by tissue engineering? Progress in Retinal and Eye Research 2000; 19: 497-527.

Gillies ER and Frechet JMJ. Dendrimers and dendritic polymers in drug delivery. Drug Discovery Today 2005; 10: 35-43.

Gobin AS and West JL. Effects of epidermal growth factor on fibroblast migration through biomimetic hydrogels. Biotechnology Progress 2003; 19: 1781-1785.

Gomez N and Schmidt CE. Nerve growth factor-immobilized polypyrrole: bioactive electrically conducting polymer for enhanced neurite extension. Journal of Biomedical Materials Research 2006; 81: 135-149.

Gorbet MB and Sefton MV. Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes. Biomaterials 2004; 25: 5681-5703.

Gospodarowicz D and Cheng J. Heparin Protects Basic and Acidic FGF from Inactivation. Journal of Cellular Physiology 1986; 128: 475-484.

Grabarek Z and Gergely J. Zero-length crosslinking procedure with the use of active esters. Analytical Biochemistry 1990; 185: 131-135.

Grainger DW, Knutson K, Kim SW and Feijen J. Poly(dimethylsiloxane)-poly(ethylene oxide)-heparin block copolymers II: Surface characterization and *in vitro* assessments. Journal of Biomedical Materials Research 1990; 24: 403-431.

Grant MB, Khaw PT, Schultz GS, Adams JL and Shimizu RW. Effects of epidermal growth factor, fibroblast growth factor, and transforming growth factor-beta on corneal cell chemotaxis. Investigative Ophthalmology and Vision Science 1992; 33: 3292-3301.

Griffith M, Li F, Lohman C, Sheardown H, Shimmura S and Carlsson DJ. Tissue Engineering of the Cornea. In: Scaffolding in Tissue Engineering. Editors: Ma PX and Elisseeff J. USA: CRC press book; 2006 p.413-423.

Griffith M, Hakim M, Shimmura S, Watsky MA, Li F, Carlsson D, Doillon CJ, Nakamura M, Suuronen E, Shinozaki N, Nakata K and Sheardown H. Artificial human corneas: scaffolds for transplantation and host regeneration. Cornea 2002; 21: S54-S61.

Gutowska A, Bae YH, Jacobs H, Mohammad F, Mix D, Feijen J and Kim SW. Heparin release from thermosensitive polymer coatings: *In vivo* studies. Journal of Biomedical Materials Research 1995; 29: 811-821.

Hacket JM, Lagali N, Merrett K, Edelhausser H, Sun Y, Gan L, Griffith M and Fagerholm P. Biosynthetic corneal implants for the replacement of pathologic cornea tissue: Performance in a controlled rabbit alkali burn model. Investigative Ophthalmology and Visual Science 2011; 52: 651-657.

Hanson SR and Harker LA. Chapter 4.5: Blood coagulation and blood–materials interactions. In: Biomaterials science. Editors: Ratner BD, Hoffman AS, Schoen FJ and Lemons JE. San Diego: Academic Press; 1996. p.193-199.

Hassell JR, Cintron C, Kublin C and Newsome DA. Proteoglycan changes during restoration of transparency of corneal scars. Archives of Biochemistry and Biophysics 1983; 222: 362-369.

Haude M, Konorza TF, Kalnins U, Erglis A, Saunamaki K, Glogar HD, Grube E, Gil R, Serra A, Richardt HG, Sick P and Erbel R. Heparin-coated stent placement for the treatment of stenoses in small coronary arteries of symptomatic patients. Circulation 2003; 107: 1265-1270.

He W, Yong T, Teo WE, Ma Z, and Ramakrishna S. Fabrication and endothelialization of collagen-blended biodegradable polymer nanofibers: potential vascular graft for blood vessel tissue engineering. Tissue Engineering 2005; 11: 1574-1588.

Heemskerk JWM, Kuijpers MJE, Munnix ICA and Siljander PRM. Platelet collagen receptors and coagulation: A characteristic platelet response as possible target for antithrombotic treatment. Trends in Cardiovascular Medicine 2005; 15: 86-92.

Hennink WE and Feijen J. Interaction of antithrombin III with preadsorbed albumin-heparin conjugates. Biomaterials 1984; 5: 264-268.

Hennink WE, Kim SW and Feijen J. Inhibition of surface induced coagulation by preadsorption of albumin-heparin conjugates. Journal of Biomedical Materials Research 1984; 18: 911-926.

Higashiyama S, Lau K, Besner GE, Abraham JA and Klagsbrun M. Structure of heparin-binding EGF-like growth factor: Multiple forms, primary structure, and glycosylation of the mature protein. The Journal of Biological Chemistry 1992; 267: 6205-6212.

Hinrichs WLJ, ten Hoopen HWM, Wissink MJB, Engbers GHM and Feijen J. Design of a new type of coating for the controlled release of heparin. Journal of Controlled Release 1997; 45: 163-176.

Hongo M, Itoi M, Yamaguchi N and Imanishi J. Distribution of epidermal growth factor (EGF) receptors in rabbit corneal epithelial cells, keratocytes and endothelial cells, and the changes induced by transforming growth factor-beta 1. Experimental Eye Research 1992; 54: 9-16.

Huang-Lee LL, Cheung DT and Nimni ME. Biochemical changes and cytotoxicity associated with the degradation of polymeric glutaraldehyde derived crosslinks. Journal of Biomedical Materials Research 1990; 24: 1185-1201.

Huntington JA. Serpin structure, function and dysfunction. Journal of Thrombosis and Haemostasis 2011; 9: 26-34.

Imanishi J, Kamiyama K, Iguchi I, Kita M, Sotozono C and Kinoshita S. Growth factors: Importance in wound healing and maintenance of transparency of the cornea. Progress in Retinal and Eye Research 2000; 19: 113-129.

Ito Y. Covalently immobilized biosignal molecule materials for tissue engineering. Soft Matter 2008; 4: 46-56.

Iwamoto R and Mekada E. Heparin-binding EGF-like growth factor: A juxtacrine growth factor. Cytokine and Growth Factor Reviews 2000; 11: 335-344.

Jee KS, Park HD, Park KD, Kim YH and Shin JW. Heparin conjugated polylactide as a blood compatible material. Biomacromolecules 2004; 5: 1877-1881.

Jester JV, Petroll WM and Cavanagh HD. Corneal stromal wound healing in refractive surgery: the role of myofibroblasts. Progress in Retinal and Eye Research 1999; 18: 311-356.

Jordan SW and Chaikof EL. Novel thromboresistant materials. Journal of Vascular Surgery 2007; 45: 104A-115A.

Joung YK, Bae JW and Park KD. Controlled release of heparin-binding growth factors using heparin-containing particulate systems for tissue regeneration. Expert Opinions in Drug Delivery 2008; 5: 1173-1184.

Kanematsu A, Marui A, Yamamoto S, Ozeki M, Hirano Y, Yamamoto M, Ogawa O, Komeda M and Tabata Y. Type I collagen can function as a reservoir of basic fibroblast growth factor. Journal of Controlled Release 2004; 99: 281-292.

Kanematsu A, Yamamoto S, Ozeki M, Noguchi T, Kanatani I, Ogawa O and Tabata Y. Collagenous matrices as release carriers of exogenous growth factors. Biomaterials 2004; 25: 4513-4520.

Kauhanen P, Kovanen PT and Lassila R. Coimmobilized native macromolecular heparin proteoglycans strongly inhibit platelet-collagen interactions in flowing blood. Arteriosclerosis, Thrombosis and Vascular Biology 2000; 20: e113-e119.

Keller KM, Keller JM and Kuhn K. The C-terminus of type I collagen is a major binding site for heparin. Biochimica et Biophysica Acta: General Subjects 1986; 882: 1-5.

Keuren JFW, Wielders SJH, Driessen A, Verhoeven M, Hendriks M and Lindhout T. Covalently-bound heparin makes collagen thromboresistant. Arteriosclerosis, Thrombosis and Vascular Biology 2004; 24: 613-617.

Keuren JFW, Wielders SJH, Willems GM, Morra M, Cahalan L, Cahalan P and Lindhout T. Thrombogenicity of polysaccharide-coated surfaces. Biomaterials 2003; 24: 1917-1924.

Kim YJ, Kang IK, Huh MW and Yoon SC. Surface characterization and *in vitro* blood compatibility of poly(ethylene terephthalate) immobilized with insulin and/or heparin using plasma glow discharge. Biomaterials 2000; 21: 121-130.

Klement P, Du YJ, Berry L, Andrew M and Chan AK. Blood-compatible biomaterials by surface coating with a novel antithrombin-heparin covalent complex. Biomaterials 2002; 23: 527-535.

Klenkler BJ and Sheardown H. Growth factors in the anterior segment: role in tissue maintenance, wound healing and ocular pathology. Experimental Eye Research 2004; 79: 677-688.

Klenkler BJ, Griffith M, Becerril C, West-Mays JA and Sheardown H. EGF-grafted PDMS surfaces in artificial cornea applications. Biomaterials 2005; 26: 7286-7296.

Klenkler BJ and Sheardown H. Characterization of EGF coupling to animated silicone rubber surfaces. Biotechnology and Bioengineering 2006; 95: 1158-1166.

Klenkler BJ, Dwivedi D, West-Mays JA and Sheardown H. Corneal epithelial cell adhesion and growth on EGF-modified animated PDMS. Journal of Biomedical Materials Research Part A 2010; 93A: 1043-1049.

Kobsa S and Saltzman WM. Bioengineering approaches to controlled protein delivery. Pediatric Research 2008; 63: 513-519.

Koenig A, Norgard-Sumnicht K, Linhardt R and Varki A. Differential interactions of heparin and heparan sulphate glycosaminoglycans with the selectins: Implications for the use of unfractionated and low molecular weight heparins as therapeutic agents. The Journal of Clinical Investigation 1998; 101: 877-889.

Khor E. Methods for the treatment of collagenous tissue for bioprostheses. Biomaterials 1997; 18: 95-105.

Krishnaswamy A, Lincoff AM and Cannon CP. The use and limitations of unfractionated heparin. Critical Pathways in Cardiology 2010; 9: 35-40.

Kuhl PR and Griffith-Cima LG. Tethered epidermal growth factor as a paradigm for growth factor-induced stimulation from the solid phase. Nature Medicine 1996; 2: 1022-1027.

Lagali NS, Griffith M, Shinozaki N, Fagerholm P and Munger R. Innervation of tissue-engineered corneal implants in a porcine model: A 1-year *in vivo* confocal microscopy study. Investigative Ophthalmology and Visual Science 2007; 48: 3537-3544.

Langer R. Tissue Engineering. Molecular Therapy 2000; 1: 12-15.

Langer R and Vacanti JP. Tissue Engineering. Science 2003; 260: 920-926.

Lavik E and Langer R. Tissue Engineering: Current state and perspectives. Applications in Microbiology and Biotechnology 2004; 65: 1-8.

Lee SJ. Cytokine delivery and tissue engineering. Yonsie Medical Journal 2000; 41: 704-719.

Lee MK and Lander AD. Analysis of affinity and structural selectivity in the binding of proteins to glycosaminoglycans: development of a sensitive electrophoretic approach. Proceedings of the National Academy of Sciences of the United States of America 1991; 88: 2768-2772.

Leslie-Barbick JE, Shen C and West JL. Micron-scale spatially patterned, covalently immobilized vascular endothelial growth factor on hydrogels accelerates endothelial tubulogenesis and increases cellular angiogenic responses. Tissue Engineering Part A 2011a; 17: 221-229.

Leslie-Barbick JE, Saik JE, Gould DJ, Dickonson ME and West JL. The promotion of microvascular formation in poly(ethylene) glycol diacrylate hydrogels by an immobilized VEGF-mimetic peptide. Biomaterials 2011b; 32: 5782-5789.

Letic-Gavrilovic A, Piattelli A and Abe K. Nerve growth factor beta (NGF beta) delivery via a collagen/hydroxyapatite (Col/HAp) composite and its effects on new bone ingrowth. Journal of Materials Science: Materials in Medicine 2003; 14: 95-102.

Li F, Carlsson D, Lohmann C, Suuronen E, Vascotto S, Kobuch K, Sheardown H, Munger R, Nakamura M and Griffith M. Cellular and nerve regeneration within a biosynthetic extracellular matrix for corneal transplantation. Publications of the National Academy of Sciences 2003; 100: 15346-15351.

Li F, Griffith M, Li Z, Tanodekaew S, Sheardown H, Hakim M and Carlsson DJ. Recruitment of multiple cell lines by collagen-synthetic copolymer matrices in corneal regeneration. Biomaterials 2005; 26: 3093-3104.

Li JP and Vlodavsky I. Heparin, heparan sulphate and heparanase in inflammatory reactions. Thrombosis and Haemostasis 2009; 102: 823-828.

Li Q, Weng J, Mohan RR, Bennett GL, Schwall R, Wang ZF, Tabor K, Kim J, Hargrave S, Cuevas KH and Wilson SE. Hepatocyte growth factor and hepatocyte growth factor receptor in the lacrimal gland, tears, and cornea. Investigative Ophthalmology and Vision Science 1996; 37: 727-739.

Liu W, Deng C, McLaughlin CR, Fagerholm P, Lagali NS, Heyne B, Scaiano JC, Watsky MA, Kato Y, Munger R, Shinozaki N, Li F and Griffith M. Collagen-phosphorylcholine interpenetrating network hydrogels as corneal substitutes. Biomaterials 2009; 30: 1551-1559.

Liu W, Merrett K, Griffith M, Fagerholm P, Dravida S, Heyne B, Scaiano JC, Watsky MA, Shinozaki N, Lagali N, Munger R and Li F. Recombinant human collagen for tissue engineered corneal substitutes. Biomaterials 2008; 29: 1147-1158.

Liberelle B, Boucher C, Chen J, Jolicoeur M, Durocher Y and De Crecenzo G. Impact of epidermal growth factor tethering strategy on cellular response. Bioconjugate Chemistry 2010; 21: 2257-2266.

Lin Q, Yan J, Qiu F, Song X, Fu G and Ji J. Heparin/collagen multilayer as a thromboresistant and endothelial coating for intravascular stent. Journal of Biomedical Materials Research, Part A 2011; 96A: 132-141.

Liu Y, Gan L, Carlsson DJ, Fagerholm P, Lagali N, Watsky MA, Munger R, Hodge WG, Priest D and Griffith M. A simple, cross-linked collagen tissue substitute for corneal implantation. Investigative Ophthalmology and Visual Science 2006a; 47: 1869-1875.

Liu Y, Griffith M, Watsky MA, Forrester JV, Kuffova L, Grant D, Merrett K and Carlsson DJ. Properties of porcine and recombinant human collagen matrices for optically clear tissue engineering applications. Biomacromolecules 2006b; 7: 1819-1828.

Lu Q, Zhang S, Hu K, Feng Q, Cao C and Cui F. Cytocompatibility and blood compatibility of multifunctional fibroin/collagen/heparin scaffolds. Biomaterials 2007; 28: 2306-2313.

Lutolf MP and Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nature Biotechnology 2005; 23: 47-55.

Madhavan K, Belchenko D, Motta A and Tan W. Evaluation of composition and crosslinking effects on collagen-based composite constructs. Acta Biomaterialia 2010; 6: 1413-1422.

Malafaya PB, Silva GA and Reis RL. Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. Advanced Drug Delivery Reviews 2007; 59: 207-233.

Mann BK, Schmedlen RH and West JL. Tethered TGF-b increases extracellular matrix production of vascular smooth muscle cells. Biomaterials 2001; 22: 439-444.

Markiewski MM, Nilsson B, Ekdahl KN, Mollnes TE and Lambris JD. Complement and coagulation: strangers or partners in crime? Trends in Immunology 2007; 28: 184-192.

Marui A, Kanematsu A, Yamahara K, Doi K, Kushibiki T, Yamamoto M, Itoh H, Ikeda T, Tabata Y and Komeda M. Simultaneous application of basic fibroblast growth factor and hepatocyte growth factor to enhance the blood vessels formation. Journal of Vascular Surgery 2005; 41: 82-90.

Massague J and Pandiella A. Membrane anchored growth factors. Annual Reviews in Biochemistry 1992; 62: 515-541.

McGuigan AP and Sefton MV. The thrombogenicity of human umbilical vein endothelial cell seeded collagen modules. Biomaterials 2008; 29: 2453-2463.

McLaughlin CR, Acosta MC, Luna C, Liu W, Belmonte C, Griffith M and Gallar J. Regeneration of functional nerves within full thickness collagen-phosphorylcholine corneal substitute implants in guinea pigs. Biomaterials 2010; 31: 2770-2778.

McLaughlin CR, Fagerholm P, Muzakare L, Lagali N, Forrester JV, Kuffova L, Rafat MA, Liu Y, Shinozaki N, Vascotto SG, Munger R and Griffith M. Regeneration of cornea cells and nerves in an implanted collagen corneal substitute. Cornea 2008; 27: 580-589.

McCaffrey TA, Falcone DJ and Du B. Transforming growth factor-beta 1 is a heparin-binding protein: identification of putative heparin-binding regions and isolation of heparins with varying affinity for TGF-beta 1. Journal of Cellular Physiology 1992; 152: 430-440.

McPherson JM, Sawamura SJ, Condell RA, Rhee W and Wallace DG. The effects of heparin on the physicochemical properties of reconstituted collagen. Collagen and Related Research 1988; 1: 65-82.

Meek KM and Boote C. The organization of collagen in the corneal stroma. Experimental Eye Research 2004; 78: 503-512.

Merrett K, Fagerholm P, McLaughlin CR, Dravida S, Lagali N, Shinozaki N, Watsky MA, Munger R, Kato Y, Li F, Marmo CJ and Griffith M. Tissue-engineering recombinant human collagen-based corneal substitutes for implantation: performance of type I versus type III collagen. Investigative Ophthalmology and Visual Science 2008; 49: 3887-3894.

Merrett K, Griffith CM, Deslandes Y, Pleizier G, Dube MA and Sheardown H. Interactions of cornea cellswith transforming growth factor beta 2-modified poly dimethyl siloxane. Journal of Biomedical Materials Research Part A 2003; 67A: 981-993.

Merrett K, Liu W, Mitra D, Camm KD, McLaughlin CR, Liu Y, Watsky MA, Li F, Griffith M and Fogg DE. Synthetic neoglycopolymer-recombinant human collagen hybrids as biomimetic crosslinking agents in corneal tissue engineering. Biomaterials 2009; 30: 5403-5408.

Mohan RR, Liang Q, Kim WJ, Helena MC, Baerveldt F and Wilson SE. Apoptosis in the cornea: further characterization of Fas/Fas ligand system. Experimental Eye Research 1997; 65: 575-589.

Mroczkowski B, Reich M, Whittaker J, Bell GI and Cohen S. Expression of human epidermal growth factor precursor cDNA in transfected mouse NIH 3T3 cells.

Proceedings of the National Academy of Sciences of the United States of America 1988; 85: 126-130.

Mulloy B and Linhardt RJ. Order out of complexity--protein structures that interact with heparin. Current Opinion in Structural Biology 2001; 11: 623-628.

Mulloy B and Rider CC. Cytokine-proteoglycan interactions: Biology and structure. Biochemical Society Translations 2006; 34: 409-413.

Najjam S, Mulloy B, Theze J, Gordon M, Gibbs R and Rider CC. Further characterization of the binding of human recombinant interleukin 2 to heparin and identification of putative binding sites. Glycobiology 1998; 8: 509-516.

Nakamura M and Nishida T. Differential effects of epidermal growth factor and interleukin 6 on corneal epithelial cells and vascular endothelial cells. Cornea 1999; 18: 452-458.

Nanba D and Higashiyama S. Dual intracellular signaling by proteolytic cleavage of membrane-anchored heparin-binding EGF-like growth factor. Cytokine and Growth Factor Reviews 2004; 15: 13-19.

Nelson RM, Cecconi O, Roberts WG, Aruffo A, Linhardt RJ and Bevilacqua MP. Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. Blood 1993; 82: 3253-3258.

Nillesen STM, Geutjes PJ, Wismans R, Schalkwijk J, Daamen WF and van Kuppevelt TH. Increased angiogenesis and blood vessel maturation in acellular collagen-heparin scaffolds containing both FGF2 and VEGF. Biomaterials 2007; 28: 1123-1131.

Nimni ME. Polypeptide growth factors: targeted delivery systems. Biomaterials 1997; 18: 1201-1225.

Nishi E and Klagsbrun M. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a mediator of multiple physiological and pathological pathways. Growth Factors 2004; 22: 253-260.

Nishida T, Nakamura M, Murakami J, Mishima H and Otori T. Epidermal growth factor stimulates corneal epithelial cell attachment to fibronectin through a fibronectin receptor system. Investigative Ophthalmology and Visual Science 1992; 33: 2464-2469.

Norgard-Sumnicht K and Varki A. Endothelial heparan sulphate proteoglycans that bind to L-selectin have glucosamine residues with unsubstituted amino groups. The Journal of Biological Chemistry 1995; 270: 12012-12024.

Nur-E-Kamal A, Ahmed I, Kamal J, Babu AN, Schindler M and Meiners S. Covalently attached FGF-2 to three-dimensional polyamide nanofibrillar surfaces demonstrates enhanced biological stability and activity. Molecular and Cellular Biochemistry 2008; 309: 157-166.

Ojeda JL, Ventosa JA and Piedra S. The three-dimensional microanatomy of the rabbit and human cornea: A chemical and mechanical microdissection-SEM approach. Journal of Anatomy 2001; 199: 567-576.

Olander B, Wirsen A and Albertsson AC. Silicone elastomer surface functionalized with primary amines and subsequent coupled with heparin. Biomacromolecules 2003; 4: 145-148.

Olde Damink LHH, Dijkstra PJ, van Luyn MJA, van Wachem PB, Nieuwenhuis P and Feijen J. Cross-linking of dermal sheep collagen using a water-soluble carbodiimide. Biomaterials 1996; 17: 765-773.

Olson ST, Richard B, Izaquirre G, Schedin-Weiss S and Gettins PGW. Molecular mechanisms of antithrombin-heparin regulation of blood clotting proteinases: a paradigm for understanding proteinase regulation by serpin family protein proteinase inhibitors. Biochimie 2010; 92: 1587-1596.

Omidi Y, Hollins AJ, Drayton RM and Akhtar S. Polypropylenimine dendrimer-induced gene expression changes: The effect of complexation with DNA, dendrimer generation and cell type. Journal of Drug Targeting 2005; 13: 431-443.

Oravecz T, Pall M, Wang J, Roderiquez G, Ditto M and Norcross MA. Regulation of anti-HIV-1 activity of RANTES by heparan sulphate proteoglycans. Journal of Immunology 1997; 159: 4587-4592.

Orwin EJ, Borene ML and Hubel A. Biomechanical and optical characteristics of a corneal stromal equivalent. Journal of Biomechanical Engineering 2003; 125: 439-444.

Orwin EJ and Hubel A. *In vitro* culture characteristics of corneal epithelial, endothelial and keratocyte cells in a native collagen matrix. Tissue Engineering 2000; 6: 307-319.

Parish CR. The role of heparan sulphate in inflammation. Nature Reviews in Immunology 2006; 6: 633-643.

Parsons TJ, Haycraft DL, Hoak JC and Sage H. Diminished platelet adherence to type V collagen. Arteriosclerosis, Thrombosis and Vascular Biology 1983; 3: 589-598.

Petitou M, Casu B and Lindahl U. 1976-1983, a critical period in the history of heparin: the discovery of the antithrombin binding site. Biochimie 2003; 85: 83-89.

Pieper JS, Hafmans T, van Wachem PB, van Luyn MJA, Brouwer LA, Veerkamp JH and van Kuppevelt TH. Loading of collagen-heparin sulphate matrices with bFGF promotes angiogenesis and tissue regeneration in rats. Journal of Biomedical Materials Research 2001; 62: 185-194.

Petite H, Rault I, Huc A, Menasche P and Herbage D. Use of the acyl azide method for cross-linking collagen-rich tissues such as pericardium. Journal of Biomedical Materials Research 1990; 24: 179-187.

Prasad CK and Krishnan LK. Regulation of endothelial cell phenotype by biomimetic matrix coated on biomaterials for cardiovascular tissue engineering. Acta Biomaterialia 2008; 4: 182-91.

Princz MA and Sheardown H. Heparin-modified dendrimer crosslinked collagen matrices for the delivery of basic fibroblast growth factor (FGF-2). Journal of Biomaterials Science, Polymer Edition 2008; 19: 1201-1218.

Prockop DJ and Kavirikko KI. Collagens: Molecular biology, diseases, and potentials for therapy. Annual Reviews in Biochemistry 1995; 64: 403-434.

Proulx S, d'Arc Uwamaliya J, Carrier P, Deschambeault A, Audet C, Gaisson CJ, Geurin SL, Auger FA and Germain L. Reconstruction of a human cornea by self-assembly approach of tissue engineering using the three native cornea cell types. Molecular Vision 2010; 16: 2192-2201.

Prywes R, Livneh E, Ullrich A and Schlessinger J. Mutations in the cytoplasmic domain of EGF receptor affect EGF binding and receptor internalization. The EMBO Journal 1986; 5: 2179-2190.

Quinsey NS, Greedy AL, Bottomley SP, Whisstock JC and Pike RN. Antithrombin: in control of coagulation. The International Journal of Biochemistry and Cell Biology 2004; 36: 386-389.

Raab G and Klagsbrun M. Heparin-binding EGF-like growth factor. Biochimica et Biophysica Acta 1997; 1333: F179-F199.

Rafat M, Li F, Fagerholm P, Lagali NS, Watsky MA, Munger R, Matsuura T and Griffith M. PEG-stabilized carbodiimide crosslinked collagen-chitosan hydrogels for corneal tissue engineering. Biomaterials 2008; 29: 3960-3972.

Ramsden L and Rider CC. Selective and differential binding of interleukin (IL)-1 alpha, IL-1 beta, IL-2 and IL-6 to glycosaminoglycans. European Journal of Immunology 1992; 22: 3027-3031.

Ratner BD and Bryant SJ. Biomaterials: Where we have been and where we are going. Annual Reviews in Biomedical Engineering 2004; 6: 41-75.

Rau JC, Beaulieu LM, Huntington JA and Church FC. Serpins in thrombosis, hemostasis and fibrinolysis. Journal of Thrombosis and Haemostasis 2007; 5: 102-115.

Rosenberg RD and Damus PS. The purification and mechanism of action of human antithrombin-heparin cofactor. The Journal of Biological Chemistry 1973; 248: 6490-6505.

Ruberti JW and Zieske JD. Prelude to corneal tissue engineering: gaining control of collagen organization. Progress in Retinal and Eye Research 2008; 27: 549-577.

Ruderman RJ, Wade CWR, Shepard WD, and Leonard F. Prolonged resorption of collagen sponges: vapor-phase treatment with formaldehyde. Journal of Biomedical Materials Research 1973; 7: 263–265.

Saik JE, Gould DJ, Watkins EM, Dickinson ME and West JL. Covalently immobilized platelet-derived growth factor-BB promotes angiogenesisin biomimetic poly(ethylene) glycol hydrogels. Acta Biomaterialia 2001; 7: 133-143.

Saksela O, Moscatelli D, Sommer A and Rifkin DB. Endothelial Cell-Derived Heparan Sulfate Binds Basic Fibroblast Growth Factor and Protects it from Proteolytic Degradation. The Journal of Cell Biology 1988; 107: 743-751.

Salek-Ardakani S, Arrand JR, Shaw D and Mackett M. Heparin and heparan sulphate bind interleukin-10 and modulate its activity. Blood 2000; 96: 1879-1888.

Saltzman WM and Olbricht WL. Building drug delivery into tissue engineering. Nature Reviews: Drug Discovery 2002; 1: 177-186.

Sask KN, McGlung WG, Berry LR, Chan AKC and Brash JL. Immobilization of an antithrombin–heparin complex on gold: Anticoagulant properties and platelet interactions. Acta Biomaterialia 2011; 7: 2029-2034.

Sask KN, Zhitomirsky I, Berry LR, Chan AKC and Brash JL. Surface modification with an anti-thrombin-heparin complex for anticoagulation: studies on a model surface with gold as substrate. Acta Biomaterialia 2010; 6: 2911-2919.

Schneider MR and Wolf E. The epidermal growth factor receptor ligands at a glance. Journal of Cellular Physiology 2009; 218: 460-466.

Schroeder-Tefft JA, Bentz H and Estridge TD. Collagen and heparin matrices for growth factor delivery. Journal of Controlled Release 1997; 49: 291-298.

Schultz GS and Wysocki A. Interactions between extracellular matrix and growth factors in wound healing. Wound Repair and Regeneration 2008; 17: 153-162

Sefton MV, Gemmell CH and Gorbet MB. What really is blood compatibility? Journal of Biomaterials Science, Polymer Edition 2000; 11: 1165-1182.

Selvan RS, Ihrcke NS and Platt JL. Heparan sulphate in immune responses. The Annuals of the New York Academy of Sciences 1996; 797: 127-139.

Shah A, Brugnano J, Sun S, Vase A and Orwin E. The development of tissue-engineered cornea: Biomaterials and culture methods. Pediatric Research 2008; 63: 535-544.

Sheardown H, Clark H, Wedge C, Apel R, Rootman D and Cheng YL. A semi-solid drug delivery system for epidermal growth factor in corneal epithelial wound healing. Current Eye Research 1997; 16: 183-190.

Sheardown H and Cheng YL. Tear EGF concentration following corneal epithelial wound creation. Journal of Oculare Pharmacology and Therapeutics 1996; 12: 239-243.

Sheardown H, Wedge C, Chou L, Apel R, Rootman DS and Cheng YL. Continuous epidermal growth factor delivery in corneal epithelial wound healing. Investigative Ophthalmology and Vision Science 1993; 34: 3593-3600.

Shen YH, Shoichet MS and Radisic M. Vascular endothelial growth factor immobilized in collagen scaffold promotes penetration and proliferation of endothelial cells. Acta Biomaterialia 2008; 4: 477-89.

Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J and Klagsbrun M. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. Science. 1984; 223: 1296-1299.

Silva AKA, Richard C, Bessodes M, Scherman D and Merten OW. Growth factor delivery approaches in hydrogels. Biomacromolecules 2009; 10: 9-18.

Singh AB and Harris RC. Autocrine, paracrine and juxtacrine signaling by EGFR ligands. Cellular Signalling 2005; 17: 1183-1193.

Sinha VR and Trehan A. Biodegradable microspheres for protein delivery. Journal of Controlled Release 2003; 90: 261-280.

Skinner MP, Fournier DJ, Andrews RK, Gorman JJ, Chesterman CN and Berndt MC. Characterization of human platelet GMP-140 as a heparin-binding protein. Biochemical and Biophysical Research Communications 1989; 164: 1373-1379.

Smith BAH and Sefton MV. Thrombin and albumin adsorption to PVA and heparin-PVA hydrogels 1: Single protein isotherms. Journal of Biomedical Materials Research 1992; 26: 947-958.

Smith BAH and Sefton MV. Thrombin and albumin adsorption to PVA and heparin-PVA hydrogels 2. Competition and displacement. Journal of Biomedical Materials Research 1993; 27: 89-95.

Spillmann D, Witt D, and Lindahl U. Defining the interleukin-8-binding domain of heparan sulphate. The Journal of Biological Chemistry 1998; 273: 15487-15493.

Steffens GCM, Yao C, Prevel P, Markowicz M, Schenck P, Noah EM and Pallua N. Modulation of angiogenic potential of collagen matrices by covalent incorporation of heparin and loading with vascular endothelial growth factor. Tissue Engineering 2004; 10: 1502-1509.

Stefonek-Puccinelli TJ and Masters KS. Regulation of cell signaling and function via changes in growth factor presentation. Annual International Conference of the IEEE Engineering in Medical and Biology Society 2009; 2009: 1167-1171.

Stompro BE, Hansbrough JF and Boyce ST. Attachment of peptide growth factors in implantable collagen. The Journal of Surgical Research 1989; 46: 413-421.

Stringer SE, Forster MJ, Mulloy B, Bishop CR, Graham GJ and Gallagher JT. Characterization of the binding site on heparan sulphate for macrophage inflammatory protein 1alpha. Blood 2002; 100: 1543-1550.

Sun B, Chen B, Zhao Y, Sun W, Chen K, Zhang J, Wei Z, Xiao Z and Dai J. Crosslinking heparin to collagen scaffolds for the delivery of human platelet-derived growth factor. Journal of Biomedical Materials Research Part B 2009; 91B: 366-372.

Sun JS, Lin FH, Wang YJ, Huang YC, Chueh SC and Hsu FY. Collagen-hydroxyapatite/tricalcium phosphate microspheres as a delivery system for recombinant human transforming growth factor-beta 1. Artificial Organs 2003; 27: 605-12.

Tabata Y. Tissue regeneration based on growth factor release. Tissue Engineering 2003; 9: S5-S15.

Taipale J and Keski-Oja J. Growth factors in the extracellular matrix. The Journal of the Federation of American Societies for Experimental Biology 1997; 11: 51-59.

Takaoka K, Koezuka M and Nakahara H. Telopeptide-depleted bovine skin collagen as a carrier for bone morphogenetic protein. Journal of Orthopaedic Research 1991; 9: 902-907.

Tanaka Y, Adams DH and Shaw S. Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. Immunology Today 1993; 14: 111-115.

Tanzi MC. Bioactive technologies for hemocompatibility. Expert Reviews of Medical Devices 2005; 2: 473-492.

Tay SW, Merrill EW, Salzman EW and Lindon J. Activity towards thrombin-antithrombin of heparin immobilized on two hydrogels. Biomaterials 1989; 10: 11-15.

Teixeira S, Yang L, Dijkstra PJ, Ferraz MP and Monteiro FJ. Heparinized hydroxyapatite/collagen three-dimensional scaffolds for tissue engineering. Journal of Biomedical Materials Research 2010; 21: 2385-2392.

Thompson SA, Higashiyama S, Wood K, Pollitt SA, Damm D, McEnroe G, Garrick B, Ashton N, Lau K, Hancock N, Klagsburn M and Abraham JA. Characterization of sequences within heparin-binding EGF-like growth factor that mediate interaction with heparin. The Journal of Biological Chemistry 1994; 269; 2541-2549.

Thorslund S, Sanchez J, Larsson R, Nikolajeff F and Bergquist J. Bioactive heparin immobilized onto microfluidic channels in poly(dimethylsiloxane) results in hydrophilic surface properties. Colloids and Surfaces B: Biointerfaces 2005; 46: 240-247.

Tigli RS, Kazaroglu NM, Mavis B and Gumusderelioglu M. Cellular behaviour on epidermal growth factor (EGF)-immobilized PCL/gelatin nanofibrous scaffolds. Journal of Biomaterials Science 2011; 22: 207-223.

Tolino MA, Block ER and Klarlund JK. Brief treatment with heparin-binding EGF-like growth factor, but not with EGF, is sufficient to accelerate epithelial wound healing. Biochimica et Biophysica Acta 2011; 1810: 875-878.

Tyan YC, Liao JD, Wu YT and Klauser R. Anticoagulant activity of immobilized heparin on the polyprolyene nonwoven fabric surface depending upon the pH of processing environment. Journal of Biomaterials Applications 2002; 17; 153-178.

Uebersax L, Merkle HP and Meinel L. Biopolymer-based growth factor delivery for tissue repair: From natural concepts to engineered systems. Tissue Engineering Part B 2009; 15: 263-289.

Uludag H, Friess W, Williams D, Porter T, Timony G, D'Augusta D, Blake C, Palmer R, Biron B and Wozney J. rhBMP-collagen sponges as osteoinductive devices: effects of *in vitro* sponge characteristics and protein pI on *in vivo* rhBMP pharmacokinetics. Annuals of the New York Academy of Sciences 1999; 875: 369-378.

Vaisman N, Gospodarowicz D and Neufeld G. Characterization of the receptors for vascular endothelial growth factor. Journal of Biological Chemistry 1990; 265: 19461-19466.

van Delden CJ, Lens JP, Kooyman RPH, Engbers GHM and Feijen J. Heparinization of gas plasma-modified polystyrene surfaces and the interactions of these surfaces with proteins studies with surface plasmon resonance. Biomaterials 1997; 18:845-852.

van Gulik TM, Christiano RA, Broekhuizen AH, Raaymakers EL and Klopper PJ. A tanned, sheep dermal collagen graft as a dressing for split-skin graft donor sites. The Netherlands Journal Surgery 1989; 41: 65-67.

van Wachem PB, Plantinga JA, Wissink MJB, Beernink R, Poot AA, Engbers GHM, Beugeling T, van Aken WG, Feijen J and van Luyn MJA. *In vivo* biocompatibility of carbodiimide-crosslinked collagen matrices: Effects of crosslink density, heparin immobilization, and bFGF loading. Journal of Biomedical Materials Research 2001; 55: 368-378.

van Wachem PB, van Luyn MJ, Olde Damink LH, Dijkstra PJ, Feijen J and Nieuwenhuis P. Biocompatibility and tissue regenerating capacity of crosslinked dermal sheep collagen. Journal of Biomedical Materials Research 1994; 28: 353-363.

van Wachem PB, Zeeman R, Dijkstra PJ, Feijen J, Hendriks M, Cahalan PT and van Luyn MJ. Characterization and biocompatibility of epoxy-crosslinked dermal sheep collagens. Journal of Biomedical Materials Research 1999; 47: 270-277.

Vasudev SC, Chandy T and Sharma CP. Development of chitosan/polyethylene vinyl acetate co-matrix: controlled release of aspirin-heparin preventing cardiovascular thrombosis. Biomaterials 1997; 18: 375-381.

Wade JD, Domagala T, Rothacker J, Catimel B and Nice E. Use of thiazolidine-mediated ligation for site specific biotinylation of mouse EGF for biosensor immobilization. Letters in Peptide Science 2002; 8: 211-220.

Wan M, Baek DK, Cho JH and Kang IK. *In vitro* blood compatibility of heparin-immobilized polyurethane containing ester groups in the side chain. Journal of Materials Science: Materials in Medicine 2004; 15: 1079-1087.

Wang X, Kamiyama K, Iguchi I, Kita M and Imanishi J. Enhancement of fibronectin-induced migration of corneal epithelial cells by cytokines. Investigative Ophthalmology and Vision Science 1994; 35: 4001-4007.

Weadock K, Olson RM and Silver FH. Evaluation of collagen crosslinking techniques. Biomaterials, Medical Devices, and Artificial Organs 1983; 11: 293-318.

Webb LM, Ehrengruber MU, Clark-Lewis I, Baggiolini M and Rot A. Binding to heparan sulphate or heparin enhances neutrophil responses to interleukin 8. Proceedings of the National Academy of Sciences of the United States of America 1993; 90: 7158-7162.

Weitz DS and Weitz JI. Update on heparin: what do we need to know? Journal of Thrombosis and Thrombolysis 2010; 29: 199-207.

Weitz JI. Low-molecular-weight heparins. The New England Journal of Medicine 1997; 337: 688-698.

Wells MR, Kraus K, Batter DK, Blunt DG, Weremovitz J, Lynch SE, Antoniades HN and Hansson HA. Gel matrix vehicles for growth factor application in nerve gap injuries repaired with tubes: A comparison of Biomatrix, collagen and methylcellulose. Experimental Neurology 1997; 146: 395-3402.

Whitcher JP, Srinivasan M and Upadhyay MP. Corneal blindness: a global perspective. Bulletin of the World Health Organization 2001; 79: 214-221.

Wilson SE, He YG, Weng J, Li Q, McDowall AW, Vital M and Chwang EL. Epithelial injury induces keratocyte apoptosis: hypothesized role for the interleukin-1 system in the modulation of corneal tissue organization and wound healing. Experimental Eye Research 1996; 62: 325-327.

Wilson SE, He YG, Weng J, Zieske JD, Jester JV and Schultz GS. Effect of epidermal growth factor, hepatocyte growth factor, and keratocyte growth factor, on proliferation, motility and differentiation of human corneal epithelial cells. Experimental Eye Research 1994a; 59: 665-678.

Wilson SE, Liang Q and Kim WJ. Lacrimal gland HGF, KGF, and EGF mRNA levels increase after corneal epithelial wounding. Investigative Ophthalmology and Vision Science 1999; 40: 2185-2190.

Wilson SE, Mohan RR, Mohan RR, Ambrosio R Jr, Hong JW and Lee JS. The corneal wound healing response: Cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells. Progress in Retinal and Eye Research 2001; 20: 625-637.

Wilson SE, Schultz GS, Chegini N, Weng J and He YG. Epidermal growth factor, transforming growth factor alpha, transforming growth factor beta, acidic fibroblast growth factor, basic fibroblast growth factor, and interleukin-1 proteins in the cornea. Experimental Eye Research 1994b; 59: 63-72.

Wissink MJB, Beernink R, Pieper JS, Poot AA, Engbers GHM, Beugeling T, van Aken WG and Feijen J. Immobilization of heparin to EDC/NHS-crosslinked collagen: Characterization and *in vitro* evaluation. Biomaterials 2001; 22: 151-163.

Wissink MJB, Beernink R, Poot AA, Engbers GHM, Beugeling T, van Aken WG and Feijen J. Improved endothelialization of vascular grafts by local release of growth factor from heparinized collagen matrices. Journal of Controlled Release 2000; 64: 103-114.

Wolf-Brandstetter C, Lode A, Hanke T, Scharnweber D and Worch H. Influence of modified extracellular matrices on Ti6AL4V implants on binding and release of VEGF. Journal of Biomedical Materials Research 2006; 79A: 882-894.

Wu B, Gerlitz B, Grinnel BW and Meyerhoff ME. Polymeric coatings that mimic the endothelium: combining nitric oxide release with surface-bound active thrombomodulin and heparin. Biomaterials 2007; 28: 4047-4055.

Wu Y, Simonovsky FI, Ratner BD and Horbett TA. The role of adsorbed fibrinogen in platelet adhesion to polyurethane surfaces: A comparison of surface hydrophobicity, protein adsorption, monoclonal antibody binding, and platelet adhesion. Journal of Biomedical Materials Research 2005; 74A: 722-738.

Wu Z, Zhou Y, Li N, Huang M, Duan H, Ge J, Xiang P and Wang Z. The use of phospholipase A2 to prepare acellular porcine corneal stroma as a tissue engineering scaffold. Biomaterials 2009; 30: 3513-3522.

Xiao J, Duan H, Liu Z, Wu Z, Lan Y, Zhang W, Li C, Chen F, Zhou Q, Wang X, Huang J and Wang Z. Construction of the recellularized corneal stroma using porous acellular corneal scaffold. Biomaterials 2011; 32: 6962-6971.

Xu KP, Ding Y, Ling J, Dong Z and Yu FS. Wound-induced HB-EGF ectodomain shedding and EGFR activation in corneal epithelial cells. Investigative Ophthalmology and Visual Science 2004a; 45: 813–820.

Xu KP, Riggs A, Ding Y and Yu FS. Role of ErbB2 in corneal epithelial wound healing. Investigative Ophthalmology and Visual Science 2004b; 45: 4277– 4283.

Yang C, Hillas PJ, Baez JA, Nokelainen M, Balan J, Tang J, Spiro R and Polarek JW. The application of recombinant human collagen in tissue engineering. Biodrugs 2004; 18: 103-119.

Yao C, Roderfeld M, Rath T, Roeb E, Bernhagen J and Steffens G. The impact of proteinase-induced matrix degradation on the release of VEGF from heparinized collagen matrices. Biomaterials 2006; 27: 1608-1616.

Yayon A, Klagsbrun M, Esko JD, Leder P and Ornitz DM. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 1991; 64: 841-848.

Yin J and Yu FS. Rho kinases regulate corneal epithelial wound healing. American Journal of Physiology: Cell Physiology 2008; 295: C378–C387.

Yoshioka R, Shiraishi A, Kobayashi T, Morita S, Hayashi Y, Higashiyama S and Ohashi Y. Corneal epithelial wound healing impaired in keratinocyte-specific HB-EGF-deficient mice *in vivo* and *in vitro*. Investigative Ophthalmology and Visual Science 2010; 51: 5630-5639.

Yu FX, Yin J, Xu K and Huang J. Growth factors and corneal wound healing. Brain Research Bulletin 2010; 81: 229-235.

Yuan S, Szakalas-Gratzl G, Ziats NP, Jacobsen DW, Kottke-Marchant K and Marchant RE. Immobilization of high-affinity heparin oligosaccharides to radiofrequency plasma-modified polyethylene. Journal of Biomedical Materials Research 1993; 27: 811-819.

Zeeman R, Dijkstra PJ, van Wachem PB, van Luyn MJ, Hendriks M, Cahalan PT and Feijen J. Crosslinking and modification of dermal sheep collagen using 1, 4-butanediol diglycidyl ether. Journal of Biomedical Materials Research 1999; 46: 424-433.

Zhou Z and Meyerhoff ME. Preparation and characterization of polymeric coatings with combined nitric oxide release and immobilized active heparin. Biomaterials 2005; 26: 6506-6517.

3 SCOPE

For the success of tissue engineered cornea equivalents (TECE) or blood-contacting biomaterials, the use of natural polymers and biomimetic modification strategies may enhance tissue-host interactions. The use of collagen-based biomaterials, with or without heparin addition, could mimic the extracellular cell environment, for enhanced *in vivo* compatibility.

Many tissue engineering applications require a substrate with enhanced strength, which has been shown to be possible with dendrimer crosslinked collagen gels. The efficacy of this crosslinking technology was further validated with commercially available collagen-based materials [Chapter 4: Paper 1], as determined via transmittance, water uptake, differential scanning calorimetry, collagenase stability and *in vitro* cell compatibility.

To continue the theme of dendrimer crosslinked collagen gels as TECE materials, growth factor incorporation was investigated with epidermal growth factor (EGF) and heparin-binding EGF (HB-EGF), as a method for improving device epithelialization and subsequent host integration. However, given the short half lives of these growth factors, an effective growth factor delivery system is necessary to protect growth factor bioactivity. As heparan sulphate proteoglycans sequester and release heparin-binding growth factors *in vivo*, the use of heparinized dendrimer crosslinked collagen gels for HB-EGF delivery would provide prolonged, controlled delivery, while maintaining growth factor effectiveness [Chapter 5: Paper 2]. Alternatively, tethering EGF or HB-EGF to dendrimer crosslinked gels could also supply growth factor stimulation in a manner that maintains bioactivity, while stimulating growth factor receptors continually with minute concentrations [Chapter 6: Paper 3]. Growth factor uptake and bioactivity was assessed with radiolabeled growth factor and through *in vitro* epithelial cell culture, respectively.

Finally, dendrimer crosslinked collagen gels, with pre-activated heparin were investigated as a tissue engineered blood-contacting biomaterial [Chapter 7: Paper 4], as we hypothesized that biomaterial induced coagulation is not only influenced by an anticoagulant surface, but also by the underlying material and that improved blood-

biomaterial interactions may be achieved by utilizing a natural polymer that emulates biomimetic properties. Pre-activation of heparin was utilized to increase heparin gel content, while antithrombotic properties were evaluated via antithrombin and fibrinogen adsorption and plasma recalcification times.

4 PAPER 1: MODIFIED DENDRIMER CROSSLINKED COLLAGEN-BASED MATRICES

4.1 Summary

Authors:

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Publication information:

Accepted to Journal of Biomaterials Science, Polymer Edition (16 pages).

Objectives:

To validate the use of dendrimer collagen crosslinking with commercially available collagen-based materials, including solutions of highly purified bovine and human collagen, and human extracellular matrix.

Main Scientific Contributions

- Synthesized dendrimer crosslinked collagen-based gels with commercially available collagen-based products and demonstrated that the process previously developed could be used generically.
- Investigated the integrity of dendrimer crosslinked collagen-based gels via transmittance, water uptake, differential scanning calorimetry and collagenase digestion.
- Developed tissue culture techniques for successfully seeding cells on dendrimer crosslinked collagen-based gels to demonstrate cell compatibility of 3T3 fibroblasts.
- Modified the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) collagen matrix assay to dissolve dendrimer crosslinked collagen-based materials to quantify cell proliferation.

Modified Dendrimer Crosslinked Collagen-Based Matrices

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Short Title:
Dendrimer Collagen-Based Matrices

4.2 Abstract

Dendrimer crosslinking has been achieved with pepsin digested over 80% type I bovine collagen to create strong hydrogels with good cell compatibility. Herein we investigate the use of commercially available collagen-based products with the dendrimer crosslinking technology. Specifically PureCol® (PC), a 97% bovine type I collagen, human collagen (HC), and human extracellular matrix (hECM) were concentrated, and then crosslinked with polypropyleneimine octaamine generation two dendrimers using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) chemistry. PC gels with 30 and 20 mg/mL bovine collagen were fabricated, and despite similar concentrations to >80% type I bovine collagen dendrimer crosslinked gels (CG), PC gels demonstrated increased swelling and decreased stability, as determined with collagenase digestion. The highly purified bovine (PC) and human sourced-collagen (HC) gels were similar in performance, but not as stable as the CG gels, which may correlate to the manufacturer's collagen purification and storage. Finally, the addition of hECM components to PC to create PC-hECM gels, resulted in a looser gel network, compared to heparinized dendrimer crosslinked bovine >80% type I collagen gels (CHG). However all collagen-based gels supported 3T3 fibroblast cell growth over 4 days, indicating these gels may be suitable for tissue engineering applications.

Keywords: collagen; dendrimers; extracellular matrix; heparin.

4.3 Introduction

Collagen, one of the most abundant proteins in the body, provides extracellular biological and structural support in soft and hard connective tissues [1]. Uniquely consisting of three polypeptide chains of glycine-X-Y amino acid sequences wrapped into a triple helical structure, 28 different types of collagen have been discovered to date [2]. The most predominant, collagen type I is fibrillar, and found in the skin, tendon, bone, cornea, dentin, fibrocartilage, large vessels, intestine, and uterus [3]. It mediates cell adhesion via interactions with over 50 molecules including glycosaminoglycans, proteins and growth factors [4]. Of the glycine-X-Y sequence, collagen type I usually has proline and hydroxyproline in the X and Y positions, respectively [5].

Collagen type I, from animal or recombinant technology sources, is widely utilized in cosmetic, biomedical and tissue engineering applications, particularly in applications where porous, three-dimensional structural matrices are required for cell support [6,7] including bone, cartilage, skin, cardiovascular, corneal, urinary and renal tissue engineering applications [1,2,8].

Collagen extraction and isolation from animal derivatives, via enzymatic digestion, cleave natural fibril crosslinks and remove telopeptide regions, resulting in a mildly immunogenic collagenous material that does not mimic its previously observed strength, and for use in biomaterial and tissue engineering applications, it may collapse or degrade too quickly upon *in vivo* implantation [3]. Thus, prior to use as a tissue engineering scaffold, chemical crosslinking techniques are necessary to increase the mechanical and biological stability of collagen-based materials, while maintaining *in vivo* compatibility.

Current chemical crosslinking techniques used to increase matrix strength include formaldehyde, glutaraldehyde, polyepoxy, hexamethylenediisocyanate, carbodiimide and acyl azide [3,9]. Formaldehyde [3,10,11,12] and glutaraldehyde [3,13,14] crosslink collagen lysine or hydroxylysine groups with tyrosine, glutamine or asparagine, but result in heterogeneous, brittle and toxic collagen-based constructs [14,15,16]. Hexamethylene diisocyanate activates collagen lysine groups for amide bond formation [17,18], while collagen carboxylic acid groups have been modified to contain reactive acyl azide end

groups that could bind amine groups [19]. However, collagen matrices crosslinked with hexamethylenediisocyanate, acyl azide, or glutaraldehyde, were found to be cytotoxic following implantation in rats [20]. Crosslinking of the lysine or hydroxylysine residues with a poly(epoxy) compound, 1,4-butanediol diglycidyl ether, was found to lead to materials which demonstrated good biocompatibility in rats [21,22].

Collagen crosslinked with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) forms zero-length crosslinks within the collagen matrix, whereby carboxyl groups of glutamic or aspartic acid are activated by EDC to form a highly reactive O-acylisourea ester intermediate, which when stabilized with N-hydroxysuccinimide (NHS), reacts with lysine amine groups, creating stable amide bonds [23,24]. Collagen matrices fabricated in this manner, and those combined with other extracellular matrix components, demonstrated increased strength, compared to collagen thermal gels, and good *in vitro* and *in vivo* compatibility [24-30]. However, collagen EDC/NHS crosslinking is limited by the number of amine groups available within the collagen molecule, typically 30 per 1000, compared to the 120 per 1000 carboxylic acid groups [24,31]. Furthermore, not all carboxylic acid groups are activated by EDC which further decreases crosslinking density [31].

Thus, to increase the number of amine groups available for crosslinking, multifunctional amine-terminated dendrimers were crosslinked with collagen type I using EDC/NHS chemistry [31]. The resulting materials demonstrated decreased water uptake, increased denaturation temperatures and increased collagenase stability, suggesting a stronger network compared to thermal and EDC/NHS crosslinked collagen gels [31]. Additional gel characterization revealed increased transmittance, mechanical strength, and glucose permeability, which supported cornea epithelialization *in vitro* [32]. Cornea cell compatibility of the dendrimer crosslinked collagen gels was further enhanced by biological functionalization with laminin-based cell adhesion peptides [33,34], and heparin for the delivery of basic fibroblast growth factor [35].

The previously mentioned collagen-based crosslinked materials were fabricated from dermal sheep collagen [24], porcine type I collagen [29], recombinant human type I

collagen [30], and bovine type I collagen [31]. As the collagen source, extraction and purification methods have been shown to alter the collagen ultrastructure of telopeptides and fibrils, and affect subsequent gel thermal and enzymatic stability following chemical crosslinking [7], it is clear that there is a need for evaluation of the various commercial collagen-based materials to distinguish the effect of the differences in collagen source and purification methods. This characterization in turn will influence the design process and applicability of collagen-based tissue engineering applications.

Herein, we explore the various commercially available collagen-based products, the kind gift of Allergan Inc. (Irvine, CA, USA), and currently available through Advanced BioMatrix (www.advancedbiomatrix.com). PureCol® (PC) is 99.9% pure bovine atelocollagen, containing 97% type I collagen with the remainder type III collagen, supplied 3 mg/mL in 0.01 N HCl (pH 2). Sourced from human neo-natal fibroblast cells, human collagen (HC), now marketed as VitroCol^{TM} by Advanced BioMatrix, is >99.9% pure human collagen supplied at a concentration of 3 mg/mL in 0.01 N HCl (pH 2), and is comprised of >97% type I collagen with the remainder type III collagen. Human extracellular matrix (hECM) was analyzed by Allergan to contain type I collagen, elastin, sulphated glycoaminoglycans (GAGs) and fibronectin, listed in order of highest to lowest content (composition not specified). Following dendrimer crossinking, we compare (1) bovine collagen to human collagen; (2) collagen gel concentrations; and (3) the effect of the incorporation of extracellular matrix components on the properties of the matrix produced.

4.4 Materials and Methods

4.4.1 Materials

Concentrated collagen suspensions of pepsin digested bovine cornium purified type I collagen, with less then 20% type III collagen, PureCol® (PC), human collagen (HC) and human extracellular matrix (hECM), were all generous gifts from Allergan Inc (Irvine, CA).

Polypropyleneimine octaamine generation two dendrimer (G2) was purchased from SyMO-Chem (Eindhoven, The Netherlands). Cell culture materials, including Dulbecco's

Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Penicillin (10,000 U/mL)-Streptomycin (10,000 ug/mL), TrypLE^{TM} Express, and Dulbecco's phosphate buffer saline (PBS, no magnesium or calcium) were purchased from Invitrogen Canada Inc. (Burlington, ON). Calcium chloride ($CaCl_2$) was purchased from Caledon Laboratory Chemicals (Georgetown, ON). Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (Oakville, ON) or EMD Chemicals Inc. (Gibbstown, NJ).

4.4.2 Collagen Gel Fabrication

Solutions of PC, HC and hECM were received acidified in 0.01 N HCl (pH 2). Solutions were freeze dried and reconstituted in PBS (pH 7.4) to a concentration of 66 mg/mL as in our previous studies. The gels were then fabricated based on a method adapted from previous work [31,35]. Briefly, collagen suspensions were diluted with phosphate buffer solution (PBS) and acidified with 1N HCl prior to crosslinking, if necessary. Crosslinking was achieved through EDC/NHS chemistry, using a second generation polypropyleneimine octaamine dendrimer (molar ratio of collagen$_{COOH}$: EDC:NHS was 1:1:1) for amplification of the amine groups in the collagen to facilitate crosslinking. Briefly, the acidified collagen solutions were combined with 200 μL of the crosslinking solution (75 mg EDC + 45 mg NHS + 38 mg G2 dendrimer dissolved in 1 mL endotoxin-free water (EFW)). Where necessary, the suspension pH was adjusted to 5.5 with 1N NaOH to stabilize the EDC/NHS reaction [24]. To ensure complete chemical crosslinking, the mixtures were pressed between glass plates and left overnight at 4^{o}C. Gels were rinsed well, dried and stored at room temperature prior to testing. Surfaces were reconstituted in Milli-Q water prior to testing. Gels were compared to previously fabricated dendrimer crosslinked collagen (CG) gels, and heparinized dendrimer crosslinked collagen (CHG), containing either 204.5 (CHG-30H) or 20.45 (CHG-30L) μg heparin per mg collagen, all fabricated with bovine type I collagen (<20% type III collagen) [35].

4.4.3 Transmittance

Transmittance of the hydrated gels (1 mm thick) was determined by measuring absorbance within the visible range of wavelengths (410 – 700 nm) using a BioRad 550 plate reader.

4.4.4 Water uptake

Water content of the collagen gels was used 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 24 hrs, 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:

$$\text{Water Uptake (\%)} = [\ (W_w - W_d) / W_w\ ] * 100\%$$

4.4.5 Differential Scanning Calorimetry

Denaturation temperatures for collagen materials, used to provide information about the degree of crosslinking [36,37], were determined by differential scanning calorimetry (DSC) using a TA instruments DSC 2910. Collagen gels were immersed in endotoxin free water (EFW) for 2 hrs, dabbed dry, and transferred to aluminum hermetic pans. They were then heated at a 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.

4.4.6 Collagenase Digestion

Gels were exposed to collagenase solution to assess degradation and biological stability through hydroxyproline content. Degradation was quantified through hydroxyproline analysis, as it is a major component of collagen released upon degradation [31,38,39]. Collagen gels (0.64 cm diameter) were submersed for 1 hour in 500 µL 0.1 M TRIS/HCl (pH 7.4) containing 0.05 M $CaCl_2$, then exposed to 100 µL of 200 U collagenase (EC 3.4.24.3; 266 U/mg; 7.52 mg/mL) for 24 hrs at 37°C. At this time, the reaction was terminated with 200 µL 0.25M EDTA and samples were placed on ice

for 10 minutes. Samples were centrifuged (5 min, 5000 rpm) at room temperature, and the supernatant analyzed for hydroxyproline content. Aliquots of 200 µL were autoclaved at 121°C for 20 min, then oxidized with 450 µL Chloramine T (0.056 M in 50% 2-propanol and 50% acetate/citric acid buffer) for 25 min at room temperature, and subjected to 500 µL Ehrlich's reagent (1M p-di-methyl-aminobenzaldehyde in 2:1 v/v 2-propanol/perchloric acid) for 20 min at 65°C. Samples (200 µL) were read at 540 nm & 700 nm, and compared to hydroxyproline standards (1 – 10 000 µg/mL).

4.4.7 Cell Viability

Cell viability was evaluated through culture of 3T3 fibroblasts onto 0.64 cm diameter collagen-based gels. Cells were grown in DMEM containing fetal bovine serum (1:10) and Penicillin-Streptomycin antibiotic (1:100). Prior to cell culture studies, the gels were soaked in 1 mL PBS containing 100 µL antibiotic (10:1) for 24 hrs. Gels were seeded with approximately 10,000 cells/gel for 3.5 hours, followed by addition of serum containing DMEM. Fresh medium was added every 2 days. Cell morphology was verified by microscopy (Axiovert 200) using a 10x objective on Day 2 and Day 4 of culture.

After 4 days, cell growth was assessed with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay [40,41]. Briefly, 150 µL of MTT solution (0.4 mg/mL MTT, 0.2 µm sterile filtered, in DMEM containing serum and antiobiotic) was added to the cells and the plate was covered and incubated for 4.5 hrs. The gels were then dissolved with 100 µL collagenase (345 U/mg; 7.71 mg/mL) in 200 µL TRIS/HCl (15.76 mg/mL) containing 0.05M $CaCl_2$ for 3.5 hours. Following centrifugation (10 min, 5000 rpm), the supernatant was removed and the pellet reconstituted in 500 µL DMSO to dissolve the formazan precipitate. Following agitation to dissolve the pellet, 200 µL was transferred to a 96-well plate and absorbance read at 595 and 700 nm.

4.4.8 Statistical Analysis

Data are presented as mean values with variability expressed as standard deviations, unless otherwise stated. Statistical significance was determined using a single factor ANOVA test on two means ($p<0.05$).

4.5 Results

4.5.1 Collagen Gel Fabrication

The collagen-based gels prepared are summarized in Table 4-1. Gels with collagen concentrations of 30 and 20 mg/mL were fabricated from PureCol® (PC) collagen, which consists of >97% type I bovine collagen, while human collagen (HC) gels were fabricated with a collagen concentration of 20 mg/mL. Interestingly, human extracellular matrix components (hECM) would form a gel only with the further addition of collagen, namely PureCol®, resulting in a gel with a final protein concentration of 20 mg/mL (PC-hECM-20; containing 30% hECM & 70% PureCol® with 14 mg/mL PureCol). For comparison, dendrimer crosslinked bovine collagen (CG) gels, with >80% type I collagen, were prepared with collagen concentrations of both 30 mg/mL (CG-30) and 20 mg/mL (CG-20). In addition heparinized dendrimer crosslinked >80% bovine type I collagen (CHG) gels with 204 or 20.45 mg heparin per mg collagen (CHG-30H & CHG-30L, respectively) were prepared to be compared to PC-hECM-20 gels, the latter of which contains type I collagen, elastin, sulphated glycosaminoglycans (GAGs) and fibronectin.

4.5.2 Transmittance

The transmittance of the dendrimer crosslinked collagen-based gels is shown in Figure 4-1. Transmittance increased with decreasing collagen concentration, as CG-30 and PC-30 gels containing 30 mg/mL of protein were found to be more opaque than CG-20 ($p<0.05$) and PC-20 ($p<0.5$) gels, respectively, which contain 20 mg/mL collagen. Gels fabricated with >97% bovine type I collagen (PC) were significantly more transparent than those prepared with the less pure >80% bovine type I collagen (CG) at a lower collagen concentration of 20 mg/mL ($p<0.05$). Transparency differences became insignificant when the gels were prepared with collagen at a concentration of 30 mg/mL ($p<0.15$). Human collagen (HC-20) had the highest transmittance, which was significantly different from the bovine-sourced PC and CG gels with similar collagen concentration of 20 mg/mL ($p < 0.05$).

The incorporation of hECM components in PC-hECM-20 gels further increased opacity, compared to PC-20 ($p<0.05$). Similar results were obtained with the addition of

heparin; the heparinized dendrimer crosslinked collagen gels (CHG) fabricated with 204.5 (CHG-30H) or 20.45 (CHG-30L) mg heparin per mg collagen were more opaque than CG-30 ($p<0.05$).

4.5.3 Water uptake

Water uptake of dendrimer crosslinked collagen-based gels, shown in Figure 4-2, was determined from dry and wet weights of dried and re-hydrated collagen materials. Gels containing highly purified collagen, namely PC-20, PC-30, HC-20, and PC-hECM-20 had high swelling values, between 94.82 ± 0.80 % and 98.75 ± 0.92 %.

Dendrimer crosslinked bovine collagen gels prepared with higher collagen concentrations (30 mg/mL; PC-30 & CG-30) swelled significantly less than gels with lower collagen amounts (20 mg/mL; PC-20 & CG-20) as expected. Highly purified bovine collagen gels with >97% collagen (PC) took up significantly more water compared to CG gels, containing 80% type I collagen, regardless of gel concentration ($p<10^{-5}$). Significantly high swelling was also observed with human collagen (HC) relative to the bovine-sourced collagen gels PC-20 and CG-20 ($p<0.09$). The addition of heparin (CHG) resulted in greater swelling compared to unheparinized CG-30 gels ($p<0.0005$). Similarly, human ECM containing gels (PC-hECM-20) swelled to a greater extent than the dendrimer crosslinked collagen gels with similar protein content (CG-20; $p<0.0002$ & PC-20; $p<0.15$) as well as the heparinized gels (CHG-30H & CHG-30L; $p<0.005$).

4.5.4 Differential Scanning Calorimetry

The denaturation temperatures for the collagen gels, found through differential scanning calorimetry (DSC), are shown in Figure 4-3. Denaturation temperatures for all gels were between 96-98^{o}C, and comparable to that of CG-30 as in our previous studies ($p>0.05$). The exception was the PC-30, which showed significantly higher denaturation temperatures compared to that of CG-30 ($p=0.03$), likely related to collagen purity.

4.5.5 Collagenase Digestion

Gels were exposed to collagenase solution to assess degradation and biological stability through hydroxyproline content. Degradation was quantified through hydroxyproline analysis, as it is a major component of collagen released upon degradation [31,38,39]. Following degradation, the hydroxyproline content for 0.64 cm diameter dendrimer crosslinked >80% bovine type I collagen (CG-30) gels was 31.69 ± 19.90 μg/mL. Hydroxyproline content of all gels is shown as relative denaturation in relation to CG-30 in Figure 4-4.

Significantly higher levels of degradation were observed with highly purified collagen gels containing >97% type I collagen and lower collagen concentration of 20 mg/mL (PC-20), compared to CG-20 ($p<0.0008$) not surprisingly. Heparinization did not affect hydroxyproline content, and hence relative degradation, as CHG-30H and CHG-30L were not significantly different than CG-30 ($p=0.5$). Despite the low appearance of hydroxyproline with the extracellular matrix gel (PC-ECM-20), the observed stability may be correlated to a lower amount of collagen in these gels, and hence hydroxyproline, within the gel matrix. The human collagen gel (HC-20) had significantly lower relative denaturation then the dendrimer crosslinked >80% bovine type I collagen gel with a similar collagen concentration, CG-20 ($p<0.004$).

4.5.6 Cell Viability

To assess cellular response to the various gels, 3T3 fibroblast cells were seeded onto the dendrimer crosslinked collagen-based gels for 4 days. Following MTT analysis, cell proliferation was assessed and compared to the CG-30 gels; results are shown in Figures 4-5 and 4-6. There was no significant difference in cell growth among gels with varied collagen purity ($p>0.05$), except between >80% type I collagen (CG-30) and >97% type I collagen (PC-30) at the high collagen concentrations of 30 mg/mL ($p<0.009$). Heparinization of dendrimer crosslinked bovine >80% type I collagen gels (CHG) had no effect on cell growth, compared to CG-30 gels ($p>0.05$). Furthermore, the extracellular matrix gel (PC-hECM-20) did not show altered cell proliferation compared to dendrimer

crosslinked gels with similar protein content (20 mg/mL; CG-20 & PC-20 & HC-20; $p>0.05$).

4.6 Discussion

Dendrimer crosslinking, with EDC/NHS, was achieved with all commercially available collagen-based materials. However, somewhat surprisingly, hECM required further addition of collagen for complete gel crosslinking. The composition of the hECM is known to include type I collagen, elastin, GAGs and fibronectin, although the specific amounts were not specified by the manufacturer. It must therefore be assumed that there were insufficient carboxylic acid groups within the hECM mixture to react fully with the amine-terminated dendrimer and allow for complete crosslinking.

Transmittance increased with decreasing collagen concentration, as gels containing 30 mg/mL collagen (CG-30 and PC-30) were found to be more opaque than gels with 20 mg/mL collagen (CG-20 and PC-20). The gels fabricated from PureCol® collagen and human ECM components (PC-hECM-14) had increased opacity relative to gels with 20 mg/mL collagen, which is not unexpected as hECM is comprised of type 1 collagen, elastin, sulphated GAGs and fibronectin. Sulfated GAGs in particular have been shown to affect collagen fibril assembly and opacity [42,43], while fibril diameter and orientation can also affect binding and recognition sites for ligands, cells, and platelets [44]. Elsewhere, collagen gels fabricated with serum demonstrated decreased transmittance [45], while heparinized dendrimer collagen gels had increased transmittance with decreased heparin content [35].

The high denaturation temperatures of the collagen-based gels, determined via DSC, are indicative of dendrimer-collagen bonds [31]. The large error bar of CHG-30H is due to an outlier; the small sample size utilized for the DSC pan may not have contained adequate amounts of dendrimer-collagen bonds for detection.

High degradation based on hydroxyproline content corresponded to the dendrimer-collagen crosslinks, and are in accordance with our previous observations for dendrimer crosslinked collagen gels [31,35]. As the collagen gel materials are all >80% collagen

type I, the hydroxyproline content was assumed to be similar. Despite the formation of stronger dendrimer-collagen crosslinks, as determined via DSC, the highly purified bovine >97% type I collagen (PC) and human collagen (HC) gels generally had increased swelling and collagen degradation, compared to bovine >80% type I collagen (CG), indicating a looser, more delicate network structure. Ultimately, it may have been useful to obtain information about the mechanical properties of these gels, but these gels did not have adequate mechanical handling strength to be subjected to traditional mechanical testing techniques.

The decreased enzymatic stability may be correlated to purification processing. The collagen products, PC and HC, as received from the manufacturer are highly purified and stored in acidic solution. It is hypothesized that harsher purification and storage conditions may degrade more of the collagen chain structure and as such the crosslinking of the collagen is compromised.

Elsewhere, type I acid solubilized and pepsin digested atelocollagen in fibril and afibrillar form, crosslinked with EDC/NHS, demonstrated ultrastructural and stability differences, hypothesized to be from extraction and purification methods [7]. Thus, we suggest that collagen-based materials intended for tissue engineering applications would benefit from long-term refrigerated storage in concentrated amounts, without exposure to acidic solutions, as was the received storage conditions of the bovine collagen utilized in CG and CHG gels. In addition, to obtain the high gel concentrations (3%), PC, HC and hECM were freeze-dried prior to gel fabrication, which may have further damaged collagen fibrils. Freeze-dried collagen matrices demonstrated weaker *in vivo* compatibility, compared to fresh-frozen collagen matrices, as evident via accelerated graft remodeling of rat tendons [46].

Despite increased swelling and collagen digestion, PC, HC and ECM-based gels support cell growth of 3T3 fibroblasts over 4 days; however to a lower extent than CG gels. Previously, CG gels, intended for tissue engineered corneal applications, were shown to support corneal epithelial cell proliferation [32] and following the addition of laminin-based peptides, enhanced epithelial cell stratification and dorsal neurite

extentions were observed [33,34]. Addition of cell binding motifs did not significantly adversely affect gel properties [33], and these motifs may be incorporated into these commercially collagen-based materials for enhanced cell-matrix interactions.

Collagen based materials have been investigated for bone, cartilage, skin, tendon, urinary, renal, corneal and cardiovascular tissue engineering [1,8]. The commercially available collagen-based materials may be suitable for certain tissue engineering applications where high stability is not required. Purecol, also referred to as Vitrogel, was utilized as a cell delivery scaffold for Schwann cells for nerve regeneration [47], transfected fibroblasts for neural axon growth following rat spinal cord injury [48] and bone marrow stromal cells in rats [49]. Basic fibroblast growth factor was delivered via Purecol matrices to assess angiogenesis in rats [50]. Furthermore, Purecol was utilized for *in vitro* cellular assays to investigate matrix invasion of astrocytoma cells [51], metabolic and cilio-toxicity on nasal cells [52], and cancer cell penetration [53,54]. Furthermore, dendrimer crosslinking of these collagen-based materials, in addition to the development of mechanically strong matrices, may be suitable for incorporation of cell binding motifs to further enhance matrix-cell interactions.

4.7 Conclusions

The collagen crosslinking method utilizing amine-terminated dendrimers can be used to generate collagen-based gels from a variety of commercially available materials including those from different sources and containing different components. The resulting gels had properties which demonstrated their suitability for certain tissue engineering applications. However, as was evident with hECM-based gels, the amplification with additional carboxylic acid functionality may be necessary in some cases, to generate gels with enhanced stability. Furthermore, processing of the materials and storage in acidic medium may have a detrimental effect on materials properties.

4.8 Acknowledgements

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4.9 References

1. P.B. Malafaya, G.A. Silva and R.L. Reis. *Adv Drug Deliv Rev* 59, 207 (2007).
2. Yang, P.J. Hillas, J.A. Baez, M. Nokelainen, J. Balan, J. Tang, R. Spiro and J.W. Polarek. *Tissue Eng* 18, 103 (2004).
3. W. Friess. *Eur J Pharma Biophar* 45, 113 (1998).
4. G.A. DiLullo, S.M. Sweeney, J. Korkko, L. Ala-Kokko and J.D. San Antonio. *J Biol Chem* 277, 4223 (2002).
5. D.J. Prockop and K.I. Kavirikko. *Annu Rev Biochem* 64, 403 (1995).
6. Sachlos and J.T. Czernuszka. *Eur Cell Mater* 5, 29 (2003).
7. R.S. Walton, D.D. Brand and J.T. Czernuszka. *J Mater Sci: Mater Med* 21, 451 (2010).
8. L. Cen, W. Liu, W. Cui, W. Zhang and Y. Cao. *Pediatr Res* 63, 492 (2008).
9. E. Khor. *Biomaterials* 18, 95 (1997).
10. R.J. Ruderman, C.W.R. Wade, W.D. Shepard and F. Leonard. *J Biomed Mater Res* 7, 263 (1973).
11. H. Uludag, W. Friess, D. Williams, T. Porter, G. Timony, D. D'Augusta, C. Blake, R. Palmer, B. Biron and J. Wozney. *Ann N Y Acad Sci* 875, 369 (1999).
12. K Madhaven, D Belchenko, A. Motta and W. Tan. *Acta Biomater* 6, 1413 (2010).
13. D.T. Cheung and M.E. Nimni. *Connect Tissue Res* 10, 201 (1982).
14. K. Weadock, R.M. Olson and F.H. Silver. *Biomater Med Devices Artif Organs* 11, 293 (1983-1984).
15. Cooke, R.F. Oliver and M. Edward. *Br J Exp Pathol* 64, 172 (1983).
16. L.L.H. Huang-Lee, D.T. Cheung and M.E. Nimni. *J Biomed Mater Res* 24, 1185 (1990).
17. T.M. van Gulik, R.A. Christiano, A.H. Broekhuizen, E.L. Raaymakers and P.J. Klopper. *Neth J Surg* 41, 65 (1989).
18. M.L. Jarman-Smith, T. Bodamyali, C. Stevens, J.A. Howell, M. Horrocks, J.B. Chaudhuri. *J Mater Sci Mater Med* 15, 925 (2004).
19. H. Petite, I. Rault, A. Huc, Ph. Menasche and D. Herbage. *J Biomed Mater Res* 24, 179 (1990).
20. P.B. van Wachem, M.J.A. van Lyun, L.H.H. Olde Damink, P.J. Dijkstra, J. Feijen and P. Nieuwenhuis. *J Biomed Mater Res* 28, 353 (1994).
21. R. Zeeman, P.J. Dijkstra, P.B. van Wachem, M.J.A. van Lyun, M. Hendriks, P.T. Cahalan and J. Feijen. *Biomaterials* 20, 921 (1999).

22. P.B. van Wachem, R. Zeeman, P.J. Dijkstra, J. Feijen, M. Hendriks, P.T. Cahalan and M.J.A. van Lyun. *J Biomed Mater Res* 47, 270 (1999).

23. Z. Grabarek and J. Gergely. *Anal Biochem* 185, 131 (1990).

24. L.H.H. Olde Damink, P.J. Dijkstra, M.J.A.van Luyn, P.B. van Wachem, P. Nieuwenhuis and J. Feijen. *Biomaterials* 17, 765 (1996).

25. J.S. Pieper JS, A. Oosterhof, P.J. Dijkstra, J.H. Veerkamp and T.H. van Kuppevelt. *Biomaterials* 20, 847 (1999).

26. P. Buma, J.S. Pieper, T. van Tienen, J.L.C. van Susante, P.M. van der Kraan, J.H. Veerkamp, W.B. van den Berg, R.P.H. Veth and T.H. van Kuppevelt. *Biomaterials* 24, 3255 (2003).

27. M.J. Wissink, R. Beernink, J.S. Pieper, A.A. Poot, G.H. Engbers, T. Beugeling, W.G. van Aken and J. Feijen. *Biomaterials* 22, 151 (2001).

28. P.B. van Wachem, J.A. Plantinga, M.J. Wissink, R. Beernink, A.A. Poot, G.H. Engbers, T. Beugeling, W.G. van Aken, J. Feijen J and M.J. van Luyn. *J Biomed Mater Res* 55, 368 (2001).

29. Y. Liu, L. Gan, D.J. Carlsson, P. Fagerholm, N. Lagali, M.A. Watsky, R. Munger, W.G. Hodge, D. Priest and M. Griffith. *Invest Ophthalmol Vis Sci* 47, 1869 (2006).

30. Y. Liu, M. Griffith, M.A. Watsky, J.V. Forrester, L. Kuffova, D. Grant, K. Merrett and D.J. Carlsson. *Biomacromolecules* 7, 1819 (2006).

31. X. Duan and H. Sheardown. *J Biomed Mater Res* 75A, 510 (2005).

32. X. Duan and H. Sheardown. *Biomaterials* 27, 4608 (2006).

33. X. Duan, C. McLaughlin, M. Griffith and H. Sheardown. *Biomaterials* 28, 78 (2007).

34. X. Duan and H. Sheardown. *J Biomater Sci Polym Ed* 18, 701 (2007).

35. M.A. Princz and H. Sheardown. *J Biomater Sci Polymer Edn* 19, 1201 (2008).

36. S.D. Figueiro, A.A. Macedo, M.R. Melo, A.L. Freitas, R.A. Moreira, R.S. de Oliveira, J.C. Goes and A.S. Sombra. *Biophys Chem* 120, 154 (2006).

37. J.M. Lee, C.A. Pereira, D. Abdulla, W.A. Naimark and I. Crawford. *Med Eng Phys* 17, 115 (1995).

38. H. Stegemann and K. Stalder. *Clin Chim Acta* 18, 267 (1967).

39. G.K. Reddy and C.S. Enwemeka. *Clin Biochem* 29, 225 (1996).

40. Leonardi, V. Papa, I. Fregona, P. Russo, G. De Franchis and G. Milazzo. *Cornea* 25, 85 (2006).

41. Ono, H. Kanno, A. Hayashi, S. Nishimura, Y. Kyuma, H. Sato, S. Ito, N. Shimizu, C.C. Chang, G. Gondo, I Yamamoto, T. Sasaki and M. Tanaka. *Int J Clin Oncol* 12, 125 (2007).

42. J.M. McPherson, S.J. Sawamura, R.A. Condell, W. Rhee and D.G. Wallace. *Col Relat Res* 1, 65 (1998)

43. J.M. McPherson, P.W. Ledger, G. Ksander, S.J. Sawamura, A. Conti, S. Kncaid, D. Michaeli and R.A. Clark. *Col Relat Res* 8, 83 (1988).

44. J.P. Orgel, O. Antipova, I. Sagi, A. Bitler, D. Qiu, R. Wang, Y. Xu and J.D. San Antonio. *Connect Tissue Res* 52, 18 (2011).

45. V.M. Borderie, N. Mourra and L. Laroche. *Graefes Arch Clin Exp Ophthalmol* 237, 861 (1999).

46. Y. Toritsuka, K. Shino, S. Horibe, N. Nakamura, N. Matsumoto and T. Ochi. *J Orthop Res* 15, 294 (1997).

47. G.R. Evans, K. Brandt, S. Katz, P. Chauvin, L. Otto, M. Bogle, B. Wang, R.K. Meszlenyi, L. Lu, A.G. Mikos and C.W. Patrick Jr. *Biomaterials* 23, 841 (2002).

48. Y. Hayashi, J.S. Shumsky, T. Connors, T. Otsuka, I. Fischer, A. Tessler and M. Murray. J Neurotrauma 22, 1267 (2005).

49. H. Yoshihara, J.S. Shumsky, B. Neuhuber, T. Otsuka, I. Fischer and M. Murray. *Brain Res* 1119, 65 (2006).

50. C.C. Sieber, L.T. Sumanovski, M. Stumm, M. van der Kooij and Battegay E. *J Hematol* 34, 644 (2001).

51. R.D. Maestro RD, R. Shivers, W. McDonald and A.D. Maestro. *J Neurooncol* 53, 87 (2001).

52. R.U. Aqu, M. Jorissen, T. Willems, P. Augustijns, R. Kinget and N. Verbeke. *J Pharm Pharmacol* 53, 1447 (2001).

53. X. Yang, E.D. Staren, J.M. Howard, T. Iwamura, J.E. Bartsch and H.E. Appert. *J Surg Res* 98, 33 (2001).

54. K.L. Sodek KL, T.J. Brown and M.J. Ringuette. *BMC Cancer* 8, 223 (2008).

4.10 Figures

Figure 4-1. Transmittance of dendrimer crosslinked collagen-based gels. Dendrimer crosslinked gels fabricated with >97% bovine type I collagen (PC) are significantly more transparent than less pure 80% bovine type I collagen (CG) at lower collagen concentration of 20 mg/mL ($p<0.05$), but this is not as apparent at 30 mg/mL ($p<0.15$). Human collagen (HC-20) has the highest transmittance, which is significantly different from the bovine-sourced PC and CG gels with similar collagen concentration of 20 mg/mL ($p<0.05$). The incorporation of extracellular matrix (ECM) components further increases opacity, which is also evident with heparinized dendrimer crosslinked collagen gels (CHG) fabricated with 204.5 (CHG-30H) or 20.45 (CHG-30L) mg heparin per mg collagen.

Figure 4-2. Water uptake of dendrimer crosslinked collagen-based gels. Dendrimer crosslinked bovine collagen gels with higher collagen concentration (30 mg/mL; PC-30 & CG-30) swells less then gels with lower collagen amounts (20 mg/mL; PC-20 & CG-20). Highly purified bovine collagen gels with >97% collagen (PC) uptake significantly more water compared to CG gels, which contain 80% type I collagen, regardless of gel concentration ($p<0.05$). High swelling is also observed with human collagen (HC) compared to the bovine-sourced collagen gels PC-20 and CG-20 ($p<0.05$). The addition of heparin (CHG) results in greater swelling compared to unheparinized gels (CG-30; $p<0.05$), while human extracellular matrix (ECM) gels swell greater then dendrimer crosslinked collagen gels with similar protein content (CG-20; $p<0.05$ & PC-20; $p<0.15$) and heparinized gels (CHG; $p<0.05$).

Figure 4-3. Thermal stability of dendrimer crosslinked collagen-based gels. Denaturation temperatures are similar regardless of collagen purity or concentration and heparin incorporation ($p<0.03$).

Figure 4-4. Hydroxyproline content following collagenase digestion of dendrimer crosslinked collagen-based gels, as normalized to CG-30 gel. Higher hydroxyproline content is observed with highly purified collagen gels containing lower collagen concentration (PC-20; $p<0.05$). Heparinization does not affect hydroxyproline content, and hence relative denaturation. Despite the low appearance of hydroxyproline with the extracellular matrix gel (PC-ECM-20), the observed stability may be correlated to a lower amount of collagen within the gel matrix. The human collagen gel (HC-20) has a lower relative denaturation then the dendrimer crosslinked collagen gel with a similar collagen concentration (CG-20; $p<0.05$).

Figure 4-5. Cell proliferation of 3T3 fibroblasts on dendrimer crosslinked collagen-based gels, compared to CG-30. There is no significant difference of growth between varied collagen purity, except between >80% type I collagen (CG) and >97% type I collagen (PC) at high collagen concentrations of 30 mg/mL ($p<0.05$). Heparinization of dendrimer crosslinked collagen gels (CHG) does not enhance or hinder cell growth ($p<0.05$). The extracellular matrix gel (PC-ECM-20) does not have altered cell proliferation compared to dendrimer crosslinked gels with similar protein content (20 mg/mL; CG-20 & PC-20 & HC-20; $p<0.05$).

Figure 4-6. Cell proliferation of 3T3 fibroblasts on dendrimer crosslinked collagen-based gels (optical density 10x) on CG-20, PC-20 and PC-hECM-20 gels after 2 days and 4 days of growth.

Figure 4-1.

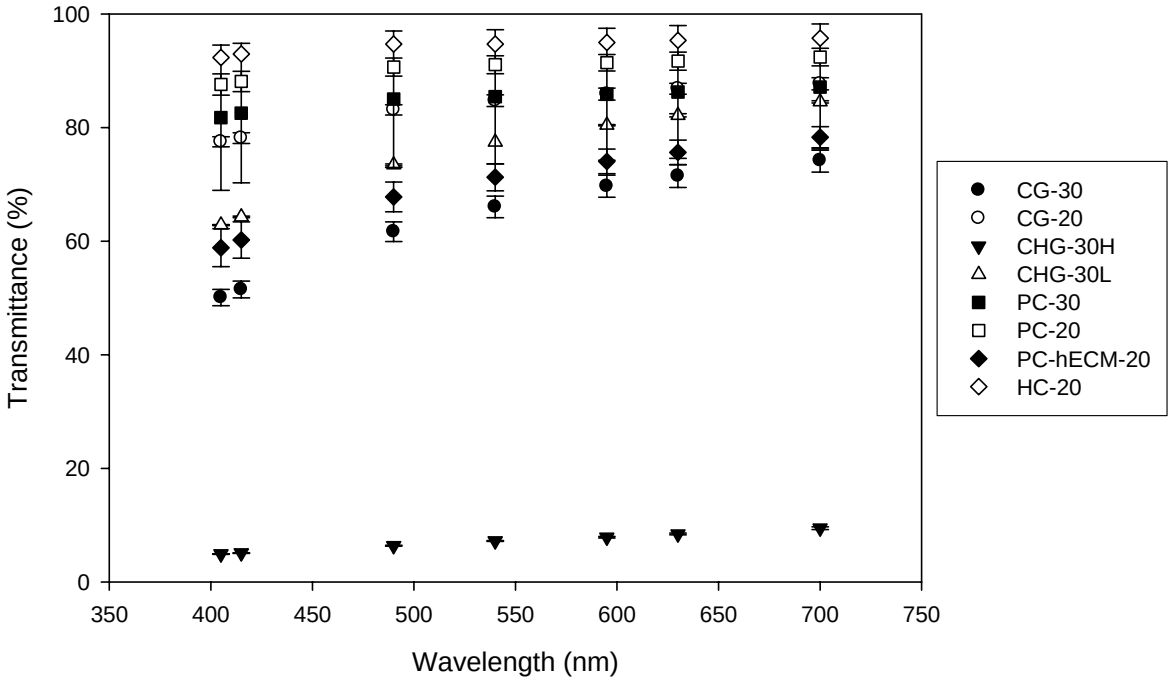

Transmittance (%)
Wavelength (nm)
0
20
40
60
80
100
350
400
450
500
550
600
650
700
750
CG-30
CG-20
CHG-30H
CHG-30L
PC-30
PC-20
PC-hECM-20
HC-20

Figure 4-2.

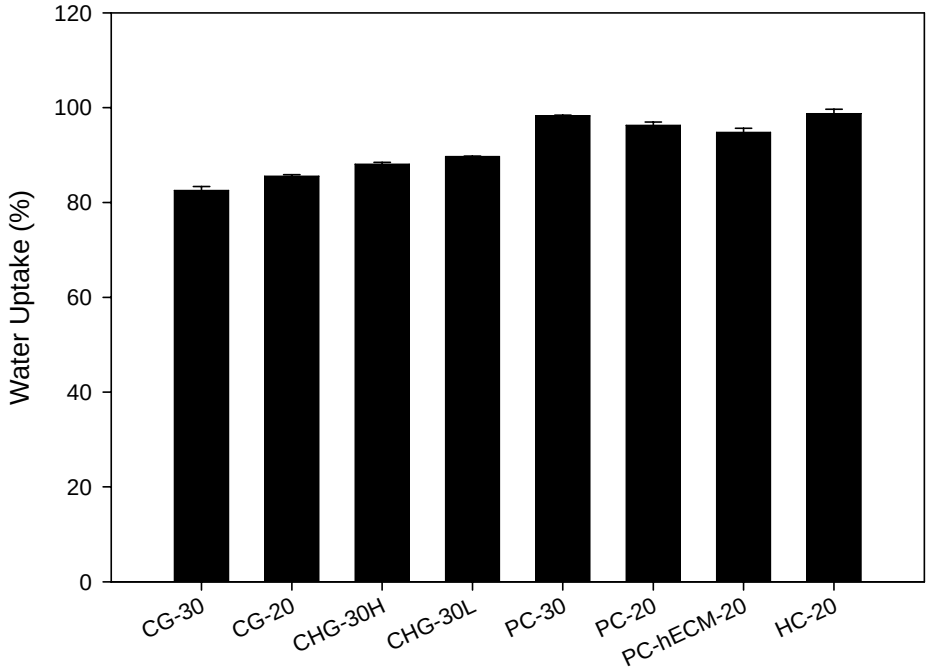

Water Uptake (%)
120
100
80
60
40
20
0
CG-30
CG-20
CHG-30H
CHG-30L
PC-30
PC-20
PC-hECM-20
HC-20

Figure 4-3.

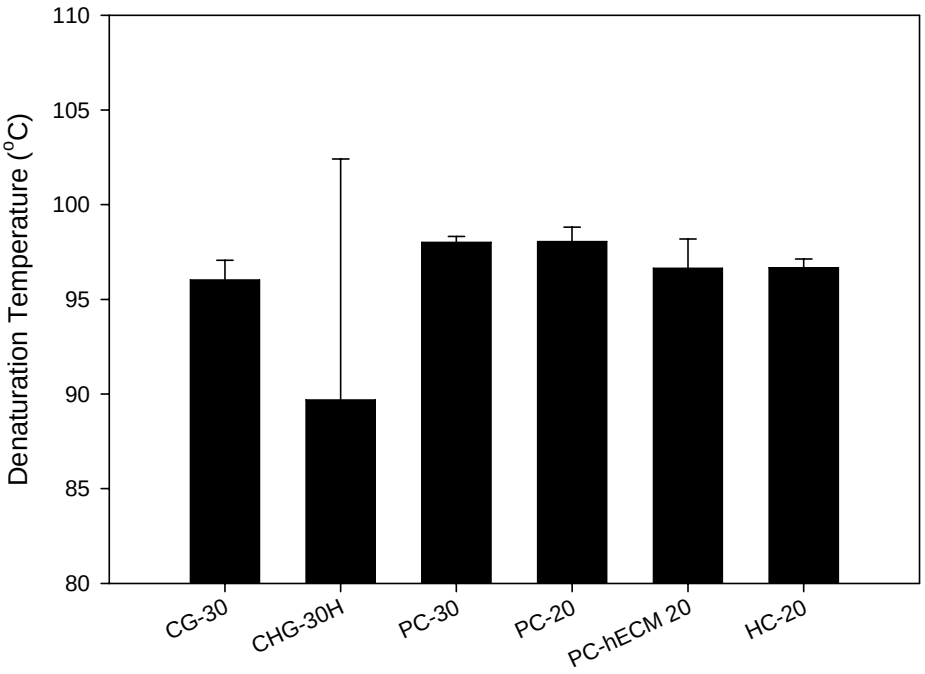

Denaturation Temperature (°C)
110
105
100
95
90
85
80
CG-30
CHG-30H
PC-30
PC-20
PC-hECM 20
HC-20

Figure 4-4.

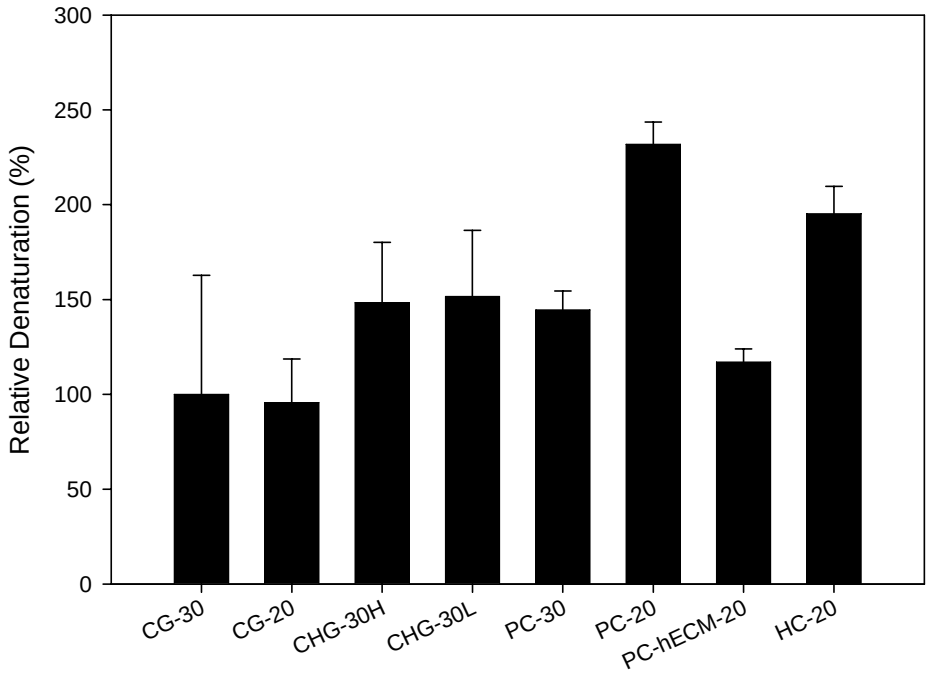

Relative Denaturation (%)
300
250
200
150
100
50
0
CG-30
CG-20
CHG-30H
CHG-30L
PC-30
PC-20
PC-hECM-20
HC-20

Figure 4-5.

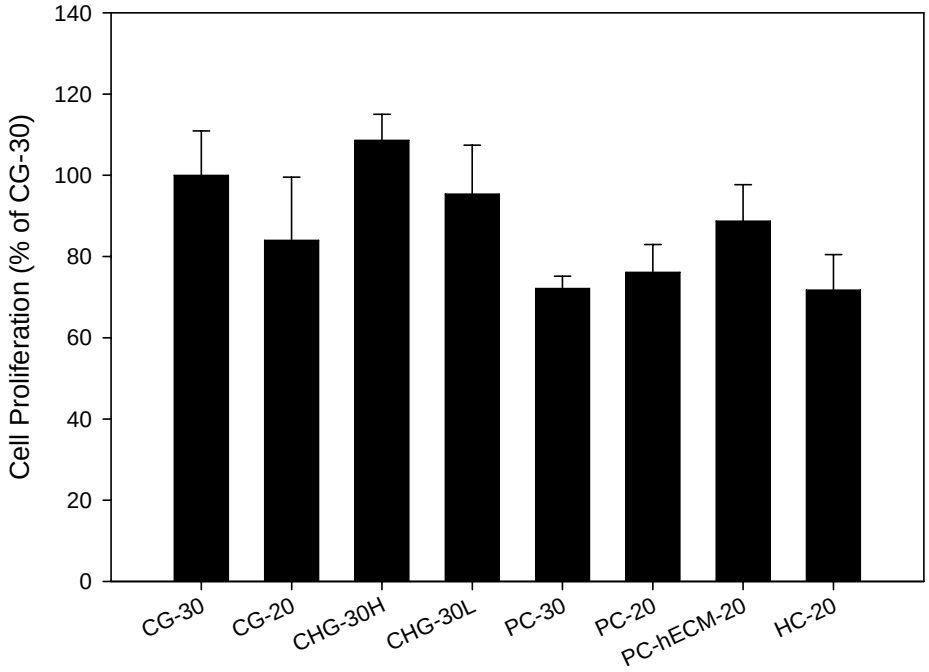
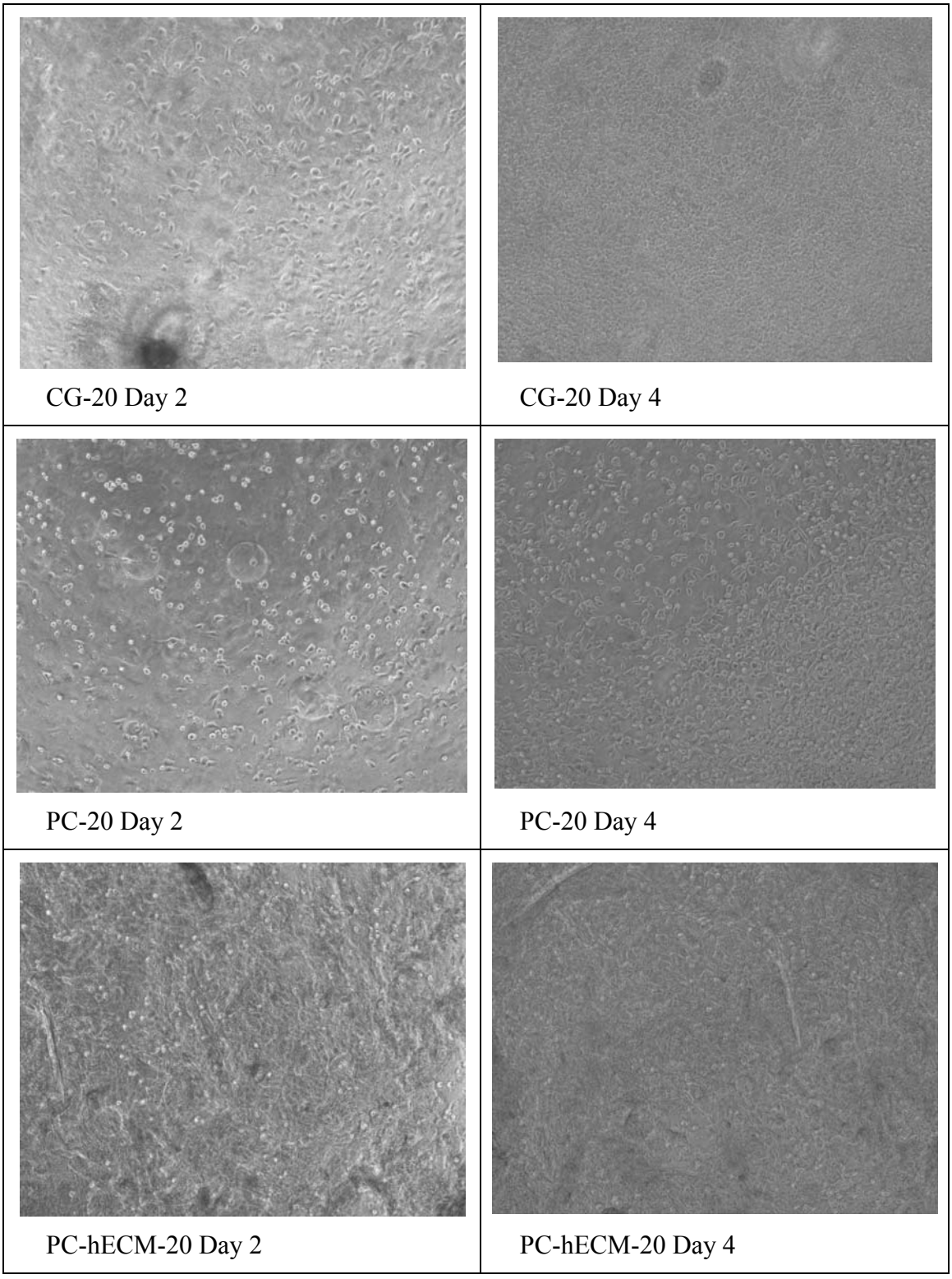

Cell Proliferation (% of CG-30)
140
120
100
80
60
40
20
0
CG-30
CG-20
CHG-30H
CHG-30L
PC-30
PC-20
PC-hECM-20
HC-20

Figure 4-6.


CG-20 Day 2
CG-20 Day 4
PC-20 Day 2
PC-20 Day 4
PC-hECM-20 Day 2
PC-hECM-20 Day 4

4.11 Tables

Table 4-1. Dendrimer crosslinked collagen-based gel recipes.

Gel	Collagen Source	Collagen (mg/mL)	Heparin (µg heparin/mg collagen)
CG-30	Bovine Type I Collagen	30	--
CG-20	Bovine Type I Collagen	20	--
CHG-30H	Bovine Type I Collagen	30	204.5
CHG-30L	Bovine Type I Collagen	30	20.45
PC-30	Bovine PureCol®	30	--
PC-20	PureCol®	20	--
HC-20	Human Collagen	20	--
PC-hECM-20	70% PureCol® & 30% Human extracellular matrix	20*	N/A[t]

*Collagen concentration represents collagen content from PureCol® (14 mg/mL) and total protein content of hECM (6 mg/mL). hECM contains type 1 collagen, elastin, sulphated GAG and fibronectin in order of highest to lowest amounts.

[t]Not available.

5 PAPER 2: HEPARIN MODIFIED DENDRIMER CROSSLINKED COLLAGEN MATRICES FOR THE DELIVERY OF HEPARIN-BINDING EPIDERMAL GROWTH FACTOR (HB-EGF)

5.1 Summary

Authors:

Marta A. Princz and Dr. Heather Sheardown.

Publication Information:

Not yet submitted.

Objectives:

To investigate the potential of heparin-binding epidermal growth factor (HB-EGF) for mediating interactions with human corneal epithelial cells (HCEC) and comparing its efficacy to epidermal growth factor (EGF) *in vitro*. Furthermore, heparinized dendrimer crosslinked collagen gels, intended for use as a TECE, are utilized for delivery of HB-EGF in a sustained manner.

Main Scientific Contributions:

- Validated that HB-EGF stimulates human cornea epithelial cell (HCEC) proliferation in a dose-dependent manner.
- Demonstrated the prolonged and sustained delivery of HB-EGF from CHG gels, a heparinized delivery system, which to our knowledge has not been published previously.
- CHG gels seeded with HCEC demonstrated maintained bioavailability and bioactivity of HB-EGF following gel uptake and release *in vitro*.

Heparin Modified Dendrimer Crosslinked Collagen Matrices for the Delivery of Heparin-Binding Epidermal Growth Factor (HB-EGF)

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Short Title:

Heparinized Dendrimer Collagen Matrices

5.2 Abstract

A tissue engineered corneal equivalent (TECE) requires host integration to achieve adequate anchorage and long-term device stability. Corneal integration, through epithelialization and stromal integration can be manipulated by growth factors. We investigated the potential of heparin-binding epidermal growth factor (HB-EGF) for mediating interactions with human corneal epithelial cells (HCEC) and compared its efficacy to epidermal growth factor (EGF) *in vitro*. Furthermore, we utilized heparinized dendrimer crosslinked collagen gels, intended for use as a TECE, for delivery of HB-EGF in a sustained manner. HCEC were exposed to HB-EGF at varying concentrations between 0.1 to 1000 ng/mL. Cell proliferation increased with growth factor concentration up to a concentration of 50 ng/mL, suggesting growth factor receptor down-regulation at higher HB-EGF concentrations. Response to HB-EGF was comparable to EGF at low concentrations of 0.1 and 1 ng/mL but at a concentration of 10 ng/mL, HB-EGF induced significantly better proliferation than EGF. Proliferation was found to be dependent on the initial seeding density. Heparinized dendrimer crosslinked collagen (CHG) gels were capable of HB-EGF uptake, which was influenced by heparin concentration within the gel, growth factor concentration and exposure time to the growth factor. HB-EGF release followed first order kinetics, with approximately 90% of the growth factor released after 2 weeks. Growth factor stability was verified with *in vitro* HCEC culture studies. Bioavailabilty was maintained in the gels through heparin interaction. Overall, HB-EGF induced proliferation of HCEC *in vitro* and can be released from heparinized collagen gels making it potentially suitable for promoting epithelialization of TECEs.

Keywords: collagen; dendrimers; heparin-binding epidermal growth factor (HB-EGF); heparin; corneal epithelium; tissue engineered corneal equivalents (TECE).

5.3 Introduction

Tissue engineered cornea equivalents (TECE) combine extracellular matrix components with immortalized corneal cell lines to create an artificial corneal implant modeled after corneal wound healing [1]. The success of a TECE requires cytocompatibility and host integration, through sustained anterior epithelialization for prevention of epithelial cell downgrowth to inhibit device extrusion, stromal integration for device anchorage, and maintenance of corneal endothelial and nerve cells [2,3,4]. Manipulation of the cornea following a TECE implantation may be achieved by utilizing extracellular matrix components and growth factors that are active in corneal wound healing [5,6]. Epithelial wound healing is greatly influenced by members of the epidermal growth factor (EGF) family [7]. It is well known that EGF can augment corneal wound healing, which has been demonstrated elsewhere via controlled delivery devices [8,9,10] and tethering [11,12,13]. As EGF influences corneal epithelial cells through the EGF receptor (EGFR) [14], it is reasonable to hypothesize that other growth factors that interact with this receptor would also promote epithelialization of a TECE.

Heparin-binding epidermal growth factor (HB-EGF), a 9.5 kDa member of the EGF family, is active in corneal epithelial wound healing, and as its name suggests, binds heparan sulphate proteoglycans and EGFR [15,16,17]. HB-EGF has been previously shown to enhance proliferation with human cornea epithelial cells to a similar degree as EGF [18], while mouse epithelial cornea cells demonstrated accelerated proliferation, migration and adhesion [19]. Furthermore, anti-HB-EGF antibodies administered *in vitro* inhibited wound closure in a rabbit cornea, which was not observed with anti-EGF [20]. Thus, HB-EGF may be an effective growth factor to enhance TECE epithelialization with the potential for delivery and maintenance of activity through its heparin binding moieties.

As the cornea is comprised mainly of collagen [21], many collagen-based TECEs are currently being investigated [22,23,24,25]. Our group has developed a collagen crosslinking technique involving multifunctional dendrimers [26,27]. These dendrimer crosslinked collagen gels intended for TECE applications have adequate suture strength

[28] and the potential for the incorporation of biological functionality using, for example, cell adhesion peptides [29,30]. Heparinization of these gels resulted in basic fibroblast growth factor (FGF-2) uptake and release [31], which could be suitable for stromal ingrowth following TECE implantation for enhanced device anchorage.

Herein, we validated the use of HB-EGF for HCEC stimulation, and compared HCEC response with that obtained for epidermal growth factor (EGF). In addition, we investigated the use of heparinized dendrimer crosslinked collagen gels for uptake and delivery of HB-EGF, with the ultimate goal of stimulating human cornea epithelial cell proliferation (HCEC).

5.4 Materials and Methods

5.4.1 Materials

Concentrated collagen suspensions of pepsin digested bovine cornium purified >80% type I collagen, with the remainder type III collagen, were the generous gift of Allergan Medical (Fremont, CA). Polypropyleneimine octaamine generation two dendrimers were purchased from SyMO-Chem (Eindhoven, The Netherlands). HB-EGF and EGF were purchased from R&D Systems (Minneapolis, MN). Unless otherwise stated, other reagents were purchased from Sigma-Aldrich (Oakville, ON) or EMD Chemicals Inc. (Gibbstown, NJ).

5.4.2 HB-EGF and HCEC Proliferation

HCEC proliferation as a function of HB-EGF concentration, heparin addition, and initial seeding density were assessed. Cells were seeded, exposed to solutions of HB-EGF, EGF or heparin for 24 hours, and growth assessed after 48 hours with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay [32]. Specifically, 150 μL of MTT solution (0.4 mg/mL MTT, 0.2 μm sterile filtered, in KSFM containing pituitary extract, EGF and antibiotic) was added to cells, the plate covered and incubated for 24 hrs. Following the addition of 500 μL of DMSO, the plate was shaken for 10 minutes to dissolve the formazan precipitate, and the absorbance of 200 μL of the resulting solution was read 595 and 700 nm.

5.4.3 Collagen Gel Fabrication

Heparinized dendrimer crosslinked collagen gels were fabricated as previously described [31]. Briefly, the collagen suspension, as received (78.8 mg/mL) was diluted with phosphate buffered saline (PBS) and endotoxin free water (EFW), and acidified with 1N HCl. Unfractionated heparin (30 mg/mL) was added to the collagen solution. Crosslinking was achieved by adding second generation polypropyleneimine octaamine dendrimer, with ethyl dimethyl carbodiimide (EDC) and N-succinimide (NHS) in a molar ratio of 1:1:1 EDC:NHS:collagen$_{COOH}$. The resultant collagen suspensions were pressed between glass plates, on ice, and incubated overnight at 4^{o}C. Gels were punched, soaked in EFW for 24 hrs to remove any unbound heparin and dendrimer, and dried prior to use.

Heparinized gels contained 204.5 (CHG-204) or 20.45 (CHG-20) μg heparin per mg collagen. Dendrimer crosslinked collagen (CG) gels without heparin and thermal collagen (COLL) gels were fabricated as controls. The thermal collagen gels were prepared by adjusting the pH of acidified collagen suspensions to 7.4 and incubating at 37^{o}C overnight. All gels contained 30 mg/mL collagen.

5.4.4 ^{125}I Labelled HB-EGF Incorporation

HB-EGF was reconstituted in 150 mM PBS buffer (pH 7.4), and radiolabelled via the IODOGEN method (Pierce, USA) [11]. Briefly, the growth factor (250 μL) was combined with 10 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 PBS buffer over a 36 hour period. Free unbound isotope amount was determined by trichloroacetic acid precipitation and remained <10% in all studies.

Dried gels were exposed to ^{125}I labelled HB-EGF (^{125}I-HB-EGF, 5 and 10 μg/mL) to measure growth factor uptake. Following swelling for different time periods, surfaces were rinsed three times in PBS for 1 min, dabbed dry and read for activity using a gamma counter (Wallac Wizard 3" 1480 Automatic Gamma Counter, Perkin Elmer Life Sciences).

5.4.5 ^{125}I-HB-EGF Release Studies

Radioactive HB-EGF containing gels were placed into 1 mL of fresh PBS buffer, in a 37^{o}C shaking waterbath. Release samples were obtained at regular intervals and radioactivity measured with a gamma counter and translated to concentrations of ^{125}I-HB-EGF.

5.4.6 HB-EGF Cell Bioactivity on CHG Gels

HB-EGF cell bioactivity was determined through culture with an immortalized human cornea epithelial cell line [4]. Cells were grown in Keratinocyte Serum-Free Medium (KSFM) containing bovine pituitary extract (25 mg; 0.05 w/v) and epidermal growth factor (EGF; 2.5 μg; 0.005 w/v), along with penicillin-streptomycin antibiotic (1:100). Prior to cell culture studies, gels were soaked in 1 mL PBS containing 100 μL antibiotic (10:1) for 24 hrs. Dried gels were exposed to either PBS or HB-EGF (10 ng/mL) for 24 hours. Gels (5/16") were seeded with 20 μL of 10,000 cells, incubated for 2 hours to allow cells to adhere, followed by the addition of 200 μL of serum- and EGF-free KSFM. Fresh serum- and EGF-free medium (200 μL) was added every 2 days.

After 6 days, cell proliferation on the gels was assessed with a modified MTT assay [33]. Briefly, 150 μL of MTT solution (0.4 mg/mL MTT, 0.2 μm sterile filtered, in KSFM containing pituitary extract, EGF and antiobiotic) was added to cells and incubated for 24 hrs. The gels were then dissolved with 200 μL collagenase (345 U/mg; 15.42 mg/mL) in 400 μL TRIS/HCl (15.76 mg/mL) containing 0.05M $CaCl_2$ for 2.5 hours. Following centrifugation (10 min, 10,000 rpm), the supernatant was removed and the pellet reconstituted in 250 μL DMSO to dissolve the formazan precipitate. Following agitation to dissolve the pellet, 200 μL was transferred to a 96-well plate and absorbance read at 540 and 700 nm (Biorad 550 Plate Reader).

5.4.7 Statistical Analysis

Data are presented as mean values with variability expressed as standard deviations, unless otherwise stated. Statistical significance was determined using a single factor ANOVA test on two means ($p<0.05$).

5.5 Results

5.5.1 HB-EGF & HCEC Proliferation

The effect of HB-EGF on HCECs was assessed *in vitro* by seeding 10,000 cells in a 48-well tissue culture treated plate (13,333 cells/cm^2), followed by 24 hrs of exposure to varying concentrations of HB-EGF or EGF under serum-free conditions. Proliferation was determined following 48 hours of growth. As shown in Figure 5-1 (n = 9; mean ± SEM), HCEC proliferation was enhanced by the addition of both growth factors ($p<0.05$). However, following exposure to 10 ng/mL HB-EGF, significantly increased cell growth was observed to a greater degree than EGF ($p=0.006$). Higher HB-EGF concentrations with HCECs were also examined; the results are shown in Figure 5-2. At higher HB-EGF concentrations of 50, 100, 500 and 1000 ng/mL, HCEC proliferation was diminished compared to 10 ng/mL HB-EGF. Furthermore, a difference in cell response in relation to cell density was observed. Thus, various cell densities were tested, as shown in Figure 5-3. At concentrations of 1 and 10 ng/mL HB-EGF, cell densities of 7500 and 10,000 cells/cm^2 demonstrated the greatest degree of cell growth among the densities and concentrations examined. The lowest and highest cell densities tested, namely 6667 and 13,333 cells/cm^2, had similarly increased cell growth compared to PBS controls, but cells grew to a lesser degree than those seeded at cell densities of 7500 and 10,000 cells/cm^2.

Finally, HCEC proliferation (13,333 cells/cm^2) was measured following exposure to 10 ng/mL HB-EGF in the presence of free heparin (1 to 10,000 μg/mL), as shown in Figure 5-4 (n = 9; mean ± SEM). Heparin, regardless of concentration, had no effect on cell proliferation, even in the presence of 10 ng/mL HB-EGF.

5.5.2 ^{125}I Labelled HB-EGF Uptake into Collagen Gels

Dried heparinized dendrimer crosslinked collagen (CHG) gels were soaked in 1 mL of either 5 or 10 μg/mL ^{125}-I-HB-EGF for either 24 hrs or 4 days at 4^{o}C to assess the amount of growth factor that could be incorporated into the materials. As shown in Figure 5-5, heparinization allowed for greater growth factor retention, as CHG gels retained on average 10 fold more ^{125}-I-HB-EGF than CG gels ($p<0.005$). Growth factor uptake was also augmented by increasing growth factor concentration and exposure time during gel

soaking, resulting in uptake amounts ranging between 33.85 ± 5.02 and 68.81 ± 2.27 ng for CHG gels. Varying heparin amounts within CHG gels to 204.5, 20.45 & 2.05 μg heparin per mg collagen (CHG-204, -20, and -2, respectively) also altered growth factor uptake, as shown in Figure 5-6, and demonstrated the ability to manipulate growth factor retention by adjusting heparin content ($p<0.05$).

5.5.3 ^{125}I-HB-EGF Release Studies

Release of ^{125}I-HB-EGF from heparinized dendrimer crosslinked collagen (CHG) gels, following exposure to 5 or 10 μg/mL growth factor, is shown in Figure 5-7. Nearly all growth factor was released over 4 weeks, regardless of the initial growth factor concentration or exposure time. After 12 days of release, CHG gels released between 77.42 ± 0.65% and 93.72 ± 1.57% ^{125}I-HB-EGF (averaging 87.51 ± 7.77%). Surprisingly, heparinization of the gels did not affect growth factor release, which is further illustrated in Figure 5-8, where CHG gels fabricated with varying heparin amounts, namely 204.5, 20.45, or 2.04 μg heparin per mg collagen, exposed to 10 μg/mL ^{125}-I-HB-EGF for 4 days, released on average 95.54 ± 4.27% growth factor independent of the heparin concentration.

5.5.4 HB-EGF Cell Bioactivity on CHG Gels

HCECs were seeded onto gels, soaked in either PBS or 10 ng/mL HB-EGF, and their proliferation was assessed after 6 days via the MTT assay; results are shown in Figure 5-9 ($n = 18$; mean ± SEM). A gel soak solution of 10 ng/mL HB-EGF was chosen, instead of the gel uptake and release experimental values of 5000 and 10,000 ng/mL, so that the gels would release a total growth factor amount suitable for stimulation of HCEC proliferation while avoiding receptor down-regulation.

HCEC proliferation was significantly higher on CG and CHG-204 gels ($p<0.05$), compared to thermal collagen (COLL) gels soaked in PBS. HB-EGF incorporation into the gel matrix further increased cell growth on COLL gels, and CHG-204, compared to their PBS counterpart, suggesting sustained growth factor bioactivity ($p=10^{-6}$). Growth factor incorporation did not affect cell growth on CG gels ($p<0.05$).

5.6 Discussion

HB-EGF is a mitogen for smooth muscle cells, keratinocytes and BALB-3T3 cells and promotes neurite growth and outgrowth [34], but is not mitogenic for capillary endothelial cells [35]. In one study, HB-EGF was found to be more mitogenic than EGF, with smooth muscle cells [35]. However, Wilson *et al*. (1994) reported similar proliferation and migration effects between EGF and HB-EGF with primary HCEC culture seeded at 4000 cells/cm^2 after 3 weeks of culture [18]. Here, we observed enhanced HCEC proliferation (13,333 cells/cm^2 initial seeding density) in response to HB-EGF at a concentration of 10 ng/mL, compared to HCEC exposed to EGF at the same concentration (Figure 5-1). However, at a lower seeding density (6667 cells/cm^2), the proliferative response to HB-EGF is not as pronounced (Figure 5-3). Differences in cell proliferation from HB-EGF may rely on cell seeding density, in addition to varying experimental conditions (e.g. length of experiment, serum-affects, etc.). This is in accordance with Wilson *et al.* also suggesting that HCEC migration and proliferation in response to growth factors may be related to the number of cell-cell contacts [18]. This is supported by recent work which suggests that HB-EGF is localized to cell-cell contact sites through heparan sulphate proteoglycans [36], and the interaction of HB-EGF with integrins [37]. Furthermore, HB-EGF signaling was enhanced, in other work, by the presence of cell surface heparan sulphate proteoglycans [15], which is in accordance with our observed results of moderately increased HCEC proliferation following exposure to 10 ng/mL HB-EGF and heparin. The proliferative response from HB-EGF at 10 ng/mL was greater than HCEC exposed to KFSM with serum (Figure 5-1), which could be due to competitive binding between heparin-binding and other growth factors for cell surface receptors, as has been modeled elsewhere with FGF-2, HB-EGF and EGF [38]. HCEC proliferation showed the expected response in relation to growth factor concentration (Figure 5-2), with a dose-dependent increase to a concentration of approximately 10 ng/mL and little or no response above this concentration. This is potentially indicative of receptor down-regulation in this system, although further studies would be necessary to confirm this.

HB-EGF was successfully loaded into and released from heparinized dendrimer crosslinked collagen (CHG) gels. HB-EGF release from such systems would mimic corneal wound healing, whereby ectodomain bound HB-EGF is shed resulting in autocrine and paracrine activation of EGF receptor for epithelial cell migration and wound closure [20]. This, to our knowledge, is the first time that the effect of this growth factor on HCEC has been examined using a heparinized delivery system. In other studies, where HB-EGF has been released from a lyophilized type I collagen matrix; release was rapid but growth factor activity was not shown [39].

Growth factor uptake and retention was related to heparin concentration within the gel, growth factor exposure time and growth factor soak concentration. This corresponds to previous work, whereby similarly prepared CHG gels were exposed to basic fibroblast growth factor (FGF-2) [31]. HB-EGF release was first order, with an initial burst of protein, indicative of surface adsorbed protein that remains after gel rinsing, and followed by slow release of the protein from within the gel matrix [40]. The amount of protein released from all CHG gels averaged 90%, regardless of gel heparinization. This is more rapid than FGF-2 release [31], which may be attributable to differences in growth factor molecular weight (MW_{HBEGF} = 9.5 kDa; $MW_{FGF\text{-}2}$ = 16 kDa) as well as differences in the binding affinity of the growth factors for heparin [41].

There is evidence of increased FGF-2 stability in the presence of heparan sulphate proteoglycans [42]; a similar effect may be expected with HB-EGF. The HB-EGF released from the CHG gels was able to stimulate cell proliferation to a greater extent than HB-EGF released from CG gels containing no heparin, demonstrating the ability of the incorporated heparin to stabilize the growth factor. There is some growth factor stabilization with CG gels, as type I collagen is capable of FGF-2 binding [43], which is further supported by bioactive HB-EGF released from collagen thermal gels.

Taken together, this work suggests that HB-EGF may be a potent mitogen for corneal tissue engineering particularly when delivered from a heparinized delivery system which will maintain HB-EGF bioactivity allowing for enhanced epithelial cell proliferation and which may enhance HB-EGF uptake allowing for better control over release.

5.7 Conclusions

Soluble HB-EGF enhanced proliferation of HCECs *in vitro* to a greater degree than EGF at 10 ng/mL. Heparinized dendrimer crosslinked collagen (CHG) gels successfully retained and released HB-EGF. While release rate and duration were not improved by the presence of the heparin, growth factor uptake and bioactivity were enhanced, with release being maintained for more than 4 weeks. Our *in vitro* work with HCEC indicated that released HB-EGF is suitable for cell proliferation; however it is unknown what concentrations within the gel would be optimal for adequate TECE host integration. However, given the ability to tailor these gel matrices with different amounts of HB-EGF, it is reasonable to assume proper release rates can be achieved and TECE host integration is possible.

5.8 Acknowledgements

NSERC is recognized for funding. Lina Liu is acknowledged for aiding with radiolabeling experiments, while Henry Fung is recognized for his assistance in performing cell culture experiments on gels.

5.9 References

1. Duan X, Klenkler BJ, Sheardown H. Progress in the development of a corneal replacement: keratoprostheses and tissue-engineered corneas. *Expert Rev Med Devices* 2006; 3: 59-72.
2. Chirila TV, Hicks CR, Dalton PD, Vijayasekaran S, Lou X, Hong Y, Clayton AB, Ziegelaar BW, Fitton JH, Platten S, Crawford GJ, Constable IJ. Artificial Cornea. *Prog Polym Sci* 1998; 23: 447-473.
3. George A, Pitt WG. Comparison of corneal epithelial cellular growth on synthetic cornea materials. *Biomaterials* 2002; 23: 1369-1373.
4. Griffith M, Li F, Lohman C, Sheardown H, Shimmura S, Carlsson DJ. Tissue Engineering of the Cornea. *Scaffolds in Tissue Engineering*. U.S.A.: Ma PX and Elisseeff J.; 2005: 403-413.
5. Zieske JD. Extracellular matrix and wound healing. *Curr Opin Ophthalmol* 2001; 12: 237-241.
6. Imanishi J, Kamiyama K, Iguchi I, Kita M, Sotozono C, Kinoshita S. Growth factors: importance in wound healing and maintenance of transparency of the cornea. *Prog Retin Eye Res* 2000; 19: 113-129.
7. Reid TW. Growth control of cornea and lens epithelial cells. *Prog Retin Eye Res* 1994; 13: 507-554.
8. Sheardown H, Clark H, Wedge C, Apel R, Rootman D, Cheng YL. A semi-solid drug delivery system for epidermal growth factor in corneal epithelial wound healing. *Curr Eye Res* 1997; 16: 183-90.
9. Schultz CL, Morck DW. Contact lenses as a drug delivery device for epidermal growth factor in the treatment of ocular wounds. *Clin Exp Optom* 2010; 93: 61-65.
10. Hori K, Sotozono C, Hamuro J, *et al*. Controlled-release of epidermal growth factor from cationized gelatin hydrogel enhances corneal epithelial wound healing. *J Control Release* 2007; 118: 169-176.
11. Klenkler BJ, Griffith M, Becerril C, West-Mays JA, Sheardown H. EGF-grafted PDMS surfaces in artificial cornea applications. *Biomaterials* 2005; 26: 7286-7296.
12. Klenkler BJ, Sheardown H. Characterization of EGF coupling to aminated silicone rubber surfaces. *Biotechnol Bioeng* 2006; 95: 1158-1166.
13. Klenkler BJ, Dwivedi D, West-Mays JA, Sheardown H. Corneal epithelial cell adhesion and growth on EGF-modified aminated PDMS. *J Biomed Mater Res A* 2010; 93: 1043-1049.
14. Yu FS, Yin J, Xu K, Huang J. Growth factors and corneal epithelial wound healing. *Brain Res Bull* 2010; 81: 229-235.

15. Nishi E, Klagsbrun M. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a mediator of multiple physiological and pathological pathways. *Growth Factors* 2004; 22: 253-260.

16. Thompson SA, Higashiyaman S, Wood K, Pollitt NS, Damm D, McEnroe G, Garrick B, Ashton N, Lau K, Hancock N, Klagsburn M, Abraham JA. Characterization of sequences within heparin-binding EGF-like growth factor that mediate interaction with heparin. *J Biol Chem* 1993; 269: 2541-2549.

17. Iwamoto R, Mekada E. Heparin-binding EGF-like growth factor: a juxtacrine growth factor. *Cytokine Growth Factor Rev* 2000; 11: 335-344.

18. Wilson SE, He YG, Weng J, Zieske JD, Jester JV, Schultz GS. Effect of epidermal growth factor, hepatocyte growth factor, and keratinocyte growth factor, on proliferation, motility and differentiation of human corneal epithelial cells. *Exp Eye Res* 1994; 59: 665-678.

19. Yoshioka R, Shiraishi A, Kobayashi T, Morita S, Hayashi Y, Higashiyama S, Ohashi Y. Corneal epithelial wound healing impaired in keratinocyte-specific HB-EGF-deficient mice *in vivo* and *in vitro*. *Invest Ophthalmol Vis Sci* 2011; 51: 5630-5639.

20. Xu KP, Ding Y, Ling J, Dong Z, Yu FX. Wound-induced HB-EGF ectodomain shedding and EGFR activation in corneal epithelial cells. *Invest Ophthalmol Vis Sci* 2004; 45: 813-820.

21. Meek KM, Boote C. The organization of collagen in the corneal stroma. *Exp Eye Res* 2004; 78: 503-512.

22. Germain L, Auger FA, Grandbois E, Guignard R, Giasson M, Boisjoly H, Guérin SL. Reconstructed human cornea produced *in vitro* by tissue engineering. *Pathobiology* 1999; 67: 140-147.

23. Orwin EJ, Borene ML, Hubel AJ. Biomechanical and optical characteristics of a corneal stromal equivalent. *Biomech Eng* 2003; 125: 439-444.

24. Xu YG, Xu YS, Huang C, Feng Y, Li Y, Wang W. Development of a rabbit corneal equivalent using an acellular corneal matrix of a porcine substrate. *Mol Vis* 2008; 14: 2180-2189.

25. Bentley E, Murphy CJ, Li F, Carlsson DJ, Griffith M. Biosynthetic corneal substitute implantation in dogs. *Cornea* 2010; 29: 910-916.

26. Duan X, Sheardown H. Crosslinking of collagen with dendrimers. *J Biomed Mater Res* 2005; 75A: 510-518.

27. Princz MA, Sheardown H. Modified dendrimer crosslinked collagen matrices. *J Biomat Sci Polymer Edn* 2011; Submitted: 21 pages.

28. Duan X, Sheardown H. Dendrimer crosslinked collagen as a corneal tissue engineering scaffold: mechanical properties and corneal epithelial cell interactions. *Biomaterials* 2006; 27: 4608-4617.

29. Duan X, McLaughlin C, Griffith M, Sheardown H. Biofunctionalization of collagen for improved biological response: scaffolds for corneal tissue engineering. *Biomaterials* 2007; 28: 78-88.

30. Duan X, Sheardown H. Incorporation of cell-adhesion peptides into collagen scaffolds promotes corneal epithelial stratification. *J Biomater Sci Polymer Edn* 2007; 18: 701-711.

31. Princz MA, Sheardown H. Heparin-modified dendrimer cross-linked collagen matrices for the delivery of basic fibroblast growth factor (FGF-2). *J Biomater Sci Polym Edn* 2008; 19: 1201-1218.

32. Leonardi A, Papa V, Fregona I, Russo P, De Franchis G, Milazzo G. In vitro effects of fluoroquinolone and aminoglycoside antibiotics on human keratocytes. *Cornea* 2006; 25: 85-90.

33. Ono A, Kanno H, Hayashi A, Nishimura S, Kyuma Y, Sato H, Ito S, Shimizu N, Chang CC, Gondo G, Yamamoto I, Sasaki T, Tanaka M. Collagen gel matrix assay as an *in vitro* chemosensitivity test for malignant astrocytic tumors. *Int J Clin Oncol* 2007; 12: 125-130.

34. Zhou Y, Besner GE. Heparin-binding epidermal growth factor-like growth factor is a potent neurotrophic factor for PC12 cells. *Neurosignals* 2010; 18: 141-151.

35. Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* 1991; 251: 936-939.

36. Prince RN, Schreiter ER, Zou P, Wiley HS, Ting AY, Lee RT, Lauffenburger DA. The heparin-binding domain of HB-EGF mediates localization to sites of cell-cell contact and prevents HB-EGF proteolytic release. *J Cell Science* 2010; 123: 2308-2318.

37. Nakamura K, Iwamoto R, Mekada E. Membrane-anchored heparin-binding EGF-like growth factor (HB-EGF) and diphtheria toxin receptor-associated protein (DRAP27)/CD9 form a complex with integrin alpha 3 beta 1 at cell-cell contact sites. *J Cell Biol* 1995; 129: 1691-1705.

38. Forsten-Williams K, Chu CL, Fannon M, Buczek-Thomas JA, Nugent MA. Control of growth factor networks by heparan sulphate proteoglycans. *Ann Biomed Eng* 2008; 36: 2134-2148.

39. Kanematsu A, Yamamoto S, Ozeki M, Noguchi T, Kanatani I, Ogawa O, Tabata Y. Collagenous matrices as release carriers of exogenous growth factors. *Biomaterials* 2004; 25: 4513-4520.

40. Saltzman WM. Controlled Drug Delivery Systems. *Drug Delivery: Engineering Principles for Drug Therapy*. Oxford University Press, New York, NY; 2001: 235-280.

41. Chu CL, Goerges AL, Nugent MA. Identification of common and specific growth factor binding sites in heparan sulphate proteoglycans. *Biochemistry* 2005; 44: 12203-12213.

42. Gospodarowicz D, Cheng J. Heparin protects basic and acidic FGF from inactivation. *J Cell Physiol* 1986; 128: 475-484.

43. Kanematsu A, Marui A, Yamamoto S, Ozeki M, Hirano Y, Yamamoto M, Ogawa O, Komeda M, Tabata Y. Type I collagen can function as a reservoir of basic fibroblast growth factor. *J Control Rel* 2004; 99: 281-292.

5.10 Figures

Figure 5-1. HCEC proliferation of 13333 cells/cm^2 (10,000 cells in 48-well plate) in response to growth factors, in comparison to cells exposed to PBS. Concentrations of 0.1, 1 or 10 ng/mL HB-EGF and EGF increased HCEC proliferation ($p=0.001$ and $p=0.0005$, respectively). Following exposure to 10 ng/mL, HB-EGF significantly increased cell growth to a greater degree than EGF ($p=0.006$). Data are shown as mean ± SEM ($n = 9$).

Figure 5-2. HCEC proliferation of 6667 and 13333 cells/cm^2 (5000 and 10,000 cells, respectively, in 48-well plate) in response to varying concentrations of HB-EGF (0.1-1000 ng/mL) in serum-free media, compared to cells exposed its PBS control well. Cell growth to HB-EGF was dose dependent up to 10 ng/mL, after which a down-regulation of cell growth was observed. Data are shown as mean ± SEM ($n = 9$).

Figure 5-3. HCEC proliferation in response to 0.1, 1, 10 ng/mL HB-EGF at varying cell densities, 6667, 7500, 10000 or 13333 cells/cm^2; each is shown in comparison its PBS control well. The response to HB-EGF is related to cell density, with 6667 and 10,000 cells/cm^2 demonstrating the greatest amount of HCEC proliferation. Data are mean ± SEM ($n = 9$).

Figure 5-4. HCEC proliferation in response to either 0 or 10 ng/mL HB-EGF with or without free heparin (1 to 10,000 μg/mL), compared to the PBS control well. There is a minor increase in HCEC proliferation in the presence of heparin, although this difference is not significant ($p>0.05$). Data are mean ± SEM ($n=9$).

Figure 5-5. Radiolabeled ^{125}I-HB-EGF uptake into heparinized dendrimer crosslinked collagen (CHG) gels, compared to unheparinized dendrimer crosslinked collagen (CG) gels following exposure to 5000 or 10000 ng/mL growth factor for 24 hours or 4 days ($p<0.005$). Heparinization allows for greater growth factor uptake, which can be manipulated by growth factor exposure concentration and exposure time.

Figure 5-6. Radiolabeled ^{125}I-HB-EGF uptake into heparinized dendrimer crosslinked collagen (CHG) gels with varying heparin content of 204.5, 20.45 or 2.05 μg heparin per mg collagen. Growth factor retention decreased with decreasing gel heparinization ($p<0.05$).

Figure 5-7. Release of ^{125}-I-HB-EGF from heparinized dendrimer crosslinked collagen (CHG) and unheparinized dendrimer crosslinked collagen (CG) gels after exposure to (a) 5000 ng/mL or (b) 10000 ng/mL growth factor for 24 hours or 4 days. Heparinization was found not to affect growth factor release.

Figure 5-8. Release of ^{125}-I-HB-EGF from heparinized dendrimer crosslinked collagen (CHG) with 204.5, 20.45 or 2.05 μg heparin per mg collagen, shown in (a) percent release (%) and (b) cumulative release (ng). Adjusting the amount of heparin within the CHG gels does not affect growth factor fractional release, but the amount of absolute growth factor released can be tailored.

Figure 5-9. HCEC proliferation on heparinized dendrimer crosslinked collagen (CHG) gels, containing either 204.5 or 20.45 μg heparin per mg collagen (CHG-204 & CHG-20, respectively), compared to collagen thermal gels (COLL) and dendrimer crosslinked collagen (CG) gels. There is increased cell proliferation on CHG and COLL gels after soaking gels in 10 ng/mL HB-EGF ($p<0.009$). Data are shown as mean ± SEM ($n = 18$).

Figure 5-1.

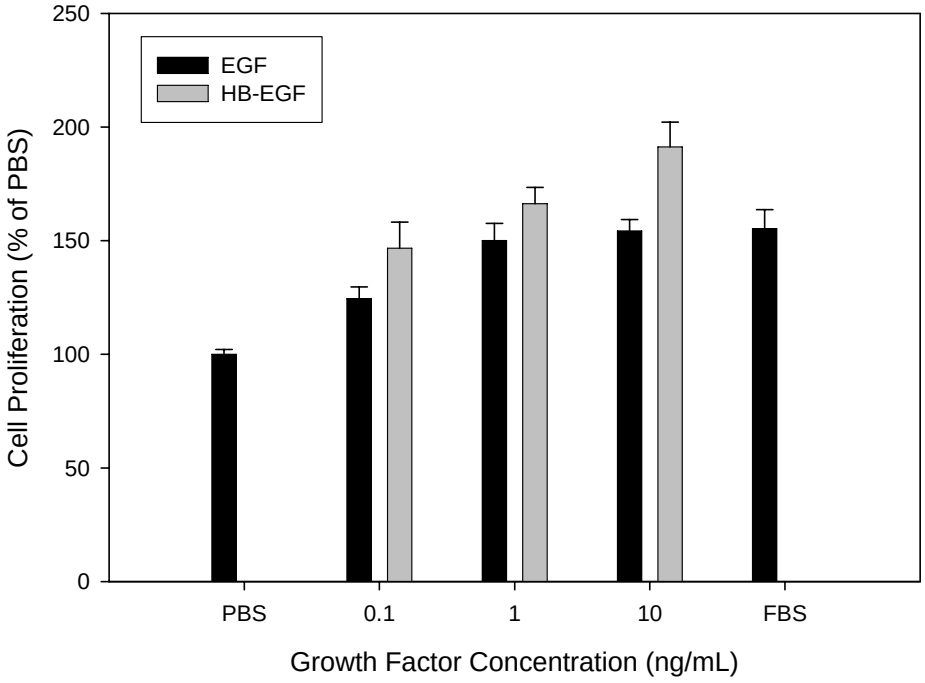

250
200
150
100
50
0
EGF
HB-EGF
Cell Proliferation (% of PBS)
PBS
0.1
1
10
FBS
Growth Factor Concentration (ng/mL)

Figure 5-2.

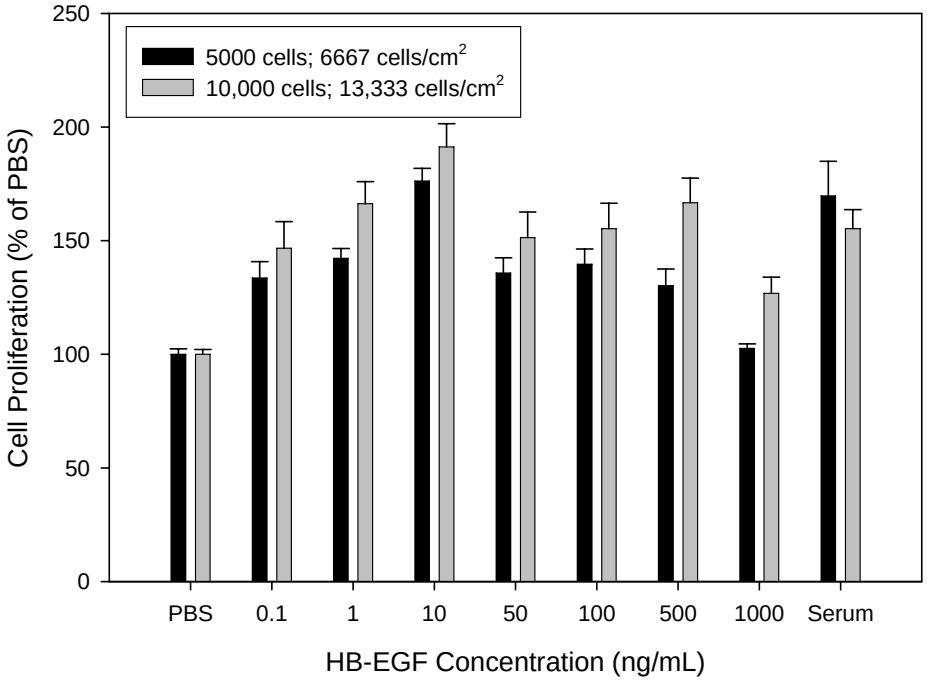

5000 cells; 6667 cells/cm2
10,000 cells; 13,333 cells/cm2
Cell Proliferation (% of PBS)
250
200
150
100
50
0
PBS
0.1
1
10
50
100
500
1000
Serum
HB-EGF Concentration (ng/mL)

Figure 5-3.

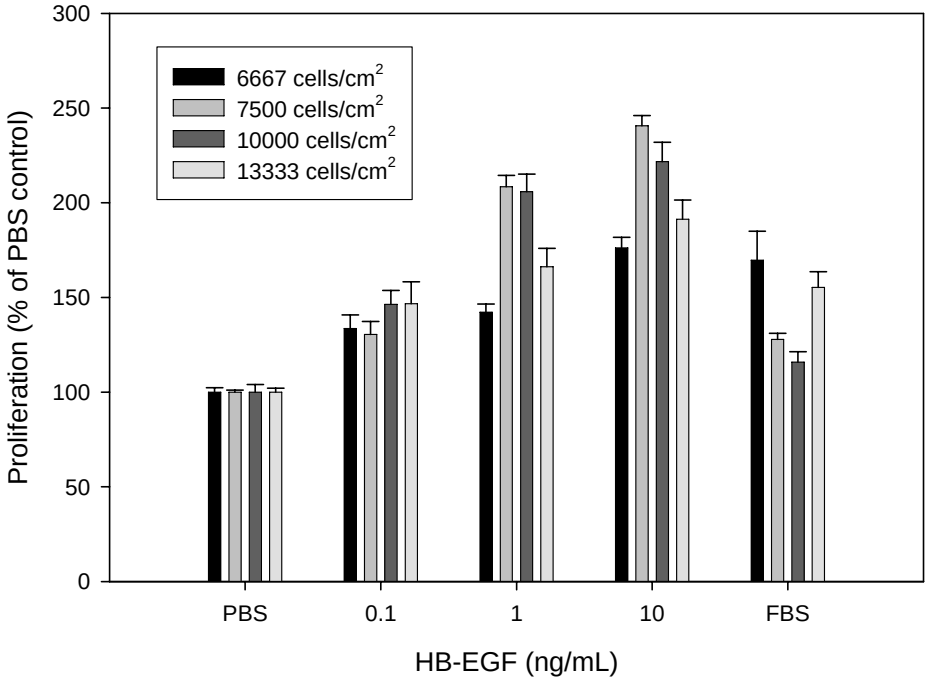

300
250
200
150
100
50
0
Proliferation (% of PBS control)
6667 cells/cm2
7500 cells/cm2
10000 cells/cm2
13333 cells/cm2
PBS
0.1
1
10
FBS
HB-EGF (ng/mL)

Figure 5-4.

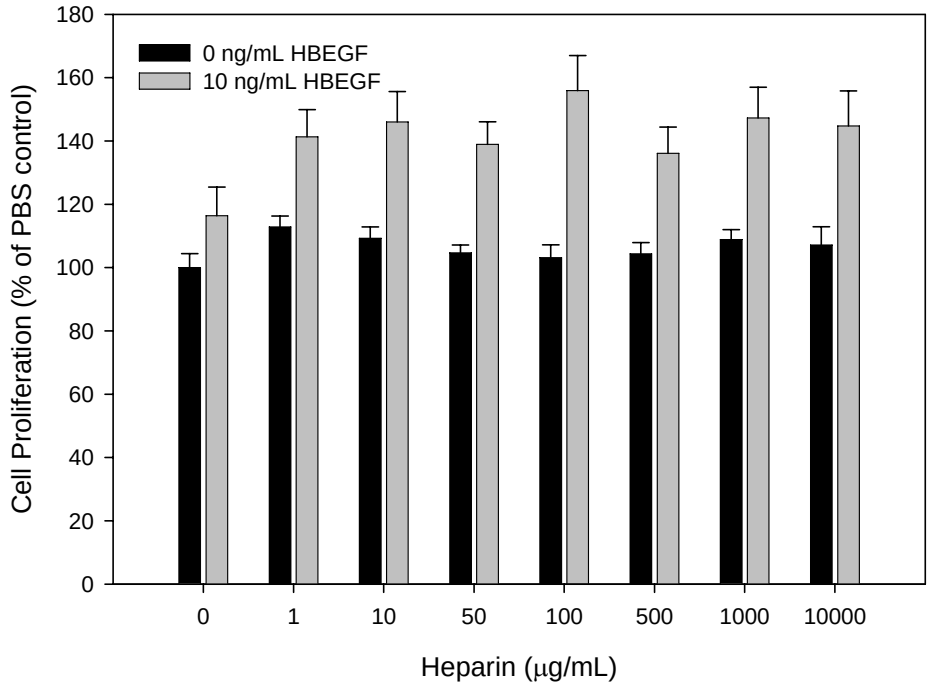

0 ng/mL HBEGF
10 ng/mL HBEGF
Cell Proliferation (% of PBS control)
180
160
140
120
100
80
60
40
20
0
0
1
10
50
100
500
1000
10000
Heparin (μg/mL)

Figure 5-5.

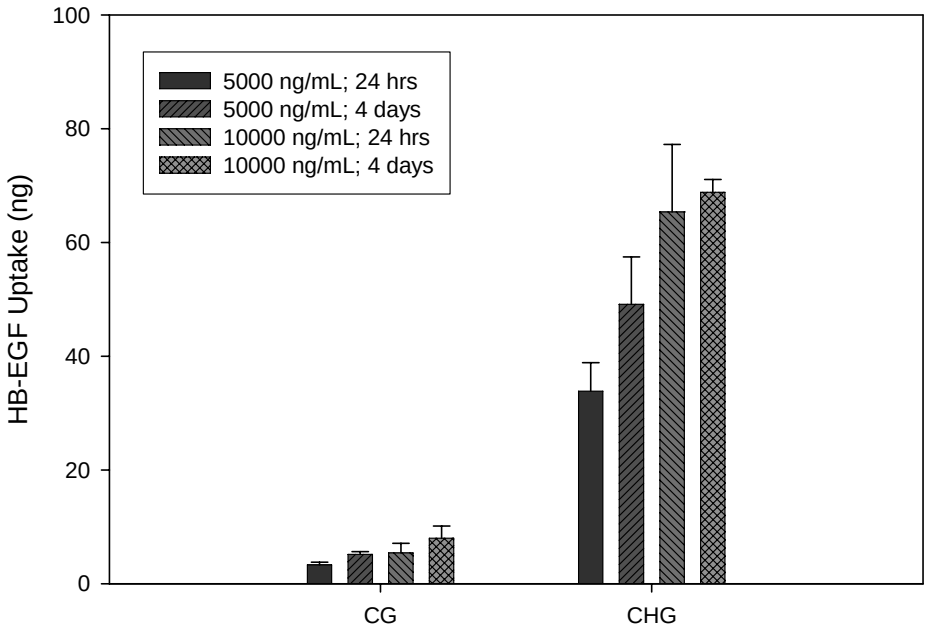

100
80
60
40
20
0
HB-EGF Uptake (ng)
5000 ng/mL; 24 hrs
5000 ng/mL; 4 days
10000 ng/mL; 24 hrs
10000 ng/mL; 4 days
CG
CHG

Figure 5-6.

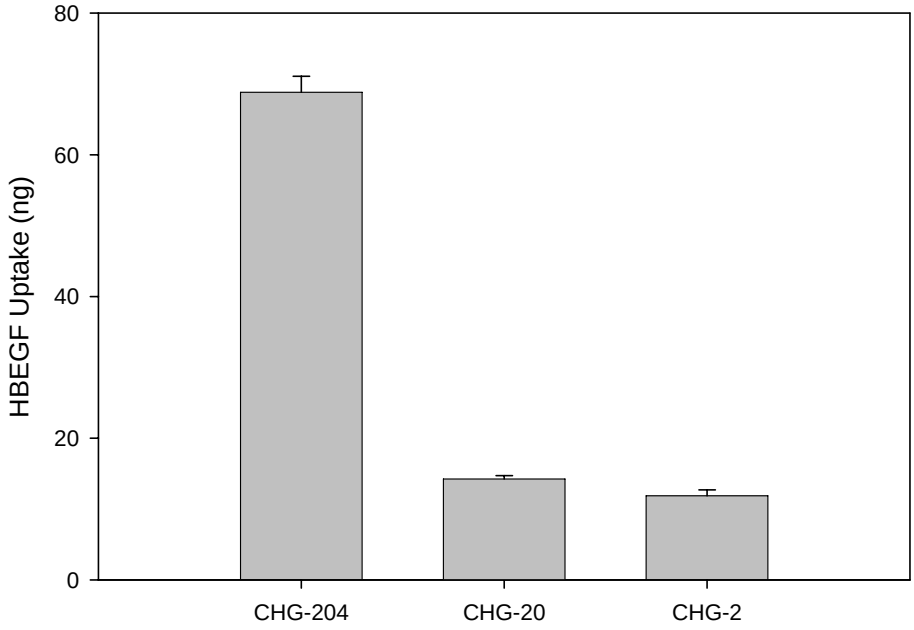


Figure 5-7(a).

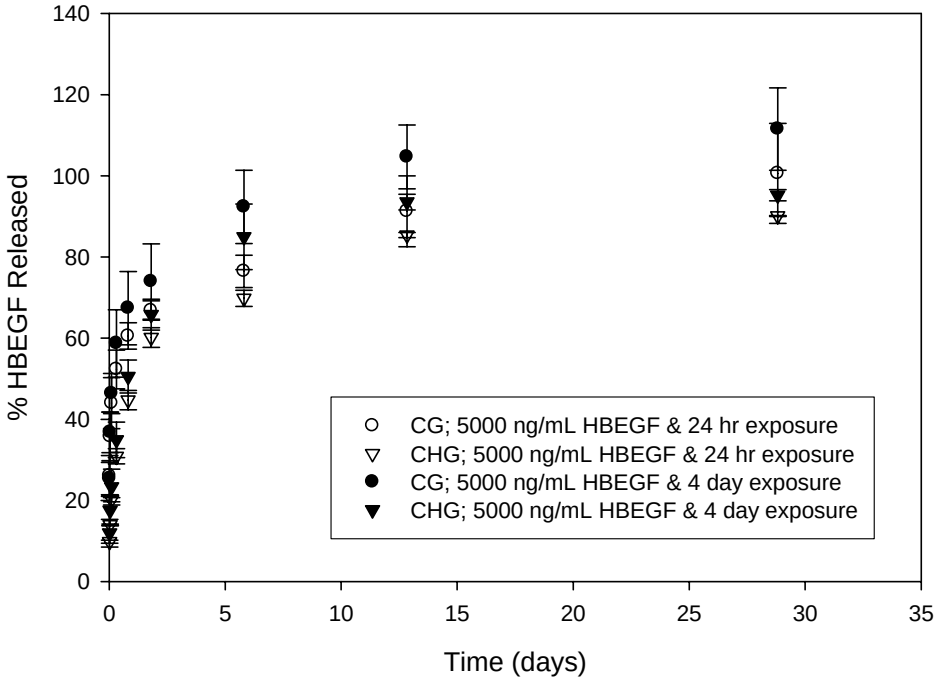

% HBEGF Released
Time (days)
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30
35
CG; 5000 ng/mL HBEGF & 24 hr exposure
CHG; 5000 ng/mL HBEGF & 24 hr exposure
CG; 5000 ng/mL HBEGF & 4 day exposure
CHG; 5000 ng/mL HBEGF & 4 day exposure

Figure 5-7(b).

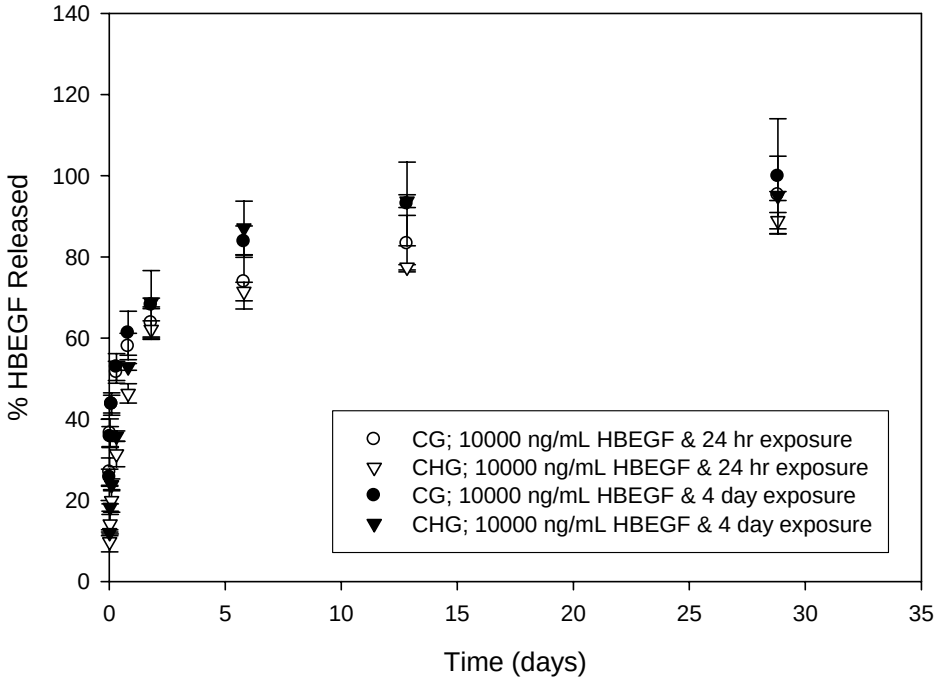

% HBEGF Released
Time (days)
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30
35
CG; 10000 ng/mL HBEGF & 24 hr exposure
CHG; 10000 ng/mL HBEGF & 24 hr exposure
CG; 10000 ng/mL HBEGF & 4 day exposure
CHG; 10000 ng/mL HBEGF & 4 day exposure

Figure 5-8(a).

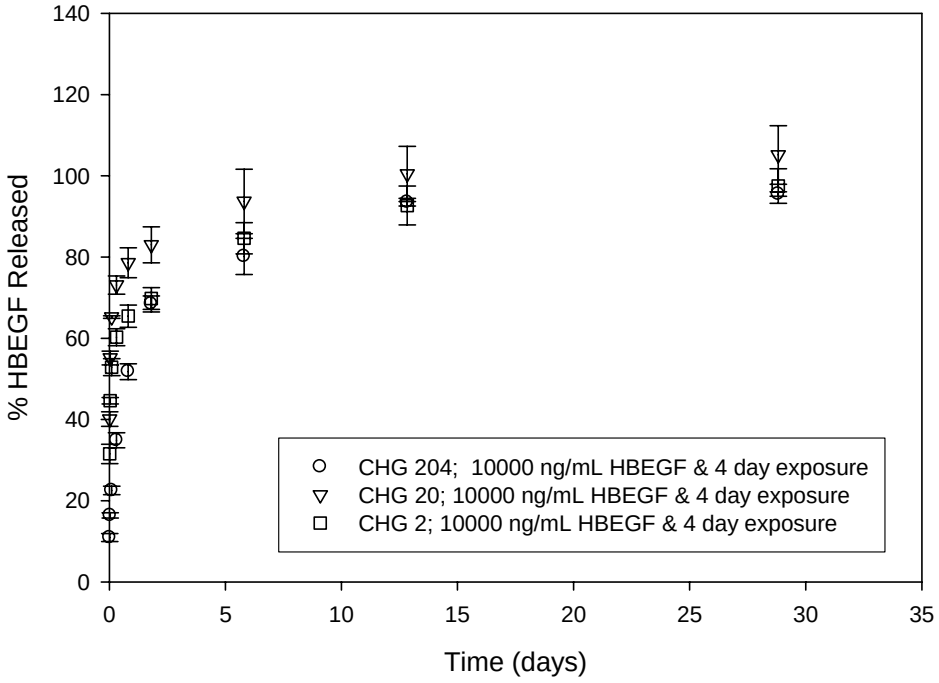

% HBEGF Released
Time (days)
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30
35
CHG 204; 10000 ng/mL HBEGF & 4 day exposure
CHG 20; 10000 ng/mL HBEGF & 4 day exposure
CHG 2; 10000 ng/mL HBEGF & 4 day exposure

Figure 5-8(b).

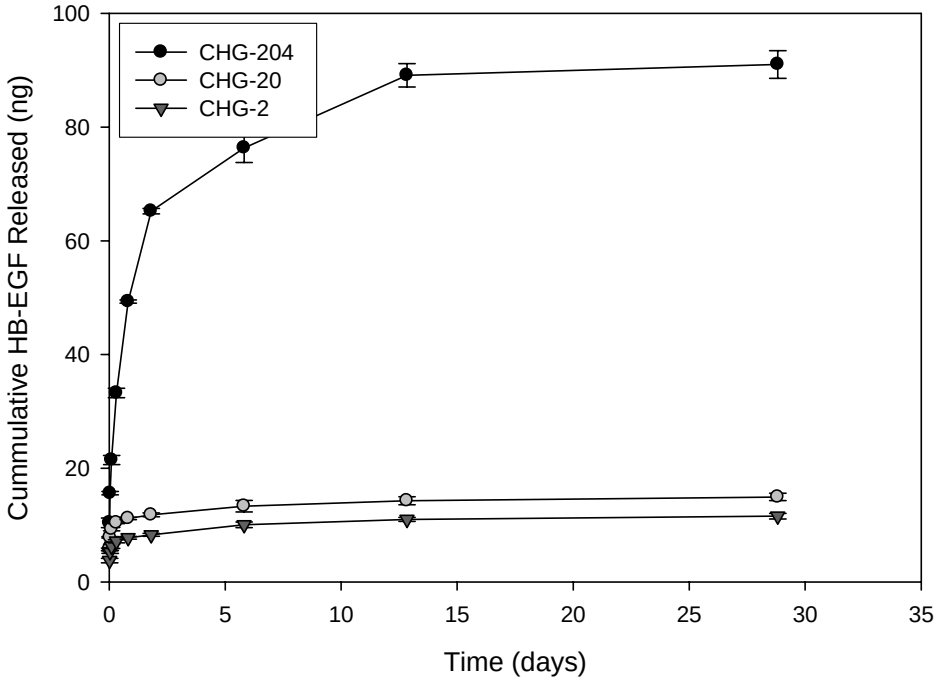
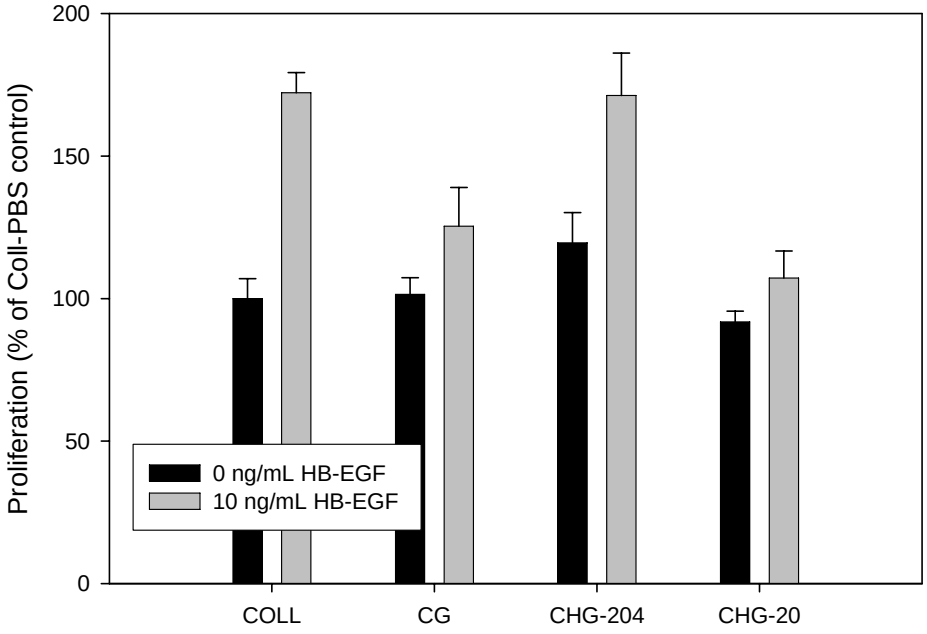

CHG-204
CHG-20
CHG-2
Cummulative HB-EGF Released (ng)
Time (days)
0
20
40
60
80
100
0
5
10
15
20
25
30
35

Figure 5-9.


200
150
100
50
0
Proliferation (% of Coll-PBS control)
0 ng/mL HB-EGF
10 ng/mL HB-EGF
COLL
CG
CHG-204
CHG-20

6 PAPER 3: TETHERING OF EPIDERMAL GROWTH FACTOR FAMILY MEMBERS TO DENDRIMER CROSSLINKED COLLAGEN GELS

6.1 Summary

Authors:

Marta A. Princz and Dr. Heather Sheardown.

Publication Information:

Not yet submitted.

Objectives:

Tethered epidermal growth factor (EGF) and heparin-binding epidermal growth factor (HB-EGF) to dendrimer crosslinked collagen (CG) gels, either during or following gel fabrication, to enhance growth factor (GF) bioavailability and bioactivity.

Main Scientific Contributions:

- Fabricated dendrimer crosslinked collagen (CG) gels with tethered growth factor in either step-wise or bulk conjugation.
- CG gels with tethered growth factor were seeded with HCEC to investigate the bioavailability and bioactivity of tethered HB-EGF or EGF *in vitro*.

Tethering of Epidermal Growth Factor Family Members to Dendrimer Crosslinked Collagen Gels

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Short Title:
Tethered Growth Factors to Dendrimer Collagen Matrices

6.2 Abstract

To enhance growth factor (GF) bioavailability and bioactivity, epidermal growth factor (EGF) and heparin-binding epidermal growth factor (HB-EGF) were tethered to dendrimer crosslinked collagen (CG) gels, either during or following gel fabrication. Tethering of the growth factors was achieved through carbodiimide chemistry, with ethyl dimethyl carbodiimide (EDC) and N-hydroxysuccinimide (NHS), either via step-wise modification, with gels soaked in activated growth factor solutions, or via bulk modification with various growth factor solutions added to collagen suspension prior to full dendrimer crosslinking of CG gels. Growth factor solutions for CG bulk modification were comprised of either: GF; GF with EDC and NHS; or GF with dendrimer (G2), EDC and NHS. Conjugation of EGF and HB-EGF to CG and thermal collagen (COLL) gels was tunable in relation to the original concentration, 1000 or 10,000 ng/mL, of activated GF exposed to gels. Slightly higher amounts of tethered EGF, compared to HB-EGF, were initially obtained following step-wise conjugation, but differences were not evident following extensive rinsing, indicating the presence of both adsorbed and absorbed protein as well as tethered growth factor. Final tethered GF amounts ranged between 0.01-0.63 ng and 2.24-58.9 ng for ¼" gels (1 mm thick) exposed to 1000 and 10,000 ng/mL GF, respectively. As with step-wise modification, bulk modification of CG gels resulted in GF retention that could be tailored by adjusting the amount of GF added to the collagen suspension during gel fabrication. However, the various GF solutions (GF, GF/EDC/NHS or GF/G2/EDC/NHS) utilized for CG bulk modification did not differ in terms of GF incorporation, as conjugated GF amounts averaged 0.11 ± 0.03 ng GF ($p > 0.05$) per ¼" gel. Regardless of the CG bulk tethering method, rough 2-5% of the original growth factor amount was covalently attached to the gels following extensive rinsing. Bioactivity of EGF and HB-EGF was enhanced following step-wise conjugation to gels, as was verified with proliferation of human corneal epithelial cells (HCEC); however, bulk modification of CG gels, with tethered GF, only maintained HCEC proliferation. Bulk modification fabrication methods likely impede GF stability and receptor

availability. Thus step-wise conjugation is a favourable method for HB-EGF and EGF tethering to CG gels.

Keywords: collagen; dendrimers; heparin-binding epidermal growth factor (HB-EGF); epidermal growth factor (EGF); corneal epithelium; tissue engineered corneal equivalents (TECE).

6.3 Introduction

Growth factors (GF) greatly influence cell migration, proliferation and stratification, extracellular matrix deposition, and are critical to wound healing and tissue maintenance [1]. Epidermal growth factor (EGF) is a 53 amino acid protein [2] highly active in cornea wound healing, particularly through epithelial cell autocrine and paracrine mechanisms with the EGF receptor (EGFR) [3,4]. Heparin-binding EGF (HB-EGF), also a member of the EGF family [5], is active in cornea epithelial wound healing via two EGFRs, as an autocrine, paracrine and juxtacrine activator [6,7,8,9]. In addition, HB-EGF houses a heparin-binding motif which binds heparin and cell surface heparan sulphate proteoglycans, which can enhance its receptor binding [10].

Given the short half lives of GF (EGF has a limited plasma half life of 2.52 minutes [11]), large size and resulting slow tissue penetration, and their fairly non-selective potency, localized and controlled long-term delivery is preferred to achieve the desired degree of tissue regeneration [12]. Current delivery methods include soluble delivery via encapsulation or entrapment from reservoir devices, microspheres and hydrogels, and tethering proteins directly to a substrate [11]. Tethering may be advantageous in controlling GF concentration through prevention of diffusion to surrounding tissues, cell uptake and degradation, and providing prolonged activation of cell signaling [13]. Covalent protein attachment to various substrates has been achieved through step-wise, bulk or micropatterning techniques, whereby GF are bound directly or via spacer chains to the substrate, or modified via protein-engineering to be substrate-reactive or –bound [14].

Direct binding of proteins to substrates has been explored by binding the amino acid sequence Arginine-Glycine-Aspartic acid (RGD) to amine-terminated poly(lactic-glycolic acid) [15], EGF and RGD to amine-terminated chitosan [16], and EGF to amine-terminated poly(caprolactone)-gelatin [17]. Furthermore GF tethering was achieved, via carbodiimide chemistry, with bone morphogenic protein (BMP) grafted onto polyethylene terephthatalate [18] and collagen [19], and nerve growth factor to a poly(caprolactone)-PEG copolymer [20], while VEGF was tethered to collagen matrices [21,22]. Despite the

occurrence of heterogeneous surface coverage and variability in GF orientation, direct tethering via carbodiimide reaction is simple, inexpensive and usually performed under mild, aqueous conditions, resulting in maintained GF bioactivity [23].

EGF has been tethered via star poly(ethylene oxide) to aminosilane-modified glass [13], and via poly(ethylene) glycol (PEG) spacer to polydimethylsiloxane [24,25,26,27] and polycaprolactone [28]. PEG spacers have also been utilized to tether basic fibroblast growth factor [29], transforming growth factor beta [30,31], platelet-derived growth factor [32], vascular endothelial growth factor (VEGF) [33], and a VEGF-mimicking peptide [34], as have cell adhesion peptides [35]. While other spacer tethering mechanisms include EGF bound to a biotin-thiazolidine ring capable of binding streptavidin [36], to an amine-reactive ester and phenyl azide heterobifunctional crosslinker on polystyrene [37], to amine-modified polycaprolactone and to gelatin nanofibrillar scaffolds via suberic acid bis(N-hydroxyl-succinimide ester) [18]. Furthermore, collagen scaffolds were modified with sulphhydryl groups for activation with VEGF conjugated to a heterobifunctional maleimide and a succinimide compound [38]. Although the use of spacer molecules can achieve specific GF orientation and improve the stability and availability of the GF to its receptor through reduced steric hindrance, it can also result in heterogeneous surface coverage and non-specific adsorption of the GF [39]. Furthermore PEGylated GF may not elicit the desired cellular response, as EGF covalently attached to PEG maintained proliferative activity, but required the cell adhesion peptide sequence, Arginine-Glycine-Aspartic acid-Serine (RGDS), to enhance migration in one study [40].

We have developed dendrimer crosslinked collagen (CG) gels [41], intended as a scaffold for a tissue engineered corneal equivalent (TECE). These materials demonstrated transparency, sufficient tensile strength and suturability [42], and functionality for direct protein conjugation. Laminin-based cell adhesion sequences were immobilized via step-wise conjugation onto CG gels, with the resulting materials demonstrating enhanced human cornea epithelial cell (HCEC) adhesion and proliferation, in addition to stimulating neurite extension [43]. CG gels were also modified during fabrication,

whereby laminin-based peptides were bound directly to dendrimer prior to gel crosslinking, for enhanced HCEC stratification [44]. However, given the role of growth factors in tissue engineering, it is also potentially desirable to provide cells with growth factor stimulation. We have previously incorporated heparin into these gels and demonstrated that HB-EGF could be released [45]. However, given the potential need for long term growth factor stimulation, in the present work, we investigate step-wise and bulk tethering of EGF and HB-EGF to CG gels to enhance GF availability and stability for enhanced HCEC proliferation, with the ultimate goal of augmenting TECE integration with host epithelial tissue.

6.4 Materials and Methods

6.4.1 Materials

Concentrated collagen suspensions of pepsin digested bovine cornium purified >80% type I collagen, with the remainder type III collagen, were the generous gift of Allergan Medical (Fremont, CA). Polypropyleneimine octaamine generation two dendrimer (G2) was purchased from SyMO-Chem (Eindhoven, The Netherlands). HB-EGF and EGF were purchased from R & D Systems (Minneapolis, MN). Unless otherwise stated, other reagents were purchased from Sigma-Aldrich (Oakville, ON) or EMD Chemicals Inc. (Gibbstown, NJ).

6.4.2 Collagen Gel Fabrication

Dendrimer crosslinked collagen (CG) gels were fabricated as previously described [46]. Briefly, the collagen suspension, as received (78.8 mg/mL) was diluted with phosphate buffered saline (PBS) and endotoxin free water (EFW), and acidified with 1N hydrochloric acid (HCl). Crosslinking was achieved by adding second generation polypropyleneimine octaamine dendrimer (G2) (10:1 weight ratio of G2:collagen), with ethyl dimethyl carbodiimide (EDC) and N-hydroxysuccinimide (NHS) in a molar ratio of 1:1:1 EDC:NHS:collagen$_{COOH}$. The resultant collagen suspensions were pressed between glass plates, on ice, and incubated overnight at 4^{o}C.

Thermal collagen (COLL) gels, fabricated as controls, were prepared by adjusting the pH of acidified collagen suspensions to 7.4 and incubating at 37^{o}C overnight.

Gels were punched into discs (1/4" or 5/16" diameter, 1 mm thick), soaked in EFW for 24 hrs to remove any unbound components, and dried prior to use. All gels contained 30 mg/mL collagen.

6.4.3 ^{125}I Labelling of Growth Factors

HB-EGF and EGF were reconstituted in 150 mM PBS (pH 7.4), and radiolabelled via the IODOGEN method (Pierce, USA) [24]. Briefly, GF (1000 μg/mL) was combined with 10 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 PBS over a 36 hour period. Free unbound isotope amount was determined by trichloroacetic acid precipitation and remained <10% in all studies.

6.4.4 Growth Factor Tethering after Gel Fabrication (step-wise conjugation)

HB-EGF or EGF was tethered in a step-wise manner, whereby GF was first activated with EDC/NHS, and then reacted with gels. Solutions of 1 mL PBS containing EDC/NHS and either 1000 ng/mL GF (1:1:1 molar ratio GF:EDC:NHS) or 10,000 ng/mL GF (1:0.1:0.1 molar ratio GF:EDC:NHS) were combined for 10 minutes, and then dried COLL and CG gels were added and incubated for 24 hrs at 4^{o}C.

6.4.5 Growth Factor Tethering during Gel Fabrication (bulk conjugation)

Bulk modification of gels with HB-EGF or EGF was achieved by adding various GF solutions into the collagen suspension prior to full gel dendrimer crosslinking. Three GF solutions were utilized: (1)GF; (2)GF/EDC/NHS; or (3)GF/EDC/NHS/G2. The first GF solution contained 1593 or 2390 ng/mL of HB-EGF or EGF, of which 225 μL was added to the collagen suspension, to result in 10 or 15 ng GF per ¼" gel. The second GF solution consisted of 2390 ng/mL GF, along with 75 μL EDC and NHS (molar ratio 1:1:1 GF:EDC:NHS), of which 150 μL was combined with the collagen suspension to result in ¼" gels with 10 ng/mL GF. The third GF solution combined various GF concentrations (358.8, 500, 1838 ng/mL) with EDC, NHS and G2 dendrimer (GF:EDC:NHS:G2 molar ratio of 1:0.1:0.1:0.1), resulting in gels with 10, 13 or 49 ng GF per ¼" gel.

6.4.6 Growth Factor Retention

Gels containing the GFs were assessed for retention by measurement of radioactivity, after preparation using radiolabeled HB-EGF or EGF. Gels were rinsed three times for 30 seconds in 1 mL PBS. In addition, gels were placed into 1 mL of fresh PBS (buffer refreshed periodically) in a 37^{o}C shaking waterbath, and GF release quantified to assess GF retention. Radioactivity was determined using a gamma counter (Wallac Wizard 3" 1480 Automatic Gamma Counter, Perkin Elmer Life Sciences).

6.4.7 Growth Factor Bioactivity of Gels

Tethered GF bioactivity in the gels was determined through *in vitro* culture with an immortilized human cornea epithelial cell (HCEC) line [47]. HCEC were maintained in Keratinocyte Serum-Free Medium (KSFM) containing bovine pituitary extract (25 mg; 0.05 w/v) and EGF (2.5 μg; 0.005 w/v), along with penicillin-streptomycin antibiotic (1:100).

Prior to cell culture studies, COLL and CG gels (5/16") intended to be step-wise modified were first soaked in 1 mL EFW for 24 hrs, then sterilized in PBS containing 100 μL antibiotic (10:1) for an additional 24 hrs, and then dried. Following exposure of dried gels to GF, EDC and NHS for modification, the gels were rinsed in 1 mL PBS for 24 hrs to remove residually adsorbed and absorbed GF.

Gels that were fabricated with GF, via bulk modification, either alone or with a combination of EDC, NHS and/or G2, prior to cell seeding were rinsed in 1 mL PBS for 24 hrs to remove adsorbed and absorbed GF, and then sterilized in PBS containing 100 μL antibiotic (10:1) for an additional 24 hrs.

All gels were seeded with a 20 μL suspension containing 10,000 cells in medium containing no EGF and incubated for 2 hours to allow cells to adhere. This was followed by the addition of 200 μL of serum-free and EGF-free KSFM. Fresh serum-free and EGF-free medium (200 μL) was added every 2 days. After 6 days, cell proliferation was assessed with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay [48,49]. Briefly, 150 μL of MTT solution (0.4 mg/mL MTT, 0.2 μm sterile filtered,

in KSFM containing pituitary extract, EGF and antiobiotic) was added to cells, the plate covered and incubated for 24 hrs. The gels were then dissolved with 200 µL collagenase (345 U/mg; 15.42 mg/mL) in 400 µL TRIS/HCl (15.76 mg/mL) containing 0.05M $CaCl_2$ for 2.5 hours. Following centrifugation (10 min, 10,000 rpm), the supernatant was removed and the pellet reconstituted in 250 µL DMSO to dissolve the formazan precipitate. Following agitation to dissolve the pellet, 200 µL was transferred to a 96-well plate and absorbance read at 540 and 700 nm (Biorad 550 Plate Reader).

6.4.8 Statistical Analysis

Data are presented as mean values with variability expressed as standard deviations, unless otherwise stated. Statistical significance was determined using a single factor ANOVA test on two means ($p<0.05$).

6.5 Results

HB-EGF and EGF were successfully incorporated into dendrimer crosslinked collagen (CG) and thermal (COLL) gels via step-wise carbodiimide reaction with GF, EDC and NHS. In addition, bulk modification of collagen suspensions with either: (1) GF alone; (2) GF, EDC and NHS; or (3) GF, EDC, NHS, and G2 was achieved prior to full gel dendrimer crosslinking. All gels fabricated with tethered GF are summarized in Table 6-1, along with their final GF uptake amounts after gels (¼" gels; 1 mm thick) were soaking in PBS for 40 hours.

6.5.1 Growth Factor Tethering after Gel Fabrication

Retention of HB-EGF and EGF to step-wise modified COLL or CG gels, ¼" diameter, after soaking in a GF, EDC and NHS solution (1000 ng/mL GF; 1:1:1 molar ratio) is shown in Figure 6-1. Initially GF uptake was comparable among COLL gels ($p = 0.13$), while CG gels had higher EGF uptake than HB-EGF uptake ($p < 0.05$). However, much of the GF was lost after three rinses and soaking for 40 hrs in PBS solution, resulting in a comparable loss (79.90 ± 9.26% after 40 hrs) of protein regardless of GF or gel type ($p = 0.06$). This resulted in a total amount of GF associated with the gels at this time point of 0.12 ± 0.04 ng/gel on average, with no difference between the two growth factors examined.

A higher GF concentration of HB-EGF or EGF (10,000 ng/mL), along with EDC and NHS (1:0.1:0.1 molar ratio), was exposed to COLL or CG gels. Figure 6-2 shows GF retention after exposure to GF, EDC and NHS, after rinsing 3 times in PBS, and after 40 hrs in PBS solution. As with lower concentrations, initial GF uptake was comparable among COLL gels ($p = 0.06$), while CG gels had higher EGF uptake than HB-EGF uptake ($p < 0.05$). The 52.14 ± 12.25% loss of GF after 40 hrs among the gels was similar ($p = 0.02$), resulting in 0.35 ± 0.07 ng/gel on average, regardless of the type of GF examined.

With both GF soak concentrations of 1000 and 10000 ng/mL, the amount of GF retained within the gels after 40 hrs is less than 1%, and ranges between 0.01-0.63 ng and 2.24-58.9 ng for gels exposed to 1000 and 10,000 ng/mL GF, respectively, suggesting that the growth factor is not likely to be tethered in this case but can be effectively be released from the gels.

6.5.2 Growth Factor Tethering during Gel Fabrication

Three bulk modification methods for GF addition during gel fabrication were utilized. The first method was to add GF solution to the collagen suspension, and then crosslink the GF-collagen solution with dendrimer, EDC and NHS. The amount of GF incorporated after crosslinking, rinsing and 40 hrs in PBS within the gels, designed to contain 10 or 15 ng per ¼" gel, is shown in Figure 6-3. HB-EGF and EGF retention was dependent upon the initial loading concentration, with less than 2% GF uptake from the original soak solution. Higher amounts of HB-EGF compared to EGF were retained within CG-15 and CG-10 gels ($p = 0.0002$ and $p = 0.002$, respectively). Following rinsing and 40 hrs in PBS, less than 20% of the GF amount was lost, ranging between 0.1-0.4 ng per ¼" gel demonstrating successful tethering into the gels.

In the second method, HB-EGF or EGF was incorporated into the collagen suspension with EDC and NHS (1:1:1 GF:EDC:NHS molar ratio) or with dendrimer, EDC and NHS (1:1:1:1 GF:EDC:NHS:G2 molar ratio), and then crosslinked fully with dendrimer, EDC and NHS. Figure 6-4 summarizes the amount of GF retained using the three bulk modification types of CG gels, when the GF was added during fabrication (10 ng per ¼"

gel). Initially HB-EGF and EGF incorporation was significantly different among the fabrication methods ($p_{HB\text{-}EGF}$ = 0.004 and p_{EGF} = 0.01, respectively); however, following rinses and 40 hrs of exposure to PBS, the amount of GF retained within all gels was similar (0.11 ± 0.03 ng; $p > 0.05$), despite the averaged 24.50 ± 9.72% loss of protein. Roughly 1% of GF was retained from the original GF solution following 40 hrs of release in PBS.

GF retention, following the third method of bulk fabrication with different GF amounts, whereby GF:EDC:NHS:G2 was added to collagen suspensions prior to crosslinking with G2, EDC and NHS, is shown in Figure 6-5. Solutions of GF:EDC:NHS:G2 with varying GF concentrations resulted in gels with 49 (1:0.1:0.1:0.1 molar ratio), 13 (1:0.1:0.1:0.1 molar ratio) or 10 (1:1:1:1 molar ratio) ng per ¼" gel. Initially GF retention is dependent on the amount of GF added, followed by protein losses of 31.18 ± 11.65% and 51.17 ± 26.05%, after rinsing and 40 hrs, respectively. Between 2-5% of the original GF/EDC/NHS/G2 solution added was detected within these gels. Higher amounts of EGF are retained within the higher GF concentrated gels, while gels with 10 ng per ¼" gel have more HB-EGF.

6.5.3 HB-EGF Cell Bioactivity on CHG Gels

To ascertain the bioactivity of tethered GF, HCEC were seeded onto gels and their proliferation assessed after 6 days with the MTT assay. While underlying interactions with the collagen gels may impact the interactions, it is believed that by culturing the cells in the absence of exogenous growth factor, it is possible to determine whether there were negative effects on growth factor bioavailability and bioactivity. HCEC grown on COLL and CG gels fabricated and then exposed step-wise to EDC/NHS activated 1000 ng/mL HB-EGF or EGF for 24 hrs is shown in Figure 6-6 (n = 9; SEM). HCEC growth was enhanced significantly on COLL gels with GF ($p_{HB\text{-}EGF}$ = 0.004 and $p_{EGF} = 3x(10)^{-5}$), compared to CG gels ($p_{HB\text{-}EGF}$ = 0.07 and p_{EGF} = 0.029). EGF had a slightly greater affect on proliferation compared to HB-EGF, but the difference was not significant (p_{COLL} = 0.11 and p_{CG} = 0.39).

HCEC proliferation on bulk modified gels containing HB-EGF or EGF that was added during gel fabrication, as compared to COLL and CG gels without GF, is shown in Figure 6-7. Gels were fabricated with GF (10 or 15 ng/gel) alone, GF, EDC and NHS (10 ng/gel; 1:1:1 GF:EDC:NHS molar ratio) or GF with dendrimer, EDC, NHS (10 ng/gel; 1:1:1:1 molar ratio GF:EDC:NHS:G2). Cell growth on bulk modified gels was not significantly different among all gel types ($p > 0.05$).

6.6 Discussion

GF regulate wound healing and tissue regeneration through cell proliferation, migration, and differentiation [23] and are therefore considered essential components of tissue engineering scaffold materials. Their potency is greatly influenced by their availability within the extracellular matrix, but they are prone to rapid denaturation and degradation. As such, GF tethering that is able to control, maintain and prolong GF potency is an attractive alternative to soluble delivery mechanisms.

Despite the occurrence of heterogeneity of GF orientation, steric hindrance or variability in GF location on or within the substrate, water-soluble carbodiimide chemistry is considered stable, simple, and inexpensive [23]. It has been utilized to tether VEGF [21,22], EGF [28], BMP-2 [18,19] and NGF [20] to various substrates, including collagen matrices and was therefore selected in the current work for effecting tethering.

Covalent attachment can be achieved with EDC whereby the zero-length crosslinker activates carboxylic acid groups into an amine-reactive intermediate, which, stabilized by NHS, can form stable amide bonds [50]. Our dendrimer crosslinked collagen (CG) gels have potential for protein tethering, through carbodiimide chemistry, as demonstrated previously with tethered laminin-based cell adhesion peptides, which promoted adhesion, proliferation and stratification of HCEC [43,44]. Since tethered EGF has been shown to be as, or more, effective than soluble EGF [13], it was hypothesized that tethering of EGF and HB-EGF into CG gels would provide improved GF biostability for enhanced HCEC proliferation with these gels.

Two methods were explored for GF incorporation: the first was step-wise introduction of GF into gels with EDC/NHS following gel fabrication, while the second method was bulk modification, whereby GF solutions were added to the collagen suspension prior to gel crosslinking with dendrimers, EDC and NHS. The second method of bulk modification was expanded to include 3 incorporation mechanisms that differed according to the GF solution added: GF was added on its own, with EDC/NHS, or with dendrimer, EDC and NHS.

In the step-wise method, pre-fabricated CG and thermal collagen (COLL) gels were reacted with EGF or HB-EGF solutions containing EDC and NHS. Following exposure to 1000 or 10,000 ng/mL GF, with EDC/NHS, both gel types demonstrated GF retention, although EGF was initially retained to a greater degree than HB-EGF. This is not surprising, as the mechanism for attachment is diffusion dependent, and the molecular weight and size of EGF (53 AA; MW = 6 kDa) [51] is smaller than HB-EGF (88 AA; MW = 9.5 kDa) [52].

COLL gels retained more GF than CG gels, which may correlate to the number of amine (NH_2) and carboxylic acid (COOH) groups available for carbodiimide reaction, although not all may react via EDC/NHS. It has been shown that our COLL gels have 87 COOH per 1000 amino acid residues available for reaction, compared to 40 COOH per 1000 amino acid residues available within CG gels [41]. The total number of amine residues within COLL and CG gels is estimated to be 30 per 1000 and 126 per 1000 [41], respectively. However, we estimated the number of available amine groups within CG gels to be 40 per 1000, as determined via ninhydran assay (data not shown). The step-wise procedure initially favours COOH activation of GF for amide reaction to the gels, but given the comparable reactive amine groups, it is likely that COOH activation of the gels correlates to differences in COLL and CG gel GF retention.

Following the step-wise tethering reaction, a loss of GF is observed for all gels after rinsing and 40 hrs in PBS, which indicates there is also not unexpectedly physically adsorbed and absorbed GF present. As step-wise tethering was performed under physiologically relevant conditions (pH 7.4) to preserve GF stability, and since the

carbodiimide chemistry utilized has shown to be optimal at pH 5 [50], it is likely that not all GF COOH groups were activated. Elsewhere, only 5% of the calculated surface coverage for available amine groups of the laminin peptide sequence Tyrosine-Isoleucine-Glysine-Serine-Arginine (YIGSR) on CG gels was achieved following reaction with EDC/NHS (pH 5.5) [43].

Despite the nanogram magnitude of tethered GF present within COLL and CG gels, after step-wise conjugation, and the likelihood of heterogeneous GF orientation, both gels following exposure to 1000 ng/mL GF, were able to significantly increase HCEC proliferation following 6 days of culture. EGF demonstrated slightly greater potency than HB-EGF, which correlates to higher gel retention (Figure 6-1), and perhaps to more favourable interaction with the EGF receptor, as cell response to growth factors is dependent on availability, surface density and orientation [53]. The degree of HCEC proliferation in response to tethered HB-EGF or EGF observed here is in accordance with our previous work, whereby HCEC proliferation increased by ~1.5x following exposure to 0.1 ng/mL soluble HB-EGF or EGF [45].

Elsewhere, step-wise conjugation of EGF to PEG, and then tethering onto plasma treated PDMS substrates resulted in < 20 ng/cm^2 bound EGF [24,25] following exposure to solutions of 1-10 μg/mL EGF, correlating to <1% grafting efficiency. These EGF-tethered substrates demonstrated increased HCEC proliferation and adhesion, compared to PDMS [27].

Although more favourable results with COLL gels with step-wise tethering were observed, compared to CG gels, they lack the stability and strength required for TECE applications. Thus, to increase the amount of EGF or HB-EGF tethered into CG gels, bulk modification was examined, whereby solutions of GF, GF/EDC/NHS, or GF/EDC/NHS/G2 were added to the collagen suspension prior to complete gel crosslinking with dendrimer, EDC and NHS. Adding GF solutions during fabrication may weaken gel integrity, as was observed elsewhere when slightly lower, but not significant, denaturation temperatures and mechanical strength following YIGSR modification occurred [43]. Here, mechanical testing was not performed, but differences in gel strength

were not observed during gel handling. There was also no difference in GF retention among the three incorporation methods, suggesting that activation of the GF with EDC/NHS or tethering to G2 with EDC/NHS was not necessary at these low concentrations. However, higher GF concentrations may warrant the use of direct tethering prior to full gel crosslinking.

GF retention during bulk modification could be tailored by adjusting the amount of GF added to the gels, as is evident in Figures 6-3 and 6-5. However, the calculated amount of GF originally added to the gels is considerably more than the actual amount of GF detected, between 1-5% depending on the incorporation method. This protein loss could be due to GF degradation during crosslinking or non-specific protein adsorption to gel manufacturing supplies as well as to the gels. Furthermore, had the reacted GF and G2 solutions been purified prior to reaction, protein retention could have been higher. Protein retention with laminin-peptide sequences, when bound first to G2, was between 24-30% when laminin-peptide-G2 solutions were purified and concentrated, and resulted in microgram quantities of protein per milligram of collagen and enhanced HCEC stratification [44]. Despite this, small but measurable amounts of growth factor could be added to the gels using this method.

HCEC proliferation on CG gels with GF added during fabrication (bulk modification), regardless of the GF incorporation type, was not augmented. Acidic and ionic conditions can alter GF electrostatic interactions, thus affecting GF conformation and activity, as was observed by Chiu and colleagues, when VEGF potency was hindered following immobilization to collagen under acidic conditions (MES, pH 4.2) [21]. Furthermore, VEGF activated with EDC/NHS and then added to collagen and crosslinked did not improve cell proliferation, as it did with step-wise conjugation of VEGF to activated collagen sponges. Thus it is likely that our acidic crosslinking conditions (pH 5.5) during bulk fabrication interfered with GF orientation and activity. Thus step-wise GF tethering with EGF and HB-EGF to CG gels is favourable, compared to bulk tethering, for HCEC proliferation. Elsewhere, step-wise conjugation was utilized with succinylated collagen activated with EDC/NHS (pH 3) prior to BMP conjugation [19], and step-wise

modification of collagen sponges with VEGF was determined to be favourable over bulk modification[21].

Finally, activation of GF with EDC/NHS could have resulted in interference of amino acid groups active in GF cell receptor binding. The C-terminus or aspartate or glutamate residues are capable of EDC activation for amine reaction, while the N-terminus or lysine residues can bind carboxylic acid groups on the substrate [23]. The amino acid sequences of human EGF (#P01133; Asn971-Arg1023) and human HB-EGF (#Q99075; Asp63-Leu148) purchased from R&D Systems (Minneapolis, MN), and manufactured in accordance with Protein Knowledgebase (UniProtKB), are illustrated in Figure 6-8. The EGFR binding domain of EGF (Ser972-Gln1013) and HB-EGF (Lys104-His144), containing the EGF-family characteristic six cysteine sequence $CX_7CX_{4-5}CX_{10}CXCX_8C$ [54], along with the HSPG receptor binding site for HB-EGF (Lys93-Lys113) [5], contain lysine, aspartic acid and glutamic acid, which can react via carbodiimide chemistry. To estimate the probability of tethered GF affecting receptor binding [21], the number of available COOH and/or NH_2 groups within the GF receptor binding domains was divided by the total number of amino acids in the binding domain (Table 6-2). The calculated probability of EGFR interference following COOH binding of EGF and HB-EGF are 14.4% and 21.8%, respectively, while HSPG binding interference with tethered HB-EGF is 47.8%. Furthermore, EGFR interference from NH_2 binding is 2.4% for EGF and 9.8% for HB-EGF. This also suggests greatly likelihood of HB-EGF heterogeneity within CG gels. These probability differences between EGF and HB-EGF coincide with the observed HCEC proliferation results, whereby HB-EGF demonstrated decreased bioactivity compared to EGF.

6.7 Conclusions

HB-EGF and EGF were incorporated via carbodiimide chemistry into dendrimer crosslinked (CG) and thermal (COLL) collagen gels in a step-wise manner, or added during CG fabrication for bulk modification. The amount of retained GF could be tailored based on the initial loading concentration, however physically adsorbed and absorbed protein was also evident. Step-wise GF addition to fabricated gels was able to stimulate

HCEC proliferation, whereas GF addition during gel fabrication (bulk modification) did not improve GF bioavailability or maintain GF bioactivity, suggesting that step-wise conjugation of GF to CG gels could be the preferred method to maintain GF bioactivity for TECE applications.

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6.9 References

1. Yu FX, Yin J, Xu K and Huang J. Growth factors and corneal epithelial wound healing. Brain Research Bulletin 2010; 81:229-235.
2. Carpenter G and Cohen S. Epidermal growth factor. J Biol Chem 1990; 265: 7709-7712.
3. Boonstra J, Rijken P, Humbel B, Cremers F, Verkleij A, and van Bergen en Henegouwen P. The epidermal growth factor. Cell Biol Int 1995; 19: 413-430.
4. Klenkler BJ and Sheardown H. Growth factors in the anterior segment: role in tissue maintenance, wound healing and ocular pathology. Exp Eye Res 2004; 79: 677-688.
5. Raab G and Klagsbrun M. Heparin-binding EGF-like growth factor. Biochim Biophys Acta 1997; 1333: F179-F199.
6. Xu KP, Ding Y, Ling J, Dong Z and Yu FX. Wound-induced HB-EGF ectodomain shedding and EGFR activation in corneal epithelial cells. Invest Ophthalmol Vis Sci 2004; 45: 813-820.
7. Yoshioka R, Shiraishi A, Kobayashi T, Morita S, Hayashi Y, Higashiyama S and Ohashi Y. Corneal epithelial wound healing impaired in keratinocyte-specific HB-EGF-deficient mice *in vitro* and *in vitro*. Invest Ophthalmol Vis Sci 2010; 51: 5630-9.
8. Block ER, Matela AR, SundarRaj N, Iszkula ER and Klarlund JK. Wounding induces motility in sheets of corneal epithelial cells through loss of spatial constraints: role of heparin-binding epidermal growth factor-like growth factor signaling. J Biol Chem 2004; 279: 24307-24312.
9. Iwamoto R and Mekada E. Heparin-binding EGF-like growth factor: a juxtacrine growth factor. Cytokine Growth Factor Rev 2000; 11: 335-344.
10. Nishi E and Klagsburn M. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a mediator of multiple physiological and pathological pathways. Growth Factors 2004; 22: 253-260.
11. Baldwin SP and Satlzman M. Materials for protein delivery in tissue engineering. Adv Drug Deliv Rev 1998; 33: 71-86.
12. Babensee JE, McIntire LV and Mikos AG. Growth factor delivery for tissue engineering. Pharm Res 2000; 17: 497-504.
13. Kuhl PR and Griffith-Cima LG. Tethered epidermal growth factor as a paradigm for growth factor-induced stimulation from the solid phase. Nat Med 1996; 2: 1022-1027.
14. Ito Y. Covalently immobilized biosignal molecule materials for tissue engineering. Soft Matter 2008; 4: 46-56.

15. Yoon JJ, Song SH, Lee DS, and Park TG. Immobilization of cell adhesive RGD peptide onto the surface of highly porous biodegradable polymer scaffolds fabricated by a gas foaming/salt leaching method. Biomaterials 2004; 25: 5613-5620.

16. Tigli RS, and Gumusderelioglu M. Evaluation of RGD- or EGF-immobilized chitosan scaffolds for chondrogenic activity. Int J Biol Macromol 2008; 43: 121-128.

17. Tigli RS, Kazaroglu NM, Mavis B, and Gumusderelioglu M. Cellular behavior on epidermal growth factor (EGF)-immobilized PCL/gelatin nanofibrous scaffolds. J Biomater Sci 2011; 22: 207-223.

18. Zouani OF, Chollet C, Guillotin B and Durrieu MC. Differentiation of pre-osteoblast cells on poly(ethylene terephthalate) grafted with RGD and/or BMPs mimetic peptides. Biomaterials 2010; 31: 8245-8253.

19. Yamachika E, Tsujigiwa H, Shirasu N, Ueno T, Sakata Y, Fukunaga J, Nizukawa N, Yamada M and Sugahara T. Immobilized recombinant human bone morphogenetic protein-2 enhances the phosphorylation of receptor-activated Smads. J Biomed Mater Res 2009; 88A: 599-607.

20. Cho YI, Choi JS, Jeong SY and Yoo HS. Nerve growth factor (NGF)-conjugated electrospun nanostructures with topographical cues for neuronal differentiation of mesenchymal stem cells. Acta Biomater 2010; 6: 4725-4733.

21. Chiu LLY, Weisel RD, Li RK and Radisic M. Defining conditions for covalent immobilization of angiogenic growth factors onto scaffolds for tissue engineering. J Tissue Eng Regen Med 2011; 5: 69-84.

22. Miyagi Y, Chiu LLY, Cimini M, Weisel RD, Radisic M and Li RK. Biodegradable collagen patch with covalently immobilized VEGF for myocardial repair. Biomaterials 2011; 32: 1280-1290.

23. Masters KS. Covalent growth factor immobilization strategies for tissue repair and regeneration. Macromol Biosci 2011; 11: 1149-1163.

24. Klenkler BJ, Griffith M, Becerril C, West-Mays JA and Sheardown H. EGF-grafted PDMS surfaces in artificial cornea applications. Biomaterials 2005; 26: 7286-7296.

25. Klenkler BJ and Sheardown H. Characterization of EGF coupling to aminated silicone rubber surfaces. Biotechnol Bioeng 2006; 95: 1158-1166.

26. Hong C, Brook MA, Sheardown H, Chen Y and Klenkler BJ. Generic bioaffinity silicone surfaces. Bioconjug Chem 2006; 17: 21-28.

27. Klenkler BJ, Dwivedi D, West-Mays JA and Sheardown H. Corneal epithelial cell adhesion and growth on EGF-modified aminated PDMS. J Biomed Mater Res A 2010; 93: 1043-1049.

28. Choi JS, Leong KW and Yoo HS. In vivo wound healing of diabetic ulcers using electrospun nanofibers immobilized with human epidermal growth factor (EGF). Biomaterials 2008; 29: 587-596.

29. DeLong SA, Moon JJ, and West JL. Covalently immobilized gradients of bFGF on hydrogel scaffolds for directed cell migration. Biomaterials 2005; 26: 3227-3234.

30. Mann BK, Schmedlen RH, and West JL. Tethered-TGF-beta increases extracellular matrix production of vascular smooth muscle cells. Biomaterials 2001; 22: 439-444.

31. Bentz H, Schroeder JA and Estridge TD. Improved local delivery of TGF-beta2 by binding to injectable fibrillar collagen via difunctional polyethylene glycol. J Biomed Mat Res 1998: 39: 539-548.

32. Saik JE, Gould DJ, Watkins EM, Dickinson ME and West JL. Covalently immobilized platelet-derived growth factor-BB promotes angiogenesis in biomimetic poly(ethylene glycol) hydrogels. Acta Biomater 2011; 7: 133-143.

33. Leslie-Barbick JE, Shen C and West JL. Micron-scale spatially patterned, covalently immobilized vascular endothelial growth factor on hydrogels accelerates endothelial tubulogenesis and increases cellular angiogenic responses. Tissue Eng Part A 2011; 17: 221-229.

34. Leslie-Barbick JE, Saik JE, Gould DJ, Dickinson ME and West JL. The promotion of microvasculature formation in poly(ethylene glycol) diacrylate hydrogels by an immobilized VEGF-mimetic peptide. Biomaterials 2011; 32: 5782-5789.

35. DeLong SA, Gobin SA and West JL. Covalent immobilization of RGDS on hydrogel surfaces to direct cell alignment and migration. J Control Release 2005; 109: 139-148.

36. Wade JD, Domagala T, Rothacker J, Catimel B and Nice E. Use of thiazolidine-mediated ligation for site specific biotinylation of mouse EGF for biosensor immobilization. Letters in Peptide Science 2002; 8: 211-220.

37. Stefonek-Puccinelli TJ and Masters KS. Regulation of cell signaling and function via changes in growth factor presentation. Conf Proc IEEE Eng Med Biol Soc 2009; 2009: 1167-1171.

38. He Q, Zhao Y, Chen B, Xiao Z, Zhang J, Chen L, Chen W, Deng F and Dai J. Improved cellularization and angiogenesis using collagen scaffolds chemically conjugated with vascular endothelial growth factor. Acta Biomater 2011; 7: 1084-1093.

39. Liberelle B, Boucher C, Chen J, Jolicoeur M, Durocher Y and De Crecenzo G. Impact of epidermal growth factor tethering strategy on cellular response. Bioconjug Chem 2010; 21: 2257-2266.

40. Gobin AS and West JL. Effects of epidermal growth factor on fibroblast migration through biomimetic hydrogels. Biotechnol Prog 2003; 19: 1781-1785.

41. Duan X and Sheardown H. Crosslinking of collagen with dendrimers. J Biomed Mater Res A 2005; 75: 510-518.

42. Duan X and Sheardown H. Dendrimer crosslinked collagen as a corneal tissue engineering scaffold: mechanical properties and corneal epithelial cell interactions. Biomaterials 2006; 27: 4608-4617.

43. Duan X, McLaughlin C, Griffith M and Sheardown H. Biofunctionalization of collagen for improved biological response: scaffolds for corneal tissue engineering. Biomaterials 2007; 28: 78-88.

44. Duan X and Sheardown H. Incorporation of cell-adhesion peptides into collagen scaffolds promotes corneal epithelial stratification. J Biomater Sci Polymer Ed 2007: 18: 701-711.

45. Princz MA and Sheardown H. Heparin Modified Dendrimer Crosslinked Collagen Matrices for the Delivery of Heparin-Binding Epidermal Growth Factor (HB-EGF). J Biomed Mat Res Submitted 2011.

46. Princz MA and Sheardown H. Heparin-modified dendrimer cross-linked collagen matrices for the delivery of basic fibroblast growth factor (FGF-2). J Biomater Sci Polymer Edn 2008; 19: 1201-1218.

47. Griffith M, Li F, Lohman C, Sheardown H, Shimmura S and Carlsson DJ. Tissue Engineering of the Cornea. In: Scaffolding in Tissue Engineering. Editors: Ma PX and Elisseeff J. USA: CRC press book; 2006 p.413-423.

48. Leonardi A, Papa V, Fregona I, Russo P, De Franchis G and Milazzo G. In vitro effects of fluoroquinolone and aminoglycoside antibiotics on human keratocytes. Cornea 2006; 25: 85-90.

49. Ono A, Kanno H, Hayashi A, Nishimura S, Kyuma Y, Sato H, Ito S, Shimizu N, Chang CC, Gondo G, Yamamoto I, Sasaki T and Tanaka M. Collagen gel matrix assay as an *in vitro* chemosensitivity test for malignant astrocytic tumors. Int J Clin Oncol 2007; 12: 125-130.

50. Grabarek Z and Gergely J. Zero-length crosslinking procedure with the use of active esters. Anal Biochem 1990; 185: 131-135.

51. R&D Systems. Recombinant Human EGF Carrier Free (236-EGF) 2010. In accordance with In accordance with Protein Knowledge (UniProtKB #P01133).

52. R&D Systems. Recombinant Human HB-EGF Carrier Free (259-HE/CF) 2010. In accordance with Protein Knowledge (UniProtKB #Q99075).

53. Hubbell JA. Bioactive biomaterials. Curr Opin Biotech 1999; 10: 123-129.

54. Schneider MR and Wolf E. The epidermal growth factor receptor ligands at a glance. J Cell Physiol 2009; 218: 460-466.

6.10 Figures

Figure 6-1. HB-EGF or EGF (ng) remaining in thermal collagen (COLL) or dendrimer crosslinked collagen (CG) gels, ¼" diameter, after soaking in a solution containing GF, EDC and NHS (1:1:1 molar ratio; 1000 ng/mL GF), after rinsing 3 times in PBS, and after 40 hrs in PBS solution. After soaking in solution, GF uptake was comparable among COLL gels ($p = 0.13$), while CG gels had higher EGF uptake than HB-EGF uptake ($p < 0.05$). There is loss of GF after rinsing and 40 hrs in PBS solution. The 79.90 ± 9.26% loss of GF after 40 hrs is comparable regardless of GF or gel type ($p = 0.06$).

Figure 6-2. HB-EGF or EGF (ng) remaining in thermal collagen (COLL) or dendrimer crosslinked collagen (CG) gels, ¼" diameter, after soaking in a solution containing GF, EDC and NHS (1:0.1:0.1 molar ratio; 10 000 ng/mL GF), after rinsing 3 times in PBS, and after 40 hrs in PBS solution. After soaking in solution, GF uptake was comparable among COLL gels ($p = 0.06$), while CG gels had higher EGF uptake than HB-EGF uptake ($p < 0.05$). There is loss of GF after rinsing and 40 hrs in PBS solution. The 52.14 ± 12.25% loss of GF after 40 hrs among the gels is not comparable ($p = 0.02$).

Figure 6-3. HB-EGF or EGF (ng) remaining in dendrimer crosslinked (CG) gels, after adding GF solution (15 or 10 ng per ¼" gel) prior to gel crosslinking, after 3 PBS rinses, and after 40 hrs in PBS solution. GF retention is dependant on loading concentrations, with higher HB-EGF incorporation evident compared to EGF ($p_{CG\text{-}15} = 0.0002$ and $p_{CG\text{-}10} = 0.002$).

Figure 6-4. HB-EGF or EGF (ng) remaining in dendrimer crosslinked (CG) gels, after adding GF solution (10 ng per ¼" gel) prior to crosslinking, after 3 PBS rinses, and after 40 hrs in PBS solution. GF was added on its own (GF), with EDC and NHS (1:1:1 molar ratio GF:EDC:NHS), or with dendrimer, EDC and NHS (1:1:1:1 molar ratio GF:EDC:NHS:G2). With respect to the fabrication method, HB-EGF and EGF incorporation was initially different ($p_{HB\text{-}EGF} = 0.004$ and $p_{EGF} = 0.01$, respectively); however, following rinses and 40 hrs of exposure to PBS, the amount of GF retained within all gels was similar (0.11 ± 0.03 ng; $p > 0.05$).

Figure 6-5. HB-EGF or EGF (ng) remaining in dendrimer crosslinked (CG) gels, after adding GF solution (48.9, 13.35 or 10 ng per ¼" gel) prior to crosslinking, after 3 PBS rinses, and after 40 hrs in PBS solution. GF was added prior to crosslinking with EDC, NHS and dendrimer (G2) in a 1:0.1:0.1:0.1 molar ratio of GF:EDC:NHS:G2. GF incorporation is tunable depending on the amount of GF initially added.

Figure 6-6. HCEC proliferation after 6 days on thermal collagen (COLL) or dendrimer crosslinked collagen (CG) gels following step-wise exposure to 1000 ng/mL HB-EGF or EGF, with EDC/NHS, for 24 hrs (n = 9, SEM). For COLL gels, cell growth was enhanced following exposure to GF ($p_{HB\text{-}EGF} = 0.004$ and $p_{EGF} = 3x(10)^{-5}$), compared to CG gels ($p_{HB\text{-}EGF} = 0.07$ and $p_{EGF} = 0.029$). EGF had a higher HCEC proliferation compared to HB-EGF, but the difference was not significant ($p_{COLL} = 0.11$ and $p_{CG} = 0.39$).

Figure 6-7. HCEC proliferation after 6 days on dendrimer crosslinked collagen (CG) gels following bulk modification with HB-EGF or EGF solutions added prior to gel

crosslinking, as compared to thermal collagen (COLL) or dendrimer crosslinked collagen (CG) gels without GF (n = 9, SEM). GF solutions were: GF (GF; 15 or 10 ng/gel) added on its own, GF with EDC and NHS (1:1:1 molar ratio GF:EDC:NHS), or GF with dendrimer (G2), EDC and NHS (1:0.1:0.1:0.1 molar ratio GF:EDC:NHS:G2). Cell proliferation was similar, regardless of GF incorporation, and comparable to COLL and CG gels without GF ($p > 0.05$).

Figure 6-8. Amino acid sequences of human EGF (#P01133; Asn971-Arg1023) and human HB-EGF (#Q99075; Asp63-Leu148) illustrating the EGF-like domains (Ser972-Gln1013 and Lys104-His144), for EGF and HB-EGF respectively), the heparin-binding site for HB-EGF (Lys93-Lys113), and amino acid residues of lysine (K; yellow), aspartic acid (D; blue), glutamic acid (E; orange) and cysteine (C; grey).

Figure 6-1.

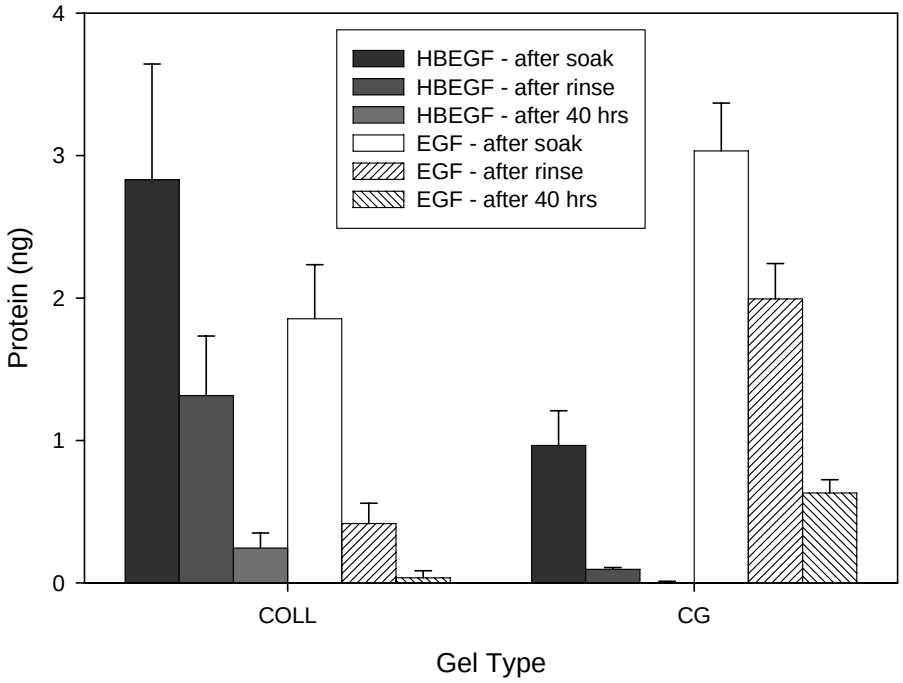

HBEGF - after soak
HBEGF - after rinse
HBEGF - after 40 hrs
EGF - after soak
EGF - after rinse
EGF - after 40 hrs
Protein (ng)
0
1
2
3
4
COLL
CG
Gel Type

Figure 6-2.

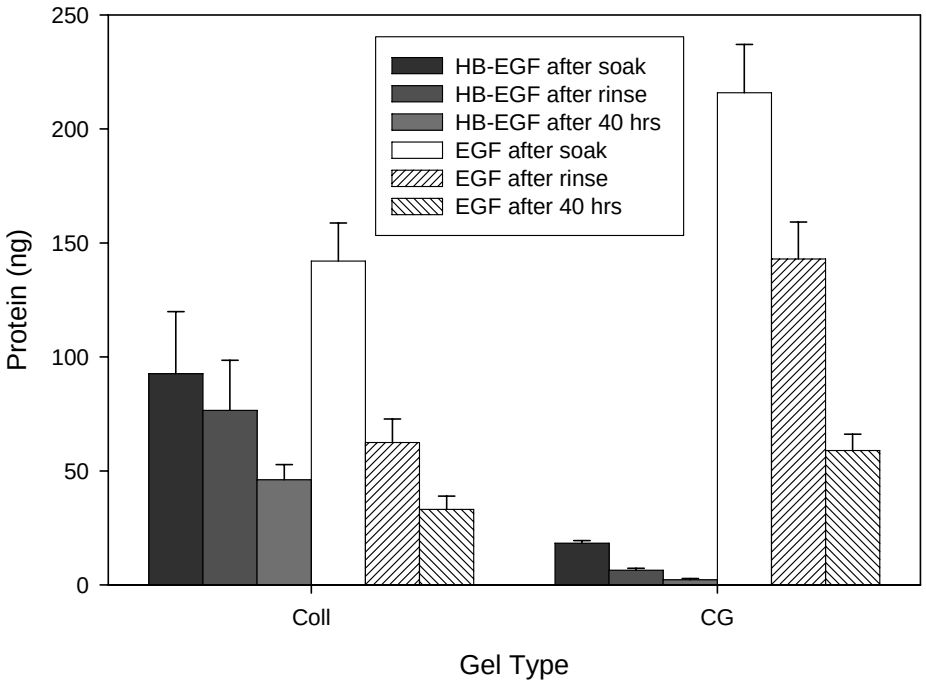

250
200
150
100
50
0
Protein (ng)
HB-EGF after soak
HB-EGF after rinse
HB-EGF after 40 hrs
EGF after soak
EGF after rinse
EGF after 40 hrs
Coll
CG
Gel Type

Figure 6-3.

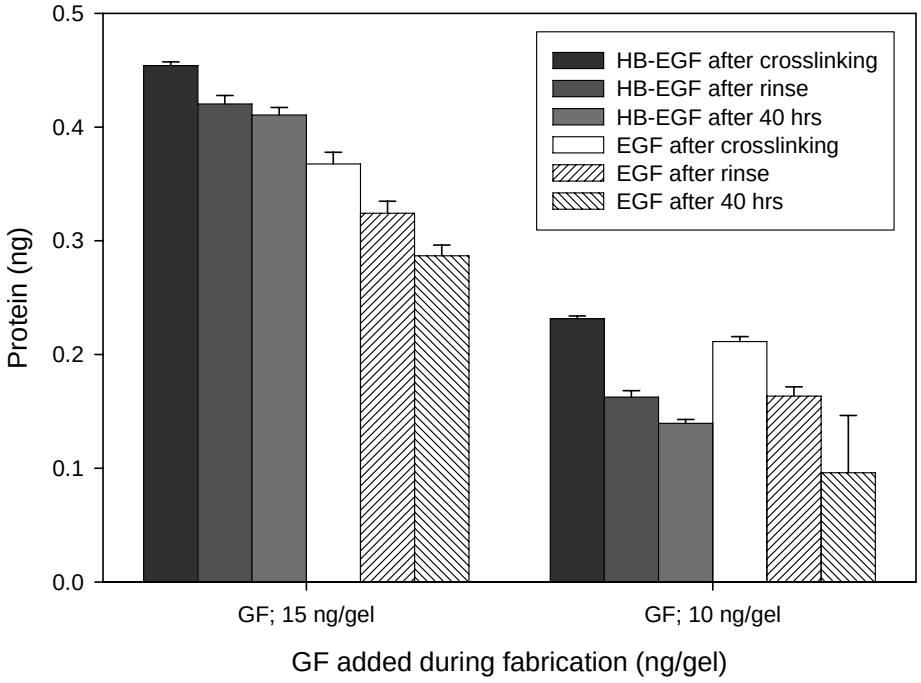

0.5
0.4
0.3
0.2
0.1
0.0
Protein (ng)
HB-EGF after crosslinking
HB-EGF after rinse
HB-EGF after 40 hrs
EGF after crosslinking
EGF after rinse
EGF after 40 hrs
GF; 15 ng/gel
GF; 10 ng/gel
GF added during fabrication (ng/gel)

Figure 6-4.

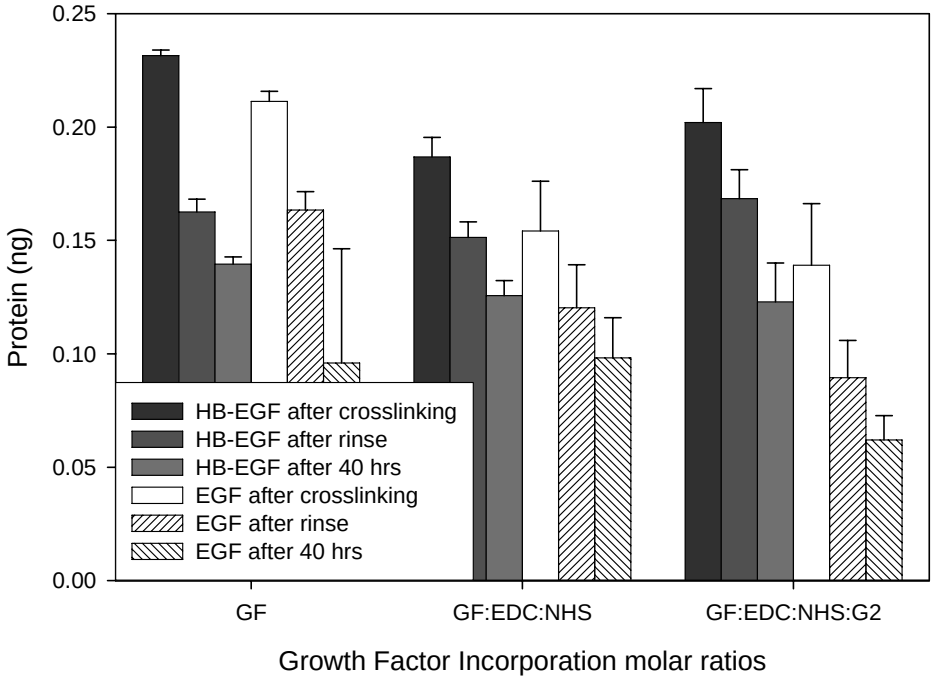

0.25
0.20
0.15
0.10
0.05
0.00
Protein (ng)
HB-EGF after crosslinking
HB-EGF after rinse
HB-EGF after 40 hrs
EGF after crosslinking
EGF after rinse
EGF after 40 hrs
GF
GF:EDC:NHS
GF:EDC:NHS:G2
Growth Factor Incorporation molar ratios

Figure 6-5.

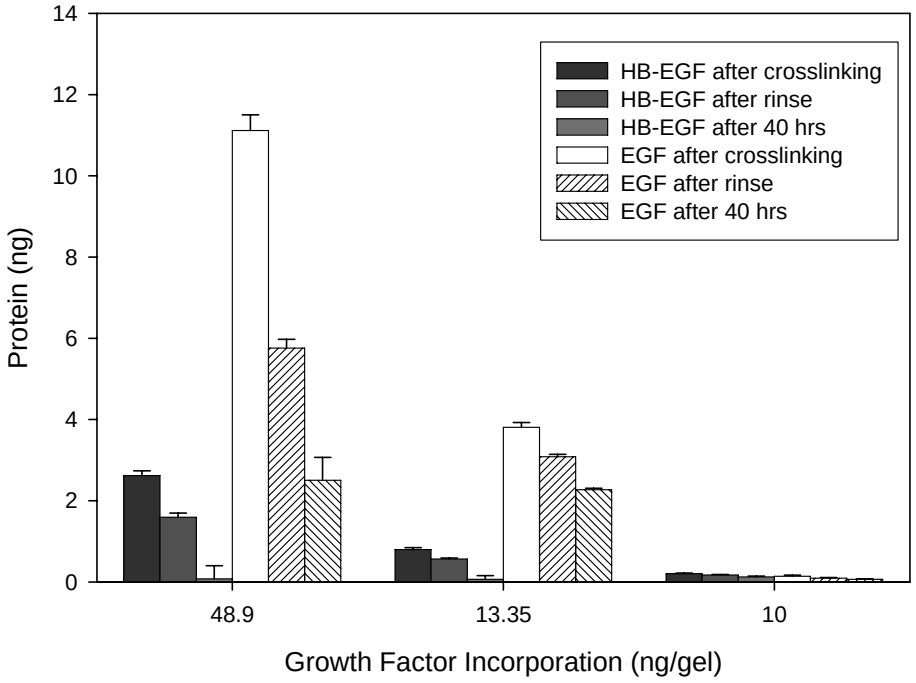

14
12
10
8
6
4
2
0
Protein (ng)
HB-EGF after crosslinking
HB-EGF after rinse
HB-EGF after 40 hrs
EGF after crosslinking
EGF after rinse
EGF after 40 hrs
48.9
13.35
10
Growth Factor Incorporation (ng/gel)

Figure 6-6.

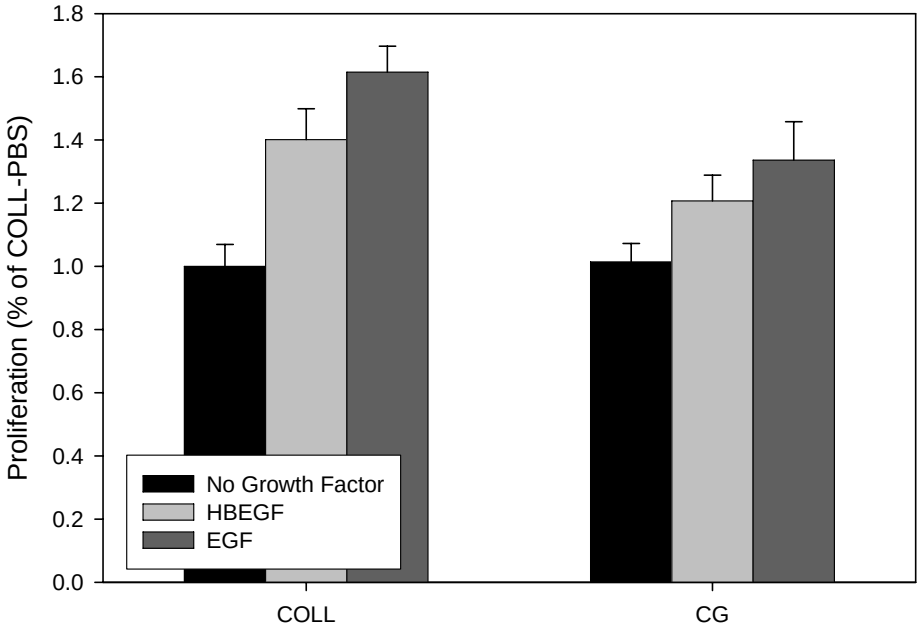

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Proliferation (% of COLL-PBS)
No Growth Factor
HBEGF
EGF
COLL
CG

Figure 6-7.

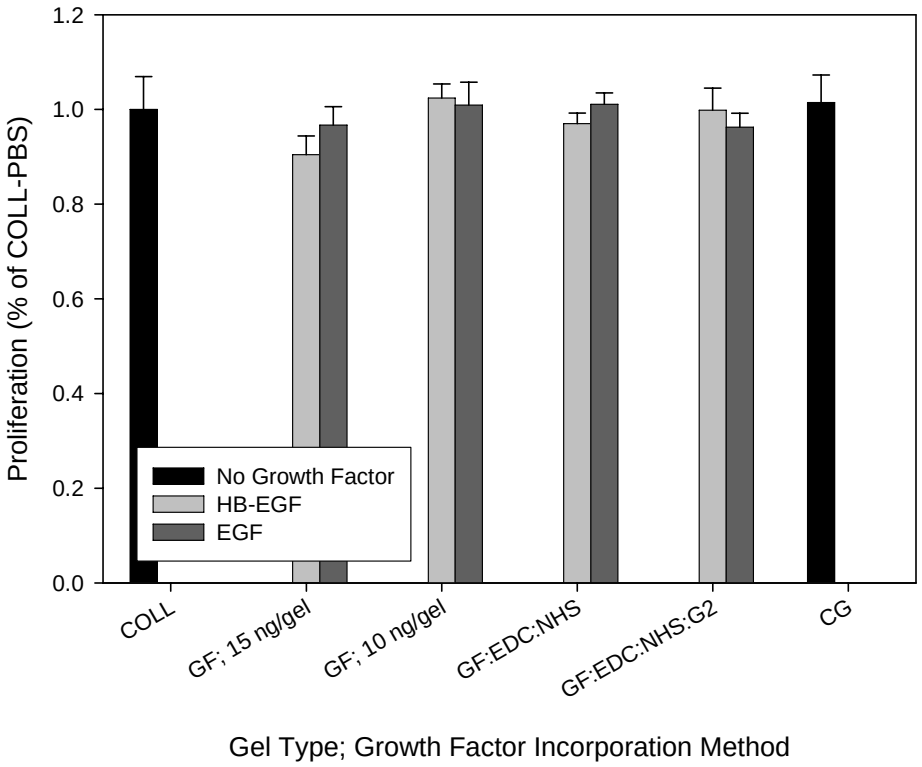
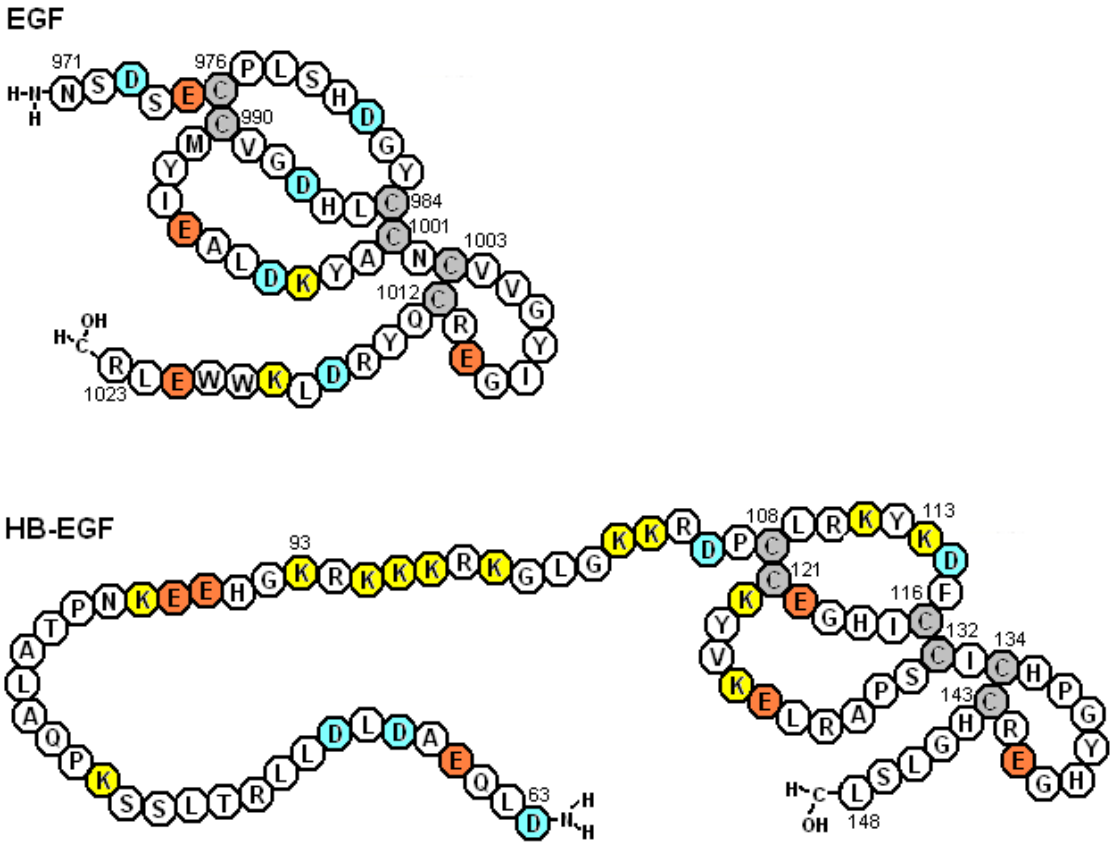

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Proliferation (% of COLL-PBS)
No Growth Factor
HB-EGF
EGF
COLL
GF; 15 ng/gel
GF; 10 ng/gel
GF:EDC:NHS
GF:EDC:NHS:G2
CG
Gel Type; Growth Factor Incorporation Method

Figure 6-8.


EGF
971
976
990
984
1001
1003
1012
1023
HB-EGF
93
108
113
121
116
132
134
143
148
63

6.11 Tables

Table 6-1. Dendrimer crosslinked collagen gels fabricated with tethered GF (¼" gels; 1 mm thick).

		EGF		HB-EGF	
		GF Added	GF Uptake (ng/gel)*	GF Added	GF Uptake (ng/gel)*
Step-wise tethering	COLL	1000 ng/mL	0.04 ± 0.05	1000 ng/mL	0.25 ± 0.11
	CG	1000 ng/mL	0.63 ± 0.09	1000 ng/mL	0.01 ± 0.02
	COLL	10 000 ng/mL	33.1 ± 5.77	10 000 ng/mL	46.1 ± 6.67
	CG	10 000 ng/mL	59.0 ± 7.23	10 000 ng/mL	2.24 ± 0.54
Bulk Tethering	GF	10 ng/gel	0.10 ± 0.05	10 ng/gel	$0.14 \pm 3.3e^{-3}$
	GF	15 ng/gel	$0.29 \pm 9.5e^{-3}$	15 ng/gel	$0.41 \pm 6.7e^{-3}$
	GF:EDC:NHS	10 ng/gel	0.10 ± 0.02	10 ng/gel	$0.13 \pm 3.3e^{-3}$
	GF:EDC:NHS:G2	10 ng/gel	0.06 ± 0.01	10 ng/gel	0.13 ± 0.02
	GF:EDC:NHS:G2	13.35 ng/gel	2.27 ± 0.04	13.35 ng/gel	0.06 ± 0.09
	GF:EDC:NHS:G2	48.9 ng/gel	2.50 ± 0.56	48.9 ng/gel	0.07 ± 0.32

*Uptake was quantified after gels were soaked in PBS for 40 hours.

Table 6-2. Probability of receptor interference with EGF and HB-EGF.

	EGF			HB-EGF		
	COOH	**NH_2**	**Total AA**	**COOH**	**NH_2**	**Total AA**
Entire Sequence[t]	10	3	53	12	14	86
EGFR binding domain	5	1	41	5	4	42
EGFR Interference (%)*	**12**	**2.4**	**14.4**	**12**	**9.8**	**21.8**
HSPG binding domain	n/a	n/a	n/a	1	9	21
HSPG Interference (%)*	**n/a**	**n/a**	**n/a**	**4.8**	**43**	**47.8**

*Calculated by dividing the number of carboxylic acid (COOH) or amine (NH_2) containing amino acids within binding domain by the total number of amino acids (AA) within the binding domain, as represented in Figure 8.
[t]Including the N- and C-termini.

7 PAPER 4: PRE-ACTIVATED HEPARINIZED DENDRIMER CROSSLINKED COLLAGEN MATRICES FOR IMPROVED BLOOD-CONTACTING BIOMATERIALS

7.1 Summary

Authors:

Marta A. Princz, Kyla N. Sask, Dr. John L. Brash and Dr. Heather Sheardown.

Publication Information:

Not yet submitted.

Objectives:

Prepared dendrimer crosslinked collagen gels with heparin pre-activated with carbodiimide chemistry (PH gels) to increase heparin content for enhanced blood-contacting biomaterial applications.

Main Scientific Contributions:

- Fabricated dendrimer crosslinked collagen gels with heparin pre-activated with carbodiimide (PH gels).
- Demonstrated protein sorption of antithrombin with PH gels and unactivated-heparinized dendrimer crosslinked collagen (CHG) gels, to quantify heparin and antithrombin interactions. Radiolabeling was performed by Kyla Sask.
- Heparinization of dendrimer crosslinked collagen gels demonstrated prolonged clotting times, particularly CHG gels, validating their use for blood-contacting applications. Clotting experiments were performed by Kyla Sask.

Pre-Activated Heparinized Dendrimer Crosslinked Collagen Matrices for Improved Blood-Contacting Biomaterials

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Short Title:
Pre-activated Heparinized Dendrimer Crosslinked Collagen Gels

7.2 Abstract

Collagen gels have potential as tissue engineering scaffolds, but are prone to adverse interactions when used in blood contacting applications. We previously developed a series of heparin containing collagen materials that may be useful in this context. In the present work dendrimer crosslinked collagen (CG) gels, prepared with heparin that was pre-activated using carbodiimide chemistry to increase heparin content (PH gels), were compared to CG gels prepared with un-activated heparin (CHG gels). Two heparin concentrations were investigated: 204.5 and 20.45 μg per mg collagen. The addition of pre-activated heparin did not compromise gel crosslinking or integrity, as assessed by light transmission, water uptake and collagenase digestion. PH gels released less of their heparin load compared to CHG gels. However, heparin-antithrombin (AT) interactions were reduced in the PH gels compared to CHG gels. CHG-204 gels sorbed 106.52 ± 7.83 and 125.32 ± 3.89 μg AT/cm^3 from phosphate buffered saline (PBS) and platelet poor plasma (PPP), respectively, while PH gels, regardless of heparin content, averaged 65.84 ± 16.61 and 61.01 ± 13.43 μg AT/cm^3 from PBS and PPP, respectively. Fibrinogen sorption was lower on CG gels than on thermal collagen (COLL) gels, but was not greatly influenced by heparinization. Reduced plasma clotting times were observed for heparinized gels compared to COLL and CG gels, Differences were not significant among the different PH gels. Of the materials investigated, the CHG-204 gels showed the longest plasma clotting times; indeed two of the three gels tested caused no observable clotting over the hour-long experiment. It is suggested that pre-activation of heparin with EDC/NHS resulted in tightly bound heparin that was not able to bind AT and inhibit thrombin. CHG-204 gels performed particularly well in these experiments and warrant further investigation for blood-contacting applications.

Keywords: collagen; dendrimers; heparin; EDC; NHS.

7.3 Introduction

Upon implantation of any foreign material into vascularized tissue, proteins adsorb non-specifically, typically leading to adhesion and activation of platelets and leukocytes, coagulation and complment activation [1]. Approaches to antithrombotic biomaterials include chemical modification, macromolecule immobilization, and the use of biomembrane-mimetic surfaces [2] to i) prevent nonspecific protein adsorption; ii) promote the adsorption of specific proteins that favour a desired biological response; iii) target molecules that inactivate activators of the coagulation or complement cascades, e.g. anticoagulants and platelet inhibitors [3]; iv) dissolve thrombi through activation of the fibrinolytic system [4].

Heparin, a potent anticoagulant, is a highly sulphated, linear polysaccharide that binds antithrombin (AT) to facilitate the inactivation of thrombin and other coagulation factors, including factor IXa, factor Xa, factor XIa, factor XIIa and kallikrein [5,6]. As heparin has a short circulatory half-life [7], stabilization methods are appealing to ensure long-term anticoagulation, while maintaining a therapeutic window and avoiding adverse side affects.

To reduce thrombosis on biomaterials, heparin has been used either in the surface bound state or as an agent to be released into the blood. Numerous synthetic and natural materials have been heparinized, including poly(ethylene-vinyl)-acetate [8,9,10], poly(ether-urethane) [11], cellulose [12], polypropylene [13], polyethylene (PE) [14], polylactic acid [15], and poly(dimethyl-siloxane) (PDMS) [16,17]. To introduce a non-fouling function in addition to reducing thrombosis, heparin has also been immobilized using poly(ethylene glycol) as a spacer, for example to PE [18], polystyrene [19], poly(ethylene-terephthalate) [20], PDMS [21], poly(vinyl chloride), poly(urethane) [22,23,24] and stainless steel [25]. Heparin has been used as a conjugate with albumin [26,27,28] for reduced cell adhesion, and with AT for improved anticoagulation properties [29,30,31]. Most of these heparinized materials have been shown to have promising antithrombotic characteristics *in vitro*, but less so *in vivo* or clinically (e.g. heparin-coated stents) [32,33].

We hypothesized that biomaterial induced coagulation is not only influenced by the surface, but also by the underlying material (unpublished observations), and that improved blood-biomaterial interactions may be achieved using naturally occurring biopolymers. Collagen, an abundant extracellular structural component, is a popular tissue engineering substrate [34] due to its ability to mediate cell adhesion [35], sequester growth factors and bind glycosaminoglycans [36]. Despite its thrombogenic properties due to platelet adhesion and activation [37,38], collagen in combination with heparin has been used as a biomaterial for decreased thrombogenesis since it has been shown that platelet activation may be reduced with heparin [39].

Type I collagen contains high and low affinity binding sites capable of binding heparin [40]. Heparin covalently immobilized to collagen via carbodiimide chemistry, showed increased thrombin inhibition, decreased contact activation, and decreased platelet adhesion, spreading and aggregation compared to unmodified thermal collagen gels, suggesting that the sites involved in heparin binding to the gel are also used in platelet binding [41]. Collagen bound to periodate oxidized heparin exhibited AT binding, decreased thrombin generation, and moderately reduced platelet activation, even though it supported platelet adhesion [42]. Layer-by-layer deposits of heparin and collagen on stainless steel had prolonged clotting times, and decreased platelet adhesion and activation; the layers also facilitated endothelialization [43].

We have previously developed dendrimer crosslinked collagen (CG) gels with enhanced stability compared to thermal collagen gels or collagen gels crosslinked with either N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) or glutaraldehyde [44,45]. These materials were further enhanced through covalent attachment of laminin-based adhesion peptides [46,47], epidermal growth factor (EGF), and heparin-binding EGF (HB-EGF) [48]. In addition, CG gels containing heparin were prepared for the controlled delivery of basic fibroblast growth factor (FGF-2) [49] and HB-EGF [50]. In the latter studies, heparin was passively added to (mixed with) the collagen suspension prior to crosslinking, resulting in materials with both immobilized and releasable heparin.

In the present study, we investigated covalent immobilization of heparin onto CG gels, by pre-activation of the heparin with EDC/NHS, with the expectation of increased heparin content (via tighter binding) and improved blood responses. With increased heparin content, it is hypothesized that gels should be antithrombotic due to increased uptake of AT and reduced non-specific protein adsorption (e.g. fibrinogen) relative to collagen gels without heparin or with lower heparin content.

7.4 Materials and Methods

7.4.1 Materials

Concentrated collagen suspensions of pepsin digested bovine cornium purified type I collagen, with less than 20% type III collagen, were a generous gift from Allergan Medical (Fremont, CA). Polypropyleneimine octaamine generation two dendrimer was purchased from SyMO-Chem (Eindhoven, Netherlands). Human antithrombin and human fibrinogen (plasminogen depleted) were purchased from Affinity Biologicals (Ancaster, ON) and Enzyme Research Laboratories (South Bend, IN), respectively. Unless otherwise stated, other reagents were purchased from Sigma-Aldrich (Oakville, ON) or EMD Chemicals Inc. (Gibbstown, NJ).

7.4.2 Collagen Gel Preparation

Dendrimer crosslinked pre-activated heparinized collagen (PH) gels were prepared based on a method adapted from previous work [49]. Briefly, the as-received collagen suspension (78.8 mg/mL), was diluted with phosphate buffered saline (PBS) and endotoxin free water (EFW), and acidified with 1 N hydrochloric acid (HCl). Pre-activated heparin (PH) solution was added to the collagen suspension (204.5 or 20.45 μg heparin per mg collagen). PH solutions consisted of heparin, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) in a molar ratio 1:1:1 ($heparin_{COOH}$:EDC:NHS). Crosslinking was achieved by adding second generation polypropyleneimine octaamine dendrimer (G2) (10:1, G2:collagen) to EDC and NHS in a molar ratio of 1:1:1 EDC:NHS:$collagen_{COOH}$. Where necessary, the pH of the gel suspension was adjusted to 5.5 with 1 N sodium hydroxide (NaOH) or 1 N HCl to facilitate the EDC/NHS reaction [51]. The resultant PH gel suspensions were

pressed between glass plates, on ice, and incubated overnight at 4°C. Gels were punched into 1/4" diameter discs, with a thickness of 1 mm, air dried, and stored at room temperature until needed. Where specified, rinsed gels were soaked for 24 h in EFW to remove any unbound or unreacted components prior to experiments.

PH gels were compared to heparinized dendrimer crosslinked collagen gels (CHG) where the heparin was not pre-activated with EDC/NHS and was added to the collagen suspension prior to dendrimer crosslinking. Gels were prepared with two heparin concentrations (204.5 and 20.45 μg heparin per mg collagen) denoted by CHG-204 and CHG-20, respectively. Other control gels included dendrimer crosslinked collagen (CG) gels prepared without heparin, and thermally crosslinked collagen (COLL) gels where collagen suspensions were brought to pH 7.4 and incubated at 37°C.

7.4.3 Light transmission

Light transmission measurements on rinsed hydrated gels were performed to assess whether pre-activated heparin affected collagen gel fibril assembly relative to un-activated heparin. Absorbance was measured in the visible range (410 – 630 nm) using a BioRad 550 plate reader.

7.4.4 Water uptake

Water uptake of previously rinsed gels was used to assess gel crosslinking. Following dry weight (W_d) measurements on air dried gels, gels were swollen in Milli-Q water for 24 h, wicked dry to remove surface moisture, and the wet weight (W_w) determined. The water uptake was calculated according to Eq. 1:

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

7.4.5 Collagenase Digestion

To further assess gel crosslinking and biostability, collagenase digestion was measured by quantification of hydroxyproline [52,53]. Rinsed collagen-based gels (1/4" discs) were immersed for 1 h in 500 μL 0.1 M Tris/HCl containing 0.05 M $CaCl_2$, then exposed to 200 U collagenase for 24 h at 37°C. The reaction was terminated by adding

200 μL 0.25M EDTA and samples were placed on ice. Following centrifugation (5 min, 5000 rpm) supernatants were analyzed for hydroxyproline content. Aliquots (100 μL) were autoclaved at 121°C for 20 min, then oxidized with 450 μL Chloramine T (0.056 M in 50% 2-propanol and acetate/citric acid buffer) for 25 min at room temperature and treated with 500 μL Ehrlich's reagent (1 M p-di-methyl-aminobenzaldehyde in 2-propanol/perchloric acid 2:1 v/v) for 20 min at 65°C. Absorbance was read at 540 nm and compared to hydroxyproline standards (2 – 200 μg/mL) and normalized to CHG relative degradation.

7.4.6 Heparin Gel Retention

Release of free heparin from the gels was examined to estimate the concentration of immobilized heparin. Hydrated collagen gels (not rinsed) were immersed in PBS (pH 7.4) and shaken in a waterbath at 37°C. Samples were taken periodically and stored at 4°C prior to determination of heparin concentration using an adapted version of the toluidine blue assay [54,55]. Briefly, in a 5 mL test tube, 0.75 mL of sample or standard heparin solution (0 – 2 μg/mL heparin in PBS) was mixed with 0.75 mL of 2% NaCl and 0.15 mL of toluidine blue solution (0.005% toluidine blue dye with 0.2% NaCl and 0.01 N HCl). Following addition of 1 mL n-hexane, the mixture was shaken for an additional 30 s. The solution was allowed to phase separate, after which the aqueous layer was removed and absorbance at 630 nm measured. Absorbance values were compared to those of standard heparin solutions and were normalized against CG gels that contained no heparin.

7.4.7 Antithrombin & Fibrinogen Sorption

Antithrombin (AT) and fibrinogen (Fg) were radiolabelled with $Na^{125}I$ for protein sorption experiments. AT was radiolabelled with $Na^{125}I$ via the Iodogen method (Iodination Reagent, Pierce Biotechnology, Rockford, IL) [56]. Briefly, AT (100 μg; 1 mg/mL) was combined with 5 μL of ^{125}I (500 μCi per 5 μL) in an Iodogen coated vial and stirred for 15 min at room temperature. The solution was subsequently dialyzed 3 times against PBS buffer over an 18 h period to remove unbound isotope. Fg (10 mg; 10 mg/mL) was radiolabelled using the iodine monochloride (ICl) technique [57]. Free

iodide was removed using an AG 1-X4 column with PBS buffer. Free unbound isotope was determined by trichloroacetic acid precipitation and was <2% in all studies.

Rinsed hydrated gels were exposed to ^{125}I labelled protein in PBS buffer (1% ^{125}I-AT or 2% ^{125}I-Fg in unlabeled protein solutions) or platelet poor plasma (PPP; 5% radioactive protein), with protein concentrations at approximate physiological concentrations (0.15 mg/mL AT; 2 mg/mL Fg). For PPP experiments, whole blood was collected from healthy volunteers (McMaster University ethics approval #04-046) into acid citrate dextrose and the platelets removed by centrifugation. Plasma samples were pooled, and the pool was aliquoted and stored at -70^{o}C until used.

Gels were incubated with radiolabeled AT or Fg solutions for 3 h, rinsed three times for 5 min in PBS buffer, and wicked dry prior to analysis. Radioactivity was determined using a Wallac Wizard 3" 1480 Automatic Gamma Counter (Perkin Elmer Life Sciences) and converted to quantity of protein by comparison with standards.

7.4.8 Plasma clotting times

The ability of heparinized gels to inhibit clotting was assessed by measuring the clotting times of recalcified citrated plasma in the presence of the gels, as described elsewhere [58,59]. Rinsed hydrated gels were incubated in 100 µL citrated PPP for 10 min at 37^{o}C in a 96 well-plate (non-tissue culture treated). Following addition of 100 µL 0.025M $CaCl_2$, 100 µL was transferred to a fresh well and absorbance read at 405 nm (340 ATTC plate reader, SOFT 2000 software, SLT Lab Instruments) at 30 s intervals over 1 h. From the absorbance-time curves the time at which absorbance began to increase ("clot initiation time") and the time at which the absorbance was halfway between the initial and final values ("time to half maximum") were determined.

7.4.9 Statistical Analysis

The data are presented as means ± standard deviation, unless otherwise stated. Statistical significance was determined with a single factor ANOVA test on two means, with $p<0.05$.

7.5 Results

7.5.1 Collagen Gel Preparation

Dendrimer crosslinked collagen gels (CG) were modified with heparin (CHG gels) or heparin pre-activated with EDC/NHS (PH gels). Table 7-1 summarizes the five sets of conditions used for adding pre-activated heparin to the PH gels, and preparation details for COLL, CG and CHG. Heparinized gels were prepared with 204 or 20.45 μg heparin per mg collagen.

7.5.2 Light Transmission

Transmittance data (calculated from absorbance in the range 405-700 nm) for PH gels and CHG gels are shown in Figure 7-1. Transmittance ranged from 13.20 ± 8.50 to 33.44 ± 15.10% for gels with 204 μg heparin per mg collagen, while gels with 20.45 μg heparin per mg collagen had transmittance in the range of 49.66 ± 6.52% to 75.23 ± 4.93%. Transmittance was higher at the lower heparin content ($p<10^{-4}$). PH-204 gels, except PH-5-204, had higher transmittance than CHG-204 gels ($p<0.05$), whereas the opposite was observed at the lower heparin concentration. Although transmittance varied among different PH preparation conditions, no clear trend was evident on the effect of the heparin incorporation method.

7.5.3 Water uptake

Water uptake (Figure 7-2) was determined gravimetrically for the dendrimer crosslinked gels. The average water uptake for the heparinized gels was 85.62 ± 3.70%. Water uptake of gels containing 204 μg heparin per mg collagen was was lower than that of gels containing 20.45 μg heparin per mg collagen, (83.66 ± 2.65% and 87.59 ± 3.71% respectively) regardless of the method of heparin addition ($p<0.05$). PH gels had lower uptake compared to their CHG counterparts ($p<10^{-5}$), except PH-5-20 gels, which had significantly higher water uptake (93.70 ± 0.09%; $p=10^{-10}$). Among the PH gels, PH-3 had the lowest water uptake (82.31 ± 0.69%), although differences were not always significant among PH gels.

7.5.4 Collagenase Digestion

Dendrimer crosslinked collagen-based gels were treated with collagenase for 24 h to assess biological stability based on hydroxyproline release. Gel stability data, expressed as relative denaturation (% of CHG), of PH gels and CHG gels, prepared with 204 or 20.45 μg heparin per mg collagen, are shown in Figure 7-3. Heparin content did not always significantly affect relative denaturation ($0.07<p<0.76$). CHG gels were digested to a greater extent than PH gels ($p<0.075$), except PH-1-20, PH-5-20 and PH-5-204 ($p=0.054$, 0.062, and 0.073, respectively). Differences among PH preparation methods were not significant. CHG-204 gels were ~20% more stable then COLL gels ($p=0.06$), and 15% less stable than CG gels ($p=0.47$) (data not shown).

7.5.5 Heparin Release

Gels were placed in 2 mL PBS buffer (pH 7.4) and the releasates periodically sampled and assayed for heparin content (toluidine blue assay) as a means of determining heparin retention following crosslinking. Data were normalized against CG gels to account for any leached materials that might affect the colorimetric assay. The percent and total amount of heparin released from PH and CHG gels, prepared with either 204.5 or 20.45 μg heparin per mg collagen, are shown in Figures 7-4a and 7-4b, respectively. Heparin release appeared to follow a first-order release profile, with much of the unbound heparin released within the first 48 h.

Although the gels with higher heparin content released greater quantities of heparin than those with lower heparin content, the percent release was lower for the higher heparin content gels. CHG-204 gels released a greater fraction of their heparin than PH-204 gels (~10% and <3%, respectively, ($p<0.005$) while CHG-20 and PH-20 gels released <1% and ~9%, respectively (not significant, $p<0.3$) (data not shown for 20 mg gels). PH-204 gels released more heparin than PH-20 gels: 175.30 ± 29.42 μg/cm^3 and 56.75 ± 21.08 μg/cm^3, respectively. For the PH gels, heparin release was generally not different for the different preparation methods.

7.5.6 Antithrombin (AT) & Fibrinogen (Fg) Sorption

To assess protein uptake, gels were exposed to solutions (PBS) of radiolabelled AT or Fg, and to plasma spiked with the labelled proteins. Figure 7-5a shows sorption of AT from PBS and Figure 7-5b shows uptake from plasma. CHG-204 gels sorbed greater quantities of AT than controls (COLL, $p<10^{-5}$; CG, $p<10^{-3}$). CHG-204 gels sorbed 106.52 ± 7.83 and 125.32 ± 3.89 μg/cm^3 from PBS and PPP, respectively. The PH-204 gels sorbed less AT than CHG-204 in PBS ($10^{-4}<p<10^{-3}$) and PPP ($10^{-5}<p<10^{-3}$). Differences in AT sorption between CHG-20 and PH-20 gels were not always significant, whether from buffer or plasma, or among the various PH preparation methods, with PH gels averaging AT uptake of 63.43 ± 14.90 μg/cm^3.

Figure 7-6a shows sorption of fibrinogen from PBS and Figure 7-6b shows uptake from plasma. Compared to CHG-204 gels, COLL gels sorbed significantly higher amounts of Fg ($p_{PBS}<10^{-3}$; $p_{PPP}<10^{-7}$) from buffer, while CG gels sorbed higher amounts from PBS ($p=10^{-6}$), but similar amounts from PPP (p=0.1). CHG-204 gels sorbed 112.24 ± 3.64 and 467.46 ± 10.30 μg/cm^3 Fg in PBS and PPP, respectively. Fg sorption was lower at the higher heparin content ($p<10^{-4}$) from PBS, but not from plasma. In both PBS and plasma, Fg sorption was not always different for CHG vs. PH gels, nor were trends evident among the PH gel preparation methods. Uptake for PH-204 gels averaged 126.19 ± 56.24 and 477.39 ± 60.49 μg/cm^3 from PBS and PPP, respectively, while PH-20 gels averaged 391.48 ± 127.07 and 465.45 ± 36.37 μg/cm^3.

7.5.7 Plasma clotting times

Plasma clotting data based on measuring change in absorbance over time in presence of the gels are shown in Figure 7-7. The times at initiation of clotting after re-calcification and the times to half maximum absorbance are summarized in Table 7-2.

The CHG gels with 204 μg heparin showed significantly reduced time to half maximum compared to the COLL (p=0.03) and CG gels (p=0.04), although clot initiation times were similar. Furthermore, of the three CHG-204 gels tested, only one showed any clot formation at all during the 60 min experiment. Among most of the heparinized gels, those having higher heparin content showed slightly higher (not significant) time to half

maximum (p_{CHG}=0.07; 0.06<p_{PH}<0.6) and delayed clot initiation (p_{CHG}=0.09; 0.08<p_{PH}<0.5), although this trend was not evident for the PH-3 gels. Compared to PH gels, CHG gels showed longer time to half maximum (0.04<p_{204}<0.2; 0.2<p_{20}<0.6) and clotting initiation times (0.08<p_{204}<0.2; 0.1<p_{20}<0.9), but the differences were not always significant. Time to half maximum and clot initiation times for the various PH preparation methods did not differ (0.08<$p_{PH\text{-}204}$<0.8; 0.4<$p_{PH\text{-}20}$<1).

7.6 Discussion

Heparin has been shown to mitigate the prothrombotic properties of collagen [42]. Thus, dendrimer crosslinked collagen (CG) gels were combined with heparin pre-activated with EDC/NHS to increase heparin content (via binding strength), while maintaining gel integrity, for enhanced antithrombotic properties.

The stability and integrity of pre-activated heparinized dendrimer crosslinked collagen (PH) gels was assessed via light transmission, water uptake, collagenase digestion and heparin release. Light transmission was used to investigate whether pre-activated heparin affects collagen fibril assembly to a greater extent than unactivated heparin. Heparinization of collagen gels has been shown to alter fibrillogenesis, resulting in a dose-dependent increase in opacity and increased fibril diameter and stability [49,60]. In the present work it was found, not unexpectedly, that gels with higher heparin content had lower light transmission. PH-204 gels had higher transmission than CHG-204, suggesting that pre-activation with EDC/NHS further affects collagen fibril assembly during gel crosslinking. Further investigation to confirm fibril diameter and orientation are warranted, as collagen fibril assembly can also affect hemostasis [61].

With the exception of PH-5-20, the PH gels showed tighter crosslinking compared to the CHG gels as evidenced by lower water uptake and collagenase digestion. The preparation method for PH-5 gels differed from those of the other PH gels in that pure dendrimer was added to the collagen suspension, followed by pre-activated heparin and then EDC/NHS. For the other PH gels solutions of dendrimer, EDC, and NHS were combined in a single operation (see Table 7-1). Furthermore the PH-5 gels were

crosslinked with only half the typical EDC/NHS concentration which may have resulted in decreased crosslinking, greater swelling and lower resistance to enzymatic attack.

As hypothesized, pre-activation of heparin prior to gel integration resulted in greater heparin retention within the gels. Thus PH-204 gels released significantly less heparin than CHG-204 gels (<3% and 10%, respectively); however, the difference was not as great at the lower heparin concentration. It has been reported elsewhere that when collagen and heparin solutions were reacted with EDC the gels released 10-14% over 4 h [62], while carbodiimide crosslinked collagen gels exposed to heparin, EDC and NHS, released 5-7% of the heparin load [41]. It has been suggested that heparin release greater than 40 ng/cm^2/min is required for maintenance of antithrombotic properties in a catheter exposed to blood at high flowrate [63]. However, we hypothesized that immobilized heparin could also promote reduced coagulation, provided it can interact with AT.

Adsorption of AT and Fg was measured in PBS to estimate adsorption capacities, and in PPP to investigate selectivity, in particular selectivity for AT as a measure of potential anticoagulant activity. Antithrombin (AT), an inhibitor of thrombin and other coagulation proteinases, is potentiated *in vivo* by heparin released from mast cells and heparan sulphate released from endothelial cells, through a common pentasaccharide sequence [64]. AT sorption studies were performed to verify heparin-AT binding following gel crosslinking; thermal collagen (COLL) and dendrimer crosslinked collagen (CG) gels, both prepared without heparin, served as controls. Little AT sorption was evident for COLL gels, while CG gels showed significant AT sorption, suggesting an interaction between AT and PPI dendrimers.

Significantly higher AT sorption was observed on CHG-204 gels compared to PH gels, suggesting decreased heparin-AT interactions in PH, possibly due to tight binding or a specific orientation of heparin within the gel matrix, rendering the pentasaccharide sequence necessary for heparin-AT binding inaccessible.

Fibrinogen adsorbed on blood-contacting surfaces causes platelet adhesion and activation via ligand receptor interactions [65]. Heparinized gels with 204 μg heparin per

mg collagen showed decreased Fg sorption in PBS compared to COLL and CG gels. The decrease was less for gels containing 20.45 μg heparin per mg collagen. COLL gels showed dramatically increased Fg sorption from PPP compared to all dendrimer crosslinked gels, in agreement with decreased Fg sorption on COLL gels compared to carbodiimide crosslinked gels observed elsewhere [66]. In PPP, differences in Fg sorption between CG, PH and CHG gels were not significant, suggesting that heparin (activated or not) did not affect Fg interactions. Whether the various crosslinking methods affect Fg orientation, and platelet aggregation and activation, remain to be investigated.

Plasma clotting in response to the gels was assessed in terms of time to clot initiation and time to half maximum absorbance. COLL gels showed the most rapid clot initiation, as expected based on the pro-thrombotic nature of collagen, followed by CG gels, and heparinized gels with heparin content of 20.45 μg per mg collagen. For PH-204 gels, clot initiation times were not different for all of the formulations, while CHG-204 gels showed significantly delayed clot initiation. Furthermore, of the three CHG-204 gels tested, only one showed any clot formation at all over the 60 min of the experiment, further suggesting that in CHG gels heparin-AT interactions are more efficient than in PH gels.

As has been reported elsewhere, collagen with bound periodate-oxidized heparin adsorbed ~1.36 μg/cm^2 AT and showed clot initiation times of 20-25 minutes [42], and stents coated with alternating layers of heparin and collagen (> 3 bilayers) showed clotting times greater than 1 h [43].

Heparin's anticoagulant activity has been shown to be hindered by EDC/NHS when used in excess [67]. Even though the molar ratio of EDC/NHS to heparin carboxylic acid groups (heparin$_{COOH}$) in the PH solutions was 1:1:1 (EDC:NHS:heparin$_{COOH}$), additional EDC/NHS was in dendrimer crosslinking solutions. The EDC:heparin$_{COOH}$ molar ratios in the PH gels were thereby increased to ~5:1 and 50:1, respectively for the 204 and 20.45 μg heparin per mg collagen gels. This may explain the low AT sorption and extended plasma recalification times for these gels, even though not all EDC added will bind to and activate heparin, as collagen and dendrimer may also react. Carbodiimide crosslinked

collagen gels for reaction with heparin were found to be optimized at EDC:heparin$_{COOH}$ ratios of 0.2-0.4 [41].

Despite the increase in heparin content among PH gels, its necessity may be unwarranted for blood-material interactions, especially when heparin interaction with AT is required. CHG-204 gels showed moderate heparin retention but substantial AT sorption and prolonged plasma clotting times; thus further investigation of CHG-204 gels is warranted.

7.7 Conclusions

Dendrimer crosslinked collagen (CG) gels were modified with heparin that was pre-activated with EDC/NHS to increase gel heparin content (via binding strength) (PH gels), and compared to heparinized gels, prepared with "passively added" heparin (CHG gels). The incorporation of pre-activated heparin did not compromise gel crosslinking or integrity, and >97% of the heparin was bound within the gel matrix. Heparinization of CG gels resulted in high AT sorption, with PH gels showing lower AT sorption than CHG gels, possibly due to different heparin binding mechanisms. Reduced plasma clotting times were observed for the heparinized gels compared to controls. The CHG-204 gels in particular demonstrated good *in vitro* anticoagulant properties and warrant further investigation for blood-contacting applications.

7.8 Acknowledgements

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7.9 References

1. Sefton MV, Gemmell CH and Gorbet MB. What really is blood compatibility? J Biomater Sci Polymer Edn 2000; 11: 1165.
2. Courtney JM, Lamba NMK, Gaylor JDS, Ryan CJ and Lowe GDO. Blood-contacting biomaterials: bioengineering viewpoints. Artificial Organs 1995; 19: 852.
3. Courtney JM and Forbes CD. Thrombosis on foreign surfaces. British Medical Bulletin 1994; 50: 966-981.
4. Brash JL. Exploiting the current paradigm of blood-material interactions for the rational design of blood-compatible materials. J Biomater Sci Polymer Edn 2000; 11: 1135.
5. Day JRS, Landis RC and Taylor KM. Heparin is much more than just an anticoagulant. Journal of Cardiothoracic and Vascular Anesthesia 2004; 18: 93-100.
6. Krishnaswamy A, Lincoff AM and Cannon CP. The use and limitations of unfractionated heparin. Crit Pathways in Cardiol 2010; 9: 35-40.
7. Weitz DS and Weitz JI. Update on heparin: what do we need to know? J Thromb Thrombolysis 2010; 29: 199-207.
8. Vasudev SC, Chandy T and Sharma CP. Development of chitosan/polyethylene vinyl acetate co-matrix: controlled release of aspirin-heparin preventing cardiovascular thrombosis. Biomaterials 1997; 18: 375-381.
9. Smith BAH and Sefton MV. Thrombin and albumin adsorption to PVA and heparin-PVA hydrogels I: Single protein isotherms. J Biomed Mater Res 1992; 26: 947-958.
10. Smith BAH and Sefton MV. Thrombin and albumin adsorption to PVA and heparin-PVA hydrogels 2. Competition and displacement. J Biomed Mater Res 1993; 27: 89-95.
11. Gutowska A, Bae YH, Jacobs H, Mohammad F, Mix D, Feijen J and Kim SW. Heparin release from thermosensitive polymer coatings: In vivo studies. J Biomed Mater Res 1995; 29: 811-821.
12. Hinrichs WLJ, ten Hoopen HWM, Wissink MJB, Engbers GHM and Feijen J. Design of a new type of coating for the controlled release of heparin. Journal of Controlled Release 1997; 45: 163-176.
13. Tyan YC, Liao JD, Wu YT and Klauser R. Anticoagulant activity of immobilized heparin on the polyprolyene nonwoven fabric surface depending upon the pH of processing environment. Journal of Biomaterials Applications 2002; 17; 153-178.
14. Cornelius RM, Sanchez J, Olsson P and Brash JL. Interactions of antithrombin and proteins in the plasma contact activation system with immobilized functional heparin. J Biomed Mater Res 2003; 67A: 475-483.

15. Jee KS, Park HD, Park KD, Kim YH and Shin JW. Heparin conjugated polylactide as a blood compatible material. Biomacromolecules 2004; 5: 1877-1881.

16. Olander B, Wirsen A and Albertsson AC. Silicone elastomer surface functionalized with primary amines and subsequent coupled with heparin. Biomacromolecules 2003; 4: 145-148.

17. Thorslund S, Sanchez J, Larsson R, Nikolajeff F and Bergquist J. Bioactive heparin immobilized onto microfluidic channels in poly(dimethylsiloxane) results in hydrophilic surface properties. Colloids and Surfaces B: Biointerfaces 2005; 46: 240-247.

18. Yuan S, Szakalas-Gratzl G, Ziats NP, Jacobsen DW, Kottke-Marchant K and Marchant RE. Immobilization of high-affinity heparin oligosaccharides to radiofrequency plasma-modified polyethylene. J Biomed Mater Res 1993; 27: 811-819.

19. Byun Y, Jacobs HA, Fiejen J and Kim SW. Effect of fibrinogen on the binding of antithrombin III to immobilized heparin. Journal of Biomedical Materials Research 1996; 30: 95-100.

20. Kim YJ, Kang IK, Huh MW and Yoon SC. Surface characterization and *in vitro* blood compatibility of poly(ethylene terephthalate) immobilized with insulin and/or heparin using plasma glow discharge. Biomaterials 2000; 21: 121-130.

21. Chen H, Chen Y, Sheardown H and Brook MA. Immobilization of heparin on a silicone surface through a heterobifuncitonal PEG spacer. Biomaterials 2005; 26: 7418-7424.

22. Wan M, Baek DK, Cho JH and Kang IK. In vitro blood compatibility of heparin-immobilized polyurethane containing ester groups in the side chain. Journal of Materials Science: Materials in Medicine 2004; 15: 1079-1087.

23. Zhou Z and Meyerhoff ME. Preparation and characterization of polymeric coatings with combined nitric oxide release and immobilized active heparin. Biomaterials 2005; 26: 6506-6517.

24. Wu B, Gerlitz B, Grinnel BW and Meyerhoff ME. Polymeric coatings that mimic the endothelium: combining nitric oxide release with surface-bound active thrombomodulin and heparin. Biomaterials 2007; 28: 4047-4055.

25. Chuang TW, Lin DT and Lin FH. Immobilization of NaIO4-treated heparin on PEG-modified 316L SS surface for high anti-thrombin-III binding. J Biomed Mater Res 2008; 86A; 648-661.

26. Engbers GHM, Dost L, Hennink WE, Aarts PAMM, Sixma JJ and Feijen J. An *in vitro* study of the adhesion of blood platelets onto vascular catheters: Part I. J Biomed Mater Res 1997; 21: 613-627.

27. van Delden CJ, Lens JP, Kooyman RPH, Engbers GHM and Feijen J. Heparinization of gas plasma-modified polystyrene surfaces and the interactions of these surfaces

with proteins studies with surface plasmon resonance. Biomaterials 1997; 18:845-852.

28. Bos GW, Scharenborg NM, Poot AA, Engbers GHM, Beugeling T, van Aken WG and Feijen J. Blood compatibility of surfaces with immobilized albumin-heparin conjugate and effect of endothelial cell seeding on platelent adhesion. J Biomed Mater Res 1999; 47: 279-291.
29. Klement P, Du YJ, Berry L, Andrew M and Chan AK. Blood-compatible biomaterials by surface coating with a novel antithrombin-heparin covalent complex. Biomaterials 2002; 23: 527-535.
30. Du YJ, Brash JL, McClung G, Berry LR, Klement P and Chan AK. Protein adsorption on polyurethane catheters modified with a novel antithrombin-heparin covalent complex. J Biomed Mat Res A 2007; 80: 216-225.
31. Sask KN, Zhitomirsky I, Berry LR, Chan AK and Brash JL. Surface modification with an anti-thrombin-heparin complex for anticoagulation: studies on a model surface with gold as substrate. Acta Biomater 2010; 6: 2911-2919.
32. Haude M, Konorza TF, Kalnins U, Erglis A, Saunamaki K, Glogar HD, Grube E, Gil R, Serra A, Richardt HG, Sick P and Erbel R. Heparin-coated stent placement for the treatment of stenoses in small coronary arteries of symptomatic patients. Circulation 2003; 107: 1265-1270.
33. Jordan SW and Chaikof EL. Novel thromboresistant materials. J Vasc Surg 2007; 45: 104A-115A.
34. Friess W. Collagen: biomaterial for drug delivery. European Journal of Pharmaceutics and Biopharmaceutics 2001; 45: 113-136.
35. Pang Y and Greisler HP. Using a type I collagen-based system to understand cell-scaffold interactions and to delivery chimeric collagen-binding growth factors for vascular tissue engineering. Journal of Investigative Medicine 2010; 58: 845-848.
36. Salchert K, Streller U, Pompe T, Herold N, Grimmer N and Werner C. In vitro reconstitution of fibrillar collagen type I assemblies at reactive polymer surfaces. Biomacromolecules 2004; 5: 1340-1350.
37. Parsons TJ, Haycraft DL, Hoak JC and Sage H. Diminished platelet adherence to type V collagen. Arterioscler Thromb Vasc Biol 1983; 3: 589-598.
38. Heemskerk JWM, Kuijpers MJE, Munnix ICA and Siljander PRM. Platelet collagen receptors and coagulation: A characteristic platelet response as possible target for antithrombotic treatment. Trends Cardiovasc Med 2005; 15: 86-92.
39. Kauhanen P, Kovanen PT and Lassila R. Coimmobilized native macromolecular heparin proteoglycans strongly inhibit platelet-collagen interactions in flowing blood. Arterioscler Thromb Vasc Biol 2000; 20: e113-e119.

40. Keller KM, Keller JM and Kuhn K. The C-terminus of type I collagen is a major binding site for heparin. Biochimica et Biophysica Acta: General Subjects 1986; 882: 1-5.

41. Wissink MJB, Beernink R, Pieper JS, Poot AA, Engbers GHM, Beugeling T, van Aken WG and Feijen J. Immobilization of heparin to EDC/NHS-crosslinked collagen: Characterization and *in vitro* evaluation. Biomaterials 2001; 22: 151-163.

42. Keuren JFW, Wielders SJH, Driessen A, Verhoeven M, Hendriks M and Lindhout T. Covalently-bound heparin makes collagen thromboresistant. Arterioscler Thromb Vasc Biol 2004; 24: 613-617.

43. Lin Q, Yan J, Qiu F, Song X, Fu G and Ji J. Heparin/collagen multilayer as a thromboresistant and endothelial coating for intravascular stent. J Biomed Mater Res A 2010; 96A: 132-141.

44. Duan X and Sheardown H. Crosslinking of collagen with dendrimers. J Biomed Mater Res (2005) 75A: 510-518.

45. Duan X and Sheardown H. Dendrimer crosslinked collagen as a corneal tissue engineering scaffold: Mechanical properties and corneal epithelial cell interactions. Biomaterials (2006) 27: 4608-4617.

46. Duan X and Sheardown H. Incorporation of cell-adhesion peptides into collagen scaffolds promotes corneal epithelial stratification. J Biomater Sci Polymer Edn 2007; 18: 701-711.

47. Duan X, McLaughlin C, Griffith M and Sheardown H. Biofunctionalization of collagen for improved biological response: Scaffolds for corneal tissue engineering. Biomaterials 2007; 28: 78-88.

48. Princz MA and Sheardown H. Tethering of Growth Factors to Dendrimer Crosslinked Collagen Gels. In Preparation 2011.

49. Princz MA and Sheardown H. Heparin-modified dendrimer crosslinking collagen matrices for the delivery of basic fibroblast growth factor (FGF-2). J Biomater Sci Polym Edn 2008; 19: 1201-18.

50. Princz MA and Sheardown H. Heparin Modified Dendrimer Crosslinked Collagen Matrices for the Delivery of Heparin-Binding Epidermal Growth Factor (HB-EGF) J Biomed Mat Res; Submitted 2011.

51. Grabarek Z and Gergely J. Analytical Biochemistry 1990; 185: 131.

52. Stegemann H and Stalder K. Determination of hydroxyproline. Clin Chim Acta 1967; 18: 267.

53. Reddy GK and Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem 1996; 29: 225.

54. MacIntosh FC. A colorimetric method for the standardization of heparin preparations. Biochem J 1941; 35: 776-782.

55. Smith PK, Mallia AK and Hermanson GT. Colorimetric method for the assay of heparin content in immobilized heparin preparations. Analytical Biochemistry 1980; 109: 466-473.

56. Salacinski PRP, McLean C, Sykes JEC, Clement-Jones VV and Lowry PJ. Iodination of proteins, glycoproteins, and peptides usinga solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3a,6a-diphenyl glycoluril (iodogen). Analytical Biochemistry 1981; 117: 136-146.

57. Wagner MS, Horbett TA and Castner DG. Characterizating multicomponent adsorbed protein films using electron spectroscopy for chemical analysis, time-of-flight secondary ion mass spectrometry, and radiolabeling: capabilities and limitations. Biomaterials 2003; 24: 1897-1908.

58. Sask KN, McClung GW, Berry LR, Chan AKC and Brash JL. Immobilization of an antithrombin-heparin complex on gold: Anticoagulant properties and platelet interactions. Acta Biomaterials 2011; 7: 2029-2034.

59. McGuigan AP and Sefton MV. The thrombogenicity of human umbilical vein endothelial cell seeded collagen modules. Biomaterials 2008; 29: 2453-2463.

60. McPherson JM. The effects of heparin on the physiochemical properties of reconstituted collagen. Coll Relat Res 1988; 1: 65-82.

61. Orgel JP, Antipova O, Sagi I, Bitler A, Qiu D, Wang R, Xu Y and San Antonio JD. Collagen fibril surface displays a constellation of sites capable of promoting fibril assembly, stability and hemostasis. Connect Tissue Res 2011; 52: 18-24.

62. Raghunath K, Biswas G, Panduranga Rao K, Joseph KT and Chvapil M. Some characteristics of heparin-collagen complex. Journal of Biomedical Materials Research 1983; 17: 613-621.

63. Basmadjian D and Sefton MV. Relationship between release rate and surface concentration for heparinized materials. J Biomed Mater Res 1983; 17: 509-518.

64. Olson ST, Richard B, Izaguirre G, Schedin-Weiss S and Gettins PGW. Molecular mechanisms of antithrombin-heparin regulation of blood clotting proteinases: A paradigm for understanding proteinase regulation by serpin family protein proteinase inhibitors. Biochimie 2010; 92: 1587-1596.

65. Horbett TA. Chapter 13: Principles underlying the role of adsorbed plasma proteins in blood interactions with foreign materials. Cardiovasc Pathol 1993; 2 Suppl: 137S-148S.

66. Nam K, Kimura T and Kishida A. Physical and biological properties of collagen-phospholipid polymer hybrid gels. Biomaterials 2007; 28: 3153-3162.

67. Oliviera GB, Carvalho LB Jr and Silva MPC. Properties of carbodiimide treated heparin. Biomaterials 2003; 24: 4777-4783.

7.10 Figures

Figure 7-1. Light transmission of pre-activated heparinized dendrimer crosslinked collagen (PH) gels, containing either 204.5 or 20.45 μg heparin per mg collagen, compared to heparinized dendrimer crosslinked collagen (CHG) gels where the heparin was not pre-activated with EDC/NHS. Transmittance was higher at the lower heparin content ($p<10^{-4}$). For gels with pre-activated heparin, improved transmittance was observed at high heparin concentration (PH-204 gels) ($p<0.05$). Although some variation in transmittance was evident among the various preparation methodologies, no clear trend is evident among the PH gels.

Figure 7-2. Water uptake (%) of heparinized dendrimer crosslinked collagen gels, prepared with pre-activated heparin (PH) or unactivated heparin (CHG), at concentrations of 204.5 or 20.45 μg heparin per mg collagen. Higher heparin content gels swelled to a lesser extent than gels with lower heparin content ($p<0.05$). All PH gels had lower swelling compared to their CHG gel counterpart ($p<10^{-5}$), except PH-5-20 gels, which were significantly more swelled ($p=10^{-10}$). Among the PH preparation methods, PH-3 had the least amount of water uptake, although swelling differences were not always significant between PH gels.

Figure 7-3. Relative degradation (%), following collagenase digestion measured by hydroxyproline release, of pre-activated heparinized dendrimer crosslinked collagen (PH) and unactivated heparinized dendrimer crosslinked collagen (CHG) gels, containing either 204.5 or 20.45 μg heparin per mg collagen. Denaturation was not significantly affected by heparin content ($0.07<p<0.76$). PH gels demonstrated less degradation than CHG gels, but the differences were not always significant. There was no denaturation difference among PH preparation methods ($p_{PH-204}=0.26$ and $p_{PH-20}=0.40$).

Figure 7-4. Heparin stability within pre-activated heparinized dendrimer crosslinked collagen (PH) and heparinized dendrimer crosslinked collagen (CHG) gels, containing either 204.5 or 20.45 μg heparin per mg collagen, shown as (a) percent heparin released, and (b) total heparin released ($\mu g/cm^3$). Data were normalized against unheparinized dendrimer crosslinked collagen (CG) gels. PH gels retained more heparin than CHG gels. Heparin stability within PH gels did not differ significantly ($p>0.05$) according to PH preparation method.

Figure 7-5. Sorption of AT ($\mu g/cm^3$) from (a) PBS, (b) platelet poor plasma (PPP), into pre-activated heparinized dendrimer crosslinked collagen (PH) gels and unactivated heparin dendrimer crosslinked collagen (CHG) gels, containing either 204.5 or 20.45 μg heparin per mg collagen, compared to thermal collagen (COLL) and dendrimer crosslinked collagen (CG) gels, prepared without heparin. CHG gels sorbed higher amounts of AT, compared to COLL ($p<10^{-5}$) and CG ($p<10^{-3}$) gels. PH-204 gels sorbed less AT, compared to CHG-204 gels ($10^{-4}<p_{PBS}<10^{-3}$), although differences were not as pronounced in PPP ($10^{-5}<p_{PPP}<10^{-3}$). Differences in AT sorption were not distinct between CHG-20 and PH-20 gels, or among the various PH preparation methods.

Figure 7-6. Sorption of Fg (μg/cm^3), from (a) PBS, (b) platelet poor plasma (PPP), into pre-activated heparinized dendrimer crosslinked collagen (PH) gels and unactivated heparin dendrimer crosslinked collagen (CHG) gels, containing either 204.5 or 20.45 μg heparin per mg collagen, compared to thermal collagen (COLL) and dendrimer crosslinked collagen (CG) gels, prepared without heparin. COLL gels sorbed high amounts of Fg, compared to CHG-204 gels ($p_{PBS}<10^{-3}$; $p_{PPP}<10^{-7}$), while heparinization of CG gels only affected Fg sorption in PBS. Decreased Fg sorption was observed by increasing heparin content ($p<10^{-4}$), but this trend was not evident in PPP. Fg sorption did not consistently vary between CHG and PH gels, nor were visible sorption trends evident among PH gels.

Figure 7-7. Plasma recalification of pre-activated heparinized dendrimer crosslinked collagen (PH) gels and unactivated heparinized dendrimer crosslinked collagen (CHG) gels, containing either (a) 204.5 or (b) 20.45 μg heparin per mg collagen, shown along with thermal collagen (COLL) and dendrimer crosslinked collagen (CG) gels, prepared without heparin (n=3). Heparinization reduced the times to half maximum and clot initiation times, with CHG-204 gels having the longest times, although differences were not always significant, as only one CHG-204 gel formed a clot over the examined time period.

Figure 7-1.

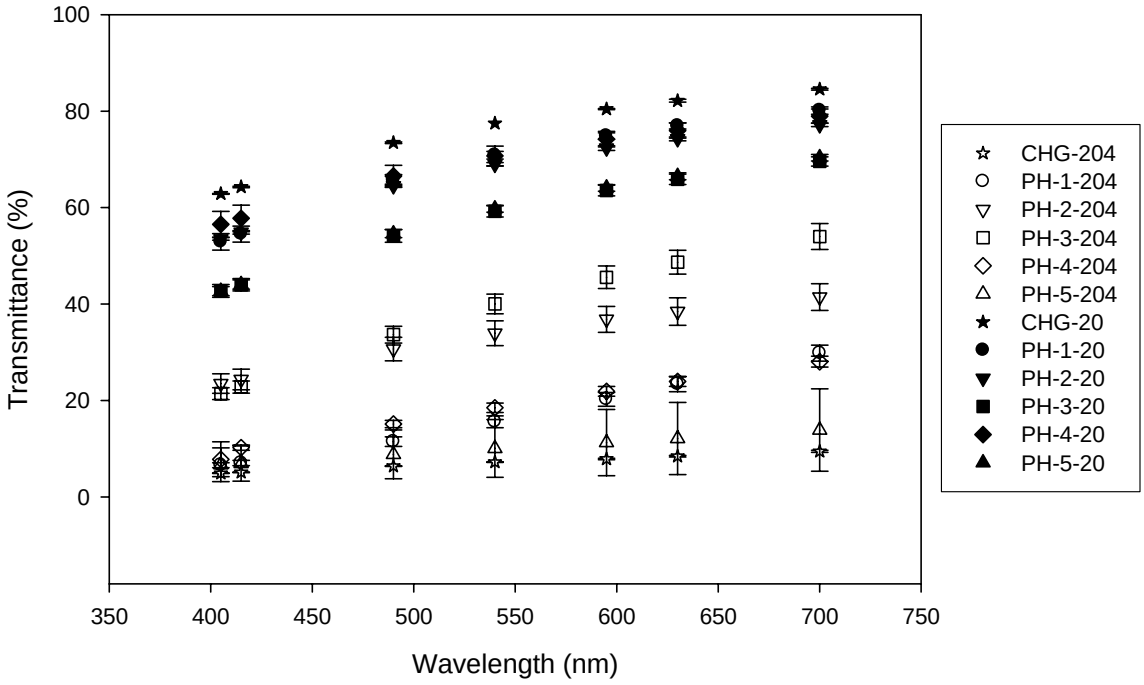

Transmittance (%)
100
80
60
40
20
0
350
400
450
500
550
600
650
700
750
Wavelength (nm)
CHG-204
PH-1-204
PH-2-204
PH-3-204
PH-4-204
PH-5-204
CHG-20
PH-1-20
PH-2-20
PH-3-20
PH-4-20
PH-5-20

Figure 7-2.

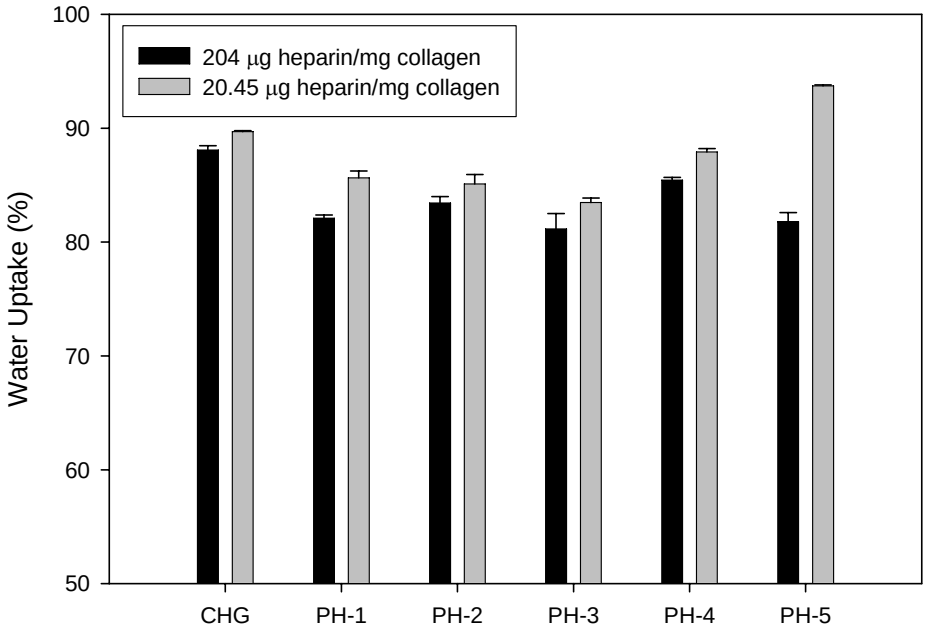

100
90
80
70
60
50
Water Uptake (%)
204 μg heparin/mg collagen
20.45 μg heparin/mg collagen
CHG
PH-1
PH-2
PH-3
PH-4
PH-5

Figure 7-3.

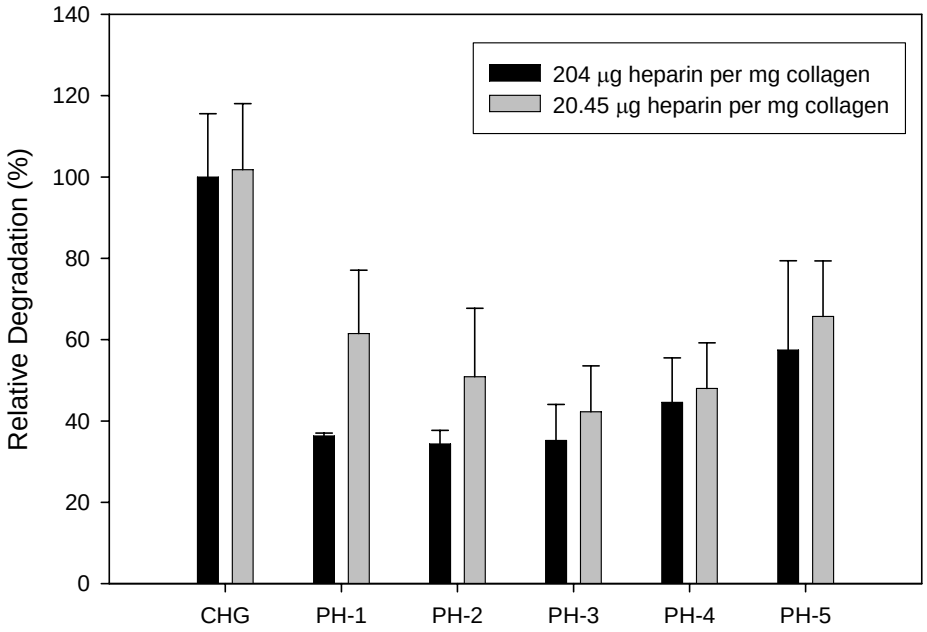

140
120
100
80
60
40
20
0
Relative Degradation (%)
204 μg heparin per mg collagen
20.45 μg heparin per mg collagen
CHG
PH-1
PH-2
PH-3
PH-4
PH-5

Figure 7-4a.

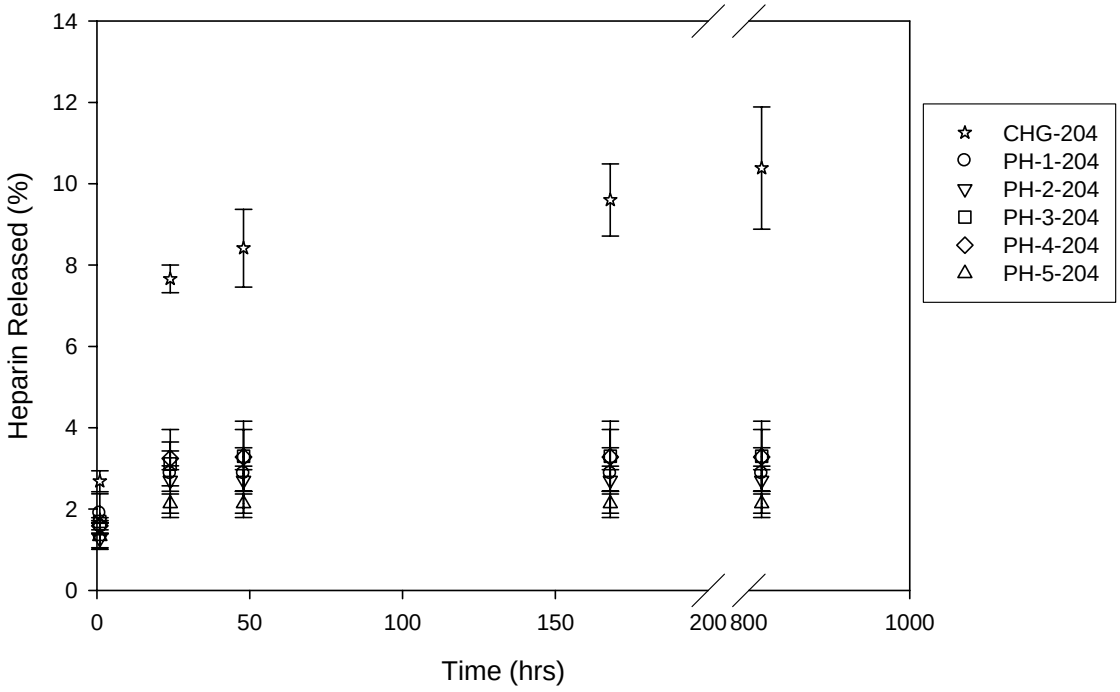

Heparin Released (%)
Time (hrs)
0
2
4
6
8
10
12
14
0
50
100
150
200
800
1000
CHG-204
PH-1-204
PH-2-204
PH-3-204
PH-4-204
PH-5-204

Figure 7-4b.

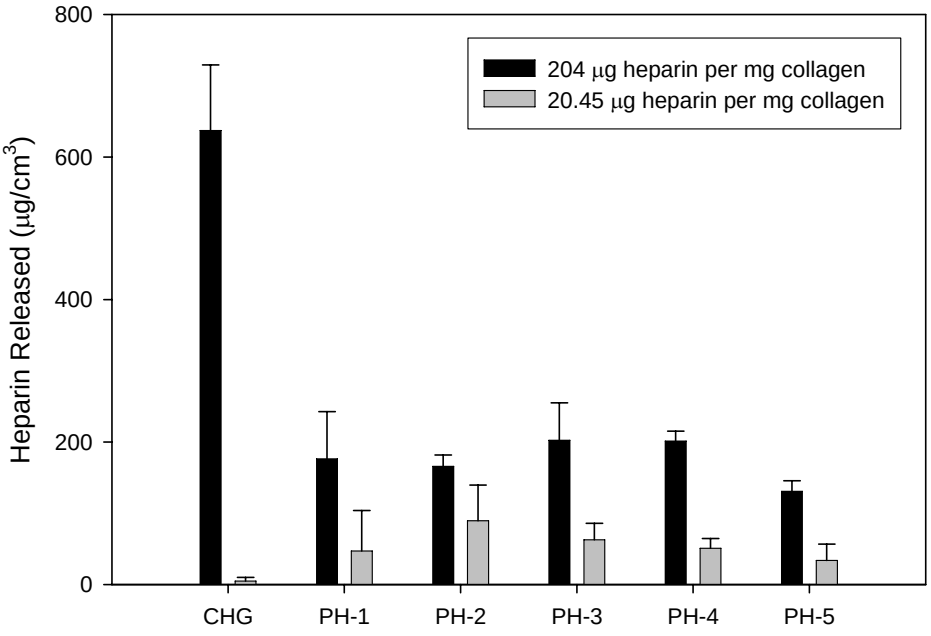

800
600
400
200
0
Heparin Released (μg/cm3)
204 μg heparin per mg collagen
20.45 μg heparin per mg collagen
CHG
PH-1
PH-2
PH-3
PH-4
PH-5

Figure 7-5a.

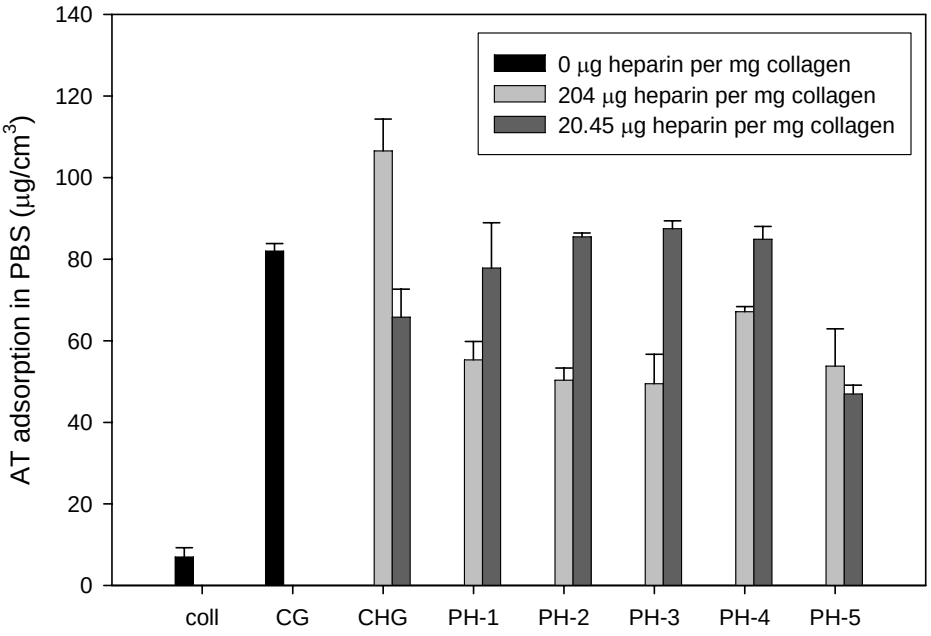

AT adsorption in PBS (μg/cm3)
0 μg heparin per mg collagen
204 μg heparin per mg collagen
20.45 μg heparin per mg collagen
0
20
40
60
80
100
120
140
coll
CG
CHG
PH-1
PH-2
PH-3
PH-4
PH-5

Figure 7-5b.

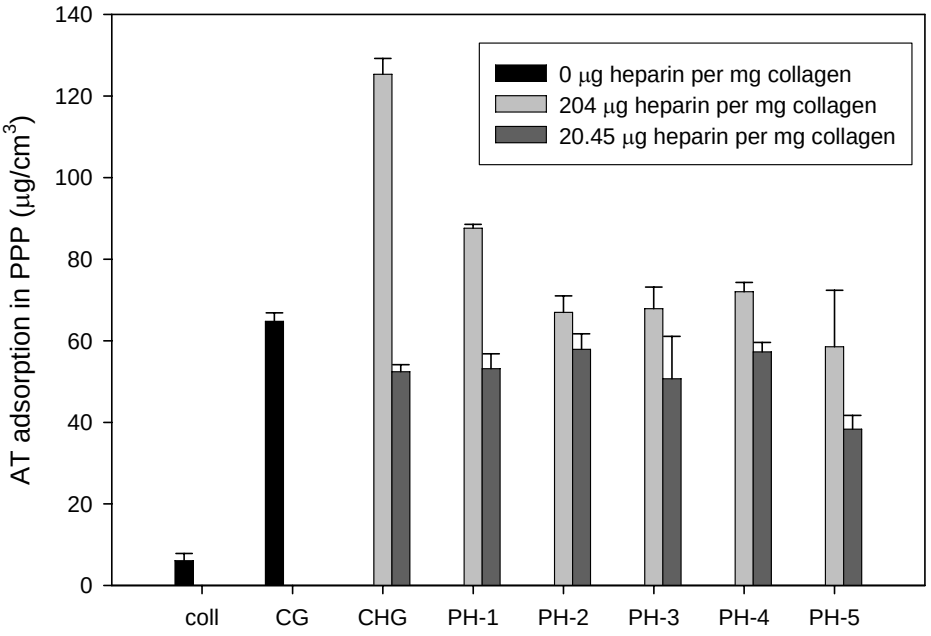

AT adsorption in PPP (μg/cm3)
0 μg heparin per mg collagen
204 μg heparin per mg collagen
20.45 μg heparin per mg collagen
0
20
40
60
80
100
120
140
coll
CG
CHG
PH-1
PH-2
PH-3
PH-4
PH-5

Figure 7-6a.

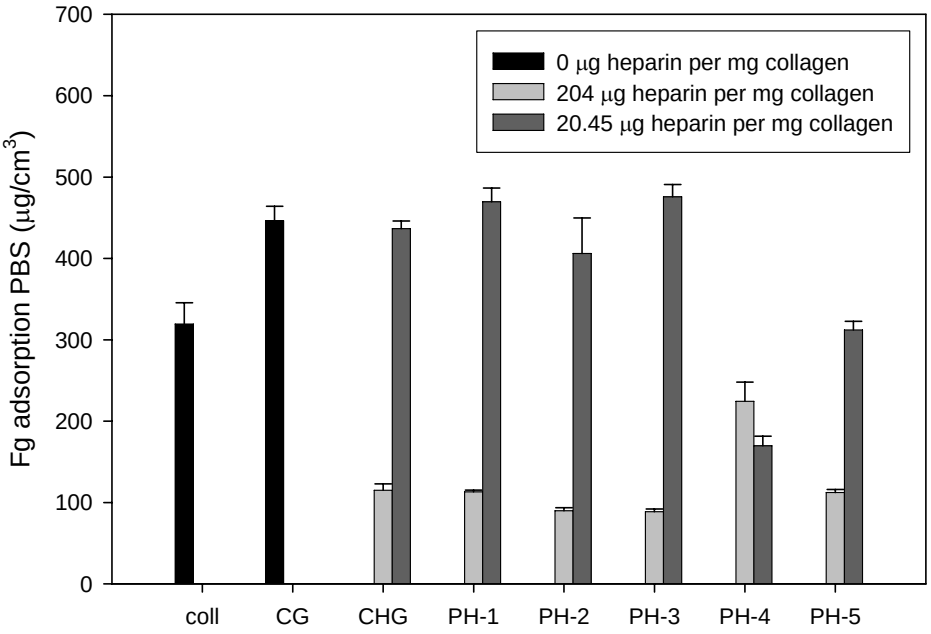

700
600
500
400
300
200
100
0
Fg adsorption PBS (μg/cm3)
0 μg heparin per mg collagen
204 μg heparin per mg collagen
20.45 μg heparin per mg collagen
coll
CG
CHG
PH-1
PH-2
PH-3
PH-4
PH-5

Figure 7-6b.

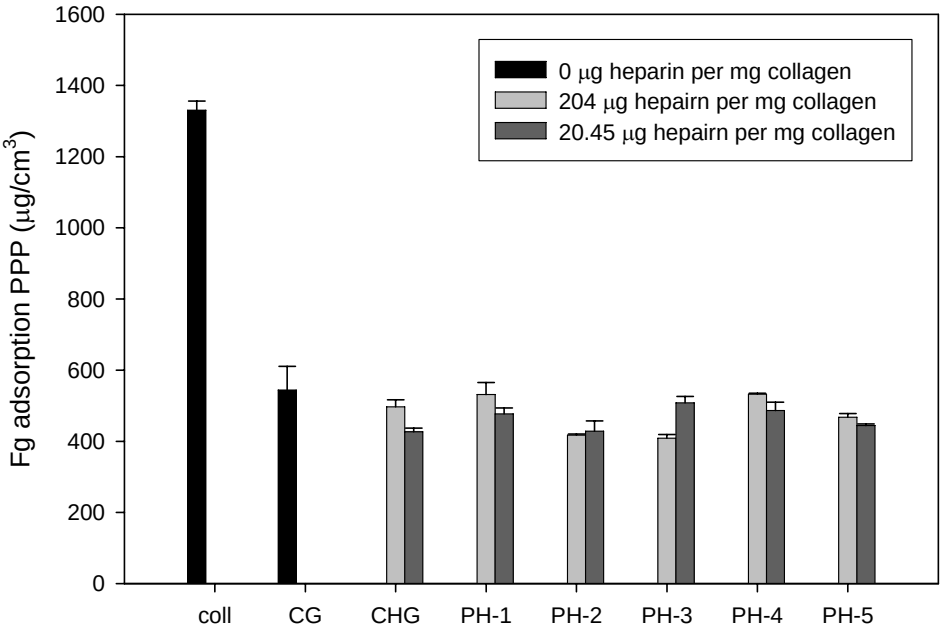

1600
1400
1200
1000
800
600
400
200
0
Fg adsorption PPP (μg/cm3)
0 μg heparin per mg collagen
204 μg hepairn per mg collagen
20.45 μg hepairn per mg collagen
coll
CG
CHG
PH-1
PH-2
PH-3
PH-4
PH-5

Figure 7-7a.

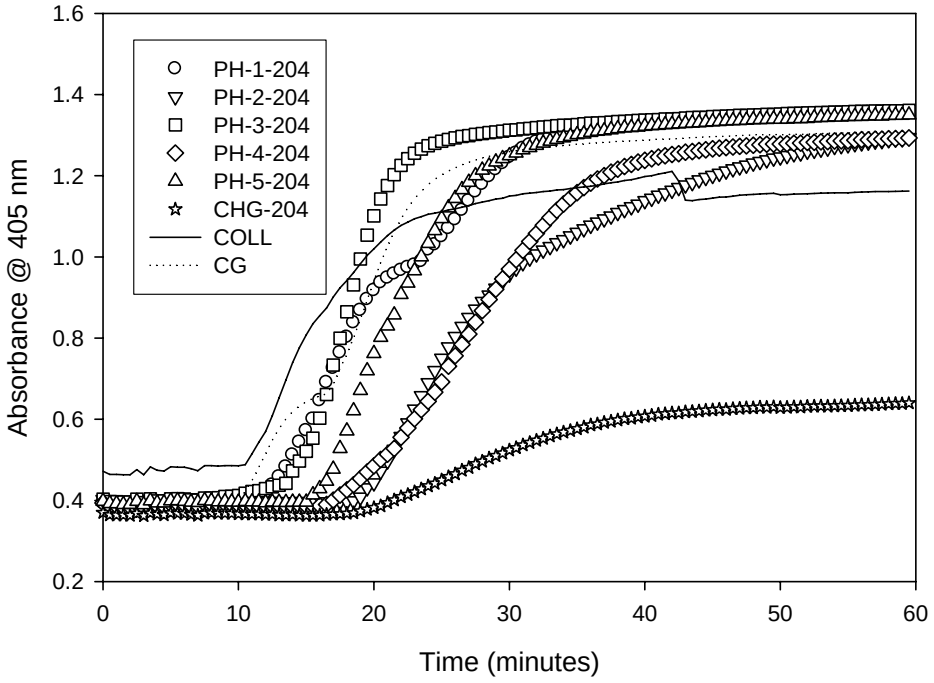
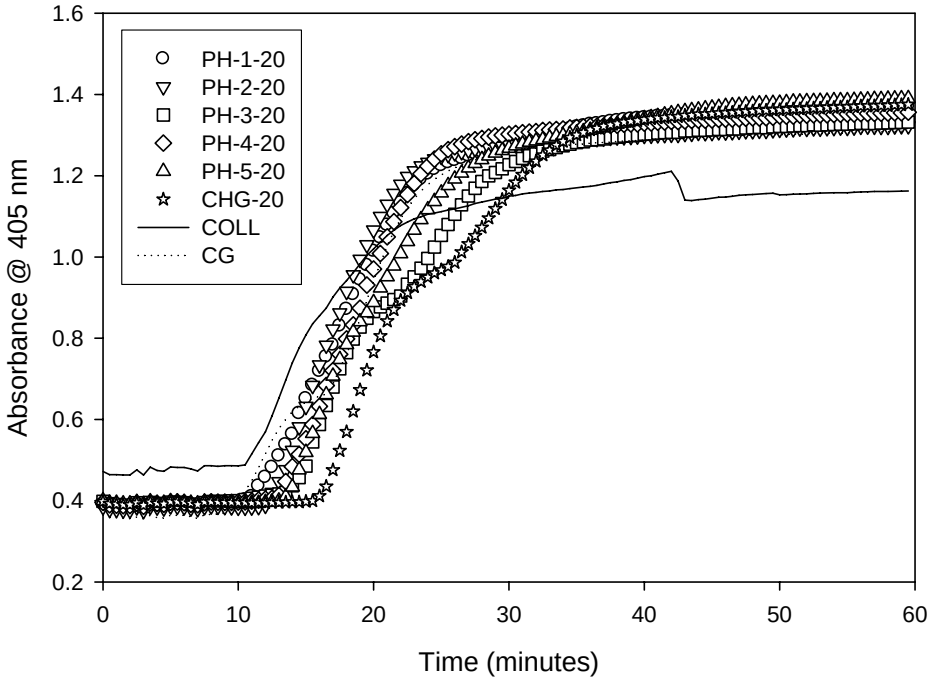

Absorbance @ 405 nm
Time (minutes)
PH-1-204
PH-2-204
PH-3-204
PH-4-204
PH-5-204
CHG-204
COLL
CG
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60

Figure 7-7b.


Absorbance @ 405 nm
Time (minutes)
PH-1-20
PH-2-20
PH-3-20
PH-4-20
PH-5-20
CHG-20
COLL
CG
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60

7.11 Tables

Table 7-1. Dendrimer crosslinked collagen gels containing pre-activated heparin (PH) and control gel formulations.

Pre-Activated Heparinized Gels:		
Gel ID	**Crosslinking Methodology**	
PH-1	(collagen suspension + 200 µL PH) + 200 µL $G2^t$ solution	[C + PH] + G2
PH-2	(200 µL PH + 200 µL $G2^t$) + collagen suspension	[PH + G2] + C
PH-3	(200 µL PH + 100 µL $G2^t$) + 100 µL $G2^t$ + collagen suspension	[PH + ½G2] + C + ½G2
PH-4	(collagen suspension + 100 µL PH) + (100 µL PH + 100 µL $G2^t$) + 100 µL $G2^t$	[C + ½PH] + [½PH + ½G2] + ½G2
PH-5[a]	(collagen suspension + 200 µL $G2^*$) + 200 µL PH + 100 µL EDC /NHS	[C + G2*] + PH + ½EDC
Control Gels:		
COLL	Collagen suspension (thermally crosslinked)	[C]
CG	Collagen suspension + 200 µL $G2^t$	[C + G2]
CHG	Collagen suspension + 450 µL Hep + 200 µL $G2^t$	[C + Hep] + G2

$G2^t$ = (75 mg EDC + 45 mg NHS + 38 mg dendrimer) in 1 mL EFW
G2* = 38 mg/mL dendrimer in EFW
Hep = 30 mg/mL heparin in EFW
PH => for 204 µg heparin per mg collagen: (67.5 mg heparin + 15 mg EDC + 9 mg NHS) in 1 mL EFW
=> for 20 µg heparin per mg collagen: (6.8 mg heparin + 1.5 mg EDC + 0.9 mg NHS) in 1 mL EFW
EDC = (75 mg EDC + 43 mg NHS) in 1 mL EFW
[a] = only half the EDC/NHS crosslinker amount, compared to other chemically crosslinked gels

Table 7-2. Plasma Recalcification Times (n=3).

	Time to half maximum (min)			Time to clot initiation (min)		
	Heparin content (µg heparin per mg collagen)					
	0	**204.5**	**20.45**	**0**	**204.5**	**20.45**
Plasma	38.53±17.7			23.50±13.1		
COLL	16.20±4.68			12.83±3.21		
CG	17.54±4.80			14.33±4.19		
CHG		28.13[a]	23.03±5.60		6.33±11.0[a]	16.50±1.00
PH-1		20.25±6.64	17.48±2.71		16.50±6.56	13.17±3.40
PH-2		29.70±9.20	17.28±2.97		22.17±5.51	14.17±2.57
PH-3		17.97±2.01	20.15±4.86		15.17±1.53	15.83±5.39
PH-4		26.53±4.16	18.50±3.44		21.00±4.27	15.50±3.00
PH-5		21.17±2.95	19.39±3.55		17.67±3.40	15.50±3.46

[a]Only one of the three CHG-204 samples formed a clot.

8 CONCLUSIONS AND FUTURE WORK

In this thesis, dendrimer crosslinked collagen gels were explored for tissue engineering applications. Collagen crosslinking was achieved using polypropyleneimine octaamine generation two dendrimers (G2) with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS).

The efficacy of the dendrimer crosslinking technique was demonstrated with three commercially available collagen-based products, which varied according to collagen purity and source (Paper 1). Given the looser networks of collagen gels fabricated from highly purified and acidified collagen solutions, we hypothesized that the commercially available collagen-based materials had compromised stability stemming from manufacturer purification methods and acidic storage solutions. In addition, to obtain the 3% gel concentrations, the commercially available collagen-based products were freeze-dried prior to gel fabrication, which may have further damaged collagen fibrils. Thus, we recommend that collagen-based materials intended for high tensile tissue engineering applications would benefit from long-term refrigerated storage in concentrated amounts, without exposure to acidic solutions.

These commercially available collagen-based materials may have suitable cell compatability for certain tissue engineering applications, such as degradable cell delivery vehicles, as 3T3 fibroblast cell growth was supported over 4 days. However, the commercially-available collagen-based products did not induce cell proliferation to the same extent as gels fabricated from less purified collagen, presumably due to the presence of cell stimulating factors. Therefore the addition of cell binding motifs into these commercially collagen-based materials may lead to enhanced cell-matrix interactions and may be useful for controlling cell interactions.

Further evaluation of the potential of dendrimer crosslinked collagen gels was performed using a novel growth factor system (Paper 2), whereby heparinized dendrimer crosslinked collagen (CHG) gels were utilized for delivery and release of heparin-binding epidermal growth factor (HB-EGF) for increasing human cornea epithelial cell (HCEC) proliferation, to ultimately aid in the epithelialization of tissue engineered corneal

equivalents (TECE) through enhanced device-host integration and retention. The potential of HB-EGF for mediating HCEC proliferation was verified, and shown to be dependent on growth factor concentration and cell seeding density. Thus, HB-EGF may be a potent mitogen for corneal tissue engineering via epithelial cell manipulation. Uptake of HB-EGF in CHG gels was influenced by the heparin concentration within the gel, growth factor concentration and exposure time to the growth factor, while HB-EGF delivery from CHG gels was prolonged and sustained, and the bioactivity of the growth factor was maintained, likely via heparin-growth factor interactions.

As HB-EGF induced proliferation of HCEC *in vitro* and can be released from heparinized collagen gels, it may be suitable for promoting epithelialization of TECE. Thus it is recommended that further work with CHG gels delivering HB-EGF be explored *in vitro* to evaluate epithelial stratification, migration and proliferation on a larger time scale. *In vivo* experimentation could also investigate CHG implantation, inflammation and corneal tissue regeneration. Furthermore, it may be advantageous to combine basic fibroblast growth factor and HB-EGF delivery for enhanced TECE performance through epithelialization and stromal anchorage.

To investigate other avenues of enhanced growth factor bioavailability and bioactivity, different methods of covalent attachment of HB-EGF and EGF, via carbodiimide chemistry, into dendrimer crosslinked collagen (CG) gels were examined and the effect on HCEC proliferation was examined (Paper 3). Growth factors were either tethered in a step-wise manner to CG gels with EDC and NHS, or via bulk modification whereby growth factor solutions, either combined with EDC, NHS or dendrimer, were incorporated into the gel matrix prior to full gel crosslinking with dendrimers.

Following step-wise tethering of HB-EGF or EGF within CG gels, HCEC proliferation was significantly increased *in vitro*, even though the likelihood of heterogeneous growth factor orientation could have interfered with growth factor receptor binding. However, HCEC proliferation on CG gels with GF added during fabrication via bulk modification, regardless of the GF incorporation type, was not augmented. Thus, it is likely that our acidic crosslinking conditions during bulk fabrication interfered with GF

stability, orientation and activity. Furthermore, activation of GF with EDC/NHS could have resulted in interference of amino acid groups active in GF cell receptor binding. As such, it can be concluded that step-wise tethering of EGF and HB-EGF to CG gels is favourable, compared to bulk tethering, for HCEC proliferation. Furthermore, step-wise activation of collagen with EDC/NHS prior to growth factor addition may facilitate amine reaction of the growth factor, and result in lower interference with receptor binding domains. Also, immobilization or molecular spacer chemistries that target a specific growth factor orientation to maintain receptor binding domains may be advantageous. Alternatively, protein engineering mechanisms could be used to produce growth factors with covalent attachment binding motifs.

Finally, since heparin is an effective anticoagulant, the dendrimer crosslinked collagen gels were fabricated with heparin pre-activated with carbodiimide (PH gels), for increased heparin retention (Paper 4), to decrease the thrombotic nature of collagen and improve blood compatibility and demonstrate other potential tissue engineering applications of these materials. The addition of pre-activated heparin did not compromise gel crosslinking or integrity, and resulted in high heparin gel retention. However, reduced heparin-antithrombin (AT) interactions were evident in the PH gels, and did not greatly prolong plasma clotting times, compared to CHG gels, suggesting that pre-activation of heparin with EDC/NHS resulted in tightly bound heparin that was not able to bind AT and inhibit thrombin formation. Thus, despite the increased heparin content among PH gels, the necessity of high heparin content may be unwarranted for blood-material interactions, especially when heparin interaction with AT is required. As such, immobilization chemistries that maintain heparin-AT interactions may be favourable, such as end-point immobilization.

As CHG gels demonstrated the longest plasma clotting times, with two of the three gels tested causing no observable clotting over the hour-long experiment, they warrant further investigation for blood-contacting applications, via longer clotting experiments, anti-factor Xa activity and platelet adhesion and activation. Furthermore, CHG gels could be combined with other antithrombotic components for increased potency. For instance,

heparinized collagen matrices could be prepared to release vascular endothelial growth factor and/or basic fibroblast growth factor to manipulate endothelial cells for reduced antithrombotic and proangiogenic characteristics. Similarly, platelet integrin antagonists, or other antithrombic pharmaceuticals, may be incorporated within this system for reduced platelet binding and activation.

Overall, it can be concluded that dendrimer crosslinking via carbodiimide chemistry is feasible for a host of commercially-available collagen-based products and tissue engineering applications, provided there are sufficient, undamaged collagen fibrils available for crosslinking. In addition, the amine-terminated dendrimer crosslinking technology may be applied to other proteins, such as hyaluronic acid or other extracellular matrix components, as long as there are sufficient amine and carboxylic acid groups for high density crosslinking. Ultimately the protein source and purity will rely on the specific tissue engineering application. For corneal tissue engineering, heparinized dendrimer crosslinked collagen gels could support epithelialization of TECE through manipulation of HCEC with heparin-binding proteins, such as HB-EGF. Alternatively dendrimer crosslinked collagen gels can be step-wise modified with HB-EGF or EGF to influence HCEC proliferation. Finally, tissue engineering applications that require good blood-material interactions would benefit from heparinized dendrimer crosslinked collagen gels, as was observed via inhibited clot formation, provided that heparin-AT interactions are maintained. Thus, the use of these dendrimer crosslinked materials, whether in combination with growth factor delivery, for tissue engineering applications, including TECE or blood-contacting biomaterials, warrants further investigation.

9 APPENDIX I - MATERIALS AND METHODS

9.1 Materials

Concentrated collagen suspensions of pepsin digested bovine cornium purified type I collagen, with less then 20% type III collagen, PureCol® (PC), human collagen (HC) and human extracellular matrix (hECM), were all generous gifts from Allergan Inc (Irvine, CA). Polypropyleneimine octaamine generation two dendrimer (G2) was purchased from SyMO-Chem (Eindhoven, The Netherlands).

Cell culture materials, including Dulbecco's Modified Eagle Medium (DMEM), keratinocyte serum-free medium (KSFM), endotoxin free water (EFW), fetal bovine serum (FBS), Penicillin (10,000 U/mL)-Streptomycin (10,000 μg/mL), TrypLETM Express, and Dulbecco's phosphate buffered saline (DPBS, no magnesium or calcium) were purchased from Invitrogen Canada Inc. (Burlington, ON). Heparin-binding epidermal growth factor (HB-EGF) and epidermal growth factor (EGF) were purchased from R&D Systems (Minneapolis, MN). Human anti-thrombin and human fibrinogen (plasminogen depleted) were purchased from Affinity Biologicals (Ancaster, ON) and Enzyme Research Laboratories (South Bend, IN), respectively.

Calcium chloride ($CaCl_2$) was purchased from Caledon Laboratory Chemicals (Georgetown, ON). Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (Oakville, ON) or EMD Chemicals Inc. (Gibbstown, NJ).

9.2 Collagen Gel Formulations

Collagen gel fabrication was based on previous formulations (Duan X and Sheardown H. 2005; Princz MA and Sheardown H. 2008). Gel solutions were mixed carefully, to avoid air bubble formation, within two 3 mL male luer-lok syringes, joined via a polypropylene female luer lug style connector (Value Plastics; Fort Collins, Colorado), as shown in Figure 9-1.

Dendrimer crosslinking solutions contained N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) in a molar ratio of 1:1:1 (EDC:NHS:collagen$_{COOH}$) with collagen carboxylic acid groups, and second generation

polypropyleneimine octaamine dendrimer (G2) (weight ratio of 10:1 G2:collagen). To facilitate EDC/NHS crosslinking, the pH of collagen gel suspensions were adjusted to 5.5, if necessary, with 1 N sodium hydroxide (NaOH) or 1 N hydrochloric acid (HCl) to stabilize the EDC/NHS reaction (Grabarek Z and Gergely J. 1990). All gel recipes are summarized in Table 9-1.

Figure 9-1. Syringe mixing system for collagen gel formation.

Following crosslinking between glass plates, gels were punched into 1/4" or 5/16" diameter discs, with a thickness of 1 mm, air dried, and stored at room temperature until needed. Where specified, rinsed gels were soaked for 24 hours in EFW to remove any unbound or unreacted components prior to experimentation.

Table 9-1. Collagen gel formulations.

Gel Name	Collagen Conc.	Collagen added	HCl added	EFW added	PBS added	Crosslinker solution	Crosslinker added	Heparin Conc.	Heparin added	NaOH added	Target pH
	(mg/mL)	µL	µL	µL	µL		µL	mg/mL	µL	µL	
COLL	66	1000	110	640	350	-	0	-	0	100	7
CG	66	1000	100	550	350	G2	200	-	0	0	5.5
CHG-204 CHG-30H	66	1000	100	100	350	G2	200	30	450	0	5.5
CHG-20	66	1000	100	100	350	G2	200	3	450	0	5.5
CHG-2 CHG-30L	66	1000	100	100	350	G2	200	0.3	450	0	5.5

Gel Legend:
COLL = Thermal collagen gel
CG = Dendrimer/EDC/NHS crosslinked collagen gel
CHG = Heparinized dendrimer/EDC/NHS crosslinked collagen gel. fabricated with either 204, 20.45 or 0.2 µg heparin per mg collagen

Crosslinker Solution:
G2 = (75mg EDC + 43mg NHS + 38mg G2) in 1mL EFW

9.2.1 Collagen Thermal (COLL) Gels

Thermal collagen (COLL) gels were prepared by adjusting the pH of acidified collagen suspensions to 7.4. The resultant collagen suspensions (30 mg/mL collagen) were pressed between glass plates and incubated at 37^{o}C overnight.

9.2.2 Dendrimer Crosslinked Collagen (CG) Gels

Dendrimer crosslinked collagen (CG) gels were fabricated by diluting the initial collagen suspension (66 mg/mL) with phosphate buffered saline (PBS) and EFW, and acidified with 1N hydrochloric acid (HCl). Crosslinking was achieved by G2 (weight ratio of G2:collagen was 10:1), with EDC and NHS (EDC:NHS:collagen$_{COOH}$ molar ratio of 1:1:1). The resultant collagen suspension was pressed between glass plates, on ice and incubated overnight at 4^{o}C. Gels had a final collagen concentration of 30 mg/mL.

9.2.3 Heparinized Dendrimer Crosslinked Collagen (CHG) Gels

Collagen solution (66 mg/mL) was diluted with PBS and EFW, and acidified with 1N HCl, as shown in Table 9-1. Unfractionated heparin (30, 3 or 0.3 mg/mL) was added to the collagen solution. Crosslinking was achieved by adding G2, EDC and NHS (molar ratio of 1:1:1 EDC:NHS:collagen$_{COOH}$; weight ratio of G2:collagen was 10:1). Heparinized gels contained 204.5 (CHG-204 or CHG-30H), 20.45 (CHG-20 or CHG-30L), or 0.2 (CHG-0.2) μg heparin per mg collagen. The resultant collagen suspensions were pressed between glass plates, on ice, and incubated overnight at 4^{o}C. Gels had a final collagen concentration of 30 mg/mL.

9.2.4 Allergan Gels

Solutions of PureCol® (PC), human collagen (HC) and human extracellular matrix (hECM) were received acidified in 0.01 N HCl (pH 2). Solutions were freeze dried and reconstituted in PBS (pH 7.4) to a concentration of 66 mg/mL. Collagen suspensions were diluted with PBS and acidified with 1N HCl prior to crosslinking, as per CG gel recipe (see Table 9-1). Crosslinking was achieved through EDC/NHS chemistry (molar ratio of collagen$_{COOH}$:EDC:NHS was 1:1:1), combined with G2 (weight ratio of 10:1 G2:collagen).

Table 9-2. Dendrimer crosslinked collagen-based gel recipes.

Gel	Collagen Source	Collagen (mg/mL)
PC-30	Bovine PureCol®	30
PC-20	PureCol®	20
HC-20	Human Collagen	20
PC-hECM-20	70% PureCol® & 30% Human extracellular matrix	20*

*Collagen concentration represents collagen content from PureCol® (14 mg/mL) and total protein content of hECM (6 mg/mL). hECM contains type 1 collagen, elastin, sulphated GAG and fibronectin in order of highest to lowest amounts.

Where necessary, the suspension pH was adjusted to 5.5 with 1N NaOH. The resultant collagen suspensions were pressed between glass plates, on ice, and incubated overnight at 4^{o}C. Gels had a final collagen concentration of 30 or 20 mg/mL, as shown in Table 9-2.

9.2.5 Dendrimer Crosslinked Pre-activated Heparinized Collagen (PH) gels

Collagen solution (66 mg/mL), was diluted with PBS and EFW, and acidified with 1 N HCl. Then pre-activated heparin (PH) solutions (67.5 or 6.8 mg/mL heparin) were added to the collagen suspension. PH solutions consisted of heparin, EDC and NHS (molar ratio of 1:1:1 heparin$_{COOH}$:EDC:NHS). Gel crosslinking was achieved by adding G2 (weight rator of G2:collagen was 10:1), with EDC and NHS (molar ratio of 1:1:1 EDC:NHS:collagen$_{COOH}$), as shown in Table 9-3.

Where necessary, the pH of the gel suspension was adjusted to 5.5 with 1 N NaOH or 1 N HCl. The resultant collagen suspension was pressed between glass plates, on ice and incubated overnight at 4^{o}C. PH gels had a final collagen concentration of 30 mg/mL, containing either 204.5 (PH-204) or 20.45 (PH-20) μg heparin per mg collagen

Table 9-3. Dendrimer crosslinked collagen gels containing pre-activated heparin (PH) and control gel formulations.

Pre-Activated Heparinized Gels:		
Gel ID	**Crosslinking Methodology**	
PH-1	(collagen suspension + 200 μL PH) + 200 μL $G2^t$ solution	[C + PH] + G2
PH-2	(200 μL PH + 200 μL $G2^t$) + collagen suspension	[PH + G2] + C
PH-3	(200 μL PH + 100 μL $G2^t$) + 100 μL $G2^t$ + collagen suspension	[PH + ½G2] + C + ½G2
PH-4	(collagen suspension + 100 μL PH) + (100 μL PH + 100 μL $G2^t$) + 100 μL $G2^t$	[C + ½PH] + [½PH + ½G2] + ½G2
PH-5[a]	(collagen suspension + 200 μL $G2^*$) + 200 μL PH + 100 μL EDC /NHS	[C + G2*] + PH + ½EDC
Control Gels:		
COLL	Collagen suspension (thermally crosslinked)	[C]
CG	Collagen suspension + 200 μL $G2^t$	[C + G2]
CHG	Collagen suspension + 450 μL Hep + 200 μL $G2^t$	[C + Hep] + G2

$G2^t$ = (75 mg EDC + 45 mg NHS + 38 mg dendrimer) in 1 mL EFW
G2* = 38 mg/mL dendrimer in EFW
Hep = 30 mg/mL heparin in EFW
PH => for 204 μg heparin per mg collagen: (67.5 mg heparin + 15 mg EDC + 9 mg NHS) in 1 mL EFW
=> for 20 μg heparin per mg collagen: (6.8 mg heparin + 1.5 mg EDC + 0.9 mg NHS) in 1 mL EFW
EDC = (75 mg EDC + 43 mg NHS) in 1 mL EFW
[a] = only half the EDC/NHS crosslinker amount, compared to other chemically crosslinked gels

9.2.6 Growth Factor Tethering after Gel Fabrication (Step-wise Conjugation)

HB-EGF or EGF was tethered in a step-wise manner to COLL and CG gels, whereby the growth factor (GF) was first activated with EDC/NHS, and then reacted with gels. Solutions of 1 mL PBS containing EDC/NHS and either 1000 ng/mL GF (1:1:1 molar ratio GF:EDC:NHS) or 10,000 ng/mL GF (1:0.1:0.1 molar ratio GF:EDC:NHS), as summarized in Table 9-4, were combined for 10 minutes, followed by the addition of dried COLL and CG gels, and incubation for 24 hrs at 4°C.

9.2.7 Growth Factor Tethering during Gel Fabrication (Bulk Conjugation)

Bulk modification of CG gels with HB-EGF or EGF was achieved by adding various GF solutions into the collagen suspension prior to full gel dendrimer crosslinking. Three GF solutions were utilized: (1)GF; (2)GF/EDC/NHS; or (3)GF/EDC/NHS/G2. The first

GF solution contained 1593 or 2390 ng/mL of HB-EGF or EGF, of which 225 µL was added to the collagen suspension, to result in 10 or 15 ng GF per ¼" gel. The second GF solution consisted of 2390 ng/mL GF, along with 75 µL EDC and NHS (molar ratio 1:1:1 GF:EDC:NHS), of which 150 µL was combined with the collagen suspension to result in ¼" gels with 10 ng/mL GF. The third GF solution combined various GF concentrations (358.8, 500, 1838 ng/mL) with EDC, NHS and G2 dendrimer (GF:EDC:NHS:G2 molar ratio of 1:0.1:0.1:0.1), resulting in gels with 10, 13 or 49 ng GF per ¼" gel. All gels were then crosslinked with G2, EDC and NHS (molar ratio $collagen_{COOH}$:EDC:NHS of 1:1:1; weight ratio of G2:collagen was 10:1). The resultant collagen suspension was pressed between glass plates, on ice and incubated overnight at 4°C. Final GF concentrations within CG gels are summarized in Table 9-4.

Table 9-4. Dendrimer crosslinked collagen gels fabricated with tethered GF (¼" gels; 1 mm thick).

		EGF Added	HB-EGF Added
Step-wise tethering	COLL	1000 ng/mL	1000 ng/mL
	CG	1000 ng/mL	1000 ng/mL
	COLL	10 000 ng/mL	10 000 ng/mL
	CG	10 000 ng/mL	10 000 ng/mL
Bulk Tethering	GF	10 ng/gel	10 ng/gel
	GF	15 ng/gel	15 ng/gel
	GF:EDC:NHS	10 ng/gel	10 ng/gel
	GF:EDC:NHS:G2	10 ng/gel	10 ng/gel
	GF:EDC:NHS:G2	13.35 ng/gel	13.35 ng/gel
	GF:EDC:NHS:G2	48.9 ng/gel	48.9 ng/gel

9.3 Transmittance

Transmittance measurements were calculated from the observed gel absorbance measured within the visible range of wavelengths (410 – 630 nm) using a BioRad 550 plate reader.

9.4 Water uptake

Water uptake of previously rinsed collagen-based gels was used to characterize gel crosslinking. Following dry weight (W_d) measurements of air dried gels, gels were swelled in Milli-Q water for 24 hours, wicked dry to remove surface moisture, and the

wet weights (W_w) determined. The water uptake was calculated according to the following formula:

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

9.5 Differential Scanning Calorimetry

Denaturation temperatures for collagen materials, used to provide information about the degree of crosslinking (Lee JM *et al*. 1995; Figueiro SD *et al*. 2006), 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 then heated at a 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.

9.6 Collagenase Digestion

Collagenase digestion was utilized to further assess gel crosslinking and biological stability through quantification of hydroxyproline (Stegemann H and Stalder K. 1967; Reddy GK and Enwemeka CS. 1996). Rinsed collagen-based gels (1/4") were immersed for 1 hour in 500 µL 0.1 M TRIS/HCl containing 0.05 M $CaCl_2$, then exposed to 200 U collagenase for 24 hrs at 37°C. After this time, the reaction was terminated with 200 µL 0.25M EDTA and the samples placed on ice. Following centrifugation (5 min, 5000 rpm), sample supernatants were analyzed for hydroxyproline content. Aliquots (100 µL) were autoclaved at 121°C for 20 min, then oxidized with 450 µL Chloramine T (0.056 M in 50% 2-propanol and acetate/citric acid buffer) for 25 min at room temperature and subjected to 500 µL Ehrlich's reagent (1 M p-di-methyl-aminobenzaldehyde in 2-propanol/perchloric acid 2:1 v/v) for 20 min at 65°C. Samples (200 µL) were read at 540 nm and compared to hydroxyproline standards (2 – 200 µg/mL) and normalized to CHG degradation.

9.7 Heparin Gel Retention

Release of free heparin from the collagen-based gels was examined in order to assess the amount of immobilized heparin. Hydrated collagen gels (not rinsed) were immersed in PBS (pH 7.4) and shaken in a waterbath, at 37^{o}C. Samples were taken periodically and stored at 4^{o}C prior to analysis for heparin concentration via the toluidine blue assay.

9.7.1 Toluidine Blue Assay

Heparin concentrations were quantified using an adapted version of the toluidine blue assay (MacIntosh FC. 1941; Smith PK *et al*. 1980). Briefly, in a 5 mL test tube, 0.75 mL of sample or standard heparin solution (0 – 2 μg/mL heparin in PBS was mixed with 0.75 mL of 2% NaCl and 0.15 mL of toluidine blue solution (0.005% toluidine blue dye with 0.2% NaCl and 0.01 N HCl). Following addition of 1 mL n-hexane, the mixture was shaken for an additional 30 seconds. The solution was allowed to phase separate, after which time, the aqueous layer was removed and analyzed spectrophotometrically at 630 nm. Sample absorbance values were standardized against known standard heparin solutions, and pre-activated heparinized gels were normalized against CG gels that contained no heparin.

9.8 Growth Factor ^{125}I-Radiolabeling

Growth factor radiolabeling with ^{125}I followed the IODOGEN method (Iodination Reagent, Pierce Biotechnology, USA) (Salacinski PRP *et al*. 1981). HB-EGF and EGF were reconstituted in 150 mM PBS buffer (pH 7.4) prior to radiolabeling. Then, GF (1000 μg/mL) was combined with 10 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 PBS over a 36 hour period. Free unbound isotope amount was determined by trichloroacetic acid precipitation and remained <10% in all studies.

9.8.1 ^{125}I Labelled Growth Factor Uptake & Release

Dried gels were exposed to ^{125}I-GF solution to measure growth factor uptake and release. Following swelling for either 24 hours or 4 days, surfaces were rinsed three times in PBS for 1 min, dabbed dry and read for activity using a gamma counter (Wallac Wizard 3" 1480 Automatic Gamma Counter, Perkin Elmer Life Sciences). To assess GF

release, ^{125}I-GF containing gels were placed into 1 mL of fresh PBS buffer, in a 37^{o}C shaking waterbath. Release samples were obtained at regular intervals and radioactivity measured with a gamma counter and translated to concentrations of ^{125}I-GF. Released GF, where appropriate, was translated to GF retention.

9.9 Antithrombin & Fibrinogen ^{125}I-Radiolabeling

Anti-thrombin (AT) and fibrinogen (Fg) were radiolabeled with Na^{125}I for protein sorption experiments. AT was radiolabelled with Na^{125}I via the Iodogen method (Iodination Reagent, Pierce Biotechnology, USA) (Salacinski PRP *et al*. 1981). Briefly, AT (100 μL; 1 mg/mL) was combined with 5 μL of ^{125}I (500 μCu per 5 μL) in an Iodogen coated vial and stirred for 15 minutes at room temperature. The solution was subsequently dialyzed 3 times with PBS buffer over an 18 hour period to remove unbound isotope. Fg (10 mg; 10 mg/mL) was radiolabelled using the iodine monochloride (ICl) technique (Wagner MS *et al*. 2003). Free iodine was removed using a gold 1-X4 column with PBS buffer. Free unbound isotope amount was determined by trichloroacetic acid precipitation and remained <2% in all studies for both AT and Fg.

9.9.1 ^{125}I-Labelled Antithrombin & Fibrinogen Sorption

Rinsed hydrated PH gels were exposed to ^{125}I labelled protein in PBS buffer (2% ^{125}I-Fg or 1% ^{125}I-AT in unlabeled protein solutions) or platelet poor plasma (PPP; 5% radioactive protein), with protein concentrations representing physiological concentrations (0.15 mg/mL AT; 2 mg/mL Fg). For PPP experiments, whole blood was collected from healthy volunteers into acid citrate dextrose, pooled, platelets removed, aliquoted and stored at -70^{o}C until used. Gels were incubated with radiolabeled AT or Fg solutions for 3 hrs, rinsed three times for 5 minutes in PBS buffer, and wicked dry prior to analysis. Protein sorption to gels was assessed using a Wallac Wizard 3" 1480 Automatic Gamma Counter (Perkin Elmer Life Sciences).

9.10 Plasma Recalcification

The ability of PH gels to inhibit clotting was assessed with plasma recalcification, as described elsewhere (McGlung WG *et al*. 2001; McGuigan AP and Sefton MV. 2008; Sask KN *et al*. 2011). Rinsed hydrated gels were incubated in 100 μL PPP (pooled from

healthy donors, anti-coagulated with acid-citrate-dextrose and stored at -70^{o}C) for 10 minutes at 37^{o}C in a 96 well-plate (non-tissue culture treated). Following addition of 100 µL 0.025M $CaCl_2$, 100 µL was transferred to a fresh well and absorbance read at 405 nm (340 ATTC plate reader using SOFT 2000 software, SLT Lab Instruments) for 30 second intervals over 1 hour. The plasma recalcification curves were analyzed for the time at which absorbance began to increase continuously ("clot initiation time") and for the time at which the absorbance values were between the initial and maximum absorbance ("time to half maximum").

9.11 Cell Culture

All cells were grown in 48-well tissue culture plates at 37^{o}C with 5% CO_2. Cell culture medium always contained antibiotic.

9.11.1 3T3 Fibroblasts Cell Culture

3T3 fibroblast cells (ATCC) were grown in DMEM containing fetal bovine serum (1:10) and penicillin-streptomycin antibiotic (1:100).

9.11.2 HCEC Cell Culture

Human cornea epithelial cells (HCEC), an immortalized cell line (Griffith M *et al*. 2003), were grown in KSFM containing bovine pituitary extract (25 mg; 0.05 w/v) and EGF (2.5 µg; 0.005 w/v), along with penicillin-streptomycin antibiotic (1:100).

9.12 HCEC Proliferation in Relation to HB-EGF, EGF and Heparin

HCEC proliferation as a function of HB-EGF (0.1-1000 ng/mL), EGF (0.1-10 ng/mL), heparin (1 to 10,000 µg/mL), and initial seeding density (6667, 7500, 10000 or 13333 cells/cm^2) was assessed. Cells were seeded over 24 hrs in KSFM containing bovine pituitary extract and EGF, rinsed with serum-free KSFM, exposed to solutions of HB-EGF, EGF or heparin, under serum-free conditions, for an additional 24 hours, and growth assessed after 48 hours with a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay.

9.12.1 MTT Assay with HCEC

Cell growth was assessed with a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay (Leonardi A *et al*. 2006). The MTT assay is a colorimetric method based on the reduction of yellow MTT to purple formazan dye by viable cell's mitochondria. Briefly, 150 µL of MTT solution (0.4 mg/mL MTT, 0.2 µm sterile filtered, in KSFM containing pituitary extract, EGF and antibiotic) was added to cells, the plate covered and incubated for 24 hrs. Following the addition of 500 µL of DMSO, the plate was shaken for 10 minutes to dissolve formazan precipitate, and the absorbance of 200 µL of the resulting solution was read 595 and 700 nm (Biorad 550 Plate Reader).

9.13 Cell Viability on Collagen-based Gels

Cell viability was assessed on various collagen-based gels. Prior to cell culture studies, gels were sterilized in 1 mL PBS containing 100 µL antibiotic (10:1) for 24 hrs. The cell seeding procedure is illustrated in Figure 9-2. Briefly, sterilized gels, either 1/4" and 5/16" in diameter, were placed into the well of a 48- or 12-well plate, respectively. Stainless steel no.8 nuts, previously rinsed thoroughly with 70% ethanol, were used to weight collagen-based gels.

Centrifugation for 5 minutes at 1500 rpm resulted in a tight seal between the nut and gel, so that pipetted cell suspensions remained on the gel surface. Following incubation for over 2 hours, 5 minutes of centrifugation at 1000 rpm was repeated to complete cell adhesion. The nut was removed, serum- and growth factor-free medium was added, and the plate incubated at 37^{o}C for the remainder of the experiment. Typically, fresh serum- and growth factor-free medium was added every 2 days. Experiments differed according to cell type, seeding density, and length of experiment.

Following cell seeding onto collagen-based gels, cell proliferation was assessed with a modified MTT collagen matrix (MTT-c) assay, outlined in section 4.14, designed to assess cell growth on collagen substrates (Ono A *et al.* 2007).

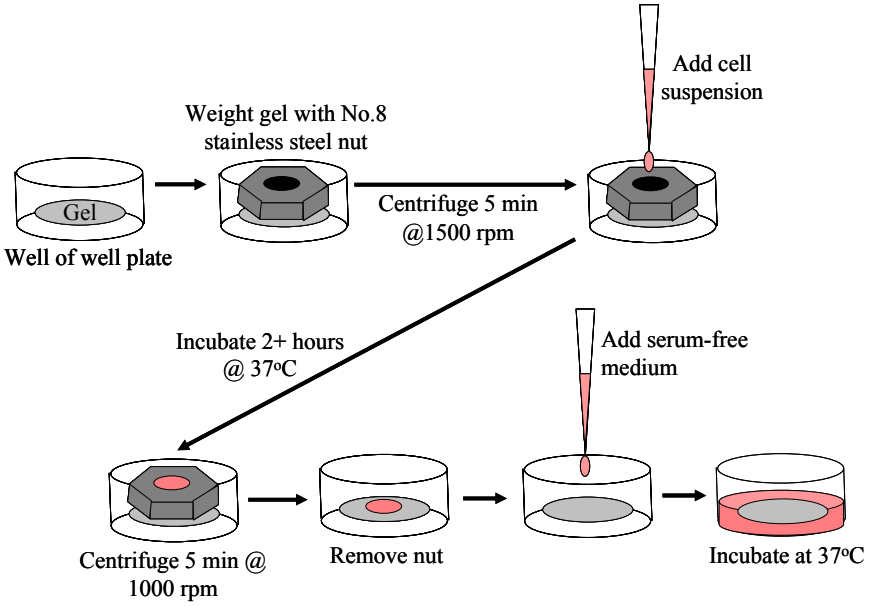


Figure 9-2. Cell seeding procedure for collagen-based gels.

9.13.1 3T3 Fibroblast Viability on Allergan Gels

Allergan gels (1/4" diameter discs) were assessed for cell viability with 3T3 fibroblasts after 4 days of culture. Prior to cell culture studies, the gels were soaked in 1 mL PBS containing 100 µL antibiotic (10:1) for 24 hrs. Allergan gels were seeded with 50 µL (10,000 cells/gel) for 3.5 hours, followed by addition of serum containing DMEM. Fresh medium was added every 2 days. Cell morphology was verified by microscopy (Axiovert 200) using a 10x objective on Day 2 and Day 4 of culture, while cell growth was assessed with the MTT-c assay (see section 9-14).

9.13.2 HCEC Viability on CHG Gels Releasing HB-EGF

HB-EGF bioactivity was determined through cell culture with HCEC. Prior to cell culture studies, gels were soaked in 1 mL PBS containing 100 µL antibiotic (10:1) for 24 hrs. Dried gels were exposed to either PBS or HB-EGF (10 ng/mL) for 24 hours. Gels (5/16" diameter discs) were seeded with 20 µL of 10,000 cells in KSFM (with serum and EGF), incubated for 2 hours to allow cells to adhere, followed by the addition of 200 µL of serum- and EGF-free KSFM. Fresh serum- and EGF-free KSFM (200 µL) was added every 2 days. After 6 days, cell proliferation was assessed with the MTT-c assay (see section 9-14).

9.13.3 HCEC Viability with Tethered Growth Factor on COLL or CG Gels

Bioactivity of tethered GF in collagen-based gels was determined through *in vitro* culture with HCEC. Prior to cell culture studies, COLL and CG gels (5/16" diameter discs) intended for step-wise modification were first soaked in 1 mL EFW for 24 hrs, then sterilized in PBS containing 100 µL antibiotic (10:1) for an additional 24 hrs, and then air-dried. Following exposure of dried gels to GF, EDC and NHS for modification, the gels were rinsed in 1 mL PBS for 24 hrs to remove residually sorbed GF. CG gels that were fabricated with tethered GF, via bulk modification, either alone or with a combination of EDC, NHS and/or G2, prior to cell seeding were rinsed in 1 mL PBS for 24 hrs to remove adsorbed and absorbed GF, and then sterilized in PBS containing 100 µL antibiotic (10:1) for an additional 24 hrs.

All gels were seeded with a 20 µL suspension containing 10,000 cells in KSFM (with serum and EGF), and incubated for 2 hours to allow cells to adhere. This was followed by the addition of 200 µL of serum-free and EGF-free KSFM. Fresh serum- and EGF-free KSFM (200 µL) was added every 2 days. After 6 days, cell proliferation was assessed with the MTT-c assay (see section 9-14).

9.14 The MTT-c Assay

To assess cell proliferation on collagen-based gels, a modified version of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) collagen matrix (MTT-c) assay was utilized, as illustrated in Figure 9-3 (Ono A *et al*. 2007).

Following cell viability experimentation, gels were exposed to MTT solution at 37^{o}C, transferred to a microcentrifuge tube, dissolved with collagenase in a TRIS/HCl and $CaCl_2$ solution. Following isolation of the formazan precipitate via centrifugation, the pellet is dissolved in DMSO, and the solution absorbance detected at 540 and 700 nm. The MTT-c assay was optimized in relation to cell type, and differed according to MTT solution and incubation times.

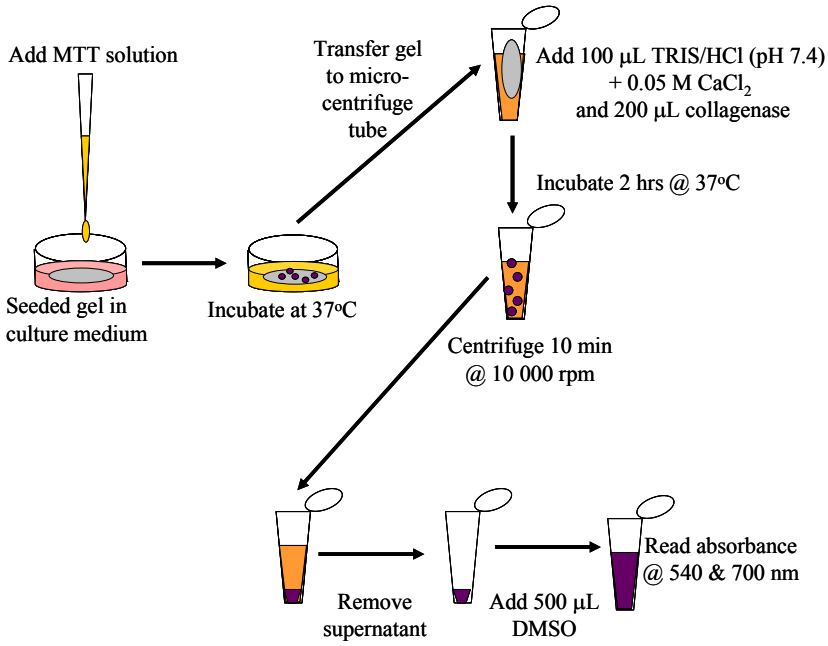


Figure 9-3. The MTT-c Assay, simplified (Ono A *et al.* 2007).

9.14.1 MTT-c Assay with 3T3 Cells Cultured on Allergan Gels

MTT solution, 150 µL, (0.4 mg/mL MTT, 0.2 µm sterile filtered, in DMEM containing serum and antiobiotic) was added to cells, the well-plate covered and incubated for 4.5 hours. The collagen-based gels were then dissolved with 100 µL collagenase (345 U/mg; 7.71 mg/mL) in 200 µL TRIS/HCl (15.76 mg/mL) containing 0.05M CaCl2 for 3.5 hours. Following centrifugation (10 min, 5000 rpm), the supernatant was removed and the pellet reconstituted in 500 µL DMSO to dissolve the formazan precipitate. Following agitation to dissolve the pellet, 200 µL was transferred to a 96-well plate and absorbance read at 595 and 700 nm (Biorad 550 Plate Reader).

9.14.2 MTT-c Assay with HCEC Cultured on Collagen-based Gels

MTT solution, 150 µL, (0.4 mg/mL MTT, 0.2 µm sterile filtered, in KSFM containing pituitary extract, EGF and antiobiotic) was added to cells, the plate covered and incubated for 24 hours. The gels were then dissolved with 200 µL collagenase (345 U/mg; 15.42 mg/mL) in 400 µL TRIS/HCl (15.76 mg/mL) containing 0.05M CaCl2 for 2.5 hours. Following centrifugation (10 min, 10,000 rpm), the supernatant was removed and the pellet reconstituted in 250 µL DMSO to dissolve the formazan precipitate. Following

agitation to dissolve the pellet, 200 μL was transferred to a 96-well plate and absorbance read at 540 and 700 nm (Biorad 550 Plate Reader).

9.15 Statistical Analysis

Data are presented as mean values (n=3) with variability expressed as standard deviations, unless otherwise stated. Cell culture experiments had a larger sample size (n=9) with variability expressed as standard error. Statistical significance was determined with a single factor ANOVA test on two means ($p<0.05$).

9.16 Materials & Method References

Duan X and Sheardown H. Crosslinking of collagen with dendrimers. Journal of Biomedical Materials Research 2005; 75A: 510-518.

Figueiro SD, Macedo AA, Melo MR, Freitas AL, Moreira RA, de Oliveira RS, Goes JC and Sombra AS. On the dielectric behaviour of collagen-algal sulphated polysaccharide blends: effect of glutaraldehyde crosslinking. Biophysical Chemistry 2006; 120: 154-159.

Grabarek Z and Gergely J. Zero-length crosslinking procedure with the use of active esters. Analytical Biochemistry 1990; 185: 131-135.

Griffith M, Li F, Lohman C, Sheardown H, Shimmura S and Carlsson DJ. Tissue Engineering of the Cornea. In: Scaffolding in Tissue Engineering. Editors: Ma PX and Elisseeff J. USA: CRC press book; 2006 p.413-423.

Lee JM, Pereira CA, Abdulla D, Naimark WA and Crawford I. A multi-sample denaturation temperature tester for collagenous biomaterials. Medical Engineering & Physics 1995; 17: 115-121.

Leonardi A, Papa V, Fregona I, Russo P, De Franchis G and Milazzo G. *In vitro* effects of fluoroquinolone and aminoglycoside antibiotics on human keratocytes. Cornea 2006; 25: 85-90.

MacIntosh FC. A colorimetric method for the standardization of heparin preparations. The Biochemical Journal 1941; 35: 776-782.

McClung WG, Clapper DL, Hu SP and Brash JL. Lysine-derivatized polyurethane as a clot lysing surface: conversion of adsorbed plasminogen to plasmin and clot lysis *in vitro*. Biomaterials 2001; 22: 1919-1924.

McGuigan AP and Sefton MV. The thrombogenicity of human umbilical vein endothelial cell seeded collagen modules. Biomaterials 2008; 29: 2453-2463.

Ono A, Kanno H, Hayashi A, Nishimura S, Kyuma Y, Sato H, Ito S, Shimizu N, Chang CC, Gondo G, Yamamoto I, Sasaki T and Tanaka M. Collagen gel matrix assay as an *in vitro* chemosensitivity test for malignant astrocytic tumors. International Journal of Clinical Oncology 2007; 12: 125-130.

Princz MA and Sheardown H. Heparin-modified dendrimer crosslinked collagen matrices for the delivery of basic fibroblast growth factor (FGF-2). Journal of Biomaterials Science, Polymer Edition 2008; 19: 1201-1218.

Reddy GK and Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. Clinical Biochemistry 1996; 29: 225-229.

Salacinski PRP, McLean C, Sykes JEC, Clement-Jones VV and Lowry PJ. Iodination of proteins, glycoproteins, and peptides usinga solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3a,6a-diphenyl glycoluril (iodogen). Analytical Biochemistry 1981; 117: 136-146.

Sask KN, McClung GW, Berry LR, Chan AKC and Brash JL. Immobilization of an antithrombin-heparin complex on gold: Anticoagulant properties and platelet interactions. Acta Biomaterials 2011; 7: 2029-2034.

Smith PK, Mallia AK and Hermanson GT. Colorimetric method for the assay of heparin content in immobilized heparin preparations. Analytical Biochemistry 1980; 109: 466-473.

Stegemann H and Stalder K. Determination of hydroxyproline. Clinica Chimica Acta 1967; 18: 267-273.

Wagner MS, Horbett TA and Castner DG. Characterizating multicomponent adsorbed protein films using electron spectroscopy for chemical analysis, time-of-flight secondary ion mass spectrometry, and radiolabeling: capabilities and limitations. Biomaterials 2003; 24: 1897-1908.