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Interactions of Corneal Cells with Transforming Growth Factor- β
Modified Poly(Dimethyl Siloxane) Surfaces

By

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A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial
fulfillment of the requirements for the degree of

Master of Applied Science, Chemical Engineering

in the

DEPARTMENT OF CHEMICAL ENGINEERING
UNIVERSITY OF OTTAWA

Ottawa, Canada

October 23, 2002

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0-612-76614-4

Statement of Contributions of Collaborators

I am the author of this thesis. My supervisors Dr. H. Sheardown and Dr. M. Dubé provided scientific guidance and editorial comments. The results generated by this project were used to compose the two manuscripts found in this thesis in chapters five and six. They will be submitted for publication to the Journal of Biomedical Materials Research (top journal in Materials Science: Biomaterials, with an impact factor of 1.9000 (ISI 2000)). During the course of this thesis a third manuscript was also submitted, entitled "Surface analysis methods for characterizing polymeric biomaterials", co-authored by K. Merrett, R.M. Cornelius, W.G. McClung, L.D. Unsworth and H. Sheardown. This manuscript has been accepted for publication in the Journal of Biomaterials Science Polymer Edition 2002. The following outline specifies the contributions that I made and indicates those areas of research that were assisted:

- Development of the growth factor attachment method based on literature was performed at the University of Ottawa.
- Growth factor surface modification of aminated PDMS surfaces was performed at the University of Ottawa.
- Advancing Water Contact Angles were performed at the University of Ottawa.
- Radioiodination and Surface Modification with Iodinated Proteins were performed with Rena Cornelius at McMaster University.
- Atomic Force Microscopy was performed with Rena Cornelius at the Brockhouse Institute for Materials Research, McMaster University.
- X-ray Photoelectron Spectroscopy was performed by Gerry Pleizer and Yves Deslandes at the National Research Council of Canada (under a contract between Dr. H. Sheardown and the NRC).
- SDS PAGE and Immunoblotting: due to the large number of samples and large time commitment, a preliminary SDS PAGE and immunoblot test was performed with Rena Cornelius at McMaster University in order that I could learn the technique. Additional samples were tested and analysed by Monique Bergeron at McMaster University.
- Cell Culturing including cell thawing, seeding, trypsinization was performed under the supervision of Dr. M. Griffith and Dr. L. Gan at the Eye Institute, University of Ottawa.
- Cell Culture Analysis including cell fixing, haemotoxylin and eosin staining, capturing of digital cell images by light microscopy and analysis

using Northern Eclipse software was performed under the supervision of Dr. L. Gan at the Eye Institute, University of Ottawa.

Abstract

Although the growth of native corneal epithelial cells over the anterior surface of an artificial corneal implant is desired, the growth of these cells on the interface located between the implant and the stromal layer of the host eye tissue (i.e. epithelial cell downgrowth) poses a significant problem to be overcome in developing a suitable implant. In this study the growth factor surface modification of a polymer substrate was examined as a means of inhibiting the proliferation of corneal epithelial cells while promoting corneal stromal cell growth. Two separate studies were conducted in which transforming growth factor- β 1 (TGF- β 1) and transforming growth factor - β 2 (TGF- β 2) respectively, were immobilized via a bifunctional poly (ethylene glycol) (PEG) spacer, MW 3400, to poly(dimethyl siloxane) surfaces (PDMS) that had been aminated by the plasma polymerization of allylamine.

The modified surfaces were chemically and biologically characterized. The desired modifications were confirmed by advancing water contact angles, x-ray photoelectron spectroscopy (XPS), and atomic force microscopy (AFM). Immobilization of the growth factor on the surfaces was confirmed by modifying the aminated PDMS surfaces with radiolabelled TGF- β 2. While the amount of growth factor covalently bound on the surface was difficult to quantify due to the strong interactions between the growth factor and the PEG layer as well as the substrate surface, differences in the modified surfaces determined by advancing water contact angles, XPS and AFM demonstrated that a significant amount of TGF- β was present on the PDMS surface following the surface modification procedure.

The effect of the surface modification with TGF- β 1 and TGF- β 2 respectively, on interactions with corneal epithelial and corneal stromal cells was examined using *in vitro* cell culture. TGF- β 1 and TGF- β 2 surface concentrations as well as culture in the absence and presence of serum and other adhesive proteins were examined. SDS PAGE and immunoblotting were used to examine the patterns of protein adsorption. Corneal stromal cells and corneal epithelial cells cultured on the TGF- β 2 modified surfaces gave results consistently opposite to those expected. Stromal cell growth appeared to be inhibited by the presence of TGF- β 2, while epithelial cell growth appeared to be promoted. A TGF-

β 2 concentration effect was noted with both cell types in the absence of serum, with higher TGF- β 2 concentrations on the modified surfaces resulting in increased cell surface coverage. The observed cell growth appeared to be the result of interactions between the cells and active TGF- β 2, since the addition of anti-TGF- β 2 antibodies added to the culture medium resulted in cell surface coverage similar to that observed on the control surfaces. In contrast to the effects observed when the corneal cells were cultured in the presence of immobilized TGF- β 2, the presence of immobilized TGF- β 1 appeared to be mildly stimulatory for corneal stromal cell growth, and mildly inhibitory for corneal epithelial cell growth. A concentration effect was noted for stromal cells in the presence of serum. SDS PAGE and immunoblotting results demonstrated that any differences in cell coverage on the modified surfaces in the presence of serum were the result of interactions with TGF- β and not the result of differences in serum protein adsorption, as all of the TGF- β modified surfaces showed similar protein adsorption patterns and levels.

These studies demonstrate that there are differences in the activity of the two TGF- β isoforms, TGF- β 1 and TGF- β 2, as illustrated by the interactions between the immobilized growth factor and corneal epithelial and corneal stromal cells under various cell culture conditions. Furthermore, the results suggest that surface modification with TGF- β 1 may prove successful in modulating corneal epithelial cell growth at the interface between the device and host tissue, although optimization of the system may be required.

Résumé

La formation d'une couche de cellules cornéennes épithéliales à l'interface entre un implant cornéen artificiel et le tissu oculaire de l'hôte pose un problème qui doit être adressé si un implant approprié peut être développé. Le présent travail consiste en la modification en surface d'un substrat polymérique avec un facteur de croissance, dans le but d'inhiber la croissance de cellules cornéennes épithéliales tout en promouvant la croissance de cellules cornéennes stromales. Deux études séparées ont été menées. Les facteurs de croissance TGF- β 1 et TGF- β 2, respectivement, ont été immobilisés sur des surfaces de poly diméthyl siloxane (PDMS) aminées par polymérisation plasmatique à l'allylamine, par le biais d'un poly (éthylène glycol) (PEG) bifonctionnel (MM 3400). Les surfaces modifiées ont été caractérisées au point de vue chimique et biologique. Plusieurs techniques telles que l'angle de contact, la spectroscopie XPS et la microscopie à force atomique (AFM) ont été utilisées pour déterminer si les modifications avaient en effet eu lieu. La présence de facteurs de croissance à la surface du polymère a été confirmée par l'utilisation d'un facteur de croissance radiomarké (TGF- β 2). La quantité de TGF- β lié de façon covalente à la surface a été difficile à quantifier étant donné de fortes interactions entre les facteurs de croissance étudiés et la couche de PEG, ainsi que la surface du polymère. Malgré tout, une différence a été notée entre les surfaces modifiées et la surface-témoin. Ceci suggère qu'une quantité significative de TGF- β se trouve à la surface du PDMS suivant la modification. L'effet des facteurs de croissance à la surface du PDMS sur l'interaction avec les cellules cornéennes épithéliales et stromales a été examiné *in vitro* par culture cellulaire. Les cellules ont été cultivées sur des surfaces présentant une variété de concentrations de TGF- β 1 ou TGF- β 2. Les études biologiques ont été effectuées en présence et en l'absence de sérum et autres protéines adhésives. Les profils d'adsorption des protéines à la surface du polymère ont été déterminés par des méthodes d'électrophorèse (SDS PAGE, immunoblotting). Les résultats obtenus lors des essais biologiques sur les surfaces recouvertes de TGF- β 2 ont été contraires aux attentes. La croissance des cellules stromales a semblé être inhibée par le TGF- β 2 tandis que la croissance des cellules épithéliales a semblé être promue. La concentration de TGF- β 2 en surface a semblé avoir un effet sur la croissance des deux

types de cellules étudiées, en l'absence de sérum. Une augmentation de la concentration du facteur de croissance a produit une hausse du nombre de cellules recouvrant la surface. Cet effet semble être le résultat d'interactions entre les cellules et le TGF- β 2 actif car l'addition de facteur anti-TGF- β 2 au milieu de culture a donné des résultats similaires à ceux obtenus pour la surface-témoin. Par contraste, la présence de TGF- β 1 à la surface du PDMS a eu un effet légèrement stimulant sur la croissance des cellules stromales et légèrement inhibant sur la croissance des cellules épithéliales. Un effet lié à la concentration superficielle de TGF- β 1 a été noté sur la croissance des cellules stromales, en présence de sérum. Les profils d'adsorption de protéines étaient semblables pour toutes les surfaces modifiées étudiées. Les résultats d'électrophorèse semblent indiquer qu'en présence de sérum, les différences entre le nombre de cellules recouvrant les surfaces modifiées s'expliquent par les interactions avec le TGF- β et non par la présence de protéines adsorbées sur la surface. Ces études démontrent qu'il existe des différences entre les activités biologiques des deux isoformes de TGF- β (TGF- β 1 et TGF- β 2), tel qu'illustré par les interactions entre les facteurs de croissance immobilisés en surface et les cellules cornéennes épithéliales et stromales pour divers milieux de culture. En outre, les résultats obtenus suggèrent que la modification d'une surface avec le TGF- β 1 puisse être un moyen efficace de contrôler la croissance des cellules cornéennes épithéliales à l'interface entre un implant cornéen et le tissu hôte, bien que l'optimisation du système puisse être nécessaire.

Acknowledgements

I wish to express my gratitude to my co-supervisors, Dr. Heather Sheardown and Dr Marc Dube, for their inspiring conversations, support and guidance during my thesis. I also wish to thank Rena Cornelius for her assistance with the radiolabelling and AFM studies, Monique Bergeron for her assistance with the SDS PAGE and immunoblotting, Dr. May Griffith for her assistance with the cell culture studies, Dr. Leisha Gan for her assistance with the cell study analysis and Dr. Gerry Pleizier for the XPS.

The Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged for funding.

In addition, I would like to thank my colleagues from the Department of Chemical Engineering and the Eye Institute at the University of Ottawa and the Department of Chemical Engineering and Pathology and Molecular Medicine at McMaster University.

Finally, my warmest thanks go to my husband Chris and our two sons, Marcus and Eric.

Table of Contents

Statement of Contributions of Collaboration	ii
Abstract	iii
Résumé	vi
Acknowledgements	viii
Table of Contents	ix
List of Figures	xii
List of Tables	xii
Nomenclature	xiii
Abbreviations	xiii
Definitions	xiii
1.0 Introduction	1
2.0 Background	3
2.1 A Human Cornea	3
2.2 An Artificial Cornea	4
2.3 Healing of a Corneal Wound	5
2.3.1 Growth Factors	6
2.4 Transforming Growth Factor Beta	7
2.4.1 TGF- β - Protein Extracellular Matrix Interactions	9
2.4.2 TGF- β – Cell Receptor Interactions	9
2.4.3 Functions of TGF- β	11
2.5 TGF- β in the Cornea	12
2.6 Growth Factor Delivery Systems	16

2.7 Substrate Surface Modification by Cold Gas Plasma Polymerization	18
2.7.1 Plasma Polymerization of Allylamine	19
3.0 Scope of Project	20
4.0 Materials and Methods	23
4.1 Substrate Preparation	23
4.1.1 Poly(dimethyl siloxane)	23
4.1.2 Amination of PDMS by Cold Gas Plasma Polymerization	23
4.2 Surface Modification of Aminated Poly(dimethyl siloxane)	24
4.2.1 Attachment of TGF- β to Poly(dimethyl siloxane)	24
4.3 Chemical Surface Characterization	26
4.3.1 Water Contact Angles	27
4.3.2 X-ray Photoelectron Spectroscopy	28
4.3.3 Radioiodination	29
4.3.4 Atomic Force Microscopy	30
4.4 Biological Surface Characterization	30
4.4.1 Cell Culture Studies	31
4.4.2 Protein Adsorption by SDS PAGE and Immunoblotting	32
5.0 Paper 1: Interactions of Corneal Cells with Transforming Growth Factor-β2 Modified Poly(Dimethyl Siloxane) Surfaces	34
6.0 Paper 2: Interactions of Corneal Cells with Transforming Growth Factor-β1 Modified Poly(Dimethyl Siloxane) Surfaces	77
7.0 Conclusions and Recommendations	97
7.1 Conclusions	97
7.2 Recommendations	99

Appendix A Mediums, Reagents and Solutions	101
Appendix B Determination of Free Iodide Concentration by Trichloroacetic Acid Precipitation of the Protein	103
Appendix C Cell Culturing Procedures	105
Appendix D Haemotoxylin and Eosin Staining Procedure	106
Appendix E SDS PAGE and Immunoblotting Procedures	108
Appendix F Summary of SDS PAGE and Immunoblotting Results for TGF- β 2	113
References	114

List of Tables

Table 1. Modified surfaces prepared for surface characterization.	26
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List of Figures

Figure 1. The requirements for a successful artificial corneal implant.	1
Figure 2. A cross section of the human eye.	3
Figure 3. The three major layers of the human cornea.	4
Figure 4(a). The primary and secondary structure of TGF- β .	8
Figure 4(b). The three-dimensional structure of TGF- β 2.	8
Figure 5. TGF- β activates signaling by binding to and bringing together a pair of type I and type II receptors.	10
Figure 6. A schematic illustration between a cell and growth factors immobilized to the surface of the polymer substrate.	17
Figure 7. An artificial corneal implant and cell interactions.	20
Figure 8. The chemical structure of poly(dimethyl siloxane).	23
Figure 9. Schematic of the microwave frequency cold gas plasma reactor.	24
Figure 10(a). The chemical structure of N-hydroxy succinimidyl ester of PEG butanoic acid.	25
Figure 10(b). The chemical structure of N-hydroxy succinimidyl ester of methoxy PEG butanoic acid.	26

Nomenclature

Abbreviations

AFM = Atomic force microscopy
ALK= automated lamellar keratoplasty (ALK)
DMEM = Dubellco's modified eagle medium
ECM = extracellular matrix
EGF = Epidermal growth factor
FBS = fetal bovine serum
bFGF = basic fibroblast growth factor
FGF-1 = acidic fibroblast growth factor-1
FGF-2 = basic fibroblast growth factor -2
H&E = haemotoxylin and eosin
HGF = hepatocyte growth factor
ITS = insulin-transferrin-selenium
KGF = keratinocyte growth factor
KFSM= keratinocyte serum-free medium
LAP = latent associated peptide
LASIK = laser in situ keratomileusis
RNA = ribonucleic acid
PBS = phosphate buffered saline
PCR = Polymerase chain reaction
PDGF = platelet-derived growth factor PDGF
PDMS = poly(dimethyl siloxane)
PEG = poly ethylene glycol
PRK = excimer laser photoreactive keratectomy
RGD = peptide sequence in fibrinogen
SDS = sodium dodecyl sulphate
TGF- α = transforming growth factor- α
TGF- β = Transforming growth factor – beta
XPS = X-ray photoelectron spectroscopy
VEGF = vascular endothelial growth factor
YIGSR = peptide sequence in laminin

Definitions

apoptosis: the most common form of physiological (as opposed to pathological) cell death.

autocrine: target cell is the same cell that secreted the growth factor.

basement membrane: part of the epithelium that serves as the foundation on which epithelial cells anchor and organize themselves.

cell differentiation: a process in the development of a multicellular organism by which cells become specialized for particular functions.

cell migration: the movement of a population of cells from one place to another.

cell proliferation: increase in cell number by division.

chemoattractant: a substance that elicits accumulation of cells.

collagen: major structural protein of the extracellular matrix.

cytokine: soluble protein or glycoprotein produced by cells, which act as chemical communicators between cells. (Many cytokines are growth and/or differentiation factors.)

endocrine: target cell is distant from the cell that secreted the growth factor.

extracellular matrix: any material produced by cells and secreted into the surrounding medium, but usually applied to the non-cellular portion of tissues.

fibronectin: glycoprotein of high molecular weight that occurs in an insoluble form in the extracellular matrix.

glycosaminoglycans: polysaccharide side-chains of proteoglycans.

glycoprotein: proteins with covalently attached sugar units, bonded either via the OH group of serine or threonine or through the amide of NH₂ of asparagines.

growth factor: protein that regulates various cell functions such as growth, differentiation, secretion and apoptosis.

hyaluronic acid: polymer that forms the core of complex proteoglycan aggregates found in the extracellular matrix.

hypertrophy: excessive enlargement.

immortalized cells: cells that have had their lifespans extended without changing the phenotype (e.g. morphology, behaviour, physiology).

immunogenicity: the property of being able to evoke an immune response within an organism.

intracrine: growth factor/receptor complex is internalized by the target cell.

juxtacrine: target cell is apposed to growth factor/receptor complex.

keratin: a strong protein substance forming the basis of nails, hair etc.

keratinized: any tissue that contains an abundance of keratin proteins.

keratoprosthesis: an implantable artificial cornea.

laminin: link protein present in the extracellular matrix which induces the adhesion and spreading of many cell types.

lysosome: membrane-bounded cytoplasmic organelle containing a variety of hydrolytic enzymes.

mesenchymal: gives rise to connective tissues, blood and blood vessels, lymphatic system and cells of the phagocyte system.

messenger RNA: single-stranded RNA molecule that specifies the amino acid sequence of one or more polypeptide chains.

paracrine: target cell is nearby the cell the secreted the growth factor.

polymerase chain reaction: the first practical system for *in vitro* amplification of DNA.

proteoglycan: a high molecular weight complex of protein and polysaccharide, characteristic of structural tissues but also present on cell surfaces.

receptor down-regulation: down-regulation occurs when receptors on a cell surface bind to a molecule (e.g. a growth factor) and are internalized leaving only a few receptors available on the surface for additional binding.

tenascin: protein of the extracellular matrix.

transformed cells: cells that have been immortalized and have experienced a change in phenotype.

1.0 Introduction

An implantable artificial cornea or keratoprosthesis could serve as either a temporary or permanent corneal substitute for those individuals whose sight has been affected by corneal damage and for whom other treatment options such as the implantation of human donor tissue are likely to fail or are not available. In North America, corneal wounds account for approximately 37% of all visual disabilities and 23% of all medical consultations for ocular problems (Germain et al., 2000). Severe recurrent and persistent corneal wounds are caused most commonly by ocular diseases and damage such as recurrent erosion, mild chemical burns, severe aqueous tear deficiency, superficial herpetic infections, neuroparalytic cornea, autoimmune diseases, and stromal ulcerations due to viral or bacterial infections or severe burns (Germain et al., 2000). Despite currently available treatments, corneal wounds may persist for long periods of time or may frequently recur, resulting in extensive scarring and eventual loss of vision. In these situations a keratoprosthesis may be a patient's only hope for restoring vision.

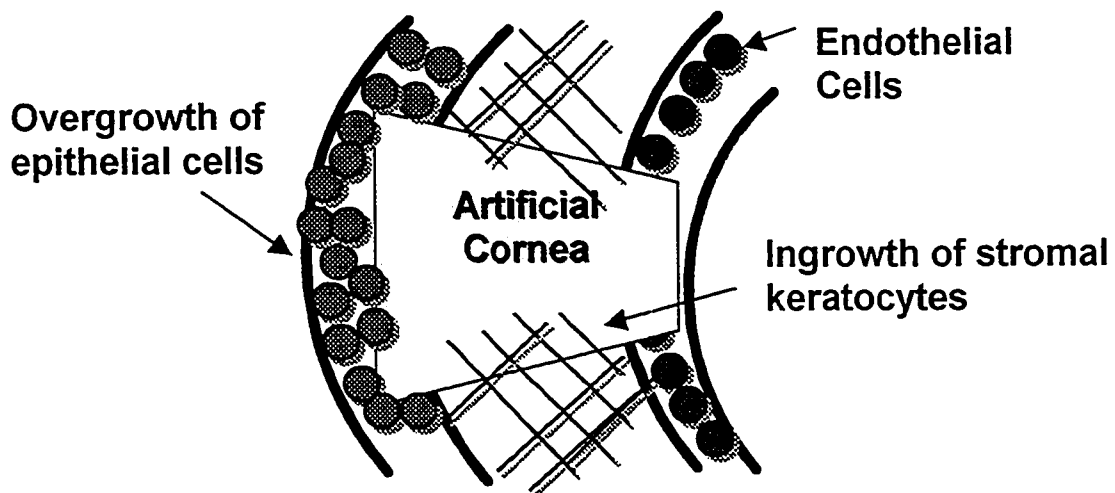


Figure 1. A suitable artificial corneal implant requires the ingrowth of stromal keratocytes for anchoring the implant in the host tissue and the overgrowth of corneal epithelial cells to provide a smooth continuous layer for ocular clarity and to provide protection to the underlying structures of the eye.

A successful artificial cornea, once implanted into the native host tissue, must interact with different corneal cell types. As shown in Figure 1, a suitable corneal implant requires

the ingrowth of stromal keratocytes for anchoring the implant in the host eye tissue and the overgrowth of corneal epithelial cells to provide a smooth continuous layer for ocular clarity and to provide protection to the underlying structures of the eye (Hicks et al., 1994; Leibowitz et al., 1994; Lloyd et al., 2001; Maldonado and Furcht, 1995; Wilson et al., 1992). Although the growth of native corneal epithelial cells over the anterior surface of an artificial corneal implant is desired, the growth of these cells on the interface located between the implant and the stromal layer of the host eye tissue (i.e. epithelial cell downgrowth) poses a significant problem to be overcome in preventing corneal implant failure related to necrosis and extrusion. In this study we examined the use of growth factor surface modification of the corneal implant substrate material as a means of inhibiting the downgrowth of corneal epithelial cells.

2.0 Background

2.1 A Human Cornea

The cornea is the principle refracting component of the human visual system and is located in the outer-tissue coat of the eye as shown in Figure 2. Its transparency and avascularity provide optimum light transmittance. The anterior surface of the cornea is covered in tear film, and the posterior surface borders the aqueous-filled anterior chamber.

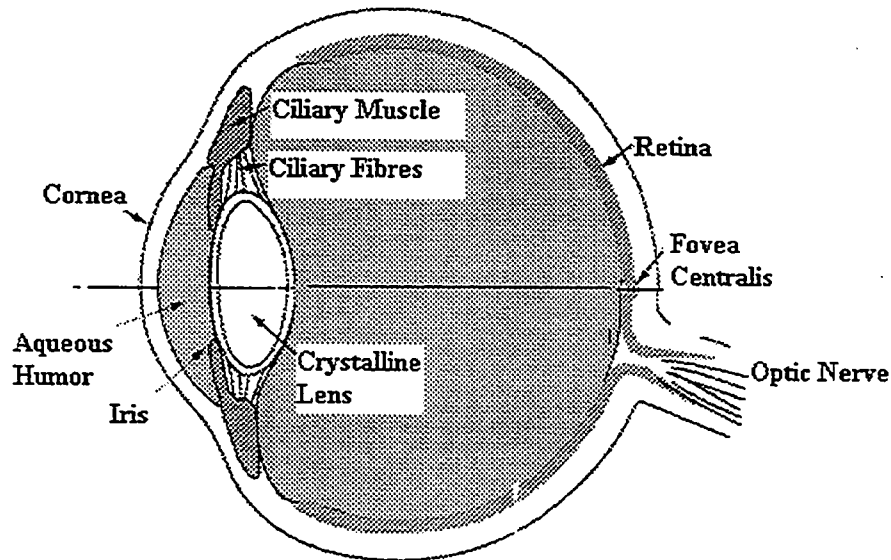


Figure 2. The cornea is located in the outer-tissue coat of the eye. Its transparency and avascularity provide optimum light transmittance. The anterior surface of the cornea is covered in tear film, and the posterior surface borders the aqueous-filled anterior chamber. (Reproduced from a drawing by Carl Nave, Department of Physics and Astronomy, Georgia State University, Atlanta Georgia, 2002. Used by permission.)

The cornea is about 0.5 mm thick in the central part. It consists of three cellular layers as shown in Figure 3. Five to six epithelial cell layers form the outer epithelial layer centrally whereas peripherally, the number of cell layers is greater (up to 10). The middle or stromal layer consists of Types I, IV, and VI collagens, glycosaminoglycans and mucopolysaccharides and keratocytes (Marshall et al., 1991(a); Marshall et al., 1991(b); Newsome et al., 1982; Zimmerman et al., 1986). The inner layer consists of a monolayer of corneal endothelial cells. Two important membranes are also present. These are Bowman's

membrane located between the epithelial and stromal cell layers and Descemet's membrane located between the stromal and the endothelial cell layers.

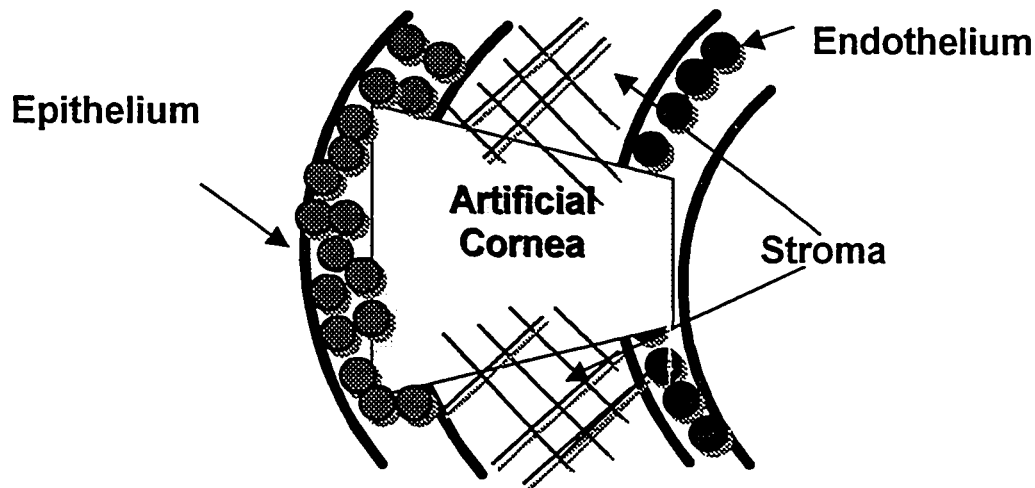


Figure 3. The cornea consists of three primary layers. As shown in the diagram, these are the epithelium, stroma and endothelium.

Corneal epithelial cells are nonkeratinized, stratified squamous cells derived from surface ectoderm. Basal epithelial cells proliferate throughout life, migrating from the basal layer to the surface where they eventually shed into the tear film (Wilson et al., 1992). A smooth, continuous layer of epithelial cells provides not only a protective layer to the underlying cornea but also participates in the maintenance of the basement membrane and the optical transparency of the underlying stroma (Maldonado and Furcht, 1995; Wilson et al., 1992). Keratocytes and endothelial cells are derived from neural crest cells. Keratocytes present in the stromal layer produce the specialized extracellular matrix responsible for corneal transparency. The endothelial cells forming the endothelium are involved in the maintenance of corneal transparency by regulating the degree of corneal hydration (Wilson et al., 1992; Imanishi et al., 2000).

2.2 An Artificial Cornea

In the 1950's, synthetic materials were introduced as potential keratoprosthesis materials. Numerous hydrophilic materials that are transparent (or become transparent after implantation in the host tissue), and possess appropriate refractive indices (Legeais et al.,

1994) have since been investigated (Chirila et al., 1995; Jacob-LaBarre et al., 1990; Kain and Thoft, 1987; Lambert and Grandon, 1978; Legeais et al., 1992; Legais et al., 1997; Trinkaus-Randall et al., 1988). The effects of the material's surface properties on epithelial cell attachment, spreading and growth have been previously investigated (Cameron et al., 1988; Evans et al., 1998; Pettit et al., 1990). Substrate surface composition has been modified by the immobilization of proteins (Aucoin et al. 2002; Evans et al., 2001; Johnson et al., 2000; Kobayashi and Ikada, 1991; Massia and Hubell, 1990; Theissen et al., 1999; Trinkaus-Randall et al., Xie et al., 1997) such as the RGD peptide sequence (Hern et al., 1997) and the YIGSR peptide sequence (Merrett et al., 2001), either by passive adsorption or covalent attachment. Plasma modification of the substrate surface has also been investigated (Chang et al., 1998; Gerenser et al., 2000; Hsiue et al., 1998; Latkany et al., 1997; Lee et al. 1996). Adsorption of serum proteins or the specific adsorption of collagen or fibronectin has been widely used to promote the migration of stromal keratocytes into the pores of polymeric materials (Chirila et al., 1996; Legeais et al., 1997; Trinkaus-Randall et al., 1990).

While significant progress has been made in the development of surfaces that promote the adhesion of both cell types, the down-growth of corneal epithelial cells at the interface of the artificial corneal implant and the native host tissue remains a significant problem to be overcome in developing a functional corneal implant that is firmly anchored in the host tissue.

2.3 Healing of a Corneal Wound

Healing of the tissue surrounding a corneal wound following injury or surgery is necessary for restoring corneal integrity, preventing infection and maintaining or restoring vision. The healing of a corneal wound is a complex biological process that can be divided into two major healing processes: epithelial wound healing and stromal wound healing. Although a complex interplay of many factors must occur during these two processes, the major events involved in the two healing processes are the migration and proliferation of epithelial cells over an epithelial wound, and the activation, migration and proliferation of keratocytes at a stromal wound site (Wilson et al., 1992). Human corneal endothelial cells have limited capacity to proliferate following wounding, and therefore endothelial defects are closed by a combination of cell hypertrophy and migration (Wilson et al., 1992).

The corneal wound healing process is believed to involve a variety of extracellular molecules, and growth factors (Andreson et al., 1996; Andreson et al., 1998; Er and Uzmez, 1998; Gibson and Inatomi, 1995; Grant et al., 1992; Imanishi et al., 2000; Sheardown et al., 1996; Schultz et al., 1994; Wilson et al., 1994). For example, wound healing results in increases of types III, IV, and VII collagen, fibronectin, laminin, proteoglycan, tenascin, keratan sulfate, and hyaluronic acid (Fantes et al., 1990; Soya et al., 1997; Tuft et al., 1989). Numerous growth factors including epidermal growth factor (EGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), fibroblast growth factor-1 (FGF-1 or acidic FGF), fibroblast growth factor-2 (FGF-2 or basic FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor- α (TGF- α), and transforming growth factor- β (TGF- β) have been shown to play important roles (Deuel and Kawahara et al., 1991; Grant et al., 1992; Imanishi et al., 2000; Li and Tseng, 1995; Li and Tseng, 1997; Long et al., 2000; Mita et al., 1998, Schultz et al., 1994; Sheardown et al., 1996) in the healing process. Growth factors may therefore have the potential to modulate the cellular activities of corneal epithelial cells and stromal cells.

2.3.1 Growth Factors

Growth factors are potent peptide or glycoprotein regulatory factors that transmit signals to modulate cellular activities. These factors can act to stimulate or inhibit cellular proliferation, differentiation, migration, adhesion, gene expression, and the secretion and action of other growth factors. Many cell types can produce the same growth factor and the same growth factor can act on many cell types with the same or different effects. Furthermore, different growth factors can share the same biological effect (Babensee et al., 2000). The relative functional potencies of growth factors are not dictated by molecular interactions alone, but also by other parameters such as the absolute number of receptors, the ability of receptor down-regulation, growth factor availability and the requirements for receptor activation (Dinbergs et al., 1996).

It is known that signal transduction by growth factors requires binding to a specific receptor on the surface of target cells to form a complex, and that the complex aggregates on the cell surface before being internalized into the cell (Ito, 1998; Tuft et al. 1993). The internalized complex is then decomposed in the lysosomes (i.e. down regulated) (Ito, 1998). The signal transduced thus stimulates the transcription of other signaling molecules and

mediates the complex biological responses associated with such cellular processes as inflammation and wound healing (Dinbergs et al., 1996; Tuft et al., 1993).

However for many growth factors, it is not known whether internalization is essential to transduce the signal to the cellular nucleus. If the events that occur on the cell surface are sufficient for signal transduction to occur, then biomaterials with immobilized growth factors could have the potential to modulate cellular activities (e.g. wound healing) (Ito, 1998). Furthermore, the mechanisms of action of many growth factors are such that immobilization of the growth factor on a biomaterial surface may result in an enhancement of its efficacy due to the absence of such effects as receptor down-regulation.

2.4 Transforming Growth Factor Beta (TGF- β)

The transforming growth factor- β s (TGF- β s) are a superfamily of structurally related multifunctional proteins that are involved in the regulation of cell growth and function, particularly during the repair of tissues (Wakefield et al., 1988). This superfamily includes the bone morphogenic proteins, the embryonic growth factors inhibin, activin and Mullerian substance as well as transforming growth factor beta (TGF- β) (Massague, 1998; Sporn and Roberts, 1989). The 12.5 kDa monomer of TGF- β is synthesized as the COOH-terminal 112 amino acids of a 390 amino acid precursor (Sporn et al., 1987). It has conserved loci of seven cysteines within the amino acid sequence. Six of these cysteines form a rigid central structure or a cysteine knot, which provides the growth factor with optimal stability and surface exposure to interact with cell receptors (Nimni, 1997). In its active form, TGF- β is a dimer with a molecular weight of 25 kDa. The primary and secondary structure of TGF- β 1 and the three dimensional structure of TGF- β 2 are shown in Figures 4 a) and b) respectively.

TGF- β was originally purified from nonneoplastic sources such as the kidney, placenta and blood platelets (Roberts et al., 1981; Roberts et al., 1983; Frolik et al., 1983; Assoian et al., 1983). This originally described form of TGF- β , now referred to as TGF- β 1, is only one of a number of closely related proteins that have been designated as TGF- β 2, TGF- β 1.2, TGF- β 3, TGF- β 4, and TGF- β 5 (Ahrendt et al., 1998). Three of these isoforms TGF- β 1, TGF- β 2, and TGF- β 3 are found in all mammalian species (Nimni, 1997). TGF- β 1 and TGF- β 2 have been found in the highest concentration in platelets and in mammalian

bone. Many other cell types produce these two isoforms in smaller amounts (Gombotz et al., 1994; Wakefield et al., 1988). The mature form of TGF- β 2 has 74% sequence homology to the TGF- β 1 isoform (Hartsough et al., 1997). The heterodimer, TGF- β 1.2, has so far been found only in small amounts in porcine platelets. TGF- β 3 has been detected in human, porcine, and avian sources, mainly in cells of mesenchymal origin, suggesting a different role for this protein other than for TGF- β 1, or TGF- β 2. The mature form of the TGF- β 3 protein contains a high degree of similarity with both the TGF- β 1 (78%) and the TGF- β 2 (84%) proteins (Hartsbough et al., 1997).

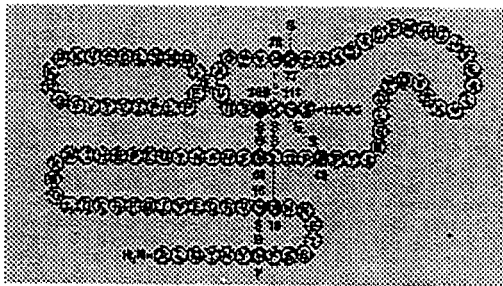


Figure 4(a). The primary and secondary structure of TGF- β 1. Cysteine residues are indicated in black along with the four intrachain and one interchain disulfide bonds ((Reproduced from Mire-Sluis, A. and Thorpe, R. Cytokines. Academic Press, San Diego, 1998. Used by permission.)

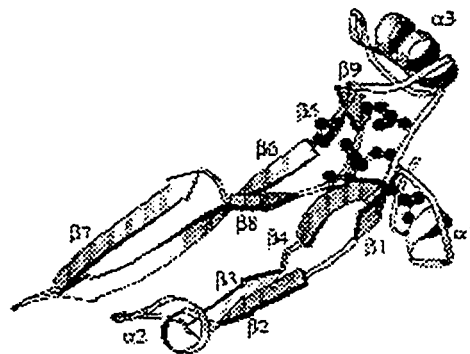


Figure 4(b). The three dimensional structure of TGF- β 2. A schematic diagram of a TGF- β 2 subunit showing the helix bundle formation (α = α -helices; β = β - sheets). (Reproduced from Mire-Sluis, A. and Thorpe, R. Cytokines. Academic Press, San Diego, 1998. Used by permission.)

In cultured cells, the three TGF- β isoforms generally exhibit the same overall cellular effects, although usually with distinct potencies (Hartsbough et al., 1997). However there are reports of differential responses of cells to TGF- β 1 and TGF- β 2 (Sporn et al., 1987). This phenomenon has been attributed to altered receptor binding properties or alterations in receptor-specific intracellular signaling components (Hartsbough et al., 1997).

TGF- β is usually secreted from cells in the inactive form, a complex of dimeric mature TGF- β bound to the latent associated peptide (LAP). This latent form is less sensitive to destruction than the bioactive form. The inactive form is activated by dissociation of dimeric mature TGF- β from the latent complex (Imanishi et al., 2000). The bioactive form of TGF- β therefore consists of a homodimer of two 12.5 kDa peptides joined by a sulphidryl bond to form a 25 kDa molecule (Clark and Coker, 1998). The mechanism(s) that activates TGF- β *in vivo* is not known, but may be protease mediated. *In vitro* activation may occur by proteolysis by plasmin or cathepsin D, heat treatment, extremes of pH, or chaotropic agents (Lyons et al, 1990).

2.4.1 TGF- β - Protein and TGF- β - Extracellular Matrix Interactions

The charge and hydrophobic properties that make TGF- β susceptible to degradation enable high affinity binding to extracellular components such as type IV collagen (Paralkar et al., 1991), fibronectin (Fava et al., 1987) and the proteoglycans (Andres et al., 1992) as well as serum proteins (Dinbergs et al., 1996; O'Connor et al., 1987). While binding to some molecules including fibronectin and alpha-fetoprotein, does not affect TGF- β activity, others result in its rapid neutralization. Binding to decorin (Yamaguchi et al., 1990) for example adversely affects the activity of the growth factor. TGF- β is rapidly (< 3 minutes) cleared when bound to α_2 -macroglobulin (LaMarre et al., 1991), leading to the suggestion that the availability of bioactive TGF- β *in vivo* is likely restricted to the local environment and that this binding occurs to regulate the biological activity of TGF- β in tissues or body fluids (Ruscetti et al., 1998).

2.4.2 TGF- β – Cell Receptor Interactions

TGF- β is a soluble growth factor that exerts its biological effect by binding to specific cell surface receptors on target cells. Most cells have 3 types of TGF- β receptors at the cell surface, which regulate the effects of TGF- β on growth and differentiation (Obata et al., 1995). Studies have shown that TGF- β signals through a heteromeric receptor complex

consisting of the type I and type II transmembrane serine/threonine kinases (Padgett et al., 1997; Yingling et al., 1995). The type I receptor (MW 65kDa) is an effector protein, which determines the specificity of signals, while the type II receptor (MW 85 to 95 kDa) is a primary binding protein for the ligand (Lee et al., 1999). Both type I and type II receptors are believed to be imperative for proper signaling to initiate the biological activities of TGF- β (Chen et al., 1993; Hartsbough et al., 1997). It is believed that TGF- β activates signaling by binding to and bringing together pairs of type I and type II receptors (Massague, 1998). Binding of TGF- β to type I and type II receptors is shown in Figure 5. One popular model explaining receptor activation by TGF- β is as follows (Hartsbough et al., 1997). TGF- β binds initially to the type II receptor, which then recruits the type I receptor. When TGF- β is bound to both type I and type II receptors, the type II receptor phosphorylates the type I receptor. The type I receptor then propagates the signal, likely by phosphorylating and activating downstream cytoplasmic components. The type III receptor (or betaglycan) (MW 280 to 330 kDa) functions mainly as a storage protein and is responsible for presenting the ligand to the signaling receptors (Miyazono, 1997). It is not likely that the type III receptor mediates any of the known biological activities of TGF- β (Chen et al., 1993). How TGF- β beta then exerts its variety of activities at the receptor level remains largely unknown (Lee et al., 1999). TGF- β has been shown to reach its half maximal receptor binding within 30 - 60 minutes and saturation within 4 hours (Massague and Like, 1985).

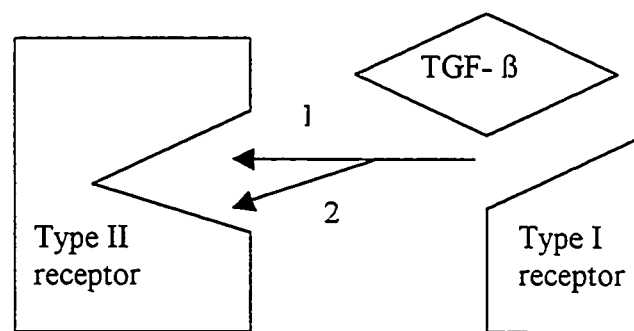


Figure 5. TGF- β activates signaling by binding to and bringing together pairs of type I and type II receptors. (Adapted from Massague, 1998.)

2.4.3 Functions of TGF- β

Literature reports that TGF- β functions in the regulation of cell proliferation, migration, differentiation, and apoptosis of various cells including corneal epithelial cells, stromal cells, and endothelial cells (Mita et al., 1998; Obata et al., 1995; Sporn et al., 1987). TGF- β regulates the enhancement of extracellular matrix production, the inhibition of proteolytic degradation of newly formed matrix proteins and the expression of integrin family adhesion molecules (Edwards et al., 1987; Miyazono et al., 1994). TGF- β has also been reported to antagonize or modulate the actions of other cytokines and growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (Battegay et al., 1990; Lucas et al., 1991; Mishima et al., 1992; Wakefield et al., 1988). Additionally, TGF- β is recognized as an immunosuppressive factor *in vivo* (Sporn and Roberts, 1989).

Studies have shown that TGF- β is a bifunctional regulator in that it may either stimulate or inhibit cellular proliferation. *In vitro*, TGF- β increases cell proliferation in freshly isolated osteoblast-like cells (Lomrie and Marie, 1990) and the administration of TGF-beta *in vivo* stimulates osteogenesis adjacent to the injection site (Rosen et al., 1994). A single application of TGF- β applied directly to experimentally induced skull defects in rabbits induces a dose-dependent increase in intramembranous osteogenesis (Beck et al., 1991). The proliferative response induced by TGF- β in mesenchymal cells may be mediated indirectly through autocrine secretion of platelet-derived growth factor (PDGF) (Battegay et al., 1990). Antiproliferative effects have also been reported for TGF- β . TGF- β inhibits the proliferation of vascular endothelial cells *in vitro* (Gajdusek et al., 1993; Heimark et al., 1986; Frater-Schroeder et al., 1986), although *in vivo* studies have shown the contrary (Roberts et al., 1986). TGF- β also inhibits the proliferation of mononuclear cells including helper and cytotoxic T lymphocytes, natural killer cells, and macrophages (Lucas et al., 1991) as well as lymphoid (Ruscetti and Palladino, 1991), hematopoietic cells (Keller and Ruscetti, 1992) and satellite cells (Velleman et al., 1999). Most importantly to the current work, TGF- β has been reported to inhibit the proliferation of cultured epithelial cells including cells of human bronchial epithelium (Getten et al., 1986), rat intestinal crypt epithelium (Kurokawa et al., 1987) and corneal epithelium (Foreman et al., 1996; Kruse and Tseng, 1994; Mishima et al. 1992; Nishida et al., 1993).

The observed effects of TGF- β may depend on the specific target cell, stage of differentiation, conditions of cell culture, the presence of other growth-regulatory molecules, and the sequence of their addition (Battegay et al., 1990; Sporn et al., 1987). However, in general, TGF- β seems to stimulate the proliferation of cells of mesenchymal origin (e.g. osteoblasts) and inhibit all non-transformed epithelial cells (Ahrendt et al., 1998; Amdreson et al., 1997; Hayashi et al., 1989; Masui et al., 1986; Moses et al., 1990; Nimni, 1997; Tucker et al., 1984). Various cell types (e.g. Mv1Lu cells) have also been shown to change their morphology in the presence of TGF- β , although it is not known how TGF- β induces the change or what cellular components or factors lead to the morphological changes (Lee et al., 1999).

Enhanced expression of TGF- β in the adult has been observed during processes such as tissue repair, bone remodeling and inflammation (Mishima et al., 1992; Er and Uzmez, 1998; Rosen et al., 1994; Lee et al., 1999). TGF- β , which is stored at high levels in blood platelets (Assoian et al., 1983; Assoian and Sporn, 1986), and monocytes (Assoian et al., 1987) is delivered to sites of injury where it can bind to fibronectin (Mooradian et al., 1989). TGF- β also stimulates collagen and fibronectin synthesis by fibroblasts (Ignatz and Massague, 1986; Laiho et al., 1986; Roberts et al., 1986) and enhances secretion of plasminogen activator inhibitor and therefore, decreases plasminogen activator activity, thereby decreasing the proteolysis of the extracellular matrix (Laiho et al., 1986; Laiho and Keski-Oja, 1986).

2.5 TGF- β in the Cornea

A number of research groups have demonstrated that transforming growth factor-beta (TGF- β) plays a role in corneal inflammatory and wound healing processes (Er and Uzmez, 1998; Grant et al., 1992; Kokawa et al., 1996; Ma and Bazen, 2000; Nishida et al., 1994; Obata et al., 1995; Obata et al., 1999; Schultz et al., 1994; Sheardown and Cheng, 1996). Polymerase chain reaction (PCR) has been used to demonstrate that TGF- β 1 and TGF- β 2 precursor mRNA is expressed in *in vivo* keratocytes and corneal endothelial cells (Nishida et al., 1994; Imanishi et al., 2000). PCR has also been used to demonstrate that human corneal epithelial cells produce TGF- β 1 mRNA in culture and *ex vivo*. Primary

cultures of corneal fibroblasts were also shown to produce mRNA coding for TGF- β 1 (Wilson et al., 1992; Wilson et al., 1994).

Although the presence of mature TGF- β 1 and TGF- β 2 was not originally detected in corneal epithelium and stroma (Pasquale et al., 1993), later studies have shown that the two isoforms are immunolocalized to both the epithelium and keratocytes in intact, normal human cornea (Wilson et al., 1994; Nishida et al., 1994; Peress and Perillo, 1994). TGF- β 2 has been shown to be synthesized, and released by corneal epithelial cells in culture (Strissel et al., 1995) and has been identified as the major inhibitor of collagenase synthesis by corneal stromal cells in culture (Strissel et al., 1995). Keratocytes have not been shown to produce TGF- β 2. Epithelial cells and keratocytes have not been shown to produce TGF- β 1 (Stamer et al., 2001). TGF- β 2 is also a major component of aqueous and vitreous humor, where it exists primarily in activated form (Jampel et al., 1990). Biologically active TGF- β 1 and TGF- β 2 are also present in human tear fluid indicating that this growth factor may also have biological functions on the ocular surface (Gupta et al., 1996).

TGF- β type I and type II receptors have been shown to be expressed by the corneal and conjunctival epithelium, corneal keratocytes, corneal endothelium, as well as the epithelium of the iris and ciliary body, epithelium of the lens, sensory retina, excluding the outer segments of photoreceptors, retinal pigment epithelium and vascular cells (Obata et al., 1995; Obata et al., 1999).

The major events that occur during epithelial wound healing are the migration and proliferation of epithelial cells. TGF- β 1 has been reported to inhibit the rate of epithelial healing in corneal organ culture systems (Foreman et al., 1996; Nishida et al., 1993). TGF- β 1 and TGF- β 2 in a serum-free, defined clonal growth assay have been reported to cause a significant, dose-dependent decrease of both colony formation and proliferation in corneal epithelial cultures (Kruse and Tseng, 1993). Other *in vitro* studies also indicate that TGF- β inhibits the rate of corneal re-epithelialization (Foreman et al., 1996; Mishima et al., 1992; Nishida et al., 1993). The effects of topical TGF- β 2 either alone or in combination with fibronectin on corneal epithelial wound healing *in vivo* have been compared. The results of this study suggest that it may also enhance corneal wound healing *in vivo*, partly by stimulating the migration of corneal cells to the wound site (Er and Uzmez, 1998), similar to its observed stimulation of corneal epithelial cell migration *in vitro* (Er and Uzmez, 1998).

Furthermore, the results suggest a synergistic effect between fibronectin and TGF- β 2, since the combination of TGF- β 2 and topical fibronectin facilitated corneal wound healing more than either alone (Er and Uzmez, 1998).

The major events that occur during repair of a stromal wound are the activation of keratocytes (adjacent to the wound) to corneal fibroblasts, which are then transformed into myofibroblasts that close the wound by contraction (Taliana et al., 2000). TGF- β 1 has been shown to play an important role in keratocyte activation and myofibroblast transformation *in vivo* (Jester et al., 1997) as well as in the early recruitment of keratocytes to the wound site (Andresen and Ehlers, 1998). In cultures of corneal keratocytes it has been demonstrated that TGF- β 1 increases cell proliferation (Ohji et al., 1993), enhances extracellular matrix collagen and fibronectin synthesis (Jester et al., 1996), and inhibits the expression of matrix metalloproteinases, collagenase and stromelysin (Fini et al., 1992; Girard et al., 1991). TGF- β 1 has also been shown to be a more effective chemoattractant for keratocytes at a 1,000 fold lower concentration than either EGF or bFGF (Grant et al., 1992). Injury has been observed to increase the level of expression of TGF- β 1 mRNA (Song et al., 2000). The half-life of TGF- β 1 mRNA was observed to be two times greater in wounded cultures, indicating that injury itself maintained growth factor expression while cell migration was present (Song et al., 2000). However, TGF- β has also been shown to be a potent inhibitor of keratocyte migration in a collagen gel invasion assay, although the presence of the growth factor in 10% serum has been shown to increase keratocyte proliferation (Andresen et al., 1997).

The effect of the TGF- β isoforms on *in vitro* corneal cell proliferation and migration is controversial. Differing cell culture conditions (Battegay et al., 1990), cell type and state of differentiation (Ruscetti et al., 1998), TGF- β concentration (Gadjusek et al., 1993) and secondary activation of counter regulatory cytokine systems present in the *in vitro* environment (Pascal et al., 1999) may account for discrepancies reported for observed *in vitro* effects.

Discrepancies reported between the *in vitro* and *in vivo* effects of TGF- β may be in part due to interactions between TGF- β and other growth factors present in the environment. TGF- β has been shown to induce anchorage-independent growth of some types of normal fibroblasts in semi-solid media, when added in combination with growth factors such as

epidermal growth factor (EGF) (Sporn et al., 1987). Exposure of quiescent human fibroblasts to TGF- β in the presence of other growth factors including basic fibroblast growth factor (bFGF) and EGF has been shown to result in the inhibition of collagenase induction with a synergistic increase in tissue inhibitor of metalloproteinase (TIMP), which plays a fundamental role in cell growth regulation and extracellular matrix degradation. This result suggests that TGF- β exerts a selective effect on extracellular matrix deposition by modulating the action of other growth factors (Edwards et al., 1987). TGF- β 1 has also been shown *in vitro* to turn off transcription and protein expression of hepatocyte growth factor/scatter factor (HGF/SF) and keratinocyte growth factor (KGF) (Li and Tseng, 1997). In culture, TGF- β 1 has been shown to enhance the keratocyte growth promoting effects of EGF (Hongo et al., 1992). TGF- β 1 has also been shown to exhibit an inhibitory effect on EGF and FGF stimulated corneal epithelial cell cultures (Kruse and Tseng, 1993). The independent and combined effects of TGF- β and EGF on rabbit corneal epithelial cells in cell and organ culture have been investigated (Mishima et al., 1992). It was found that TGF- β in the absence of EGF inhibited the proliferation of corneal epithelial cells, but it did not affect corneal epithelial migration (Mishima et al., 1992). More recent literature looking at the effects of TGF- β in the presence of other growth factors show that both TGF- β 1 and TGF- β 2 inhibit, in a dose-dependent manner, corneal epithelial cell proliferation promoted by KGF and HGF, but weakly inhibited the cell proliferation promoted by EGF. The inhibitory effect of TGF- β 2 was observed to be stronger than that of TGF- β 1 (Honma et al., 1997).

The use of techniques such as excimer laser photoreactive keratectomy (PRK), automated lamellar keratectomy (PRK), automated lamellar keratoplasty (ALK) and laser in situ keratomileusis (LASIK) have provided researchers with information about the growth factor response to wound healing in the human cornea. For example, excimer laser ablation studies have shown the possibility of involvement of TGF- β in the corneal wound healing processes, including corneal epithelial cell migration and corneal stromal cell reaction (Mita et al., 1998). However, these studies have not confirmed whether the observed reactions were specific for excimer laser ablation or whether TGF- β affects the epithelial and stromal cells directly or indirectly (Mita et al., 1998). Other groups who are interested in the effects of TGF-beta on stromal haze following excimer laser photoreactive keratectomy have found

that TGF-beta may be involved in stromal healing and corneal haze formation (Chen et al., 2000; Faktorovich et al., 1999; Myers et al., 1997; Tuft et al., 1993).

2.6 Growth Factor Delivery Systems

It has been suggested that the ideal growth factor delivery system is one that can provide a “tunable” amount of growth factor, which can ensure that an appropriate number of growth factor receptor complexes are formed at the cell surface during the requisite time period for signal transduction (Kuhl and Griffith-Cima, 1996). This type of system may be achieved by immobilizing the growth factor to an insoluble polymer surface via a covalent bond. Binding of the growth factor to specific cell receptors is believed to still occur as long as the mobility and the biological activity of the immobilized growth factor are retained (Hern et al., 1997; Ito et al., 1998; Kuhl and Griffith-Cima, 1996; McLean et al., 2000). The amount of the growth factor immobilized on the surface is a key factor in this type of system, since it has been shown in numerous studies (Sheardown and Cheng, 1996) that growth factor efficacy occurs within a very narrow concentration range. Covalent binding of a growth factor to an insoluble substrate may also provide a means of controlling the local concentration of growth factor delivered to a target tissue by limiting diffusive spread, cell uptake, and degradation of the growth factor at the implant site (Babensee et al., 2000; Kuhl and Griffith-Cima, 1996).

The biological activity of the growth factor in such a system is dependent on the signaling mechanism. For example, if the growth factor-receptor complex requires internalization to transduce the signal to the cellular nucleus, an immobilized growth factor may be unable to exert its biological activity. However, if the events that occur on the cell surface are sufficient for signal transduction to occur, then biomaterials with immobilized growth factors could have the potential to modulate cellular activities. A schematic illustration of the binding between a cell and an immobilized growth factor is shown in Figure 6.

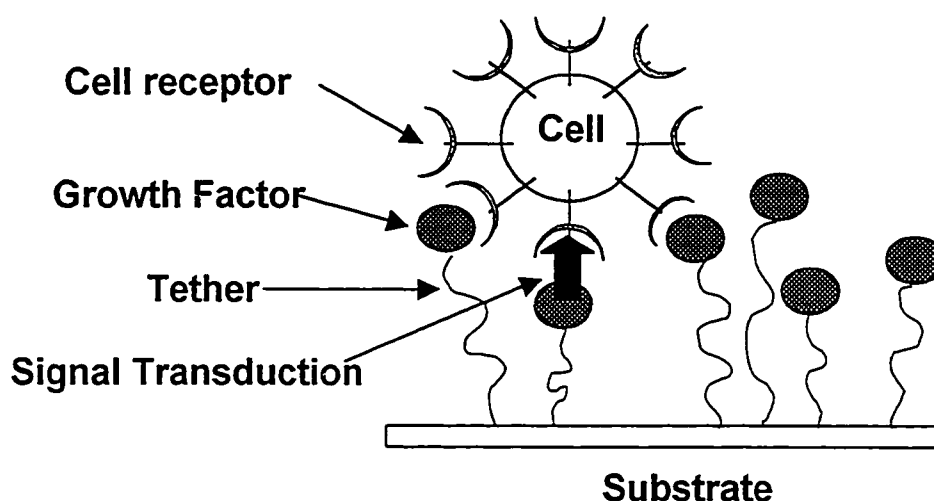


Figure 6. A schematic illustration between a cell and growth factors immobilized to the surface of the polymer substrate. It is not known whether internalization of the growth factor-receptor complex is essential to transduce the signal to the cellular nucleus. If the events that occur on the cell surface are sufficient for signal transduction to occur, then biomaterials with immobilized growth factors could have the potential to modulate cellular activities. (Adapted from Ito et al., 1994 and Ito, 1998.)

The feasibility of tethering a growth factor to a substrate via a PEG spacer has been previously demonstrated. Proteins bound to PEG have been shown to maintain their main biological functions to a much greater extent than proteins in solution (Zalipsky and Harris, 1992; Veronese, 2001). Furthermore, the high flexibility of the PEG chain in aqueous solution has been shown to contribute to the ready availability of the immobilized protein and therefore facilitates the cross-linking of the growth factor-receptor complex (Ito et al., 1994). PEG is also known to be protein and cell repulsive (Harris, 1992); this results in the ligand and cell interacting in a specific manner, as desired, without nonspecific interactions with the PEG spacer (Sofia et al., 1990). It is noteworthy that PEG-tethered proteins have been shown to have dramatically reduced immunogenicity (Zalipsky et al., 1992).

Epidermal growth factor, EGF, has been tethered via PEG-based polymers to a substrate with good retention of activity as assessed by both mitogenic and morphological assays (Kuhl and Griffith-Cima, 1996). Specifically, mouse EGF was covalently coupled to aminosilane-modified glass via star poly(ethylene oxide) (PEO) by either a “surface-first” approach, in which tethers were first linked to the surface and EGF was subsequently coupled to a fraction of the remaining star arms, or by a “solution-first” method, in which

EGF was coupled to the PEO star tethers in solution and the EGF-PEO conjugate was then covalently linked to the surface. An important facet of this study was that this group found that the mere presence of EGF on the surface (e.g. adsorbed EGF) was insufficient to elicit biological response. It is therefore likely that the conformation of physically adsorbed EGF is unsuitable for stimulating specific cell receptors or that receptor mobility is important in the signaling process for stimulating biological response.

TGF- β has been contained in a hydrogel using a PEG tether in order to develop a bioactive scaffold material that will support biospecific cell adhesion and encourage robust extra cellular matrix synthesis (Mann et al., 2001). In order to contain the growth factor in the hydrogel, TGF- β was first covalently tethered to acryloyl-PEG-N-hydroxysuccinimide; this conjugate was added to a polymer-cell solution that was then photopolymerized. The reaction was run at a pH of 8.5 to provide for better conversion. The pH was then adjusted to pH 7.4 before conducting cellular activity studies (West, 2001). This study found that the presence of tethered TGF- β resulted in a significant increase in matrix production over the same amount of soluble, unmodified TGF- β incorporated into the same scaffold. This result may be due to diffusion of soluble TGF- β into the culture medium, whereas tethered TGF- β remains within the scaffold since it is covalently bound.

An injectable formulation containing TGF- β bound to fibrillar collagen via a PEG tether has been developed to facilitate tissue repair (Bentz et al., 1998). Optimal covalent binding of TGF- β to collagen was achieved by reacting the growth factor with either the N-hydroxysuccinimidyl ester of poly (ethylene glycol) bis-glutarate, or poly (ethylene glycol) bis-propionate, admixing the PEG-attached TGF- β , and reacting with fibrillar collagen. *In vivo* and *in vitro* testing showed that the growth factor remained active in both the formulations. The results of this study show that covalent binding of TGF- β to collagen via a PEG spacer stabilizes *in vivo* growth factor responses and therefore results in a superior activity and delivery of TGF- β .

2.7 Substrate Surface Modification by Cold Gas Plasma Polymerization

The immobilization of a ligand to an insoluble polymer surface via a covalent bond may require prior modification of the polymer surface to produce surface reactive functional groups. Cold gas plasma polymerization is a common means for carrying out this type of

modification. Plasma is a “quasi-neutral gas of charged and neutral particles with high energy” (Grill, 1994). Low temperature or cold plasmas are most commonly generated with radio frequency (13.56 MHz) excitation of gases at low pressure. However, microwave frequencies (e.g. 2.45 GHz) can also be used to generate cold plasmas (Wickson and Brash, 1999) with good success. Microwave frequency plasmas have been shown to yield a higher concentration of active species per unit power than plasmas generated with lower frequencies (Moisan and Wertheimer, 1993). Under optimal conditions, plasma components will polymerize and attach chemically to the substrate surface resulting in a thin, pinhole free, and highly crosslinked polymer film of irregular structure with no identifiable repeat units (Yasuda, 1985).

This technique has been widely used in the modification of polymeric materials for biomedical applications. For example, the plasma polymerization of a selected monomer on a surface is a proven method to produce a reactive surface with desired functionality while not affecting the bulk properties (Wickson and Brash, 1999). The grafted functional groups on the surface are then free to react with other molecules for further surface modification.

2.7.1 Plasma Polymerization of Allylamine

The cold gas plasma polymerization of the monomer, allylamine, can be used to produce a reactive surface with amine groups. The high energy associated with the allylamine plasma may affect other bond sites other than the C=C bond such as the C-N bond region, so that rearrangements of monomer atoms are likely to occur. Therefore, some functional groups that are not expected to be observed under conventional polymerization methods have been observed to appear such as the $-\text{CH}=\text{NH}$ group (Krishnamurthy and Karnei, 1989.) The mechanism of plasma polymerization of allylamine is not known precisely (Krishnamurthy and Karnei, 1989).

3.0 Scope of Project

Unlike a human donor cornea, which possesses a natural substrate for cell growth, an artificial cornea must have surface characteristics that promote the adhesion and proliferation of the native corneal cells. Substrate surface modification techniques have been previously investigated to promote the growth of epithelial cells over the anterior surface of an artificial corneal implant to create a smooth continuous cellular layer for ocular clarity and to provide protection to the underlying structures of the eye. Techniques have also been investigated to promote the ingrowth of stromal keratocytes to firmly anchor an implant in the host eye tissue. Although the growth of native corneal epithelial cells over the anterior surface of an artificial corneal implant is desired, the growth of these cells on the interface located between the implant and the stromal layer of the host eye tissue (i.e. epithelial cell downgrowth) poses a significant problem to be overcome in developing a suitable implant. In this study we examined the use of growth factor surface modification of the corneal implant substrate material as a means of inhibiting the downgrowth of corneal epithelial cells.

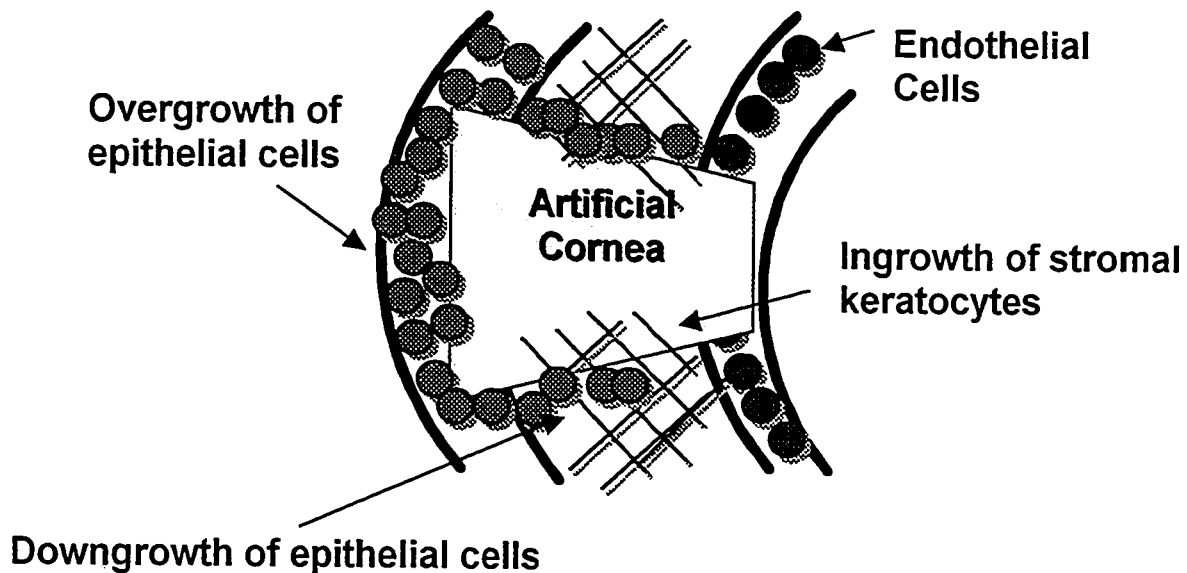


Figure 7. Although the overgrowth of native corneal epithelial cells is desired, the growth of these cells on the interface located between the implant and the stromal layer of the host eye tissue poses a significant problem to be overcome in developing a suitable implant.

For many growth factors, it is not known whether internalization of the growth factor complex is essential to transduce the signal to the cellular nucleus. As mentioned earlier, if the events that occur on the cell surface are sufficient for signal transduction to occur, then biomaterials with immobilized growth factors could have the potential to modulate cellular activities such as cell migration and proliferation. In addition, it has been shown that by immobilizing a growth factor on the surface, it may be possible to control the local concentration of the growth factor delivered to the surrounding tissue by limiting diffusive spread, cell uptake and degradation of the growth factor, and therefore may significantly enhance the bioactivity of the growth factor.

Literature reports that the growth factor known as TGF- β participates in the regulation of cell proliferation, migration, differentiation, and apoptosis of various cells including corneal epithelial cells, stromal cells, and endothelial cells. Studies have also shown that TGF- β can be bifunctional, in that it may either stimulate or inhibit cell proliferation. For example, TGF- β seems to stimulate the proliferation of cells of mesenchymal origin (e.g. osteoblasts) and inhibit all non-transformed normal epithelial cells. Most importantly to the current work, both messenger RNA and active TGF- β have been reported to be present in the corneal epithelial and stromal layers, where the active form of has been reported to inhibit local epithelial cell proliferation.

In cultured cells, the TGF- β isoforms generally exhibit the same overall cellular effects. However there are reports of differential responses of cells to TGF- β 1 and TGF- β 2. For this reason the effects of immobilized TGF- β 1 and TGF- β 2 on the cellular activities of corneal epithelial cells and stromal keratocytes were examined. Specifically, the growth factor was tethered to an insoluble polymer substrate via a covalent bond. The surface properties of the modified substrate were characterized by advancing water contact angles, x-ray photoelectron spectroscopy (XPS), and atomic force microscopy (AFM). Immobilization of the growth factor on the surfaces was confirmed by modifying the aminated PDMS surfaces with radioiolabelled TGF- β 2. The effect of the surface modification with TGF- β 1 and TGF- β 2 respectively, on interactions with corneal epithelial and corneal stromal cells was examined using *in vitro* cell culture. TGF- β surface concentrations as well as culture in the absence and presence of serum and other adhesive proteins were examined. SDS PAGE and

immunoblotting was used to examine the patterns of protein adsorption on the various modified surfaces.

4.0 Materials and Methods

4.1 Substrate Preparation

4.1.1. Poly(dimethyl siloxane) (PDMS)

Poly(dimethyl siloxane), as shown in Figure 8, was chosen as the substrate based on its ophthalmic compatibility, transparency, oxygen permeability and previous application in keratoprosthesis applications (Hsuie et al., 1998; Chang et al., 1998). The porosity and therefore the nutrient permeability of this material is not sufficient for long term implantation (Aucoin et al., 2002). However, modifications to the material are possible and may render it suitable for implantation. Furthermore, the surface modification procedure (as given below) should yield relatively substrate independent surfaces and can thus be applied to other substrate choices.

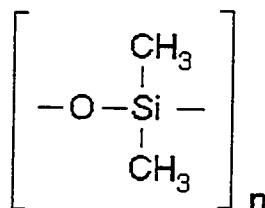


Figure 8. The chemical structure of poly(dimethyl siloxane). Poly(dimethyl siloxane) was chosen as the substrate based on its ophthalmic compatibility, transparency, oxygen permeability and previous application in keratoprosthesis applications.

4.1.2 Amination of Poly(dimethyl siloxane) by Cold Gas Plasma Polymerization

A schematic diagram of the microwave glow discharge apparatus is shown in Figure 9. Poly(dimethyl siloxane) was aminated using microwave frequencies by the cold gas plasma polymerization of allylamine. Plasma polymerization was performed at McMaster University. Poly(dimethyl siloxane) (PDMS, Dow Corning, Toronto Canada) membranes were first prepared according to directions provided by the manufacturer. The monomer and curing agent were mixed in a 10:1 molar (m:m) ratio and placed under vacuum for approximately 1 hour to remove entrapped air. The mixture was then placed in a plastic petri dish and allowed to cure overnight. Surfaces were aminated on both sides by the microwave frequency plasma polymerization of allylamine (Wickson and Brash, 1999). The reactor was

evacuated to a pressure of 40 $\mu\text{m Hg}$ and the argon flow started at a rate of 235 scc/m (standard cubic centimeters per minute). At a pressure of approximately 60 $\mu\text{m Hg}$, the glow discharge was initiated at a power of 20 W for 5 minutes. The allylamine flow was subsequently initiated at a flow rate of 0.9 sccm for 10 minutes. Surfaces were rinsed extensively with distilled water, dried under vacuum overnight and placed in sealed containers until use.

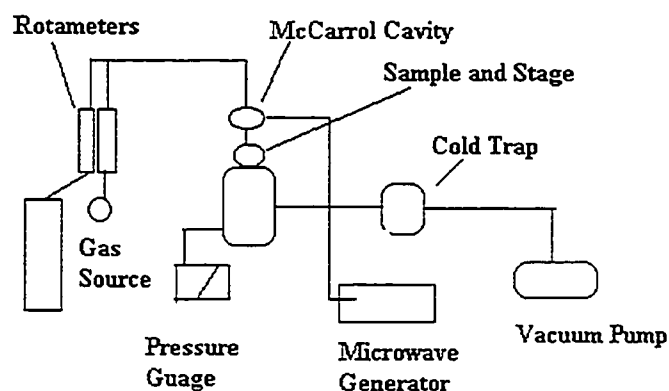


Figure 9. Schematic of the microwave frequency cold plasma reactor. (Adapted from Wickson and Brash, 1999).

4.2 Surface Modification of Aminated Poly(dimethyl siloxane)

4.2.1 Attachment of TGF β to Poly(dimethyl siloxane)

Surface modification of PDMS with TGF- β via a PEG spacer was performed based on the method of Bentz *et al.* (Bentz et al., 1998). The reaction conditions were optimized to minimize (i) PEG hydrolysis, (ii) the binding of TGF- β to both active terminal sites of the bifunctional PEG spacer, and (iii) the binding of TGF- β to itself (Bentz et al., 1998). Using this method, Bentz *et al.* found that the TGF- β remained active after the attachment of the growth factor to the PEG spacer and the resulting PEG-TGF- β complex was fully stable in phosphate buffered saline at 4 $^{\circ}\text{C}$ for up to four weeks.

PEG, an uncharged hydrophilic polymer that typically increases the solubility and stability of molecules to which it is attached (Zalipsky and Harris, 1992), was selected as the spacer. TGF- β is known to be poorly soluble in aqueous solutions near neutral pH, resulting

in rapid aggregation and therefore inactivation. Furthermore, proteins bound to PEG have been shown to not only maintain their main biological activities such as receptor recognition but are typically more stable than their soluble counterparts (Zalipsky and Harris, 1992; Veronese, 2001). The high flexibility of the PEG chain in aqueous solution has been shown to contribute to the ready availability of the immobilized protein and therefore facilitates the cross-linking of the growth factor-receptor complex (Ito et al., 1994). PEG is also well known to be protein and cell repulsive. This results in the ligand of choice, in this case the growth factor, and the cell interacting in a specific manner, without nonspecific interactions occurring with the PEG spacer (Sofia et al., 1990). It is noteworthy that PEG-tethered proteins have been shown to have dramatically reduced immunogenicity (Zalipsky et al., 1992).

Lyophilized transforming growth factor β (TGF- β) (R&D Systems, Minneapolis, MN) was immobilized to aminated PDMS surfaces via a stable bifunctional poly (ethylene glycol) (PEG) spacer made by using the succinimidyl derivative of PEG butanoic acid (SBA₂-PEG) (Shearwater Polymers Inc., Huntsville, AL), MW 3400. For the purposes of comparison, the succinimidyl derivative of PEG propionic acid (SPA₂-PEG) (Shearwater Polymers Inc., Huntsville, AL) was also used. The N-hydroxysuccinimidyl ester of methoxy poly (ethylene glycol) butanoic acid, (Methoxy-PEG-SBA) (Shearwater Polymers Inc., Huntsville, AL) MW 5000, was used as the control. The chemical structures of N-hydroxy succinimidyl ester of PEG butanoic acid and N-hydroxy succinimidyl ester of methoxy PEG butanoic acid are given in Figures 10(a) and 10(b) respectively.

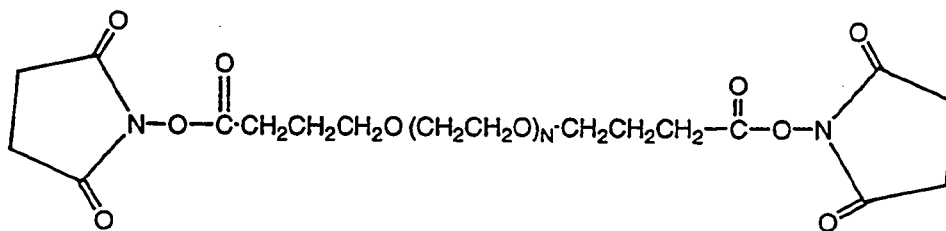


Figure 10(a). The chemical structure of N-hydroxy succinimidyl ester of PEG butanoic acid.

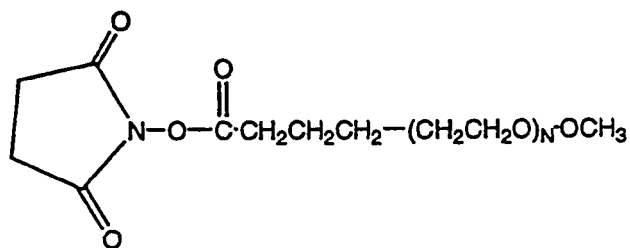


Figure 10(b). The chemical structure of N-hydroxy succinimidyl ester of methoxy PEG butanoic acid.

The growth factor was attached to the PEG by dissolving lyophilized TGF- β in HCl (pH 2.0). An aqueous solution of propylene glycol (Sigma Chemical Co., St. Louis MO) and 10x phosphate buffered saline (PBS) (Appendix A) was added so that the final solution consisted of 40% propylene glycol in PBS (pH 7.3). A 1:50 molar excess of SBA₂PEG or methoxy PEG-SBA dissolved in ice cold HCl (pH 2.0) was then added to the TGF- β solution. After reacting for 10 minutes, the aminated PDMS surfaces were exposed to the solution for an additional 2 hours at room temperature. Following the reaction, surfaces were extensively rinsed with 10x PBS and stored at 4- 6 °C in 10x PBS until characterized. Studies were carried out with both major TGF- β isoforms, TGF- β 1 and TGF- β 2. Table 1 delineates the various surfaces prepared in this study.

Surface	Explanation
PDMS	Poly(dimethyl siloxane) surfaces
Aminated PDMS	PDMS aminated by microwave frequency plasma polymerization of allyl amine
SBA-PEG-methoxy + TGFbeta	SBA-PEG-methoxy+ TGF-beta immobilized on surface of aminated PDMS.
SBA-PEG-SBA	SBA-PEG-methoxy immobilized on surface of aminated PDMS.
SBA-PEG-SBA + TGFbeta	SBA-PEG-SBA+TGF-beta immobilized on surface of aminated PDMS.
TGF-beta adsorbed	Aminated PDMS surface exposed to TGF-beta

Table 1. Modified surfaces prepared for chemical and biological surface characterization.

4.3 Chemical Surface Characterization

Characterization of the chemical surface properties of a biomaterial is an essential step for correlating surface modifications with biological performance such as biocompatibility and adhesion. In this study, surface properties were characterized by advancing water

contact angles, x-ray photoelectron spectroscopy (XPS), and atomic force microscopy (AFM). Immobilization of the growth factor on the surfaces was confirmed by modifying the aminated PDMS surfaces with radioiodinated TGF-β2.

4.3.1 Water Contact Angles

Contact angle measurement is straightforward technique revealing surface energetic information related to the outermost surface layer and surface areas of a few mm². For example, the surface free energy of a solid surface can be investigated by measuring the contact angle of a sessile drop of a test liquid. According to the Young equation the surface free energy of the solid can be derived from the cosine of this angle and the surface tension of the test liquid (Gibbs, 1961):

$$\cos \theta = \frac{\gamma_{sv} - \gamma_{sl}}{\gamma_{sv}} \dots\dots\dots(3)$$

θ contact angle

γ interfacial tensions sv (solid-vapour) sl (solid-liquid)

Measurements are commonly used to study changes in biomaterial surface composition caused by surface modification techniques in terms of changes in the relative hydrophobicity or hydrophilicity of the surface. The hysteresis observed when comparing contact angles during increase and decrease of the test droplet volume enables one in certain cases to draw conclusions on the surface heterogeneity (Werner and Jacobasch, 1999). For example, contact angle hysteresis has been reported to occur with surfaces that are rough, chemically heterogeneous or contaminated with surface-active agents (Werner and Jacobasch, 1999).

In this study, contact angle measurements were used to monitor changes in the relative hydrophobicity or hydrophilicity of the modified PDMS surfaces. Advancing contact angles were measured using the sessile drop method using a Rank Scherr Tumico bench top goniometer. All surfaces were air dried prior to measurement. Distilled water was used with a drop volume of approximately 10 µl.

4.3.2 X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) is used to determine the elemental composition of solid surfaces. All elements, except hydrogen and helium, can be detected with a sensitivity of about 0.1 atom percent (Jablonski, 2000; Ratner et al., 1993; Werner and Jacobasch, 1999). The sampling depth (which is related to the inelastic mean free path of photoelectrons in the surface region) typically varies from 4 to 20 Angstroms depending on the photoelectron energy and on the solid studied (Jablonski, 2000).

The principle of the XPS method is based upon the emission of electrons from matter in response to irradiation of the surface by a beam of monochromatic X-rays. These electrons are emitted with energy characteristic of the atoms from which they emerged (Ratner et al., 1993) and detection of these electrons can provide information about both the elemental composition of the surface as well as the binding characteristics of the elements in question. Two energies of X-rays are commonly used in experimental practice: 1253.6 eV (Mg K alpha) and 1486.6 eV (Al K alpha). The kinetic energy of the emitted electrons is smaller than the exciting energy, and these electrons have little ability to penetrate matter. Therefore, only those electrons emitted from the outermost 80 Å can escape the surface and be counted (Jablonski, 2000; Ratner et al., 1993). Surface characterization of plasma treated surfaces by XPS has been well established (Chilkoti et al., 1991; Daw et al., 1998; Iucci et al., 2000; Johnston and Ratner, 1996).

In this study, XPS was used to obtain qualitative information pertaining to changes in elemental surface composition that occurred following modification of the PDMS surfaces. XPS analysis was performed at the National Research Council of Canada. The surface of the samples was analyzed using a KRATOS AXIS HS x-ray photoelectron spectrophotometer (Kratos, Manchester UK). The size of the analyzed area was approximately 1 mm². Monochromatized Al K α radiation was used for excitation and a 180° hemispherical analyzer with a three channel detector was employed. The x-ray gun was operated at 15 kV and 20 mA. The spectrophotometer was operated in Fixed Analyser Transmission (FAT) mode throughout the study using electrostatic magnification. Surface and high-resolution spectra were collected using 160 and 20 eV pass energy respectively. The pressure in the analyzer chamber was 10⁻⁸ to 10⁻⁹ torr. An electron flood gun was used to neutralize the charge during the experiment. Binding energies were referenced to the carbon-carbon bond that was

assigned a binding energy of 285 eV. Atomic composition was estimated using standard software provided with the instrument using the following sensitivity factors: 0.25 for C1s, 0.66 for O1s and 0.42 for N1s relative to F1s at 1.00. Peak deconvolution was performed using the software provided with the instrument.

4.3.3 Radioiodination

Radioiodination is a common radiolabelling procedure that provides excellent sensitivity in the detection of immobilized and adsorbed proteins on biomaterial surfaces (Regoeczi, 1984). Radioiodination involves the introduction of radioactive iodine into tyrosine, an amino acid found in most proteins and growth factors. Iodination takes place at the positions ortho to the hydroxyl group on tyrosine; mono- or di-substitution may also occur (Regoeczi, 1984).

In this study, TGF- β 2 was radioiodinated with ^{125}I using the IODO-GEN method (Pierce, U.S.A). Briefly, the desired amount of IODO-GEN iodinating reagent was coated onto a reaction vial from a chloroform solution. A ratio of 1 μg of IODO-GEN reagent was used per 10 μg of growth factor. The reaction vial was rinsed with 10x PBS to remove loose microscopic flakes of iodinating reagent. In order to assume 100% labeling, 2 μg of TGF- β 2, dissolved in 100 μl HCl and 150 μl 10x PBS, was added to the reaction vial containing the radiolabelled iodine, ^{125}I . The reaction was allowed to proceed for 15 minutes. Since some of the ^{125}I may remain unreacted and a small amount of active species may also remain, the solution was removed from the reaction vial and placed into a dialysis cassette (Pierce, USA). Free iodide was removed by overnight dialysis against cold PBS with three changes of buffer using a dialysis membrane that was pre-coated with albumin. The free iodide concentration was determined by trichloroacetic acid (TCA) precipitation (Appendix B). The ^{125}I labeled TGF- β was then reacted with the appropriate PEG spacer followed by exposure to the aminated PDMS surfaces. TGF- β associated with the surface was then evaluated by counting using a gamma counter.

4.3.4 Atomic Force Microscopy

Atomic force microscopy creates three-dimensional images of the sample surface by monitoring the minute forces of interaction experienced between the sample surface and a probe as it scans the surface (Amato, 1997; Vansteenkiste et al., 1998). The forces of interaction may be repulsive or attractive and this gives rise to the different modes of

operation of the AFM. Mode selection depends on the sample properties and the experimental objectives (Revenko, 2000).

In this study the tapping mode was used. In tapping mode, the cantilever is vibrated and at the lowest point in the modulation cycle, the tip touches the sample surface. This mode combines the high-resolution capabilities of the traditional contact mode but is not destructive (Vansteenkiste et al., 1998). Tapping mode AFM has been proven successful for the high-resolution studies. For example titanium surfaces with adsorbed human plasma fibrinogen have been successfully imaged with tapping mode AFM (Cacciafesta et al., 2000).

Tapping mode atomic force microscopy was performed at the Brockhouse Institute for Materials Research at McMaster University using a Digital Instruments Nanoscope III. Scanning sizes of 10 μm x 10 μm were used on each sample. The scan rate was 1 Hz.

4.4 Biological Surface Characterization

The effect of immobilized TGF- β on interactions with corneal epithelial and corneal stromal cells was examined using *in vitro* cell culture. TGF- β surface concentrations as well as culture in the absence and presence of serum and other adhesive proteins were examined. SDS PAGE and immunoblotting were used to examine the patterns of protein adsorption on the various modified surfaces.

4.4.1 Cell Culture Studies

The effect of the modification with TGF- β 1 and TGF- β 2 respectively on interactions with corneal epithelial and corneal stromal cells was examined using *in vitro* cell culture. Cell culture was performed at the Eye Institute at the University of Ottawa. All materials used in cell culture, if not received sterile from the manufacturer, were sterilized by autoclaving.

Polymer samples were placed in the wells of 24 or 48 well tissue culture plates. The samples were treated with 10 $\mu\text{g/ml}$ gentamycin in 0.2M phosphate buffered saline (Appendix A) for 48 hours. This was followed by a second rinse with 10 $\mu\text{g/ml}$ gentamycin in 0.2M phosphate buffered saline prior to culture. Human corneal epithelial cells from established cell lines and human corneal stromal cells from either low passage or established cell lines were seeded on the surfaces at a density of 10^4 cells per well. (All cells were

initially removed from cryogenic storage, thawed and seeded in culture flasks prior to use. Protocols for thawing and seeding are found in Appendix C.) For corneal epithelial cells either Keratinocyte Serum-Free Medium (Gibco, Invitrogen Corporation, Grand Island NY), supplemented with bovine pituitary extract (20-30 g/ml) and rEGF (0.1-0.2 ng/ml), or SHEMA medium was used. Stromal cells were cultured in either Gibco's Modified Eagle Medium (DMEM) (Gibco, BRL, Life Technologies, Grand Island NY) supplemented with insulin-transferrin-selenium (1000 μ l/l) (ITS) and gentamycin, or DMEM supplemented with ITS (1000 μ l/l), gentamycin and fetal bovine serum (100 ml/l) (FBS). Preparation protocols for cell culture mediums are given in Appendix A. Additional surfaces were preadsorbed with laminin based on positive results with YIGSR and related peptides in previous work [Merrett et al., 2001] for a period of two hours prior to culture under serum free conditions. Samples were examined on a daily basis for cell attachment, spread and proliferation. For further examination, the cells were fixed in 4% paraformaldehyde (PFA) (Appendix A) for 20 minutes. The fixed cells were stained with haematoxylin and eosin (H&E). Details of reagents and the staining protocol are found in Appendix D. Cell surface density was assessed using digital images captured by light microscopy and analysed morphometrically using the Northern Eclipse software to obtain fractional surface coverage in a blinded fashion.

4.4.2 Protein Adsorption by SDS PAGE and Immunoblotting

SDS PAGE and immunoblotting techniques were used to identify proteins that adsorb to modified PDMS surfaces following exposure of the surfaces to normal pooled plasma. These results can be used to predict the protein adsorption that will occur on the modified PDMS surfaces in serum containing media under cell culture conditions if, for example, the surfaces are precultured with corneal cells prior to implantation. Furthermore, the protein composition of the tears is similar to plasma (Sheardown and Cheng, 1996) and the information gained from plasma adsorption experiments can be extrapolated to predict the response of the surfaces to the tears following implantation.

SDS PAGE is a gel electrophoresis technique, which is commonly used for separating proteins in a mixture. When this method is combined with the use of sodium dodecylsulphate (SDS), the binding of the SDS to the protein in large amounts allows for the separation of proteins based on molecular weight only, avoiding the secondary effect of charge. Following

incubation with SDS, the proteins are loaded onto a two-phase polyacrylamide gel and subjected to an electric field. The electric field acts on the negative charges of the proteins conferred by the binding of the SDS, causing the proteins to move through the gel at a rate proportional to their molecular weight.

A two-phase gel was used in the current work to improve the resolution of protein separation. The first phase, the stacking gel, is composed of large pores and serves to concentrate the proteins into tight bands to provide the sufficient differentiation after separation. The second phase of the gel is the separation gel that is composed of smaller pores. The flow of larger proteins is retarded while the smaller proteins flow more easily through the gel. The result is a gradient with low molecular weight proteins concentrated at the cathode and high molecular weight proteins concentrated at the anode. A low molecular weight tracer is added to the proteins and indicates that separation is complete when it reaches the cathode. Upon staining, bands corresponding to specific proteins can be seen and the proteins can be identified by molecular weight. The gels can also be probed with monoclonal antibodies to determine the presence or absence of specific proteins in the immunoblot procedure (Cornelius and Brash, 1993).

SDS PAGE and immunoblotting were performed at McMaster University. Platelet poor ACD plasma prepared from freshly drawn human blood (25 donors), aliquoted and stored at -70°C , was used. While it is possible that differences will exist in between the bovine serum used for culture and the human plasma used for examining protein adsorption, we believe that the results using human plasma will be fairly representative of those in culture. Furthermore, well established protocols and antibodies are available in our laboratory for examining human proteins. Surfaces previously equilibrated in phosphate buffered saline (PBS), pH 7.4 for 20 hours were exposed to plasma for 3 hours under static conditions at room temperature. Following adsorption, samples were rinsed extensively with PBS and exposed to a 2% sodium dodecyl sulfate (SDS) solution (24 h, 4°C) to elute the adsorbed protein. The eluates were stored at -70°C until needed. Reduced SDS PAGE (12% gels) and immunoblot analysis were performed on the eluted proteins (Cornelius and Brash, 1993). The proteins were transferred to immobilon PVDF membranes (Milipore, Bedford MA) and stained using a stabilized gold sol (Protogold, Cedarlane Laboratories, Hornby, Ontario Canada). Primary antibodies to various plasma proteins, the majority of which were

in the form of fractionated antisera developed in goats, were used at a dilution of 1:1000. Optical scans of the gold stained gels and immunoblots were analyzed using whole band analysis software (Bioimage, Ann Arbor MI) to determine molecular weights and intensities. Details of the reagents and of the electrophoresis and immunoblotting procedure are found in Appendix E.

5.0 Paper I

Interactions of Corneal Cells with Transforming Growth Factor- β 2 Modified Poly (Dimethyl Siloxane) Surfaces

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Running Title: Corneal cell interactions with TGF- β modified surfaces

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Abstract

The downgrowth of corneal epithelial cells at the interface of an artificial corneal implant and the host eye tissue poses a significant problem to be overcome in developing a successful implant. As a means of inhibiting the proliferation of the epithelial cells on the stromal surface of the implant, we immobilized transforming growth factor beta-2 (TGF- β 2) via a bifunctional poly (ethylene glycol) (PEG) spacer to poly (dimethyl siloxane) surfaces (PDMS) that had been aminated by the plasma polymerization of allylamine.

Immobilization of the growth factor on the modified surfaces was confirmed by modifying the aminated surfaces with radiolabelled TGF- β 2. The modified surfaces were also chemically characterized by advancing water contact angles, x-ray photoelectron spectroscopy (XPS), and atomic force microscopy (AFM). While the amount of growth factor covalently bound on the surface was difficult to quantify due to the strong interactions between the growth factor and the PEG layer as well as the substrate surface, differences in the modified surfaces determined by advancing water contact angles, XPS and AFM suggest that a significant amount of TGF- β was present on the PDMS surface following the surface modification procedure.

The interactions between modified TGF- β 2 surfaces with corneal epithelial and corneal stromal cells were examined using *in vitro* cell culture. TGF- β 2 surface concentrations as well as culture in the absence and presence of serum and other adhesive proteins were examined. SDS PAGE and immunoblotting were used to examine the patterns of protein adsorption. Corneal stromal cells and corneal epithelial cells cultured on the TGF- β 2 modified surfaces gave results consistently opposite to those expected. The most notable and surprising feature of these results was the lack of adhesion of the stromal cells with surface coverage averaging only 3-5%. In comparison, corneal epithelial cell growth appeared to be promoted by the presence of the growth factor and showed a much higher level of adhesion with cell surface coverage averaging 50-65%. A TGF- β 2 concentration effect was noted with both cell types in the absence of serum, with higher TGF- β 2 concentrations on the modified surfaces resulting in increased cell surface coverage. The observed cell growth appeared to be the result of interactions between the cells and active TGF- β 2 since the addition of anti-TGF- β 2 added to the culture medium resulted in cell surface coverage similar to those on the control surfaces. SDS PAGE and immunoblotting results demonstrated that any differences in cell surface coverage on the modified surfaces in the

presence of serum were the result of interactions with TGF- β 2 and not the result of differences in serum protein adsorption, as all of the TGF- β 2 modified surfaces showed similar protein adsorption patterns and levels.

Keywords: TGF- β , keratoprosthesis, corneal epithelium, corneal stroma, PEO, surface modification, poly (dimethyl siloxane)

Introduction

Due to the unique structural, biological and physiological features of the cornea, a successful artificial corneal implant should perform a number of functions. Appropriate interactions with the major cellular components of the native cornea, specifically the epithelium and the stroma are of particular interest. It has been widely reported (1-3) that an artificial cornea or keratoprosthesis should support the ingrowth of stromal keratocytes for anchoring the implant in the host eye tissue and the overgrowth of corneal epithelial cells to provide a smooth continuous layer for ocular clarity and to provide protection to the underlying structures of the eye (4). Adsorption of serum proteins or specific adsorption of collagen or fibronectin has been widely used to promote the migration of stromal keratocytes into the pores of a polymeric support and the subsequent production of collagen (5-7). Techniques to promote epithelialization have included surface modification with cell adhesion proteins (8-18), either by passive adsorption or covalent attachment, or by plasma modification of the optic surface (19-23). While significant progress has been made in the development of surfaces that promote the adhesion of both cell types, the problem of epithelial cell downgrowth at the interface between the corneal implant and the native eye tissue has not been adequately addressed. This remains a significant problem to be overcome in order to reduce implant failure. In the current work, we address this problem through the use of growth factor modified implant surfaces as a means of modulating interactions between the substrate and the major cellular components of the host tissue.

Growth factors have been shown to play a significant role in corneal wound healing in a number of different studies (24-31). While epidermal growth factor (EGF) has been most widely studied and found to significantly increase corneal epithelial wound healing rates, corneal epithelial and stromal cells have been shown to interact with a wide spectrum of other growth factors including basic and acidic fibroblast growth factor (bFGF and aFGF), transforming growth factor-alpha (TGF- α) and transforming growth factor-beta (TGF- β) (29-40). Of primary interest to our current study are the reports that TGF- β is a bifunctional regulator of cell growth in that it may either stimulate or inhibit proliferation, depending on the cell type, culture conditions, and the presence of other growth-regulatory molecules (41,42,43). In general, TGF- β appears to stimulate the proliferation of fibroblasts and cells of mesenchymal origin (41,44) and to inhibit non-transformed cells of epithelial origin (41,42,45,46). TGF- β has also been

reported to interact with the activity of other growth factors including EGF (28, 48-51) and to regulate the formation of extracellular matrix and inhibit proteolytic degradation of newly formed matrix proteins and the expression of integrin family adhesion molecules (41,47).

A number of research groups have demonstrated that mature TGF- β (29,52,53) is present in the human cornea. The corneal epithelium and corneal keratocytes have been shown to express TGF- β mRNA (28-30,32-34) and both TGF- β type I and type II receptors (54-56). In addition, the use of antibodies against the latency-associated peptide (LAP) region has shown that TGF- β -LAP is localized in all layers of the corneal epithelium (28). Corneal epithelial cells have been shown to synthesize and release TGF- β (57). The rate of corneal epithelial cell proliferation in *in vitro* systems such as organ-cultured corneas has been shown to decrease in the presence of TGF- β (48,58, 59,60). TGF- β has also been shown to inhibit corneal cell growth promoted by the presence of EGF, FGF and KGF (28,48,60,61,62). In the stroma, TGF- β expression and TGF- β receptors have been shown to increase following excimer laser keratectomy suggesting that TGF- β may also be involved in stromal wound healing (63,64). These studies have not confirmed whether the observed reactions were specific for excimer laser ablation and whether TGF- β affects the stromal cells directly or indirectly. TGF- β has also been shown to participate in the activation and myofibroblast transformation of cultured rabbit corneal keratocytes *in vivo* (65,66) as well as in the early recruitment of keratocytes to the wound area (67).

Evidence currently suggests that TGF- β modulates corneal wound healing possibly by acting as an inhibitor of cell migration and mitosis (48,59,60,68) although these studies are controversial (24,25). In cultured cells, the 3 mammalian isoforms, TGF- β 1, TGF- β 2 and TGF- β 3, generally exhibit the same overall cellular effects, although with distinct potencies (46). However, there are reports of differential responses of cells to TGF- β 1 and TGF- β 2, (28,46,69,70) which may be attributed to altered receptor binding properties or alterations in receptor-specific intracellular signaling components (46).

Based on the evidence in current literature, we hypothesized that TGF- β has the potential to modulate corneal cell activity. However, TGF- β has a short half-life and is prone to denaturation making it a difficult protein to deliver to a target tissue *in vivo*. Immobilization of growth factors such as TGF- β to biomaterial surfaces has been shown to potentially expedite

their use in delivery applications by enhancing not only their stability but also their bioactivity (18) and temporal and spatial availability in the extracellular environment (71). Tethered EGF has been shown to be as effective as soluble EGF in eliciting DNA synthesis and cell rounding responses in primary rat hepatocytes under different surface conditions (72). TGF- β 2, delivered via covalent attachment to fibrillar collagen, has been shown to retain activity for significantly longer than TGF- β 2 simply mixed with the collagen matrix *in vitro* and to give a longer lasting fibroblastic response *in vivo* (73). Mann et al. (74), using tethered TGF- β 1, demonstrated that matrix production by vascular smooth muscle cells was increased on surfaces containing covalently bound adhesive ligands with the presence of 0.04 pmol/mL TGF- β 1. Furthermore, tethering TGF- β 1 resulted in a significant increase in matrix production over the same amount of soluble TGF- β 1. In the current study, we immobilized TGF- β 2 to aminated polydimethyl siloxane surfaces and examined the interactions of these modified surfaces with corneal epithelial and stromal cells *in vitro*.

Materials and Methods

Surface Preparation

Poly (dimethyl siloxane) (PDMS, Dow Corning, Toronto Canada) membranes were prepared according to directions provided by the manufacturer. Briefly, the monomer and curing agent were mixed in a 10:1 (m:m) ratio and placed under vacuum for approximately 1 hour to remove entrapped air. The mixture was then placed in a plastic petri dish and allowed to cure for a period of at least 48 hours. Surfaces were aminated on both sides for subsequent modification using microwave frequency plasma polymerization of allylamine. The reactor was evacuated to a pressure of 40 mm Hg and the argon flow started at a rate of 235 sccm (standard cubic centimeters per minute). At a pressure of approximately 60 mm Hg, the glow discharge was initiated at a power of 20 W for 5 minutes. The allylamine flow was subsequently initiated at a flow rate of 0.9 sccm for 10 minutes. Amination conditions were selected based on the work of Wickson and Brash (75). Following amination, the surfaces were rinsed extensively with distilled water, dried under vacuum overnight and placed in sealed containers until use. Subsequent surface modification with TGF- β 2 was based on the method of Bentz *et al.* (73). Lyophilized TGF- β 2 (R&D Systems, Minneapolis, MN) was dissolved in HCl (pH 2.0). This was added to an aqueous solution of propylene glycol (Sigma Chemical Co., St. Louis MO) and

10x phosphate buffered saline (PBS) so that the final solution consisted of 40% propylene glycol in PBS (pH 7.3). A 1:50 molar excess of a succinimidyl derivative of PEG butanoic acid (SBA₂-PEG) (Shearwater Polymers Inc., Huntsville, AL), MW 3400, dissolved in ice cold HCl (pH 2.0) was added to the TGF- β solution. An N-hydroxysuccinimidyl ester of methoxy poly (ethylene glycol) butanoic acid, (methoxy-SBA-PEG) (Shearwater Polymers Inc., Huntsville, AL), MW 5000, was used as a control. Additional controls consisted of surfaces modified by TGF- β 2 in the absence of PEG and by PEG in the absence of TGF- β 2. After reacting for 10 minutes, the aminated PDMS surfaces were exposed to the solution for additional 2 hours at room temperature. Following reaction, surfaces were extensively rinsed with 10x PBS and stored at 4 – 6 degree C until characterized.

¹²⁵I Labeled TGF- β Immobilization

To characterize the binding of TGF- β 2 to the modified surfaces, the growth factor was radiolabelled with ¹²⁵I using the IODO-GEN (Pierce, USA) method. Free iodide was removed by overnight dialysis against cold PBS with three changes of buffer. The ¹²⁵I labeled TGF- β 2 was then reacted with the surfaces as above.

Surface Characterization

Advancing sessile drop water contact angles were measured on the unmodified, aminated and growth factor modified PDMS surfaces with a Rank Scherr Tumico 22-2000 series 14-inch horizontal beam bench top comparator. Milli-Q water (18 M Ω) was used with a drop volume of approximately 10 μ L.

X-ray photoelectron spectroscopy (XPS) analysis was performed at the National Research Council of Canada. The surface of the samples was analyzed using a KRATOS AXIS HS x-ray photoelectron spectrophotometer (Kratos, Manchester UK). The size of the analyzed area was approximately 1 mm². Monochromatized Al K radiation was used for excitation and a 180° hemispherical analyzer with a three channel detector was employed. The x-ray gun was operated at 15 kV and 20 mA. The spectrophotometer was operated in Fixed Analyser Transmission (FAT) mode throughout the study using electrostatic magnification. Surface and high-resolution spectra were collected using 160 and 20 eV pass energy respectively. The pressure in the analyzer chamber was 10⁻⁸ to 10⁻⁹ torr. An electron flood gun was used to neutralize the charge during the experiment. Binding energies were referenced to the carbon-carbon bond that was assigned a binding energy of 285 eV. Atomic composition was estimated

using standard software provided with the instrument using the following sensitivity factors: 0.25 for C1s, 0.66 for O1s and 0.42 for N1s relative to F1s at 1.00. Peak deconvolution was performed using the software provided with the instrument.

Tapping mode atomic force microscopy (AFM) was performed at the Brockhouse Institute for Materials Research at McMaster University using a Digital Instruments Nanoscope III. Scanning sizes of 10 mm x 10 mm were used on each sample with a scan rate of 1 Hz.

Cell Culture Studies

The effect of the modification with TGF- β 2 on interactions with corneal epithelial and corneal stromal cells was examined using *in vitro* cell culture. Polymer samples were placed in the wells of 24 or 48 well tissue culture plates. The samples were treated with 10 μ g/ml gentamycin in 0.2M phosphate buffered saline for 48 hours. This was followed by a second rinse with 10 μ g/ml gentamycin in 0.2M phosphate buffered saline prior to culture. Human corneal epithelial cells from established cell lines and human corneal stromal cells, from either low passage or established cell lines were seeded on the surfaces at a density of 10^4 cells per well. For corneal epithelial cells either Keratinocyte Serum-Free Medium (Gibco, Invitrogen Corporation, Grand Island NY), supplemented with bovine pituitary extract (20-30 g/ml) and rEGF (0.1-0.2 ng/ml) or SHEM medium was used. Stromal cells were cultured in either Dubellco's Modified Eagle Medium (DMEM) (Gibco, BRL, Life Technologies, Grand Island NY) supplemented with insulin-transferrin-selenium (1000 μ l/l) (ITS) and gentamycin or DMEM supplemented with ITS (1000 μ l/l), gentamycin and fetal bovine serum (100 ml/l) (FBS). Additional surfaces were preadsorbed with laminin based on positive results with YIGSR and related peptides in previous work (16,17) for a period of two hours prior to culture under serum free conditions. Samples were examined on a daily basis for cell attachment, spread and proliferation. For further examination, the cells were fixed in 4% paraformaldehyde (PFA) for 20 minutes. The fixed cells were stained with haemotoxylin and eosin (H&E). Cell surface density was assessed using digital images captured by light microscopy and analysed morphometrically using the Northern Eclipse software to obtain fractional surface coverage in a blinded fashion.

SDS PAGE and Immunoblotting

SDS PAGE and immunoblotting were used to examine the patterns of protein adsorption on the various modified surfaces in order to provide information about the adsorption of various

serum proteins on the various surfaces and to examine whether differences between the surfaces cultured with cells in the presence of serum could be correlated to adsorption of specific proteins. Platelet poor ACD plasma prepared from freshly drawn human blood (25 donors), aliquoted and stored at -70°C was used. While it is possible that differences will exist between the bovine serum used for culture and the human plasma used for examining protein adsorption, we believe that the results using human plasma will be fairly representative of those in culture. Furthermore, well established protocols and antibodies are available in our laboratory for examining human proteins. Surfaces previously equilibrated in PBS buffer, pH 7.4 for 20 hours were exposed to plasma for 3 hours under static conditions at room temperature. Following adsorption, samples were rinsed extensively with PBS and exposed to a 2% sodium dodecyl sulfate (SDS) solution (24 h, 4°C) to elute the adsorbed protein. The eluates were stored at -70°C until needed. Reduced SDS PAGE (12% gels) and immunoblot analysis were performed on the eluted proteins (76). The proteins were transferred to immobilon PVDF membranes (Milipore, Bedford MA) and stained using a stabilized gold sol (Protogold, Cedarlane Laboratories, Hornby, Ontario Canada). Primary antibodies to various plasma proteins, the majority of which were in the form of fractionated antisera developed in goats were used at a dilution of 1:1000. Optical scans of the gold stained gels and immunoblots were analyzed using whole band analysis software (Bioimage, Ann Arbor MI) to determine molecular weights and intensities.

Results

Water Contact Angles

Advancing water contact angles measured on the various modified surfaces are summarized in Figure 1. It can be seen that modification with the amine groups by plasma polymerization of allylamine results in a significant decrease in the water contact angles relative to the unmodified PDMS surface, from approximately 95° to less than 20° . Surface modification with SBA₂-PEG alone, or with a combination of SBA₂-PEG and TGF- β 2 also resulted in significant differences in contact angles relative to the PDMS surface ($\alpha < 0.05$) with a higher water contact angle measured on the SBA₂-PEG+TGF- β 2 modified surfaces. Differences between the SBA₂-PEG+TGF- β 2 surface and the aminated control as well as the SBA₂-PEG only surface and the aminated control were also significant ($\alpha < 0.05$).

Modification of Surfaces with ^{125}I TGF- β 2

Surfaces were modified with ^{125}I labeled TGF- β 2 to quantify the amount of covalently bound growth factor present on the modified PDMS surfaces. Free iodide, determined by TCA precipitation, was less than 2%. The results are summarized in Figure 2 as the amount of TGF- β 2 on the PDMS surfaces for the various surface modifications studied. High levels of TGF- β 2 were noted on all the surfaces following an extensive PBS rinse suggesting high levels of TGF- β 2 adsorption. This high level of adsorption is not surprising given the sticky nature of this protein. On the PEG+TGF- β 2 modified surfaces it is likely that a fraction of the protein is also entrapped in the PEG layer and is not on the surface. A fraction of the TGF- β 2 was removed from all the modified surfaces following an overnight sodium dodecyl sulfate (SDS) rinse. Given that a smaller fraction of the radioactivity was removed by the SDS rinse on the surfaces to which some of the TGF- β should be covalently bound via the bifunctional SBA₂-PEG or SPA₂-PEG spacers, it seems likely that the attachment method results in some covalent attachment of the protein, although the high levels of adsorbed TGF- β 2 make it difficult to quantify the amount that is covalently bound.

X-ray Photoelectron Spectroscopy

Representative XPS results on the TGF- β 2 modified surfaces are summarized in Table 1. In all cases, at least three different surfaces for each modification were examined to determine whether the surfaces were consistent. Unmodified PDMS showed expected results for the carbon, oxygen and silicon signals. The ratio is not exactly what would be expected; this is due to the hydrophobic XPS environment. Compared to the unmodified PDMS, significant differences in the surface composition were noted following amination. As expected, there is a significant decrease in the Si2p signal, as well as the appearance of a N1s signal. Changes are also noted in the C1s envelope, with an increase in the signal consistent with C-N at approximately 286 eV and the appearance of peaks consistent with C-O at 287.2 eV and 288.8 eV. The latter two are likely the result of a small amount of air contamination in the reactive plasma environment. While these changes are consistent with polymerization of allylamine on the surface, the N1s level is significantly less than has been noted previously in our labs using a polyethylene (PE) substrate. This is likely the result of differences in the surface properties of the two polymers. Surface modification under conditions that would be expected to result in the surface adsorption of TGF- β 2 (e.g. modification with TGF- β 2 in the absence of SBA₂-PEG)

results in a decrease in the nitrogen and oxygen signals and a decrease in the carbon signal relative to the aminated surface. This result is somewhat surprising given that the radiolabeled TGF- β 2 results show significant adsorption of the protein, and may be due to differences in adsorption between the surfaces. The Si2p signal on these surfaces has increased to a level similar to that noted on the unmodified control surface. A slight difference in the C1s envelope is also noted on this surface relative to the aminated substrate, although the changes are neither dramatic nor conclusive. It should be noted that these surfaces should also contain a methoxy-SBA-PEG layer that should lead to an increase in the carbon and oxygen signal relative to the PDMS control. It seems likely that TGF- β 2 is embedded in this PEG layer and that this may be the reason for the relatively low N1s signal on these surfaces. This result was obtained consistently on repeated surfaces. Surface modification with SBA₂-PEG but with no TGF- β 2 resulted in a surface with a lower total carbon, but higher total oxygen relative to the aminated PDMS control. The increase in the O1s signal in particular is consistent with surface modification with PEG. Also notable is the continued presence of a lower but still significant N1s signal that is likely occurring due to the aminated substrate. Modification of the surface with TGF- β 2 covalently bound to the SBA₂-PEG spacer resulted in a significant increase in the N1s signal to 2.5 atom %, approximately double that noted on the aminated surface alone. Further evidence of the presence of the growth factor on the surface is the C1s envelope which contains a significant amount of C-OR (5.3 atom%) and C(OOR) (1.7 atom %). It should be noted that it is likely that the TGF- β 2 is also embedded in a PEG layer on these surfaces, although it is hoped that a significant fraction of the growth factor is covalently attached to the PEG spacers.

Atomic Force Microscopy

Atomic force microscopy images of the various modified surfaces are shown in Figure 3. Compared to the unmodified PDMS surface shown in Figure 3(a), significant roughness and an altered morphology can be seen on the aminated surface shown in Figure 3(b), demonstrating that there has been some surface alteration as a result of the plasma reaction. This is likely due to the presence of a multi-layer of plasma-polymerized allylamine on the surface. Immobilization of SBA₂-PEG on the aminated PDMS surface also results in a change, with a decrease in the roughness as seen in Figure 3(c). Differences were also observed between the aminated surfaces and the surfaces modified by both TGF- β 2 in the absence of PEG as seen in

Figure 3(d), SBA₂-PEG+TGF- β 2 as seen in Figure 3(e) and methoxy-SBA-PEG+ TGF- β 2 as seen in Figure 3(f), with significantly more roughness noted on the surfaces exposed to the growth factor. Large aggregates were noted on the aminated surfaces modified with TGF- β 2. The size of these aggregates is consistently on the order of 1 μ m, and may represent aggregated TGF- β 2.

Cell Culture Studies

Results from the cell culture studies for TGF- β 2 modified surfaces and cells grown in serum free medium are summarized in Figure 4. The most notable and surprising feature of these results is the lack of adhesion of the stromal cells with surface coverage averaging only 3-5%. In comparison, the corneal epithelial cells showed a much higher level of adhesion on these surfaces, averaging 50-65%. There was an observed difference in the surface coverage of both epithelial and stromal cells on the modified surfaces in comparison to the aminated surface as expected. It is surprising that there was a high level of adhesion to the SBA₂-PEG modified surface, particularly given that the surfaces were incubated in serum free medium. However, it is notable that the surface with covalently attached TGF- β 2 showed the highest level of adhesion.

Since the adsorption of serum proteins may promote the initial adhesion of the cells to the surfaces, the effect of culture in the presence of 10% serum was examined on various surfaces. Surfaces modified using either a high or low concentration of TGF- β 2 in the reaction solution were also examined. Figure 5 summarizes results of epithelial cell coverage to the various surfaces in the presence of serum, laminin and anti-TGF- β 2, respectively. The results for different TGF- β 2 concentrations used in the modification procedure are shown in Figure 6. A comparison between epithelial cell surface coverage using identical surfaces (high and low concentrations of SBA₂-PEG+TGF- β 2 solution) prepared on the same day and cultured at the same time shows higher coverage in the presence of serum. It is also interesting to note that the presence of serum seems to mask the effect of the growth factor, with similar levels of epithelial cell coverage seen on surfaces modified with both high and low TGF- β 2 solution concentrations. In the absence of serum, a definite surface concentration effect was noted, with a higher amount of TGF- β 2 resulting in a higher level of cell coverage. Surprisingly, the adsorption of laminin to the surfaces prior to culture, while resulting in an increase in the epithelial cell coverage relative to the controls, did not provide the dramatic effect expected based on our previous results

(16,17). In all cases, except in the presence of anti-TGF- β 2 antibodies, the epithelial cell coverage was considerably greater than on the aminated controls. The effect of the TGF- β 2 and its activity on the surface is clearly demonstrated with a decrease in coverage in the presence of anti-TGF- β 2 under serum free conditions.

Stromal cell coverage to the same surfaces is summarized in Figure 7. While the trends were similar to those noted with the epithelial cells, coverage is much lower with these cells. It is interesting to note however, that in the presence of the anti-TGF- β 2 antibody, there was a decrease in the level of coverage, suggesting that the growth factor is having some effect in this system. Again, it can be seen that in the presence of serum, the effect of TGF- β 2 surface concentration seems to be masked, but that a small concentration effect occurs in the absence of serum. Laminin adsorption in this case did not affect surface coverage at all, with levels similar to the aminated control, as expected would be expected for stromal cells.

SDS PAGE and Immunoblotting

Since serum-containing medium was used in some of the studies to improve cell adhesion, the potential surface specific differences in protein adsorption and their effect on cell adhesion under serum containing conditions were examined. While cells were grown in the presence of fetal bovine serum, protein adsorption studies were done using human plasma due to the ready availability of antibodies to human proteins in our laboratories. It is expected that the behaviour of bovine proteins will be similar and that adsorption from serum will be similar to adsorption from plasma. Immunoblots of elutes following plasma protein adsorption to the various modified surfaces are summarized in Figure 8. While there are significant differences between the unmodified and aminated surfaces and the PEG modified surfaces, there are not notable differences between the various TGF- β 2 modified surfaces. In general, there were more proteins and larger quantities of proteins found on the SBA₂-PEG + TGF- β 2 surface, as well as the methoxy-SBA-PEG+TGF- β 2 and TGF- β 2 adsorbed surfaces than on either the PDMS control or on the aminated control. Of specific interest, fibronectin was noted only on the surfaces with TGF- β 2, but was not found on the aminated or on the unmodified PDMS. While vitronectin was found in similar quantities on all surfaces, it was only activated on the TGF- β 2 modified surfaces. Many of the coagulation proteins, including Factor XI and Factor XII were found in greater quantities on the surfaces with TGF- β 2. Similar differences were noted with the

complement proteins, with none or only small quantities on the unmodified and aminated surfaces, but significantly more on the three TGF- β 2 modified surfaces. Of ophthalmic interest, plasminogen, which has been shown to be present in significant quantities in tears, was also found only on the TGF- β 2 modified surfaces, but not on the two controls. From these results, it seems likely that any differences in cell coverage on the modified surfaces in the presence of serum are the result of interactions with the TGF- β 2 and not the result of differences in serum protein adsorption, as all of the TGF- β 2 modified surfaces showed similar protein adsorption patterns and levels.

Discussion

It is clear that for long-term success, synthetic polymer based tissue substitutes must interact with the cells of the surrounding host tissue. This requires the presence of appropriate bioactive functionality mimicking that normally present *in vivo*. Covalent attachment of cell adhesion peptides and proteins has been widely used to promote adhesion of different cell types to biomaterial surfaces. Recently, surface modification with growth factors has been shown to promote desirable interactions between the cellular component of a tissue substitute and the material component (72-74,77). In the cornea, complex interactions between various cell types and the corneal implant must be maintained in order to achieve required implant anchoring and optical properties. In order to promote corneal epithelial cell adhesion to the anterior surface of an artificial corneal implant, we have previously modified our substrate surfaces with cell adhesion peptides, including cell adhesion peptide combinations with reasonable success (16,17). However, the problem of corneal epithelial cell downgrowth at the interface of the implant and the host tissue was not addressed in our previous studies. In the current work, we hypothesized that modification of the stromal contacting surface of the implant with appropriate growth factors may help to inhibit corneal epithelial cell downgrowth. Based on current literature, TGF- β 2 was selected as the growth factor for immobilization due to its reported ability to stimulate the growth of corneal stromal cells while inhibiting the growth of corneal epithelial cells (48,58-60,65,66, 68-70).

The surface modification procedure developed by Bentz et al. (73) was modified to allow us to immobilize TGF- β 2 to aminated PDMS substrates. TGF- β 2 and the bifunctional SBA2-PEG were first placed in solution to allow the growth factor to covalently attach to the PEG

spacer. The aminated PDMS surfaces were then exposed to this solution, allowing the unreacted terminal SBA group of the bifunctional SBA₂-PEG spacer, as well as any unreacted SBA₂-PEG to be immobilized on the aminated surface. The reaction conditions were optimized to minimize SBA₂-PEG hydrolysis, the extent of TGF- β 2 attachment to both ends of the SBA₂-PEG spacer and binding of TGF- β 2 to itself by using a molar excess of SBA₂-PEG to TGF- β 2, low temperature conditions and a short initial reaction time between the PEG spacer and the growth factor. The rapid kinetics of the reaction between the terminal SBA group of the bifunctional PEG and amine groups of the growth factor (73) should permit a significant amount of the TGF- β 2 to be covalently attached to PEG during a short reaction time. The non-reacted SBA group of the bifunctional SBA₂-PEG spacer is then free to attach to the aminated PDMS surface in the longer reaction period following.

The presence of TGF- β 2 on the surfaces was confirmed by modifying the aminated PDMS surfaces using radiolabelled growth factor. These results suggest that a significant fraction of the TGF- β 2 may be adsorbed to the surfaces. This is expected given that the charge and hydrophobic properties of TGF- β 2 enable high affinity binding of the this growth factor on numerous surfaces. However, given that a smaller fraction of the radioactivity was removed by extensive rinsing on the surfaces to which some of the TGF- β 2 should be covalently bound via the bifunctional SBA₂-PEG or SPA₂-PEG spacer, it seems likely that the attachment method results in some covalent attachment of the protein. The radiolabelling results suggest that the maximum surface density of covalently bound TGF- β 2 on these surfaces is the on the order of 0.5 $\mu\text{g}/\text{cm}^2$, although the unknown high levels of adsorption make it difficult to accurately quantify the amount that is truly covalently bound. This estimated quantity is higher by a factor of 300 on a molar basis, than the EGF immobilization results reported by Kuhl and Griffith-Cima (72). This difference in estimated quantities may be attributed to the molecular weight difference between the two growth factors, the well-known surface binding affinity of TGF- β 2 and our demonstrated high levels of TGF- β 2 surface adsorption and absorption.

Additional surface characterization by advancing water contact angles, XPS and AFM clearly demonstrated differences between the various modified surfaces and suggest that a significant amount of TGF- β 2 was present on the PDMS surfaces following the modification procedure. A significant decrease in advancing water contact angle following surface amination

of the PDMS surface as well as a significant increase in advancing water contact angle following exposure of the aminated surface to SBA₂-PEG+TGF- β 2 demonstrate that the PDMS surface was modified at each stage of the modification procedure. Changes in the XPS results, including the decrease in the Si2p signal and the appearance of a significant N1s peak as well as changes in the C1s envelope, which contains a significant amount of C-OR and C-(OOR) provide further evidence of the presence of TGF- β 2 on the surfaces, although again it is difficult to quantify the amounts that are actually covalently attached versus the amount of TGF- β 2 adsorbed to the surfaces. Differences in the C1s envelope and a lower N1s contribution was observed on the methoxy-SBA-PEG+TGF- β 2 surfaces compared to the SBA₂PEG+TGF- β 2 providing additional evidence that the high level of radioactivity associated with the methoxy-SBA-PEG+TGF- β 2 surfaces is the likely the result of growth factor entrapped within the PEG layer.

AFM scans showed changes in the surface morphology of the various modified surfaces. Aminated surfaces were considerably rougher than the unmodified PDMS. Roughness decreased substantially with SBA₂-PEG modification and increased on the surfaces exposed to TGF- β 2. However, again it was difficult to determine whether there were differences between the surfaces with adsorbed TGF- β 2 compared to the surfaces exposed to SBA₂-PEG+TGF- β 2.

TGF- β 2 was selected as the growth factor for immobilization due to its reported ability to stimulate the growth of corneal stromal cells while inhibiting the growth of corneal epithelial cells (48,58-60, 65, 66, 68-70). The TGF- β 2 isoform was selected for immobilization based on reports of higher levels of TGF- β 2 receptor localization in the healing cornea (63) and potential greater inhibitory effects on EGF, KGF, or FGF stimulated corneal epithelial cells compared to the inhibitory effects of TGF- β 1 (28,62). All cells in the current work were grown in EGF containing medium.

However, when the TGF- β 2 modified surfaces were cultured *in vitro*, the expected interactions between the corneal cells and the immobilized growth factor were not observed. Rather than being inhibited by the presence of the growth factor, relative to the unmodified surfaces, coverage with corneal epithelial cells increased on the growth factor modified surfaces. Stromal cell growth appeared to be inhibited by the presence of TGF- β 2. The observed cell growth appears to be the result of interactions between the cells and active TGF- β 2 since the addition of a stoichiometric excess of anti-TGF- β 2 antibody added to the both the epithelial cell

and stromal cell culture medium, respectively, resulted in a decrease in cell coverage. In both cases, cell coverage returned to levels similar to those noted on the control surfaces. A TGF- β 2 surface concentration effect was noted with both cell types in the absence of serum, with higher levels of surface coverage noted on surfaces generated using higher concentrations of TGF- β 2 in the reaction mixture.

As suggested by Kuhl and Griffith-Cima (72) cell adhesive properties are generally not attributed to growth factors. We therefore examined the cell interactions with the various modified surfaces in the presence of 10% serum. Stromal cell coverage on these surfaces remained low even with the presence of the serum. The observed concentration effect observed for epithelial cells cultured in the absence of serum was no longer seen when serum was added to the culture medium. Surprisingly, preadsorption of laminin to the substrates was significantly less effective at promoting corneal epithelial cell coverage, despite our previous studies that showed that covalent immobilization of the laminin binding peptide YIGSR resulted in significant enhancement of epithelial cell adhesion (16). As expected, laminin preadsorption had little if any effect on the coverage of stromal cells. Immunoblot results for protein adsorption from plasma appear to demonstrate that any differences in cell coverage on the modified surfaces cultured in the presence of serum are the result of interactions with the growth factor and not the result of differences in serum protein adsorption.

Based on the numerous factors that may play a role in the *in vitro* and *in vivo* effects of TGF- β on corneal cell proliferation and migration, it is not surprising that the reported results in literature are controversial. For example, TGF- β was shown to enhance wound strength and stimulate epithelial cell growth *in vivo* in contrast to the results of inhibition of epithelial cell growth *in vitro* (78). Epithelial cells grown as anchorage dependent monolayers *in vitro* were inhibited by TGF- β , but the same cells were stimulated by TGF- β treatment when grown in an anchorage independent manner (82). Discrepancies in the results may be caused by differing cell culture conditions (49) cell type and state of differentiation (79), TGF- β concentration (80) and possible interactions between TGF- β 2, the extracellular matrix and other growth factors present in the environment (61,81). For example, the presence of TGF- β 1 may be required for the higher levels of TGF- β 2 receptors noted in the healing cornea (63). And an interaction effect between TGF- β and other growth factors, specifically epidermal growth factor (EGF), keratocyte

growth factor (KGF) and hepatocyte growth factor (HGF) has been suggested (62) with TGF- β inhibiting the mitosis of cell growth promoted by these growth factors. Interactions with an appropriate extracellular matrix may also affect TGF- β signaling. These discrepancies may also be possibly due to the use of different methods, animal species or clinical situations used in the various *in vivo* studies (24,60,65,66).

It is also possible that the substrate, following amination and exposure to SBA2-PEG and TGF- β 2 may be adversely affecting the adhesion and growth of corneal stromal cells resulting in stromal cell surface coverage on the order of only a few percent after 1 week of incubation. It is interesting to note that differences in stromal cell surface coverage between the aminated surfaces exposed to TGF- β 2 are very minimal. Given this lack of stromal cell growth in combination with the surface analysis and cell culture results of this study, which clearly demonstrate that active TGF- β 2 is present on the modified aminated PDMS surfaces, it may be that immobilized TGF- β 2 is not the appropriate method of growth factor delivery to corneal stromal cells.

It is also interesting to note, that in the stroma, there is evidence that TGF- β may not be directly responsible for stimulating growth. It has been suggested that treatment of healing corneas with TGF- β results in an increase in the production of platelet-derived growth factor (PDGF) by corneal epithelial cells, which in turn stimulates stromal cell growth. Furthermore, TGF- β has been suggested to result in the differentiation of stromal cells from fibroblast to myofibroblasts.

The results of this study suggest that an alternative modification procedure will be necessary to develop a surface that will inhibit epithelial cell proliferation while promoting stromal cell adhesion and ingrowth. Further examination of alternative modification procedures and more complex delivery systems will be the focus of future studies. In this study, TGF- β 2 modified surfaces were cultured using either corneal epithelial cells or stromal cells in isolation. Therefore, it is also of interest to study the interactions of the growth factor modified materials with both corneal epithelial and stromal cells simultaneously in an environment that takes into account the extracellular milieu of these cells in their *in vivo* environment using an organ culture system.

Conclusions

The surfaces of poly (dimethyl siloxane) were modified by covalently attaching TGF- β 2 in an attempt to generate a surface with stromal stimulating and epithelial inhibiting properties for use as a synthetic artificial cornea. While the amounts of covalently attached growth factor were difficult to quantify due to strong interactions between the growth factor, the PEG layer, and the substrate surface differences in the modified surfaces determined by advancing water contact angles, XPS and AFM suggest that a significant amount of growth factor was present on the polymer surface. However, when corneal epithelial and stromal cells were grown on the various modified surfaces, cell interactions with the surfaces were consistently opposite to those expected. Stromal cell growth seemed to be inhibited, with cell coverage less than 5% after 1 week of incubation. Epithelial cell growth seemed to be promoted by the presence of the growth factor, with coverage on the order of 50% or more after 1 week of growth. This growth seemed to be stimulated by the presence of the growth factor since the presence of a stoichiometric excess of anti-TGF- β 2 antibodies gave cell coverage similar to those on the control surfaces. The presence of serum proteins did not seem to affect these results with either cell type. Furthermore, a TGF- β 2 concentration effect was noted with both cell types in the absence of serum, with high TGF- β 2 concentrations on the surface resulting in higher cell coverage on the surfaces. It is clear that alternative modification procedures and more complex delivery systems may be required in order to develop growth factor modified surfaces that can successfully modulate the interactions between the cellular components of the cornea and an artificial corneal implant.

Acknowledgements

The technical assistance of Rena Cornelius and Monique Bergeron are gratefully acknowledged. Funding was provided by the Natural Sciences and Engineering Research Council of Canada.

Figure Legends

Figure 1. Advancing water contact angles measured on PEG and TGF- β 2 modified aminated PDMS surfaces. Surface modification with PEG alone, or with a combination of PEG and TGF- β 2 also resulted in significant differences in contact angles relative to the PDMS surface ($\alpha < 0.05$).

Figure 2. Surfaces were modified with ^{125}I labeled TGF- β 2. The results are summarized as the amount of TGF- β present on the modified PDMS surfaces. These results suggest that it is likely that the immobilization method results in covalent attachment of TGF- β 2. The high levels of adsorbed TGF- β 2 make it difficult to quantify the amount of covalently bound protein. The PEG layer may reduce non-specific TGF adsorption.

Figure 3. Atomic force microscopy images of the various modified PDMS surfaces. Aminated surfaces were considerably rougher than the unmodified PDMS. Roughness decreased significantly with SBA2-PEG modification and increased on the surfaces exposed to TGF- β 2.

Figure 4. Summary of the results from the cell culture studies for TGF- β 2 modified surfaces and cells grown in serum free medium. The most notable and surprising feature of these results is the lack of adhesion of the stromal cells with surface coverage averaging only 3-5%. In comparison, the corneal epithelial cells showed a much higher level of adhesion on these surfaces, averaging 50-70%.

Figure 5. Summary of the results of epithelial cell coverage on the various surfaces cultured using different cell culture conditions. An interaction between active TGF- β 2 and the epithelial cells is demonstrated by the significant decrease in cell surface coverage observed following the addition of anti-TGF- β to the culture medium under serum free conditions. The adsorption of laminin to the surfaces prior to culture, while resulting in an increase in the epithelial cell coverage relative to the controls, did not provide the expected increase in cell adhesion.

Figure 6. Summary of the results of epithelial cell coverage on the surfaces modified using different TGF- β 2 concentrations cultured both in the absence and in the presence of serum. In the absence of serum, a surface concentration effect was noted, with higher amounts of TGF- β 2 resulting in higher levels of cell coverage.

Figure 7. Summary of stromal cell coverage on the various modified surfaces cultured using different cell culture conditions. While the trends were similar to those noted with the epithelial

cells, coverage is significantly lower with these cells. An interaction between TGF- β 2 and the stromal cells is demonstrated by the significant decrease in coverage observed in the presence of anti-TGF- β under serum free conditions. Again, it can be seen that in the presence of the serum, the effect of TGF- β 2 surface concentration seems to be masked, but that a small concentration effect occurs in the absence of serum. Laminin adsorption in this case did not affect surface coverage at all, with levels similar to the aminated control, as expected would be expected for stromal cells.

Figure 8. Summary of the immunoblots of elutes following plasma protein adsorption to the various modified surfaces. While there are significant differences between the unmodified and aminated surfaces and the PEG- modified surfaces, there are not notable differences between the various TGF- β 2 modified surfaces.

Figures

Figure 1.

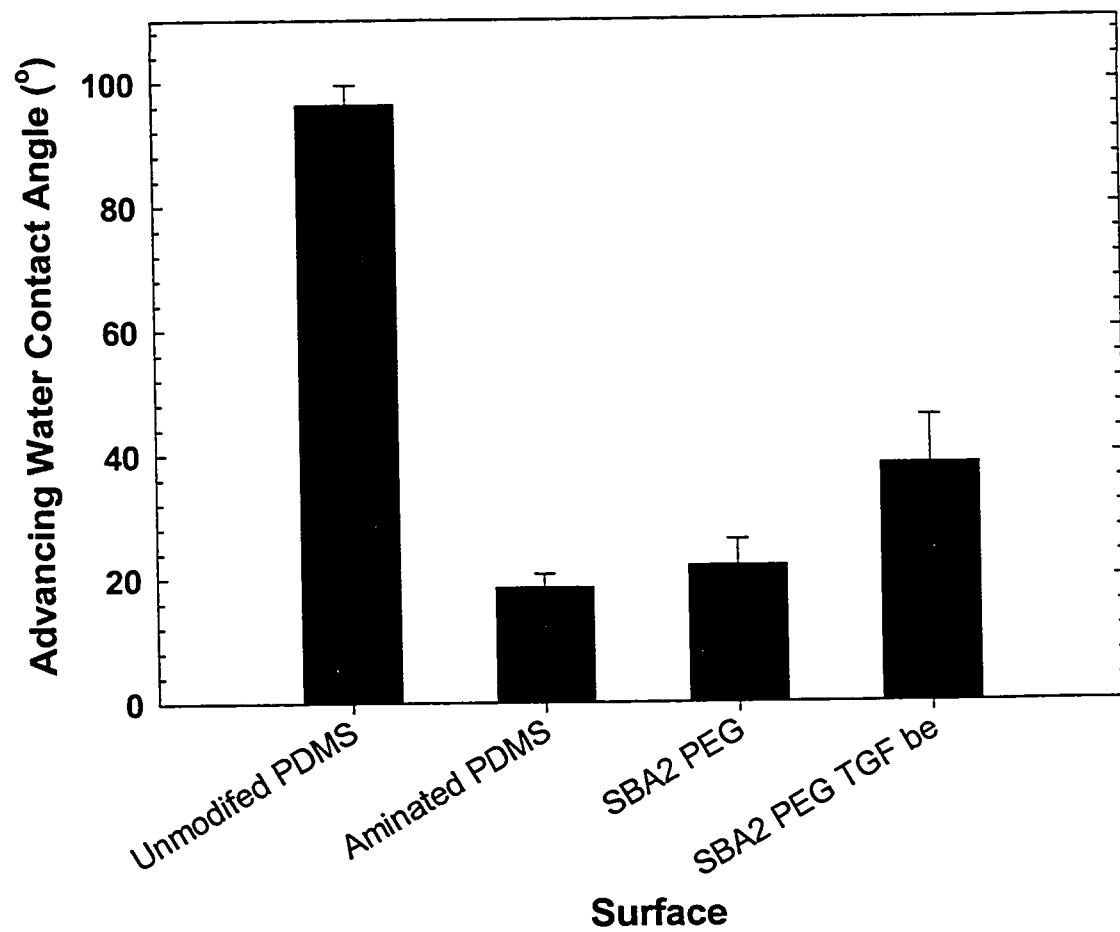


Figure 2.

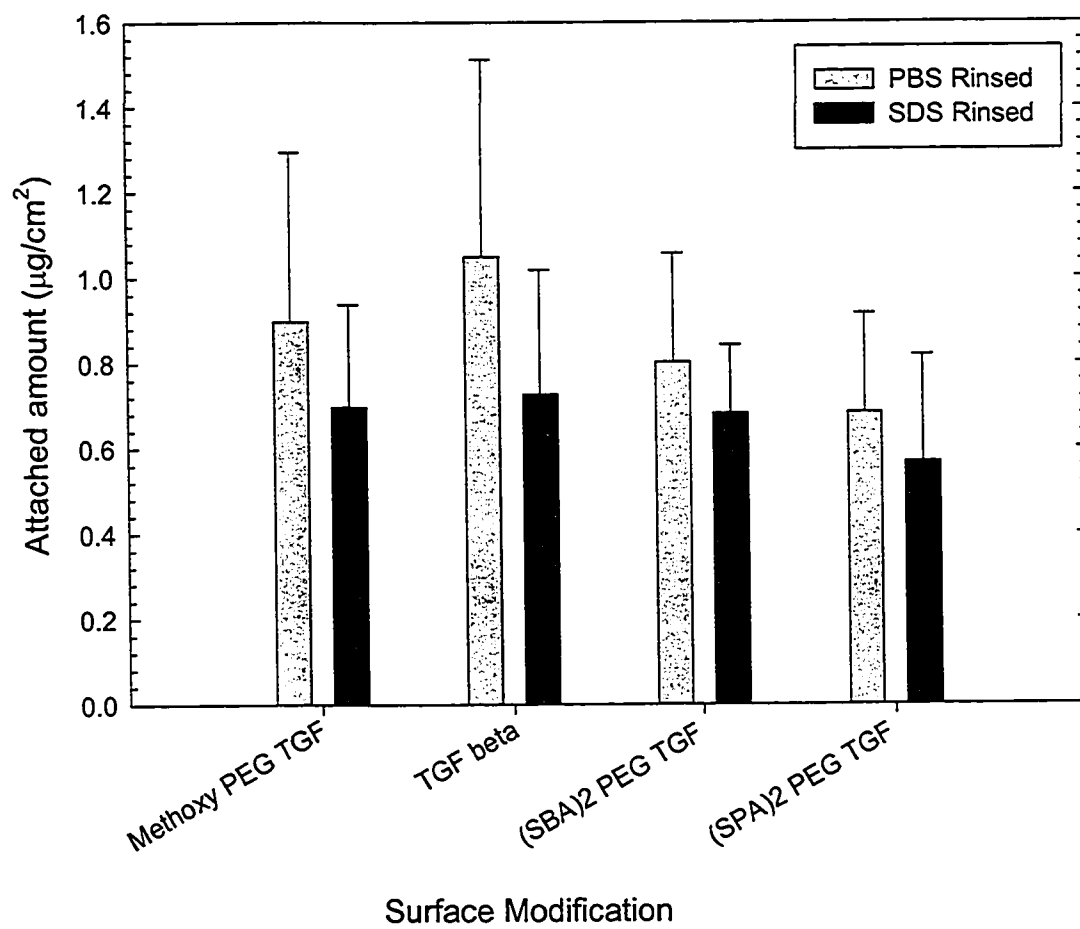
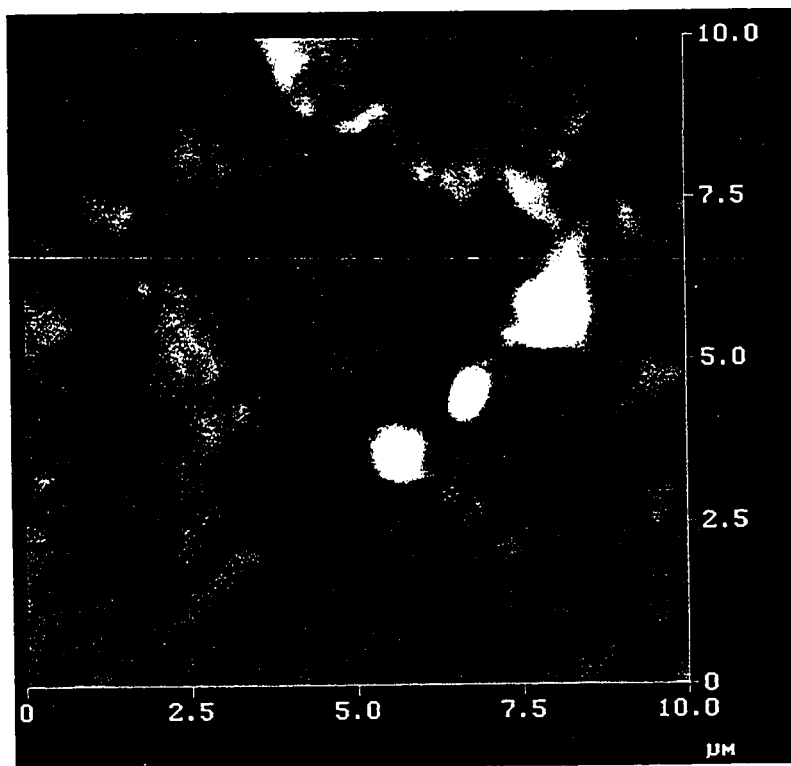
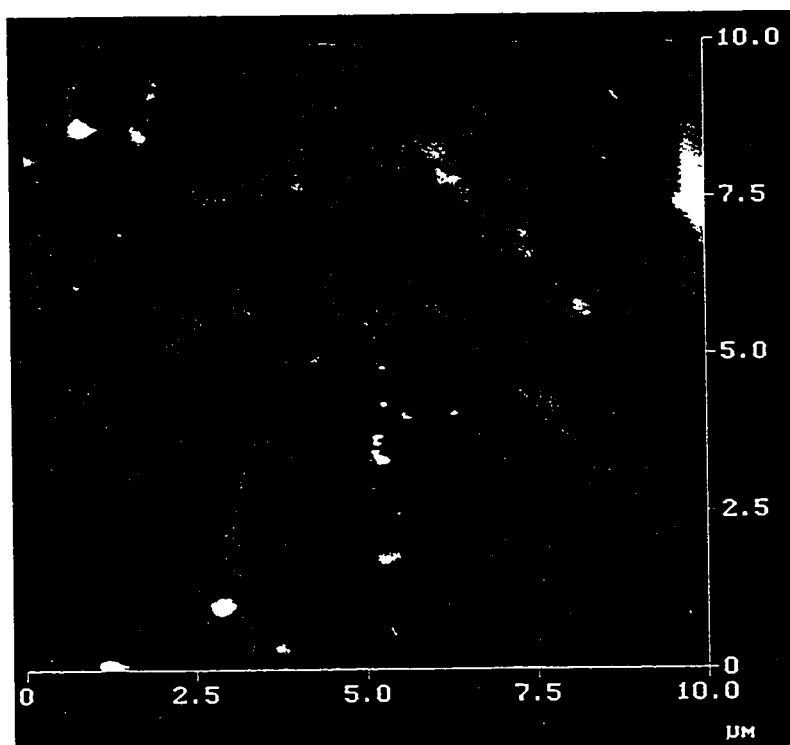


Figure 3.

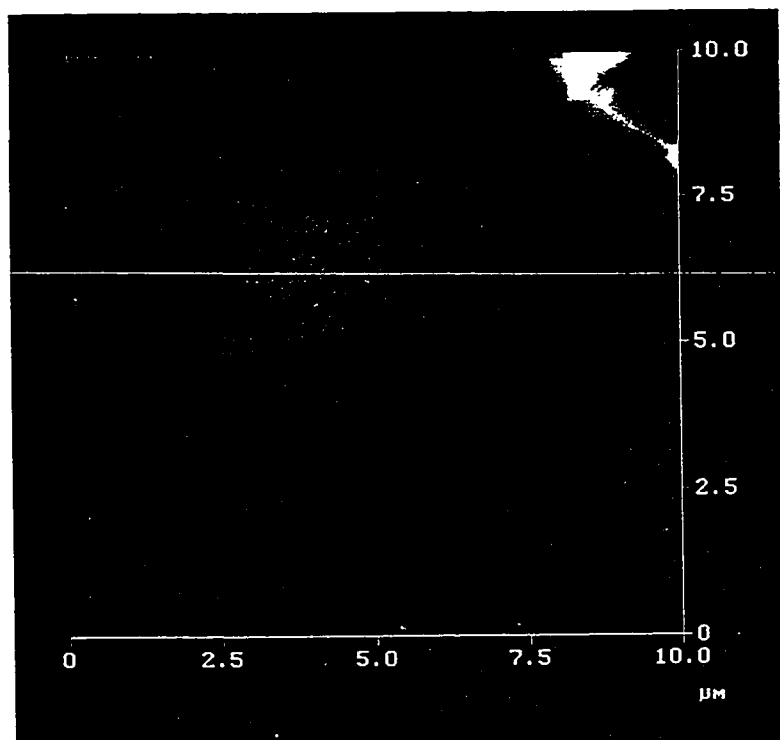
(a) Unmodified PDMS



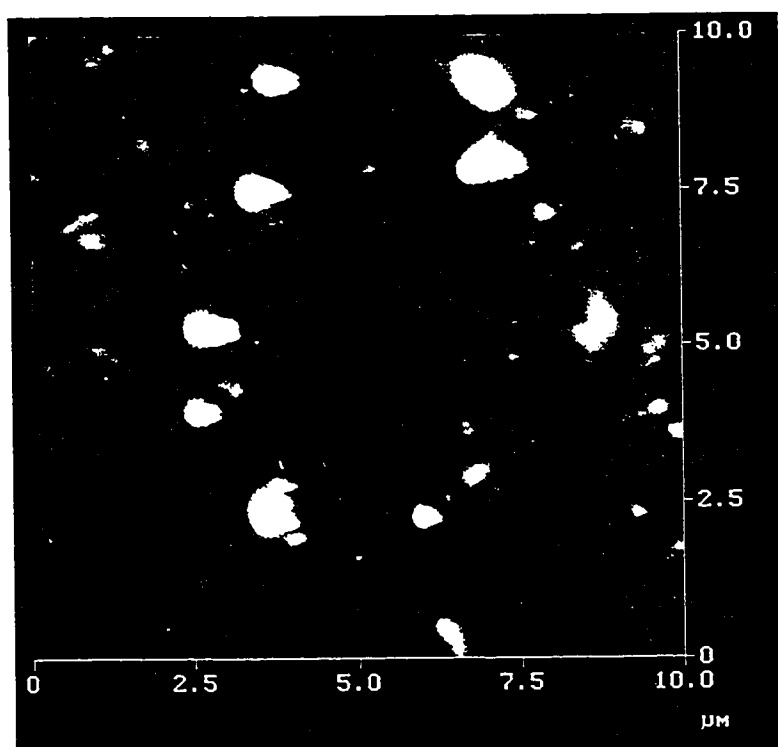
(b) aminated PDMS



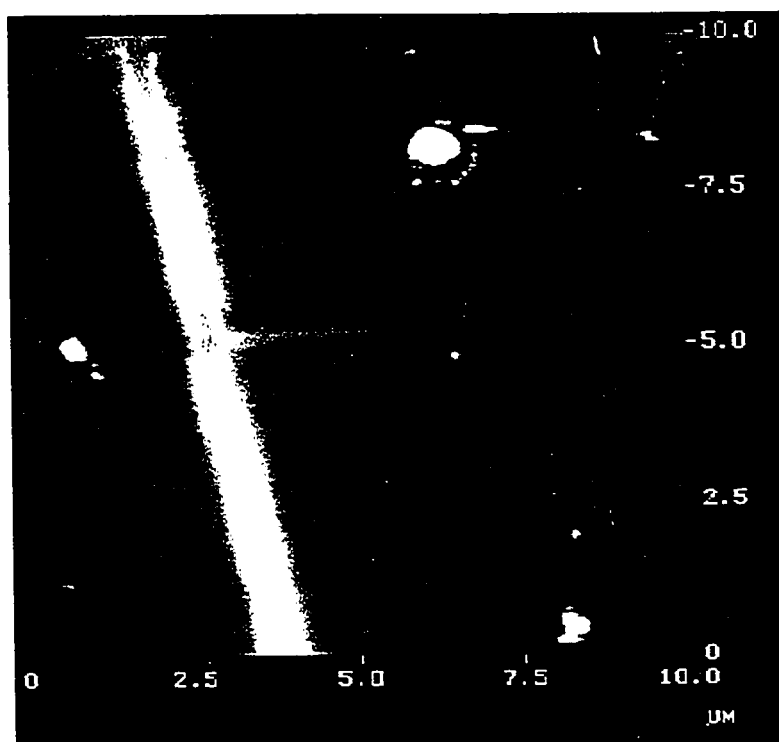
(c) aminated PDMS exposed to SBA₂-PEG



(d) aminated PDMS exposed to TGF- β 2



(e) aminated PDMS exposed to SBA2-PEG + TGF- β 2



(f) aminated PDMS exposed to methoxy-SBA-PEG + TGF- β 2

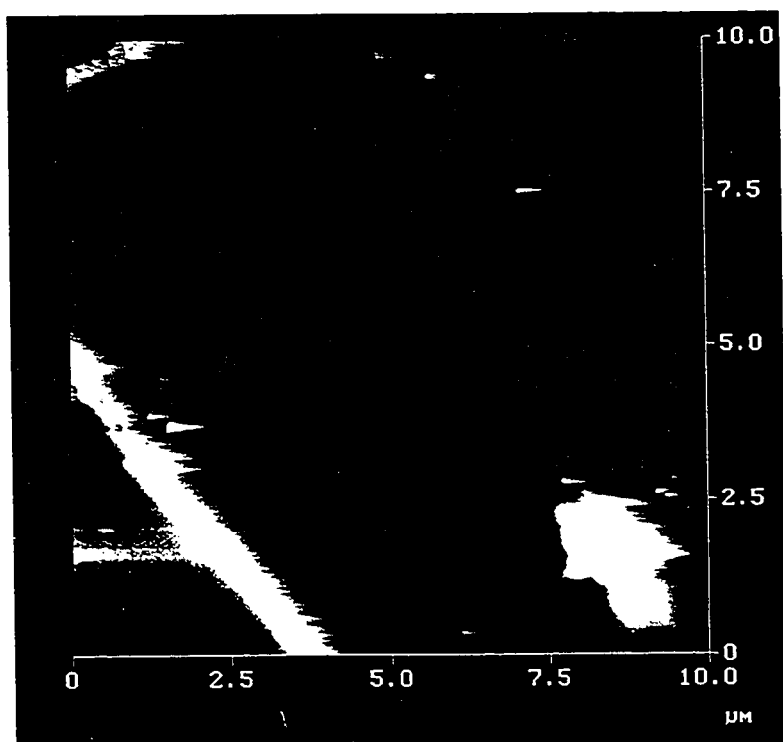


Figure 4.

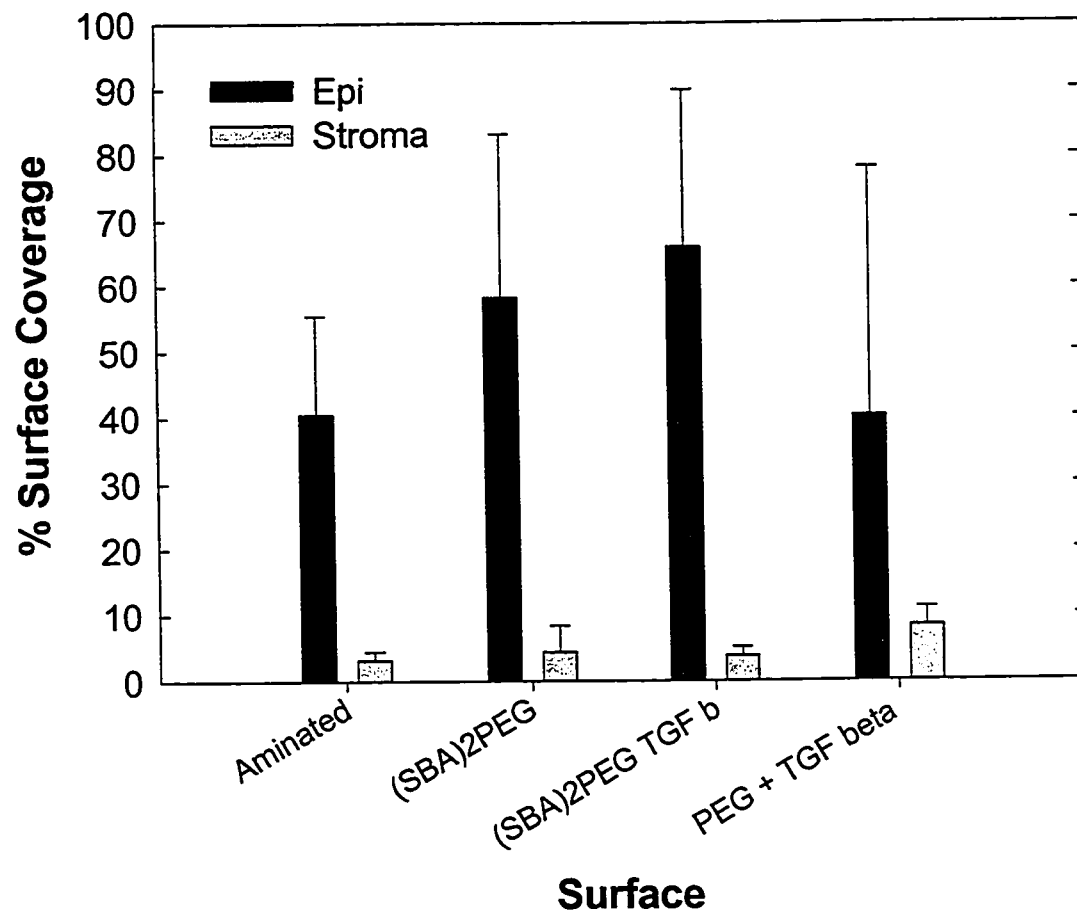


Figure 5.

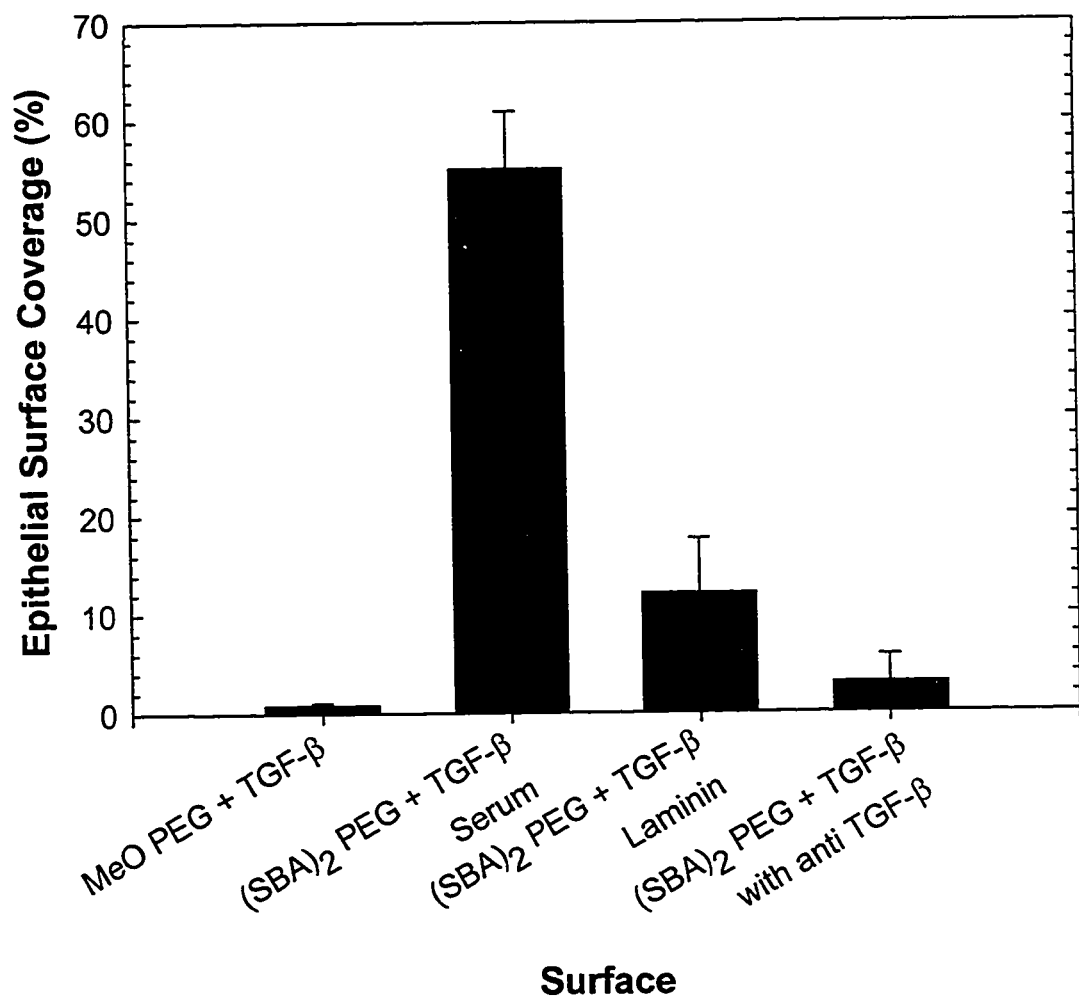


Figure 6.

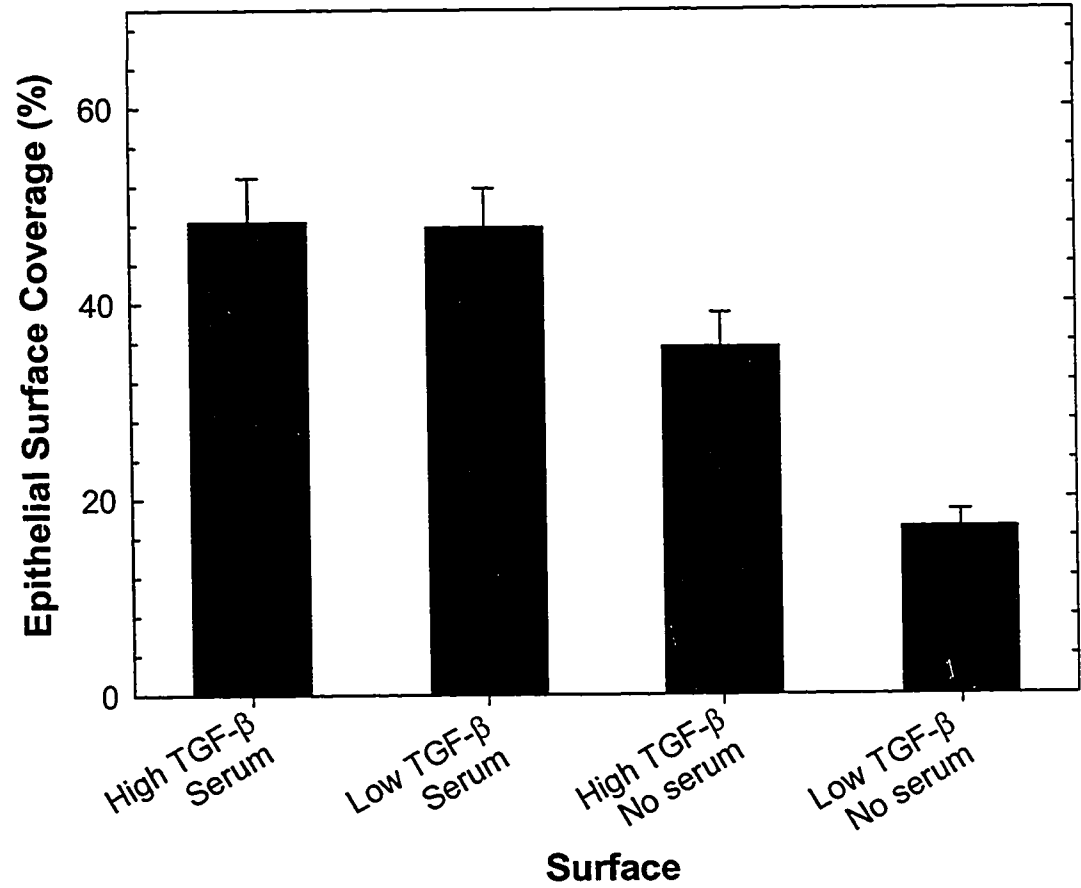


Figure 7.

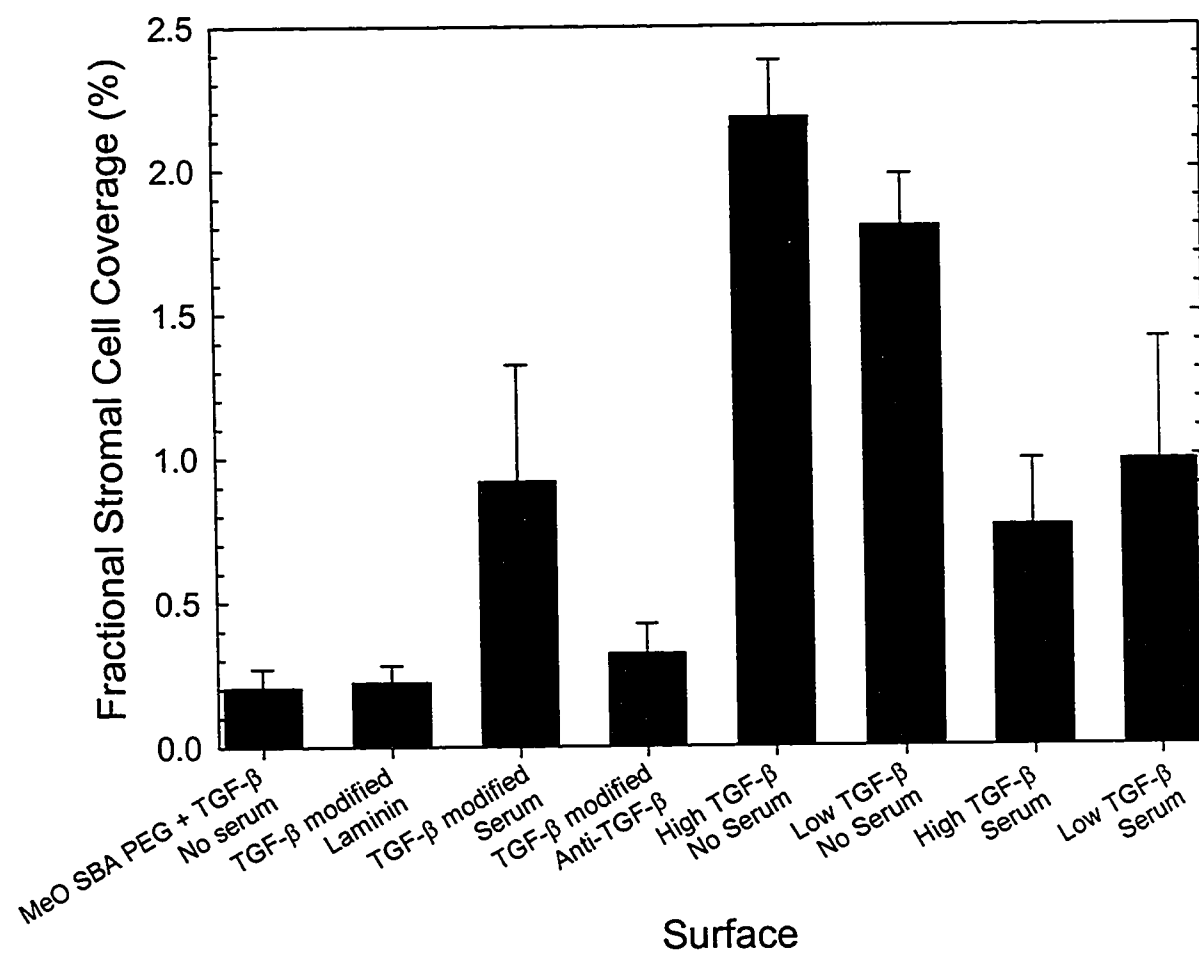
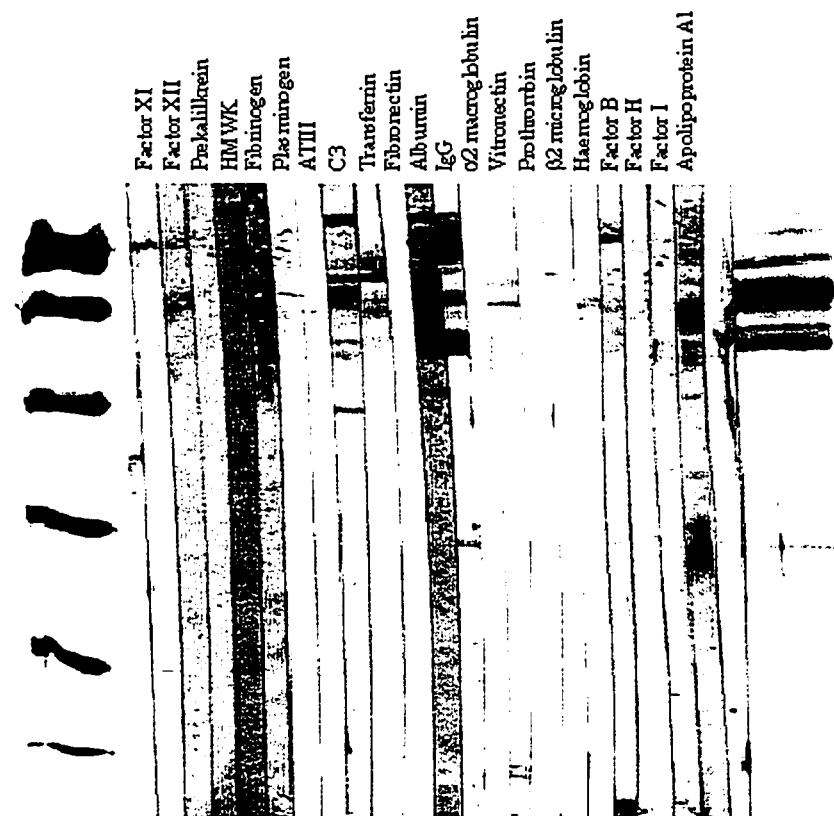
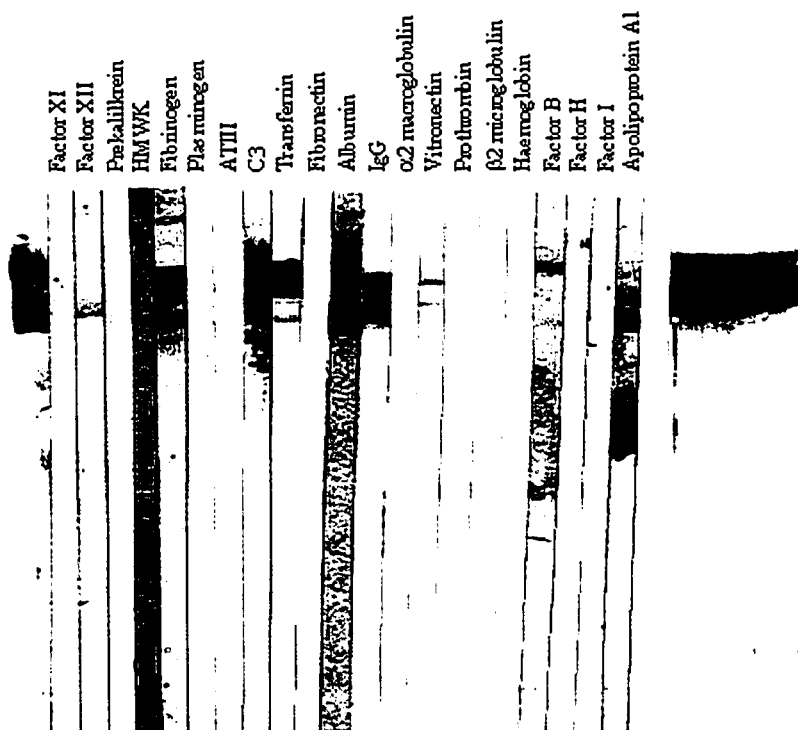


Figure 8.

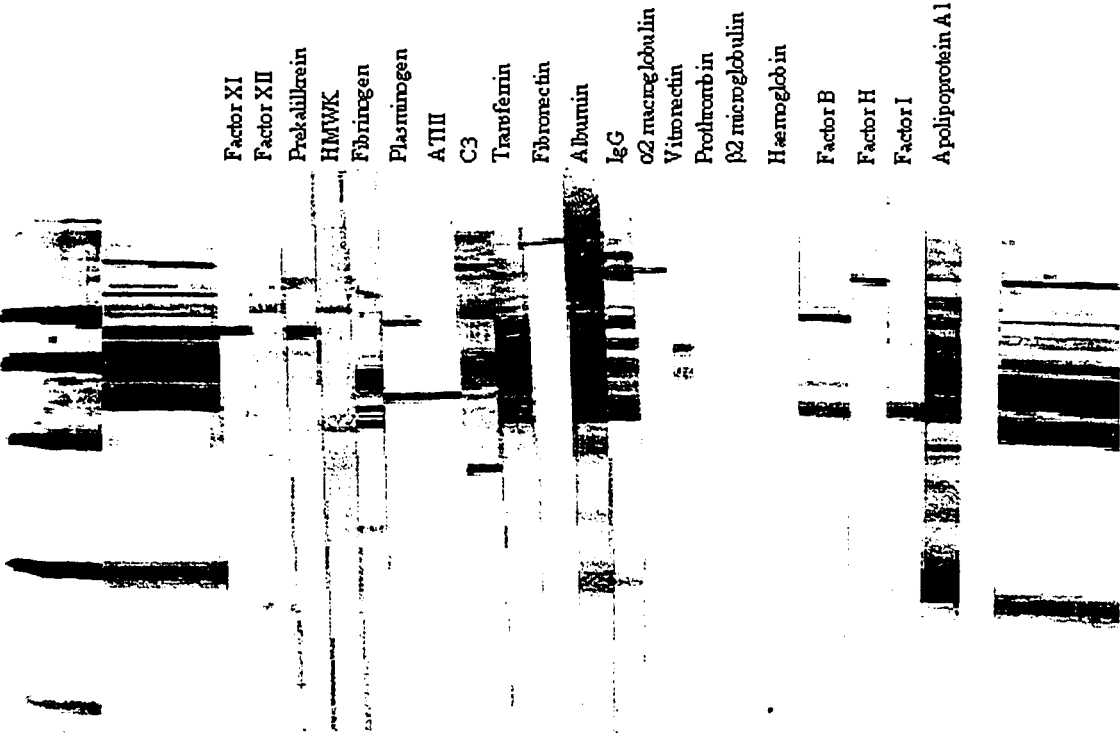
(a) unmodified PDMS



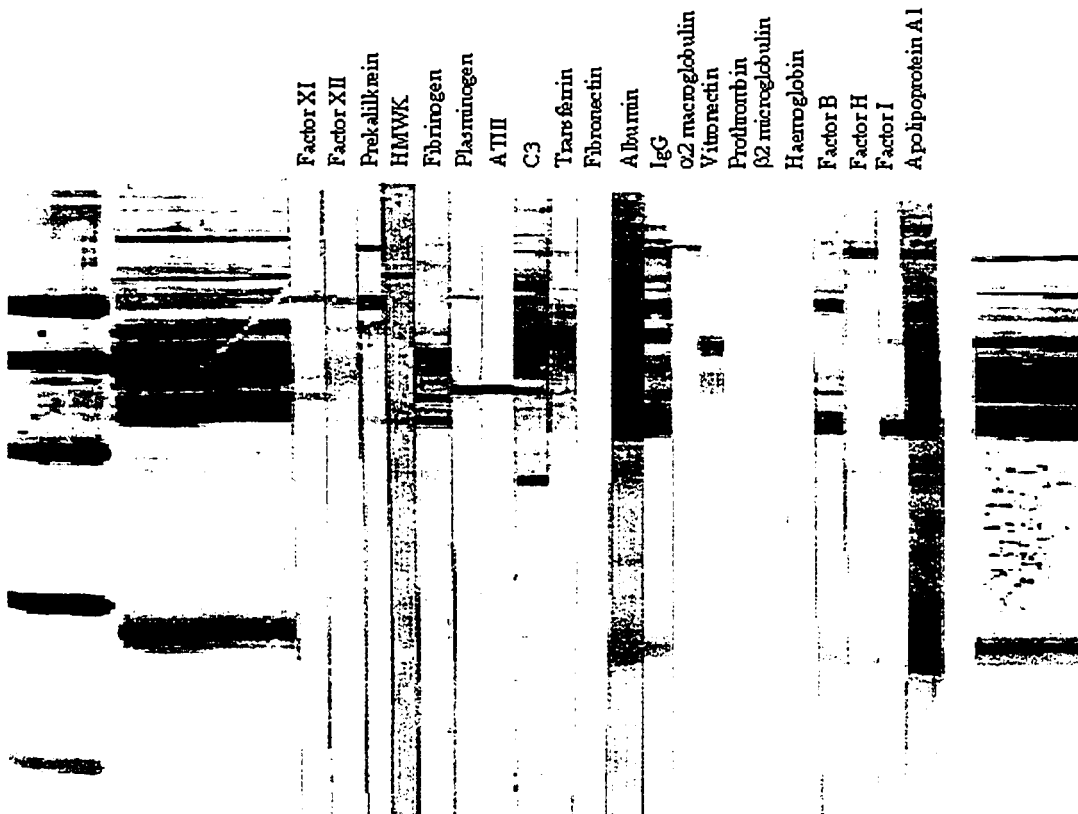
(b) aminated PDMS



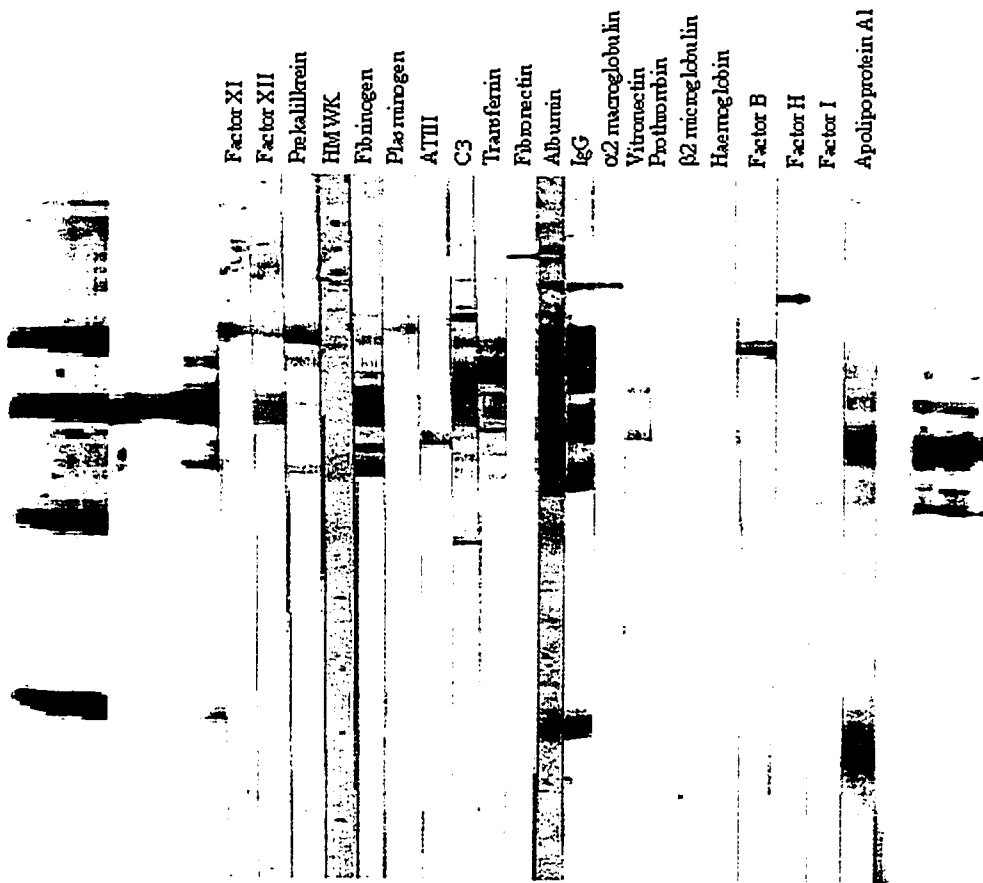
(c) aminated PDMS exposed to methoxy-SBA-PEG+TGFβ2



(d) aminated PDMS exposed to SBA2-PEG+TGFβ2



(e) aminated PDMS exposed to TGF β 2



Tables

Table 1: Summary of the XPS results for the modified aminated PDMS surfaces. Modification of the surface with TGF- β 2 covalently bound to the SBA₂PEG spacer resulted in a significant increase in the N1s signal to 2.5 atom %, approximately double that noted on the aminated surface alone. Further evidence of the presence of the growth factor on the surface is the C1s envelope which contains a significant amount of C-OR (5.3 atom%) and C(OOR) (1.7 atom %).

Sample	C1s					O1s	Si2p	N1s
	Total	285.0 C-C	285.8 C-N	287.2 C-OR	288.8 C(OOR)			
Unmodified PDMS	40.4	37.7	2.7			33.8	25.6	
Aminated PDMS	44.0	33.2	5.3	3.3	2.2	32.2	14.2	1.4
TGF- β adsorbed	37.8	32.1	4.0	1.7		35.7	26.1	0.4
(SBA) ₂ PEG	34.2	29.9		3.6	0.8	39.6	25.3	1.0
(SBA) ₂ PEG + TGF β	36.6	29.6		5.3	1.7	38.2	22.1	2.5

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6.0 Paper II

Interactions of Corneal Cells with Transforming Growth Factor- β 1 Modified Poly (Dimethyl Siloxane) Surfaces

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Running Title: Corneal cell interactions with TGF- β modified surfaces

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Abstract

A successful artificial corneal implant will require a stromal contacting surface with properties that will inhibit the downgrowth of corneal epithelial cells while promoting stromal cell ingrowth. As means of developing such a surface, we immobilized transforming growth factor beta-1 (TGF- β 1) via a bifunctional poly (ethylene glycol) (PEG) spacer to poly (dimethyl siloxane) surfaces (PDMS) that had been aminated by the plasma polymerization of allylamine. The modified surfaces were chemically and biologically characterized. X-ray photoelectron spectroscopy (XPS) analysis provided evidence for the successful immobilization of the growth factor on the PDMS surface. Furthermore, the magnitude of the results were consistent with those noted on the TGF- β 2 modified surfaces, which we examined in our previous study, suggesting that the isoforms show similar reactivity in the environment studied. In contrast to the observed effects of immobilized TGF- β 2 previously examined, the presence of immobilized TGF- β 1 appeared to be mildly stimulatory for corneal stromal cell growth, and mildly inhibitory for corneal epithelial cell growth. Under serum containing conditions the cell surface coverage on the surfaces cultured with corneal stromal cells appeared to be increasing as a function of time, although the coverage at day 7 remained relatively low at 30-50%. Furthermore, the growth of corneal epithelial cells on these same surfaces appeared to be inhibited, with diminishing cell counts and diminishing surface coverage as a function of time. After 7 days of culture, epithelial cell surface coverage was on the order of 5-10%. A concentration effect was noted for stromal cells in the presence of serum. This study suggests that TGF- β 1 may prove successful in modulating corneal epithelial and stromal cell growth although optimization of the system may be necessary.

Keywords: TGF- β 1, poly (dimethyl siloxane), corneal epithelial cells, corneal stromal cells, artificial cornea

Introduction

One of the main challenges in the development of an artificial corneal implant involves the regulation of corneal stromal and corneal epithelial cell proliferation. It has been shown in various studies (1-3) that the ingrowth of corneal stromal cells is required to firmly anchor the implant in the surrounding host eye tissue. In addition, the overgrowth of corneal epithelial cells is required to provide a smooth continuous layer for ocular clarity and the maintenance of an appropriate tear layer, as well as for inhibiting bacterial and microbial invasion (4). Substrate surface modification techniques have been previously investigated to promote the ingrowth of stromal cells and the overgrowth of epithelial cells (5-22). However, the downgrowth of the corneal epithelium at the interface of the implant and the host eye tissue remains to be a significant problem as it ultimately leads to implant failure. Therefore, we have hypothesized that it may be necessary to develop a device with a surface that interacts differently with the major cellular components of the host eye tissue. Specifically, the device will require an anterior surface that has properties that will promote the adhesion and growth of corneal epithelial cells, and a stromal contacting surface with properties that will inhibit the downgrowth of the corneal epithelium while promoting corneal stromal cell ingrowth. In the current work, we have growth factor modified our substrate surface as a means of achieving these requirements.

Transforming growth factor-beta (TGF- β) is a protein that is involved in the regulation of cell growth and function, particularly during the repair of tissues (23). The three main mammalian isoforms of TGF- β are TGF- β 1, TGF- β 2, and TGF- β 3. The mature form of TGF- β 1 has 74% sequence homology to TGF- β 2 (24). It is widely accepted that TGF- β is a bifunctional regulator of cell growth in that it has the ability to stimulate the growth of fibroblasts and cells of mesenchymal origin and to inhibit the growth of non-transformed epithelial cells (25-28). In the cornea, TGF β 1 and TGF- β 2 have been most widely studied (29-43). There are reported differential responses of cells to TGF- β 1 and TGF- β 2, which may be attributed to altered receptor binding properties or alterations in receptor-specific intracellular signaling components (24, 44-46). Of primary interest to our current study are the reports that corneal epithelial cells cultured *in vitro* may be inhibited by the presence of TGF- β 1 or TGF- β 2 in the culture medium (29,31,33,42,43).

TGF- β has a short half-life and is prone to denaturation making it a difficult protein to deliver in a predetermined amount to a target site *in vivo*. However, it has been shown that by

immobilizing a growth factor on the surface of a substrate, it may be possible to control the local concentration of the growth factor delivered to the tissue by limiting diffusive spread, cell uptake and the degradation of the growth factor (47,48). Furthermore, immobilization may significantly enhance the bioactivity of the growth factor (48) as well as its temporal and spatial ability in the extracellular environment (17). The feasibility of immobilizing a growth factor to a substrate without loss of bioactivity has been previously demonstrated. Kuhl and Griffith have tethered epidermal growth factor (EGF) via a poly (ethylene glycol) (PEG) linkage and have showed that tethered EGF was as effective as soluble EGF in eliciting DNA synthesis and cell rounding responses of primary rat hepatocytes (49). Bentz *et al.* (50) have covalently bound TGF- β 2 to collagen matrices via a PEG linkage and demonstrated that the growth factor was fully active following binding and that covalent binding of the growth factor resulted in prolonged responses *in vivo* as well as improved stability *in vitro*. Mann *et al.* (51) have demonstrated that 0.04 pmol/mL of tethered TGF- β 1 increased matrix production by vascular smooth muscle cells compared with the same amount of soluble TGF- β 1.

We have previously examined the interactions of corneal cells with surface immobilized TGF- β 2 and have shown that while the growth factor on the surface was active, the interactions with corneal cells were contrary to those expected. Specifically, we observed the enhancement of corneal epithelial cell growth and the inhibition of corneal stromal cell adhesion and growth. Since there are reported differential responses of cells to TGF β 1 and TGF- β 2 (24, 44-46,59) we immobilized TGF- β 1 to poly (dimethyl siloxane) surfaces and examined the interactions of these modified surfaces with corneal epithelial and corneal stromal cells *in vitro*.

Materials and Methods

Surface Preparation

Surfaces were prepared as in our previous work (15,16). Poly (dimethyl siloxane) (PDMS, Dow Corning, Toronto Canada) membranes were prepared according to directions provided by the manufacturer. The monomer and curing agent were mixed in a 10:1 (m:m) ratio and placed under vacuum to remove entrapped air. The mixture was then placed in a plastic petri dish and allowed to cure for at least 48 hours. Surfaces were then aminated on both sides for subsequent modification by microwave frequency plasma polymerization of allylamine using conditions described by Wickson and Brash (60). Following amination, the surfaces were rinsed

extensively with distilled water, dried under vacuum overnight and placed in sealed containers until use. Subsequent surface modification with TGF- β 1 was based on the method of Bentz *et al.* (50). Lyophilized TGF- β 1 (R&D Systems, Minneapolis MN) was dissolved in HCl (pH 2.0) and diluted if necessary to give a TGF- β 1 concentration range between 1 μ g/ml to 0.4 μ g/ml. This was added to an aqueous solution of propylene glycol (Sigma Chemical Co., St. Louis MO) and 10x phosphate buffered saline (PBS) so that the final solution consisted of 40% propylene glycol in PBS (pH 7.3). A 1:50 molar excess of a succinimidyl derivative of PEG butanoic acid (SBA₂PEG, Shearwater Polymers, Huntsville AL), MW 3400, was added to the TGF- β 1 solution. An N-hydroxysuccinimidyl ester of methoxy (ethylene glycol) butanoic acid (methoxy-PEG-SBA, Shearwater Polymers, Huntsville AL), MW 5000, was used as a control. Additional controls consisted of surfaces modified with TGF- β in the absence of PEG and surfaces modified with SBA₂-PEG in the absence of TGF- β . After reacting for 10 minutes, the aminated PDMS surfaces were exposed to the solution for an additional 2 hours at room temperature. Following the reaction, surfaces were extensively rinsed with 10x PBS and stored at 4-6 degree C until characterized.

Surface Characterization

X-ray photoelectron spectroscopy (XPS) analysis was performed at the National Research Council of Canada. The surface of the samples was analyzed using a KRATOS AXIS HS x-ray photoelectron spectrophotometer (Kratos, Manchester UK). The size of the analyzed area was approximately 1 mm². Monochromatized Al K radiation was used for excitation and a 180° hemispherical analyzer with a three channel detector was employed. The x-ray gun was operated at 15 kV and 20 mA. The spectrophotometer was operated in Fixed Analyser Transmission (FAT) mode throughout the study using electrostatic magnification. Surface and high-resolution spectra were collected using 160 and 20 eV pass energy respectively. The pressure in the analyzer chamber was 10⁻⁸ to 10⁻⁹ torr. An electron flood gun was used to neutralize the charge during the experiment. Binding energies were referenced to the carbon-carbon bond that was assigned a binding energy of 285 eV. Atomic composition was estimated using standard software provided with the instrument using the following sensitivity factors: 0.25 for C1s, 0.66 for O1s and 0.42 for N1s relative to F1s at 1.00. Peak deconvolution was performed using the software provided with the instrument.

Cell Culture Studies

The effect of the modification with TGF- β 2 on interactions with corneal epithelial and corneal stromal cells was examined using *in vitro* cell culture. Polymer samples were placed in the wells of 24 or 48 well tissue culture plates. The samples were treated with 10 μ g/ml gentamycin in 0.2M phosphate buffered saline for 48 hours. This was followed by a second rinse with 10 μ g/ml gentamycin in 0.2M phosphate buffered saline prior to culture. Human corneal epithelial cells from established cell lines and human corneal stromal cells, from either low passage or established cell lines were seeded on the surfaces at a density of 10^4 cells per well. Corneal epithelial cells were cultured in either Keratinocyte Serum-Free Medium (Gibco, Invitrogen Corporation, Grand Island NY), supplemented with bovine pituitary extract (20-30 g/ml) and rEGF (0.1-0.2 ng/ml) or SHEM medium. Stromal cells were cultured in either Dubellco's Modified Eagle Medium (DMEM) (Gibco, BRL, Life Technologies, Grand Island NY) supplemented with insulin-transferrin-selenium (1000 μ l/l) (ITS) and gentamycin or DMEM supplemented with ITS (1000 μ l/l), gentamycin and fetal bovine serum (100 ml/l) (FBS). Additional surfaces were preadsorbed with laminin based on positive results with YIGSR and related peptides in previous work (15,16) for a period of two hours prior to culture under serum free conditions. Samples were examined on a daily basis for cell attachment, spread and proliferation. For further examination, the cells were fixed in 4% paraformaldehyde (PFA) for 20 minutes. The fixed cells were stained with haematoxylin and eosin (H&E). Cell surface density was assessed using digital images captured by light microscopy and analysed morphometrically using the Northern Eclipse software to obtain fractional surface coverage in a blinded fashion.

Results

X-Ray Photoelectron Spectroscopy

XPS results for the various modified surfaces are summarized in Table 1. As expected, on the aminated surface, there was a decrease in the Si2p and the appearance of a significant N1s signal relative to the unmodified PDMS control. The continued presence of a O1s signal on these surfaces is likely a result of both the oxygen present in the underlying PDMS substrate as well as air contamination in the plasma reactor. Following exposure of the aminated PDMS to SBA₂-PEG, the oxygen signal increased and the nitrogen signal decreased relative to the

aminated surfaces as expected. The increase in the O1s signal was greater at a more surface specific takeoff angle, providing additional evidence of the success of the modification procedure. Furthermore, there was a significant change in the high resolution C1s envelope with the disappearance of the signal at 287.9 eV (urea) and an increase in the signal at 285.0 eV (C-C). Reaction with SBA₂-PEG + TGF- β 1 resulted in an expected increase in the nitrogen signal and an increase in C-N and C(OOR) contribution, relative to the SBA₂-PEG modified surface. The N1s signal in particular showed an increase with an increase in the takeoff angle suggesting that the protein is on the surface. The magnitudes of the signals are generally consistent with our previous studies using TGF- β 2 for surface modification as expected. Exposure of aminated PDMS with methoxy-SBA-PEG +TGF- β 1 results in an increase in the N1s signal at the higher takeoff angle, suggesting that TGF- β 1 is present on the surface and may be absorbed in the PEG layer as observed in our previous study. Modification of the surface with TGF- β 1 in the absence of the SBA₂-PEG spacer also shows a result that is consistent with our previous TGF- β 2 results. While the growth factor present on the aminated PDMS surfaces exposed to TGF- β 2 in the absence of PEG should not be covalently attached, it is well known that TGF- β is a sticky protein and we therefore expect high levels of adsorption.

Cell Culture Studies

Growth of epithelial cells and corneal stromal cells on the various modified surfaces as a function of time is summarized in Figures 1 and 2 respectively. Differences in cell surface coverage between the two cell types are not as pronounced with TGF- β 1 modification as compared to the observed surface coverage obtained in the presence of TGF- β 2. Additional differences were also noted in this study relative to our previous work with TGF- β 2. Under serum containing conditions to promote cellular adhesion, with both high and low concentrations of TGF- β 1 immobilized on the surfaces, the coverage on the surfaces cultured with corneal stromal cells appears to be increasing as a function of time as expected, although the coverage at day 7 still remains relatively low at 30-50%. Furthermore, there is some inhibition of the growth of corneal epithelial cells on these same surfaces, with diminishing cell counts and diminishing surface coverage as a function of time. At the day 7 time point with high and low concentrations of TGF- β 1 immobilized on the surfaces cultured in the presence serum, the surface coverage with corneal epithelial cells was less than 5%, in direct contrast to the results noted with the

TGF- β 2 modified surfaces, which showed 40-60% surface coverage at the 7 day time point. With both cell types, there were not significant differences noted when high versus low concentrations of TGF- β 1 were used in the modification procedure except at the 7 day time point with stromal cells. The stromal cells cultured in the absence of serum showed very low counts and minimal surface coverage at the 7 day time point, which is somewhat surprising since these cells in the native cornea are avascular and have been shown to survive for extended periods of time in serum free medium (61).

It is interesting to note that the epithelial cells grown in the absence of serum resulted in higher cell surface coverages than those grown in a serum-containing environment. This may be due to the ability of corneal epithelial cells to produce their own matrix (62), which may be preferable for cell adhesion and growth factor recognition. Comparing the stromal cell surface coverage obtained in the presence of serum to the results obtained in the absence of serum suggests that, unlike corneal epithelial cells, which have been shown to be able to produce their own extracellular matrix, stromal cells require the presence of some exogenous adhesion molecules. In all cases, the cells grown in the absence of serum showed little or no coverage at the end of the 7 day time period.

Based on previous results from our labs, which suggest that the presence of laminin binding peptides may affect the growth of epithelial cells (15,16) both cell types were grown on surfaces that had been preadsorbed with laminin for a period of 2 hours prior to the introduction of cells. From Figure 1, it is clear that the presence of the laminin had a significant effect on the epithelial cells. Cells which were grown with no serum but with laminin were present in significantly greater numbers relative to those grown with serum, particularly at the later time points. Not surprisingly, the presence of the laminin had little or no effect on the stromal cells, although the cells grown in the presence of serum and laminin showed poorer coverage at 7 days than those grown in serum only, suggesting that adhesion to laminin may not be preferable for the maintenance of these cells.

Discussion

Inhibition of corneal epithelial cell downgrowth remains to be a significant problem in the development of an artificial corneal implant. In the current work, we examined the TGF- β 1 surface modification of an aminated PDMS substrate and examined the interactions of these

modified surfaces on corneal epithelial and stromal cells proliferation *in vitro*. TGF- β 1 has been reported to inhibit corneal epithelial cell growth (29,31,33,35) despite the fact that neither corneal epithelial nor corneal stromal cells have been shown to produce TGF- β 1 (64). Since this growth factor has a short *in vivo* half-life of less than 30 minutes, is prone to denaturation and high affinity binding to extracellular matrix components (51), covalent immobilization of the growth factor was chosen as the preferred delivery system in order to enhance both the stability and bioactivity of TGF- β 1 (51).

XPS analysis of the surfaces provides evidence for the successful immobilization of the growth factor to the PDMS substrate. As expected, increases in the N1s signal were noted on the surfaces with attached TGF- β 1. Furthermore, the magnitude of the results were very consistent with those noted on the TGF- β 2 modified surfaces, suggesting that the proteins show similar reactivity in the environment studied. Based on these results and our previous studies with ^{125}I labeled TGF- β 2, it is reasonable to estimate that the levels of TGF- β 1 on the surfaces were on the order of $\mu\text{g}/\text{cm}^2$. Although TGF- β has been shown to act in solution at concentrations of ng/nl, the physiological concentration of TGF- β 1 at a cell surface *in vivo* is not known, therefore, it is difficult to determine how much growth factor should be immobilized on the substrate surface.

In our cell culture studies, we examined the effect of changing the TGF- β concentration in the solution in order to better determine the surface density for optimum cellular response. In contrast to our previous studies with immobilized TGF- β 2 that showed that immobilized TGF- β 2 stimulated corneal epithelial cells, the interactions of corneal cells with immobilized TGF- β 1 were closer to what was expected based on the reports in current literature that suggest that TGF- β may have an inhibitory effect on corneal epithelial cells. While corneal epithelial cells initially showed reasonable surface coverage on the TGF- β 1 modified surfaces, cell coverage was found to decrease as function of time in culture. After 7 days of culture in the presence of serum, the cell surface coverage of the surfaces with epithelial cells was 5-10%. This is in contrast to our 7 day culture results with TGF- β 2 modified surfaces, which showed surface coverage on the order of 40-60%. Furthermore, a mild stimulatory effect of these surfaces on corneal stromal cells was also noted. In contrast to our previous studies, which showed stromal cell surface coverage of

less than 10% in all cases, culture of stromal cells on the TGF- β 1 modified surfaces in the presence of serum after 7 days resulted in cell surface coverage on the order of 30%.

It seems clear from these studies that the actions of TGF- β 1 and TGF- β 2 on corneal cells are different. While there is evidence in the literature that suggests that this may be the case (24,44-46,59), this study shows clearly that the two growth factors have different effects on corneal cells cultured under identical conditions despite the seemingly similar surface concentrations of the two growth factors. It should be noted however that the literature is not clear on the effects of these factors in the cornea, and it seems that the conditions under which the cells are cultured may significantly affect the results (63). Furthermore, where TGF- β has been shown in some cases to be inhibitory for a cell line *in vitro*, its effect *in vivo* has been shown to be exactly the opposite (29,31,33,35,64). It is our expectation that the surfaces will be preincubated with the appropriate cellular components prior to implantation to improve the chances of success. Therefore, the surfaces should be examined under the appropriate *in vivo* conditions in order to better understand the properties that will be necessary for successful implantation.

Conclusions

The effect of the immobilization of TGF- β 1 on corneal epithelial and corneal stromal cells was examined. TGF- β 1 was immobilized on the surface of aminated PDMS via a PEG spacer. Similar to previous studies with TGF- β 2, XPS results provided strong evidence for growth factor immobilization. In particular, there was a change in the N1s signal and in the high resolution C1s envelope on the various modified surfaces. The results also indicate significant adsorption of the growth factor on the modified surfaces. Based on the XPS results and the results of previous studies using TGF- β 2, the levels of growth factor associated with the surfaces is on the order of $\mu\text{g}/\text{cm}^2$. However, unlike our previous results with immobilized TGF- β 2, TGF- β 1 was mildly stimulatory for corneal stromal cells and was inhibitory for corneal epithelial cells. After 7 days of culture, surface coverage with corneal epithelial cells was on the order of 10% or less, which is significantly lower than the surface coverage of 30-60% noted when the surface was modified with TGF- β 2. Stromal cell surface coverage on the TGF- β 1 modified surfaces was approximately 30% compared to 10% on the TGF- β 2 modified surfaces. Therefore,

it is clear from this work that there are differences in the activity of the two growth factors in the cornea. In conclusion, the results of the current study seem to suggest that surface modification with TGF- β 1 may prove successful in modulating corneal epithelial cell growth at the interface between the device and native tissue. Future studies will focus on examining the effects of these surfaces in corneal organ culture and *in vivo*.

Acknowledgements

The technical assistance of Dr. Leisha Gan is gratefully acknowledged. Funding support was provided by NSERC.

Figure Legends

Figure 1. Summary of the results of epithelial cell coverage on the various modified surfaces. There is some inhibition of the growth of corneal epithelial cells on these same surfaces, with diminishing cell counts and diminishing surface coverage as a function of time. Culturing of the TGF- β 1 modified surfaces in the presence serum resulted in less than 5 % cell surface coverage after 7 days. This result is in direct contrast to the results noted with the TGF- β 2 modified surfaces, which showed 40-60% surface coverage after same time period.

Figure 2. Summary of the results of stromal cell coverage on the various modified surfaces. Under serum containing conditions to promote cellular adhesion, the stromal cells appear to increasing as a function of time on TGF- β 1 modified surfaces as expected, although the coverage at day 7 still remains relatively low at 30-50%.

Figure 1.

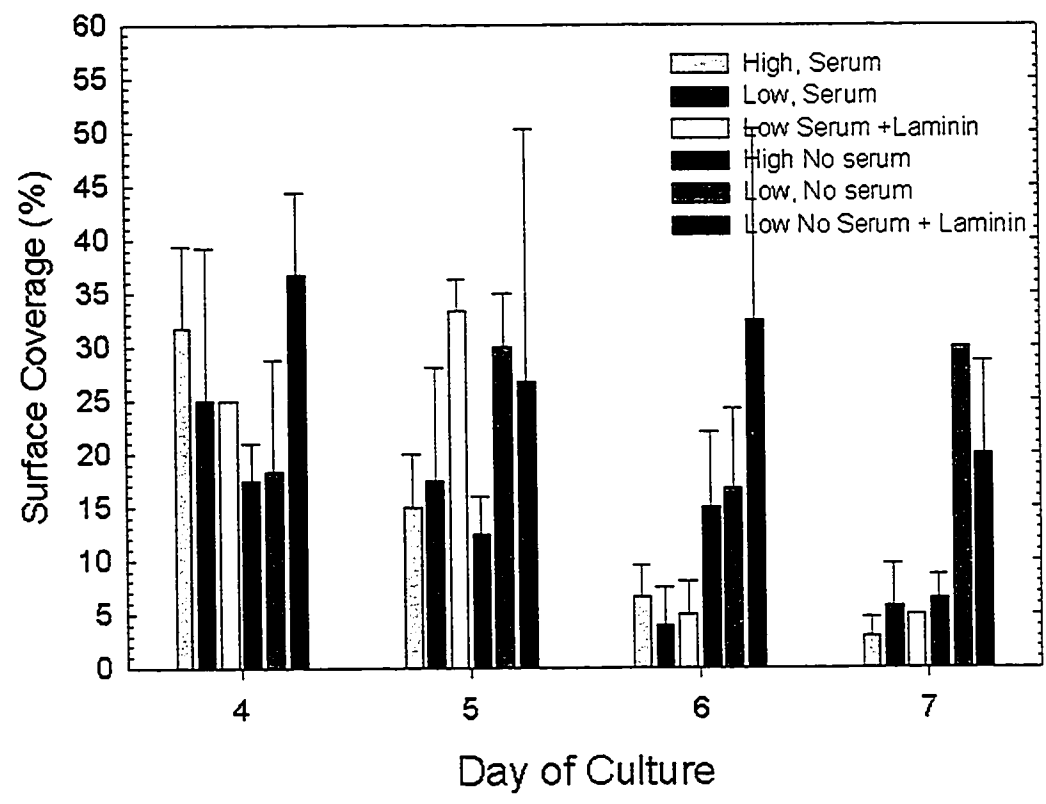


Figure 2.

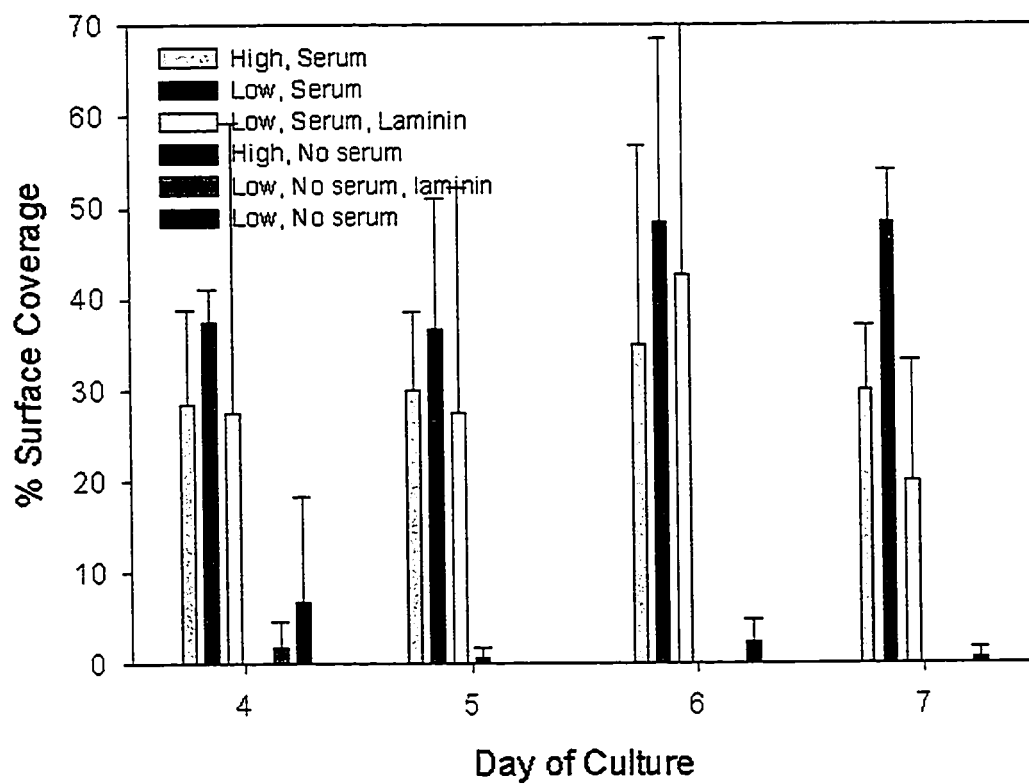


Table 1: Summary of XPS Results for Modified Surfaces

Sample	Takeoff angle	C1s					O1s	Si2p	N1s
		Total	285.0	286.6	287.9	289.2			
Unmodified PDMS	0	40.4	37.7	2.7			33.8	25.6	
Aminated PDMS	0	50.8	35.9	8.4	4.2	2.4	24.3	16.9	7.9
SBA ₂ PEG	0	49.1	43.9	3.8		1.4	26.2	23.3	1.4
	70	43.7	38.0	4.9		0.9	31.8	22.5	2.0
SBA2PEG + TGF- β 1	0	51.4	45.4	4.0		2.0	24.6	21.7	1.6
	70	49.4	38.5	6.8		4.1	26.5	16.5	4.2
MeO SBA PEG +TGF- β 1	0	51.4	46.6	3.2		1.6	24.3	21.8	1.8
	70	47.2	38.6	5.5		3.0	26.7	16.0	3.0
TGF- β 1 adsorbed	0	51.1	47.1	2.7		1.3	25.4	22.3	0.9
	70	49.9	40.2	6.2		3.5	28.1	17.8	3.7

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7.0 Conclusions and Recommendations

7.1 Conclusions

The surfaces of poly(dimethyl siloxane) (PDMS) were modified by the covalent attachment of TGF- β in order to generate a surface with properties that will inhibit the proliferation of corneal epithelial cells while promoting corneal stromal cell growth. The successful development of a surface that interacts with the major cellular components of the cornea in this way will make a strong contribution to the development of a successful artificial corneal implant. Specifically, such a surface would promote the ingrowth of corneal stromal cells to firmly anchor the implant in the surrounding host eye tissue and inhibit the downgrowth of epithelial cells thereby minimizing necrosis and implant extrusion.

Our first study looked at the immobilization of TGF- β 2 on the surface of aminated PDMS and the interactions of these modified surfaces with corneal epithelial and corneal stromal cells. While the amount of growth factor covalently bound on the surface was difficult to quantify due to the strong interactions between the growth factor and the poly ethylene glycol (PEG) layer as well as the substrate surface, differences in the modified surfaces determined by water contact angles, X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM) suggest that a significant amount of TGF- β 2 was present on the PDMS surface following the modification procedure.

Based on the reports in current literature, the hypothesis that TGF- β 2 may have the ability to promote stromal cell growth while inhibiting epithelial cell growth *in vitro* was developed. However, corneal stromal cells and corneal epithelial cells cultured on the TGF- β 2 modified surfaces gave results consistently opposite to those expected. Stromal cell growth appeared to be inhibited by the presence of TGF- β 2, with cell surface coverage less than 5% after 1 week of incubation. Epithelial cell growth appeared to be promoted by the presence of TGF- β 2, with cell surface coverage averaging 50-70% after 1 week of incubation. A TGF- β 2 concentration effect was noted with both cell types in the absence of serum, with higher TGF- β 2 concentrations on the modified surfaces resulting in increased cell surface coverage. The observed cell growth appears to be the result of interactions between the cells and active

TGF- β 2 since the addition of a stoichiometric excess of anti-TGF- β 2 antibodies added to the culture medium resulted in cell surface coverage similar to those observed on the control surfaces.

SDS PAGE and immunoblotting results appear to demonstrate that any differences in cell coverage on the modified surfaces in the presence of serum are the result of interactions with the TGF- β 2 and not the result of differences in serum protein adsorption, as all of the TGF- β 2 modified surfaces showed similar protein adsorption patterns and levels.

Since there are reported differential responses of cells to TGF- β 2 and TGF- β 1, TGF- β 1 was immobilized on the surface of aminated PDMS surfaces in our second study and the interactions of these modified surfaces with corneal epithelial and corneal stromal cells *in vitro* were examined. Similar to our first study with TGF- β 2, XPS results provided strong evidence for the presence of the growth factor on the aminated PDMS surface following the modification procedure. However, in contrast to our first study, the presence of immobilized TGF- β 1 appeared to be mildly stimulatory for corneal stromal cell growth and mildly inhibitory for corneal epithelial cell growth. Under culture conditions in the presence of serum, the cell surface coverage for the stromal cells appeared to be increasing as a function of time, although the surface coverage after 1 week of incubation remained relatively low. Under identical conditions, epithelial cell counts decreased resulting in diminishing surface coverage as a function of time after 1 week of incubation. In the presence of serum, a concentration effect was noted for the stromal cells only after 1 week of incubation. It is interesting to note that epithelial cells when compared to stromal cells grown under identical conditions have higher cell surface coverage when cultured in the absence of serum compared to those cells cultured in the presence of serum after 1 week of incubation. This suggests that stromal cells, unlike epithelial cells that have been shown to produce their own extracellular matrix, may require the presence of some exogenous adhesion molecules.

In conclusion, these two studies demonstrate that there are differences in the activity of the two TGF- β isoforms, TGF- β 1 and TGF- β 2, as illustrated by the interactions between the immobilized growth factor and corneal epithelial and corneal stromal cells under various cell culture conditions. Furthermore, the results of the second current study appear to suggest that surface modification with TGF- β 1 may prove successful in modulating corneal epithelial cell growth at the interface between the device and native tissue.

7.2 Recommendations

As the physiological concentration of TGF- β at a cell surface *in vivo* is not known it is difficult to predetermine how much growth factor should be immobilized on the surface. Furthermore, it has been speculated that the presence of TGF- β *in vivo* stimulates the migration of cells towards an area of higher growth factor concentration and once the cells reach the zone of higher of TGF- β concentration, both cell migration and proliferation become inhibited, so that cells may devote their energy to perform differentiated functions (Moses et al., 1990). At the lower TGF- β concentration, TGF- β may act as an indirect mitogen of the same cell type resulting in cell proliferation. In order to fully examine the bifunctional effects of immobilized TGF- β *in vitro* it will be necessary to look at the effects of the growth factor over a much wider range of concentrations.

The local availability (i.e. the local concentration) of TGF- β may be modulated by the presence of matrix proteins and other substances. For example, TGF- β has been shown to inhibit cell growth in the presence of 2-5 % serum, but to stimulate growth of the same cell type in the presence of 10% serum (Assoian and Sporn, 1986; Vivien et al., 1990). These results may in part be explained by the presence of α_2 -macroglobulin in serum, which differentially binds to TGF- β_1 and TGF- β_2 thereby inhibiting growth factor activity. In order to determine if the percentage of serum used in the culture conditions is affecting the outcome of the cell culture experiments, the effect of immobilized TGF- β on corneal cell growth should be studied using varying percentages of serum in the culture medium. In addition, the effects of TGF- β may be studied in the presence of other growth factors such as EGF, HGF and KGF as well as their sequence of addition to the cell culture medium.

It is clear that the amount of growth factor delivered to the cell surface is an important parameter in terms of the effects that the growth factor will have on cell growth. However, for many growth factors, including TGF- β , it is not known if optimal signal transduction between the growth factor and the cell requires internalization of the growth factor-receptor complex. In the current work, TGF- β was immobilized on the surface via an insoluble PEG spacer. Although some of the TGF- β was either strongly adsorbed on the surface or absorbed in the PEG layer, the evidence suggests that some quantity of TGF- β was covalently bound. This mode of delivery assumes that the events that occur on a cell surface

are sufficient for signal transduction to occur. An alternative method of modification would be to immobilize TGF- β via a labile spacer such as a synthetic peptide that is easily degraded by an enzyme (Seltzer et al., 1989; West and Hubbell, 1999) permitting the release of the growth factor over a suitable period of time. This mode of delivery would permit internalization of the growth factor-receptor complex thereby permitting signal transduction to the cellular nucleus to occur.

In our current studies, the TGF- β modified surfaces were cultured using either corneal epithelial cells or corneal stromal cells. However, experiments with isolated cell types exposed to growth factors *in vitro* may lead to contradictory results (Nathan and Sporn, 1991). Definitive evidence for the given bioactivity of a growth factor and the way it modulates the activity of specific cell type requires not only further *in vitro* tests but also tests in organ culture as well as in intact organisms. The TGF- β modified surfaces should therefore be cultured in an environment that takes into account the extracellular milieu of the cells of interest in their *in vivo* environment using a corneal organ culture system and if results appear promising, should be further tested in an appropriate animal model.

Appendix A

Mediums, Reagents and Solutions

Dubellco's (DMEM) medium

Follow instructions for DMEM on package (comes in powder).

Per 1 litre add:

ITS	1000 μ l
FBS	100 ml
Gentamycin	20 drops

Store at 4°C.

4% Paraformaldehyde (PFA) in 0.1M phosphate buffer (PBS)

Make up an 8% stock solution:

- paraformaldehyde 8g
- ddH₂O 50 ml

1. Add PFA to warmed PBS (60°C) and stir.
2. When solution is almost clear, add 1 drop of sodium hydroxide (NaOH).
3. Cool to room temperature.
4. Store at 4°C (or at -20°C for longer storage).
5. Thaw out (heat to 60°C) and add to equal amount of 0.2M phosphate buffer, pH 7.2-7.4 to make a 4% PFA working solution.

Phosphate-buffered saline (PBS), 0.2M

Na ₂ HPO ₄	14.8g
KH ₂ PO ₄	4.3g
NaCl	7.2g
ddH ₂ O	500ml

Phosphate Buffered Saline (PBS)

1.32 g Disodium hydrogen phosphate
0.345 g Sodium dihydrogen phosphate
8.5 g Sodium chloride
Fill to 1 L with distilled water.
Adjust pH to 7.38.

Phosphate Buffered Saline with Sodium Iodide (PBS-NaI)

1.32 g	Disodium hydrogen phosphate
0.345 g	Sodium dihydrogen phosphate
8.08 g	Sodium chloride
1.09 g	Sodium iodide

Fill to 1 L with distilled water.

Adjust pH to 7.4.

SHEM medium

To make 1 litre:

DMEM (low glucose)/F12(1:1)	820ml
15% FBS	150ml
10 ng/ml EGF	10 µl of 1µg/µl stock
5 µg/ml insulin	1 ml of 1000X ITS
0.1 µg/ml Cholera toxin A	1 ml of 100 µg/ml stock
DMSO 0.5%	5 ml (filter through syringe)
L-glutamax 5mM	25 ml of 200 mM stock
Gentamycin	10 drops

Filter prior to use.

Tris Buffered Saline (TBS)

6.05 g	Tris
8.76 g	NaCl

Fill to 1 L with distilled water.

Adjust pH to 7.4.

Appendix B

Determination of Free Iodide Concentration by Trichloroacetic Acid (TCA) Precipitation of the Protein

- Into two groups of 3 vials (Group A and B), 0.9 mL of a 1% (w/v in MilliQ water) bovine serum albumin (BSA) and 0.1 mL of the iodinated protein solution were added.
- To 3 of the vials (Group B), 0.5 mL of TCA was added. These vials were mixed with a vortex and left to stand for 10 minutes. After the ten minutes, the three vials were spun in a microcentrifuge for 1 minute.
- To a third set of 3 vials (Group C), 0.5 mL of buffer (PBS-NaI) was added to each.
- 0.5 mL of the supernatant from the precipitated Group B vials was added to the Group C vials.
- The vials from both Group A and Group C were counted in the gamma counter for 1 minute. The free iodide content was calculated as follows (equation 4):

$$\%FreeIodide = \left(\frac{3 \times GroupC_{AVERAGE}}{GroupA_{AVERAGE}} \right) \times 100 \quad (1)$$

Appendix C

Cell Culturing Procedures

Cell Thawing

Note: If more than one cryovial is to be thawed, thaw one cryovial at a time and keep other cryovials in liquid nitrogen until ready for use.

After the flasks after equilibrated for 30 minutes:

1. Remove cryovial from storage.
2. Holding the cryovial, dip the bottom $\frac{3}{4}$ of the cryovial in a 37 °C water bath and swirl gently for 1-2 minutes until the contents are thawed. When the last sliver of ice melts remove it. Thawing the cells for more than 3 minutes results in less than optimal results.
3. Remove the cryovial immediately, wipe it dry and transfer to a sterile field where equilibrated flasks are waiting. Rinse the cryovial with 70% ethanol, then wipe to remove excess.

Cell Seeding

After cells are thawed:

1. Remove the cap, being careful not to touch the interior threads with your fingers.
2. Using a micropipette with a 1000 ml tip set to 800 ml, put the tip into the cryovial and resuspend the cells.
3. Dispense an equal amount of cells into the flasks.
4. Replace the cap or cover of the flasks and gently rock the vessels to evenly distribute the cells.
5. Return the culture vessels to 37 °C, 5% CO₂ incubator. Lay them flat on the shelf, providing the largest surface for cells to attach.
6. Change the growth medium the day after the seeding, then every other day thereafter while examining them daily until use. Note: If the cells are allowed to become overconfluent and stay at confluence for more than 2 days, they can suffer irreversible contact inhibition.

Preparing Cells for Culturing of Sample Surfaces

1. Allow 1x trypsin to thaw and warm to 37 °C.
2. Remove appropriate growth medium from 4 °C storage and allow to start warming to room temperature.
3. Aspirate the medium from the culture vessel containing the cells to be cultured.
4. Rinse the cells with 5 ml of room temperature PBS (calcium and magnesium free).
5. Aspirate the PBS from the flask.

6. Add 1 ml of 1X trypsin to each flask and rock the flask to make sure the cells come into contact with the trypsin. Allow the trypsinization to continue until the majority of the cells are rounded up. At this point, rap the flask against the palm of your hand to release the majority of cells from the culture surface.
7. After the cells are release, neutralize the trypsin in the flask with 8-9 mls of room temperature cell culture medium.
8. Transfer the detached cells to a sterile 50 ml centrifuge tube.
9. Centrifuge the harvested cells at 1500 rpm for 5 minutes to pellet the cells.
10. Aspirate most of the supernatant, being careful not to aspirate the cell pellet.
11. Dilute the cells in the desired amount of growth medium.
12. Mix the diluted cells to ensure a uniform suspension.
13. Use a hemacytometer to determine the total number of cells in the total suspension volume. This value will you allow you to make any required dilutions prior to culturing the sample surfaces.

Appendix D

Haematoxylin and Eosin Staining Procedure

Eosin-Phloxine B

1% Eosin Stock

Eosin Y (water soluble)	1.0 g
ddH ₂ O	100 ml

1% Phloxine B

Phloxine B	1.0 g
ddH ₂ O	100 ml

Working Solution

Eosin stock	100 ml
Phloxine stock	10 ml
95% ethanol	720 ml
glacial acetic acid	5 ml

Modified Mayer's Haematoxylin

haematoxylin	4.0 g
ddH ₂ O	1.0 L
sodium iodate	0.3 g
ammonium alum	50.0 g
citric acid	1.5 g
chloral hydrate	75.0 g

1. Dissolve alum in water; do not use heat.
2. Add haematoxylin, iodate, citric acid, and chloral hydrate, in order.
3. Filter through coarse filter paper.

Staining Procedure

All surfaces were left in wells.

1. ddH ₂ O	1 min
2. Haematoxylin	15 min
3. ddH ₂ O	2 min
4. Acid alcohol(1% conc. HCL in 70% EtOH (v/v))	6 rinses
5. ddH ₂ O	2 min

6. Scotts modified tap water	1 min
7. ddH ₂ O	10 min
8. Eosin-phloxine solution	3 min
9. 70% ethanol	10 min

Appendix E

SDS PAGE and Immunoblot Procedures

Polyacrylamide Gel Preparation (9% or 12% separating gel, 4% stacking gel)

The acrylamide/bis solution is prepared by dissolving the following reagents in distilled water, diluting to 100 mL and filtering the final solution:

Acrylamide	29.2 g
N,N'-Methylenebisacrylamide	0.8 g

The reagents for the 9% and 12% separating gel were mixed and degassed for 15 min at room temperature:

<i>Separating Gel</i>	<i>9%</i>	<i>12%</i>
Distilled water	4.4 mL	3.35 mL
1.5 M Tris, pH 8.8	2.5 mL	2.5 mL
10% (w/v) SDS	0.1 mL	0.1 mL
30% (w/v) Acrylamide/Bis	3.0 mL	4.0 mL

Immediately prior to casting the gel, the following reagents are added to initiate polymerization in the above mixture:

10% (w/v) ammonium persulfate (fresh)	50 μ L
TEMED	5 μ L

The casting plates were cleaned with distilled water and 95% ethanol. Once dry, the plates were inserted into the casting assembly. The assembly was then secured to the casting stand. Using a syringe, the gel plates were filled with polymerising 9% or 12% acrylamide solution, leaving enough space to pour the stacking gel. After 2 min, a small quantity of water was layered over the gel. The gel was allowed to polymerize for 1 h.

The reagents for the 4% stacking gel were mixed and degassed for 15 min at room temperature:

Distilled water	3.0 mL
0.5 M Tris, pH 6.8	1.2 mL
10% (w/v) SDS	0.1 mL
30% (w/v) Acrylamide/Bis	0.65 mL

Immediately prior to casting the gel, the following reagents are added to initiate polymerization in the above mixture:

10% (w/v) ammonium persulfate (fresh)	25 μ L
TEMED	5 μ L

Using a syringe, the remainder of the gel plates was filled with polymerizing 4% acrylamide solution. An appropriate comb was added and the gel allowed to polymerize for 1 h.

Sample Preparation

The sample buffer used in sample preparation consists of the following reagents, mixed and stored at 4°C in 225 µL aliquots:

Distilled water	4.0 mL
0.5 M Tris, pH 6.8	1.0 mL
10% (w/v) SDS	1.6 mL
Glycerol	0.8 mL

Immediately prior to use, the following reagents are added to an aliquot, yielding tracking dye (TD):

2-Mercaptoethanol	30 µL
0.5% (w/v) Bromophenol blue	30 µL

Samples and standards used for SDS-PAGE only are prepared as follows:

1 µL SDS-PAGE MW Standards, Low Range, 10 µL TD
10 µL Protein sample, 10 µL TD
7.5 µL Prestained SDS-PAGE Standards, Low Range

Samples and standards used for western blotting are prepared as follows:

1 µL SDS-PAGE MW Standards, Low Range, 10 µL TD
150 µL Protein sample, 100 µL TD
7.5 µL Prestained SDS-PAGE Standards, Low Range

Once mixed, the samples are placed in a 95°C water bath for 10 min.

Electrophoresis

Once the gel polymerization was complete, the combs were gently removed and the wells rinsed with distilled water. The gels were removed from the casting stand and placed into the clamp assembly. The assembly was then placed into the buffer chamber. A 5X stock solution of electrophoresis buffer was prepared by mixing the following reagents in distilled water and diluting to 1 L (Note: the pH of this solution should be 8.3 ± 0.3):

Tris Base	15 g
Glycine	72 g
SDS	5 g

Just prior to use, the 5X stock solution was diluted to 1X with distilled water. The upper buffer chamber was filled to a level 3 mm below the edge of the outer (long) glass plate with electrophoresis buffer. The lower buffer chamber was filled to a level that covered the bottom 1 cm of the gel. The comb was subsequently removed and the well flushed with transfer buffer. The sample was then loaded into the wells and a potential difference of 200 V applied across the gel for approximately 1 h. When performing an immunoblot, a small quantity of pyronin Y dye (dissolved in sample buffer) was layered into the wells just before the tracking dye had reached the bottom of the separating gel. Electrophoresis was stopped once the pyronin Y dye had reached the top of the separating gel.

Gel Equilibration

Transfer buffer was prepared by mixing the following reagents in distilled water and diluting to 1 L (Note: the pH of this solution should be 8.3 ± 0.3):

Tris Base	3.03 g
Glycine	14.4 g
Methanol (HPLC grade)	200 mL

The gels were removed from the electrophoresis assembly and equilibrated in fresh cold (4°C) transfer buffer for 30 min.

Electrophoretic Transfer

Immobilon (PVDF) membranes were cut to gel-size, prewetted in methanol (1-3 seconds), incubated in water (1-2 min) and soaked in transfer buffer (15 min). The gels and membranes were loaded in the transfer cassettes according to specifications and placed in the transfer chamber. The chamber was then filled with transfer buffer so that the entire gel surface was covered. A potential difference of 100V (200 mA) was applied for 1 h. The membranes can then immediately be stained with colloidal gold or dried and used for immunoblot analysis.

Gold Staining

The PVDF membranes were washed two times in phosphate buffered saline (PBS), pH 7.4. PBS was prepared by mixing the following reagents in distilled water, adjusting the pH to 7.4 and diluting to 1 L:

Na ₂ HPO ₄	1.32 g
NaH ₂ PO ₄ ·H ₂ O	0.345 g
NaCl	8.5 g

The membranes were then incubated in 0.3% (v/v) Tween 20 solution in PBS for 1 h at 20°C to block unbound membrane sites. This was followed by three further 5 minutes washings with this blocking solution. The membranes were then rinsed in water three times for 1 min.

The membranes were then placed in Protogold solution and stained for 1 to 4 h. Following the staining, the membranes were rinsed extensively with distilled water and air dried.

Immunoblotting

The sections of the membrane containing MW markers lanes and a small section of the sample lane were removed to be stained with the gold staining procedure described above.

Tris-buffered saline (TBS) was prepared as follows:

50 mM Tris
150 mM NaCl

Adjust pH to 7.4

The remainder of the membrane was sliced into 3 mm strips. The strips were pre-wet in methanol, rinsed in distilled water and placed into plastic wells. In order to block unbound membrane sites and prevent non-specific binding, the strips were incubated for 1 h in 5% (w/v) dry skim milk in TBS, pH 7.4 with gentle agitation. This treatment was followed by three 5 min rinses in 0.1% (w/v) dry skim milk in TBS.

Each strip was then incubated for 1 h in 1 mL 1% (w/v) dry skim milk and 0.05% (v/v) Tween 20 in TBS with a 1/1000 dilution of the primary antibody to the protein of interest. This treatment was followed by three 5 min rinses in 0.1% (w/v) dry skim milk in TBS. Each strip was then incubated for 1 h in 1 mL 1% (w/v) dry skim milk and 0.05% (v/v) Tween 20 in TBS with a 1/1000 dilution of the alkaline phosphatase-linked secondary antibody. Again followed three 5 min rinses in 0.1% (w/v) dry skim milk in TBS. Finally, the strips were incubated for up to 4 h with a solution to develop the colour reaction and detect the bands. The buffer for this solution is prepared by dissolving the following reagents in distilled water, adjusting the pH to 9.8 and diluting to 100 mL:

NaHCO ₃	840 mg
MgCl ₂ ·6H ₂ O	20 mg

The final solution is prepared by mixing 1 mL NBT stock (30 mg NBT in 1 mL 70% DMF in distilled water) and 1 mL BCIP stock (15 mg BCIP in 1 mL DMF) in 100 mL buffer. This reaction was terminated by rinsing the strips in distilled water twice for 5 min.

Appendix F

Summary of the SDS PAGE and Immunoblot Results for TGF- β 2

No	Protein	Unmodified 1	Unmodified 2	Aminated 3	(SBA)2- PEG+TGF	Met-SBA- PEG+TGF	TGF
1	Factor XI		X		XX	XX	XX
2	Factor XII	X	XX	X	XX	XX	XX
3	Prekallikrein		X		XX	XX	XX
4	HMW/K				XX	XX	X
5	Fibrinogen	XX	XX	XX	XXX	XXX	XXX
6	Plasminogen			X	XX	XX	XX
7	ATIII	X	X	X	XX	XX	XX
8	C3		XXX	XX	XXX	XXX	XXX
9	Transferrin	XX	XXX	XX	XXX	XXX	XXX
11	Fibronectin				X	XX	XX
12	Albumin	XXX	XXX	XXX	XXX	XXX	XXX
13	IgG	XXX	XXX	XXX	XXX	XXX	XXX
15	alpha-2-macro				X	XX	XX
16	Vitronectin	XX	XX	XX	XX	XX	XX
18	Prothrombin				X		X
19	beta-2-microglobulin						
20	Haemoglobin	XX	X	XX		X	X
23	Factor B	XX	XX	XX	XXX	XXX	XX
24	Factor H			XX	XXX	XX	XX
25	Factor I	X	X		XXX	XX	X
30	Apolipoprotein	XXX	XXX	XXX	XXX	XXX	XXX
0	None						

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