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MULTIFUNCTIONAL BIOMIMETIC MATERIALS FOR CORNEAL REGENERATION

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Multifunctional Biomimetic Materials for Corneal Regeneration

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ABSTRACT

The cornea is the outermost layer of the eye, which is responsible for transmitting 95% of the incident light to the retina for vision and provides 70% of the focusing power of the eye. Corneal disease is a primary cause of blindness worldwide. Replacing the pathologic cornea with a donor cornea is the most accepted treatment, but there is a severe shortage of donor tissue, resulting in an extensive waiting list for transplantation of over 10 million people. In this thesis, we worked on the development of artificial corneas to solve the donor shortage issue. Although an artificial cornea made from carbodiimide crosslinked recombinant human collagen developed within our lab was successfully transplanted into 10 patients in a clinical trial, this material was not tough enough to withstand severe disease conditions where inflammation is present, and where enzymes secreted can cause premature implant degradation. To improve mechanical strength and material stability, a secondary network of 2-methacryloyloxyethyl phosphorylcholine (MPC) biopolymer was incorporated within the collagen hydrogel, forming an interpenetrating network (IPN). High resolution transmission electron microscopy showed that the implants comprised loosely bundled collagen filaments. X-ray scattering further revealed that the collagen fibrils within the implants were uniaxially oriented, whereas a biaxial alignment is present within the human cornea. This fibril arrangement resulted in highly transparent implants that transmitted virtually all incoming light of visible spectra together with a large proportion of UV light. This study is critical in a sense that it strongly suggests that all patients transplanted with this artificial cornea should take the precaution to use UV protection prior to re-growth of the epithelium, which is known to absorb harmful UV rays. To determine the utility of the implants for clinical use, we showed that they could be cut with a femtosecond laser. Laser excision of diseased patient tissue avoids damage to the surrounding healthy tissue, thereby circumventing excessive, undesirable inflammatory responses associated with the manual surgical technique while the cutting of a matched implant allows for precise host-graft apposition and seamless regeneration. We also showed that the surface of the implants could be modified to enhance rapid and stable epithelial growth. We demonstrated that we could pattern the implants surfaces using microcontact printing with fibronectin as "ink". The dimensions of the patterned stripes were important in controlling corneal epithelial cell behavior including proliferation. This is important to ensure rapid wound healing and hence, an overall superior clinical outcome.

In all of the above materials, the collagen was crosslinked with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC)/N-hydroxysuccinimide (NHS). EDC is a zero-length crosslinker and while it produces a sufficiently robust hydrogel for clinical implantation, suturability was still an issue. To enhance suturability, we evaluated the effects of an epoxy-based crosslinker, 1,4-Butanediol diglycidyl ether (BDDGE), which has been shown to result in collagen hydrogels with enhanced elasticity. As neuronal ingrowth into the hydrogels and epithelial cell coverage are important considerations in achieving regeneration, we examined the effects

of incorporation of short cell adhesive laminin peptides within the BDDGE-crosslinked hydrogels. We showed that incorporation of YIGSR and IKVAV peptides enhanced the proliferation of corneal epithelial cells and neuronal progenitor cells, respectively.

Although artificial corneas made from collagen have been successfully tested in the clinic, animal-derived collagens, in general, come from very heterogeneous sources and carry a risk of pathogen transmission. Use of recombinant human collagens mitigates those issues but just like native collagens; they are large macromolecules, relatively inert and therefore difficult to chemically alter to design in new functionalities. They are difficult and hence expensive to produce. Collagen-like peptides (CLP), also known as collagen mimetic peptides, are relatively short sequences that have been designed to replicate and reproduce the function of full-length collagen. We examined the safety and efficacy of one such CLP that we had conjugated to polyethylene glycol-maleimide (PEG) as implants for promoting corneal regeneration in mini-pig models. This CLP-PEG implants promoted the regeneration of corneal epithelial and stromal cells from endogenous progenitors, as well as cornea nerves to form a stable neo-cornea. The use of fully synthetic materials that can be produced under a tightly controlled environment such as CLP-PEG mitigates safety issues associated with native collagen from animal or human sources, as well as makes production sufficiently cost-effective to allow for future scale-up.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which, will be referred to by their Roman numerals in the main text.

I. The structural and optical properties of type III human collagen biosynthetic corneal substitutes.
Sally Hayes, Phillip Lewis, **M. Mirazul Islam**, James Doutch, Thomas Sorensen, Tomas White, May Griffith, and Keith M. Meek.
Acta Biomater. 2015 Oct 1; 25: 121–130.

II. Functional fabrication of recombinant human collagen-phosphorylcholine hydrogels for regenerative medicine applications.
M. Mirazul Islam*, Vytautas Cėpla*, Chaoliang He*, Joel Edin, Tomas Rakickas, Karin Kobuch, Živilė Ruželė, W. Bruce Jackson, Mehrdad Rafat, Chris P. Lohmann, Ramūnas Valiokas*, May Griffith*.
Acta Biomater. 2015 Jan;12:70-80. *equal contribution.

III. Epoxy cross-linked collagen and collagen-laminin Peptide hydrogels as corneal substitutes.
Li Buay Koh*, **Mohammad Mirazul Islam***, Debbie Mitra*, Christopher W. Noel*, Kimberley Merrett, Silvia Odorcic, Per Fagerholm, William. Bruce Jackson, Bo Liedberg, Jaywant Phopase*, and May Griffith*.
J Funct Biomater. 2013 Aug 28;4(3):162-77. *equal contribution.

IV. Cathelicidin LL-37 and HSV-1 Corneal Infection: Peptide Versus Gene Therapy.
Chyan-Jang Lee, Oleksiy Buznyk,* Lucia Kuffova,* Vijayalakshmi Rajendran, John V. Forrester, Jaywant Phopase, **Mohammad M. Islam**, Mårten Skog, Jenny Ahlqvist, and May Griffith
Transl Vis Sci Technol. 2014 May; 3(3): 4. *equal contribution.

V. Self-assembled collagen-like-peptide implants as alternatives to human donor corneal transplantation.
M. Mirazul Islam,* R. Ravichandran,* D. Olsen, M. K. Ljunggren, Per Fagerholm, C. J. Lee, M. Griffith* and J. Phopase.* Manuscript. *equal contribution.

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LIST OF ABBREVIATIONS

WHO	World Health Organization
HSV	Herpes simplex virus
PK	Penetrating keratoplasty
APC	Antigen-presenting cells
KPro	Keratoprosthesis
PHEMA	Poly(2-hydroxyethyl methacrylate)
PHEA	Poly(hydroxyethyl acrylate)
OOKP	Osteo-Odonto-keratoprosthesis
PMMA	Polymethylmethacrylate
SDS	Sodium dodecyl sulfate
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide
NHS	N-hydroxysuccinimide
RHC	Type III recombinant human collagen
IPN	Interpenetrating polymer network
MPC	2-methacryloyloxyethyl phosphorylcholine
PEGDA	Poly(ethylene glycol) diacrylate
APS	Ammonium persulphate
TEMED	N,N,N,N tetramethylethylenediamine
CLP	Collagen-like peptides
MES	2-morpholinoethane sulfonic acid monohydrate
BDDGE	1,4-Butanediol diglycidyl ether
YIGSR	Tyrosine-Isoleucine-Glycine-Serine-Arginine
IKVAV	Isoleucine-Lysine-Valine-Alanine-Valine
HPLC	High performance liquid chromatography
PEG	Polyethylene glycol
TEM	Transmission electron microscopy
DSC	Differential scanning calorimeter
FTIR	Fourier transform infrared spectroscopy
CD	Circular dichroism
HCEC	Human corneal epithelial cells

GFP	Green fluorescence protein
KSFM	Keratinocyte serum-free medium
EGF	Epidermal growth factor
BPE	Bovine pituitary extract
NDC	Neuroblastoma cells
ISO	International Organization for Standardization
DLKP	Deep lamellar keratoplasty
HAM	Human amniotic membrane
IVCP	*In-vivo* confocal microscopy
AFM	Atomic-force microscopy
RMS	Root mean square
GLP	Good Laboratory Practice
UV	Ultraviolet
μCP	Micro contact printing
ECM	Extracellular matrix
SLET	Simple limbal epithelial transplantation

1   INTRODUCTION

REGENERATIVE MEDICINE

Regeneration or regrowth of lost or damaged tissues or organs is a part of life. An adult human body has stem cells in virtually every organ that, in theory, have the capacity to replace themselves. Although organs like the liver and skin do have the regenerative capacity, in most organs, however, the resident stem cells on their own are not sufficient to achieve the repair needed to prevent organ failure in cases of disease or extensive damage. Hence, in humans, regeneration is limited.

Regenerative Medicine has been defined as a "branch of medicine that develops methods to regrow, repair or replace damaged or diseased cells, organs or tissues[1]". As such, it includes the science of developing therapeutic stem cells and tissue engineering. This field holds the promise of restoring damaged tissues and organs by stimulating the body's own repair mechanisms to functionally heal previously irreparable tissues or organs.

If the patients' own endogenous or autologous cells were used therapeutically, then immune rejection issues would be circumvented. The use of acellular biomaterials that can promote endogenous regeneration is an option that has been gaining momentum. The use of such an approach alleviates the need for expensive cleanrooms that are needed for cell expansion[2]. In this thesis, I will mostly discuss the regenerative approaches of making artificial cornea using biomaterials that promote endogenous regeneration.

1.1   CORNEA

The cornea is the transparent covering and the main refractive element of the eye. It is responsible for transmission of light to the retina. The human cornea is composed of three primary layers, an outermost epithelium layer, a middle stroma containing keratocytes and an innermost, single layer of endothelial cells[3]. Two acellular layers separate these cellular layers; Bowman's layer that separates epithelium and stroma, and Descemet's layer which separate stroma and the endothelium. There is an

additional, recently discovered, acellular layer termed as the pre-Descemet's layer (Dua's layer) that lies adjacent to the Descemet's layer[4].

The corneal epithelium is a stratified, non-keratinizing epithelium. Centrally, it is five cell layers thick, whereas, in the periphery, it is up to 10 cell layers thick. Cells of the epithelial layer are self-renewing, and their stem cells are located basally as will as in the limbus, which is a ring of tissue immediately adjacent part to the peripheral cornea[5].

Keratocytes or stromal cells are neural crest-derived mesenchymal cells that make up approximately 3% of the total stromal volume[6]. The rest of the stroma comprises extracellular matrix (ECM) macromolecules consisting of mainly collagen and proteoglycans. Functional properties of the healthy cornea largely depend on the structure of corneal stroma, which is around 500um thick (90% of the total cornea) and highly hydrated (78% water). Highly specific arrangement of collagen fibrils within the stroma is the reason behind corneal transparency[7]. Unlike corneal epithelial cells, stromal cells are not self-renewing and they remain quiescent throughout life. Upon injury to the stroma, keratocytes transform into mitotically active fibroblasts[8, 9], while ECM secreted by the fibroblasts[10] form a scar within the cornea that can lead to vision loss. More recent studies have shown that stromal cells with stem cell specific markers have been isolated from the corneal limbal resign. These cells have the ability to differentiate into keratocytes and are most likely stem cells[11, 12].

The corneal endothelium is a single layer of non-proliferative cells[13]. The density of the cells is $2\text{-}5\text{x}10^3$ cells/mm^2 in the normal human cornea[14]. The endothelium functions to maintain the hydration of cornea by pumping out excess water from the corneal stroma through their Na, K-ATPase pump[15]. In case of the loss of endothelial cells during the aging or wounding process, the exposed area becomes covered by increasing the overall cell size and altering the shape to fit the gap[16]. Recent studies showed that endothelial progenitor cells exist in the corneal periphery and have the potential to differentiate into functional corneal endothelial cells[17, 18].

The cornea is the most densely innervated surface tissue of human body. Most of the corneal nerves are sensory in origin and originate from the ophthalmic branch of the trigeminal nerve. Corneal nerves lose their perineurium and myelin sheaths as they enter the vicinity of the limbus before entering into the cornea. They form a nerve

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plexus that lies parallel to the surface of the cornea[19]. Corneal nerves play an important role in sensory function, maintaining the homeostasis of the corneal epithelium, tear production and blinking[20]. The cornea does not have blood vessels. The nutritional requirement is fulfilled by the tears[21], aqueous humor[22] and also neurotrophins from its nerve supply. The highly hydrated and porous structure of the cornea allows for diffusion of solutes and nutrition throughout the structure[23]. The cornea is immunologically privileged[24] and is, therefore, a perfect model for studying transplantation and tissue engineering.

1.2   CORNEAL DISEASES

According to World Health Organization (WHO), 285 million people throughout the world are visually impaired, and among them, 39 million are blind[25]. One of the major causes of blindness worldwide is corneal disease, leading to loss of corneal transparency and deteriorating vision. There are a wide variety of infectious and inflammatory eye diseases that cause corneal scarring and may result in total blindness. Ulceration and trauma cause cornea related monocular blindness to 1.5–2.0 million new cases every year[26].

Microbial attack is a common cause of corneal disease. Endogenous antimicrobial peptides or proteins such as lysozyme, lactoferrin, phospholipase A2, defensins, and cathelicidins are present in the tear film. These provide host defense against microbial attack[27-31]. Alteration of this host defense mechanism makes the cornea susceptible to microbial attack.

Herpes simplex virus (HSV) infection is one of the prime causes of corneal disease and loss of vision. HSV is a neurotrophic virus that infects the skin, mouth mucous membrane, genitalia and eyes. HSV serotype 1 (HSV-1) is commonly associated with corneal infections. Up to 500,000 cases per year of HSV infection in the cornea are reported in the USA alone. HSV-1 infection is the most frequent cause of corneal blindness in North America and reports of visual disability are as high as 40%[32]. The pathogenesis of the HSV-1 virus is complex. Initial exposure generally results in a primary infection during which the clinical signs can include the followings: 1) Infectious epithelial keratitis in which corneal epithelial ulcers (surface lesions) are

present; 2) Neurotrophic keratopathy in which abnormal corneal innervation and poor tear production occur. The infection produces a non-healing surface ulcer that can progress into the deeper layers and cause corneal perforation; 3) Stromal keratitis that can be either necrotizing or non-necrotizing; and 4) Endothelialitis, which is an immune reaction at the level of the endothelium that may occur by months to years of HSV infection. After recovery from primary infection, the virus establishes latency in the trigeminal ganglion that supplies sensory neurons to the ocular surface[33] and also likely, within the cornea[34]. Prompts for reactivation are diverse and nonspecific including stress, immunosuppression and sunlight. The recurrent disease is generally more severe, involving the stroma as well as epithelium, leading to Herpes Simplex Keratitis (HSK).

Bacterial and fungal keratitis are also common corneal diseases that can cause damage to the cornea and lead to blindness[35]. Most keratitis is associated with the use of contact lenses, e.g. infection caused by the bacterium, *Pseuromonas aeruginosa*[36] and the fungus, *Fusarium solani*[37]. Infective crystalline keratopathy is caused by *Viridans streptococci*[38], *Staphylococcus epidermidis*[39] and *Candida albicans*[40]. Trachoma, which is caused by the bacterium *Chlamydia trachomatis*, is related to corneal inflammation and scarring that affects 5 million people worldwide[26]. In most of the cases, bacteria form the biofilm, which makes them more resistant to conventional treatment[41].

Keratoconus is another common and non-infectious-based corneal disease that is characterized by non-inflammatory and progressive thinning of the cornea. Genetic as well as environmental factors have been associated with this disease[42]. Keratoconus causes visual interference with multiple images and sensitivity to light[43].

1.3 CORNEAL TRANSPLANTATIONS AND ISSUES

The most widely accepted treatment for corneal blindness is transplantation of a full thickness healthy donor cornea after removal of the damaged tissue; a process termed as penetrating keratoplasty (PK)[44]. Unfortunately, the supply of donor tissue is substantially less than the demand for transplantation that has resulted in 10 million

untreated patients worldwide, with an additional 1.5 million new patients every year[26].

Apart from the donor shortage, donor corneal grafting is contraindicated in a proportion of patients reasons such as autoimmune situations, chemical burns, and infections[45]. Graft failure due to the graft rejection remains the most challenging complication in corneal transplantation. The Swedish register transplantation database shows that the 2-year rejection rate of transplanted donor corneas is quite low, at 15% with an overall complication rate of 26%[46]. The survival rate of corneal grafts, however, decreases over time to 62% at 10 years and 55% at 15 years[47].

Although the healthy cornea is immune privileged, corneas that are inflamed and neovascularized are no longer immune privileged leading to graft rejection. The loss of immune privilege is probably due to infiltration of blood vessels and lymphatics into the cornea. Rejection of a corneal graft is a CD4+ T cell-mediated response[48]. In the case of inflammation, host antigen-presenting cells (APC) are attracted into the stroma, and due to the local production of pro-inflammatory cytokines, major histocompatibility complex antigen gets overexpressed on grafted corneal cells. The up-regulation of alien histocompatibility complex triggers host immune system. This immune reaction against donor antigens results in rejection of corneal graft[49].

Donor-cornea derived infection is another serious complication associated with transplantation of human donor corneas[50]. HSV-1 DNA isolated and characterized from donor corneas before and after corneal transplantation confirmed the transmission of HSV-1 through transplantation from donor to host[51]. Bacterial and fungal post-keratoplasty endophthalmitis is another common complication that appears after five days of donor transplantation[52]. Processing and proper screening of donor corneas can reduce the complications of infection related to donor cornea transplantation. Screening is an expensive procedure, with processing fees in the USA around 2.5-3.5 thousand US dollars per cornea[45]. Infection has been implicated in graft rejection. For example, corneal transplantation is the treatment of choice for HSV-induced corneal blindness[47] despite a poor prognosis (22% success at 5 years[53]) compared to 73% success at 5 years for non-HSV grafts[47]. After recovery from the primary infection of HSV, this virus may remain in the trigeminal ganglion as a latent state[54]. The virus

can reactivate after transplantation with a donor cornea and cause the same disease to the newly transplanted cornea[55].

1.4   PROPOSED ALTERNATIVES TO HUMAN DONOR CORNEAS

There has been a long history of research into the development of alternatives to human corneas, both artificial as well as natural alternatives.

Xenografts have been tested since the 1800s. In 1838, Richard Sharp Kissam transplanted a cornea from a 6 months old pig into the cornea of a young Irishman, James Dunn, who suffered from a central leucoma. Within two weeks of transplantation, the cornea became opaque and was absorbed within one month[56]. During the same year, a sheep xenograft cornea was grafted into a human patient[57]. In this case, the grafted cornea became opaque. In all instances of xenografts, the implants failed due mainly to host immune reaction against the graft[58]. In addition, cross-species diseases are also a major complication of xenograft transplantation. To date, xenografts are still being tested. However, they are now treated to remove the cellular components to prevent immune rejection and screened to prevent pathogen transmission.

Decellularized organs for potential transplantation have become popular in recent time due to their ability to retain the native ECM of the target organ[59]. Decellularized corneas have been studied to evaluate their potential as grafts in same or cross-species[60]. There have been a number of methods developed to decellularize corneas[61, 62]. Most of the methods used 1) 0.1% sodium dodecyl sulfate (SDS)[63] for 7 hours, 2) hypoxic nitrogen (N_2) for 7days[64] or 3) hypertonic NaCl for 48 hours[65]. The human cornea was decellularized and evaluated for the reconstruction of corneal epithelium and stroma, *in vitro*[61]. In 2015, decellularized porcine corneas have been successfully transplanted into patients in clinical evaluation. Forty-seven patients with the fungal corneal infection were transplanted with the decellularized porcine cornea. Up to 6 months of post operation, there was no recurrence of infection and corneas became re-epithelialized. Visual improvement was reported for 72% of the patients although neovascularization was reported in 53% of the patients[66]. Decellularized tilapia fish scale-derived extracellular matrix has also been tried in a rat

model to evaluate the biocompatibility. The short term (21days) result ended up with the haziness, neovascularization around the sutures, obscuring the pupil, melting of the anterior corneal lamella and local swelling; depending on the place in the cornea the scale was transplanted[67]. But in traumatic perforations in a mini-pig model, fish scale cornea showed promising results in a very short-term study (3-4 days) with only mild to moderate swelling in the perforated cornea[68].

Stem cell treatment, in contrast to decellularized corneas, is another commonly accepted therapy, particularly with problems related to deficiency of the stem cells of the corneal epithelium, i.e. limbal stem cell deficiency[69]. Limbal stem cell deficiency is overcome by grafting small pieces of the health eye's limbus to the damaged eye[70]. More recently, limbal stem cells have been cultured *in vitro* to expand their numbers and then grafted into cell deficient eye[71]. Autologous grafts obtained from the healthy contralateral corneas have worked very well[72]. Burn related destruction of the lumbus resulting in limbal stem cell deficiency has been successfully treated with autologous cultured limbal stem cells from contralateral eye[72] cultured on human amniotic membranes[73] or fibrin substrates[74]. Within our group, we have shown that a stratified epithelium layer can be reconstructed by seeding limbal cells on top of collagen-based materials[75]. The expansion of stem cells requires the use of certified cleanrooms following Good Manufacturing Practice (GMP) guidelines. This therefore limits the ability to perform stem cell transplantations to large, affluent tertiary healthcare centers. Recently, however, a technique known as Simple Limbal Epithelial Transplantation (SLET) was developed to allow for transplantation of stem cells without the need for expansion of stem cells. In this technique, a healthy limbal (2x2mm) part is divided into small pieces and expanded *in vivo* in the stem cell-deficient eye with the help of amniotic membrane and fibrin glue[76]. Two-amniotic membranes are also used to sandwich harvested limbal stem cell to protect the cells from hostile microenvironment[77]. Multicenter clinical trial with autologous SLET technique showed the 83.8% success rate with the complete epithelization and avascular corneal surface within 6 months post-operation[78].

Artificial corneas known as keratoprostheses (KPro's) have been in development for over 200 years. In 1789, French scientist, Pellier de Quengsy proposed replacement of opaque corneas using a glass alternative[79]. In 1855, a quartz crystal implant was first transplanted into a human[80]. In the second half of the 20th century, core-and-skirt

designs for artificial corneas became common. This design comprised of a transparent plastic optical core (e.g. poly (methyl methacrylate)) and a porous skirt of different materials. The most successful among this approach is the Boston KPro. In this KPro, a donor cornea is positioned between the front and back plates[81]. Boston KPros' are well retained. Development of glaucoma, retroprosthetic membrane formation, and persistent epithelial defects, however, remain as postoperative difficulties encountered[82, 83]. Other problems include the need for lifetime antibiotics, and in a proportion of patients, immune suppression is required.

The AlphaCor is another well-known KPro that used in the clinic[84]. In this KPro, both optic and skirt are made from poly (2-hydroxyethyl methacrylate) (PHEMA). The optic is solid, but the skirt contains interconnecting pores that allow biointegration with adjacent corneal tissue. The retention rates of AlphaCor after 1, 2 and 3 years of transplantation were 87%, 58%, and 42%, respectively. Due to the low water content of PHEMA based AlphaCor, the KPro has reduced permeability to glucose and other solutes, which may account for the decreasing implant survival over time. In order to increase the water content of PHEMA-containing implants, methacrylic acid monomers were incorporated into the PHEMA. The surface of this KPro was treated with laminin and fibronectin, which increased corneal epithelial growth *in vitro*[85, 86]. In another approach, KPro was designed to selectively assist different corneal cells growth. In this approach optic core was made of a dual network of poly (ethylene glycol) and poly (acrylic acid) (PEG/PAA) to support epithelization and skirt is made of microperforated poly(hydroxyethyl acrylate) (PHEA) that encourages stromal tissue integration[87]. *In vivo* biocompatibility of PEG/PAA based materials were tested in rabbit cornea[88].

The osteo-odonto-keratoprosthesis or OOKP is another type of KPro that contains dental tissue enveloped with autologous oral mucosal cells[80, 89]. OOKP implantation requires a two-stage surgical procedure. The first stage involves the harvesting of a monoradicular tooth to prepare osteo-odonto-lamina. A hole is drilled through dentine, and poly(methyl methacrylate) (PMMA) optic cylinder is placed into the hole. This implant then placed into a submuscular pouch within the oral mucosa for 2-4 months. By this time, the OOKP becomes vascularized to maintain a blood supply. During the second stage, the graft is removed and placed in the eye after removing the cornea up to Bowman's layer. OOKP implantation is a complicated procedure, but the survival rates

are high as it can withstand hostile ocular environment and in a severely dry eye condition[90].

1.5 HUMAN RECOMBINANT COLLAGEN BASED ARTIFICIAL CORNEAS

As collagen is the main competent of the corneal extracellular matrix, artificial corneas made from collagen have garnered a lot of interest as alternatives to human donor corneas. The main source of collagen is extracted animal protein, although recombinantly produced collagen is now available. To give mechanical strength and feasibility for transplantation, collagen is cross-linked by different mechanisms[91-94]. The most conventional crosslinking method is using with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS)[95]. The EDC cross-linking reaction starts with the activation of the carboxylic acid groups of Asp or Glu residues of collagen by EDC. The reaction of the carboxylic acid with EDC gives O-acylisourea, an intermediate reactive group. O-acylisourea is very unstable to hydrolysis. NHS converts the O-acylisourea group into a NHS-activated carboxylic acid group. The resultant then reacts with amine groups of lysine and the final product will be zero length cross-linked collagen[96]. Optically transparent and cell friendly corneal implants were made from porcine and bovine collagen and transplanted into animal models[97, 98]. However, porcine collagen-based corneas when grafted in mice showed immunogenic reaction[99]. Animal-derived collagen comes from heterogeneous sources, and because of the different levels of processing and screening in each different source, great care needs to be taken due to the risk of transmitting diseases[100] as well as provoking immune responses in the host[101].

The use of recombinant human collagen mitigates the heterogeneity and pathogen transmission issue. Recombinant human collagen production has been developed using mammalian cell culture systems[102], insect cell cultures[103], silkworm[104], tobacco plants[105] and yeast[106, 107]. Only mammalian cells expressed full-length hydroxylated collagen. All other expression systems are lack proline hydroxylation result in the production resulting in an unstable collagen that is readily degraded by proteolytic degradation[108]. In the yeast, *Pichia pastoris*, co-expression of prolyl 4-hydroxylases with the collagen gene facilitates the production of stable collagen in a

recombinant manner[107, 109]. *P. pastoris* has been used to express type I, II and III collagen[110]. Recombinant human collagen-based artificial corneas have been made from both type I and III collagen. Both types of collagen showed similar mechanical properties and *in-vivo* biocompatibility in mini-pigs although type III implants were superior in optical clarity[111]. Type III recombinant human collagen (RHC) based artificial corneas have been tested in a Phase I clinical trial on 10 patients in Sweden. RHC was crosslinked with EDC/NHS and fabricated into implants. They were grafted into 10 patients. Nine patients had keratoconus while the tenth had a central corneal scar[44]. The implants promoted regeneration of corneal epithelium, stroma, and nerves from the patients' endogenous cells. The grafted corneal implants have remained stable for four years without any rejection and without sustained immune suppression. At the 4-year follow-up (**Fig. 1**), the implanted cornea remained stably integrated into the host eye[112]. *In vivo* confocal microscopy showed that corneal cells had populated the implant (**Fig. 2**).
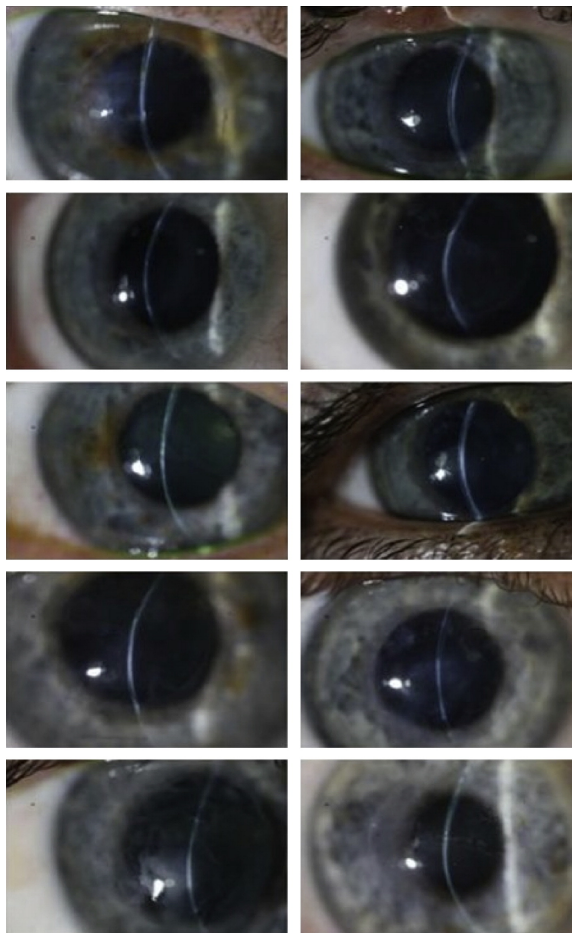

Figure 1. Slit lamp biomicroscopy images of the corneas of all 10 patients transplanted with recombinant human collagen implants at 4 years post-operation (From Biomaterials. 2014 Mar;35(8):2420-7).
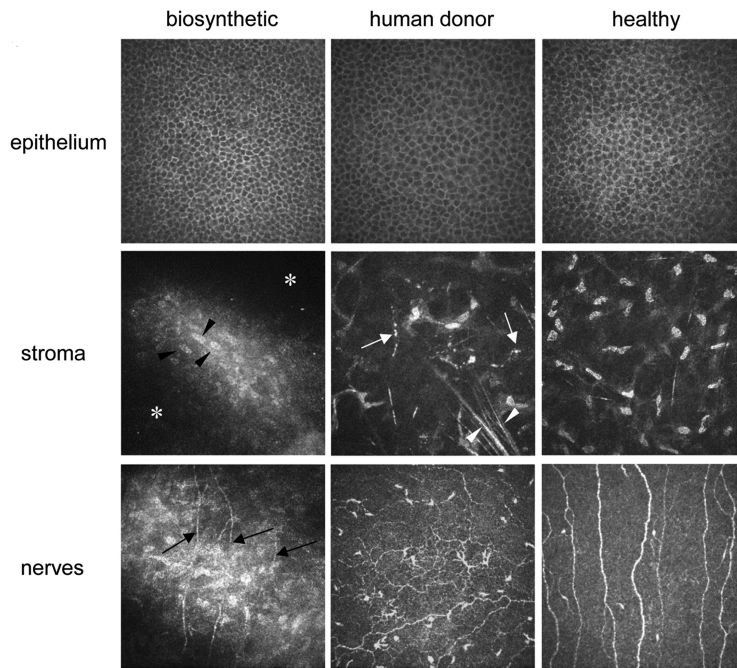
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Figure 2. Typical *in vivo* confocal microscopic appearance of four-year post-operation, unoperated and normal corneas. (From Biomaterials. 2014 Mar;35(8):2420-7)

One patient had undergone re-grafting as he could not be properly fitted with contact lenses for visual acuity. Histopathology of the neo-cornea obtained showed that it had a normal corneal structure, although a part of corneal implant still remained[113].

To improve the functionality and increase resistance to enzymatic degradation in the severe disease condition, a new artificial cornea was developed using interpenetrating polymer networks (IPN) of collagen and phosphorylcholine. These implants were tested in mini-pig, guinea pig and rabbit models[114-116]. In that case 2-methacryloyloxyethyl phosphorylcholine (MPC) was polymerized with poly(ethylene glycol) diacrylate (PEGDA) with the help of ammonium persulphate (APS) and N,N,N,N tetramethylethylenediamine (TEMED)[116].

1.6   ARTIFICIAL CORNEAS BASED ON PEPTIDE ANALOGS OF COLLAGEN

About 90% of the world's visually impaired live in low-income nations[25]. Production and purification of RHC in yeast is an expensive process that makes the price of the artificial cornea unreachable to the most needy individuals. Therefore, an alternative that could replace RHC with the same physicochemical and biological properties would

be a huge advancement. Collagen-like peptides (CLP) or collagen mimetic peptides have been developed as functional alternatives of collagen [117].

2 AIMS OF THIS PROJECT

Artificial corneas have been developed as an alternative to donor human corneas and recently, our group has shown that implants based on recombinant human collagen have successfully promoted regeneration of corneal tissues and nerves in a small clinical trial. However, these implants are not amenable to grafting in patients with severe pathologies. We hypothesize that the structural integrity and mechanical properties can be enhanced in several ways, ranging from incremental changes by incorporation of a second network of materials, peptides to surface modification and surgical handling techniques. We also hypothesize that the functional properties of full-length collagen can be replicated using shorter peptide analogs to collagen.

The specific aims of this thesis were therefore to:

- characterize the ultrastructural properties of collagen versus collagen-MPC cornea implants to understand how they can be improved
- optimize the collagen-MPC formulation by enhancing the mechanical and chemical properties by examining the effects of a new epoxy-based crosslinker, and incorporation of laminin-derived cell adhesion peptides, YIGSR and IKVAV
- optimize the functionality of collagen-MPC implants by surface modification using microcontact printing, and evaluating its performance as an implant by examining the effects of laser-cutting in potential surgical use
- evaluate a collagen-like peptide hydrogel as an analog to collagen-based hydrogels *in vitro* and *in vivo*

3 MATERIALS AND METHODS

Please refer to Papers I-V for the detail description of the materials and methods used in this thesis.

3.1 ETHICAL CONSIDERATIONS

Paper IV

Dr. Lucia Kuffova, University of Aberdeen, UK, performed the animal studies, had a permit from the Ophthalmic Research and Animal License Act (United Kingdom). Project license number was 60/3890.

Paper V

After ethical approval from the local ethical committee, Linköpings Djurförsöksetiska Nämnd (Dnr 52-15), and in compliance with the Swedish Animal Welfare Ordinance and the Animal Welfare Act, materials were implanted in the rats subcutaneously.

After permission from Stockholms Norra Djurförsöksetiska Nämnd (N204/13), materials were transplanted into pig corneas.

3.2 RHC-MPC ARTIFICIAL CORNEAL IMPLANT

RHC used in for the papers was purchased from FibroGen Inc. (San Francisco, USA) and 3H Biomedical (Uppsala, Sweden). For making hydrogels, approximately 500 mg of RHC solution was buffered with 150μl of 0.625M 2-morpholinoethane sulfonic acid monohydrate (MES) buffer. MPC solution in MES was added into a syringe mixing system (**Fig. 3**) for through mixing.
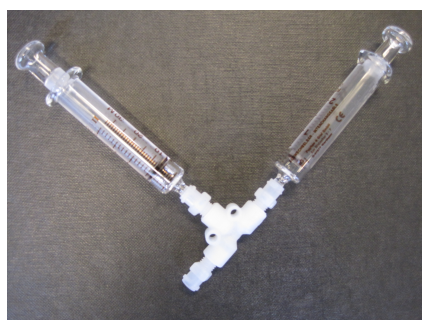

Figure 3. Syringe mixing system

Figure 4. Jigs with 500 μm moulds

The MPC:RHC (w/w) ratio used was 1:2. PEGDA was then added (PEGDA:MPC (w/w) = 1:3). Calculated volumes of 4% (w/v) APS solution in MES and 2% (v/v) TEMED solution in MES were added sequentially (APS:MPC (w/w) = 0.03:1, APS:TEMED (w/w) 1:0.77). Calculated amounts of NHS (10% (w/v) in MES) and EDC (5% (w/v) in MES) solutions were added and the reactants were thoroughly mixed at $0^{o}C$ (EDC:RHC-NH$_2$ (mol:mol) = 0.4:1, EDC:NHS (mol:mol) = 1:1). The final mixed solution was immediately cast into cornea-shaped moulds (12 mm diameter, 500 μm thick) (**Fig. 4**).

In paper II, RHC-MPC hydrogels were made with slight modifications. Different RHC:MPC (w/w) ratios were used as 1:1, 2:1 and 4:1. EDC:RHC-NH$_2$ (mol:mol) ratios were 0.3-1.5:1. APS:MPC (w/w) ratio was 0.015:1. For microcontact printing, RHC:MPC (w/w) ratio of 1:2 with EDC:RHC-NH$_2$ of 0.4:1 was used.

3.3 EPOXY-CROSSLINKED COLLAGEN HYDROGELS WITH INCORPORATED LAMININ PEPTIDES

1,4-Butanediol diglycidyl ether (BDDGE), a bi-functional epoxy crosslinker, was used to crosslink an aqueous solution of type 1 porcine collagen (10% w/v) with the intention of enhancing the mechanical properties of the hydrogel by increasing its elasticity (Paper III). BDDGE was used alone to crosslink at basic pH and in the combination of EDC/NHS at acidic pH. The same syringe mixing system as above was used to thoroughly mix up the hydrogel components with sodium bicarbonate buffer for hydrogels made under basic pH conditions, and MES for the acidic conditions. For hydrogels prepared under the basic conditions, 1 molar equivalent (relative to the collagen lysine residues) of BDDGE was used. For acidic conditions, 0.4 molar equivalents of BDDGE were used together with 0.5 molar equivalent of EDC/NHS. The final products of both formulations were then cast into moulds and cured within a humidified chamber for 24 and 72 hours for the basic and the acidic conditions, respectively. Under the acidic condition, 0.3 molar equivalent Cu$^+$ was used to catalyze the formation of the amide bonds with epoxides. YIGSR and IKVAV, small cell adhesive peptides derived from laminin were incorporated into the acidic hydrogel at 0.001 molar equivalent concentrations to collagen amine, to increase cellular biocompatibility properties of the hydrogel.

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3.4 SYNTHESIS OF COLLAGEN-LIKE PEPTIDE (CLP) AND FABRICATION OF HYDROGEL

3.4.1 Synthesis of CLP-PEG

A 38 amino acid long CLP peptide, CG- (Pro-Lys-Gly)$_4$(Pro-Hyp-Gly)$_4$(Asp-Hyp-Gly)$_4$, was synthesized using a Symphony automated peptide synthesizer (Protein Technologies Inc., Tucson, AZ, U.S.A.) using standard fluorenylmethoxycarbonyl (Fmoc) chemistry. The resulting peptides were split from the resin by treatment with a mixture of trifluoroacetic acid (TFA), water and triisopropylsilane (TIS) (95:2.5:2.5 v/v; 10 mL per gram of polymer) for 2h at ambient temperature. The CLP was then precipitated by the addition of cold diethyl ether, followed by centrifugation and lyophilization. Purification was done using reversed-phase High Performance Liquid Chromatography (HPLC) on a semi-preparative C-18 column (Grace Vydac, Helsingborg, Sweden).

The solution of CLP and 8-Arm PEG-Maleimide was mixed at the molar ratio of PEG-Maleimide:CLP=1:5. After 4 days of continuous stirring the mixture was dialyzed through a dialysis membrane (12-14,000 molecular weight, Spectrum Laboratories, Inc., CA, US). After dialysis, the solution was lyophilized to obtain solid CLP-PEG.

3.4.2 Fabrication of hydrogel

To fabricate corneal implants, 500mg of 12% (w/w) CLP-PEG was taken into a 2ml glass syringe. Calculated volumes of NHS and then EDC were added to the syringe mixing system we used previously. Depending on the molar equivalent ratio of EDC to the amine of CLP-PEG, three different types of hydrogels were made; CLP-PEG-NH$_2$: EDC=1:0.5, CLP-PEG-NH$_2$: EDC=1:1 and CLP-PEG -NH$_2$: EDC=1:2. The molar ratio of EDC: NHS was 1:1. As a negative control of in-vitro biocompatibility, hydrogels were made by 8-Arm PEG-Maleimide and 8-Arm PEG-Thiol together at a molar ratio 1:1 at 37°C. CLP-PEG-NH$_2$: EDC=1:2 was used for further characterizations and animal studies.

3.5   CHARACTERIZATION

Structural and optical properties of the RHC-MPC corneal implants were examined in Paper I, using transmission electron microscopy (TEM), X-ray scattering, spectroscopy, and refractometry.

Light transmission, refractive index and water content of the implants were measured. Thermal stability of the materials was measured by differential scanning calorimeter (DSC, Q20, TA Instruments, New Castle, UK). Tensile strength, elongation at break and elastic modulus was measured (not as a part of the Quality control for clinical use) by Instron machine (Biopuls, High Wycombe, UK). Fourier transform infrared spectroscopy (FTIR, VERTEX 70, Bruker, Billerica, MA, USA) confirmed the presence of different functional groups present on the surface of the materials.

The feasibility of using femtosecond laser assisted cutting for RHC-MPC hydrogels was tested, to determine whether the hydrogels were amenable to the laser-assisted surgical grafting of corneal hydrogel implants.

To confirm the conjugation of CLP with PEG-Maleimide, ^{1}H NMR spectra was measured using an Oxford 300 MHz (Varian, CA, US) spectrometer at room temperature. CLP was dissolved in D_2O (1mg/0.1ml) whereas CLP-PEG and 8-Arm PEG-Maleimide was dissolved in C_2D_6OS (1mg/0.1ml) (Paper V).

Triple helicity of CLP-PEG was confirmed using circular dichroism (CD) on a Chirascan CD Spectrometer (Applied Photophysics Ltd, Surrey, UK). CLP-PEG was dissolved in water at a concentration 0.5% (w/v). The solution was scanned at a range of wavelengths from 180 to 260nm at 25^oC, and its ellipticity, θ (mdeg) was recorded (Paper V).

3.6   COLLAGENASE STUDY

Enzymatic degradation of hydrogels was performed using collagenase from *Clostridium histolyticum* (Sigma-Aldrich). Small pieces of hydrogels were placed in 5U/ml collagenase solution in 0.1M tris-HCL (pH 7.4) containing 5mM $CaCl_2$. The hydrogels were incubated at 37^oC and the collagenase solution was changed at every 8 hours. The gels were weighed at specific time pauses after removal of surface water.

The enduring mass of the hydrogels was traced as a function of time, comparative to their original hydrated weight.

Residual mass % = Wt / Wo %

Wt is the weight of hydrogel at a certain time point, and Wo is the initial weight of the hydrogel.

3.7 CELL CULTURE

Human corneal epithelial cells (HCEC) and GFP-HCEC (green fluorescence protein-tagged Human corneal epithelial cells) were used to evaluate the proliferation of cells on the hydrogels. Cells were maintained in KSFM supplemented with epidermal growth factor (EGF) and bovine pituitary extract (BPE) at 37°C incubator. Images of cultured cells were taken at different time point by using fluorescence microscope (AxioVert A1, Carl Zeiss, Göttingen, Germany). Tissue culture plate (TCP) was used as a positive control for culturing HCEC.

In Paper III, HCEC was seeded on different hydrogel and MTS was done to examine the proliferation of the cells. To evaluate the effect of incorporation of laminin peptides, HCECs and rodent hybrid dorsal root ganglia- neuroblastoma cells (NDC)[118] cells were seeded on peptides containing hydrogels and proliferation was measured over time.

3.8 IMMUNOHISTOCHEMISTRY

For immunohistochemistry (Paper II) HCECs were cultured on hydrogels for four days. Then cells were fixed, permeabilization and block. Cells were incubated with anti-proliferating cell protein Ki67 antibody (Sigma–Aldrich, MO, USA), anti-focal adhesion kinase (FAK) antibody (Abcam, Cambridge, UK) and anti-integrin beta 1 (integrin β1) antibody (Abcam, Cambridge, UK) followed by incubation with secondary antibody, Alexa Fluor-594 (Invitrogen, Oregon, USA). A confocal microscope (LSM700, Carl Zeiss, Göttingen, Germany) was used to visualize and capture the images.

3.9 *IN VIVO* BIODEGRADATION

All materials of 10mm diameter and 500um thick were implanted subcutaneously into three Albino Wistar rats (Charles River, USA) after anesthetized with Isoflurane (5% for induction and 2% for maintenance). The subchronic effect of the implants and overall biodegradation was evaluated according to the ISO 10993-6 (International organization of standardization, Tests for local effects after implantation, Biological evaluation of medical devices, Geneva, Switzerland) guideline. For implantation, two small incisions were made paravertebrally on the backs of the each rat. Implants were inserted, and the wound was closed with 2-3 interrupted sutures. After 90 days, the animals were euthanized, and the implanted materials were removed from the host together with surrounding tissue for histopathological analyses.

3.10 *IN VIVO* IMPLANTATION OF CORNEAL CONSTRUCTS IN ANIMAL CORNEAS

With a license from Home Office, UK, RHC-MPC implants were tested for their ability to tolerate the adverse environment within mice with HSK corneas. Mice were infected with HSV-1 viruses at six weeks before implantation and had developed HSK. 1.5mm diameter of hydrogels was grafted into corneas that had HSK and retained using continuous sutures.

RHC-MPC corneal implants were also tested in corneas of mini-pigs. With ethical approval (N204/13) from the regional animal experimental ethics committee in Stockholm (North), RHC-MPC & CLP cornea shape materials (500μm thick and 6.5mm diameter) were transplanted into the cornea of Göttingen mini-pigs (Ellegaard, Denmark). A Swedish Medical Products Agency contract research laboratory, Adlego Biomedical AB, 75103 Uppsala, Sweden, conducted the entire experiment (Study plan: AB13-32). Two groups of four female mini-pigs in each group, aged 5-6 months at the beginning of the procedure were grafted with corneal implants comprising RHC-MPC crosslinked with EDC/NHS and CLP-PEG. Prior to the surgery, animals were anesthetized with isoflurane (0.5-3%, IsoFlo vet, Abbott Laboratories, UK) in oxygen and air. Tetracain 1% eye drop (Chauvin Pharmaceuticals Ltd, UK) was applied on ocular surface as topical anesthesia. Each pig cornea was cut circularly with a trephine

to a depth of 500um and 6.50mm diameter, and the corneal bottom was manually dissected and removed. Then implant material was cut with a 6.75mm diameter trephine and placed on the surgically removed corneal wound bed, as in deep lamellar keratoplasty (DLKP). All implants were covered with the human amniotic membrane (HAM) (St:Erik's Eye Hospital, Stockholm) and the implants were kept in place using overlying sutures. Upon completion of the surgery, antibacterial and anti-inflammatory ophthalmic suspensions (3mg/ml dexamethasone and 1mg/ml tobramycine, Alcon, Sweden) were administered. The maintenance dose was 1 drop, 3 times daily for 5 weeks. The unoperated eye of each pig served as a control.

The mini-pigs were examined at 3, 6, 9 and 12 months post-operation. Clinical examinations conducted at these times included slit lamp biomicroscopy (using a Kowa SL-15 Portable Slit Lamp, Kowa company, Ltd., Aichi, Japan), Schirmer's tear test, intraocular pressure (IOP; measured using a Tonovet Tonometer, Icare Finland Oy, Finland), corneal touch sensitivity using aesthesiometry (measured using a Cochet-Bonnet aesthesiometer, Handaya Co., Tokyo, Japan), corneal thickness measurement by Handy Pachymeter SP-100 (Tomey, AZ, USA), and *in vivo* confocal microscopy (IVCM; using a Heidelberg HRT3 Rostock Cornea Module, Heidelberg Engineering GmbH, Dossenheim, Germany). The McDonald-Shadduck scoring system was used to score results of the slit lamp evaluation[119]. IVCM images were used to analyze different cell types, tissue structure, and density of nerve[120].

At 12 months post-operation, the animals were euthanized with an overdose of pentobarbital (Allfatal vet 100mg/ml, Omnidea, Sweden). Both operated and control unoperated corneas were harvested for further evaluation.

3.11 BIOCHEMICAL ANALYSIS ON EXCISED PIG CORNEAS

Biopsies (3 mm) from within the operated area and from unoperated control corneas were collected for biochemical analyses. Samples were suspended in 10 mM HCl. Pepsin (Sigma Aldrich, USA) was added to a final concentration of 1 mg/mL from a 10 mg/mL stock solution prepared just before use. The samples were digested with pepsin at 2-8°C for 96 hours, and the soluble fraction was recovered by centrifugation. An aliquot of the pepsin soluble fraction was mixed with NuPAGE 4X LDS sample buffer

(Life Technologies), denatured at 75°C for 8 minutes and analyzed on a 3 - 8% Tris-acetate gels under non-reducing conditions or 4 -12% Bis-Tris gels (Life Technologies) under reducing conditions. Proteins were visualized by staining with Gelcode Blue. Precision Plus Markers and porcine skin type I collagen were used as molecular weight standards. To determine whether there were quantitative differences in the amounts of type I and type V collagens in operated and unoperated corneas, densitometric scans of the stained gels were made to obtain relative numerical units using a GE Healthcare Image Quant 350. ANOVA or pairwise t-tests were performed to determine statistical differences using GraphPad Prism 5.

4 SUMMARY OF THE RESULTS

4.1 CHARACTERIZATION OF RHC-MPC IMPLANTS

4.1.1 Physical and mechanical characterization

RHC-MPC hydrogels had a refractive index of 1.334, which is very similar to the hydrated human cornea and identical to that of water. These hydrogels transmitted 92% of incident light with 500nm wavelength spectra. Wide-angle X-ray scattering showed the uniaxial orientation of collagen throughout RHC-MPC.

Formulations with a 2:1 ratio of RHC:MPC were optimal for fabrication of implants. Changes in MPC content did not change the properties of the hydrogels mechanically. Increased collagen content increased the tensile strength of the RHC-MPC hydrogels but resulted in overall stiffer and less elastic constructs.

The thermal degradation temperature of RHC-MPC hydrogels was lower than human cornea but far above than usual body temperature. FTIR analysis confirmed the IPN formation showing the peaks for both collagen and MPC of the surface of the implants, which resemble by the increased stability against collagenase degradation.

4.1.3 *In vivo* compatibility

RHC-MPC implants were transplanted in herpetic mice model (Paper IV) and the results showed that implants remain in the host for 22 days whereas allograft got rejection by day 15-post operation.

In paper V, the subcutaneous study in rat showed that the RHC-MPC materials did not trigger the immune reaction against the materials. Implantation into pig corneas showed stable integration with infiltration of corneal cells in the materials. Biochemical analysis of excised cornea confirmed the presence of α1 and α2 of collagen type 1; and α1 of collagen type V.

4.2   SURFACE MODIFICATION OF RHC-MPC IMPLANTS

The microcontact printing had only a minor effect on the optical properties of the hydrogels. The percentage of transmitted light measured for implants with 30μm stripes was 99% with a backscatter of 1.7%; while the 200μm striped samples showed light transmission of 96% and backscatter of 0.6%. Atomic force microscopy (AFM) analysis showed that the printed areas did not affect the overall surface properties of the implants. The height difference between patterned and unpatterned areas was negligible.

Analysis of the patterns showed that the highest number of cells was found on the 200μm patterned strips. However, immunohistochemical analysis showed the highest expression of integrin β1, FAK, and Ki67 at 30μm patterned stripes.

4.3   EPOXY CROSS-LINKED COLLAGEN HYDROGELS

4.3.1   Physical and mechanical characterization

BDDGE crosslinking under basic conditions allowed the formation of collagen hydrogels. Under acidic conditions, both BDDGE and EDC/NHS were needed for gelation. The optical and thermal properties were similar for both BDDGE and EDC/NHS crosslinked hydrogels, but the denaturation temperature increased for BDDGE hydrogels compare to EDC/NHS crosslinked control hydrogels. The mechanical properties differed between the acidic, and basic BDDGE crosslinked hydrogel. Hydrogels made under of acidic conditions showed 147% elongation at break while that made under basic conditions showed 14.02% elongation. Results from femtosecond laser assisted cuts revealed that BDDGE crosslinked hydrogel could cut precisely using the laser.

4.3.2 *In vitro* biocompatibility

Cell proliferation was decreased in BDDGE crosslinked hydrogels. However, upon adding laminin peptides, YIGSR increased proliferation of HCEC while IKVAV increased NDC proliferation.

4.4   CLP-PEG HYDROGELS

4.4.1 Physical and mechanical characterization

^{1}H NMR spectra of 8-Arm PEG-Maleimide and CLP-PEG confirmed the conjugation of CLP with PEG-Maleimide. Peaks at 6.5-6.6 at PEG-Maleimide spectra attributed to the proton attached to the -C=C- group of Maleimide on PEG. CD of CLP-PEG showed the triple helix characteristic with a positive peak at 221nm at 25°C. CLP-PEG was possible to crosslink with EDC/NHS and formed the stable hydrogel that holds the structure of the artificial cornea. CLP-PEG hydrogels were >90% transparent and water content was also over 90%. CLP-PEG hydrogels were completely resistance to collagenase degradation at high concentration of EDC.

4.4.2 *In vitro* biocompatibility

In vitro biocompatibility of the hydrogels was tested against HCEC and result showed that no cytotoxicity of the materials with the confluence of cells within 5 days at the initial of 5000 cells on 96 well place.

4.4.3 *In vivo* compatibility

Results from the subcutaneous study revealed that, up to 12 weeks of transplantation, CLP-PEG showed complete biocompatibility without inflammation and fibrosis. Artificial corner made from CLP-PEG implanted in mini-pig and examined over 12 months at GLP facilities. *In-vivo* confocal microscopy showed corneal cells growth in the materials including subepithelial nerve. Biochemical analysis on the excised corneas showed the presence of type I and V collagens in quantities resembling those of the untreated control corneas as well as RHC-MPC benchmarks.

5 DISCUSSION

5.1 PHYSICAL AND MECHANICAL PROPERTIES

Optical characterization performed in Paper I was crucial for using RHC-MPC as a corneal implant, and the results showed the implants were as transparent or more transparent than human corneas. The reasons for being transparent are high water content, narrow aligned collagen fibers and a uniform refractive index. Being cell-free, RHC-MPC implants were also found to be transparent to UV light that is unlike to normal human cornea, which is populated by cells. In particular, the corneal epithelium absorbs UV light and thereby protects the inner components of the eye against potentially harmful UV rays. These results suggest that patients transplanted with RHC-MPC implants should use UV protection during the early stages of regeneration until they become populated by in-growing endogenous host cells. In Paper II, SEM results suggested that RHC-MPC hydrogels had a lamellar organization of collagen fibrils with interpenetrating networks that were more closely mimics the microstructure of the native cornea than RHC alone.

5.1.1 *In vivo* biocompatibility in animal models

Previously, it was shown that incorporation of MPC into RHC hydrogels allowed the implants to withstand in-growth of neovascularization in rabbit models of alkali burns[115]. More recent reports show that MPC has anti-inflammatory properties[121, 122]. Incorporation of MPC into the RHC implant also enhanced the mechanical properties of the implants. Although the stability of RHC-MPC implants was increased in case of enzymatic degradation, the overall strength of the implant remains far weaker than native cornea[123]. The lower mechanical strength could be accounted for by the absence of proteoglycans, the absence of Bowman's membrane, the small diameter of aligned uniaxial collagen fibrils and the overall low concentration of collagen within the hydrogel.

When transplanted into HSV mice (Paper IV), RHC-MPC implants survived 7 days longer than allograft although this difference was not significant due mainly to the small sample size. However, this result indicates implants were at least comparable to allograft to stand against the hostile environment in HSV cornea.

In Paper V, implantation of the RHC-MPC hydrogels into rats confirmed that the implants were fully biocompatible. When implanted into the corneas of mini-pigs, there were fine blood vessels found in early post-operation condition but these retreated by 6 months post-operation. By 12 months post-operation, the initially cell-free corneal implants became populated by epithelial and stromal cells. They were also reinnervated.

5.2   MICROCONTACT PRINTING ON RHC-MPC HYDROGELS

Microcontact printing (μCP) has been used to integrate biologically active macromolecules onto the surfaces of solid substrates without the loss of their activity[124, 125]. Microcontact printing has been mainly described as patterning on hard surfaces such as glass, silicone (e.g. polydimethylsiloxane (PDMS)) and silicon oxide, polystyrene, PMMA (polymethylmethacrylate) and gold[124-126]. In Paper II, we achieved reproducible μCP on thick, highly hydrated RHC-MPC hydrogels, using fibronectin, as "ink". Fibronectin is a large glycoprotein present in the basement membranes of the corneal epithelium[127]. Cell attachment to fibronectin has been shown to trigger β1 integrin-FAK signaling to initiate proliferation of metastatic lung cancer cells [128] and in our case, HCECs. Expression of β1 integrin, FAK and Ki67 was upregulated in cells growing on 30 μm stripes over broader 200 μm stripes or unpatterned surfaces. This showed that the width of the patterned fibronectin stripes was able to influence cell behavior.

5.3   BDDGE CROSSLINKED HYDROGELS

Epoxy-containing crosslinkers such as BDDGE have been shown to crosslink collagen producing hydrogels. In this thesis, crosslinking porcine type 1 collagen with BDDGE resulted in hydrogels with enhanced elastic properties. BDDGE can crosslink at acidic pH in the presence of a catalyst as mentioned in other published report; Cu (Copper) catalyzes the formation of secondary amine bond when epoxide reacts with amine[129]. BDDGE was used to crosslink collagen at acidic condition together with a secondary crosslinker, EDC/NHS. As an alternative approach, BDDGE was used to crosslink collagen at basic pH as isoelectric point of collagen lysine is basic, which facilitates the

crosslinking reaction through amine and epoxide[129, 130]. IKVAV and YIGSR, laminin peptides were incorporated into the hydrogel in acidic condition to enhance the cell attachment and proliferation, as they improve the cellular response of the biomaterials[131]. IKVAV showed increased neuronal cell viability and differentiation[132]; and YIGSR showed improved cell attachment[133] together with enhance collagen synthesis in human dermal fibroblasts[134]. In our case, like other published reports, IKVAV and YIGSR enhanced NDC and HCEC proliferation, respectively.

5.4   CLP-PEG HYDROGELS

O'Leary and co-workers (2011) described a 36 amino acid peptide, Pro-Lys-Gly)$_4$(Pro-Hyp-Gly)$_4$(Asp-Hyp-Gly)$_4$ that mimics collagen fibrils[135]. However, that peptide was only able to self-assemble into a soft hydrogel. For translational to clinical application, we needed a more robust hydrogel. We modified the O'Leary collagen-like-peptide by the addition of two extra amino acids – a glycine and a cysteine to allow covalent attachment to an 8-armed polyethylene glycol maleimide (PEG). The resulting CLP-PEG hydrogels were transparent and mechanically strong enough for grafting into host corneas. The implants showed stable and functional integration into the corneas of mini-pigs. Corneal epithelial and stromal tissue as well as extracellular matrix components and nerves had grown into the implants. The CLP-PEG hydrogels were therefore able to function, as successful scaffolds that promoted regeneration, just like implants made from RHC. For translation into clinical application, the use of fully synthetic CLP-PEG hydrogels will avoid potential problems such as transmission of viruses and other pathogens. As substitutes of RHC, CLP-PEG is potentially easier to handle and would allow for future functionalization.

6 CONCLUSION AND FUTURE PERSPECTIVES

This thesis describes the results from interdisciplinary research, combining biomaterials research, tissue engineering and biology to find out a solution for corneal blindness. Biomaterials based on collagen, the major ECM component of the human body have been successfully stimulating tissue regeneration in clinical trials. Collagen derived from animal or human cadaveric sources, however, suffer from batch to batch heterogeneity and carry a risk of pathogen transmission. We avoided these problems by developing implants based on RHC:

In particular, our optimized RHC-MPC implants stimulated the regeneration of corneal epithelium, stroma, and nerves in mini-pig corneas as well as corneas of HSK mice. These RHC-MPC implants were amenable to cutting with a femtosecond laser, and also to microcontact printing.

Nevertheless, like native collagen, RHC is large, relatively inert and difficult to handle, leading to the development of a range of CLPs. A comparison of the versatility and functionality of CLP-PEG analogs showed that these hydrogels were comparable to that of full-length collagen as scaffolds for promoting regeneration *in vivo* in mini-pigs.

I feel extremely fortunate to be a part of these interesting projects, and I am hopeful that these works will be helpful for removing the word blindness due to corneal disease. I also wish these works will be able to bring back light to millions of unprivileged people throughout the world.

7 MY SCIENTIFIC CONTRIBUTION

There is a colossal gap between the supply and demand for the good quality human donor corneas to replace diseased or damaged ones. I started the cornea research with the eagerness to solve the donor shortage problem by using artificial corneas. During the process, I have both optimized and developed new artificial corneas for future clinical use to combat conditions that are currently difficult to treat or deemed untreatable.

For my thesis, I had optimized the RHC-MPC formulation that is now in clinical evaluation (ClinicalTrials.org identifier NCT02277054). I have also enhanced the mechanical properties of these implants by improving the elasticity using a BDDGE crosslinker. This resulted in a new version of the artificial cornea that would be more suturable and therefore easier for the surgeon to handle during transplantation.

The production cost of RHC in yeast in a recombinant way is very expensive which ultimately increase the production costs of the artificial corneas made from these materials. In this thesis, I have developed an alternative artificial cornea that is made using collagen-like peptides. This fully synthetic implant will ultimately reduce the production cost of each artificial cornea. This will allow for potential scaling-up and large scale manufacturing of cost-efficient artificial corneas that can in the future be used to treat patients in developing nations.

I feel extremely privileged to be a part of these challenging projects, and I am hopeful that these implants developed during the course of my Ph.D. work will help eradicate blindness due to corneal disease. My goal is to bring back light to millions of unsighted people worldwide, and in particular, those who are underprivileged and who would benefit from the scale-up of cost-effective artificial corneas.

8 POPULAR SUMMARY

The cornea is an important part of the eye for vision. It is optically clear and functions like a window to the eye, letting light into the interior for vision. Corneal diseases are one of the major causes of blindness throughout the world. The main treatment still today for cornea blindness is the replacement of the diseased or damaged cornea with a human donor cornea by transplantation. However, the number of available donated corneas is severely limited compared to the demand in most countries. Even if donor corneas were readily available, cornea transplantation is not always successful as reactivation of diseases can occur after transplantation and can cause problems to the transplanted cornea. In addition, corneas with severe pathologies that include inflammation are often at high risk of rejection of the corneal grafts. As an attempt to solve the cornea shortage or rejection problem, an artificial corneal implant was being developed in this thesis. Crosslinked collagen and phosphorylcholine together were used to develop a transparent, hydrated implant with the shape and size of a human cornea. Collagen was used as the main ingredient because the human cornea is made up of mostly collagen. When transplanted into mini-pig corneas, the newly developed corneas were stable. The cells of the epithelium and stroma and nerves had regrown into the implants. The newly regenerated corneas showed functionality that approached that of normal corneas. We were also able to surface modify the materials using a printing technique. We printed stripes with a protein named fibronectin, and showed that thin stripes grew faster than thick stripes and unmodified surfaces. Although recombinant human collagen-based implants work well, its production is difficult and expensive. In addition, collagens are large and inert entities. In this thesis, we developed collagen-like peptide based corneal implants, which were easy and inexpensive to produce. The artificial cornea made from short peptide analogs of collagen was well tolerated in mini-pig corneas and promoted in-growth of host cells into the implants. The cells proliferated and produced new extracellular matrix to form a new cornea. Our all findings appear to be promising as potential implants to treat blindness and supplement the shortfall of human donor corneas.

9 ACKNOWLEDGEMENTS

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