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Development and Characterization of Peptide Modified Bioactive Membrane for Regeneration of Corneal Endothelial Tissue

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by

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Development and Characterization of Peptide Modified Bioactive Membrane for Regeneration of Corneal Endothelial Tissue

Abstract

Because of the drawbacks related with the current corneal transplantation techniques such as donor shortage, rejection or disease transmission risk, there is still an urgent requirement in order to produce new treatments for regenerating corneal tissue. Specially, it is also necessary to overcome the problems related with the blindness and vision losses originating from damage to corneal endothelium. By considering these, corneal tissue engineering might possess an encouraging potential to ensure alternative and innovative approaches toward the development of effective corneal tissue equivalents. Peptides allow to the creation of biomimetic and bioactive material surfaces, thus peptide-modified scaffolds have become a popular subject of research in tissue engineering. Herein, our objective was to synthesize the corneal endothelium tissue-specific peptides, to conjugate these peptides on created polyvinyl alcohol (PVA)-xanthan gum (XG) membranes, to characterize the peptide conjugation and also to define the influence of those peptide conjugated structures on cellular viability to utilize in corneal endothelium tissue engineering. First, for confirming the peptide conjugation on developed PVA-XG membranes, peptide conjugated samples were characterized based on the Scanning Electron Microscopy (SEM), Energy Dispersive X-ray (EDX) Spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR) and X-

ray Photoelectron Spectroscopy (XPS) analysis. Characterization findings of the current study showed that synthesized peptides were successfully conjugated onto the PVA-XG membranes. Moreover, the influence of these peptide-modified membranes on the viability and proliferation of human bone marrow derived mesenchymal stem cells (hMSCs) was assessed with respect to the MTT analysis and Live/Dead staining assay. Findings of the cell viability experiments revealed that the developed peptide conjugated membrane structures did not cause a toxical effect on hMSCs. Overall, results of this study indicated that bioactive peptide modified PVA-XG membranes possess the potential to be utilized for tissue engineering of corneal endothelium. Also, it is strongly believed that such results from this thesis study will aid and shed light on the further research relating to the regeneration of corneal endothelium.

Keywords: Corneal tissue engineering, Corneal endothelium, Peptide conjugation, Bioactive membrane, Characterization

Kornea Endotel Dokusunun Rejenerasyonu İçin Peptit Modifiye Biyoaktif Membranın Geliştirilmesi ve Karakterizasyonu

Öz

Mevcut kornea transplantasyonu teknikleriyle ilgili donör eksikliği, ret veya hastalık bulaşma riski gibi dezavantajlar nedeniyle, kornea dokusunun rejenerasyonu için yeni tedavilerin üretilmesi için hala acil bir ihtiyaç bulunmaktadır. Özellikle, kornea endoteli hasarından kaynaklanan körlük ve görme kayıplarına bağlı problemlerin de üstesinden gelinmesi gerekmektedir. Bunları göz önünde bulundurarak, kornea dokusu mühendisliği, etkili kornea dokusu eşdeğerlerinin geliştirilmesine yönelik alternatif ve yenilikçi yaklaşımlar sağlama konusunda umut verici bir potansiyele sahip olabilir. Peptitler, biyomimetik ve biyoaktif malzeme yüzeylerinin oluşturulmasına izin verir, bu nedenle peptid ile modifiye edilmiş yapı iskeleleri, doku mühendisliğinde popüler bir araştırma konusu haline gelmiştir. Burada, amacımız, kornea endoteli dokusuna özgü peptitlerin sentezlenmesi, bu peptitlerin oluşturulan polivinil alkol (PVA)-ksantan sakızı (KS) membranları üzerine konjuge edilmesi, peptit konjugasyonunun karakterize edilmesi ve ayrıca kornea endotel doku mühendisliğinde kullanım için bu peptit konjuge yapıların hücresel canlılık üzerindeki etkisinin tanımlanmasıdır. İlk olarak, geliştirilen PVA-KS membranlar üzerindeki peptit konjugasyonunu doğrulamak için, peptit konjuge numuneler Taramalı Elektron Mikroskobu (TEM), Enerji Dağılımlı X-Işını Spektroskopisi (EDX), Fourier Dönüşümlü Kızılötesi

Spektroskopisi (FTIR) ve X-ışını Fotoelektron Spektroskopisi (XPS) analizleri ile karakterize edildi. Mevcut çalışmanın karakterizasyon sonuçları, sentezlenen peptitlerin PVA-XG membranlara başarıyla konjuge edildiğini gösterdi. Ayrıca, bu peptit ile modifiye edilmiş membranların insan kemik iliğinden türetilmiş mezenkimal kök hücrelerin (iMKH'ler) canlılığı ve çoğalması üzerindeki etkisi, MTT analizi ve Canlı/Ölü boyama tahliline göre değerlendirildi. Hücre canlılığı deneylerinin bulguları, geliştirilen peptit konjuge membran yapılarının iMKH'ler üzerinde toksik bir etkiye neden olmadığını ortaya koydu. Genel olarak, bu çalışmanın sonuçları, biyoaktif peptit modifiye PVA-XG membranların, kornea endoteli doku mühendisliği için kullanılma potansiyeline sahip olduğunu göstermiştir. Ayrıca, bu tez çalışmasının bu tür sonuçlarının kornea endotelinin rejenerasyonu ile ilgili daha sonraki araştırmalara yardımcı olacağına ve ışık tutacağına kuvvetle inanılmaktadır.

Anahtar Kelimeler: Kornea doku mühendisliği, Kornea endoteli, Peptit konjugasyonu, Biyoaktif membran, Karakterizasyon

To my family. . .

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List of Abbreviations

3D	Three-Dimensional
ATPase	Adenosine Triphosphatase
CE	Corneal Endothelium
CECs	Corneal Endothelial Cells
CTE	Corneal Tissue Engineering
DBCs	Decellularized Bovine Corneas
DIEA	N,N-Diisopropylethylamine
DMEK	Descemet's Membrane Endothelial Keratoplasty
DMF	N,N-Dimethylformamide
DMSO	Dimethyl Sulfoxide
DSAEK	Descemet Stripping with Automated Endothelial Keratoplasty
DSEK	Descemet’s Stripping and Endothelial Keratoplasty
ECM	Extracellular Matrix
EDC	1-ethyl-(dimethylaminopropyl) carbodiimide
EDX	Energy Dispersive X-Ray
EK	Endothelial Keratoplasty
FECD	Fuchs Endothelial Corneal Dystrophy
FITC	Fluorescent Isothiocyanate
Fmoc	9-Fluorenylmethoxycarbonyl
FTIR	Fourier Transform Infrared Spectroscopy

HBTU	O-benzotriazole-N,N,N’,N’-tetramethyluronium-hexafluoro-phosphate
hCECs	Human Corneal Endothelial Cells
hMSCs	Human bone marrow derived mesenchymal stem cells
HOBt	Hydroxybenzotriazole
LM-5	Laminin-5
MBHA	4-Methylbenzhydrylamine
MCECs	Monkey Corneal Endothelial Cells
MES	2-Morpholinoethanesulfonic acid
MIDAS	Metal Ion Dependent Adhesion Site
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NHS	N-hydroxy-sulfosuccinimide
PAA	Poly (acrylic acid)
PBS	Phosphate Buffer Solution
PEG	Poly (ethylene glycol)
PEGDA	Polyethylene (glycol) diacrylate
pεK	Poly-ε-lysine
PHEMA	Poly (2-hydroxyethyl methacrylate)
PK	Penetrating Keratoplasty
PLGA	Poly(lactic-co-glycolic acid)
PNiPAAm	Poly (N-isopropylacrylamide)
PVA	Polyvinyl Alcohol
RNA	Ribonucleic Acid
SEM	Scanning Electron Microscope
TE	Tissue Engineering
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane
UV	Ultraviolet

WHO	World Health Organization
XG	Xanthan gum
XPS	X-ray Photoelectron Spectroscopy

List of Symbols

μm	Micrometer
mm	Milimeter
mmol	Millimole
g	Gram
ml	Milliliter
MW	Molecular Weight
°C	Degree Celcius
θ	Contact Angle
mM	Millimolar
α	Alpha
β	Beta
γ	Gamma

Chapter 1: Introduction

1.1 Biology of Cornea Tissue

1.1.1 Structure and Properties of Cornea

Cornea is known to create anterior part of eye's outside casing and it safeguards eye's inner contents and ensures nearly two thirds of refractive capacity of eye [1]. Cornea can be thought as a window to eye, which is also the powerful focusing component of eye [2]. It is also involved in refraction of light into retina, it lets to take visual informations from outside environment [3].

Cornea is a thin, transparent, smooth, avascular, and greatly innervated tissue. Cornea is convex, aspherically shaped and it is directly open to the outside environment [4]. It is continual through eye's white portion, named as sclera, and semi-transparent tissue, named as conjuctiva [4]. The boundary of cornea where it is continuing with sclera, is named as limbus. Limbus of the cornea is greatly vascularized and includes pluripotent stem cells reservoir [4]. In addition, corneal surface which is exposed to outside environment is covered via a tear film and inside surface of it is straightly contacts with fluid, aqueous humor [4].

Cornea consists of greatly structured, membrane bound however acellular relatively, and transparent collagenous tissue type which connects more dispersed and opaque sclera part at limbus [5]. As an information about dimensions, cornea's thickness progressively enhances from center to periphery, and that is seen with corneal curvature, that is biggest at center and tiniest at limbus [4]. Normal cornea tissue is known to measure 9–11 mm vertically and 11–12.5 mm horizontally in adult humans,

and thickness enhances from 0.5 to 0.6 mm centrally, resembling to a credit card thickness, to 0.6–0.8 mm peripherally [6, 7].

Mechanical strength and transparency are the most significant features of the cornea, and they are also significant factors while reconstructing damaged cornea [2]. In most mammals, cornea is the only tissue that needs a remarkable tensile strength with a greatly defined shape and optical clarity [1]. For optimal transparency, definite control of corneal hydration via endothelial cell layer is needed and any harm or endothelial dysfunction might cause corneal edema and visual impairment [6]. The strength of cornea is arising from nearly 200 collagen lamellae that are criss-crossing cornea within distinct directions. Also that collagen-rich layer consists of nearly 90% of corneal thickness and named "stroma proper layer" [2].

Cornea tissue possesses a complicated avascular structure that is composed of three major layers (the epithelium, stroma, and endothelium) and three major types of cell (epithelial, fibroblasts/keratocytes and endothelial) [8]. Normal corneal tissue does not possess a blood vessel supply, thus it is an avascular tissue of body. Although cornea is avascular, cornea's epithelial and endothelial cells are known to be metabolically active and actively found in wound recovery. These cell layers take blood elements and some other needs from blood vessels of interior and exterior carotid arteries. Aqueous humor provides glucose and little quantity of oxygen that is needed via cornea [4]. Even though normal human cornea has an avascular structure, it is dependent on elements of blood for remaining healthy. Those elements are given by tiny vessels found at outmost side of cornea and elements that are given via end branches of facial and ophthalmic arteries by aqueous humor and tear film [9]. Cornea takes most of oxygen from air exposure, oxygen that is absorbed in tear layer diffuses to corneal epithelial cells, and also expose of covered tear layer found on surface of cornea is required for some properties such as smoothness, integrity, oxygen supply [4].

Corneal nerves and sensation are known to be obtained from nasociliary branch of first division of trigeminal nerve. Also, corneal tissue is one of tissues that have the most highly innervated structure and most sensitive tissues. Moreoever cornea includes autonomic sympathetic nerve fibers [9]. It is greatly innervated with nerve endings, by

a 300-400 times higher in density relative to skin. Long ciliary nerves, sympathetic autonomic nerve fibers, and sensory nerves supply cornea with nerves [4].

1.1.2 Functions of Cornea

Cornea possesses the properties such as being transparent, avascular, and greatly innervated [10]. The natural cornea tissue is important in ensuring three major functional features to ocular optic system as protection (transport/mechanical protecting), transmission and refraction of incoming light through retina [5]. Besides transmitting and refracting light, the principal roles of cornea include protecting the eye from mechanical harms, ultraviolet (UV) light, or infection [10]. Also other two important roles are carried out by human cornea; the first one is that cornea creates anterior part of the outside casing of eye and keeps the inside part of eye from harm from outside, while the second function is that cornea is the eye's only strongest focusing component [2]. Cornea is known to ensure approximately 80% refractive power of eye. Due to those critical roles of cornea, it is both mechanically strong and transparent [2]. Healthy cornea tissue has the role of transmitting of nearly 75% of light to lens, also ensures an acute seeing which is dependent on collagen fibers and its water amount [5, 11].

1.1.3 Layers of Cornea

Human cornea tissue is created from 5 different layers, these include 3 cellular (epithelium, stroma, endothelium) and 2 interface (Bowman membrane, Descemet membrane) layers (Figure 1.1) [2, 9, 12, 13].

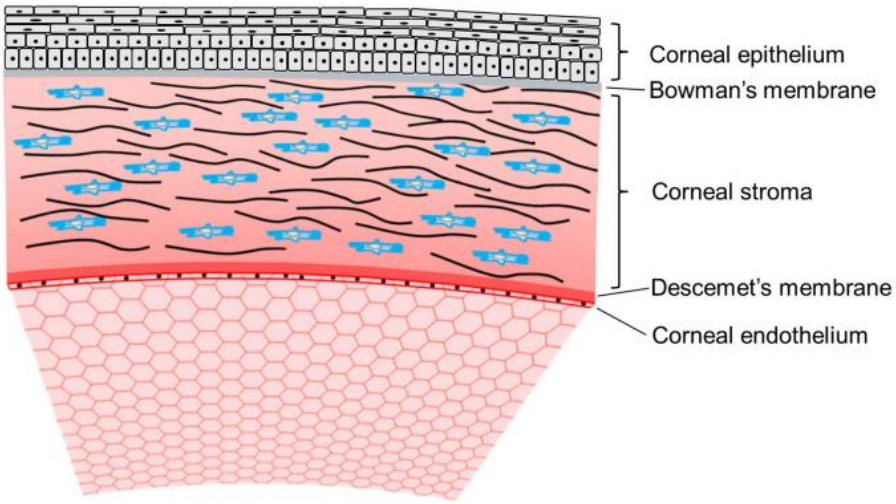

Figure 1.1: Cross-sectional representation of cornea [10]

1.1.3.1 Epithelium

Embryologically, epithelium layer of cornea is known to be derived from surface ectoderm between 5 and 6 weeks of gestation [13]. This layer forms the first barrier structure to outer surroundings, and this structure is a complementary portion of tear film-cornea interface that is important for eye's refractive functions. Epithelium can be defined as a stratified, non-keratinizing squamous stratum that is known via high uniformity from limbus to limbus [9]. Epithelium layer is coated by tear film that is crucial in terms of optically for smoothing out of microirregularities found on anterior part of it. In conjuction with cornea, air-tear film interface, ensures two thirds of eye's refractive ability [9]. Furthermore, tear film is able to safeguard corneal surface from microbial invasion, chemical, toxic, and foreign-body harms, and it can supply some immunological and growth factors which are crucial for epithelial reproduction, health and restoration [9]. Whenever eyes are open, epithelium takes oxygen from environment, and whenever eyelids are covered, oxygen is taken from tear film by surface conjunctival capillaries. Also epithelium gets glucose mostly via diffusion from aqueous humor [13].

Epithelium layer possesses 5-7 cellular layers through 3 different greatly differentiated and also self-renewing cell categories as the strata germinatum, daughter/wing cells, and squamous cells [12]. Also a normal epithelium founds on basement membrane made up of laminin, collagen IV, recognizable hemidesmosomes and anchoring fibrils [5]. Corneal epithelium is a layer that is a ‘‘tight’’, stratified, squamous, multilaminar

epithelium consisting of three different cell layers. Stratum germinatum is the back most layer and it is the unique epithelial cell layer able to go through mitosis. Middle layer of it consists of daughter or wing cells that are forced anteriorly throughout the epithelial desquamation. Squamous cells superficial layer creates the full tight junctions that form prime chemical and antigen shielding set in cornea [5].

Corneal epithelial cells possess mean life time of 7 to 10 days, regularly go through involution, apoptosis, and desquamation [9]. That means corneal epithelial cells are capable of regenerating each 7–10 days by centripetal migration beginning from limbus, found on corneal rim at the joint of sclera and cornea, and limbus includes corneal epithelial stem cells that are crucial cells for homeostasis state [10].

Epithelium's superficial layer includes straight polygonal cells, and more deep layers include cuboidal cells, and posterior most layer is known as basal layer and it is mostly composed of single layer of columnar epithelium [13]. The most surface corneal epithelial cells create an average of 2 to 3 layers of flat polygonal cells [9]. Wing or suprabasal cells help 2- to 3-cell thick layer and show tight junction complexes among cells [14]. The deepest cellular stratum of epithelium is basal layer. Basal cells are connected to basement membrane via hemidesmosomal network. Apart from stem cells and temporary amplifying cells, basal cells are only corneal epithelial cells able to undergo mitosis. Epithelial basement membrane, having a 0.05 μm thickness, includes collagen IV and laminin that is secreted via basal cells [9]. Basal epithelial cells symbolize the posterior most stratum of epithelium. These cells use hemidesmosomes for attaching to underlying basement membrane and stroma [14].

There is an anterior lamina lucida and posterior lamina densa, lamina lucida is constructed by laminins, and lamina densa is mainly made of heparan sulfate proteoglycans, collagens, nidogens, and laminin [14]. Laminin is known as the most common protein apart from collagen, and from a structural view, laminin protein is able to self-assemble into sheets and helps to epithelial basement membrane's embryological development [14].

When the functions of epithelium layer is considered, it takes a crucial task in vision since the most of eye's refractive power happens at interface of air and tear film [14]. Corneal epithelium's major roles are to create a barrier structure for avoiding the

entering of toxins and some microbes to epithelium, to combine with tears for ensuring smooth stratum for refraction of light, and also to transfer the water and soluble elements in or out of stroma [10, 15]. Moreover, epithelium's barrier function is crucial for immune regulating and avoiding of pathogen input in cornea [14]. Epithelium layer has a transparent construction, and conduces to cornea's smooth refractive surface, thus assissts in ensuring a clear sight. Epithelium is able to keep safe the stroma layer through creating a physical barrier toward outside media. Also, this structure avoids the motion of tears from ocular surface to stroma [13]. The major task of epithelium is to preserve the stroma layer from incursion via limiting unknown elements going into eye [12].

1.1.3.2 Bowman's Layer

There is the Bowman's layer among the epithelium and stroma layer [10]. This bowman's layer can be described as a transparent tissue sheet found below the epithelium's basement membrane. This layer is less complicated when compared to others with randomly organized collagen fibers [12, 16]. Bowman layer is found front of stroma and this is not a true membrane but rather the acellular condensate of most front part of stroma. It is nearly 15 μm in thickness and it gives assistance to the cornea to sustain form of it. However when it is destroyed, that smooth layer will not regenerate and might generate a scar [9]. Bowman's layer has 8–15 μm thickness, it is a fibrillar material's amorphous band found under basal corneal epithelial cells. It is a non-regenerative layer type, and it creates a border among corneal stroma and epithelial cells, also preserves the form of cornea. Bowman's layer includes short collagen I fibrils found in proteoglycan matrix [4].

We can describe Bowman's layer as a specified layer of collagen and this layer is known to be acellular, except for nerve axons which widen through epithelium [13]. It is known to possess various pores letting the passage of nerve bundles [17]. Collagen I and III are predominant in that layer [13]. It is created mostly of collagen I and III, and types V and VI, that create fibrils having a 20–25 μm diameter. Separate collagen fibrils connects for forming a sheet nearly 8–12 μm [10, 18]. Bowman's layer's thickness reduces by age, and it is known that this layer does not regenerate after a trauma and removal [10, 17].

Although certain task of Bowman's membrane is unknown, it is recommended that Bowman's layer might behave like a physical barrier which guards subepithelial nerve plexus [13]. Moreover, this layer acts like a barrier structure for avoiding straight traumatic linkage of the epithelium with stroma, thus stromal wound recovery is fast, and finally assists in sustaining transparency [17]. Bowman's membrane is acellular, and for this reason it performs like a biological barrier for spreading of viral infection, since viruses need cells for spreading [19].

1.1.3.3 Stroma

Corneal stroma has a sandwiched structure between Bowman's layer and Descemet's membrane, it corresponds to 95% of corneal thickness. This layer is made up of Schwann cells, neural tissue, fibroblastic cells, and keratocytes. Stroma's major part is composed of collagen fibrils, collagen type I, which ensure mechanical strength of cornea [4]. Collagen fibers are organized within parallel bundles named as fibrils, and those fibrils are packaged in parallel layers or lamellae, and also stroma includes 200 to 250 distinct lamella [9]. Collagen fibrils include collagen I within a heterodimeric complex with type V. Those complexes are enveloped via proteoglycans, keratan sulfate or chondroitin sulfate/dermatan sulfate, that assissts regulation of hydration and constructional features [9]. Keratocytes are the main cells found in stroma layer and they are included in sustaining the extracellular matrix (ECM) media. These cells are capable of producing collagen and glycosaminoglycans [9].

It is important to highlight that the stroma symbolizes one of the most greatly innervated tissues within body [14]. Stroma layer ensures most of biomechanical power and creates more than 90% of the whole corneal thickness. Also higher than 97% of the stroma is made up of collagen fibers and ECM proteins that are formed via relatively scarce keratocytes [6]. Collagen fibers of stromal layer are exactly positioned for optimizing the visible light transmission [6]. Besides holding nearly 90% of cornea's total thickness, stromal layer possesses the most organized ECM within body [20]. It is also important to know that stroma layer is in charge of entire strength and form of cornea [21]. Furthermore, any deterioration to stroma through a disease or an injury leads to permanent blindness [12].

Adult stroma is about 500 μm in thickness, relatively acellular (3–10% quiescent corneal keratocytes by volume), and it is made up of hydrated type I/V heterotypic collagen fibrils (15% wet weight)'s aligned arrays, glycosaminoglycan molecules keratan sulfate, dermatan sulfate (1% wet weight), several proteoglycan core proteins and some others like laminin, collagen type VI, and fibronectin [5].

The stroma of corneal tissue consists of both cellular (keratocytes) and extracellular elements [13]. Keratocytes which are the main cells of stroma layer, can preserve entirety of that layer, creating collagen, matrix metalloproteinases, glycosaminoglycans [14]. On the other hand, ECM keeps an important portion of stroma, and it consists of macromolecules organized network. ECM consists of fibrous proteins (collagen, laminin, fibronectin) and polysaccharide glycosaminoglycans (dermatan sulfate, chondroitin sulfate, keratin sulfate) [13]. In the organization of stroma layer, there is a meshwork of collagen fibers and ground substance, having an ECM consisting of water, glycoproteins, inorganic salts, and proteoglycans [14]. Predominant collagen type found in stroma is collagen type I, with type III and V collagen these create collagen fibrils [10].

The corneal stroma provides some important biochemical features of cornea such as tensile strength, stability and transparency [10]. Stroma layer ensures constructional assistance to cornea and transparency via making the transition of light easy throughout the collagen fibrils's framework, avoiding the scattering [14]. In order for appropriate functioning of stroma in the transition of light, corneal tissue should handle scattering from keratocytes, from which light's scattering is obviously estimated throughout in vivo confocal microscopy [14]. Also the property of avascularity of stroma is required for making transparency easy, and also the creation of antiangiogenic factors helps for sustaining balance with proangiogenic factors [14]. Moreover, besides the role of surface immune regulating via epithelium, stroma takes a central part in corneal immunity [14].

1.1.3.4 Descemet's Membrane

Descemet's membrane can be defined as 10 μm thick with amorphous structure and unbanded. Throughout the developmental process, it is known to be discontinuous, homogeneous and acellular and by stages it turns into Descemet's membrane [4]. Pre-

Descemet layer, which is an anchoring zone of interwoven collagen fibers, is recognized to interface among the stroma and the Descement membrane which is corneal endothelium (CE)'s basement membrane [6]. From starting within utero at 8 week level, endothelial cells continually secrete Descemet membrane. Descemet structure might be accumulate up to 10 μm thick by age [9].

In the Descemet's membrane, there are collagen type VIII, XII, vitronection, laminin, nidogens, perlecan, and fibronectin, while type IV collagen is the predominant one [22]. Unlike the Bowman's layer of cornea, the thickness of Descemet's membrane enhances by stages from birth to adulthood, among 5–10 μm. This membrane is known to be secreted via endothelial cells for creating basement membrane for endothelial cells to anchor [23]. Although Descemets membrane is a thin barrier, it is also a strong barrier towards the infection and damage, thus helps to preserve corneal curvature. It is known to be derived from secretions of endothelial cells and capable of regenerating following the infection [12, 24].

Descemet membrane is defined as endothelium layer's basement membrane, and it is known to measure about 3 μm thick in children, by stages thickens to 10 μm in adults. It is made up of two different layers; one is the anterior banded layer that is builded up via collagen lamellae and proteoglycans, other is the posterior nonbanded layer that is put down via endothelial cells and also become thicker with decades [14]. As an additional information, resembling to stroma layer, laminin, fibronectin, heparan sulfate, keratan sulfate, and dermatan sulfate exist, and proper hydration is needed for sustaining clarity [14]. Importantly, Descemet membrane gives assisstance in the preservation of corneal dehydration [14].

1.1.3.5 Endothelium

Embryologically, CE is known to be derived from neural crest. CE is made up of a single layer of cells creating back surface of cornea [13]. The human endothelium is a monolayer, that seems like a honeycomb-like mosaic if looked from posterior [9]. Endothelium's basal surface includes several hemidesmosomes which encourage the attachment to Descemet membrane [9]. Endothelium layer, which is very thin, is the innermost layer of corneal tissue. This layer is comprised of nearly interdigitated cells that are found in a mosaic pattern of mainly hexagonal forms [12].

CE is made up of hexagonal-like endothelial cells which form a cell's packed monolayer onto Descemet membrane [3]. The CE can be defined as a transporting monolayer of nearly 400,000 hexagonal cells, which are 20 μm across, 4–6 μm in height [5]. In the absence of endothelium layer, cornea shows a native propencity for imbibing the fluids, resulting in swelling, opacity, and blindness [5]. Human CE is comprised of single stratum of hexagonal cells having a thickness of about 5 μm [10]. As seen in Figure 1.2, corneal endothelial cells (CECs) are polygonally shaped and specialized cells and exist on the interior surface of cornea tissue with anterior chamber [25]. Adjoining endothelial cells are known with lateral gap and tight junctions, they are vertically binded to Descemet's membrane via some hemidesmosomes [10]. Anterior to tight junctions, besides them, gap junctions exist in endothelium helping electrical coupling of endothelial cells [14].

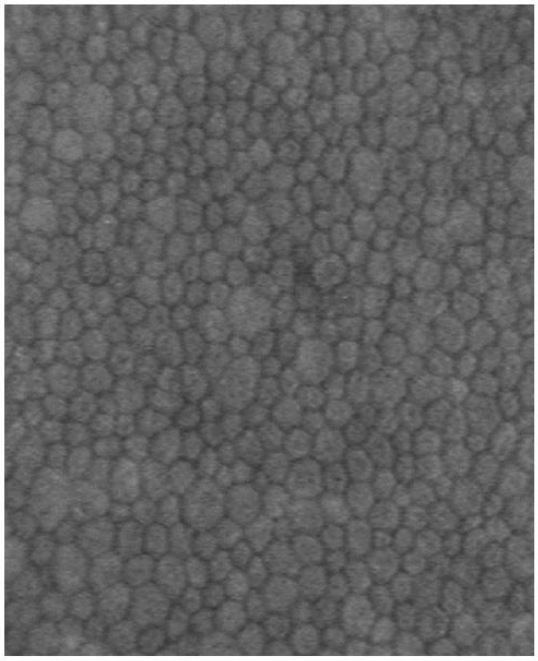

Figure 1.2: Confocal microscopy image of human CE shows one monolayer of straight cells that are found with hexagonal arrangement. Various larger cells conduce to small changeability in size (polymegethism) and shape (polymorphism) [14]

It is necessary to maintain the cornea clean because of the pumping excessive fluid out of stroma. That function avoids stromal swelling and hazing, also avoids opaque stromal layer creation [12]. During usual stromal deturgescence circumstances, in a relative stromal dehydration state, arrangement of collagen fibers presents the needed transparency and curvature that is required to vision. Nevertheless, stroma layer could

not correctly accomplish this task without the endothelium, that is able to control stromal dehydration, and thus for preserving corneal properties it provides proper distance between stromal collagen fibers [3]. It is known that CECs are responsible for regulation of corneal stromal hydration through acting like an inactive barrier to absorption of fluid from aqueous humor and through actively pumping fluid out of cornea [6]. Besides behaving like a barrier for restraining fluids into cornea tissue, endothelial cells are able to avoid extreme levels of hydration (78% water content) of stroma layer, in this way it sustains stromal deturgescence through a passive pump-leak mechanism which passes some ions and water from hypotonic stroma to hypertonic aqueous humor [26]. In other words, with the actions of endothelial cells, stroma layer is preserved in relatively deturgesced status (78% water content). That dehydration process is carried out via a pump–leak mechanism when fluids exit from stroma layer down the osmotic gradient from relatively hypo-osmotic stroma to relatively hypertonic aqueous humor. That motion of passive bulk liquid does not need energy, however there is also some energy-needing procedures of ions transportation for creating osmotic gradient. The most significant ion carriage mechanisms include membrane-bound Na^+ and K^+-adenosine triphosphatase (ATPase) parts and intracellular carbonic anhydrase path. Activities and functions inside those systems creates ions net flow from stroma to aqueous humor [9].

Tight junctions allow endothelium to act as a leaky barrier, since these have the function of controlling the transmembrane circulation of molecules like bicarbonate ions, glucose, sodium, chloride, among aqueous humor and stroma layer, in conjuction with distinct transporters, like Na^+/K^+ ATPase pump and the sodium bicarbonate cotransporter, that withstand to the flow of water in stroma through pumping the ions and some other solutes in aqueous humor. In this way, endothelium like a leaky-pump barrier, retains stroma's water amount at enough stages in order to maintain transparency [3, 27, 28].

Human endothelial cells demonstrate a restricted ability for dividing in vivo since these are mainly arrested in G1 phase [3]. When endothelium layer is generated, those endothelial cells become mitotically inactive. At first, human CE contains about 4500 cells mm^{-2}, but endothelial cells entire number reduces by age and cell surface area enhances on the other hand [10, 29]. Since these cells are mitotically inactive, when

the endothelial cell number is reduced, for preserving tissue integrity, surrounding cells infiltrate by degrees to the empty area [10]. The entire mechanism is demonstrated to be related to Na^+, K^+–ATPase pumps which reside in basolateral membrane [30].

From life's second to eight decades, density of cells decrease from 3000 to 4000 cells/mm^2 to about 2600 cells/mm^2, and also hexagonal cells percentage decreases from nearly 75% to nearly 60% [9, 31]. In a normal cornea tissue, centric endothelial cell density reduces at a mean ratio of 0.6% for each year [9]. Importantly, CECs of adult humans are arrested in G1 phase of cell cycle, these cells have restricted regenerative ability, therefore endothelial dysfunction or any injury leads to the corneal edema, and that situation changes the positioning of stromal collagen fibers, lowering the corneal transparency [6].

1.2 Functions of Corneal Endothelium

The innermost stratum of cornea tissue, CE, has the function of preserving transparency and thickness via active Na^+ /K^+ pumps and leakage barrier role [6, 32]. Endothelium layer takes a crucial role in maintaining the stromal dehydration. Preservation of this hydration is dependent on tight junctions between endothelial cells and the pump role related to Na^+/K^+-ATPase and bicarbonate-dependent Mg^{2+}-ATPase. Endothelial insufficiency occurs via corneal edema, it symbolizes the net inflow of aqueous fluid in cornea with a greater ratio than the quantity that is pumped out throughout certain duration [14]. Also, endothelial cells minimal number is needed for ensuring enough pump role, while endothelial cell density declines from at birth about 3000–4000 cells/mm^2 to 2500 cells/mm^2 in late adulthood [14].

The cornea's transparency is sustained via adjusting stromal hydration with barrier and pump roles of CE tissue [33]. CE is significant for preserving transparency and nutrition of corneal tissue by 'pump-leak' action [10]. CECs have an important role in actively pumping the fluids for sustaining the cornea's semi-dehydrated status and maintain the transparency [25]. Each of these CECs are able to control the hydration via function of pumping water from stroma in aqueous humor, in this way it preserves mechanical strength [34]. Major function of CE is pumping surplus fluid from cornea

to inside the anterior chamber and also transition of solutes and nutrients to cornea's superficial layer, so that it preserves hydration and nutrition, that process is named as "pump-leak hypothesis," which holds cornea in a slightly dehydrated status and this is needed for optical transparency property [13].

In short, the natural CE that is in the form of a monolayer, takes an important part in maintaining visual acuity through sustaining hydration and thickness of stroma. If the endothelium with enough cell density is intact, it might let cornea for preserving clarity [35].

1.3 Endothelial Cell Loss

In contrast to epithelium layer, endothelial cells don't show mitosis in vivo. Therefore, exchange of endothelial cell function from injury and cell death happens not by division of cells but migration. Also, focal loss of cells centrally, thereby, ends up with adjacent endothelial cells centripetal migration, followed by tight junctions creation and restorating of pump functions, later on remodeling of endothelial construction from expanded and unevenly formed endothelial cells toward more hexagonal form [14].

As it was stated before, endothelial cells do not possess a mitotic activity in vivo. At birth cell density is nearly 3500 cells/mm^2, on the other hand that value reduces by stages during the life nearly at 0.6% for each year. It had been noticed that if the endothelial cell number of eyes is under 500 cells/mm^2, the possibility of corneal edema creation might be arised [9].

An enhancement in cell dimension (polymegathism) and an enhancement in change of cell shape (pleomorphism) relate to decreased capability of eye's endothelial cells for deturgescing cornea [9]. As mentioned earlier, endothelial cell numbers usually decline by age, inflammation, some diseases, and trauma, while remaining cells are able to "stretch", taking the area of these deteriorated endothelial cells, and via replacing these pathways, residual cells increase in size (polymegathism) and no more possess hexagonal shape (pleomorphism) [9]. Shortly, throughout a disorder, cells enhance in size (polymegathism) and demonstrate a shape change (pleomorphism) for satisfying for spaces created by degenerating cells [4].

Although at stage of birth, mean density of human corneal endothelial cells (hCECs) is nearly 5000 cells/mm^2, because of the restricted mitotic potential of them, total number of these cells reduces with age [34, 36]. In the absence of the probability to replace the dead endothelial cells, density of endothelial cells reduces at 0.3% ratio per year, with average endothelial cells density nearly 3000 cells/mm^2 in adults [37]. Therefore, for preserving active cell monolayer, endothelium utilizes the ways of cell migration and contiguous cell spreading [38]. Because of this, endothelium of adults seems like a cells sheet that is deployed after cobblestone pattern formed via changes in shape (pleomorphism) and dimension (polymegathism) [37]. Since hCECs are known to possess poor capability for regeneration in vivo, when injured via trauma or diseases this is compensated through migration and enlargement of remaining hCECs [32, 36, 39]. In short, when an injury or a harm take place on CECs, these cells rather than via mitosis division, are known to be repaired by cell enlargement and migration [34, 36]. Thus any harm to endothelial cells ends up with a permanent destruction causing to corneal edema (swelling) and blindness [12]. Once the density of CECs drops under 500 cells/mm^2 level, either because of a hereditary disorder or aging, cells can not make up for the loss through normal repair mechanisms, and unfortunately this damage stays permanent [40]. When the density of endothelial cells is under a crucial grade (400 cells/mm^2), some cascade reactions such as stroma edema, bullous keratopathy, vision loss, and corneal opacification would take place [32, 41].

1.4 Corneal Diseases and Current Treatments

It is known that diseases and damages are secondly most frequent reason of blindness influencing more than 10 million people worldwide [12]. Some infectious or non infectious disorders may lead to serious visual impairments requiring medical application for preserving vision [2]. Some examples of main causes of blindness worldwide include onchocerciasis, corneal dystrophies, corneal ulceration, xerophthalmia, trachoma [42].

Also there are some corneal endothelial diseases which need corneal transplant, and these involve Fuchs' dystrophy, iridocorneal endothelial syndrome, some intermediate forms, posterior polymorphous dystrophy, congenital hereditary endothelial dystrophy, and bullous pseudophakic keratopathy [43]. Corneal endothelial dystrophy

is an important parameter in serious visual distruption [34]. For example, Fuchs endothelial corneal dystrophy (FECD) which is one widespread corneal problem, is known by progressive lose of endothelial cells, thickening of Descemet's membrane's, depositing of guttae [34]. Moreover, in United States, that disorder is well recognized to happen in 4% of population above 40 years of age [44].

Whenever CECs number turns into inadequate, corneal endothelial dysfunction problem develops, and then results in corneal stroma edema [35]. Once the cornea has a disorder or injured, cornea could be removed surgically and then changed by the tissue from a donor [45]. Recently, usual treatment for corneal endothelial dysfunction has become full-thickness corneal transplantation, but that therapy possesses some disadvantages such as restricted donor cornea availability, rejection of allograft, and following failures of graft in specific situations [35].

For restoring the vision for people who suffer from endotheliopathies, corneal transplantation is the only accessible therapy [46]. Once endothelium has a failure, vision of the patient might be damaged, and sometimes in serious situations, some surgically operation might be needed for replacing injured part by a novel one through keratoplasty [3]. Although the major therapy existing for restoring the CE is keratoplasty, that process bears various problems such as donor tissue deficiency, post-surgical difficulties related with utilization of drugs for preventing immune rejection, and an important enhancement in glaucoma occurrence [43].

Penetrating keratoplasty (PK) utilized to become the most widespread method to treate the cornea's irreversible opacification ten years ago [47]. PK includes replacement of cornea's full thickness, and it has the prime way in order to restore the clarity of cornea and vision [45]. However, this procedure debilitate the eye's structural integrity, and can unfavorably change its optical properties [45].

Currently, when working with disordered CECs, endothelial keratoplasty (EK), like Descemet stripping with automated endothelial keratoplasty (DSAEK) and Descemet's membrane endothelial keratoplasty (DMEK), was substituted the PK operation like a preferential therapy [47, 48]. Although PK is known as the most widespread keratoplasty technique utilized for treating cornea's irreversible opacification, whenever working with disordered hCECs, Descemet's stripping and endothelial

keratoplasty (DSEK) procedure, has been replaced the PK [49]. In the process of DSEK, problematic hCECs with underlying basement membrane (Descemet's membrane) of them, are peeled from stroma in physical means, and donor tissue, involving a slim stratum of posterior stroma, Descemet's membrane and healthful hCECs, was placed [49].

In USA, at least 50,000 corneal transplants were known to be carried out yearly, and more than 50% included the EK surgical operation [47]. Also when compared against to PK, EK surgery usually ends up with a quicker visual rehabilitation, higher refractive result, and less post-surgery associated difficulties because of ultrathin graft [50]. Nevertheless, human CE tissue is very thin (around 5 μm thick) and sensitive, and this enhances the problems for transferring monolayer cell sheet directly into eye [47].

It is known that DSAEK is the choosen technique for the corneal endothelial shortage, however this method needs a donor cornea [33]. Recently, surgical applications like Descemet's stripping EK focus on transplanting of CE, giving more suitable visual outcomes however still bearing the requirement of donor tissue [43]. The major issue with EK is postoperative cell loss comparable to or greater than which is seen in PK procedure [43]. Procedures including Descemet's membrane endothelial keratoplasty and Descemet's membrane automated endothelial keratoplasty were also evolved, presenting better visual outcomes, having lesser scarring and lesser optical stromal aberrations [43]. Also those methods give the probability to replace CE by an endothelium that is reconstructed with bioengineering method. Nevertheless, clinically existing processes with artificial corneas possess some restrictions like retroprosthetic membrane's inflammation and glaucoma, and they are divided for patients who are at high risk [43].

For getting over the donor cornea deficiency, cultured hCEC sheets transplantation has been studied in experimental works [33]. Corneal transplantation do not continue forever, and though DSEK is known as superior than PK, still there is a need for improving [49]. On the other hand, cell therapy strategies have been investigated as an alternate way, which includes isolation of hCECs, reproduction in vitro, and later transplantation of it in anterior chamber of eye for replacing disordered cells [49]. At this point, there are two techniques explored; first one includes the harvesting of a

sheet of hCECs, by utilizing thermal reversible culture plane, then transporting this cell sheet in eye, while second one includes seeding of hCECs on a slim scaffold construct directly, and placing cell-scaffold structure in eye. Although both of these methods have demonstrated hope, second technique is appealing because of easier handling ensured via more strong scaffold [49].

1.5 The Need for Corneal Transplantation

Recently, cornea is the most frequently transplanted tissue worldwide [2]. Some factors such as aging, trauma, and disease can cause loss in CECs number. When the number of CECs is critically decreased, cornea tissue no longer have the property of optical clarity because of oedema and causes blindness [25]. It is well recognized that hCECs do not possess the capability for regenerating in vivo, therefore once the cell number is severely reduced, for restoring the vision corneal transplantation is required. When CECs lose their function, corneal transplantation is necessary [25].

Depending on the surgically replaced tissue amount, corneal transplantations are categorized into two major classes. The first one is the PK where the whole cornea is replaced by transmitter cornea. On the other hand, in lamellar keratoplasty which is a newer process, surgeon replaces only the injured layers of cornea with donor graft, and healthy portion of cornea tissue is remained as not impaired. During the lamellar keratoplasty procedure, unity of cornea and neighbouring tissues are maintained, thus generally a better visual recovery is obtained. However, due to the rejection of graft or late graft insufficiency, poor results can be seen [2]. Moreover, based on the World Health Organization (WHO) report, because of the corneal donor deficiency, 15–20% of patients who are in requirement of transplantation left untreated [51].

Endothelial cell density might be importantly reduced because of an accidental or surgical trauma, endothelial dystrophies, earlier PK or EK, refractive surgery, stress reasoned via some diseases like diabetes or glaucoma [39]. Whenever there is a very low endothelial cell density, cornea loses the barrier function and also more amount of fluid enters into the cornea than could be taken out by ionic pumps activity [39].

The CE gives assistance to sustain corneal transparency by barrier and ionic pump functions of it. For preserving transparency, endothelial cell density has to be higher

than a critical value, generally 400-500 cells/mm^2 [39]. However, that transparency function might be compromised, and this leads to a serious losing in endothelial cell density, bullous keratopathy, visual acuity loss, or corneal edema [39]. Existing treatments like PK and several EK forms are able to restore corneal clarity, but these methods might possess some problems and there might be a need for re-grafting or other therapies [39]. Moreover, since deficiency of healthy donor corneas to be utilized in keratoplasty is enhancing worldwide, it is an urge to develop novel therapies for restoring corneal clearness which is lost because of endothelial dysfunction [39]. In short, the research in this topic has been focused on investigating alternate strategies to in vivo trigger the temporary corneal endothelial cell division or for in vitro reproduction of healthy endothelial cells for use in corneal bioengineering [39].

1.6 Corneal Tissue Engineering

For corneal degeneration, also aging is an important factor. Based on the severity of injury, for corneal disorders, conservative treatments and donor tissue transplantation are the mostly used therapies. Because of the donor tissue shortage and infection or tissue rejection probability, native capability of corneal cells and layers for regenerating has caused to explore new approaches and therapies [8].

In order for decreasing the necessity for donor cornea and to prevent the complications related to existing corneal transplantation techniques, tissue engineering (TE) can be an alternate for corneal endothelial regeneration. TE can be described like an interdisciplinary science domain which implements the rules and principles of engineering and life sciences for developing biological tissue substitutes which are able to restore, sustain, or enhance the role of tissue [52]. A TE approach involves the three key elements which are tissue scaffold, cells and signaling molecules like growth factors or genes [53]. TE provides an alternative and innovative approach by utilizing biomaterials that are created with cultured cells for replacing injured tissue and also for regaining the role of native tissue [47].

Since corneal transplantation still includes the disadvantages of donor scarcity, rejection possibility and infectious disease transmitting, there is still an increasing necessity for novel approaches for corneal regeneration. Within this perspective,

corneal tissue engineering (CTE) rised up with the purpose of creating synthetic corneas or some other tissue-engineered constructs which promote good corneal regeneration, thus eliminating the major drawbacks of allogeneic corneal transplants [54]. Because there is a significant lack of newly harvested donated corneas and an enhancing population with corneal disorder, and standard heterografts carry an uncertain possibility of immune rejection, it is critical and necessary to develop a corneal analogous for replacing diseased corneal tissue. Currently, for these reasons lots of scientists have been focused on the area of CTE and made significant progress [55]. CTE has arised like a viable technique for developing corneal equivalents, also the creation of scaffolds having mechanical qualities and transparency comparable to the native corneal tissue is critical for corneal tissue regeneration [55]. The prime way is the production of scaffolds which could imitate the ECM of natural cornea tissue, and for that aim, several techniques and materials have been investigated to evaluate their use in CTE [55].

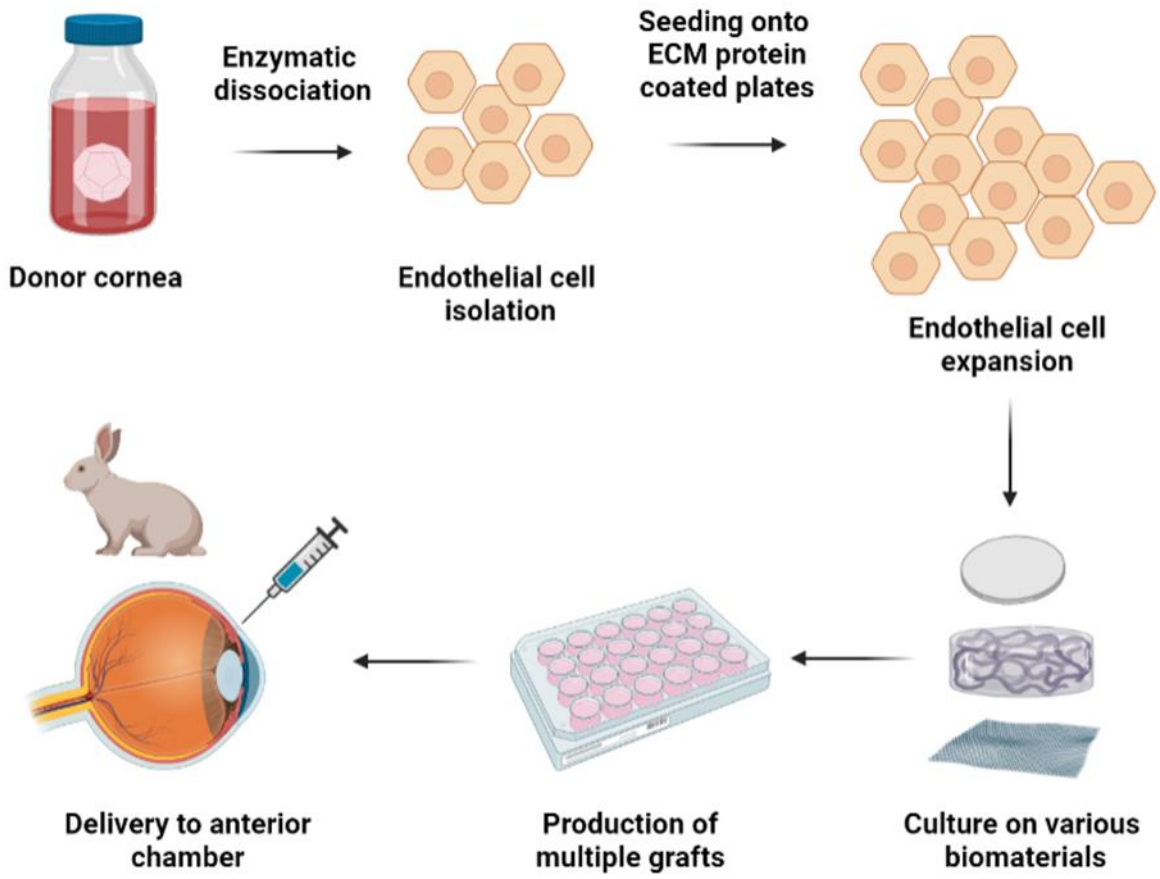

Figure 1.3: TE of cornea. Cadaveric donor cornea proper for study is obtained. hCECs are isolated with determined procedures and expanded in number in in vitro culture. When the number of cells becomes enough, cells are taken onto a scaffold

that could be biologically obtained, synthetic or semi-synthetic. Then, grafts might be generated as a confluent monolayer of cells on the biomaterials. Those grafts might then be given into anterior chamber of animal with the ultimate aim of transplantation into humans [56]. Figure is created with BioRender.com

The steps of TE of cornea tissue is demonstrated in Figure 1.3. In CTE, by utilizing appropriate biomaterials, cells and signals, the aim was to create a perfect biocompatibility and a good surrounding that mimics the in vivo. Engineered three-dimensional (3D) scaffolds can not only replace an injured cornea, by ensuring mechanical and structural stability, but they can also create right milieu for cells to rebuild the tissue [54]. A scaffold is an engineered substitute that could imitate the ECM of natural tissue and mimic in vivo environment, assisting cells to multiply, migrate and produce microenvironment [54, 57].

There are some properties that an optimal corneal scaffold should have [54, 58]; it should

1) be transparent for restoring the vision of patient,
2) be biologically compatible and assisst attachment, reproduction and migration of cells,
3) possess resembling biomechanical features to human cornea for preserving its form, important for optimum vision, and give response to intraocular pressure fluctuations,
4) maintain smooth surface of it for preventing light scattering,
5) possess biodegradation features which are matching the period of tissue remodeling and biointegration,
6) possess a refractive index resembling to cornea,
7) have proper porosity and proper diffusion for nutrients, as well as acting like microbial barrier, and
8) become cost-effective by means of production procedure and implementation.

An alternate to corneal transplantation can be TE of CE that includes scaffold-based and scaffold-free approaches (Figure 1.4). The revolutionary scaffold-free way can be defined as cell sheet engineering, that usually forms cell layers enclosured via an intact ECM with customizable release from stimuli-responsive surface. Although in the researches, these scaffold-based or scaffold-free methods have been recognized for giving charming findings, for translating them from bench to clinical experience, still some problems remain [59].

Scaffold-free methods generate cell sheets via implementing proliferative and differentiated hCECs from in vivo resources on a stimuli-responsive plane [59]. For treating the corneal endothelial diseases, cell therapy like an alternate way can be performed. During cell therapy method, suspension cells are generally transferred via injection, that is less invasive and is an easier process, but trypsinization gives damage to integration of cell surface proteins, that are necessary in cell-cell and cell-ECM interplays. Despite novel hopes with cell therapy, because of restrictions in clinical implementation and some difficulties, using scaffolds like carriers and a microenvironment for CE regeneration is essential [59].

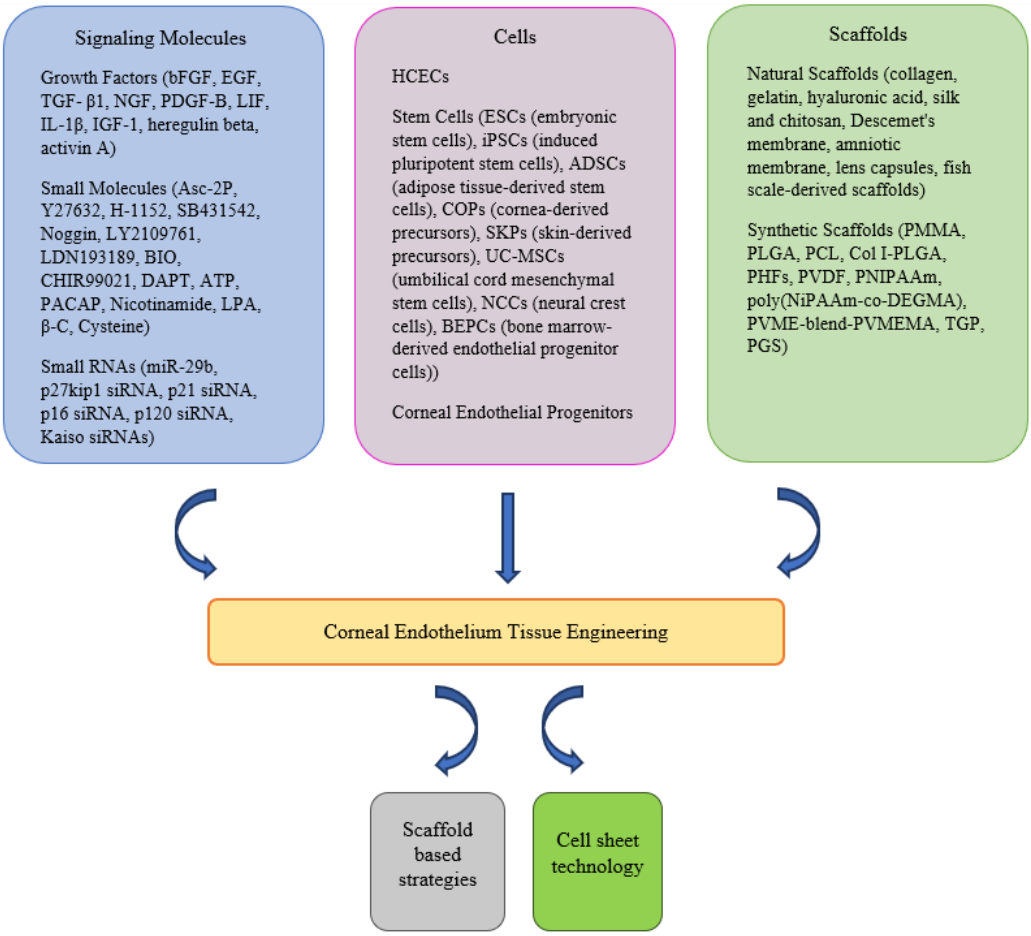

Figure 1.4: Cells, signaling molecules and scaffolds are the three components of TE of CE. Combinations of those three components could be utilized for treating CE disorders via cell therapy (cells / cells + signaling molecules), cell sheet (cell layer + deposited ECM), and scaffold-based therapy (cells + scaffolds / cells + signaling molecules + scaffolds) [59]

Signaling molecules can be written as necessary elements of TE, and those molecules involve small molecules (chemical compounds), small Ribonucleic Acids (RNA), growth factors, and cytokines. For regulating cellular reproduction, differentiation, attachment, survival, and migration, those signaling elements are able to trigger cell-signaling pathways modulation [59].

Since hCECs are not optimal cells for in vitro expansion because of lower reproduction rate of them, alternate cell lineages having the capability for differentiating into hCECs are chosen, with properties like availability, huge subculture capability, good proliferation, and low immunogenicity. Primary hCECs, corneal endothelial progenitors, and stem cells can be written as the major probable sources for creation of hCECs. Nevertheless, implementation of them is difficult because of producing

proper methods for cell isolation, maintenance, and expansion for assissting integrated structure and function properties [59].

Successful CTE necessitates the use of proper scaffolds that allow cells to reproduce, arrange natural ECM, and also recapitulate original corneal structure and functions [60]. Scaffolds as biomaterials behave like a temporary ECM for ensuring proper microenvironment for cells and assisst enough signaling for attachment, migration, reproduction, and differentiation and also for regulation of tissue formation [59].

Optimal scaffold construct for hCECs's tranplantation should imitate the features of Descemet's membrane, involving resembling softness, topography, and surface signals as well as clearness, allowing gas permeability, supplying water and nutrient, absence of cytotoxicity, biological degradability, biocompatibility, simple handling during surgery, integrity to Descemet's membrane, simple manufacturing, and ensuring an optimal microenvironment for hCECs growth, reproduction, adhesion, function, and long-term survival [49, 59, 61]. Also, for transplanting hCECs, scaffold should have the properties of being transparent, non-cytotoxic, possess proper mechanical features, and being permeable to water and nutrients [49]. Furthermore, ideal scaffold has to assisst the growth, function, and survival of hCECs, and also should be integrated in surrounding tissue in an easy way. Lastly, scaffold should be adequately durable for simple handling, nevertheless be flexible sufficiently for being folded to assisst in the transplantation process [49].

Tissue-engineered endothelium should have some properties such as being hugely functional, given on a biologically compatible carrier construct, being in a format that is convenient to surgical methods existing for transplantation. Also this transplant should not influence the biomechanical features of receiver cornea. In addition, corneal transplant requires to regard the optical properties of usual cornea, meaning transparency, with minimum triggered bigger order optical aberrations, ametropia or astigmatism, for permitting a good visual acuity and huge contrast sensitivity. Moreover, tissue-engineered endothelial transplant requires to become long lasting [46].

1.7 Biomaterials for Corneal Tissue Engineering

Biomaterials are utilized within the body to replace, repair, support or augment the harmed organs, body parts, tissues or the missing functions of body, partially or completely [62]. Three main design requirements which the native cornea should fulfill are protecting brittle intraocular contents, providing the property of transparency to visible light, and creating approximately excellent optical interface for refracting the light on retina [5]. Biomaterials in CTE have to show various important properties such as transparency, mechanical integrity, biological compatibility and slow biodegradation, to be utilized in vivo [63]. In last years, distinct corneal tissue substitutes have been produced via utilizing amniotic membrane [64-66], chitosan [67], fibrin [68, 69], caprolactone [70], collagen hydrogel [71], and some other biomaterials. Though these corneal graft materials demonstrate good biocompatibility property, native cornea's microstructure is not mimicked entirely, thus scientists are still investigating novel materials with optimum structures for clinical corneal transplantation [72].

In the field of ophthalmology, scaffolds investigated for corneal substitution could be classified into three parts: natural-based, synthetic and hybrid materials [54].

1.7.1 Natural-Based Scaffolds

Natural-based biomaterials are known to provide inherent biocompatibility and biodegradability property with an important level of biomimetic features and biological roles [54]. Mainly explored natural-based corneal scaffolds can be written as protein or polysaccharide based types. An example of the mainly investigated protein-based scaffold constructs for artificial cornea is the collagen. It arises from its biocompatibility, little toxicity, studied physical, immunological, structural, and chemical properties with preserving RGD sequences in own structure which triggers attachment of cell to scaffold [54]. Gelatin, fibrin and silk can be classified as other protein-based biopolymers which have studied like corneal substitutes. Although each class have distinct properties, mechanical features of them are known to be inferior when compared with that of natural cornea and be uncapable for assisting disordered cornea's integrity [54]. Polysaccharide-based materials such as chitosan, dextran,

alginate, chondroitin sulfate, and hyaluronic acid are also studied for CTE applications [54]. Although they have higher mechanical and optical features, they are inadequate for ensuring 3D microenvironment for effectual attachment and reproduction of cells, thus polysaccharide-based materials have not been capable for providing effectual resolution for corneal substitute [54].

Natural scaffolds come from animals and have various advantages such as satisfactory bioactivity, biocompatibility, biodegradability, as well as noncytotoxic impact because of resemblance to original tissue's ECM that allows them for triggering biological recognition, cell attachment, and function [59]. However, these natural scaffold constructs show weak mechanical features and optical transparency, also might transfer infections and cause rejection [59].

1.7.1.1 Natural-Polymer Scaffolds

Natural polymers which are utilized for hCECs contain chitosan, collagen, hyaluronic acid, gelatin, and silk [59].

Collagen: Collagen I and V are the major proteins found in cornea tissue of humans [73]. Various characteristics like optimum biodegradability, biocompatibility, cost-effectivity, being nontoxic, and little immunogenicity, recommend that collagen material might be a proper scaffold element for corneal endothelial TE [59]. Common implementation of collagen, specifically type I collagen, in TE field is partly because of its simplicity of manufacturing. Thus, collagen is essential for synthesizing biomimetic corneal stroma equivalents like major element of human stromal tissue, also it is known to be convenient with corneal cells and nerve regrowth in vivo [10]. On the other hand, the prime disadvantage is the inadequate mechanical toughness and elasticity [10]. Collagen scaffold shows weak mechanical construction and is fastly degraded via proteases due to the huge water content [74].

Gelatin: Compatibility, flexibility, transparency, biodegradability, and cell attachment (as a consequence of RGD), represent gelatin like an optimal construct for CE, however mechanical strength of gelatin is not adequate for transferring throughout the surgery [59, 75]. Gelatin can be defined as a potentially beneficial biomaterial in TE implementations, since it is resembling to the ECM proteins chemically, possesses

little antigenicity, plentifulness, cost effectiveness, and reachable functional groups which could let chemical modifications [76]. Kimoto et al. prepared a spherically curved gelatin hydrogel sheet, then seeded monkey corneal endothelial cells (MCECs) on those sheets, and MCEC-spherically curved gelatin hydrogel sheet structures were transplanted in monkeys having bullous keratopathy, and reported that MCEC-spherically curved gelatin hydrogel sheet structure was effectual in monkey model of bullous keratopathy. Also spherically curved gelatin sheet accomplishes close attachment to posterior corneal surface with no wrinkling and might conduce to CEC sheets clinical transplanting [77].

Hyaluronic acid: Hyaluronic acid can be described as a natural linear polysaccharide having some important features like biocompatibility and biodegradability for TE of CE. It is the element found in eye exist in aqueous humor and vitreous body. Moreover, some trade materials obtained from hyaluronic acid are utilized for reducing hCECs injured throughout cataract surgery [59]. On the other hand, because of fast degradation in aqueous media and weakness mechanically, hyaluronic acid is a poor carrier material for transferring hCECs [59, 78].

Silk: Silk fibroin can be defined as a structural protein that is acquired from cocoon of silkworm bombyx mori and it is commonly utilized for TE, due to its non-immunogenic answer, controllable degradation ratios and mechanical features. From the perspective of corneal engineering, innate optical clarity of silk fibroin provides it as an encouraging nominee [10]. Silk and silk-derived fibroin provide perfect biocompatibility, transparency, mechanical strength, good oxygen/water permeability, biodegradability, and noncytotoxicity, that suggest this materials for tissue bioengineering [59]. As an example, silk-fibroin constructs modified by collagen IV enhance hCEC's reproduction, adhesion, and confluency with an appropriate polygonal morphology [45]. Although silk fibroin, which is a native fibrin derived from silk possesses little immunogenicity, good transparency, and it is nontoxic and shows a controlled degradation ratio, it possesses small elasticity and thus it could readily show surface fractures throughout the manufacturing and utilization procedures [56].

Chitosan: Chitosan as a biomimetic polysaccharide which is acquired from chitin, is an element of crustaceans exoskeleton [56]. Chitosan is known to be a biocompatible,

biodegradable, and cost-effective material. It is also a nonantigenic biomaterial and could simulate ECM [59]. Amino groups on the outside surface of chitosan films decrease hCECs reproduction and viability ratio. Also, it suppresses maturation in these cells, and fragility of it is a problem in clinic [59]. For corneal bioengineering, chitosan has utilized like a material for amniotic membrane like a cell carrier for reconstructing ocular surface or endothelium. Despite chitosan has showed good promise for the field of corneal bioengineering, it is more proper to be used as mixed, rather than pure constructs [10].

1.7.1.2 Natural-Membrane Scaffolds

Natural membrane scaffold constructs for transplanting hCECs include Descemet's membrane, decellularized corneal-derived ones, decellularized human crystalline lens capsules, decellularized fish scale-derived constructs, and amniotic membrane [59].

Descemet's membrane: Descemet's membrane is recognized as the first scaffold utilized for seeding of hCECs [79]. Because the Descemet's membrane is known as a native substrate for hCEC by means of structural, physical, topographic, and chemical properties, this material is one of the greatest options for TE of CE, but important restriction of it is the donors availability [59, 80].

Amniotic membrane: Human amniotic membrane is performed in various ways such as intact construction, basement layer, decellularized and lyophilized forms utilized for creating scaffold constructs for culture of hCECs [59]. Human amniotic membranes take the advantage of antimicrobial, anti-inflammatory, antiangiogenic, and antifibrosis activities, also it is biocompatible having low immunogenicity, that triggers attachment, reproduction, and differentiation of cells. These advantages recommend this material as a proper construct for TE of CE [59]. Amniotic membrane which is a native, inert and, non-cytotoxic biological material, possesses a big benefit since this material decreases the probability of potential rejecting of grafts because of its proven biocompatibility property in ocular applications [56]. Besides these good properties, there are some restrictions like poor mechanical features, low biodegradation ratio, corneal calcification, infection transmitting, low optical clarity, immune rejection, fast detachment following the transplantation, and etc. [59, 81]. It also bears some other restrictions like availability from donor bank, and also since the

transparency of CE is important for functioning, another concern is the semiopaque nature of the amniotic membrane [56].

Decellularized corneal-derived scaffold: Because of the resembling mechanical and optical features as compared with native cornea and minimal immunogenicity, decellularized cornea has demonstrated promise for corneal scaffold production. However, the scarcity of appropriate corneal tissue as a need for human decellularized cornea continues to be a stumbling block, hence most researches have used porcine corneas or bovine corneas like raw material [10]. Distinct decellularized corneal substitutes are utilized for human implementation, involving decellularized porcine corneas [82], decellularized human corneal stromal lamellae [83], and etc.. Although they are utilized for seeding and growing of hCECs, clinical implementation of those scaffolds cause corneal edema relief [84]. Despite decellularized corneal stroma ensures proper mechanical features and native recognition signals which trigger cellular migration, attachment, reproduction, and junction, the implementation of decellularized cornea scaffold constructs is restricted because of donors low amount, defective ECM and recognition signals [59, 85]. Bayyoud et al. studied the decellularized bovine corneas (DBCs) like a carrier for culture and for transplanting hCECs. hCECs created continual, viable, polygonal monolayer having an average cell density of 2380 ± 179 cells/mm^2 on DBCs. It is reported that hCECs phenotypical features on DBCs suggest that hCEC sheets are able to sustain an intact barrier and ionic pump task in vitro, and thus DBCs may be an encouraging material for hCECs ex vivo expansion [86].

Decellularized human crystalline lens capsules: Despite lens capsules have the property of biocompatibility, innate transparency, proper thickness, availability, endogenous source, and resistance to enzymatic degrading, they are restricted because of some reasons like low diameter, probability of infection transmitting [59, 87]. The fundamental problem with using the human anterior lens capsule like a scaffold construct for TE is that it is a biological material that relies significantly upon the presence of eye donors [56].

Decellularized fish scale-derived scaffolds: Fish scale is made of 3D micropatterned type I collagen, that shows resembling template to human cornea and might ensure cost-effective and accessible scaffold constructs for CTE. Decellularized fish scale can

be performed to corneal epithelial and stromal cells with proper properties such as optimal reproduction, good optical clarity, mechanical stability, and biocompatibility. [59]. Lin et al. aimed to produce a new scaffold arised from fish scales, like an alternate substance having enough mechanical capacity for corneal regenerative implementations. In that study, fish scales were acellularized, decalcified and developed to collagen scaffolds, and rabbit corneal cells were cultivated on these constructs. They show evidences for applicability of this scaffold like a pattern for growing and migrating of corneal cells, as a result it is written that fish scale-derived construct could be fabricated as a potential material for CTE [88]. In vitro experiments have demonstrated corneal epithelial cells cytocompatibility on those heterogeneously templated constructs. Also transparency and accessibility of it, coarsely 200 scales from a single fish, provides it an appealing biologically compatible material for the production of corneal epithelial cell grafts. On the other hand, a recent study recommended that fish scale derived construct in its existing shape might not become optimal for developing tissue engineered corneal endothelial structures [89].

1.7.2 Synthetic Scaffolds

Synthetic scaffolds can be defined as fascinating materials with some properties, involving mass production, optimum chemical construction, optimum structure and form, optical clarity, lengthy durability, mechanical strength, absence of infection transmitting and immunogenicity [59]. Also synthetic scaffolds are costly effective alternates to natural scaffolds [59]. Nevertheless, in the nonexistence of cell-recognition cues, there is not an identifiable bioactivity like reproduction, differentiation, and attachment of seeded cells. Also those scaffold constructs could be unsatisfactorily integrated into host tissue [59].

Polyethylene glycol (PEG), acrylate-based polymers, polyesters, polydimethylsiloxane, polyvinyl alcohol (PVA)-based polymers and polyamides are the major explored synthetic ones for corneal substitutes [54]. Several synthetic materials involving PVA, poly (2-hydroxyethyl methacrylate) (PHEMA), polyethylene (glycol) diacrylate (PEGDA), poly (lactic-co-glycolic acid) (PLGA) and poly (ethylene glycol)/poly (acrylic acid) (PEG/PAA)-based hydrogels have been employed in corneal bioengineering, like substrates for corneal cells or artificial cornea

substitute materials [10]. Although showing great permeability, tunable mechanical and optical features, all those materials show low cell-adhesive function in the absence of a surface modification [10]. Their biomimetic features, which are needed for cell attachment, reproduction, and effective integration with host tissue, are still required to be improved importantly upon they can be used in clinical settings. Besides non-biodegradable property of them, their incapability for carrying cells and biointegrating throughout the healing and remodeling of tissue remains like the major problem [54].

Stimuli responsive polymer materials like poly (N-isopropylacrylamide) (PNiPAAm) and PNiPAAm-based copolymers are also progressively utilized, due to thermoresponsive hydrophobic and hydrophilic features of them, that allows separation of cell sheets with no usage of enzymatic digestion method. Also capability for creating carrier-free cell sheets provides it a potential method for corneal epithelium and endothelium engineering [10].

Though both of the synthetic and natural-based scaffolds propose an attempting way for producing artificial corneas, this kind of scaffold constructs have the deficiency of complexity of corneal tissue's 3D microenvironment by means of not only physical and chemical features but also compositional properties, microarrangement, alignment, and directionality found in human corneal stroma [54]. Moreover, xenogeneic tissues and organs generally include cellular antigens, and they could be understood like foreign via the host body's tissue and as a result they cause inflammatory reaction or immune-mediated rejection issue [54].

1.8 The Materials Used in This Thesis Study: Polyvinyl Alcohol (PVA) and Xanthan Gum (XG)

PVA can be defined as a favorable biodegradable, biocompatible, and hydrophilic synthetic polymer material which has been broadly included in diverse parts of the biomedical area [90]. PVA as a water-soluble material, consists of a lot of polar hydroxyl groups (-OH) in repeating units of linear hydrocarbon chain, that has

demonstrated unbelievable implementations in fields of medicine, pharmacy, and bioengineering [91].

PVA-based hydrogel structures possess a huge water content with great swelling capability, and they are fabricated via diverse cross-linking procedures [91]. Chemical cross-linking might happen via several ways like free-radical polymerization, enzymatic reactions, high energy irradiation, and chemical agents that usually create covalent bridges between polymer chains of PVA [92]. Moreover, the crystalline structure of PVA polymer attracts attention for creating crystalline zones as physical cross-links mesh in the hydrogel, that might be produced via the recurring freeze-thaw cycles [93].

Because of the unique properties of PVA, it has been utilized in the applications of TE recently, for developing artificial organs [94, 95]. PVA-based hydrogel structures are a colloidal dispersion with 3D networks. Also, PVA possesses a huge potential because of its high-water absorption, biocompatibility, low toxicity, good mechanical features (elastic modulus, mechanical strength), and proper physicochemical characteristics [96-98]. PVA hydrogel offered possibilities as a replacement for cartilage tissue [99], heart valves [100], arterial phantoms [101], corneal implants [102], electroskins, supercapacitors, fuel cells, and actuators [103, 104]. However, PVA hydrogels used for tissue-engineered scaffold constructs frequently require additional modification for enhancing cell-matrix interplays with regard to following cell reproduction and differentiation [91].

Xanthan gum (XG) has extensively been utilized like an additive in several industrial and biomedical implementations including food and its packaging, drug delivery, petroleum, water-based paints, oil-recovery, cosmetics, toiletries, and construction and building materials [105]. Specifically, XG has received a lot of attention as a biomaterial for fabrication of scaffold structures (ECM) for applications of TE, in recent days [105]. For instance, xanthan-based hydrogels are known as attractive substances that should be investigated as tissue scaffolds since they are biocompatible and biodegradable [106]. It is recognized that for appropriately regenerating the injured tissues, XG-based scaffold constructs should possess 3D microenvironment ensuring proper cell-matrix interplays with nano topology, proper stiffness, and surface chemistry [107]. As an important information, XG itself creates a weak gel-

like construction by double helical conformations in the existence of certain bivalent cation. Therefore, XG's stiffness or the mechanical stability with other features might be manipulated utilizing other materials or reinforcements [105].

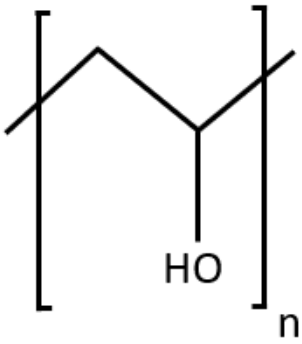

Figure 1.5: Chemical structure of PVA

PVA, which is a synthetic polymer, and XG, which is a natural polymer, have been utilized together for creating hydrogels [108-111]. PVA is known as a long-chain polymer that is synthesized via hydrolysis of polyvinyl ester (normally polyvinyl acetate) [108]. PVA possesses the features between those of rubbers and plastics, and has various properties involving high mechanical strength, low cost, chemical stability, low toxicity, and proper biocompatibility, thus it is studied in a lot of biomaterials [112-114]. Moreover, the PVA is a water-soluble semicrystalline material including various hydroxyl groups that might be readily stimulated for creating a stable hydrogen bonding meshwork. Figure 1.5 represents the chemical structure of PVA polymer. Considering all these, it can be written that PVA is a proper polymer for creating hydrogen bonding hydrogels [108]. But there are some disadvantages of single-mesh hydrogels, like PVA, which are weak mechanical features and slow reaction on swelling of them [115]. Therefore, for enhancing these properties, multicomponent networks have been studied [116]. XG can be described as an inherently occurring extracellular heteropolysaccharide, and XG is a fermentation substance of Xanthomonas campestris, an aerobic and Gram-negative pathogen [117]. XG is usually thought as a non-gelling polymer material, while it might stimulate synergic impacts with many biocompatible polymers, ending up with greater viscosity in blend or in creation of a gel [108, 118, 119]. The chemical structure and functional groups of XG is demonstrated in Figure 1.6. As shown in Figure 1.6, the macromolecules of XG are plentiful by means of hydroxyl, carboxyl, and other functional groups [120]. For this reason, it can be considered that blending of PVA and XG polymers with a

certain ratio with the specific circumstances of synthesis (temperature or time of reaction) could produce composite hydrogels with perfect features [108].

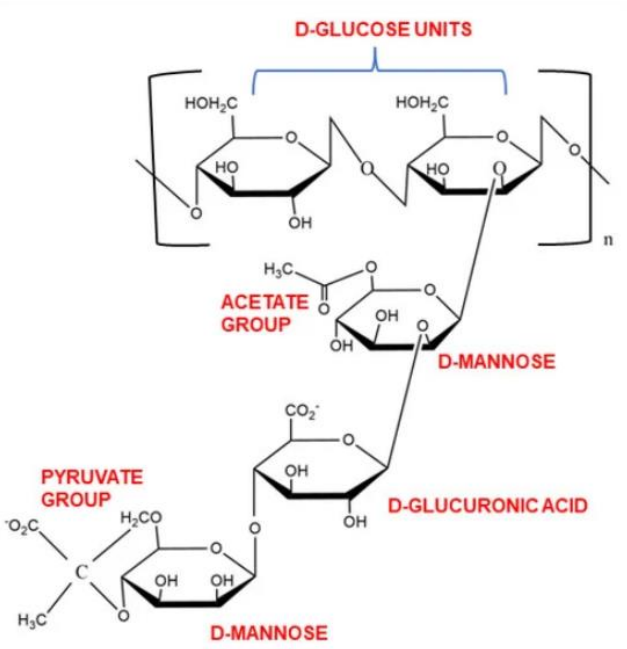

Figure 1.6: Chemical structure and functional groups of XG [117]

PVA-based hydrogel structures lack sufficient mechanical flexibility, frequently showing fragility, thus, for obtaining needed properties of gels, PVA polymer is usually utilized in combination with other polymers [121]. As it was expressed before, PVA involves various hydroxyl groups which might be readily stimulated for creating a steady hydrogen bonding network. On the other hand, XG polymer is also plentiful in terms of hydroxyl, carboxyl, and some other functional groups, being it a polymer having the capability to interplay with PVA, also XG is known as biologically compatible [108, 109]. However, XG could not ensure a proper environment because of its poor physical strength [122]. Within the light of these informations, it can be concluded that combining these two polymers and exposing to freeze/thaw cycles is a potential approach to create a biomaterial with appropriate mechanical characteristics [109]. Furthermore, the existence of hydroxyl groups in both of PVA and XG polymers can be considered as an advantageous property as the hydroxyl groups exhibited huge degrees of cell attachment [123].

1.9 Development of Peptide Modified Bioactive Scaffolds

Biocompatibility is a very crucial property of TE scaffolds since this feature permits suitable interactions of cells with scaffolds, meaning that biocompatible materials are non-toxic and able to ensure perfect cell attachment, spreading, reproduction, and differentiation [124-127]. Although polymer scaffolds are usually non-toxic, in some situations, the surface features (topography, charge, wettability) of polymer-based scaffolds might not ensure proper assistance for adhesion, reproduction, and differentiation of cells, and this also slows down tissue regeneration [124]. For increasing the biological features and biocompatibility, this kind of biomaterials should be enriched or modified by bioactive molecules, ECM proteins, growth factors, cytokines, adhesive peptides, and hormones [124]. At this point, it is important to know that the bioactive molecules possess an excellent impact on both scaffold features and cellular behaviors [124]. Bioactive molecules can be described as biological factors or signals which enhance the features of biomaterials, assist cellular activities, and thus result in better regeneration of tissue of interest [125, 126, 128]. Therefore, enrichment of scaffold materials by bioactive molecules encourages the biocompatibility, increases the cell adhesion, reproduction, and differentiation.

Significant progresses in the areas of biomaterials and TE have caused various surface functionalization methods directed through the production of biomimetic materials that mimic the features of natural ECMs. ECM proteins arrange cellular behaviors via the interplays with cell-surface receptors, like integrins, ending up with tissue-specific cell spreading, differentiation, migration, tissue assembly and also cell-to-cell communications. Receptor identification of binding areas on ECM proteins is known to be greatly adjusted and is based on some structural properties of binding motifs, ligand density, ECM's structural entirety, and spatial orientation [129]. Moreover, integrin receptors take a role in cell-cell attachment and cell-pathogen attachment, besides mediating interplays of cells and ECM [129, 130].

Whenever biological materials are subjected to biological medias, proteins of ECM are non-precisely adsorbed on surface of almost whole the materials, later on cells implicitly interplay with surface of biological material via adsorbed ECM proteins

[131]. Designing biomimetic materials aims to obtain the materials such that they are able to leading certain cellular actions and guiding novel tissue creation mediated via certain interplays, that could be manipulated via changing the design features in place of via non-precisely adsorbed ECM proteins [131]. Materials biomolecular recognition via interested cells has been obtained via surface and bulk modification of biomaterials by chemical/physical techniques by bioactive elements like a natural long chain of ECM proteins, and also short peptide sequences deriving from original ECM proteins which could cause certain interplays with cell receptors [131]. Numerous functions of ECM in tissues may be mimicked by the biomimetic materials. For instance, immobilizing the signaling peptides makes the biomaterials surface cell adhesive which had previously been inherently non-adhesive to cells [132].

The implementation of biomimetic properties is very significant in the field of TE and defines the biointegration of artificial corneas with host corneal tissue [54]. When designing ideal biomaterials, since transmitting the necessary signals to the cells, cell-scaffold interactions, and resulting cellular responses are very critical, functionalization of biomaterials are of great importance. The inclusion of peptide molecules to materials could also cause the material to be degradable via certain protease enzymes [133] or stimulate responses from cells which might not be existing in a local natural tissue [134]. Peptide-modified scaffolds may ensure biological signals for cell-matrix interplays for encouraging the tissue growth [131]. When tissue scaffolds are modified by some bioactive elements like ECM proteins or peptides, this might direct and activate some intended signalling pathways and in this way increases the role of scaffold constructs [135].

It is known that modifying the biomaterials surface by proper bioactive peptide molecules may further increase the functions of biomaterials [136]. Bioactive peptide molecules can be utilizied for modifying the scaffold surface in order to ensure desired biological ligands which might increase the attachment, differentiation and reproduction of cells and thus the interplays between cells and scaffold [135]. It is well recognized that peptides allow the fabrication of biologically active and biomimetic materials, and the materials functionalized by peptides may be more effective for tissue formation and regeneration. Biomimetic materials enriched by these bioactive

molecules might be utilized as scaffold constructs that probably act like artificial ECM ensuring proper biological signals for directing novel tissue creation [131].

Particularly, using a short peptide sequence for modifying surface has advantages over using natural ECM proteins' long chain [131]. Natural ECM proteins have a tendency to become randomly folded following the adsorption to biomaterial surface, suchlike that receptor binding spaces are not continuously sterically obtainable [131]. On the other hand, short peptides are known to be relatively more steady throughout the modification procedure than long chain proteins suchlike that almost whole the modified peptide molecules are obtainable for binding of cells [131]. Moreover, the short peptide sequences could be produced in greater quantities and at a lower cost in laboratories [131]. Utilizing peptides rather than native proteins are advantageous also because of some other reasons such as peptides could be created synthetically and their chemical synthesis ensures accurate control of chemical constitution of them, and as a result of that prevents from the pathogenic contamination coming from animal sources, and also manipulation of peptides during grafting is easy. In addition, peptides show higher resistance than high-molecular weight proteins, to denaturation that is resulting from pH or temperature changes [136].

Methods for enhancing modulation of interactions between cell and material resulted in the creation of synthetic bioadhesive cues for modifications of biomaterial surface. Different binding motifs of ECM have explained and included in amino acids short linear sequences. As an important knowledge, the most frequently utilized peptides are arised from proteins involving fibronectin (e.g. RGD, KQAGDV, REDV and PHSRN), laminin (e.g. IKLLI, LRE, LRGDN, PDGSR, IKVAV, LGTIPG, and YIGSR), collagen (e.g. DGEA, GFOGER) and elastin (e.g. VAPG) [129]. From those peptides, the most commonly utilized one is the arginine–glycine–aspartate (RGD), which is a omnipresent receptor adhesion motif present in nearly all ECM proteins [129]. Increased cell spread on RGD-functionalized material was first presented via Cook et al. within the scope of a study where RGD sequence was applied to covalently modify poly(lactic acid-co-lysine) [137].

The peptide sequences studied in this thesis were determined because of the design and simulation studies of corneal endothelial tissue-specific peptides that encourage the adhesion of cells by utilizing the computational biology techniques. Thus, the goal

was artificially synthesizing the peptides with the same functionality as proteins that enable specialized cells to adhere to the CE.

The honeycomb morphology of CECs directly affects the transparency and function of the cornea. It is known that in addition to the developed membrane, the special laminin protein in the Descement layer, which is found only in the corneal tissue, is also effective in driving the CECs into a hexagonal honeycomb-shaped morphology. At the point of determining which peptides to investigate, using the computational and structural biology approach, a method was applied to identify and model these tissue-specific peptides in the laminin protein in the Descement membrane to which the CECs are attached. Briefly, this method includes first the modeling of laminin-5 (LM5) and integrin-α3β1 protein complexes by simulating the structures available, then calculating how the γ-terminus of LM5 binds to the Metal Ion Dependent Adhesion Site (MIDAS) on integrin-α3β1, using modeled complexes and biochemical data available in the literature, and finally designing peptides that can mimic the γ-terminus of LM5 or bind better from γ-terminus of LM5. As a result of this method, peptide sequences defined by detecting the active sequences of LM5 protein were used to modify the membrane surface.

1.10 Aim of The Study

For overcoming the problems related to CE tissue and for preventing the blindness and vision losses originating from the corneal endothelial damage, there is an urgent need for exploring novel strategies in addition to the current corneal transplantation techniques. TE as an alternative to existing therapies might ensure promising approaches for regeneration of CE. As far as we know, there is not any reported data associated with the characterization and evaluation of corneal endothelial tissue specific peptide-modified PVA-XG hydrogel membranes for use in CTE applications. Within this context, the major aims of this thesis study are to develop and characterize the corneal endothelial tissue specific peptide-modified bioactive membrane for use as a scaffold for CE and also to define the influence of the developed peptide-conjugated hydrogel membrane on the viability and proliferation of mesenchymal stem cells.

The major objectives of this thesis can be written as below;

- Synthesis of corneal endothelial tissue-specific LM5-derived peptides,
- Production of PVA-XG membranes,
- Peptide conjugation to the membrane surface,
- Characterization of peptide conjugation,
- Maintaining in vitro mesenchymal stem cell culture,
- Performing MTT assay and Live and Dead staining analysis,
- Investigation of how the produced peptide-conjugated membrane affects cell viability and proliferation, and
- Determination of bioactivity of this developed membrane structure.

It is thought that the developed bioactive membrane structures can lead to new studies and guide further research in CTE applications. It is aimed that the produced structures will stimulate cell adhesion and proliferation and will possess a favorable influence on the regeneration of corneal endothelial tissue, when used as TE scaffold in CTE applications.

Chapter 2: Materials and Methods

2.1 Peptide Synthesis

For performing the solid phase peptide synthesis process, all of the peptide synthesis chemicals were purchased from AAPPTEC (Louisville, KY, USA). Peptide sequences defined via detecting the active sequences of the LM5 protein, were synthesized on 4-methylbenzhydrylamine (MBHA) resin (0.67 mmol/g loading capacity) utilizing the 9-fluorenylmethoxycarbonyl (Fmoc) chemistry as explained by using the automated peptide synthesis device (AAPPTEC Focus Xi, Louisville, KY, USA) which is shown in Figure 2.1 [135].

Figure 2.1: Automated peptide synthesis device used for performing solid phase peptide synthesis

Peptide sequences that were identified by detection of the active sequences of the LM5 protein were "QQELAQTNYCGPPC (hereafter denoted by P1)", "RGDVAVKIYCGPPC (hereafter denoted by P2)", and "KNELAQTNYCGPPC (hereafter denoted by P3)" (K:Lysine, P:Proline, L:Leucine , C:Cysteine, Y:Tyrosine, N:Asparagine, D:Aspartic acid, T:Threonine, A:Alanine, E: Glutamic acid,

Q:Glutamine, R: Arginine, V:Valine, I:Isoleucine, G:Glycine). Briefly, resin was swelled via keeping it within *N*, *N*-dimethylformamide (DMF) during 30 minutes and then rinsed by DMF. Throughout the synthesis, amino acid coupling was carried out with Fmoc-protected amino acid (2 equiv), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; 2 equiv), hydroxybenzotriazole (HOBt; 2 equiv), and *N*, *N*-diisopropylethylamine (DIEA; 4 equiv) in DMF for 3 hours. 20% piperidine in DMF solution was used for removing Fmoc-protecting group during 30 minutes. In every coupling and deprotection steps, Ninhydrin test (Kaiser test) was done for controlling the presence of unreacted amines. After aminoacid coupling step, in case the test results were positive, resin was rinsed by DMF and the reactions were performed again till a negative result was taken. In case the test results were negative, resin was rinsed first with DMF, after that it was left within 20% piperidine/DMF for 30 minutes for removing Fmoc-protecting group, and finally it was washed with DMF. To guarantee that the Fmoc protecting group had been removed, the ninhydrin test was repeated and if no positive results were found, incubation in 20% piperidine/DMF solution was continued until a positive result was obtained. Negative and positive Kaiser test results are demonstrated in Figure 2.2.

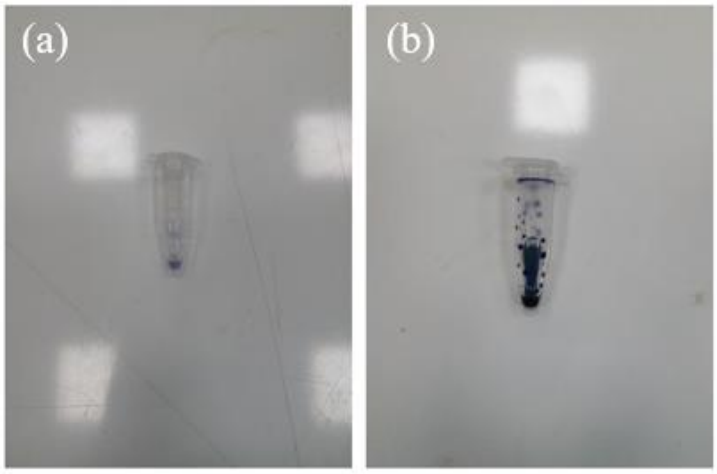

Figure 2.2: Images of the results obtained from a Kaiser test, (a) Negative result of Kaiser test, (b) Positive result of Kaiser test

The remaining amino acids were added to the peptide sequence with the same procedure. When all of the amino acids were coupled to desired sequence, for cleaving the peptide from resin, trifluoroacetic acid (TFA): triisopropylsilane (TIPS): H_2O solution at ratio of 95:2.5:2.5 was used. Peptide solution was poured into cold diethyl ether and rinsed with cold diethyl ether for three times. Following the centrifugation

of suspension, supernatant part was discarded. Lastly, lyophilization of solid part was performed by freeze-drying and resulting peptide was obtained in the powder form (Biobase Biodustry Bk-FD10P, Shandong, China). The lyophilizer that was used for freeze-drying is shown in Figure 2.3.

Figure 2.3: The lyophilizer device used for freeze-drying method

2.2 Biomembrane Fabrication

PVA-XG based membrane production was carried out following a previously used method [109]. The hydrogel to be developed included PVA-XG polymers at a ratio of 95:5, and the concentration of polymer mixture was 15%. As demonstrated in Figure 2.4, firstly, the powder of PVA polymer (MW 31,000-50,000, %87-89 hydrolyzed) was added into the mixture of deionized water/dimethyl sulfoxide (DMSO) (20% DMSO, 80% deionized water) for preparing 50 ml solution, and this solution was stirred using a magnetic stirrer at 85–90 °C and 700 rpm during 30 minutes. Next, the XG powder (Xanthan gum from *Xanthomonas campestris*) was slowly and with a controlled manner distributed into the PVA solution, and for fully and homogeneous incorporation and dispersion, this solution was left to swell during 24 hours by continuing the stirring at 85–90 °C. Following this step, the solution was stirred at room temperature and 700 rpm during 15 minutes. The obtained mixture was placed in 48-well plates using a pasteur pipette in equal amounts in each well. The solutions in well plates were kept at atmospheric pressure for 2 hours and allowed to be

degassed. Lastly, solutions were freezed at -20 °C for 12 hours. Then, these frozen samples were thawed at 25 °C during 2 hours. This freeze/thaw cycle was performed for 3 times.

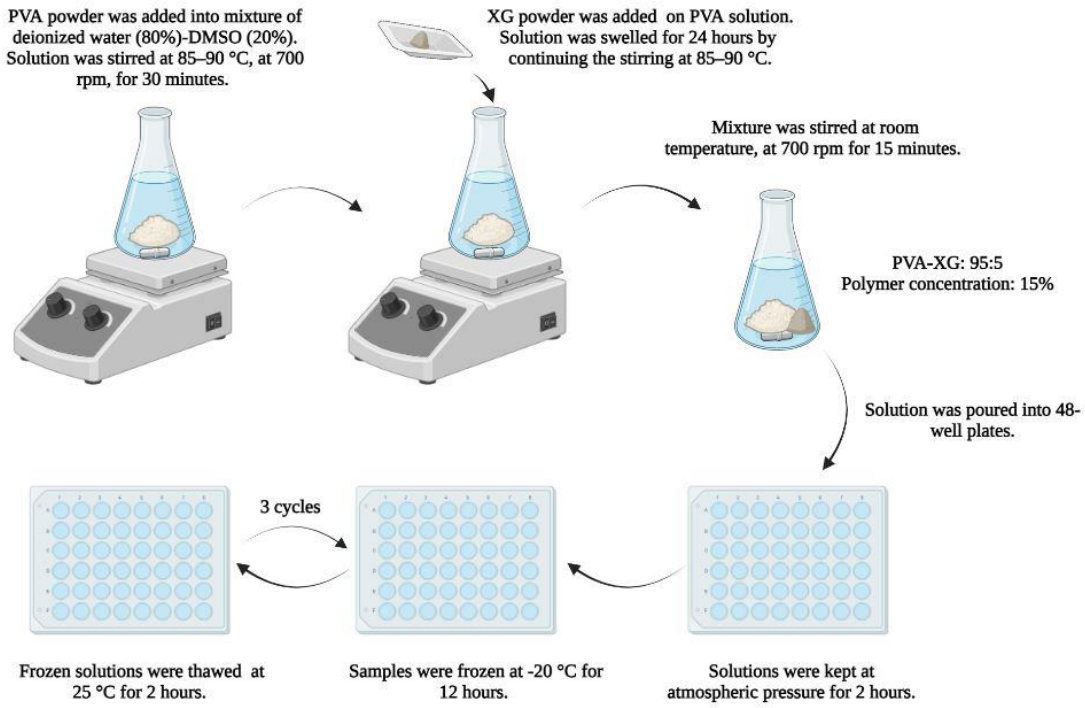

Figure 2.4: Schematic diagram of membrane production procedure. Figure is created with BioRender.com.

2.3 Peptide Conjugation to Biomembrane Surface and Characterization

Carboxylic acid functional groups in the membrane structure allow covalent modification with different types of peptides. Conjugation of synthesized peptides to the membrane surface was carried out utilizing the procedure produced by Deng et al. [138]. Briefly, the membrane was modified with 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC), a water-soluble carbodiimide, to create amide links among the peptide involving amine groups and carboxyl motifs in the polymeric membrane structure, and then with *N*-hydroxy-sulfosuccinimide (NHS) to increase the efficiency of the amide bond formed. For this process, 2 mM EDC and 5 mM NHS were made ready in 0.1 M 2-Morpholinoethanesulfonic acid (MES) buffer solution, and given on the membrane, and membrane was incubated at 37 ºC during 40 minutes in the incubator. After incubation period, 1 mM peptide was dissolved in Phosphate Buffered

Saline (PBS) and given on the membrane. Conjugation reaction was performed by incubating the samples at 4 ºC during 24 hours. Lastly, peptide modified membranes were washed with PBS before characterization.

Membrane-peptide conjugation was characterized by applying a beforehand utilized procedure [139]. In short, labeling of the synthesized peptides was done with the fluorescent isothiocyanate (FITC) molecule prior to detachment from the resin. The labeling reaction took place among the amine groups in the peptide structure and the succinimidyl ester group of the FITC molecule. Later on, conjugation on membrane was made utilizing FITC-labeled P1 peptide, and with an inverted fluorescent microscope (Olympus, CKX41), the FITC-Peptide-Membrane surface was imaged.

The surface topography of the peptide-conjugated structures was observed with a scanning electron microscope (SEM) (FEI QUANTA 250 FEG). Also, the energy dispersive X-ray (EDX) analysis was performed to observe the elemental composition of developed membranes. In addition, the surface chemistry of membranes was characterized using the methods followed by Horzum et al. in previous studies by Fourier Transform Infrared Spectroscopy (FTIR) analysis and X-ray photoelectron spectroscopy (XPS) [140, 141]. For FTIR analysis, Perkin Elmer Spectrum Two FTIR Spectrometer having a spectral resolution of 4 cm^{-1} was used. XPS analysis was performed using Thermo Scientific K-Alpha at EGE MATAL-EU Central Research and Analysis Laboratory Application and Research Center. The measurements were performed on at least 3 randomly selected areas with an Al-Kα monochromatic X-ray source and using high vacuum [140, 142].

2.4 Cell Culture and Cell Seeding

Human bone marrow derived mesenchymal stem cells (hMSCs) (HMSC-AD-500, CLS cell lines Service, Lot #102, Eppelheim, Germany) were cultivated in the basal medium (DMEM integrated with 10% FBS, 100 µg/mL streptomycin, 250 ng/mL fungizone, 100 units/mL penicillin, and 50 µg/mL gentamicin). The growth medium of cultures was replaced with the fresh medium in each of two days. Whenever the cells achieve 80-90% confluency, by utilizing a 0.25% trypsin/EDTA solution for removing the cells from the surface of flask, cells were passaged at a suitable passage

number, and hMSCs were taken into a new flask. For creating enough stocks in the related passage number, hMSCs were stored by freezing under controlled conditions within the freezing media, and then placed in the liquid nitrogen tank at -196 °C (ThermoScientific, Bio-cane 47).

For seeding of hMSCs on the membrane surface, the membranes were sterilized by UV radiation in the biological safety cabinet for 1 hour. Then, every membrane was seeded with hMSC suspension (5×10^4 cells/cm^2) within basal medium. Lastly, the plate was put in the incubator and hMSCs were cultivated in the 5% CO_2 incubator for up to 7 days at 37 °C.

2.5 Cell Proliferation Analysis

We evaluated the cell proliferation by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Vybrant® MTT Cell Proliferation Assay Kit, Invitrogen, Waltham, MA, USA) test on days 1, 4 and 7 as beforehand defined [143]. In brief, 10% MTT dye within growth media was given on cells found on membranes and incubated for 4 hours in the incubator at 37 °C. For dissolving the formed formazan crystals, the medium was changed with DMSO (Sigma-Aldrich, Steinheim, Germany) and incubated during 15 minutes in a shaker. Then a microplate reader (Biotek Synergy HTX, Winooski, VT, USA) was utilized to measure the absorbance at 570 nm.

2.6 Cell Viability Analysis

To observe the cell viability of seeded hMSCs, we conducted Live and Dead staining assay on day 7. The Viability/Cytotoxicity Assay Kit for Animal Live and Dead Cells (Biotium, Inc. Hayward, MN, USA) was utilized to assess the cell viability. In brief, firstly the culture medium was removed from hMSC seeded membranes. Membranes were rinsed with PBS twice. Then, the viability/cytotoxicity assay solution, including Calcein acetoxymethyl ester (Calcein AM; 4 mM) and Ethidium homodimer III (EthD-III; 2 mM) within PBS, was added on seeded hMSCs, and they were kept in the incubator for 45 minutes. Finally, live cells (green) stained by Calcein AM and dead cells (red) stained by EthD-III, were imaged with the fluorescent microscope (Olympus CKX41, Tokyo, Japan).

2.7 Statistical Analysis

At least three samples from each group were tested. All obtained data were statistically analyzed utilizing one-way analysis of variance (ANOVA) (SPSS 12.0, SPSS GmbH, Germany) and the Student–Newman–Keuls method as a post hoc test. Meaningful distinctions among groups were defined at p values at least less than .05. (* $p < .05$, ** $p < .01$, *** $p < .001$).

Chapter 3: Results

3.1 Fabrication of PVA-XG Hydrogel Membranes

Hydrogel membranes were successfully fabricated using a water-soluble polymer, PVA, and XG biomaterial. The photographic images of transparent membranes are shown in Figure 3.1. The membranes had good transparency, flexibility, and smooth surface after being conditioned in a fume hood for a week. They were subjected to first peptide conjugation and then different analyses to obtain their morphology, structure, and cell proliferation.

Figure 3.1: Photographs of the produced PVA-XG hydrogel membranes

3.2 Characterization of Peptide Conjugation on Membrane Surface

3.2.1 Conjugation of FITC Labeled Peptide

The confirmation of the peptide conjugation on the surface of PVA-XG membranes was evaluated by observing the FITC intensity utilizing a fluorescent microscope. In short, 1 mM FITC-labeled P1 peptide was conjugated on PVA-XG membranes by using EDC/NHS chemistry method. As demonstrated in Figure 3.2, the fluorescence was seen with P1-conjugated membrane, and as we expect no fluorescence was observed for non-peptide conjugated membrane, confirming that the fluorescence seen in the image of P1-conjugated structure came from the FITC label.

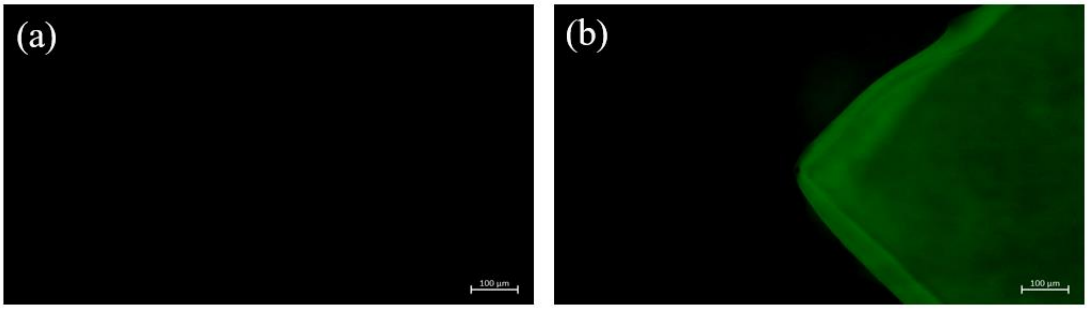

Figure 3.2: Fluorescent microscopy images of membranes, (a) Non-peptide conjugated PVA-XG membrane (control), (b) Fluorescein isothiocyanate (FITC) labeled peptide conjugated PVA-XG membrane (Scale bar represents 100 µm)

3.2.2 Scanning Electron Microscopy (SEM) Analysis

The morphological characterization of PVA-XG and peptide-conjugated PVA-XG membranes was investigated by SEM analysis. Figure 3.3 represents the SEM images of PVA-XG membranes. Based on these images, it is observed that after peptide conjugation, membrane surfaces were coated by a thin layer of peptide material.

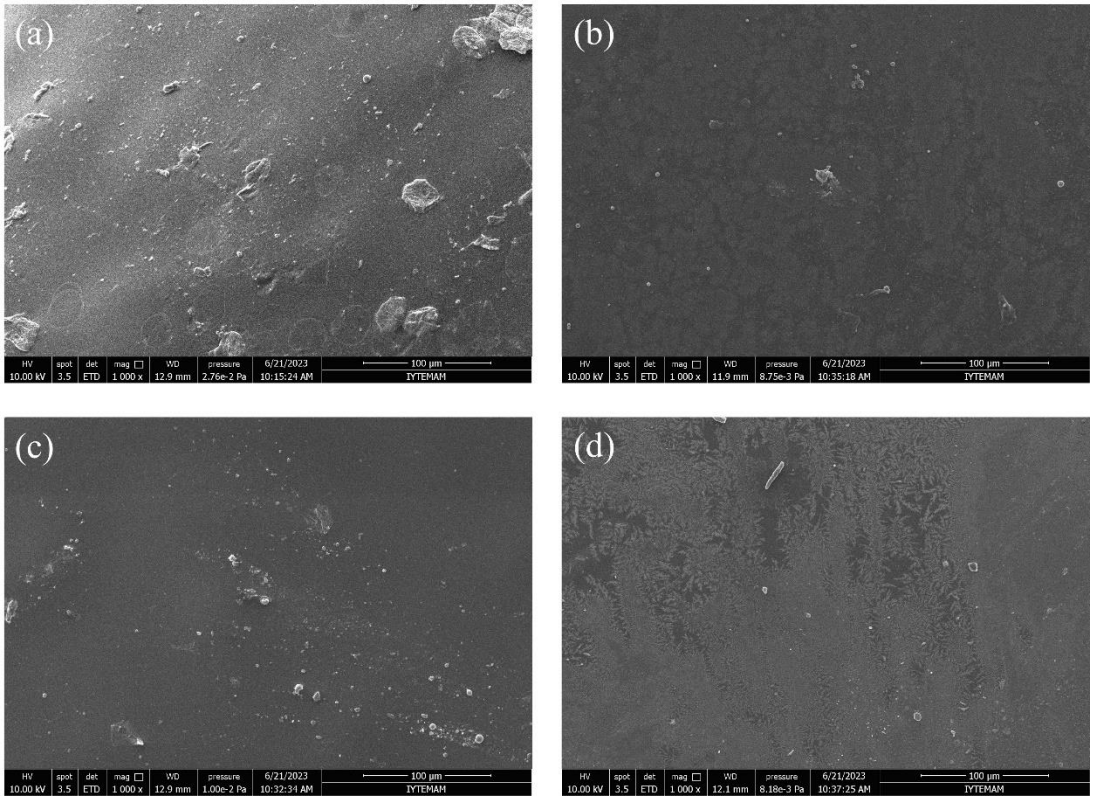

Figure 3.3: SEM images of PVA-XG membranes, (a) Non-peptide conjugated PVA-XG membrane (control), (b) P1-conjugated PVA-XG membrane, (c) P2-conjugated PVA-XG membrane, (d) P3-conjugated PVA-XG membrane (Scale bar represents 100 µm)

3.2.3 Energy Dispersive X-ray (EDX) Analysis

The corresponding distribution of C, O, and N elements of the PVA-XG and peptide-conjugated PVA-XG membranes was analyzed by EDX measurements. Figure 3.4 shows the EDX mapping images for the membranes. After peptide conjugation, N element appeared on the surface of the membranes.

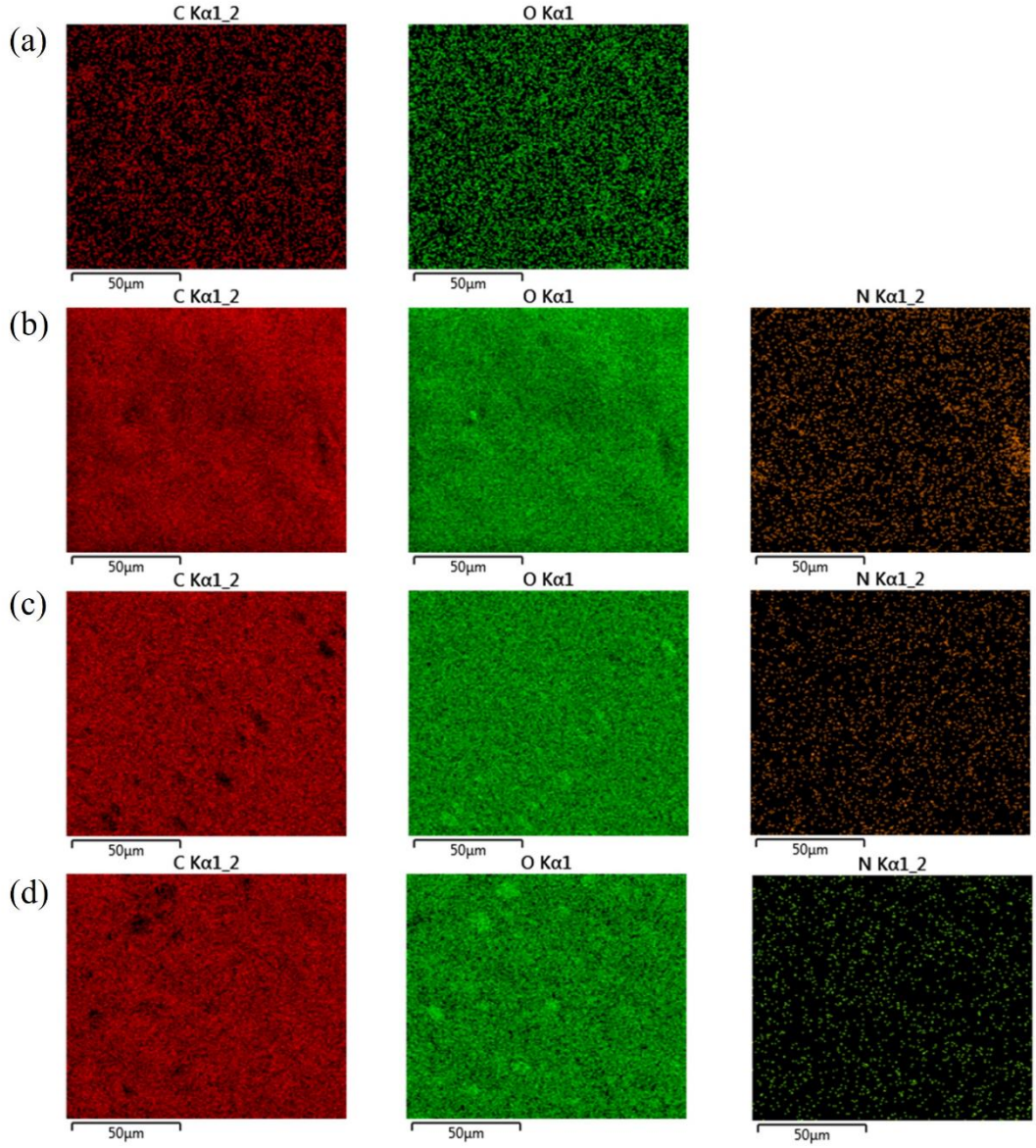

Figure 3.4: EDX mapping analysis images of PVA-XG membranes, (a) Non-peptide conjugated PVA-XG membrane (control), (b) P1-conjugated PVA-XG membrane, (c) P2-conjugated PVA-XG membrane, (d) P3-conjugated PVA-XG membrane

Table 3.1 demonstrates the weight and atomic percantages of the elements found on the surface of the PVA-XG and peptide-conjugated PVA-XG membranes. Unlike the control group membrane, it was observed that the N element was homogeneously distributed in the structure of all the peptide-conjugated membranes.

Table 3.1: Elemental percentage compositions of PVA-XG and peptide-conjugated PVA-XG membranes

Membranes	Elements					
	C		O		N	
	Atomic %	Wt %	Atomic %	Wt %	Atomic %	Wt %
PVA-XG	51.99	44.84	48.01	55.16	-	-
P1-conjugated PVA-XG	61.61	54.71	37.59	44.47	0.80	0.82
P2-conjugated PVA-XG	57.82	50.74	41.89	48.97	0.29	0.29
P3-conjugated PVA-XG	57.30	50.22	42.29	49.36	0.41	0.42

3.2.4 Fourier-Transform Infrared Spectroscopy (FTIR) Analysis

FTIR spectroscopy measurement was utilized for further confirming the peptide conjugation on the developed membranes. Figure 3.5 demonstrates the FTIR spectra of PVA and PVA-XG membranes (a), and peptide-conjugated PVA-XG membranes (b).

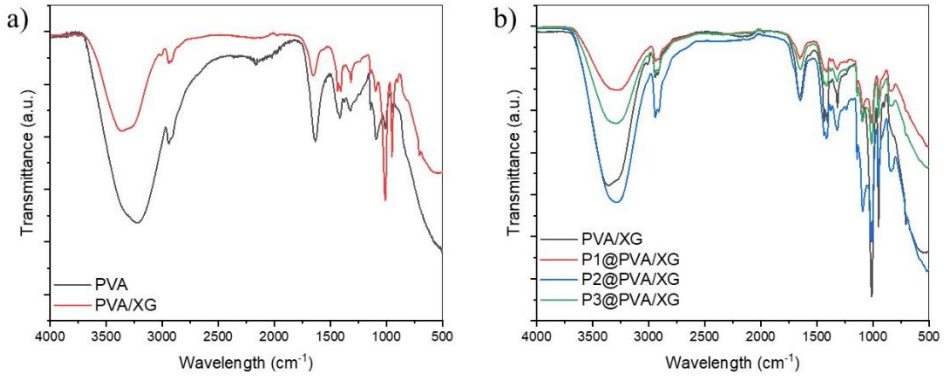

Figure 3.5: FTIR spectra of the (a) PVA and PVA-XG membranes, (b) peptide conjugated PVA-XG membranes

3.2.5 X-ray Photoelectron Spectroscopy (XPS) Analysis

XPS analysis of PVA-XG membranes after peptide conjugation was carried out to observe the surface composition. Figure 3.6 shows the XPS survey spectra and Table 3.2 lists the surface chemical compositions for PVA-XG and peptide-conjugated PVA-

XG membranes. In detail, carbon 1s (C1s), oxygen 1s (O1s), and nitrogen 1s (N1s) spectra of the membranes are shown in Figure 3.7.

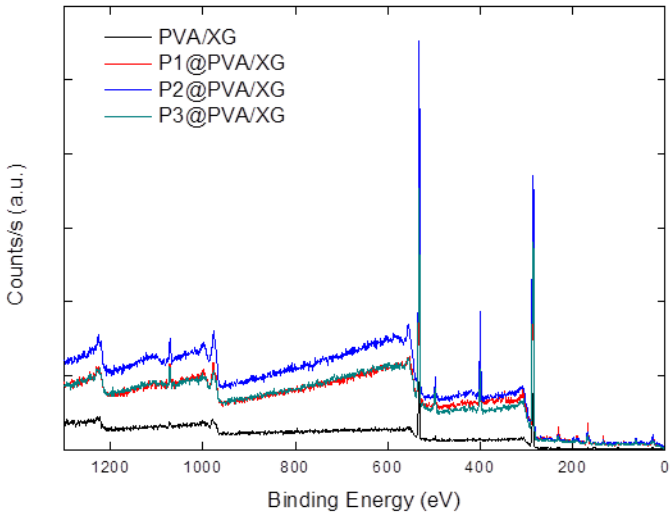

Figure 3.6: XPS survey spectrum

Table 3.2: Relative elemental concentrations of the PVA-XG and peptide conjugated PVA-XG membranes

	C	O	N	Si, S, P, Cl, Na	N/C	O/C
Control	68.51	28.28	-	3.21	-	0.4128
P1@PVA/XG	63.56	27.99	3.60	4.85	0.0566	0.4404
P2@PVA/XG	60.97	27.16	9.18	2.69	0.1506	0.4455
P3@PVA/XG	63.88	25.65	7.02	3.45	0.1099	0.4015

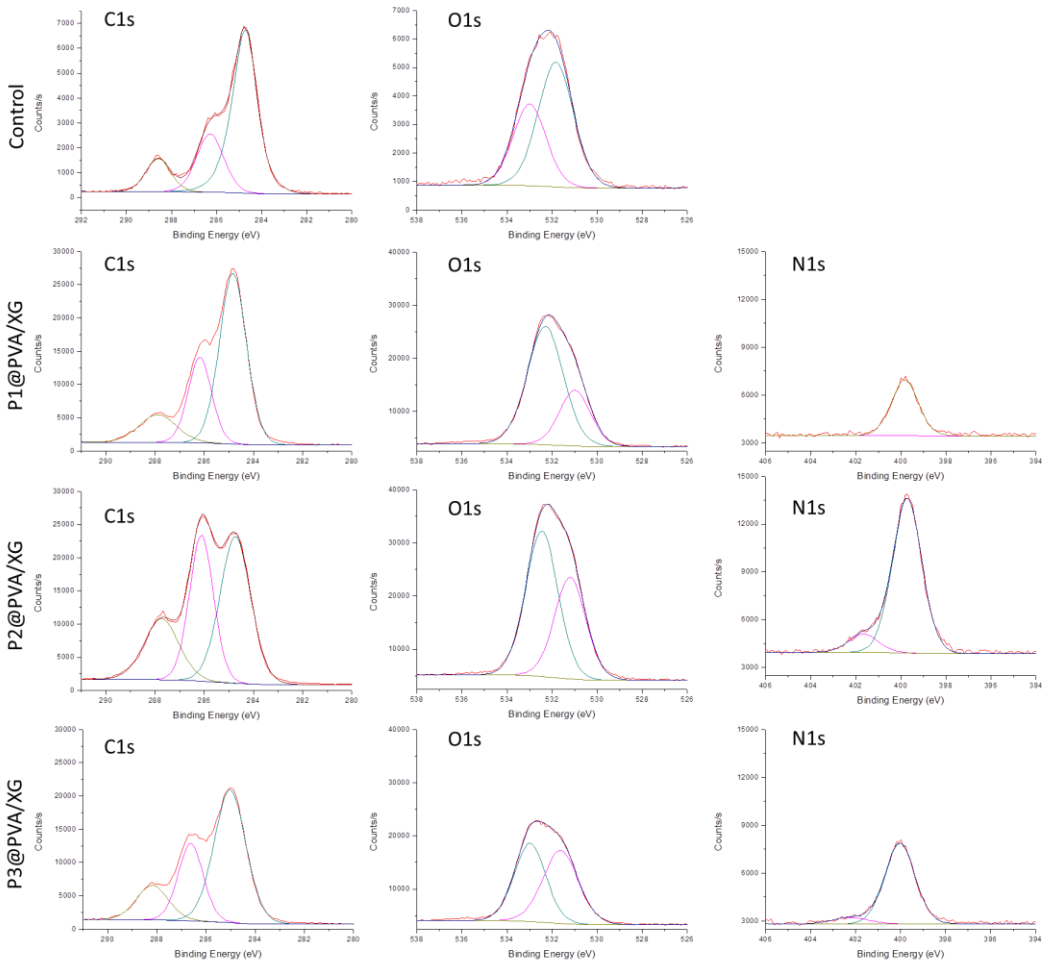

Figure 3.7: C1s, O1s, and N1s spectra of the PVA-XG and peptide conjugated PVA-XG membranes

3.3 Cell Proliferation Analysis

MTT analysis was conducted for evaluating the hMSCs viability and proliferation on PVA-XG membranes during the 1., 4., and 7. days of incubation (Figure 3.8). According to the findings from MTT assay, in Figure 3.8, it was shown that the cell number of control, P1-conjugated, P2-conjugated, and P3-conjugated membranes slightly increased with the incubation time for 7 days. These cell numbers in each group recommended that the produced PVA-XG membranes did not possess a toxical impact on the seeded cells. hMSCs seeded on PVA-XG hydrogel membranes were able to maintain their viability and proliferate. However, the cell number for P2-conjugated membranes was greater than that of for control, P1 conjugated, and P3-conjugated groups in all time points. For example, at Day 7, the cell number on P2-conjugated membranes was 403726,38 ± 22058,38, while the cell numbers were

238288,53 ± 25894,62, 359609,62 ± 31746,914, and 207278,923 ± 31650,1909 on control, P1-conjugated, and P3-conjugated membranes, respectively.

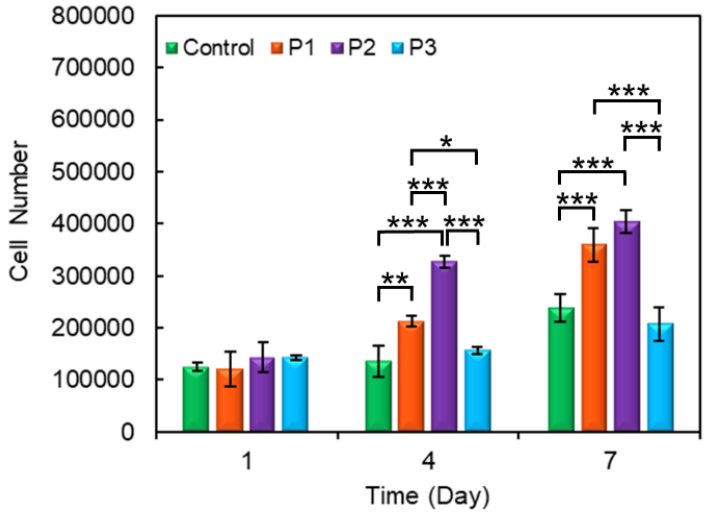

Figure 3.8: Cell number of hMSCs seeded on control, P1-conjugated, P2-conjugated, and P3-conjugated membranes and incubated in basal medium for 7 days. Error bars show mean ± SE (n = 3) (significant differences were defined by one-way ANOVA [Newman–Keuls multiple comparison test, * p < 05, **p < .01, ***p < .001])

3.4 Live&Dead Cell Viability Analysis

To determine the viability of hMSCs seeded on control, P1-conjugated, P2-conjugated, and P3-conjugated membranes, Live&Dead cell staining analysis was applied on day 7 of incubation. As the result of Live&Dead assay, images which were taken by using fluorescent microscope are shown in Figure 3.9. In these fluorescent microscopy images, green color symbolizes the live cells, and red color symbolizes the dead cells. As it is observed from Figure 3.9, peptide conjugated PVA-XG membranes showed higher green color intensity.

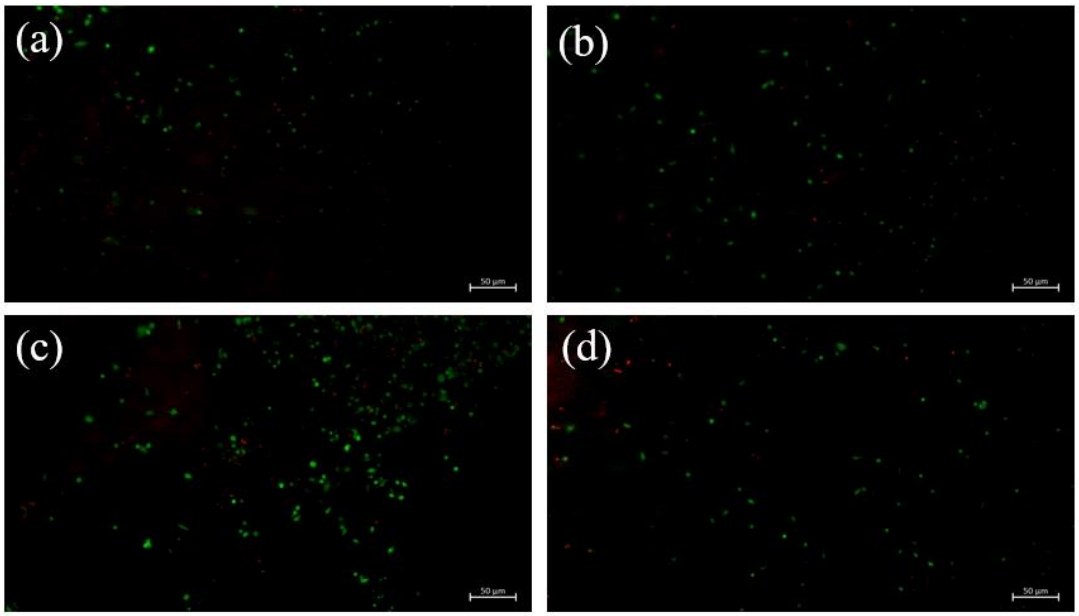

Figure 3.9: Viability analysis of hMSCs seeded on membranes at day 7, (a) Control, (b) P1-conjugated, (c) P2-conjugated, and (d) P3-conjugated membranes. Live cells and dead cells are stained with Calcein-AM (green) and EthD-III (red), respectively (scale bar represents 50 µm)

Chapter 4: Discussion

The cornea as the transparent window found at the forepart of an eye serves as a barrier to shield the eye from UV radiation and particle invasion in addition to aiding in the focus of light onto the retina [9, 144]. Corneal tissue has layers of epithelium, stroma, and endothelium. If there is a damage to one of these layers, the current treatment option is a partial or full-thickness corneal transplant from a cadaveric donor [145]. With enhancing cell loss, the CE, one of the layers of it, becomes dysfunctional, resulting in corneal edema and severe vision impairment [146]. hCECs do not possess the capability for regenerating in vivo, thus when the cell number is significantly decreased, to restore the vision, corneal transplantation is required [25]. The damaged CE is currently replaced using donor allogeneic tissue [146]. Nevertheless, it is known that there is a donor cornea shortage for the transplantation process, with over 10 million people found on the transplant waiting list worldwide [147]. Moreover, the possibility of tissue rejection, infection, and the procedure's high overall cost are further drawbacks [148].

All of these shortcomings have led to developments in the methods of biomaterials and TE for ensuring proper alternative treatment options [144]. TE perspectives toward corneal regeneration have been extensively investigated by researchers [144]. To decrease the need for available donor cornea and for avoiding the problems associated with the current corneal transplantation procedures, TE methods and approaches might be potential and promising alternatives for the regeneration of CE tissue.

Synthetic, semi-synthetic, biologically derived materials are used for corneal endothelial cell culture and TE. Synthetic, semi-synthetic or natural hydrogels have the potential to be used as corneal epithelial barriers and have distinctive structures and behaviors in response to internal and/or external stimuli. They are obtained using

physical and chemical cross-linking methods that the complexation occurs through hydrophilic ionic interactions. The freeze/thaw as a physical technique, includes exposing a polymer dispersion in the vehicle, which is often aqueous, to freezing, generally below −15 °C, for an amount of time that varies on the kind of material and concentration of polymer used. After that, the obtained structure is exposed to a thawing period, which is usually performed at room temperature. This process turns into cyclical, and depending on the goal it is preserved as many times as needed [109, 149]. Additionally, in the procedure of freeze/thaw, the crystallization of water forms high polymer concentrations in interstitial domains, and these highly concentrated domains' polymer chains may crystallize, eventually resulting in the gelation of the entire system [109, 150]. Here, we used the freeze-thaw method to fabricate membrane structures that are intended for being used as scaffolds for CE. We utilized the optimized ratio of 95:5 for the manufacturing of PVA:XG hydrogels, with the concentration of polymer mixture as 15% [109]. The freezing time of scaffolds was 12 hours, and the freeze/thaw cycle number was three.

PVA as a synthetic polymer material has the property of hydrophilicity (-OH groups), a degree of biodegradability, and also perfect biocompatibility [151]. PVA-based hydrogels were identified as hopeful biomaterials and proper nominees for TE implementations and could be handled to serve several crucial functions [151]. Because of its very good biocompatibility, PVA polymer has received a lot of attention in the biomedical area, particularly in the implementations of TE [152, 153]. However, since there are some drawbacks, such as the lack of cell-adhesive features, PVA materials require further modification for intended and targeted aims [151].

The development of biomimetic materials for TE applications has gained increasing popularity recently [131]. Using peptides as bioactive molecules have been the focus of studies from different research areas. Peptides are promising components of TE scaffolds since they allow to the development of biomimetic and bioactive material surfaces that improve important cellular activities such as adhesion, migration, growth, reproduction, and differentiation. Recently, biomaterials that are modified with peptides are being studied for use in CTE practices. For instance, Duan et al. chemically attached the YIGSR to dendrimers, then used the YIGSR attached dendrimers like collagen crosslinkers, integrating this peptide in collagen gels bulk.

Then YIGSR was also attached to the dendrimer crosslinked collagen gels surface. It was reported that incorporating YIGSR, a model cell adhesion peptide, in bulk, and YIGSR surface modification encouraged the attachment and reproduction of human corneal epithelial cells and neurite extension from dorsal root ganglia [154]. Gil et al. prepared an RGD-coupled silk protein-biomaterial lamellar system, then evaluated the effect of RGD-coupling on reproduction, organization of ECM, orientation, and human cornea fibroblast gene expression. It was reported that surface modification with RGD increased the cell adhesion, alignment, reproduction, and collagens (type I and type V) and some proteoglycans expression [60]. Moreover, Kennedy et al. developed synthetic peptide hydrogel by utilizing poly-ε-lysine (pεK), cross-linked with octanedioic-acid, material for CECs growth and CE grafts. Functionalizing this pεK hydrogel by synthetic cell-binding peptide H-Gly-Gly-Arg-Gly-Asp-Gly-Gly-OH (RGD) or α2β1 integrin recognition sequence H-Asp-Gly-Glu-Ala-OH (DGEA) ended up with the increased adhesion of primary porcine CECs with RGD only. It was reported that the modification of the pεK surface by RGD, enhanced the attachment and expansion of primary porcine CECs, also at 5 weeks those cells possessed a monolayer by ZO-1 and Na^+/K^+ATPase expression, showing the creation of tight junctions and functional pumps existence, respectively [146].

In the present work, the aim was to produce and characterize the corneal endothelial tissue specific peptide-modified bioactive PVA-XG hydrogel membranes for the use as scaffolds in CTE applications. Firstly, we used the FITC labeling for confirming the peptide conjugation on PVA-XG membranes. FITC-labeled P1 peptide was conjugated on developed membranes, and FITC intensity was observed by using a fluorescent microscope. Figure 3.2 shows the fluorescent microscopy images of membranes. As shown in Figure 3.2, while fluorescence was seen for the P1-conjugated membrane, no fluorescence was seen for the non-peptide-conjugated membrane. This indicates that the fluorescence intensity that was observed in the image of the P1-conjugated sample was due to the presence of the FITC label.

To observe the surface topography of the developed PVA-XG membranes, the SEM imaging method was used. Figure 3.3 shows the SEM images for PVA-XG, P1-conjugated PVA-XG, P2-conjugated PVA-XG, and P3-conjugated PVA-XG membranes. According to these images, membrane surfaces after peptide conjugation

were coated by a thin layer of peptide material, confirming that peptide conjugation was successful.

EDX microanalysis is known as a method of elemental analysis related to electron microscopy using the production of distinctive X-rays which indicate the presence of elements exist in the examined samples [155]. Additional elemental analysis and quantification of existing elements on the surface of PVA-XG membranes were performed with EDX measurements. EDX mapping analysis images for PVA-XG membranes are represented in Figure 3.4. Based on these images, it is observed that there is not any EDX mapping image for the N element in the control group membrane, only mapping images for C and O elements were presented. On the other hand, for the peptide conjugated membranes there are also EDX mapping images for the N element in addition to the images for C and O elements. Moreover, these mapping images for the N element show that the peptide material was homogenously distributed on the surface of the membranes.

In the Results section, the Table 3.1 shows the weight and atomic percentages of the existing elements on the surfaces of control, P1-conjugated, P2-conjugated, and P3-conjugated PVA-XG membranes, respectively. From this table, it is clearly observed that while C and O elements are found on the surfaces of all membrane groups, N element is present only on the surfaces of P1-conjugated, P2-conjugated, and P3-conjugated PVA-XG membranes, as a distinction from control group membrane. These data from both EDX mapping images and the EDX elemental composition table indicate that the N element is present in the structure of all three peptide-conjugated membranes, and this also confirms the successful peptide conjugation.

In most cases, whenever two different polymer solutions are combined, diffusion of a flexible polymer chain, like PVA, in a structured one, like XG, which has rigid chains in double helix construction is participated via the breaking up of preexisting poor bonds, production of novel ones, lastly, of structural rearrangement of whole macromolecular structure within solution [156].

FTIR spectroscopy is a commonly utilized method for investigating macromolecular structures and for detecting the interplays of polymer chains [156]. The large quantity of hydroxyl groups of PVA and XG polymers results in the creation of intermolecular

interplays in physical blends of them according to the hydrogen bonding between hydroxyl and carbonyl/carboxylate groups of XG and hydroxyl and acetate groups of PVA [156]. Figure 3.5 demonstrates the FTIR spectra of PVA, PVA-XG membranes (a), and peptide-conjugated PVA-XG membranes (b). It is known that individual XG is plentiful in acetyl, carboxylic acid, hydroxyl groups [157]. PVA also possesses a large quantity of hydroxyl and residual ester groups [158]. For membrane production, XG polymer was incorporated in the PVA solution, and following the freeze-thaw procedure, a gel was obtained. FTIR spectra of the formed gel of PVA-XG demonstrates unique and distinctive properties, which verifies the interaction between PVA and XG [108]. In addition, peptide conjugation on PVA-XG membranes was similarly confirmed.

In Figure 3.5, the initial characteristic is observed in 3600-3000 cm^{-1} region which is related to -OH group stretching vibration [108]. The -OH group signal seems in PVA at 3228 cm^{-1} and seems in PVA-XG at 3363 cm^{-1}. A significant decreased intensity of the bands located in the region of 3600 to 3000 cm^{-1} indicates that the hydrogen bonding interplays in primary and secondary alcohol groups [159-161]. Upon freeze-thaw process of PVA-XG mixture, the hydroxyl and carboxyl groups found in the XG molecule and hydroxyl group in PVA polymer create a 3D structure through hydrogen bond, resulting in the formation of a physical intersecting PVA-XG hydrogel [108]. XG's molecular weight is great adequately, thus it does not spread out of the gel [108]. The following describes how the cross-linking process works during freezing and thawing procedure: first, throughout the freezing procedure, the free water molecules within the polymer mixture freeze and push the polymer chains. Then, that situation causes PVA–XG's big local concentration that eases the creation of hydrogen bonds among the polymer chains and the creation of crystal nucleus. Later on, via repetition of freeze-thaw cycles, microcrystals proceed to enhance in size and in the end produce a stable 3D net structure [108]. A newly formed =C–H stretching of aromatic ring because of XG is observed at 3014 cm^{-1}. The second characteristic band is observed in the region of 1815-1630 cm^{-1} which is related to the –C=O group stretching vibration [108]. The –C=O group signal seems in PVA polymer at 1637 cm^{-1} and in PVA-XG polymer at 1655 cm^{-1}. The bands at 2943 cm^{-1} are attributed to the symmetrical stretching CH_2 vibrations, while the bands at 1324 cm^{-1} are related to O-H bending (in plane) in both spectra. The bending vibration

of CH_2 is observed at 1422 cm^{-1} for PVA, while the C-O-H bending (1440 and 1395 cm^{-1}), O-H bending (out-of-plane) (707 cm^{-1}) vibrations are observed for PVA-XG. The bands at 1091, 1012, and 948 cm^{-1} are attributed to C–O stretching, =C-H & $=CH_2$ bending vibrations, respectively.

In addition to the non-peptide conjugated control group membrane which is PVA-XG, P1, P2, and P3 peptide sequences were then separately conjugated on the surfaces of the control membranes. A variation in the absorption bands at 3600–3000 cm^{-1} and 1265–1000 cm^{-1} was seen in P1-conjugated, P2-conjugated and P3-conjugated membranes because of the influence of nitrogen introduction resulting from peptide conjugation [162]. It was observed that around at 3000-3500 cm^{-1}, the OH-derived band shifted after peptide conjugation. Moreover, the other bands observed at 840 cm^{-1} (NH_2 & N-H wagging), 1140 cm^{-1}, may be because of the newly formed N-H and C-N bands caused by peptide conjugation.

XPS is a surface-sensitive technique, with an average depth of analysis is approximately 5 nm, that ensures element-specific chemical composition. XPS survey spectra of PVA-XG and peptide-conjugated PVA-XG membranes are given in Figure 3.6 and the surface chemical compositions are listed in Table 3.2. Apart from C, O, and N, the detected other elements (Sulfur (S), Phosphorus (P), Chlorine (Cl), and Sodium (Na)) probably result from crosslinking agents and buffer solution. The peptide conjugation of the PVA-XG membrane surface was observed through the change in the elemental composition, which became evident with the additional nitrogen photoemission in the spectra (Figure 3.7). C 1s emission spectra were deconvoluted into three peaks: aliphatic C-C, C-O/C-N species, and O=C-O/O=C-N groups (the left column of Figure 3.7). Two photoemission peaks were observed in the O 1s spectra indicating the decreasing electron density at the ester oxygen environments, O=C-O (O=C and C-O) (the middle column of Figure 3.7). A significant increase in intensity was observed in the C 1s and O 1s signals after peptide conjugation. Furthermore, N1s photoemission signals appeared at ~400 eV (O=C-N) and 402 eV ($C-NH_3^+$), which are characteristic of an amide bond and conjugated-peptide side chains [163] (the left column of Figure 3.7). $C-NH_3^+$ signal could not be detected for P1-conjugated membrane. In the presence of a protonated amino group (and consequently a positive charge), there may be enhanced electrostatic interactions

with the negatively charged membrane. Conversely, here the absence of such protonated groups signify reduced interaction with the membrane, and thus less nitrogen content observed. The N/C and O/C ratios are the highest in the P2-conjugated membrane (Table 3.2), confirming a higher quantity of N and O atoms on the surface.

As it is stated before, in addition to the produced membrane structure, the special laminin protein in the Descement layer, which is found only in the corneal tissue, is also effective in driving the CECs into a hexagonal honeycomb-shaped morphology. Herein, to determine which peptide sequences will be investigated, by using the computational and structural biology approach, a method was applied to identify and model these tissue-specific peptides in the laminin protein in the Descement membrane to which the CECs are attached. As the result of this method, peptide sequences determined by detecting the active sequences of LM5 protein were used for membrane conjugation.

Laminin protein includes three chains, α, β, and γ, that assemble in a cross-shaped heterotrimer [164]. It is known to regulate different cellular functions via interaction with integrins [164]. Laminins help to cell-adhesive activity of basement membrane, and reveals some cellular responses by interplays with cell surface receptors, involving integrins [164]. Yamaguchi et al. aimed to explore LM5 and its receptors expression by hCECs and whether the recombinant human LM5 affects hCECs attachment, reproduction and migration [165]. Their results showed that adult HCECs expressed LM5 receptor α3β1 integrin, however not LM5 oneself, and importantly more number of cells turn into adherent to recombinant LM5 (1.0 μg/mL)-modified dishes than to uncoated ones. It was reported that hCECs express the LM5 receptor α3β1 integrin, and recombinant LM5 encourages the attachment, average proliferation and migration of these hCECs. Herein, we used non-peptide conjugated membrane as the control group, and we used each of three different LM5-derived peptide-conjugated membranes as experimental groups. It was also aimed to evaluate and compare the influence of these three LM5-derived peptides on the proliferation and viability of hMSCs.

The effect of LM5-derived peptide conjugated PVA-XG membranes on the proliferation of hMSCs was determined with MTT assay on 1., 4., and 7. days of incubation. As an important note, for membrane fabrication, to obtain 50 ml of PVA

solution, first we used a mixture of 80% DMSO and 20% deionized water. However, when we performed cell seeding on the membranes prepared by this solution and did MTT analysis, it was observed that there was not a purple color change after addition of DMSO, indicating that these membranes did not encourage the proliferation of seeded cells, instead they showed a toxical effect on cells. This toxical effect is thought be reasoned from the high percentage of DMSO in the solution. Therefore, we decided to decrease the concentration of DMSO solvent, and for membrane production we used a mixture of 20% DMSO and 80% deionized water.

As observed from Figure 3.8, for all the groups, control, P1-conjugated, P2-conjugated, and P3-conjugated membranes, the cell number increased slightly by incubation time throughout 7 days. These enhanced cell numbers in all groups recommend that the PVA-XG membranes did not cause a toxic impact on hMSCs. It was thought that cells could adhere on the surface of PVA-XG membrane structures and had enough amount of area for their proliferation. Moreover, MTT assay results revealed that the cell numbers for P2-conjugated membranes were higher than the cell numbers for other three group membranes in all time points. P2-conjugated PVA-XG membranes easied the hMSCs proliferation significantly when compared to control, P1-conjugated, and P3-conjugated groups during incubation. This finding suggests that the peptide sequence P2 induces cell proliferation more, and P2-conjugated membranes provided more proper environment for assisting the proliferation of hMSCs. The more potential of P2 in cell proliferation might be associated with the presence of RGD epitope in the original sequence of P2, which is not found in other peptide sequences P1 and P3. RGD is found within the main ECM components, involving laminin and fibronectin proteins, and acts like an adhesive ligand to integrins, and also it has been shown to possess an effect on behaviors of human corneal epithelial cells [166-169]. Scientists reported that to make the biomaterial surfaces biologically activated, arginine-glycine-aspartic acid (RGD) peptide was used for immobilization on surface of biomaterials for inducing cell adhesion via the certain interplays with integrin receptors [170]. It is known that RGD peptide binding to integrin receptors takes an important part in the cell adhesion mechanism. Also, RGD has been demonstrated to enhance cellular intensity and migration in microenvironment. In tissue or cell implants, RGD sequence does not only enhance tissue growth but also declines the interface fibrous tissue [171-173]. Recent works

have shown that cell adhesion via RGD-integrin binding and following focal adhesion creation possess a straight effect on actin cytoskeleton creation and contraction [174-176], also that interaction suggested as the major mechanism by which cells are affected via biophysical features of extracellular environment of them [177-179]. In short, in order to functionalize a material surface, using RGD peptide increases the cell alignment, adhesion, reproduction, expression of collagen I and V and several proteoglycans [180].

On the day 7 of cell incubation, Live/Dead staining assay was performed for evaluating the cells viability on PVA-XG membranes. In Figure 3.9, the images demonstrated that for all the membrane groups there were both live cells and dead cells, which were indicated with green color and red color, respectively. However, green color intensity was greater for P1, P2, and P3-conjugated membranes as compared with the control group membrane. This indicates that LM5-derived peptide-conjugated membranes have higher number of living cells and therefore viability of cells was superior on them. Our findings from Live/Dead staining analysis revealed that peptide-conjugated PVA-XG membranes could ensure an appropriate microenvironment for the seeded hMSCs to live and stay alive.

Chapter 5: Conclusion

The major aim of this thesis is to fabricate peptide-modified bioactive membranes for the purpose of use in the regeneration of corneal endothelium tissue. To eliminate the disadvantages of current corneal transplantation methods, CTE aims to produce synthetic corneal tissue equivalents. However, synthetic scaffold constructs lack cell-recognition signals, thus they are not originally bioactive materials. CTE also aims to develop biomimetic scaffolds made up of biocompatible materials. At this point, the functionalization of synthetic scaffolds by biomimetic peptides is of great importance. Biomimetic peptides can be used to increase the bioactivity of synthetic scaffolds for the applications of CTE. Furthermore, scaffolds modified with biomimetic peptides could mimic the natural environment of cornea ECM, and thus enhance the functions of the scaffold and also the cellular activities such as adhesion, proliferation, and differentiation. In the current thesis study, first the corneal endothelium tissue-specific peptides were synthesized by applying the solid phase peptide synthesis method, and PVA-XG membranes were fabricated by applying the freeze-thaw method. It was found that the synthesized peptides were successfully modified on the PVA-XG membrane structures, confirmed by SEM, EDX, FTIR, and XPS measurements. Besides the characterization studies related to the peptide conjugation, the influence of the LM5-derived peptide-modified PVA-XG membranes on the viability and proliferation of hMSCs was assessed via MTT and Live&Dead staining assays. The results from these experiments demonstrated that the peptide-conjugated membranes did not cause a toxical impact on hMSCs indicating that the developed scaffold constructs might be considered as biocompatible and might be a candidate for TE applications of CE. Moreover, our findings revealed that the peptide sequence of P2 possessed more potential in promoting cellular viability and proliferation. In summary, for better and more efficient regeneration of CE, developing peptide-functionalized

biomaterials is of great importance to increase the bioactivity of synthetic scaffold structures in TE. Therefore, it is strongly believed that such outcomes from this thesis will assist and shed light on further studies about CE tissue regeneration. As the studies about the production and characterization of peptide-modified bioactive scaffold materials increase, it will be easier to transfer these biomaterials to the clinic and convert them into clinically usable form.

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Appendix

Publications from the Thesis

Conference Papers

1. Sunal, G., Pulat, G., Akgun, I. H., Guven, S., Yildiz, U. H., Karaman, O., & Horzum, N. (2022, October). Biomimetic peptide-conjugated membranes for developing an artificial cornea. In *2022 Medical Technologies Congress (TIPTEKNO)* (pp. 1-4). IEEE.

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Publications:

1. Günnur Pulat, Gülşah Sunal, Ozan Karaman, "Doku Mühendisliği ve Rejeneratif Tıp", Biyomedikal Mühendisliğinin Temelleri, ed. İsmail Devecioğlu (Nobel Akademik Yayıncılık, Ekim, 2022), 373-397.
2. Sunal, G., PULAT, G. O., & Karaman, O. (2022). Enhanced Proliferation of Human Mesenchymal Stem Cells by Self-Assembled Peptide Hydrogel Modified with Heparin Mimetic Peptide. *Avrupa Bilim ve Teknoloji Dergisi*, (41), 59-66.
3. Sezer, B., Özcan, Ş., Bilgiç, E., Sunal, G., Pulat, G., Usta, Y. H., & Karaman, O. (2022, October). Peptide-Based Bioink Development for Custom-made Bioprinter with Specialized Nozzle Design. In *2022 Medical Technologies Congress (TIPTEKNO)* (pp. 1-4). IEEE.

4. Utar, B., Sunal, G., Pulat, G., Karaman, O. (May, 2022). Human Mesenchymal Stem Cells Osteogenic Differentiation on Dental Barrier Membrane. International Conference on Medical Devices (ICMD). DOI:10.54856/jiswa.202205206.

5. Sunal, G., Onak, G., Gökmen, O., Namlı, İ., & Karaman, O. (2020, November). Determination of the Effect of Glycosaminoglycan Mimetic Peptide Hydrogels on Cell Viability for Cartilage Tissue Engineering Applications. In *2020 Medical Technologies Congress (TIPTEKNO)* (pp. 1-4). IEEE.