Biomimetic peptide-conjugated membranes for developing an artificial cornea

[1]Gülşah Sunal, [1]Günnur Pulat, [2]İsmail Hakkı Akgün, [3]Sinan Güven, [4]Ümit Hakan Yıldız, [1]Ozan Karaman*, [5]Nesrin Horzum*

[1]*Department of Biomedical Engineering, İzmir Katip Çelebi University, İzmir, Turkey*

[2]*Department of Bioengineering, Ege University, İzmir, Turkey*

[3]*Izmir Biomedicine and Genome Center, İzmir, Turkey*

[4]*Department of Chemistry, Izmir Institute of Technology, İzmir, Turkey*

[5]*Department of Engineering Sciences, İzmir Katip Çelebi University, İzmir, Turkey*

**Correspondence: ozan.karaman@ikcu.edu.tr, nesrin.horzum.polat@ikcu.edu.tr*

2022 Medical Technologies Congress (TIPTEKNO) | 978-1-6654-5432-2/22/$31.00 ©2022 IEEE | DOI: 10.1109/TIPTEKNO56568.2022.9960151

***Abstract*—The corneal endothelium is composed of a single layer of specialized endothelial cells, protecting, and nourishing the inner surface of the cornea. Corneal endothelial cells do not proliferate after birth and their number decrease with age. Trauma, inflammation, or surgical intervention can cause cell loss. When damage is extensive and the density of corneal endothelial cells decreases to a critical level, it results in corneal edema and vision loss. Besides them, when corneal endothelium has irreversible damage, the only treatment way is corneal transplantation. But there are some drawbacks such as finding donors, immune reactions, and the number of patients waiting on the transplantation lists for years. Tissue engineering approaches can provide promising alternatives for the regeneration of corneal endothelium tissue. Peptides can be used to modify and functionalize the scaffolds, allowing for the production of bioactive and biomimetic surfaces. Peptide-modified scaffold surfaces might direct and enhance the behaviors of cells. In this study, the aim was to functionalize the polycaprolactone (PCL) membranes with tissue-specific peptides and to characterize the peptide-conjugated membranes by Fourier-Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), and X-ray Photoelectron Spectroscopy (XPS) analysis. The synthesized peptides were successfully conjugated on the PCL biomembranes.**

Keywords—tissue engineering; corneal endothelium; peptide synthesis; characterization

I. INTRODUCTION

The corneal endothelium is composed of hexagonal-like endothelial cells that create a packed monolayer of cell onto the Descemet membrane [1]. The corneal endothelium consists of a single layer of specialized endothelial cells, protecting and nourishing the inner surface of the cornea. This layer also functions as a pump, regulating the osmotic balance of the cornea and providing its transparency. Whenever endothelium is created, endothelial cells turn mitotically inactive. The human corneal endothelium at first includes nearly 4500 cells mm^{-2}, but the total number of endothelial cells lowers with age and cell surface area enhanced [2, 3].

The number of endothelial cells generally declines with age, trauma, inflammation, and some diseases. On the other hand remaining cells can stretch and get the space of degenerated endothelial cells, and also by taking place of that pathways, remaining cells expand in size (polymegathism) and no longer have hexagonal shape (pleomorphism) [4].

Corneal endothelial damage caused by the decrease in cell density in the corneal endothelium is tried to be compensated by increasing the pump and barrier function of the corneal endothelial cells that remain behind and provide the transparency of the cornea. When the density of corneal endothelial cells decreases to a critical level (generally 500 cells/mm^2), this feature disappears, and corneal edema and severe visual impairment begin to be observed [4].

According to the data of the World Health Organization (WHO), corneal diseases are in 4th place among the diseases that cause blindness all over the world. When irreversible damage occurs in the corneal endothelium, the only treatment option is surgery, namely corneal transplantation. Corneal endothelial damage, which is one of the most common vision losses in the clinic, can only be treated with an endothelial layer (Endothelial keratoplasty) transplanted from another person today. The main disadvantage of this treatment is the limited donor finding and immune reaction.

Tissue engineering ensures an alternative approach to repair and regeneration of body tissues via using biomaterials, cells, and signaling molecules. Biomaterials are utilized for replacing, repairing, or augmenting the diseased or damaged organs, tissues, parts, or the missing functions of the body, in a partial or complete manner [5]. Biopolymers are generally preferred as 3-dimensional scaffold materials in tissue engineering applications

978-1-6654-5432-2/22/$31.00 ©2022 IEEE

and the goal is to support cell attachment and create a physical form.

Biomimetic approaches allow directing cellular behaviors on tissue scaffolds made of biomaterials. At this point, the use of peptides as bioactive molecules has gained importance. Peptides obtained from functional sequences of proteins are used in the development of bioactive surfaces that enhance cellular response, as they can be designed for many different target molecules. Peptide structures have two main sources: peptides derived from natural proteins of plants, animals, or humans and produced by chemical synthesis. Nature-derived peptides have some important disadvantages, such as the variety in each batch, difficulty in obtaining, and the risk of contamination. Peptides obtained by chemical synthesis, on the other hand, have important advantages such as not having these problems, being able to be modified according to the desired functional structure, and enabling the production in high quantities with the same purity by scaling up the synthesis process [6]. With the new developments in peptide synthesis technology, which started with solid-phase peptide synthesis and continued until today, the use of functional peptides in bioactive material surface development has increased by reducing the cost of peptide synthesis and increasing the synthesis efficiency [7, 8]. A high biological response can be obtained by modifying bioinert scaffolds using functional peptides to be developed in the active sequences of specific proteins in the extracellular matrix of different tissues. Karaman *et al*. observed a significant increase in the amount of CaP crystals attached to nanofibers by the biomineralization method because of the conjugation of peptide epitopes containing functional sequences of osteonectin and osteopontin proteins, which are common in the structure of bone tissue, to PLGA nanofibers. With this developed method, it has been reported that peptide epitope conjugated groups significantly increase the osteogenic differentiation of mesenchymal stem cells [9].

In this study, it was aimed to develop an artificial corneal endothelium tissue, to conjugate specific peptides to these produced membranes, and to characterize them. Corneal endothelium-specific peptide sequences were determined because of the design and simulation of peptides that promote tissue-specific cell attachment using computational biology methods. Subsequently, it was aimed to artificially synthesize peptides with co-functionality with proteins that ensure the attachment of specialized cells in the corneal endothelium. Membranes to mimic corneal endothelium tissue were successfully functionalized by peptide conjugation, and the influence of peptide conjugation to membrane surface was examined.

II. METHOD

A. Peptide Synthesis

The peptide sequences determined by detecting the active sequences of the laminin-5 protein are "PPGCYNTQALEQQ", "PPGCYNTQALEDQQ", and "PPGCYNTQALEDNQ" (P: Proline, G: Glycine, C: Cystine, Y: Tyrosine , N: Asparagine, T: Threonine, Q: Glutamine, A: Alanine, L: Leucine, E: Glutamate, D: Aspartate), and they were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on 4-Methylbenzhydrylamine (MBHA) (0.67 mmol g^{-1}) resin with an automated peptide synthesis device (AAPPTEC FocusXi, Louisville, KY, USA) [10]. Firstly, 100 mg of resin was swelled by soaking in 3 ml of *N, N*-dimethylformamide (DMF) for 30 minutes and then washed again with DMF (2x3 ml). All amino acids were coupled with Fmoc-protected amino acid derivatives, O-Benzotriazole-*N, N, N′, N′*-tetramethyluronium-hexafluoro-phosphate, hydroxybenzotriazole and, *N, N*-diisopropylethylamine in DMF for 3 hours. A solution formed with 20% piperidine in DMF was used to remove the Fmoc-protecting group for 30 minutes. At each amino acid coupling and deprotection step, the reactions were checked by the Kaiser test. To separate the peptide from the resin, the resin was left in a mixture of 95% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% H_2O for 2 hours. Then, the solution was poured into cold ether, and washed 3 times with cold ether. The suspension was then centrifuged, and the supernatant was removed. After the remaining ether was removed by vacuum evaporator, the solid part was lyophilized by freeze drying method (Biobase Biodustry Bk-FD10P, Shandong, China) [11, 12].

B. Production of Biomembranes

ω-Dihydroxy Polycaprolactone (PCL) was synthesized according to the previously used protocol [13]. Dihydroxy PCL (0.052 or 0.104 g for 5 or 10 wt%, PCL content) dissolved in dichloromethane (15 ml), then glycerol ethoxylate (0.62 ml, 0.70 mmol) and sebacoyl chloride (0.30 ml, 1.42 mmol) was added to dihydroxyl PCL solution and the mixture was stirred well and left for 1 hour at room temperature. Then, 7.5 ml of the solution was transferred to a glass petri dish and kept in a vacuum oven (20 mbar) at 60 °C for 30 minutes. The biomembrane was removed from the petri dish, washed with deionized water, and transferred to a 1:1 tetrahydrofuran:deionized water solution.

C. Conjugation of Peptides onto Biomembranes

Conjugation of synthesized peptides onto the biomembrane surface was performed using the protocol developed [14]. 2 mM 1-ethyl-(dimethylaminopropyl) carbodiimide and 5 mM *N*-hydroxy-sulfosuccinimide were prepared in 0.1 M 2- (*N*-morpholino) ethanesulfonic acid buffer, added to the membrane, and kept in an incubator at 37 ºC for 40 minutes. Then, 1 mM peptide was dissolved in phosphate buffered saline (PBS) and added to the membrane, and the conjugation reaction was carried out by keeping it at 4 ºC for 24 hours.

D. Characterization of Peptide-Conjugated Biomembranes

The characterization of the membrane-peptide conjugation was performed according to the previously used protocol [15]. Briefly, the synthesized peptides were labeled with the fluorescent isothiocyanate (FITC) molecule before being cleaved from the resin molecule. Membrane conjugation was then performed using FITC-labeled peptides and the produced FITC-Peptide-Membrane surface was visualized with an inverted fluorescent microscope (Olympus, CKX41). Surface topography of the peptide-conjugated membranes was observed by SEM. (Carl Zeiss 300VP, Germany) at 3 kV accelerating voltage after coating with gold (QUORUM; Q150 RES; East Sussex; United Kingdom) at 20 mA for 60 seconds. In addition, the surface chemistry was characterized by FTIR (Nicolet iS5, Termo Scientifc, Madison, WI, USA) and XPS (Thermo Scientific K-Alpha) using the methods followed in previous studies [16-18].

III. Results And Discussion

A. Characterization of Peptide-Conjugated Biomembranes

The activity studies with the highest peptide concentration membrane surface were performed. Therefore, peptides labeled with FITC were prepared at a pre-validated concentration (10 mM) and conjugated onto the biomembrane surface. Fig. 1. shows that peptides were successfully conjugated to the biomembrane surface.

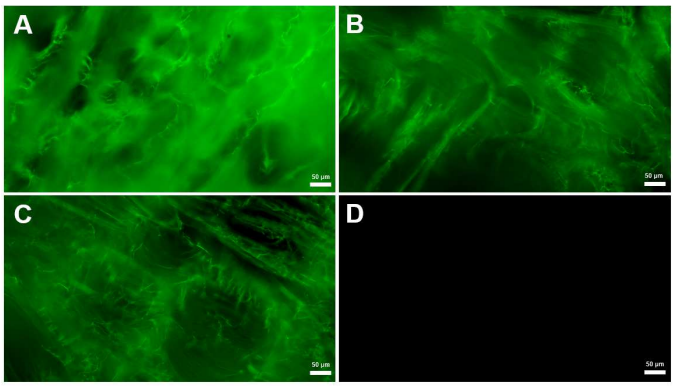

Fig. 1. Fluorescence microscopy images of FITC-labeled peptide-conjugated membranes (Scale bar = 50 µm). Membrane conjugated with 10 mM of A. PPGCYNTQALEQQ, B. PPGCYNTQALEDQQ, C. PPGCYNTQALEDNQ, D. Non-conjugated PCL control group membrane.

FTIR spectra of membranes was shown in Fig. 2. The band in the FTIR spectrum at 1700 cm^{-1} corresponds to the carbonyl (C = O stretch) group of the carboxylic acid. The intensity of this band increased after peptide conjugation and a shift was observed especially for the PPGCYNTQALEDQQ peptide. Also, C-N stretching due to the aliphatic amine group was observed at 1187 cm^{-1}, and bands of C-O stretching were observed at 1090 cm^{-1}. These values revealed that peptide conjugation onto the biomembrane was successful.

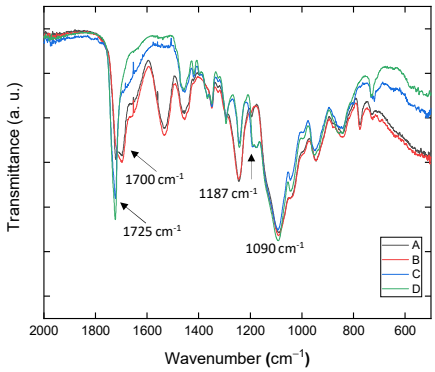

Fig. 2. FTIR spectra of PCL membranes A. Control group, B. PPGCYNTQALEQQ, C. PPGCYNTQALEDQQ, D. PPGCYNTQALEDNQ peptide-conjugated membranes

Fig. 3. shows the morphological structure and atomic composition of PCL membranes. All samples were washed with PBS for peptide conjugation. The salt crystals appearing on the surface are thought to originate from the washing process. The smooth PCL surface (Figure 3A) became rough with a thin layer of peptide material after peptide conjugation (Figure 3B-D). Also, the percentage of nitrogen in the atomic compositions of the membranes appears to increase after peptide conjugation, indicating that the conjugation was successful. The nitrogen composition of the membrane conjugated with PPGCYNTQALEDQQ and PPGCYNTQALEDNQ is higher than the membrane conjugated with PPGCYNTQALEQQ due to the presence of aspartic acid and asparagine.

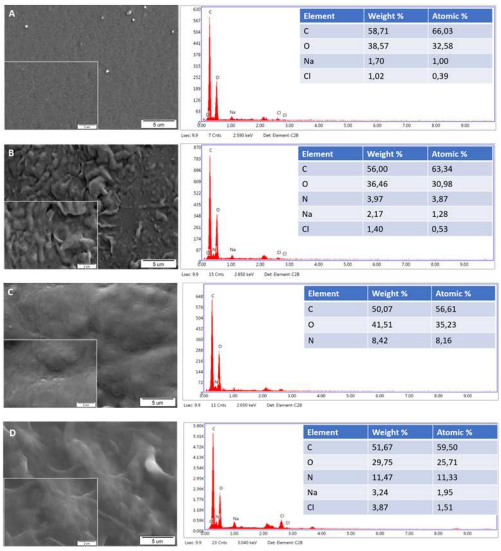

Fig. 3. SEM images and EDX results of PCL membranes. A. Control group, B. PPGCYNTQALEQQ, C. PPGCYNTQALEDQQ, D. PPGCYNTQALEDNQ conjugated membranes

C1s narrow region XPS spectra of PCL and peptide-conjugated PCL membranes are shown in Fig. 4. In the spectrum of PCL in Fig. 4A, the 3 peaks of carbon oxidation 284.6 eV, 286.2 eV and 288.6 eV correspond to C–H or C–C bonds, C–O bond, and C=O bond, respectively. After peptide conjugation (Fig. 4B–D), C–N, and N–C=O (amide) bonds caused shifting of the C–C/H, C–O, and C=O bands. In particular, the C=O band shifted to higher binding energy compared to PCL. This indicates that peptide conjugation, that is, nitrogen bonding, takes place over the carbonyl.

IV. Conclusion

This study aimed to develop an artificial corneal endothelium with biomimetic peptides and perform its characterization. Membrane structures to mimic corneal endothelium tissue were produced and biomimetic peptides were successfully conjugated onto membranes. The developed bioactive peptide-conjugated membranes may be good biomaterials to be used as scaffolds and they might provide an optimal microenvironment for corneal endothelial cells under in vitro conditions. Considering the increasing need for cornea, the number of waiting patients and the developments in the world in the artificial cornea, it is seen that the artificial corneal endothelium developed is of great importance.

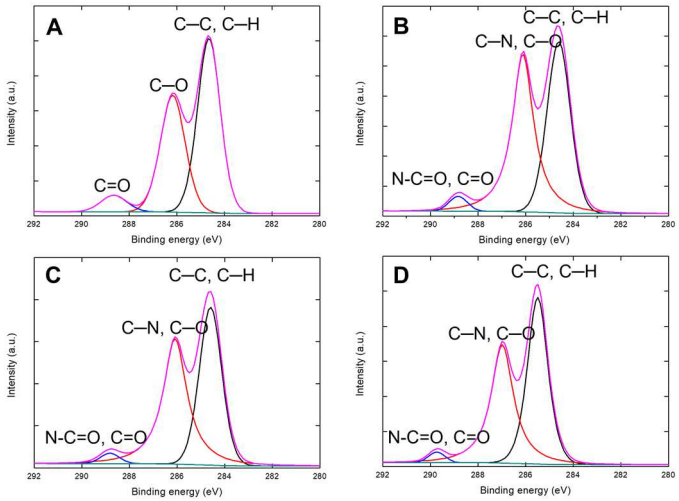

Fig. 4. C1s narrow region XPS spectra of PCL and peptide-conjugated PCL membranes A. PCL, B. PPGCYNTQALEQQ, C. PPGCYNTQALEDQQ, D. PPGCYNTQALEDNQ conjugated membranes

The present study will ensure a crucial direction for future studies on corneal endothelial tissue regeneration. Furthermore, it is planned to perform viability experiments with stem cells, and it is aimed to contribute to the literature at the point of closing a major deficiency in the field of ophthalmology with the developed bioactive peptide modified-artificial corneal endothelium tissue.

ACKNOWLEDGMENT

The authors thank to the Scientific and Technological Research Council of Turkey (TUBITAK) (1003 Project numbers: 218S991, 219S989, and 318S275).

REFERENCES

[1] S. Rodríguez-Fernández *et al.*, "Current development of alternative treatments for endothelial decompensation: Cell-based therapy," *Experimental Eye Research,* vol. 207, p. 108560, 2021.

[2] Z. Chen *et al.*, "Biomaterials for corneal bioengineering," *Biomedical Materials,* vol. 13, no. 3, p. 032002, 2018.

[3] A. Laule, M. K. Cable, C. E. Hoffman, and C. Hanna, "Endothelial cell population changes of human cornea during life," *Archives of ophthalmology,* vol. 96, no. 11, pp. 2031-2035, 1978.

[4] D. W. DelMonte and T. Kim, "Anatomy and physiology of the cornea," *Journal of Cataract & Refractive Surgery,* vol. 37, no. 3, pp. 588-598, 2011.

[5] N. Köse, "Biyomalzemeler ve İmplantlara Biyolojik Yanıt," *Türk Ortopedi ve Travmatoloji Birliği Derneği,* 2013.

[6] M. J. Webber, J. A. Kessler, and S. I. Stupp, "Emerging peptide nanomedicine to regenerate tissues and organs," *Journal of internal medicine,* Review vol. 267, no. 1, pp. 71-88, Jan 2010, doi: 10.1111/j.1365-2796.2009.02184.x.

[7] P. Vlieghe, V. Lisowski, J. Martinez, and M. Khrestchatisky, "Synthetic therapeutic peptides: science and market," *Drug discovery today,* vol. 15, no. 1, pp. 40-56, 2010.

[8] H. Acar *et al.*, "Self-assembling peptide-based building blocks in medical applications," *Advanced drug delivery reviews,* 2016.

[9] O. Karaman, A. Kumar, S. Moeinzadeh, X. He, T. Cui, and E. Jabbari, "Effect of surface modification of nanofibres with glutamic acid peptide on calcium phosphate nucleation and osteogenic differentiation of marrow stromal cells," *Journal of tissue engineering and regenerative medicine,* vol. 10, no. 2, 2016.

[10] G. Onak, O. Gökmen, Z. B. Yaralı, and O. Karaman, "Enhanced osteogenesis of human mesenchymal stem cells by self-assembled peptide hydrogel functionalized with glutamic acid templated peptides," *Journal of Tissue Engineering and Regenerative Medicine,* vol. 14, no. 9, pp. 1236-1249, 2020.

[11] K. Karaman, A. Kumar, X. He, and E. Jabbari, "Glutamic acid grafted nanofibers as a biomimetic template for mineralization and osteogenic differentiation of mesenchymal stem cells," in *JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE*, 2012, vol. 6: WILEY-BLACKWELL 111 RIVER ST, HOBOKEN 07030-5774, NJ USA, pp. 200-200.

[12] O. Karaman, A. Kumar, S. Moeinzadeh, X. He, T. Cui, and E. Jabbari, "Effect of surface modification of nanofibres with glutamic acid peptide on calcium phosphate nucleation and osteogenic differentiation of marrow stromal cells," *Journal of tissue engineering and regenerative medicine,* vol. 10, no. 2, pp. E132-E146, 2016.

[13] B. Ozcelik *et al.*, "Biodegradable and biocompatible poly (ethylene glycol)-based hydrogel films for the regeneration of corneal endothelium," *Advanced healthcare materials,* vol. 3, no. 9, pp. 1496-1507, 2014.

[14] Y. Deng *et al.*, "Peptide-decorated polyvinyl alcohol/hyaluronan nanofibers for human induced pluripotent stem cell culture," *Carbohydrate polymers,* vol. 101, pp. 36-39, 2014.

[15] O. Karaman, A. Kumar, S. Moeinzadeh, X. He, T. Cui, and E. Jabbari, "Effect of surface modification of nanofibres with glutamic acid peptide on calcium phosphate nucleation and osteogenic differentiation of marrow stromal cells," *Journal of tissue engineering and regenerative medicine,* Jul 30 2013, doi: 10.1002/term.1775.

[16] N. Horzum *et al.*, "Controlled surface mineralization of metal oxides on nanofibers," *RSC Advances,* vol. 5, no. 47, pp. 37340-37345, 2015.

[17] N. Horzum, M. M. Demir, M. Nairat, and T. Shahwan, "Chitosan fiber-supported zero-valent iron nanoparticles as a novel sorbent for sequestration of inorganic arsenic," *RSC Advances,* vol. 3, no. 21, pp. 7828-7837, 2013.

[18] X. Gao *et al.*, "Osteoinductive peptide-functionalized nanofibers with highly ordered structure as biomimetic scaffolds for bone tissue engineering," *International journal of nanomedicine,* vol. 10, p. 7109, 2015.