Construction and Evaluation of Collagen-Based Corneal Grafts Using Polycaprolactone To Improve Tension Stress

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ABSTRACT: The emergence of innovative surgical procedures using partial thickness corneal transplant has created a need for the development of corneal grafts to replace pathologic corneal tissue. Corneal repair materials have been successfully prepared in the past 10 years, but they were difficult to be used in clinics because of the unbearable tension caused by interrupted suture during routine surgery. However, polycaprolactone (PCL), a medical polymer material, can solve this problem. Therefore, a hierarchical collagen (Col)-based corneal graft with curvature, consisting of a transparent core part composed of collagen in the center and a mechanically robust fixed part containing collagen and polycaprolactone in the edge, was used as a potential corneal graft for corneal repair and regeneration in this study. The hierarchical collagen-based corneal grafts [collagen–polycaprolactone (Col–PCL) membranes] that are capable of mimicking the native cornea were developed based on chemical and thermal crosslinking mechanisms. The water adsorption of Col–PCL membranes could reach over 80% similar to that of human cornea, and its swelling could reach over 400%. More importantly, the formed Col–PCL membranes could resist a larger tensile strength (1.1 ± 0.03 MPa) before rupturing in comparison with pure collagen membranes and polycaprolactone membranes. Furthermore, the biodegradable Col–PCL membranes could facilitate cell adhesion and proliferation as well as cell migration (exhibiting epithelial wound coverage in <5 days), which showed promise as corneal grafts for cornea tissue engineering.

1. INTRODUCTION

Cornea is a delicate tissue that are crucial for normal vision. Different from many other tissues, cornea is an avascular, hierarchical, and transparent tissue on the surface of human eyes.1 Damage from mechanical, thermal, or chemical injuries and microbial infections frequently leads to the dysfunction of cornea, resulting in the appearance of grayish-white opacification in the transparent cornea, vision loss, and even blindness.1–4 At present, the donor corneal graft is a widely accepted therapeutic method because of its accessibility and immune privilege.1 However, donors are inadequate and difficult to match patients.5 The emergence of innovative surgical procedures using partial thickness corneal transplants has created a need for the development of corneal grafts to replace pathologic corneal tissue. This procedure has been called anterior lamellar keratoplasty (ALKP).6–8 Therefore, many researchers have attempted to develop corneal grafts using natural macromolecule materials to replace pathologic corneal tissue via ALKP.1,9 The corneal stroma, which accounts for approximately 90% of the cornea, mainly composes of aligned collagen (Col) I fibrils and quiescent keratocytes.10–12 Bionic corneal structures are the key to the development of successful corneal products. In addition, the cornea repair materials should possess good biocompatibility and nutrition permeability, appropriate optical performance and mechanical strength.13–15 Although many cornea repair materials have been successfully obtained in a variety of ways,1,9,14–19 the main problem is that they are difficult to apply in the clinical suture.

Collagen (Col), a natural biopolymer, is used widely as a biomaterial in medical areas, with biocompatibility, biodegradability, and biological activity,20–24 which makes Col possible to be excellent candidates for the tissue repair material. Meanwhile, it is also the main component of corneal stroma. However, the poor mechanical toughness and elasticity limited its clinical application in our previous work.16,17,25,26 Therefore, Col is often accompanied by other materials to counteract its poor mechanical properties. Another material which was successfully used in tissue repair and functional reconstruction, showing good results, was polycaprolactone (PCL).27–31 PCL is a hydrophobic and semicrystalline polymer, exhibiting more advantages, including versatile mechanical performance, slower
degradation rate, and lesser inflammation. These advantages have a potential significance on the biomedical applications of PCL, such as sutures and fixation devices. However, it still has its own limitations, such as high hydrophobicity, poor cell attachment, and proliferation. To solve these limitations, Ma’s team reported on biocomposites of both synthetic and natural polymers that can be identified and metabolized in the biological environment.

In previous work, collagen as a cornea repair material could hardly bear interrupted stitching during routine surgery, which limited its clinical application. In this study, our aim is to solve the problem of low mechanical strength and difficult suture in cornea transplant operation. Col–PCL membranes were prepared using chemical crosslinking and thermal crosslinking methods during membrane formation, consisting of a transparent core part composed of collagen in the center and a mechanically robust fixed part containing collagen and PCL in the edge. The fixed part is convenient for suturing during operation. After the preparation of Col–PCL membranes, the physical properties of the membranes were characterized in optical transparency, water content, and tensile strength. The in vitro cytocompatibility of the membranes to human corneal epithelial cells (HCECs) was evaluated. Finally, the in vitro ability of re-epithelialization has been examined using an ex vivo corneal graft culture model.

2. RESULTS AND DISCUSSION

2.1. Macroscopic and Microscopic Morphology Characteristics. Because of the special structure of corneal stroma, designing corneal structure is the key to the development of a successful corneal product to repair a corneal anterior lamellar defect. In this study, a rehydrated Col–PCL composite membrane with curvature and transmittance was successfully prepared (Figure 1) and is shown in Figure 2A. It consisted of a transparent core part composed of collagen in the center and a mechanically robust fixed part containing Col and PCL in the edge. The core part provided optical performance for vision, and the fixed part provided interrupted suture during surgery. Figure 2E shows the surface
of a dry Col–PCL membrane, which presented a lower and smoother core part composed of Col and a higher and rough fixed part composed of Col–PCL, but the core part and fixed part had the same plane after absorbing water. A lamellar structure similar to native cornea was discovered in Figure 2B,C,F,G. The Col–PCL membrane (Figure 2C,G) had a small amount of reconstructive PCL that showed a dense structure compared to the Col membrane (Figure 2B,F). At 120°, liquid PCL infiltrated into the collagen membrane. The PCL infiltrating into collagen provides mechanical properties for the surgical suture, which is mainly due to the mechanical properties of PCL itself. After returning to room temperature, PCL existed in the form of a reconstructive solid state. Group PCL exposed a dense structure (Figure 2D,H), which was not conducive to air permeability and nutrient transport. Interestingly, the dense structure was similar to where indicated by the red arrow in Figure 2C,G. Although a dense structure similar to PCL appears, it was small and did not affect air permeability and nutrient transport of the material itself. Therefore, a small amount of the reconstructive PCL may be more suitable for this study.

2.2. Physical Characterization. Transparency is critical to the functionality of corneal grafts and is related to the collagen organization, which contributes to the transparency with its water-retaining proteoglycans.41,42 Clinically, the degree of corneal injury is different for each patient, so corneal grafts should be of different thickness and size for patients to choose. All dry membranes in this study were about 40 ± 5 μm. Col membranes as a positive control represented the core part of the Col–PCL membrane, which had higher transparency values than the fixed part of Col–PCL membranes (Figure 3A). The fixed part of dry Col–PCL membranes with 40 μm thickness was 60% transparent at 780 nm and the fixed part of wet Col–PCL membranes with 400 μm thickness was only
42% transparent at 780 nm, because their internal structure was damaged after swelling. Therefore, it is impossible to achieve minimal functional transparency at a thickness of human corneal thickness with these grafts. Importantly, it only provides surgical suture, not vision. However, the core part of Col–PCL membranes was the Col with 40 μm thickness, which was 90% transparent at 780 nm in a dry environment. Within the same 400 μm thickness, wet Col membranes were 78% transparent at 780 nm. The transparency of Col membranes in the visible range was lower than that of native cornea (87%).

The cornea is a water-bearing tissue with moisture retention capacity. Before surgery, the corneal grafts will be rehydrated until it reaches saturation. After rehydration for 60 min, the quality of PCL membranes remained constant because of its hydrophobicity (Figure 3B); the saturation curves of Col and Col–PCL membranes had a common feature that was oversaturated (10–30 min) before reaching stable saturation (30–60 min). Therefore, the corneal grafts only need to be rehydrated 30 min before surgery. Moreover, quality of Col membranes after rehydration was higher than Col–PCL membranes, which was mainly due to the higher content of Col in Col membranes.

The water adsorption and swelling properties play an important role in maintaining the structural stability of corneal grafts, which is in favor of cell migration, adhesion, and proliferation. To further evaluate the physical properties of Col, Col–PCL, and PCL membranes, water adsorption and swelling were characterized because they can judge the hydrophilic properties of materials. After 24 h of incubation in NS, the water adsorption of Col and Col–PCL membranes could reach over 80%, while those of PCL membranes were only less than 20% (Figure 3C) and there were significant differences between them, but there was no significant difference between Col and Col–PCL. Therefore, Col and Col–PCL membranes demonstrated similar water adsorption to human cornea (78–82%).

Furthermore, the swelling of Col, Col–PCL, and PCL membranes was measured under the same conditions. The swelling of Col and Col–PCL membranes could reach over 400%, while those of PCL membranes were only less than 30% (Figure 3D). No significant difference was observed in Col and Col–PCL, but there were significant differences between them and PCL. Therefore, Col–PCL membranes with excellent moisture retention capacity can maintain water balance and provide a water environment for cell adhesion and proliferation.

Besides the above-mentioned transparency and swelling properties, the mechanical properties of corneal grafts also play an important role in surgical suture. Therefore, the mechanical properties were characterized using a method of the simulated tension of suture during surgery. The native corneas usually underwent environmental shear or tensile stress resulting from the blink and the intraocular pressures.

Furthermore, surgical implantation of a corneal graft requires tensile strength to support suturelike native tissue. Therefore, the tensile properties of Col–PCL membranes were tested and compared with Col and PCL. An engineered suture pull-out test (Figure 3F) found that Col–PCL membranes could withstand a larger tensile strength (1.1 ± 0.03 MPa) than Col and PCL membranes before rupturing and the differences were significant (Figure 3E). The results showed that the mechanical properties of Col membranes were enhanced by the melted PCL, and the tensile strength of Col–PCL membranes was close to the sum of Col and PCL. Meanwhile, the results suggested that they have the potential to bear routine interrupted suture in clinics.

**2.3. In Vitro Degradation.** Controllable corneal graft degradation kinetics are also very crucial for the regeneration of cornea. It would be beneficial if the implanted corneal graft is degraded and replaced by normal cornea tissue. Col is usually degraded by collagenase, which is present in tear films. To investigate the degradation time of the prepared corneal grafts in vitro, the membranes were incubated in phosphate-buffered saline (PBS) containing 0.017 ng/mg of collagenase type I at 37 °C. Figure 4 depicts the degradation profiles of all membranes (Col, Col–PCL, and PCL) with different incubation times. After continuous incubation for 8 h, the Col membrane lost nearly half of its mass and degraded the most. This may be because the reconstructive PCL prevents the rapid degradation of Col. After 24 h, the mass of Col membranes remained 20.45%, Col–PCL membranes remained 13.86%, and PCL membranes did not change. Col–PCL membranes stabilized in 24 h, and the quality remained unchanged in the following time. Moreover, Col membranes could maintain degradation for 48 h; Col–PCL membranes could maintain degradation for 24 h; and PCL membranes could not degrade. Therefore, the enzymatic degradation of the membranes suggests that PCL with slow degradation is feasible as a fixing part and that may be used for biomedical applications.

**2.4. Cell Proliferation and Adhesion.** Cell proliferation and adhesion are essential for corneal grafts; thus, cell proliferation and adhesion assay were carried out on the as-prepared membranes. A cell counting kit-8 (CCK-8) kit was used to quantitatively estimate the proliferation and adhesion of HCECs as shown in Figure 5A,B. In cell proliferation experiments, the proliferation rate of cells on the three membranes was close to each other after 5 days of culture and there were no significant differences. At day 3, the proliferation rate of cells on PCL membranes was the lowest compared to that of Col and Col–PCL membranes due to the low adhesion rate of PCL. However, the cell proliferation rates of all membranes were lower than that of the control group, because some cells floated on the cell culture plate when planting cells. Moreover, optical density (OD) values increased with the incubation time. To evaluate the cell adhesion of
Col−PCL membranes, the OD values of HCECs remaining on the membrane after washing with PBS are plotted in Figure 5B. After incubation for 1 day, the cell adhesion on the three membranes was different and there were no significant differences between them. However, there was a significant difference between the control group and PCL membranes. The results indicated that PCL membranes have relatively poor cell adhesion, which is consistent with the results of cell proliferation. To further evaluate cell adhesion of the three membranes, the cell morphology adhered to the membranes was obtained by SEM (Figure 5C). Compared with PCL membranes, HCECs could spread well with a fibroblastic morphology. All of these results were consistent with the quantification of HCECs to estimate their proliferation and adhesion and illustrated the potential use of Col−PCL membranes for tissue engineering applications.

2.5. Live/Dead Cell Viability Assay. Studying the impact of the membrane on HCEC viability is of great interest to biocompatibility of membranes. To perform the assay, HCECs were used to evaluate cell viability on the membranes by means of calcein acetoxymethyl (calcein-AM)/propidium iodide (PI) double staining performed at 5 days (Figure 6). HCECs adhering to the membranes were homogenously distributed and retained high cell viability over 5 days postculture (Figure 6A). In addition, minimal cell death was shown in all groups. At the same magnification, the cell adhesion area on the PCL

![Figure 5](image5.png)

**Figure 5.** Cell proliferation and adhesion assay of Col, Col−PCL, and PCL membranes. (A) Cell proliferation of Col, Col−PCL, and PCL membranes for day 1, 3, and 5. (B) Number of HCECs adhering to membranes for 1 day. (C) SEM images from HCECs adhering to membranes. Data are means ± SD, n = 3, ***p < 0.001, **p < 0.01.

![Figure 6](image6.png)

**Figure 6.** Viability of HCECs on the membranes. (A) Live/dead assay of HCECs stained by calcein-AM/PI. (B) Quantified cell viability using ImageJ software.
membrane was significantly smaller than that on Col and Col–PCL membranes. These results were consistent with the cell adhesion experiments in Figure 5B,C. Cell viability in different groups was quantified using ImageJ. All membranes showed nearly 100% cell viability, and there were no significant differences.

2.6. Ex Vivo Cell Migration. Epithelial cells maintain a balance between limbal stem function, tear quantity and quality, eyelid anatomy and function, and corneal sensitivity. Moreover, Col–PCL membranes could facilitate HCEC adhesion, proliferation, and migration in vitro. Therefore, the prepared Col–PCL membranes dramatically increase the choice of artificial corneal grafts for the regeneration of cornea.

4. MATERIALS AND METHODS

4.1. Materials. Collagen extracted from bovine tendon was supplied by Pudaot Liansin Biotech, Co., Ltd. (Guangzhou, China). PCL (Mw 40 kDa) was purchased from Haishan Technology Co., Ltd. (Wuhan, China). The CCK-8 kit was purchased from Dojindo Laboratories (Japan). Fetal bovine serum (FBS), phosphate-buffered saline (PBS, pH = 7.4), and Dulbecco’s modified Eagle’s medium (DMEM)-basic (1×) was provided from Gibco. N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (≥98.0%) (EDC) and N-hydroxysuccinimide (98.0%) (NHS) was purchased from Sigma-Aldrich (Shanghai, China). Collagenase I from clostridium histolyticum was supplied by QiYun Biotechnology Co., Ltd. (Guangzhou, China). Sodium fluorescein ophthalmic test papers were purchased from Tianjin Jingming New Technology Development Co., Ltd. (China, Tianjin). All other chemical reagents were of analytical grade and obtained from commercial sources.

4.2. Preparation of Col and Col–PCL Membranes. Col membranes were prepared as described in a previously published protocol. The crosslinking agents EDC and NHS were added to 0.1 M HCl with a mass ratio of Col/(EDC/NHS) = 6:1 and stirred slowly at 4 °C for 8 h, in which the molar ratio of EDC to NHS was 4 to 1. After fully crosslinking, the homogeneous mixture was returned to room temperature and centrifuged to remove bubbles, following which the resulting mixture was poured into a disposable culture dish and air-dried in a clean bench. After washing, the air-dried membranes were put into a vacuum drying oven for thermal crosslinking. In this study, EDC/NHS is used to activate the carbonyl group when an amide bond is formed and generate synthetic crosslinks that form a bridge between amino acids without affecting any chemistry to the collagen molecule. The preparation process of Col–PCL membranes is shown in Figure 1. Briefly, Col from fresh bovine tendon was dissolved in 0.01 M HCl solution at 4 °C, and the final concentration of Col was determined to be 6.5 mg/mL. The formed Col solution was then crosslinked by EDC and NHS, in which the molar ratio for the EDC to NHS was 4:1, and the Col to EDC/NHS mass ratio was 6:1. The EDC-mediated coupling reaction proceeded at 4 °C with stirring for 8 h. After the reaction was complete, the resulting mixture was placed at room temperature for 4 h and then centrifuged to remove bubbles. Immediately, the precursor solution was dispensed into a disposable culture dish and air-dried in a clean bench. PCL rings (outer diameter, 10 mm; inner diameter, 5 mm; thickness, 30–50 μm) were obtained by dissolving with dichloromethane, air drying, and tailoring. The obtained PCL membrane was placed into a part of the precursor solution until Col–PCL membranes were formed by air drying. The obtained Col and Col–PCL membranes were rinsed with deionized water five times to remove salt from the membrane. After the membranes were air-dried again, they were then put into a specific mold and hot-pressed in a vacuum drying oven for 24
h at 120 °C. The obtained Col and Col–PCL membranes were stored in a desiccator for further characterization.

4.3. Macroscopic and Microscopic Morphology of the Membranes. The macroscopic morphology of the membranes was obtained by a digital camera, and the hierarchical structure was imaged using field-emission scanning electron microscopy (Philips Electronics N.V. Holland, SEM). Prior to the observation, the prepared membrane was quickly fractured in liquid nitrogen to observe the sectional morphology; the formed cross-section was then sputter-coated with platinum for observation.

4.4. Physical Characterization. Following immersion in normal saline (NS) for 2.5 h, transparency of the dry and wet membranes was determined by an ultraviolet–visible spectrophotometer (UNICO, China) at room temperature with the spectral range from 380 to 780 nm.

Water saturation was determined by the weight of the membranes and absorbed NS at different time points. Considering the small mass of the membranes, three homologous membranes were set to a sample and each batch was performed in triplicate. The equilibrated water content of the membranes was measured by the following steps. First, the membranes were weighed \( W_0 \) and immersed in NS for 24 h at 37 °C. Subsequently, the membranes were taken out from the NS and the superficial NS was removed with filter papers and then weighed \( W \). The swelling ratio and water adsorption of the membranes were determined using the following equations

\[
\text{swelling ratio} \ (\%) = \frac{W - W_0}{W_0} \times 100\%
\]

\[
\text{water adsorption} \ (\%) = \frac{W - W_0}{W} \times 100\%
\]

Tensile testing was performed on materials using DMA (Instron Corporation, Issaquah, WA) with a load velocity of 0.05 mm/s. In this study, the suture tension during surgery was measured by a microplate reader (Thermo 3001, America).

4.5. In Vitro Degradation Experiment. Considering the weight of the membranes, two membranes with a diameter of 10 mm were defined as a sample. After samples \( W_0 \) and 70 \( \mu \)m cell filters \( W_1 \) were weighed respectively, the samples were put into the cell filters, and then the cell filters with samples were placed in collagenase type I solution \( 0.017 \text{ ng/mL} \). The cell filters with samples were removed from collagenase type I solution every 8 h, dried with filter paper, and weighed \( W_2 \). The residual quality of samples in collagenase type I solution was calculated by the following equations

\[
\text{membrane quality} = W_2 - W_1 - W_0
\]

4.6. Cell Proliferation and Adhesion Assay. Cell proliferation onto the membranes was assessed by the CCK-8 kit. Briefly, the samples sterilized by UV were put into 10 mm were de

48-well plates with DMEM-basic (1 × 10^6) and then the wells were rinsed with PBS thrice after adding 100 \( \mu \)L of staining solution, cell-seeded membranes were incubated for 15 min at 37 °C, in the dark. After PBS washing, live (green stain) and dead (red stain) cells were imaged using a confocal laser scanning microscope (Leica TCS SP8). Finally, cell viability was quantified by the proportion of live cells to total cells, using ImageJ software.

4.7. Determination of Cell Viability. The calcein acetoxyethyl (calcein-AM)/(propidium iodide) PI double stain kit (Dojindo Laboratories, Japan) was employed to determine the viability of HCECs on the membranes for 5 days.

4.8. Ex Vivo Cell Migration. All experimental procedures followed the Association for Research in Vision and Ophthalmology (ARVO) Statement that permits the Use of Animals in Ophthalmic and Vision Research, as well as the local ethical rules. Rabbit eyes were obtained from New Zealand white rabbits of euthanasia. Corneal grafts (Col–PCL membranes) of 7.25 mm diameter were surgically implanted into an isolated corneal anterior lamellar defect model (anterior wound diameter 7 mm). The samples were cultured in complete medium for 5 days. Re-epithelialization was tracked using sodium fluorescein ophthalmic test paper.

4.9. Statistical Analysis. Samples of at least \( n = 3 \) were used for data analysis. All data were presented as means ± standard deviations and statistical difference (p value) and analyzed using one-way analysis of variance. \( p < 0.05 \) was defined as statistically significant and marked with an asterisk.
or interpretation of data. X.S., W.S., and L.R. designed the study. X.Y. contributed to the preparation of materials. All authors have substantially contributed to the study and have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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