T-style keratoprosthesis based on surface-modified poly(2-hydroxyethyl methacrylate) hydrogel for cornea repairs

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Abstract

Corneal disease is a common cause of blindness, and keratoplasty is considered an effective treatment method. However, there is a severe shortage of donor corneas worldwide. This paper presents a novel T-style design of a keratoprosthesis and its preparation methods, in which a mechanically and structurally effective artificial cornea is made based on a poly(2-hydroxyethyl methacrylate) hydrogel. The porous skirt was modified with hyaluronic acid and cationized gelatin, and the bottom of the optical column was coated with poly(ethylene glycol). The physical properties of the T-style Kpro were analyzed using ultraviolet and visible spectrophotometry and electron scanning microscopy. The surface chemical properties were characterized using Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy. The surface modification in the spongy skirt promoted cell adhesion and prevented retroprosthetic membrane formation. Through improved surgical techniques, the novel T-style keratoprosthesis provides enough mechanical stability to facilitate long-term biointegration with the host environment. In vivo implantation experiments showed that the T-style keratoprosthesis is a promising cornea alternative for patients with severe limbal stem cell deficiency and corneal opacity.

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1. Introduction

Corneal disease is a leading cause of blindness, affecting tens of millions of people worldwide. Corneal transplantation using human donor tissues is the standard procedure to treat corneal diseases and restore sight. Because of the insufficient supply of donor tissues, especially in developing countries, and the high risk for graft failure in patients with severe limbal stem cell deficiency, some promising cornea alternatives, such as artificial corneas or a keratoprosthesis (Kpro), have been developed in recent decades [1–5].

Although hard materials such as glass, ceramics, plastics and alloys were initially used to prepare artificial corneas [2,6], soft and wet materials (such as hydrogels or hydrogel-based composites) have been popular in recent years because of their good biocompatibility and functionality [7–14]. Artificial corneas made of hydrogels are closer to the ideal artificial cornea than those made with traditional materials; ideal artificial corneas have the following advantages: sufficient strength for suturing, optical transparency, capability of robust biointegration with surrounding ocular tissue, permeability to nutrients and sufficient support for surface epithelialization [7,15]. Among the several kinds of artificial corneas made with hydrogels, the most successful is the AlphaCor Kpro, which has undergone extensive clinical investigation with human patients since 1998 [16–18]. In the AlphaCor device, both the optic column and the porous skirt are composed of poly(2-hydroxyethyl methacrylate) (PHEMA) hydrogels, which have good biocompatibility in the cornea [9]. The optic column is optically clear and allows light into the eye. The porous skirt remains enclosed within the corneal stroma, thus allowing invasion by corneal stromal cells [19]. The PHEMA hydrogel offers high water content, which plays an important role in ophthalmic applications. The hydrogel’s high water content increases the permeability of nutrients such as small proteins and saccharides, which are particularly important to promote the regrowth of the outermost corneal cell layer [20–22], and prevents some molecular events (e.g. calcification), which lead to the implantation failure of corneal prosthetics [23]. However, hydrogel’s high water content, which is based on a large number of micropores in PHEMA sponges,
leads to inadequate sewing performance because PHEMA sponges possess inherently poor mechanical strength. The insufficient surgical sutures decrease the overall mechanical stability and induce corneal stromal melting and AlphaCor Kpro extrusion.

Mechanical strength and the overall mechanical stability can be strengthened by increasing the degree of polymerization and adjusting the kind and quantity of crosslinking agents [24]. However, it can decrease the water content in the artificial cornea. To obtain sufficient mechanical stability based on the biointegration of artificial corneas with high water content, it is necessary to improve the artificial cornea design, the skirt's affinity and the surgical techniques. Therefore, we designed a T-style artificial cornea in which the outstanding optical column could be used to position and fix the artificial cornea in the corneal pocket. In addition, the porous skirt was clamped between two cornea lamellas with the sandwich model. This design decreases the seam strength and increases the overall mechanical stability. The T-style artificial cornea follows a core–skirt model in which an optically transparent column permits vision while a porous skirt allows tissue integration by cell and tissue ingrowths. The T-style artificial cornea could be designed to resist adverse events including core–skirt separation, extrusion and corneal stromal melting. The T-style Kpro is prepared using a polymerization of 2-hydroxyethyl methacrylate monomers based on our designed mold; the resulting PHEMA Kpro is easily modified with different functional groups [25,26]. In this paper, we describe improvements to the cellular affinity of artificial cornea made by coating the skirt surface with biocompatible, cytophilic and hydrophilic natural polymers. Hyaluronic acid (HA) and cationized gelatin (CG) were chosen for their polymer electrolyte characteristics, including opposite charges to form a surface coating through a layer–by–layer (LBL) self-assembly method [27,28]. For clinical investigations based on AlphaCor Kpros, another factor affecting long-term vision restoration is retroprosthetic membrane formation, which results from the cellular attachment on the optical column of AlphaCor Kpros [29]. We attempt to modify the posterior surface of the optic column with a poly(ethylene glycol) (PEG) layer, which could be used to inhibit cellular attachment and proliferation [30–33], thus preventing the retroprosthetic membrane formation that can lead to optical column opacification and visual loss. The novel T-style design and the multifunctional surface modifications of this artificial cornea would overcome the shortcomings of AlphaCor Kpro to improve the overall mechanical stability and long-term biointegration with the host environment.

The novel T-style design of an artificial cornea and its preparation methods, in which a mechanically and structurally effective artificial cornea is made with PHEMA hydrogel. The physical properties of the T-style Kpro, including the optic column's light transmittance and the spongy skirt microstructure, were analyzed by infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS). The biocompatibility, cell attachment, mechanical stability and long-term biointegration with the host environment of the T-style arti-

cial cornea were also investigated using in vitro and in vivo experiments.

2. Materials and methods

2.1. Materials

This process used 2-hydroxyethyl methacrylate (HEMA) (J & K Chemical Ltd.); poly(ethylene glycol) diacrylate (PEG-DA, Mw 700), N, N'-methylenebis (acrylamide) (BIS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC), Dulbecco's Modified Eagle Media (DMEM/F12) media, fetal bovine serum (FBS), 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (D2959), l-alanine, gelatin (Type A, 300 bloom, derived from acid-cured porcine skin), 2,4,6-trinitrobenzenesulfonic acid and methylthiazol tetrazolium (MTT) assay (all from Sigma-Aldrich Chemical Co.); hyaluronic acid (HA, MW 1.40 MDa), dimethyl sulfoxide (DMSO), 2, 2’-azobis(2-methylpropionitrile) (AIBN) and sodium chloride (NaCl) (all from Shanghai Aladdin Industrial Inc.); ethylenediamine (SinoPharm Chemical Reagent Co.); and γ-aminopropyltriethoxysilane (KH550) (Shanghai Yaohua Chemical Corporation, China). The NaCl was screened and collected in 80–100 μm sizes. The drugs included penicillin/streptomycin (North China Pharmaceutical Group Corporation); ketamine hydrochloride (Jiangsu Hengrui Medicine Co., Ltd.); xylazine (Shanghai Sangon Biological Engineering Ltd.); erythromycin eye ointment (Shanghai General Pharmaceutical Co., Ltd.); tobramycin/dexamethsone eye drops (S.A. Alcon-Couvreur N.V.); 0.15% tetracycline eye ointment (Guzhzhou Baiyunshan Pharmaceutical Group Corporation); and 1% tetracaine hydrochloride eye drops (EENT Hospital).

2.2. Synthesis of cationized gelatin

The preparation of CG was carried out as described in previous reports [27,34]. Ethylenediamine (4.0 g) and EDAC (2.0 g) were added to 100 ml of a phosphate-buffered solution (PBS) (pH = 7.4) containing 2.0 g of gelatin, and the pH of the solution was adjusted to 5.0 by adding 5 M of HCl. The mixture was agitated at 37 °C for 24 h and dialyzed against double-distilled water (DDW) for 48 h at 4 °C. The dialyzed solution was freeze-dried (−40 °C) for 24 h to obtain the CG. The conventional trinitrobenzene sulfonate (TNBS) method was used to determine the number of amino groups introduced into the gelatin [35].

2.3. Preparation of the artificial cornea

The HEMA prepolymer was synthesized through the incomplete polymerization of HEMA monomers. First, 20 g HEMA, 20 g ethanol and 0.01 g AIBN were added to a three-necked flask using a condenser pipe and mechanical agitation. The bottle was heated to reflux under N₂ protection for over 30 min. The solution's viscosity gradually increased, and the reaction was stopped when numerous bubbles appeared in the viscous solution.

Next, 4.5 g NaCl and 1.0 g honey were added to a 5 ml bottle, and the mixture was stirred. Then, 0.5 g HEMA prepolymer, 0.01 g BIS and 0.005 g AIBN were dissolved in 1 ml ethanol. The prepolymer solution was then added to the bottle with the NaCl and honey, and the mixture was stirred.

The Kpro device polymerization and molding processes were carried out sequentially in the same mold. The cylindrical mold, designed by us and shown in Fig. 1, was made of Teflon and consisted of an annular head (A) and base (B). To produce the spongy rim, the mixture including NaCl, honey and the HEMA prepolymer with BIS and AIBN was added into an empty space (D) created by assembling and clamping together the head (A) and base (B) (Fig. 1a). During this process, slightly more mixture than the space could hold was added. The plunger (C) was then inserted slowly through the central hole of the head (A) and pushed carefully until it touched the base (B). During this operation, the excess mixture was forced out through the edge of the head (A) and the base (B) to the empty space (E). The clamped system was maintained at 50 °C for 6 h until the spongy annular rim was completed.

In the second stage, the plunger (C) was removed, the empty cylindrical space (G) (Fig. 1b) was cleared of debris, and the HEMA monomer solution with BIS and AIBN was added to this space to produce the central column. At this time, the space (D) was full of the spongy polymer produced in the first stage. A plunger (F) of a specific length was slowly inserted until its shoulder stopped at the top surface of the head (A). The excess liquid was forced out through the channel of the plunger (F) and head (A). The system was immediately placed in an oven and maintained at 50 °C for 15 h.

At the end of the second stage, the mold was opened and the resulting polymeric device, which consisted of a circular transparent region surrounded by a white spongy ring, was removed. The polymeric
Then dipped into 0.1% (w/w) CG aqueous solution for 1 min and rinsed with pure water. The samples were treated with 1% (w/w) HCl aqueous solution for 20 min at 25 °C, placed into 0.1% (w/w) HA aqueous solution for 20 min at 37 °C for 24 h. The LBL technique was used to modify the surface of PHEMA skirt[27]. The samples used to determine the equilibrium water content (EWC) were prepared using the same mixture containing NaCl, honey and the HEMA prepolymer with BIS and AIBN. Following polymerization, the sponge specimens were kept at equilibrium in deionized water for 2 weeks with daily water changes. Prior to weighing, the hydrated samples were gently blotted with tissue paper. The specimens were then dried under a vacuum at 37 °C for 48 h. The EWC in weight percentage was calculated using the following equation: EWC (%) = 100% × (Ww − Wd)/Ww, where Ww and Wd are the weight of a fully swollen specimen and of the same specimen after vacuum drying, respectively. The results were the average values of four measurements for each sponge.

2.4. Surface modification of the spongy skirt

2.4.1. Surface modification of the spongy skirt

The PHEMA spongy skirt was treated with plasma (HPD-280, Suman Co. Ltd., Nanjing, China) for 60 s at 100 V, dipped into a 0.1% (w/w) KH550 ethanol solution for 1 min, and dried at 110 °C for 2 h. The sample was washed with a 75% alcohol aqueous solution and then dried at 37 °C for 24 h. The LBL technique was used to modify the surface of the PHEMA skirt[27]. The samples were treated with 1% (w/w) HCl aqueous solution for 20 min at 25 °C, placed into 0.1% (w/w) HA aqueous solution for 1 min and rinsed with pure water. The samples were then dipped into 0.1% (w/w) CG aqueous solution for 1 min and rinsed again. Next, 10 bilayers of HA and CG were prepared by repeating the deposition process described above 10 times. The samples were dried at 37 °C for 48 h.

2.4.2. Surface modification of the optic column

The 10% PEG-DA (Mw 700) aqueous solution was used to modify the PHEMA central column of the artificial cornea. The initiator (D2959, 1% (w/w) to PEG-DA) was also added to the precursor solution. The bottom of the optic column was submerged into the above PEG-DA precursor solution, and N2 gas was used to remove the excess liquid. The sample was maintained in UV light (365 nm, SB-100P/F, Spectronics Corporation, USA) for 5 min to polymerize the PEG layer (the reaction product of PEG-DA). The sample was placed in distilled water, where it was stored for at least 1 week with daily water changes.

2.5. Characterization of the artificial cornea

2.5.1. Equilibrium water content

The samples used to determine the equilibrium water content (EWC) were prepared using the same mixture containing NaCl, honey and the HEMA prepolymer with BIS and AIBN. Following polymerization, the sponge specimens were kept at equilibrium in deionized water for 2 weeks with daily water changes. Prior to weighing, the hydrated samples were gently blotted with tissue paper. The specimens were then dried under a vacuum at 37 °C for 48 h. The EWC in weight percentage was calculated using the following equation: EWC (%) = 100% × (Ww − Wd)/Ww, where Ww and Wd are the weight of a fully swollen specimen and of the same specimen after vacuum drying, respectively. The results were the average values of four measurements for each sponge.

2.5.2. Scanning electron microscope (SEM) images

A scanning electron microscope (SEM) (TESCAN VEGA 3, Brno, Czech Republic) was used to observe the surface characteristics of the Kpro optic column and the pore structure of the spongy skirt. In this process, 2-mm-thick slices were excised along the diameter. The specimens were dehydrated with increasing concentration gradients of ethanol (from 35% to 100%). The collapse of pores in the spongy material should be avoided in the treatment course. After drying, the specimens were coated with a 10 nm layer of gold in a vacuum evaporator, and the images were then taken using SEM.

2.5.3. Fourier transform infrared spectrometer spectra

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was used to characterize the surface chemical properties of the PHEMA film, the PEG film, the PHEMA film modified with PEG, and the PHEMA film modified with the LBL course. The spectra were acquired on a Thermo Scientific Nicolet 6700 spectrometer using a diamond ATR crystal and a MCT/A detector over a range of 650–4000 cm⁻¹ at a resolution of 4 cm⁻¹ averaged over 64 scans. For ATR-FTIR measurement, the PHEMA, PEG, PHEMA–PEG and PHEMA–LBL films were prepared using polymerization and modification courses. The samples were kept in deionized water for 2 weeks with daily water changes and then dried under a vacuum for 48 h. The KBr disc method was only used for the gelatin and the cationized gelatin, which was obtained through a freeze-drying course.

2.5.4. X-ray photoelectron spectroscopy

The X-ray photoelectron spectroscopy (XPS) experiments were carried out in a RBD-upgraded PHI-5000C ESCA system (Perkin Elmer) with MgKα radiation (hν = 1253.6 eV). In general, the X-ray anode was run at 250 W and the voltage was kept at 14.0 kV with a detection angle of 54°. The pass energy was fixed at 23.5, 46.9 or 93.9 eV to ensure sufficient resolution and sensitivity. The base pressure of the analyzer chamber was approximately 5 × 10⁻⁸ Pa. The samples used for XPS...
were the same as those used for ATR-FTIR and were washed with anhydrous ethanol and dried under a vacuum for 48 h.

2.5.5. Light transmission

The transparency of the PHEMA optic column in the artificial cornea was examined before and after PEG modification by scanning within a range of wavelengths (250–800 nm) with a UV–vis spectrophotometer (Biomate 3S, Thermo Scientific).

2.6. Cell adhesion

Corneal stromal cells were isolated from New Zealand white rabbits as described in Alaminos [36]. We studied cell adhesion on the surfaces of the optic column and the spongy skirt in the artificial cornea. To simplify the sample preparation, we prepared the PHEMA flat films and modified their surface properties to study cell adhesion instead of the optic column and the spongy skirt in the artificial cornea. Three samples were prepared: (1) PHEMA flat film; (2) PHEMA flat film modified with PEG; and (3) PHEMA flat film modified with HA and CG using an LBL course. Cell adhesion behaviors on these different surfaces were investigated. To compare the cell adhesion results, we also set tissue culture polystyrene (TCP) as a control. The samples were sterilized in 75% ethanol for 10 min and then washed in sterile PBS six times for 30 min at a time. Before the cells were cultured, the sample was fixed into a 12-well plate with polytetrafluoroethylene (PTFE) rings. Only a 1.5 cm² area was used to culture cells and study cell adhesion. The cells were harvested and seeded onto the sample surface at the same density of 3500 cells/cm². The cells were cultured in an incubator at 37 °C and 5% CO₂ for 48 h. After that, the media were removed and the sample surface was washed with PBS. A MicroScopics inverted microscope (Leica DM 3000B) with a charge-coupled device (CCD) (Leica DFC 500) was used to view and capture the cultured cells.

Cell viability and proliferation were assessed with an MTT assay [27]. The optical density (OD) value of absorbance was measured with a Synergy H1 Microplate Reader (BioTek, America); the obtained value was 490 nm. Differences in the OD value between the experimental and control groups were analyzed statistically using a paired sample t-test. The statistical significance level was set at 0.05.

2.7. Implantation of the artificial cornea

The rabbits used in the study came from the Experimental Animal Centre (Chinese Academy of Sciences, Shanghai, China). All the experimental protocols and animal care complied with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and with the guidelines of the Animal Care and Use Committee of Fudan University (Shanghai, China). In this study, 10 mature male New Zealand rabbits (mean weight 1.5 kg) were used. A corneal alkali burn model was established, followed by the implantation of the artificial corneas.

2.7.1. Rabbit corneal alkali burn model

The alkali injury course used was similar to the procedure established by Hackett [37]. The rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (40 mg/kg body weight) and xylazine (4 mg/kg body weight). In addition, they were given local anesthetic (tetracaine hydrochloride eye drops 1%). The experiment was carried out on each animal’s right eye. To create the chemical burn, a circular filter paper 10 mm in diameter was soaked in 1 M NaOH for 5 s and applied to the corneal surface for 60 s. Then, the corneal surface was washed with 20 ml of a 0.9% sodium chloride solution. Tobramycin/dexamethsone eye drops were then applied to the cornea, and the eyelid was taped shut for 1 h. Over the next month, the erythromycin eye ointment was applied once a day. The rabbits with stable corneal scarring and neovascularization nebula were selected for the corneal implantation experiment. The courses and results of the corneal alkali burn model are shown in Fig. 2.

2.7.2. Kpro implantation

The implantation procedures were divided into two stages, with an interval of 3 months between stages. In stage I, the lamellar cornea was cut annularly at 1 mm within the limbus and the anterior lamellar was partially opened; one-third of the anterior lamellar remained at the nasal quadrant. An indentation was made in the central area of the corneal posterior lamella using a trephine 4 mm in diameter. Then, the stromal tissue within the indentation range was removed. The artificial cornea was positioned with its optic column passing through the pore of the corneal posterior lamella. The necessary cataract surgery and partial iris excision were performed concurrently with the Kpro implantation, and the anterior lamellar cornea was sutured in situ.

The schematic diagram for the Kpro implantation is shown in Fig. 3. Stage II was carried out 3 months later. Stromal tissue anterior to the optic column was removed to centrally expose the device as a full-thickness corneal replacement, while its skirt remained biomaterial within the stromal pocket.

Tobramycin/dexamethsone eye drops were used four times a day, and 0.15% tetracycline eye ointment was applied each night for 4–6 weeks after the stage II operation. At 6 months after the stage II operation, all rabbits were euthanized with an overdose of anesthetic agent, and their eyes were enucleated for histology.

The healing process, iris synechia, angle structure and inflammatory reaction were investigated using slit-lamp microscopy and anterior segment optical coherence tomography (AS-OCT) (Carl Zeiss Meditec, Jena, Germany) at 1, 2 and 3 months after stage I and 1 week, 3 and 6 months after stage II.

Cell adhesion on the bottom of the optical column was studied using SEM. For SEM imaging, the samples were fixed in glutaraldehyde for 1 h and then dehydrated with increasing concentration gradients of ethanol (from 35% to 100%). Finally, the samples were dried using a mixture of hexamethyldisilazane (HMDS) and ethanol (1:1, v/v) for 1 h followed by 100% HMDS for 5 min. After drying, the samples were sputter-

![Fig. 2. Corneal alkali burn model in rabbits. (a) The circular filter paper with a sodium hydroxide solution was placed onto the central surface of the rabbit’s cornea; (b) the circular filter paper was removed, and white opacity was present; and (c) stable corneal scarring and neovascularization nebula were formed.](image-url)
coated with a 10 nm gold layer in a vacuum evaporator and then examined using SEM.

The adhesion relationship between the skirt and cornea and the growth of cells and blood vessels in the skirt were studied using hematoxylin and eosin (HE) stains. Immediately after euthanasia, the sample specimens were cut and fixed in 10% formalin for 48 h before being embedded in paraffin wax. The samples were sectioned with a 5-μm thickness perpendicular to the longitudinal axis of the graft. These sections were treated with HE for histological evaluation. The HE images were visualized using inverted light microscopy (Leica DM 3000B).

3. Results and discussion

The study explored the feasibility of preparing a T-style Kpro based on a PHEMA hydrogel with high water content and a high-volume fraction of porosity. Surface modifications were performed to regulate cell adhesion on different functional areas of the artificial cornea. The T-style Kpro presented acceptable physical properties, surface chemical properties and biocompatibility during in vitro and in vivo experiments. Through its improved surgical techniques, the novel T-style Kpro could provide sufficient mechanical stability and long-term biointegration with the host environment.

3.1. Synthesis and characterization of cationized gelatin

In this study, we synthesized the CG based on natural gelatin and measured the number of amino groups introduced into the gelatin using the conventional TNBS method. Calculations based on the calibration curve prepared using β-alanine showed that the amino group contents of the native gelatin and the CG were 0.44 mmol g\(^{-1}\) and 0.71 mmol g\(^{-1}\) respectively, which demonstrated that some amino groups were successfully introduced to the native gelatin. That was, the native gelatin was successfully cationized.

In addition, we also validated the in vitro cytotoxicity of the synthesized CG by culturing corneal stromal cells on surfaces coated with CG and natural gelatin and by assessing the cell viability and proliferation with an MTT assay; the results are shown in Fig. 4. These results revealed that both the CG and gelatin were favorable for corneal stromal cell attachment and growth. No significant difference was found between the number of cells cultured on the CG surface and the gelatin surface after 24 h. The comparison verified that the CG had no cytotoxicity in vitro, which was consistent with previous reports [27,38].

The surface chemistry of the gelatin sample was changed because more amino groups were introduced onto the surface. This modification was favorable for efficiently attracting particular proteins by hydrogen bonding and inducing more positively charged groups (amino group cationation) to enhance the interactions between the material surfaces and the negatively charged cells [38].

3.2. Preparation and surface modification of the artificial cornea

The HEMA monomer was incompletely polymerized to synthesize the PHEMA prepolymer. Additional crosslinking agents and initiators were added to further polymerize the HEMA prepolymer into the PHEMA hydrogel. NaCl and honey were selected to form pores in the spongy skirt. The prepolymer solution was kneaded with the mixture of NaCl and honey. The T-style Kpro was formed in the mold. The spongy skirt was prepared through further reactions of the HEMA prepolymer. A slight excess of the prepolymer mixture was added to make the mold fill completely. Pressure was added to ensure that the plunger met the mold base tightly. Before the polymerization of the optic column, the debris in the cylindrical space (G) [Fig. 1] was cleared to ensure that the cylindrical space was empty; otherwise, the debris makes the optic column opaque. The spongy skirt was not removed from the mold to control the accurate position and surface structure of the porous skirt. After polymerization, the mold was opened and the resulting Kpro sample was removed; the sample comprised a circular transparent region surrounded by a white spongy ring. The sample was placed in distilled water for at least 1 week with daily water changes. The pore-forming agents (NaCl and honey) were dissolved in water, and the spongy skirt of the artificial cornea was formed. NaCl was used to provide sufficient micropore sizes, and honey was used to facilitate the interconnection among micropores in the interior and on the surface of the spongy skirt.

Synthetic polymers permit a high level of control over mechanical properties, geometry shapes and process ability, which are all beneficial when preparing an artificial cornea. In this process, both the optic column and the spongy skirt were made of 2-hydroxyethyl methacrylate and the artificial cornea was chemically identical. During the course of polymerizing the optic column, the HEMA monomer solution could penetrate along the boundary into the porous skirts to a certain distance and form a specific structure of interpenetrating polymer networks (IPN) of the two polymers through polymerization.

The artificial cornea prepared is shown in Fig. 5.
The transparent optic column and the spongy skirt are clearly shown in Fig. 5; the transition between the two parts is relatively smooth and does not show a well-defined separation. This specific IPN structure ensures the mechanical properties and structure stability of the T-style artificial cornea. However, the in vivo long-term stability and vision restoration are mainly determined by ocular biocompatibility and adequate host integration of the artificial cornea, which are based on ingrowths of cells and tissue into the spongy skirt. To promote cell adhesion and tissue ingrowths, the Kpro skirts were modified using HA and CG, which are popular polymers in the study of the interactions between cells and materials [38–40]. In this study, we used HA and the synthesized CG to modify the spongy skirt using the LBL technique, in which the positive charges of CG showed an electrostatic interaction with the negative charges of HA. Before the LBL process, plasma modification and KH550 were used to introduce a covalent bond-anchored surface layer of amino groups on the surface of the PHEMA spongy skirt. The LBL process then began with HA self-assembly by an electrostatic interaction. The surface modification with 10 bilayers of HA and CG was enough to cover the surface of the PHEMA spongy skirt. The CG was the top layer after the LBL process; it showed some positive charge on the surface and could improve cell adhesion on the PHEMA spongy skirt.

The retroprosthetic membrane formation on the optic column surface occurs due to cell adhesion and leads to the opacification of the artificial cornea’s optic column. To solve this problem, the optic column surface was modified with PEG, which effectively resists cell adhesion. During the modification course, only the bottom of the optic column was modified with PEG and N2 gas was used to remove the excess liquid to make the modified layer thinner. The PEG macromonomer attached to the optic column surface could penetrate into the PHEMA hydrogel to a certain distance and form a specific IPN structure with the PHEMA polymer through polymerization [41]. Although the specific IPN structure layer of the PEG and PHEMA was very thin, it was enough to ensure that the PEG layer remained stable on the surface of the optic column, even though the PHEMA–PEG samples were placed in distilled water for at least 1 week with daily water changes.

3.3. Equilibrium water content

The PHEMA Kpro swells in water to form a hydrogel. The water uptake of the PHEMA optic column was 82 ± 3%, which was close to that of a human’s natural cornea (78%) [42]. The water uptake in the PHEMA optic column depended on the components in the polymerization mixture, especially the quantitative variation of the crosslinking agents. The water content was stable for some crosslinking agents, dosages and polymerization methods.

The size of the optic column increased to 22 ± 2% with PHEMA swelling. The PEG film prepared from the 10% PEG solution had a swelling ratio of 20 ± 1%, which was similar to that of the PHEMA optic column. The PEG modification layer was prepared based on a suitable PEG-DA concentration and showed a swelling ratio similar to the PHEMA optic column. The similar swelling ratio meant that the modification was successful and that the wrinkles and desorption of the PEG layer, which formed because of different swelling ratios, were avoided. More importantly, the smooth modified layer made the optic column adequately clear, which ensured the positive visual performance of the Kpro.

3.4. SEM

The surface of the optic column and the microstructure of the spongy skirt were characterized using SEM. The modified optic column and spongy skirt were also observed. The SEM results are shown in Fig. 6.

The SEM results revealed that the PHEMA optic column was flat and smooth (Fig. 6a). When the optic column was modified with the PEG graft, it remained flat and smooth, and no wrinkles or desorption on the optic column surface were observed (Fig. 6b), thus showing that the coating of the PEG layer was stable in distilled water for at least 1 week. If the PEG layer had not remained stable on the surface of the PHEMA column, the PHEMA–PEG sample would have shown a very rough or wrinkled surface. The flat and smooth surface of the PHEMA–PEG sample was mainly due to the similar swelling ratio (see Section 3.3) and the specific IPN structure (see Section 3.2) of the PHEMA and the PEG modification layer. The appearances of the PHEMA spongy skirts before and after modification with HA and CG using the LBL technique are shown in Figs. 6c and d. After the LBL procedure, the surfaces of the spongy skirts (Fig. 6d) were more uneven and rough than those of the spongy skirts before the modification. Pores that were 80 μm or larger in the spongy skirt were obvious, and the skirt pores appeared to be well interconnected.
3.5. ATR-FTIR

Fig. 7 shows the ATR-FTIR spectra of the four films: PHEMA, PEG, PHEMA modified with PEG and PHEMA modified with HA and CG using LBL technique.

The ATR-FTIR spectra for the PEG, PHEMA and PHEMA–PEG samples were analyzed, and the peaks at 2883, 1724 and 1247 cm$^{-1}$ were the signals from the CH$_2$, C=O and C–O groups in the PHEMA and PEG layers (PEG-DA), respectively (Fig. 7a). The peak at 2985 cm$^{-1}$ responded to CH$_3$ in the PHEMA, which remained unchanged in the modification course because no CH$_3$ group was present in the PEG layer. Therefore, we can calculate the ratio of the specific peaks at 2883 and 2985 cm$^{-1}$ in the PHEMA and PHEMA–PEG spectra, respectively. In the present study, we use OriginPro 8 software to pick multiple peaks from the FTIR curves to fit Gaussian peak function. Before peak resolution and fitting, the baseline is corrected by “Auto Create” method in the 2700–3050 cm$^{-1}$ region using OriginPro 8 software. The fitted curves are shown in Fig. 7b and c. The adjusted R-square values are

![Figure 7: ATR-FTIR spectra. (a) Curves of PEG, PHEMA and PHEMA–PEG; (b) fitted curve based on specific peaks in the PHEMA spectra; (c) fitted curve based on specific peaks in the PHEMA–PEG spectra; and (d) curves of PHEMA and PHEMA-LBL.](image-url)
0.9963 (PHEMA curve) and 0.9960 (PHEMA–PEG curve), respectively. The peak height ratio ($H'_{ps-1}/H'_{ps-3}$) at 2883 and 2985 cm$^{-1}$ in the PHEMA spectrum is 2.4, while the height ratio ($H'_{ps-1}/H'_{ps-3}$) in the PHEMA–PEG spectrum is 3.2. The peak integral area ratio ($A'_{ps-1}/A'_{ps-3}$) at 2883 and 2985 cm$^{-1}$ in the PHEMA spectrum is also calculated as 7.9, while the area ratio ($A'_{ps-1}/A'_{ps-3}$) in the PHEMA–PEG spectrum is 11.1. Based on the increased intensity ratios of the specific peaks at 2883 and 2985 cm$^{-1}$ before and after PEG modification, we can conclude that the surface chemical groups changed during the PEG modification course, i.e. the PHEMA optical column was successfully coated with the PEG layer.

Fig. 7d shows the ATR-FTIR spectra of the PHEMA and the PHEMA-LBL. The peaks at 3200–3500 and 1540–1660 cm$^{-1}$ correspond to the peaks of the hydroxyl bonds and amide bands, respectively. A comparison of the two infrared spectra shows that the intensity of the peaks at these points was significantly higher for the PHEMA-LBL with a composite coating of HA and CG. For the LBL layer, new vibration peaks at 1651 (−CONH−) and 1558 cm$^{-1}$ (−NH$_2$) indicated that the CG was successfully anchored on the PHEMA surface.

3.6. X-ray photoelectron spectroscopy

XPS is widely used to provide both qualitative and quantitative information for the surface chemistry of a material in its ‘as received’ state or after certain treatments. The alteration of the elemental contents of the PHEMA sample surface before and after LBL modification was determined using XPS. The contents of C, O, N, Si and Cl on the sample surfaces were analyzed; the results are shown in Table 1.

As shown in Table 1, the analysis of atomic compositions indicated that the N percentage on the PHEMA-LBL surface with HA and CG was significantly higher than the percentage for the PHEMA, which is because of the amine groups within the CG. The intensity increases for the Si and Cl elements in the PHEMA-LBL surface were mainly because of the silane coupling agent (KH550) and HCl used in the LBL process. These results were consistent with similar measurements reported by Li et al. [27]. The XPS dates and the ATR-FTIR spectra (Fig. 7a in Section 3.5) provide that a successful LBL coating was present on the PHEMA surface.

3.7. Light transmission

Fig. 8 shows the light transmittance curves of the PHEMA optic column before and after modification with PEG. Fig. 8 shows that visible light transmittance through the PHEMA Kpro optic column in the wavelength ranging from 500 to 700 nm is more than 90%, which is consistent with similar measurements reported by Gulsen and Chauhan [43] and approximates that of the natural cornea [44]. The PHEMA Kpro optic column modified with PEG had a high visible light transmittance (~82%) in the visible light wavelength range, although it was slightly less than that of the unmodified PHEMA optic column (~90%) [13]. The modification process did not cause any obvious destruction on the smooth surface of the optic column, which was in agreement with the SEM results (see Section 3.4). Under UV light with wavelengths ranging from 350 to 400 nm, the PHEMA hydrogel modified with PEG showed better blocking ability than that of the pure PHEMA hydrogel, which is crucial to an ideal artificial cornea because it prevents UV damage to internal eye tissue.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>C (%)</th>
<th>O (%)</th>
<th>N (%)</th>
<th>Si (%)</th>
<th>Cl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHEMA (%)</td>
<td>71.62</td>
<td>27.41</td>
<td>0.24</td>
<td>0.72</td>
<td>0.03</td>
</tr>
<tr>
<td>PHEMA-LBL (%)</td>
<td>64.39</td>
<td>24.23</td>
<td>8.31</td>
<td>2.68</td>
<td>0.40</td>
</tr>
</tbody>
</table>

LBL, layer-by-layer modification; C, carbon; O, oxygen; N, nitrogen; Si, silicon; Cl, chlorine.

3.8. Cell adhesion

The cell adhesion results for the different biomaterial surfaces are shown in Fig. 9. The results show that these materials were not toxic to corneal stromal cells and had good biocompatibility.

Fig. 9a shows that the cells adhered well on the TCPS surface. Fig. 9b reveals that the cells adhered to some extent on the pure PHEMA surface. In contrast with the pure PHEMA surface, the cells did not adhere to the PEG-modified PHEMA surface shown in Fig. 9c. PEG is a well-known biomaterial that prevents the non-specific adsorption of proteins and cells [30,31]. Fig. 9c shows that the PEG layer successfully remained on the PHEMA surface and effectively resisted cell adhesion even in later cell experiments. On the other hand, more cells adhered to the PHEMA-LBL surface with HA and CG, which proved that the HA and CG grafted to the PHEMA surface successfully and promoted cell adhesion.

Kpro implantation

In this study, 10 Kpros were transplanted successfully into the rabbits’ corneal lamellar pockets. After the transplants, 3 months were provided to improve wound healing in the split cornea and allow biointegration of the stromal pocket and the Kpro’s spongy skirt. An open angle structure, decreased iris synechia and a normal position were found in seven cases in the study (Fig. 10). However, corneal stromal melting was found in two cases 1 month after stage I, followed by spontaneous extrusion and secondary endophthalmitis. In one other
case, elevated intraocular pressure and corneal protrusion were found 2 months after stage I; the angle structure closure and iris synechia were identified using AS-OCT. This case also demonstrated spontaneous extrusion of the artificial cornea 2 weeks after the stage II operation.

Fig. 10b and d show that the implanted artificial cornea had an open angle structure in the normal position and was biointegrated with surrounding tissue. At 2 weeks after the stage II operation, the opened pore of the anterior lamellar began to close gradually; this pore disappeared in 1 week in most cases. Therefore, the anterior lamellar above the optic column was removed again to expose the implant device. In all cases, no retroprosthetic membrane formation occurred until the implant experiments were terminated 6 months after the stage II surgery, when all the rabbits were euthanized and their eyes were enucleated for histology.

The results of the histological examination and the SEM photo are shown in Fig. 11.

Fig. 11a and b show that a large amount of the host corneal tissue grew into the pores of the Kpro’s spongy skirt. Histologically, the inflammatory reaction was not severe, and the Kpro implant and the host corneal tissue connected well. In Fig. 11c, no obvious complications can be seen in the posterior segment of the rabbit’s eye, which shows that implanting the T-style Kpro is safe in rabbit eyes. The SEM photo in Fig. 11d shows that no cells or tissues grew on the posterior part of the Kpro, which proves that an optic column modified with PEG can effectively resist cell adhesion on the implant surface in vivo.

The insufficient surgical fixation of an artificial cornea does not benefit tissue healing. Long-term vision restoration depends on adequate host integration, which is based on cell and tissue ingrowths into the spongy skirt and the retention of the artificial cornea in the host [19, 45]. Uncontrolled cell growth and debris adsorption on the column’s bottom should be discouraged to maintain its optical clarity, while the porous skirt of the Kpro should be encouraged to promote cell and tissue ingrowths. The increased ingrowth of cells and tissues produces a firm bond between the corneal tissue and the peripheral prosthetic material. It can thus prevent some complications, including tissue melting.

Fig. 9. Comparisons of cell adhesion characteristics: (a) cells adhering on the TCPS surface; (b) cells adhering on the PHEMA surface; (c) cells adhering on the PHEMA–PEG surface; (d) cells adhering on the PHEMA-LBL surface; and (e) MTT test results for (a), (b), (c) and (d). *, significance at p < 0.05 vs. TCPS as control; **, significance at p < 0.05 vs. the PHEMA surface as control.
and the subsequent extrusion of the implant. Compared with AlphaCor Kpro, the artificial cornea with a novel T-style design and multifunctional surface modifications improves the overall mechanical stability and long-term biointegration with the host environment. In addition, our artificial cornea based on soft PHEMA hydrogels has better biocompatibility and functionality (robust biointegration, permeability to nutrients and sufficient support for epithelialization) than some hard artificial corneas, such as Boston keratoprosthesis [46], which has an outstanding optic column and is made of poly(methyl methacrylate) (hard plastic).

The opened pore of the anterior lamellar closed gradually and disappeared after the stage II operation mainly due to the insufficient cell-adhesion resistance of the anterior surface of the artificial cornea to corneal stromal cells. In future work, we will solve this problem using surface modification.

4. Conclusions

This paper presents a novel T-style design of an artificial cornea and its preparation methods, in which a mechanically and structurally effective artificial cornea is made based on a PHEMA hydrogel. Selective surface modifications effectively promoted cell adhesion on the spongy skirt of the Kpro, which improved biointegration and prevented cell adhesion and retroprosthetic membrane formation on the Kpro’s optic column. The T-style Kpro presented acceptable physical properties, surface chemical properties and biocompatibility during in vitro and in vivo experiments. Through improved surgical techniques, the novel T-style Kpro provides enough mechanical stability to facilitate long-term biointegration with the host environment. In vivo implantation experiments showed that the T-style artificial cornea is a promising corneal implant.
alternative and can be used for corneal regeneration in patients with severe limbal stem cell deficiency and corneal opacity.

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