

# Functional biomedical polymers for corneal regenerative medicine

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Dedicated to Professor Teiji Tsuruta on the occasion of his 88th birthday (Beiju).

## Abstract

Recent progress in biomedical polymer science has greatly contributed to rapid development of corneal regenerative medicine. In the past decades, scientists have achieved several major breakthroughs in corneal tissue reconstruction. Studies regarding the findings of core-and-skirt keratoprostheses for visual rehabilitation, biosynthetic tissue replacements for corneal transplantation, and thermo-responsive cell-detachable substrates for corneal cell sheet engineering have been reported by several groups of investigators. This brief overview focuses on the contributions of functional polymers in the applications of corneal regenerative medicine. The keratoprosthetic devices developed by our group using heterobifunctional silicone rubber membranes grafted with different bioactive functional groups showed promising results in animal studies. In addition, the fabrication and transplantation of bioengineered human corneal endothelial cell sheets by utilizing the functional biomedical polymers such as poly(*N*-isopropylacrylamide) and gelatin are discussed, especially for the significance of gelatin in the development of a potential intraocular delivery system for cell sheet grafts.

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## 1. Introduction

In the human body, the eye is one of the most complex and remarkable organs. The cornea is the transparent circular part of the front of the eyeball

that covers the pupil, iris, and anterior chamber. It is composed of five distinct anatomic layers including epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium. Corneal transplantation may restore vision when the cornea has become opacified as a result of hereditary diseases, infection, or injury. However, the shortage of donor corneal tissue has driven the expansion of research on keratoprosthesis for treatment of

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corneal blindness. Functional biomedical polymers have attracted much attention for their potential applications in the field of keratoprosthetic devices. In recent years, the development of biomimetic materials as tissue-engineered corneal replacements also showed very encouraging results. On the other hand, scientists have now achieved a major breakthrough in corneal epithelial regeneration. The revolutionary design of thermo-responsive cell harvest system enables novel cell sheet techniques for engineering tissue replacements without scaffolds. Carrier-free and sutureless transplantation of autologous oral mucosa epithelial cell sheets may represent an effective way to treat patients with several bilateral limbal stem cell deficiency. Although the improvements in ocular surface reconstruction are impressive, the occurrence of corneal opacities is closely related to endothelial dysfunction in most patients requiring corneal transplantation. It is expected that the cell sheet-based therapy will give promising hope of a possible treatment of corneal endothelium deficiency. This overview will provide an insight into the various functional biomedical polymers that are useful in corneal regenerative medicine.

## 2. Keratoprosthesis

When a progressive corneal ulceration or a penetrating corneal injury with tissue loss occurs, emergency penetrating keratoplasty or lamellar keratoplasty is carried out to restore ocular integrity and to avoid further complications, such as extensive chamber angle synechia, angle closure glaucoma, and endophthalmitis. Although corneal transplantation has a high success rate, the shortage of donor corneas remains a worldwide problem. The development of artificial corneas (keratoprostheses) is a promising alternative to obtain biosynthetic tissue replacements for corneal transplantation. In 1771, French ophthalmologist Guillaume Pellier de Quengsy [1] first suggested inserting a glass plate into opaque corneas to restore clear vision. The first experimental implantation was performed by von Nussbaum and Nepomuk [2] in 1856, using rabbits as an experimental model. In 1859, human implantation was first attempted when Heusser [3] placed a glass implant into the cornea of a 19-year-old patient. Polymer has become the most popular substance of choice for keratoprosthesis research following World War II. The keratoprosthesis developed by Stone

and Herbert [4] consisted of a central optical core with a marginal part for anchoring of a perforated plastic. They found that the fragments made of poly(methyl methacrylate) (PMMA) were well tolerated in the cornea. In the 1960s, investigators focused on the design of core-and-skirt keratoprosthetic models. A plastic fiber meshwork supporting plate, i.e., Cardona implant, was first studied for keratoprosthesis [5,6]. The second generation of keratoprosthesis, Girard keratoprosthesis, was reported based on the modification of the original Cardona implant [7]. In addition to PMMA, other materials such as silicone [8] and ceramic [9] have also been used for preparation of keratoprosthetic devices. Although the design of keratoprosthesis has significantly improved, the persistence of a high incidence of failure suggests that successful development of keratoprosthetic implants remains an extremely difficult task [10]. Previous attempts failed due to erosion and necrosis of the adjacent tissue, chronic inflammation, and epithelialization of the anterior chamber [11–13]. Optimally, the anterior surface of a keratoprosthesis should support the adherence and proliferation of corneal epithelial cells, subsequently resulting in an intact epithelial layer which is continuous with the surrounding host epithelium. A continuous layer of epithelial cells permits maintenance of the normal precorneal tear film, ensures a good optical surface, and provides a barrier against microbial invasion.

In 1991, Kirkham et al. [14] found that the PMMA intracorneal keratoprosthesis coated with type I collagen could be used to improve the biocompatibility of an implant. In another study, Ikada et al. [15] reported that poly(vinyl chloride) (PVC) hydrogel immobilized with collagen in contact lens could feasibly be used as keratoprosthetic devices. A variety of synthetic polymers such as poly(vinyl alcohol-co-vinyl acetate) copolymer [16], polybutylene/polypropylene blend [17,18], poly(tetrafluoroethylene) (PTFE) [19,20], polyurethane [21], polysiloxane [22], and poly(2-hydroxyethyl methacrylate) (PHEMA) [23–25] have recently been investigated for their potential use in keratoprosthesis. The preparation of artificial cornea has already been studied in our laboratory for quite some time [26–31]. It is found that four important issues must be addressed if an ideal keratoprosthesis is to be obtained. First, the anterior surface of implant is required to be completely covered with epithelium by promotion of cell growth. Secondly, epithelial downgrowth has to be suppressed when

the implant is kept in the living cornea for a long period of time. Thirdly, the keratoprosthetic materials must be highly biocompatible with the host cornea and have high permeability to gas or nutrients. Finally, the process of wound healing has to be considered because the implant needs to be tightly fixed on the host cornea. On the basis of the above considerations, we have developed a variety of silicone rubber (SR) membranes grafted with different bioactive functional groups using plasma chemistry. At the early stage of our study, a plasma polymerized HEMA film was prepared by plasma deposition polymerization onto an elastic material, SR [26,27]. The introduction of PHEMA onto a hydrophobic support could provide an adequate surface for improving rabbit corneal epithelial cell attachment and growth. Later, it was demonstrated that HEMA monomer could be successfully immobilized onto either SR or a plastic material, poly(4-methyl-1-pentene) (TPX) by using plasma-induced graft polymerization [28]. The PHEMA-grafted SR or PHEMA-grafted TPX films were used as culture supports for rabbit corneal epithelial cells. After 3 days in culture, a significant higher cell attachment and growth was observed on the PHEMA-grafted SR films than those on the PHEMA-grafted TPX samples. On the other hand, a highly biocompatible homobifunctional membrane made of PHEMA-*g*-SR-*g*-PHEMA was developed by plasma-induced graft polymerization, and the biological properties of modified SR membrane were investigated *in vitro* and *in vivo* to determine the possible use as keratoprosthetic device [29,30]. The attachment and growth of rabbit corneal epithelial cells onto PHEMA-*g*-SR-*g*-PHEMA (grafting density  $75 \mu\text{g}/\text{cm}^2$ ) membranes was enhanced. The depth of anterior chamber was maintained 2 weeks after implantation with a SR grafted with PHEMA (grafting density  $210 \mu\text{g}/\text{cm}^2$ ). In 1998, we further designed a novel heterobifunctional SR membrane as a new generation of artificial cornea (Fig. 1). The heterobifunctional SR membranes were prepared by grafting different functional polymers (i.e., (2-methacryloyloxyethyl) (MPC), acrylic acid (AAc) and collagen) on each side of a SR membrane [31]. This type of membrane was developed with upper-side favoring cell attachment and growth whereas the lower-side suppressing the adhesion strength of either cells or proteins. *In vitro* cytocompatibility studies showed excellent affinity on SR-*g*-PHEMA and SR-*g*-PAAc-collagen, whereas SR-*g*-PMPC exhibited poor affinity for

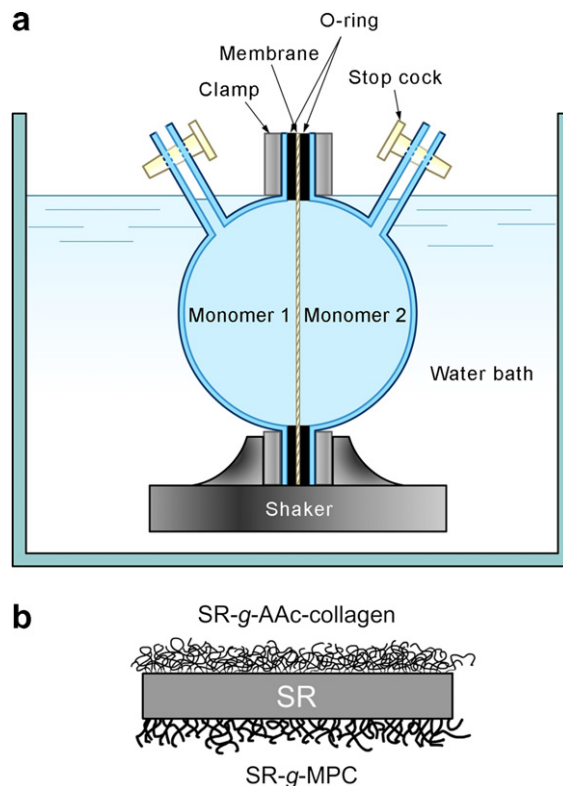


Fig. 1. (a) Schematic illustration of a reactor for the preparation of heterobifunctional membrane. (b) A representative heterobifunctional SR membrane grafted with different functional groups on each side of the substrate.

rabbit corneal epithelial cells. In an *in vivo* study, the anterior chamber formed and corneal epithelial cells completely covered the PMPC-*g*-SR-*g*-PAAc-collagen heterobifunctional membrane 3 weeks postoperatively. Biological analyses indicated that the heterobifunctional membrane seemed to be potentially applicable for an artificial cornea. Recently, Sheardown [32] have shown that the polydimethylsiloxane (PDMS) surfaces could be modified with epidermal growth factor by plasma polymerization of allylamine via a homobifunctional poly(ethylene glycol) (PEG) spacer in order to improve the growth of corneal epithelial cells.

Tissue engineering has emerged over the past few years as a promising technology for corneal regenerative medicine. Many researchers have made great efforts to take use of the biomedical polymer materials to design and create corneal tissue replacements. Griffith and coworkers [33] have reported a technique to prepare biosynthetic extracellular matrix (ECM) macromolecules that perform as physiologically functional corneal substitutes. A

copolymer poly(*N*-isopropylacrylamide-*co*-acrylic acid-*co*-acryloxysuccinimide) (PNIPAAm-*co*-AAc-*co*-ASI) was synthesized by free radical copolymerization of its three monomers in dioxane. The hydrated collagen and NIPAAm copolymer-based hydrogels were further fabricated by mixing neutralized 4% (wt/wt) bovine atelocollagen with purified copolymer (collagen/copolymer = 1.4:1, wt/wt) at 4 °C. It was found that the grafting of the laminin adhesion pentapeptide motif, YIGSR, to the hydrogels significantly promoted epithelial stratification and neurite in-growth. The resultant combinations of biological and synthetic mimics of ECM macromolecules may provide optically clear and mechanically stable hydrogels to be suitable for use in corneal tissue engineering. Later, the tissue substitutes made of cross-linked porcine type I collagen were developed by the same group for corneal implantation [34,35]. In another study, Cao and coworkers [36] demonstrated that the polyglycolic acid (PGA) fibers could be used as a scaffold for tissue engineering of nearly transparent corneal stroma. Intrastromal implantation of PGA scaffold implants bearing corneal stromal cells is a useful procedure for corneal stromal tissue reconstruction. These studies represent a promising new direction for bioengineering of corneal tissue replacements.

### 3. Corneal cell sheet engineering

Corneal epithelial cell transplantation has gained more attention in the past decade. In 1997, Pellegrini et al. [37] first reported a clinical application of autologous cultivated corneal epithelium to treat patients with corneal–limbal epithelial defects. Later, several investigators have shown that the biological tissue materials such as amniotic membrane can be used as a matrix carrier for *ex vivo* expanded corneal epithelial stem cells as bioengineered ocular surface [38–40]. Cultivated limbal epithelial stem cell transplantation in conjunction with amniotic membrane has been proven to be an effective technique for the therapeutic treatment of ocular surface diseases, such as Stevens–Johnson syndrome and chemical/thermal burns. In addition to amniotic membrane, other carrier substrates such as fibrin gel [41] and chitosan-coated alginate membranes [42] have recently been investigated for their potential use in corneal epithelial regeneration. Although cultivated cell transplantation using carrier substrates can strengthen the tissue-engineered epithelial grafts, the presence of carrier materials

postoperatively may decrease optical transparency and pose a risk of inflammatory reaction [43]. To overcome these limitations, Nishida et al. [44] recently fabricated bioengineered corneal epithelial cell sheets by using temperature-responsive PNIPAAm-grafted polystyrene (PS) culture surfaces. Functional corneal epithelial sheet grafts are successfully transplantable without any carriers, producing corneal surface reconstruction in a rabbit model.

The concept of cell sheet engineering was proposed by Okano and colleagues to represent a novel technology for tissue regeneration without artificial scaffolds using temperature-responsive culture dishes [45]. They have demonstrated that the cultured cells could adhere and proliferate on the hydrophobic PNIPAAm-grafted surfaces at 37 °C, and spontaneously detach from the hydrophilic surfaces due to abrupt hydrated transition of polymer chains when the medium temperature was lowered to a level below the lower critical solution temperature of PNIPAAm (i.e., 32 °C) [46]. The temperature-responsive culture dishes, which are fabricated by electron beam irradiation, are very effective to harvest cells without damage [47]. A technique of temperature-modulated cell adhesion/detachment using PNIPAAm-based substrates has also been disclosed in other studies. Takezawa et al. [48] found that when detached from substratum containing the PNIPAAm, the monolayered sheet of human dermal fibroblasts gradually aggregated and finally formed a multicellular spheroid. Ratner and coworkers [49] reported that the removal of bovine aortic endothelial cells via low-temperature liftoff from plasma polymerized PNIPAAm-treated surfaces is less damaging to the ECM proteins remaining at the surface than enzymatic or mechanical detachment methods. Kanamori and coworkers [50] developed a novel selective cell-separation method based on using a PNIPAAm-grafted membrane containing adsorbed monoclonal antibody specific to the target cell. Since the novel cell harvest system is advantageous for tissue engineering applications, a wide range of thermo-responsive materials such as graft copolymers of PNIPAAm with PEG [51], PNIPAAm-grafted gelatin [52], block copolymers of poly(NIPAAm-*co*-AAc)-*b*-poly(L-lactic acid) (PLLA) [53], methylcellulose [54], pluronic [55], and PNIPAAm/clay nanocomposite [56] have been explored as potential cell-detachable substrates. By avoiding enzymatic treatment (i.e., trypsinization), the bioengineered cell sheets fabri-

cated from thermo-responsive culture supports substantially retain their cellular activity, organization, function, and ECM integrity. A recent attempt has been made by Nishida et al. [57] to reconstruct ocular surface by sutureless transplantation of tissue-engineered cell sheets composed of autologous oral mucosal epithelium. The results of this work were very encouraging since the visual acuity of patients who received cell sheet grafts was significantly improved. The carrier-free cell sheet engineering technique may overcome the drawbacks associated with conventional corneal transplantation surgery, which remains the most common form of treatment for corneal epithelial dysfunction. However, in more than half of cases requiring corneal transplantation, the endothelial cell layer is the only corneal component that requires replacement [58]. Therefore, corneal endothelial reconstruction is an attractive research field in tissue engineering since it is of high clinical importance.

The corneal endothelium is a thin cell monolayer that forms the posterior boundary of the cornea and maintains corneal deturgescence and clarity [59]. Human corneal endothelial cells (HCECs) do not proliferate *in vivo* and the number of these cells decreases gradually with aging [60]. The limited regenerative capacity of human corneal endothelium may cause irreversible corneal edema and loss of vision. In the mid to late 1970s, corneal endothelial transplantation was first attempted by Maurice and coworkers [61] who directly injected a suspension of cultured CECs into the rabbit anterior chamber to repopulate the damaged corneal endothelium. However, that trial was limited because only scattered clumps of endothelial cells randomly attached to the targeted cornea and to other normal ocular tissues such as the iris and lens. In order to overcome the problems associated with isolated cell injection, several investigators have reported a method to transplant CECs expanded *ex vivo* on different carrier substrates made of biopolymers (gelatin [62], collagen-coated dextran [63], collagen [64]), synthetic polymer materials (methyl methacrylate/*N*-vinyl pyrrolidone copolymers [65]), and biological tissue materials (Descemet's membrane [66], amniotic membrane [67]). In 1980, Maurice's group [62] prepared a thin gelatin film about 1  $\mu\text{m}$  thick by cross-linking with glutaraldehyde, and the resulting transparent, wrinkle-free, water-permeable substrate was used to support the growth of tissue cultured CECs. However, such a system would involve the use of cyanoacrylate adhesives, which

may result in unstable attachment of the carrier substrate to the recipient corneal stroma. In 1994, Mohay et al. [65] have investigated the feasibility of using hydrogel lens as cell carrier device in a feline or rabbit allogeneic transplantation model. They found that the hydrogel device was mechanically stable to allow for easy handling of fragile cell grafts, but the implantation of a foreign body carrier may potentially induce a rejection reaction. Recently, the human amniotic membrane without amniotic epithelium has also been applied as a carrier for cultivated HCEC transplantation in a rabbit model [67]. It is noted that the problems with a tissue regeneration technique using CEC carrier substrates may include poor graft–host integration, optical interference, risk of foreign body reaction, and disturbance of endothelial cell function because of the presence of carrier materials between the implanted cells and the host tissue.

To overcome these limitations, we have developed the cell sheet engineering technique for the treatment of corneal endothelium deficiency. A novel strategy for corneal endothelial reconstruction using functional biomedical polymers is shown in Fig. 2 [68]. By means of plasma chemistry, the polyethylene (PE) surfaces were modified with 1.6  $\mu\text{g}/\text{cm}^2$  of PNIPAAm. Untransformed adult HCECs derived from eye bank corneas were plated on the thermo-responsive PNIPAAm-grafted culture substrates and incubated for 3 weeks at 37 °C. When the surrounding temperature was lowered to 20 °C, the confluent cultures were harvested as intact cell monolayers. The characteristics of tissue-engineered HCEC sheets are similar to those observed in the native endothelium of eye bank donor corneas [69]. Fabrication of bioengineered HCEC sheets from thermo-responsive PNIPAAm-grafted culture dishes [70] or poly(NIPAAm-*co*-diethyleneglycol methacrylate (DEGMA)) substrates [71] have also been recently reported by other groups. They confirmed proper structure and function of the thermally detached HCEC monolayers. While these findings suggest that transplantable HCEC sheets can feasibly be used as tissue equivalents for replacing compromised endothelium, it is apparent that the technology of cell sheet transplantation allows us to achieve targeted delivery to the corneal posterior surface. Cell sheet delivery is another very important issue for potential tissue engineering applications. Despite having a tissue-like architecture, the thermally detached cell sheets were easily wrinkled and folded during



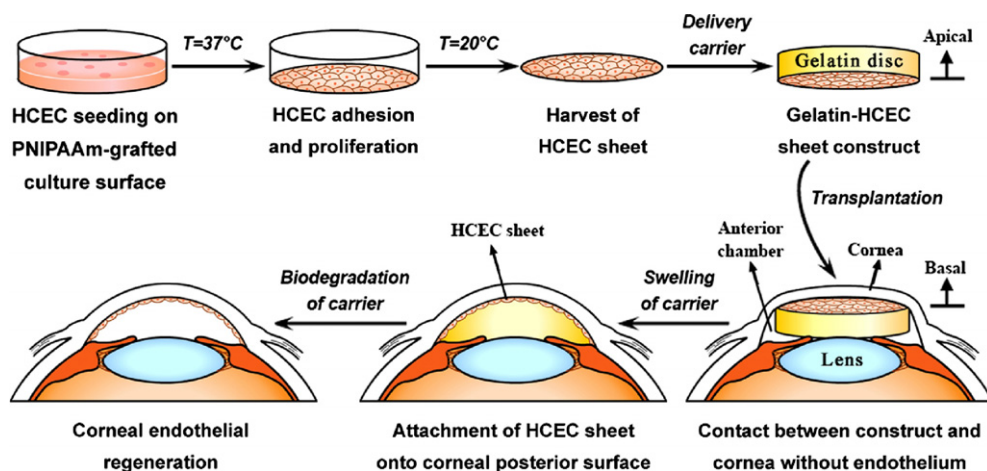


Fig. 2. Schematic representation of a novel strategy for corneal endothelial reconstruction using functional biomedical polymers.

removal of the thermo-responsive culture substrates [72]. In the field of cell sheet transfer, Okano and coworkers [73] have introduced the hydrophilically modified poly(vinylidene difluoride) (PVDF) membranes as a supporter, which renders for the three-dimensional manipulation of cardiomyocyte sheets into layered constructs. Recently, a doughnut-shaped PVDF supporter has been successfully used for cultured epithelial cell sheet transplantation [57]. However, intraocular grafting is different from the trials in corneal epithelial cell therapy because the anterior chamber is perfused with large amounts of tissue fluid, which may cause unstable attachment of implanted cell sheets to lesion sites. In these cases, it is necessary to provide a temporary support structure for enhancing the graft–host integration during and after intraocular delivery of bioengineered cell monolayers. By avoiding permanent residence of foreign supporting materials in the host, we have utilized the biodegradable and cell-adhesive gelatin discs as a supporter for transportation and surgical handling of well-organized cell sheets [74]. The gelatins with a negative charge and higher molecular weight show the stable mechanical property, appropriate biodegradability, and acceptable biocompatibility, making these materials interesting candidates for intraocular delivery of thermally detached HCEC sheets [68]. A long-term (i.e., 6 months) study in a rabbit model strongly suggested that the corneal clarity was restored by implanting the bioengineered HCEC sheets and we have recently confirmed [75] that the cultured HCECs and functional biomedical polymers, PNIPAAm and gelatin, hold high promise to treat corneal endothelial failure.

#### 4. Conclusion

Although the concept of keratoprosthesis has been studied for decades, continued development of ophthalmic biomedical materials have expanded the scope of applications of corneal tissue replacements. The use of synthetic polymers to replace corneal tissue and restore ocular integrity is a complex issue. In this article, we have shown that the hetero-bifunctional SR membranes are very promising candidates as keratoprosthesis materials in corneal replacement. On the other hand, considering that the cells are essential components of corneal tissues, we aimed to fabricate tissue-engineered cell sheets as close to the native tissues as possible. This review has demonstrated the versatility and utility of thermo-responsive cell-separation systems in corneal tissue engineering applications. By taking advantage of noninvasive cell harvest technique, the functional bioengineered cell sheets composed of either oral mucosal epithelium or corneal endothelium can be successfully fabricated from the PNIPAAm-grafted culture surfaces. In light of the studies discussed in this review, the thermo-responsive material has proven to be a potential biomedical material to be used in cell sheet engineering. It is also noted that the gelatin has functionality for intraocular delivery of cell sheet grafts. Upon exposure to the aqueous humor, the swelling of gelatin occurred, which allows the attachment and spread of HCEC sheets on the lesion area of rabbit cornea. The advantage of implantation of a gelatin disc may be that it improves the capability of cell sheet integration into host tissue. Since the foreign supporting materials are substantially completely degraded and

absorbed *in vivo*, we believe that this novel cell therapy technique will have a high success rate in treating CEC loss. Despite encouraging results from experimental work and clinical trials with regard to the cell sheet-based therapy, more studies are necessary to further evaluate the clinical application feasibility of functional biomedical polymers.

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