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Biocompatibility of chemically cross-linked gelatin hydrogels for ophthalmic use

Jui-Yang Lai

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Abstract Biocompatibility is a major requirement for the development of functional biomaterials for ophthalmic applications. In this study, we investigated the effect of cross-linker functionality on ocular biocompatibility of chemically modified gelatin hydrogels. The test materials were cross-linked with glutaraldehyde (GTA) or 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC), and were analyzed using in vitro and in vivo assays. Primary rat iris pigment epithelial cultures were incubated with various gelatin discs for 2 days, and the cellular responses were monitored by cell proliferation, viability, and pro-inflammatory gene and cytokine expression. The results demonstrated that the cells exposed to EDC cross-linked gelatins had relatively lower lactate dehydrogenase activity, cytotoxicity, and interleukin-1β and tumor necrosis factor-α levels than did those to GTA treated samples. In addition, the gelatin implants were inserted in the anterior chamber of rabbit eyes for 12 weeks and characterized by clinical observations and scanning electron microscopy studies. The EDC cross-linked gelatin hydrogels exhibited good biocompatibility and were well tolerated without causing toxicity and adverse effects. However, a significant inflammatory reaction was elicited by the presence of GTA treated materials. It was noted that, despite its biocompatibility, the potential application of non-cross-linked gelatin for local delivery of cell and drug therapeutics would be limited due to rapid dissolution in aqueous environments. In conclusion, these findings suggest ocular cell/tissue response to changes in cross-linker properties. In comparison to GTA treatment, the EDC cross-linking is more suitable for preparation of chemically modified gelatin hydrogels for ophthalmic use.

J.-Y. Lai (✉)
Institute of Biochemical and Biomedical Engineering, Chang Gung University, 33302 Taoyuan, Taiwan, Republic of China
e-mail: jylai@mail.cgu.edu.tw

J.-Y. Lai
Biomedical Engineering Research Center, Chang Gung University, 33302 Taoyuan, Taiwan, Republic of China

J.-Y. Lai
Molecular Medicine Research Center, Chang Gung University, 33302 Taoyuan, Taiwan, Republic of China

1 Introduction

Recent progress in biomaterial science has stimulated the development of ocular tissue engineering and regenerative medicine [1]. In 2003, Li et al. reported a technique to prepare biosynthetic extracellular matrix macromolecules that perform as physiologically functional corneal substitutes [2]. The hydrated collagen and *N*-isopropylacrylamide (NIPAAm) copolymer-based hydrogels containing laminin adhesion pentapeptide motif (i.e., YIGSR) are optically clear and may be used to promote epithelial stratification and neurite in-growth during ocular surface reconstruction. In 2004, Nishida et al. fabricated bioengineered oral mucosal epithelium from thermo-responsive PNIPAAm-grafted culture surfaces and demonstrated significant benefits of cell sheet transplantation in a clinical trial [3]. More recently, our group has presented a series of experiments examining the role of functional biomaterials and the effect of the cell sheet grafts to enhance corneal endothelial regeneration [4–7]. Intraocular delivery of bioengineered human corneal endothelium by means of multifunctional gelatin carriers holds promise to become a useful alternative to full-thickness penetrating or lamellar

keratoplasty. On the other hand, because of its thermoreversible gelation property, gelatin has potential for use as an encapsulating matrix in retinal sheet transplantation [8, 9].

Gelatin is a naturally occurring biopolymer derived from collagen, which is the major protein component of the skin, bone and connective tissue of all animals. As a protein-based biomaterial, gelatin is biodegradable and has excellent bioaffinity. Therefore, it has received much attention for pharmaceutical applications in ophthalmology. Using a desolvation method, Vandervoort and Ludwig have prepared gelatin nanoparticles for topical administration as eye drops [10]. The encapsulation of either a hydrophilic (pilocarpine HCl) or a hydrophobic (hydrocortisone) drug into these bioadhesive nanoparticles may prolong their residence time on the ocular surface and enhance their therapeutic efficacy. Recently, Hori et al. proposed a new ophthalmic drug delivery system composed of biodegradable hydrogels of cationized gelatin [11]. The controlled release of epidermal growth factor from the gelatin matrices placed over a corneal epithelial defect results in accelerated wound healing in rabbits. In addition, a study from Sakai et al. demonstrated that intravitreally injected basic fibroblast growth factor-impregnated gelatin nanoparticles may prevent photoreceptor degeneration by inhibiting apoptosis in Royal College of Surgeons (RCS) rats because of targeting and sustained release of growth factors [12]. These studies implicated that the gelatin carriers could be developed to increase the bioavailability and retain the desirable properties of the drugs.

Gelatin is known to exhibit rapid dissolution in aqueous environments, thereby limiting its biomedical applications [13]. Physical or chemical cross-linking treatment can be employed to improve the material stability against enzymatic degradation. In general, physical cross-linking methods (including UV irradiation and dehydrothermal treatment) do not cause potential harm, but a major problem with this technique is due to the difficult control of the cross-linking density of the gelatin matrices [14]. By contrast, chemical cross-linking is a more efficient method involving the use of cross-links which join gelatin molecules by creating covalent bonds. Various cross-linking agents including glutaraldehyde (GTA) [14, 15], epoxy compounds [16], and carbodiimides [17, 18] have been investigated for chemical modification of gelatin. Among these reagents, GTA is a non zero-length cross-linker that reacts with the ε-amino groups to create Schiff bases between polymer chains [19]. The gelatin molecules are bridged by cross-linking reagents. Although aldehydes have high reactivity and are frequently used in the cross-linking of proteinaceous materials, they may be associated with problems such as toxicity and calcification [20]. In comparison to GTA, 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) is a zero-length cross-linker that activates carboxylic acid groups and then forms the amide bonds with the amino groups of gelatin [18]. This modification technique is considered less toxic since it cross-links without incorporation of foreign structures into the biopolymers. However, EDC over certain concentrations may pose a risk of cytotoxicity [16]. Therefore, the biocompatibility of chemically cross-linked gelatin hydrogels should be investigated to evaluate their suitability for ophthalmic applications.

In general, biocompatibility is governed mainly by the interface between foreign materials and host living cells/tissues [21]. We have previously shown that cross-linking agent type gives influence on the biocompatibility of cell sheet delivery carriers [22]. The GTA cross-linked hyaluronic acid hydrogels in contrast to EDC treated samples are toxic towards rabbit corneal endothelial cells. It has been documented that the aldehyde groups introduced in the gelatin materials can be quenched with glycine [23]. In the present work, the GTA cross-linked gelatin hydrogels were treated with glycine to reduce the toxicity of aldehyde compounds. The in vitro biocompatibility of gelatin materials cross-linked with GTA or EDC was analyzed using rat iris pigment epithelial (IPE) cell cultures. The cell proliferation, viability, and pro-inflammatory gene and cytokine expression were studied to give insight into the effects of chemical cross-linkers on cellular responses to gelatin. In addition, we performed a study in a rabbit model to examine the in vivo biocompatibility of chemically cross-linked gelatin hydrogels for ophthalmic use. The test materials were implanted into the anterior chamber of rabbit eyes for 12 weeks and characterized by clinical observations and scanning electron microscopy (SEM) studies.

2 Materials and methods

2.1 Materials

Gelatin (Type A; 75–100 Bloom; isoelectric point 7.0–9.0), glutaraldehyde, and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS, pH 7.4) was obtained from Biochrom AG (Berlin, Germany). Ham's F-12 nutrient mixture (Ham's F-12), trypsin-ethylenediaminetetraacetic acid (EDTA), and TRIzol reagent were purchased from Gibco-BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) and the antibiotic/antimycotic (A/A) solution (10,000 U/ml penicillin, 10mg/ml streptomycin, and 25 μg/ml amphotericin B) were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). All the other chemicals were of reagent grade and used as received without further purification.

2.2 Preparation of gelatin hydrogels

The gelatin hydrogels were prepared by solution casting methods as we have described elsewhere [5, 24]. In brief, an aqueous solution of 10 wt% gelatin was cast into a polystyrene planar mold (5 × 5 cm^2, 1.5 cm depth), and air-dried for 3 days at 25°C to obtain hydrogel sheets (approximately 750 μm in thickness).

2.3 Cross-linking of gelatin hydrogels by GTA or EDC

The gelatin hydrogels were cross-linked with GTA by directly immersing the samples in an ethanol/water mixture (8:2, v/v) of 50 mM GTA. The cross-linking reaction was allowed to proceed at 25°C for 80 min and the resulting hydrogel sheets were then placed in 100 mM glycine aqueous solution at 37°C for 1 h to block unreacted residual GTA [25]. For EDC cross-linking, the gelatin hydrogels were immersed in an ethanol/water mixture (8:2, v/v, pH 4.75) of 50 mM EDC for 24 h at 25°C. After reaction, the resulting hydrogel sheets were thoroughly washed with double-distilled water to remove excess EDC. Subsequently, the GTA and EDC cross-linked samples were dried in vacuo for 24 h. Using a 7-mm diameter corneal trephine device, the hydrogel sheets were cut out to obtain three groups of gelatin discs: (1) Gel: non-cross-linking; (2) GTA-Gel: GTA cross-linking; (3) EDC-Gel: EDC cross-linking. The gelatin hydrogels were sterilized in a 70% ethanol solution overnight and thoroughly rinsed in sterilized PBS for use in the in vitro and in vivo biocompatibility tests.

The water content of gelatin hydrogels was determined to evaluate their extent of cross-linking [25]. For measurements, each test gelatin disc was immersed in double-distilled water at 37°C with reciprocal shaking (50 rpm) in a thermostatically controlled water bath. After 6 h, the swollen hydrogel discs were removed from the swelling medium, blotted with tissue paper to remove excess water on the surface, and weighed immediately. The water content (%) of the test sample was defined as $((W_s - W_i)/W_s) \times 100$, where W_s is the weight of the swollen test sample and W_i is its initial dry weight. Results were averaged on four independent runs.

2.4 Iris pigment epithelial cell cultures

All animal procedures were approved by the Institutional Review Board and were performed in accordance with the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-eight Long-Evans rats (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan, ROC) were used for this study. Primary rat IPE cells were prepared according to previously published procedures [9]. Briefly, the eyes were enucleated immediately after the death of the animals. The iris was removed from the anterior segment with forceps and incubated in a solution containing trypsin–EDTA for 20 min at 37°C. The iris tissues were then placed in regular growth medium containing Ham's F-12, 15% FBS, and 1% A/A solution. The IPE cells were gently separated from the stroma by microdissection with a flame-polished pipette, and a cell pellet was collected via centrifugation. Thereafter, rat IPE cells were resuspended and maintained in regular growth medium. Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed twice weekly until the cells had reached confluence. Cells were subcultured by treating with trypsin–EDTA and seeded at a 1:3 split ratio. Only second-passage cells were used for experiments.

2.5 In vitro biocompatibility tests

The sterilized gelatin hydrogels were completely dissolved in fresh culture medium at 37°C. After 48 h of incubation with medium containing gelatin materials, the cultures were examined by various techniques. The gelatin was presented as a soluble protein on the cell layers during in vitro biocompatibility testing. Rat IPE cells in regular growth medium without gelatins served as control groups.

2.5.1 Cell proliferation assays

Rat IPE cells with a density of 5 × 10^4 cells/ml were seeded into 24-well plates by 1 ml/well, and incubated at 37°C overnight to allow cell attachment. After 2 days of culture with gelatin, cell proliferation was determined by lactate dehydrogenase (LDH) assay (Promega, Madison, WI, USA), which measures cell number via total cytoplasmic LDH released upon cell lysis [26]. Attached cells were incubated in lysis solution for 50 min at 37°C, and centrifuged at 2000 rpm for 4 min. Subsequently, 50 μl/well supernatants were transferred to a fresh 96-well plate, and mixed with 50 μl of LDH assay solution. After incubation at room temperature for 30 min, the reaction was terminated by the addition of 1 N HCl. The data of absorbance readings at 490 nm were measured using the Multiskan Spectrum Microplate Spectrophotometer (ThermoLabsystems, Vantaa, Finland), and referenced to a standard curve of absorbance versus cell number as determined by hemacytometer. All experiments were performed in quadruplicate.

2.5.2 Cell viability assays

Rat IPE cells were seeded in 24-well plates containing regular growth medium and allowed to grow to near

confluence. After 2 days of culture with gelatin, cell viability was determined using a membrane integrity assay, the Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR, USA) which contains calcein AM and ethidium homodimer-1 (EthD-1). It depends on the intracellular esterase activity to identify the living cells, which cleaves the calcein AM to produce a green fluorescence. In dead cells, EthD-1 can easily pass through the damaged cell membranes to bind to the nucleic acids, yielding a red fluorescence. After washing three times with PBS, the cultures were stained with a working solution consisting of 2 μl of EthD-1, 1 ml of PBS, and 0.5 μl of calcein AM. Under fluorescence microscopy (Axiovert 200 M; Carl Zeiss, Oberkochen, Germany), three different areas each containing approximately 500 cells were counted at 100 × magnification. All experiments were performed in duplicate, and the viability of the cell cultures was expressed as the average ratio of live cells to the total number of cells in these six different areas.

2.5.3 *Pro-inflammatory gene and cytokine expression analyses*

Rat IPE cells were grown to near confluence on 24-well plates in regular growth medium. Following 2 days of culture with gelatin, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) expression were detected at both messenger RNA (mRNA) and protein levels. Total RNA was isolated from rat IPE cells with TRIzol reagent according to the manufacturer's procedure. Reverse transcription of the extracted RNA (1 μg) was performed using ImProm-II (Promega) and Oligo$(dT)_{15}$ primers (Promega). The sequences of the primer pairs for each gene are listed in Table 1. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was performed on a LightCycler instrument (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions with FastStart DNA Master SYBR Green I reagent (Roche Diagnostics). Each sample was determined in triplicate and the results for IL-1β and TNF-α were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. On the other hand, aliquots of the supernatant were collected to measure the IL-1β and TNF-α levels. The release of IL-1β and TNF-α from cultivated cells into the conditioned medium was detected by the Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) specific for rat IL-1β and TNF-α. Cytokine bioassays were performed according to the manufacturer's instructions. Photometric readings at 450 nm were measured using the Spectrophotometer (ThermoLabsystems). Results were expressed as pg/ml. All experiments were conducted in quadruplicate.

2.6 Animals and surgery

Twenty-four adult New Zealand white rabbits (National Laboratory Animal Breeding and Research Center), weighing from 3.0 to 3.5 kg and 16–20 weeks of age, were used for in vivo biocompatibility testing. Animals were healthy and free of clinically observable ocular surface disease. Surgical operation was performed in the single eye of animals, with the normal fellow eye. In the three test groups (Gel, GTA-Gel and EDC-Gel) of animals (six rabbits/group), the gelatin implants were inserted in the anterior chamber of the eye. The remaining six rabbits received no implant (only corneal/limbal incision) and served as a control group (Ctrl).

The rabbits were anesthetized intramuscularly with 60 mg/kg body weight of ketamine hydrochloride (Imalgene; Merial, Lyon, France) and 10 mg/kg body weight of xylazine hydrochloride (Chanazine; Chanelle Pharmaceuticals, Loughrea, Co. Galway, Ireland), and topically with two drops of 0.5% proparacaine hydrochloride ophthalmic solution (Alcaine; Alcon-Couvreur, Puurs, Belgium). After disinfection and sterile draping of the operation site, the pupil was dilated with one drop of 1% atropine sulfate ophthalmic solution (Oasis, Taipei, Taiwan, ROC), and a lid speculum was placed. Under the surgical microscope (Carl Zeiss, Oberkochen, Germany), the cornea was penetrated near the limbus by using a slit knife. Then, the corneal/limbal incision was enlarged to 7.5 mm with corneal scissors to allow the insertion of material implants in the anterior chamber. The incision site was finally closed with 10-0 nylon sutures, and 1% chlortetracycline hydrochloride ophthalmic ointment (Union Chemical & Pharmaceutical, Taipei, Taiwan, ROC) was immediately applied to the ocular surface.

2.7 In vivo biocompatibility tests

To determine the implant-tissue interaction in the anterior chamber, the rabbits were anesthetized under the same

Table 1 Sequences of primers used in gene expression analyses

Genes	Forward (5′-3′)	Reverse (5′-3′)
IL-1β	GATGATGACGACCTGCTAGTGT	GGATTTTGTCGTTGCTTGTCTC
TNF-α	AGGAGGAGAAGTTCCCAAATG	GGTTGTCTTTGAGATCCATGC
GAPDH	GTGATGCTGGTGCTGAGTATGT	ATGCATTGCTGACAATCTTGAG

conditions as for surgery. Ophthalmic evaluations were performed before and immediately after surgical insertion of material implants. Subsequently, we examined the bilateral eyes of 24 rabbits at postoperative 6, 12 h, and daily for 4 weeks, and thereafter twice weekly for 12 weeks.

2.7.1 Clinical observations

At each study point, the intraocular pressure (IOP) was measured using a Schiotz tonometer (AMANN Ophthalmic Instruments, Liptingen, Germany), calibrated according to the manufacturer's instructions. For each IOP determination, five readings were taken on each eye, alternating the left and right eyes, and the mean was calculated. The IOP values of the contralateral normal eyes were used as baseline readings. Data were expressed as the difference from baseline values at each time point. Central corneal thickness (CCT) was determined using an ultrasonic pachymeter (DGH Technology, Exton, PA, USA) with a hand-held solid probe. During the measurements, the probe tip of the pachymeter was held perpendicular on the central cornea. An average of ten readings was taken.

2.7.2 Scanning electron microscopy studies

The corneal samples were processed for SEM observations in conformity with described procedures [6]. At the end of experiments, the animals were euthanized with CO_2 gas. The excised rabbit corneas were fixed with 2% GTA in 0.1 M cacodylic acid buffer (pH 7.4), and then post-fixed in 1% osmium tetroxide. After fixation, the tissues were dehydrated in a graded series of ethanol solutions and dried with carbon dioxide in a critical point dryer (Balzers, Liechtenstein). The specimens were gold coated in a sputter coater (Hitachi, Tokyo, Japan) before examination under a Hitachi S-3000N SEM with an accelerating voltage of 10 kV.

2.8 Statistics

Results were expressed as mean ± standard deviation. Comparative studies of means were performed using one-way analysis of variance (ANOVA). Significance was accepted with $P < 0.05$.

3 Results and discussion

3.1 Cross-linking of gelatin hydrogels by GTA or EDC

Gelatin is a highly hygroscopic molecule which is composed of 20 amino acid residues in different proportions. We have previously shown that gelatin can absorb a tremendous amount of water at up to 10 times its weight [5]. The mechanism of hydration of gelatin is a capillary phenomenon of water molecules penetrating the tiny interstices of triple-helical fibrils in the gelatin matrix [27]. In this study, the gelatin discs largely disintegrated into small fragments after 6 h of swelling, and their water contents were limited. To overcome the problems imposed by the very rapid dissolution of gelatin materials, the GTA or EDC was used for chemical modification. The water content of cross-linked gelatin hydrogels was determined as a measure to compare them in terms of extent of cross-linking [25]. Our results indicated that after 80 min of treatment with 50 mM GTA, the cross-linked gelatin hydrogels have water content of about 92%. To achieve the same extent of cross-linking, the gelatin samples must be incubated with 50 mM EDC for 24 h. This implies that the extent of cross-linking of gelatin hydrogels may depend on the chemistry of cross-linking agents. Sung and coworkers have compared the cross-linking efficiency of different reagents for gelatin microspheres [28]. The degrees of cross-linking for both the GTA and genipin treated samples were approximately 60% by adjusting their cross-linking duration for 45 min and 72 h, respectively, which suggested that the GTA is more reactive than genipin during chemical cross-linking. Similar to their findings, our results indicate that the GTA produces stronger cross-linking to gelatin molecules than using EDC. To examine the effects of chemical cross-linkers on biocompatibility of gelatin hydrogels, the GTA and EDC treated materials with the same extent of cross-linking in the present work were characterized by various in vitro and in vivo assays.

3.2 Cell proliferation assays

The LDH assay measures LDH released into the culture supernatant upon damage of cell membranes. Therefore, an increase in LDH release reflects increased dead cells. Figure 1 shows the results of quantitative analysis for rat IPE cell growth. After 2 days of culture, the cell number in control group reached about $5.5 \pm 0.2 \times 10^4/cm^2$ compared with the initial cell seeding density i.e., $2.5 \times 10^4/cm^2$. Similar results were found in Gel and EDC-Gel groups, which indicate that the exposure to non-cross-linked and EDC cross-linked gelatin hydrogels did not significantly affect cell proliferation ($P > 0.05$). However, the cells of GTA-Gel group showed significantly lower density than did those of control group ($P < 0.05$). It implied that the GTA cross-linked gelatin hydrogels could markedly reduce the proliferative capacity of rat IPE cells.

Burugapalli et al. have demonstrated that the GTA cross-linked tissue matrices fail to support the proliferation of murine 3T3 fibroblasts [29]. Normally, cells are cultured

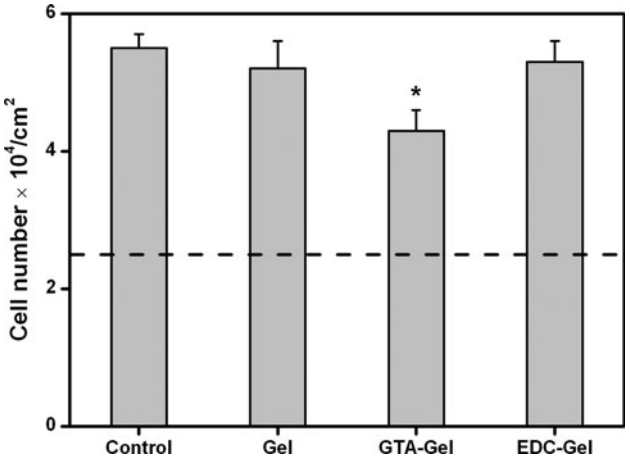

Fig. 1 Cell proliferation assay of rat IPE cell cultures incubated in the presence of various dissolved gelatin materials for 2 days at 37°C. The dash line represents the initial cell seeding density ($2.5 \times 10^4/cm^2$). An *asterisk* indicates statistically significant differences (* $P < 0.05$; $n = 4$) as compared to controls (without materials)

in vitro in an atmosphere of 5% CO_2 in the presence of media containing nutrients. It has been reported that the ionic interactions between added biopolymers and nutrients in culture media affect the regulation of cell activity and growth. The nutrient components (i.e., basic fibroblast growth factor and transforming growth factor-β) could be easily enveloped in the molecules of negatively charged hyaluronic acid, thereby leading to less cell proliferation [30]. Gelatin is zwitterionic, carrying both free carboxyl and free amine functionalities. EDC cross-linking involves the reaction between free carboxylic acid groups and amino groups of gelatin to form amide bonds, and this process reduces equal amounts of both functional groups in gelatin. In contrast, GTA reacts primarily with the reactive groups, generally free amino groups in lysine residues. Thus, the GTA cross-linked gelatin hydrogels contain larger amounts of free carboxylic acid groups. The imbalance of functional groups may influence the strength of ionic interactions and cause insufficient nutrient availability to the cultured IPE cells.

3.3 Cell viability assays

Figure 2 shows representative photographs of rat IPE cells labeled with Live/Dead stain, where the live cells fluoresce green and the dead cells fluoresce red. Large percentage of live cells was observed in the control and Gel groups after 2 days of culture on 24-well plates (Fig. 2a–d). The number of red-stained nuclei in the GTA-Gel groups increased with respect to all groups, indicating that the GTA cross-linked gelatin hydrogels induced significant cytotoxicity (Fig. 2e, f). The IPE cultures exposed to EDC-Gel samples maintained good viability with only a few dead cells (Fig. 2g, h). Figure 3 shows the mean percentage of live cells as determined by the Live/Dead assay. The cell viability did not show a significant difference between the control (98.9 ± 0.8%), Gel (97.4 ± 0.9%), and EDC-Gel groups (97.1 ± 1.3%; $P > 0.05$). However, the exposure of cells to GTA-Gel samples could cause significant reduction of viability (about 22% compared to control groups) in rat IPE cultures ($P < 0.05$). These data suggest that the gelatin hydrogels cross-linked with EDC have better cytocompatibility than those with GTA.

In vitro cytotoxicity testing aims to screen the biocompatibility of surgical implant materials and biomedical devices. Our previous studies have shown that the biocompatibility of gelatin is affected by the intrinsic nature of the material including charge, molecular weight, and Bloom index [5, 9]. The viability of retinal pigment epithelial cell line cultures exposed to gelatin (Bloom index = 75–100) was assessed by Live/Dead stain and found to be 97–99% [21]. The results of this study also demonstrated a relatively high viability of rat IPE cells after exposure to the gelatin with the same Bloom strength. The cytotoxicity of cross-linked biopolymers appears to be associated with specific linkages present in the cross-linking structure. While cross-linking of gelatin with GTA involves the formation of short aliphatic chains or pyridinium compounds [23], the cross-linking by carbodiimide occurs with no added bridging moieties [18]. Amide-type cross-linkers such as EDC may offer the advantage of lower toxicity and better compatibility over GTA [31]. In our experiments, obvious cell death was observed in the cultures exposed to GTA-Gel samples for 2 days. The findings clearly demonstrated that GTA cross-linking greatly affects the interaction between gelatin hydrogels and rat IPE cells.

3.4 Pro-inflammatory gene and cytokine expression analyses

The IL-1β and TNF-α expression at both mRNA and protein levels were characterized using the techniques of quantitative real-time RT-PCR and ELISA. Figure 4 shows the pro-inflammatory gene expression of rat IPE cells exposed to various gelatin hydrogels for 2 days. There was no statistically significant difference in the IL-1β gene expression between the control, Gel and EDC-Gel groups ($P > 0.05$). However, in the GTA-Gel groups, the IL-1β expression was significantly higher than that of the other groups ($P < 0.05$). Similarly, no significant change in TNF-α expression was found for the non-cross-linked and EDC cross-linked gelatin samples. The TNF-α gene expression remained significantly higher than the other groups ($P < 0.05$). On the other hand, Fig. 5 shows the rat

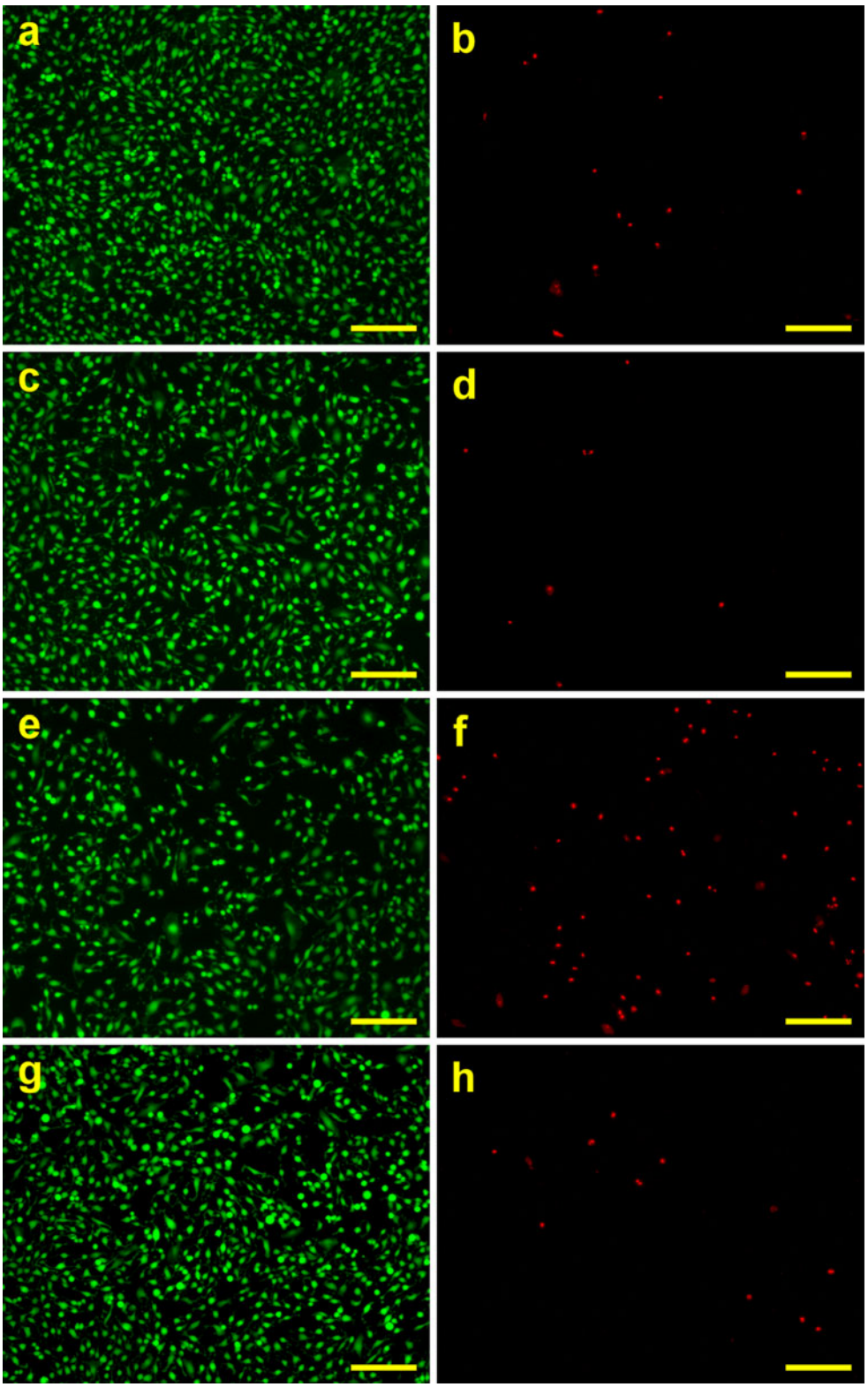

Fig. 2 Cell viability of rat IPE cultures was determined by staining with Live/Dead Viability/Cytotoxicity Kit in which the live cells fluoresce green and the dead cells fluoresce red. *Green* (**a**, **c**, **e**, **g**) and *red* (**b**, **d**, **f**, **h**) fluorescence images of cells in **a**, **b** controls (without materials) after exposure to various dissolved gelatin materials of **c**, **d** Gel; **e**, **f** GTA-Gel; and **g**, **h** EDC-Gel for 2 days at 37°C. Scale bars: 100 μm

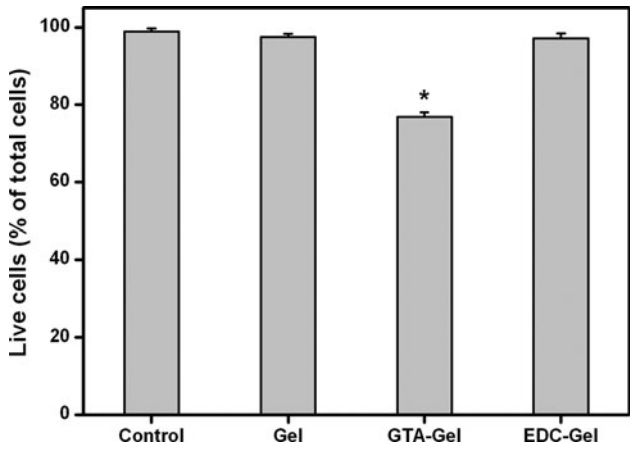

Fig. 3 Mean percentage of live cells in the rat IPE cultures exposed to various dissolved gelatin materials as measured by the Live/Dead assay. An *asterisk* indicates statistically significant differences (* $P < 0.05$; $n = 6$) as compared to controls (without materials)

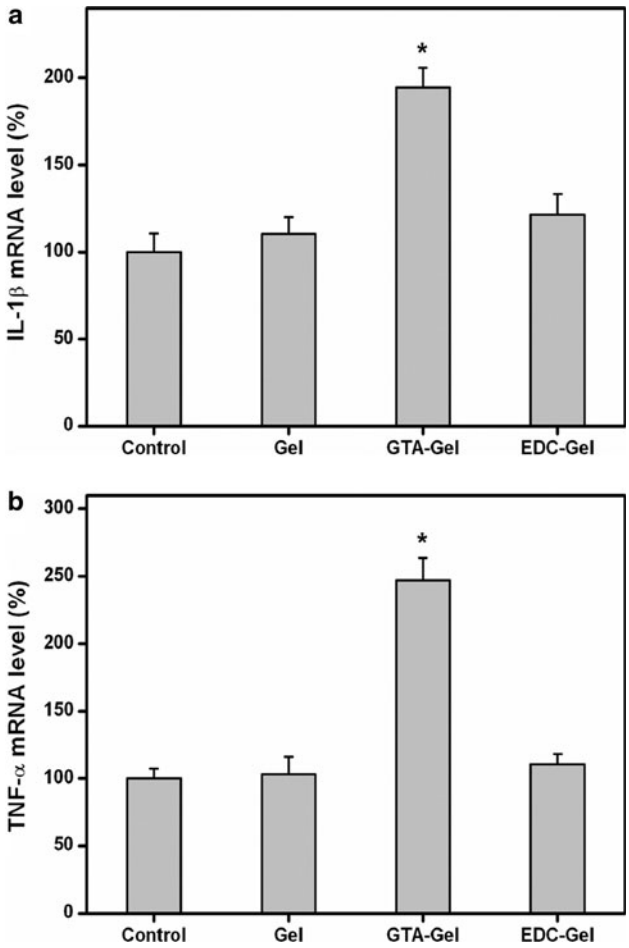

Fig. 4 Gene expression of **a** IL-1β and **b** TNF-α in rat IPE cells incubated with various dissolved gelatin materials for 2 days by real-time RT-PCR. Normalization was done by using GAPDH. Data in the experimental groups are percentages relative to that of control groups (without materials). An *asterisk* indicates statistically significant differences (* $P < 0.05$; $n = 3$) as compared to controls

IPE cell secretion of IL-1β and TNF-α in response to various gelatin hydrogels. The measured concentration of IL-1β in the GTA-Gel groups was 326.4 ± 39.1 pg/ml, which was significantly higher than those in the control (158.3 ± 30.6 pg/ml), Gel (177.1 ± 29.8 pg/ml), and EDC-Gel (192.5 ± 23.2 pg/ml) groups ($P < 0.05$). When compared to the controls, the cells exposed to the non-cross-linked and EDC cross-linked gelatin hydrogels did not show a significant difference in TNF-α gene expression ($P > 0.05$). The GTA cross-linked gelatin hydrogels induced significantly higher levels of TNF-α in rat IPE cultures. These findings suggest that while the culture with GTA cross-linked gelatin hydrogels up-regulates pro-inflammatory gene expression and stimulates pro-inflammatory cytokine production, the exposure of IPE cells to EDC treated samples does not promote inflammation.

Several investigators have demonstrated that analysis of pro-inflammatory gene and cytokine expression is a powerful method for studying biomaterial biocompatibility. In 2003, Brodbeck et al. identified hydrophilic surface chemistries as having significant effects on leukocyte cytokine responses in vivo by decreasing the expression of inflammatory and wound healing cytokines by inflammatory cells adherent to the biomaterial as well as present in the surrounding exudate [32]. Later, Kim et al. investigated the temporal cytokine expression profile from human THP-1 monocytes exposed to phagocytosable Ti particles and to Ti discs of comparable surface roughness [33]. Their study seems to show that the cells treated by disc samples may produce in many instances a higher cytokine expression than did particles. A recent report from Sandeman et al. indicated that hydroxyapatite coated carbon matrices may suppress excessive inflammation by adsorption of the cytokine IL-8 into the porous, internal carbon structure and these materials can provide a suitable environment to enhance in-growth of corneal cells without inducing further inflammation [34]. In studies using human corneal endothelial cells [5] and retinal pigment epithelial cell lines [21], our group has previously measured secretion of IL-6 in response to gelatin materials. Pro-inflammatory cytokines such as IL-1β and TNF-α have been found to be involved in the local reaction of retinal tissues after the early days of transplantation of rat IPE cells into the sub-retinal space [35]. Thus, in the present work, the expression of cytokines IL-1β and TNF-α by rat IPE cells interacting with the materials was used as an indication of the inflammatory potential of the chemically cross-linked gelatin hydrogels. Our results show that the chemical cross-linkers have a profound effect on the cellular inflammatory reactions to gelatin.

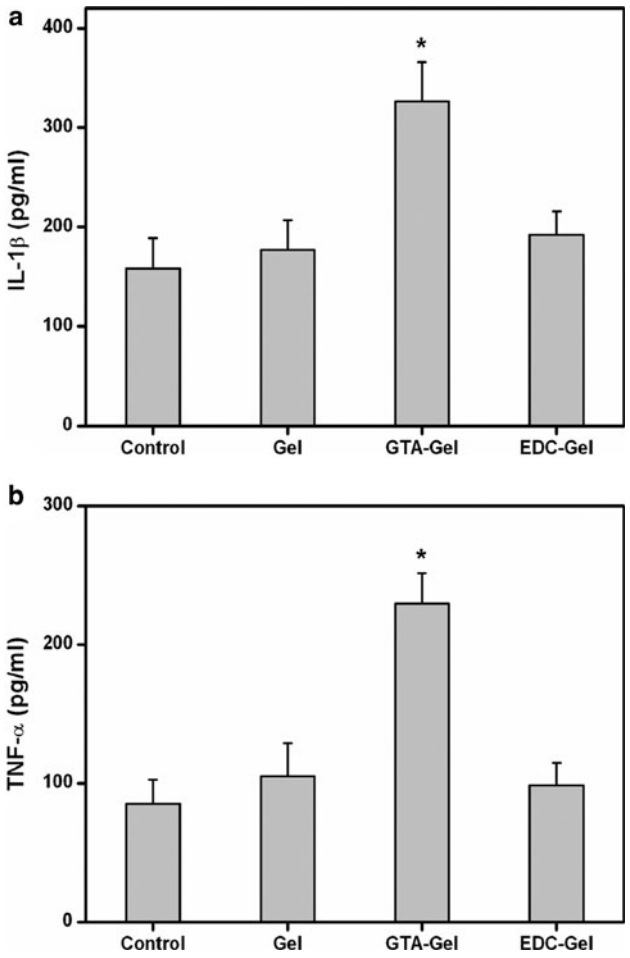

Fig. 5 Level of **a** IL-1β and **b** TNF-α released from rat IPE cell cultures after incubation with various dissolved gelatin materials for 2 days. An *asterisk* indicates statistically significant differences (* $P < 0.05$; $n = 4$) as compared to controls (without materials)

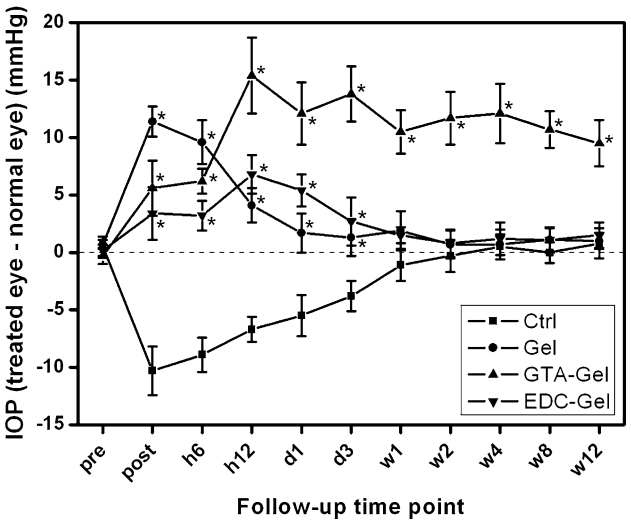

Fig. 6 Measurements of IOP after insertion of various gelatin implants in the anterior chamber. An *asterisk* indicates statistically significant differences (* $P < 0.05$; $n = 6$) when compared with Ctrl (sham-operated rabbits). Follow-up time point: preoperation (pre); postoperation (post); hour (h); day (d); week (w)

3.5 Clinical observations

In this study, the IOP and CCT were measured to evaluate the ocular biocompatibility of chemically cross-linked gelatin implants. Figure 6 shows the IOP profile of the four groups. Mean baseline IOP values ranged from 16.4 to 18.9 mmHg. There was a sharp fall in the IOP of Ctrl groups immediately after surgery, presumably due to the loss of a large amount of the aqueous humor through the corneal/limbal incision. The IOP changes reverted to baseline level within 1 week, indicating healing of incision wounds with time. By contrast, the IOP values were increased by the implantation of various gelatin samples into the anterior chamber of the eye. It is speculated that a complete filling of the graft site with gelatin hydrogel may generate excessive extrusion pressures. Another possibility is the continued presence of foreign materials that increases aqueous humor outflow resistance in the trabecular meshwork. Our data showed that different gelatin implants resulted in different extent of increase in IOP. In the Gel groups, the hydrogel swelling was extremely rapid, thereby causing elevated IOP immediately after surgery. The subsequent in vivo degradation of the biopolymer decreased the pressure in the anterior chamber. Within postoperative 1 day, the IOP changes reverted to baseline level. In both the GTA-Gel and EDC-Gel groups, the IOP peaked at 12 h after surgery. The delayed increase in IOP was probably due to inhibited swelling of chemically cross-linked gelatin hydrogels. It was noted that the IOP in the EDC-Gel groups gradually decreased and returned to the baseline values 1 week postoperatively. However, in rabbits receiving GTA-Gel implants, the IOP was maintained at a high level (i.e., about 30 mmHg) during the follow-up period of 12 weeks. It implied that the GTA cross-linked gelatin samples and their degradation products caused elevated pressure from aqueous humor circulation.

Figure 7 shows the mean CCT changes of the surgical corneas of the four groups. The mean preoperative CCT of all 24 eyes was 420.8 ± 21.1 μm. During the whole follow-up period, the CCT values in the Ctrl groups were maintained in the normal range. The results indicate that the sham operation (i.e., only corneal/limbal incision) causes no significant CCT changes. Nonetheless, it was evident that the non-cross-linked and chemically cross-linked gelatin implants in the anterior chamber contributed to corneal thickness variations. The cornea consists of collagen fibers and is a flexible membrane of high tensile strength. The rigidity of material implants may allow the mechanical stretching of corneal fibers and the reduction of

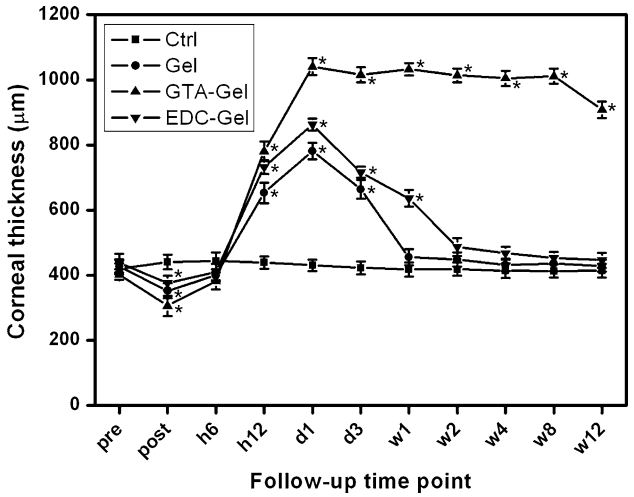

Fig. 7 Measurements of CCT after insertion of various gelatin implants in the anterior chamber. An *asterisk* indicates statistically significant differences (* $P < 0.05$; $n = 6$) when compared with Ctrl (sham-operated rabbits). Follow-up time point: preoperation (pre); postoperation (post); hour (h); day (d); week (w)

corneal thickness. Therefore, the CCT values in the three test groups were decreased immediately after surgery. Our data showed that this temporary corneal deformation disappears on swelling of the gelatin. In both the Gel and EDC-Gel groups, the CCT peaked at postoperative 1 day and then declined. Two weeks later, the thickness of EDC-Gel groups returned to nearly original values. By contrast, the CCT in the GTA-Gel groups was increased to a value larger than 1000 μm and remained at a high level throughout the experimental time period. The findings suggest that the exposure to the GTA-Gel implants caused continued corneal edema.

After surgical insertion into the eye, the test materials came into contact with physiological fluids and anterior segment tissues. We therefore performed IOP and CCT measurements. The IOP has been used as an indicator of the effect of implants on the balance between aqueous humor secretion and drainage [24]. It is known that glaucoma patients usually have a documented history of elevated IOP (i.e., >21 mmHg) [36]. In comparison to the critical value, the elevated IOP induced by the GTA-Gel samples is smaller, but is sufficient to cause a high risk of ocular disease progression. When implanted in the anterior chamber of rabbit eyes, the GTA cross-linked gelatin hydrogels may be harmful to ciliary body and trabecular meshwork cells, thereby affecting the regulation of aqueous humor production and its outflow through the canal of Schlemm. In addition, corneal thickness changes generally reflect alterations in hydration and metabolism [37]. It has been reported that increased IOP itself, if present for at least 3 days, damages the corneal endothelium and reduces its cell density [38]. The results of clinical observations indicate that the long-term elevated IOP caused by the implantation of GTA-Gel discs may lead to significant corneal edema. The EDC cross-linked gelatin implants in the anterior chamber are well tolerated, triggering an acute inflammatory reaction that quickly resolves.

3.6 Scanning electron microscopy studies

SEM studies were performed at 12 weeks after disc implantation to examine the morphological characteristics of rabbit corneal endothelium. Figure 8 shows representative SEM images from each group. In the control groups, the endothelial cells on the Descemet's membrane packed together and had distinct borders (Fig. 8a). Both microvilli and cilia could be observed on the apical cell surface of native corneal endothelium. These cells were of relatively uniform size, with an average diameter of 20 μm. It is known that the normal corneal endothelial cells exhibit a typical hexagonal shape and form a single continuous monolayer [6]. Our results indicate that the sham operation does not affect cell morphology or lead to endothelial damage. After exposure to non-cross-linked gelatin implants, the corneal endothelial cells showed similar morphological features to those in the control rabbits (Fig. 8b). However, in the GTA-Gel groups, the hexagonal cell shape was severely affected (Fig. 8c). A large number of malformed cells were found which frequently lost their contacts with other cells. It was noted that the corneal endothelium surrounding the EDC cross-linked gelatin hydrogels did not reveal any morphological abnormalities, indicating good biocompatibility in vivo (Fig. 8d). The endothelial cells of EDC-Gel-implanted rabbits exhibited significantly higher percent hexagonality than did those of GTA-Gel-implanted rabbits.

Many of the biomaterials currently in use as medical devices (i.e., therapeutic implants and interventional devices) have been tested through various methodological studies using in vitro cell cultures [39, 40] and in vivo animal models [41, 42]. Biocompatibility assessment of newly developed materials is needed to analyze their potential for ophthalmic use. Huhtala et al. have evaluated the cytocompatibility of degradable poly(lactic-*co*-glycolic acid) based biopolymers in five ocular cell line cultures [43]. Einmahl et al. also studied the ocular biocompatibility of poly(ortho ester) after subconjunctival, intracameral, intravitreal, and suprachoroidal injections in rabbit eyes [44]. In this work, using primary rat IPE cell culture system and anterior chamber of rabbit eyes model, we investigated the in vitro and in vivo biocompatibility of chemically cross-linked gelatin hydrogels. The results suggest that while the EDC cross-linked gelatin materials are satisfactorily biocompatible, the GTA treated samples do not seem to be tolerated.

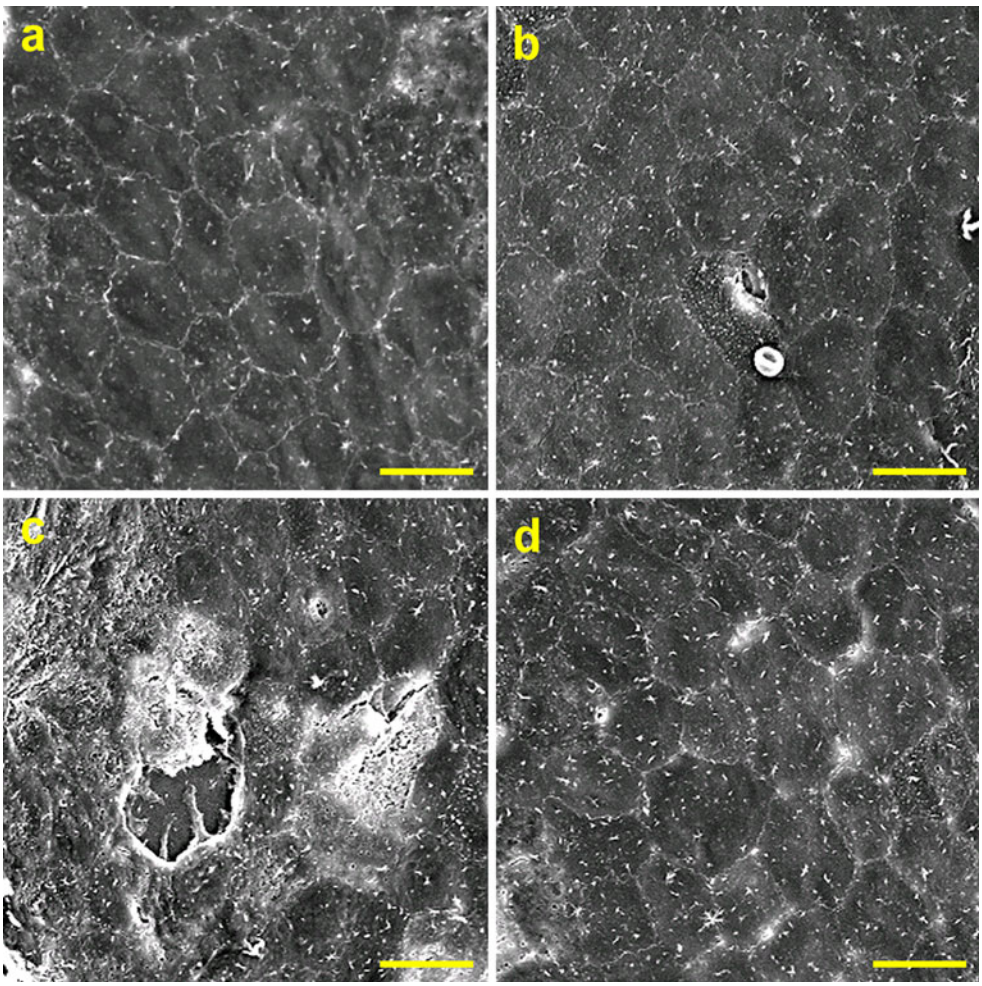

Fig. 8 Representative SEM images of corneal endothelium 12 weeks after insertion of various gelatin implants in the anterior chamber of rabbit eyes. **a** Ctrl (sham-operated), **b** Gel, **c** GTA-Gel, and **d** EDC-Gel groups. Scale bars: 20 μm

The toxicity of cross-linking agents presents a major drawback in the use of gelatins as ophthalmic biopolymers, because their seepage into body fluids can be detrimental to living cells/tissues, even at low concentrations [45]. In the gelatin matrix, the carbodiimides do not remain as part of the cross-links, but rapidly react to form urea derivatives that have very low toxicity [46]. By contrast, GTA has very reactive properties and is easily interacted with cell surface, thereby leading to apoptosis and cytotoxicity [47]. Several methods have been used to minimize the adverse biological effects of GTA. The inhibition of calcification of GTA-fixed porcine aortic valve cusps with ethanol pretreatment can be enhanced through the use of sodium borohydride, which reduces the exposed aldehyde groups to hydroxyl groups [48]. However, it should be noted that the treatment with sodium borohydride will not only reduce the aldehydes but also the Schiff bases. In another study, Lee et al. have demonstrated that the additional grafting of sulfonated poly(ethylene oxide) or heparin following chemical fixation may improve biocompatibility and calcification resistance of bovine pericardium [49]. The GTA cross-linked gelatin films are treated with glycine solution since the aldehyde groups can react with the amino groups of glycine, resulting in conversion of the aldehyde into a carboxyl group [23]. In the present work, to block unreacted aldehyde groups remaining in the GTA cross-linked hydrogels, the gelatin materials were immersed in 100 mM glycine aqueous solution at 37°C for 1 h. Nevertheless, the GTA-Gel samples were less biocompatible than their EDC-Gel counterparts. As already mentioned, the presence of larger amounts of carboxylic acids in the GTA cross-linked gelatins probably affected the cell-material interactions. Because the use of GTA involves very complex reactions, the possibility of producing polymeric forms of GTA cannot be excluded during the cross-linking [19, 23]. Although capping of residual aldehyde groups of GTA with glycine can reduce the toxicity of cross-linker, this reaction is less effective due to that the formation of Schiff base is an equilibrium process

and glycine may be released, exposing again the aldehydes. Other groups have developed alternative cross-linkers, including genipin [50] and malic acid derivative [51] for cross-linking of collagenous biomaterials.

4 Conclusions

Biocompatibility is a major requirement for the development and applications of functional biomaterials. In this paper, the biocompatibility of GTA and EDC cross-linked gelatin hydrogels were analyzed using in vitro and in vivo ocular assays. The understanding of the cell/tissue-material interactions by which chemical cross-linkers affect ocular biocompatibility will facilitate the engineering of the appropriate cross-linked gelatin materials to enhance the local delivery of cell and drug therapeutics. The present study demonstrates that the cells of EDC-Gel group have relatively lower LDH activity, cytotoxicity, and IL-1β and TNF-α levels than did those of GTA-Gel group. In addition, the EDC-Gel discs exhibit good biocompatibility and are well tolerated without causing toxicity and adverse effects. However, a significant inflammatory reaction is elicited by the presence of GTA-Gel samples. In conclusion, these findings suggest ocular cell/tissue response to changes in cross-linker functionality. In comparison to GTA treatment, the EDC cross-linking is more suitable for preparation of chemically modified gelatin hydrogels for ophthalmic use.

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