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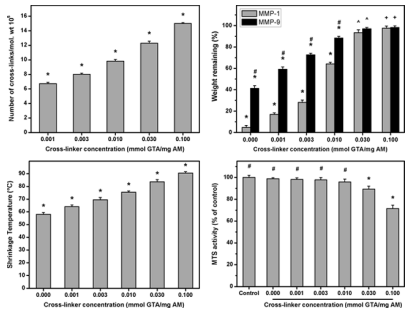
Chemical cross-linker concentration has a marked influence on the interrelationship between cross-linking structure, molecular stability, and cytocompatibility of glutaraldehyde-treated amniotic membrane for a limbal stem cell niche.



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ARTICLE TYPE

# Interrelationship between cross-linking structure, molecular stability, and cytocompatibility of amniotic membranes cross-linked with glutaraldehyde of varying concentrations

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The aim of this study was to investigate the interrelationship between cross-linking structure, molecular stability, and cytocompatibility of glutaraldehyde (GTA)-treated amniotic membrane (AM). With increasing cross-linker concentration from 0.001 to 0.1 mmol GTA/mg AM, the cross-linking index and number of cross-links per unit mass significantly increased. Inversely, the average molecular weight of polymer chains between two consecutive junctions was significantly decreased with an increase in cross-linking density. The shrinkage temperature and weight remaining of the chemically modified collagenous tissues were respectively used for the assessment of their resistance to thermal denaturation and enzymatic degradation. Our results demonstrated that the collagen molecular stability was enhanced considerably in the presence of higher amount of non-zero-length cross-linkers. The AM matrices treated with low cross-linker concentrations (i.e., < 0.03 mmol GTA/mg AM) displayed good compatibility with human corneal epithelial cells. In contrast, the samples with the greatest extent of cross-linking may cause marked alteration in cell morphology and decrease in cell viability. Although the biological tissues cross-linked with GTA still possessed anti-inflammatory actions, the interleukin-6 cytokine levels in lipopolysaccharide-stimulated cells were increased with increasing cross-linking density. The up-regulated stemness gene expression was positively correlated with the degree of GTA cross-linking, suggesting the chemical cross-linking structure-mediated preservation of limbal epithelial progenitor cells. It is concluded that during cross-linking treatment, the GTA concentration is critical to tailor the properties of chemically modified AM for a limbal stem cell niche.

## Introduction

In clinical ophthalmology, the amniotic membrane (AM) is a valuable tissue material that has low immunogenicity, anti-inflammatory, anti-angiogenic, and anti-scarring properties.[1] For the past decade, this biological tissue has been considered as an artificial matrix for corneal cell cultivation and tissue engineering. The group of Tseng has reported that AM is a stem cell niche that can support the expansion of corneal epithelial progenitor cells.[2] Nakamura et al. have also examined the feasibility of introducing sterilized, freeze-dried AM as a substrate for the growth of autologous corneal epithelial cells for ocular surface reconstruction.[3] To maintain the characteristic cell morphology and keratocan expression, Espana et al. have developed a method of using the stromal matrix of AM for proliferating human corneal keratocytes in serum.[4] Later work from the same group has revealed that the promoter activity of transforming growth factor-β2 is down-regulated when murine corneal keratocytes are expanded on AM substrates.[5] For the treatment of corneal diseases caused by endothelial dysfunctions, AM has been used as a biomaterial carrier of human corneal endothelial cells.[6] More recently, Fan et al. have performed tissue reconstruction by the transplantation of human corneal endothelial cells cultured on denuded AM and found that the tissue-engineered corneal endothelium is able to restore tissue function in a cat model.[7]

Although the AM displays several advantages as an important biological tissue material, its ophthalmic application is sometimes limited due to poor biostability. The manipulation of soft and fragile AM is technically not easy to implement. As reported in the literature,[8] the exposure of fresh amnions to the endogenous enzyme can contribute to the rapid degradation (i.e., within 7 days). In particular, the accelerated disintegration of these biological specimens is noted when used in certain corneal diseases causing increased action of tissue collagenase.[9] It is highly desirable to improve the material stability against enzymatic cleavage. Given that the AM is primarily made of collagen, the induced formation of peptide linkages between amino acids is a promising way to reinforce the structural strength of the biological tissue. We have previously proposed the use of carbodiimide as a chemical cross-linker for the modification of AM.[10] The generation of amide bonds (i.e., cross-links) within the AM collagen is achieved with the release of water-soluble urea by-products that have very low

cytotoxicity.[11,12] But the carbodiimide cross-linking of AM is ineffective in preventing significant weight loss of proteinaceous matrices after 4 weeks of degradation.[13]

Because of its high performance for collagen stabilization, glutaraldehyde (GTA) is another class of cross-linker frequently used in the chemical modification of biological tissue materials such as vascular grafts, heart valves, and elastic cartilages.[14] The two aldehyde groups of the GTA can react with free amino groups of the polypeptide chains to form the Schiff bases, resulting in the cross-linking of bioprostheses. We have shown that the cross-linking agent type has an important influence on the characteristics of modified biomaterials. In comparison with its carbodiimide counterpart at the same cross-linker concentration, the GTA treatment is able to render the gelatin network more stable against mechanical stress and enzymatic degradation.[15] Furthermore, GTA also tends to produce stronger cross-linking to hyaluronic acid molecules because the treated hydrogel discs have lower water content than carbodiimide cross-linked materials.[16] These observations suggest the possibility that GTA may be an efficient cross-linker for improving the biostability of AM. Nevertheless, the protein-based biomaterials treated with GTA may pose a risk for toxicity in living cells/tissues.[17] Hence, after AM collagen cross-linking, the residual aldehyde groups are capped with glycine, which can further reduce the cytotoxicity of chemical compounds.[18]

During the cross-linking process, the concentration of cross-linkers is a crucial parameter in determining the applicability of the chemically modified biomaterials. The effect of carbodiimide concentration on the preparation of gelatin membranes for retinal sheet encapsulation and transplantation has been investigated by us.[19] The delivery efficiency of carrier system is significantly enhanced with increasing cross-linker concentration from 0.001 to 0.02 mmol carbodiimide/mg gelatin membrane. It is also noted that the treatment with 0.1-0.4 mmol carbodiimide/mg gelatin membrane may lead to poor biocompatibility although these high cross-linker concentrations are beneficial to provide a stable encapsulating structure for preventing the disruption of embedded tissue laminate. It motivates our group to explore the interrelationship between cross-linking structure, molecular stability, and cytocompatibility of chemically modified AM matrices. The purpose of this work was to optimize the procedure of GTA cross-linking of AM for the fabrication of limbal epithelial cell (LEC) scaffolds. After the treatment with varying cross-linker concentrations, the AM samples were evaluated by means of ninhydrin assays and cross-linking density measurements in order to study changes in cross-linking degree. Additionally, the shrinkage temperature and weight remaining of the chemically modified collagenous tissues were respectively used for the assessment of the stability against thermal denaturation and enzymatic degradation. The in vitro biocompatibility of GTA cross-linked AM materials was analyzed by using corneal and limbal epithelial cell cultures. The cellular responses were monitored by cell viability, anti-inflammatory activity, and stemness gene expression.

## Experimental

### Materials

This study followed the tenets of the Declaration of Helsinki involving human subjects and received approval from the Institutional Review Board of our institution. Human AM tissues (i.e., the innermost layer of the placental membranes) were obtained with informed consent at the time of elective cesarean section from mothers when human immunodeficiency virus, syphilis, and hepatitis B and C had been excluded by serologic tests. The separation of AM was carried out using blunt dissection to cut the tissue samples approximately 2 cm from the placental disc. The average thickness of AM samples used in this work was 100 μm. Glutaraldehyde (GTA), ninhydrin reagent, matrix metalloproteinase-1 (MMP-1, EC 3.4.24.7), MMP-9 (EC 3.4.24.35), and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from J.T.Baker (Phillipsburg, NJ, USA). Deionized water used was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Phosphate-buffered saline (PBS, pH 7.4) was purchased from Biochrom (Berlin, Germany). Balanced salt solution (BSS, pH 7.4) was obtained from Alcon Laboratories (Fort Worth, TX, USA). Dispase II was purchased from Roche Diagnostics (Indianapolis, IN, USA). FNC Coating Mix (i.e., a fibronectin/collagen mixture) was obtained from Athena ES (Baltimore, MD, USA). Dulbecco's modified Eagle's medium (DMEM), keratinocyte serum-free medium (KSFM), Ham's F-12 nutrient mixture (Ham's F-12), gentamicin, 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, 10 mg/ml streptomycin and 25 μg/ml amphotericin B) were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). 24-well tissue culture polystyrene (TCPS) plates (Falcon 353047) were purchased from Becton Dickinson Labware (Franklin Lakes, NJ, USA). All the other chemicals were of reagent grade and used as received without further purification.

### Preparation of glutaraldehyde cross-linked amniotic membranes

The AM samples were aseptically washed three times with PBS containing 1% A/A solution and 50 μg/ml of gentamicin according to the protocols reported previously.[20] The membranes were immersed with sequential concentrations of DMSO, followed by freezing and storing at -80°C in DMEM containing 50% glycerol. After a further incubation with 0.02% EDTA at 37°C, the AM was denuded of its amniotic epithelial cells by gentle scraping. For GTA cross-linking, the membranes were immersed in 30 ml of PBS containing cross-linking agent under gentle shaking. The cross-linking reaction was allowed to proceed at 25°C for 24 h and the resulting membranes were then placed in 100 mM glycine aqueous solution at 37°C for 1 h to block unreacted residual GTA.[17] To obtain AM samples with varying degrees of cross-linking, the cross-linker concentration was altered in the range of 0-0.1 mmol GTA/mg AM. In this study, the biological tissue materials modified with cross-linker concentration of 0.001 mmol GTA/mg AM was designated as G/A001.

### Ninhydrin assays

The ninhydrin assay was used to determine the amount of free

amino groups of each AM. The test samples were weighed and heated with a ninhydrin solution for 20 min. After the test solution was cooled to room temperature and diluted in 95% ethanol, the optical absorbance of the solution was recorded with a UV-visible spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 570 nm using glycine at various known concentrations as standard.[21] The amount of free amino groups in the AM before ($C_b$) and after ($C_a$) cross-linking is proportional to the optical absorbance of the solution. The extent of cross-linking of the AM was calculated as cross-linking index (%) = (($C_b$-$C_a$)/$C_b$) × 100. Results were the average of five independent measurements.

**Cross-linking density measurements**

The cross-linked structure of the chemically modified AM such as degree of cross-link and average molecular weight of polymer chains between two consecutive junctions was analyzed according to the method reported previously.[22] After immersion in deionized water for 12 h at 25°C, the membrane samples (20 mm × 10 mm) were mounted between two clamps of an Instron Mini 44 universal testing machine (Canton, MA, USA). The lower clamp was then adjusted downward until the sample was just in tension and the unstressed length was noted. Following determination of mechanical properties, the test specimens were removed from the clamps and blotted with tissue paper, and the density was determined by the specific gravity bottle method. A graph of $\sigma$ against ($\alpha$-$\alpha^{-2}$) would be a straight line with the slope giving $RT\rho V^{1/3}/M_c$, where $\sigma$ = the force per unit area of the swollen unstretched sample; $\alpha$ = extension ratio; $R$ = gas constant; $T$ = absolute temperature; $\rho$ = density of sample; $V$ = volume fraction; and $M_c$ = average molecular weight of the chains between cross-links. The number of cross-links per unit mass would be given by $(2M_c)^{-1}$. Results were the average of five independent measurements.

**Differential scanning calorimetry**

The thermal property of the chemically modified AM was investigated using a DSC 2010 differential scanning calorimeter (TA Instruments, New Castle, DE, USA). The test samples (5 mg) were hermetically sealed in aluminum pans for DSC experiments. Programmed heating was carried out at 5°C/min in the temperature range from room temperature to 110°C with an empty aluminum pan as the reference probe. The shrinkage temperature ($T_s$) of AM, which is a measure of the resistance against thermal denaturation, was determined as the onset value of the occurring endothermic peak.[10] Results were averaged on four independent runs.

**In vitro degradability**

To measure the extent of degradation, each test AM (1 × 1 $cm^2$) was first dried to constant weight ($W_i$) in vacuo and was immersed in 1 ml of BSS containing 12 μg MMP-1 or MMP-9 at 37°C with reciprocal shaking (50 rpm) in a thermostatically controlled water bath. Degradation medium was replaced weekly with fresh buffer solution containing the same concentration of enzyme.[23] At different time intervals, the membrane samples were taken out and washed with deionized water. The degraded samples were further dried in vacuo and weighed to determine the dry weight ($W_d$). The percentage of weight remaining (%) was calculated as ($W_d/W_i$) × 100. Results were the average of four independent measurements.

**Cell viability assays**

In this study, HCE-2 cells, a human corneal epithelial cell line (ATCC No. CRL-11135), were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were seeded on tissue culture plastics precoated with FNC Coating Mix, and maintained in regular growth medium containing KSFM, 0.05 mg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, 500 ng/ml hydrocortisone, and 0.005 mg/ml insulin. Cultures were incubated in a humidified atmosphere of 5% $CO_2$ at 37°C. The medium was changed twice a week. Cells were subcultured by trypsinization at a split ratio of 1:3.

A single extract of the test article was prepared using regular growth medium.[24] The extracts were obtained by incubation of the UV-sterilized AM materials with culture medium at 37°C for 24 h with an extraction ratio of 0.2 g/ml. Each test extract was then placed onto HCE-2 cell cultures with a seeding density of 5 × $10^4$ cells/well. After a 3-day incubation at 37°C in the presence of 5% $CO_2$, the qualitative and quantitative assays were performed to examine the cellular responses to chemically cross-linked AM. The cells in regular growth medium without contacting material samples served as control groups.

Cell morphology was observed by phase-contrast microscopy (Nikon, Melville, NY, USA).[25] Furthermore, cell viability was estimated using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation MTS Assay (Promega, Madison, WI, USA), in which MTS tetrazolium compound is bio-reduced by cells to form a water-soluble colored formazan.[26] The amount of colored product is proportional to the number of metabolically active cells. 100 μl of the combined MTS/PMS (20:1) reagent was added to each well of the 24-well plate, and incubated for 3 h at 37°C in a $CO_2$ incubator. The data of absorbance readings at 490 nm were measured using the Multiskan Spectrum Microplate Spectrophotometer (ThermoLabsystems, Vantaa, Finland). All experiments were performed in quadruplicate, and the results were expressed as relative MTS activity when compared to control groups.

**Anti-inflammatory activity studies**

HCE-2 cells (5 × $10^4$ cells/well) were seeded in 24-well plates containing regular growth medium and incubated overnight. For LPS stimulation, the medium was replaced with the fresh medium containing 1 μg/ml LPS.[13] Using cell culture inserts (Falcon 3095, Becton Dickinson Labware), each well of a 24-well plate was divided into two compartments.[27] A sterilized membrane sample was placed into the inner well of the double-chamber system to examine the LPS-stimulated cultures after exposure to chemically cross-linked AM materials. Unstimulated and LPS-stimulated HCE-2 cells without contacting the test samples served as the negative control (NC) and positive control (PC) groups, respectively.

After 3 days of incubation, the release of interleukin-6 (IL-6) 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 human IL-6.[28] Aliquots of the supernatant from each well were collected, and cytokine bioassays were performed according to the

manufacturer's instructions. Photometric readings at 450 nm were measured using the Multiskan Spectrum Microplate Spectrophotometer (ThermoLabsystems). Results were expressed as pg/ml. All experiments were conducted in quadruplicate.

**Stemness gene expression analyses**

The rabbit corneoscleral rims were used to culture LECs. To disperse the cells, the corneoscleral rims were treated with dispase II at 37°C, followed by incubation with trypsin-EDTA solution. The cultures were maintained with supplemental hormonal epithelial medium (SHEM), which was made of an equal volume of HEPES-buffered DMEM containing bicarbonate and Ham's F-12, 0.5% DMSO, 2 ng/ml mouse epidermal growth factor, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 0.5 μg/ml hydrocortisone, 30 ng/ml cholera toxin A subunit, 5% FBS, 50 μg/ml gentamicin, and 1.25 μg/ml amphotericin B.

Rabbit LECs ($5 \times 10^4$ cells/well) were seeded into 24-well TCPS plates (control groups) and various sterilized AM materials. After incubation in SHEM at 37°C for 5 days, the total RNA was isolated from cells with TRIzol reagent according to the manufacturer's procedure.[29] Reverse transcription of the extracted RNA (1 μg) was performed using ImProm-II (Promega) and Oligo$(dT)_{15}$ primers (Promega). The primers used to amplify the rabbit ABCG2 complementary DNA (cDNA) were 5'-GAGAGCTGGGTCTGGAAAAAGT-3' (sense) and 5'-ATTCTTTTCAGGAGCAGAAGGA-3' (antisense). The sequences of the primer pair used to amplify the internal control cDNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were 5'-TTGCCCTCAATGACCACTTTG-3' (sense) and 5'-TTACTCCTTGGAGGCCATGTG-3' (antisense). Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was performed on a Light-Cycler instrument (Roche Diagnostics) according to the manufacturer's instructions with FastStart DNA Master SYBR Green I reagent (Roche Diagnostics). Each sample was determined in triplicate, and the gene expression results were normalized to the level of GAPDH mRNA.

**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 $p < 0.05$.

## Results and discussion

**Preparation of glutaraldehyde cross-linked amniotic membranes**

Cross-linking is a medical procedure that can strengthen the chemical bonds in the cornea and stabilize the tissue collagen. More recently, Hashemi et al. have reported the long-term results of corneal collagen cross-linking in keratoconus patients and suggested that riboflavin in combination with UV light is effective in preventing disease progression, without raising safety concerns.[30] To enhance its performance as a limbal stem cell scaffold for ophthalmic tissue engineering applications, the biological tissue material such as AM has been chemically modified in our laboratory using exogenous cross-linker.[10] In general, two major categories of methods are available for biomaterials prepared in the presence of cross-linking agents. These are solution casting and film immersion techniques.[31] Since the AM has a membrane structure, the solution casting method is not suitable to treat the collagenous material studied. Therefore, we cross-link the AM by directly incubating the biological tissue in buffered solution containing GTA. This film immersion process has also been described for the chemical cross-linking of prefabricated biopolymer membranes made of gelatin,[32] hyaluronic acid,[33] and chitosan.[34]

**Ninhydrin assays**

Ninhydrin (i.e., 2,2-dihydroxy-1,3-indanedione) is a compound that specifically reacts with free amino acids to form a purple product.[35] Since GTA cross-linking involves the consumption of free amino groups of lysine or hydroxylysine amino acid residues of the polypeptide chains, a quantitative colorimetric method based on the ninhydrin detection was used to determine the cross-linking index of proteinaceous matrices. In this work, the extent of cross-linking of chemically modified AM was investigated as a function of GTA concentration (Fig. 1). The results indicate that the cross-linking index significantly increased with increasing cross-linker concentration from 0.001 to 0.1 mmol GTA/mg AM ($p < 0.05$). Our previous study has also demonstrated that the extent of cross-linking of carbodiimide-treated AM may increase with an increase in the amount of cross-linker molecules in the reaction system.[10] However, the most complete cross-linking reaction between tissue collagen and chemical cross-linker occurs at the concentration of 0.05 mmol carbodiimide/mg AM, but not for the case of 0.25 mmol carbodiimide/mg AM, indicating that the addition of excessive amounts of carbodiimide to AM does not further promote the cross-linking reaction. In contrast, the results of the present study show that the maximum value of cross-linked amino groups in tissue collagen molecules is approximately 80% when the concentration reaches the highest level (i.e., 0.1 mmol GTA/mg AM). This finding supports the general notion that GTA (i.e., non-zero-length cross-linker)-mediated reaction mechanism is different from that observed in carbodiimide (i.e., zero-length cross-linker) chemistry.

**Cross-linking density measurements**

During the reaction of AM with carbodiimide, the aggregation of collagen microfibrils is noted, suggesting that the matrix nanostructure is sensitive to cross-linker molecules.[13] Given that the formation of cross-links in the chemically modified tissue collagen may lead to remarkable change in the cross-linked structure, it is very important to determine the average molecular weight of the chains between cross-links and the number of cross-links per unit mass. To the best of our knowledge, the role of GTA cross-linking in the modulation of the cross-linked structure of the AM matrices has not been investigated. As shown in Fig. 2a, the $M_c$ significantly decreased with increasing concentration of cross-linking agent from 0.001 to 0.1 mmol GTA/mg AM ($p < 0.05$), indicating that the increase in the collision frequency of tropocollagen molecules may affect the average molecular weight of polymer chains between two consecutive junctions. Additionally, the number of cross-links per unit mass in the G/A001, G/A003, G/A010, G/A030, and G/A100 groups was 6.73 ± 0.18, 8.02 ± 0.16, 9.83 ± 0.23, 12.30 ± 0.27, and 15.01 ± 0.12, respectively (Fig. 2b). Our results demonstrate that the

amount of cross-links formed between the tissue collagen chains is significantly increased with increasing concentration of cross-linking agent from 0.001 to 0.1 mmol GTA/mg AM ($p < 0.05$). The findings of the degree of cross-link support the data from the ninhydrin assays. On the other hand, Charulatha et al. have shown that the maximum value of cross-linking density for the GTA-treated bovine Achilles tendon collagen is 13.[36] The results obtained in the current study are compatible with this earlier report.

**Differential scanning calorimetry**

The thermal stability of collagenous biomaterials is usually determined by using DSC which measures the thermal transitions related to the shrinkage temperature changes of the samples.[37] In this study, the resistance against thermal denaturation of chemically modified AM was investigated as a function of GTA concentration, and the results are shown in Fig. 3. As compared with their non-cross-linked counterparts, the GTA-treated AM had significantly higher $T_s$ ($p < 0.05$), irrespective of cross-linker concentration (also see Fig. S1, ESI†). Several investigators have reported that GTA cross-linking is able to enhance the hydrothermal stability of triple-helical regions of tissue collagen. Spoerl et al. demonstrated that while untreated control corneas exhibited maximum shrinkage at 70.3 ± 0.9°C, the corneal strips cross-linked with 0.1% GTA had maximum shrinkage temperature of 89.7 ± 1.7°C.[38] A study from Yoshioka et al. showed that the native and GTA-treated bovine pericardium had $T_s$ of 63.6 ± 0.5 and 85.8 ± 0.3°C, respectively.[39] Olde Damink et al. further examined the effect of GTA concentration on the shrinkage behavior of sheep dermal collagen and found a positive correlation between the amount of cross-linker and $T_s$.[40] In accordance with this previous work, our present case indicated that for the collagenous tissue materials cross-linked with GTA, increasing chemical cross-linker concentration led to a significant increase in $T_s$ from 64.1 ± 1.3°C to 90.5 ± 1.1°C ($p < 0.05$). For the first time, this study reported that the thermal stability of GTA-treated AM matrices strongly depends on their cross-linking density.

**In vitro degradability**

It is known that MMPs can break down the protein molecules and degrade the collagen framework.[41] Here, the biostability of chemically modified AM was investigated by performing the degradation test under the action of collagenases such as MMP-1 and MMP-9.[13] To evaluate the in vitro enzymatic degradability of collagenous biomaterials, we monitored the mass change of the GTA-treated AM. After 2 weeks of incubation in BSS containing MMP-1, the remaining weight in the G/A000, G/A001, G/A003, G/A010, and G/A030 groups was 13.5 ± 1.7, 31.7 ± 3.0, 51.4 ± 2.5, 73.6 ± 2.8, and 96.1 ± 2.3%, respectively (Fig. 4a). The values showed significant differences between these groups ($p < 0.05$). In addition, during the digestion of biological tissue materials by means of MMP-9, the increase in chemical cross-linker concentration from 0.001 to 0.03 mmol GTA/mg AM led to significant enhancement of resistance to collagenolytic degradation. It was also noted that when the cross-linking density reached the highest level, no further increase in remaining weight of the degraded samples was observed, irrespective of enzyme type. The results indicate that the amount of cross-links formed between the tissue collagen chains is sufficient to provide protective barriers shielding AM from enzymatic attack. As shown in Fig. 4b, the weight remaining after 4 weeks of exposure to MMP-1 in the G/A000, G/A001, G/A003, and G/A010 groups was 4.7 ± 1.9, 16.9 ± 1.5, 28.2 ± 2.1, and 64.2 ± 1.4%, respectively, which was significantly lower than their counterparts incubated for a short period i.e., 2 weeks ($p < 0.05$). In the case of MMP-9, a similar trend was also found for the effect of cross-linker concentration on remaining weight variation. The findings of the present work reveal that the GTA-treated AM materials with lower cross-linking density undergo progressive degradation with time, suggesting limited biostability. For both G/A030 and G/A100 groups, the biological tissues show almost no weight loss during the degradation period of 4 weeks. Therefore, the GTA concentration may play an important role in controlling the enzymatic degradability of chemically modified AM.

**Cell viability assays**

Given that AM is one of the most commonly reported biological tissues for the development of corneal epithelial cell culture platform,[42,43] we tested the in vitro biocompatibility of GTA cross-linked AM materials by using HCE-2 cells (i.e., a human corneal epithelial cell line). Fig. 5 shows representative phase-contrast micrographs of HCE-2 cell cultures after incubation for 3 days at 37°C with extract medium conditioned with various AM samples. In the control groups, the cells in regular growth medium without contacting biological tissues appeared healthy and exhibited typical corneal epithelial morphological characteristics. The HCE-2 cell cultures in the G/A000, G/A001, G/A003, and G/A010 groups did not reveal any morphological abnormalities, indicating good cell viability. In contrast, the AM cross-linked with GTA of higher concentration presented different cell growth patterns. Some malformed cells were found in the G/A030 groups. In particular, the exposure of cell cultures to the sample G/A100 revealed a significant amount of dead corneal epithelial cells.

Further quantitative analysis for human corneal epithelial cell growth was carried out by MTS assay (Fig. 6). During 3 days of culture in regular growth medium, the HCE-2 cells actively proliferated. The mitochondrial dehydrogenase activity (MTS activity) in the control, G/A000, G/A001, G/A003, and G/A010 groups was 100.0 ± 1.9, 98.8 ± 1.0, 98.2 ± 1.4, 97.9 ± 2.0, and 95.8 ± 2.6%, respectively, which did not demonstrate significant statistical differences ($p > 0.05$). The results indicate that these GTA cross-linked AM samples are compatible toward human corneal epithelial cells without causing toxicity. As compared to the control groups, the activity level of the G/A030 groups was significantly reduced by about 10.7% ($p < 0.05$). In addition, the HCE-2 cells exposed to sample G/A100 were less metabolically active than those from all the other groups ($p < 0.05$). We have previously demonstrated that the time for GTA cross-linking of AM is a crucial parameter in the determination of cytotoxicity and inflammation.[20] The MTS activity of biological tissues treated with cross-linker of 0.05 mmol GTA/mg AM for 24 h is 80.4 ± 2.2%. It is noted that for the same reaction duration treatment, the AM samples cross-linked with lower concentrations (i.e., ≤ 0.03 mmol GTA/mg AM) can significantly enhance the proliferative capacity of the HCE-2 cell line cultures.

The findings of the present work also suggest that the test extracts prepared from GTA-treated AM matrices with the highest cross-linking density possess inhibitory effects on the corneal epithelial cell growth. One possible explanation for our observations is that the remaining aldehyde groups in the AM having relatively high cross-linking degree may induce self destruction of the cells,[44] thereby contributing to the decrease in biocompatibility of collagenous tissue materials.

**Anti-inflammatory activity studies**

It is known that the AM materials contain various anti-inflammatory proteins.[45] In our laboratory, the ocular tissue responses to AM have been tested by using the anterior chamber of a rabbit eye model.[46] The continued presence of AM samples within an intraocular space does not cause inflammation for the studied period of three years after the implants have been placed. Here, LPS-stimulated human corneal epithelial cells were used to assay the anti-inflammatory activity of GTA cross-linked AM materials (Fig. 7). After 3 days of incubation, the supernatants from untreated (NC) and LPS-stimulated (PC) groups were analyzed by ELISA to detect pro-inflammatory cytokine levels. The IL-6 concentration in the media of the NC groups was 43.4 ± 7.9 pg/ml, which was significantly lower than those of the PC (1528.0 ± 40.1 pg/ml) groups ($p < 0.05$). Our result of up-regulated production of IL-6 is in agreement with general behavior of LPS as a stimulus for cytokine release. Additionally, the measured concentration of IL-6 in the G/A000 (408.3 ± 18.5 pg/ml) groups was significantly lower than those of the PC groups, indicating the anti-inflammatory activity of non-cross-linked AM matrices. Although the biological tissues cross-linked with GTA still possessed anti-inflammatory properties, the pro-inflammatory cytokine level significantly increased with increasing cross-linker concentration from 0.001 to 0.1 mmol GTA/mg AM ($p < 0.05$). It has been documented that GTA molecules can induce a marked stimulation of IL-6 production and elicit an appreciable cellular inflammatory response.[34,47] This may account for the negative correlation between the cross-linking density and anti-inflammatory activity in our analysis of GTA-treated AM materials.

**Stemness gene expression analyses**

The design of functional biomaterials for corneal regenerative medicine has received much attention in the past few years.[48] As a valuable ophthalmic biomaterial, the AM has already been proposed as a limbal stem cell niche.[2] This biological tissue is able to maintain the undifferentiated precursor cell phenotype and support the regeneration of corneal epithelium. In this study, the gene expression of ABCG2 (i.e., a marker of stemness in limbal epithelia) was measured after 5 days of LEC growth on the AM samples treated with varying GTA concentrations. As shown in Fig. 8, the expression in the control groups was 100% at mRNA level detected by quantitative real-time RT-PCR. The LECs in the G/A000 (180.0 ± 12.1%) groups had relatively higher ABCG2 levels than did those of control groups ($p < 0.05$). The results indicate that as compared with the TCPS control, the AM substrate is more appropriate to create a LEC culture platform. Additionally, the stemness gene expression in the G/A000 groups was significantly lower than those in the G/A001 (279.2 ± 20.6%), G/A003 (391.5 ± 28.0%), G/A010 (641.7 ± 36.2%), G/A030 (983.5 ± 37.4%), and G/A100 (1946.1 ± 42.5%) groups ($p < 0.05$), suggesting the GTA cross-linking-mediated preservation of epithelial progenitor cells. For the AM materials treated with GTA, increasing chemical cross-linker concentration may further promote the stem cell marker expression. The development of biomaterial scaffolds that govern cell adhesion, proliferation, and differentiation is of great importance in the field of tissue engineering.[49] More recently, Rowland et al. have investigated the influence of cross-linking of cartilage-derived matrix scaffolds on the chondrogenic differentiation of adult human bone marrow-derived stem cells.[50] Their study suggests that the physical cross-linking methods can impart sufficient cross-linking degree to the biological tissue scaffolds to allow them to inhibit cell-mediated contraction of porous materials, thereby retaining chondrogenic potency. In contrast, the technique involving the use of chemical reagents may produce extensive cross-linking that masks the cell-matrix interactions and tends to attenuate chondrogenic differentiation in the fabricated scaffolds. Our findings are compatible with this prior research and emphasize that during cross-linking treatment, the GTA concentration is critical to tailor the properties of chemically modified AM to be suitable for application to LEC cultivation. In addition, the present study shows that the GTA-treated AM matrices with high cross-linking density may provide a better environment for limbal stem cell niche.

## Conclusions

There has been an increased interest in the development of chemically cross-linked AM as a limbal stem cell niche because this modified biomaterial has an appropriate stability for ophthalmic applications. In summary, we sucessfully demonstrated the cross-linking of AM collagen with GTA of varying concentrations. For the first time, this study reported that the increase in the collision frequency of tropocollagen molecules may affect cross-linking structure of GTA-treated AM matrices. In addition, the tissue collagen stability, anti-inflammatory activity, and stemness gene expression strongly depended on the cross-linking density. In vitro biocompatibility studies also showed that the samples treated with low cross-linker concentrations (i.e., < 0.03 mmol GTA/mg AM) are well-tolerated by the human corneal epithelial cell cultures. Taken together, our findings suggest an interrelationship between cross-linking structure, molecular stability, and cytocompatibility of GTA-treated AM materials.

~~In this work, we explored the interrelationship between cross-linking structure, molecular stability, and cytocompatibility of AM materials cross-linked with GTA of varying concentrations. Our results indicated that the cross-linking index and number of cross-links per unit mass significantly increased with increasing cross-linker concentration from 0.001 to 0.1 mmol GTA/mg AM. Inversely, the average molecular weight of polymer chains between two consecutive junctions was significantly decreased with an increase in cross-linking density. DSC measurements and in vitro degradation tests demonstrated that the collagen molecular stability was enhanced considerably in the presence of higher amount of non-zero-length cross-linkers. The GTA-treated AM matrices with the greatest extent of cross-linking may cause marked alteration in cell morphology and decrease in cell~~

viability, indicating poor compatibility with human corneal epithelial cell cultures. It was also found that although the biological tissues cross-linked with GTA still possessed anti-inflammatory actions, the IL-6 cytokine levels in LPS-stimulated cells were increased with increasing cross-linking density. The up-regulated stemness gene expression was positively correlated with the degree of GTA cross-linking, suggesting the chemical cross-linking structure-mediated preservation of limbal epithelial progenitor cells. It is concluded that during cross-linking treatment, the GTA concentration is critical to tailor the properties of chemically modified AM for a limbal stem cell niche.

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## Notes and references

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**Figure Captions**

**Fig. 1** Cross-linking index of AM as a function of GTA concentration. Values are mean ± standard deviation ($n$ = 5). *$p$ < 0.05 vs all groups.

**Fig. 2** Cross-linked structure of AM as a function of GTA concentration. (a) $M_c$ and (b) number of cross-links per unit mass. Values are mean ± standard deviation ($n$ = 5). *$p$ < 0.05 vs all groups.

**Fig. 3** Shrinkage temperature of AM as a function of GTA concentration. Values are mean ± standard deviation ($n$ = 4). *$p$ < 0.05 vs all groups.

**Fig. 4** Weight remaining of various AM samples after incubation at 37°C for (a) 2 and (b) 4 weeks in BSS containing MMP-1 or MMP-9. Values are mean ± standard deviation ($n$ = 4). *$p$ < 0.05 vs all groups; ^$p$ < 0.05 vs all groups except 0.100 mmol GTA/mg AM; $^{+}p$ < 0.05 vs all groups except 0.030 mmol GTA/mg AM (compared only within MMP-1 or MMP-9 group). $^{\#}p$ < 0.05 indicates statistically significant differences from the MMP-1 group (compared only within each cross-linker concentration group).

**Fig. 5** Phase-contrast micrographs of HCE-2 cell cultures. The pattern of cell growth in (a) controls (without materials) after incubation for 3 days at 37°C with extract medium conditioned with various AM samples (b) G/A000, (c) G/A001, (d) G/A003, (e) G/A010, (f) G/A030, and (g) G/A100. Scale bars: 100 μm.

**Fig. 6** Cell proliferation assay of HCE-2 cell cultures incubated with extract medium conditioned with various AM samples for 3 days. Results are expressed as percentage of controls (MTS activity of cells cultured in the absence of materials). Values are mean ± standard deviation ($n$ = 4). *$p$ < 0.05 vs all groups; $^{\#}p$ < 0.05 vs 0.030 mmol GTA/mg AM and 0.100 mmol GTA/mg AM groups.

**Fig. 7** Level of IL-6 released from HCE-2 cell cultures after incubation with various AM samples for 3 days. Unstimulated and LPS-stimulated cells without contacting the test materials were the NC and PC groups, respectively. Values are mean ± standard deviation ($n$ = 4). *$p$ < 0.05 vs all groups.

**Fig. 8** Gene expression level of ABCG2 in rabbit LECs grown on various AM samples for 5 days, measured by real-time RT-PCR. Normalization was done by using GAPDH. Data in the experimental groups are percentages relative to that of control groups (cells cultured on TCPS in the absence of AM materials). Values are mean ± standard deviation ($n$ = 3). *$p$ < 0.05 vs all groups.

**Fig. 1**

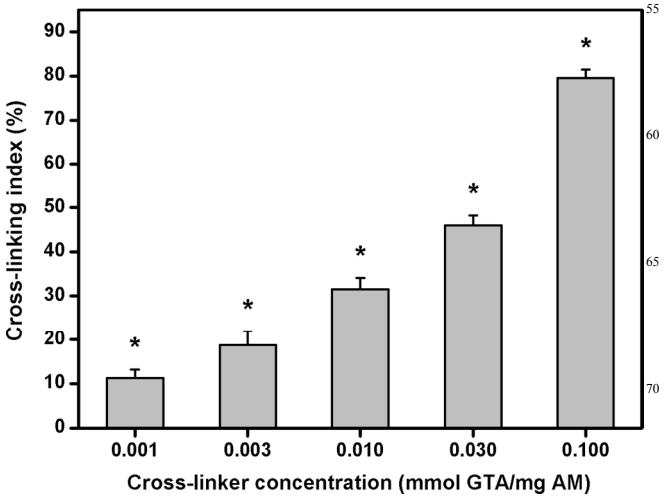
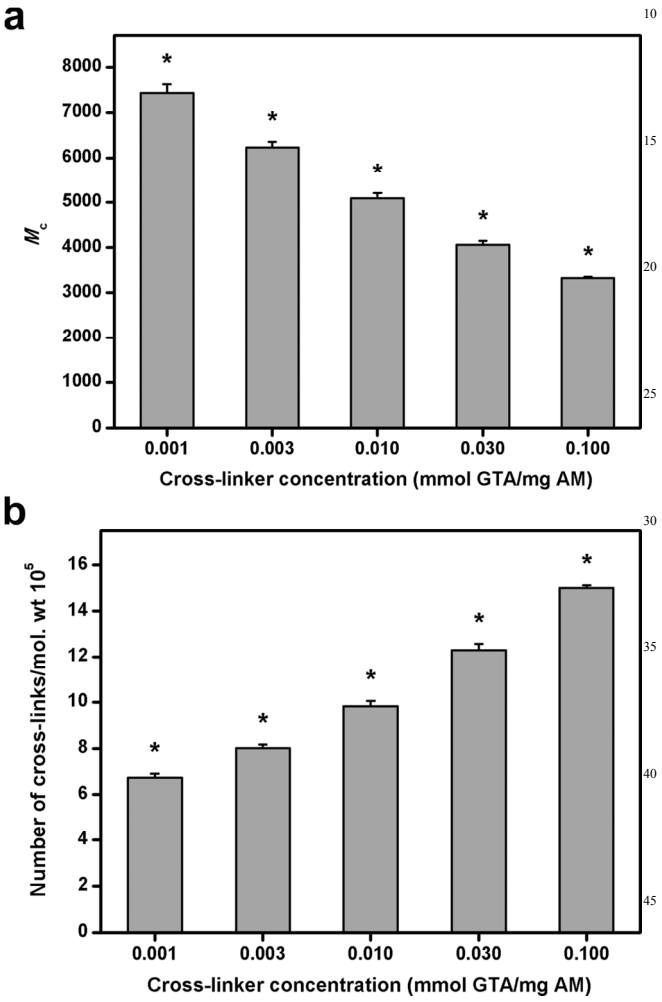


Fig. 2



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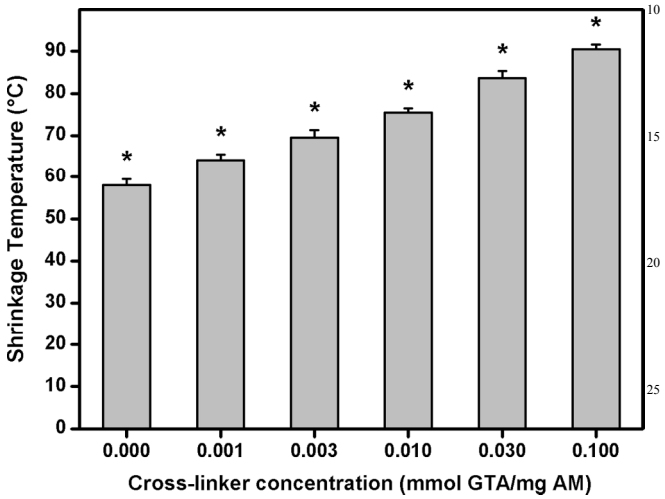
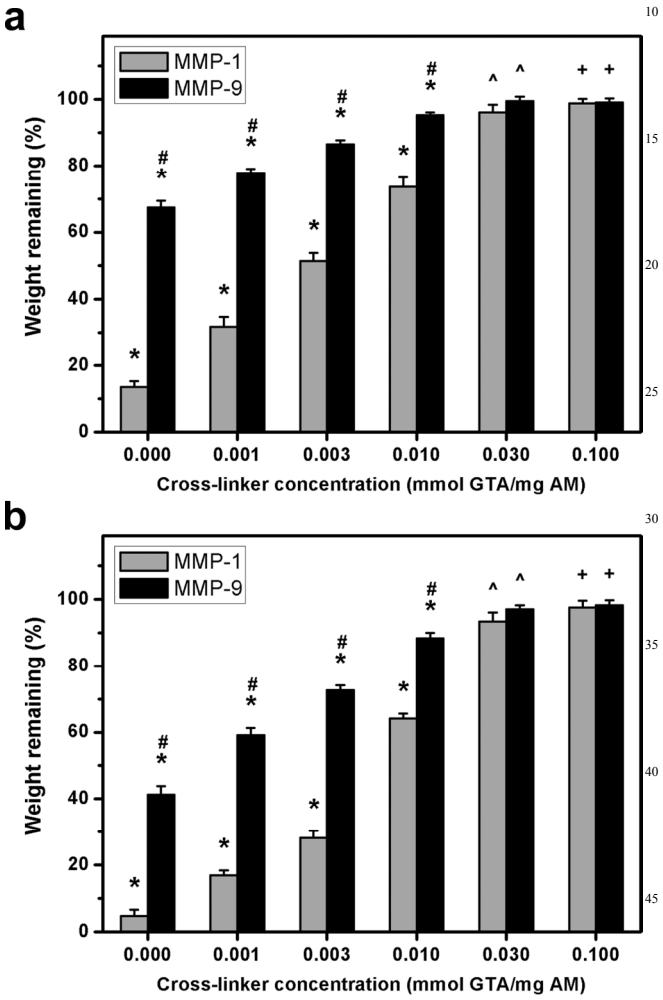


Fig. 4



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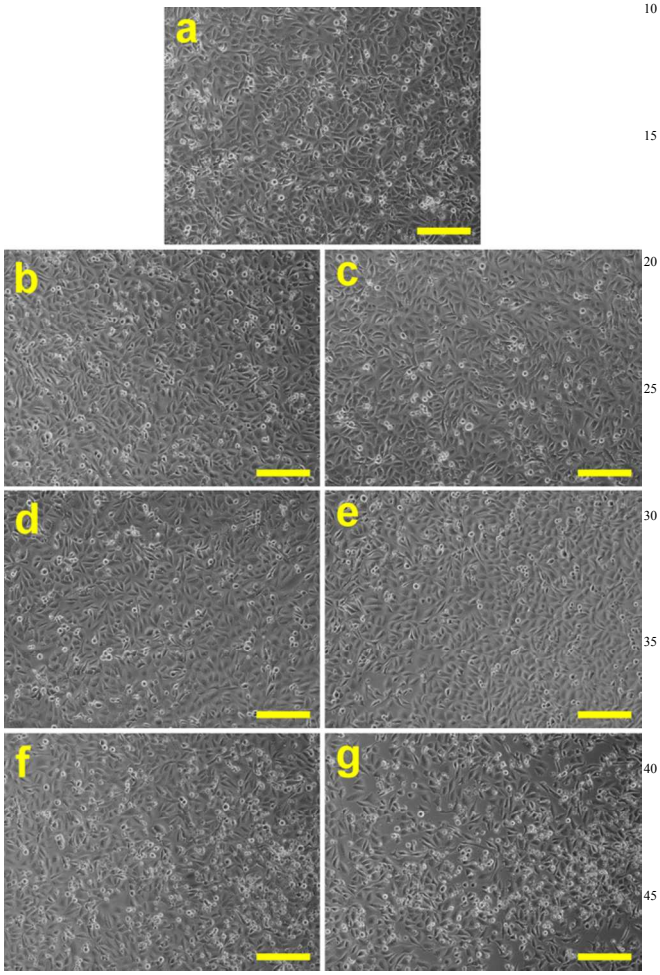

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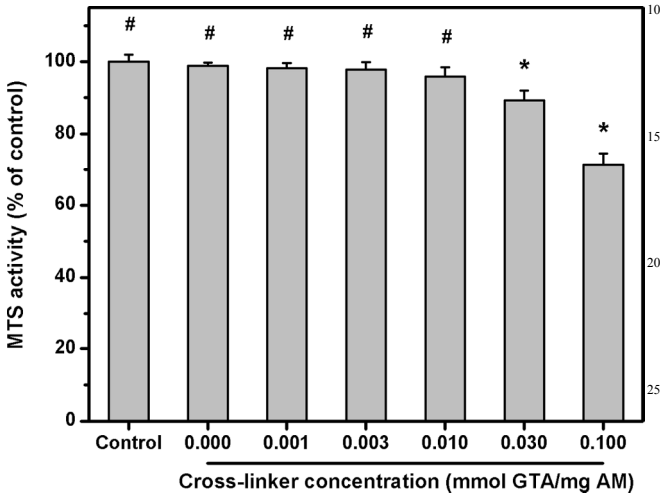

Fig. 7

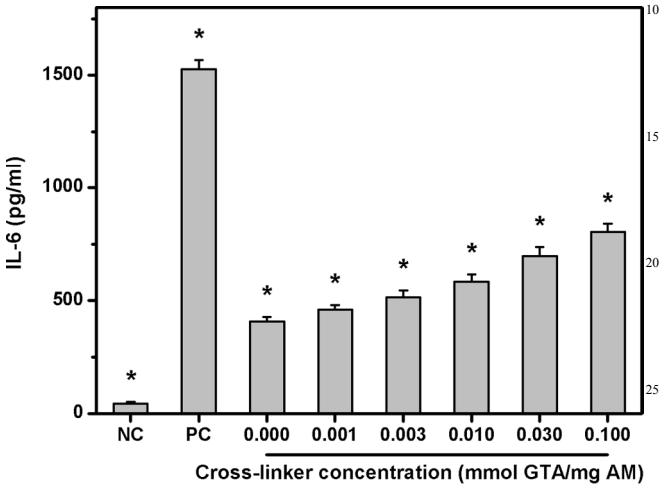

Fig. 8