

Development of Contact Lens-Shaped Crosslinked Amniotic Membranes for Sutureless Fixation in the Treatment of Ocular Surface Diseases

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Purpose: To develop a new method of manufacturing contact lens-shaped crosslinked amniotic membranes (AMs) using glutaraldehyde (GA) and dialdehyde starch (DAS) as crosslinking agents.

Methods: Amniotic membranes were placed on a curved plastic mold and crosslinked with either 4.5% DAS or 1% GA, after which their physical properties and biological safety were evaluated.

Results: The tensile strength of the GA- and DAS-crosslinked samples was much increased compared with that of normal AMs. Neither crosslinking process affected AM transparency. Although the GA-crosslinked AM showed better enzymatic resistance, its physiological structure was severely damaged after the crosslinking process. On the other hand, compared with the GA-crosslinked AM, the DAS-crosslinked AM showed higher growth factor concentrations and better biocompatibility, similar to normal AMs. In addition, the DAS-crosslinked AM was effective in the recovery of corneal epithelial wounds and was well maintained over 3 days without decentration or degradation on the ocular surface in human subjects.

Conclusions: Contact lens-shaped AMs were successfully prepared with crosslinking agents. Crosslinking with DAS did not affect the structural properties or biological activity of the AMs, and the improved mechanical properties helped the AM to maintain its curved shape. This crosslinking method allowed us to transplant AMs into patients' eyes without sutures.

Translational Relevance: Sutureless fixation of contact lens-shaped AMs would be very convenient and safe for the treatment of corneal surface disease.

Introduction

The amniotic membrane (AM) is the innermost thin and translucent membrane of the placenta with a thickness of 0.02 to 0.5 mm. It serves as a fetal barrier against various infections and provides an immune response that may be transmitted from the mother. In addition, the AM functions as a barrier to epithelial cells and as a basement membrane, fibroblast, or sponge layer, characterized by the absence of rejection even if transplanted.¹

AM was first used for therapeutic applications on the skin by Davis in 1910.² Although de Rotth³ first announced the ophthalmological use of human fetal membranes in 1940, it has not been used clinically, and no reports have been published in the ophthalmic area. However, Kim and Tseng⁴ reported successful reconstruction of the ocular surface using AM in a rabbit model of corneal epithelial defects. Since then, many researchers have subsequently reported the use of AMs as a biological bandage and as an aid to physiological wound healing.⁵⁻¹¹ The AM offers several advantages for ocular surface reconstruction in various diseases

due to its transparent structure and lack of immunologic rejection, which means it can be used as a mediator for growth, migration, and adherence of cornea and conjunctival epithelial cells.¹² In addition, the AM promotes epithelialization and exhibits antifibrotic, antiinflammatory, antiangiogenic, and antimicrobial properties.¹³ These effects are associated with various factors, such as epidermal growth factor (EGF); EGF receptor (EGFR); keratinocyte growth factor; tumor growth factor (TGF)- α , TGF- β 1, and TGF- β 2; vascular endothelial growth factor; insulin growth factor (IGF); hepatocyte growth factor; and tissue inhibitor of metalloproteinase 1 secreted by the AM.^{12,14} Moreover, the composition of the AM basement membrane is very similar to that of the collagen of the cornea and conjunctiva and supports the growth of epithelial cells.¹⁵ All of these advantages have deemed AM useful for corneal injury and ulcer treatment.¹²

Generally, for the treatment of ocular surface diseases, the AM is fixed through surgical suturing. To fix the AM on an ocular surface by sutures, a proper anesthetic method is required. Normally, topical anesthesia can be applied for the fixation; however, in some cases, invasive anesthetic methods such as peribulbar anesthetic injection or general anesthesia may be necessary. During the process of suturing, subconjunctival hemorrhage can occur, and suture-related corneal scars or induced astigmatism can result when the AM is fixated to the cornea. Suture-related infection or inflammation is rare but can occur. Furthermore, most of sutures ultimately must be removed, much to the patient's discomfort.¹⁶

Products have been developed to compensate for the difficulties of this operation. A commercial product utilizing AM has been marketed as solving problems related to suturing. PROKERA (Biotissue, Miami, FL, USA), a plastic O-ring-fixed AM,¹⁷⁻¹⁹ has eliminated the greatest disadvantages of suturing, but some problems still exist. The use of an O-ring comprised of poly(methyl methacrylate), also known as acrylic or acrylic glass, causes discomfort in patients during insertion of the O-ring due to the difference between the size of the ring and the eyeball.²⁰ For these reasons, it would be more appropriate to use a contact lens-shaped membrane without a ring that matches the size of the patient's eyeball.

The authors have developed a contact lens type of AM that maintains the properties of the AM and compensates for the disadvantages of PROKERA. Because the AM is very thin, it is difficult to maintain its shape. We hypothesize that the crosslinking method we have developed will be suitable for making contact lens-shaped AMs, as it increases the AM mechanical stiffness, helping it to retain a curved, three-dimensional contact lens shape.²¹ Among the

various crosslinking agents, glutaraldehyde (GA) is one most commonly used to immobilize biological tissues; however, its clinical applications are limited due to high calcification levels and cytotoxicity, which could cause undesirable reactions in patients.²² Dialdehyde starch (DAS) is known to exhibit good biocompatibility and biodegradability, and DAS-fixed tissues do not release DAS, suggesting that the hydrolysis product is nontoxic. Moreover, this crosslinking approach offers substantial biomechanical strength and resistance to enzymatic degradation compared with normal AMs.²³⁻²⁵ For these reasons, DAS as a crosslinking agent holds promise for the development of bio-implants. In this study, GA- and DAS-crosslinking techniques were used to produce contact lens-shaped AMs that allow AM implantation on the ocular surface without the need for any surgical procedure to treat ocular surface disease. This study was also designed to analyze the efficacy and safety of GA- and DAS-crosslinked AMs as dressings for treatment of the ocular surface.

Materials and Methods

This study was conducted in accordance with the tenets set forth in the Declaration of Helsinki and was approved by the Institutional Review Board, Kyungpook National University Hospital. For in vivo efficacy testing using animal models, the experiments were in agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, were approved by the Institutional Animal Care and Use of Committee of Daegu-Gyeongbuk Medical Innovation Foundation (DGMIF), and were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (DGMIF-1804170300).

Preparation and Crosslinking of AM

We purchased AMs from MS Bio Co., Ltd. (Busan, Republic of Korea), under the management of good tissue practice guidelines. A plastic mold 13.5 mm in diameter used for contact lens manufacturing served as the template. AM tissues were removed with sterilized tweezers and hydrated in a sterile physiological saline solution for 10 minutes. The AM was placed on the curved fixation frame with the basement membrane side exposed. The moisture was removed with moisture absorption paper, and the AM was allowed to dry naturally. A second AM was placed on top of the dried AM and allowed to dry naturally.

Normal AMs were used as controls immediately after hydration without the use of crosslinking agents. The AMs were crosslinked using GA or DAS as the crosslinking agent. GA (Merck KGaA, Darmstadt, Germany) was dissolved in a solution of 20% dextran (Sigma-Aldrich, St. Louis, MO, USA) and PBS at a concentration of 1% (v/v). The AMs were crosslinked by incubation in a GA solution for 5 minutes at room temperature. We completely dissolved DAS (BOC Sciences, Shirley, NY, USA) in PBS at a concentration of 4.5% (w/v), and the pH of the solution was adjusted to 7.4. The AMs were crosslinked in DAS solution at room temperature for 30 minutes and treated with 1% sodium borohydride/absolute ethanol (EtOH) at room temperature for 1 minute to stabilize the reaction. Each crosslinked AMs was cut into the shape of a contact lens with a diameter of 13 mm using a laser cutting system (Time-Bandwidth Products AG, Zurich, Switzerland). The AMs were washed several times with 0.2-M glycine in PBS.

Measurement of Tensile Strength and Elasticity

The crosslinked and normal AMs were cut with a razor blade into 5 × 15-mm rectangles. The thickness of the samples was measured by obtaining a microscopic image of the lateral cross-section using SmartDrop (Femtobiomed Inc., Seongnam, Republic of Korea). The mechanical tensile test was performed using an in-house test machine with a resolution of 0.1 N, as previously described but with a modification for sample clamping.²⁶ Briefly, both ends of the sample were clamped, and the sample was pulled at a constant speed of 10 mm/minute until it broke, resulting in a force–displacement curve. The stress–strain curve, ultimate tensile strength, and elastic modulus were calculated from the force–displacement result.

Measurement of Transmittance of AM

The crosslinked AMs and normal AMs were cut into circular shapes using a 6-mm biopsy punch (Kai Corporation, Tokyo, Japan). The test samples were placed inside a 96-well plate. Excessive fluid around the samples was removed with blotting paper. The absorbance of the samples was measured with a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at wavelengths ranging from 300 to 700 nm at 10-nm intervals. The transmittance of the samples was calculated from the result using the following equation:

$$T = 10^{-A} \times 100\% \quad (1)$$

where T and A represent the transmittance and absorbance, respectively.

Histology

Because the AM was too thin to be analyzed, three sheets of AM were layered and embedded using the Tissue-Tek O.C.T. Compound (Sakura Finetek Japan, Tokyo, Japan). The specimens were cut to a thickness of 7 μm and placed on Fisherbrand Superfrost Plus Microscope Slides (Thermo Fisher Scientific, Waltham, MA, USA). After they dried for 30 minutes at room temperature, the samples were fixed on a slide for 5 minutes with 95% EtOH. Hematoxylin–eosin (H&E) staining was performed, followed by observation using optical microscopy.

Collagen type IV, a constituent protein of the basement membrane, was identified by immunohistochemistry. The tissues attached to the slides were washed with PBS and treated with a blocking solution containing 1% bovine serum albumin for 1 hour to inhibit any non-specific reactions. The samples were incubated overnight with a primary anti-collagen type IV antibody (1:200; Abcam, Cambridge, UK) at 4°C. After washing, Alexa Fluor 488 conjugated anti-rabbit antibody (1:200; Abcam) was used as a secondary antibody for 1 hour at room temperature. Nuclear staining was performed using 4',6-diamidino-2-phenylindole.

Measurement of Contained Growth Factors in AM

Crosslinked AMs and normal AMs were placed in 6-well plates; 1 ml of serum-free Gibco Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific) was then added to each well. Each piece was 9 cm² in size. The supernatants incubated for 24 hours in serum-free medium were obtained to determine the protein content of the supernatants and the growth factors present in each sample. The analysis of growth factor levels was carried out using a Quantibody Human Growth Factor Array kit (RayBiotech, Peachtree Corners, GA, USA), following the manufacturer's protocols. Briefly, after a 30-minute incubation at room temperature with sample diluent, the glass chips were washed five times, and each well arrayed with human growth factor antibodies was overlaid with 100 μl of medium. After overnight incubation at 4°C and extensive washing, the detector antibody was added for 1 hour and then washed away, and Alexa Fluor 488 conjugated streptavidin was added for 2 hours at room temperature. The signals were

scanned with a fluorescence laser scanner. Each sample was prepared in quadruplicate. Growth factors were quantified with the Q-Analyzer tool (#QAH-GF-1-SW; RayBiotech) against a standard curve set for each growth factor, with a five-point serial dilution of growth factor standards.

Biocompatibility of Crosslinked AM

The 9-mm² pieces of normal AMs and crosslinked AMs were placed in 35-mm cell culture dishes coated with collagen type I and lightly dried to facilitate attachment. The human corneal epithelia (HCE) cell line was cultured in Gibco DMEM: Nutrient Mixture F-12, supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA) and 1% penicillin/streptomycin (Gibco). The cell cultures were incubated for 24 hours at 37°C with humidified atmosphere containing 5% CO₂.

Calcein-acetoxymethyl/ethidium homodimer was used to confirm cell viability. Live cells were identified by the presence of intracellular esterase activity, as indicated by a green fluorescence produced by the addition of calcein-acetoxymethyl. Ethidium homodimer enters cells with damaged membranes and binds to nucleic acids, producing a red fluorescence in dead cells. The LIVE/DEAD Viability/Cytotoxicity Kit assay was prepared at concentrations according to the manufacturer's directions (Invitrogen, Waltham, MA, USA). This solution was added directly to the cell culture medium, which was incubated for 50 minutes at room temperature. At the end of this time, the cells were observed by fluorescence microscope. The number of cells in a unit area (500 × 500 μm) was counted for five images acquired randomly, and the relative adhesion rate of cells stained for calcein-acetoxymethyl was determined.

Wound Healing Assay

To harvest the conditioned media, 3 × 3-cm pieces of normal AMs and DAS- and GA-crosslinked AMs were placed in each well and maintained for 24 hours in DMEM/F-12 supplemented with 1% FBS. The culture supernatants from each different group were collected and stored at -80°C until use.

HCE cells were grown to confluence in 96-well ImageLock Microplates coated with collagen type 1 (Essen BioScience, Inc., Pittsfield Charter Township, MI, USA). Cells were gently washed with PBS and switched to 1% FBS medium for 4 hours. Consistent scratching wounds were made using the Essen BioScience WoundMaker to create a cell-free area, based on the technique described previously.²⁷ After-

ward, cells were washed twice with PBS to remove debris, and prepared conditioned media from each group was applied. Each well was automatically imaged every 3 hours within a CO₂ incubator, and the relative wound area was measured using IncuCyte software (Essen BioScience). The cell proliferation rate was measured as previously described.^{28,29}

Degradation Assay

Collagenase assays were performed to confirm whether the hardened AMs were resistant to degradation. GA- and DAS-crosslinked AMs and normal AMs in the form of 1 × 1-cm pieces were placed in a 0.1% collagenase solution (Roche, Mannheim, Germany) and observed at room temperature. The grid paper was attached to a dish, and the size of the remaining AMs retained after degradation was calculated.

In Vivo Efficacy Test

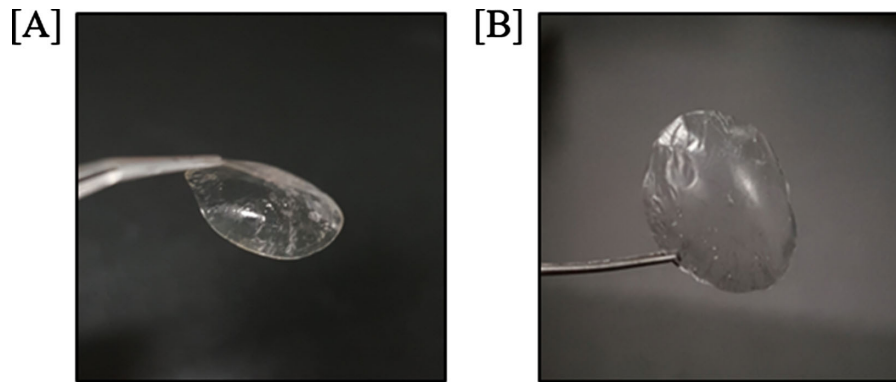
Animal experiments were carried out using New Zealand white rabbits (Orientbio, Gyeonggi-do, Korea) weighing 2.0 to 3.0 kg. Rabbits were anesthetized with an intramuscular injection of ketamine (15 mg/kg) and xylazine (5 ml/kg). Proparacaine (Alcaine; Alcon, Ft. Worth, TX, USA) was then applied to each cornea as anesthesia. After instillation with 30% alcohol for 15 seconds, the corneal epitheliums were removed from limbus to limbus with a scalpel blade. A rabbit-eye, DAS-crosslinked AM contact lens was placed on a rabbit's cornea, and tarsorrhaphy was performed to prevent loss of the AM due to blinking. The control group received tarsorrhaphy without any treatment. After 24 hours, the rabbit corneas were examined via fluorescein staining and slit-lamp to identify corneal epithelial defects. The areas of corneal epithelial defects were measured using Image J software (National Institutes of Health, Bethesda, MD, USA), and the healing rates were calculated.

Fitting Test

We conducted a pilot observation study for a wearing period of 7 days to determine the safety and wearability of DAS-crosslinked AMs in human subjects. The contact lens-type AMs were obtained from the tissue bank MS Bio Co. Six patients received the DAS-crosslinked AMs after we obtained their informed consent (Table). Each DAS-crosslinked AM was applied to the ocular surface after washing with Hank's Balanced Salt Solution (Thermo Fisher Scientific). The crosslinked AMs were fitted on the ocular surface and covered with bandage contact

Table. Diagnosis and Application Data

Patient	Diagnosis	Eye	Duration (d)	Wearability of Crosslinked AM
1	Band keratopathy	Right	7	Good
2	Band keratopathy	Right	4	Good (AM fell out after 4 d)
3	Chemical burn	Left	7	Good
4	Neurotrophic keratitis	Left	7	Good
5	Neurotrophic keratitis	Left	7	Good
6	Neurotrophic keratitis	Right	7	Good

**Figure 1.** Crosslinked AMs were processed into contact lens-shaped membranes by GA crosslinking (A) and DAS crosslinking (B).

lenses (Acuvue; Johnson & Johnson Vision Care, Inc., Jacksonville, FL, USA). After fitting, slit-lamp examination (Model 900 BQ; Haag-Streit, Bern, Switzerland) and ophthalmic coherence tomography (OCT) were performed to assess the centration and degree of contact of each AM. All OCT imaging was performed using spectral-domain OCT (Spectralis, Heidelberg Engineering, Heidelberg, Germany; Spectral OCT/SLO, OTI Ophthalmic Technologies, Inc., Toronto, Canada).

Statistical Analysis

The experimental data were expressed as means \pm standard deviation. Statistical significances of differences were determined by the Student's unpaired *t*-test, and $P < 0.05$ was considered statistically significant. Analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Crosslinking of AM

Both GA- and DAS-crosslinked contact lens-type AMs are shown in Figure 1. In both cases, the contact lens shape was maintained after the crosslinking proce-

dure. The AMs were cryopreserved at -80°C in a 1:1 mixture of glycerol and Gibco DMEM, phenol red-free.

Measurement of Ultimate Tensile Strength and Elasticity

The stress-strain curves showed that the mechanical properties of the AMs were significantly improved by the chemical crosslinking and changed according to the type of crosslinking agent (Fig. 2A). The ultimate tensile strengths of the GA- and DAS-crosslinked samples were 13.0 ± 1.4 MPa and 5.7 ± 1.2 MPa, four and two times higher than the normal AM (3.1 ± 0.3 MPa), respectively ($P < 0.005$ and $P < 0.05$, respectively) (Fig. 2B). The GA- and DAS-crosslinked samples also showed a remarkable increase of elastic moduli (46.5 ± 9.8 MPa and 12.9 ± 3.5 MPa, respectively) as compared with the normal AMs (8.5 ± 1.7 MPa) ($P < 0.005$) (Fig. 2C). These results show that the GA- and DAS-crosslinked AMs demonstrated a significant increase in mechanical strength as compared with the normal AMs.

Measurement of Transmittance

Measurements of transparency from 300 to 700 nm revealed that the normal AMs had the highest trans-

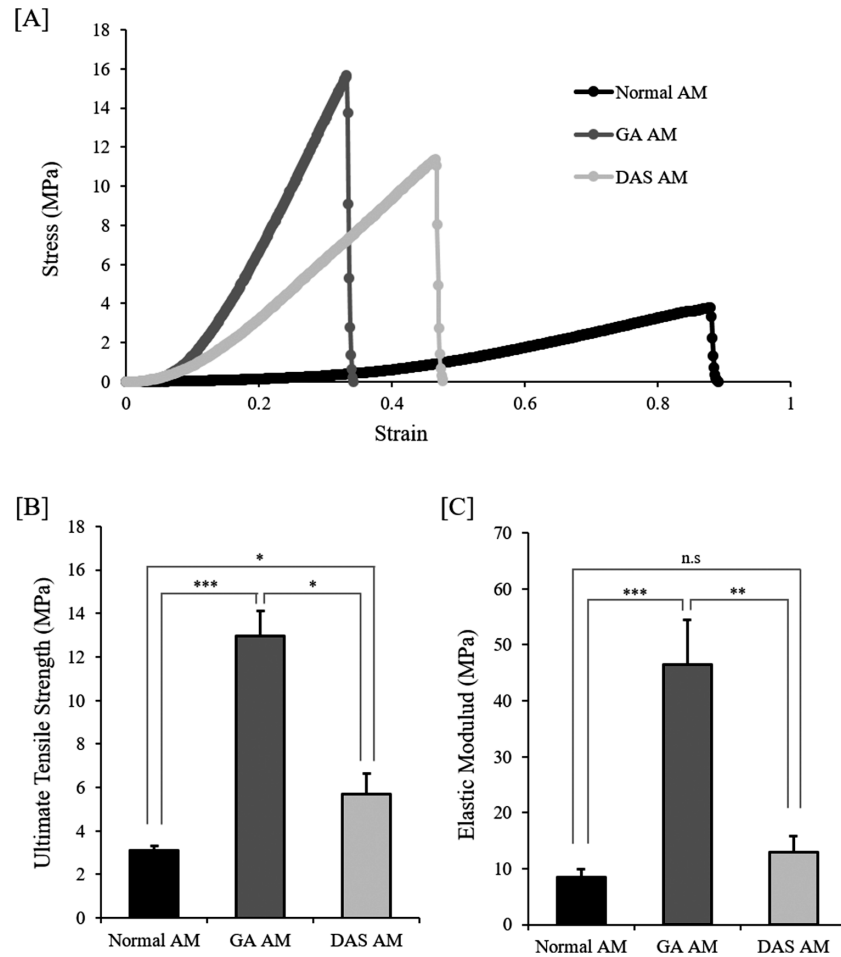


Figure 2. For tensile strength and elasticity measurement, the membrane was pulled in both directions at a constant force until it broke. The force per hour was measured and recorded (A). The largest value of stress was recorded for tensile strength (B); the slopes of the graph represent the elastic modulus (C). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0001$; n.s., not significant.

parency (Fig. 3A). At a wavelength of 550 nm, the most sensitive wavelength for human eyes, normal AMs had the highest transparency (Fig. 3B); however, no statistical significance ($P < 0.05$) was observed. These results indicate that the GA and DAS crosslinking did not affect the transparency of the AMs.

Histologic Analysis

After crosslinking, H&E staining was performed to analyze the structural integrity of the AMs and determine their resistance to the action of destructive crosslinking agents. In GA-crosslinked AMs (Fig. 4B), the epithelial layer was thin and not clearly differentiated from the basement membrane layer. In the DAS-crosslinked AMs (Fig. 4C), the epithelial layer and basement membrane layer were similar to those observed in normal AMs.

The basement membrane of AMs is comprised of collagen type IV, which makes up the basement membrane of most stromal cells.³⁰ Collagen type IV was well maintained in DAS-crosslinked AMs as compared with normal AMs (Figs. 4D, 4F); however, no expression of collagen type IV was detected in GA-crosslinked AMs (Fig. 4E). These results indicate that the agent DAS had no effect on the structural integrity of the AMs.

Measurement of Growth Factors in Crosslinked AMs

The GA- and DAS-crosslinked AMs were evaluated for concentrations of growth factors (Fig. 5). The levels of growth factors such as EGF, TGF- β 1, EGFR, IGF-1, bone morphogenetic protein 5 (BMP-5), phosphatidylinositol-glycan biosynthesis

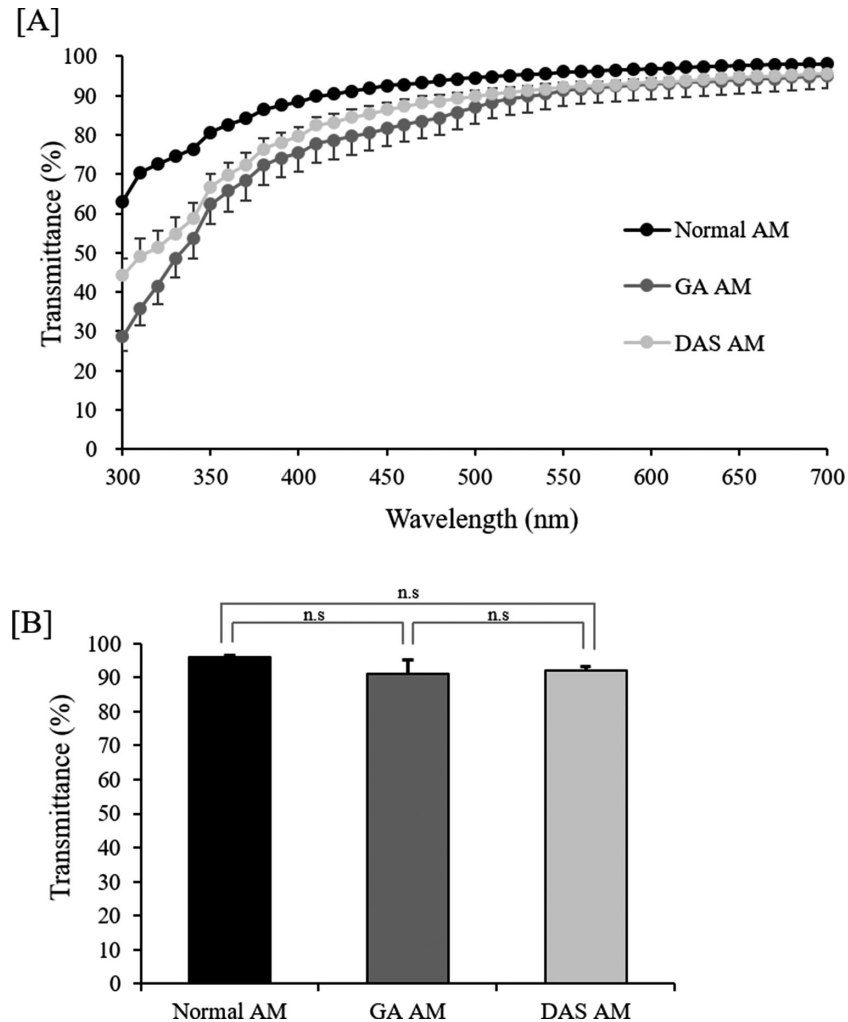


Figure 3. Measurement of AM transparency. (A) Optical transmittance of normal and crosslinked AMs at wavelengths from 300 to 700 nm. (B) Transparency of normal and crosslinked AMs at 550 nm. n.s., not significant.

class F (PIGF) protein, and growth hormone (GH, somatotropin) in each supernatant were measured.

The EGF concentrations of the normal and DAS-crosslinked AM conditioned media were 15.1 ± 1.1 pg/ml and 13.9 ± 1.2 pg/ml, respectively, three time higher than the concentration for the GA-crosslinked AMs (5.8 ± 0.3 pg/ml) ($P < 0.001$). Also, the normal and DAS-crosslinked AMs showed a remarkable increase of TGF- β 1 (22577.9 ± 990.7 pg/ml and 15920.5 ± 503.9 pg/ml, respectively) as compared with the GA-crosslinked AMs (7899.1 ± 227.4 pg/ml) ($P < 0.001$). The levels of growth factor for normal and DAS-crosslinked AMs were higher than for GA-crosslinked AMs. EGFR concentrations for normal and DAS-crosslinked AMs were significantly higher (1884.0 ± 497.1 pg/ml and 677.9 ± 60.1 pg/ml, respectively) than for the GA-crosslinked AMs (227.0 ± 10.8 pg/ml) ($P < 0.001$). Similarly, DAS-crosslinked AMs

had a higher concentration of IGF-1 than did GA-crosslinked AMs (normal AM, 2261.1 ± 37.4 pg/ml; GA-crosslinked AM, 945.3 ± 61.8 pg/ml; and DAS-crosslinked AM, 1198.5 ± 77.1 pg/ml) ($P < 0.001$).

The BMP-5 concentration for normal AMs was 18668.4 ± 2642.4 pg/ml, which was statistically different from the concentration for GA-crosslinked AMs (8685.9 ± 720.9 pg/ml) ($P < 0.05$) but was not statistically different from the concentration for DAS-crosslinked AMs (10497.9 ± 815.1 pg/ml) ($P < 0.05$). For DAS-crosslinked AMs, the concentrations of insulin (normal AM, 4361.6 ± 269.2 pg/ml; GA-crosslinked AM, 2276.2 ± 178.1 pg/ml; and DAS-crosslinked AM, 3361.7 ± 133.1 pg/ml), PIGF (normal AM, 161.8 ± 14.1 pg/ml; GA-crosslinked AM, 94.8 ± 2.3 pg/ml; and DAS-crosslinked AM, 117.1 ± 8.2 pg/ml), and GH (normal AM, 1032.3 ± 69.5 pg/ml; GA-crosslinked AM, 769.9 ± 89.7 pg/ml; and DAS-

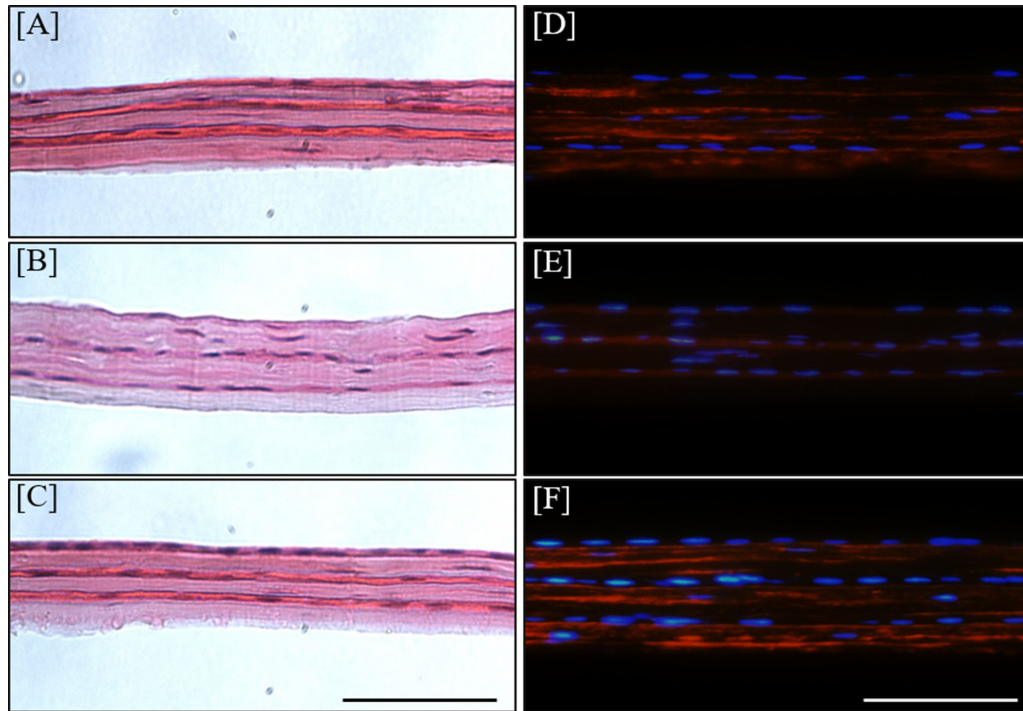


Figure 4. Histology and expression of collagen IV in AMs. Normal AMs (A), GA-crosslinked AMs (B), and DAS-crosslinked AMs (C) were stained with H&E. Normal AMs (D), GA-crosslinked AMs (E), and DAS-crosslinked AMs (F) were immunolabeled with collagen type IV. Scale bar: 50 μm .

crosslinked AM, 871.6 ± 39.4 pg/ml) were higher compared with GA-crosslinked AMs ($P < 0.05$) but did not differ statistically from normal AMs ($P < 0.001$). Overall, the retention of growth factors was found to be higher in DAS-crosslinked AMs than in GA-crosslinked AMs.

Biocompatibility of Crosslinked AM

After the AMs were incubated for 24 hours, cell viability for the normal and crosslinked AMs was evaluated based on the epithelial cell attachment rate observed on tissue culture dishes coated with collagen type I. As shown in Figure 6, when cell relative adhesion rates were 100.0% for normal AMs, cell viability values for the GA- and DAS-crosslinked AMs were $30.8 \pm 7.4\%$ ($P < 0.001$) and $81.9 \pm 9.6\%$ ($P < 0.05$), respectively (Fig. 6A). The images were captured after the 24 hours of incubation followed by treatment with calcein-acetoxymethyl (Figs. 6B–6D). The number and proportion of adherent cells for the DAS-crosslinked AMs and the normal AMs were similarly high ($P < 0.05$), and the GA-crosslinked AMs were reported to attach to significantly fewer cells ($P < 0.001$).

Wound Healing Assay

Using an in vitro wound healing model of HCE cells, we investigated the effects of corneal epithelial cell migration on the three conditions: normal AMs and DAS- and GA-crosslinked AMs (Fig. 7). Representative micrographs of wound analysis over time are shown in Figure 7A. Wounds treated with normal AMs were completely recovered at 24 hours, and considerable cell migration occurred for the DAS-crosslinked AMs. On the other hand, little cell migration was observed for the GA-crosslinked AM group over time. When comparing the relative adhesion rates of cell migration at specific times (Fig. 7B), we found that for the normal AM group ($30.4 \pm 5.0\%$ at 6 hours, $68.0 \pm 3.1\%$ at 12 hours, and $98.7 \pm 3.7\%$ at 24 hours) the scratches had almost recovered over time. The DAS-crosslinked AM group did not recover as much as the normal AM group, but they recovered significantly ($23.7 \pm 3.4\%$ at 6 hours, $35.6 \pm 6.6\%$ at 12 hours, and $52.9 \pm 4.5\%$ at 24 hours). The lowest levels of cell migration rate were observed in GA-crosslinked AM conditioned media ($3.4 \pm 0.7\%$ at 6 hours, $6.0 \pm 1.0\%$ at 12 hours, and $8.4 \pm 1.1\%$ at 24 hours), which was a statistically significant ($P < 0.05$) difference from the normal and DAS-crosslinked AM groups. In

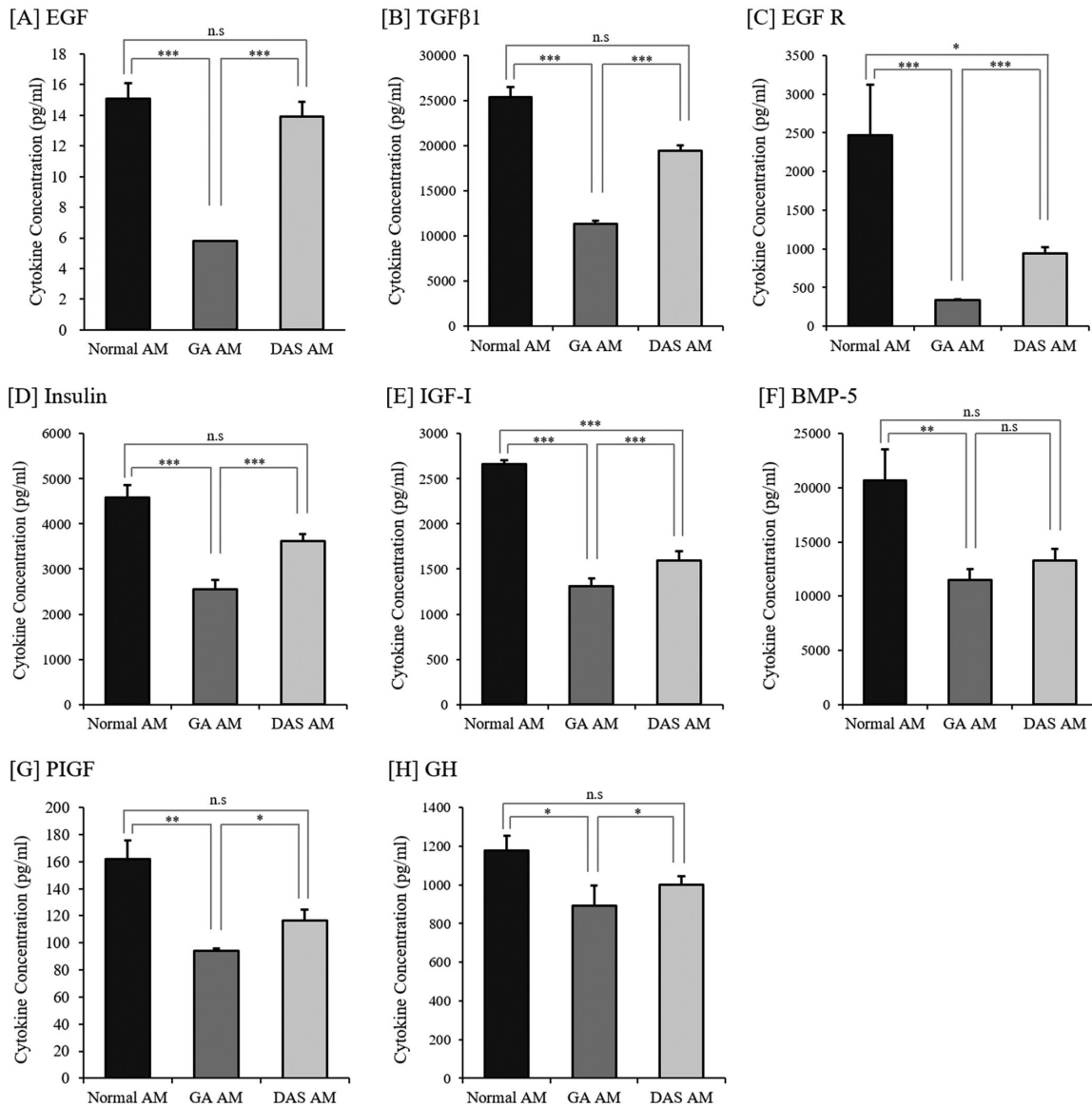


Figure 5. Measurement of growth factors secreted from normal AMs and from GA- and DAS-crosslinked AMs: (A) EGF, (B) TGF- β 1, (C) EGFR, (D) insulin, (E) IGF-1, (F) BMP-5, (G) PIGF, and (H) GH. DAS AM and GA AM indicate DAS-crosslinked AMs and GA-crosslinked AMs, respectively. * $P < 0.05$, ** $P < 0.05$, *** $P < 0.001$; n.s., not significant.

particular, it was confirmed that the DAS-crosslinked AM group did not differ in a statistically significant way compared with the normal AM group at 6 hours.

Collagenase Assay

As shown in Figure 8, the GA- and DAS-crosslinked AMs were more resistant to the action of the degrading enzyme than the normal control AMs. After 24 hours of incubation, the normal AMs remained at $70.3 \pm 1.3\%$, but the GA- and DAS-crosslinked AMs remained at $99.7 \pm 0.5\%$ and $85.0 \pm 2.5\%$, respec-

tively. The GA- and DAS-crosslinked AMs also showed higher resistance ($98.7 \pm 0.5\%$ and $65.0 \pm 1.6\%$, respectively) compared with the normal AMs ($50.0 \pm 0.8\%$) after 48 hours of incubation ($P < 0.005$). Samples of the GA- and DAS-crosslinked AMs demonstrated resistance of $98.0 \pm 0.8\%$ and $51.7 \pm 1.7\%$ over 72 hours, 3 times and 1.5 times greater than for the normal AMs ($30.3 \pm 1.7\%$), respectively ($P < 0.001$). Based on these results, crosslinked AMs can be expected to remain on the ocular surface for a relatively longer time. GA-crosslinked AMs were more resistant than DAS-crosslinked AMs to the action of the degrading enzyme.

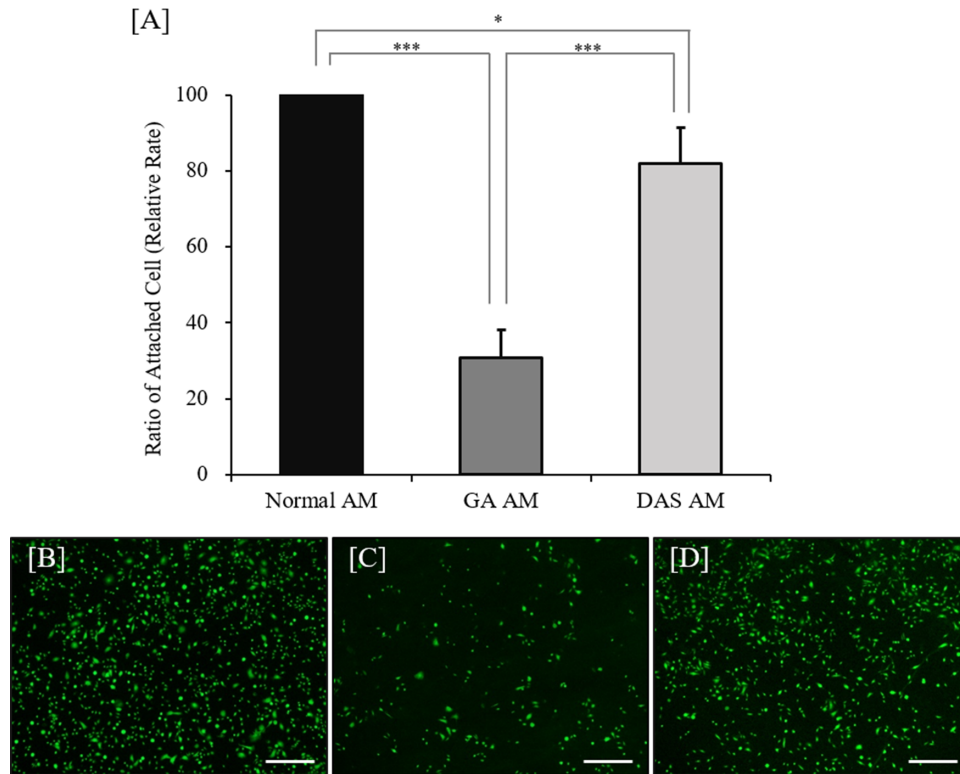


Figure 6. Biocompatibility test. HCE cells were attached to each AM type. The attached cells were stained with calcein-acetoxymethyl after 24 hours, and the cell number was counted in a unit area of $500 \times 500 \mu\text{m}$ (A). Ratios between normal AMs and crosslinked AMs were compared. Images show HCE cells stained with calcein-acetoxymethyl cultured on normal AMs (B), GA-crosslinked AMs (C), and DAS-crosslinked AMs (D). * $P < 0.05$, *** $P < 0.0001$. Scale bar: $500 \mu\text{m}$.

In vivo Experiment Using the Rabbit Corneal Epithelial Defect Model

After treatment for 24 hours, DAS-crosslinked AMs were well maintained on the rabbit corneas, and the areas of epithelial damage were reduced for eyes treated with crosslinked AMs compared with the control AMs (untreated) (Fig. 9). The wound area treated with the DAS-crosslinked AMs was 64.0%, which was reduced more quickly compared with the control AMs (89.2%). Thus, DAS-crosslinked AMs can be expected to promote faster recovery of the corneal epithelium than the control.

Fitting Test

Slit-lamp and OCT images confirmed that DAS-crosslinked AMs were securely attached to the human cornea (Fig. 10). DAS-crosslinked AMs covered with bandage contact lenses were maintained on the ocular surface for 7 days. Of the six patients who participated in the pilot study, five patients showed very good tolerance and retained the crosslinked AMs for 7 days without decentration; one crosslinked AM was lost out

of a patient's eye after 4 days without ocular injury, perhaps due to irregularities in the corneal epithelium. The results of this experiment indicate that the durability of sutureless fixation of contact lens-type AMs is similar to that of conventional AM fixation.

Discussion

A 10-0 nylon suture is widely employed to secure an AM to the ocular surface; however, such a suture limits the use of AMs because of the various suture-related side-effects.¹⁴ PROKERA was developed to address the problems caused by the use of sutures,^{17,18} but its fixation ring interferes with the ability of the AM to attach tightly to the ocular surface, and a discrepancy in size between the conjunctival sac and the ring causes discomfort to patients. It would definitely be of value to develop a contact lens-shaped AM that fits the native cornea without a supporting device.

This study applied the crosslinking agents of GA and DAS to create contact lens-shaped AMs. The sequential process of drying and crosslinking

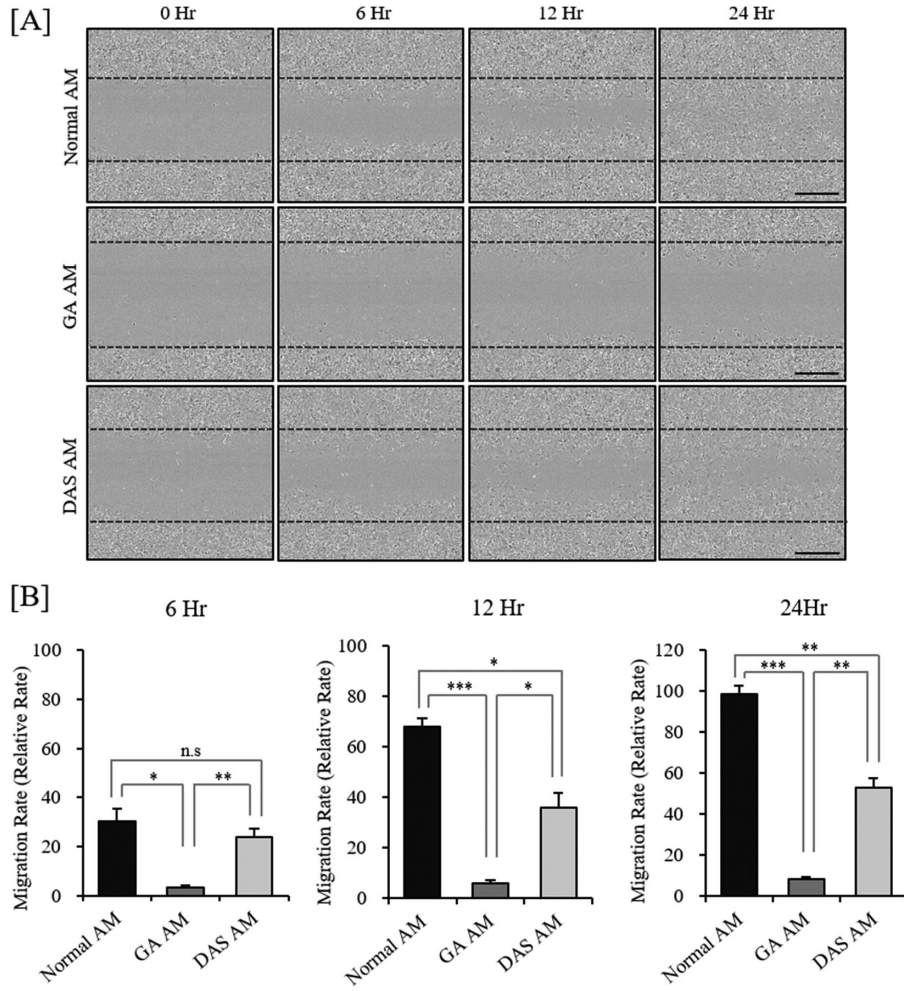


Figure 7. Wound healing assay. Wound closure rates were evaluated in the in vitro scratch assay. HCE cells were treated with normal or GA- or DAS-crosslinked AM conditioned medium. (A) Representative micrographs of the wound closure assays in the various media. (B) Relative rates of migration rates for monolayer cultures of HCE cells at 6, 12, and 24 hours. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n.s., not significant. Scale bar: 300 μm .

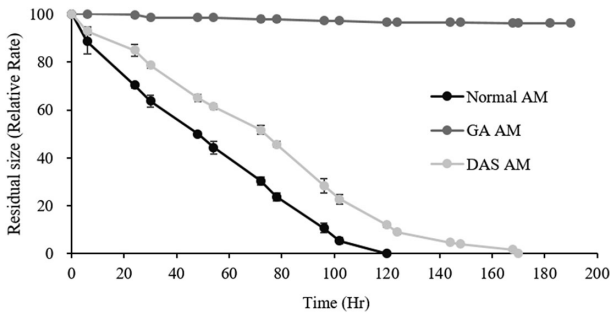


Figure 8. Degradation of AMs following crosslinking. GA- and DAS-crosslinked AMs showed an increase in resistance to degradative enzymes as compared with untreated AMs.

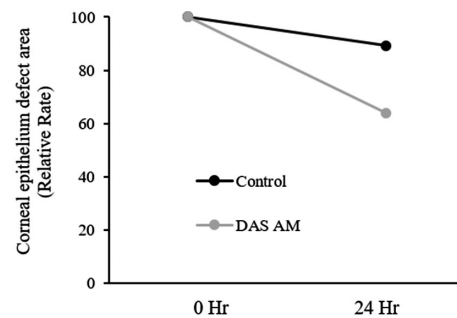


Figure 9. Comparison of recovery following corneal epithelial damage. After 24 hours of treatment, the area of epithelial damage was reduced for eyes treated with DAS-crosslinked AMs compared with the control (untreated) AMs.

on the curved mold successfully generated three-dimensionally curved AMs with significant improvement in mechanical properties. The ultimate tensile strength and elastic modulus significantly increased

for both GA- and DAS-crosslinked AMs as compared with normal AMs and were sufficient for maintaining the contact lens shape. We observed that DAS-

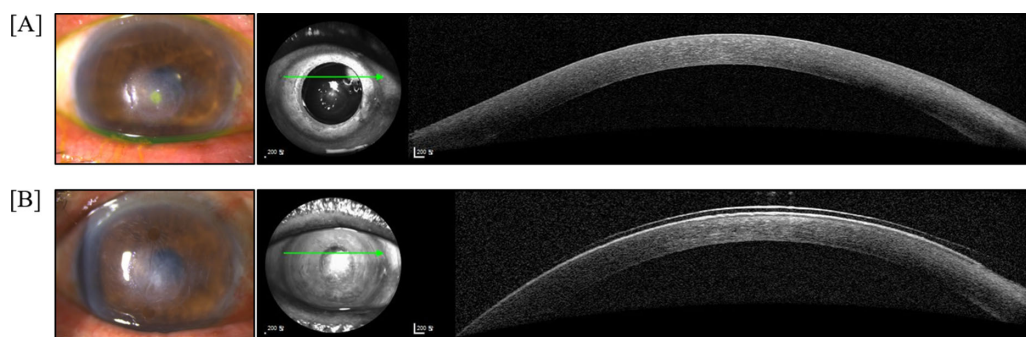


Figure 10. Slit-lamp examination and OCT images of DAS-crosslinked AM on a human corneal surface: (A) before wearing and (B) after wearing.

crosslinked AMs did not last as long as the GA-crosslinked AMs, but they did not degrade over any longer period than normal AMs. This suggests that DAS-crosslinked AMs can prolong application times by delaying by approximately 2 to 3 days the usual dissolving and disappearance of AMs during corneal damage healing.

In contrast, the GA-crosslinked AMs seemed to lose much of their biologic activity, although they showed very high tensile strain and resistance to collagenase and so would not be expected to dissolve for a long time after ocular surface insertion. In the histological analysis of the GA-crosslinked AMs, basement membranes and epithelial layers were indistinguishable and no collagen type IV was detected, indicating that GA crosslinking affects the biological composition of the AM. On the other hand, the DAS crosslinking process had no effect on the biological composition and activity of the AM, and the structure of the AM after crosslinking was intact, as evident from the histological analysis.

Compared with GA-crosslinked AMs, *in vitro* experiments indicated that the DAS-crosslinked AMs displayed a high relative adhesion rate of cells that was comparable to that of normal AMs, and they appeared to have the same wound healing rate as normal AMs at 6 hours after amnion culture treatment. This suggests that DAS-crosslinked AM treatment may be suitable early in the treatment of ocular surface disease. Moreover, *in vivo* experiments confirmed that DAS-crosslinked AMs promoted the recovery of rabbit corneal injury and were maintained for more than 7 days during human ocular surface fitting. This observation suggests that DAS-crosslinked AMs are nontoxic and may be suitable as a dressing for ocular surface treatment.

In conclusion, the contact lens-shaped AM dressing prepared with the crosslinking agent DAS exhibits excellent transparency and tensile strength and offers several competitive advantages, without any struc-

tural destruction. In particular, the DAS-crosslinked AMs showed excellent biocompatibility, as well as high growth factor content, and they can be expected to be used effectively in the treatment of various ocular surface diseases. In addition, DAS-crosslinked AMs can eliminate a number of surgery-related issues, such as hemorrhage, suture complications, having to reoperate, suture removal, and operation time. DAS-crosslinked AMs have the advantage of being able to be applied without anesthesia, and repeat transplantation is possible, if necessary. The DAS-crosslinked AMs can safely deliver the benefits of AMs without damaging the patient's corneal surface, so these AMs should be effective for treating ocular surface disease.

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