Light-Initiated Bonding of Amniotic Membrane to Cornea

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PURPOSE. Suturing amniotic membrane to cornea during surgery is time consuming, and sutures may further damage the eye. The authors introduce a novel sutureless, light-activated technique that securely attaches amnion to cornea through protein-protein crosslinks.

METHODS. Cryopreserved human amniotic membrane, stained with Rose Bengal (RB), was placed over a full-thickness wound in deepithelialized rabbit cornea and was treated with green laser. The intraocular pressure that broke the seal (IOPs) was measured, and adhesion was measured with a peel test. The influences on bonding strength of fluence, irradiance, RB concentration, and amnion surface bonded were measured. Epithelial cell migration on treated amnion and keratocyte viability after bonding were also measured. The involvement in the bonding mechanism of oxygen, singlet oxygen, and association of RB with stromal collagen was investigated.

RESULTS. Sealing amniotic membrane over cornea using 0.1% RB and 150 J/cm² at 532 nm produced an IOP of 261 ± 77 mm Hg ex vivo and 448 ± 212 mm Hg in vivo. The ex vivo IOPs increased with increasing fluence (50–150 J/cm²). Equivalent IOPs were produced for bonding basement membrane or stromal amnion surfaces. The bonding treatment was not toxic to keratocytes but slightly reduced the migration of corneal epithelial cells on amnion ex vivo. Mechanism studies indicated that RB forms two complexes with amnion stromal collagen, that bonding requires oxygen, and that singlet oxygen mediates protein crosslinking.

CONCLUSIONS. A rapid, light-activated technique produces strong, immediate bonding between amnion and cornea and merits further evaluation for ocular surface surgeries. (Invest Ophthalmol Vis Sci. 2011;52:9470–9477) DOI:10.1167/ iovs.11-7248

Amniotic membrane is frequently used in corneal and scleral surgery as a temporary patch or a reconstructive graft.1 Use of amniotic membrane transplantation (AMT) in-clude covering persistent epithelial defects, pterygium surgery, and ocular surface reconstruction in stem cell deficiency. Amniotic membrane is composed of a single layer of epithelial cells attached to a basement membrane that lies over a stromal layer containing primarily types I and III collagen, proteoglycans, and fibroblasts. Currently amnion is sutured to the cornea, a time-consuming process requiring high skill to place hair-fine sutures. In addition, suturing may injure the eye and, because sutures act as a foreign body, can lead to persistent inflammation, infection, and granuloma.1,2

We have evaluated an additional application of AMT, namely, sealing amniotic membrane over penetrating corneal wounds using a novel light-activated technology called photochemical tissue bonding (PTB). PTB produces an immediate seal between tissue surfaces without additional glues or proteins. Covalent crosslinks are formed that bridge proteins between the tissue surfaces by a photochemical, nonthermal mechanism.3–12 After applying a photoactive dye, the tissue surfaces are placed in contact, and the area is treated with a green laser to activate the dye and initiate the bonding chemistry. The dye, Rose Bengal (RB), is approved by the US Food and Drug Administration for the diagnosis of ocular surface damage.

A rabbit eye model of penetrating eye injury was chosen to challenge the ability of PTB to form a strong and secure seal between amniotic membrane and the corneal surface. In this study we identified the PTB treatment conditions that produced strong bonding, evaluated potential side effects, and investigated mechanisms for crosslink formation between amnion and corneal surface proteins.

MATERIALS AND METHODS

Materials

Frozen albino rabbit eyes from Pel-Freeze Biologicals (Rogers AR) were used at room temperature. Rose Bengal (95%; Sigma-Aldrich, St. Louis, MO) was a 0.1% wt/vol solution in phosphate-buffered saline (PBS; Sigma-Aldrich). Fibrin sealant (Tisseel) was from Baxter (Deerfield, IL). Nitrogen (Ultra High Purity) and oxygen (99.5%) were from Airgas (Cambridge, MA). New Zealand White rabbits (weight range, 2–2.5 kg) were purchased from Charles River Laboratories (Wilmington MA). The in vivo study was approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Amniotic membrane was obtained from scheduled caesarean deliveries, as we have done previously.9 This research adhered to the tenets of the Declaration of Helsinki. The amnion epithelium was removed with trypsin (0.25%, 90 minutes, 37°C) and light rubbing. Amnion was stored on nitrocellulose paper at −80°C in 1:1 glycerol/Dulbecco’s modified Eagle’s medium (DMEM) with 1% penicillin-streptomycin and 0.05% amphotericin B (Sigma-Aldrich).


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Absorption Spectra

Spectra of RB on amnion or on amnion bonded to cornea were measured on glass slides using a microplate reader (Spectramax M5; Molecular Devices, Sunnyvale CA). To correct for scattering from these tissues, spectra of amnion or cornea (without RB) were subtracted.

Association of Rose Bengal with Amnion

Amnion samples (1.1–1.3 mg) were stained on the stromal surface with 0.1% RB (5 minutes) and were briefly washed with PBS before absorption spectra were measured. Samples were then placed individually in 1 mL PBS and kept in the dark at room temperature until absorption spectra were measured. After each measurement, the samples were placed in fresh PBS.

Preparation of Amnion Patch

Amniotic membrane was first rinsed in PBS for 45 minutes to remove glycerol from the storage medium, then transferred from the nitrocellulose backing to Parafilm with the stromal surface upward and allowed to dry. A 13-mm diameter circle was cut from the amnion and Parafilm backing (Fig. 1A). RB solution (0.1% in PBS, ~200 μL) was placed on the amnion stromal surface for 5 minutes (Fig. 1B), and excess was removed to produce slightly moist, but not wet, amnion. The dye-stained amnion was peeled from the backing (Fig. 1C).

Bonding Amniotic Membrane to Rabbit Cornea

The same procedure for photobonding amnion to cornea was followed for both ex vivo and in vivo rabbit eyes, except for the differences noted. In vivo, the nictitating membrane was displaced and held with a suture (Fig. 1D). The epithelial layer was removed with 70% ethanol (10–15 seconds), and a full-thickness incision was made in the central cornea (ex vivo: V-shaped, 90° angle, 2-mm arms; in vivo, linear 5 mm) (Fig. 1E). The RB-stained amnion was placed with its stromal surface in contact with the cornea, and wrinkles were removed (Figs. 1G, 1H). A 4-mm diameter pupil-blocking opaque white disc was placed over the center cornea (Fig. 1I) before irradiation (Fig. 1J). A green laser (OcuLight OR KTP; Iridex Corp., Mountain View, CA) emitting cw 532 nm radiation was used for irradiances = 0.25 W/cm². A 532 nm cw Nd/YAG laser (Aura i; Laserscope, San Jose, CA) was used to deliver 0.5 W/cm². The beam was transmitted through a 600-μm optical fiber and passed through a microscope objective (20× or 40×) to produce a homogeneous beam at the corneal surface. Laser power was measured with a spectroradiometer (SPR-01; Luzchem, Ottawa, ON, Canada). The amnion surface was lightly misted with water every 90 seconds during irradiations.

To evaluate the influence of oxygen on photobonding, the cornea was stained instead of the amnion because RB photobleaches more rapidly in the presence of oxygen. Thus, more green light would have reached the cornea-amnion interface through the RB-stained amnion in the presence of oxygen. The deep epithelialized corneal surface was...
placed in 1% RB for 2 minutes and then briefly washed. After an incision was made and the amnion patch was placed, the eye was placed in a 3-cm-diameter, 7.5-cm tall plastic cylinder. To maintain humidity, water-saturated gases were used to purge the cylinder before (10-minute) and during irradiation. The amnion-covered cornea was irradiated through the plastic top (150 J/cm², 0.25 W/cm²).

When H₂O and D₂O were compared, the corneal surface was immersed in either D₂O or H₂O for 30 minutes before the incision. The amnion was treated for 5 minutes with 0.1% RB prepared in either D₂O or H₂O.

For bonding with fibrin sealant (Tissel; Baxter), 11 μL thrombin solution was spread on the amniotic membrane and 11 μL fibrinogen solution was spread on the cornea, as described previously. The amnion was then placed over the V-shaped wound in the cornea, and any wrinkles were removed. The eye was allowed to stand at least 15 minutes before bonding strength was measured.

### Measurement of Bonding Strength

Bonding strength between the amnion and cornea was determined by slowly infusing PBS into the anterior chamber through a 22-gauge needle inserted into the cornea ~1 mm anterior of the limbus and parallel to the iris. A mini-infuser (Genie Plus Infusion/Withdrawal Pump, Kent Scientific, Torrington, CT) and a pressure transducer (IsoTec; Harvard Apparatus, Holliston, MA) were connected by a T-couple to the needle. The increase in IOP was measured immediately after photobonding by the method used previously. The amnion surface was wetted before measurement to ensure that drying of the amnion on the cornea did not contribute to the bonding strength. The pressure attained immediately before fluid leaks from under the amnion, the leak pressure (IOPₗ), is a measure of bonding strength. For in vivo studies, the animals were euthanized immediately after the measurement.

Adhesion was also measured using a peel test. A 5-mm wide, 20-mm long strip of amnion was bonded to a 6-mm wide and 10-mm long strip of cornea using PTB or fibrin sealant (Tissel, Baxter). The bonded overlap area measured 5 mm × 10 mm (Supplementary Material and Fig. S1, http://www.iovs.orglookup/suppl/doi:10.1167/iovs.11-72418/DSupplemental). The PTB bonding procedure mimicked that used to seal amnion to the intact cornea as described. A previously described procedure was followed for bonding amnion to cornea with fibrin sealant. Equal volumes (7.5 μL) of thrombin and fibrinogen solutions were applied to the amnion and corneal surfaces, respectively, which were then placed in tight contact. After at least 1 hour, the force generated while peeling amnion from the cornea was measured using a universal testing system (Nano UT; Surface Systems and Technology GmbH, Hueckelhoven, Germany) with a separation rate of 5.0 mm/min. The mean force (milliNewtons [mN]) generated while peeling was measured using a universal testing system (Nano UT; Surface Systems and Technology GmbH, Hueckelhoven, Germany) with a separation rate of 5.0 mm/min.

### Keratocyte Viability

RB-stained amnion and a 4-mm opaque disc were placed on the central corneas of freshly harvested rabbit eyes, which were then exposed to either 100 or 200 J/cm². The amnion was removed, and the eye was maintained in organ culture in DMEM at 37°C and 5% CO₂. After 24 hours, the corneal sections were fixed in 10% formalin,paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Vertical sections (5 μm) contained both the irradiated peripheral and the light-blocked central areas. The keratocytes in 12 areas, each measuring 0.25 mm², adjacent to the anterior corneal surface were counted in both the non-light-treated area and the surrounding irradiated areas.

### Migration of Corneal Epithelial Cells on Photocrosslinked Amnion

Amnion was treated with 0.1% RB for 5 minutes before brief washing and irradiation with fluence of 532 nm between 0 and 150 J/cm². Excess RB was removed by soaking amnion in PBS for 18 hours. Immortalized human corneal-limbus epithelial cells (4 × 10⁴) were placed on the basement membrane of deepithelialized amnion within a 6-mm cloning ring for 3 hours. At 24 to 96 hours after the ring was removed, the distance from the ring to the edge of the migrating cells was measured at six evenly separated locations on the circumference of the circle.

### Statistical Analysis

Student’s t test for unpaired data was used to compare groups; significance was set at P < 0.05.

### Results

### Rose Bengal Associates with Amniotic Membrane Components

The amount of RB associated with amnion was calculated from the absorbance of RB at 532 nm after treating the stromal surface with RB (0.05% and 0.1%, ~200 μL) for 5 or 10 minutes and briefly washing. As shown in Figures 2A and 2B, the RB absorption approximately doubled between 5 and 10 minutes of staining. The mean RB concentration was estimated using an absorption coefficient for RB at 532 of 3.9 × 10⁻³ M⁻¹ · cm⁻¹ (measured for RB bound to type I collagen in solution, Y. Tang, unpublished result, 2010). Because amnion demonstrates a location-dependent variation in thickness, the absorption by RB is expected to vary. Using a mean amnion thickness of 50 μm, the stromal RB was calculated to be 2.56 and 5.12 mM after 5 minutes of staining with 0.05% and 0.10% RB, respectively. These RB concentrations are approximately five-fold greater than the RB staining solutions (0.05% and 0.10% correspond to 0.5 and 1 mM RB), indicating that RB complexes with components in amnion.

To further investigate this association, amnion was stained with 0.1% RB for 5 minutes and then was incubated in PBS for varying times. The decrease in RB absorption over time indicated that a portion of the RB diffused from the membrane (Fig. 2C). After 30 minutes, only approximately 25% of the RB was retained in the membrane. When the staining period was 10 minutes, the initial absorbance was higher, but the final amount of RB retained was the same (Fig. 3C, inset). Thus, RB appears to associate with amnion collagen in tight-binding sites that retain RB even after extended washing and in a larger number of loose-binding sites.

### Bond Strength Increases with Fluence

The relationship between fluence and bond strength was measured using three fluences, 50, 100, and 150 J/cm², delivered in 3.3, 6.7, and 10 minutes, respectively, to amnion stained with 0.1% RB. Controls were amnion stained with RB but not irradiated, unstained amnion treated with 150 J/cm², and amnion sutured to the cornea with eight nylon sutures. As shown in Figure 3A, strong bonding was produced using all three fluences; the mean IOPₗ values of 95 to 261 mm Hg are significantly higher than the normal IOP of human eyes (~20 mm Hg). The IOPₗ produced by 100 and 150 J/cm² differed significantly from the control (P < 0.001) and from each other (P = 0.03). Bonding strength for the controls were all <20 mm Hg. Fibrin sealant produced an IOPₗ of 66.6 ± 18.8 mm Hg, significantly different from the control (P < 0.05).

The adhesion of amnion to cornea was also measured using a 180° peel test (Fig. 3B). The force generated while peeling amnion from cornea after bonding with PTB was greater than that for the control using either 100 or 50 J/cm² (P < 0.0005). The adhesion strength after bonding amnion to cornea with fibrin sealant did not differ significantly from the control (P = 0.085).

Although the trend in the results in Figure 3A suggests that fluences higher than 150 J/cm² might produce even stronger
bonding, RB was destroyed (i.e., photobleached) during the irradiation as seen by the decrease in the RB absorption (Fig. 3C), suggesting that higher fluences would not proportionally increase the IOP\textsubscript{T}.

**Relationships among Irradiance, Temperature, and Bonding**

Higher irradiance delivers the same fluence in a shorter time according to the relationship: Irradiance (W/cm\textsuperscript{2}) × Time (s) = Fluence (J/cm\textsuperscript{2}). However, it might also produce a damaging temperature increase. We measured IOP\textsubscript{T} after delivering 100 J/cm\textsuperscript{2} at irradiances varying by a factor of 4, which required 13.3, 6.7, and 3.3 minutes of irradiation, respectively. Surface temperature was measured during the irradiations with an infrared thermometer (model 572; Fluka, Mississauga, ON, Canada). As shown in Figure 3D, the mean IOP\textsubscript{T} for the three irradiances were 206 to 304 mm Hg, with no significant differences between these values.

The maximum temperatures attained using 0.125, 0.250, and 0.500 W/cm\textsuperscript{2} were 22.2°C, 27.8°C, and 36.7°C, respectively (Fig. 3D); thus, a substantial increase over the control (18.3°C) was produced only by the highest irradiance. However, even 36.7°C is much lower than what is used for thermal laser welding (75°C).\textsuperscript{17,18} To eliminate any potential thermal effect, 0.25 W/cm\textsuperscript{2} was used throughout these studies.

**Other Factors Influencing Bond Strength**

A fixed fluence (100 J/cm\textsuperscript{2}) and irradiance (0.25 W/cm\textsuperscript{2}) were used to test variables that might affect the IOP\textsubscript{T}. A higher RB concentration might increase bond strength because of greater light absorption (Fig. 2) and more photocrosslinking sites. As shown in Figure 4A, the IOP\textsubscript{T} using 0.10% or 0.05% RB did not differ (P > 0.05). The amnion surface in contact with the cornea might also have affected the IOP\textsubscript{T}. RB was applied to the stromal surface of deepithelialized amnion, and either the stromal or the basement membrane surface was in contact with the deepithelialized cornea. The IOP\textsubscript{T} did not differ (Fig. 4B, second and third bars), indicating that RB applied to the stromal surface penetrates the basement membrane surface. When the epithelial layer was not removed, applying RB to the epithelial face and placing the epithelial face in contact with the cornea during irradiation produced an IOP\textsubscript{T} that did not differ from the control (Fig. 4B, fourth bar). Finally, RB-stained amnion was washed for 1 hour to remove loosely associated RB before irradiation. The IOP\textsubscript{T} did not differ from the control IOP\textsubscript{T} (Fig. 4B, fifth bar), indicating that photoactivation of the tightly associated RB in the amnion was not sufficient to produce strong bonding. Further studies are required to assess the effect on photobonding of the known variation in amnion thickness and transparency.\textsuperscript{16}

**In Vivo Bonding of Amniotic Membrane to Rabbit Cornea**

The bonding procedure shown in Figure 1 was used with 50, 100, or 150 J/cm\textsuperscript{2} (3.3, 6.7, and 10 minutes’ irradiation, respec-
Strong immediate bonding was produced at all fluences, and the bond strength increased with fluence (Fig. 5). Controls (RB-stained amnion not irradiated or unstained amnion irradiated with 150 J/cm²) did not bond. Photobonding amnion to cornea was not toxic to keratocytes (Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7248/-/DCSupplemental).

Corneal Epithelial Cell Migration on Photocrosslinked Amnion

Because RB diffuses the stroma, green light may crosslink multiple stromal proteins, including those in the basement membrane. The migration of corneal epithelial cells involves interaction with basement membrane proteins and is influenced by the properties of the surface\(^{19,20}\); therefore, we tested whether PTB treatment might alter the migration of these cells on amnion. As shown in Figure 6, the PTB treatment conditions used for bonding, 100 and 150 J/cm², decreased the extent of migration by approximately 15% to 30%.

Mechanism for Photobonding Amnion to Cornea

Photoactivated RB generates singlet oxygen (\(_{1}^{1}O_2\)), a reactive oxygen species that initiates protein-protein crosslinking by oxidizing amino acid side chains, especially histidine.\(^{21-23}\) Oxidized histidine then reacts with certain amino acids, mainly lysine, to form protein-protein crosslinks. Photexcited RB may also transfer an electron to or from certain amino acids (AA) to form radical ions.\(^{24}\) Crosslinks may form without oxygen when the protonated AA\(^+\) combines or when AA\(^-\) reacts with oxygen to form products that subsequently lead to pro-
tein-protein crosslinks. These processes are shown in Supplementary Fig. S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7248/-/DCSupplemental.

To determine whether photobonding requires oxygen, irradiation was carried out in air, oxygen, or nitrogen atmospheres. Control corneas were irradiated in oxygen but were not stained with RB. Irradiations in air and oxygen produced IOPL of 178 ± 110 and 208 ± 81 mm Hg, respectively (Fig. 7A). Irradiation in nitrogen produced a substantially lower IOPL (66 ± 44 mm Hg) that did not differ from the control, indicating that oxygen participated in at least a portion of the reactions leading to covalent crosslinks.

To test for the involvement of ¹⁰², we used the inherently longer lifetime of ¹⁰₂ in D₂O than in H₂O, which will lead to increased crosslink formation. As shown in Figure 7B, using D₂O produced a higher IOPL irradiation than using H₂O. Thus, the protein photocrosslinking responsible for bonding between amnion and cornea is mediated, at least partially, by ¹⁰₂.

**DISCUSSION**

These studies demonstrate that a light-activated technology that joins tissue surfaces by forming molecular crosslinks between proteins can securely attach amniotic membrane to the corneal surface. The seal formed was immediate and strong enough to seal penetrating eye wounds. The seal strength

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**FIGURE 4.** Factors potentially influencing seal strength for bonding amnion to rabbit eyes ex vivo. (A) RB concentration used to stain amnion for 5 minutes was varied. NS = P > 0.05. (B) Influence on IOPL of the surface stained with RB, the surface bonded to the cornea, and removal of loosely bound RB. Amnion-covered corneas treated with 100 J/cm² and 0.25 W/cm² in all experimental groups. S, stromal surface stained, then bonded; BM, stromal surface stained but basement membrane surface bonded; EPI, epithelial layer stained, then bonded; RB eluted, loosely bound RB removed by soaking in PBS for 1 hour, then stromal surface bonded. Mean ± SD shown; n = 5. *P < 0.05 compared with no light group.

**FIGURE 5.** In vivo bonding of RB-stained amnion to corneal surface of rabbit eyes. Varying fluences were delivered at 0.25 W/cm² after staining with 0.1% RB for 5 minutes. No RB control, unstained amnion irradiated with 150 J/cm²; n = 5. *P < 0.05 compared with no RB control.

**FIGURE 6.** Migration of corneal epithelial cells on RB- and light-treated amniotic membrane. Cells attached to irradiated (0.1% RB, 100 and 150 J/cm²) and control amnions were allowed to migrate away from a circle, and the migration distance was measured. Mean ± SD, cumulative results from three experiments with duplicate samples. *P < 0.05.
been shown to be phototoxic to keratocytes, whereas our increased with increasing fluence and required a short irradiation time.

Sutureless attachment of amnion to cornea has the advantages of being rapid, forming an immediate water-tight seal and not causing additional damage to the cornea compared with the use of sutures. Although fibrin glue has been used for sealing amnion to the ocular surface and is used for low-tension applications, clinical experience indicates that it has insufficient bonding strength required for large lacerations and stellate lacerations. The results of this study using a V-shaped incision mimicking a large irregular laceration support this experience. Fibrin sealant produced a lower bonding strength (Fig. 3A) and adhesion strength (Fig. 3B) than PTB. In addition, fibrin adhesive is sticky, difficult to use on cornea, and must be prepared immediately before use. PTB can be simplified for clinical application by using prestained, dry-stored amnion discs and a compact, inexpensive, non–laser light source (e.g., LED) that can deliver higher irradiances that shorten the irradiation time.

Photosensitized crosslinking of collagen is well established and is under evaluation for the treatment of keratoconus using riboflavin-5-phosphate (RF-5P). RB also photosensitizes cross-link formation in collagen gels and scaffolds. Our results indicate that RB will not damage keratocytes (Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7248/). We have previously shown that PTB was not phototoxic to dermal cells in vivo when PTB was used to seal skin wounds. A small decrease in the extent of corneal epithelial cell migration was observed (Fig. 6). Whether this response will be observed in vivo must be tested. The opaque disc over the central cornea effectively kept the irradiance below established damage thresholds. Because, to bond the tissue surfaces, the protein-protein cross-links must form between amino acids on the external surface of collagen fibers.

The full-thickness wounds used for this study are frequent in both civilian and military populations, in which they constitute approximately 50% of the eye wounds in current wars. Sealing amnion with PTB over these difficult-to-suture wounds has distinct advantages. In addition, our results suggest that PTB can be used for sealing amnion in corneal surgery, including pterygium excision, fornical reconstruction, corneal melting syndromes, and attachment of composite limbal stem cell and amnion grafts. We have initiated a detailed study of longer term biological responses to photo-bonding amnion to cornea.

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