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Collagen Gels Crosslinked by Photoactivation of Riboflavin for the Repair and Regeneration of Corneal Defects

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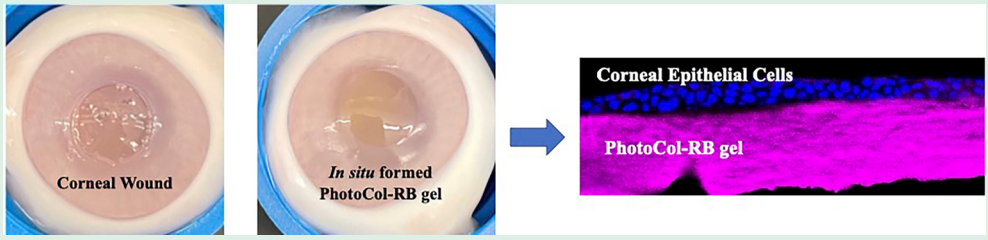

ABSTRACT: Bioengineered corneal tissue is a promising therapeutic modality for the treatment of corneal blindness as a substitute for cadaveric graft tissue. In this study, we fabricated a collagen gel using ultraviolet-A (UV-A) light and riboflavin as a photosensitizer (PhotoCol-RB) as an *in situ*-forming matrix to fill corneal wounds and create a cohesive interface between the crosslinked gel and adjacent collagen. The PhotoCol-RB gels supported corneal epithelialization and exhibited higher transparency compared to physically crosslinked collagen. We showed that different riboflavin concentrations yielded gels with different mechanical and biological properties. *In vitro* experiments using human corneal epithelial cells (hCECs) showed that hCECs are able to proliferate on the gel and express corneal cell markers such as cytokeratin 12 (CK12) and tight junctions (ZO-1). Using an *ex vivo* burst assay, we also showed that the PhotoCol-RB gels are able to seal corneal perforations. *Ex vivo* organ culture of the gels filling lamellar keratectomy wounds showed that the epithelium that regenerated over the PhotoCol-RB gels formed a multilayer compared to just a double layer for those that grew over physically cross-linked collagen. These gels can be formed either *in situ* directly on the wound site to conform to the geometry of a defect, or can be preformed and then applied to the corneal wound. Our results indicate that PhotoCol-RB gels merit further investigation as a way to stabilize and repair deep and perforating corneal wounds.

KEYWORDS: *bioengineered cornea, riboflavin, collagen, wound healing, UV-A light*

■ INTRODUCTION

A leading cause of vision impairment is trauma to the cornea—the transparent outermost tissue of the eye that provides the majority of the visual pathway's refractive power. The most common curative treatment to restore vision is a full or partial thickness keratoplasty with corneal tissue from a cadaveric donor.[1−3] Unfortunately, fewer than 2% of the 12.5 million patients worldwide suffering from corneal blindness have access to the human graft tissue required for this sight-restoring corneal transplant.[1] Bioengineered corneal tissue substitutes are a promising avenue to meet this major global clinical need. But while there has been substantial progress toward designing preformed, biosynthetic buttons, they still require meticulous lamellar dissection of the host corneal tissue, precise sizing, and numerous sutures for proper placement. Therefore, in cases of corneal perforation localized to a well-defined area of the tissue, there is a major clinical need to functionally stabilize, restore transparency, and support healing without the immediate need for a corneal transplant.

The current point-of-care treatment for corneal perforations is the off-label use of cyanoacrylate glue to stabilize the wound and prevent fluid egress. However, cyanoacrylate glue creates an opaque plaque and is only a temporary measure to seal the defect prior to a therapeutic corneal transplant.[4] Recently, approaches using three-dimensional matrix therapies including hydrogels have been introduced for corneal regeneration.[5−11] Hydrogels are water-swollen polymer networks that can be designed to provide structural support to a defect and recapitulate biochemical features of the native extracellular matrix. Photocrosslinkable methacrylated or acrylated gelatin-based gels activated by ultraviolet (UV) or visible light have recently been reported for the purpose of filling stromal defects of the cornea.[5,6] We have previously shown that growth factors can be conjugated to collagen matrix by using riboflavin and

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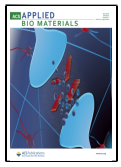

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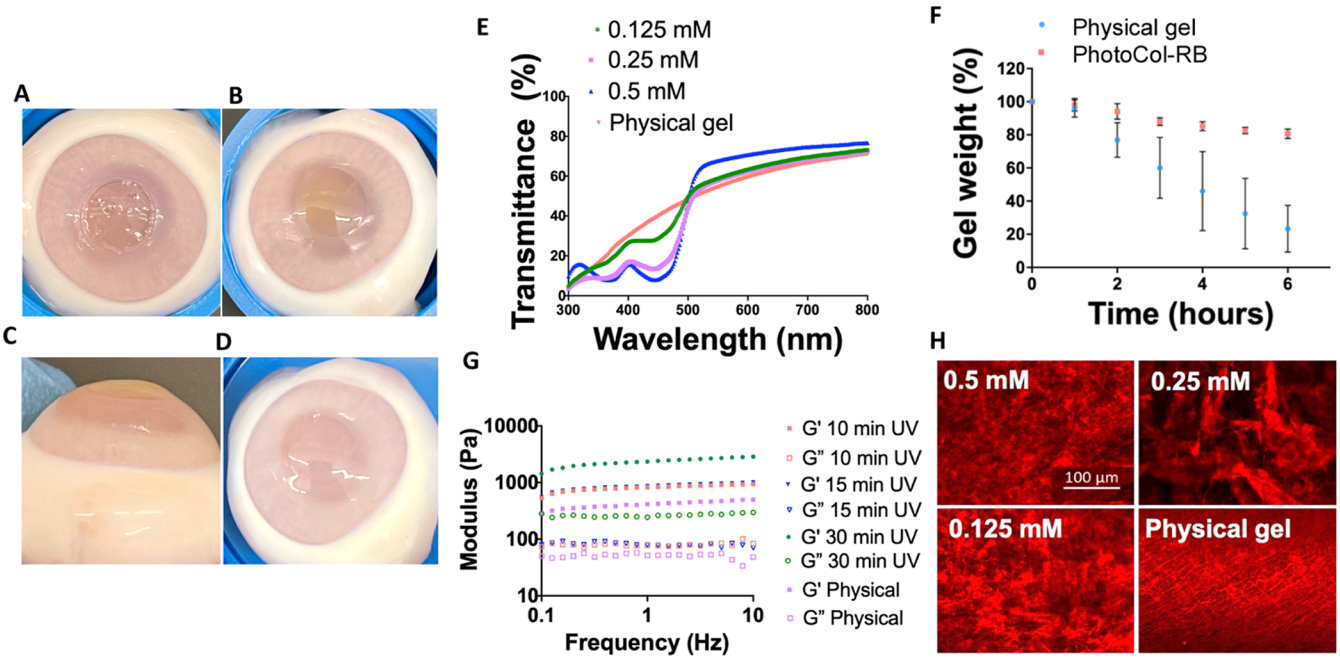

Figure 1. Properties of the PhotoCol-RB gel. (A) Rabbit corneas exhibit a rough surface following keratectomy. (B) The PhotoCol-RB gels photo-cross-linked with UV light have a faint yellow color on the rabbit cornea due to the presence of riboflavin. (C) The PhotoCol-RB gel helps restore the native corneal curvature. (D) The PhotoCol-RB gel becomes transparent after incubation in PBS. (E) Light transmittance of collagen gels with different riboflavin concentrations of 0.125, 0.25, and 0.5 mM. (F) Photoactivated and physical collagen gel degradation after incubation with collagenase over 6 h. (G) PhotoCol-RB gel modulus after exposure to different UV light times of 10, 15, and 30 min. Filled and open symbols represent the storage modulus (G') and loss modulus (G''), respectively. (H) Photoactivated and physical collagen gel structures with different riboflavin concentrations.

blue light.[12,13] This suggests that the use of riboflavin as a photosensitizer can be a strategy to create an adhesive linkage between the gel applied to the perforated area and the healthy surrounding corneal tissue for optimal sealing capacity. Furthermore, we have reported on several in situ-forming hydrogels based on reactive conjugates of collagen and hyaluronic acid that fill deep stromal defects and foster epithelialization both in vitro and in vivo.[14−18]

To address the unmet need for gels with both regenerative and adhesive properties for the repair of corneal perforations, we have developed an *in situ*-forming gel composed of native, unconjugated collagen type I cross-linked by riboflavin photoactivated with UV-A light. This system has key translational advantages to fill and regenerate corneal defects. First, collagen type I is the most abundant protein in the cornea and is known to promote corneal regeneration.[19] Second, this technology combines three elements already approved by the FDA for human use in other applications: collagen type I, riboflavin, and UV-A light. Collagen type I is used in currently FDA-approved devices such as dermal fillers.[20] Riboflavin and UV-A light have been FDA-approved together for corneal crosslinking (CXL) procedures to treat keratoconus through a crosslinking mechanism for native collagen *in situ* that is well-documented.[21] Here, we leverage the same cross-linking technique used in clinical CXL to cross-link exogenous collagen applied to the cornea into a stable gel that conforms to the shape and size of the defect. Since the native cornea is collagen-rich, the photoactivated riboflavin may also facilitate cross-linking between exogenous collagen and endogenous collagen present in the wound bed to not only provide a substrate for wound healing, but also promote wound sealing.

A therapeutic strategy that can fill deep corneal wounds and seal perforations without the use of sutures while rapidly restoring a transparent stroma to the cornea would be of great benefit to patients with corneal trauma. Here, we present *in vitro* experiments demonstrating gel strength, transparency, and cell viability as well as *ex vivo* experiments showing the performance of this material as a tissue sealant for the cornea.

■ RESULTS

Characterization of PhotoCol-RB Gels. *Transparency.* Rabbit corneas that underwent a keratectomy had a rough surface (Figure 1A). PhotoCol-RB gels formed *in situ* exhibited a yellow color immediately after gelation (Figure 1B), which is indicative of the presence of riboflavin.[22] The PhotoCol-RB gels restored the normal, smooth curvature of the cornea when applied to the stromal wounds (Figure 1C). After incubation in PBS for 1 h, the yellow color from the riboflavin washes away, yielding a transparent gel (Figure 1D). Compared to the physical collagen gels, the PhotoCol-RB gels exhibit greater transparency, confirmed through evaluation of the gel's light transmittance using spectrophotometry (Figure 1E). The concentration of riboflavin did not significantly impact the transmittance of light through the gels at wavelengths between 500 and 800 nm. PhotoCol-RB gels exhibited transparency levels greater than 50% between 500 and 600 nm and above 60% at wavelengths of 600−800 nm. Physical collagen gels exhibited transparency lower than 50% between 500 and 600 nm and around 50% at wavelengths of 600−800 nm. After 1 and 2 days of incubation in PBS, the PhotoCol-RB gels' transparency improved for wavelengths in the range of 400−500 nm compared to before PBS incubation. In addition,

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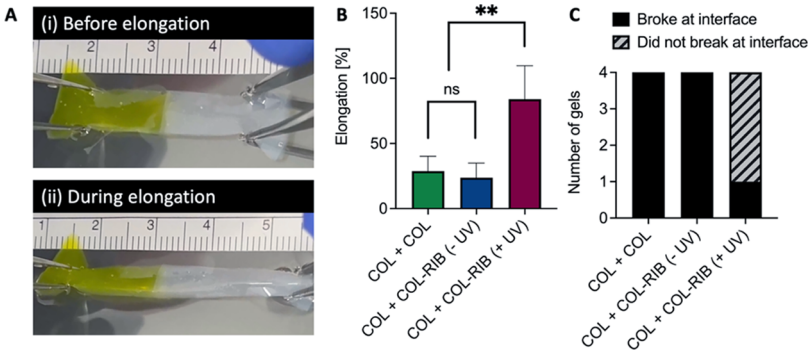

Figure 2. Riboflavin-induced photocrosslinking of collagen facilitates adhesion of the gel to physical collagen. (A) Dual-material gels with photoactivated collagen (yellow, 0.25 mM riboflavin, 10 min UV light exposure) cast next to physical collagen (white) can be elongated while maintaining a cohesive interface. (B) PhotoCol-RB cast next to collagen and photo-cross-linked with UV light (COL + COL-RIB (+UV)) significantly increases the maximum elongation possible before gel fracture (**$p = 0.0015$) compared to the control conditions of physical collagen cast next to physical collagen (COL + COL) and collagen with 0.25 mM riboflavin cast next to physical collagen but not exposed to UV light for photo-cross-linking (COL + COL-RIB (−UV)). (C) All replicates of COL + COL gels and COL + COL-RIB (−UV) gels broke first at the interface between the cast gels ($n = 4$), while COL + COL-RIB (+UV) gels did not break at the interface for three of the four gel replicates.

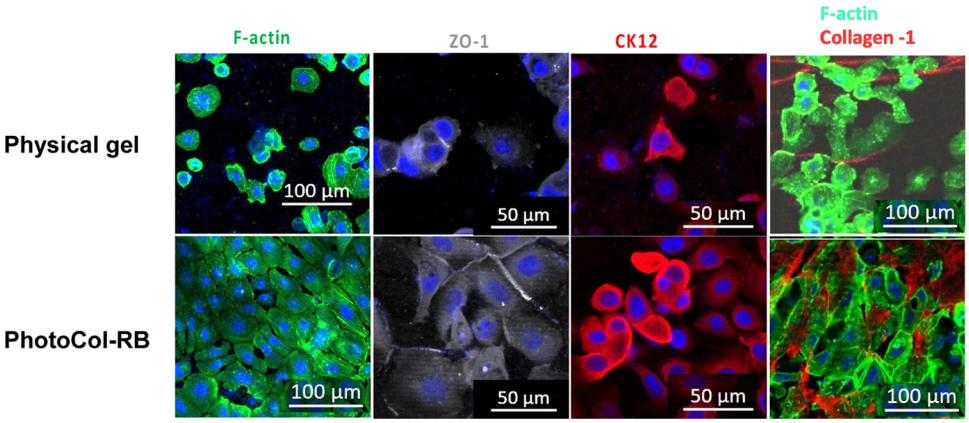

Figure 3. Corneal epithelial cell morphology and phenotype on physical and PhotoCol-RB gels. Cells were stained with DAPI for the nuclei (blue), phalloidin for the F-actin stress fibers (green), and antibodies against ZO-1 (gray), CK12 (red), and collagen type 1 (red).

PhotoCol-RB gel transparency after incubation in PBS was significantly greater than that of physical collagen (Figure S1).

Degradability. In the presence of 0.4% of collagenase, PhotoCol-RB gels lost about 20% of their initial mass as a result of degradation over 6 h compared to 80% of the initial mass for the physical collagen gels (Figure 1F). This is consistent with prior reports that CXL improves corneal tissue resistance to enzymatic degradation.[12]

Mechanical Properties. The storage modulus of the PhotoCol-RB gels was dependent on the UV light exposure time. UV light exposure times of 10 and 15 min resulted in gels with storage moduli (G') of about 1 kPa, while 30 min of UV light exposure resulted in gels with storage moduli greater than 2 kPa. In comparison, the physical collagen gel had a storage modulus of around 0.5 kPa (Figure 1G). To evaluate the collagen fibrillar microstructure after cross-linking with different concentrations of riboflavin, the collagen was stained using an anticollagen type 1 antibody and imaged using fluorescence microscopy (Figure 1H). The collagen crosslinked with riboflavin and UV-A light showed a less compact structure and thicker fibers compared to the physical collagen gel. Photocrosslinking collagen with riboflavin at a concentration of 0.25 mM appeared to give the largest fiber diameter compared to riboflavin concentrations of 0.5 mM and 0.125 mM.

Adhesion. Riboflavin-induced photocrosslinking of collagen facilitates adhesion to physical collagen gels. These gels can be elongated while maintaining a cohesive interface, demonstrating adhesion between the collagen materials (Figure 2A). Specifically, gels formed from photoactivated collagen (0.25 mM riboflavin) cast adjacent to physical collagen gels and photo-cross-linked with UV light for 10 min (COL + COL-RIB (+UV)) may be elongated 84% of their initial length before fracture, which is significantly greater ($p = 0.0015$) than the elongation possible for physical collagen cast adjacent to physical collagen (COL + COL) or photoactivated collagen (0.25 mM riboflavin) cast adjacent to physical collagen but not exposed to UV light (COL + COL-RIB (−UV)) (Figure 2B). The COL + COL gels and the COL + COL-RIB (−UV) gels had maximum elongations before fracture of 29 and 24% of their initial length, respectively (ns). Furthermore, the COL + COL gels and the COL + COL-RIB (−UV) gels all fractured first at the interface between the materials ($n = 4$), while the COL + COL-RIB (+UV) gels did not break at the interface for three of the four gel replicates, highlighting the cohesiveness of the interface that is enabled by the riboflavin-induced photo-cross-linking of the collagen (Figure 2C).

hCECs Spread on PhotoCol-RB Gel and Express Epithelial Cell Markers. Human corneal epithelial cells (hCECs) were seeded on PhotoCol-RB gels and physical

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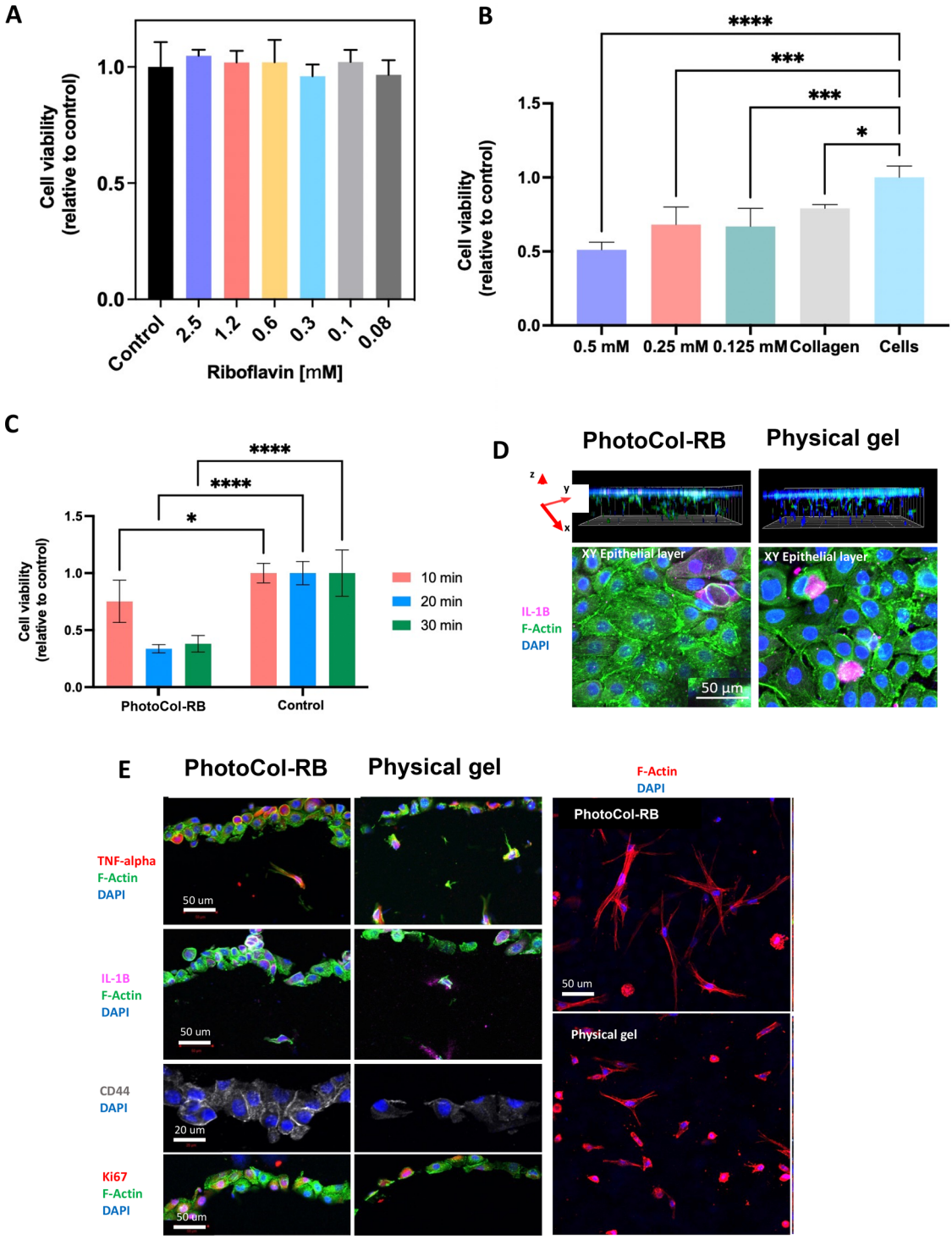

Figure 4. Biocompatibility of PhotoCol-RB gels. (A) Riboflavin treatment at different concentrations without UV activation. (B) *In situ*-forming PhotoCol-RB gel after exposure to UV light for 10 min. Collagen with 0.5 mM riboflavin significantly decreased cell viability compared to the control (****$p < 0.001$). Riboflavin concentrations of 0.25 and 0.125 mM had similar cell toxicity to each other (ns) compared to the control (***$p < 0.05$). Cells treated with collagen solution and exposed to UV light for 10 min showed decreased cell viability compared to control (*$p = 0.01$). (C) Increased exposure time to UV light to form the PhotoCol-RB gels. UV light exposure of 10 min caused more toxicity to the cells compared to the control of cells without UV light exposure (*$p = 0.01$). UV light exposure times of 20 and 30 min decreased cell viability (****$p < 0.001$). (D) A 3D view shows MSCs encapsulated in and hCECs seeded on the PhotoCol-RB gels and physical collagen gels. Expression of IL-1B was evaluated. F-actin (green), IL-1B (magenta), and DAPI (blue). (E) Cross sections of the gels show the expression of inflammatory markers (IL-1B and TNF-α) as well as CD44 and Ki67. F-actin (red) expression for encapsulated MSCs can be observed. TNF-α (red), IL-1B (magenta), CD44 (gray), Ki67 (red), F-actin (green), and DAPI (blue).

collagen gels for subsequent phenotypic and morphological evaluation. This assay was performed to understand if corneal epithelial cells can proliferate and maintain their phenotype when migrating over these gels. Phalloidin staining showed

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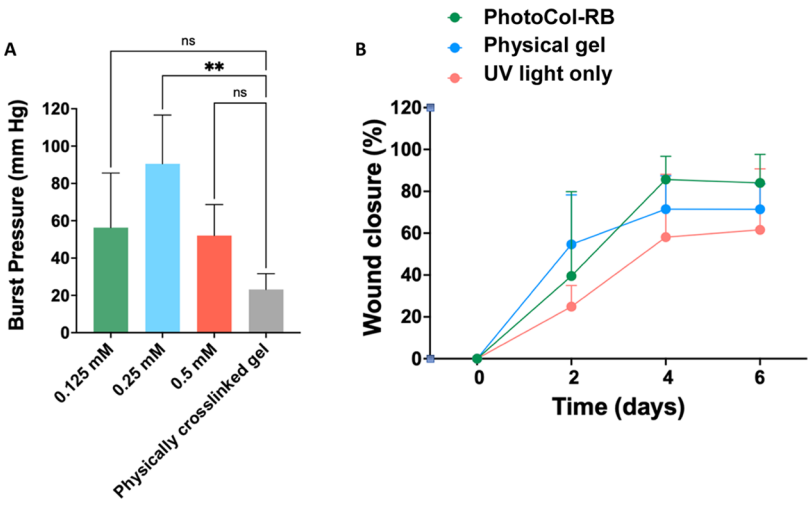

Figure 5. PhotoCol-RB gels improve corneal burst pressure and wound healing. (A) Burst pressure of physical gel, and PhotoCol-RB gels with riboflavin concentrations of 0.125, 0.25, and 0.5 mM. The burst pressure for the PhotoCol-RB gel with 0.25 mM riboflavin was statistically different from the physical collagen gel (**p = 0.002). (B) Corneal wound healing after the application of the PhotoCol-RB gel, physical collagen gel, exposure to UV light without collagen gel, and no treatment (saline only) (n = 4 for each group). The wound closure was evaluated over 6 days.

that cells seeded on PhotoCol-RB gels spread evenly over the gel surface forming a monolayer, while few cells were observed for the physically cross-linked collagen gels (Figure 3). Cells seeded on photochemically crosslinked gels expressed greater tight junction protein (ZO-1) and cytokeratin 12 (CK12) compared to cells seeded on physical collagen gels (Figure 3). Many hCECs growing on thick collagen fibers from PhotoCol-RB gels were observed compared to a few cells growing on the thin collagen fibers from physically crosslinked collagen gels (Figure 3).

PhotoCol-RB Gel Preserves Corneal Cell Bioactivity. To evaluate the potential toxicity of the photoactivated collagen gels, we first determined the toxicity of riboflavin toward the cultured corneal epithelial cells. We evaluated hCEC viability cultured for 12 h in riboflavin solutions with concentrations ranging from 0.08 to 2.5 mM riboflavin. Riboflavin, at any given concentration, was not toxic to the cells compared to the control of hCECs with no riboflavin in the cell culture medium (Figure 4A). Next, we aimed to determine the toxicity of the PhotoCol-RB gel itself. The hCECs were covered with a solution of collagen alone or collagen with different concentrations of riboflavin and exposed to UV light for 10 min. Figure 4B indicates that significant cytotoxicity was observed by the *in situ*-forming gel with riboflavin at a concentration of 0.5 mM ($p < 0.001$), compared to cells only exposed to UV light. PhotoCol-RB gels formed with riboflavin at concentrations of 0.25 and 0.125 mM were moderately toxic to the cells compared to the control ($p < 0.05$). Exposure of the cells to the collagen gel pre-cursor solution and UV light with no riboflavin reduced cell viability compared to controls (p = 0.01). Next, we determined the effect of UV exposure time on cell toxicity using a fixed riboflavin concentration of 0.25 mM (Figure 4C). Gels formed by UV light exposure for 20−30 min significantly reduced cell viability ($p < 0.001$) compared to cells exposed to UV light for 20 and 30 min without the presence of gels. As observed before, gels formed *in situ* through riboflavin exposure for 10 min imparted some toxicity to the cells compared to the control of cells only exposed to UV light for 10 min.

Next, we used a co-culture system to evaluate the viability of corneal MSCs cultured within the *in situ*-forming photoactivated and physical collagen gels in the presence of hCECs. Toward this end, MSCs were encapsulated within the PhotoCol-RB gels with a riboflavin concentration of 0.25 mM and a UV light exposure time of 10 min. For the physical collagen gels, MSCs were encapsulated and then similarly exposed to UV light for 10 min. Then, hCECs were seeded on the gels. A three-dimensional view of Figure 4D shows that MSCs were successfully encapsulated and were able to spread in both the PhotoCol-RB and physical collagen gels in the presence of hCECs. The inflammatory response of the MSCs to the gels in the presence of hCECs was evaluated by staining the cells with IL-1B and TNF-α (Figure 4D) antibodies. IL-1B and TNF-α staining were present in MSCs for both photoactivated and physical collagen gels.

Figure 4E shows hCECs growing on the surface of the PhotoCol-RB and physical collagen gels in the presence of MSCs. The cell area of hCECs on the PhotoCol-RB appears larger, with a greater degree of confluence compared to cells on the physical collagen gels. hCECs seeded on physical collagen gels were able to proliferate, as indicated by the expression of Ki67, but did not form a confluent monolayer. A cross-sectional view of the gels shows that hCECs seeded on PhotoCol-RB gels were more proliferative, resulting in a large number of cells that formed a double layer compared to the single layer grown on the physical collagen gels. The hCECs were stained with IL-1B, TNF, CD44, and Ki67 antibodies to evaluate the behavior of the cells in a coculture system. hCECs were stained with CD44 and Ki67 to evaluate cell proliferation since the expression of these proteins is related to the increase in cell proliferation. The hCECs seeded on both PhotoCol-RB gels and physical gels showed the expression of IL-1B and TNF-α. CD44 receptors and Ki67 were highly expressed in cells seeded on the PhotoCol-RB gels compared to cells seeded on physical collagen gels.

PhotoCol-RB Gels Seal Corneal Wounds. PhotoCol-RB gels were evaluated for their capacity to serve as a corneal sealant compared to physical collagen gels. Burst pressure was measured after application of the gels to corneal incisions

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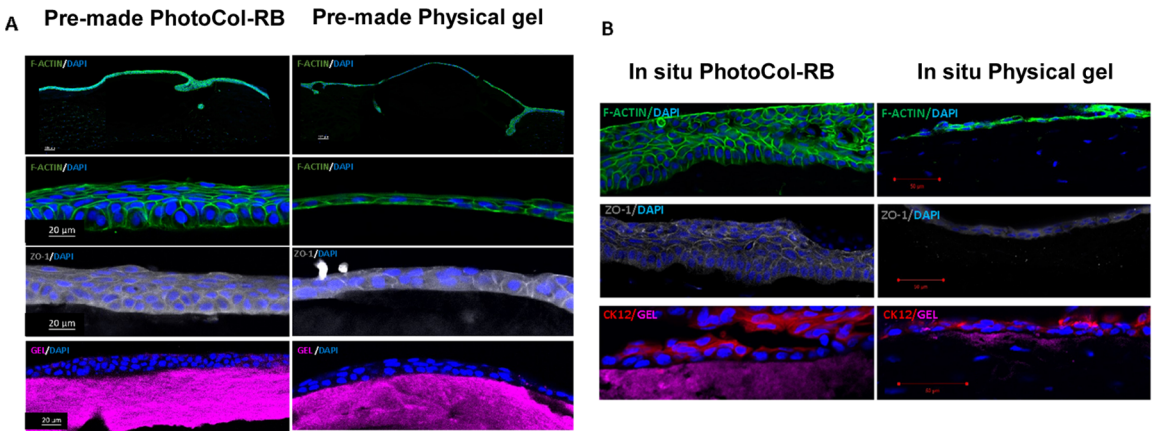

Figure 6. (A) Premade and (B) *in situ*-formed PhotoCol-RB gels supported multilayer re-epithelialization in an *ex vivo* rabbit model of lamellar keratectomy. Host cells were able to migrate and form a multilayer on PhotoCol-RB gels compared to double-layer formation for both premade and *in situ*-formed physical collagen gels. Expression of ZO-1 was observed for the epithelial layer over all types of gels. CK12 was expressed for both *in situ*-formed PhotoCol-RB and physical gels. The collagen gels (magenta) are still present after 3 days in culture. F-actin (green), ZO-1 (gray), collagen type 1 (magenta), and CK12 (red).

(Figure 5A). Physical collagen gels provided the lowest burst pressure of about 25 mm Hg compared to the PhotoCol-RB gels. The burst pressure for the PhotoCol-RB at concentrations of 0.125 or 0.5 mM riboflavin was around 55 mm Hg, double the value for the physical gels. PhotoCol-RB with 0.25 mM riboflavin provided the highest burst pressure of about 90 mm Hg. The burst pressure of collagen cross-linked with 0.25 mM riboflavin was statistically greater than that of physical collagen gels ($p = 0.002$).

PhotoCol-RB Gels Support Epithelialization *Ex Vivo*. Corneal wound healing in an *ex vivo* rabbit eye model of lamellar keratectomy was evaluated after application of the gels over 6 days. On day 2, about 50% of the wound was closed for the physical collagen gels, compared to 40% of the wound for the corneas that received the PhotoCol-RB gels (Figure 5B). The wounded corneas that were exposed to UV light only or that did not receive any treatment showed slower wound closure, about 10 and 20%, respectively, on day 2. On day 4, more than 80% of the wound was closed for the corneas that received the PhotoCol-RB gel, compared to 70% for the corneas that received the physical gel. The wound healing for the corneas exposed to UV light only or that did not receive treatment improved from day 2 to day 4 but both were still less than 60% healed. At day 6, the wound healing for the corneas that received gels was similar to day 4 and was about 60% for the no treatment group and 40% for the corneas exposed to UV light only.

***In Situ*-Formed and Premade PhotoCol-RB Gels Support Epithelialization after 3 Days in an Organ Culture.** An *ex vivo* rabbit corneal organ culture system was used to evaluate the capacity of the PhotoCol-RB gels to support re-epithelialization of the ocular surface. Toward this end, an anterior keratectomy 5 mm in diameter was performed, and both premade and *in situ*-formed PhotoCol-RB gels or physical gels were applied within the defects to reconstitute a smooth, continuous contour to the corneas. After 3 days in culture, the rabbit corneas were fixed and analyzed. Immunohistochemical evaluation showed that for both premade and *in situ*-formed PhotoCol-RB and physical collagen gels, the epithelial layer from the rabbit cornea was able to proliferate and migrate, covering the entire wound area as shown by F-actin staining (Figure 6). The epithelial cells on both premade and *in situ*-formed photoactivated gels formed multiple cell layers, indicating faster wound healing compared to the double epithelial layer formed over both premade and *in situ*-formed physical collagen gels. The epithelial layer stained positively for the tight junction marker zonula occludens-1 (ZO-1) for both types of gels and expressed CK12 for both *in situ*-formed gels. The gels were observable throughout the wounds with the epithelia overlying them.

■ DISCUSSION

Corneal crosslinking (CXL) using UV light with riboflavin as a photosensitizer is the standard of care treatment for patients with keratoconus. The covalent crosslinks formed between the collagen side chains and proteoglycan core proteins from the corneal stroma increase the stiffness of the cornea and reduce corneal thinning.[13] In the past few years, chemically cross-linked collagen gels have demonstrated potential as scaffolds for corneal cell regeneration.[7,23,24] Previous studies have evaluated the mechanical properties of collagen gels with riboflavin as a photosensitizer as a function of UV light exposure time and collagen concentration.[13,25] Most of these studies focused on bone tissue engineering, and few explored the use of collagen riboflavin gels for corneal regeneration. Recently, Lei et al. showed that electrically-assembled collagen riboflavin materials sutured to rabbit corneas allowed stromal integration and reconstruction.[26] In other work, Zhao et al. showed that collagen riboflavin corneal substitute material sutured to rabbit corneas promoted re-epithelialization.[27] Here, we aimed to form an *in situ* UV-cross-linked collagen with riboflavin as a photosensitizer to support two wound stabilizing functions: wound sealing and epithelial healing, both of which are important in the setting of a perforating wound. We compared PhotoCol-RB gels to physical collagen gels to demonstrate how the riboflavin-mediated crosslinking reaction impacted the ability of applied collagen to seal incisions and provide a substrate that supports epithelialization.

First, we demonstrated that PhotoCol-RB gels exhibit enhanced transparency compared to physical collagen gels overall in the visible light regime (Figure 1), though the temporary presence of riboflavin decreased the gel transparency in the range of 400−500 nm until it had completely diffused out from the gel. The modulus of the PhotoCol-RB

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gels was dependent on the UV light exposure time. Increasing UV light exposure time from 10 to 30 min not only increased the storage modulus of the gels (Figure 1G) but also increased cytotoxicity (Figure 4C). Previous studies have found similar mechanical results: Ahearne et al. showed that increased exposure of collagen and riboflavin to UV light statistically increased the storage modulus of the gel.[13] We showed that the storage modulus of the gels exposed to 10 and 15 min of UV light was very similar, and therefore we chose to work with 10 min of exposure time to form the gels to strike a balance between cytotoxicity and robust gel formation.

Furthermore, we demonstrated that the PhotoCol-RB gels (0.25 mM riboflavin, 10 min UV light exposure) formed a cohesive interface when crosslinked adjacent to a physical collagen gel, indicating adhesion between the materials. The strength of this adhesion was likely due to crosslinking between the riboflavin-laden collagen and the physical collagen gel. This photocrosslinking occurs at the interface between the materials, which likely also includes intermixing of the collagen macromers to form a region of interpenetration of the two adjacent collagen networks. Since the most abundant protein in the native cornea is collagen, these results indicate that with our technology, the riboflavin may induce crosslinking between the exogenous riboflavin-laden collagen and the endogenous collagen present in the wound bed to facilitate robust wound sealing.

We found that PhotoCol-RB gels formed using 10 min of UV light exposure caused less cytotoxicity than longer durations of UV light exposure and still had enhanced mechanical properties compared to physical collagen gels. The UV light exposure time in the clinic is 30 min (370 nm, 3 mW/cm^2), which is longer and more intense than what we used in this work. The fibrillar structure of the collagen gels varied as a function of both riboflavin concentration and UV exposure time. Interestingly, collagen crosslinked with a riboflavin concentration of 0.25 mM had fibers with larger diameters compared to collagen with riboflavin concentrations of 0.125 and 0.5 mM.

For proper wound healing, corneal epithelial cells must be able to spread over the gels and maintain their normal phenotype. The hCECs seeded on physical collagen gels had a smaller cell area. For both gels, the hCECs expressed ZO-1 and CK12 markers. ZO-1 and CK12 are characteristic corneal epithelial cell markers that indicate differentiation into a mature epithelial phenotype.[23] The hCECs were able to spread, form a confluent epithelial layer, and express mature epithelial markers on PhotoCol-RB gels, suggesting that this gel had suitable material properties for corneal re-epithelialization.

Previous reports have shown that the combination of 30 min of UV light and riboflavin leads to an intense process of apoptosis of keratocytes.[28,29] This known cytotoxicity of the riboflavin-mediated cross-linking is probably related to the production of singlet reactive oxygen species that are fundamental for the formation of covalent bonds between the collagen molecules.[28,30] Armed with this knowledge, we aimed to evaluate cell viability upon treatment with only riboflavin, collagen, and the *in situ* PhotoCol-RB gels using different UV light exposure times. Our results suggest that riboflavin exposed to UV light caused varying degrees of cell toxicity. Specifically, increasing riboflavin concentration from 0.25 to 0.5 mM resulted in more significant cell toxicity upon exposure to UV light. Similarly, increasing UV light exposure time from 10 to 30 min also caused more significant cell toxicity. We therefore decided to use a UV light exposure time of 10 min to form the PhotoCol-RB gels since it facilitated the lowest impact on cell viability.

We used a co-culture system to evaluate the behavior of corneal cells in the PhotoCol-RB gels. Previous studies found that the cross-linking mechanism used for gelation can induce proinflammatory cytokine expression by iris-pigmented epithelial cells.[31] Here, encapsulated MSCs in PhotoCol-RB gels expressed proinflammatory markers (IL-1B and TNF-α), but inflammatory markers were also observed for the MSCs encapsulated in the physical collagen gels. This suggests that the PhotoCol-RB gels, upon UV light exposure, did not induce the expression of proinflammatory cytokines. Similar results were found by Wilson et al. where keratocyte death by apoptosis upon exposure to UV light and riboflavin leads to a relatively lower increase in proinflammatory proteins.[32] The encapsulated MSCs were able to spread within the PhotoCol-RB gels producing F-actin stress filaments (Figure 4E). MSCs encapsulated within physical gels were also able to spread, though most remained rounded (Figure 4E). The hCECs seeded on the gels encapsulated with MSCs expressed IL-1B and TNF-α, but the expression of these markers was found for both physical and photoactivated gels. The hCECs on PhotoCol-RB gels formed a double epithelial layer, suggesting faster cell proliferation, which is ideal to promote corneal regeneration. Previous studies found that the expression of CD44 proteins was related to increased corneal epithelial cell proliferation.[33] In this study, epithelial cells expressed CD44 and Ki67, suggesting that the gel can provide a suitable substrate for both types of corneal cells. The hCECs seeded on physical collagen gels formed a single epithelial layer and expressed CD44 and Ki67 markers to a lower degree, suggesting that the cells proliferated less compared to the hCECs on the PhotoCol-RB gels.

Additionally, we aimed to investigate the burst pressure provided by the PhotoCol-RB gels upon corneal perforation. The rupture pressure in mm Hg for a normal human eye is about 2175 mm Hg and decreases after corneal wounds. Riboflavin concentration was found to influence burst pressure. The highest burst pressure was achieved by using riboflavin at a concentration of 0.25 mM in the collagen. This result is probably due to the gel structure that was obtained after using different concentrations of riboflavin as shown in Figure 1H. The collagen fibers had different diameters, but further studies need to be performed to investigate this result. The normal intraocular pressure is 10−21 mm Hg. A clinically used sealant for surgical corneal incisions is ReSure, a biodegradable sealant made of poly(ethylene glycol)-based gel.[34] ReSure provides ample protection against elevated intraocular pressure but has no known bioregenerative properties.[35] In our experiments, we measured the burst pressure for PhotoCol-RB gels to be approximately 90 mm Hg (Figure 5A), which is similar to the values reported using ReSure on clear corneal incisions.[34] The PhotoCol-RB gels thus appear to provide a clinically relevant sealant effect in addition to its support for corneal cell regeneration.

Finally, we evaluated the host−scaffold response using an *ex vivo* model of lamellar keratectomy. Organ culture systems are an intermediate model between cell culture and animal studies and have been shown to be a reproducible method to study corneal wound healing.[36−38] Corneas that received the PhotoCol-RB gel resulted in faster corneal epithelial wound

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healing compared to the corneas that received the physical collagen gel. Rabbit corneal epithelial cells migrated over the PhotoCol-RB gel, formed a multilayer, and maintained normal epithelial cell phenotype, which was confirmed by the expression of ZO-1. These results indicate that the mechanical and biological properties of the PhotoCol-RB gels are well-suited to foster corneal wound repair compared to no treatment or physically crosslinked collagen gels. The PhotoCol-RB gel here facilitated epithelialization by virtue of being biologically favorable and sufficiently stable and rigid for epithelial cells to proliferate and migrate over its surface. In addition, encapsulated stromal cells were able to spread within the gel *in vitro*. This suggests that the crosslinked structure of the gel facilitates stromal cell migration, spreading, and matrix remodeling.

■ CONCLUSIONS

Given the challenges of controlling deep and perforating corneal injuries and the global shortage of donor corneal graft tissue, there is a major clinical need of bioengineered strategies to stabilize and repair corneal wounds. An ideal repair modality would be highly biocompatible and allow for tissue regeneration and would also be highly transparent to allow for clear vision. In this work, we have demonstrated that *in situ*-forming collagen gels crosslinked by photoactivated riboflavin support epithelial growth on their surface and stromal cell growth within their bulk. This biomaterial strategy is designed to allow for both sealant and regenerative benefits in the treatment of deep and perforating corneal wounds without requiring sutures or a more invasive procedure such as a therapeutic graft. Further work is merited to evaluate both the wound sealing and healing properties of the material through *in vivo* corneal injury studies.

■ MATERIALS AND METHODS

PhotoCol-RB Gel Development. Neutralized collagen solutions were created from 6 mg/mL type I bovine telocollagen (Advanced Biomatrix, 5225) and a neutralization solution containing sodium hydroxide (75 μL), deionized water (1450 μL), and 10× phosphate-buffered saline (PBS, 500 μL). The telocollagen and neutralizing solution were mixed at a 5:1 ratio for a final collagen concentration of 5 mg/mL using a vortex orbital shaker to ensure complete neutralization. In this study, three concentrations of riboflavin 5′-phosphate sodium salt hydrate (Sigma-Aldrich, R7774-10G) for the photoactivated collagen gels were evaluated: 0.125, 0.25, and 0.5 mM riboflavin. To create these collagen riboflavin solutions, the riboflavin was pipetted into vials containing neutralized collagen and mixed using a vortex orbital shaker for complete homogenization. A control solution of neutralized telocollagen did not contain any riboflavin and physically cross-linked into a gel. All solutions were exposed to UV light (UVP Upland, CA) with a wavelength of 365 nm and an intensity of 1.8 mW/cm^2 for either 10, 15, or 30 min to cross-link the PhotoCol-RB.

PhotoCol-RB Gel Light Transmittance. The light transmittance of the collagen samples with three concentrations of riboflavin (0.125, 0.5, and 0.5 mM) and the control telocollagen gel sample without riboflavin was analyzed as previously described.[24] Briefly, the transparency of the gels was determined with a microplate reader (Tecan Microplate Reader, Morrisville, NC) upon exposure of the gels to a 365 nm UV light for 10 min. The absorbance (A) from 300 to 800 nm was measured immediately after gel formation, and the light transmittance (T) was calculated using the relationship $A = 2 - \log_{10}(\%T)$.

PhotoCol-RB Gel Rheology. The storage and loss moduli of the collagen gels were evaluated using an ARES-G2 rheometer (TA Instruments, New Castle, DE) with a 25 mm parallel plate according to previous work.[7] PhotoCol-RB gels and physical gels were exposed to 10, 15, or 30 min of UV light. Time sweeps were performed at 25 °C for 1 h at 1% strain and a 1 Hz oscillatory frequency. Frequency sweeps were performed from 0.1 to 10 Hz with a fixed 1% strain.

Degradation Assay. A degradation assay was performed on photoactivated (0.25 mM riboflavin) and physical collagen gels using a solution of 0.4% collagenase from Clostridium type 1 (Sigma-Aldrich, C0130) in keratinocyte SFM (KSFM) cell medium (Gibco, 10724−011). We chose to work with a higher concentration (0.4%) of collagenase to show the relative degradation rates and not absolute degradation. Three replicates of the photoactivated and physical collagen gels were exposed to the collagenase for 6 h on a shaker at room temperature. The vials with the samples were weighed before being exposed to the collagenase solution and at the end of every hour after removal of the collagenase solution. Fresh collagenase solution was added between measurements, and the process was continued for 6 h until the collagen gel had completely degraded.

Adhesion Assay. Adhesion tests were performed to assess the suitability of PhotoCol-RB to adhere to physical collagen, the primary protein component of the native cornea. The integrity of the interface between PhotoCol-RB and physical collagen was tested with dual-material dogbone constructs commonly used for tensile measurements. Dogbone-shaped molds were cut from 2.5 mm thick silicone sheets (Electron Microscopy Sciences, 70338-25) and firmly pressed onto glass coverslips to form a tight seal. The silicone in the dogbone shape was cut in half, and one-half was reinserted into the mold as a plug. Physical collagen was cast into the open half of the mold and cross-linked at 37 °C for 5 min. The silicone plug was then removed, and collagen with 0.25 mM riboflavin was cast into the other half of the mold and cross-linked under UV light for 10 min. Following cross-linking, the dual-material dogbone gels were removed from the molds and submerged in a PBS bath. The percent elongation of the gels before fracture was determined for each gel using methods as previously described.[39] Briefly, the gels were manually stretched with forceps, recording the initial length of the gel (L_0) and the maximum length of the gel before fracture (L). The maximum percent elongation was calculated as $[(L - L_0)/L_0] \times 100\%$. The approximate applied strain rate during elongation of the gels was 0.2−0.3 mm/s. However, as the gels are viscoelastic materials, analysis at various controlled strain rates would be interesting to explore in the future. For each gel, we also recorded whether the fracture occurred at the interface between the materials. Controls were (1) physical collagen gels cast in both halves of the mold and (2) physical collagen in the first half and collagen with 0.25 mM riboflavin in the second half of the mold but without UV light exposure for riboflavin-induced photo-cross-linking. For each condition, $n = 4$ gels.

Burst Assay. *Ex vivo* burst experiments on pig corneas were performed to determine the burst pressure of PhotoCol-RB gels with riboflavin concentrations of 0.125, 0.25, and 0.5 mM. In order to measure the burst pressures of the PhotoCol-RB gel, a custom-designed burst pressure apparatus was used in which air was continuously pumped into porcine globes using a syringe pump and an in-line manometer. The burst pressure was defined as the peak pressure prior to wound leakage for all experiments.[34] A control of physical collagen gel was also examined for comparison. In brief, a folded Kimwipe was placed at the bottom of each well in a 6-well plate, with the cornea of the pig eye facing upward. A corneal perforation was created by inserting a 19-G needle through the cornea. Each gel sample was applied in 20−50 μL volumes to four pig eyes with the stimulated corneal perforation. After applying the PhotoCol-RB gel solution, the eyes were exposed to 10 min of UV light. A 23-G needle was then inserted at the edge of each cornea until the tip neared the center of the cornea. The needle was connected to a burst pressure assay apparatus (Kent Scientific Corporation), which pumped in water with fluorescein sodium salt (Sigma-Aldrich, F6377) at 1 mL/min. The peak pressure each eye could withstand before any leakage from the corneal wound or a significant drop in pressure was recorded.

Human Corneal Epithelial Cell (hCEC) and Human Corneal Mesenchymal Stromal Cell (c-MSC) Culture. Human corneal

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mesenchymal stromal cells (c-MSCs) were isolated from human corneas using methods as previously described.[40] Briefly, human corneas were kindly donated from Emmecell (Menlo Park, CA) and used to harvest the c-MSCs. The endothelial layer was removed, followed by the central corneal button using a trephine (8 mm). The remaining corneo-scleral rims were cut into three pieces that were placed epithelial side down on a 6-well tissue culture plastic (TCP) plate. The segments were attached to the TCP after air-drying for 2 min. Next, drops of c-MSCs minimum essential medium Eagle's (Corning, 15012CV) medium containing 10% fetal bovine serum (FBS, Gibco), 1% antibiotic antimycotic solution (Sigma-Aldrich), 1% nonessential amino acid solution (Sigma-Aldrich), and 1% Glutamax (Thermo Scientific) were added to the cornea-scleral rim segments. Drops of c-MSC medium were added twice a day until the cells formed a matrix fully attached to the TCP. Next, 1−2 mL of c-MSCs medium was added every 3 days until the cells reached confluency. Once the cells were about 50% confluent, the segments were removed, and the cells were trypsinized. Culture media was changed every 4 days, and cells were subcultured by digestion with 0.25% trypsin (Sigma-Aldrich) at 90% confluence and plated into 75-cm^2 T-flasks at passages 1, 2, and 3. Cells were cultured in c-MSC medium and used between passages 3 and 6 for *in vitro* experiments.

Telomerase human corneal epithelial cells (hCECs) were kindly donated by Djalilian's laboratory from the University of Chicago, Illinois. The hCECS were cultured in keratinocyte SFM (KSFM) cell medium (Gibco, 10724−011) containing 0.0005% insulin, 1% hydrocortisone, and 1% penicillin−streptomycin (PS). Before plating, flasks were first coated with 3 mL of FNC coating mix (Athena Environmental Sciences, 0407) for 3 min at room temperature.

To thaw vials of MSCs and hCECs, the vials were heated in a water bath at 37 °C. Cells were then transferred to a conical tube containing their respective cell culture medium (10 mL) and centrifuged for 5 min at 200 RCF (g) and 23 °C. The cell medium was then aspirated, and the cells were resuspended in 1 mL of their cell culture medium by gently pipetting to thoroughly distribute the cells in the medium. The cells were transferred to the flask with 15 mL of their cell culture medium. The cell medium was changed every other day, and cells were passaged after reaching 90% confluence.

To passage cells, the cell culture medium was aspirated from the flasks, and the cells were washed with PBS. The cells were trypsinized with 4 mL of 0.05% trypsin−EDTA for 5−10 min at 37 °C. Once cells were detached, 6 mL of cell culture medium with 10% FBS was added, and the cell suspension was transferred to a conical tube. Cells were centrifuged for 5 min at 200 RCF (g) and 23 °C. The medium was aspirated from the conical tube, and 1 mL of fresh cell culture medium was added to the cells by pipetting 5 times to mix the cells. Cells were counted by mixing 50 μL of the cell suspension with 50 μL of Trypan Blue and pipetting the solution into the counting chambers of a hemocytometer. Cells were then counted on an automated cell counter machine (Countess II FL, Life Technologies). The cells were replated in flasks at 1×10^6 cells/mL and cultured with the above-stated procedure.

Immunostaining of Cultures in PhotoCol-RB and Physical Collagen Gels. A coculture system was created to mimic the stromal and epithelial layers of the native cornea. Human corneal (MSCs) were encapsulated in PhotoCol-RB gels (0.25 mM riboflavin) and physical collagen gels at a cell density of 1×10^6 cells/mL. Specifically, 50 μL of the cell-laden solutions was cast in a 96-well plate and exposed to UV light for 10 min. After gel formation, 100 μL of hCEC suspended in medium at a cell density of 1×10^5 cells/mL was pipetted on top of each gel. The system was incubated for 5 days, changing the cell medium every other day. The cell medium was then removed, and 300 μL of 4% paraformaldehyde (PFA) was added to each well for 15 min to fix the samples. After removal of the PFA, the wells were washed with PBS for 5 min. 300 μL of blocking solution was added to each well for 30 min to prevent nonspecific binding. Primary antibodies anti-IL1 (Abcam, ab226918, 1:100 dilution), TNF-α (Abcam, ab66579, 1:100 dilution), and Ki67 (Abcam, ab15580, 1:100 dilution) were added to the gels overnight at 4 °C. The next day, the gels were washed 3 times in PBS for 5 min each. Secondary antibodies (1:1000) were added at room temperature for 2 h. Then, Phalloidin (1:40 dilution in PBS) was added to the gels for 30 min. The gels were washed with PBS, and DAPI (1:1000 dilution in PBS) was added for 5 min. Samples were washed with PBS and then mounted on slides to be imaged. This same immunostaining process was also repeated with anticollagen type 1 (Abcam, ab6308) and ZO-1 AlexaFluor 488 (Invitrogen, 339188) under the same conditions to mark the collagen gel location and examine epithelial cell phenotype.

Immunostaining was also performed on the formed gels to evaluate the microstructure of the collagen fibrils. Three concentrations of riboflavin were used in the PhotoCol-RB gels: 0.125, 0.25, and 0.5 mM riboflavin. A physical collagen gel was used as a control. The gels were exposed to UV light for 10 min. The gels were then fixed, permeabilized, stained with anticollagen type 1 antibody as above, and imaged with confocal microscopy (Zeiss, LSM T-PMT).

Riboflavin and PhotoCol-RB Gel Cytotoxicity. hCECs were seeded in a 96-well plate (1×10^4 cells/well) for 24 h. Six concentrations of riboflavin solutions (0.08, 0.1, 0.3, 0.6, 1.2, and 2.4 mM) were applied in volumes of 100 μL to the seeded cells. A control consisting of hCEC cells and cell culture medium with no riboflavin was also analyzed. The cytotoxicity of the riboflavin concentrations after 24 h was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cellular metabolic activity, and the absorbance at 570 nm was measured in a microplate reader relative to the control solution.

The toxicity of the riboflavin-induced photo-cross-linking of the collagen gels was also evaluated for hCECs seeded in a 96-well plate. 24 h after seeding, the cell culture medium was removed, and either no gel, a 0.25 mM PhotoCol-RB gel (100 μL), or a physical collagen gel (100 μL) was cast on top of the cells. Each well plate was exposed to UV light for 10, 20, or 30 min to evaluate the effect of UV light exposure time on the cytotoxicity of the gel environment for the hCECs. An MTT assay was performed again, with cell metabolic activity measured directly after exposure of the well plates to UV light relative to the control of the seeded hCECs with no gel.

Organ Culture. In order to evaluate the efficacy of the PhotoCol-RB gels for promoting wound closure, an organ culture of rabbit corneas was used as an *ex vivo* model. A lamellar keratectomy with an 8 mm trephine was performed on the rabbit corneas. The corneas were cut from the eyes leaving a 2 mm thick ring of sclera, removing both the lens and iris. The corneas were washed once in a betadine solution and twice in PBS solution containing 1% PS. The corneas were then mounted on agar plugs. Agar plugs were formed by microwaving 2 g of agar in 50 mL of medium containing DMEM/F12 (Ham, 1:1, 1×, Gibco, 02151), L-glutamine, 15 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), insulin−transferrin−selenium (ITS) (0.05%), and penicillin−streptomycin (PS) (1%) for 30 s. After that, 25 mL of DMEM/F12 medium was added to 50 mL of medium containing agar. This mixture was poured into agar molds and left at room temperature until solidified. Agar molds were made as previously described.[41] Briefly, poly-(dimethylsiloxane) (PDMS) was used to create a base to prepare the agar plugs. The base part and curing agent of Sylgard 184 (Dow Corning, Midland, MI) were mixed as a 10:1 weight ratio, and 10 mL round-bottom tubes were inserted inside the PDMS precuring mixture. The tubes were removed after PDMS curing, and the PDMS mold was autoclaved before use. After casting the agar within the PDMS mold, the solidified agar plugs were transferred to a 12-well plate. After mounting the rabbit corneas on the agar plugs, the DMEM/F12 cell culture medium was added until it touched the sclera surrounding the cornea. This medium was changed daily. 0.25 mM PhotoCol-RB and physical collagen gels, both premade (8 mm) and *in situ*-formed (20 μL), were applied to the keratectomy area. A control of no treatment was also examined. Samples with *in situ*-formed gels were exposed to UV light for 10 min and then placed into the incubator. Corneas were stained with fluorescein until they were distinctly fluorescent green. Images of fluorescein wound healing were taken on days 0, 2, 4, and 6.

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Following 6 days of organ culture, the corneas were removed from the agar plugs and placed in 4% PFA in a new 12-well plate to fix the tissue. The next day, the corneas were transferred to 10% sucrose and left overnight at room temperature. The same process was repeated using 20 and 30% sucrose. Then, corneas were then submerged and frozen in optimal cutting temperature (OCT) compound, and slides were prepared by slicing cross sections of the cornea with a cryostat. A blocking buffer containing 0.25% of Triton-X and 0.25% of goat serum was added for 30 min, followed by washing with PBS (1×) and drying. A solution of Phalloidin (1:40 dilution in PBS) was added for 30 min, then removed, and washed with PBS (1×). DAPI (1:1000 dilution in PBS) was then added for 5 min, again washing once with PBS (1×). Slides were then mounted onto a coverslip for imaging with confocal microscopy.

Statistical Analyses. Statistical analyses were performed using GraphPad Prism ver. 9.1.0 (216). The data were represented as mean ± SD, and p-values < 0.05 were considered statistically significant. Ordinary one-way analysis of variance (ANOVA) was used to detect statistical differences for the assays followed by Sidak's multiple comparison and Dunnett's multiple comparison test or uncorrected Fisher LSD.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsabm.3c00015.

PhotoCol-RB and physical collagen gel light transmittance (A) right before gel formation, (B) after 1 day of incubation in PBS, and (C) after 2 days of incubation in PBS (Figure S1) (PDF)

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Author Contributions

G.M.F.C., D.M., and S.H. designed the project; G.M.F.C., L.G.B., A.A., A.M., Y.A.S., F.C., and C.L. carried out the experiments; G.M.F.C. and L.G.B. analyzed the data; G.M.F.C., L.G.B., A.A., and A.M. wrote the paper.

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Notes

The authors declare the following competing financial interest(s): The authors declare that D. Myung has a patent application pending on photocrosslinked biomaterials for ocular wound healing.

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