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Immobilization of Growth Factors to Collagen Surfaces Using Pulsed Visible Light

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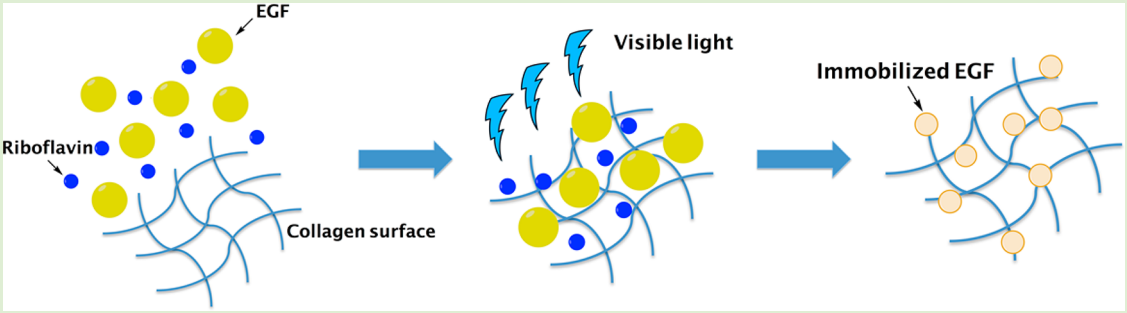

ABSTRACT: In the treatment of traumatic injuries, burns, and ulcers of the eye, inadequate epithelial tissue healing remains a major challenge. Wound healing is a complex process involving the temporal and spatial interplay between cells and their extracellular milieu. It can be impaired by a variety of causes including infection, poor circulation, loss of critical cells, and/or proteins, and a deficiency in normal neural signaling (e.g., neurotrophic ulcers). Ocular anatomy is particularly vulnerable to lasting morbidity from delayed healing, whether it be scarring or perforation of the cornea, destruction of the conjunctival mucous membrane, or cicatricial changes to the eyelids and surrounding skin. Therefore, there is a major clinical need for new modalities for controlling and accelerating wound healing, particularly in the eye. Collagen matrices have long been explored as scaffolds to support cell growth as both two-dimensional coatings and substrates, as well as three-dimensional matrices. Meanwhile, the immobilization of growth factors to various substrates has also been extensively studied as a way to promote enhanced cellular adhesion and proliferation. Herein we present a new strategy for photochemically immobilizing growth factors to collagen using riboflavin as a photosensitizer and exposure to visible light (~458 nm). Epidermal growth factor (EGF) was successfully bound to collagen-coated surfaces as well as directly to endogenous collagen from porcine corneas. The initial concentration of riboflavin and EGF as well as the blue light exposure time were keys to the successful binding of growth factors to these surfaces. The photocrosslinking reaction increased EGF residence time on collagen surfaces over 7 days. EGF activity was maintained after the photocrosslinking reaction with a short duration of pulsed blue light exposure. Bound EGF accelerated in vitro corneal epithelial cell proliferation and migration and maintained normal cell phenotype. Additionally, the treated surfaces were cytocompatible, and the photocrosslinking reaction was proven to be safe, preserving nearly 100% cell viability. These results suggest that this general approach is safe and versatile may be used for targeting and immobilizing bioactive factors onto collagen matrices in a variety of applications, including in the presence of live, seeded cells or in vivo onto endogenous extracellular matrix collagen.

INTRODUCTION

The cornea is the dome-shaped, outermost tissue of the eye and is composed of epithelial, stromal, and endothelial layers, as well as Bowman's layer and Descemet's membrane. To maintain its transparency, the cornea must remain avascular and heal without scarring. The main function of the corneal epithelium is to act as a barrier against physical trauma, chemicals, and pathogens.[1] Therefore, it is the most common site of primary eye injuries. When burns, infections, or nerve damage affect the cornea, the compromised epithelium heals more slowly, putting the deep structures of the cornea at risk.[1] This delay in healing is directly associated with pain and risk of vision loss. Accordingly, corneal pathologies are a major cause of blindness worldwide and represent a major economic burden.[2]

A variety of physical and chemical coupling strategies have been previously explored to immobilize growth factors onto surfaces.[3−5] Here we introduce a novel strategy to covalently couple epidermal growth factor (EGF) to collagen substrates, with the ultimate goal of enhancing corneal wound healing.

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Human EGF is a polypeptide consisting of 53 amino acid residues, including one methionine, two lysines, and six cysteines that form three disulfide bonds.[6] Because of the possibility of chemical modifications to the amino acids mentioned above, various studies have explored the covalent attachment of EGF to surfaces in order to enhance cell proliferation.[7−10] However, these strategies require the use of various chemicals in which the safety has not been completely understood. Also, these reactions are often slow, laborious, and may be difficult to translate in vivo. To address these problems, here we develop an in situ strategy to immobilize EGF to collagen surfaces and endogenous stromal collagen by using riboflavin as a photosensitizer and blue light for illumination. Coupling growth factors in this fashion is advantageous for the following reasons. First, riboflavin is already approved by the FDA for use in human corneal cross-linking procedures in combination with ultraviolet A (UVA) light.[11] Second, blue light is used ubiquitously in ophthalmology to visualize fluorescein staining of corneal wounds and is safer compared to UVA light. Finally, the use of light provides spatiotemporal control over the reaction, limiting the reaction to just the location and duration of light exposure. Our approach seeks to chemically bind EGF to collagen surfaces in order to provide a cytocompatible substrate, either ex vivo as a cell scaffold or carrier or directly in vivo as a direct modification of tissue, that enhances cell proliferation.

EXPERIMENTAL SECTION

Blue Light. A Blue Dental Wireless/Cordless LED Cure Curing Light Lamp, spectrum 420−480 nm was used as light source in this study. The intensity of blue light was measured as a function of distance from the light source using a digital light meter (Dr. Meter LX1330B Digital Illuminance/Light Meter, Dr. Meter). The light intensity applied for all the assays was 100 mW/cm^2 by setting a fixed distance from the light source. In addition, blue light was used in pulse mode, meaning that, for each second of exposure, the light was 0.5 s on and 0.5 s off. The riboflavin used for all assays was from Sigma-Aldrich solubilized in Dulbecco's phosphate-buffered saline (D-PBS) or keratinocyte serum-free medium (KSFM) (Thermo Scientific).

Mass Spectrometry. Mass spectrometry was performed to observe the effects of blue light-exposed EGF with riboflavin on molecular weight. To begin, riboflavin was added to 10 μg/mL EGF solution, to achieve a final concentration of 0.025 mM. Then blue light was applied to the solution for 10 s in pulse mode. The resultant EGF was analyzed by matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry (Perspective Voyager-DE RP Biospectrometry instrument, Framingham, MA) at the Stanford Protein and Nucleic Acid Biotechnology Facility (Stanford, CA).

EGF Activity Evaluation by EGF/EGFR Binding Assay. An EGF/EGFR binding assay was performed to evaluate the activity of blue light-exposed EGF. First, riboflavin was added to 10 μg/mL EGF solution, so that the final concentration was 0.025 mM. Then, blue light was exposed to the mixture for 5, 10, and 20 s in pulse mode. The resultant EGF solution was dialyzed using a dialysis cassette kit overnight at 4 °C. To evaluate EGF bioactivity, we followed the protocol provided in the AlphaLISA EGF and EGFR Binding Kit. Briefly, anti-human IgG Fc-specific AlphaLISA acceptor beads and EGFR-Fc were mixed according to the instructions and incubated for 30 min at room temperature. The solution was added to each well containing 1−100 μg/mL blue light-exposed EGF with biotinylated EGF, and then incubated for 30 min at room temperature. Streptavidin-coated donor beads were added to each well and incubated for 30 min at room temperature. Then the AlphaLISA optical signal resulting from each well was measured at 615 nm using AlphaLISA mode on the TECAN M1000 Pro (San Jose, CA).

Enzyme-Linked Immunosorbent Assay (ELISA). EGF ELISA was performed as outlined in the kit protocol with some minor modifications. Before the ELISA procedure, the EGF was immobilized onto the surface. Collagen I bovine protein solution (0.1 mg/mL) (Thermo Scientific) was placed in a 96-well plate and incubated overnight at 4 °C, in order to coat the surface of the well plate with collagen. Riboflavin solution (0.0025−0.25 mM) containing 1 μg/mL EGF was added to wells and incubated for 15 min at room temperature. Then blue light was applied (5−20 s in pulse mode) to each well without washing. For physical adsorption, 1 μg/mL EGF was added after collagen coating for 60 min at room temperature.

The ELISA procedure was carried out by adding biotinylated antibody reagent and diluted streptavidin-horseradish peroxidase (HRP) in phosphate-buffered saline (PBS) to the wells. The solution was incubated for 2 and 1 h, respectively. After each step, including EGF immobilization and ELISA, all the wells were washed three times using the washing buffer in the kit. For the color development, tetramethylbenzidine (TMB) solution was added and incubated in the dark for 30 min at room temperature. Stop solution was then added to terminate the reaction. The absorbance from each well was measured at 550 nm using a SpectraMax M Series Multi-Mode microplate reader (Sunnyvale, CA). For quantification of immobilized EGF, a standard curve of EGF ELISA was obtained following the established protocol of instruction, and the concentration of EGF was calculated.

Surface Plasmon Resonance (SPR). A Biacore X100 (GE Healthcare Life Science, Pittsburgh, PA, USA) was used for monitoring EGF binding on the collagen surface. HBS-EP (0.01 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at pH 7.4, 0.15 M NaCl, 3 mM EDTA (ethylenediaminetetraacetic acid), 0.005% v/v Surfactant P20) buffer was used as running buffer. On a CM5 chip, an EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride)/NHS (*N*-hydroxysuccinimide) amine coupling kit was used to make an active NHS surface, and then 0.1 mg/mL collagen I solution was injected. To deactivate unreacted NHS, 1.0 M ethanolamine-HCl was injected after collagen immobilization. Then, the SPR chip was removed from the equipment. Riboflavin solution (0.025 mM) containing 10 μg/mL of EGF was dropped onto the collagen coated chip surface and blue light was exposed for 20 s in pulse mode. After gentle washing, the SPR chip was remounted on the equipment, and running buffer and PBS were flowed in to observe dissociation of immobilized EGF. The response curve and values were obtained by subtraction of reference surface signal and analyzed using Biacore X100 evaluation software (GE Healthcare Life Science, Pittsburgh, PA).

Atomic Force Microscopy (AFM). An AFM system (NX10, Park Systems) equipped with tapping mode AFM was used to observe the surface topography of two-dimensional collagen surfaces after exposure to blue light and riboflavin, as well as after EGF coupling. Soft AFM cantilevers with spring constants of ∼0.1 N/m were driven at ∼6 kHz during tapping mode scans. All images were acquired at room temperature with 5-μm scan width, 0.25 Hz scan rate, and set point equal to 40−50% of the free amplitude. Image analysis was performed using XEI Software (Park Systems). The 0-point was set as mean of the heights, and the root-mean-square roughness was obtained via XEI.

Western Blot. The corneal epithelial layer from porcine eyes was removed via scraping with a #10 Bard-Parker blade. The exposed stromal layer was then air-dried. Next, a solution of riboflavin 0.025 mM and EGF-fluorescein isothiocyanate (FITC) 100 μg/mL dissolved in PBS was applied to the stromal surface. After 15 min, pulsed blue light was applied for 5 s and then the cornea was washed three times with 1 mL of PBS. The other group received only the topical administration of EGF-FITC and after 2 min the cornea was washed three times with 1 mL of PBS. The corneas were then cut and snap frozen at −80 °C. Then, 100 μL of radioimmunoprecipitation assay buffer (RIPA buffer, Thermo Scientific) was added to the tissue, and the mixture was vortexed and sonicated for 5 min. Next, the tissue in RIPA buffer was centrifuged at 1500 rpm for 5 min at 4 °C. The supernatant was collected, and total protein concentration was determined by using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). After determining total protein concentration, NuPAGE lithium dodecyl sulfate (LDS) sample buffer

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(Thermo Scientific) and NuPAGE sample reducing agent (Thermo Scientific) were added to the samples as specified by the manufacturer. The samples were then heated at 70 °C for 10 min. The denatured proteins were applied in a NuPAGE Novex 4−12% Bis-Tris protein gels. Electrophoresis was performed at 150 V for 40 min.

Next, the proteins in the gel were transferred to an Immobilon-P Membrane, (polyvinylidene difluoride (PVDF), 0.45 μm, 26.5 cm × 3.75 m roll) for 1 h at 30 V. The membrane was then washed three times with tris-buffered saline 1× with 0.5% polysorbate 20 (TTBS) (Thermo Scientific) for 5 min and blocked with bovine serum albumin (BSA) 3% in TTBS 1× for 1 h. Anti-FITC horseradish peroxidase (HRP)-conjugated antibody (Abcam) was added overnight in blocking solution (1:1000). The following day, the membrane was washed with TTBS 1× three times for 5 min. The chemiluminescence substrate (Thermo Scientific) was added, and the bands were visualized by using a Luminescent Image Analyzer, ImageQuant LAS 4000. The intensity of the EGF-FITC band was quantified by ImageJ software. The normalized data was obtained by dividing the intensity of the EGF-FITC band by that of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) internal standard protein.

Cross-Linking of EGF to Endogenous Collagen from Porcine Cornea. Porcine corneas were prepared and treated as described above. The corneas were then cut and fixed with 4% paraformaldehyde (PFA). After 2 h, the PFA solution was removed and the corneas were placed in 10% sucrose for 2 h and then 20% sucrose for another 2 h. Next, the corneas were put in tissue freezing medium (Leica) and sectioned for histological evaluation.

Tissue sections were washed three times with PBS and a solution of 4′,6-diamidino-2-phenylindole (DAPI) in PBS (1:1000) was added for 5 min. After washing, the sections were mounted and observed using confocal microscopy (ZEISS LSM 880).

Release of EGF from Riboflavin/Blue Light Cross-Linked Hydrogels. Collagen solution (5 mg/mL) was neutralized by adding 200 μL of a cocktail solution consisting of water, PBS 10×, and sodium hydroxide (NaOH). EGF was then added to the collagen solution to a final concentration of 10 μg/mL. Riboflavin was added to the neutralized solution of collagen and EGF (500 μL) to obtain a final concentration of 0.025 mM. Blue light was exposed to the solution of collagen, riboflavin and EGF for 20 s. The hydrogel was then added to a PBS solution with or without collagenase type 1 (Sigma-Aldrich) at different concentrations, 0.1 and 0.2%, to evaluate EGF release over 5 days. At each day, the solution was removed and a fresh solution with or without collagenase was added.

Cell Culture. Primary corneal epithelial cells (keratinocytes) were obtained from rabbit's corneas following a described protocol with few modifications.[12] First, plates were precoated with a solution of collagen and fibronectin (1:1) and BSA (1%) for 1 h at 37 °C. Rabbits' eyes were then sterilized in 10% betadine and washed with PBS, in the presence of antibiotic and antimycotic. The corneas were placed epithelial side down on a sterile surface, and the endothelial layer was removed. Then, the cornea was cut into triangular shaped wedges. The segments were placed upside down on the precoated plates. The tissue was allowed to dry for 20 min, and then one drop of Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM:F12, 1:1) containing HEPES and L-glutamine (Thermo Scientific), fetal bovine serum (FBS) (15%), dimethyl sulfoxide (DMSO) (0.5%), Cholera Subunit A (1 μg/mL), EGF (10 ng/mL), and insulin (5 μg/mL) was added to each segment. On the next day, 1 mL of medium was added to each well. On day 5, the segments were removed and the medium was changed to keratinocyte serum-free medium (KSFM) containing bovine pyruvate extract (BPE) and EGF. After confluence, the cells were subcultured and used at passage two to four.

Primary corneal stromal cells (keratocytes) were also obtained from rabbit's corneas following a previously published protocol.[12] First, plates were precoated with gelatin and water (1:1). After sterilization of the eyes, the cornea was cut and both the epithelium and endothelium layer were mechanically removed. The cornea was cut into pieces and digested with 0.25% collagenase at 37 °C for 2 h. The pieces of stroma were placed in the gelatin precoated plates and teased apart with forceps. After that, 1 mL of medium DMEM/F12 with HEPES buffer containing 10% of FBS and insulin-transferrin-selenium (ITS) premix (0.5%) (Thermo Scientific) was added and the plates were incubated overnight. The following day, the matrix debris was separated by using a plastic cell sieve (20 μm pores). The suspension was centrifuged, and the cells were resuspended in DMEM/F12 medium containing HEPES buffer and ITS. After confluence, the cells were subcultured and used at passage four through six.

Corneal Epithelial and Stromal Cell Toxicity. Polystyrene plates (48 wells) were coated with collagen solution (Sigma-Aldrich) for 30 min at 37 °C. Riboflavin solution (0.025 mM) containing 10 μg/mL EGF, solubilized in D-PBS, was added to the collagen coated surfaces. After 15 min, each well was exposed to pulsed blue light for 5 s, and the plates were washed three times with D-PBS. Other conditions included a D-PBS solution containing only 0.025 mM of riboflavin or exposure to only pulsed blue light for pulsed 5 s. After the treatments were performed, keratinocytes were seeded on the plates at a concentration of 5×10^4 cells/mL. After 24 and 72 h, cell viability was assessed via Live/Dead staining (Thermo Scientific), following the manufacturer's instructions.

Keratocytes were seeded on precoated polystyrene plates (48 wells) at a concentration of 5×10^4 cells/mL overnight. The next day, the riboflavin and EGF were dissolved in KSFM to a final concentration of 0.025 mM and 10 μg/mL, respectively. The plate was placed at 37 °C for 15 min. Other conditions included a solution containing only 0.025 mM of riboflavin in KSFM or wells exposed to only pulsed blue light for 5 s. After that, the cells were washed three times with media. After 24 and 72 h, cell viability was assessed via Live/Dead staining, following the manufacturer's instructions. The number of viable and dead cells was analyzed using ImageJ software. Cell viability was obtained by dividing the number of live cells to number of total cells. Each condition was performed in triplicate and averaged from three distinct experiments.

Corneal Epithelial Cell Proliferation. Polystyrene 96-well plates were coated with collagen solution for 30 min at 37 °C. Next, 50 μL of a D-PBS solution containing 0.025 mM riboflavin and 10 or 1 μg/mL EGF was applied to the surfaces. A D-PBS solution containing only riboflavin (0.025 mM) was also added to different wells. After 15 min, pulsed blue light was applied for 5 s to the wells containing the different solutions. The wells were washed three times with D-PBS.

Keratinocytes growth medium was substituted for KSFM medium without EGF and BPE and the cells were incubated overnight. The following day, 4 mL of trypsin (0.25%) was added to the cells for 2 min and then neutralized by the addition of 6 mL of medium containing serum. The cells were centrifuged at 1000 rpm for 5 min. The medium was aspirated and the cells were resuspended in KSFM medium, without BPE and EGF. The cells were plated on the pretreated surfaces at a density of 1000 cells per well. KSFM medium without BPE and EGF was added to each well, and for the soluble EGF group, the growth factor was added to a final concentration of 10 or 1 μg/mL. Cell proliferation was assessed using a Cell counting kit-8 (Sigma-Aldrich) following the manufacturer's instructions. The data was obtained in triplicate for each condition and normalized dividing the absorbance from the treated group by that of the no treatment group.

Corneal Epithelial Cell Morphology and Phenotype. A Lab-Tek Chamber Slide system was coated with collagen at 37 °C for 30 min. Then, D-PBS solution containing 0.025 mM of riboflavin and 10 μg/mL of EGF was added to the wells. After 15 min, pulsed blue light was applied for 5 s, then the wells were washed with D-PBS three times. Next, D-PBS solution containing 1% BSA was added for 1 h at room temperature, then the wells were washed three times with D-PBS. Keratinocytes were added to the treated wells following the same procedure described above. At 24 h, the wells were washed with D-PBS and fixed with 4% PFA for 15 min. After washing with D-PBS, the wells were blocked and the cells permeabilized for 30 min with 1% BSA and 0.1% Triton-X. Next, Alexa Fluor Phalloidin 647 was added and incubated for 30 min. After washing three times with D-PBS, DAPI was added for 5 min in PBS solution (1:1000). The cells were mounted, and the morphology was observed using confocal

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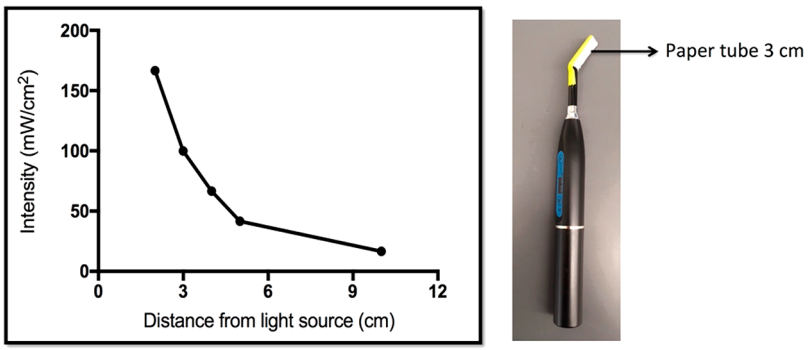

Figure 1. Blue light intensity as a function of the distance from light source and blue light apparatus.

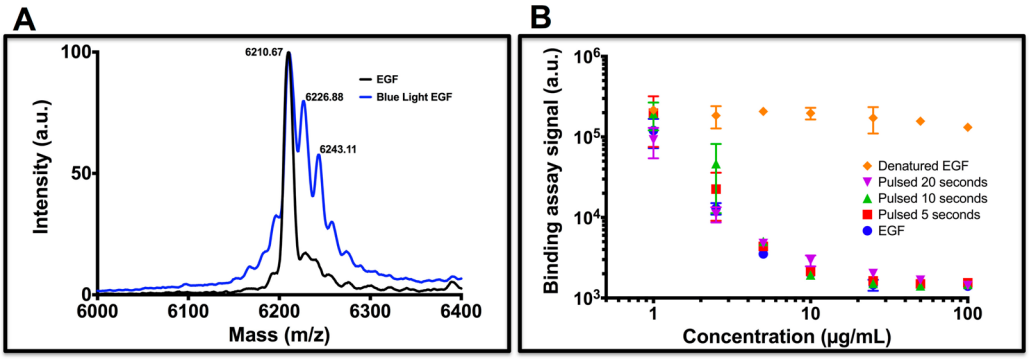

Figure 2. EGF mass spectra before and after exposure to riboflavin (0.025 mM) and blue light (pulsed 10 s) (A). Binding assay signal after exposing to riboflavin (0.025 mM) and blue light at different exposure times (pulsed 5, 10, and 20 s), $n = 3$ (B).

microscopy. To quantify the cell area, ImageJ software was used. The data is presented as the average from three different experiments.

For the cell phenotype assay, the treatment was performed in glass plates as described above. In this case, BSA 1% was not added. Keratinocytes were seeded as described above. At 72 h, the wells were washed with D-PBS and fixed with 4% PFA for 15 min. After washing with D-PBS, the wells were blocked and the cells permeabilized for 30 min, with 1% BSA and 0.1% Triton-X. Mouse primary antibody anti-rabbit cytokeratin (CK3/12) and rabbit anti-ZO-1 were added and incubated overnight at 4 °C in blocking solution. The next day, the wells were washed with D-PBS and the secondary antibody, Alexa Fluor 594 anti-mouse or Alexa Fluor 546 anti-rabbit, was added and incubated for 2 h in blocking solution. After 2 h, DAPI was added and incubated for 5 min (1:1000). Cells phenotype was by confocal microscopy.

Statistical Analyses. For statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test and unpaired *t* test was used to compare the results obtained using GraphPad Software. In these comparisons, a *p*-value less than 0.05 was used to indicate a statistically significant difference.

RESULTS

Blue Light. Blue light intensity was evaluated at a distance ranging from 2 to 10 cm (Figure 1). By increasing the distance from the source, the light intensity decreased considerably. To apply a light intensity of 100 mW/cm^2, we maintained a constant working distance of 3 cm by adding a paper tube spacer to the end of the light source.

EGF Binding to EGF Receptor Is Preserved after Exposure to Blue Light in the Presence of Riboflavin. EGF molecular weight was evaluated by mass spectrometry after exposing to pulsed blue light for 10 s (5 s total exposure) in a solution containing 0.025 mM of riboflavin (Figure 2A). Compared to native EGF, two new peaks with higher m/z (6226.88; 6243.11) are formed after blue light exposure. However, the native EGF m/z (6210.67) is maintained after blue light exposure. The percentage of unmodified EGF was about 40%. The newly formed peaks represent about 60% of the total EGF. No EGF-to-EGF cross-linking was observed.

To investigate EGF bioactivity, a binding assay to the EGF receptor was performed (Figure 2B). For this assay, biotinylated EGF and nonbiotinylated EGF were used. Mixing them together results in competitive binding of the EGF receptor. In this regard, the expected signal decreases after exposure to streptavidin-coated donor beads. First, the EGF riboflavin solution was dialyzed after exposure to blue light, to eliminate any unreacted riboflavin. The dialysis was an important step since the yellow color of riboflavin would interfere with the binding assay reading. Denatured EGF was unable to bind to the EGF receptor, indicated by the fact that the signal was not decreased by any of the denatured EGF concentrations, thus allowing it to serve as a negative control. Native, nonbiotinylated EGF decreased the signal as a result of competitive binding to the EGF receptor. Similar results were observed for nonbiotinylated EGF exposed to blue light; thus, blue light exposure for 5, 10, or 20 s did not interfere with EGF receptor binding.

Photocrosslinking Reaction Successfully Immobilizes EGF to Collagen-Coated Surfaces. EGF attachment to collagen-coated surfaces after photochemical reaction was monitored by surface plasmon resonance (SPR) and enzyme-linked immunosorbent assay (ELISA) (Figure 3). The photochemical reaction led to an increase of about 200 response units (RU) on the collagen-coated SPR chip without dissociation, showing that EGF was immobilized on the surface (Figure 3A). Generally, the response unit in SPR represents the

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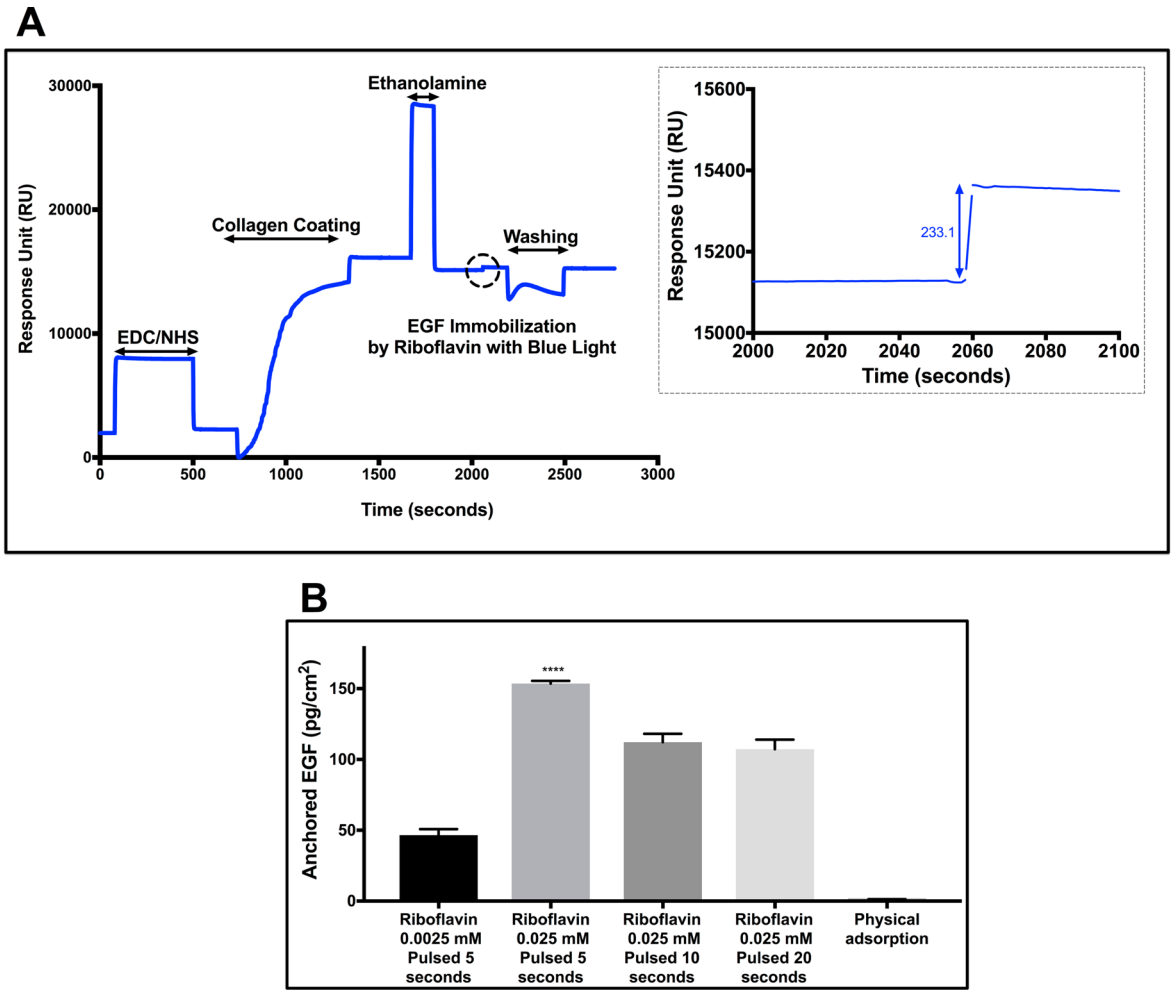

Figure 3. SPR of collagen-coated surface showing increased response units after the photocrosslinking reaction pulsed blue light for 20 s (A). Immobilized EGF on collagen-coated surfaces at different riboflavin concentrations (0.0025 and 0.025 mM) and blue light exposure times (pulsed for 5, 10, and 20 s) (B), as detected by ELISA, $n = 3$, ****$p < 0.0001$.

change in resonance angle by surface coverage, so the change of response units indicates mass differences on the surface.[13] ELISA results confirmed the binding of EGF to collagen-coated polystyrene surfaces (Figure 3B). Riboflavin at a lower concentration (0.0025 mM) was less efficient at binding EGF to the surface. The highest binding was achieved by riboflavin at an intermediate concentration (0.025 mM). Blue light exposure time also played an important role in immobilizing EGF to collagen surfaces. An exposure time of 5 s of pulsed blue light provided the best attachment results for EGF, while increased exposure times (10 and 20 s) resulted in less bound EGF to the collagen surface. The physical adsorption of EGF did not result in the detectable binding of the growth factor to the surface.

The surface topography was measured using atomic force microscopy (AFM) (Figure 4). Collagen-coated surfaces without riboflavin and blue light exposure showed a root-mean-square roughness (Rq) of 0.843 nm (Figure 4A). The effect of a 20 s, pulsed blue light exposure alone was analyzed, and there was no significant topographical change observed without riboflavin (Figure 4B). The Rq of a photocrosslinked collagen surface (i.e., exposure to riboflavin and pulsed blue light for 5 s) without EGF was 2.249 nm (Figure 4C), while the same treatment with EGF was 4.988 nm (Figure 4E), an approximate doubling in size of surface topography features. Longer blue light exposure time led to an increase in surface roughness (Figure 4D and F). The Rq of surfaces treated for 20 s of pulsed blue light without and with EGF was 7.706 and 8.534 nm, respectively; thus, there were negligible changes in surface topography with or without EGF when subjected to longer light exposure times.

Ex Vivo Immobilization of EGF to Endogenous Corneal Collagen by Blue Light Photocrosslinking Reaction. We next evaluated whether our strategy could immobilize EGF to the endogenous collagen of the porcine corneal stroma. We treated excised and de-epithelialized corneas with EGF-FITC to distinguish it from the native EGF present in the cornea. To evaluate the presence or absence of EGF-FITC, we used an anti-FITC HRP-conjugated antibody for Western blot analysis. For the photocrosslinking conditions, EGF-FITC in a riboflavin solution was first applied for 15 min, and then pulsed blue light was applied for 5 s. For topical administration without photocrosslinkingg, EGF-FITC was applied for 2 min to simulate topical administration of an eye drop. For both conditions, the corneas were washed abundantly with PBS. By Western blot analysis, a strong band was observed for EGF-FITC immobilized by photoreaction, whereas topical administration of EGF-FITC showed a very weak band (Figure 5A), which was statistically significant by band density quantification. No EGF-FITC was detectable for the EGF-FITC free group.

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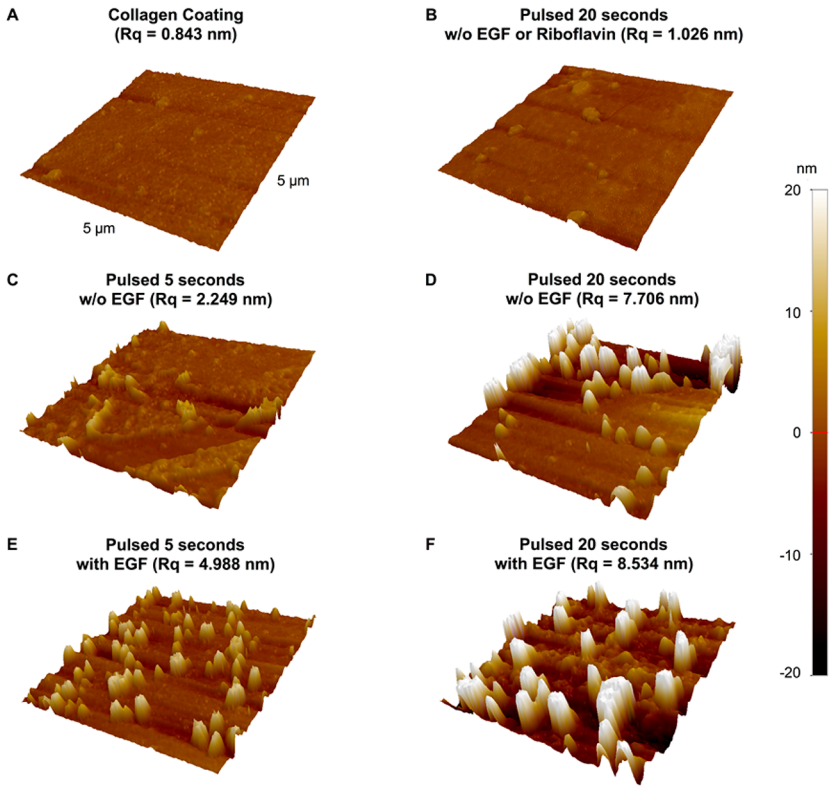

Figure 4. Topography of dry collagen-coated surfaces measured by AFM. (A) Collagen coating without treatment, (B) after blue light exposure alone for 20 s without EGF or riboflavin, (C) pulsed blue light exposure with riboflavin for 5 s but without EGF, (D) pulsed blue light exposure with riboflavin for 20 s without EGF, (E) pulsed blue light exposure with riboflavin for 5 s with EGF, and (F) pulsed blue light exposure with riboflavin for 20 s with EGF.

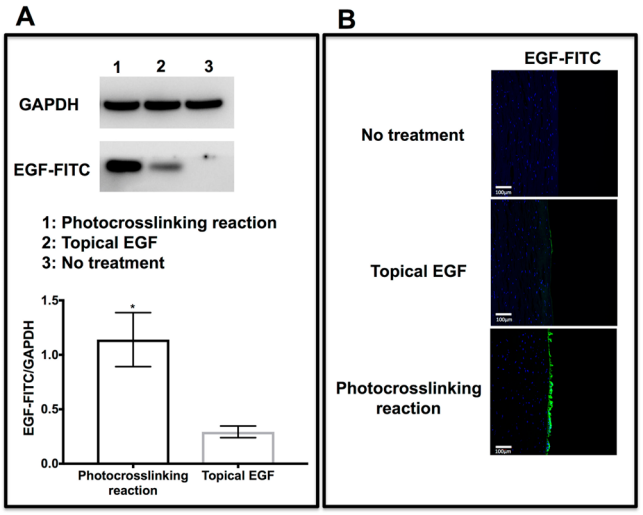

Figure 5. (A) Western blot showing the intense band related to the immobilization of EGF-FITC to the cornea after photocrosslinking reaction (p = 0.0420) (B). Confocal microscopy of porcine corneas showing an abundance of immobilized EGF-FITC on the stromal surface after photocrosslinking reaction. Scale bar 200 μm.

The presence of immobilized EGF-FITC on the corneal tissues was also confirmed by direct confocal microscopy. A strong fluorescence signal was observed on the stromal layer for the group in which EGF-FITC was immobilized by photoreaction, while a weak signal was observed for topically applied EGF-FITC without the photoreaction (Figure 5B).

Stability of Immobilized EGF on Collagen-Coated Polystyrene Surfaces. The long-term, in vitro stability of EGF immobilized onto collagen-coated polystyrene surfaces was investigated by ELISA (Figure 6). For a low riboflavin concentration (0.0025 mM), the amount of EGF was maintained from day 0 to day 1, and then slowly decreased on subsequent days. In the best photocrosslinking condition, 0.025 mM riboflavin and 5 s pulsed blue light exposure, the amount of bound EGF dropped from day 0 to day 1, and then was maintained at a constant level through day 7. In contrast, with the same riboflavin concentration but longer pulsed blue light exposure times (10 and 20 s), the EGF concentration not only decreased from day 0 to day 1, but continued decreasing over the following days. For an even higher riboflavin concentration (0.25 mM) with 10 s of pulsed blue light exposure, the temporal EGF profile was similar to that of the intermediate riboflavin concentration.

Release of EGF from Collagen Hydrogels. The release of EGF from collagen gels was investigated both in the absence and presence of collagenase (Figure 7). Without enzymatic treatment, EGF is released relatively slowly from the collagen matrix in pure PBS, with less than 1% EGF released at 70 h. The addition of collagenase (0.1%) enhanced the release of EGF, with about 2.5% EGF released at 70 h. Using a higher concentration of enzyme (0.2%) further increased the EGF release rate, with about 4.5% EGF released at 70 h.

Riboflavin and Blue Light Are Noncytotoxic. Cytotoxicity was evaluated using Live/Dead assay in two different cell lines at 24 and 72 h (Figure 8). First, we investigated the toxicity that each variable would provide, and then we evaluated the full photocrosslinking reaction in the presence of EGF.

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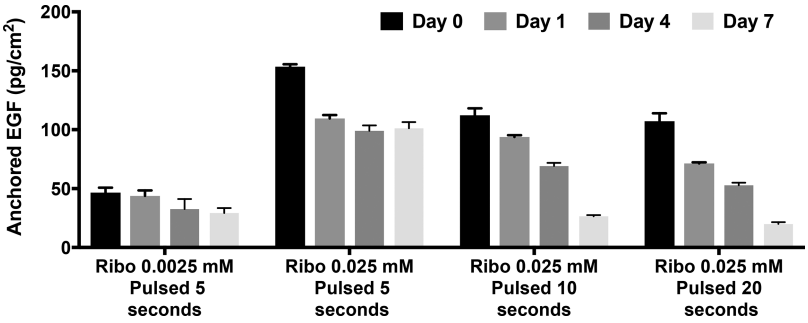

Figure 6. Stability of photocrosslinked EGF on collagen-coated surfaces during in vitro incubation over 7 days with varying riboflavin concentrations (0.0025, 0.025, and 0.25 mM) and blue light exposure times (pulse 5, 10, and 20 s), as detected by ELISA.

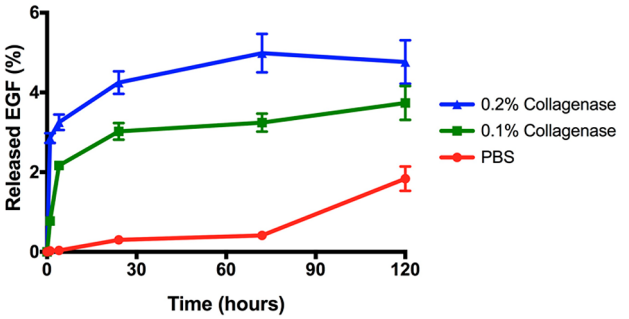

Figure 7. EGF released from riboflavin/blue light cross-linked collagen hydrogels in 0.1% collagenase, 0.2% collagenase, and PBS solution.

After coating the plate with collagen, primary cultured keratocytes were exposed directly to pulsed blue light for 5 s and 0.025 mM riboflavin in KSFM solution. Cell viability was nearly 100% after 24 and 72 h, showing that riboflavin in combination with blue light was not toxic to the cells. We next performed the photocrosslinking reaction in the presence of EGF. No cytotoxicity was observed, and cell viability was maintained near 100% for 72 h (Figure 8A).

To simulate in vivo conditions, corneal epithelial cells were seeded onto wells where EGF was previously immobilized by the photocrosslinking reaction. In this case, we aimed to evaluate whether the treated surfaces were cytocompatible. Exposure to pulsed blue light for 5 s did not impact cell survival. Similarly, riboflavin in combination with blue light

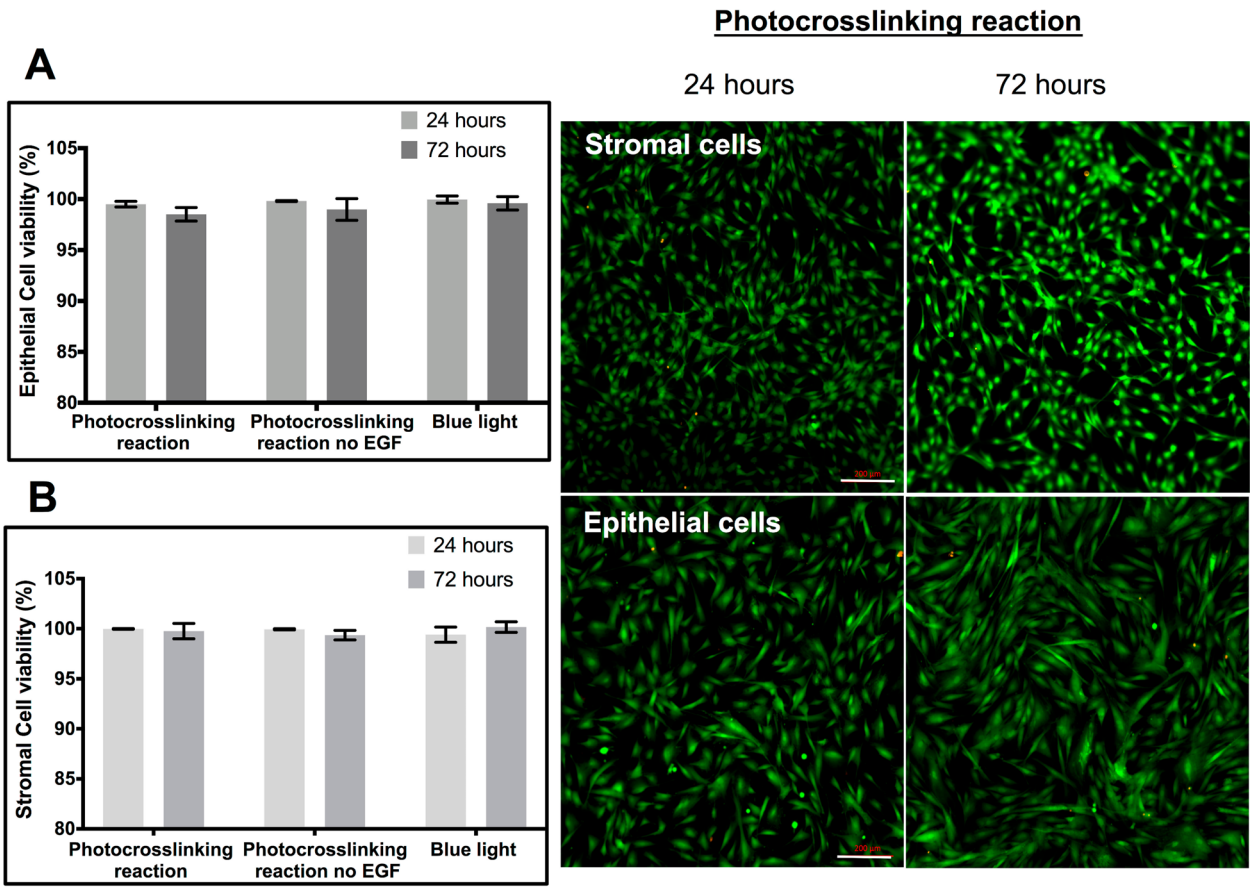

Figure 8. Corneal stromal (A) and epithelial (B) cell viability (quantified in left panels, representative micrographs in right panels) after treatment with blue light, blue light with riboflavin, or the full photocrosslinking reaction in the presence of EGF. Scale bar 200 μm.

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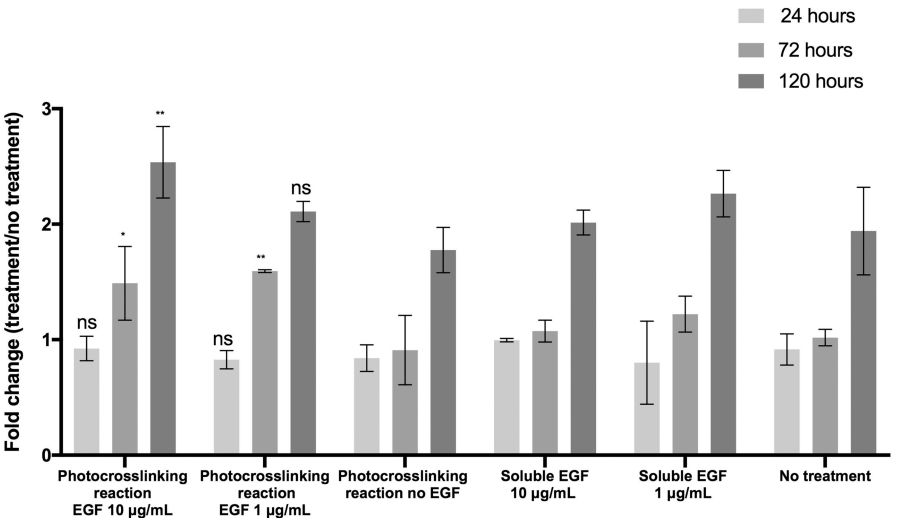

Figure 9. Epithelial cell proliferation assay. Corneal epithelial cells were cultivated in KSFM medium without BPE and EGF for 120 h on collagen-coated surfaces (cells), on collagen-coated surfaces with soluble EGF (10 and 1 μg/mL), on riboflavin/blue light treated, collagen-coated surfaces, and on collagen-coated surfaces treated with blue light to immobilize EGF from solutions with initial concentrations of 10 and 1 μg/mL. $n = 3$, $0.01 < p < 0.05$ (*) and $p < 0.01$ (**).

showed high cell viability. Finally, when exposed to the photocrosslinking reaction in the presence of EGF, keratinocytes remained nearly 100% viable over 72 h (Figure 8B).

Corneal Epithelial Cell Proliferation Is Increased in the Presence of Bound EGF. Corneal epithelial cell proliferation was evaluated within an interval of 24−120 h (Figure 9). Cells with soluble EGF (at equal concentrations as those used to prepare the EGF-immobilized substrates) and EGF-free groups were seeded on collagen-coated surfaces as controls. Two EGF concentrations were evaluated (1 and 10 μg/mL). After seeding the cells, the medium was changed at 48 and 96 h. At each medium change, new soluble EGF was added to the cells for the EGF group only. At 24 h of incubation, cell proliferation was the same for all analyzed groups. After 72 h, cells seeded on EGF-immobilized surfaces showed increased proliferation compared to soluble EGF and EGF-free groups. At 120 h of incubation, cells seeded on EGF-immobilized surfaces prepared with higher EGF concentrations (10 μg/mL) outperformed EGF-immobilized surfaces prepared with lower EGF concentration (1 μg/mL). Soluble EGF did not significantly augment cell proliferation compared to the EGF-free group. Cells seeded on riboflavin/blue light treated surfaces without immobilized EGF showed no increased proliferation compared to the other groups.

Corneal Epithelial Cell Morphology and Phenotype on EGF-Immobilized Surfaces. Corneal epithelial cell morphology was investigated in the presence of soluble EGF and EGF immobilized by photocrosslinking reaction (Figure 10). After 24 h of incubation, no statistically significant changes in cell morphology were observed between immobilized and soluble EGF samples. However, a dramatic morphological change was observed for cells in the immobilized EGF and the EGF-free groups. In the absence of EGF, the cells exhibited a round shape. In contrast, cells from the immobilized EGF group showed dense actin filaments, filopodia, and extended lamellipodium, with a statistically significant increase in cell spreading ratio.

Corneal epithelial cell phenotype was characterized by immunofluorescence staining using antibodies specific for keratinocytes (CK3/12) and zonula occludens-1 (ZO-1), a tight junction protein (Figure 10B). The plate surface was coated with collagen, and EGF was immobilized by photochemical reaction before cell seeding. For the soluble EGF group, the cells were seeded on collagen surface, and soluble EGF was added. No EGF treatment was used as the control. Cells from all three groups were able to express CK3/12 after 24 h, but the EGF-free group showed reduced CK3/12 expression. All groups were able to form tight junctions after 72 h.

DISCUSSION

EGF has been extensively studied, and one of its main characteristics is to improve cell proliferation, migration, adhesion, and differentiation.[14,15] Accordingly, the application of EGF in severe corneal wounds is an attractive strategy to increase the rate of wound healing. However, topical eye drops of EGF have not been clinically successful. This may be because, as any other drug applied by eye drops to the cornea, the retained concentration is small, decreases quickly, and typically requires frequent administration. Maintaining growth factor concentrations at the ocular surface requires frequent applications. In addition to limited bioavailability, local depletion of growth factors via endocytosis also occurs, thus decreasing its concentration even more.[16] To overcome these problems, herein we provided a cytocompatible strategy to directly immobilize EGF to collagen surfaces by photochemical reaction. We aimed to provide a growth factor-enriched surface that leads to the increase of corneal epithelial cell proliferation. The immobilization of EGF could augment EGF availability and reduce the endocytosis rate while providing a growth factor reservoir at wound sites. Clinically, this could potentially decrease the need for frequent eye drop administration while achieving the same or better effects.

Numerous strategies to covalently bind EGF to surfaces have been studied.[3−5] Many strategies require complex reactions that would be difficult to translate in vivo. In the present study, we chose to immobilize EGF to collagen surfaces using the FDA-approved photosensitizer riboflavin in combination with visible blue light, which is used ubiquitously in ophthalmology as a diagnostic aid to measure intraocular pressure, diagnose

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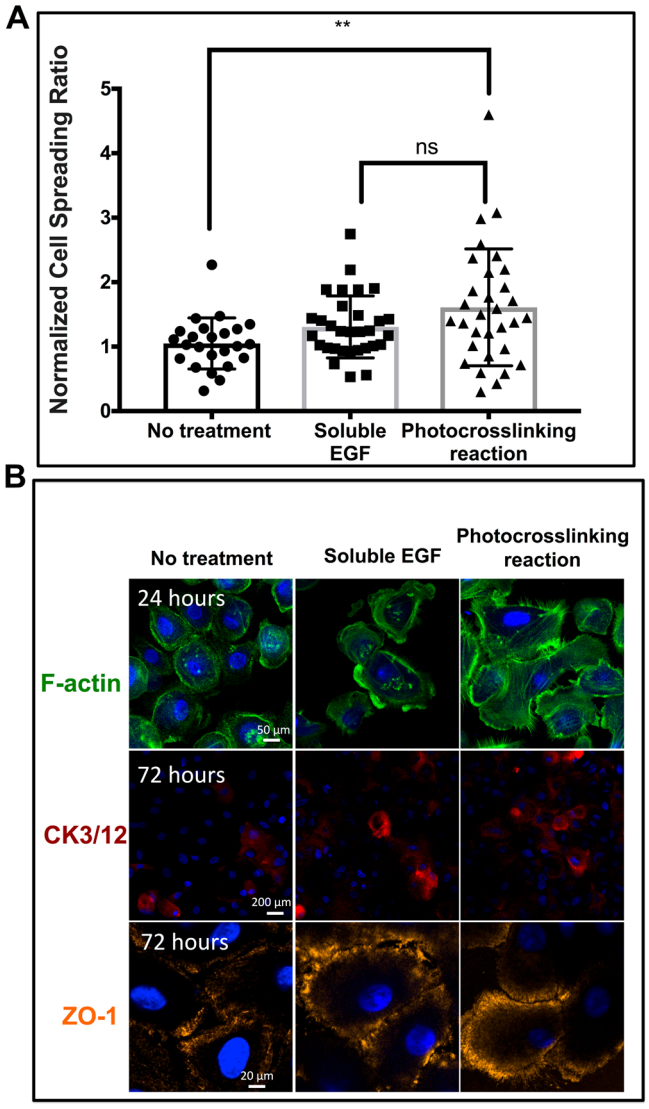

Figure 10. Morphological study of corneal epithelial cells in no treatment, soluble EGF, and immobilized EGF groups cultured in KSFM medium without BPE and EGF for 24 or 72 h. The graph represents the quantitative analysis of cell spreading area of each cell from the different treatments after 24 h, $^{**}p < 0.05$ (A). Cells were stained for F-actin with Alexa Fluor 647 phalloidin (green), CK3/12 (red), and ZO-1 (yellow) (B).

epithelial defects, and observe retinal vascular abnormalities in conjunction with fluorescein. Collagen is present in the stromal layer of the cornea, thus socollagen-coated polystyrene surfaces served as an in vitro model substrate. For the photocrosslinking, the only external chemical used was riboflavin. Riboflavin has already been approved by the United States Food and Drug Administration (FDA) for corneal collagen cross-linking using UVA light, in order to increase the mechanical strength of ectatic corneas, and in particular keratoconic corneas.[11] However, it has been shown that corneal epithelial cells and stroma are sensitive to UVA light exposure.[11,17] To improve the safety profile of the photocrosslinking reaction, here we chose a less cytotoxic energy source—blue light—instead of UVA light.[18]

Previously EGF has been shown to lose its bioactivity when covalent coupling strategies are performed to bind it to surfaces.[19,20] For example, tethering strategies that rely on reaction with the primary amines on lysine 28 and lysine 48 from human EGF were found to decrease EGF bioactivity.[20] Activity loss is the main concern when working with human EGF. To this end, we first verified the changes on EGF molecule and activity after the photocrosslinking reaction. After riboflavin excitation, the singlet oxygen species produced are expected to react with histidine amino acids from collagen and EGF, thus cross-linking the growth factor into the matrix.[21,22] We were interested in observing whether the EGF chains would cross-link with each other in solution to form dimers, trimers or more. Interestingly, no cross-linking between EGF molecules was observed after the photocrosslinking reaction (Figure 2A). However, two new peaks with higher molecular weights were formed. The mass increase in the EGF molecule was about 16 and 32 Da, which corresponds to one and two molecules, respectively, of oxygen incorporated into the EGF. These new peaks show the reaction of the two EGF histidine residues with the singlet oxygen forming the imidazolone ring.[21] These newly formed peaks correspond to more than 50% of the soluble EGF. To investigate if this modification would impact EGF bioactivity, we performed a binding assay to EGF receptor. Notably, EGF activity after exposure to blue light and riboflavin was not reduced (Figure 2B), which suggests that histidine residue modification does not interfere with binding to the EGF receptor.[7] Furthermore, EGF activity was independent of various blue light exposure times, demonstrating that the range of blue light exposure intensities and durations used in these experiments does not cause degradation or denaturation of EGF. Hence, our results show an advantage over the strategies that require reaction with amines. These data also suggest that the blue light/riboflavin strategy may be amenable to immobilization of other types of growth factors for future applications.

Next, we aimed to investigate whether the photocrosslinking reaction would be able to immobilize EGF to collagen surfaces. It is well-known that UV light-activated riboflavin leads to a dose-dependent cross-linking of corneal collagen.[23,24] Although UV light is the most effective and established means of inducing this cross-linking, blue light has also been shown to initiate collagen cross-linking, although to a lesser extent.[11,23,24] This is based on the experimental observation that the tensile modulus of corneal stroma is increased to a lower degree with blue light versus UV light for a given riboflavin concentration.[24] Furthermore, Ibusuki et al. showed that chondrocytes could be encapsulated within collagen gels using blue light and riboflavin.[18] With SPR, we observed a layer increase of about 200 response units after the addition of EGF and the photocrosslinking reaction facilitated by riboflavin, to a collagen-gold surface (Figure 3A). This result confirmed that EGF was attached to the collagen surface upon blue light and riboflavin treatment. We further aimed to investigate by ELISA if shorter blue light exposure times could bind EGF to collagen-coated polystyrene surfaces. Interestingly, shorter exposure times were more effective at immobilizing EGF onto collagen surfaces (Figure 3B). This is likely caused by changes in the underlying collagen network structure due to collagen-collagen cross-linking, as discussed in more detail below in the interpretation of the AFM data.

As expected, EGF that was physically adsorbed without any photocrosslinking reaction did not result in any attachment of the growth factor to the surface (Figure 3B). This result strongly suggests that the photocrosslinking reaction results in covalent tethering between EGF and the collagen matrix. We

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further evaluated the extent to which riboflavin concentration might impact EGF immobilization on collagen surfaces. A lower riboflavin concentration (0.0025 mM) led to significantly less EGF immobilization on collagen (Figure 3B). Thus, a minimum concentration of 0.025 mM riboflavin is likely required to complete the photocrosslinking reaction efficiently.

We next characterized the topography of the various collagen surfaces using AFM. Interestingly, the surface roughness of collagen was drastically increased with pulsed blue light exposure for 20 s compared to 5 s in the absence of EGF (Rq of 7.7 and 2.2 nm, respectively, Figure 4). This increase in surface roughness is likely due to an increase in collagen cross-linking. For UV-based, riboflavin-initiated cross-linking of corneal collagen, there is a known time-dependence, with longer exposure times leading to increased collagen cross-linking.[23,24] These AFM observations are consistent with the "condensation" phenomenon seen in the collagen matrix of the vitreous humor, where collagen fibrils aggregate over time and form relatively insoluble clusters that are perceived visually as "floaters."[25] We hypothesize that increased collagen cross-linking may lead to enhanced collagen aggregation into larger-sized surface structures.

We then evaluated changes in collagen surface roughness upon photocrosslinking in the presence of EGF. Substrates exposed to pulsed blue light for 5 s substantially increased their surface roughness in the presence of EGF (5.0 vs 2.2 nm, Figure 4). The dimension of EGF is approximately 3.3 × 3.3 × 2.2 nm,[26] so these data suggest that EGF has been efficiently immobilized onto the surface of the collagen.[26] In contrast, for substrates exposed to pulsed blue light for 20 s, the surface roughness in the presence or absence of EGF did not appreciably change (8.5 vs 7.7 nm), suggesting that EGF immobilization was less efficient on these surfaces. These AFM data are consistent with the previous ELISA data that also demonstrated less efficient EGF immobilization for longer blue light exposure times (Figure 3B). Thus, it appears that increased collagen-collagen cross-linking may inhibit effective EGF-collagen cross-linking.

Once we determined the best conditions to immobilize EGF in vitro, we next used ex vivo porcine corneas to investigate the attachment of EGF to the endogenous collagen of corneal tissue. Because EGF is known to be present in healthy corneas, we evaluated the attachment of EGF to the stromal surface by conjugating the growth factor with FITC. EGF-FITC was then immobilized to the cornea by the photocrosslinking protocol described herein. Both Western blot and confocal microscopy showed the successful attachment of EGF-FITC to endogenous collagen, while topically applied EGF provided minimal EGF attachment (Figure 5). These results show that an exposure time of 5 s is appropriate to immobilize EGF to a broad variety of different collagen surfaces. Taken together, our data show that blue light-activation of riboflavin immobilizes EGF to plated (Figure 3) and stromal (Figure 5) collagen, but does not cause EGF-EGF intermolecular cross-linking as evidenced by the lack of dimer and trimer formation (Figure 2A).

The stability of immobilized EGF was evaluated next. Ideally, the EGF would be maintained during the time necessary to close the wound. EGF immobilized using 0.025 mM riboflavin and a pulsed blue light exposure of 5 s maintained a constant EGF concentration from day 1 to day 7 (Figure 6). The initial decrease in EGF concentration from day 0 to day 1 is likely due to a rapid initial release of loosely bound, physically adsorbed EGF from the collagen matrix. From days 1 to 7, the sustained concentration of EGF is suggestive of effective covalent coupling between the growth factor and the substrate in vitro. Consistent with our ELISA and AFM data (Figures 3B and 4), the less effective photocrosslinking reaction conditions (i.e., those with longer pulsed blue light exposure times) led to less stable EGF immobilization (Figure 6). In vivo, a number of proteases are present in corneal tissue that would likely result in decreased EGF concentration over time, and future studies will be performed to evaluate this release rate in a preclinical model of corneal wound closure. To simulate the presence of endogenous proteases, we demonstrated that collagenase could effectively release the immobilized EGF in a dose-dependent manner (Figure 7).

Cell viability of the photocrosslinking reaction was assessed using two primary rabbit corneal cell types: corneal epithelial and stromal cells. To evaluate any potential in vitro toxicity for keratocytes, plates were precoated with collagen, then the cells were seeded, and the photocrosslinking reaction was performed. Cytotoxicity was determined by using Live/Dead assay. We first investigated if blue light (pulsed 5 s) and riboflavin (0.025 mM) were harmful to the cells. As expected for riboflavin, no in vitro cytotoxicity was observed at 24 and 72 h (Figure 8). Previous studies also showed the absence of toxicity for riboflavin at concentrations varying from 0.1 to 1 mM and the combination of riboflavin with visible light, for 40 s, did not induce toxicity.[18] However, increasing the exposure time from 40 to 300 or 600 s gradually decreased cell viability.[18] Our results confirmed previous observations that shorter exposure time is not harmful to the cells.[18] In addition, our light intensity was lower (100 mW/cm^2) compared to that used in previous work (500 mW/cm^2).[18] Furthermore, we saw that the addition of EGF to the photocrosslinking reaction also maintained cell viability at values higher than 95% for all times evaluated (Figure 8). To investigate effects on corneal epithelial cells, collagen surfaces were first photochemically reacted with EGF and then seeded with cells. The cells were able to attach to the modified surfaces, and, similarly to stromal cells, no cytotoxicity was observed after 24 and 72 h. Cell viability was greater than 95% for all conditions (Figure 8).

Next, we evaluated whether the photoimmobilized EGF would retain its bioactivity by increasing cell proliferation. Cells seeded on the EGF immobilized surfaces outperformed the cells cultured with no EGF at 72 and 120 h (Figure 9). For comparison, we tested proliferation in the presence of soluble EGF added to the medium at 1 and 10 μg/mL final concentrations. These EGF concentrations were selected to be identical to those used in the preparation of the immobilized EGF substrates. We saw that soluble EGF was less efficient in increasing cell proliferation compared to the photocrosslinked EGF. This indicates that immobilized EGF retains its bioactivity and is a viable strategy to enhance cell proliferation. Previous work that tethered EGF to polyethylene terephthalate films through Kcoil peptides similarly found that tethered EGF stimulated human corneal epithelial cell growth more than soluble EGF and no treatment groups at 26 h.[10] In another study, EGF tethered via poly(ethylene oxide) (PEO) to polymeric substrates enhanced mesenchymal stem cell proliferation after 7 days in culture.[27] The enhanced proliferation provided by tethered EGF compared to soluble EGF may be due to prolonged phosphorylation of EGF receptors.[10,27] Soluble EGF provides a transient Erk1/2 phosphorylation pattern lasting for minutes, while tethered EGF prolongs the Erk1/2 phosphorylation pattern for hours.[10]

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As expected, decreasing the level of tethered EGF (by changing the initial EGF solution concentration from 10 to 1 μg/mL), decreased the cell proliferation response. Importantly, control collagen surfaces treated with riboflavin and blue light only (in the absence of EGF), did not impact cell proliferation, further supporting the cytocompatibility of the photocrosslinking reaction.

We next evaluated the effects of immobilized EGF on cell morphology. In this assay, after EGF treatment of the surfaces, we added 1% BSA for 1 h to avoid nonspecific cell binding and protein absorption. Lamellipodia and filopodia projections were present in the soluble EGF and immobilized EGF groups, but not the EGF-free group (Figure 10). This indicates cell migration and spreading. Dense F-actin polymerization was observed for the immobilized EGF group as well as an increased cell spreading ratio. Previous studies have shown that immobilized growth factors can increase actin polymerization and lamellipodia extensions.[10,27] Previously, immobilized EGF has been reported to support the spreading and focal adhesion formation of human epithelial cells, while soluble EGF treatment results in rounded, low-adhesive cells.[28] Other studies have shown the increased spreading ratio and increased cell adhesion of mesenchymal stem cells when seeded on immobilized EGF.[27,28]

Corneal epithelial cell differentiation is characterized by the expression of a cornea-specific keratin pair, keratin 12 and keratin 3.[29,30] Improper expression of cytokeratin (CK) 3/12 leads to the formation of a fragile epithelial layer. Additionally, the formation of tight junctions is required for proper epithelial barrier function. Thus, we aimed to investigate whether our EGF-immobilized surface would facilitate expression of both CK3/12 and tight junctions. We compared cells seeded on surfaces with no EGF, soluble EGF, or immobilized EGF after 72 h in medium without BPE/EGF. ZO-1 expression was observed for all groups, showing the ability of corneal epithelial cells to form tight junctions over the modified surface. Expression of CK3/12 was observed for cells in the soluble EGF group and the immobilized group, while lower CK3/12 expression was observed for the EGF-free group. These data suggest that the immobilization of EGF to the collagen surface provided a suitable environment for normal growth and cellular differentiation.

CONCLUSION

Here we reported a new strategy to bind growth factors to collagen surfaces by riboflavin and blue-light-mediated growth factor immobilization. This approach is versatile and has the main advantage of being performed in situ using only riboflavin, an FDA-approved photosensitizer, without apparent loss of bioactivity within a specific range of conditions. We have identified photochemical reaction conditions that efficiently result in EGF immobilization while still enabling EGF receptor binding. The photocrosslinking reaction enabled successful EGF tethering both to collagen-coated substrates in vitro and on ex vivo cornea. Riboflavin concentration and blue light exposure time were shown to be key variables for the successful immobilization of EGF to the collagen surfaces. The bioactivity of immobilized EGF was preserved, and surface-immobilized EGF provided an environment that induced faster cell proliferation and enhanced cell spreading. Additionally, cell phenotype and viability were preserved after the photocrosslinking reaction. Finally, these results bode well for the safety profile for using this strategy in vitro for the preparation of cell scaffolds and carriers as well as in situ on wounded corneas and other tissues.

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Notes

The authors declare no competing financial interest.

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