Bioactive Materials 40 (2024) 417–429

Contents lists available at ScienceDirect

Bioactive Materials

journal homepage: www.keaipublishing.com/en/journals/bioactive-materials

Photoactivated growth factor release from bio-orthogonally crosslinked hydrogels for the regeneration of corneal defects

Nae-Won Kang [a], Youngyoon Amy Seo [a], Kevin J. Jackson [a], Kyeongwoo Jang [a], Euisun Song [a], Uiyoung Han [a], Fang Chen [a], Sarah C. Heilshorn [b], David Myung [a,c,d,*]

[a] Department of Ophthalmology, Byers Eye Institute at Stanford University, School of Medicine, Palo Alto, CA 94304, United States
[b] Department of Materials Science and Engineering, Stanford University, Stanford, CA 94305, United States
[c] Department of Chemical Engineering, Stanford University, Palo Alto, CA 94305, United States
[d] VA Palo Alto HealthCare System, Palo Alto, CA 94304, United States

ARTICLE INFO

Keywords:
In situ-forming hydrogel
Bio-orthogonally crosslinked hydrogel
Corneal regeneration
Photo-responsive release
Growth factor delivery

ABSTRACT

In situ-forming hydrogels are an attractive option for corneal regeneration, and the delivery of growth factors from such constructs have the potential to improve re-epithelialization and stromal remodeling. However, challenges persist in controlling the release of therapeutic molecules from hydrogels. Here, an *in situ*-forming bio-orthogonally crosslinked hydrogel containing growth factors tethered *via* photocleavable linkages (PC-HACol hydrogel) was developed to accelerate corneal regeneration. Epidermal growth factor (EGF) was conjugated to the hydrogel backbone through photo-cleavable (PC) spacer arms and was released when exposed to mild intensity ultraviolet (UV) light (2–5 mW/cm^2, 365 nm). The PC-HACol hydrogel rapidly gelled within a few minutes when applied to corneal defects, with excellent transparency and biocompatibility. After subsequent exposure to UV irradiation, the hydrogel promoted the proliferation and migration of corneal epithelial cells *in vitro*. The rate of re-epithelialization was positively correlated to the frequency of irradiation, verified through *ex vivo* rabbit cornea organ culture studies. In an *in vivo* rat corneal wound healing study, the PC-HACol hydrogel exposed to UV light significantly promoted re-epithelialization, the remodeling of stromal layers, and exhibited significant anti-scarring effects, with minimal α-SMA and robust ALDH3A1 expression. Normal differentiation of the regenerated epithelia after healing was evaluated by expression of the corneal epithelial biomarker, CK12. The remodeled cornea exhibited full recovery of corneal thickness and layer number without hyperplasia of the epithelium.

1. Introduction

Corneal defects resulting from traumatic injuries can lead to complications such as stromal thinning, corneal perforation, and scarring which ultimately lead to severe visual impairment [1]. While corneal transplantation stands out as the most effective treatment option for visually significant scarring, its success hinges on the availability of graft tissue, surgical expertise, and specialized equipment [2]. A promising alternative gaining attention is the use of *in situ*-forming hydrogels for corneal regeneration where a polymer matrix is formed directly upon a corneal wound. Previously, we have demonstrated the benefits of using bio-orthogonal click chemistry reactions to crosslink hydrogels in situ in order to promote corneal defect regeneration [3,4]. Strain Promoted Azide-Alkyne Cycloaddition (SPAAC), a copper-free form of click chemistry, has been shown to facilitate a supportive matrix environment for nerve and cell regeneration [5], and we have reported on its use in the synthesis of in situ-forming corneal tissue substitutes [6]. The SPAAC reaction is effectively bio-orthogonal and highly biocompatible due to thier high selectivity and lack of cross-reactivity with host tissue cells and biomolecules [7–9], thus making it ideally suited for chemical reactions taking place on a corneal where non-fibrotic and transparent wound healing is the end goal.

In conjunction with the use of hydrogels, the delivery of growth factors has emerged as a valuable strategy to augment re-epithelialization, transparency, and overall corneal regeneration [10,11]. Nerve growth factor (Cenegermin, or Oxervate) was recently

Peer review under responsibility of KeAi Communications Co., Ltd.
* Corresponding author. Ophthalmology, Byers Eye Institute at Stanford University, School of Medicine, Palo Alto, CA, United States.
E-mail address: djmyung@stanford.edu (D. Myung).

https://doi.org/10.1016/j.bioactmat.2024.05.045
Received 27 February 2024; Received in revised form 15 May 2024; Accepted 28 May 2024
Available online 20 June 2024

FDA-approved for the treatment of neurotrophic keratopathy [12]. Meanwhile, other growth factors such as Epidermal Growth Factor (EGF) and Hepatocyte Growth Factor (HGF) have been studied extensively for the purpose of corneal tissue regeneration after injury [13]. Addressing inherent challenges in using growth factors topically, such as the need for repeated administration and rapid washout from the ocular surface, hydrogel networks can serve as effective carriers for these molecules and can improve their therapeutic potential [10]. Two primary fabrication methods, physical mixture and conjugation, are commonly employed. While physically entrapping growth factors within hydrogels offer a straightforward and convenient approach, this typically results in a fast rate of release, limiting its suitability for long-term applications [14]. On the other hand, growth factor conjugation to hydrogel networks is a promising alternative but present the opposite challenge of relatively slow release due to its dependence on matrix degradation [15]. Given that normal corneal re-epithelialization typically proceeds on the order of days [16], an advanced strategy for controlled growth factor release becomes crucial to improve real-time control over dosing and, in turn, the efficiency of corneal regeneration.

Recently, photo-responsive hydrogels have emerged as a promising avenue in the realm of addressing corneal diseases [2,17,18]. These hydrogels utilize ultraviolet (UV) or visible light to induce crosslinking between polymers, thereby enhancing mechanical properties, adhesiveness, and transparency. Furthermore, the use of light is employed to exert control over the release of cargo from nanoparticles and membranes [19,20]. The key advantage of photo-responsive hydrogels lies in the potential for spatio-temporal control over release: the ability to modulate properties precisely at a desired location and time [21]. Importantly, the UV light irradiation (365 nm) conditions utilized in our study are less intense than the typical intensity of natural sunlight (<7 mW/cm^2) [22]. Of note, corneal cross-linking (CXL), an FDA-approved procedure for treating keratoconus, utilizes similar UV light exposure conditions in conjunction with riboflavin-5-phosphate to crosslink corneal collagen [23].

Here, we developed a photoactivated, bio-orthogonally crosslinked hydrogel, termed the PC-HACol hydrogel, designed for the *in situ*-gelation and controlled release of EGF in corneal defects (Fig. 1a). This hydrogel is bio-orthogonally crosslinked through the copper-free click chemistry reaction strain-promoted azide-alkyne cycloaddition (SPAAC) between a collagen-azide (Col-N_3) conjugate and a hyaluronic acid-polyethylene glycol-dibenzocyclooctyne (HA-PEG-DBCO) conjugate. EGF was first grafted to the HA-PEG-DBCO backbone *via* a photocleavable (PC) linker derived from *o*-nitrobenzene. This PC linker, sensitive to mild UV irradiation conditions (2–5 mW/cm^2), facilitates the efficient release of EGF. Each component (*i.e.*, HA-PEG-DBCO and Col-N_3) exists as a solution before being mixed (prior to administration), becoming a solid and transparent hydrogel within minutes upon administration into corneal defects followed by *in situ* gelation. We assessed the physicochemical properties and biodegradability of the PC-HACol hydrogel and evaluated cell proliferation and migration in the presence of the *in situ*-formed PC-HACol hydrogels using primary human corneal epithelial cells. The impact of multiple UV exposures on re-epithelialization were investigated in *ex vivo* rabbit eyes. We then studied *in vivo* stromal regeneration and re-epithelialization through a rat lamellar keratectomy model. The goal of our analysis was to provide proof of concept of the effectiveness and safety of our novel PC-HACol hydrogel for potential applications in corneal wound healing.

2. Materials and methods

2.1. Materials

Hyaluronic acid-Amine (MW 250 k; DoS of 50 %) was obtained from Creative PEGWorks (Durham, NC, USA). Collagen (TeloCol-10) was purchased from Advanced BioMatrix (Carlsbad, CA, USA). DBCO-PEG-NHS ester (10 k), Azido-PEG5-NHS ester (98 %), and PC Azido-PEG3-NHS carbonate ester (95 %) were purchased from BroadPharm (San Diego, CA, USA). Recombinant Human Epidermal Growth Factor Protein (EGF, carrier-free) was purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Fabrication of PC-HACol hydrogel

To fabricate the photo-cleavable and bio-orthogonally crosslinked hydrogel, we first prepared DBCO-PEG-conjugated HA (*i.e.*, HA-PEG-DBCO) and azide-conjugated collagen (*i.e.*, Col-N_3). For the synthesis of HA-PEG-DBCO, DBCO-PEG-NHS ester solution (10 k; 160 μL; 100 mg/mL in DMSO) was mixed with the hyaluronic acid amine solution (HA-NH_2; 20 mg/mL in PBS). The mixture was stirred overnight under protection from light. Then, the solution was dialyzed against water for 48 h. The water was replaced at least two times a day. The dialyzed solution was lyophilized and stored at −20 °C until use. For the synthesis of collagen-N_3 (Col-N_3), collagen solution (9 mg/mL) was neutralized with 10X PBS and 1 N NaOH, and azido-PEG5-NHS ester was diluted ten times with DMSO. The diluted azido-PEG5-NHS ester solution (20.7 μL; 100 mg/mL) was mixed with the neutralized collagen solution (1 mL) and rotated gently at 4 °C overnight under protection from light. The product solution was dialyzed against 1X PBS using Slide-A-Lyzer G2 Dialysis Cassettes (Thermo Fisher Scientific; Waltham, MA, USA) at 4 °C overnight. For the preparation of photocleavable linker-conjugated EGF, PC azido-PEG3-NHS ester (10 mg/mL) was prepared in the mixture of PBS and DMSO (1:1, v/v) under protection from light. The solution (0.5 μL) was added to the EGF solution (5 μL; 500 μg/mL in PBS). The mixture was incubated for 1 h at room temperature under protection from light. To conjugate the photocleavable linker-conjugated EGF to the polymer backbone, the mixture (4.4 μL) was added to the HA-PEG-DBCO solution (70 mg/mL in PBS; 100 μL) for 1 h at room temperature under protection from light. PC-HACol hydrogel was prepared by mixing the solution with Col-N_3 (100 μL; 9 mg/mL). DoS values of DBCO-PEG-HA and Col-N_3 were calculated by measuring the absorbance at 306 nm and 285 nm, respectively. The DoS value is defined as DBCO-or N_3-conjugated amines/total surface primary amines*100.

In this study, we prepared two kinds of control hydrogels: Physical hydrogel and Non-PC hydrogel. Physical hydrogel was prepared by physically mixing native collagen (*i.e.*, unmodified collagen), hyaluronic acid amine, and EGF. In this hydrogel, EGF was physically dispersed in hydrogel networks. Non-PC hydrogel was prepared by using azido-PEG5-NHS ester instead of PC Azido-PEG3-NHS carbonate. The fabrication processes were the same with PC-HACol hydrogel.

2.3. Characterization

To investigate the rheological properties of PC-HACol hydrogel at different fabrication conditions, we prepared four kinds of HA-PEG-DBCO solution (concentration of 10, 30, 50, and 70 mg/mL) and four kinds of Col-N_3 solution (concentration of 1, 3, 6, and 9 mg/mL). The storage and loss modulus of each condition were measured by rheometer (ARES-G2; TA instrument, New Castle, DE, USA) at varying frequencies ranging from 0.1 to 10 at the fixed oscillation strain of 1 at 37 °C. Changes of storage modulus and tan δ values over time were measured for 900 s at oscillation strain of 1 % at 37 °C. Tan δ value was defined as the ratio of loss modulus (G″)/storage modulus (G'). Digital image of PC-HACol hydrogel was taken after gelation on the background with letters. Transmittance of PC-HACol hydrogel in visible light spectrum was measured in the range from 400 nm to 800 nm. The percentage of transmittance was calculated based on the values of PBS. SEM images (Thermo Fisher Scientific Apreo S LoVac Scanning Electron Microscope) were obtained to observe the surface morphology of lyophilized hydrogel. The samples were prepared by lyophilizing the hydrogel mixture immediately after mixed and after incubated for 30 min. Biodegradability of PC-HACol hydrogel was investigated under bio-simulating conditions. The hydrogels were pre-incubated in PBS for

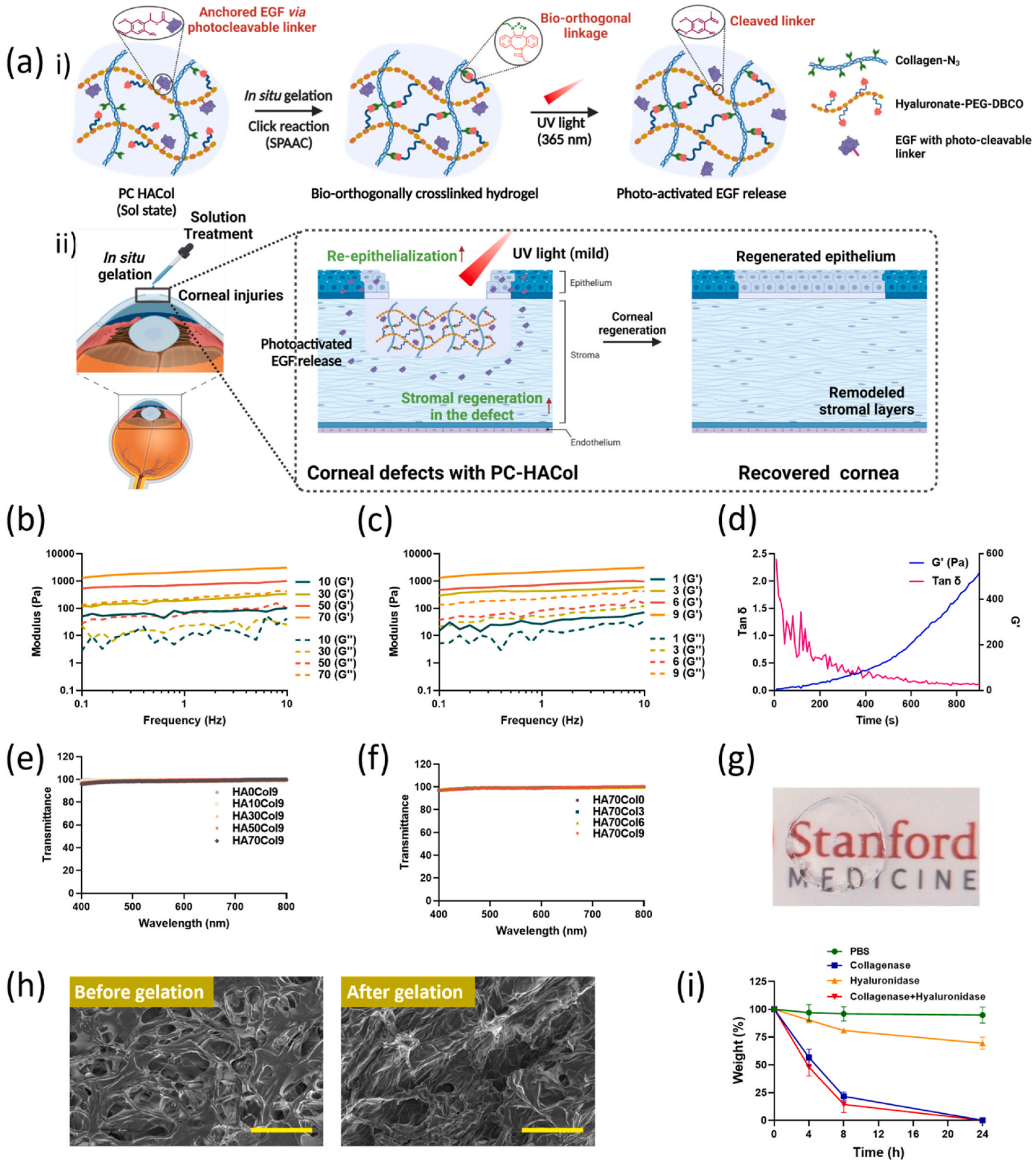

Fig. 1. Characterization of PC-HACol hydrogel. (a) Schematic illustration of PC-HACol hydrogel treatment. i) The gelation and photoactivated release of PC-HACol hydrogel. ii) The application of hydrogel pre-solution to corneal defects. iii) Wound healing processes by PC-HACol hydrogel. (b,c) Storage (G′) and loss (G″) modulus of PC-HACol hydrogel with varying b) HA-PEG-DBCO concentration and c) Col-N_3 concentration. Col-N_3 concentration in b) was fixed as 9 mg/mL, and HA-PEG-DBCO concentration in c) was fixed as 70 mg/mL. Concentrations are expressed as mg/mL. (d) The changes in Tan δ and G′ values of the PC-HACol hydrogel over time at 37 °C after mixing. (e,f) Transmittance of PC-HACol hydrogel with varying e) HA-PEG-DBCO concentration and f) Col-N_3 concentration. (g) Digital image of PC-HACol hydrogel over the background plastic with letters. (h) SEM images of PC-HACol lyophilized before and after gelation for 30 min. Scale bar: 100 μm. (i) Enzymatic biodegradation profiles of PC-HACol hydrogel in collagenase solution (≥2 CDU/mL) or hyaluronidase solution (5 U/mL) or the mixture of collagenase (≥2 CDU/mL) and hyaluronidase (5 U/mL).

one day. The pre-incubated hydrogels were weighed (W_1) and submerged in the 5 units/mL of hyaluronidase (Sigma-Aldrich) solution or 5 ≥ CDU/mL collagenase (Sigma-Aldrich) solution or the mixture of 5 units/mL of hyaluronidase and 5 ≥ CDU/mL collagenase. The hydrogels were incubated at 37 °C. As pre-determined timepoints (2, 6, 24, and 48 h), the weight of hydrogels (W_n) was measured after removing enzyme solution. The percentage weight (%) of the hydrogels at those points was calculated as $W_n/W_1 \times 100$.

2.4. In vitro EGF release

EGF release kinetics from the fabricated hydrogel was investigated *in vitro*. PC-HACol was inserted into the dialysis bag (MWCO: 12–14 kDa), and the bag was submerged in 1% BSA solution. UV light (365 nm) was irradiated at 2 mW/cm^2 for 10 min at 12 and 60 h. At 36 and 84 h, the strength of UV light was increased (5 mW/cm^2 for 10 min). The release medium (20 μL) was collected to analyze the amount of released EGF. At pre-determined timepoints (2, 12, 24, 36, 48, 60, 72, 84, and 96 h). For the Physical hydrogel, the release medium was collected at 2, 4, 8, 12, 24, 48, 72, and 96 h. The release medium was replenished with fresh medium after collection. The collected medium was analyzed using EGF ELISA kit (Abcam, Cambridge, UK). In addition to the release kinetics, the effects of irradiation time and strength were assessed. For the effects of irradiation time, PC-HACol hydrogels were irradiated for 0, 1, 5, or 10 min at 2 mW/cm^2. For the effects of irradiation strength, PC-HACol hydrogels were irradiated at 0, 1, 2, or 5 mW/cm^2 for 10 min.

2.5. Cell culture

Corneal epithelial cells (CECs; ATCC; CRL-11135) and corneal stromal stem cells (CSSCs; harvested from human donor corneas provided by Lions Eye Institute) were cultured to assess cell proliferation, migration, and cytocompatibility. CECs were cultured in keratinocyte-serum free medium supplemented with hydrocortisone (500 ng/mL), epidermal growth factor (5 ng/mL), bovine pituitary extract (0.05 mg/mL), and insulin (5 μg/mL). CSSCs were cultured in Minimum Essential Medium Eagle (Sigma-Aldrich) containing 10% fetal bovine serum, 1% antibiotic antimycotic solution (Sigma-Aldrich), 1% nonessential amino acid solution (Sigma-Aldrich) and 1% Glutamax (ThermoFisher Scientific). Cells were incubated at 37 °C in a 5% CO_2 atmosphere, and the medium was refreshed every other day. The proliferated cells were subcultured at 80% confluency using Trypsin-EDTA solution.

2.6. Cell proliferation

CECs (1×10^4 cells) were cultured on 96-well culture plates one day before treatment. Then, PC-HACol hydrogel was added to the cells at four different concentrations of PC-HACol (0, 5, 10, and 50 mg/mL). All the treated cells were incubated for two days, and their cell number was calculated using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, MD, USA). After incubation with CCK-8 solution for 2 h, the absorbance (450 nm) of the supernatant was analyzed with the microplate reader. The cell viability (%) values were calculated as (absorbance at each concentration/absorbance at 0 mg/mL) × 100.

2.7. Cell migration

The UV-sterilized parafilm piece (1.6 mm × 5.0 mm) was attached to the ground of cell culture dishes (100 mm) to mimic the artificial scratches. These dishes were sterilized using 70 % ethanol and ultraviolet UV light irradiation. CECs (3×10^5 cells) were seeded on the dishes and incubated until the cell population reached a confluency of more than 90 %. Then, the parafilm was removed from the ground to make the scratches, and PC-HACol hydrogel (60 μL) was added alongside the wounds. The dishes were irradiated by UV light (5 mW/cm^2, 10 min). In this study, PC-HACol hydrogel without UV light was employed to compare the effect of UV light. The cell migration in the wounds was monitored using an optical microscope at 0, 6, 24, and 48 h. The wounded area was measured using ImageJ software.

2.8. Cytocompatibility

The cytocompatibility of PC-HACol hydrogel was evaluated by analyzing cell proliferation on the PC-HACol hydrogel-coated ground. PC-HACol hydrogel was thinly spread on the culture dish (100 mm), and CECs (3.5×10^5 cells) were seeded on dish. The cells were incubated for one day, and the dish was irradiated by UV light (2 mW/cm^2 for 5 min). The cells were incubated one more day, and live and dead cells were monitored using the Live/Dead Viability/Cytotoxicity Kit (Invitrogen, MA, USA). The cell populations of each image were measured using ImageJ software. To visualize the cells on the hydrogel, PC-HACol hydrogel was labeled with 6-FAM NHS ester (Lumiprobe, Maryland, USA). The 6-FAM-labeled PC-HACol hydrogel-coated dishes were prepared in the same way, and the cells were cultured for two days. The images were taken using confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.9. Cell migration in 3D cell culture model

Stromal cell migration into PC-HACol hydrogel was evaluated in a 3D cell culture model. CSSCs were mixed with collagen solution at a volume of approximately 3–5 %, and the suspension was incubated on the half area of glass bottom dish (35 mm) for 30 min for gelation. Then, cell culture medium was added on the top of the gel and incubated overnight. The medium was removed from the dish and half of the dish was filled with pre-hydrogel solution of PC-HACol hydrogel. The dish was incubated for 30 min for gelation of PC-HACol hydrogel. Cell culture media was added to the hybrid hydrogel matrix cell culture system, and the dish was incubated for 7 days. The cell culture media was replaced every two days. On day 7, calcein-AM solution (Invitrogen, MA, USA) was added to the medium as a final concentration of 2 μM to label the live cells. Cells proliferated in the hydrogel matrix were visualized using confocal microscope.

2.10. Ex vivo re-epithelialization of rabbit eye

Re-epithelialization of defected corneas was investigated using rabbit eyes. *Ex vivo* rabbit eye culture model was previously established in our research group [24]. Briefly, anterior lamellar keratectomy (ALK) with a 3.5 mm trephine (Robbins Instruments, CA, USA) was performed on rabbit corneas. The corneas were cut off from the eyeballs leaving a 5-mm thick sclera, and lens and iris were removed from the corneas. The prepared corneas were washed two times with 1% penicillin-streptomycin (PS) solution and mounted on agar plugs. The agar plugs were prepared by microwaving agar powder (2 g) in organ culture medium (75 mL) containing DMEM/F12 (Gibco, MT, USA), insulin-transferrin-selenium (0.05%), and PS (1%). The agar suspension was solidified in PDMS mold for 30 min, and the prepared rabbit corneas were placed on the agar plugs to maintain the shapes of corneas. After mounting the corneas, the culture medium was added until it met the sclera. After that, PC-HACol hydrogels were applied to the defects (3–5 μL) to fill out the defects. After gelation, the hydrogels were irradiated by UV light (2 mW/cm^2 for 5 min). In this study, PC-HACol hydrogels were irradiated with three different conditions: no UV light, UV light one time on day 0, and UV light three times on day 0, 1, and 2. To monitor the re-epithelialization, the corneas were stained with fluorescein solution (1.5 %) and washed with balanced salt solution (BSS, Alcon, Geneva, Switzerland). The fluorescein-stained photos were obtained under blue light irradiation. The fluorescein-stained area (%) was calculated using imageJ software.

2.11. Animals

Brown rats (Rattus norvegicus; 150–200 g) were used in this study. Animal experiments were designed to conform with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were reviewed and approved by the Stanford University Institutional Animal Care and Use Committee (protocol #: APLAC-32765). For anesthesia, the mixture of ketamine, xylazine, and water (2:1:3, v/v/v) was injected into muscle (intramuscular injection) as a dose of 1.68 mL/kg. For the follow-up studies, rats were injected with half-dose of the mixture, and low flow of isoflurane gas was used.

2.12. In vivo corneal regeneration

For all surgeries and follow-up studies, proparacaine hydrochloride ophthalmic solution (0.5 %; Bausch and Lomb, Laval, Canada) was dropped into the eyes prior to examination. To establish corneal defect model, ALK was performed on one eye of each rat using a 2.0-mm trephine to create a deep circular cut (40–60 % depth of corneas) and a spatula to eliminate the collagen layers of stroma. PC-HACol hydrogel (2–3 μL) was treated into the defects. After gelation, the treated hydrogel was irradiated by UV light (2 mW/cm^2, 10 min). As a post-operative cares, ofloxacin ophthalmic solution (0.3 %, Bausch and Lomb) was added to the eyes to prevent bacterial infections. The defects after application of PC-HACol hydrogel were examined by optical coherence tomography (OCT; Heidelberg Engineering, Heidelberg, Germany) images and digital photographs. To assess the wound closure of the defected areas, fluorescein solution (1.5 %) was dropped into the defects and washed gently with the BSS. Fluorescein-stained areas were visualized under blue light irradiation. The percentage of fluorescein-stained area was calculated using ImageJ software. The eyes were examined on day 0, 1, 3, 5, and 7, and the eyes were dissected and cryo-sectioned for immunostaining on day 7. The cornea slices were stained with DAPI, phalloidin, anti-CK12 (Abcam) and anti-α-smooth muscle actin (anti-α-SMA; Abcam). The thickness of the epithelium and whole cornea on day 7 was measured based on confocal microscopic images of the sliced sections and OCT, respectively.

2.13. Immunohistochemical analysis

The biomarkers were analyzed at the regenerated corneas. The treated corneas were sliced and stained with various epithelial and stromal markers. The slices were stained with DAPI (1:1000, Thermo Fisher Scientific), Alexa fluor 488-conjugated anti-cytokeratin 12 (anti-CK12; 1:100, Abcam), anti-α-smooth muscle actin (α-SMA; 1:500, Abcam), anti-ALDH3A1 (anti-ALDH3A1; 1:500, Abcam), and Alexa fluor 555-conjugated anti-phalloidin (1:400, Thermo Fisher Scientific). Subsequently, the anti-α-SMA and anti-ALDH3A1 were captured with Alexa fluor 488-conjugated anti-mouse IgG (1:200, life technologies corporation, CA, USA) and Alexa fluor 555-conjugated anti-rabbit IgG (1:200, life technologies corporation), respectively. The fluorescent signals were detected with the confocal microscope (Zeiss, Jena, Germany).

2.14. Statistical analyses

All the statistical analyses were carried out using Graphpad Prism software (version 8.0.2.263). We employed one-way ANOVA followed by Tukey's post-hoc analysis for multiple comparisons. For pairwise comparisons, the significance was determined using a two-tailed unpaired *t*-test. The significance threshold between groups was set at $p < 0.05$; otherwise, labeled as "not significant (n.s.)".

3. Results and discussion

3.1. Synthesis and fabrication of PC-HACol hydrogel

For the photoactivated release of EGF from the PC-HACol hydrogel, EGF was conjugated with photo-cleavable (PC) linkers *via* primary amine (-NH_2)/N-hydroxysuccinimide (NHS) reactions. On one end of the linker was an NHS ester, allowing it to react with lysine (K) residues on human recombinant EGF. To form HA-azide, hyaluronic acid amine (HA-NH_2; degree of substitution (DoS): 50 %) was reacted with DBCO-PEG-NHS ester (10 k) to form HA-PEG-DBCO. We used PEG as a solubilizing linker to offset the increased hydrophobicity induced by DBCO conjugation. As the counter part of HA-PEG-DBCO, azide-functionalized collagen (Col-N_3) was synthesized *via* primary amine/NHS reactions. DoS values for HA-PEG-DBCO and Col-N_3 were calculated as 3.36 ± 0.25 % ($n = 3$) and 58.5 ± 13.7 % ($n = 3$), respectively. The PC linker-conjugated EGFs were conjugated to HA-PEG-DBCO through SPAAC (*i.e.*, DBCO/N_3 reaction), followed by mixed with Col-N_3. The HA-PEG-DBCO reacted with Col-N_3 through SPAAC reactions, and gelation of the mixture occurred within 5 min at 37 °C (Fig. S1).

3.2. Fabrication and characterization of PC-HACol hydrogel

Hydrogels for corneal wound healing require robust mechanical strength to maintain their structures until new stroma is regenerated. Specifically, based on our previous studies, bio-orthogonally crosslinked hydrogels for ocular administration should have a storage modulus of over 1000 Pa to maintain their structural integrity during the wound healing processes, with their modulus depending on the concentration of both azide- and DBCO-functionalized polymers [3,4]. Thus, we prepared four different concentrations of HA-PEG-DBCO (10, 30, 50, and 70 mg/mL) and Col-N_3 (1, 3, 6, and 9 mg/mL) (Fig. 1b and c). In all conditions, EGF was conjugated to HA-PEG-DBCO through the PC linker, and the HA-PEG-DBCO and Col-N_3 were mixed at equal volumes (1:1, v/v). The storage modulus of the hydrogels increased with higher concentration of HA-PEG-DBCO and Col-N_3. Notably, the hydrogel reached a storage modulus of well over 1000 Pa (~3100 Pa) when the concentrations of HA-PEG-DBCO and Col-N_3 were 70 mg/mL and 9 mg/mL, respectively. Therefore, the final hydrogel (PC-HACol hydrogel) components were determined as 70 mg/mL of HA-PEG-DBCO (with EGF conjugation) and 9 mg/mL of Col-N_3. The gelation of PC-HACol was investigated by measuring storage modulus and Tan δ values over time (Fig. 1d). The storage modulus values of the mixture continuously increased after mixing, while the Tan δ values decreased over time. Thus, the mixture of the component solutions gel in a time-dependent manner, with gelation attributed to triazole bonds formed between the DBCO and azide groups (SPAAC reaction).

A desirable characteristic of hydrogels for ocular applications is transparency, in order to facilitate clear vision. To investigate the transparency of the hydrogels composed of HA-PEG-DBCO and Col-N_3, we measured their transmittance of visible lights spanning from 400 nm to 800 nm (Fig. 1e and f). All the hydrogels exhibited high transmittance, exceeding 90 % throughout the range. Thus, the hydrogels maintained their transparency after gelation. We further examined the transparency of PC-HACol hydrogel by placing it over lettering on a plastic card (Fig. 1g). The background letters were clear and legible through the material, confirming the excellent transparency of the PC-HACol hydrogel.

In addition to its appearance, we interrogated the microstructure of the lyophilized PC-HACol hydrogel using scanning electron microscopy (SEM) (Fig. 1h). The hydrogels were lyophilized at two different times: immediately after mixing (*i.e.*, before gelation) and 30 min after mixing (*i.e.*, after gelation). In the 'before gelation' image, we observed an interconnected structure with numerous and relatively large pores throughout the material. Meanwhile, the porosity (size and number) of the matrix was reduced after gelation, due to the enhanced crosslinking

density resulting from the chemical reactions.

Hydrogels for corneal defects should be degradable for de novo stromal regeneration after re-epithelialization. As expected, the PC-HACol hydrogel exhibited both collagenase and hyaluronidase-dependent degradation patterns, suggesting their biodegradability within corneal defects. The enyzmatic degradation of PC-HACol hydrogel was evaluated in solutions containing 5 units/mL of hyaluronidase, ≥2 CDU/mL of collagenase or a combination of 5 units/mL of hyaluronidase and ≥2 CDU/mL of collagenase (Fig. 1i). The PC-HACol hydrogel was degraded both in collagenase solution and hyaluronidase solution, and their degradation rate was higher in the mixture of collagenase and hyaluronidase. Interestingly, the hydrogel was fully dissolvable in collagenase alone as well, suggesting that the collagen makes a relatively greater contribution to the mechanical integrity of the PC-HACol construct.

3.3. *In vitro* growth factor release

The release kinetics of EGF from PC-HACol hydrogel under UV irradiation was investigated *in vitro* (Fig. 2). In this study, we prepared three kinds of hydrogels: PC-HACol hydrogel, Non-PC-HACol Hydrogel, and Physical hydrogel. In the 'Non-PC-HACol hydrogel', a non-photocleavable linker (Azido-PEG5-NHS ester) was used to conjugate EGF to HA-PEG-DBCO (Fig. 2a). The 'Physical hydrogel' was composed of a physical mixture of native Col, HA-NH_2, and EGF. The Non-PC-HACol and Physical hydrogels were employed as control groups to compare the release patterns. The linker is cleaved when exposed to mild UV irradiation (Fig. 2b and S2) through a series of electron transfer steps, thereby allowing free EGF to be released from the hydrogel networks. Thus, the release rate of EGF depends on both UV irradiation and the structural integrity of the hydrogel network.

Fig. 2c illustrates the release patterns of EGF from the hydrogels. In

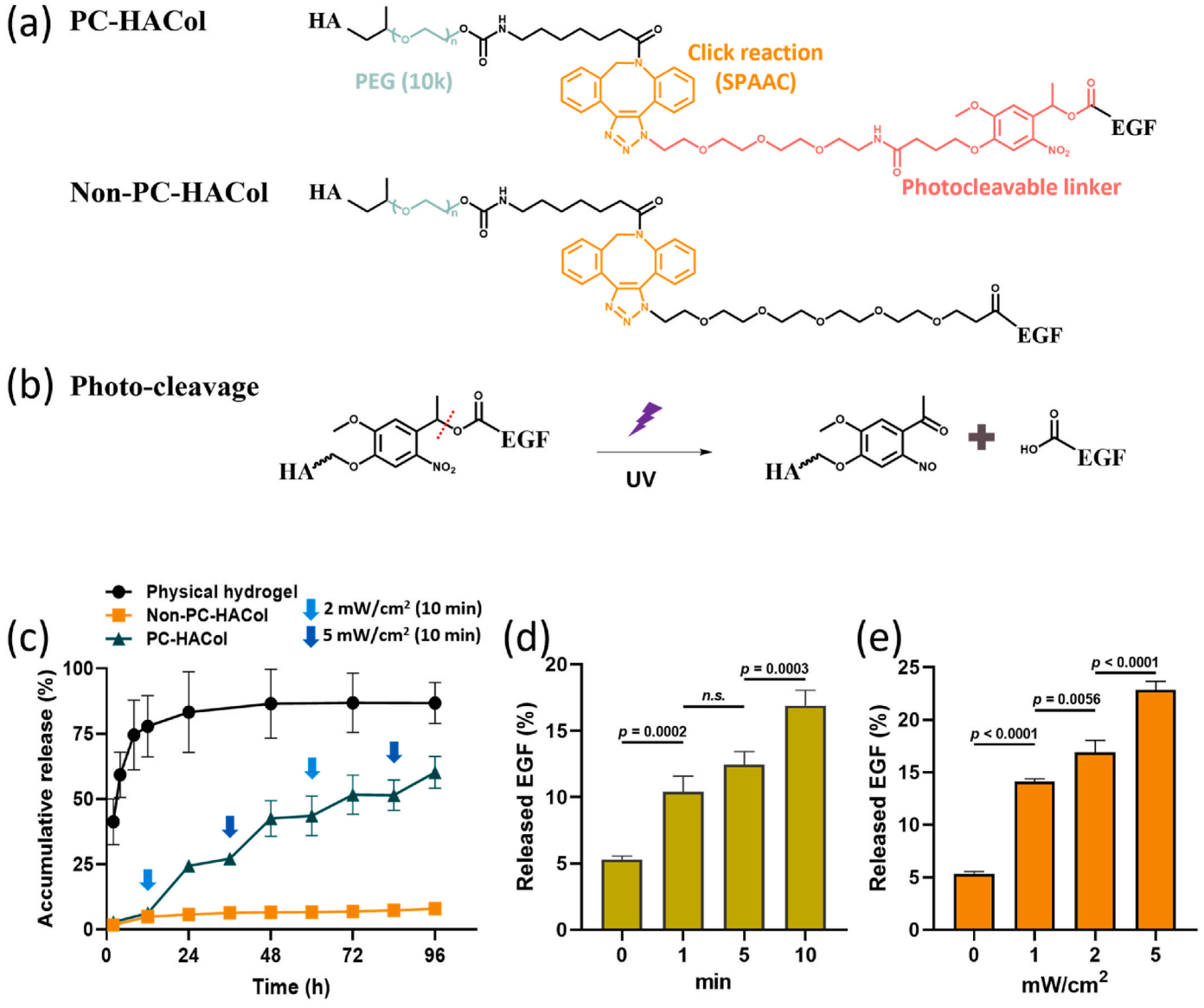

Fig. 2. *In vitro* EGF release from HACol hydrogels. (a) Structures of PC-HACol and Non-PC-HACol hydrogels. In this structure, 'HA' and 'EGF' indicates hyaluronic acid and epidermal growth factor, respectively. (b) Scheme for photo-cleavable release of EGF under mild UV irradiation. (c) *In vitro* EGF release profiles from hydrogels in 1% BSA solution ($n \geq 3$). Physical hydrogels were prepared by physically mixing the native Col, HA, and EGF. PC-HACol and Non-PC-HACol hydrogels were irradiated by UV lights four times with two different conditions. The hydrogels were irradiated at 12 and 60 h with low-strength UV light (2 mW/cm^2 for 10 min) and at 36 and 84 h with stronger UV light (5 mW/cm^2 for 10 min). (d,e) The released amount of EGF with varying d) irradiation time at 2 mW/cm^2 ($n \geq 3$) or e) UV strength for 10 min ($n \geq 3$). The amount of EGF was measured at 12 h after irradiation. The released amount of EGF at 0 min in d) is equal to that at 0 mW/cm^2 in e). n.s.: not significant.

the Physical hydrogel, EGF was released rapidly, exceeding 85 % of the accumulative release within 24 h. In contrast, the Non-PC-HACol hydrogel under UV irradiation exhibited only 5.70 ± 0.57 % release within 24 h and 7.99 ± 1.15 % release within 96 h, indicating a lack of responsiveness to UV irradiation. Notably, the PC-HACol hydrogel exhibited a controlled release pattern of EGF, which was dependent on UV irradiation. The PC-HACol hydrogel exhibited minimal release without irradiation (6.2 ± 1.8 % for 0–12 h; 2.8 ± 1.3 % for 24–36 h; 0.6 ± 0.8 % for 48–60 h; 0.6 ± 0.8 % for 72–84 h). However, it released a significantly larger amount of EGF under UV irradiation (18.2 ± 3.3 % for 12–24 h; 15.3 ± 6.7 % for 36–48 h; 5.9 ± 4.9 % for 60–72 h; 8.2 ± 1.5 % for 84–96 h) (Table S1). As the released amount would depend on the remaining amount in the hydrogels, we calculated the normalized EGF release under two different irradiation conditions (Fig. S3). Interestingly, the released amount of EGF was larger under strong UV irradiation (5 mW/cm^2 for 10 min) compared to weak irradiation (2 mW/cm^2 for 10 min). Thus, PC-HACol hydrogel was likely to exhibit a strength-dependent EGF release pattern.

To further investigate the release patterns according to irradiation conditions, we prepared hydrogels by four different irradiation times (0, 1, 5, and 10 min) at 2 mW/cm^2 (Fig. 2d) and strengths (0, 1, 2, and 5 mW/cm^2) for 10 min (Fig. 2e). Without irradiation, the PC-HACol hydrogel released 5.3 ± 0.2 % of EGF within 12 h, possibly due to unconjugated fractions during synthesis. Of note, PC-HACol hydrogel exhibited a time-dependent EGF release (10.4 ± 1.2 % at 1 min; 12.4 ± 1.0 % at 5 min; 16.9 ± 1.2 % at 10 min) and a strength-dependent EGF release (14.1 ± 0.3 at 1 mW/cm^2; 16.9 ± 1.2 % at 2 mW/cm^2; 22.9 ± 0.8 % at 5 mW/cm^2). These results suggest that EGF release can be controlled by adjusting the irradiation time and strength.

3.4. In vitro cell proliferation and migration

EGF can promote the proliferation and migration of epithelial cells at injury sites [25]. To investigate cell proliferation, primary corneal epithelial cells (CECs) were incubated for one day, followed by treatment with PC-HACol hydrogel (Fig. 3a). We assessed the relative cytotoxicity of our chosen UV light conditions to CECs (Fig. S4). The hydrogels were irradiated with UV light (2 mW/cm^2 for 5 min) after being added to the cells. To evaluate the effects of UV light, one group was prepared by adding PC-HACol hydrogel without irradiation. Cell viability (%) was calculated by comparing the values with untreated cells. Notably, at all concentrations, cell viability was measured to be higher than 100 %, indicating that, at the very least, the UV irradiation conditions being tested were not toxic to the cells. Interestingly, without UV light, PC-HACol hydrogel did not increase the cell viability at 5 and 10 mg/mL, and cell viability at 50 mg/mL was significantly lower than that with UV light. These results suggest that the activities of EGF significantly decreases when conjugated to polymer chains.

In addition to cell viability, we investigated the rate of cell migration when treated with PC-HACol hydrogel (Fig. 3b). CECs were cultured until reaching confluency exceeding 90%, and then a linear scratch was created in the middle to simulate a corneal epithelial wound. In the control group (No treatment group), the scratched area decreased to 29.9 ± 12.2 % within 48 h. Surprisingly, PC-HACol without UV light (PC-HACol group) did not exhibit significant differences (26.4 ± 9.3 % within 48 h), compared to the no treatment group. In contrast, under UV light irradiation, PC-HACol hydrogel group (PC-HACol + UV) exhibited complete wound closure within 48 h. Thus, PC-HACol hydrogel needed to be irradiated by UV light to facilitate cell migration. These results suggest that EGFs need to be released from polymer chains to function effectively.

3.5. Cytocompatibility

The cytocompatibility of the PC-HACol hydrogel was evaluated by observing cell growth on the hydrogel (Fig. 3c and d). PC-HACol hydrogel was thinly spread on the bottom of the cell culture dishes, and CECs were cultured on the hydrogel-coated dishes. In the hydrogel-uncoated dishes (No treatment group), the cells reached a population of 179 ± 28 cells/cm^2, with a dead cell population accounting for 1.4 ± 0.7 %. Similarly, cells grew on the hydrogel, exhibiting a cell population of 185 ± 45 cells/cm^2 with 2.2 ± 0.8 % of dead cells. Thus, the PC-HACol hydrogel did not prohibit cell growth. Interestingly, under UV irradiation, PC-HACol hydrogel promoted cell growth, exhibiting a significantly increased cell population of 252 ± 57 cells/cm^2 with a decreased ratio of dead cells (0.5 ± 0.4 %).

3.6. Cell migration assay in a 3D cell culture model

The migration of corneal stromal cells into PC-HACol hydrogel matrix was evaluated in a 3D cell culture model (Fig. S5). In this study, we established a hybrid cell culture system. We used a hybrid hydrogel matrix, with one half containing corneal stromal stem cells (CSSCs) encapsulated in a collagen matrix (3–5 %, v/v), while the other half consisting of PC-HACol hydrogel without cells. Cell culture medium was added on the top of the hybrid matrix to provide cells with nutrients. After incubation for 7 days, the hybrid matrix appeared to be divided into two distinct sections (Fig. 3e), with a rough surface (section A) and a smooth surface (section B), corresponding to the collagen matrix and PC-HACol matrix, respectively. To visualize the cells in the matrix, we incubated the hybrid matrix with Calcein-AM and observed the live cells using a confocal microscope with z-stack mode at four different angles (Fig. 3f). Dense and plain layers of cells were observed in the collagen matrix (section A), which originated from initially encapsulated CSSCs. Notably, the cells were also observed in the PC-HACol hydrogel matrix (section B) where cells were not initially encapsulated. Thus, the cells observed in section B originated from cells in the collagen matrix, indicating cell migration from the collagen matrix to the PC-HACol matrix. This result suggests that our PC-HACol hydrogel matrix can serve as a bed for stromal regeneration.

3.7. Ex vivo re-epithelialization in rabbit eyes

Re-epithelialization following corneal injuries is an initial and critical process for proper wound healing, serving as the eye's initial protective measure from infection and other deleterious exposures [26]. This process can be promoted by growth factors [27]. Based on the results from *in vitro* cellular studies, we hypothesized that freed EGF after photocleavage from the network would be more effective than covalently-bound EGF, and multiple UV irradiation exposures over a couple of days would sustain the release of EGF, facilitating would healing processes. To initially demonstrate the wound healing effects of the PC-HACol hydrogel, we used an *ex vivo* 3.5-mm anterior lamellar keratoplasty (ALK) model using rabbit eyes [24]. In this study, PC-HACol hydrogel was applied to the surgically prepared corneal stromal wounds and irradiated with UV light (2 mW/cm^2 for 5 min) either one or three times (Fig. 4). The control group was not subjected to light irradiation. Re-epithelialization in defects was assessed by staining the defects with fluorescein (Fig. 4a). When the hydrogel was not irradiated, the fluorescein staining persisted on the wound for five days, while eyes subjected to one-time UV irradiation (at day 0, after hydrogel administration) tended to exhibit wound closure within 2–4 days (Fig. 4b). Interestingly, PC-HACol hydrogel with three UV irradiation exposure (at day 0, 1, and 2) exhibited significantly faster re-epithelialization than that with only a one-time irradiation. These results suggest that photoactivated release of EGF in discrete doses over time increases the wound healing effects of the PC-HACol hydrogel.

3.8. In vivo wound healing effects in rats

Corneal regeneration by PC-HACol hydrogel was assessed in rats over seven days. A corneal defect model was established using a 2.0-mm

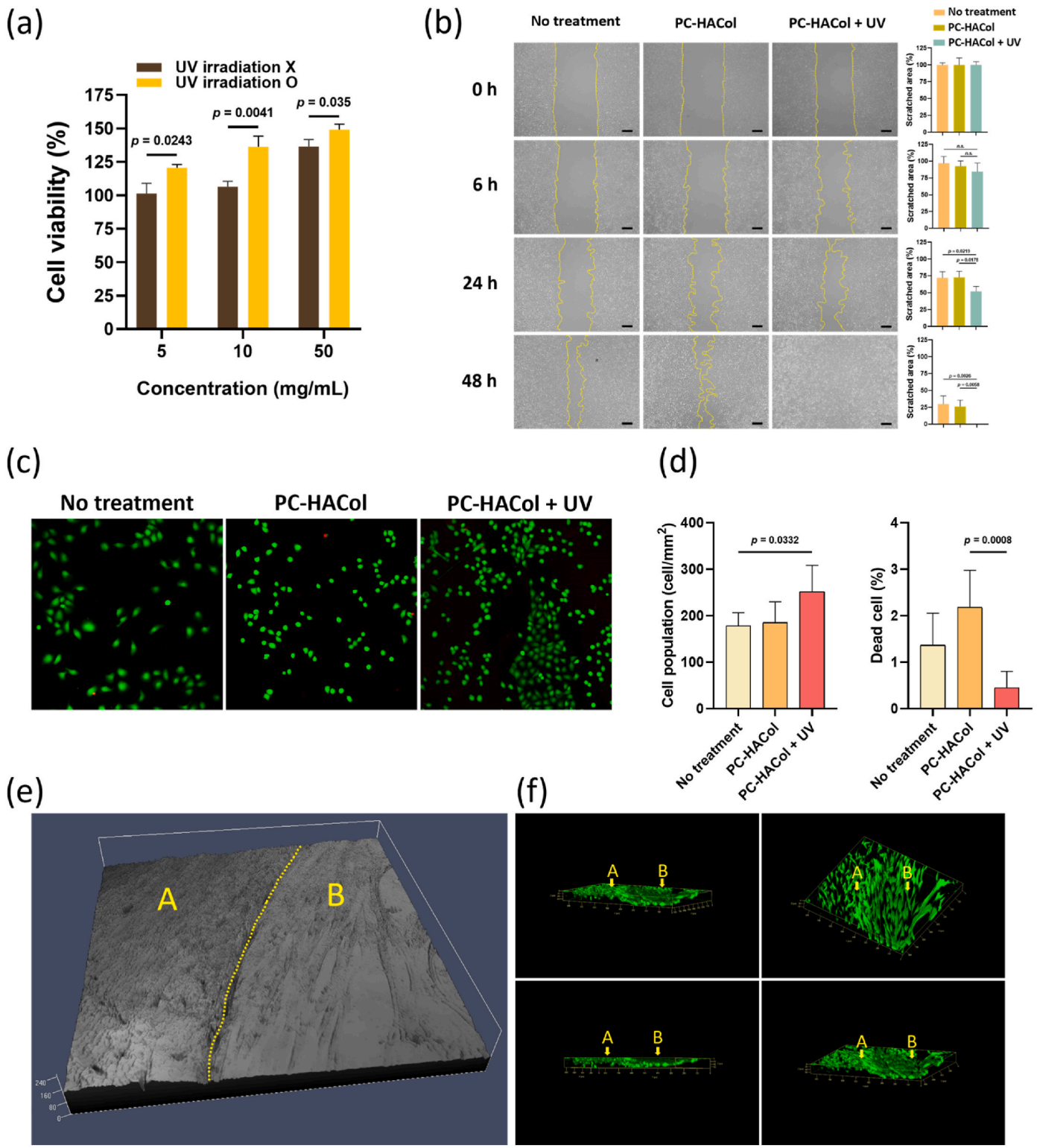

Fig. 3. *In vitro* cell proliferation and cytocompatibility using CECs and CSSCs. (a) Cell proliferation when co-cultured with PC-HACol hydrogel under or without UV irradiation (2 mW/cm^2 for 5 min) ($n = 4$). The cell viability was measured by CCK-8 assay. (b) Scratch assay for 48 h ($n = 4$). CECs were treated with PC-HACol hydrogel, and one group was subjected to UV irradiation (5 mW/cm^2 for 10 min), while the other group received no irradiation. The bar charts indicate the normalized wounded area in square micrometers relative to the area at 0 h. Scale bar: 400 μm. (c) The cytocompatibility of PC-HACol hydrogel against CECs. CECs were seeded and cultured on the PC-HACol hydrogel-coated cell culture dishes for 48 h. After incubation, live cells were stained with Calcein-AM (green color) and dead cells were stained with ethidium homodimer-1 (red color). Scale bar: 100 μm. (d) Bar charts depicting the populations of live and dead cells from c) ($n = 6$). e) Surface bright field image of 3D cell culture model. Yellow capital A and B indicate the stromal cell-encapsulated collagen hydrogel section and PC-HACol hydrogel section, respectively. f) 3D live cell images of e) with four different angles. The cells were stained with Calcein-AM (green). The images were obtained using confocal microscope with z-stack mode.

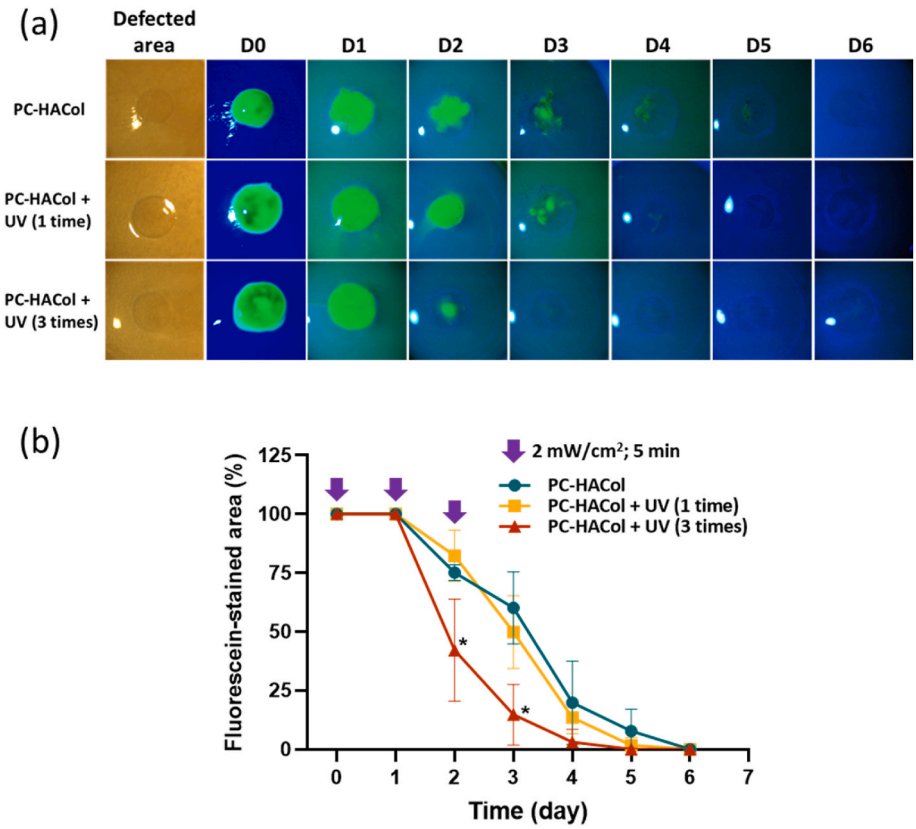

Fig. 4. *Ex vivo* re-epithelialization in rabbit eyes ($n = 3$). (a) Representative slit lamp (defected area) and fluorescein-stained images (Day 0 – Day 6) of corneal defects. PC-HACol hydrogel was applied to the wound and irradiated by UV light (2 mW/cm^2 for 5 min). UV light was applied either once on Day 0 or three times on Day 0, 1, and 2, using the same irradiation conditions. PC-HACol hydrogel without UV irradiation group was used as a control group. (b) Fluorescein-stained areas were measured for each group. *$p < 0.05$, compared to other groups.

trephine during ALK. In this study, to minimize the exposure to anesthesia for UV irradiation and to comply with relevant animal ethics, we irradiated UV light once, but for a longer duration (10 min) compared to the *ex vivo* study. Additionally, we opted for a rat model instead of a rabbit model due to its faster corneal repair processes, potentially leading to significant differences with just one round of irradiation [28]. The negligible damage to the cornea caused by the UV irradiation was verified in rats (Fig. S6). PC-HACol hydrogels were applied to the corneal defects, either with UV irradiated (2 mW/cm^2 for 10 min) or without. The PBS-treated group served as a control group. In Fig. 5a, optical coherence tomography (OCT) images of cornea revealed that, at day 0, corneal defects of 40–60 % depth were created, and the applied hydrogels were observed in the defects (marked by orange arrows). One day post-surgery, PBS-treated eyes showed no noticeable stromal regeneration, while PC-HACol hydrogel group exhibited greater corneal regeneration. Under UV irradiation, PC-HACol hydrogel group exhibited faster and more extensive regeneration than other groups. The differences in corneal regeneration in PC-HACol hydrogel groups (*i.e.*, PC-HACol and PC-HACol + UV) were more evident in the following examination, compared to the PBS group. In addition to OCT images, we monitored the appearance of the wounded eyes using slit lamp images (Fig. 5b and S7). All PBS-treated rats exhibited clinically apparent central corneal opacities. Meanwhile, the PC-HACol group also showed a degree of fibrotic healing for the first three days, but this scarring significantly diminished by day 5 and 7. With UV irradiation leading to photo-cleavage and release of EGF, PC-HACol-treated corneas exhibited markedly improved transparency.

Re-epithelialization during healing was investigated by staining the defects treated with fluorescein (Fig. 5c and d). In the PBS group, the corneal defects persisted for longer than those in the PC-HACol hydrogel group, and the fluorescein-stained area was significantly smaller in PC-HACol hydrogel group. These results were consistent with our previous reports on bio-engineered hydrogels for improving corneal regeneration [3,4,29]. Thus, the application of the PC-HACol hydrogel promoted the wound healing processes even without UV irradiation. Notably, under UV irradiation, the PC-HACol hydrogel significantly accelerated re-epithelialization, achieving complete re-epithelialization in half of the rats within one day. The other half exhibited a fluorescein staining area of less than 5 %. These results suggest that EGF released from PC-HACol hydrogel promotes the proliferation and migration of epithelial cells, resulting in faster re-epithelialization.

To further analyze the materials' effects on corneal regeneration, we measured the thickness of the cornea and epithelium (Fig. 5e and S8). At the time of surgery on day 0, approximately 50 % of the corneas were removed in all groups (Fig. 5e) through an anterior keratectomy. In the PBS group, the corneal thickness increased slowly and continuously for five days (52.5 ± 3.4 % on day 1; 48.2 ± 8.8 % on day 3; 58.7 ± 10.2 % on day 5) and dramatically increased from day 5–7 (76.3 ± 4.5 % on day 7). In contrast, the PC-HACol group exhibited markedly thicker corneal layers starting from day 1 (72.7 ± 8.6 %), and the same layers were significantly thicker in the PC-HACol + UV group (91.3 ± 12.9 %). The PC-HACol + UV group showed the highest corneal thickness (closest to native corneas) over the evaluation period, regenerating to a thickness of 101.6 ± 3.1 % by day 7. Thus, the PC-HACol hydrogel exposed to UV light exhibited significantly greater pro-regenerative effects both in terms of surface epithelialization and stromal regeneration. In addition to the cornea, we investigated the thickness of the epithelial layers because hyperplasia of the epithelium is a commonly observed abnormality during the corneal healing process particularly in the case of deeper wounds (Fig. S8) [30,31]. As expected, PBS-treated corneas

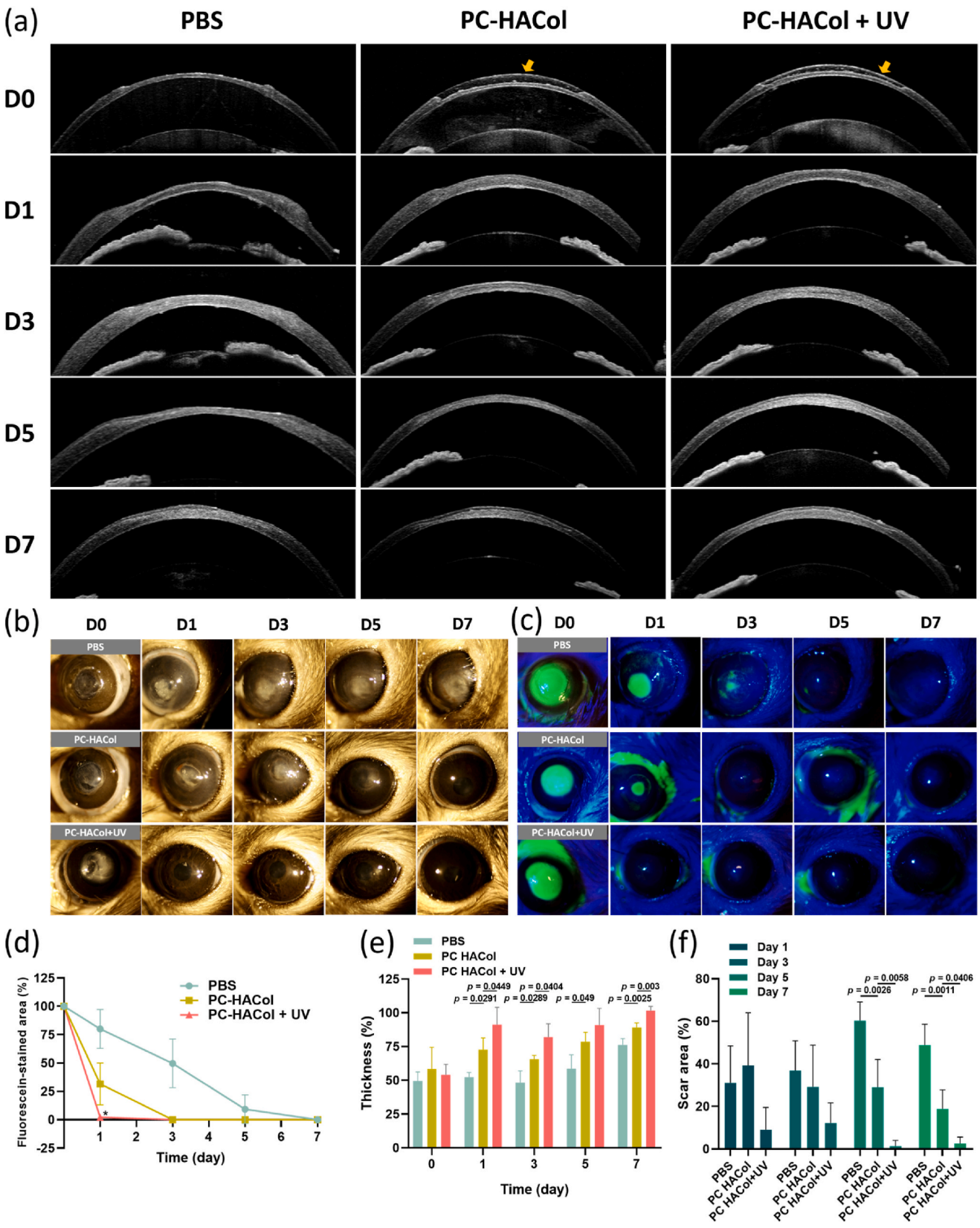

Fig. 5. *In vivo* corneal wound healing in rats ($n = 4$). (a) OCT images of PC-HACol hydrogel-treated corneas for 7 days. The images were taken on days 0, 1, 3, 5, and 7. The orange arrows indicate the applied PC-HACol hydrogels on the defects. (b) Slit lamp images of defected eyes. (c) Fluorescein-stained images of the defects. The green colors in the center of eyes indicate the stained area. (d) Fluorescein-stained area calculated from c). (e) Average thickness of the cornea in defects. The thickness was normalized by dividing it by the thickness of the normal cornea. (f) Scar area on the eyes over the evaluation period. n.s.: not significant.

exhibited a 1.53-fold thicker epithelium (52.0 ± 7.1 μm), compared to normal corneas (34.0 ± 1.6 μm). Surprisingly, this phenomenon was not observed in the PC-HACol (33.3 ± 4.0 μm) and the PC-HACol + UV (33.0 ± 1.4 μm) groups. Therefore, these results suggest that the PC-HACol hydrogel promotes a return to baseline corneal epithelial thickness after injury.

Corneal opacities were quantified using slit lamp images (Fig. 5f) as a function of % area scarring relative to the regenerated corneal surface area. All the PBS-treated eyes exhibited significant scars on the defects over the evaluation period (31.0 ± 17.4 % on day 0; 36.9 ± 14.0 % on day 3; 60.3 ± 8.8 % on day 5; and 48.9 ± 9.8 % on day 7), suggesting severe vision impairment. Surprisingly, the PC-HACol hydrogel group exhibited clinically observable scarring for three days (39.3 ± 24.7 % on day 1; 29.2 ± 19.6 % on day 3) and the scars markedly decreased from three to seven days, much improved optical clarity by day 7 (28.9 ± 13.2 % on day 5; 18.8 ± 9.0 % on day 7). Importantly, scars were rarely observed in the UV-exposed PC-HACol group over the evaluation period (9.0 ± 10.5 % on day 1; 12.3 ± 9.4 % on day 3; 1.4 ± 2.7 % on day 5; 2.6 ± 3.0 % on day 7), with a significantly smaller scar area than those in other groups. Thus, the PC-HACol hydrogel mitigated scarring during the healing processes, and UV irradiation-induced photocleavage of the EGF within the network further fortified the anti-scarring effects of the hydrogel.

3.9. Immunohistochemical analysis of treated corneal defects

The regenerated corneas on day 7 were further investigated through immunohistochemical analysis to visualize the biomarkers (Fig. 6). Fig. 6a–d depict the expression of alpha-smooth muscle actin (α-SMA)—a marker of fibrotic healing— in the regenerated stromal layers. During corneal regeneration, activated keratocytes can differentiate into α-SMA-expressing myofibroblasts, potentially leading to mild-to-severe

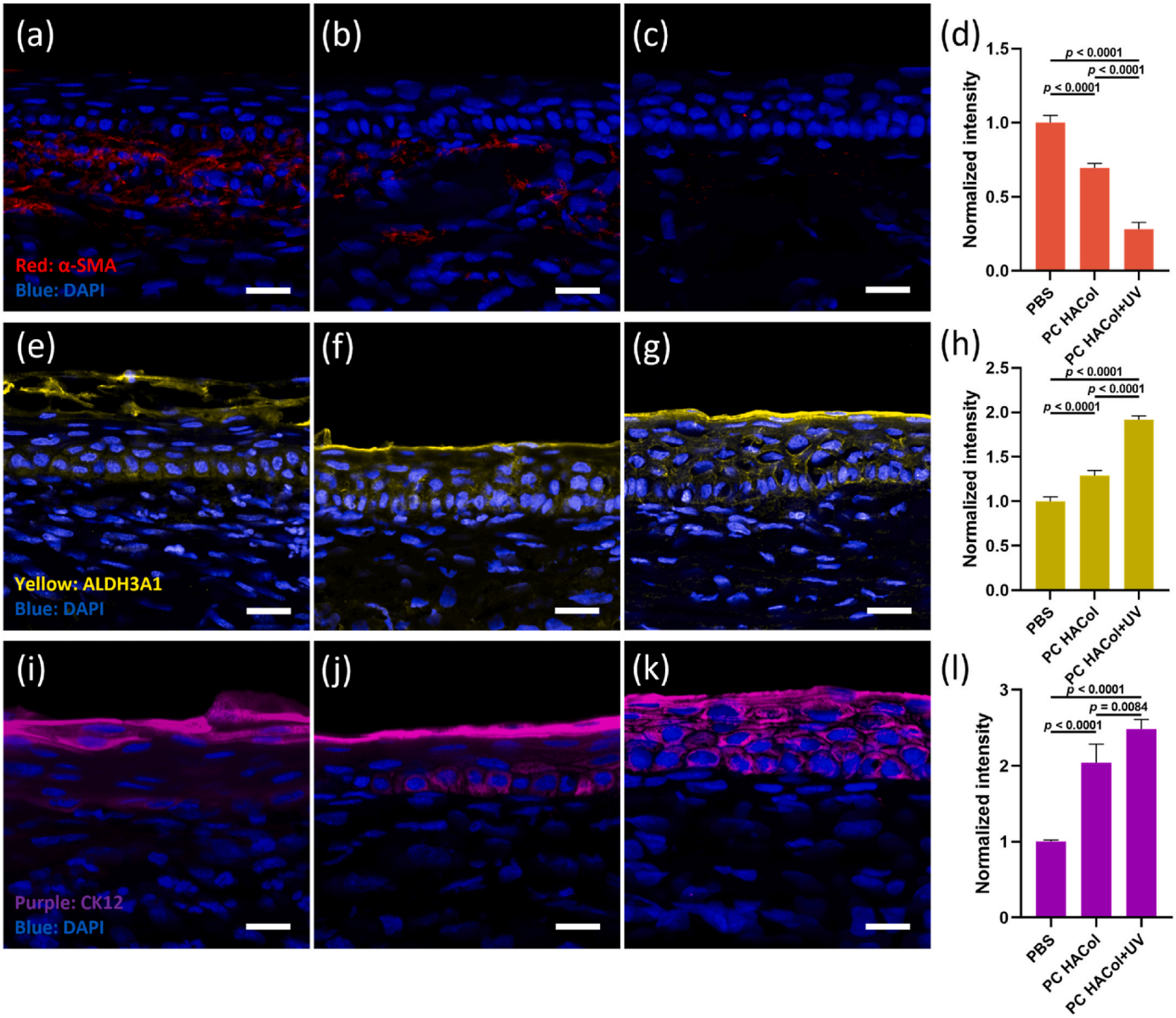

Fig. 6. Immunohistochemical analyses of corneas harvested on day 7. (a–c) α-SMA expression patterns of a) PBS group, b) PC-HACol group, and c) PC-HACol + UV group. (d) Quantification of α-SMA intensities normalized by the average value of PBS group ($n = 4$). (e–g) ALDH3A1 expression of e) PBS group f) PC-HACol group, g) PC-HACol + UV group. (h) Quantification of ALDH3A1 intensities normalized by the average value of PBS group ($n = 4$). (i–k) CK12 expression of i) PBS group, j) PC-HACol group, and k) PC-HACol + UV group. (l) Quantification of CK12 intensities normalized by the average value of PBS group ($n = 4$). The length of the scale bar is 20 μm.

scarring [32]. Importantly, PC-HACol hydrogel exhibited a markedly lower expression of α-SMA (Fig. 6b) compared to PBS (Fig. 6a), and the expression was much lower with UV irradiation (Fig. 6c and d). These results suggest a reduction in myofibroblastic activities and fibrosis in the regenerated cornea, consistent with the clear transparency observed in slit lamp images (Fig. 5b). To further investigate corneal transparency, we assessed the expression of aldehyde dehydrogenase 3A1 (ALDH3A1), a corneal crystallin and biomarker of corneal health and transparency (Fig. 6e–h) [33]. The epithelium of the corneas treated with the PC-HACol hydrogel + UV light exposure group expressed ALDH3A1 evenly in all layers (Fig. 6g), whereas the PBS-treated and PC HACol groups exhibited uneven and low expression patterns in epithelial layers (Fig. 6e and f). The intensities of ALDH3A1 signals were also significantly higher in PC HACol + UV group than the other groups (Fig. 6h). Together with the aforementioned α-SMA expression patterns, these results could explain the differences in transparency of the corneas between the groups.

Additionally, we examined cytokeratin 12 (CK12) expression patterns in the regenerated epithelium, a cornea-specific cytokeratin and cell differentiation marker in the regenerated epithelium (Fig. 6i - l). The PBS group showed a highly polarized expression pattern on superficial cells of epithelial layers (Fig. 6i). The PC-HACol group also exhibited a heterogeneous expression pattern for CK12 similar to PBS group, but this expression was also observed in the basal layers (Fig. 6j). In contrast, PC-HACol + UV group exhibited a positive and homogeneous expression of CK 12 throughout all epithelial layers, suggesting that PC-HACol hydrogel with UV light resulted in higher and normal epithelial differentiation of the regenerated layers (Fig. 6k and l), compared to other groups [34]. In addition to the epithelial markers, regenerated stromal layers were observed by staining F-actin (Fig. S9). F-actin was highly observed in the stroma close to the epithelial layers, indicating cell-matrix mechanical interactions at the regenerated site [35].

4. Conclusion

We have developed a novel, *in situ*-forming, bio-orthogonally crosslinked hydrogel capable of subsequent photoactivated release of EGF for corneal regeneration. The bio-orthogonally crosslinked hydrogel wassynthesized through SPAAC click chemistry reactions between HA-PEG-DBCO and Col-N_3, achieving rapid gelation within minutes within corneal defects. EGF was conjugated to HA-PEG-DBCO using photocleavable linkers, allowing the hydrogel to release EGF under mild UV conditions. The amount of released EGF depended on UV intensityand irradiation time. Upon UV irradiation, the PC-HACol hydrogel promoted the proliferation and migration of corneal epithelial cells with excellent biocompatibility. The rate of re-epithelialization in corneal defects was also influenced by the frequency of UV irradiation, as shown through *ex vivo* rabbit corneal organ culture studies. In rats *in vivo*, the PC-HACol hydrogel after exposure to UV light promoted significantly faster re-epithelialization without hyperplasia as well as stromal regeneration with return to baseline corneal thickness compared to treatment conditions without UV irradiation and without any gel treatment. Our results indicate that *in situ*-forming, bio-orthogonally crosslinked hydrogels with controllable growth factor release may be a promising and advanced approach for the treatment of corneal defects.

Ethics approval and consent to participate

All animal procedures were performed according to the protocol (APLAC-32765) approved by Stanford University Institutional Animal Care and Use Committee.

Data availability

The data in this work are available in the manuscript or Supplementary Information, or available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Nae-Won Kang: Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Youngyoon Amy Seo:** Visualization, Methodology, Investigation, Formal analysis. **Kevin J. Jackson:** Visualization, Methodology, Investigation, Formal analysis. **Kyeongwoo Jang:** Investigation, Formal analysis, Data curation. **Euisun Song:** Visualization, Validation, Software. **Uiyoung Han:** Visualization, Validation. **Fang Chen:** Methodology, Funding acquisition, Formal analysis. **Sarah C. Heilshorn:** Visualization, Data curation. **David Myung:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

Authors N.-W. K. and D.M. declare a patent application on the material technology described in this study.

Acknowledgements

This work was supported by a departmental core grant from Research to Prevent Blindness (RPB) as well as funding from the National Eye Institute (NIH R01 EY035697, R01 EY033363-03, K99EY034168, and P30 EY026877), Harrington Discovery Institute Scholar-Innovator Program, and the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (RS-2023-00247051). Experiments were also performed in the Stanford Nano Shared Facilities and the Stanford School of Engineering Soft Materials Facility.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2024.05.045.

References

[1] K. Sadtler, A. Singh, M.T. Wolf, X. Wang, D.M. Pardoll, J.H. Elisseeff, Design, Clinical translation and immunological response of biomaterials in regenerative medicine, Nat. Rev. Mater. 1 (2016) 16040.
[2] X. Shen, S. Li, X. Zhao, J. Han, J. Chen, Z. Rao, K. Zhang, D. Quan, J. Yuan, Y. Bai, Dual-crosslinked regenerative hydrogel for sutureless long-term repair of corneal defect, Bioact. Mater. 20 (2023) 434.
[3] F. Chen, P. Le, G.M. Fernandes-Cunha, S.C. Heilshorn, D. Myung, Bio-orthogonally crosslinked hyaluronate-collagen hydrogel for suture-free corneal defect repair, Biomaterials 255 (2020) 120176.
[4] H.J. Lee, G.M. Fernandes-Cunha, K.-S. Na, S.M. Hull, D. Myung, Bio-orthogonally crosslinked, in situ forming corneal stromal tissue substitute, Adv. Healthc. Mater. 7 (2018) 1800560.
[5] C.M. Madl, S.C. Heilshorn, Bioorthogonal strategies for engineering extracellular matrices, Adv. Funct. Mater. 28 (2018) 1706046.
[6] M. Rafat, F. Li, P. Fagerholm, N.S. Lagali, M.A. Watsky, R. Munger, T. Matsuura, M. Griffith, PEG-stabilized carbodiimide crosslinked collagen–chitosan hydrogels for corneal tissue engineering, Biomaterials 29 (2008) 3960.
[7] R. Ravichandran, M.M. Islam, E.I. Alarcon, A. Samanta, S. Wang, P. Lundstrom, J. Hilborn, M. Griffith, J. Phopase, Functionalised type-I collagen as a hydrogel building block for bio-orthogonal tissue engineering applications, J. Mater. Chem. B 4 (2016) 318.
[8] J. Rosenquist, M. Folkesson, L. Hoglund, J. Pupkaite, J. Hilborn, A. Samanta, An injectable, shape-retaining collagen hydrogel cross-linked using thiol-maleimide click chemistry for sealing corneal perforations, ACS Appl. Mater. Interfaces 15 (2023) 34407.
[9] E.M. Sletten, C.R. Bertozzi, Bioorthogonal chemistry: fishing for selectivity in a sea of functionality, Angew. Chem. Int. Ed. 48 (2009) 6974.
[10] R.C. Cooper, H. Yang, Hydrogel-based ocular drug delivery systems: emerging fabrication strategies, applications, and bench-to-bedside manufacturing considerations, J. Contr. Release 306 (2019) 29.

[11] J. Imanishi, K. Kamiyama, I. Iguchi, M. Kita, C. Sotozono, S. Kinoshita, Growth factors: importance in wound healing and maintenance of transparency of the cornea, Prog. Retin. Eye Res. 19 (2000) 113.
[12] E.D. Deeks, Y.N. Lamb, Cenegermin: a review in neurotrophic keratitis, Drugs 80 (2020) 489.
[13] H. Miyagi, S.M. Thomasy, P. Russell, C.J. Murphy, The role of hepatocyte growth factor in corneal wound healing, Exp. Eye Res. 166 (2018) 49.
[14] M.S. Kong, W.-G. Koh, H.J. Lee, Controlled release of epidermal growth factor from furfuryl-gelatin hydrogel using in situ visible light-induced crosslinking and its effects on fibroblasts proliferation and migration, Gels 8 (2022) 214.
[15] Y. Lee, S. Lim, J.A. Kim, Y.H. Chun, H.J. Lee, Development of thiol−ene reaction-based HA hydrogel with sustained release of EGF for enhanced skin wound healing, Biomacromolecules 24 (2023) 5342.
[16] A.M.S. Cheng, L. Chua, V. Casas, S.C.G. Tseng, Morselized amniotic membrane tissue for refractory corneal epithelial defects in cicatricial ocular surface diseases, Transl. Vis. Sci. Technol. 5 (2016) 9.
[17] M. Li, R. Wei, C. Liu, H. Fang, W. Yang, Y. Wang, Y. Xian, K. Zhang, Y. He, X. Zhou, A "T.E.S.T." hydrogel bioadhesive assisted by corneal cross-linking for in situ sutureless corneal repair, Bioact. Mater. 25 (2023) 333.
[18] G. Yazdanpanah, X. Shen, T. Nguyen, K.N. Anwar, O. Jeon, Y. Jian, M. Pachenari, Y. Pan, T. Shokuhfar, M.I. Rosenblatt, E. Alsberg, A.R. Djalilian, A light-curable and tunable extracellular matrix hydrogel for in situ suture-free corneal repair, Adv. Funct. Mater. 32 (2022) 2113383.
[19] N. Formina, C. McFearin, M. Sermsakdi, O. Edigin, A. Almutairi, UV and near-IR triggered release from polymeric nanoparticles, J. Am. Chem. Soc. 132 (2010) 940.
[20] B. Guo, R. Fan, S. Shen, Y. Xue, Z. Zhu, R.X. Xu, A photo-responsive membrane for tailored drug delivery with spatially and temporally controlled release, J. Mater. Chem. B 9 (2021) 8615.
[21] I. Tomatsu, K. Peng, A. Kros, Photoresponsive hydrogels for biomedical applications, Adv. Drug Deliv. Rev. 63 (2011) 1257.
[22] A.V. Parisi, D. Igoe, N.J. Downs, J. Turner, A. Amar, M.A.A. Jebar, Satellite monitoring of environmental solar ultraviolet A (UVA) exposure and irradiance: a review of OMI and GOME-2, Rem. Sens. 13 (2021) 752.
[23] M.W. Belin, L. Lim, R.K. Rajpal, F. Hafezi, J.A.P. Gomes, B. Cochener, Corneal cross-linking: current USA status report from the cornea society, Cornea 37 (2018) 1218.
[24] G.M. Fernandes-Cunha, L.G. Brunel, A. Arboleda, A. Manche, Y.A. Seo, C. Logan, F. Chen, S.C. Heilshorn, D. Myung, Collagen gels crosslinked by photoactivation of riboflavin for the repair and regeneration of corneal defects, ACS Appl. Bio Mater. 6 (2023) 1787.
[25] E. Fuchs, Scratching the surface of skin development, Nature 445 (2007) 834.
[26] G.C. Gurtner, S. Werner, Y. Barrandon, M.T. Longaker, Wound repair and regeneration, Nature 453 (2008) 314.
[27] L. Wang, X. Wu, T. Shi, L. Lu, Epidermal growth factor (EGF)-induced corneal epithelial wound healing through nuclear factor κB subtype-regulated CCCTC binding factor (CTCF) activation, J. Biol. Chem. 288 (2013) 24363.
[28] A. Loiseau, G. Raiche-Marcoux, C. Maranda, N. Bertrand, E. Boisselier, Animal models in eye research: focus on corneal pathologies, Int. J. Mol. Sci. 24 (2023) 16661.
[29] Y.H. Chun, S.-K. Park, E.J. Kim, H.J. Lee, H. Kim, W.-G. Koh, G.F. Cunha, D. Myung, K.-S. Na, In vivo biocompatibility evaluation of in situ-forming polyethylene glycol-collagen hydrogels in corneal defects, Sci. Rep. 11 (2021) 23913.
[30] M.V.D.R. Silva, J.A.D. Toledo, A.T. Ruibal, Mechanical removal of epithelial hyperplasia leads to successful treatment of irregular astigmatism, BMC Ophthalmol. 23 (2023) 122.
[31] S.E. Wilson, R.R. Mohan, J.-W. Hong, J.-S. Lee, R. Choi, R.R. Mohan, The wound healing response after laser in situ keratomileusis and photorefractive keratectomy, Arch. Ophthalmol. 119 (2001) 889.
[32] J. Chen, Z. Li, L. Zhang, S. Ou, Y. Wang, X. He, D. Zou, C. Jia, Q. Hu, S. Yang, X. Li, J. Li, J. Wang, H. Sun, Y. Chen, Y.-T. Zhu, S.C.G. Tseng, Z. Liu, W. Li, Descemet's membrane supports corneal endothelial cell regeneration in rabbits, Sci. Rep. 7 (2017) 6983.
[33] Y. Pei, R.Y. Reins, A.M. McDermott, Aldehyde dehydrogenase (ALDH) 3A1 expression by the human keratocyte and its repair phenotypes, Exp. Eye Res. 83 (2006) 1063.
[34] H.M. Reza, B.-Y. Ng, F.L. Gimeno, T.T. Phan, L.P.-K. Ang, Umbilical cord lining stem cells as a novel and promising source for ocular surface regeneration, Stem Cell Rev. and Rep 7 (2011) 935.
[35] P.B. Kivanany, K.C. Grose, M. Tippani, S. Su, W.M. Petroll, Assessment of corneal stromal remodeling and regeneration after photorefractive keratectomy, Sci. Rep. 8 (2018) 12580.