Bioactive Materials 6 (2021) 4447–4454

Contents lists available at ScienceDirect

Bioactive Materials

journal homepage: www.sciencedirect.com/journal/bioactive-materials

# A long-term retaining molecular coating for corneal regeneration

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ARTICLE INFO

*Keywords:*
Corneal regeneration
GelNB molecular coating
Long-term adhesion
Eye drops

ABSTRACT

Corneal injuries will cause corneal surface diseases that may lead to blindness in millions of people worldwide. There is a tremendous need for biomaterials that can promote corneal regeneration with practical feasibility. Here we demonstrate a strategy of a protein coating for corneal injury regeneration. We synthesize an o-nitrosobenzaldehyde group (NB)-modified gelatin (GelNB), which could adhere directly to the corneal surface with covalent bonding to form a thin molecular coating. The molecular coating could avoid rapid clearance and provide a favorable environment for cell migration, thereby effectively accelerating corneal repair and regeneration. The histological structure of the regenerated cornea is more similar to the native cornea. This molecular coating can be used conveniently as an eye drop solution, which makes it a promising strategy for corneal regeneration.

## 1. Introduction

Corneal blindness is the fourth common cause of blindness [1]. Corneal damage occurs owing to various reasons such as chemical or thermal burns, contact lens overuse, and mechanical trauma [2,3]. Reduced vision and corneal diseases, resulted from corneal integrity loss, afflicts more than 23 million individuals worldwide [4]. It is estimated that 12.7 million patients with advanced corneal injury have few choices but turn to corneal transplantation [5]. However, donor tissue shortage is the most obvious problem, with only 1 cornea available for 70 needed. The high expense of transplantation surgery is another limiting factor [6]. Notably, there are also many patients with milder symptoms at an early stage. Surgery is not necessary for them while other choices, such as the ocular drug delivery system and regenerative biomaterials could be adopted. So, designing the biomaterials that can repair the cornea is still in need [7]. And novel formulated polymers have been developed for corneal defect repair [8,9].

The corneal surface structure composition plays an important role for vision in maintaining transparency and light transmission. It is a unique type of non-keratinized epithelial cells arranged in an orderly fashion. Deficiency in corneal epithelium may cause cornea into a non-transparent condition [10]. It may further induce corneal stromal inflammation, stromal matrix breaking down, even blindness. Generally, when the cornea does not become severely damaged, the tissue has the ability to heal or repair in an appropriate environment [11]. Thus, scaffolds or biomaterials that could mimic the natural microenvironment and promote the migration of epithelial cells are often used for corneal regeneration [9].

Hydrogels are widely applied for corneal regeneration [12]. Silk fibroin hydrogel [13], poloxamer [14], polyethylene glycol (PEG) [15],

Peer review under responsibility of KeAi Communications Co., Ltd.

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https://doi.org/10.1016/j.bioactmat.2021.04.032
Received 3 February 2021; Received in revised form 26 March 2021; Accepted 17 April 2021

gellan gum [16], chitosan [17], gelatin [18] and hyaluronic acid [19] are the commonly used hydrogel materials. However, these hydrogels for ocular application still have some limitations in adhesive stability or operability. For example, the immediate gelation of the conventional F127 resulted in limited spreading and rapid clearance due to the blinking of the eyelids [20]. In one study, GelCORE was advocated as an effective corneal replacement. This hydrogel could be crosslinked *in situ* without UV light, but the eyes were directly exposed to visible light for 4 min during the application of GelCORE. Even the healthy eyes could hardly bear the long exposure time [18]. Recently, research demonstrated collagen patches had the potential for repairing corneal defects. This collagen patch forms gelation at body temperature in 5 min and has a great potential to be applied in clinics. But this collagen patch is as thick as 700 μm and may cause discomfort for patients [21].

Based on these considerations, we think that a hydrogel coating may be an efficient strategy to promote cell adhesion and immigration within tissue defects [22–24]. Collagen is the major component of the corneal extracellular matrix [26] and is essential for corneal cell attachment, migration, proliferation, and differentiation [27]. Meanwhile, our previous study demonstrated that the o-nitrosobenzaldehyde group (NB) had a strong adhesive ability to the amino groups on tissue [25]. So, in this study, we design a hydrogel coating system that consists of gelatin modified with o-nitrosobenzaldehyde groups (NB). We hypothesized that the NB-modified collagen derivative—GelNB could avoid rapid removal and provide a suitable microenvironment for corneal cell migration and regeneration. Besides, considering the eye drop system is the easiest and noninvasive way, we further designed the biomaterial into the form of fluid-phase eye drop.

Our study showed that the hydrogel coating would formed on the corneal defect after the photo-activated GelNB solution dropped to the eyes. The extended contact time and enhanced adhesion characteristics of the molecular coating were then identified by SEM and fluorescent images of explanted rabbit eyes cornea. At last, rabbit cornea defect models were used to assess its biocompatibility and effects on corneal regeneration.

## 2. Method and materials

### 2.1. Synthesis and characterization

#### 2.1.1. Synthesis of NB-COOH

NB was synthesized as previously [28]. 4-Hydroxy-3-(methoxy-D3) benzaldehyde (8.90 g, 58.5 mmol, 1.06 eq.), potassium carbonate (10.2 g, 73.8 mmol, 1.34 eq.), and 4-bromobutyric acid methyl ester (9.89 g, 55.0 mmol, 1.0 eq.) were dissolved in dimethylformamide (DMF) to react. The mixture was deposited with chilled water (200 ml) to gain the product. Then, the product was dissolved in DMF and dried.

To induce ipso substitution in 4-(4-formyl-2-methoxyphenoxy methoxyphenyl) butanoic acid methyl ester, early product (9.4 g, 37.3 mmol, 1 eq.) was added to a cool solution (−2 °C) of nitric acid (70%) for 3 h. To deposit the solid product, the mixture was filtered with chilled water and then purified in DMF. A slightly yellow powder was yielded after drying and removing the solvent under reduced pressure following hydrolysis in TFA/$H_2O$ 1:10 v/v (100 ml) at 90 °C. The resulting products were washed and dried. The product (7.4 g, 23.8 mmol, 1.0 eq.) was then dissolved in THF/EtOH 1:1 v/v. 3 h later, $NaBH_4$ (1.43 g, 35.7 mmol, 1.5 eq.) was slowly added in. Dichloromethane was used to extract the product in the aqueous layer and the organic layers were dried by magnesium sulfate. Raw products were purified by column chromatography on silica gel using DCM/MeOH = 10:1 (1% TEA) and finally to obtain a slightly yellow powder NB-COOH 5.31 g (18.6 mmol, 78.3%).

#### 2.1.2. Synthesis of GelNB

GelNB was synthesized by activating the carboxyl groups of NB-COOH and reacting them with gelatin by EDC/NHS. In brief, 10% w/v homogeneous gelatin solution (Sigma-Aldrich) was kept at 37 °C. While NB-COOH was dissolved in Dimethyl sulfoxide (DMSO). 1-(3-Dimethylaminopropyl)–3-ethylcarbodimide hydrochloride (EDC) (Aladdin) and N-hydroxysuccinimide (NHS) (Aladdin) were each added to NB-COOH DMSO solution for 10 min. The mixture was then slowly dropped into the dissolved gelatin solution to react at 45 °C for 4 h. To purify the product, GelNB solution was dialyzed against distilled water for at least 3 days. The GelNB solution was collected and lyophilized for further use. $^1$H NMR spectrum was used to verify the NB modification degree.

#### 2.1.3. Preparation and characterization of eyedrops

The freeze-dried GelNB foams were dissolved in distilled water at 37 °C to make the final eyedrop concentrations at 100 mg/ml. Once fully dissolved, it turned into a solution. The solution was photo-activated with UV light (365 nm, 30 mW/cm$^2$) for 10 s and finally turned to an amino-reactive eye drop. The samples of gelatin solution, unactivated GelNB solution, and UV-activated GelNB solution were freeze-dried in vacuum for Fourier transform infrared (FTIR)-attenuated total reflectance (ATR) spectroscopy analysis (Nicolet iS50).

### 2.2. In vitro ocular surfaces modification and integration

#### 2.2.1. SEM analysis

Scanning Electron Microscopy was used to identify the integration of coating on the cornea with the cryo-fracturing method [29]. Briefly, the samples were fixed in 2.5% glutaraldehyde overnight. Then the specimens were frozen in liquid nitrogen and cracked into small pieces with tweezers. The small pieces were washed in phosphate buffer and were treated by different concentrations of ethanol extractions (50%, 70%, 80%, 90%, 100%, 100%) for 10 min, respectively. All samples were ready for observation under an SEM (Nova Nano 450) after gold-palladium treatment (Leica ACE200).

#### 2.2.2. Fluorescence evaluation

To quantify the adhesion of the coating on the corneas, rhodamine B was introduced to assess the fluorescence intensity on the corneas. Rhodamine labeled gelatin (Rd-gelatin) and GelNB was prepared by mixing rhodamine B isothiocyanate with gelatin and GelNB at 48 °C for 48 h. The two solutions were dialyzed and lyophilized for use according to the previous methods [30]. Corneas were explanted from sacrificed rabbits and were kept in cold DPBS containing 2% antibiotics. 40 μl eye drop was dropped onto the wound. Fluorescence was envaulted in 0 h, 24 h, 48 h after eyedrop application. To simulate the wet and blinking environment *in vivo*, the corneas were immersed with PBS and put on a shaker.

#### 2.2.3. Integration mechanism study

X-ray photoelectron spectroscopy (Thermo Scientific ESCALAB 250Xi) analysis was employed to further identify the surface chemical composition of the treated tissue, using an Al K source (1486.6 eV). The corneas were treated with engineered GelNB eyedrop, gelatin, and blank. The three groups were washed in warm water. We made a detailed scanning for Nitrogen. And we used the calibration of the Carbon 1s peak (284.6 eV).

### 2.3. In vitro cell studies

#### 2.3.1. Determination of cell viability and proliferation

Human corneal epithelial cells were cultured at 37 °C and 5% $CO_2$ in Dulbecco's modified Eagle's media F12 supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin. The HCEC lines (PCS-700-010) were purchased from ATCC (VA, USA). Sterilized GelNB was added into the culture medium (10% w/v) then UV irradiated (30 mW/cm$^2$). Cells were seeded inside 24-well plates (5000 cells/500 μl/well). The cell viability was assessed with the live/dead staining assay

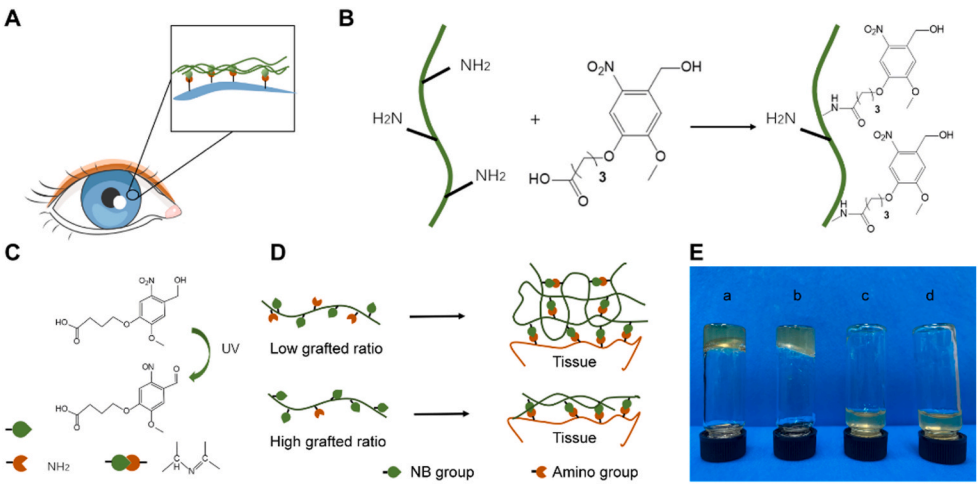


**Fig. 1. Design, synthesis, and characterization of GelNB coating for the corneal defect.**
**(A)** Schematic of the molecular coating strategy for corneal regeneration.
**(B)** Schematic of the chemical reaction for GelNB formation.
**(C)** Schematic diagram illustrating the photo-triggered chemical structures transformation of the GelNB hydrogel. O-nitrobenzene is converted to o-nitrosobenzaldehyde groups under UV exposure. Then the active aldehyde group could subsequently crosslink with amino groups to form Schiff bases.
**(D)** Schematic of GelNB forming hydrogel and coating under different feed ratios.
**(E)** Different gelling performance of GelNB with various feed ratios of NB. (a–d) represent 0.5,1,2,4 NB feed ratio of GelNB, respectively.

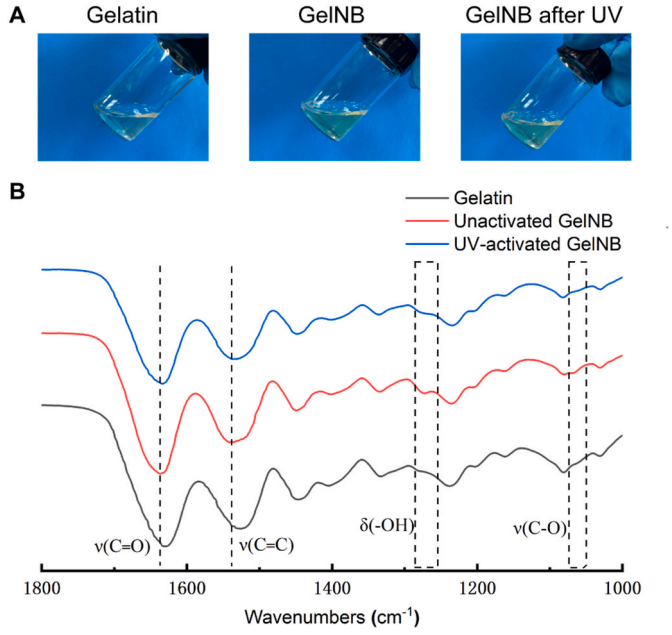


**Fig. 2. Characterization of GelNB molecular coating solution.**
**(A)** Gross view of gelatin, unactivated modified GelNB-4 molecular coating solution, and GelNB-4 solution after UV illumination.
**(B)** The ATR-FTIR spectra of gelatin, unactivated GelNB-4, and UV-activated GelNB-4.

(Dojindo, Japan) for 1, 3, and 5 days. Live and dead cells were imaged under a fluorescence microscope. To assess the proliferation and cytotoxicity under GelNB treatment, we used Cell Counting Kit-8 method. Human corneal epithelial cells grew inside 96-well cell culture plates (1000 cells/100 μl/well). Cell medium was replaced with culture medium with or without eyedrop and further incubated for 1, 3, and 7 days. The culture media was replaced with the CCK-8 reagent solution to incubate for another 3 h. Then, we measured the absorbance of the solution using a microplate reader (Spectra Max M5) at a wavelength of 450 nm [31].

### 2.3.2. Cell scratch test on coating glass slides

Sterilized clean glass slides (size: 32 mm diameter) were soaked into 1% (3-Aminopropyl) triethoxysilane (Macklin) aqueous solution for 30 min and dried [32]. The modified glass slides were then immersed in UV-activated eyedrop and gelatin respectively, and washed for scratch evaluation. Glass slides with different treatments were seeded in a 6-well plate. Human corneal epithelial cells were suspended onto the glass slides to grow until complete confluence. The cell layer cultured on the coating was scratched gently with a 1 ml pipette tip in each well. The migration of cells was taken photos after culturing for 12 and 24 h. And the distance of the gap was quantitatively evaluated using Image J.

## 2.4. In vivo function identification

### 2.4.1. Rabbit corneal surgical procedures

Adult New Zealand white rabbits were purchased from Zhejiang University. All the animals involved *in vivo* experiments were followed the Zhejiang University Ethics Committee standard guidelines (ZJU20200156). Survival surgeries were performed under aseptic conditions. After general anesthesia, a partial-thickness-keratectomy was performed to induce corneal injury in rabbit eyes. First, we made a trephination of a 3-mm biopsy punch in the center of the cornea. Then, a corneal layer board knife was used to separate the lamellar cornea. GelNB was prepared as described before [10% (w/v), 10-s UV exposure]. 40 μl of the eye drop formulation was directly dropped into the wounds. All the rabbits received penicillin by intramuscular injection. These rabbits would receive a series of eye checks at 1, 3, and 5 days after surgery. After 2 weeks, they were euthanized for further histologic analysis.

### 2.4.2. Slit lamp biomicroscopy

Photographs were taken every time after the examination of eyes with slit lamp biomicroscope (YZ5T Image System, China). Slit and broad beams were used to evaluate the transparency of the surface of the cornea and the surrounding tissue. Fluorescein staining and cobalt blue slit lamp photography were used to assess the repair of corneal epithelia over the wounds.

### 2.4.3. Histological analysis

The rabbit's corneas were fixed using 4% paraformaldehyde (PFA) for 3 days at room temperature. And then samples were dehydrated through the treatments of series graded alcohol and embedded in paraffin. Using Leica rotary microtome (Hamburg, Germany), samples of the corneal defect areas were sectioned into 7 μm thick slices for analyses of hematoxylin and eosin (H&E) staining.

### 2.4.4. Statistical analysis

The experiments involved were tested at least three times, and data were expressed as means ± SD. One-way analysis of variance (ANOVA) was performed the multiple comparison tests followed by Tukey's test. And non-paired Student's t-test was used between two groups for

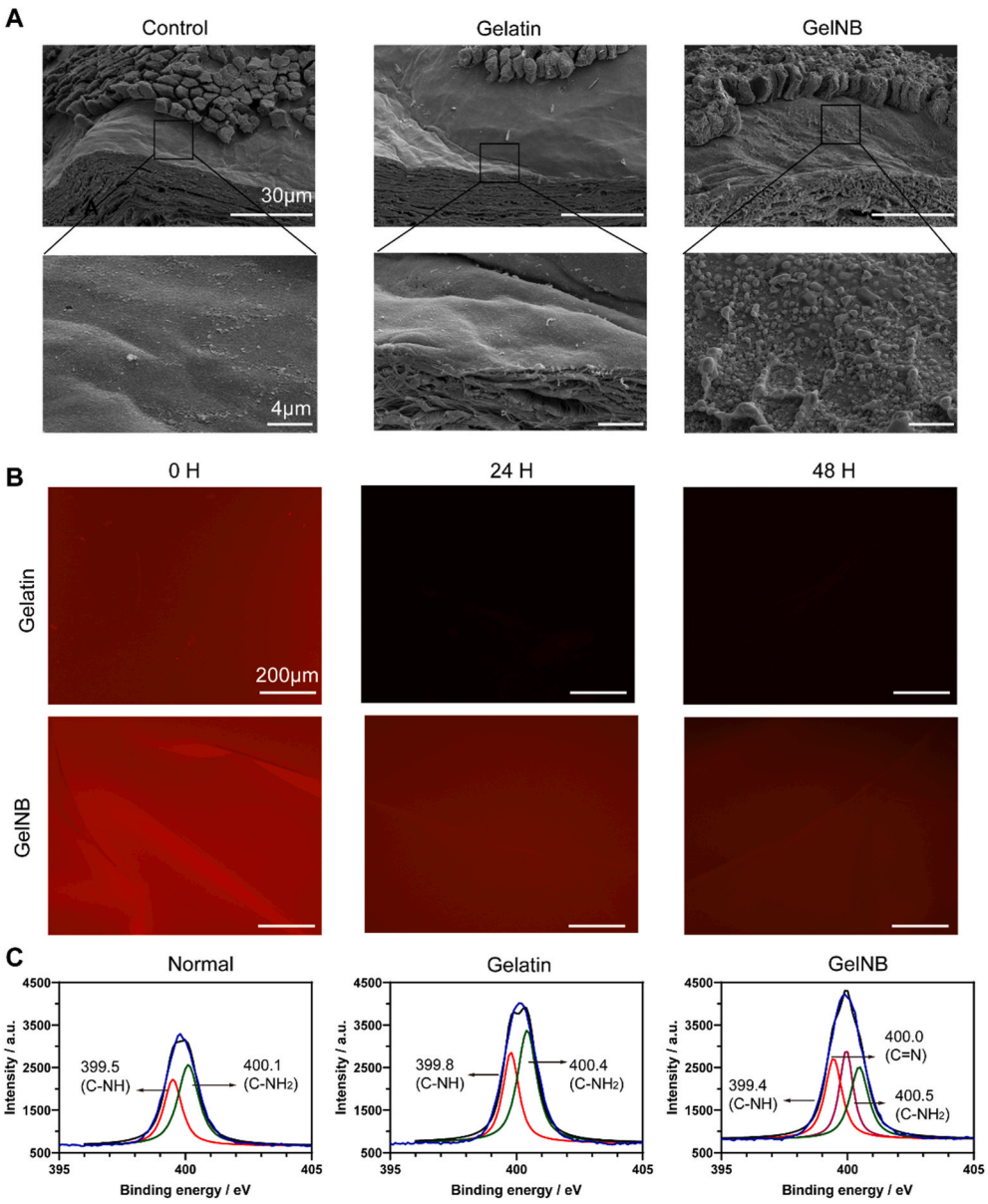


**Fig. 3. *In vitro* adhesion performance characterization of GelNB molecular coating.**

**(A)** SEM images of the injured corneal surface, gelatin, and GelNB-4 protein coating treated corneal surface. Scale bars: 30 μm (top panels); 40 μm (bottom panels, enlarged).

**(B)** Fluorescence images of labeled gelatin and GelNB-4 protein coating treated corneal surface at different time points. Scale bars: 200 μm.

**(C)** X-ray photon spectroscopy (XPS) of GelNB-4 bonding to tissue. The bond energies of the peptide -C–NH– and amino amine group C–$NH_2$ shifting respectively with the appearance of a C═N bond peak reveals the UV-induced formation of Schiff bases.

statistical analysis (GraphPad Prism 8.0, GraphPad Software). (*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001).

## 3. Results and discussion

### 3.1. Synthesis and the characteristic of the NB-functionalized eye drops

Among the treatments of choice for corneal damage, hydrogels have risen as a promising approach with efficient corneal regeneration [33,34]. However, in clinical practice, commercial hydrogels are limited by poor adhesive stability and inconvenient to use such as cyanoacrylate glue and ReSure (Ocular Therapeutix Inc.) [35,36]. These shortcomings affect the broad applications of regenerative biomaterials. As shown in Fig. 1A, we synthesized the UV-activated GelNB which could form a molecular coating for the treatment of corneal defect in form of eye drop. An efficient adhesive system was developed to promote tissue integration by grafting O-nitrosobenzaldehyde groups (NB) onto gelatin. O-nitrobenzene would be converted into NB after immediate UV illumination to crosslink with –$NH_2$ on surrounding tissue surfaces, which would be a quick and easy application in clinics [25,28,37].

To synthesize GelNB, carboxyl terminated NB (NB–COOH) were substituted by the primary amino groups of gelatin via EDC/NHS mediated reaction in Fig. 1B. After dialysis and freeze-drying, these GelNB foams were dissolved via a heating stage at 37 °C, resulting in an eye drop after rehydration to use. The final concentration of GelNB solution was 100 mg/ml.

It has been reported that in-situ UV cross-linkable hydrogels or photo-activated sealants could be applied to diverse tissue defect shapes to create patient-specific corneal scaffolds [18,38]. However, the operation is complicated and direct exposure of eyes to UV light exerts the risk of DNA damage [39]. In our work, the GelNB solution was applied onto the corneal defect just like the application of eye drops. The UV light converted the hydroxyl group on the benzene into an aldehyde group which could form the chemical bonds with amino groups on the tissue surface (Fig. 1C) which is more convenient and can avoid direct UV exposure of the eyes.

Modification of NB groups on gelatin was also confirmed through the identification of NB-related peaks and gel-related peaks in ATR-FTIR analysis (Fig. 2B). The enhancement of the primary hydroxyl group absorption bands around 1280 and 1170 $cm^{-1}$ indicated the successful

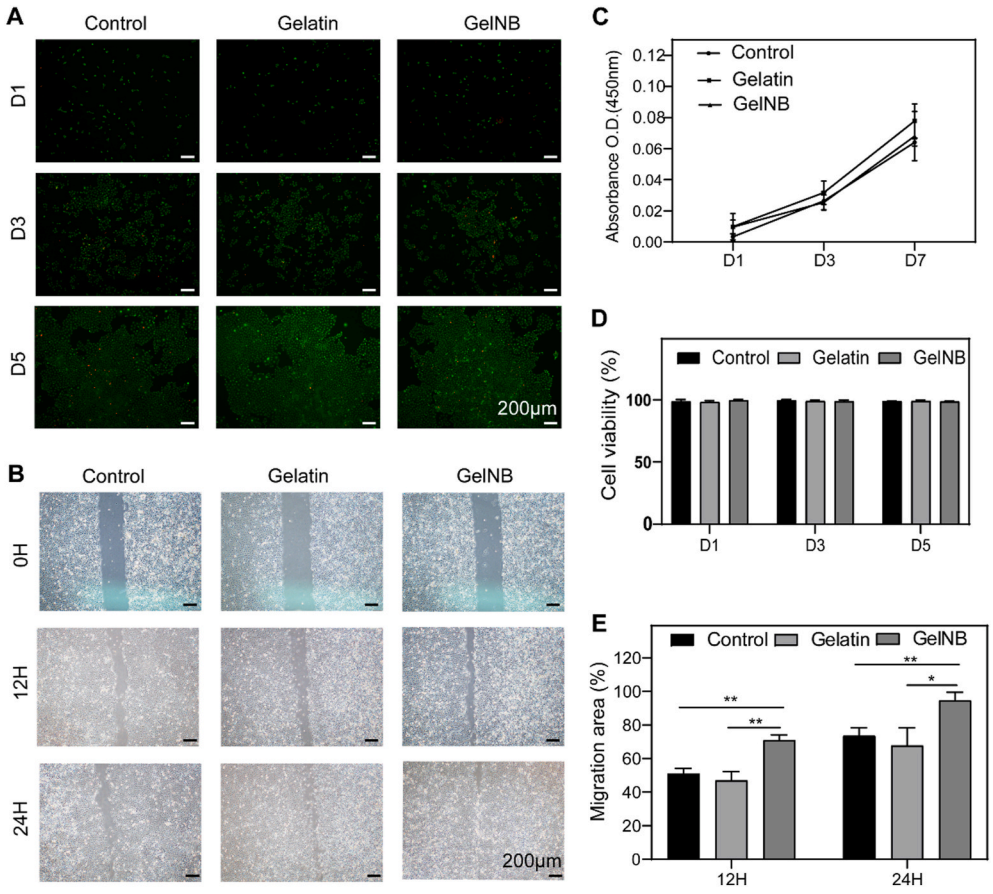


**Fig. 4. *In vitro* biocompatibility characterization.**
**(A)** Representative fluorescence images showed human corneal epithelial cells treated with or without modified gelatin on days 1, 3, and 5. Scale bars: 200 μm.
**(B)** Scratch assay images of corneal epithelial cells migration into the scratched area cultivated with gelatin and GelNB-4 coating treated samples. Scale bars: 200 μm.
**(C)** The cytotoxicity of GelNB-4 coating to human corneal epithelial cells after incubation for 1, 3, and 7 days.
**(D)** Viability quantification of the epithelial cells culture at different time points.
**(E)** Quantification of cell migration area after incubation of 12 and 24 h (*$p < 0.05$ and **$p < 0.01$; $n \geq 3$).

grafting of NB-COOH on gelatin which belonged to –OH deformation vibration and C–O stretching vibration respectively. However, these two peaks declined while the stretching vibrations of C═O (1650 $cm^{-1}$) and C═C (1550 $cm^{-1}$) were red-shifted in UV-activated GelNB. It revealed that the primary hydroxyl groups of NB were gradually transformed into phenol conjugated aldehyde groups after UV irradiation.

To ensure that the molecular coating formed without self-crosslinking after light exposure, we investigated the relationship between the feed ratio (FR) of NB and the gelling performance. The feed ratio is defined as the molar ratio between NB groups and primary amino groups in gelation [4]. And in our study, 53 mg NB with 1 g gelatin is defined as $FR_{NB} = 1$. As shown in Fig. 1E, GelNB with low FR formed a soft and weak hydrogel since there were a lot of amino groups that could react with the photo-generated aldehyde groups, while GelNB with high FR could keep a flexible fluid eye drop. Therefore, GelNB with high FR was used for further investigation. GelNB-4 used in the following study is $FR_{NB} = 4$ and the substitute rate (SR) is 36.9% with $^1H$ NMR analysis (Fig. S1).

### 3.2. Characteristic of GelNB molecular coating

Subsequently, we studied the formation and retention of molecular coatings. SEM was used to investigate the molecular coating formed on the corneal surface. The normal corneal epithelium is a stratified squamous epithelium that covers the front of the cornea. The basal columnar cell layer would anchor to the basal lamina [40]. When the corneas were damaged, the denuded basal lamina was exposed. The injured epithelial cells lost most of their microvilli. No obvious difference was found between the non-treated group and the gelatin-treated group. However, in the GelNB-4 treated group, GelNB appeared as the form of ruffles of different shapes extend out over the basal lamina (Fig. 3A). This result proved that GelNB-4 could form a molecular coating on the cornea surface. Pfister et al. reported that cellular motility appeared to be initiated by ruffling at or near the free edge of squamous epithelial cells. Ruffles and filopodia projected out ahead of the cell edge, contacted the basal lamina, and appeared capable of drawing the cells forward into the area of the defect [41]. It suggested that GelNB coating might accelerate corneal epithelial cell migration by forming ruffles over the basal lamina.

To verify the retention of hydrogel coating on the cornea surface, fluorescently labeled gelatin and GelNB-4 were used to confirm the presence of the surface coating. The eyeballs were put in a wet shaking situation to simulate the true corneal physiological environment. As shown in Fig. 3B, fluorescence was detected at 0 h in both Rhodamine labeled gelatin group and GelNB-4 groups, and the fluorescence intensity of the GelNB-4 group was significantly stronger than that of the gelatin group. After 48 h, obvious fluorescence could still be observed in the GelNB-4 group. However, no fluorescence could be detected in the gelatin group within 24 h. These results demonstrated that GelNB-4 coating could stay on the corneal surface and resist water washing, while gelatin could not. The results also suggested that the engineered GelNB-4 eye drops had the potential function under the situation with eye tears and frequent blinking.

X-ray photoelectron spectroscopy (XPS) was adopted to further detect the chemical bonds formed between GelNB-4 and tissue surface. Commercially available sausage skin was used as a tissue model. As shown in Fig. 3C, spectra of tissue alone and treated with gelatin were almost the same, only C–NH bonds and C–$NH_2$ bonds peaks could be found. However, in the GelNB-4 treated group, there is an extra peak appearing at 400.0 eV. A large amount of C═N bonds formed on the tissue after treatment with UV-activated GelNB-4 indicating that Schiff bases formed at the interface between GelNB-4 coating and tissue [43].

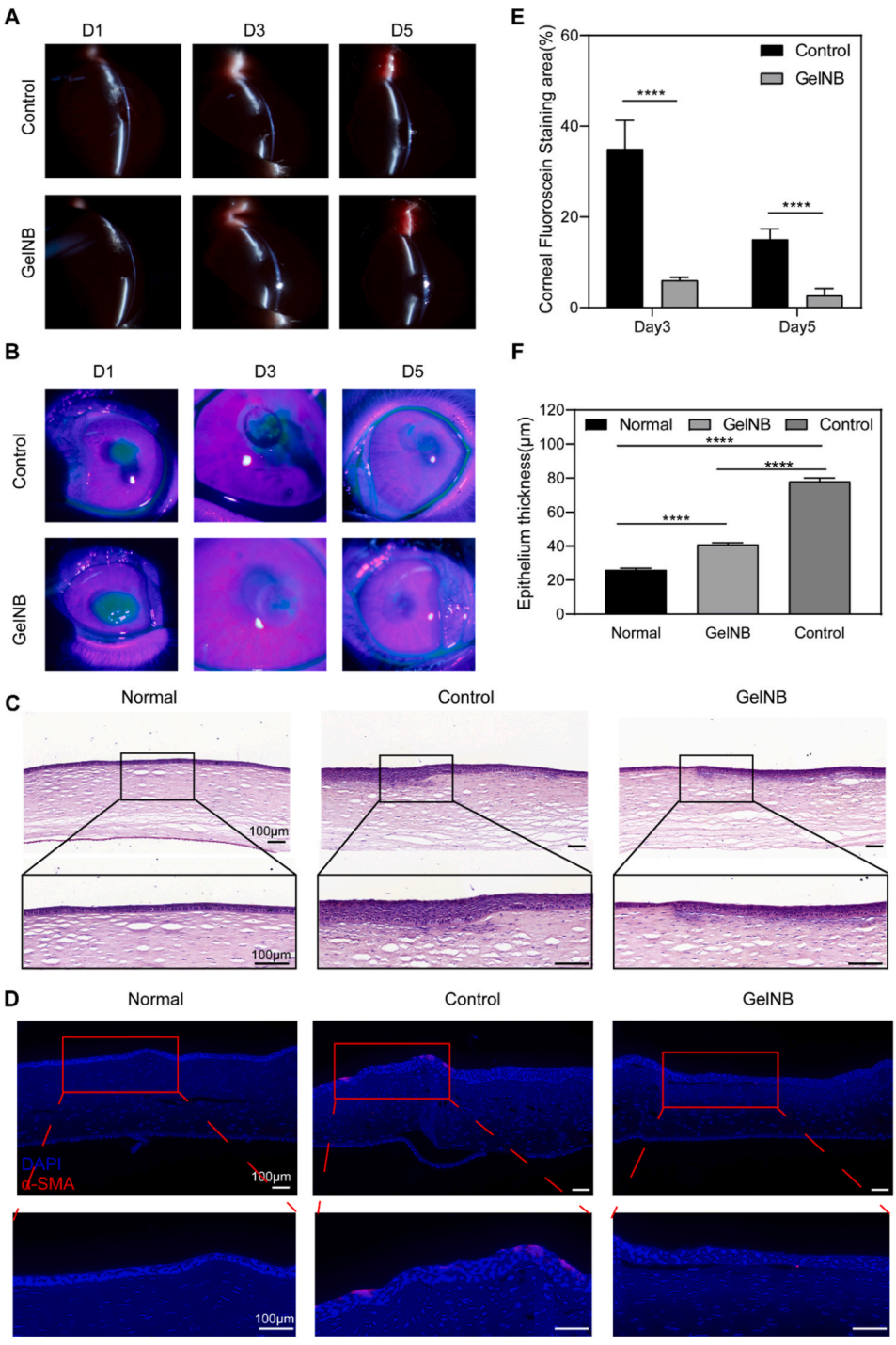


**Fig. 5.** ***In vivo*** **function of the GelNB molecular coating on the corneal defect.**
**(A)** Representative photographs taken by slit lamp demonstrated a recovery of smooth and clear slit beam of the eyes.
**(B)** Fluorescein staining imaged with cobalt blue showed the recovery of the corneal epithelial by the progressive reduction staining size after the application of GelNB-4.
**(C)** H&E stained images of rabbit cornea section for the native rabbit cornea, untreated cornea following the surgery, and corneal defect after application of UV-activated GelNB eyedrop on the defect. Scale bars: 100 μm (top panels); 100 μm (bottom panels, enlarged).
**(D)** Immunofluorescence staining of wounded cornea treated with and without GelNB (DAPI and α-SMA marker). Scale bars: 100 μm (top panels); 100 μm (bottom panels, enlarged).
**(E)** Corneal epithelial migration was quantified by comparing the size of green staining in the corneal center, with respect to day 1.
**(F)** The measurements of epithelial layer thickness in different groups obtained from histological images. (****$p < 0.0001$; $n = 3$.).

### *3.3. Evaluation of cytocompatibility and biofunction of GelNB molecular coating*

The cell viability and proliferation of the human corneal epithelial cells were assessed with the Live/Dead assay and CCK-8 assay, respectively. Live/Dead staining revealed the corneal epithelial were metabolically active. Cell viability was >98% in gelatin and modified gelatin after cultivation for 5 days (Fig. 4A&D). The result of CCK-8 test revealed that GelNB-4 had no adverse effect on cell proliferation (Fig. 4C).

The cornea has a certain ability to repair itself by recruiting surrounding epithelial cells. If biomaterials can promote the migration of surrounding cells to the defect, it would greatly promote the healing of corneal defects. Therefore, we used a cell migration test to verify whether GelNB-4 could promote cell migration. It showed that human corneal epithelial cells seeded on the surface treated with GelNB-4 migrated faster than control and gelatin treated groups, covering the scratched area in less time (Fig. 4B&E). It suggested that this coating strategy might enhance cell integration and recruit more cells *in vivo*.

New Zealand white rabbit corneal injury model was created to evaluate the repair performance of GelNB-4. After the corneal defect was created, the activated GelNB-4 solution was applied onto the defect site just like an eye drop solution (Fig. S2). We did the regular optical check for the rabbit at day 1, day 3, and day 5 after surgery, by slit lamp

examination and fluorescein eye staining.

With the aid of a slit view, we directly viewed the corneal situation. The slit beam allowed proper evaluation of the cornea. As shown in Fig. 5A, the thickness of the corneal stroma reduced after injury, and the slit beam seemed a little crude and not very clear on the defect site. 5 days after surgery, we could see a significant increase in the thickness of the corneal stroma and a uniform, smooth slit beam on the defect site in the GelNB-4 treated groups. While in the control groups, the corneal stroma did not increase much and the slit beam still seems crude and unclear.

Corneal abrasions could be easily revealed by the fluorescent staining size under a slit lamp examination with the cobalt blue filter. When treated with GelNB-4, the size of the defect decreased significantly (Fig. 5B), suggesting the migration of the epithelium. The green staining area of injury in the corneal center on the first day was measured as 100%. 3 days after surgery, the staining area reduced to 6.0 ± 0.4% in the GelNB-4 coating treated group, while the staining area of the control group only reduced to 31.9 ± 2.8%. 5 days after surgery, the corneal surface defect was almost healed in the GelNB-4 coating treated group, however, the control group still showed 16.2 ± 0.5% fluorescence remaining (Fig. 5E).

Histological H&E staining revealed that the GelNB-4 coating could induce a better corneal repair. The eyes of rabbits lost all epithelium in the defect after the surgery shown in Fig. S3. There were statistically significant differences in the thickness among the control group, GelNB-4 group, and native corneas for corneal epithelial layers (Fig. 5C). According to our measurements, the normal corneal epithelial thickness is 27–30 μm, and the average thickness of the GelNB-4 coating treated group is 40 μm, close to the normal tissue. However, the control group showed the thicker (80 μm) corneal epithelial layer, indicating heterogeneous re-epithelialization (Fig. 5F). Corneal epithelial squamous hyperplasia is characterized by epithelial hyperkeratosis, and is often associated with corneal haze and scar [44]. The results suggested defected eyes applied with GelNB-4 coating had an intact and regular epithelial layer, but the cornea of the control group had serious epithelial hyperplasia [45]. We further did the immunofiuorescence staining of alpha-smooth muscle actin (α-SMA) to analyze the myofibroblast formation and corneal scarring [7]. Persistent myofibroblasts activity may lead to corneal scarring fibrosis. The images suggested that GelNB decreased the expression of α-SMA, and did not induce significant myofibroblast activation (Fig. 5D).

Overall, the key feature of the biomaterial is the regenerative ability [46]. To repair the injured corneas, cell-based therapies also have the supreme regenerative capacity [26]. Kohji Nishida et al. transplanted stem cell sheets onto rabbit eyes to reconstruct a healthy corneal barrier [47]. These cell therapies achieved great results and demonstrated translational potential. However, the efficiency of the cell culture process (24–38 days) [47] and the high cost of cell production are still challenges for cell-based therapies [48]. In our study, GelNB can be used as easily as eye drops with a low cost. GelNB-4 coating efficiently accelerated corneal epithelium recovery and promoted regular corneal regeneration. With tightly adhesive covalent bond and extracellular matrix mimic composition, the molecular coating showed a fast and homogenous corneal regeneration. Our results showed the feasibility of a molecular coating strategy that provides a new perspective for corneal repair.

## 4. Conclusion

In summary, we have designed a molecular coating for corneal regeneration. GelNB can form a thin coating on the cornea surface and be used as easily as an eye drop solution. This coating could enhance corneal epithelial cell migration *in vitro*. An *in vivo* study identified that the molecular coating took advantage of corneal regeneration. GelNB greatly promoted self-healing and inhibited the disorder of regeneration. So, the results show a directly molecular coating on the tissue defect may be the new strategy for corneal regeneration.

## CRediT authorship contribution statement

**Yi Zhang:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Chenglin Li:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. **Qiuwen Zhu:** Conceptualization, Methodology, Investigation, Formal analysis. **Renjie Liang:** Methodology, Formal analysis, Writing – review & editing. **Chang Xie:** Methodology, Resources, Data curation. **Shufang Zhang:** Supervision, Data curation, Writing – review & editing, Funding acquisition. **Yi Hong:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition, Project administration. **Hongwei Ouyang:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing.

## Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

## Acknowledgements

This work was supported by the National Key R&D Program of China (2017YFA0104900), National Natural Science Foundation of China (31830029, 32000957, 81972053). We thank Qiaomei Tang for her help with the cell lines. We thank Zhi Peng for his help with the animal experiments. We thank Dandan Song in the Center of Cryo-Electron Microscopy (CCEM), Zhejiang University for her technical assistance on Scanning Electron Microscopy. We thank Jian Ma and Xiaomeng Wang in the Center for Drug Safety Evaluation and Research of ZJU.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2021.04.032.

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