

ACCEPTED PAPER · MAIN TEXT

Boronate ester-crosslinked GelMA composite bioink for 3D bioprinting of cell-laden corneal stroma and *in vivo* evaluation in rabbits

Paper version: Accepted Paper

Accepted Papers are manuscripts accepted for publication, encompassing all changes made following the peer review process, along with a standard cover page indicating the paper version and an “Accepted Paper” watermark, but excluding any other editing, typesetting or other changes made by AccScience Publishing and/or authors post-acceptance.

Article ID: IJB026350379

Citation: Hsueh YJ, Chen CA, Yang CY, *et al.* Boronate ester-crosslinked GelMA composite bioink for 3D bioprinting of cell-laden corneal stroma and *in vivo* evaluation in rabbits. *Int J Bioprint*. 2026. doi: 10.36922/IJB026350379

Copyright: © 2026 Author(s). This is an Open Access article distributed under the terms of the Creative Commons Attribution License, permitting distribution, and reproduction in any medium, provided the original work is properly cited.

Publisher’s Note: AccScience Publishing remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Boronate ester-crosslinked GelMA composite bioink for 3D bioprinting of cell-laden corneal stroma and *in vivo* evaluation in rabbits

Running title: Boronate-ester bioink for corneal bioprinting

Yi-Jen Hsueh^{1,2†}, Chung-An Chen^{3,4†}, Chin-Yu Yang², Chang-Hong Lin¹, Chun-Yuan Cheng⁴, Chih-Hsin Lin^{5,6,7}, Shih-Jung Liu^{4,8}, Tsung-Ting Tsai^{3,4*}, and Hung-Chi Chen^{1,2,3*}

¹ Department of Ophthalmology, Chang Gung Memorial Hospital, Linkou Branch, Taoyuan 333, Taiwan

² Center for Tissue Engineering, Chang Gung Memorial Hospital, Linkou Branch, Taoyuan 333, Taiwan

³ School of Medicine, Chang Gung University, Taoyuan 333, Taiwan

⁴ Department of Orthopaedic Surgery, Spine Section and Bone and Joint Research Center, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan

⁵ Institute of Molecular Medicine and Bioengineering, National Yang Ming Chiao Tung University, Hsinchu 300, Taiwan

⁶ Department of Dentistry, School of Dentistry, National Yang Ming Chiao Tung University, Taipei, 112, Taiwan

⁷ Department of Biological Science and Technology, College of Engineering Bioscience, National Yang Ming Chiao Tung University, Hsinchu, 300, Taiwan

⁸ Department of Mechanical Engineering, Chang Gung University, Taoyuan 333, Taiwan

[†] *These authors contributed equally to this work.*

***Corresponding author:**

Hung-Chi Chen (mr3756@cgmh.org.tw)

Tsung-Ting Tsai (tsai1129@gmail.com)

Citation: Hsueh YJ, Chen CA, Yang CY, *et al.* Boronate ester-crosslinked GelMA composite bioink for 3D bioprinting of cell-laden corneal stroma and *in vivo* evaluation in rabbits. *Int J Bioprint.* 2026. doi: 10.36922/IJB026350379

Received: February 3, 2026

1st revised: August 28, 2026

2nd revised: September 12, 2026

Accepted: September 15, 2026

Published online: September 15, 2026

Copyright: © 2026 Author(s). This is an Open Access article distributed under the terms of the Creative Commons Attribution License, permitting distribution, and reproduction in any medium, provided the original work is properly cited.

Publisher's Note: AccScience Publishing remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract

Corneal transplantation restores vision by replacing damaged corneal tissue with a donor graft, but the limited supply and variable quality of donor corneas remain major challenges. We therefore developed an engineered corneal substitute using gelatin methacryloyl (GelMA), a modified gelatin. Phenylboronic acid-functionalized gelatin (PBA@G) and poly(vinyl alcohol) (PVA) were incorporated to introduce dynamic boronate ester crosslinking and modify the rheological behavior of the hydrogel. The formulation optimized for bioprinting contained 10% GelMA, 10% PBA@G, and 1% PVA (10G10P1PVA). Scanning electron microscopy revealed an interconnected porous microstructure. The rheological behavior, optical transmittance, swelling, degradation, and printability of 10G10P1PVA were characterized. Human corneal stromal cells (CSCs) remained viable through day 7, and the CCK-8 assay showed significantly higher metabolic activity in 10G10P1PVA than in 10G. Phalloidin staining showed cell spreading and longitudinal F-actin organization. RT-qPCR showed no marked ACTA2 induction, with expression remaining below that of the myofibroblast-like reference group. The corneal stromal cell marker ALDH3A1 was expressed at higher levels in cells cultured in 10G10P1PVA than in either the 10G or 10G10P group. These findings support the short-term cytocompatibility of the newly developed bioink and selected stromal-associated features. An exploratory rabbit ALK evaluation was performed to document short-term ocular response, epithelial recovery, and stromal organization following implantation over 7 days. Together, the material and biological results support further evaluation of 10G10P1PVA as a GelMA-based composite bioink for corneal stromal bioprinting.

Keywords: Artificial corneal stroma; Bioink; Bioprinting; Biomaterials; Photo-crosslinking; 3D cell culture

1. Introduction

The cornea is the transparent anterior tissue of the eye and provides the principal refractive surface through which light enters the eye. Irreversible disruption of corneal structure or transparency can result in severe visual impairment or blindness. For advanced corneal diseases in which transparency and visual function cannot be restored by other treatments, corneal transplantation remains the mainstay of treatment. However, the global supply of donor corneas is far below clinical demand. A global survey by Gain et al. estimated that approximately 12.7 million patients were awaiting corneal transplantation, with only one donor cornea available for every 70 needed and approximately 53% of the world's population lacking access to corneal transplantation services ¹. In its 2022 Package of Eye Care Interventions, the World Health Organization also highlighted limitations in corneal donation, eye-banking capacity, and access to transplantation services in many countries. The persistent shortage of donor corneas therefore remains a major clinical impetus for the development of tissue-engineered corneal substitutes and bioengineered artificial corneas ².

The development of artificial corneas has spanned more than two decades ³. Various materials and techniques have been developed, including poly (ethylene glycol) (PEG), polycaprolactone (PCL), poly (vinyl alcohol) (PVA), and poly (lactic-co-glycolic acid) (PLGA), as well as natural macromolecules such as collagen, gelatin, alginate, and hyaluronic acid, to create functional tissues that can replace natural corneas. Additionally, decellularized corneas and self-assembled molecules have been explored. Recently, corneal bioprinting has been used to evaluate corneal tissue repair and replacement. An early report by Kim in 2014 described a 3D-bioprinted corneal stromal prototype using fibrinogen-hyaluronic acid hydrogel and limbal keratocytes. The results revealed that the cells resembled in vivo human stromal tissue both morphologically and biologically ⁴. A key consideration in corneal bioprinting is the bioink, which must balance optical transmittance, physicochemical properties, and biomechanical performance. Gelatin methacryloyl (GelMA) is a modified form of gelatin that offers excellent biocompatibility, easy customization, and a wide range of applications. GelMA-based hydrogels are characterized by their biocompatibility, biodegradability, noncytotoxicity, and nonimmunogenic properties, making them highly suitable for biomedical applications. The three-dimensional network structure of GelMA mimics that of the natural extracellular matrix, creating an ideal environment for three-dimensional cell growth. Additionally, GelMA is a versatile biomaterial that can be modified as needed for specific applications ⁵. The compressive modulus of GelMA can be precisely tuned by adjusting the level of methacryloyl substitution or by adding inorganic or organic additives ⁶. Corneal stromal biofabrication has progressed from early collagen-based cell-laden constructs ^{7,8} to GelMA-based and composite hydrogel systems, including conventional GelMA ⁹, fiber-reinforced GelMA ¹⁰, corneal decellularized extracellular matrix-GelMA ¹¹, GelMA/collagen ¹², and GelMA/carboxymethyl chitosan formulations ¹³. These studies have demonstrated advances in printability, optical properties, stromal cell phenotype, stromal architecture, and in vivo tissue response, with preclinical evaluation of 3D-printed cell-laden corneal constructs in rabbit models also reported ^{14,15}. Collectively, these findings highlight the need to balance printability, optical

transparency, material properties, and stromal cell responses when designing bioinks for corneal stromal engineering.

To improve the mechanical strength of biomaterials, several strategies can be employed, such as polymer crosslinking^{16,17}, the application of composite material structures^{18,19}, and the introduction of dynamic chemical bonds²⁰⁻²². Among these methods, biomaterials with dynamic covalent bonds (DCBs) have attracted significant attention because of their self-healing ability and potential for fabrication and synthesis. These materials can exhibit various advanced properties, including self-healing and shape memory properties, increased toughness, the capacity to relax stresses and the ability to transition between different macromolecular architectures²³. Numerous examples of dynamic covalent bonds suitable for self-healing polymers include oxime, acylhydrazone, imine, disulfide, and boronic/boronate ester bonds²⁴. Boronic/boronate esters are formed through reactions between boronic acid and 1,2- or 1,3-diols, generating boron–oxygen (B–O) bonds. Recently, some natural polymers and hydrogels have been modified with boronic/boronate esters to improve their mechanical strength, making them more resilient and durable and broadening their applications in structural materials.

The success of corneal bioprinting depends not only on the physicochemical properties of the scaffold but also on the phenotype of the incorporated corneal stromal cells (CSCs). During *in vitro* culture, CSCs may undergo phenotypic activation toward fibroblastic or myofibroblastic states, particularly under serum-rich conditions²⁵. Myofibroblastic activation is characterized by increased expression of alpha-smooth muscle actin (α -SMA), whereas less activated stromal cells retain greater expression of keratocyte-associated proteins, including corneal crystallins such as ALDH3A1. Phenotypic activation is also accompanied by changes in extracellular matrix remodeling and matrix metalloproteinase activity²⁶. Although maintaining a keratocyte-associated phenotype during *in vitro* expansion remains challenging, culture conditions that limit myofibroblastic activation may help preserve a less activated stromal phenotype. Mehta et al. reported that modulation of serum concentration and supplementation with amniotic membrane-derived factors can help preserve keratocyte-associated characteristics during CSC culture²⁵. In the present study, CSC phenotype was therefore evaluated, in addition to cell viability, as a complementary biological parameter relevant to the development of cell-laden corneal constructs.

This study aimed to develop and characterize a composite hydrogel based on boronic/boronate ester chemistry for corneal bioprinting and 3D cell culture. GelMA, PBA@G, and PVA were combined to generate a photocrosslinkable formulation, and its rheological behavior, transparency, handling characteristics, and cytocompatibility were evaluated. The resulting 10G10P1PVA formulation was the primary focus of this study. To further assess its applicability as a cell-laden construct, CSC viability, cytoskeletal organization, and phenotype-associated marker expression were evaluated. Finally, a rabbit anterior lamellar keratectomy model was used for a preliminary *in vivo* evaluation of early ocular response and the possible contribution of incorporated CSCs to corneal repair.

2. Materials and methods

2.1. Materials

Gelatin from porcine skin, polyvinyl alcohol (PVA), phenylboronic acid (PBA), N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), dimethyl sulfoxide (DMSO), sodium bicarbonate, sodium chloride (NaCl), methacrylic anhydride (MAA), glycine, deuterium oxide (D₂O), and 2,4,6-trinitrobenzenesulfonic acid (TNBS) solution (5 w/v% in water) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HCl, sodium dodecyl sulfate (SDS), and NaOH were purchased from J.T. Baker (Phillipsburg, NJ, USA). N-Morpholinoethanesulfonic acid (MES) was purchased from Acros Organics (Geel, Belgium). 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Collagenase IV was purchased from Worthington Biochemical Corp. (Vassar Ave, Lakewood, CO, USA). Dialysis bags (molecular weight cutoff of 3.5 and 12–14 kDa) were purchased from Rainbow Biotechnology, Taipei, Taiwan. A Millex-HA Filter Unit for a 33-mm-diameter sterile syringe filter with a 0.45- μ m pore size mixed cellulose ester membrane was purchased from Merck (Darmstadt, Germany). Dulbecco's phosphate-buffered saline, Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F12), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and penicillin–streptomycin were purchased from Gibco (Grand Island, NY, USA). Double-distilled water was obtained using an Arium Advance Pure Water System (Sartorius, Göttingen, Germany) and prepared with a minimum resistivity of 18.2 M Ω ·cm.

2.2. Synthesis of GelMA and PBA@G.

Gelatin was modified with MAA using an alkaline buffer solution, as described by Chou et al. and Shirahama et al., with slight modifications^{27,28}. In brief, gelatin (type A, 300 bloom) was dissolved in carbonate–bicarbonate (CB) buffer (250 mM, pH 9) and stirred at 50 °C until the solution was uniform. Next, MAA (10 w/v%; 0.04 mL per gram of gelatin) was added to the gelatin solution, which was magnetically stirred at 500 rpm for 3 hours. The pH was then adjusted to 7.4 with 6 N HCl to halt the reaction. After filtration, dialysis with Slide-A-Lyzer cassettes, and lyophilization, the GelMA samples were stored at –20 °C until further use.

PBA@G was synthesized by modifying gelatin with PBA through an NHS/EDC-mediated carbodiimide crosslinking reaction, following the procedure of Liu et al. with slight modifications²⁹. First, 1.0 g of gelatin was dissolved in 50 mL of pure water in a 45 °C bath. Additionally, 100.0 mg of PBA, 50 mg of NHS, and 100 mg of EDC were dissolved in 3 mL of DMSO at 60 °C for 5 hours for activation. The activated PBA solution was then added dropwise to the gelatin solution, and the reaction proceeded for 24 hours in a 37 °C oven. The product was dialyzed in a dialysis bag (molecular weight cutoff of 3.5 kDa; Rainbow Biotechnology, Taipei, Taiwan) for 4–5 days to remove the side products and then freeze-dried by freezing followed by lyophilization (HyperCOOL HC3055; LabTech Srl, Sorisole, Italy) for 3 days to yield a foam-like powder, which was stored at –20 °C.

2.3. Fabrication of a Photocrosslinking Device and Mixing the Hydrogel with a Photoinitiator.

A custom 3D-printed UV light-emitting device was fabricated to provide 365-nm illumination for photocrosslinking (**Fig. S1**). The housing was printed from polylactic acid using an Ender-3 V2 printer (Creality 3D, Shenzhen, China), and a modular LED was mounted and secured in the device.

For photocrosslinking, GelMA (20%, w/v), PBA@G (20%, w/v), and PVA (2%, w/v) were dissolved in ultrapure water to prepare a polymer precursor solution. An Irgacure 2959 solution (2%, w/v) was then mixed with the precursor at a 1:1 (v/v) ratio. The resulting working solution was vortexed for 30 s and incubated at 40 °C for at least 20 min. The precursor containing 1% Irgacure 2959 underwent sol–gel transition within 2 min under 365-nm irradiation.

2.4. Nuclear Magnetic Resonance Spectroscopy, Fourier Transform Infrared Spectroscopy, and Scanning Electron Microscopy.

To verify substitution, ¹H nuclear magnetic resonance (NMR) spectroscopy was performed as previously described²⁷. The gelatin, GelMA, and PBA@G samples were each dissolved to a concentration of approximately 20 mg/mL in deuterium oxide. The chemical shifts of each sample were measured at 37 °C using a 600 MHz NMR spectrometer (AVANCE III HD 600, Bruker, Switzerland).

The compositions of the gelatin, GelMA, and PBA@G samples were examined with an Agilent Cary 630 Fourier transform infrared (FTIR) instrument (Agilent Technologies Inc., Santa Clara, CA) between 4000 and 650 cm⁻¹ in attenuated total reflection mode at room temperature.

Scanning electron microscopy (SEM) was used to compare the microstructures of the crosslinked 10G, 10G10P, and 10G10P1PVA hydrogels. The hydrogels were photo-crosslinked after mixing with 1% Irgacure 2959 and exposure to 365 nm UV light for 2 min. The samples were then frozen for 2 days at –80 °C, followed by lyophilization at –50 °C for 3 days to obtain foamy fragments. SEM images were obtained via field-emission SEM (FE-SEM) (Hitachi SU8220, Tokyo, Japan).

2.5. Determination of the Degree of Functionalization.

To quantify the degree of functionalization (DoF), a TNBS assay was performed following the method of Habeeb et al., as modified by Lee and Shirahama^{28,30}. In brief, the GelMA and gelatin samples were separately dissolved in 0.1 M sodium bicarbonate buffer (pH 8.5) to a concentration of 1.6 mg/mL. Each solution (250 µL) was then mixed with a 0.2% TNBS solution (in 0.1 M sodium bicarbonate buffer), and the mixture was incubated at 37 °C for 3 hours. Next, 250 µL of 10% SDS (w/v) and 125 µL of 1 M HCl were added to each solution to stop the reaction. The absorbance of each sample was measured at 335 nm. A standard curve of glycine was then plotted to determine the amino group concentration with sample solutions at concentrations of 0, 0.8, 8, 16, 32, and 64 µg/mL.

2.6. Rheological Analysis.

The rheological properties of the gelatin, GelMA, and PBA@G hydrogels were assessed using an MCR 302e rheometer (Anton Paar Physica, Ostfildern, Germany) with a PP25 Measuring Parallel Plate accessory at DG26.7. The cylinder was tested with the P-PTD220 Air and C-PTD 200 Heating rheometer systems. The experimental conditions included a shear rate of 0.1 to 100 s⁻¹ at 20 °C for the flow curve, an amplitude sweep with 0.01 to 1000% shear strain at an angular frequency of 10 s⁻¹ at 20 °C, and a shear strain of 2% at a frequency of 1 Hz over the temperature range of 20 °C to 40 °C.

2.7. Transparency, degradation, and swelling tests.

Optical transmittance of photo-crosslinked 10G10P1PVA containing 1% Irgacure 2959 was measured at 37 °C using a Synergy HTX Multi-Mode Reader (Agilent Technologies, Inc., CA, USA). A 120 µL aliquot of the hydrogel sample was added to each well of a 96-well microplate (Corning 3635, Corning Inc., NY, USA), resulting in a hydrogel thickness of approximately 3.8 mm. Absorbance was spectrally scanned from 400 to 700 nm. A photograph showing the visibility of letters through the hydrogel was retained as a qualitative reference. The transmittance was determined using formula 1:

$$\text{Transmittance (\%)} = 10^{(2-A)} \quad (1)$$

For the in vitro degradation assay, approximately 300 µL of each hydrogel sample was lyophilized and weighed as M₀. The samples were then incubated at 37 °C in 1× PBS (pH 7.4) containing 20 µg/mL type IV collagenase. At each time point, the samples were removed, washed with pure water, lyophilized, and weighed as M_t. The degradation rate was determined using formula 2:

$$\text{Degradation rate (\%)} = [(M_0 - M_t) / M_0] \times 100\% \quad (2)$$

The swelling properties of the hydrogels were evaluated using lyophilized samples of 10% GelMA, 10G10P, and 10G10P1PVA. The initial dry weight of each sample was recorded as W_d. The samples were immersed in double-distilled water at room temperature for 24 h and subsequently removed from the solution. Excess surface liquid was gently removed, and the swollen samples were immediately weighed to obtain the swollen weight (W_s). The swelling ratio was calculated using formula 3:

$$\text{Swelling ratio (\%)} = ((W_s - W_d) / W_d) \times 100\% \quad (3)$$

2.8. Human corneal stromal cell preparation

Human corneal keratocytes were purchased from Innoprot (P10972; Bizkaia, Spain) and used as human corneal stromal cells (CSCs). Cells were initially maintained in DMEM/F12 (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) supplemented with 2% FBS (Gibco 10437028, Thermo Fisher Scientific), 1% ITS (Sigma, Merck, Darmstadt, Germany), 1.25 µg/mL amphotericin B (Invitrogen), 25 µg/mL gentamicin (Sigma), and 0.02 µg/mL EGF (PeproTech, Thermo Fisher Scientific). Upon reaching approximately 50% confluence, the cells were cultured under two different conditions to generate distinct phenotypic states, with the CSC-F culture

condition adapted from the approach described by Mehta et al.²⁵. CSCs maintained under conditions designed to limit myofibroblastic activation were designated CSC-F, whereas CSCs cultured under conditions intended to induce a myofibroblast-like phenotype were designated CSC-M. For CSC-F preparation, cells were cultured in DMEM/F12 supplemented with 5% FBS, 10 μ M SB431542 (Sigma), 10 μ M Y27632 (Sigma), and 5 μ g/mL amniotic stromal extract. For CSC-M induction, cells were cultured in DMEM/F12 supplemented with 10% FBS. The phenotypic differences between CSC-F and CSC-M were subsequently evaluated by cell morphology, α -SMA expression, MMP-2 secretion, and phenotype-related gene expression. Culture media and cells were collected for the corresponding downstream analyses.

2.9. Matrix metalloproteinase-2 (MMP-2) ELISA

An MMP-2 ELISA Kit (QuickZyme Bioscience, Leiden, Netherlands) was used to quantify MMP-2 levels in the culture media of CSC-F and CSC-M. The standards were diluted in series, and the test samples were diluted 10-fold with assay buffer. One hundred microliters of each sample was added to the MMP-2 ELISA plate and incubated at 4 °C overnight. The plate was subsequently washed four times with wash buffer, after which 50 μ L of APMA and 50 μ L of detection solution were added to measure the T0 (0-hour) value at an OD of 405 nm. The plate was then incubated at 37 °C for 4 hours to obtain the T4 data.

2.10. Evaluation of cell viability and morphology

The artificial cornea test materials were divided into three groups: 10% GelMA (10G), 10% GelMA with 10% PBA@G (10G10P), and 10% GelMA with 10% PBA@G and 1% PVA (10G10P1PVA). CSCs at a density of 1×10^6 cells/mL were mixed with the hydrogel formulations. Cell viability was evaluated on days 0, 1, and 7 using a Live/Dead Kit (IMT Formosa New Materials, Kaohsiung, Taiwan), and the stained cells were examined using a confocal microscope (Leica TCS SP8X, Wetzlar, Germany).

Cell metabolic activity was additionally assessed on day 7 using a Cell Counting Kit-8 (CCK-8) assay (IMT Formosa New Materials) according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader, and the values were normalized to those of the 10G group.

For evaluation of cell morphology and cytoskeletal organization, cell-laden hydrogels cultured for 7 days were washed with $1 \times$ PBS and fixed with 4% paraformaldehyde (PFA) containing 0.1% Triton X-100 for 60 min. After washing with PBS, the samples were permeabilized with 1% Triton X-100 in PBS for 60 min. F-actin and nuclei were subsequently stained with $1 \times$ Alexa Fluor 488 phalloidin (Invitrogen, Thermo Fisher Scientific, A12379) and DAPI in PBS for 60 min. After washing with PBS, fluorescence images were acquired by confocal microscopy.

2.11. Quantitative real-time PCR

Total RNA was isolated from the indicated CSC cultures using TRIzol reagent. Complementary DNA (cDNA) was synthesized using the PrimeScript RT Reagent Kit (TaKaRa) according to the manufacturer's instructions. Quantitative real-time PCR was performed using KAPA SYBR FAST ABI Prism 2X qPCR Master Mix (Kapa Biosystems/Roche). The expression of ACTA2, encoding α -smooth muscle actin (α -SMA), and ALDH3A1 was evaluated as markers of myofibroblastic activation and corneal stromal phenotype, respectively. ACTB was used as the internal reference gene. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences are listed in Table S3.

2.12. Western blotting

Cells were lysed in RIPA buffer supplemented with inhibitors (10 mM NaF, 10 mM Na₃VO₄, and a 1× protease inhibitor cocktail; Sigma–Aldrich). The lysates were kept on ice, transferred to microtubes, disrupted by brief sonication, and clarified by centrifugation at maximum speed for 15 min at 4 °C. The supernatants were collected as total protein extracts. The samples were separated by 10% acrylamide SDS–PAGE and electrotransferred onto PVDF membranes (MilliporeSigma, Burlington, MA, USA). The membranes were blocked with 5% skim milk in TBST (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.05% Tween-20), incubated overnight at 4 °C with the indicated primary antibodies, and then treated with the appropriate HRP-linked secondary antibodies. Immunoreactive bands were visualized using ECL reagents (GE Healthcare, Chalfont St Giles, UK).

2.13. Printing the artificial cornea with a BioX 3D printer

The artificial cornea was produced using a BIO X 3D bioprinter (Cellink, Gothenburg, Sweden). A cornea-shaped groove mold was fabricated as the base for bioprinting (Fig. S2). The scanned corneal mold was digitized and processed using 3D modeling software to generate a reverse model of the artificial cornea. Printing used a 3-mL syringe and 25 G blunt needle (nozzle diameter, 0.41 mm) at a syringe temperature of 33.5 °C, bed temperature of 15 °C, extrusion pressure of 20 psi, and printing speed of 3 mm/s; the programmed layer height was set to 50% of the nozzle diameter (0.205 mm). The Fig. S2F construct was printed as a single layer and photocrosslinked at 365 nm for 120 s. The UV wavelength and exposure time were recorded, whereas irradiation intensity was not independently measured in the present experiment.

Printing began at the center of the groove mold, within the path that expanded outward in concentric circles. After each full circle, the BioX printer was programmed to apply UV light for 10 seconds to cure the printed layer before moving on to the next printing step. Finally, a transparent, concave, disc-shaped artificial cornea was printed after several cycles and stored in 1× PBS.

2.14. Animal experiments

To evaluate the effect of the cell-seeded hydrogel on corneal repair, a rabbit anterior lamellar keratectomy (ALK) model was established. Animal experiments were conducted in compliance

with the Chang Gung Memorial Hospital Animal Research Guidelines and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Ethical approval was obtained from the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital (No. 2022022303).

For the in vivo implantation experiments, rabbit corneal stromal cells (RCSCs) were used instead of the human CSCs employed in the in vitro studies to avoid xenogeneic immune responses. RCSCs were isolated from rabbit corneal tissues according to a previously described method³¹. Briefly, central corneal buttons were prepared from Descemet membrane-stripped corneas and incubated with 5 mg/mL dispase II at 4 °C overnight. After removal of the loosened epithelial sheets, the stromal discs were cut into small pieces and digested with 2 mg/mL collagenase I for 24 hours at 37 °C. Cell pellets were collected by centrifugation at 1000 rpm for 5 minutes at 25 °C and resuspended in RCSC medium consisting of DMEM supplemented with 10% FBS and 25 µg/mL gentamicin. The cells were cultured on FNC-coated plates at 37 °C with 5% CO₂ and subcultured for preparation of the Cell-Full constructs.

A total of eight New Zealand White rabbits were used in the animal experiments associated with this study. Three rabbits were used in preliminary implantation and procedure-development experiments and were not included in the final ALK outcome evaluation. The final exploratory ALK evaluation included five rabbits: ALK without grafting (Control, n = 1), ALK followed by transplantation of an allogeneic corneal graft (Graft, n = 2), ALK followed by implantation of a cell-free 10G10P1PVA hydrogel (Cell-Free, n = 1), and ALK followed by implantation of a 10G10P1PVA hydrogel seeded with RCSCs (Cell-Full, n = 1). Rabbits included in the final ALK evaluation weighed 3.36-4.02 kg and were anesthetized by intramuscular injection of a 1:1 mixture of Zoletil (25 mg/mL) and Rompun (10 mg/mL). The anterior corneal lamella, including the epithelium and approximately 150 µm of the anterior stroma, was excised using a 7.5-mm corneal trephine. The rabbits were divided into four ALK groups: ALK without grafting (Control), and ALK followed by transplantation of an allogeneic corneal graft (Graft), a cell-free 10G10P1PVA hydrogel (Cell-Free), or a 10G10P1PVA hydrogel seeded with RCSCs (Cell-Full). External eye photographs and fluorescein staining images were obtained immediately after ALK and treatment on day 0 to document the initial surgical defect. In all four ALK groups, the surgical site was subsequently covered with amniotic membrane and secured with 10-0 sutures for protection. The eyelids were temporarily closed with 6-0 Mersilk sutures. On day 3, the eyelid sutures and amniotic membrane were removed. Corneal recovery was subsequently evaluated by external eye photography and fluorescein staining on days 4 and 7. Normal rabbit corneas without ALK were included as histological references.

2.15. Histological analysis

The corneas were harvested on day 7 after ALK for histological evaluation. Each sample was fixed in 10% buffered formalin for 24 hours, followed by dehydration, paraffin embedding, and sectioning. The tissue sections were subjected to hematoxylin and eosin (H&E) and Masson's trichrome staining. Histological sections were examined to assess epithelial continuity, anterior

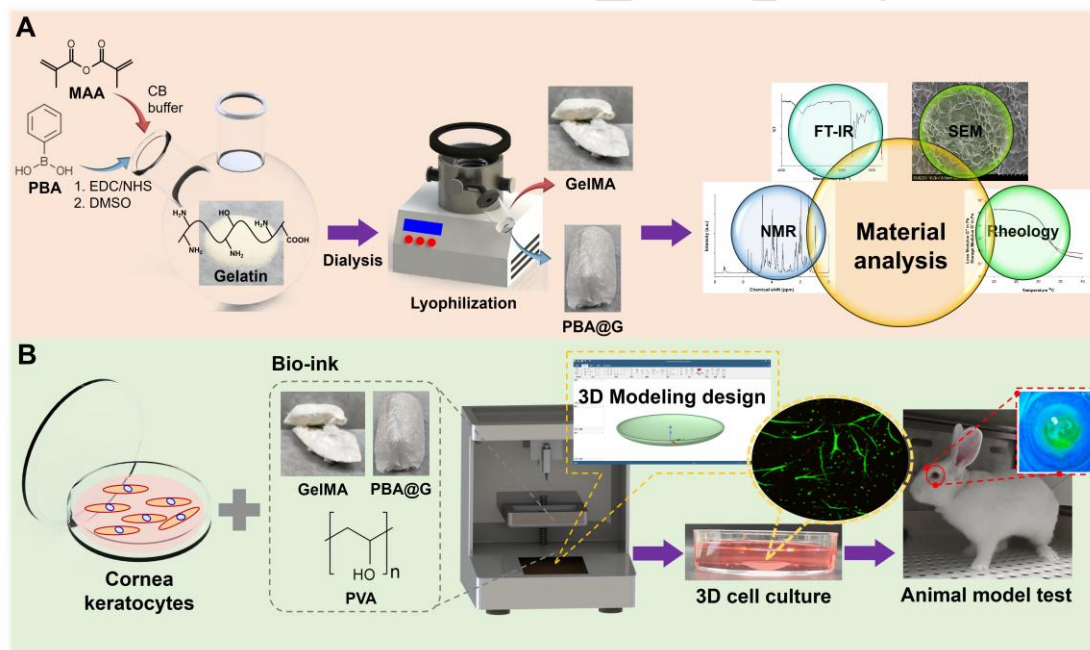
corneal contour, and stromal organization among the experimental groups. Normal rabbit corneas were processed as histological references.

2.16. Statistical analysis

Quantitative data are presented as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using Microsoft Excel. Differences between groups were evaluated using two-tailed Student's t-tests. A p value < 0.05 was considered statistically significant.

3. Results and discussion

An overview of the synthesis of GelMA and PBA@G and the in vivo corneal evaluation is shown in **Scheme 1**. One-pot MAA modification of the gelatin enabled efficient, straightforward GelMA synthesis using a CB buffer system. In addition, the gelatin was modified with PBA using the EDC/NHS method to generate the final product, PBA@G, for this study. To ensure the stability and quality of the synthesized materials, we characterized the compounds using NMR, FT-IR, and SEM to confirm their formation. The rheological, transparency, swelling, and in vitro degradation tests provided baseline characterization of the hydrogel formulations.



Scheme 1. Overview of (A) the synthesis of GelMA and the PBA@G hydrogel and (B) the examination of their biocompatibility in a rabbit model. (GelMA, gelatin-methacryloyl; MAA, methacrylic anhydride; PBA, phenylboronic acid; PBA@G, PBA-modified gelatin; PVA, polyvinyl alcohol).

3.1. Instrumentation and microscopic imaging for compound identification.

^1H NMR spectroscopy of gelatin, GelMA, and PBA@G was performed to characterize the methacrylation, and the DoF of GelMA was quantified (**Fig. 1A–B**). Within the GelMA sample,

the three central regions were analyzed to verify successful modification: (1) the characteristic peaks at approximately 5.4 and 5.7 ppm, corresponding to the methacrylamide double bond-adjacent protons; (2) the peak at 2.9–3.0 ppm, attributed to unreacted methylene protons from lysine residues; and (3) the peak at 1.8–1.9 ppm, corresponding to the methacrylamide single bond-adjacent protons. The agreement between the spectra confirmed the successful construction of the synthesized GelMA²⁸. On the other hand, distinct peaks were observed at approximately 7.5 and 7.8 ppm in the PBA@G spectrum, corresponding to the aromatic protons of the benzene ring. This peak is characteristic of the aromatic hydrogens in PBA@G and is absent in the gelatin and GelMA samples, distinguishing the molecular structure of PBA@G. In the current study, the DoF was calculated for the GelMA samples^{32,33}. The NMR results (DoF = 54.60%) and the results of the TNBS assay revealed a medium DoF of approximately 50–55% for GelMA (Table S1), indicating the high stability and repeatability of its synthesis. According to Xie et al., PBA groups in different PBA@G samples cannot be well quantified by NMR; thus, determining the DoF of PBA@G was difficult³⁴. In general, GelMA can be classified into three types (low, medium, and high) depending on the DoF, with up to 40% substituted amine groups indicating a low DoF, and at least 65% substituted amine groups indicating a high DoF. The DoF significantly affects the physical properties of GelMA, including swelling, stiffness, and enzymatic degradation, with increasing functionalization generally associated with greater stiffness and slower degradation^{35–37}. In this study, we referred to the methods of Liu et al. to modify the hydrogel formula for application in artificial corneal stroma²⁹.

FT-IR was also employed for chemical group identification. As shown in **Fig. 1C**, the broad absorption band observed in the range of 3200–3500 cm⁻¹ corresponds to the stretching vibrations of N-H and O-H bonds. Since gelatin contains amine (-NH₂) and hydroxyl (-OH) functional groups, a broad peak appeared in this wavenumber range for the gelatin sample²⁷. All three materials (gelatin, GelMA, and PBA@G) exhibited absorption peaks within this range, indicating the presence of amine and hydroxyl groups in their structures. The spectral profiles of GelMA and gelatin are relatively similar. In contrast, the shape of the peak in the PBA@G spectrum differed from those of the other two materials, which may be attributed to changes in the hydrogen bond distribution resulting from chemical modification. The characteristic peak at 1650 cm⁻¹ (amide I band) corresponds to the C=O stretching vibration, originating from the amide bonds in gelatin. The peak located at approximately 1650 cm⁻¹ is one of the characteristic absorption bands of gelatin. The presence of this absorption peak in the gelatin, GelMA, and PBA@G samples indicates that the amide bond is retained in all three materials. The absorption peak at approximately 1550 cm⁻¹ (amide II band) is typically due to the N-H bending vibration and C-N stretching vibration, and it is characteristic of the amide bond in the gelatin structure. A slight shift in this peak was observed in the GelMA and PBA@G spectra relative to gelatin, which may reflect changes associated with their respective chemical modifications.

After freeze-drying, the SEM images of three different mixed formulations were compared (**Fig. 1D–F**). All three materials exhibited porous structures following freeze-drying. Compared with the GelMA formulation, the 10G10P formulation had smaller pores. After the addition of 1%

PVA (10G10P1PVA), the pores decreased further in size and became more uniform, producing a denser microstructure than that observed in GelMA. Because SEM was performed after freeze-drying, these images characterize pore morphology but do not directly establish hydrogel mechanical strength. PBA groups can form dynamic boronate ester bonds with the diol groups of PVA. These reversible interactions may contribute to the rheological behavior of the composite hydrogel.

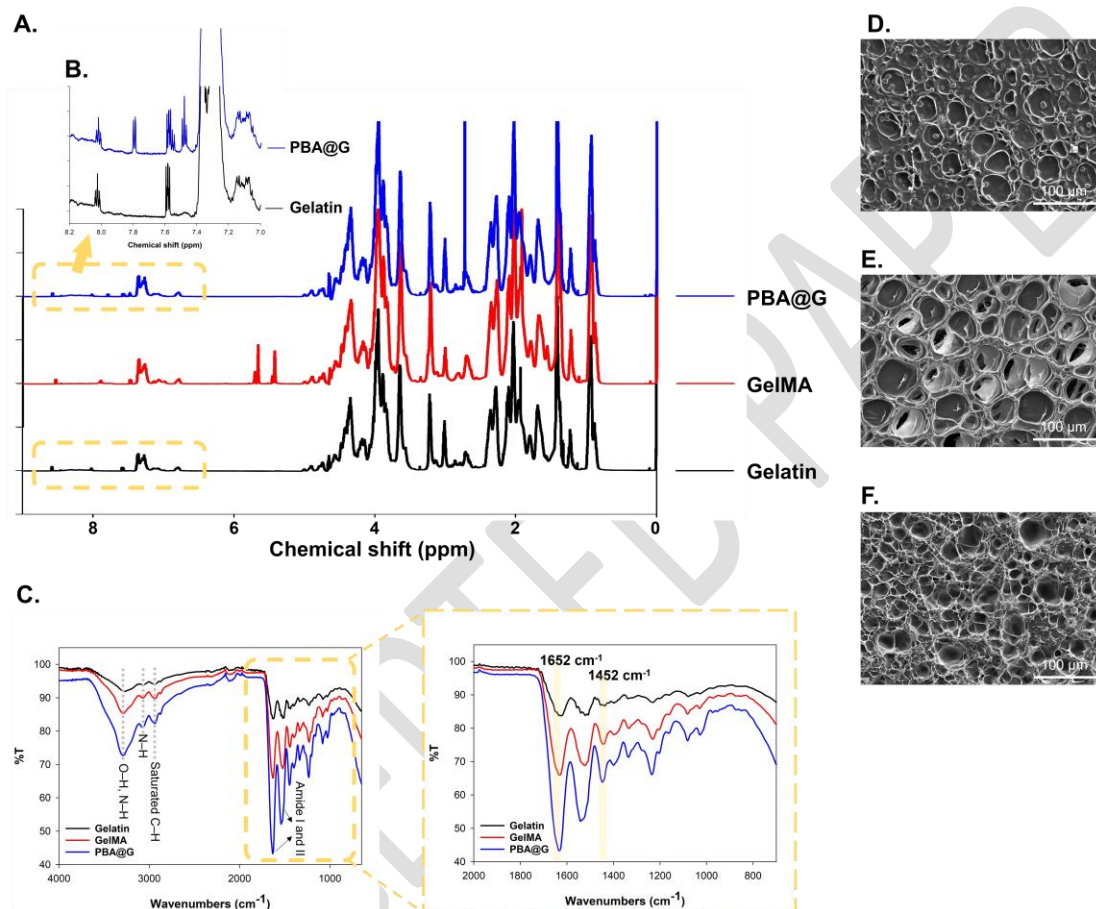


Fig. 1. (A) ¹H NMR spectra of gelatin, GelMA, and PBA@G recorded in D₂O at 37 °C. (B) Comparisons of the NMR signals between gelatin and PBA@G. (C) FT-IR spectra of gelatin, GelMA, and PBA@G measured at room temperature in attenuated total reflection mode. SEM images of the (D) GelMA, (E) 10G10P, and (F) 10G10P1PVA hydrogels. Scale bar = 100 μm.

3.2. Rheological properties, transparency, swelling properties, and degradation of the compounds *in vitro*.

As shown in **Fig. 2A–B**, the sol–gel transition temperatures ($G' = G''$) for gelatin, GelMA, PBA@G, 10G10P, and 10G10P1PVA were 33.44, 32.28, 33.66, 33.00, and 34.23 °C, respectively, indicating that all formulations were in the gel state at room temperature (25 °C). Amplitude sweep testing was used to compare the rheological response of the formulations under the tested shear conditions. The measured shear stress values for gelatin and PBA@G were 1344 and 1397 Pa,

respectively, whereas that of GelMA was 630.7 Pa; 10G10P and 10G10P1PVA showed values of 5589 and 4270 Pa, respectively. These measurements were used for formulation comparison and were not interpreted as direct measures of hydrated tensile, fracture, or implantation-related mechanical strength. The parent material used in this work was type A gelatin with a bloom factor of 300³⁸.

Spectral scanning of photo-crosslinked 10G10P1PVA at 37 °C showed that transmittance increased from 44.57% to 66.22% across the measured range of 400–700 nm, while letters remained visible through the hydrogel as a qualitative reference (**Fig. 2C**). The swelling ratios of lyophilized 10% GelMA, 10G10P, and 10G10P1PVA were $1134.54 \pm 84.72\%$, $711.94 \pm 16.99\%$, and $609.61 \pm 101.59\%$, respectively (**Table S2**). Thus, inclusion of PBA@G and PVA reduced swelling relative to GelMA alone under the tested conditions. Degradation of 10G, 10G10P, and 10G10P1PVA was evaluated in 1× PBS containing 20 µg/mL type IV collagenase at 37 °C (**Figs. 2D and S3**). Approximately 50% degradation occurred after 6 h, and complete degradation was observed by 24 h. Because this assay was performed under an accelerated enzymatic condition, these data reflect susceptibility to collagenase-mediated degradation rather than the in vivo residence time of the hydrogel. The persistence of the implanted construct was not directly quantified in the present study.

The present rheological measurements support formulation comparison for extrusion printing, while hydrated tensile, fracture, fatigue, and suture-related properties remain relevant to implantation-related mechanical assessment. The programmed single-layer height was 0.205 mm, which is of a similar order of magnitude to the approximately 150 µm of anterior stroma removed in the rabbit ALK model. However, the construct was not designed as an exact thickness-matched reconstruction, and actual hydrated construct thickness was not quantitatively measured. Quantitative line width, hydrated thickness, dimensional deviation, and construct-defect geometric matching remain relevant to further evaluation of print fidelity and implantation geometry.

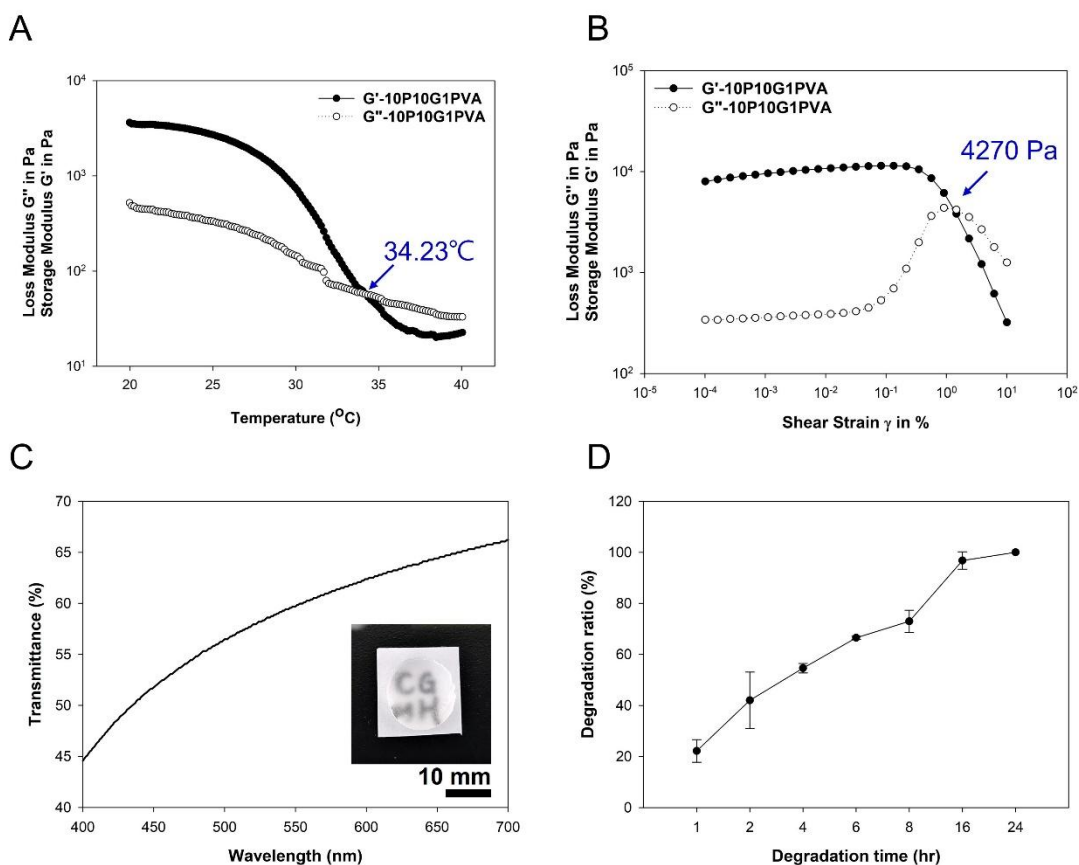


Fig. 2. Rheological analysis of 10G10P1PVA by temperature ramp (A) and amplitude sweep (B) experiments at 20°C . (C) Optical transmittance spectrum of photo-crosslinked 10G10P1PVA at 37°C over 400–700 nm. (D) Accelerated in vitro degradation of 10G10P1PVA in $1\times$ PBS containing $20 \mu\text{g/mL}$ type IV collagenase at 37°C .

3.3. Viability and phenotypic characteristics of corneal stromal cells in GelMA-based hydrogels

CSCs were encapsulated in three GelMA-based hydrogel formulations, 10% GelMA (10G), 10% GelMA with 10% PBA@G (10G10P), and 10% GelMA with 10% PBA@G and 1% PVA (10G10P1PVA), and cultured for 7 days (Fig. 3A). Live/Dead staining performed immediately after photopolymerization (day 0) showed comparable cell survival among the three formulations (10G, 59.00%; 10G10P, 66.52%; and 10G10P1PVA, 72.00%). Cell viability ranged from 56.17% to 66.79% on day 1 and from 64.82% to 78.15% on day 7 across the three formulations. Although the viability percentage in the 10G group increased numerically by day 7, Live/Dead staining alone does not distinguish cell proliferation from changes in the proportion of surviving cells; therefore, metabolic activity was evaluated independently using CCK-8.

The day-7 CCK-8 assay provided a quantitative assessment of cellular metabolic activity. Relative to the 10G group, which was normalized to 100%, the 10G10P and 10G10P1PVA groups showed relative CCK-8 levels of $144.98 \pm 23.69\%$ and $137.23 \pm 11.92\%$, respectively (Fig. 3A). The 10G10P group was numerically higher than 10G but did not reach statistical significance ($p = 0.058$), whereas the 10G10P1PVA group exhibited significantly greater metabolic activity than 10G ($p = 0.020$). Together with the Live/Dead results, these data indicate that incorporation of

PBA@G and PVA did not compromise CSC viability during the 7-day culture period and that the 10G10P1PVA formulation supported higher day-7 metabolic activity than GelMA alone.

Because the elongated morphology observed in the composite hydrogels could not by itself distinguish cell spreading from fibroblastic or myofibroblastic activation, cytoskeletal organization and phenotype-associated gene expression were further examined on day 7 (**Fig. 3B**). Phalloidin staining showed limited spreading of CSCs in 10G, whereas cells in 10G10P and 10G10P1PVA displayed a more elongated morphology with F-actin bundles predominantly oriented along the longitudinal axis of the cells; this organization was most evident in 10G10P1PVA. RT-qPCR provided molecular context for these morphological observations. ACTA2 (α -SMA) expression was markedly increased in the myofibroblast-like reference population (CSC-M), whereas all three hydrogel groups maintained low ACTA2 expression without the pronounced induction observed in CSC-M. In contrast, ALDH3A1, a keratocyte-associated corneal crystallin, was reduced in CSC-M and in the B4G12 negative control. Among the hydrogel groups, ALDH3A1 expression was similar between 10G and 10G10P but was significantly higher in 10G10P1PVA. Taken together, 10G10P1PVA supported CSC survival, metabolic activity, cell spreading, and a phenotype-associated marker profile during the 7-day culture period.

Cellular responses to the matrix must be considered in cell-laden corneal constructs. CSC morphology is strongly influenced by the culture environment and does not reliably distinguish keratocyte, fibroblastic, and myofibroblastic states. We therefore evaluated cell viability, metabolic activity, F-actin organization, and phenotype-associated gene expression rather than relying on morphology alone^{25,26}.

Live/Dead staining and the CCK-8 assay provided different but not conflicting information. Comparable Live/Dead viability indicated that the proportion of viable cells was similar among formulations, whereas the higher CCK-8 signal in 10G10P1PVA indicated greater overall metabolic activity. Because the CCK-8 signal is influenced by both viable cell number and cellular metabolic state, this finding cannot by itself be interpreted as increased cell proliferation. Phalloidin staining confirmed cell spreading and longitudinal F-actin organization within the construct. These features indicate cell-matrix interaction and cytoskeletal adaptation but do not define CSC phenotype. ALDH3A1 expression was higher in CSCs cultured in 10G10P1PVA than in those cultured in 10G or 10G10P. ALDH3A1 is a corneal crystallin expressed by native keratocytes and is reduced when human keratocytes acquire fibroblastic or myofibroblastic repair phenotypes³⁹. Because the same CSC source and culture conditions were used across formulations, this difference suggests that 10G10P1PVA provided an environment that better supported ALDH3A1 expression. The comparison with 10G10P suggests that inclusion of PVA, or the resulting PBA-PVA network, may have contributed to this response, although the responsible matrix property cannot be identified from the present data.

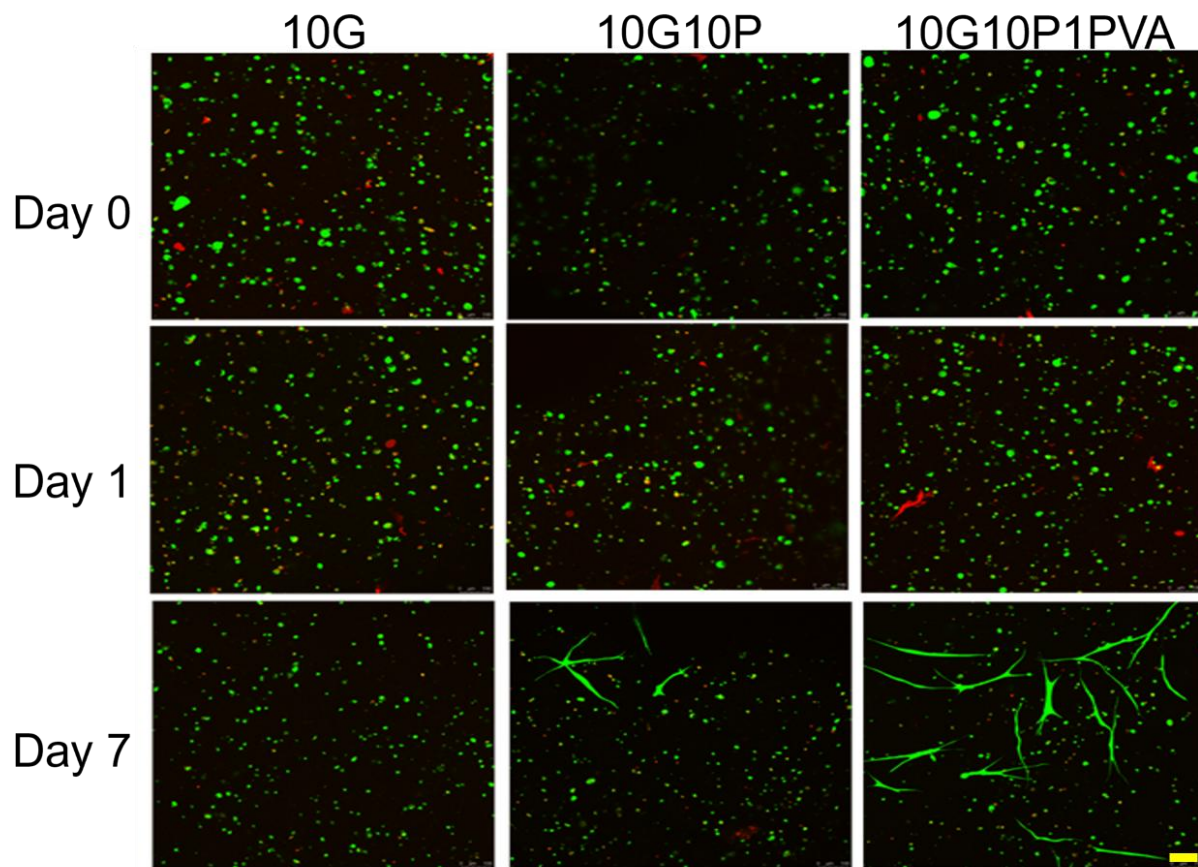
The higher ALDH3A1 expression in 10G10P1PVA was considered together with CSC survival, metabolic activity, cell spreading, and the low ACTA2 expression observed relative to CSC-M. This combined pattern indicates a more favorable short-term CSC response to

10G10P1PVA among the tested hydrogels. However, higher expression of a single marker does not establish stable maintenance of the keratocyte phenotype. The present data therefore describe phenotype-associated responses observed during the 7-day culture period rather than stable phenotype control.

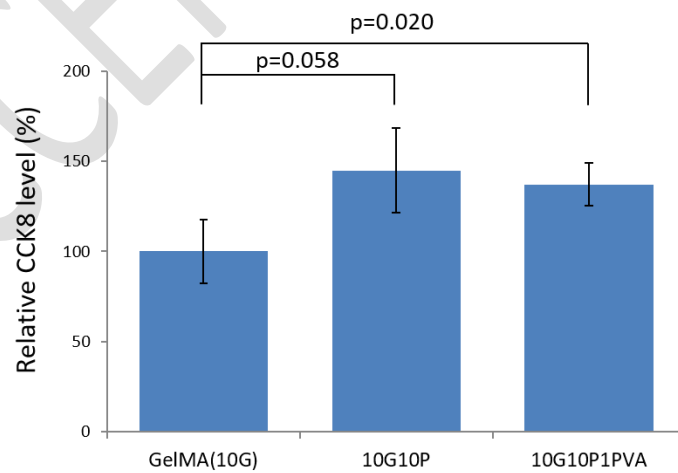
ACCEPTED PAPER

A. Cell viability and metabolic activity

Live/Dead staining

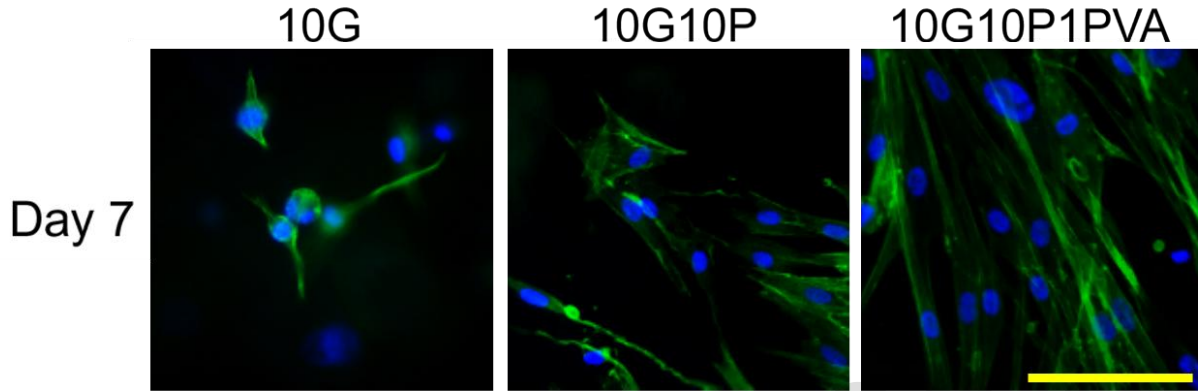


CCK-8 assay (Day 7)



B. Cytoskeletal morphology and phenotypic markers

Phalloidin staining (Day 7)



RT-qPCR (Day 7)

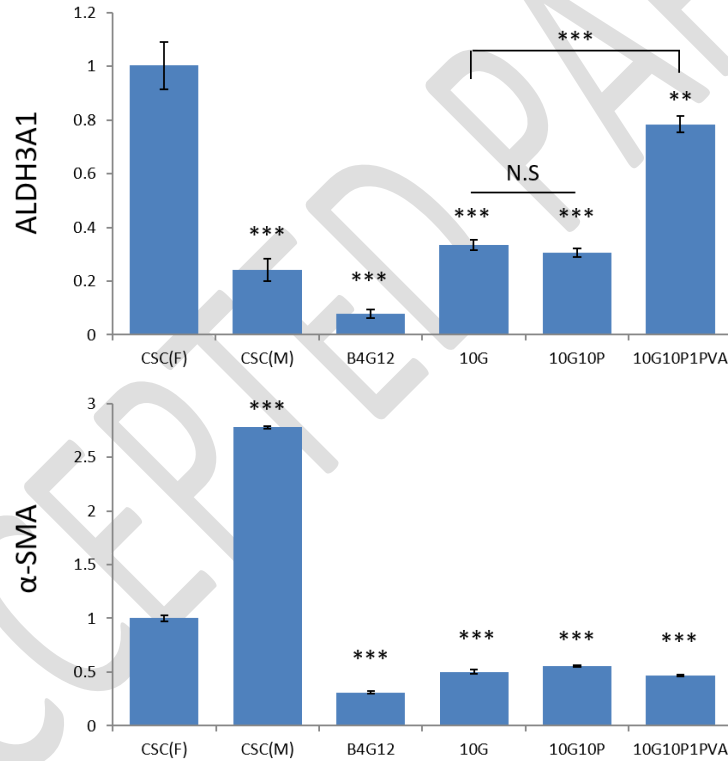


Fig. 3. Viability, cytoskeletal organization, and phenotypic marker expression of human corneal stromal cells (CSCs) cultured in GelMA-based hydrogels. (A) Representative Live/Dead staining images of CSCs cultured in 10G, 10G10P, and 10G10P1PVA hydrogels on days 0, 1, and 7. Live cells are shown in green and dead cells in red. Cell viability remained within a similar range among the three hydrogel formulations at the assessed time points. Cellular metabolic activity was quantified by CCK-8 on day 7 and normalized to the 10G group. The 10G10P group was numerically higher than 10G but did not reach statistical significance ($p = 0.058$), whereas 10G10P1PVA showed significantly greater metabolic activity than 10G ($p = 0.020$). (B) Representative day-7 phalloidin staining of F-actin (green) with DAPI nuclear counterstaining (blue). CSCs in 10G10P and 10G10P1PVA exhibited greater spreading and longitudinal F-actin organization than those in 10G. RT-qPCR analysis of ALDH3A1 and ACTA2 (α -SMA) included CSC-F and CSC-M as phenotypic reference populations; separate characterization of these reference states is shown in Fig. S4. B4G12 was included as a negative control for ALDH3A1. ALDH3A1 expression was significantly higher in

10G10P1PVA than in 10G and 10G10P, whereas the hydrogel groups did not exhibit the marked ACTA2 induction observed in CSC-M. RT-qPCR data are presented as mean $\pm$ SD. **p < 0.01 and ***p < 0.001; N.S., not significant. Scale bars = 100 μ m.

Given the potential relationship between stromal cell state and matrix remodeling, CSCs cultured under conditions intended to limit myofibroblastic activation (CSC-F) or induce a myofibroblast-like state (CSC-M) were characterized separately (Fig. S4). CSC-F and CSC-M showed distinct morphologies and α -SMA band intensities, while MMP-2 levels were numerically higher in CSC-M. These observations indicate that the culture conditions generated distinguishable reference states. Corneal keratocytes, fibroblasts, and myofibroblasts have distinct transcriptional programs and matrix-remodeling characteristics⁴⁰. MMP activity has also been shown to participate in corneal keratocyte spreading, migration, and local extracellular matrix remodeling in three-dimensional collagen matrices⁴¹. The present reference experiment did not test how either state affected the hydrogel. These in vitro reference states were not extrapolated to molecular expression in rabbit corneal tissue after implantation.

3.4. Corneal healing after anterior lamellar keratectomy in rabbits

The 10G10P1PVA hydrogel was evaluated in a rabbit anterior lamellar keratectomy (ALK) model over a 7-day postoperative period. Following removal of the corneal epithelium and anterior stroma, the defects were left without grafting (Control) or treated with an allogeneic corneal graft (Graft), a cell-free 10G10P1PVA hydrogel (Cell-Free), or a 10G10P1PVA hydrogel containing rabbit corneal stromal cells (Cell-Full). External eye photographs and fluorescein staining were used to monitor postoperative recovery (**Fig. 4**). Mild central corneal haze remained visible during the 7-day observation period, whereas no additional gross ocular abnormalities were apparent on external examination. On day 4, fluorescein retention remained evident in the Control, Cell-Free, and Cell-Full groups, whereas little to no retention was observed in the Graft group. By day 7, fluorescein retention was no longer apparent in the Control, Graft, or Cell-Full group, whereas residual staining remained detectable in the Cell-Free group. Because fluorescein staining primarily reflects epithelial barrier integrity, these observations support short-term differences in epithelial barrier recovery rather than an assessment of overall corneal healing.

All ALK groups received temporary amniotic membrane coverage and eyelid closure to protect the surgical area and implanted constructs during the early postoperative period. Amniotic membrane has been reported to provide a substrate for epithelial migration and re-epithelialization and to contain bioactive components that modulate inflammation and wound healing⁴². We therefore cannot exclude a shared effect of the amniotic membrane on the early healing environment during the first 3 days. However, the membrane and eyelid sutures were removed on day 3 in all ALK groups, making this a common early postoperative condition. Under this shared condition, the less fluorescein retention observed in the Cell-Full group than in the Cell-Free group at day 7 supports the possibility that CSCs may have provided an additional contribution to early corneal repair.

The difference between the Cell-Full and Cell-Free groups may reflect biological activity of the incorporated RCSCs in addition to the hydrogel scaffold. Corneal repair involves coordinated epithelial-stromal interactions, and stromal cells can influence epithelial migration and proliferation through paracrine signaling and extracellular matrix remodeling^{26,43}. Hepatocyte growth factor and keratinocyte growth factor are well-characterized mediators of stromal-to-epithelial signaling in the cornea⁴³. The RCSC-containing construct may therefore provide temporary cell delivery and a local cellular microenvironment during the early postoperative period. The accelerated in vitro degradation assay does not define the in vivo persistence of the construct, and a day-7 difference does not require the hydrogel to remain intact throughout the entire 7-day period. Within the present observation window, the less fluorescein retention in the Cell-Full group than in the Cell-Free group supports a possible contribution of the cellular component to early repair. Longer-term follow-up with standardized slit-lamp haze grading, OCT-based corneal thickness measurements, and corneal topography will be important to evaluate later stromal remodeling and the stability of corneal transparency, thickness, and curvature.

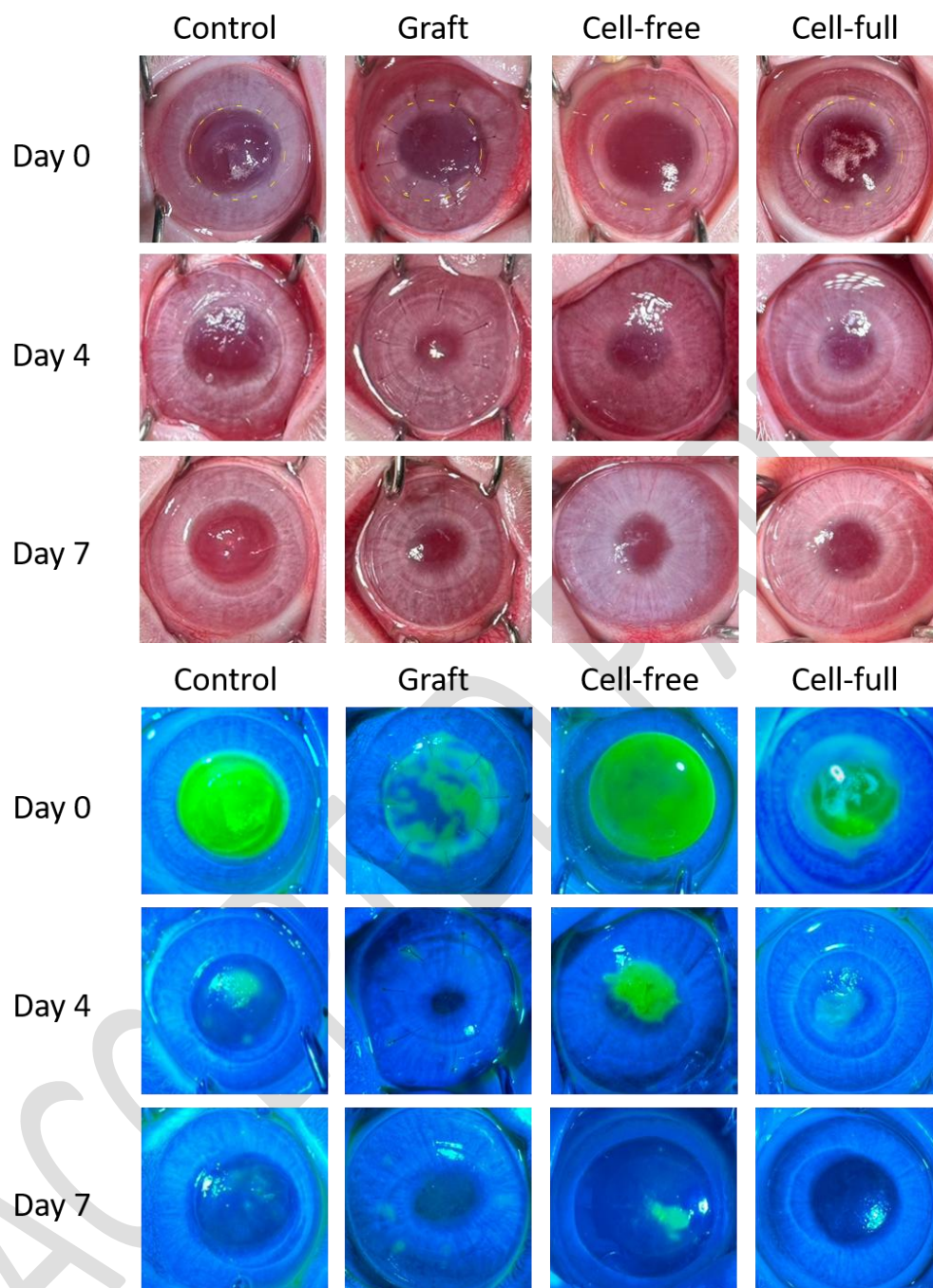


Fig. 4. Corneal healing after anterior lamellar keratectomy (ALK) in rabbits. External eye photographs (upper panel) and fluorescein sodium staining images (lower panel) are shown for the Control, Graft, Cell-Free, and Cell-Full conditions. Images were obtained on days 0, 4, and 7 after ALK, as indicated from top to bottom within each panel. Day 0 images were obtained after the respective treatment was placed and before temporary amniotic membrane coverage. Yellow dashed circles in the day 0 external photographs indicate the ALK area. The Control group underwent ALK without grafting, whereas the Graft, Cell-Free, and Cell-Full groups received an allogeneic corneal graft, a cell-free 10G10P1PVA hydrogel, or a rabbit CSC-seeded 10G10P1PVA hydrogel, respectively. On day 4, fluorescein retention remained evident in the Control, Cell-Free, and Cell-Full groups, whereas little to no retention

was observed in the Graft group. By day 7, fluorescein retention was no longer apparent in the Control, Graft, or Cell-Full group, whereas residual staining remained detectable in the Cell-Free group.

3.5. *Histological analysis*

Masson's trichrome staining was performed to evaluate corneal tissue organization 7 days after ALK (Fig. 5). These sections were used for morphological comparison of tissue architecture rather than quantitative pachymetric measurement; therefore, absolute postoperative corneal thickness was not inferred from the histological sections. Morphologically, the Normal group showed a full-thickness corneal profile, whereas the Control group showed a thinner tissue profile with an irregular anterior corneal contour after removal of the epithelium and approximately 150 μm of the anterior stroma. The Graft group exhibited a relatively smooth anterior surface and a visible graft-host interface. No complete hydrogel layer could be clearly identified in the day-7 sections of the Cell-Free or Cell-Full groups. In the Cell-Free group, the anterior contour remained markedly irregular with an uneven stromal profile, whereas the Cell-Full group showed a smoother anterior contour and more uniform stromal architecture. Continuous epithelial coverage was present in all ALK groups at day 7, although epithelial thickness and organization did not uniformly resemble those of the normal cornea. No conspicuous dense inflammatory cell accumulation was apparent within the stromal tissue in the representative sections.

The more regular tissue architecture in the Cell-Full group compared with the Cell-Free group, together with the lower fluorescein retention observed on day 7, supports a possible additional contribution of the RCSC-containing construct during early corneal repair. Because all ALK groups received the same temporary amniotic membrane coverage and eyelid closure, these differences occurred under a common postoperative healing condition. Masson's trichrome staining does not identify specific inflammatory cell populations or quantify α -SMA or MMP expression, and tissue expression of these markers was not measured in the rabbit corneas in the present study. Accordingly, the histological findings are interpreted in terms of epithelial continuity, anterior corneal contour, and stromal organization rather than tissue-level molecular regulation.

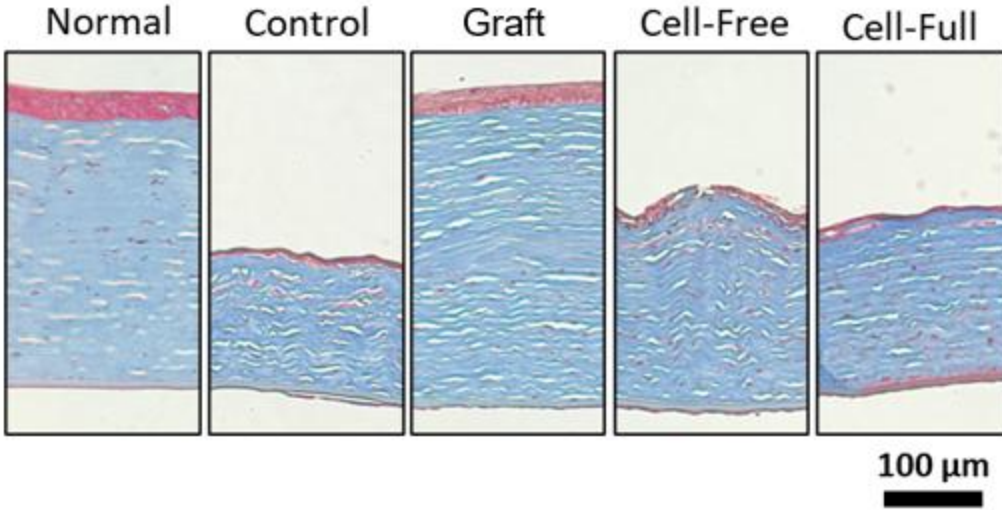


Fig. 5. Histological evaluation of corneal healing after anterior lamellar keratectomy (ALK). Representative Masson's trichrome-stained corneal sections are shown for the Normal, Control, Graft, Cell-Free, and Cell-Full conditions. Corneas from the four ALK groups were harvested 7 days after surgery, with normal cornea included as a reference. The Control group underwent ALK without grafting, whereas the Graft, Cell-Free, and Cell-Full groups received an allogeneic corneal graft, a cell-free 10G10P1PVA hydrogel, or an RCSC-seeded 10G10P1PVA hydrogel, respectively. The sections were used for morphological comparison rather than quantitative pachymetric measurement. Continuous epithelial coverage was present in all ALK groups. The Cell-Free group exhibited a markedly irregular anterior contour and uneven stromal profile, whereas the Cell-Full group showed a smoother anterior contour and more uniform stromal architecture. No conspicuous dense inflammatory cell accumulation was apparent in the representative hydrogel-treated sections. All panels are shown at the same magnification and use the same 100 μm scale bar.

To clarify the position and contribution of the present study within the literature on corneal stromal bioinks and related material systems, representative studies are summarized in Table 1. As shown in Table 1A, corneal stromal bioinks have been developed using GelMA, corneal dECM/GelMA, GelMA/collagen, GelMA/carboxymethyl chitosan, and other composite systems. Table 1B summarizes material and bioprinting precedents directly relevant to the GelMA/PBA@G/PVA design, including PVA-containing corneal hydrogels, PBA-functionalized gelatin/PVA boronate ester hydrogels, boronate ester-based printable bioink, and PBA-modified GelMA systems. These studies establish prior use of PVA in corneal hydrogels, PBA–diol dynamic networks, boronate-mediated crosslinking in printable GelMA-containing bioink, and PBA-modified GelMA. For extrusion-based corneal stromal bioprinting, however, a bioink must exhibit rheological behavior compatible with extrusion and, after printing, maintain construct geometry in an aqueous environment while providing a cell-compatible microenvironment. PBA@G and PVA can form a reversible boronate ester network through PBA–diol interactions. We therefore incorporated this dynamic network into GelMA to modulate rheological and hydration behavior while retaining GelMA photocrosslinking. Among the studies summarized, related material chemistries and boronate-based printable systems have been reported in other applications, but the GelMA/PBA@G/PVA combination has not been reported as a CSC-laden corneal stromal bioink. In addition, although some corneal bioink

studies have evaluated stromal cell phenotype, the present study incorporated assessment of both keratocyte-associated and myofibroblast-associated phenotypic responses.

The experimental findings support this design strategy. Relative to 10G, 10G10P1PVA showed a different rheological response and lower swelling under the tested conditions, while remaining suitable for extrusion and photocrosslinking into corneal constructs. The change in material composition did not compromise short-term CSC viability. Compared with 10G, 10G10P1PVA showed higher metabolic activity, and ALDH3A1 expression was higher than in both 10G and 10G10P. Thus, incorporation of PBA@G/PVA altered the material behavior of GelMA and reduced swelling without compromising short-term CSC viability, while being accompanied by higher metabolic activity and ALDH3A1 expression. The study further extended evaluation of the cell-laden formulation to an exploratory rabbit ALK model, providing short-term in vivo observations of epithelial recovery and stromal organization following implantation.

Table 1. Comparison of representative corneal stromal bioinks and material precedents relevant to the present GelMA/PBA@G/PVA formulation.

A. Representative corneal stromal bioinks: material and biological comparison

Study	Bioink / fabrication	Material / printing evaluation	Biological / in vivo evaluation
Kim et al., 2019 ¹⁵	Corneal dECM; shear-guided 3D cell printing	Transparency; collagen fibril alignment	Cell organization; matrix remodeling
Kilic Bektas and Hasirci, 2019 ⁹	GelMA; keratocyte-laden printing; photocrosslinking	Optical transmission; mechanical properties	Keratocyte viability; stromal phenotype markers
Zhang et al., 2023 ¹¹	Corneal dECM/GelMA; digital light processing	Curvature; optical and mechanical properties	Cell viability; epithelial regeneration; matrix alignment
Xu et al., 2025 ¹²	GelMA/collagen; flat and convex 3D-printed constructs	Flat/convex construct architecture	Keratocyte phenotype; fibrosis-related gene/protein expression
Basoz et al., 2025 ¹⁴	GelMA; cell-loaded and cell-free 3D-printed constructs	Printability; water content; compression; transparency	Rabbit implantation; tissue response
Vijayaraghavan et al., 2026 ¹³	GelMA/carboxymethyl chitosan; cell-laden printing; photocrosslinking	Printability; thermal stability; optical transmission	Keratocyte markers (keratan sulfate, lumican); α -SMA
Present study	GelMA/PBA@G/PVA (10G10P1PVA); CSC-laden extrusion; photocrosslinking	Rheological behavior; swelling; optical transmittance; collagenase degradation; extrusion printability	CSC viability; metabolic activity; ALDH3A1 and ACTA2 expression; exploratory in vivo evaluation

B. Material precedents relevant to the GelMA/PBA@G/PVA formulation

Study	Material system	Application	Established precedent
Han et al., 2020 ⁴⁴	Bacterial cellulose/PVA composite	Corneal stromal substitute	CSC compatibility and tissue response

Study	Material system	Application	Established precedent
Wang et al., 2021 ⁴⁵	3-CPBA-grafted gelatin/PVA; boronate ester hydrogel	Diabetic wound treatment	Reversible dynamic crosslinking; no cell-laden construct
Feng et al., 2022 ⁴⁶	HAMA-PBA/GelMA/HA-DA; dynamic crosslinking	Tissue adhesion	Boronate-mediated dynamic crosslinking in a GelMA-containing printable bioink
Chou et al., 2024 ²⁷	4-CPBA-modified GelMA; photocrosslinking	Vitreoretinal surgery	Biocompatibility; no cell-laden construct
Li et al., 2026 ⁴⁷	PBA-modified alginate/PVA	Corneal drug delivery	Responsive hydrogel behavior; no cell-laden construct

Abbreviations: GelMA, gelatin methacryloyl; dECM, decellularized extracellular matrix; PBA, phenylboronic acid; PBA@G, PBA-functionalized gelatin; PVA, poly(vinyl alcohol); CSCs, corneal stromal cells; CMCh, carboxymethyl chitosan; 4-CPBA, 4-carboxyphenylboronic acid; ALK, anterior lamellar keratectomy; HAMA-PBA, hyaluronic acid modified with methacrylate and phenylboronic acid groups; HA-DA, dopamine-modified hyaluronic acid.

4. Conclusion

In summary, we developed a 10G10P1PVA composite hydrogel and characterized its rheological behavior, optical transmittance, swelling, degradation, printability, and cytocompatibility. Among the tested formulations, 10G10P1PVA supported CSC viability over 7 days, greater day-7 metabolic activity than 10G, and higher ALDH3A1 expression than 10G or 10G10P without the marked ACTA2 induction observed in CSC-M. The exploratory rabbit ALK evaluation provided short-term qualitative observations of epithelial recovery and stromal organization following implantation. These material and biological results support further evaluation of 10G10P1PVA as a GelMA-based composite bioink for corneal stromal bioprinting.

Acknowledgments

The authors thank the Microscope Core Laboratory, Genomic Medicine Core Laboratory and Laboratory Animal Center, Chang Gung Memorial Hospital, Linkou branch, for providing technical assistance.

Funding

This study was partially supported by Chang Gung Memorial Hospital [CMRPG3Q0481] to Chen H.C. and the Taiwan National Science and Technology Council Grants [NSTC 114-2314-B-182-002 and 114-2811-B-182-014] to Tsai TT. The funding organization played no role in the design or conduct of this research.

Conflict of interest

The authors declare that there is no conflict of interest.

Author contributions

Conceptualization: Yi-Jen Hsueh, Chung-An Chen, Tsung-Ting Tsai, Hung-Chi Chen

Formal analysis: Yi-Jen Hsueh, Chung-An Chen

Funding acquisition: Tsung-Ting Tsai, Hung-Chi Chen

Investigation: All authors

Methodology: Yi-Jen Hsueh, Chung-An Chen, Chin-Yu Yang, Chang-Hong Lin, Chun-Yuan Cheng, Chih-Hsin Lin, Shih-Jung Liu

Project administration: Tsung-Ting Tsai, Hung-Chi Chen

Supervision: Tsung-Ting Tsai, Hung-Chi Chen

Validation: Yi-Jen Hsueh, Chung-An, Chen Tsung-Ting Tsai, Hung-Chi Chen

Visualization: Yi-Jen Hsueh, Chung-An Chen

Writing—original draft: Yi-Jen Hsueh, Chung-An Chen

Writing—review & editing: Tsung-Ting Tsai, Hung-Chi Chen

Ethics approval and consent to participate

This research was conducted in accordance with the principles of the Declaration of Helsinki, and it received approval from Chang Gung Memorial Hospital's Institutional Review Board (IRB No. 202001800B0).

Consent for publication

Not applicable.

Availability of data

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Further disclosure

Chung-An Chen is currently the Manager of Analytical method development group, AngenMed Therapeutics, Inc.

References

1. Gain P, Jullienne R, He Z, et al. Global Survey of Corneal Transplantation and Eye Banking. *JAMA Ophthalmol.* Feb 2016;134(2):167–73. doi:10.1001/jamaophthalmol.2015.4776
2. Guerin LP, Le-Bel G, Desjardins P, et al. The Human Tissue-Engineered Cornea (hTEC): Recent Progress. *Int J Mol Sci.* Jan 28 2021;22(3)doi:10.3390/ijms22031291
3. Germain L, Auger FA, Grandbois E, et al. Reconstructed human cornea produced in vitro by tissue engineering. *Pathobiology.* May–Jun 1999;67(3):140–7. doi:10.1159/000028064
4. Kim HK. Development of a Bioengineered Cornea Using a 3-Dimensional Bioprinting System. *Cornea.* 2014;33(Supplement 11):S12. doi:10.1097/ico.0000000000000230
5. Li P, Zhang M, Chen Z, Tian B, Kang X. Tissue-Engineered Injectable Gelatin-Methacryloyl Hydrogel-Based Adjunctive Therapy for Intervertebral Disc Degeneration. *ACS Omega.* Apr 18 2023;8(15):13509–13518. doi:10.1021/acsomega.3c00211
6. Yue K, Trujillo-de Santiago G, Alvarez MM, Tamayol A, Annabi N, Khademhosseini A. Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels. *Biomaterials.* Dec 2015;73:254–71. doi:10.1016/j.biomaterials.2015.08.045
7. Isaacson A, Swioklo S, Connon CJ. 3D bioprinting of a corneal stroma equivalent. *Exp Eye Res.* Aug 2018;173:188–193. doi:10.1016/j.exer.2018.05.010
8. Duarte Campos DF, Rohde M, Ross M, et al. Corneal bioprinting utilizing collagen-based bioinks and primary human keratocytes. *J Biomed Mater Res A.* Sep 2019;107(9):1945–1953. doi:10.1002/jbm.a.36702
9. Kilic Bektas C, Hasirci V. Cell loaded 3D bioprinted GelMA hydrogels for corneal stroma engineering. *Biomater Sci.* Dec 17 2019;8(1):438–449. doi:10.1039/c9bm01236b
10. Kong B, Chen Y, Liu R, et al. Fiber reinforced GelMA hydrogel to induce the regeneration of corneal stroma. *Nat Commun.* Mar 18 2020;11(1):1435. doi:10.1038/s41467-020-14887-9
11. Zhang M, Yang F, Han D, et al. 3D bioprinting of corneal decellularized extracellular matrix: GelMA composite hydrogel for corneal stroma engineering. *Int J Bioprint.* Jun 14 2023;9(5):774. doi:10.18063/ijb.774
12. Xu Y, Liu W, Zhao Q, et al. 3D bioprinted GelMA/collagen hydrogel for corneal stroma regeneration. *Biofabrication.* Jul 24 2025;17(4)doi:10.1088/1758-5090/ade7b2

13. Vijayaraghavan R, Loganathan S, Valapa RB. Functional 3D bioprinting with GelMA/CMCh bioinks: a supportive microenvironment for stromal keratocyte maintenance and potential corneal stromal repair. *J Mater Chem B*. Apr 22 2026;14(15):4704–4723. doi:10.1039/d5tb02459e
14. Basoz D, Akalinli A, Buyuksungur S, et al. Preclinical Testing of 3D Printed, Cell Loaded Hydrogel Based Corneal Substitutes on Rabbit Model. *Macromol Biosci*. Jul 2025;25(7):e2400595. doi:10.1002/mabi.202400595
15. Kim H, Jang J, Park J, et al. Shear-induced alignment of collagen fibrils using 3D cell printing for corneal stroma tissue engineering. *Biofabrication*. May 7 2019;11(3):035017. doi:10.1088/1758-5090/ab1a8b
16. Gao Y, Peng K, Mitragotri S. Covalently Crosslinked Hydrogels via Step-Growth Reactions: Crosslinking Chemistries, Polymers, and Clinical Impact. *Adv Mater*. Jun 2021;33(25):e2006362. doi:10.1002/adma.202006362
17. Nasra S, Patel M, Shukla H, Bhatt M, Kumar A. Functional hydrogel-based wound dressings: A review on biocompatibility and therapeutic efficacy. *Life Sci*. Dec 1 2023;334:122232. doi:10.1016/j.lfs.2023.122232
18. El Sayed MM. Production of Polymer Hydrogel Composites and Their Applications. *Journal of Polymers and the Environment*. 2023/07/01 2023;31(7):2855–2879. doi:10.1007/s10924-023-02796-z
19. Lim JYC, Goh L, Otake KI, Goh SS, Loh XJ, Kitagawa S. Biomedically-relevant metal organic framework-hydrogel composites. 10.1039/D2BM01906J. *Biomater Sci*. Apr 11 2023;11(8):2661–2677. doi:10.1039/d2bm01906j
20. Condo I, Giannitelli SM, Lo Presti D, Cortese B, Ursini O. Overview of Dynamic Bond Based Hydrogels for Reversible Adhesion Processes. *Gels*. Jul 4 2024;10(7):442. doi:10.3390/gels10070442
21. Han Y, Cao Y, Lei H. Dynamic Covalent Hydrogels: Strong yet Dynamic. *Gels*. Sep 10 2022;8(9):577. doi:10.3390/gels8090577
22. Perera MM, Ayres N. Dynamic covalent bonds in self-healing, shape memory, and controllable stiffness hydrogels. 10.1039/C9PY01694E. *Polymer Chemistry*. 2020;11(8):1410–1423. doi:10.1039/c9py01694e
23. Chakma P, Konkolewicz D. Dynamic Covalent Bonds in Polymeric Materials. *Angew Chem Int Ed Engl*. Jul 15 2019;58(29):9682–9695. doi:10.1002/anie.201813525
24. Cho S, Hwang SY, Oh DX, Park J. Recent progress in self-healing polymers and hydrogels based on reversible dynamic B–O bonds: boronic/boronate esters, borax, and benzoxaborole. 10.1039/D1TA02308J. *Journal of Materials Chemistry A*. 2021;9(26):14630–14655. doi:10.1039/d1ta02308j
25. Binte MYNZ, Riau AK, Yam GHF, Binte Halim NSH, Mehta JS. Isolation and Propagation of Human Corneal Stromal Keratocytes for Tissue Engineering and Cell Therapy. *Cells*. Jan 5 2022;11(1)doi:10.3390/cells11010178
26. Kamil S, Mohan RR. Corneal stromal wound healing: Major regulators and therapeutic targets. *Ocul Surf*. Jan 2021;19:290–306. doi:10.1016/j.jtos.2020.10.006
27. Chou HD, Chen CA, Liu HY, et al. Synthesis, Properties, and Biocompatibility of 4-Carboxyphenylboronic Acid-Modified Gelatin-Methacryloyl: A Hydrogel for Retinal Surgeries. *ACS Omega*. Oct 15 2024;9(41):42147–42158. doi:10.1021/acsomega.4c02842
28. Shirahama H, Lee BH, Tan LP, Cho NJ. Precise Tuning of Facile One-Pot Gelatin Methacryloyl (GelMA) Synthesis. *Sci Rep*. Aug 9 2016;6(1):31036. doi:10.1038/srep31036
29. Liu C, Yu Q, Yuan Z, et al. Engineering the viscoelasticity of gelatin methacryloyl (GelMA) hydrogels via small "dynamic bridges" to regulate BMSC behaviors for osteochondral regeneration. *Bioact Mater*. Jul 2023;25:445–459. doi:10.1016/j.bioactmat.2022.07.031
30. Habeeb AF. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal Biochem*. Mar 1966;14(3):328–36. doi:10.1016/0003-2697(66)90275-2
31. Hsueh YJ, Meir YJ, Lai JY, et al. Lysophosphatidic acid improves corneal endothelial density in tissue culture by stimulating stromal secretion of interleukin-1beta. *J Cell Mol Med*. Jun 2020;24(12):6596–6608. doi:10.1111/jcmm.15307

32. Walejewska E, Melchels FPW, Paradiso A, et al. Tuning Physical Properties of GelMA Hydrogels through Microarchitecture for Engineering Osteoid Tissue. *Biomacromolecules*. Jan 8 2024;25(1):188–199. doi:10.1021/acs.biomac.3c00909
33. Clerkin S, Singh K, Davis JL, et al. Tuneable gelatin methacryloyl (GelMA) hydrogels for the directed specification of renal cell types for hiPSC-derived kidney organoid maturation. *Biomaterials*. Nov 2025;322:123349. doi:10.1016/j.biomaterials.2025.123349
34. Xie W, Zhang Y, Zhang J, et al. Dynamically crosslinked protein hydrogel composite as multifunctional wound dressing for cutaneous infection. *Colloid and Interface Science Communications*. 2022/09/01/ 2022;50:100654. doi:10.1016/j.colcom.2022.100654
35. Li X, Chen S, Li J, et al. 3D Culture of Chondrocytes in Gelatin Hydrogels with Different Stiffness. *Polymers (Basel)*. Jul 26 2016;8(8):269. doi:10.3390/polym8080269
36. Ghosh RN, Thomas J, B. R V, et al. An insight into synthesis, properties and applications of gelatin methacryloyl hydrogel for 3D bioprinting. *Materials Advances*. 2023;4(22):5496–5529. doi:10.1039/d3ma00715d
37. Krishnamoorthy S, Noorani B, Xu C. Effects of Encapsulated Cells on the Physical-Mechanical Properties and Microstructure of Gelatin Methacrylate Hydrogels. *Int J Mol Sci*. Oct 12 2019;20(20):5061. doi:10.3390/ijms20205061
38. Pepelanova I, Kruppa K, Scheper T, Lavrentieva A. Gelatin-Methacryloyl (GelMA) Hydrogels with Defined Degree of Functionalization as a Versatile Toolkit for 3D Cell Culture and Extrusion Bioprinting. *Bioengineering (Basel)*. Jul 18 2018;5(3):55. doi:10.3390/bioengineering5030055
39. Pei Y, Reins RY, McDermott AM. Aldehyde dehydrogenase (ALDH) 3A1 expression by the human keratocyte and its repair phenotypes. *Exp Eye Res*. Nov 2006;83(5):1063–73. doi:10.1016/j.exer.2006.05.011
40. Poole K, Iyer KS, Schmidtke DW, Petroll WM, Varner VD. Corneal Keratocytes, Fibroblasts, and Myofibroblasts Exhibit Distinct Transcriptional Profiles In Vitro. *Invest Ophthalmol Vis Sci*. Mar 3 2025;66(3):28. doi:10.1167/iovs.66.3.28
41. Zhou C, Petroll WM. MMP regulation of corneal keratocyte motility and mechanics in 3-D collagen matrices. *Exp Eye Res*. Apr 2014;121:147–60. doi:10.1016/j.exer.2014.02.002
42. Sanders FWB, Huang J, Alio Del Barrio JL, Hamada S, McAlinden C. Amniotic membrane transplantation: structural and biological properties, tissue preparation, application and clinical indications. *Eye (Lond)*. Mar 2024;38(4):668–679. doi:10.1038/s41433-023-02777-5
43. Wilson SE, Liu JJ, Mohan RR. Stromal-epithelial interactions in the cornea. *Prog Retin Eye Res*. May 1999;18(3):293–309. doi:10.1016/s1350-9462(98)00017-2
44. Han Y, Li C, Cai Q, et al. Studies on bacterial cellulose/poly(vinyl alcohol) hydrogel composites as tissue-engineered corneal stroma. *Biomed Mater*. Apr 30 2020;15(3):035022. doi:10.1088/1748-605X/ab56ca
45. Wang Y, Wu Y, Long L, et al. Inflammation-Responsive Drug-Loaded Hydrogels with Sequential Hemostasis, Antibacterial, and Anti-Inflammatory Behavior for Chronically Infected Diabetic Wound Treatment. *ACS Appl Mater Interfaces*. Jul 21 2021;13(28):33584–33599. doi:10.1021/acsami.1c09889
46. Feng Q, Li D, Li Q, et al. Assembling Microgels via Dynamic Cross-Linking Reaction Improves Printability, Microporosity, Tissue-Adhesion, and Self-Healing of Microgel Bioink for Extrusion Bioprinting. *ACS Appl Mater Interfaces*. Apr 6 2022;14(13):15653–15666. doi:10.1021/acsami.2c01295
47. Li S, Hu X, Zhang G, et al. ROS-Triggered Disassembly of Nanoparticle-Networked Hydrogels Enables Dynamic Gating of Corneal Tight Junctions. *ACS Appl Mater Interfaces*. Aug 12 2026;18(31):42273–42288. doi:10.1021/acsami.6c06859