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RESEARCH ARTICLE

3D-printed artificial cornea featuring aligned fibrous structure and enhanced mechanical strength

Supplementary File

S1. Materials and methods

S1.1. Synthesisprocedure of monomer (NCA: L-glutamate(bn) N-carboxyl anhydride)

Six grams of L-Glutamic acid γ-benzyl [H-Glu(OBzl)-OH] (MW: 237.30 g/mol), anhydrous tetrahydrofuran (THF, 90 mL), and propylene oxide (7.8 mL) were stirred in the 3-neck round-bottom flask (500 mL) for 5 min. Then, 3.70 g of triphosgene was added to the flask and the excess of pressure inside the flask was released with a needle. The solution was stirred for 2 h at room temperature and became a clear solution. The solution was concentrated to a viscous liquid by a rotary evaporator at 30°C to remove by-products and propylene oxide. Anhydrous THF was added to 60 mL of solution and the n-hexane was added dropwise until there was precipitation inside the solution. The solution was kept at -20°C overnight for the crystallization process; on the next day, the solution was filtered with 1A filter paper to collect the white precipitate. In a dried 1000-mL conical flask, the white precipitate was dissolved in 50 mL of anhydrous THF, and n-hexane was added dropwise until the precipitation started, for the recrystallization process was completed at -20℃ overnight. The solution with white crystals was centrifuged at 5000 rpm for 10 min, the liquid was decanted, and the white crystals were dried at 40°C in a vacuum oven. The white cotton-like product of 5.45 g was obtained at a yield of 84.00%. The synthetic chemical routes are shown in **Figure S1.**

S1.2. Synthesis procedure of monomer L-lysine(Cbz) N-carboxyl anhydride

Six grams of N6-Carbobenzyloxy-L-lysine [H-Lys(Z)-OH] (MW: 280.32 g/mol), anhydrous THF (90 mL), and propylene oxide (15.7 mL) were stirred in the 3-neck round-bottom flask (500 mL) for 5 min. After that, 3.70 g of triphosgene was mixed and the excess of pressure inside the flask was released with a needle. The solution was stirred for 2 h at room temperature and become a clear solution; the solution was concentrated by a rotary evaporator at 30°C to viscous liquid to remove the remaining by-products and propylene oxide. Anhydrous THF was added to 60 mL of solution and the n-hexane

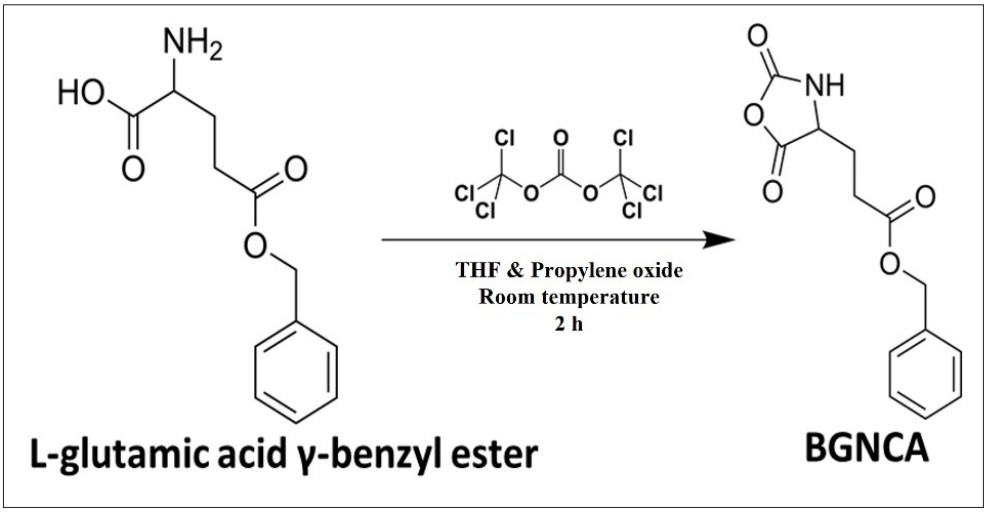

Figure S1. Synthetic route of monomer NCA: L-glutamate(bn)N-carboxyl anhydride (BGNCA).

was added dropwise until precipitation occurred inside the solution. The solution was kept at -20°C overnight for the crystallization process; on the next day, the solution was filtered with 1A filter paper to collect the white precipitate. In a dried 1000-mL conical flask, the white precipitate was dissolved in 50 mL of THF, and again, the n-hexane was added dropwise until the precipitation started, for the recrystallization process of the precipitated solution to occur under -20°C overnight. After recrystallization overnight, the recrystallized solution was centrifuged at 5000 rpm for 10 min to obtain the white precipitate, which was then dried at 40°C in a vacuum oven. The white cotton-like product obtained weighed 5.75 g. The synthesis route with chemical structure is shown in **Figure S2.**

S1.3. Synthesis of co-polymer [P ($(CBZL)_{80}$- co- $(BG)_{20}$)]

For polymerization, benzylamine was used as the initiator. For this purpose, the pure benzylamine was prepared by the distillation process, 20 mL benzylamine was dried with 5 g calcium hydride, distilled, and stored with a 4A molecular sieve. A 100-mL 3-neck flask and a 50-mL 3-neck flask were placed in a vacuum overnight. On the next day, 2.47 g and 0.53 g of monomers (LLCbz-NCA_{80} and $BGNCA_{20}$) were subjected to 3 cycles of vacuum and N_2(g) purge; afterwards, 30 mL of dry dimethylformamide (DMF) was added. On the other hand, after 3 cycles of vacuum and N_2(g) purge, 110 µL benzylamine (MW: 107.15, density: 0.981 g/mL) was diluted into 9.89 mL of dry DMF solution. The polymerization started upon the addition of diluted benzylamine solution (molar ratio 1/100, 1 mL). After reacting at room temperature for 3 days, the DMF solution was poured into a beaker containing 400 mL of ether and stirred vigorously. The whole solution turned into a turbid solution, and stirring was continued for 24 to 48 h. The solution turns crystal-clear with a layer of yellowish sticky product on the wall of the beaker. The ether solution was removed, added with 400 mL of methanol, and stirred continuously overnight. The yellowish product turned white. The methanol was removed, added with 400 mL of distilled water, pill of the white product, and stirred for one more night. Finally, the product, P($(CBZL)_{80}$- co $(BG)_{20}$) was obtained as white precipitates remaining on the filter paper after filtering the distilled water solution with 1A filter paper. The product was precipitated in cold ethyl ether, collected by filtration, and dried at 40°C in a vacuum oven. The synthetic route with chemical structure is shown in **Figure S3a**.

S1.4. Hydrolysis procedure of co-polymer [P ($(CBZL)_{80}$- co $(BG)_{20}$)] for $PLL_{80}GA_{20}$ (PG)

For this purpose, 500 mg of P ($(CBZL)_{80}$- co- $(BG)_{20}$) was dissolved with 12.5 mL of dichloroacetic acid (DCA) and 760 µL of 33 wt% HBr/acetic acid solution was added. As soon as the deprotection reaction completed after 1 h, the transparent yellowish solution transformed into a turbid, dark yellowish suspension. The process can be stopped by adding 12.5 mL of distilled water into the suspension, which transforms it into a clear, yellowish solution. Dialysis using membranes with a molecular weight range of 12,000–14,000 against 5%v /v HBr aqueous solution should be performed right away to stop the hydrolysis. After three days of dialysis against distilled water and the solution changes with fresh water every two hours, the solution in the beaker should have a pH of 7. The product was formed by freezing the aqueous solution for two days. The synthetic route is shown in **Figure S3b**.

S1.5. Preparation of bio-ink and 3D printing process

To prepare the bio-ink, a 2 wt% sodium alginate (SA) solution and a cellulose nanofiber (CNF) aqueous

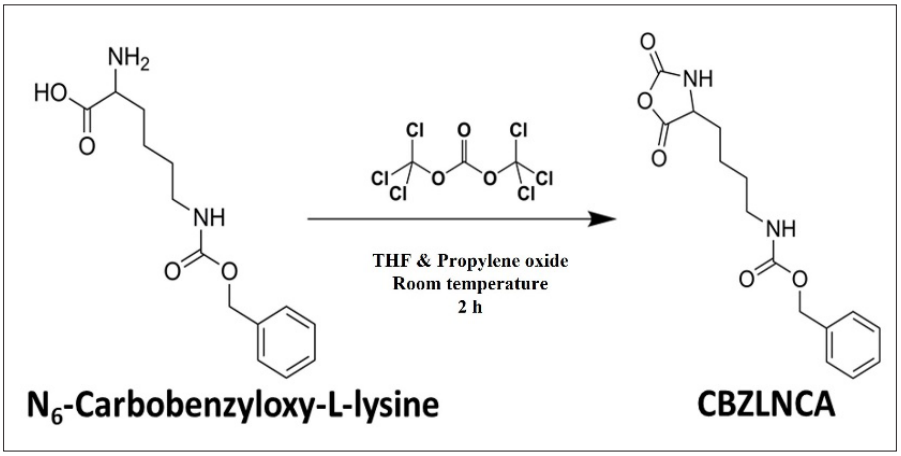

Figure S2. Synthetic route of monomer L-lysine(Cbz) N-carboxyl anhydride (CBZLNCA).

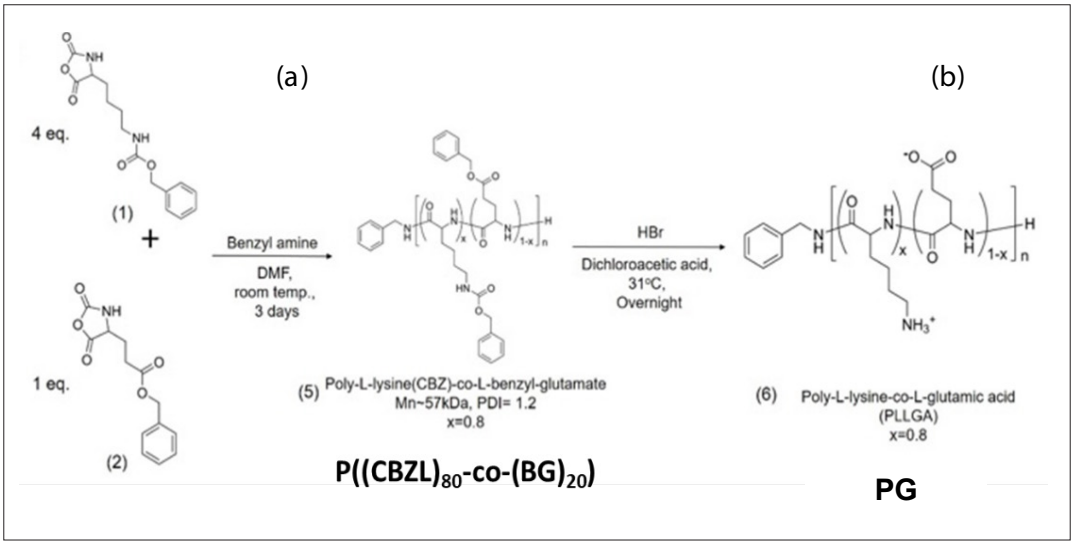

Figure S3. Synthetic routes of co-polymer [P ($(CBZL)_{80}$- co- $(BG)_{20}$)] (a) and hydrolysis of PG (b).

solution were individually prepared by stirring for 24 h. Subsequently, both solutions were combined and homogenized for 2 h. The composite solution was then stirred overnight at 300 rpm to ensure uniformity, after which the bio-ink was ready for printing. The poly-L-lysine-co-L-glutamic acid ($PLL_{80}GA_{20}$, PG) aqueous solutions were prepared with effective charge concentrations of 6.25 mM, 12.25 mM, and 25 mM, respectively. SA was selected for its excellent viscosity, which facilitates printability, in addition to its biocompatibility, hydrophilicity, and gel-forming capability, making it ideal for biofabrication. CNF was selected owing to its unique fibrous structure, high mechanical reinforcement properties, and biocompatibility. In the SA/CNF composite, SA provides excellent water retention and overall biocompatibility, while the addition of CNF enhances mechanical strength, alignment, and biodegradability. This makes the combination particularly suitable for replicating the structural and functional properties of the native cornea.

The cornea mold was designed using SolidWorks software (JonHirschtick , Massachusetts Institute of Technology), based on average adult humans' measurements. The stroma dimensions were set to 500 μm at the center and 700 μm at the periphery, with a uniform radius of 5.80 mm and a height of 3.0 mm. To produce a 3D stromal shape, these dimensions were drawn on a plane and then rotated around the y-axis. A specially designed 3D bioprinter (Allevi 2, Bioprinter, developer/manufacturer, country) was used to create the artificial cornea, utilizing an STL file for printing. The prepared bio-inks were poured into a 10 cc BD syringe, and dispensing was done using precision tips (27 G, 0.2 mm, Nordson EFD, USA). The printing process was carried out at a speed of 10 mm/s with a pressure of 40 psi, allowing for the precise formation of the corneal structure.

S2. Analytical methods

S2.1. Confirmation of chemical structure by nuclear magnetic resonance

The nuclear magnetic resonance (NMR; 400MHz, Bruker, USA) was used to characterize every chemical at National Taiwan University's Department of Chemistry. The ^{1}H-NMR sample for LLCbz-NCA and LBG-NCA required 5–10 mg of compounds in 1.5 mL of d-chloroform. The ^{1}H-NMR samples for PG consisted of 10–20 mg of compounds in 1.5 mL of d-trifluoroacetic acid, which is strong enough to hydrolyze the protection of PG precursors. Therefore, it is important to characterize the ^{1}H-NMR sample of these PG precursors as quickly as possible after they are dissolved in d-trifluoroacetic acid.

S2.2. Molecular weight by gel permeation chromatography

The molecular weight of PG was measured via gel permeation chromatography (GPC) employing DMF.The 3-5 mg of PG was dissolved in 1 mL of DMF solution. PG having an O-H or NH_2 bond should not be subjected to GPC examination.

S2.3. Functional group by Fourier-transform infrared spectroscopy

The surface functional groups of BGNCA, CBZNCA, and PG were measured in ambient conditions by means of reflective Fourier-transform infrared (FTIR) spectroscopy (PerkinElmer, country) in a solid state using an attenuated total reflectance (ATR) module. Due to the high hydrophilicity of PG and N-carboxyl anhydride (NCA)

monomers, the sample must be stored under nitrogen or vacuum.

S2.4. Stability by dynamic light scattering technique

Dynamic light scattering (Zetasizer Nano, Malvern Panalytical, USA) was used in this study to analyze zeta potential of the PG, SA, and CNF solutions. The positive and negative charges were analyzed by the zeta potential to confirm the charge for crosslinking of hydrogel. The solutions were prepared in distilled,water, diluted to 0.01 wt%, then adjusted to pH = 7 using 0.5 M NaOH.

S2.5. Rheological study

TA Instrument's AR2000 Rheometer (USA) was used to measure mechanical characteristics. In this test, hydrogel samples were created using the drop method with 1 mL of SA/CNF suspension and 2 mL of crosslinker solution. A 20 mm parallel plate with a 400 μm gap was used to characterize the mechanical characteristics at 25°C. Time scanning, frequency scanning, and strain scanning were the three categories of characterizations. Under 10Hz vibration and 0.5% strain, a time scanning test measured the storage modulus (G') versus oscillation strain. The two modules were measured as a function of frequency using frequency scanning; under 0.5% tension, the frequency scanning ranged from 0.1 to 100 Hz. The two modules were scanned for strain, and when the vibration was set to 10 Hz, the strain increased from 0.1% to 100%. At room temperature, the viscosity property was measured at shear rates of 0.01–100 s^{-1}. The inks were subjected to oscillation experiments using a constant strain of 1% and a sweep frequency of 10 Hz.

S2.6. Water content angle measurement

The water content was calculated by the weight of the dry and pristine hydrogels. For statistical analysis, the water contents of each of the three pieces that made up each hydrogel sample were measured individually. The hydrogels' pre- and post-drying weights were measured. To avoid oxidation during heating, the hydrogels were dried by freeze-drying. Hydrogel water content was measured using **Equation S1**:

$$\text{Water content} = \left(1 \frac{W_{dried\ hydrogel}}{W_{wet\ hydrogel}}\right) \times 100\% \textbf{(S1)}$$

$W_{dried\ hydrogen}$ is the hydrogel after dried it by freeze drying and $W_{wet\ hydrogen}$ is defined as hydrogel used after gelation.

S2.7. Polarized optical microscopy

Polarized optical microscopy (Germany) was used to observe the hydrogels. The hydrogel was fabricated by dropped method and co-extrusion method in distilled water to avoid deforming its microstructure. The measurements were taken in three categories: cross-polar, parallel-polar, and cross-polar + compensator.

S2.8. Live/dead and cell viability tests

We used a Live/Dead Cell Viability Kit (cat. num. L3224, Thermo Fisher, USA) to monitor the cell viability in cultured PC12 cells (USA). Fluorescent dyes used for viability determinations are chemical reagents that stain living or dead cells throught heir size differentiation or differential cell permeability. The Living dye in this commercial kit is calcein-AM, also known as acetoxymethyl ester (AM). It can pass cell membranes and be cell-penetrative when in its acetoxymethyl (AM) ester form, and afterwards it is converted back to calcein. It absorbs blue light (λ = 494 nm) and emits green light (λ = 516 nm). Therefore, it is commonly referred to as a green, fluorescent indicator. In this research, the stained PC12 cell culture performed on day 6. The staining solution was 2 μM calcein-AM and 4μMethidium homodimer-1 (EthD-1) in phosphate-buffered saline (PBS). Mostly, the staining solution should be prepared slightly more than expected—two to three wells more. In the case of a 10mL staining solution, 5 μL of the commercial calcein-AM solution and 20 μL of the commercial EthD-1 solution were added into a centrifuge tube. Then, the solution was diluted with 9.975 mL of PBS to 10 mL in total. The staining solution should be used as soon as possible to ensure the quality of dyes. Also, the staining solution and dyes should be protected from light. The medium was removed before staining. Then, the cell culture was washed twice with PBS carefully and immersed in the staining solution for 20 to 30 min. The well plate was shaken to ensure uniform cell staining. The staining process should be carried out in a darkroom. When the staining process was completed, the staining solution was removed, and the cell culture was washed twice with PBS. Before observation, we added 10 to 20 μL of PBS to prevent hydrogel from desiccation. The cells (at a density of 5000 cells per well) were plated in triplicate without any additional coatings, and the plates were coated with polystyrene (PS), and the cells were allowed to adhere for 24 h. Cell viability was evaluated on a PS cell plate (48-well). The hydrogels were initially produced in the wells, at which $n = 3$ for each of hydrogel, and then sterilized under ultraviolet (UV) radiation for 4 h. For efficient exchange of water to medium, hydrogel was pre-incubated at 37°C in RPMI-1640 for 24 h. The positive control was a tissue culture polystyrene (TCPS) plate with cell seeding, whereas the negative control or blank was a plate without cell seeding. The hydrogel was washed with 37°C PBS prior to cell seeding, and 400 μL of RPMI-1640 medium was added into each well. The alamarBlue assay was performed on days 1, 3, and 7 with alamarBlue™

cell viability reagent (Invitrogen, Lot#.2214489), diluted in DMEM (Thermofisher, USA) to 10% v/v working solution. First, the debris and medium in the gastric/intestinal cell medium were removed by a pipette tip, with gentle shaking to tilt the well plate, to avoid physical disruption to the structure of the hydrogel. Then, 400 µL of 37°C PBS was added to wash the hydrogel, followed by 400 µL of the working solution. The well plate was wrapped with aluminum foil to avoid light damage before incubating the samples for 3 h at 37°C. The working solution was then transferred to 96-well plates, and 100 µL was transferred to each well of the 96-well plates for fluorescence testing (n = 9). After fluorescence, the working solution was washed 3 times with PBS and DMEM complex, and then 400 µL of fresh RPMI-1640 medium was added to each well for 3 days of incubation. The absorbance of each well during the working solution stage was measured at 570/600 nm by microplate reader (TechComm X, NTU). Every sample requires three replicates and three data and % reduction were calculated using **Equation S2.**

% reduction of alamarBlue reagent =

$$\frac{E_{Oxi600} \times A_{570} - E_{Oxi570} \times A_{600}}{E_{red570} \times C_{600} - E_{red600} \times C_{570}} \quad \textbf{(S2)}$$

where:

E_{oxi570} = the molar extinction coefficient of oxidized Alamar Blue at 570 nm (80586)

E_{oxi600} = the molar extinction coefficient of oxidized Alamar Blue at 600 nm (117216)

E_{red570} = the molar extinction coefficient of reduced Alamar Blue at 570 nm (155677)

E_{red600} = the molar extinction coefficient of reduced Alamar Blue at 600 nm (14652)

A_{570} = absorbance of the tested well at 570 nm

A_{600} = absorbance of the tested well at 600 nm

C_{570} = absorbance of the negative control well at 570 nm

C_{600} = absorbance of the negative control well at 570 nm

S2.9. Degradation study

The degradation rate of scaffolds was assessed by soaking 20 mg of dried hydrogel samples in 10 mL of PBS solution (pH 7.4) at a temperature of 37°C over various time intervals (1, 3, 6, 9, 12, 15, 18, and 21 days). Prior to immersion in PBS, the initial weight of the samples was recorded as W_0 g. After the immersion period, the samples were extracted from the PBS and rinsed with distilled water to eliminate any buffer salts. Subsequently, the samples were placed in a freeze-drying apparatus for 24 h, after which their final weight was measured as W_1 g. The degradation rate (D) was then determined using **Equation S3.**

$$\text{Degradation (D)} = \left(\frac{W_0 - W_1}{W_0}\right) \times 100\% \quad \textbf{(S3)}$$

S2.10. Statistical analysis

All experiments were conducted in triplicates (n = 3). A one-way analysis of variance (ANOVA) of the cell viability test results was performed. A significant value of $P > 0.05$ was set for all statistical analyses. All quantitative data are presented as the mean ± standard deviation.

S3. Results and discussion

Figure S4a shows the differential scanning calorimetry (DSC) analysis and purity of the monomer with a melting point of 98°C. The surface functional groups confirmed by the FTIR study of PG NCAs reveal that the absorption peaks at 1830 cm^{-1} correspond to the carboxyl group on the N-carboxyl anhydride (NCA) ring. This peak vanishes, and a new signal of about 1650 cm^{-1} emerges after polymerization, indicating the carboxyl group of the amide backbone. We confirmed the successful polymerization of the monomers using these two peaks. Following PG deprotection, the carboxyl group signal on benzyl carbamate (Cbz), which is around 1690 cm^{-1}, disappears. This implies that the carbamate protective group was successfully deprotected, as shown in **Figure S4b**. The hydrogel was fabricated after the confirmation of the surface charge of the PG, CC, SA, and CNF (**Figure S4c**). The mechanical strength of the enhanced ionic crosslinking is shown in **Figure S4d**, and

Table S1. Mechanical properties of SA and calcium chloride

Sample name	Sample code	Effective charge concentration (mM) of crosslinker	Water content (wt%)	Storage modulus, G' (kPa) (n = 3)
SA/CC	SA/CC-1	6.25	97.11 ± 0.30	18.31 ± 1.39
	SA/CC-2	12.50	98.15 ± 0.80	38.01 ± 0.89
	SA/CC-3	25	97.35 ± 0.40	42.76 ± 0.52

Abbreviations: CC: Calcium chloride; SA: Sodium alginate.

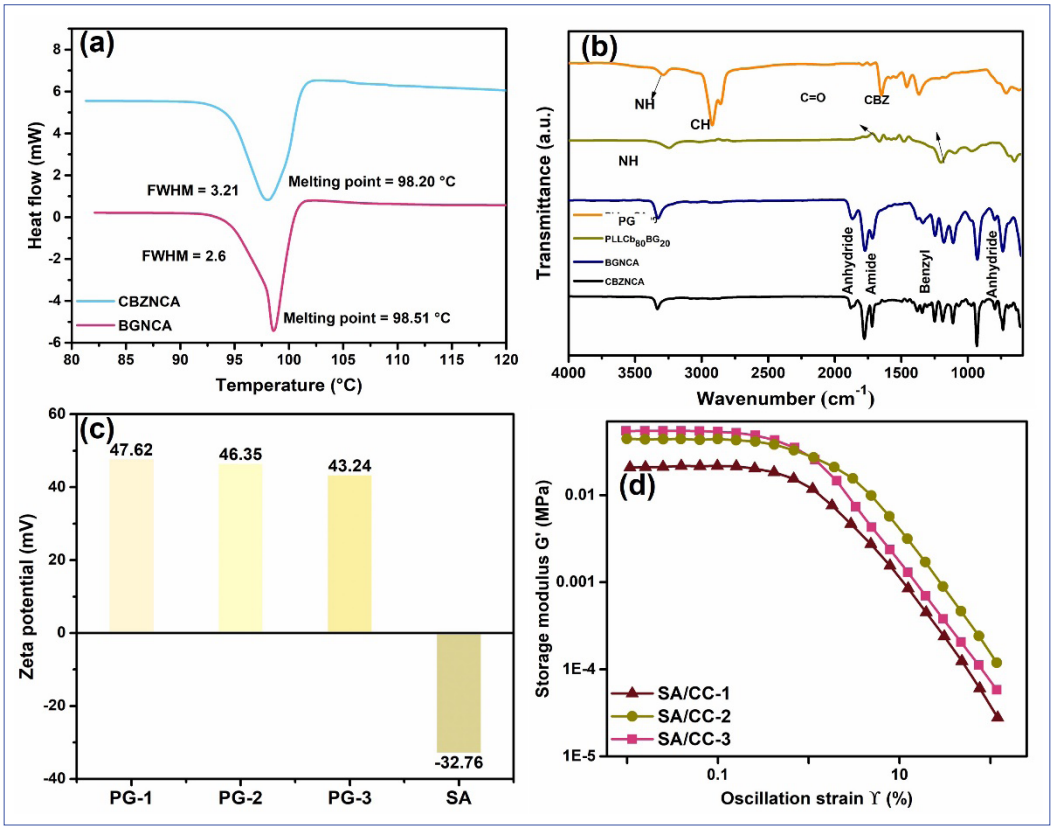

Figure S4. Physical and chemical properties. (a) DSC analysis of the monomers. (b) FTIR analysis of the monomers and PG. (c) Zeta potential of the bio-ink. (d) Mechanical properties of sodium and calcium chloride. Abbreviations: BGNCA: L-glutamate (bn) N-carboxyl anhydride; CBNZA: L-lysine(Cbz) N-carboxyl anhydride; CC: Calcium chloride; DSC: Differential scanning calorimetry; FTIR: Fourier-transform infrared spectroscopy; FWHM: Full width half maxima; PG: Poly-L-lysine-co-L-glutamic acid ($PLL_{80}GA_{20}$); SA: Sodium alginate.

the corresponding values are tabulated in **Table S1**. SA is a polysaccharide containing carboxylate groups that can interact with divalent cations, such as calcium ions (Ca^{2+}), through ionic crosslinking. The "egg-box" model brings out the structure of the ionic linking of the guluronic acid segments of the alginate chains through the calcium ions, giving a network structure. In that respect, crosslinking enhances the mechanical strength of the gel. The storage moduluswere improved by precisely regulating the effective charge concentrations of calcium ions (6.25, 12.25, and 25 mM). The storage modulus values for SA/CC-1, SA/CC-2, and SA/CC-3 were 18.31 ± 1.39 kPa, 38.01 ± 0.89 kPa, and 42.76 ± 0.52 kPa, respectively.

Nuclear magnetic resonance (^{1}H-NMR) is widely recognized as the primary means of characterization for organic compounds. It is possible to determine the chemical structure of several compounds based on the subtleties of the ^{1}H-NMR spectrum displays, including the chemical shifts, peak splitting, and peak integrals. In this experiment, PG monomers were dissolved in deuterated chloroform (d-CF), while the PG was prepared in deuterated trifluoroacetic acid (d-TFA) for ^{1}H-NMR. ^{1}H-NMR of CBZNCA, BGNCA, $PLLCbz_{80}BG_{20,}$ and PG were recorded. Only minor changes of the ^{1}H-NMR spectra were detected before and after polymerization. The main peak differences existed in amine peaks (δ = 7.06, 4.96, as shown in **Figure S5a**) and hydrolysis peaks (δ = 5.47 in **Figure S5b, c**). Being in polar solvent, the amine peak is inherently unstable because of its tendency to dissociate hydrogen atoms. Using d-TFA as solvent, the stability of the amine peak would be compromised, potentially giving discrepancies in the integral against the number of hydrogen atoms. Besides that, d-TFA is a strong acid and thus hydrolyzes both the Cbz and the benzyl ester group, which appears as a new peak at b1 (δ = 5.47). Thus, for the complete characterization of the chemical structure, the integral of the b1 peak must be counted together with that of the b_2 peak. The dramatic change in the spectrum includes, after the deprotection of $PLLCbz_{80}BG_{20}$, the disappearance of aromatic ring signals at δ = 7.3–7.4, as shown in **Figure S5d**, suggesting that the protecting

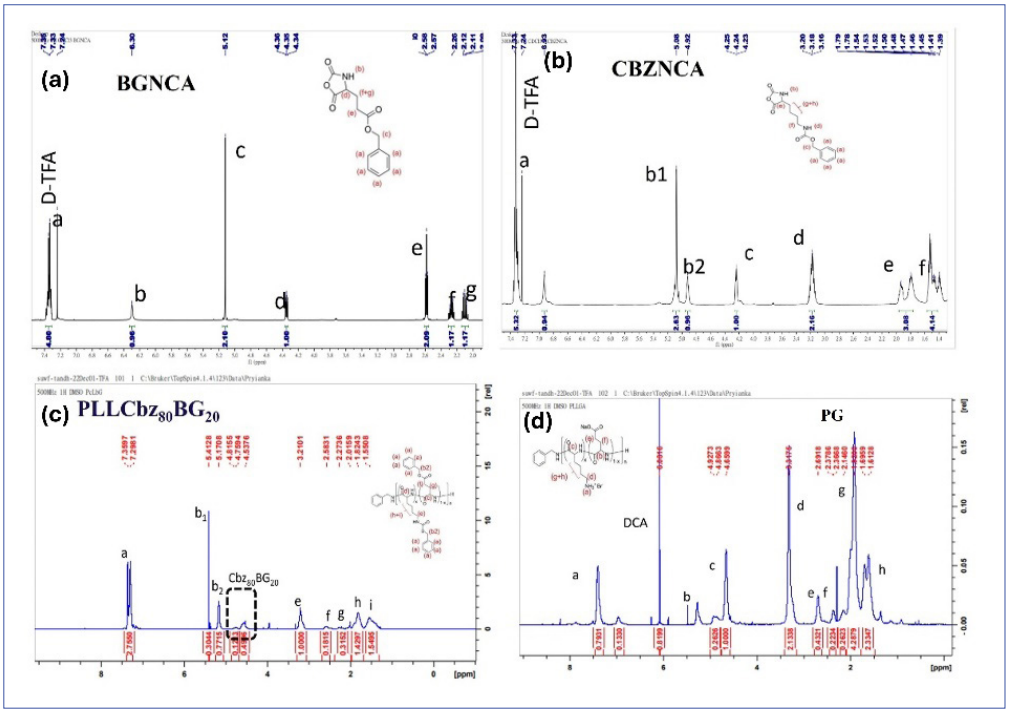

Figure S5. NMR analysis of BGNCA (a), CBZNCA (b), PLLCbz$_{80}$BG$_{20}$ (c), and PG (d). Abbreviations: BGNCA: L-glutamate (bn) N-carboxyl anhydride; CBZNCA: L-lysine N-carboxyl anhydride; DCA: Dichloroacetic acid; PG: Poly-L-lysine-co-L-glutamic acid (PLL$_{80}$GA$_{20}$); PLLCbz$_{80}$BG$_{20}$: Poly-L-lysine-L-glutamic acid.

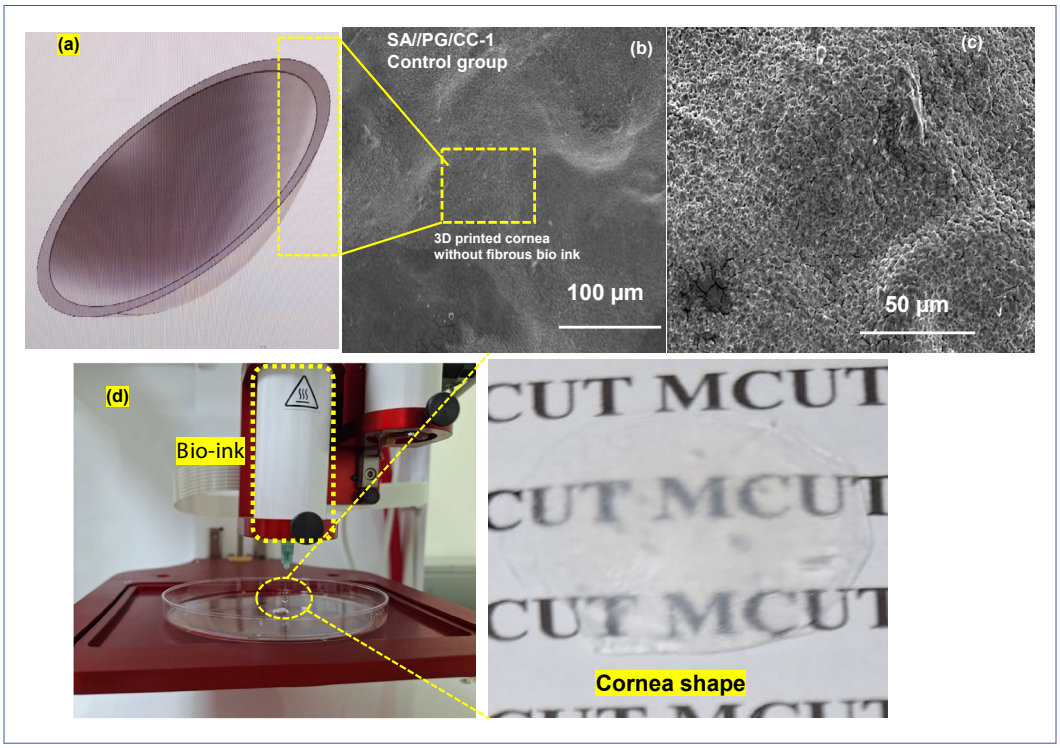

Figure S6. 3D-printed cornea. (a–c) Control group without CNF bio-ink. (d) Transparency after the printing of cornea. Abbreviations: CC: Calcium chloride; PG: Poly-L-lysine-co-L-glutamic acid (PLL$_{80}$GA$_{20}$); SA: Sodium alginate.

group was removed by hydrolysis with HBr. Moreover, the signal at about 5.2 ppm representing hydrogen on the benzyl ($-CH_2-C_6H_5$) protecting group disappeared. In brief, the spectra confirm that after deprotection, the complete removal of the benzyl carbamate and benzyl ester groups occurred. Also, aside from the peaks due to the products, there were only several peaks involving residual solvents with no signals for remaining reactants, thus suggesting product purity being well above 95% as evidenced by the previous reported literature.[1,2]

References

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