

Preparation and Characterization of Poly(γ -glutamic acid) Hydrogels as Potential Tissue Engineering Scaffolds*

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Abstract In this paper, methacrylated γ -PGA (mPGA) precursor was synthesized *via* reaction between γ -PGA and glycidyl methacrylate (GMA). Hydrogels from this precursor were prepared under 365 nm ultraviolet irradiation. The swelling behavior and mechanical properties were studied in detail as functions of the degree of substitution (DS), precursor concentration, and environmental pH. Results showed that the crosslink density, swelling kinetics and mechanical properties of the hydrogel could be tailored by adjusting the DS and concentration of the precursor as well as the environmental pH. Three-dimensional photo-encapsulation of swine cartilage chondrocytes and Live/Dead assay proved the cytocompatibility of the hydrogel.

Keywords: Poly(γ -glutamic acid); Hydrogel; Tissue engineering scaffold.

INTRODUCTION

As a naturally derived biopolymer, poly(γ -glutamic acid) (γ -PGA) is characterized by its excellent biodegradability, biocompatibility and strong hydrophilicity^[1–3]. In addition, γ -PGA has unique bioactivity, the amine group in the N-terminal γ -glutamic unit can be identified by γ -glutamyl transpeptidase in the cell membrane and thus promote the cellular uptake of drug carriers with γ -PGA moieties^[4–7]. The abundant side carboxyl groups of γ -PGA can be readily modified with bio-/chemical factors through conventional bioconjugate techniques, making it a promising candidate in biomedical field, such as drug/gene carrier and tissue engineering scaffold^[6, 7].

As a polypeptide-based polyelectrolyte, γ -PGA can be utilized to prepare hydrogels with various specific applications^[8–12], such as drug/gene carrier and tissue engineering scaffold^[13, 14]. Usually, hydrogels based on γ -PGA can be fabricated *via* γ -irradiation assisted crosslinking^[15, 16] or condensation with compounds bearing reactive groups^[17–19]. γ -Ray irradiated crosslinking is an efficient method for the preparation of γ -PGA hydrogels, with side reactions of nonselectively breaking down of polymer backbones. Condensation of γ -PGA was always followed by side reactions. Compared with the above crosslinking methods, photo-crosslinking^[20, 21], which does not involve harsh conditions such as high energy irradiation or toxic chemicals, is much more cytocompatible and can achieve better spatiotemporal control over the polymerization process and form more homogenous network structure^[22].

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In this study, methacrylated γ -PGA (mPGA) was synthesized through the reaction between glycidyl methacrylate (GMA) and γ -PGA; and γ -PGA hydrogels were then prepared via the photopolymerization of the mPGA precursor under UV irradiation. The swelling behavior, mechanical properties, and cell cytocompatibility of the hydrogel was investigated in detail.

EXPERIMENTAL

Materials

γ -PGA (Nanjing Saitaisi Biotechnology Co. Ltd., China) was extracted with methanol for three times to remove impurities and dried under vacuum before use, and its viscosity-average molar mass (M_{η}) was 2.6×10^5 g/mol as determined by viscometric measurements^[23]. GMA, 4-dimethylaminopyridine (DMAP) and 4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone (Irgacure 2959) were purchased from Aldrich and used as received. All other reagents were of analytical grade and used without purification.

Synthesis of Methacrylated γ -PGA

1.00 g of the purified γ -PGA was dissolved in 10 mL of DI water in a round-bottom flask at room temperature, followed by addition of 50.0 mg of DMAP and stirred for 1 h; afterwards, 1.00 mL of GMA was added to the reaction mixture and the reaction was vigorously stirred for additional 96 h at room temperature. The reaction mixture was precipitated drop wise into 200 mL of acetone; the precipitate was filtered, washed twice with acetone and dried in vacuum at room temperature for 24 h (yield 50%).

Preparation of γ -PGA Hydrogel

A solution containing the precursor (mPGA) at predetermined concentration and 0.05% (W/V) of Irgacure 2959 in phosphate-buffered saline (PBS) was subjected to 365 nm UV light irradiation ($30 \mu\text{W}/\text{cm}^2$) for 10 min in a cylindrical mold (diameter 5.4 mm, height 3.8 mm). After the photopolymerization, hydrogels were removed from the mold for further characterization.

Characterization of γ -PGA-GMA

Proton nuclear magnetic resonance (^1H -NMR) spectra were recorded on a Bruker spectrometer at 400 MHz using D_2O as solvent. Fourier-transformed infra-red (FTIR) spectra were recorded in the range of 500–4000 cm^{-1} on a Bruker VERTEX 70 spectrophotometer with KBr pellet at 25 °C.

Swelling Behavior

The swelling behavior of the hydrogels was investigated in different buffer solutions: 0.01 mol/L citrate-buffered saline (pH 3.0), 0.01 mol/L citrate-buffered saline (pH 4.8), 0.01 mol/L citrate-buffered saline (pH 6.4), and 0.01 mol/L PBS (pH 7.4). The ionic strength of all the buffer solutions was adjusted to 0.15 mol/L using NaCl. As polymerized γ -PGA hydrogels were immersed in the buffer solution at room temperature for 24 h to achieve equilibrium swelling (replenish fresh buffer solution every 6 h), then the samples were blotted with wet filter paper to remove excessive buffer solution on the surface and weighed (W_s); the samples were then dried in vacuum to constant weight (W_d). Swelling studies were carried out in quintuplicate.

The equilibrium swelling ratio (Q_m) of the hydrogel was calculated by the following equation:

$$Q_m = (W_s - W_d) / W_d$$

Mechanical Properties

Unconfined compression test of fully swollen hydrogels was performed on an Universal material testing machine (LLOYD, United Kingdom) at a constant compression rate of 0.5 mm/min, a LR100-Plus force transducer was used. Five duplicates were made for each sample for measurements. The compressive modulus was calculated from the slope of the initial linear region of the stress-strain curve.

Determination of the effective crosslink density

The effective crosslink density of the hydrogel was estimated from the original shear modulus, which can be derived from the compressive test according to Mooney-Rivlin equation^[24]:

$$\sigma = G(\lambda - \frac{1}{\lambda^2}) \quad (1)$$

where σ is the stress in Pascal, λ is the strain, and G is the original shear modulus.

The effective crosslink density of hydrogels can be calculated by the following equation^[25]:

$$\nu_e = G/(RT\nu_{2,s}^{1/3}\nu_{2,r}^{2/3}) \quad (2)$$

where ν_e is the effective network density in mol/m³, R is the gas constant, T is the absolute temperature, $\nu_{2,r}$ and $\nu_{2,s}$ is polymer volume fractions of hydrogel in the relaxed and swollen state, respectively; density of γ -PGA (1.42 g/cm³) was measured through density bottle method in methanol and used for the determination of $\nu_{2,r}$ and $\nu_{2,s}$.

Three-dimensional (3D) Photoencapsulation of Swine Cartilage Chondrocytes (SCCs)

A solution of mPGA precursor (10%, W/V) and Irgacure 2959 (0.05%, W/V) in sterile PBS was prepared. Immediately before photo-encapsulation, SCCs were suspended at a density of 2×10^7 cells/mL in the precursor solution. 60 μ L of the cell suspension was transferred to a cylindrical mold and exposed to 365 nm UV light with intensity of 30 μ W/cm² for 5 min. After photopolymerization, the cell-laden hydrogels were transferred to a 24-well plate containing DMEM culture medium and incubated at 37 °C in 5% CO₂ atmosphere.

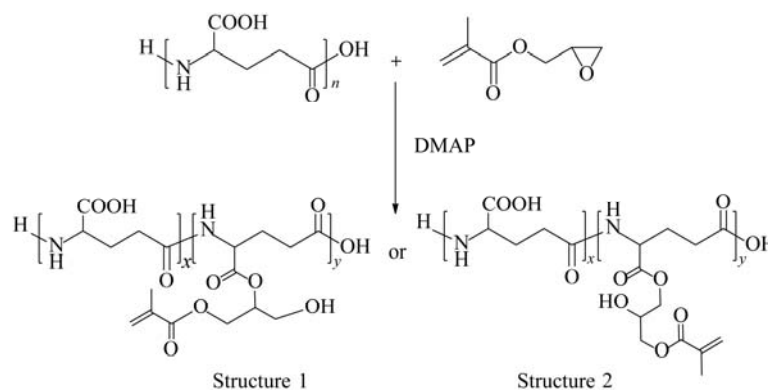
Cell Viability

Cell viability was evaluated using the Live/Dead assay kit after 24 h of photo-encapsulation according to reported protocol^[20]. The live and dead cells were observed under fluorescence microscope (OLYMPUS-LX71 Inverted microscope with U-RFL-T fluorescence lamp), from which the live cells can be seen with green fluorescence, and the dead cells with red fluorescence.

RESULTS AND DISCUSSION

Synthesis of mPGA

mPGA was prepared through the transesterification reaction between γ -PGA and GMA (Scheme 1). The molar feed ratio of γ -PGA to GMA in the reaction and the degree of substitution (DS) of methacryloyl group on mPGA are presented (Table 1). The chemical structure of the product was examined by ¹H-NMR spectrum (Fig. 1). The signals at $\delta = 5.76$ and 6.17 belong to the vinyl protons, and that at $\delta = 1.89$ can be attributed to the methyl protons, the appearance of the above peaks confirms the presence of methacryloyl groups on γ -PGA. This reaction may have two products as isomers: 3-methacryloyl-1-glyceryl and 3-methacryloyl-2-glyceryl esters^[26]. The signals at $\delta = 5.06$ and 4.02 belong to the hydrogen of the methacryloyl-1-glyceryl and methacryloyl-2-glyceryl esters^[27], respectively. The spectrum of the product suggested the presence of both of the two isomers, although it's difficult to calculate their relative ratio due to the overlap of corresponding peaks with those of



Scheme 1 Synthesis of mPGA

other protons. The degree of substitution of the methacryloyl groups (DS, the molar ratio of methacryloyl groups per glutamyl unit) was determined by ^1H -NMR spectroscopy. mPGAs with different DSs was synthesized in this study: PGA-15 and PGA-10, with DS of 15% and 10%, respectively.

Table 1. Preparation of methacrylated γ -PGA

No.	—COOH:GMA (mol:mol)	Degree of substitution (%)	Yield (%)
1	4:1	5.0	43.5
2	2:1	10.0	51.0
3	1:1	15.0	63.5

Note: the γ -PGA to GMA ($n:n$) here is the molar ratio of carboxyl groups from γ -PGA to GMA molecules.

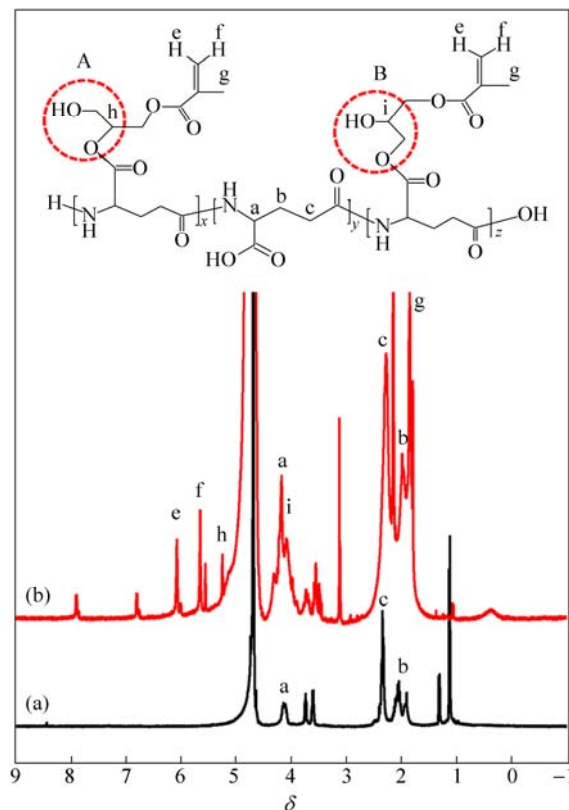


Fig. 1 ^1H -NMR spectra of (a) pristine γ -PGA and (b) mPGA

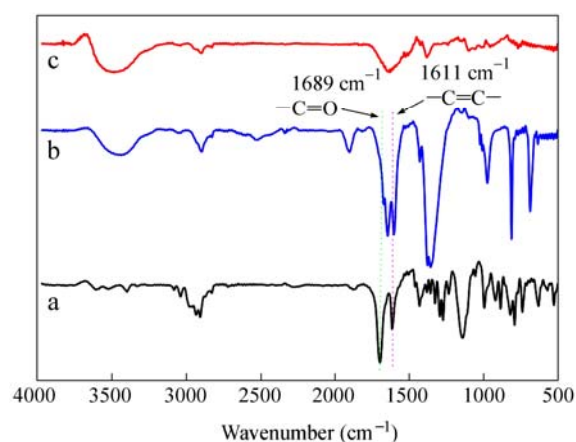


Fig. 2 FTIR spectra of (a) GMA, (b) mPGA and (c) PGA

The FTIR spectra of the product further confirmed the successful incorporation of methacryloyl groups onto the γ -PGA backbone (Fig. 2). The absorption at 1689 cm^{-1} was attributed to the carbonyl groups in a conjugated system, and the presence of C=C groups in mPGA was identified by the appearance of a peak at 1611 cm^{-1} .

Swelling Behavior

We found that the critical polymer concentration for the photopolymerization could be as low as 2% (W/V) for mPGA with 10% DS to fabricate hydrogel. The swelling behavior of γ -PGA hydrogel was investigated as a function of DS of methacryloyl group, precursor concentration and environmental pH. The effect of DS was examined using PGA-10 and PGA-15 with DS of 10% and 15% in PBS, respectively (Fig. 3a). It was observed that hydrogels from precursor with higher DS of methacryloyl groups resulted in lower swelling ratio in PBS. For example, under the same precursor concentration of 2%, the hydrogel from PGA-10 exhibited a swelling ratio of (48.38 ± 2.95) , while that from PGA-15 was (38.41 ± 2.02) ; and similar trend was observed when the precursor concentration was 3%. This can be explained by the increased crosslink density with increased DS of methacryloyl group in the precursor molecule, and higher crosslink density would lead to smaller mesh size as well as lower swelling ratio^[28].

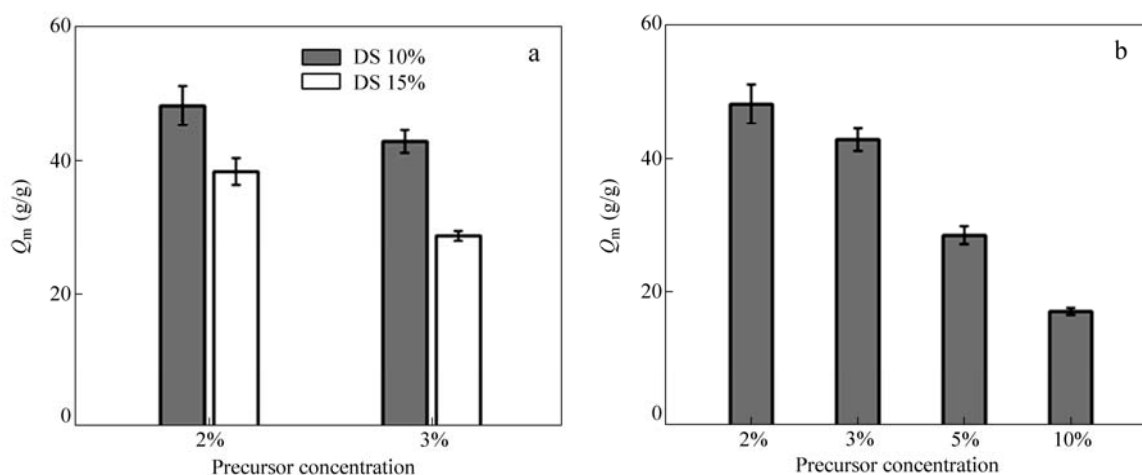


Fig. 3 The effect of (a) DS and (b) precursor concentration (DS = 10%) on the swelling ratio of γ -PGA hydrogel

Using PGA-10 as the precursor, the effect of precursors concentration on the swelling ratio of the hydrogel was studied (Fig. 3b). For hydrogels made from 2%, 3%, 5%, and 10% of PGA-10 solutions, the swelling ratios were (48.38 ± 2.95) , (42.91 ± 1.71) , (28.59 ± 1.34) , and (17.03 ± 0.51) , respectively. The swelling ratio of the γ -PGA hydrogel showed monotonic decrease upon the increase of the precursor concentration.

The γ -PGA hydrogels showed a pH responsive swelling behavior due to its unique structure (Fig. 4). It's reported that γ -PGA has pKa value in the range of 2.6~3.6^[29], the α -carboxyl groups in γ -PGA may be protonated at lower pH and de-protonated at higher pH. The γ -PGA has the helix conformation and the intermolecular interaction of hydrogen bond exists. The protonation/de-protonation of carboxyl groups can cause the formation or dissociation of inter-carboxyl hydrogen bonding^[30], which would facilitate the helix-coil transition and affect the conformational state of the polymer in aqueous^[31–33]. When the carboxyl groups are protonated, the inter-carboxyl hydrogen bonding would make the polymer chain more compact and less hydrated; once the carboxyl groups are deprotonated, the electro-repulsion forces between the negatively charged side groups would make the polymer chain less compact and highly hydrated with a random-coil conformation. Upon the pH change, protonation/de-protonation of carboxyl groups in γ -PGA hydrogel may at least alter the inter-carboxyl hydrogen bonding, and consequently lead to the increase/decrease of apparent crosslink density, and thus affect the swelling behavior of the hydrogel at different pH. To study the effect of pH on the swelling behavior of the hydrogel, the hydrogel was prepared from a 5% solution of PGA-10 precursor

and immersed in buffer solutions at equal ionic strength and different pH, *i.e.* 3.0, 4.8, 6.4, and 7.4. The swelling ratio of the γ -PGA hydrogel at pH 3.0, 4.8, 6.4, and 7.4 was found to be (6.78 ± 0.23) , (25.45 ± 0.20) , (29.41 ± 0.20) , and (28.59 ± 1.34) . Obviously, the hydrogel exhibited the lowest swelling ratio at pH 3.0, and this can be explained by the dominant inter-carboxyl hydrogen bonding at a pH lower than or close to the pK_a of γ -PGA. When increasing the pH to 4.8, at which most of the carboxyl groups was deprotonated, and the inter-carboxyl hydrogen bonding becomes weaker while the electro-repulsion between deprotonated carboxyl groups became stronger, leading to the increase of swelling ratio. Upon pH 6.4 and higher, which is far more higher than pK_a of γ -PGA, carboxyl groups would be fully deprotonated, and the electro-repulsion becomes dominant. Once γ -PGA hydrogel is fully deprotonated at pH 6.4 or higher, its swelling ratio of γ -PGA hydrogel reaches a maximum value and does not change with pH.

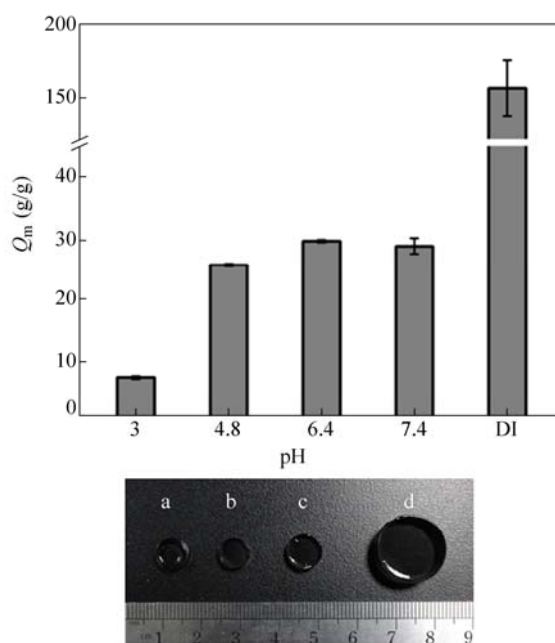


Fig. 4 Effect of pH on the swelling ratio of the hydrogel and photograph of swollen hydrogels under different pH on bench top (a, as polymerized hydrogel; b, pH 3.0; c, pH 7.4; d, DI water)

No surprisingly, the swelling ratio of γ -PGA hydrogel in DI water was 160 and much higher than those in buffer solutions. Swelling ratio of polyelectrolyte hydrogel is highly dependent on the ionic strength; in the case of γ -PGA, the shielding effect of mobile ions may weaken the inter- and/or intra-molecular interaction of the hydrogel backbone and result in reduced swelling ratio^[17, 34].

Mechanical Properties

The protonation/de-protonation of the side carboxyl groups not only affects the swelling ratio of the hydrogel, but also has significant impact on the mechanical properties of the γ -PGA hydrogel. mPGA with DS of 10% at precursor concentration of 5% (W/V) was used to study the influence of pH on the mechanical properties of the hydrogel. The mechanical properties of γ -PGA hydrogels were studied using buffer solutions at pH 3.0, 4.8, 6.4, and 7.4, respectively (Table 2).

The electro-repulsion force in polyelectrolyte hydrogels has proved to contribute to the increase of elastic modulus/stiffness, provided those hydrogels have the same or similar swelling ratio/polymer content. Surprisingly, at pH 3.0, the γ -PGA hydrogel, which bears no negative charge on the side group, exhibited significantly higher compressive modulus (48.13 ± 5.95 kPa) than that at pH 4.8 (22.42 ± 5.47 kPa), 6.4 (21.94 ± 2.44 kPa), and 7.4 (18.74 ± 3.22 kPa). This phenomenon can be attributed to the inter-carboxyl hydrogen

bonding, which acts as physical crosslinking points and increases the apparent crosslink density in addition to the co-valent crosslinking. The fracture stress, fracture strain and fracture energy of hydrogels at pH 3.0 were also much higher than those at pH 4.8, 6.4, and 7.4. This can also be explained by the inter-carboxyl hydrogen bonding, which is reversible in nature and can be temporarily broken down to sustain stress/energy dissipation^[35].

Table 2. Mechanical properties of the hydrogel at different pH (Concn. = 5%, DS = 10%)

pH	Compressive modulus (kPa)	Fracture stress (kPa)	Fracture strain (%)	Fracture energy (kJ/m ³)	Crosslink density (mol/m ³)
3.0	48.13 $\pm$ 5.95	265.71 $\pm$ 16.83	72.0 $\pm$ 1.0	40.30 $\pm$ 3.40	142.86
4.8	22.42 $\pm$ 5.47	82.41 $\pm$ 17.54	61.0 $\pm$ 1.0	11.74 $\pm$ 2.44	100.37
6.4	21.94 $\pm$ 2.44	82.50 $\pm$ 10.05	59.0 $\pm$ 1.0	11.60 $\pm$ 1.33	107.24
7.4	18.74 $\pm$ 3.22	110.15 $\pm$ 14.63	66.0 $\pm$ 2.0	14.90 $\pm$ 1.90	94.56

When γ -PGA hydrogel swells at pH 4.8 and higher pH, most of the carboxyl groups are presumably de-protonated; thus close or no significantly different compressive modulus, fracture stress, fracture strain, fracture energy and crosslink density were observed at pH 4.8, 6.4, and 7.4.

Cell Viability

The viability of the photoencapsulated SCCs in γ -PGA hydrogel was evaluated by Live/Dead assay, with pure poly(ethylene glycol) diacrylate (PEGDA-20K) hydrogels as the control, where live cells were fluoresced green and dead cells were fluoresced red. After 24 h of photoencapsulation, live/dead fluorescence staining results indicated that there was no significant difference in the viability of SCCs between the control and the hydrogels (Fig. 5). This result suggests that γ -PGA hydrogels have comparable cytotoxicity with the widely recognized PEG hydrogels.

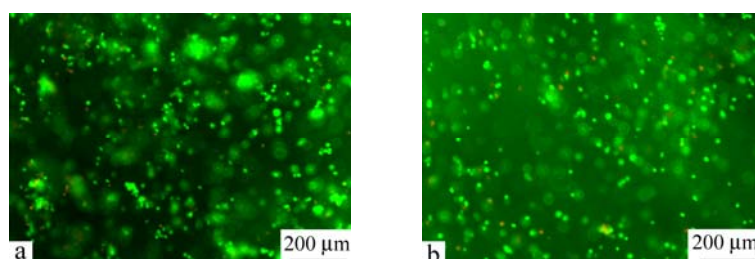


Fig. 5 Fluorescent micrographs of SCCs encapsulated γ -PGA hydrogels: (a) PEGDA hydrogel and (b) γ -PGA hydrogel

CONCLUSIONS

Hydrogel based on methacrylated poly(γ -glutamic acid) was prepared *via* photopolymerization. The properties of the hydrogel can be tuned by varying the precursor concentration as well as the environmental pH. Inter-carboxyl hydrogen bonding was proposed to be responsible for the enhanced compressive modulus, fracture strength and fracture energy at lower pH. Poly(γ -glutamic acid) hydrogels are proved to be cytocompatible toward swine cartilage chondrocytes and may be used as a potential scaffolding materials in cartilage tissue engineering.

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