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In situ injectable poly(γ-glutamic acid) based biohydrogel formed by enzymatic crosslinking

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ABSTRACT: Enzymatic crosslinking was developed to prepare *in situ* forming poly(γ-glutamic acid) (γ-PGA) based hydrogel in this study. First, the precursor of poly(γ-glutamic acid)–tyramine (γ-PGA–Ty) was synthesized through the reaction of carboxyl groups from a γ-PGA backbone with tyramine. The structure of the grafted precursor was confirmed by ^{1}H-NMR and Fourier transform infrared spectroscopy. After that, the crosslinking of the phenol-containing γ-PGA–Ty precursor was triggered by horseradish peroxidase in the presence of H_2O_2; this resulted in the formation of the γ-PGA–Ty hydrogels. The equilibrium water content, morphology, enzymatic degradation rate, and mechanical properties of the hydrogels were characterized in detail. The data revealed that the well-interconnected hydrogels had tunable water contents, mechanical properties, and degradability through adjustments of the composition. Furthermore, cell experiments proved the biocompatibility of the hydrogels by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. These characteristics provide an opportunity for the *in situ* formation of injectable biohydrogels as potential candidates in cell encapsulation and drug delivery. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42301.

KEYWORDS: biodegradable; crosslinking; gels; porous materials; properties and characterization

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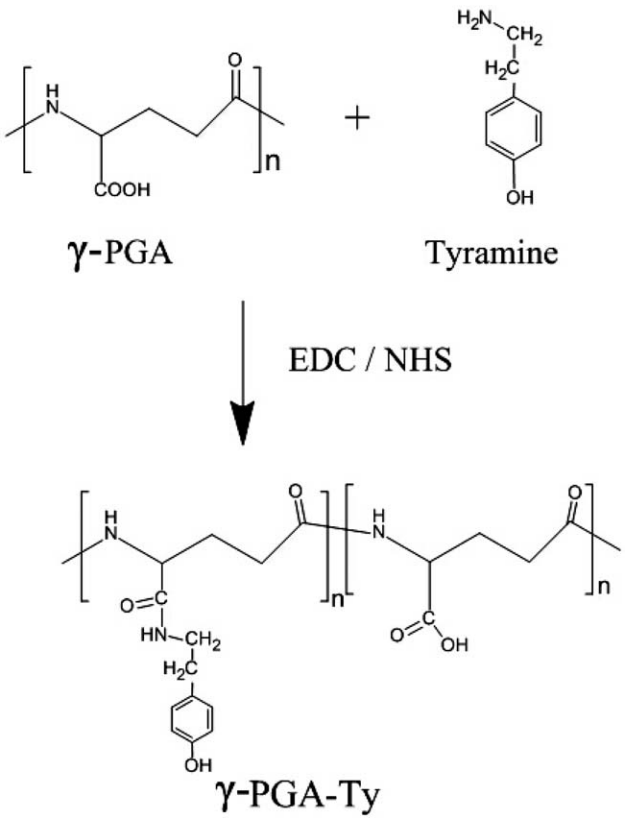
INTRODUCTION

Hydrogels are three-dimensional networks capable of absorbing large amounts of water or biological fluids; they have been used for various biomedical applications.[1–11] In recent decades, injectable hydrogels have attracted much attention because of their great success in plastic and reconstructive surgery and their potential in regenerative medicine. The *in situ* formation of injectable hydrogels not only can provide the possibility of good alignment with the wound cavity, but it also offers the advantage of minimally invasive surgery and minimizes the possibility of scar formation; this reduces the risk of infection and complications.[12–15] This has become one of the most active research fields in the last several years. For instance, Deng's group[16] explored the *in situ* formation of hydrogels using tetrazole–alkene chemistry. Li *et al.*[17] prepared starch-based *in situ* forming hydrogels by a Schiff base reaction. Click chemistry is an effective approach for developing hydrogels formed *in situ*,[18] but complicated chemical modifications are required in their polymer backbones.

Most recently, increasing interest has been devoted to enzymatically crosslinked hydrogels, mainly because of the mildness of the reaction. The majority of the enzymes involved in the crosslinking approach are the same as the enzymes that catalyze reactions that naturally occur in our bodies.[19–29] Horseradish peroxidase (HRP) is the most widely used enzyme for enzymatically crosslinked hydrogels because of its high stability and good biocompatibility. The HRP-mediated *in situ* forming hydrogels on which researchers have focused have generally been natural polymers, such as hyaluronic acid,[30,31] dextran,[27] gelatin,[32,33] poly(aspartic acid),[34] and chitosan.[35] Synthetic polymers, such as poly(L-glutamic acid)[29] and 4-arm poly(propylene oxide)-poly(ethylene oxide) (PPO-PEO) oligomer[36] have also been applied for enzymatic hydrogels. However, the clinical applications of synthetic polymer hydrogels are always restricted severely, because of their extremely complex synthesis process and the potential toxicity of the additive residues.

Poly(γ-glutamic acid) (γ-PGA) chemically is a nylon 4 derivative bearing a carboxylic side group attached to the fourth carbon of the repeating unit. It was discovered as a component of the capsule of *Bacillus anthracis* in 1935.[37] It was reported to be the main extracellular product excreted in the aerobic fermentation of organic substrates with *Bacillus subtilis*.[38] Some of the most interesting properties of γ-PGA that make it an ideal candidate for biomedical applications are its water solubility, biodegradability, edibility, and nontoxicity toward human and environment.[39–41] It has been found to have profound applications in biomedical areas.[42]

In this research, a novel *in situ* forming injectable γ-PGA biohydrogel was studied. The poly(γ-glutamic acid)–tyramine



Scheme 1. Synthesis of the γ-PGA–Ty precursor.

(γ-PGA–Ty) precursor was first synthesized with 1-ethyl-(3-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/*N*-hydroxysuccinimide (NHS) protocol under mild conditions, and its structure was subsequently confirmed by ^{1}H-NMR and Fourier transform infrared (FTIR) spectroscopy. After that, the crosslinking of the precursor was catalyzed by HRP in the presence of H_2O_2. The performances, including the equilibrium water content (EWC), morphology, enzymatic degradation rate, and mechanical properties of the biohydrogels were then examined in detail. Finally, the *in vitro* cytotoxicity of the sterilized hydrogel was also evaluated.

EXPERIMENTAL

Materials

γ-PGA (weight-average molecular weight 1000 kDa) was obtained from Shineking Biotechnology Co., Ltd. (Nanjing, China). Tyramine hydrochloride (Ty), NHS, HRP (260 U/mg), and papain (6000 U/mg) were all purchased from Sigma-Aldrich. EDC was obtained from Adamas-Beta Chemical Co (Shanghai, China). Dimethyl sulfoxide and H_2O_2 were purchased from Sinopharm Chemical Reagent Co, Ltd. (Shanghai, China).

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, L-glutamine, trypsin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Invitrogen Co. (Carlsbad, CA). L929 mouse fibroblasts cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Science). The L929 cells were cultured in complete growth culture medium prepared with DMEM supplemented with 10% fetal bovine serum, 1 m*M* L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 atmosphere. All of the other chemical reagents were supplied from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Synthesis of the γ-PGA–Ty Precursor

The γ-PGA–Ty precursor was prepared as described previously[30] with some modifications to improve the grafting ratio, as shown in Scheme 1. γ-PGA (1 g, 7.75 mmol) and Ty (1.076 g, 6.2 mmol) were dissolved in 50 mL of distilled water. EDC (4.457 g, 23.25 mmol) and NHS (2.674 g, 23.25 mmol) were then added to initiate the conjugation reaction. As the reaction proceeded, the pH of the mixture was maintained at 4.8 with 1*M* NaOH and 1*M* HCL. The reaction mixture was stirred overnight at room temperature, and then the pH was brought to 7.0. The solution was dialyzed (molecular weight cutoff = 1000 Da) successively against a 100 m*M* sodium chloride solution for 2 days, a mixture of distilled water and ethanol (3 : 1) for 1 day, and distilled water for 1 day. The purified solution was lyophilized to obtain γ-PGA–Ty with a yield of 86%. The degree of substitution[29] (the number of tyramine molecules per 100 repeating units of γ-PGA) was calculated from ^{1}H-NMR (Bruker 500-MHz, D_2O) measurements by a comparison of the ratio of the relative peak integrations of the phenyl protons at 7.0 and 6.7 ppm of tyramine and the hydrogens of two methylene at 1.8–2.3 ppm of γ-PGA. The degree of substitution was about 28%. The samples for the FTIR spectrum were obtained by the creation of 0.1 g of pellets with a ratio of KBr/sample of 99 : 1. FTIR spectroscopic analysis (Nexus 870, Nicolet) was set on transmission mode with a wave-number range between 4000 and 500 cm^{-1}.

Hydrogel Formation

The γ-PGA–Ty hydrogels were prepared with the compositions listed in Table I. Briefly, an aqueous solution composed of γ-PGA–Ty (3–7% w/v) was prepared at room temperature, and then, the solution was poured into a 5 mm high cylindrical mold. HRP was added with a final concentration of 10 or 20 U/mL, whereas H_2O_2 was added at a concentration of 10 m*M*. After that, the mixture was quickly stirred to obtain a homogeneous solution. This allowed it to form a hydrogel at room temperature in 20 min. After release from the mold, the samples, which were sterilized in 75% ethanol and then washed three times in deionized water, were used for further characterization.

Table I. Compositions of the γ-PGA–Ty Hydrogels

Sample	γ-PGA–Ty (mg/mL)	HRP (U/mL)	H_2O_2 (mmol/L)
PA10	30	10	10
PA20	30	20	10
PB10	50	10	10
PB20	50	20	10
PC10	70	10	10
PC20	70	20	10

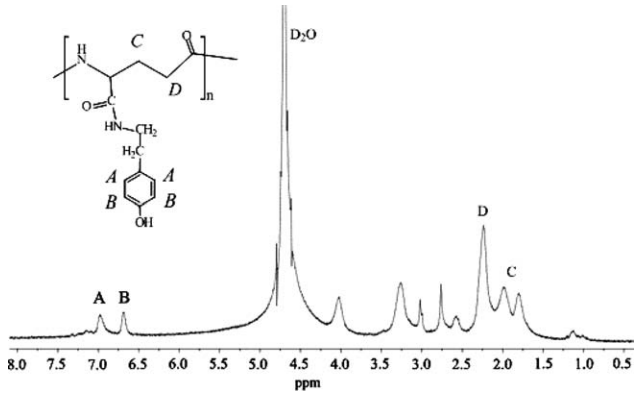


Figure 1. ^{1}H-NMR spectrum of the γ-PGA–Ty precursor.

The gelation time was established by the vial tilting method,[43] that is, with no flow within 1 min after the vial inverted regarded as the gel state. The samples with γ-PGA-Ty concentration of 30, 50 and 70 mg/ml were defined as PA, PB and PC group, respectively. The number after the term indicates different HRP concentration.

EWC

The as-prepared hydrogels (PA10, PA20, PB10, PB20, PC10, and PC20) were incubated in distilled water at room temperature for 24 h to reach their equilibrium swollen state. After excess surface water was removed, the swollen weight (W_s) of each hydrogel was recorded. Then the samples were lyophilized to complete dryness and the dry weight (W_d) of each samples was weighed. The EWC was calculated with the following equation:

$$\mathrm{EWC}=\frac{(W_s-W_d)}{W_d}$$

All of the samples were tested in triplicate for each group.

Scanning Electron Microscopy (SEM)

The morphologies of the hydrogels were characterized with SEM (Mirero AIS2100, Seoul, Korea) with PC20 as an example. The samples were placed in a −20°C freezer and lyophilized in a lyophilizer (Lyovac GT-2, Germany). The lyophilized samples were then positioned on SEM stubs with adhesive tape and sputter-coated for 1 min with a thin gold layer. The morphologies of the samples were viewed at a 10-kV accelerating voltage.

Mechanical Properties

The mechanical properties of the γ-PGA–Ty hydrogels (PA10, PA20, PB10, PB20, PC10, and PC20) were characterized by a universal testing system (YL-1109, Yuelian Testing Machines Co., Ltd., Dongguan, China) at room temperature. Cylindrical samples 5 mm in height and 10.7 mm in diameter prepared in a mold were subjected to compression tests with a compression rate of 20 mm/min. Individual compressive strength was obtained from the load–displacement curve at break. All of the samples were tested in triplicate for each group.

In Vitro Enzymatic Degradation

To test enzymatic degradation of the γ-PGA–Ty hydrogels, bulk samples were immersed in phosphate-buffered saline (PBS; 0.01*M*) containing papain (0.05 mg/mL) under constant shaking at 100 rpm for 2, 4, 8, and 24 h at 37°C to accelerate degradation. At each predetermined time interval, the samples were washed with distilled water and then lyophilized. The *in vitro* degradation rate was calculated by the dry weight after degradation (W_t) divided by the initial weight of the gel (W_0) as follows:

$$\text{Fractional mass remaining}=(W_t/W_0)\times 100\%$$

All of the samples were tested in triplicate for each group.

Cytotoxicity Evaluation

The evaluation of the cytotoxicity of the γ-PGA–Ty hydrogel was accomplished in an extracted solution of the hydrogel via MTT assay according to ISO 10993-5. Briefly, sterilized hydrogels were extracted with DMEM at an extraction ratio of 1 cm^2/mL at 37°C for 24 h. A 100-μL media suspension containing a total of 10^4 cells and 100 μL of extract solution were plated into each well of the 96-well plate and then incubated at 37°C in a 5% CO_2 atmosphere. On the 1st, 3rd, and 5th day, 20 μL of MTT (5 mg/mL in PBS) was added for 4 h to allow the formation of formazan crystals. After the removal of the supernatant, 150 μL of dimethyl sulfoxide was added to each well, and the absorbance was measured at 490 nm with an enzyme-linked immunosorbent assay reader (Elx800, Bio-Tek Instrument, Inc., Winooski, VT). The results were expressed as percentages relative to the data obtained with the blank control. Six samples were tested for each group.

RESULTS AND DISCUSSION

Synthesis of the γ-PGA–Ty Precursor

With the EDC/NHS protocol, the γ-PGA–Ty precursor was chemically coupled by the carboxylic acid groups in γ-PGA and the amine groups in Ty via the amide bonds, as shown in Scheme 1. ^{1}H-NMR was used to confirm the structure of the precursor and to determine the degree of Ty substitution, as revealed in Figure 1. The peaks at 1.8–2.3 ppm were assigned to γ-PGA methylene protons, and methine protons at 4.1 ppm were present in both the initial γ-PGA and γ-PGA–Ty precursor spectra because the γ-PGA backbone remained untouched. The success of the grafting was verified by the additional peaks

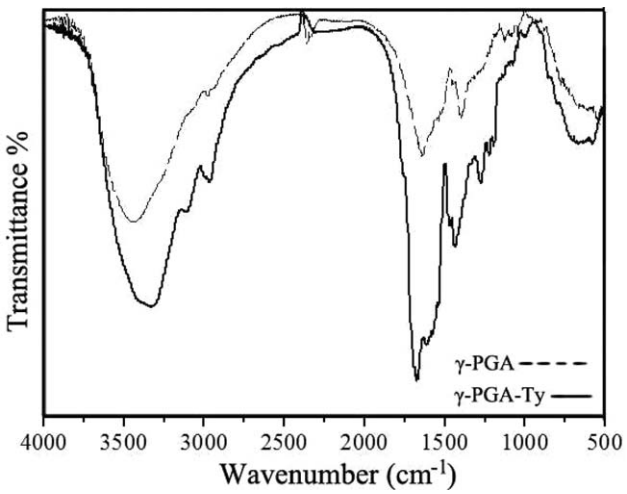
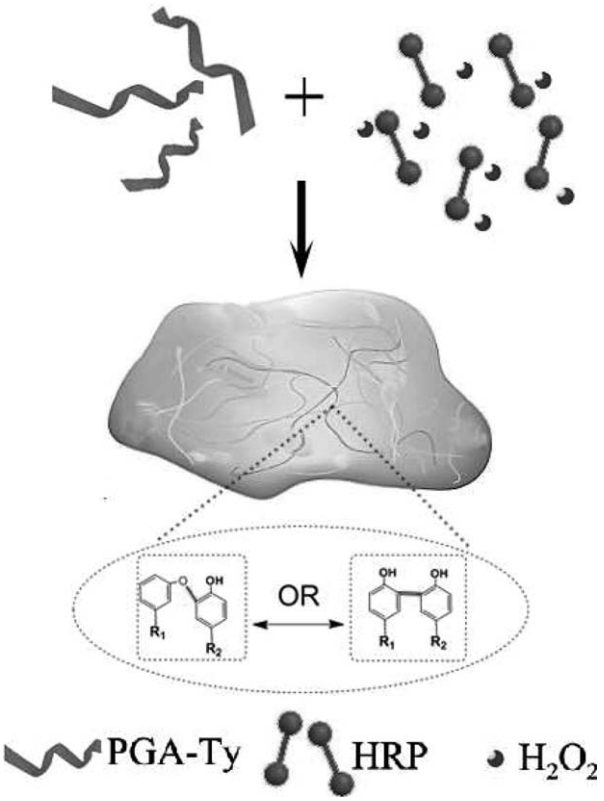


Figure 2. FTIR spectra of the γ-PGA and γ-PGA–Ty precursor.



Scheme 2. Illustration of the formation of the γ-PGA–Ty hydrogel via enzymatic crosslinking. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

corresponding to aromatic protons at 6.9 and 7.2 ppm.[30] The degree of tyramine substitution was calculated to be 28%.

The chemical structure of γ-PGA and γ-PGA–Ty was further analyzed by FTIR spectroscopy, as shown in Figure 2. The strongest absorption bands in the FTIR spectra of the γ-PGA and γ-PGA–Ty precursor were observed around 3700–3000 cm^{-1}; these corresponded to —OH stretching vibrations with a maximum at about 3400 cm^{-1}. The other main adsorption bands of the FTIR spectrum of pure γ-PGA were COO— bands with a maximum intensity at 1610 and 1400 cm^{-1}. In contrast, the FTIR spectrum of the γ-PGA–Ty precursor had characteristic peaks represented by stretching vibrations of the Ty substituents around 1700 cm^{-1} (aromatic C=C). The peaks around 1650 cm^{-1} (C=O in the amide bond) increased significantly, and this also confirmed the success of Ty grafting on the γ-PGA backbone.[22]

In Situ Forming γ-PGA–Ty Hydrogel

The formation of the γ-PGA–Ty hydrogel via enzymatic crosslinking is depicted in Scheme 2. The γ-PGA–Ty precursor solution was gently shaken during and after the addition of HRP/H_2O_2 to prevent the formation of clusters (high-crosslinking-density regions) and achieve a homogeneous structure in the final hydrogel. The γ-PGA grafted with tyramine could be crosslinked by HRP in the presence of H_2O_2. To prevent the cytotoxicity problem that H_2O_2 may cause, a low concentration of H_2O_2 (10 m*M*) was used in this study.[22,30] The principle of this kind of crosslinking reaction was confirmed, as reported in the literature,[27,44,45] and could be characterized by the appearance of two types of bonds: (1) C—C bonds between the two carbon atoms at the ortho position and (2) C—O bonds between the two carbon atoms at the ortho position and the oxygen atom from the phenolic group.

The average gelation time of enzymatically crosslinked γ-PGA–Ty hydrogel was measured to be about 10 s (10 ± 2 s) with the tilting method;[43] this was very suitable for the *in situ* forming injectable hydrogel. This *in situ* forming injectable hydrogel system not only could provide the possibility of good alignment with wound cavities inside the body, but it could also offer the advantage of minimally invasive surgery and minimize the possibility of scar formation. Furthermore, fast gel formation could also prevent the diffusion of active substances outside the gel, which is an important factor in clinical application.

EWC

Figure 3 illustrates the EWC of γ-PGA–Ty hydrogels with the compositions listed in Table I. In the first group with 10 U/mL HRP, EWC decreased with increasing γ-PGA–Ty content; this indicated that the hydrogel network formed more efficiently with increasing crosslinking density. The second group with 20 U/mL HRP exhibited a similar trend with increasing precursor content. This phenomenon was similar to results from the literature.[29,46] Among all of these groups, PC20 showed the lowest EWC value of 13.2; this could be ascribed to its highest crosslinking density. The PA10 hydrogel group having lowest precursor content and HRP concentration was formed at a low crosslinking density; this resulted in a relatively loose structure with an EWC value of 18.1. Compared with the data reported previously,[22,47] the γ-PGA–Ty biohydrogel had a tunable EWC value in a range from 13.2 to 18.1 and is expected to have biomedical applications.

Morphology

The pore size and pore interconnectivity are critical parameters in determining the performance of hydrogels in tissue engineering. The pore size affects the cell attachment, migration, morphology, and proliferation; the pore structures also have a

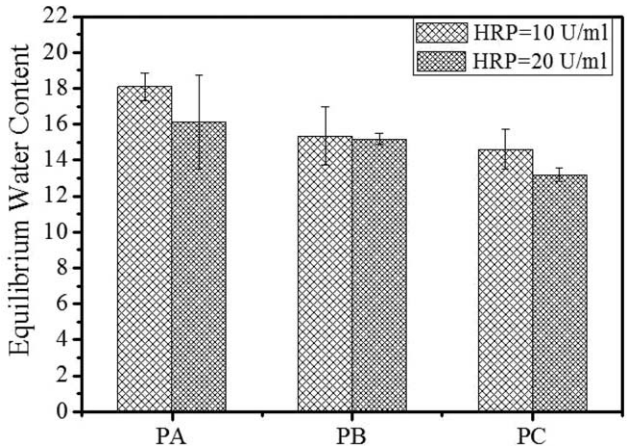


Figure 3. EWC of the PA10, PA20, PB10, PB20, PC10, and PC20 hydrogels.

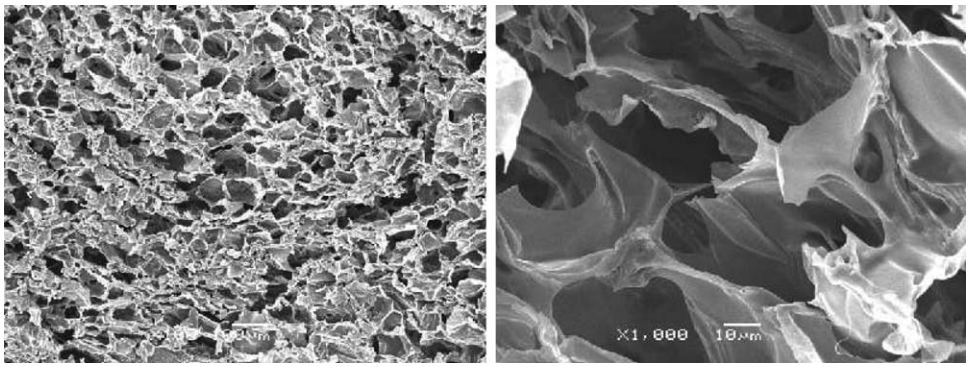


Figure 4. SEM images of the lyophilized PC20 hydrogel samples (left: 100× and right: 1000×).

strong influence on the mechanical properties of the matrix, the supply of nutrients, and the removal of waste products. To characterize the microstructures and morphologies of the lyophilized γ-PGA–Ty hydrogel, SEM was used to observe their details. As shown in Figure 4, the hydrogel of PC20 used as a sample was highly porous with a well-interconnected pore structure. It was characterized by a wide pore size distribution; the estimated pore size was in the range 10–100 μm. It is assumed that these properties are satisfactory for cell encapsulation and other biomedical applications, as reported in the literature.[48,49]

Mechanical Properties

The compressive strength of the γ-PGA–Ty hydrogels was examined with a universal tester, as shown in Figure 5. In the PA group, the structure of the hydrogel was relatively unstable because of the lower precursor concentration and crosslinking density; this resulted in a lower compression modulus. The compression modulus values of the PB and PC groups increased gradually with increasing γ-PGA–Ty concentration. The data indicated that the crosslinking density was enhanced; this resulted in a more compact hydrogel structure. These results exhibit similar trends to some reported in the literature.[50,51]

Furthermore, with increasing HRP concentration, the compression modulus of each group was obviously promoted. The compression modulus of PA20 was higher than that of PA10 by 11% (4200 vs 3800 Pa). The compression modulus of PC20 was higher than that of PC10 by 12% (11,500 vs 10,300 Pa). The data reveals that the increase in the HRP concentration enhanced the crosslinking density to form more compact and stable hydrogel networks. This was in good agreement with the results found in the EWC study. Compared with some reported hydrogels,[24,52] the γ-PGA–Ty biohydrogel in this study had a tunable compression modulus from 4 to 11.5 KPa, which is expected to be suitable for different clinical applications.

In Vitro Enzymatic Degradation

The enzymatic degradation properties of the γ-PGA–Ty hydrogels (PB20 and PC20) were characterized by the measurement of the hydrogel weight change in PBS containing 0.05 mg/mL papain at 37°C, as shown in Figure 6. This indicated that the weight of PC20 decreased 15.8% within 2 h. The PB20 decreased 23.0% within 2 h, and 65.6% remained after 24 h. We found that the PA always broke into very small pieces after a period of degradation, and the degradation data of PA could not be obtained properly. In the initial stage, the rapid degradation of the PB20 hydrogel was attributed to the relatively loose network structure in the hydrogel. The greater fluidity of free water in the PB20 group made the enzyme solution the contact of the crosslinked hydrogel much easier, and thus, it degraded rapidly. Although in the PC20 group the hydrogel with a higher precursor concentration formed a more compact structure, the

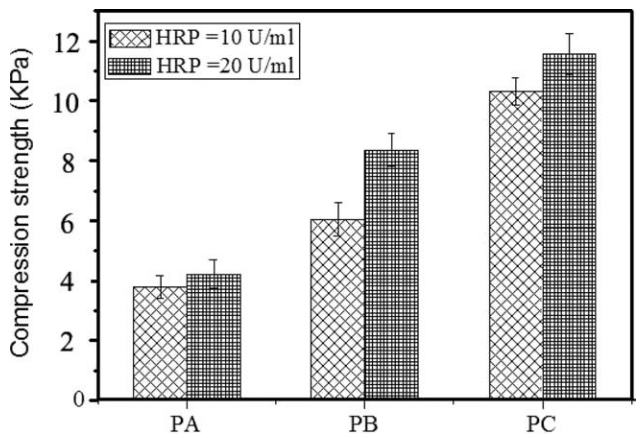


Figure 5. Compressive strength of the PA10, PA20, PB10, PB20, PC10, and PC20 hydrogels.

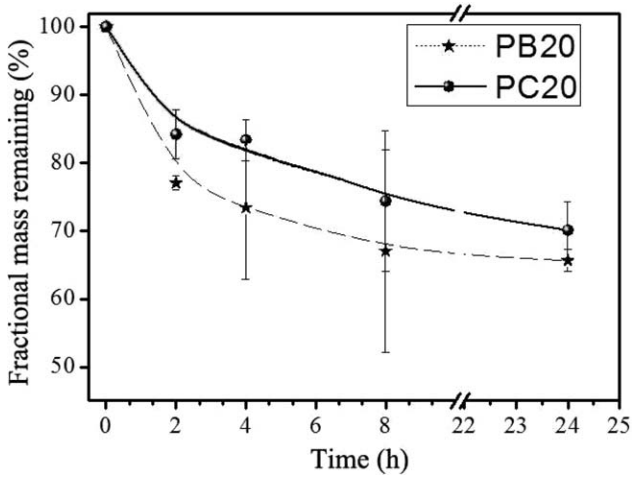


Figure 6. Degradation profiles of the PB20 and PC20 hydrogels in PBS containing 0.05 mg/mL papain at 37°C.

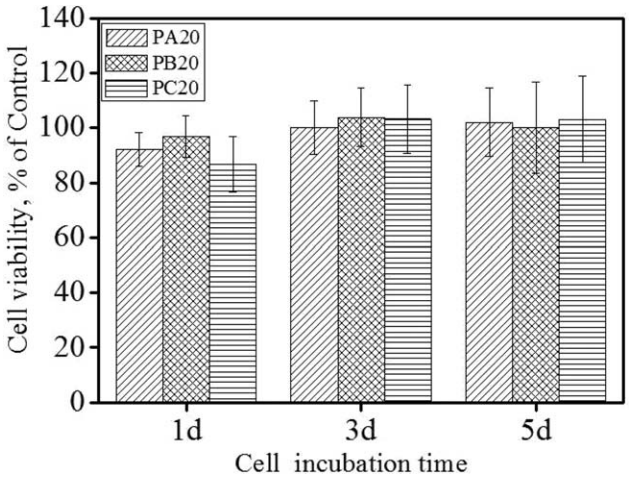


Figure 7. Cytotoxicity of the PA20, PB20, and PC20 hydrogels.

enzyme was hindered to access the cleavage sites in γ-PGA, and thus, the degradation approach of PC20 tended to be more regular and controllable. In addition, this kind of γ-PGA–Ty hydrogels had a much longer degradation time and more controllable degradability compared to our previous work.[53] One of the most important properties of γ-PGA is the degradability; this ideally places them as material of choice for tissue engineering.[42] Furthermore, this controllable degradation property makes this type of hydrogel gain good potential use in cell or drug release *in vivo*.

In Vitro Cytotoxicity

The *in vitro* cytotoxicity of the γ-PGA–Ty hydrogels was investigated with mouse fibroblast cells (L929) incubated with their extract solution for 1, 3, or 5 days. The quantitative assessments of their cytotoxicity by MTT assay are shown in Figure 7. The results reveal that there were no significant differences in the cytotoxicity among all of the γ-PGA–Ty hydrogel groups. The calculated value of relative growth rate (RGR) confirmed the biocompatible nature of the γ-PGA–Ty biohydrogels and met the requirements of a potential material for biomedical applications well.

CONCLUSIONS

The naturally derived biopolymer γ-PGA was successfully grafted with tyramine with the EDC/NHS protocol. The γ-PGA–Ty precursor was enzymatically crosslinked with HRP in the presence of a low concentration of H_2O_2 to provide a gelation time within 10 ± 2 s at room temperature. In addition, the adjustable mechanical properties, porous interconnected structure, tunable degradation, and ideal EWC of the γ-PGA–Ty biohydrogels were investigated in detail. Furthermore, the preliminary biological evaluation revealed the nature of nontoxicity of the hydrogels. All of these characteristics provided a potential opportunity for the *in situ* forming γ-PGA–Ty biohydrogels for numerous biomedical applications.

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REFERENCES

1. Deligkaris, K.; Tadele, T. S.; Olthuis, W.; van den Berg, A. *Sens. Actuators B* **2010**, *147*, 765.
2. Peppas, N. A.; Hilt, J. Z.; Khademhosseini, A.; Langer, R. *Adv. Mater.* **2006**, *18*, 1345.
3. Sidorenko, A.; Krupenkin, T.; Taylor, A.; Fratzl, P.; Aizenberg, J. *Science* **2007**, *315*, 487.
4. Oh, J. K.; Drumright, R.; Siegwart, D. J.; Matyjaszewski, K. *Prog. Polym. Sci.* **2008**, *33*, 448.
5. Nuttelman, C. R.; Rice, M. A.; Rydholm, A. E.; Salinas, C. N.; Shah, D. N.; Anseth, K. S. *Prog. Polym. Sci.* **2008**, *33*, 167.
6. Lee, K. Y.; Mooney, D. *J. Prog. Polym. Sci.* **2012**, *37*, 106.
7. Hoffman, A. S. *Adv. Drug Delivery Rev.* **2002**, *54*, 3.
8. Jia, X.; Kiick, K. L. *Macromol. Biosci.* **2009**, *9*, 140.
9. He, C.; Kim, S. W.; Lee, D. S. *J. Controlled Release* **2008**, *127*, 189.
10. Bhattarai, N.; Gunn, J.; Zhang, M. *Adv. Drug Delivery Rev.* **2010**, *62*, 83.
11. Liu, F.; Seuring, J.; Agarwal, S. *Macromol. Chem. Phys.* **2014**, *215*, 1466.
12. Zarembinski, T. I.; Doty, N. J.; Erickson, I. E.; Srinivas, R.; Wirostko, B. M.; Tew, W. P. *Acta Biomater.* **2014**, *10*, 94.
13. Kim, K. S.; Park, S. J.; Yang, J. A.; Jeon, J. H.; Bhang, S. H.; Kim, B. S.; Hahn, S. K. *Acta Biomater.* **2011**, *7*, 666.
14. Radhakrishnan, J.; Krishnan, U. M.; Sethuraman, S. *Biotechnol. Adv.* **2014**, *32*, 449.
15. Bae, K. H.; Wang, L.-S.; Kurisawa, M. *J. Mater. Chem. B* **2013**, *1*, 5371.
16. Fan, Y.; Deng, C.; Cheng, R.; Meng, F.; Zhong, Z. *Biomacromolecules* **2013**, *14*, 2814.
17. Li, Y.; Liu, C.; Tan, Y.; Xu, K.; Lu, C.; Wang, P. *Carbohydr. Polym.* **2014**, *110*, 87.
18. Takahashi, A.; Suzuki, Y.; Suhara, T.; Omichi, K.; Shimizu, A.; Hasegawa, K.; Kokudo, N.; Ohta, S.; Ito, T. *Biomacromolecules* **2013**, *14*, 3581.
19. da Silva, M. A.; Bode, F.; Drake, A. F.; Goldoni, S.; Stevens, M. M.; Dreiss, C. A. *Macromol. Biosci.* **2014**, *14*, 817.
20. Darr, A.; Calabro, A. *J. Mater. Sci. Mater. Med.* **2009**, *20*, 33.
21. Lim, K. S.; Alves, M. H.; Poole-Warren, L. A.; Martens, P. *J. Biomaterials* **2013**, *34*, 7097.
22. Kuzmenko, V.; Hägg, D.; Toriz, G.; Gatenholm, P. *Carbohydr. Polym.* **2014**, *102*, 862.
23. Lee, B. P.; Dalsin, J. L.; Messersmith, P. B. *Biomacromolecules* **2002**, *3*, 1038.
24. Joung, Y. K.; You, S. S.; Park, K. M.; Go, D. H.; Park, K. D. *Colloids Surf. B* **2012**, *99*, 102.
25. Sakai, S.; Liu, Y.; Matsuyama, T.; Kawakami, K.; Taya, M. *J. Mater. Chem.* **2012**, *22*, 1944.
26. Liu, Y.; Sakai, S.; Taya, M. *Acta Biomater.* **2013**, *9*, 6616.
27. Moreira Teixeira, L. S.; Bijl, S.; Pully, V. V.; Otto, C.; Jin, R.; Feijen, J.; van Blitterswijk, C. A.; Dijkstra, P. J.; Karperien, M. *Biomaterials* **2012**, *33*, 3164.

28. Lih, E.; Lee, J. S.; Park, K. M.; Park, K. D. *Acta Biomater.* **2012**, *8*, 3261.
29. Ren, K.; He, C.; Cheng, Y.; Li, G.; Chen, X. *Polym. Chem.* **2014**, *5*, 5069.
30. Kurisawa, M.; Chung, J. E.; Yang, Y. Y.; Gao, S. J.; Uyama, H. *Chem. Commun.* **2005**, *34*, 4312.
31. Lee, F.; Chung, J. E.; Kurisawa, M. *Soft Matter* **2008**, *4*, 880.
32. Park, K. M.; Ko, K. S.; Joung, Y. K.; Shin, H.; Park, K. D. *J. Mater. Chem.* **2011**, *21*, 13180.
33. Wang, L.-S.; Boulaire, J.; Chan, P. P. Y.; Chung, J. E.; Kurisawa, M. *Biomaterials* **2010**, *31*, 8608.
34. Sofia, S. J.; Singh, A.; Kaplan, D. L. *J. Macromol. Sci. Chem.* **2002**, *39*, 1151.
35. Jin, R.; Lin, C.; Cao, A. *Polym. Chem.* **2014**, *5*, 391.
36. Park, K. M.; Shin, Y. M.; Joung, Y. K.; Shin, H.; Park, K. D. *Biomacromolecules* **2010**, *11*, 706.
37. Bruckner, V.; Ivánovics, G.; Hoppe-Seylers, Z. *Physiol. Chem.* **1937**, *247*, 281.
38. Bovarnick, M. *J. Biol. Chem.* **1942**, *145*, 415.
39. Shih, I.-L.; Van, Y.-T. *Bioresour. Technol.* **2001**, *79*, 207.
40. Buescher, J. M.; Margaritis, A. *Crit. Rev. Biotechnol.* **2007**, *27*, 1.
41. Hoste, K.; Schacht, E.; Seymour, L. *J. Controlled Release* **2000**, *64*, 53.
42. Otanibajaj, I.; Singhal, R. *Bioresour. Technol.* **2011**, *102*, 5551.
43. Jin, R.; Moreira Teixeira, L. S.; Dijkstra, P. J.; Karperien, M.; van Blitterswijk, C. A.; Zhong, Z. Y.; Feijen, J. *Biomaterials* **2009**, *30*, 2544.
44. Fukuoka, T.; Uyama, H.; Kobayashi, S. *Biomacromolecules* **2004**, *5*, 977.
45. Moreira Teixeira, L. S.; Feijen, J.; van Blitterswijk, C. A.; Dijkstra, P. J.; Karperien, M. *Biomaterials* **2012**, *33*, 1281.
46. Gao, X.; Cao, Y.; Song, X.; Zhang, Z.; Zhuang, X.; He, C.; Chen, X. *Macromol. Biosci.* **2014**, *14*, 565.
47. Lee, F.; Chung, J. E.; Kurisawa, M. *J. Controlled Release* **2009**, *134*, 186.
48. Tan, H.; Chu, C. R.; Payne, K. A.; Marra, K. G. *Biomaterials* **2009**, *30*, 2499.
49. Al-Abboodi, A.; Fu, J.; Doran, P. M.; Tan, T. T.; Chan, P. P. *Adv. Healthcare Mater.* **2014**, *3*, 725.
50. Ng, K. W.; Torzilli, P. A.; Warren, R. F.; Maher, S. A. *J. Tissue Eng. Regen. Med.* **2014**, *8*, 164.
51. Truong, V. X.; Ablett, M. P.; Gilbert, H. T. J.; Bowen, J.; Richardson, S. M.; Hoyland, J. A.; Dove, A. P. *Biomater. Sci.* **2014**, *2*, 167.
52. Jin, R.; Lou, B.; Lin, C. *Polym. Int.* **2013**, *62*, 353.
53. Wen, C.; Lu, L.; Li, X. *Macromol. Mater. Eng.* **2014**, *299*, 504.