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Injectable Enzymatically-crosslinked Hydrogels Based on Poly(L-glutamic acid) Graft Copolymer

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Enzymatically-crosslinked injectable hydrogels based on poly(L-glutamic acid) grafted with tyramine and poly(ethylene glycol) (denoted as PLG-g-TA/PEG) were developed under physiological conditions with the presence of horseradish peroxidase (HRP) and hydrogen peroxide (H₂O₂). Their gelation time, mechanical properties, swelling behaviors and porous structure were evaluated. The hydrogels were rapidly formed with the presence of low concentrations of HRP and H₂O₂. The storage modulus of the hydrogels could be well controlled and increased with increasing the concentrations of HRP and H₂O₂. The average pore-size of the hydrogels varied from 20 to 120 µm, depending on the H₂O₂ concentration. In addition, the encapsulated L929 fibroblast cells in the PLG-g-TA/PEG hydrogels exhibited high viability and proliferation. After subcutaneous injection of the PLG-g-TA/PEG solutions containing HRP and H₂O₂ into the back of rats, the hydrogels were rapidly formed in situ. The hydrogels were found to persist for up to 10 weeks in vivo, and histological analysis indicated that the hydrogels exhibited acceptable biocompatibility. These results suggested that the biocompatible, injectable enzyme-mediated PLG-g-TA/PEG hydrogels are promising for biomedical applications including tissue engineering scaffolds and drug delivery carriers.

Introduction

Hydrogels are three-dimensional (3D) hydrophilic polymer networks retaining a large amount of water. Due to their high water contents and physical properties that resemble the native extracellular matrix (ECM), hydrogels have drawn considerable attention for biomedical applications, such as tissue engineering scaffolds and drug delivery carriers. 1-5 Injectable hydrogels exhibit unique advantages, such as excellent permeability for nutrients and metabolites, good biocompatibility and minimally invasive injection procedures. The encapsulation of cells, drugs, and bioactive molecules into the hydrogels can be easily achieved. Up to now, various crosslinking approaches, including physical and chemical crosslinking, have been developed to construct hydrogels in situ. Physically-crosslinked hydrogels are formed based on non-covalent interactions, such as ionic interactions,6, 7 hydrogen bonds,8 hydrophobic interactions,9 and stereo-complexations.10, 11 They are usually reversible networks with relatively low mechanical properties. On the other hand, chemically-crosslinked hydrogels are covalent networks formed via chemical reactions, including radical polymerization,12, 13 Michael-type addition reaction,14-16 or Schiff-base reaction.17

Recently, increasing attention has been paid to enzymatically-crosslinked hydrogels for biomedical applications due to their good biocompatibility, fast gelation process and tunable mechanical properties. Horseradish peroxidase (HRP) is the most widely used enzyme for enzymatically-crosslinked hydrogels because of its high stability and good biocompatibility.18 HRP is a hemoprotein that catalyzes the coupling of aniline or phenol derivatives via a carbon-carbon bond or a carbon-nitrogen/oxygen bond with the presence of hydrogen peroxide.18 Additionally, the physiochemical properties of hydrogels, such as gelation rate, mechanical strength and porous structure, are easy to control by modulation of the HRP activity. Considerable studies on HRP-mediated in-situ forming hydrogels have been focused on naturally derived materials, such as hyaluronic acid,19-21 alginate,22, 23 dextran,24-27 gelatin28-31 and chitosan.2-36 These hydrogels have been shown potential in biomedical applications due to their cell-interactive properties and biodegradability. Very recently, the enzymatically-crosslinked hydrogels based on synthetic polymers have attracted increasing interest due to their advantages including tunable mechanical properties and low immunogenicity. For instance, the injectable hydrogels based on tyramine-conjugated 4-arm poly(propylene oxide)-poly(ethylene oxide) (PPO-PEO) via enzyme-catalyzed crosslinking have been developed.37-40 The hydrogels displayed good cytocompatibility and degraded rapidly in 6 days in vitro.38 Among various synthetic polymers, synthetic polypeptides have attracted extensive interest due to their excellent biocompatibility,
biodegradability, and structures mimicking natural proteins.\(^{41, 42}\) In addition, the reactive side groups in some polypeptides facilitate further functionalization. These properties lead to unique advantages of polypeptides in biomedical applications. Nevertheless, to-date, reports on injectable enzymatically-crosslinked hydrogels based on polypeptides were still limited. In the present work, a type of injectable hydrogels based on poly(L-glutamic acid) grafted with tyramine and poly(ethylene glycol) (denoted as PLG-g-TA/PEG) were prepared under physiological conditions with the presence of horseradish peroxidase (HRP) and hydrogen peroxide (H\(_2\)O\(_2\)). The PLG-g-TA/PEG copolymers were synthesized via an amidation reaction. The chemical characteristics of the copolymers were characterized by \(^1\)H NMR and UV-Vis spectroscopy. The physicochemical properties of the hydrogels, such as gelation time, mechanical properties, swelling behaviors and porous structure, were studied in detail. The viability and proliferation of L929 fibroblast were still limited. In the present work, a type of injectable hydrogels based on poly(L-glutamic acid) grafted with tyramine and poly(ethylene glycol) (PLG-g-TA/PEG) was obtained by deprotection of PLBG. Briefly, PLBG (5 g) was dissolved in 50 mL of CHCl\(_3\)/COOH, followed by addition of 15 mL of HBr solution (33 wt%) in acetic acid. The reaction mixture was stirred at 30 °C for two hours. The product was obtained by precipitation in diethyl ether, washed repeatedly with diethyl ether and dried under vacuum. After dissolving the product in DMSO, the solution was dialyzed against deionized water for 3 days (MWCO 7000 Da). PLG was collected by lyophilization with a yield of 87%.

\textbf{Synthesis of poly(L-glutamic acid)-graft-tyramine/poly(ethylene glycol) (PLG-g-TA/PEG)}

PLG-g-TA/PEG copolymer was prepared by coupling PLG with TA and mPEG-NH\(_2\) via EDC/NHS activated amidation reaction. PLG (1.5 g, 11.6 mmol of COOH groups) was first dissolved in 40 mL of DMSO. The carboxyl groups of PLG were activated using EDC/NHS (COOH:EDC:NHS = 1:0.6:0.6 (molar ratio)). TA (0.5 g, 2.9 mmol) and mPEG-NH\(_2\) (7.0 g, 3.5 mmol) were then added and the resulting solution was stirred for two days at room temperature. The product was purified by dialysis against deionized water for 3 days (MWCO 7000 Da). The final product, PLG-g-TA/PEG copolymer, was collected as a white solid by lyophilization with a yield of 83%.

\textbf{Gelation time}

Enzymatically-crosslinked PLG-g-TA/PEG hydrogels (300 µL) were prepared with the presence of HRP and H\(_2\)O\(_2\) at room temperature. The final concentration of PLG-g-TA/PEG was fixed at 6.7% (w/v) (Figure S1 in Supporting Information). To evaluate the dependence of gelation time on the HRP and H\(_2\)O\(_2\) concentration, the polymer solution (200 µL, 10% (w/v)) in 0.01 M PBS (pH 7.4) was mixed with different concentrations of HRP solution (50 µL) and H\(_2\)O\(_2\) solution (50 µL) in 5 mL vials, and then the mixture was gently shaken. The gelation time of the PLG-g-TA/PEG hydrogels was determined using the vial tilting method. The sample was considered to be a gel state if no flow was observed within 30 s after inverting the vial.

\textbf{Rheological experiments}

Rheological experiments of the hydrogels were performed on a US 302 Rheometer (Anton Paar) using a parallel plate (plate diameter = 25 mm, gap = 0.5 mm) in oscillatory mode at 37 °C. For the measurements, 200 µL of the PLG-g-TA/PEG solution (10% (w/v)) was mixed with different concentrations of HRP solution (50 µL) and H\(_2\)O\(_2\) solution (50 µL) in 5 mL vials, and then placed on the plate of the rheometer immediately. The storage modulus (G') and loss modulus (G'\(_\perp\)) were recorded as a function of time at a frequency of 1 Hz and a strain of 1%. The sample was sealed by a thin layer of silicon oil to prevent the evaporation of water.

\textbf{Morphology of the hydrogels}

To measure the morphology of hydrogels, the samples were frozen rapidly by plunging them into liquid nitrogen and then freeze-dried for two days. The specimens were cross-sectioned and sputter coated
Swelling ratio

Hydrogels were prepared in vials according to the procedure above. The freeze-dried hydrogels were accurately weighed \((W_0)\) and then incubated in 10 mL of PBS solution at 37 °C. At predetermined time intervals, the buffer solutions were removed completely and the left samples were weighed \((W_t)\) to calculate the swelling ratio \((SR)\), which is defined as \((W_t - W_0)/W_0\). The experiments were performed in triplicate.

In vitro cytocompatibility

The cell viability and proliferation of the hydrogels was evaluated by 3D culture of L929 mouse fibroblasts inside the hydrogels. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, supplemented with 50 U/mL penicillin and 50 µL/mL streptomycin and incubated at 37 °C and in 5% CO₂ atmosphere. All the sample solutions were sterilized by filtering via 0.2 µm syringe filters. First, PLG-g-TA/PEG solution \((10\% \ (w/v), 200 \mu L)\) was mixed with harvested cells \((1 \times 10^5)\) cells and put into the well of a 24-well plate, and then 50 µL of HRP solution and 50 µL of PBS solution were added into the well. 1 mL of DMEM was added after the hydrogel was formed. The viability of encapsulated L929 cells at 24h was determined by using a live-dead staining kit. In brief, 500 µL of PBS solution containing 2 µM calcein AM and 4 µM propidium iodine (PI) was added and the sample was further incubated at 37 °C for 30 min. The sample was observed by an inverted microscope after removing the staining solution. Viable cells were stained green with calcein AM, and dead cells were stained red with PI. In addition, the proliferation of L929 cells in the hydrogels was assessed by cell counting kit28 (CCK28) assay. Briefly, L929 cells were seeded at a density of 10^4 cells/cm² and put into the well of a 24-well plate, and then 50 µL of HRP solution \((10\% \ (w/v), 200 \mu L)\) was mixed with harvested cells \((1 \times 10^5)\) cells) and incubated in 10 mL of PBS solution at 37 °C for another 24 h. The absorbance value at 490 nm was measured on a ELISA reader (Tecan Infinite M200). The absorbance at 450 nm was measured with an ELISA reader. Cell viability (%) was calculated according to the equation: viability (%) = (A_sample/A_control) 100%, where A_sample and A_control are the absorbance of the sample and control well (without the eluent of hydrogels), respectively. The measurements were performed in triplicate.

In vivo degradation and biocompatibility

Sprague-Dawley (SD) rats \((-200 g)\) were used for in vivo tests. Rats were anesthetized by inhalation of diethyl ether. PLG-g-TA/PEG solutions \((10\% \ (w/v), 500 \mu L\) for each sample) in PBS containing H₂O₂ \((1.6 \text{mM})\) and HRP \((2 \text{units/mL})\) were subcutaneously injected in the back of the rats. At predetermined time intervals, the rats were euthanized, and the gel status was observed. The tissues surrounding the injection sites were surgically removed and stored in neutral buffered formalin solution (NBF). The histology analysis was examined by staining with hematoxylin and eosin (H&E). The animal experiments were carried out according to the guide for the care and use of laboratory animals, provided by Jilin University, Changchun, China, and the procedure was approved by the local Animal Ethics Committee.

Results and discussion

Synthesis and characterization of PLG-g-TA/PEG copolymer

The synthetic route of PLG-g-TA/PEG copolymer is illustrated in Scheme 1. Poly(L-glutamic acid) (PLG) was synthesized by ring-opening polymerization (ROP) of BLG NCA with triethylamine as an initiator, followed by deprotection of the benzyl groups of PLBG using HBr solution \((33\% \text{w/w})\) in acetic acid. Subsequently, TA and mPEG-NH₂ were grafted to the backbone of PLG via a carbodiimide active amidation reaction. The typical ¹H NMR spectra are shown in Figure 1. The disappearance of the peak of benzyl groups in PLG suggested the successful deprotection of PLBG (Figure 1A and 1B). The weight-average molecular weight \((M_w)\) of PLG was evaluated to be \(1.3\times 10^4\) with a polydispersity index (PDI) of 1.28 by GPC. Based on the ¹H NMR spectrum of the graft copolymer (Figure 1C), in addition to the typical peaks assigned to PLG, the representative peaks ascribed to tyramine (TA) and PEG were also observed, indicating the successful synthesis of the PLG-g-TA/PEG copolymer. The grafting ratio of TA residues, defined as the number of TA moieties per 100 repeated units of L-glutamate, was calculated to be 20 by comparing the integration of the peak of TA \((-C_6H_4Cl-)\) at 6.8-7.0 ppm with that of methylene peak of glutamate unit \((-CH_2CH_2C(O)-)\) at about 2.1 ppm (Figure 1C). Similarly, the grafting ratio of mPEG-NH₂ residues was calculated to be 28 based on the NMR results. The weight contents of TA and PEG were 3.9 wt% and 79.1 wt%, respectively. The conjugation of TA onto PLG was also examined by using UV-Vis spectrometer. The PLG-g-TA/PEG aqueous solution showed a specific absorbance peak at 277 nm, which corresponds to the absorbance of phenol moieties (Figure 2a). The content of conjugated TA was 4.5 wt%, which is consistent with the NMR results. Additionally, the conformation of PLG-g-TA/PEG in aqueous solution at pH 7.4 was examined by circular dichroism spectroscopy. As shown in Figure 2b, a small positive maximum at about 217 nm and a large minimum at about 204 nm were observed, indicating that PLG-g-TA/PEG adopted a random conformation. The reason could be attributed to the fact that the residue carboxyl groups of PLG were in ionized form at pH 7.4.
Preparation and gelation time of the hydrogels

The hydrogels were prepared by HRP-mediated crosslinking of PLG-g-TA/PEG copolymer (Scheme 2). The coupling of phenol moieties in the PLG-g-TA/PEG copolymer via the carbon-carbon bonds or carbon-oxygen bonds was catalyzed by HRP and H$_2$O$_2$, leading to the formation of intermolecular covalent linkages and the rapid formation of hydrogels. The gelation time was determined by the vial tilting method. The dependence of the gelation time on the concentrations of HRP and H$_2$O$_2$ is shown in Figure 3. The gelation time reduced from 135 to 23 s as the HRP concentration increased from 1.0 to 16.7 units/mL at fixed polymer concentration of 6.7% (w/v) and H$_2$O$_2$ concentration of 1.6 mM. This should be attributed to the fact that the increase in HRP concentration accelerated the generation of phenolic free radicals. In contrast, as the H$_2$O$_2$ concentration increased from 0.8 to 8.2 mM, the gelation time prolonged from 80 to 150 s at a fixed HRP concentration of 4 units/mL. This phenomenon occurred due to the inhibition effect of excessive H$_2$O$_2$ on the activity of HRP. The influence of HRP and H$_2$O$_2$ concentrations on the gelation time was consistent with some naturally derived systems, such as carboxymethylcellulose and gelatin. Notably, the hydrogels based on PLG-g-TA/PEG have been developed by using relatively low amounts of H$_2$O$_2$, which may avoid toxic effects of H$_2$O$_2$ at high concentrations. Additionally, it was found that the gelation time of PLG-g-TA/PEG hydrogels was tunable by changing the concentrations of HRP and H$_2$O$_2$. The control of gelation time is quite important for practical applications of the hydrogels.

Rheological experiments

The viscoelastic property of PLG-g-TA/PEG hydrogels was measured in a time-controlled oscillatory mode at 37 °C. A mixture of PLG-g-TA/PEG, HRP and H$_2$O$_2$ solutions in PBS was placed on the plate of rheometer. The formation of hydrogels was detected by monitoring the variations of storage modulus (G') and loss modulus (G'') with time. As shown in Figure 4a, the G' and G'' of PLG-g-TA/PEG hydrogels crosslinked with 2 units/mL of HRP and 1.6 mM of H$_2$O$_2$ were measured as a function of time. An immediate gelation was observed as soon as the mixture was placed on the plate of rheometer. Subsequently, the storage modulus of the hydrogels gradually increased during the initial stage and eventually reached a plateau, suggesting that the crosslinking reaction was complete.

As shown in Figure 4b, the storage modulus of PLG-g-TA/PEG hydrogels strengthened from 1600 to 2300 Pa with increasing the concentration of HRP from 1 to 4 units/mL at a fixed H$_2$O$_2$ concentration of 1.6 mM. This result indicated that HRP displayed a marked effect on the mechanical properties of the hydrogels. Notably, the increase in H$_2$O$_2$ concentration led to higher storage modulus. This should be due to the fact that H$_2$O$_2$ acted as a crosslinker in this system and therefore the increase in H$_2$O$_2$ concentration led to the increase in the crosslinking density. The results of rheological tests demonstrated that the mechanical properties of the hydrogels could be controlled by varying the HRP and H$_2$O$_2$ concentrations.
The morphology of lyophilized hydrogels was observed by SEM. As shown in Figure 5, the PLG-g-TA/PEG hydrogels exhibited interconnected porous structures. It was found that the pore size of the hydrogels was markedly influenced by the H$_2$O$_2$ concentration. For instance, the average pore-size of the hydrogel crosslinked with 0.8 mM of H$_2$O$_2$ and 2 units/mL of HRP ranged from 70 to 120 µm (Figure 5a). In contrast, the inner porous structure of the hydrogel crosslinked with 1.6 mM of H$_2$O$_2$ and 2 units/mL of HRP was more compact and the average pore-size ranged from 20 to 60 µm (Figure 5b). The decrease in the pore size as the increase in H$_2$O$_2$ concentration was due to a higher cross-linking density. It is noteworthy that many studies have demonstrated that porous microstructure with suitable pore size of hydrogel matrix played a critical role in cell survival, migration, proliferation and differentiation. Furthermore, the pore size of hydrogels affected the release behavior of encapsulated drugs or bioactive molecules. In our study, the pore size of the PLG-g-TA/PEG hydrogels could be easily adjusted by the H$_2$O$_2$ concentration, which contributed to the further applications of the PLG-g-TA/PEG hydrogels.

The swelling behaviors of PLG-g-TA/PEG hydrogels formed with various HRP and H$_2$O$_2$ concentrations were determined by incubating the freeze-dried hydrogels in PBS at 37 °C. The hydrogels were weighed at predetermined time intervals to obtain the swelling ratios. As shown in Figure 6, the hydrogels had high equilibrium swelling ratio ranging from 33 to 49, suggesting that freeze-dried PLG-g-TA/PEG hydrogels absorbed plenty of water because of the hydrophilic PEG segments. It was found that the equilibrium swelling ratio reduced as the H$_2$O$_2$ concentration increased from 0.8 to 1.6 mM (Figure 6a, Figure S2 in Supporting Information). Additionally, with the increase of HRP concentration from 2 to 8 units/mL, the equilibrium swelling ratio decreased from 49 to 40 (Figure 6b). Generally, the swelling ratio is related to the crosslinking density of the hydrogels. An increase in the crosslinking density usually results in a decrease in the swelling ratio. The results suggested that, in our experimental range, the increases in the H$_2$O$_2$ and HRP concentrations led to the increase in the crosslinking density, which is coincident with the SEM observation (Figure 5).

In vitro cytocompatibility

L929 mouse fibroblasts were incubated inside the PLG-g-TA/PEG hydrogels formed with different concentrations of H$_2$O$_2$ and 2 units/mL of HRP. The cell viability was then determined by a live-dead cell staining kit. Cells were stained with calcein-AM/PI and observed by fluorescent microscope. As shown in Figure 7a, most of cells inside the hydrogels were stained green, indicating a high viability of the cells. The cell proliferation in the PLG-g-TA/PEG hydrogels was analyzed by cell counting kit-8 (CCK8) method. The L929 cells in the hydrogel with lower H$_2$O$_2$ concentration exhibited a higher proliferation rate (Figure 7b), which may due to a higher porosity. The cytotoxicity of the hydrogels and any leachable materials was also evaluated by using MTT assay. The cell viability was over 90% after incubation with the eluent of the hydrogels, suggesting good cytocompatibility of the hydrogels (Figure 7c). It is worth mentioning that the low dose of H$_2$O$_2$ used in the PLG-g-TA/PEG hydrogels and the mild enzyme-mediated gelation process should contribute to the excellent cytocompatibility of the hydrogels.

In vivo degradation and biocompatibility

In order to investigate the in vivo degradation and biocompatibility of the PLG-g-TA/PEG hydrogels, 500 µL of PLG-g-TA/PEG solution in PBS containing H$_2$O$_2$ (1.6 mM) and HRP (2 units/mL)
was injected into the subcutaneous layer of rats. It was found that the hydrogels were rapidly formed in situ after subcutaneous injection of the PLG-g-TA/PEG solutions (Figure 8). The hydrogels persisted for up to 10 weeks in vivo, and completely degraded 14 weeks post-injection. The PLG-g-TA/PEG hydrogels in subcutaneous layer of the rats were mainly degraded due to the effects of mammalian proteolytic enzymes.34, 55

![Image](https://example.com/image1)

**Fig.8** In vivo hydrogels status at different periods. PLG-g-TA/PEG solutions in PBS (10% w/v) containing H$_2$O$_2$ (1.6 mM) and HRP (2 units/mL) were subcutaneously injected into rats. Photos were taken at 15 min (0 day), 4, 10, 14 weeks.

Additionally, to investigate the biocompatibility of the hydrogels in vivo, the inflammatory response to the injected hydrogels was studied by hematoxylin and eosin (H&E) staining of the surrounding tissues at different time intervals. Elevated number of inflammatory cells were observed at first four weeks after injection (Figure 9), indicating mild inflammatory reaction in the initial stage.36, 57 Notably, inflammatory cells markedly reduced and the inflammatory reaction eliminated gradually along with the degradation of the hydrogels. Moreover, during our experiment, neither obvious tissue necrosis, edema, hyperemia and hemorrhaging, nor muscle damage were observed. Therefore, the results suggested that the PLG-g-TA/PEG hydrogels exhibited acceptable biocompatibility in vivo, indicating that the hydrogels have potential in some biomedical applications, such as tissue engineering scaffolds and carriers for long-term sustained delivery of drugs and bioactive molecules.

![Image](https://example.com/image2)

**Fig.9** Histological images of tissues around the injection sites at the back of rats (H&E staining).

Conclusions

A kind of enzyme-mediated injectable hydrogels based on poly(L-glutamic acid) grafted with tyramine and poly(ethylene glycol) (PLG-g-TA/PEG) were developed. The hydrogels were rapidly formed under physiological conditions with the presence of HRP and H$_2$O$_2$. The gelation time could be adjusted by varying the concentrations of HRP and H$_2$O$_2$. The physicochemical properties of the hydrogels, including mechanical strength, swelling ratio and porous structure, were dependent on the concentrations of HRP and H$_2$O$_2$. The in vitro formation of hydrogels in the subcutaneous layer of rats persisted for up to 10 weeks and displayed acceptable biocompatibility in vivo. Therefore, the PLG-g-TA/PEG hydrogels could be promising candidates for biomedical applications, such as tissue engineering scaffolds and carriers for long-term sustained delivery of bioactive molecules.

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Notes and references

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Enzyme-mediated injectable hydrogels based on a poly(L-glutamic acid) graft copolymer with tunable physicochemical properties, biodegradability and good biocompatibility were developed.