Preparation and Characterization of Attractive Poly(amino acid) Hydrogels Based on 2-Ureido-4[1H]-pyrimidinone

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Abstract  Self-healing hydrogels with the shear-thinning property are novel injectable materials and are superior to traditional injectable hydrogels. The self-healing hydrogels based on 2-ureido-4[1H]-pyrimidinone (UPy) have recently received extensive attention due to their dynamic reversibility of UPy dimerization. However, generally, UPy-based self-healing hydrogels exhibit poor stability, cannot degrade in vivo and can hardly be excreted from the body, which considerably limit their bio-application. Here, using poly(l-glutamic acid) (PLGA) as biodegradable matrix, branching α-hydroxy-ω-amino poly(ethylene oxide) (HAPEO) as bridging molecule to introduce UPy, and ethyl acrylate polyethylene glycol (MAPEG) to introduce double bond, the hydrogel precursors (PMHU) are prepared. A library of the self-healing hydrogels has been achieved with well self-healable and shear-thinning properties. With the increase of MAPEG grafting ratio, the storage modulus of the self-healing hydrogels decreases. The self-healing hydrogels are stable in solution only for 6 h, hard to meet the requirements of tissue regeneration. Consequently, ultraviolet (UV) photo-crosslinking is involved to obtain the dual crosslinking hydrogels with enhanced mechanical properties and stability. When MAPEG grafting ratio is 35.5%, the dual crosslinking hydrogels can maintain the shape in phosphate-buffered saline solution (PBS) for at least 8 days. Loading with adipose-derived stem cell spheroids, the self-healing hydrogels are injected and self-heal to a whole, and then they are crosslinked in situ via UV-irradiation, obtaining the dual crosslinking hydrogels/cell spheroids complex with cell viability of 86.7%±6.0%, which demonstrates excellent injectability, subcutaneous gelatinization, and biocompatibility of hydrogels as cell carriers. The novel PMHU hydrogels crosslinked by quadruple hydrogen bonding and then dual photo-crosslinking of double bond are expected to be applied for minimal invasive surgery or therapies in tissue engineering.

Keywords  2-Ureido-4[1H]-pyrimidinone (UPy); Poly(l-glutamic acid) (PLGA); Self-healable; Photo-crosslinking

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INTRODUCTION

Self-healing hydrogels with the shear-thinning property are promising injectable materials, showing great advantages in minimally invasive treatment, filling irregular defects, and loading cells or drugs, which has attracted extensive attention in recent years.\(^{[1–3]}\) Meanwhile, self-healing hydrogels have the ability to control the gelation rate on the basis of the shear-thinning property, avoiding the loss of loading cells/drugs or unnecessary premature gel formation before the sol-gel transition at the desired sites.\(^{[3]}\) They could also prevent the cell spheroids from aggregation or sedimentation in liquid precursors or cell leakage after injecting into the body,\(^{[4]}\) which are common issues of traditional injectable hydrogels. Compared with dynamic covalent cross-linked self-healing hydrogel, those constructed by noncovalent bonds usually undergo rapid sol-gel transition.\(^{[5]}\) Among the physical interactions, hydrogen bonding is considered as a controllable force to construct self-healing hydrogels. The stability and mechanical properties of hydrogen-bonding self-healing hydrogels are tunable through changing the density or bonding strength of hydrogen bonds,\(^{[6]}\) and can be enhanced by synergistic effects.\(^{[7]}\)

Ureido-4[1H]-pyrimidinone (UPy) has been widely studied in the past decades due to its ability of dimerization and self-assembly as a self-complementary quadruple hydrogen-bond unit.\(^{[8–10]}\) The hydrogen bonding motif contains the sequence of DDAA (D: Donor; A: Acceptor) and has a favorable dimerization constant (10\(^9\) L/mol) in methylbenzene.\(^{[10]}\) Currently, a library of UPy-based self-healing hydrogels with remarkable properties have been reported, including pH-responsive hydrogels,\(^{[11]}\) thermosensitive hydrogels,\(^{[12]}\) and multi-stimulus response hydrogels.\(^{[13]}\) But most of them are composed of nondegradable backbones, such as polyacrylates\(^{[14]}\) and poly(ethylene glycol) (PEG).\(^{[15]}\) Besides, the dynamic reversible crosslinking reactions of UPy-induced dimerization atten-

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uates the stability of hydrogels, and the self-healing hydrogels generally show rapid erosion. These limitations of application as tissue engineering materials invite the need to develop strategies to prepare injectable and biodegradable UPy-based hydrogels with good stability and mechanical properties. By grafting the molecules containing unsaturated bonds onto the polymer backbones, dual crosslinking network is formed in the hydrogel with improved stability. This can not only keep the biofunctionalities of implanted materials, but also meet the in vivo requirements of biomedical engineering.

Poly(ethylene glycol) (PLGA) is a preeminently biodegradable and biocompatible polypeptide. Our group has used PLGA to prepare a series of tissue engineering scaffolds applied to reconstruct the defect tissues, such as bulk hydrogels[16], injectable hydrogels[17], microgels[18] and non-immunogenicity[16,18].

Herein, in this study, UPy units were attached onto the PLGA through the connection agent of α-hydroxy-ω-amino poly(ethylene oxide) (HAPEO), and methyl acrylate polyethylene glycol (MAPEG) was introduced to the PLGA backbone to provide unsaturated bonds. The hydrogel precursor P(LGA-co-(LG-g-MAPEG)-co-(LG-g-HAPEO)-co-(LG-g-(HAPEO-g-UPy))) was obtained and marked as PMHU. The self-healing hydrogels were developed through the self-assembly of UPy dimers as crosslinked blocks with well-performed self-healing and shear-thinning properties. Ultraviolet (UV) photo-crosslinking was utilized to acquire dual crosslinking hydrogels with enhanced stability and mechanical properties. The influence of grafting ratio of MAPEG on shear-thinning and self-healing properties of the hydrogels was studied. To understand the effect of grafting ratio of MAPEG, the viscoelasticity of self-healing hydrogels and dual crosslinking hydrogels was investigated. Moreover, the impact of the grafting ratio of MAPEG on the stability of the dual crosslinking hydrogels was tested. With the aim of biomedical application, the cyto-compatibility of PMHU hydrogels was evaluated. Loading with adipose-derived stem cell spheroids, the self-healing hydrogel was injected and then crosslinked in situ via UV-irradiation. Then, the obtained dual crosslinking hydrogels/cell spheroids complex showed ideal cell viability (Scheme 1). Consequently, we hypothesized the PMHU hydrogels might be the promising biomaterials for tissue engineering.

**EXPERIMENTAL**

**Materials**

PLGA \( M_n=2.4 \times 10^5 \text{ Da}, \ PDI=1.63 \) was synthesized in the lab of our research group.\[19\] 1-Ethyl-3-dimethylaminopropyl carbodiimide hydrochloride (EDC·HCl) was obtained from GL Biochem Co., Ltd. (Shanghai, China). Diethyl ether was bought from Sinopharm Group Co., Ltd. (Shanghai, China). Cysteamine hydrochloride, N,N-dimethylformamide (DMF), azobisobutynitrile (AIBN), 2-hydroxy-4-(2-hydroxy-ethoxy)-2-methylpropionone (I2959), methanol, chloroform, anhydrous sodium sulfate (Na$_2$SO$_4$), hydroxide potassium (KOH), 4-dimethylaminopyridine (DMAP), hexyl diisocyanate (HDI), dimethyl sulfoxide (DMSO), and n-hexane were obtained from Aladdin Industrial Inc. (Shanghai, China). 2-Amino-4-hydroxy-6-methylpyrimidinone (AHMP) was bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). MAPEG \( M_n=2860 \text{ Da} \) and allyl polyethylene oxide (APEO, \( M_n=2877 \text{ Da} \) were purchased from Jiangsu Haian petrochemical plant. All of the chemical reagents were of analytic grade and employed directly without further purification.

**Synthesis of HAPEO**

HAPEO was synthesized based on the reported literature.\[20\] Briefly, APEO (10.00 g) reacted with cysteamine hydrochloride (3.17 g) in DMF (90 mL), catalyzed by AIBN (0.18 g) at 70 °C for 24 h. The mixture was precipitated twice in diethyl ether. The precipitate (10.00 g) and KOH (0.18 g) were melt in a component solvent of water (3 mL) and methanol (200 mL), and then stirred

![Scheme 1](https://doi.org/10.1007/s10118-021-2498-y)
for 1 h. The target product was extracted from the above solution with chloroform and then dried for 1 h with Na₂SO₄, followed by rotary evaporation. After precipitating in the diethyl ether, the amorphous white powder was obtained. The reaction yield was 73.1%.

**Synthesis of 2(6-Isocyanato Hexyl Amino Carboxyl Amino)-6-Methyl-[1'H] Pyrimidinone (UPy-HDI)**

Following the previous procedures, UPy-HDI was successfully synthesized. After HDI (26 mL) reacted with AHMP (2.91 g) at 90 °C for 16 h under N₂, the mixture was filtered and rotary evaporation. After precipitating in the diethyl ether, the amorphous white powder was obtained after drying in vacuum. The yield was 73.1%.

**Synthesis of MAPEG and HAPEO Grafted to PLGA (PLGA-co-(LG-g-MAPEG)-co-(LG-g-HAPEO)), Marked as PMH**

MAPEG and HAPEO were simultaneously grafted to PLGA by carbodiimide reaction. Firstly, PLGA (0.20 g), MAPEG (0.88 g), and HAPEO (0.37 g) were dissolved in 50 mL of DMSO. DMAP (0.05 g) and EDC-HCl (0.15 g) were added, followed by stirring the mixture for 24 h at 50 °C. After being dialyzed via deionized water for 7 days thoroughly, the resulting products were lyophilized. The reaction yield was 75.4%. The molar feed ratio of the carboxyl group from PLGA to MAPEG and HAPEO was fixed at 5:1 and 10:1, matching with the calculated grafting ratios as 20.0% and 10.0%, respectively. The products were analyzed by proton nuclear magnetic resonance (¹H-NMR). The grafting ratios of MAPEG and HAPEO were 18.3% and 9.3%, marked as PMH-1. Fixing the calculated grafting ratios of HAPEO at 10.0%, other grafting ratios of MAPEG, 30.0%, 40.0%, 50.0%, and 60.0%, were carried out according to the similar procedures.

**Synthesis of UPy Modified PMH (PMHU)**

PMH-1 (0.50 g) was reacted with UPy-HDI (0.08 g) in DMF (96 mL) at 90 °C for 24 h under N₂. After precipitating the mixture in diethyl ether, a white amorphous solid was obtained and then dried in vacuum. Analyzed and calculated by ¹H-NMR, the grafting ratio of UPy was 3.3%, marked as PMHU-1. The polymers produced by other molar ratios were synthesized based on the similar procedures.

**Characterizations of Polymers**

¹H-NMR spectra of PLGA, UPy-HDI, and PMHU were acquired utilizing NMR spectroscopy (AV 500 MHz, Bruker, U.S.A.) to calculate the grafting ratios of MAPEG, HAPEO, and UPy. The dissolution reagent was deuterated water (D₂O). Infrared spectroscopy (IR) spectra of PLGA, UPy-HDI, and PMHU were recorded on Fourier infrared spectrometer (Avatar 370, Nicolet, U.S.A.). The mixed powder of the dried sample and KBr was pressed into a sheet and tested in a scanning wavenumber range of 500—4000 cm⁻¹.

**Preparation of Self-healing Hydrogels**

PMHU (0.10 g), phosphate-buffered saline solution (PBS) (0.90 mL), and I2959 (2.00 mg, 2 mg/mL) were mixed, generating the hydrogel with a mass concentration of 10.0 wt%.

**Preparation of Dual Crosslinking Hydrogels**

Self-healing hydrogels were transformed into dual crosslinking hydrogels after 5 min of UV irradiation. UV lamp (Zigoo, Beijing Worldfa Glass Tools Co., Ltd., China) was utilized to provide UV irradiation in the experiments. UV intensity was 200 mW/cm², and the UV light wavelength was 365 nm.

**Rheological Behaviors of Hydrogels**

Through the oscillatory mode on the rheometer (DHR, TA, U.S.A.), rheological properties of hydrogels were examined at 37 °C. To evaluate viscoelastic behaviors of hydrogels, frequency spectrum experiment was operated at the 10% settled regular strain and the 0.1—100 rad/s angular frequency range. The alternate strain test was operated to research the self-healing properties, of which the large strain was 200% and the small strain was 1%. Viscosity-shear rate test was carried out to verify the shear-thinning property, and the shear rate was 0.001—100 s⁻¹.

**Stability and Biodegradability of Hydrogels**

Hydrogels were transferred into vials filled with PBS and put in the 37 °C incubator. PBS was refreshed every 24 h. After specific time points, the hydrogels were rinsed by deionized water. The wet weight was measured via the analytical balance with the accuracy of 0.1 mg after blotting hydrogels’ surface with filter paper. The mass of the vial was denoted as Wvial. The overall mass of the vial and the hydrogel was denoted as WHV. The overall mass of the vial and the wet weight denoted as WHV. The mass-loss rate (R) of the hydrogel could be calculated by the formula: \[ R = \left( \frac{W_{all} - W_{all \_dry}}{W_{all \_dry}} \right) \times 100\% \].

**Cytotoxicity Test**

Before the cytotoxicity study, the materials were disinfected and sterilized. Rabbit adipose stem cells (rASCs) were inoculated with the cell density of 4×10⁴ cells/200 μL solution of Dulbecco’s Modified Eagle Medium (DMEM) but 20% fetal bovine serum (FBS) in 96-well plates, followed with all-night culture. Different concentrations of I2959 (0.08—10.67 mg/mL) and different concentrations of hydrogel precursor of (0.33—67.33 mg/mL) were incubated with 5% CO₂, and DMEM was specified to be a control group. The concentrations were based on the max mass concentration of materials as 1.50%. After 24-h culture, the methyl thiazolyltetrazolium (MTT) solution was then added to each and every well, followed with another 4-h culturing. Absorbance (A) was measured on the SpectraMax M2 microplate reader from Molecular Devices in triplicate. Hence, cell survival rate = \( \frac{A_{sample}}{A_{control}} \).

**Preparation of Cell Spheroids**

Polyethylene glycol diacrylate (PEGDA, 0.400 g) and photo-initiator I2959 (0.025 g) were dissolved in 5 mL of DMSO. After UV irradiation in a glass plate (diameter 2.00 cm) for 15 min, the gel formed. To prepare PEG hydrogel, DMSO was then replaced with PBS immersion. Before cell inoculation, the hydrogel was sterilized in ethanol for 12 h and then immersed in PBS. rASCs were inoculated on the surface of hydrogel (cell inoculation density was 1.6×10⁵/cm²) and cultured for 24 h to obtain cell spheroids in a specific size (100.0—200.0 μm).

**Minimally Invasive Injection Assay and Cell Spheroid Viability in Hydrogels**

After cell spheroids were collected, 1 mL of self-healing hydrogel with a mass concentration of 10.0% was added. Cell spheroids were dispersed evenly in self-healing hydrogels by...
turbine oscillation. The hydrogel loaded with cell spheroids was put into a 1 mL sterile syringe and injected into a culture dish by the 18G needle (outside diameter=1.2 mm, injection speed=0.2 mL/s). After injecting, the complex of PMHU hydrogels loaded with cell spheroids self-healed to an integrated gel. Then, the dual crosslinking hydrogels/cell spheroids complex was obtained by secondary photo-crosslinking under UV irradiation for 5 min. The cell spheroids encapsulated in self-healing hydrogel and dual crosslinking hydrogel were cultivated respectively in the live/dead cell double staining kit for 30 min. Then, the cell spheroids were observed with a laser confocal microscope. The fluorescence area was calculated by the fluorescence analysis software Image J.

The representative infrared spectroscopy (IR) spectra of PMHU-1 are presented in Fig. S1(c) (in ESI). The absorption at 3300 cm$^{-1}$ on the spectra was from the stretching vibration of the nitrogen-hydrogen bond (N$\equiv$H) on the secondary amine of PLGA. The absorption at 2800 cm$^{-1}$ was ascribed to the oxygen-hydrogen bond (O$\equiv$H) stretching vibration of PLGA’s residual carboxyl group. The characteristic band appearing at 1680 cm$^{-1}$ was ascribed to the carbonyl group (C=O) stretching vibration on the amide bond. The absorption at 1465 and 1150 cm$^{-1}$ corresponded to the carbon-hydrogen bond (C$\equiv$H) bending vibration on the methylene group in the PEG structural unit as well as the stretching vibration of the ether bond (C$\equiv$O$\equiv$C). From the following spectra, there was no isocyanate (–N=C=O) stretching vibration absorption band at 2250 cm$^{-1}$, indicating that almost all of the hydroxyl group of PMH and the isocyanate group of UPy participated in the nucleophilic addition reaction. This proved that UPy units were successfully grafted onto the PMH backbone.

**RESULTS AND DISCUSSION**

**Synthesis and Characterization of PMHU**

The carboxyl groups of PLGA was activated by 4-dimethylaminopyridine (DMAP) and 1-ethyl-3-dimethylaminopropyl carbodiimide hydrochloride (EDC-HCl) and reacted with MAPEG and HAPEO to produce the intermediate (P(LGA-co-(LG-g-MAPEG)-co-(LG-g-HAPEO))) (marked as PMH). By the nucleophilic addition reaction via the hydroxyl group of PMH and the isocyanate group of 2(6-isocyanato hexyl amino carbonyl amino)-6-methyl-4[1H] pyrimidinone (UPy-HDI), the hydrogel precursor PMHU was prepared. The synthetic route of PMHU is exhibited in Fig. S1a (in the electronic supplementary information, ESI).

The grafting ratios of MAPEG, HAPEO, and UPy in hydrogel precursors PMHU are listed in Table S1 (ESI). In consideration of the water solubility of the hydrogel precursor, the graft ratio of HAPEO was fixed at 10.0%, and the graft ratio of UPy was selected to be 3.0%. Proton nuclear magnetic resonance ($^1$H-NMR) spectra of PLGA, UPy-HDI, and PMHU-1 are shown in Fig. S1(b) (in ESI). The single peak at $\delta_1=4.9$ ppm was the chemical shift of proton at the double bond in MAPEG, and the single peak at $\delta_2=4.1$ ppm corresponded to the chemical shift of proton at the tertiary carbon in PLGA. The single peak at $\delta_3=3.2$ ppm represented the active proton at the methylene group in HAPEO. Calculated through peak integration, the grafting ratio of MAPEG and HAPEO was 18.5% and 9.3%, which were closed to the theoretical grafting ratio of 20.0% and 10.0%, respectively. The signals at $\delta_1=5.7$ ppm corresponded to the protons of UPy and the areas of peak represented the grafting ratio of UPy units (3.3%). The other grafting ratios of MAPEG in PMHU were 25.9%, 35.5%, 45.2%, and 52.9%, and all of them were lower than the theoretical graft ratios, which might be caused by reaction steric hindrance. The $^1$H-NMR spectra of all hydrogel precursors PMHU are shown in Fig. S2 (in ESI).

**Statistical Analysis**

All of the experimental data were expressed as mean ± standard deviation (SD). Sample size ($n\geq3$) represented the number of biological replicates ($n\geq3$). Before performing a statistical test, normality and variance were checked equal. Single-factor analysis for variance (ANOVA) was employed to evaluate the statistical significance, and then Tukey’s test was used for multiple comparisons. When the significance criterion ($p$ value) was lower than 0.05, the difference of the experimental data was considered to be statistically significant ($^*, p<0.05$).

**Preparation and Characterization of Self-healing Hydrogels**

**Preparation of self-healing hydrogels**

The self-healing hydrogels were manufactured via the self-assembly of hydrogel precursor PMHU in aqueous solution. The driving force was the dimerization of UPy units, which formed the reversible noncovalent crosslinked networks. For self-healing hydrogels, the gelatinizing property of different hydrogel precursors PMHU is shown in Fig. 1, all of which could form hydrogels except PMHU-5 (52.9% of MAPEG grafting ratio). The possible reason was that as the grafting ratio of MAPEG gradually increased from 18.5% to 52.9%, the hydrophilicity of the precursor polymer steadily increased. When the grafting ratio of MAPEG was 52.9%, the precursor PMHU-5 was too hydrophobic to form a hydrogel because water molecules would interfere with hydrogen bond accumulation of UPy. Also, the increased grafting ratio of side chains might enhance the steric hindrance of the multiple-hydrogel-bond assay.

![Fig. 1](https://doi.org/10.1007/s10118-021-2498-y)  
**Fig. 1**  Vial inversion of self-healing hydrogels with different grafting ratios of MAPEG. The mass concentration of all hydrogel precursors was 10.0%.

**Measurement of self-healable and shear-thinning properties**

The dimerization of UPy units is a dynamic reversible process in aqueous solution, which can endow hydrogels with self-healable and shear-thinning properties. (Fig. 2a). When hydrogels deform, weaker bonds in the hydrogel network, such as noncovalent bonds, will be broken as sacrificial bonds.
Fig. 2  (a) Schematic illustration of dynamical association and dissociation; (b) Gel-sol conversion point of self-healing hydrogels with different grafting ratios of MAPEG; (c) Alternate step strain test of self-healing hydrogels with different grafting ratios of MAPEG.
the increase of deformation, the noncovalent bonds are gradually interrupted by the external mechanical forces. When the residual supramolecular crosslinking is insufficient to support the hydrogel network, the solid-state gel is converted into the liquid state.[28] This point is considered as the gel-sol conversion point (i.e., the storage modulus ($G'$) = the loss modulus ($G''$)). To understand the relationship between the grafting ratio of MAPEG and the gel-sol conversion point, the rheological properties of PMHU self-healing hydrogels were investigated. The mass concentration of all hydrogel precursor was 10.0%. When the grafting ratio of MAPEG was 18.5%, 25.9%, 35.5%, and 45.2%, the gel-sol conversion point was 20.1%, 90.0%, 20.1%, and 12.6%, respectively (Fig. 2b). The transition point of the self-healing hydrogels increased at first and then decreased with the upregulation of the grafting ratio of MAPEG. The results demonstrated that when the grafting ratio of MAPEG was 18.5%, the hydrophilic part was weaker than the hydrophobic self-assembly aggregations derived from the dimerization of UPy units in the hydrogel, leading to heterogeneities in hydrogel network,[27] thus other properties of this hydrogel would not be discussed further. When the grafting ratio of MAPEG increased to 25.9%, the hydrophilicity of the hydrogel precursor was improved; meanwhile, the density of aggregation in the hydrogel network decreased. The hydrogel network tended to be homogeneous, so the hydrogel showed enhanced damage resistance. However, when the grafting ratio of MAPEG further increased, the optimal balance between the hydrophilic part and hydrophobic aggregations in the hydrogel network was disrupted, and the hydrophilicity of the hydrogel further increased. Steric hindrance[23] and water molecules[14] interference broke UPy-stacked hydrogen bonds, showing lower conversion points.[27]

To quantify the self-healable and shear-thinning abilities as well as subsequent self-recovery of the hydrogels, the rheological behaviors were measured by alternating strain tests based on the gel-sol transition point of each hydrogel, from the strain of 1% to 200%. The mass concentration of all hydrogel precursor was 10.0%. When the strain switched to 200%, the $G'$ was lower than the $G''$, and the hydrogel was in the gel state. While the strain switched to 200%, the $G'$ was lower than the $G''$, and the hydrogel was converted into the liquid state. After multiple cycles, the hydrogels at different grafting ratios of MAPEG maintained their initial mechanical properties (Fig. 2c), indicating that the hydrogels showed good self-healing capabilities at different grafting ratios of MAPEG. Shear-thinning means that the weaker bonds in hydrogels are temporarily destroyed when the shear rate or shear force increases, and the viscosity of hydrogels decreases sharply.[28] As the shear rate and force continue to increase, the viscosity of hydrogel gradually decreases and eventually flattens, showing a liquid-state viscosity.[29,30] The influence of the grafting ratio of MAPEG (PMHU-2: 25.9%, PMHU-3: 35.5%, and PMHU-4: 45.2%) on shear-thinning properties of UPy-based hydrogels is shown in Fig. 3(a). The self-healing hydrogels corresponding to each grafting ratio of MAPEG had a tendency that the viscosity dropped sharply at a low shear rate, indicating that UPy dimers’ dissociation in hydrogels occurred and the hydrogels showed well shear-thinning property. At the same shear rate (0.001–0.01 s$^{-1}$), as the grafting ratio increased, the viscosity of the hydrogels decreased, similar to

![Fig. 3](https://doi.org/10.1007/s10118-021-2498-y)
The hydrogels should be stable during in aqueous solution. Additionally, with the influence of steric hindrance, the mechanical properties gradually decreased.

**Preparation and Characterization of Dual Crosslinking Hydrogels**

**Preparation of dual crosslinking hydrogels**

The UPy-based hydrogels had excellent self-healing and sheath-thinning properties via multiple-hydrogen-bond interactions and UPy dimerization. Nevertheless, while UPy dimers were transferred to solution, the strength and stability of hydrogen bonds undermined, leading to a weak and unstable hydrogel. The UPy-based hydrogels were steady in solution only for 6 h, far less than the required time of implanted materials (Fig. S3 in ESI). Therefore, for further stability improvement, the unsaturated bonds were incorporated to the UPy-based hydrogel to introduce the secondary crosslinking. After the activation of photo-initiator (i.e. 2-hydroxy-4-(2-hydroxy-ethoxy)-2-methyl-propiophenone (I2959)) at 5-min UV irradiation, the self-healing hydrogels were transformed into dual crosslinking hydrogels by photo-crosslinking of pendant methacrylate.

**Viscoelasticity of dual crosslinking hydrogels**

As shown in Fig. 5, the impact of the grafting ratio of MAPEG on the viscoelastic properties of dual crosslinking hydrogels was studied. The dual crosslinking hydrogels with MAPEG grafting ratios of 25.9%, 35.5%, and 45.2% (corresponding to PMHU-2, PMHU-3, and PMHU-4) had $G'$ values of 1614.3, 1562.1, and 786.1 Pa, respectively. The $G'$ attenuated with the increase of the MAPEG grafting ratio, which was caused by the double bond crosslinking as well as the synergetic effect of hydrophilic and hydrophobic interactions. Compared with the corresponding self-healing hydrogels, the $G'$ of all dual crosslinking hydrogels increased, indicating the introduction of the dual network improved the mechanical properties.

**Stability and degradation of dual crosslinking hydrogels**

In tissue engineering, the stability of hydrogels is as important as the mechanical property of hydrogels. In the early stage of tissue repair, hydrogels can provide an advantageous micro-environment for cell proliferation and differentiation, and also maintain the three-dimensional (3-D) structure of the new tissue in aqueous solution. The hydrogels should be stable during this period. Along with the further growth of new tissues, the temporary template is desired to erode or degrade in vivo, to make a place for tissue growth. Thus, the investigation of stability and degradation of hydrogels is necessary. Under simulated physiological conditions (phosphate-buffered saline solution (PBS), 37 °C), the stability and degradation of the dual crosslinking hydrogels were evaluated in vitro.

As revealed in Fig. 6, water absorption and swelling of the dual crosslinking hydrogels PMHU-3 and PMHU-4 occurred for 1 day due to the higher content of PEG segment. Since the dual crosslinking hydrogels PMHU-2 had a low PEG segment content, there was no apparent swelling. The dual crosslinking hydrogel PMHU-3, immersed in PBS, could maintain the shape for 8 days. This period is sufficient for cell induction and differentiation (generally 7 days). Meanwhile, compared with self-healing hydrogels, the stability of dual crosslinking hydrogels was enhanced significantly, demonstrating the introduction of the secondary covalent crosslinking network improved the stability of hydrogels regardless of soaking in solution.

The biodegradation of dual crosslinking hydrogels was evidently relative to the grafting ratio of MAPEG. The dual crosslinking hydrogel with the MAPEG grafting ratio of 35.5% (PMHU-3) showed the slowest degradation rate among the hydrogels in PBS. When the dual crosslinking hydrogels with the MAPEG grafting ratio of 25.9% (PMHU-2) and 45.2% (PMHU-4) immersed in PBS for 19 days, the degradation was almost completed, while PMHU-3 dual crosslinking hydrogel still maintained 18.9%±13.0%. The MAPEG grafting ratio-dependent degradation behaviors made it possible to meet various requirements for tissue engineering, in which case an interim carrier typically needed days or even weeks.

**Biocompatibility of the hydrogels**

The dynamic and reversible crosslinking endowed the self-healing hydrogels with both sheath-thinning and excellent self-healable properties, which made it possible to apply the self-healing hydrogels as injectable cell carriers for minimally invasive implantation. Additionally, stable and biodegradable dual crosslinking hydrogels were obtained in situ through photo-crosslinking for further tissue regeneration, which provided an ideal biomaterial for tissue engineering.

The cytotoxicity of I2959 and the hydrogel precursor PM-
Fig. 6 Effects of grafting ratio of MAPEG on the stability of dual crosslinking hydrogels: (a) gels only, (b–d) degradation in PBS on day 0/1/8, (e) degradation in PBS.

Fig. 7 MTT assay of (a) I2959 and (b) the mixture of hydrogel precursor and I2959; Hydrogels laden with cell spheroids: (c) self-healing hydrogels and (d) dual crosslinking hydrogels; (e) Cell viability.
H-U-3 of diverse concentrations was evaluated by MTT assay, employing a predetermined number of rASCs. Simultaneously, for reducing the error, rASCs fostered in DMSO was set as the positive control. As seen in Fig. 7(a), cell activity decreased gradually from 89.3%±3.2% to 21.9%±0.8% with the increase of I2959 concentration from 0.08 mg/mL to 10.67 mg/mL. In the fabrication of the dual crosslinking hydrogels, the concentration of I2959 was 1 mg/mL. The relative viabilities of rASCs in the mixture of hydrogel precursor and I2959 were between 159.7%±10.1% and 355.4%±11.2% at a wide range of concentrations (Fig. 7(b)), which showed the superior cytocompatibility.

Compared with monodisperse cells, cell spheroids are multicellular aggregates (Fig. S4 in ESI), which have a stronger resistance to harsh external environments[38] and more advantages in cell differentiation.[24] Therefore, cell spheroids were encapsulated in PMHU hydrogels instead of single rASCs. The feasibility of minimally invasive implantation and biocompatibility of secondary photo-crosslinking process by UV irradiation were verified. The hydrogel precursor PMHU-3 was used to fabricate the self-healing hydrogel, and then it was evenly mixed with cell spheroids (cell spheroids density: 5x10^5/mL). The self-healing hydrogel/cell spheroids complex was easily injected into a Petri dish through the 18G needle and then joined to form an integrated gel, indicating that the cell spheroids did not interrupt the self-healable or shear-thinning properties of the hydrogel. Afterwards, the dual crosslinking hydrogel/cell spheroids complex was obtained by 5-min UV illumination. Through the fluorescence images of cell spheroids in the hydrogel (Figs. 7c and 7d), the cell survival rate in the self-healing hydrogel and dual crosslinking hydrogel was 98.0%±10.0% and 86.7%±6.0% (Fig. 7e), respectively, which illustrated that apoptosis occurred during the UV illumination. This result suggested that the dual crosslinking hydrogel preserved most of the spheroids (86.7%±6.0% survived), which would be a promising material for tissue engineering.

CONCLUSIONS

In summary, through grafting Upy moieties onto the PLGA backbone via the connection agent of HAPEO, and introducing MAPEG into the PLGA backbone to provide unsaturated bonds, a novel hydrogel system was successfully fabricated. The PMHU self-healing hydrogels displayed well shear-thinning as well as self-healable properties as a result of the transient network, which could meet convenient injection. The G’ of the self-healing hydrogels decreased with the upregulation of the grafting ratio of MAPEG because of the balance of hydrophobic and hydrophilic segment as well as the influence of steric hindrance. However, the self-healing hydrogels could be steady in solution only for 6 h. The UV photo-crosslinking was then introduced to obtain dual crosslinking hydrogels for further stability improvement. In the same proportion, the G’ of dual crosslinking hydrogels was increased compared with the corresponding self-healing hydrogels. The improved mechanical property was attributed to the dual network derived from photo-crosslinking. The properties of hydrogels could be adjusted by controlling the proportion of MAPEG. When the graft ratio of MAPEG was 35.5%, the PMHU-3 dual crosslinking hydrogel showed the optimal stability and moderate degradative shape, which could be stable for at least 8 days in PBS. The PMHU-3 self-healing hydrogel loaded with rASCs cell spheroids was injected conveniently, and then through photo-crosslinking, the stable and biodegradable dual crosslinking hydrogel/cell spheroids complex was obtained in situ. Moreover, it showed favorable biocompatibility with a cell survival rate of 86.7%±6.0%. This novel hydrogel system can be used for minimally invasive implantation as injectable cell carriers and further tissue regeneration in situ, which will have broad applications in tissue engineering. Therefore, we expect that the PMHU hydrogels can be the promising tissue engineering biomaterials.

Electronic Supplementary Information
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