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One-Pot Synthesis of Highly Mechanical and Redox-Degradable Polyurethane Hydrogels Based-on Tetra-PEG and Disulfide/thiol Chemistry

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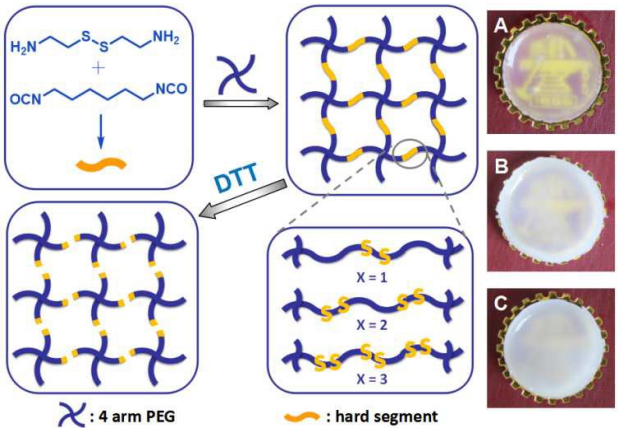
Highly mechanical hydrogels with stimuli degradability are promising scaffold materials for tissue engineering, due to their unique advantage that can keep mechanical strength in use while readily removed by external stimuli after use. However, it has always been a big challenge to integrate both high mechanical property and stimuli degradability into one single hydrogel. Herein, in this work, a series of tetra-PEG polyurethane hydrogels with tunable redox-degradability and high compressive fracture strength have been synthesized by one-pot method. The high mechanical property is owed to the extremely uniform network of tetra-PEG, and the redox-degradability is realized by using cystamine, which contains a highly DTT sensitive disulfide bond. The mechanical strength of the as-prepared hydrogels reach a megapascal range, and their complete degradation time can be flexibly adjusted from 4 to 22 days by controlling the proportion of cystamine. With the above properties, these hydrogels are believed to have potential bio-applications.

Introduction

Hydrogels are appealing for tissue engineering.[1] And stimuli-degradable hydrogels can be flexibly cleaned away by external stimulus after completing missions, and have a wide range of biological applications, including scaffolds for tissue engineering and regenerative medicine, barriers or adhesives between tissue and material surfaces, fillers for cavities caused by surgery, reservoir for drugs.[2-6] Broadly speaking, the most common stimuli-degradable hydrogels are those composed of hydrolyzable segments such as poly(lactic acid), poly(glycolic acid), poly(ε -caprolactone) with water as external stimulus. However, the slow but constant hydrolyzation process reduces the mechanical performance with time, affecting the use effect of materials. Therefore, many mechanically stable stimuli-degradable hydrogels have been designed using enzymatic hydrolysis,[7] photolytic cleavage,[8] disulfide/thiol chemistry,[9] and/or a combination of these methods.[10] Among these, disulfide/thiol chemistry method has the advantage that the disulfide bond is specifically cleavable under mild external stimulus such as thermal, pH, light, dithiothreitol (DTT) and/or glutathione (GSH).[9] Moriyama and coworkers developed a thiolated poly(ethylene glycol) (PEG) hydrogel that exhibits quick degradation after soaking in DTT solution.[12] Yang and coworkers prepared a GSH triggered degradable disulfide-containing PEG-based injectable hydrogel.[13] Fairbanks and coworkers designed a hydrogel using thiol-functionalized 4-armed PEG macromolecules, which is degradable under irradiation of low intensity UV light.[14] Li and coworkers reported a polyurethane hydrogel crosslinked by azobenzene/ α -cyclodextrin interaction and disulfide bond, which underwent degradation under both light and reductive external stimuli.[15] Though other similar hydrogel system are developed,[16-19] the major drawback of these hydrogels is lack of mechanical strength, let alone the tedious and time-consuming multi-step polymerization process.

4-armed PEG polyurethane (PU) hydrogel is one of the hydrogels with compressive strength reaching a megapascal range.[20-30]Because the tetra-PEG hydrogels have an extremely

Scheme 1. Preparation process of PEG-(S-S)$_X$-HDI hydrogels, and the corresponding hydrogel photograph with X from 1 to 3, abbreviated to X1(A), X2(B), X3(C).



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uniform network structure that can behave cooperatively, thus the mechanical strength increases greatly.[31] And there are two advantages to introduce tetra-PEG into a PU system. First, the homogeneous network of tetra-PEG hydrogel can be well preserved in PU system, because the soft segments (tetra-PEG) and hard segments must connect alternately with each other, zero self-reaction helps to avoid micro-loops and micro-defects that weaken the gels. Second, tetra-PEG can react with hard segments without any further terminal modification, due to the high reactivity and efficiency between isocyanate(-NCO) and hydroxyl(-OH) groups, greatly simplifying the difficult synthesis process.

Hence, in this work, we proposed an one-pot method to synthesize robust and redox-degradable PU hydrogels. Tetra-PEG, which endows the hydrogel with good biocompatibility, was used as soft segment; and hexamethylene diisocyanate (HDI) end-capped cystamine (Cys), which provides redox-degradability due to its disulfide bond, was used as hard segment. The facile preparation process of the PU hydrogels is briefly described in Scheme 1. First of all, Cys, the chain extender, was end-capped by HDI to form the hard segment HDI-Cys-HDI (yellow segment in Scheme 1). Then tetra-PEG was added to the mixture as the soft segment and crosslinker to get the final PU hydrogels, termed as PEG-$(S\text{-}S)_X$-HDI, where X=0, 1, 2, 3, referring to the number of S-S bonds between every two PEG segments in the network, so the series of PU hydrogels are further abbreviated to X0, X1, X2, X3, respectively.

Results and Discussion

Fourier Transform Infrared (FTIR) and Raman analysis demonstrate the successful preparation of PEG-$(S\text{-}S)_X$-HDI hydrogels. From FTIR spectra of X1 hydrogel (Fig. 1A), the disappearance of -NCO peak (2276 cm^{-1}), the emergence of -C=O peaks (1715 and 1639 cm^{-1}) and O-C-O peak (1096 cm^{-1}) indicate that HDI have completely reacted with Cys and tetra-PEG in the PU hydrogel. Since disulfide bond (S-S) is a symmetrical structure, corresponding IR absorption is hardly observed. Then the existance of disulfide bond is further proved by Raman spectrum at 537 and 580 cm^{-1} (Fig. 1B), meaning Cys (the chain extender) has been successfully incorporated into the PU hydrogels. Moreover, by normalizing the absorption intensity of Raman spectra at 844 cm^{-1} (C-O stretch of tetra-PEG) (Fig 1C), the content ratio of S-S bonds in X1, X2, X3 is confirmed approximately 1:2:3. Afterwards, exact content of S in each hydrogel is detected by Inductive Coupled Plasma Emission Spectrometer (ICP), see table 1. Ideal content of S refers to the calculated value of corresponding hydrogel with perfect structure. Conversion equals was obtained by dividing content of S with ideal content. It's found that, firstly, the corresponding S content appears to be 0.59%, 1.13%, 1.76% of X1, X2, X3, indicating the amount of S-S bonds in the hydrogels can be quantitatively controlled. Secondly, the S conversion of X1, X2, X3 reaches 92.2%, 88.2%, 91.7%, respectively, meaning Cys is used in a high efficiency.

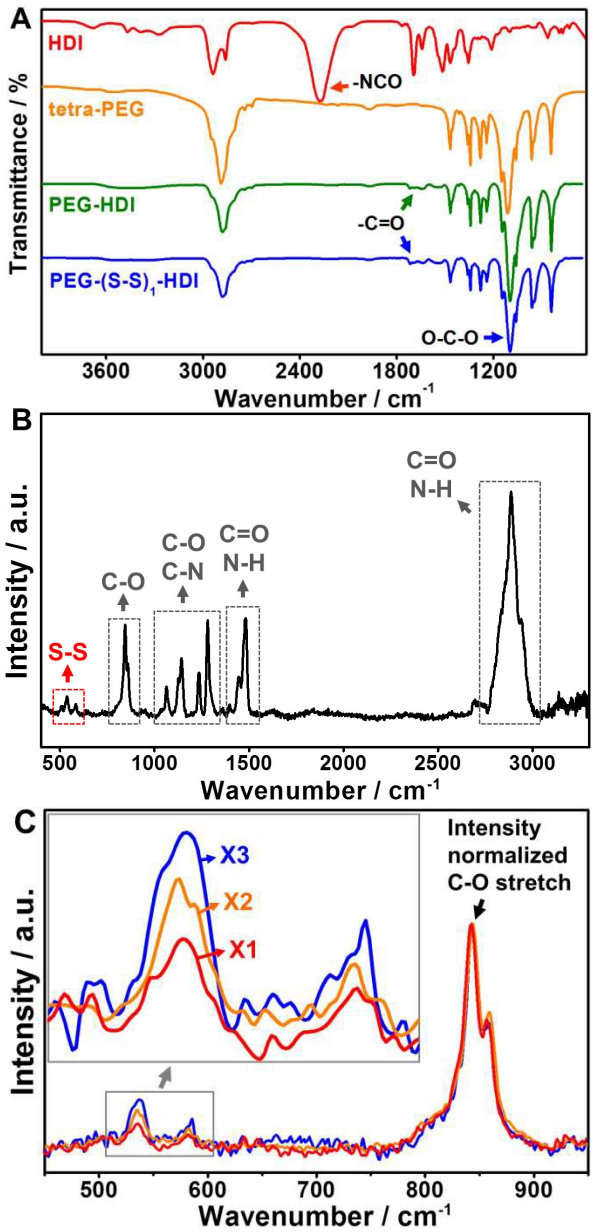

Figure 1. A) FTIR spectra of HDI, PEG, freeze-dried X0 and X1 hydrogels. B) Representative Raman spectra of X1 hydrogel, since spectra of X2 and X3 are similar with X1. C) Local enlarged Raman spectra of X1, X2, X3 hydrogels from 450 cm^{-1} to 950 cm^{-1}. The two bands in the inserted image on the left top refer to S-S stretching.

Table 1. Content of S in each PEG-$(S\text{-}S)_X$-HDI hydrogels, X from 0 to 3, tested by ICP. Conversion Equals were calculated by dividing content of S with ideal content.

	X=0	X=1	X=2	X=3
Content of S / %	0	0.59	1.13	1.76
Ideal Content of S / %	0	0.64	1.28	1.92
Conversion Equals / %	/	92.2	88.2	91.7

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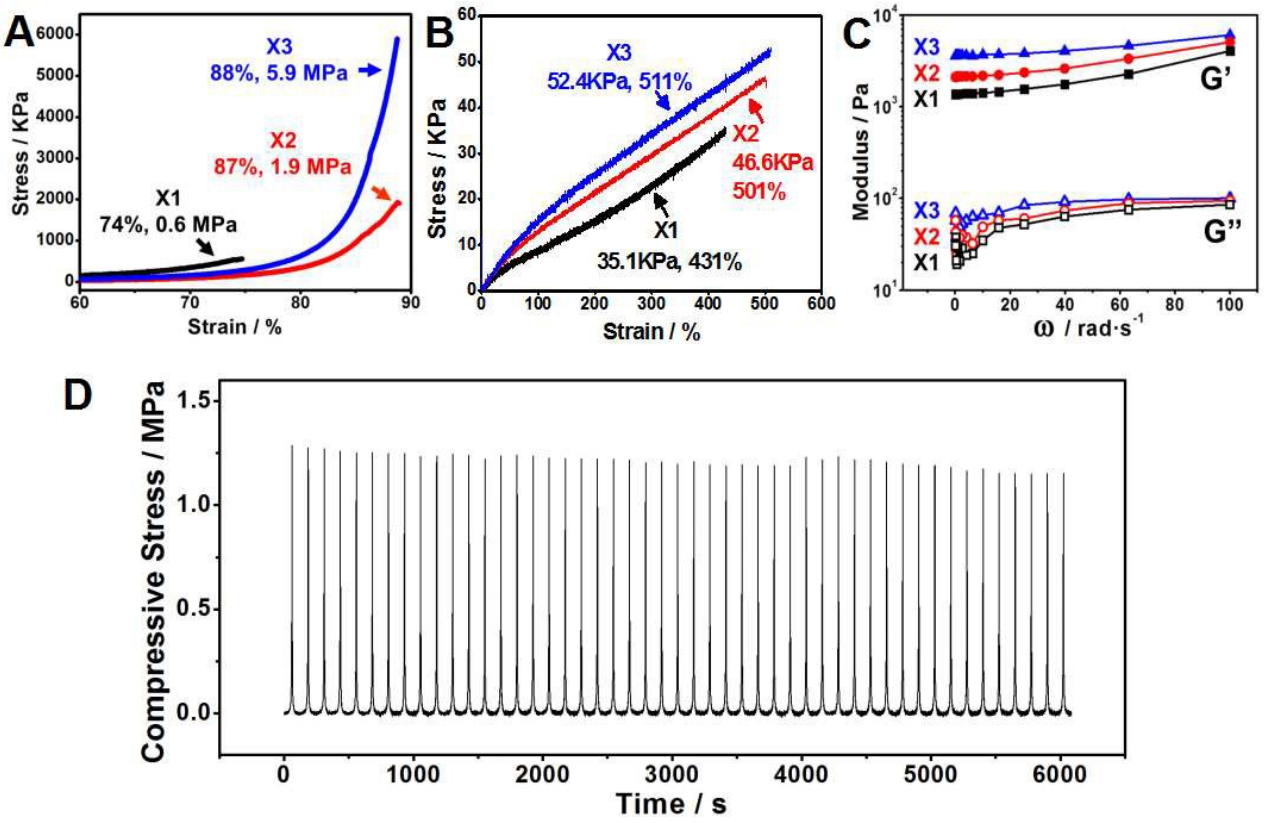

Figure 2. A)Compression stress-strain curves of X1(black), X2(red), X3(blue) hydrogels. B)Tensile stress-strain curves of X1, X2, X3 hydrogels.C)Rheology analysis of X1, X2, X3 hydrogels. D) Fatigue tests of X3 hydrogel as representative.

Random crosslinking points is one of the main reasons hinder the strength of hydrogels.[32] However, PEG-(S-S)$_X$-HDI hydrogels, whose framework consist of homogeneous 4-arm PEG, have subtly avoided this problem. Before mechanical property examination, all the samples were immersed in deionized water to reach a fully swollen state. With X increases from 1 to 3, equilibrium water content (EWC) increases from 93.93%, 94.85% to 95.01%. Fig. 2A-B shows the compressive and tensile stress-strain curve of PEG-(S-S)X-HDI hydrogels, and one can see the hydrogels present high compresion fracture strength with 0.6~5.9 MPa, 74%~88% deformation at break and high tensile fracture strength with 35~53 KPa, 431~511% deformation at break. Rheology studies show the PEG-(S-S)$_X$-HDI hydrogels are of high elasticity, as their storage modulus is two orders higher than their loss modulus (Fig. 2C). And fatigue tests reveal the PU hydrogels have good reliability. Take X3 hydrogel as an example (Fig. 2D), it can perfectly recover its mechanical property after 50-cycle testing under the condition of 0~83% deformation, 5 mm/min compression rate. We note that the content of Cys in hard segments plays a significant role in the mechanical strength of PEG-(S-S)$_X$-HDI hydrogels, which are increased with higher Cys concentration. An explanation is that higher Cys component increases the association energy of hydrophobic interaction, because water is good solvent for PEG segments but poor to the hard segments, and the large amount of extra strong hydrophobic interactions contribute a lot to the total mechanical strength. And we suppose it is the aggregation of hard segments through hydrophobic interaction that makes the hydrogel opaque (Scheme. 1A-C).

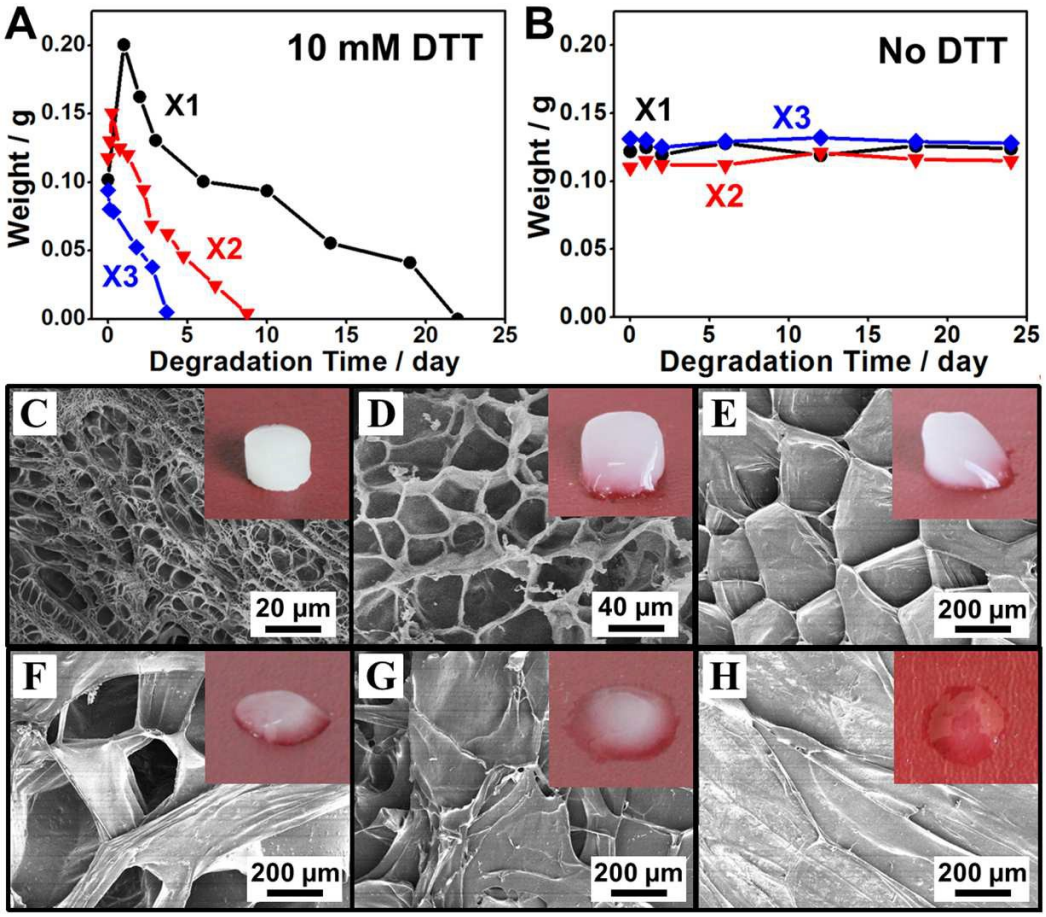

Figure 3. Degradation process of PEG-$(S\text{-}S)_X$-HDI hdyrogels. Weight loss curve of X1, X2, X3 hydrogels A) with and B) without 10 mM DTT treated. The network morphology evolution by SEM of redox-degraded X1 hydrogel at C) 0 d, D) 1 d, E) 6 d, F) 10 d, G) 14 d, H) 19 d. The insets are photographies of relevant hydrogels.

To investigate the degradability of the PEG-$(S\text{-}S)_X$-HDI hydrogel, we treated them with 10 mM DTT. First of all, quantitatively degradation process of X1, X2, and X3 hydrogels with (Fig. 3A) and without (Fig. 3B, controlled group) DTT treatment were carried out by measuring weight loss of the hydrogels. It is found that the weights of hydrogels treated by DTT decrease obviously over time, owing to the cleavage of S-S bonds in hard segments, but those of the controlled samples keep unchange, showing the hydrogels have rapid redox-degradation property. Meanwhile increasing the Cys component in the hard segments can effectively accelerate the degradation rate, for instance X1 (22 d) < X2 (9 d) < X3 (4 d). Therefore, it is possible to tune the degradation rate by controlling the proportion of Cys in the hydrogel. Once redox-degradation starts, the hydrogels turn to soft (inserted images in Fig. 3C-H) and lost mechanical strength. But the parallel samples without DTT treatment still remain high mechanical strength. This property ensures the hydrogels can preserve constant mechanical strength in use, and be easily cleaned away after use.

According to the morphology evolution in the redox-degradation process of X1 hydrogel(Fig.3C-H), The degradation pattern of PU hydrogels belongs to bulk erosion.[33] Because high hydrophilicity of PEG allows the rapid permeability of DTT solution in the hydrogel network, making the network structure gradually become larger and sparser, and eventually collapse. Finally, we evaluated the anti-adhesion property of the hydrogels, as it is important to prevent post-surgical inflammation.[34] L929 cells were cultured on X1, X2, X3 and tissue culture polystyrene (TCPS) for 24h, surfaces of substrates were observed by microscope(Fig 4. A-D). Compared to TCPS, cells adhered on the substrates of X1, X2, X3 were quite few, which illustrates the series of PU hydrogels exhibit outstanding anti-cell adhesive property. It's well known that the super-high hydrophilicity of tetra-PEG segments help to form a hydration layer on the surface of the PU hydrogels, which thereby restrain cells from attaching to the surface.[35] However, there is a trend that with the increase of Cys content, cells adhered on the surface augment, which mainly contributed by the increase of hydrophobic segments.

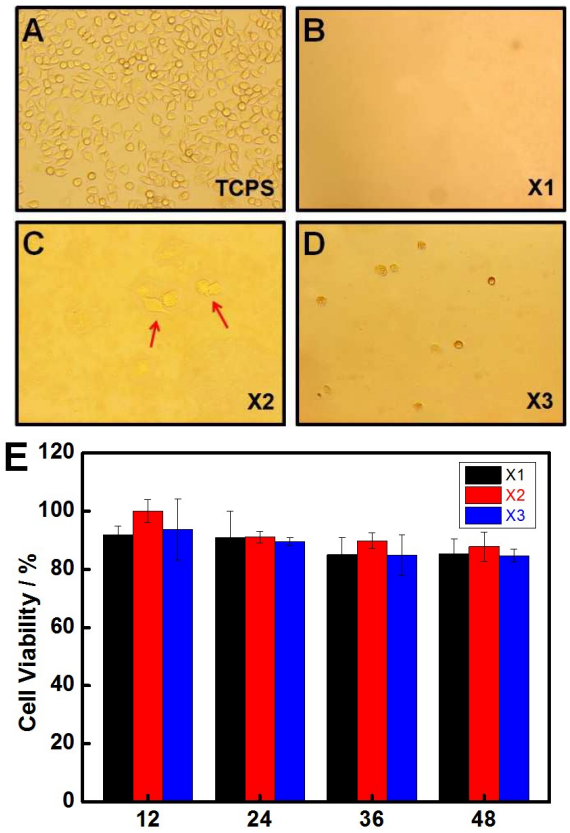

Figure 4. A-D) Morphology of L929 cells adhered on TCPS, X1, X2, X3 hydrogels after culturing for 24h. Red arrows demonstrate L929 cells adhered on the substrates. E) Cell viability after incubated in X1, X2, X3 hydrogel solutions for 24h.

Cytotoxicity analysis was also carried out, and the cell viabilities of X1, X2, and X3 all maintain at high level (>80%) after incubated together with the corresponding hydrogels for 48 hours(Fig. 4E). This experiment proves that the PU hydrogels have negligible cytotoxicity, because the hydrogel networks are composed of biocompatible tetra-PEG. This property may further make the hydrogels possible to be used as tissue filler or structural biomaterials.[36,37]

Conclusions

We used an one-pot method to successfully synthesize a series of tetra-PEG polyurethane hydrogels, which present tunable redox-biodegradability from 4 to 22 days in 10 mM DTT solution by controlling Cys content in hard segment, high compressive strength reaching 5.9 MPa, anti-cell-adhesion property and low cytotoxicity. Because the PU hydrogels can preserve high mechanical strength in use, while be completely cleaned away after use by external reducing agent such as DTT, they are promising for various bio-applications, for instance, structural biomaterial, tissue filler, barriers between material and tissue, etc.

Experimental

Materials

4-arm polyethylene glycol (4-arm PEG, Mn=20000, hydrogel value=11.2 mg KOH per g) was obtained from Xiamen Sinopeg Biotech Co. Ltd, Xiamen, China, and was dried at 85℃ under vacuum for 2 hours. Hexamethylene diisocyanate (HDI) and Cystamine dihydrochloride (Cys· 2HCl) were purchased from J&K. Catalyst, Stannous octanoate and Dichloromethane (CH_2Cl_2) were obtained from Adamas Reagent, Ltd. Besides, CH_2Cl_2 was further dried by CaH_2. Triethylamine (TEA) and ethyl alcohol absolute were purchased from Shanghai Chemical Reagent Corporation. In the biological experiments, all glassware was dried overnight under vacuum at 110 ° C before use. Bovine serum albumin (66 kDa, >98% purity), fetal bovine serum (FBS), and Dulbecco's modified Eagle medium (DMEM) were purchased from Gibco BRL. HeLa cells were obtained from the Cell Resource Center of Shanghai Biological Sciences Institutes. Water used in the experiments was purified to a resistivity higher than 18.2 MΩ· cm by using a Hitech system.

Preparation of PEG-$(S\text{-}S)_X$-HDI hydrogels

Solution of HDI in CH_2Cl_2 (0.1 g· ml^{-1}), TEA and Cys· 2HCl were added into CH_2Cl_2 (2 mL). The amount of Cys· 2HCl was determined by X value. When X=1, HDI was twice more than Cys· 2HCl in mol, when X=2, 1.5 times, when X=3, 1.33 times. Moreover, the mol of Cys· 2HCl was 2X times more than that of 4-arm PEG. Subsequently, the mixture was stirred in an ultrasonic bath (50 W, 40 kHz) at room temperature for 15 min. Then 4-arm PEG (0.2g, 0.00001 mol) was added into the mixture and after PEG was completely dissolved, solution of stannous octanoate in CH_2Cl_2 (0.1 g· ml^{-1}, 10 L) was added into. The resulting mixture was stirred in ultrasonic bath again for 15 min, and then kept at 50 ℃ for 24 hours for gelation. The resultant gels were successively dipped in ethyl alcohol absolute and deionized water, which are refreshed every day, for 3 days to remove the residual reagent completely. Finally, PEG-$(S\text{-}S)_X$-HDI hydrogels were successfully prepared.

Gel characterization

Raman Spectra of freeze-dried PEG-$(S\text{-}S)_X$-HDI gels were recorded on a Paragon 1000 (Perkin Elmer) spectrometer, and the excited wavelength at 780 nm. FTIR spectra of freeze-dried PEG-$(S\text{-}S)_X$-HDI gels, X from 1 to 3, were recorded on a Paragon 1000 (Perkin Elmer) spectrometer. Content of S in each hydrgel was examined by ICP, iCAP 6000 Radial, Thermo. Scanning electron microscopy (SEM) images of PEG-$(S\text{-}S)_X$-HDI hydrogels were obtained by a Philips Sirion 200 instrument, the hydrogel samples were wet off under liquid nitrogen, and the fracture surface was chosen to be observed under SEM. EWC of X1, X2, X3 hydrogels were calculated through the following equation:

$$EWC=(W_{wet}-W_{dry})/W_{wet}\times 100\%$$

Here Wwet refers to the weight of fully swelling sample, Wdry refers to the weight of fully dried sample.

Rheological measurements

Rheological characterization of the PEG-(S-S)$_X$-HDI hydrogels were determined by rheological measurements. The Storage and loss moduli of all PEG-(S-S)$_X$-HDI hydrogel samples were measured over the frequency range 0.1<w<100 rad· s^{-1} with the strain amplitude of 2% at 25 ℃ by a rotational rheometer (Bohlom Instruments, Advanced Rheometer) with plate/plate arrangement (25 mm in diameter and 1-1.5 mm in the gap width). Within the linear viscoelastic range, in which G' and G'' are independent of strain.

Mechanical test of hydrogels

Compression and tensile test were operate on a universal test machine(Instron 4465) at room temperature. Compression test samples were cylindrical, with 10 mm in diameter and 5~8mm in height. And the compression speed was 2 mm/min. For tensile tests, corresponding samples were 7~8 mm in diameter and about 25 mm in effective length. Elongation speed was 100mm/min. For recovery test, the compression loading rate was 5mm/min.

Degradation of PEG-(S-S)$_X$-HDI hydrogels

PEG-(S-S)$_X$-HDI hydrogels samples weight from 1.0-1.5 g were immersed in the solution of DTT (10 mM) in PBS (pH=7.4, 0.01M) at 37 ℃. The weight of samples was measured periodically, and the solution of DTT was refreshed every three days.

Biocompatibility of PEG-(S-S)$_X$-HDI hydrogels

Cell adhesion study: The three kinds hydrogels, named PEG-(S-S)$_X$-HDI (X=1,2,3), were cut into 8 mm×8 mm square, and then rinsed into the PBS solution, refreshed daily, for 3 days. The samples were sterilized before added to a 24-well plate cultured L929 cells in a density of 1×10^5 cell/well. And the culture medium of L929 cells was consist of DMEM with 1 wt% penicillin/streptomycin solution, 10 wt% FBS and 5% CO_2 at 37 ℃. After the cells were cultured for 24 hours, all the hydrogel samples were rinsed twice with PBS to remove unattached cells and observed under a Nikon-C1 laser scanning confocal microscope.

Cytotoxicity: Toxicity analysis of PEG-(S-S)$_X$-HDI (X=1,2,3) hydrogels were tested by MTT assay. 1×10^5 3T3/balb cells per well were seeded in a 96 well plate. And cells were incubated at 37℃, 5% CO_2 atmosphere for 24 h. Afterwards, PEG-(S-S)$_X$-HDI (X=1,2,3) hydrogels were respectively added in triplicate wells for each sample before incubated at 37℃, 5% CO_2 atmosphere for 24 h. After the incubation, cells were washed and replenished with fresh culture medium, which were further incubated for 2 h. Next, MTT was added to cells at a final concentration of 0.5 mg/mL and incubated for another 4 h. In order to dissolve the resulting formazan crystals, 100 μL DMF solubilization solution was added to each well. Absorbance was measured at a wavelength of 570 nm. The cell viability was assessed by dead/live double staining.

Acknowledgements

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