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Journal of Materials Chemistry B

ARTICLE

An injectable supramolecular self-healing bio-hydrogel with high stretching, extensibility, ductility and swelling ratio

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Experimental methods

1)¹H-NMR measurements

When the water content is very high, the obtained sample is sol; when the water content is relatively low, the obtained sample is gel. This kind of sol (which is similar to the gel in the chemical structure although it contains less chemical cross-linking points than the gel) can be used to conduct the NMR test to analyze the chemical structure of the hydrogel.The as-prepared hydrogels were immersed in an excess of water for 3 days to allow the residual chemicals to be released out of the gel. After freeze-drying, the structure of the as-prepared hydrogels was characterized by ^{1}H-NMR spectra performed on a Bruker 400 MHz spectrometer using 400MHz with 2 mg of dried gel dissolved in 2 mL D_2O. In all NMR measurements, chemical shifts were referenced to the solvent values.

2) In vitro degradation

0.3g PGA-Ly hydrogel was immersed in 3mL of collagenase solution of different concentration (10μg/mL, 5μg/mL, 2.5μg/mL, 1.25μg/mL, 0μg/mL in PBS, respectively) and incubated 37℃. At each time point investigated, the UV absorption for the byproducts of hydrogel degradation in the supernatant of each sample was measured using a spectrophotometer (UV-2550, Shimadzu, Japan) at 259 nm. The results were presented as a mean value with a standard deviation (n=4).

3) Cytotoxicity analysis of PGA-Ly-OSM hydrogels

The biocompatibility of hydrogels was evaluated by fibroblast cells from rats. In vitro cell experiments, all kinds of powder precursor materials were irradiated under UV light for 2 hours, and distilled water was sterilized by autoclaving. The hydrogel was prepared under aseptic conditions. And then the investigated hydrogels were coated on glass discs using a spinning coater. All the hydrogel-coated glass discs for cell culture were immersed in 75% ethanol for 2h for further sterilization and followed by rinsing three times with PBS before cell culture and then they were put in 24-well plates for cell culture at 37°C in a 5% CO_2 atmosphere with a medium replacement every 2 days. The investigated time points were days 1, 3, 5 and 7, respectively. Cell proliferation was determined by an MTT assay. After 4 hours of adding 20μL of MTT, excess medium was removed and added dimethyl sulfoxide (DMSO) to dissolve the formazan crystal. And then 100μL of DMSO was added to each well and the absorbance was measured at 450 nm with an enzyme-linked immunosorbent assay reader. The results were expressed as percentages relative to the data obtained with the blank control.

4) Hemolysis test

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The whole rabbit blood (2mL) was collected into the sterile sodium citrate solution (0.2 mL, 1 mM) to form the anticoagulant blood and it was diluted according to the volume ratio of the anticoagulant rabbit blood / saline (0.9% NaCl) (4:5). 1 ml of specimens (the byproducts of hydrogel degradation in the supernatant of each sample) in 15 mL centrifuge tubes were incubated in 10 mL saline (0.9% NaCl) at 37℃ for half-hour. 0.2 mL diluted rabbit blood was added to each centrifuge tube just mentioned. Distilled water and physiological saline were used as positive and negative controls, respectively. Samples were placed in a static incubator at 37℃ for 1 h. After that, the samples were centrifuged at 1000 r/min for 5 min. The absorbance of the supernatant was measured at 545 nm using a CliniBiol28c enzyme-linked immunosorbent assay reader. The hemolysis percentage was calculated according to the following equation:

$$\text{Hemolysis rate (\%)} = (A_2 - A_1)/(A_3 - A_1) \times 100\%$$

where A_1, A_2 and A_3 are the absorbance of the negative control, sample and positive control, respectively.

Results and discussion

1) ¹H-NMR analysis.

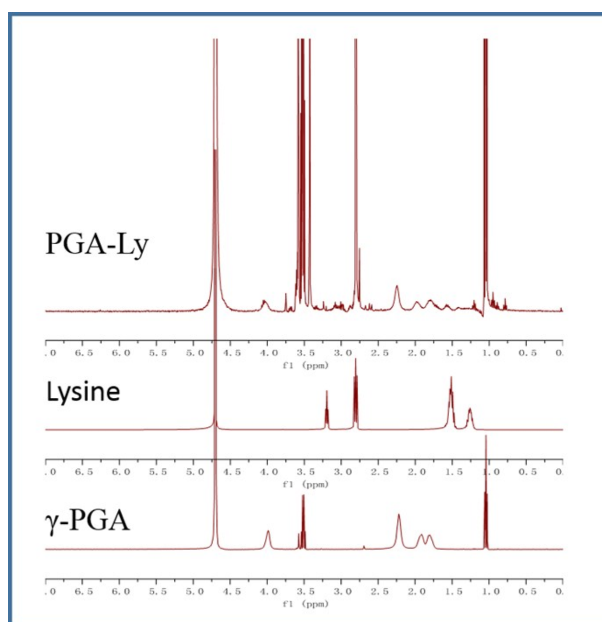

Fig.1 The ¹H-NMR spectra of (a) γ-PGA, (b) lysine, and (c) PGA-Ly hydrogel.

¹H-NMR was used to confirm the structure of the PGA-Ly hydrogel, as revealed in Fig. 4. The peaks at 1.8–2.3 ppm were assigned to PGA methylene protons, and methine protons at 4.1 ppm were present in both the initial PGA and PGA-Ly hydrogel spectra because the PGA backbone remained untouched. The peak of a $-CH_2$ next to a nitrogen atom was shifted from 3.2 ppm in lysine to 3.4 ppm in PGA hydrogel, indicating that lysine was introduced into PGA by an amide linkage.

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2) *In vitro* degradation analysis

The degradation properties of PGA-Ly hydrogels in collagenase solutions and phosphate buffered saline (PBS) were evaluated at a temperature of 37℃. Collagenase was widely distributed in the skin tissue, which can cleave the peptides in the protein molecule by acting on the L-lysine, glycine and L-citrulline residues. The results showed that the PGA-Ly hydrogel without collagenase was restively stable in the PBS compared with those with collagenase. The in vitro degradation curves of PGA-Ly hydrogels with various enzyme concentrations were depicted in Fig.2. The PGA-Ly hydrogels in the collagenase solutions lost 60-100% of weight within ten days while the PGA-Ly hydrogels in PBS just lost 47% of weight.

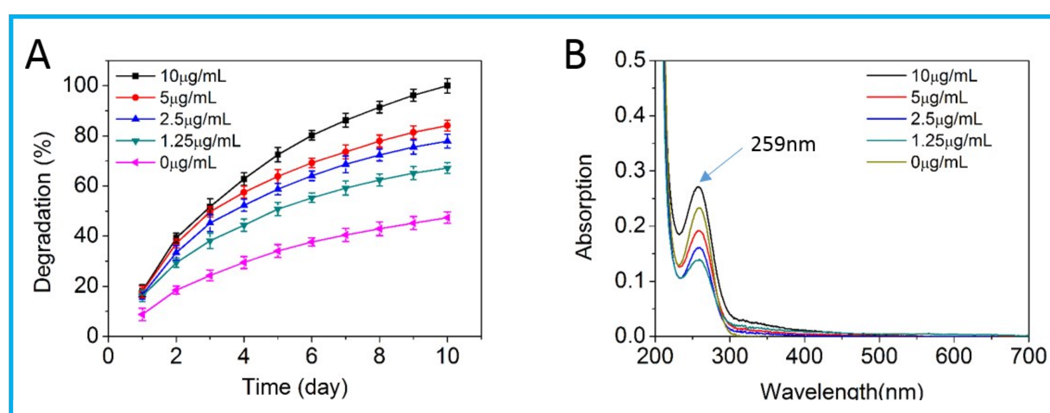

Fig.2 (A) The degradation rate of PGA-Ly hydrogels in collagenase solutions and PBS; (B) UV-vis absorbance of the degradation solutions for PGA-Ly hydrogels in collagenase solutions and PBS at the 5th day.

3) *Cytotoxicity analysis of PGA-Ly-OSM hydrogels*

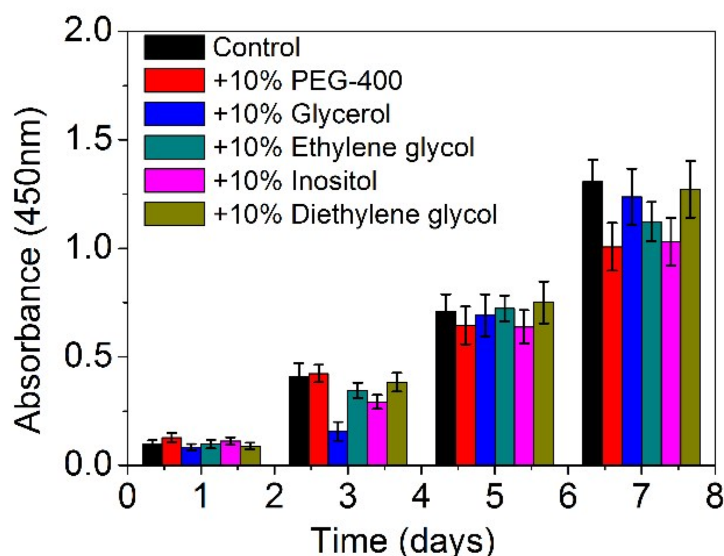

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Fig.3 Cell proliferation on PGA-Ly-OSM hydrogels evaluated by the MTT.

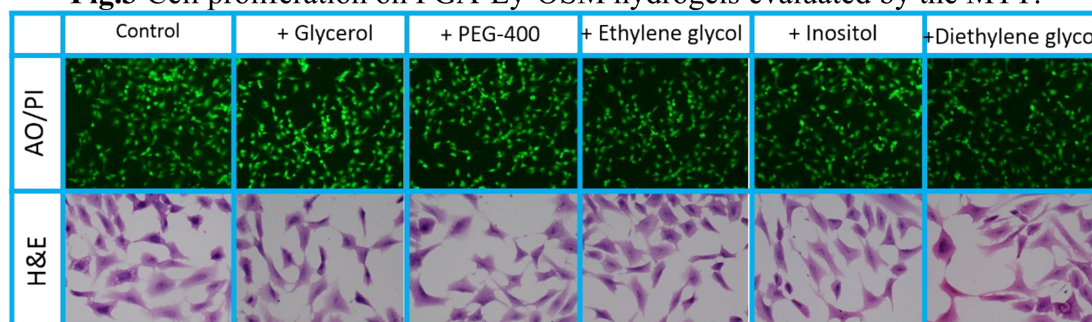

Fig.4 The images of cells under AO/PI staining (×40) and H&E staining (×100).

The cell viability of the PGA-Ly-OSM hydrogels was not statistically different from the control group at days 1, 3, 5 and 7 and thus could be considered non-toxic. Moreover, it can be seen that the cell structure was intact and that no any mutation was observed under H &E and immunofluorescence staining, which showed that the as the PGA-Ly-OSM hydrogels had no damage to the cells and other negative effects, demonstrating that they have good biocompatibility.

4) Hemolysis analysis

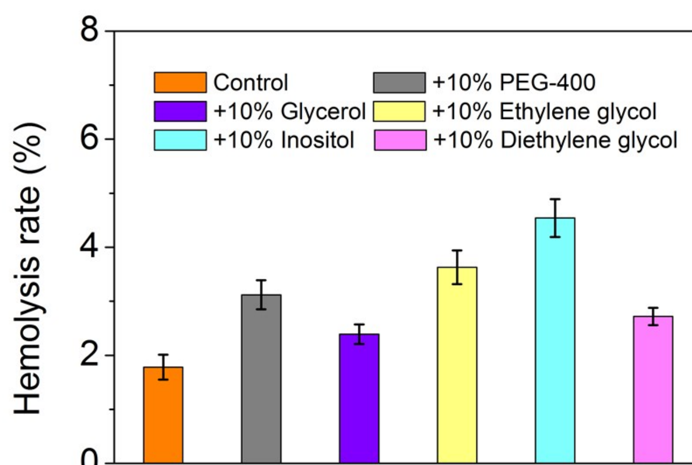

Fig.5 Hemolysis rate of PGA-Ly-OSM hydrogels

The hemolysis assay showed that the hydrogels were nonhemolytic in nature. Fig.5 shows the percentage hemolysis of blood in contact with different samples at 37℃ for 1 hour. All the samples were found to be non-hemolytic, whose percentage hemolysis was lower than the permissible level of 5%.

5) Modulus

The elastic moduli of the hydrogels were measured as the ratio of stress to corresponding strain in a material under tension.

Table1. The elastic moduli of hydrogels with various weight ratios of EDC/NHS.

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EDC/NHS	1：0.875	1：0.75	1：0.625	1：0.5
(MPa)	0.018	0.034	0.043	0.066

Table2. The elastic moduli of PGA-Ly-OSM hydrogels

(MPa)	Inositol	PEG-400	Glycerol	Ethylene glycol	Diethylene glycol
4%	0.026	0.015	0.017	0.021	0.018
6%	0.022	0.013	0.025	0.021	0.012
8%	0.018	0.011	0.013	0.011	0.008
10%	0.016	0.010	0.011	0.008	0.006

Supplementary video 1. The excellent ductility of the obtained hydrogels.

Supplementary video 2. Self-healing ability of hydrogel.

Supplementary video 3. Injectable property of hydrogels.

Supplementary video 4. The mechanical property of hydrogels after swelling.

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