Bioinspired self-degradable hydrogels towards wound sealing†

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1. Materials

Tetra polyethylene glycol and its derivatives (Tetra PEG-OH, Tetra PEG-SH, M_w = 20 kDa) were purchased from SINOPEG, China. Methoxypolyethylene glycol with thiol end groups (mPEG-SH, M_w = 2 kDa) were purchased from SINOPEG, China. The pig skin was purchased from a local grocery (Chaoshifa, Beijing). All other chemicals were purchased from Energy Chemical and used as received.

Experimental Methods

2.1 Synthesis of Acrylate terminated Tetra PEG (Tetra PEG Acr) and Acrylate terminated mPEG (mPEG Acr)

Tetra PEG Acr and mPEG Acr polymers were synthesized by esterification between PEG-OH and Acryloyl chloride. Typically, Tetra PEG-OH (10 g, 2 mmol -OH) and triethylamine (1.01 g, 10 mmol) was dissolved in 200 mL anhydrous dichloromethane (DCM) and cooled down to 0 °C for 10 min. Acryloyl chloride (0.82 mL, 10 mmol) was dissolved in 10 mL of anhydrous dichloromethane, and then was dropwise added to the mixture. The reaction was stirred for 48 h under RT. The organic phase was washed with NH_4Cl aqueous solutions for three times. The organic phase was collected and dried with anhydrous $MgSO_4$. The solvent was removed by rotary evaporation and the solid was re-dissolved in 10 mL DCM, followed by recrystallized with ethyl ether three times. Tetra PEG Acr was obtained in vacuo as an off-white powder (9.1 g, 91%). The end group conversion rate calculated by ^{1}H NMR is 97.8%.

2.2 Synthesis of the Tetra PEG SAcr hydrogel

The SAcr gel was simply fabricated by mixing the macromolecular precursor solutions with sodium carbonate. Typically, Tetra PEG-SH and Tetra PEG Acr were dissolved in physiological saline solution with the concentration to be 15 wt%, respectively. 0.15 mL of each precursor solution was pipetted and mixed in the 2 mL vial, followed by added the sodium carbonate solution (10 μL) of given concentration. The concentration of sodium carbonate in the gel directly after mixing is calculated and recorded as $c(Na_2CO_3)$.

2.3 Vial inversion tests

The gelation and liquefaction behaviors are examined by the vial inversion method. The temperature is kept at 25 °C during gel formation and liquefaction process. If no flow is observed within 10 s after inverting the vial, the formation of a hydrogel is determined. When the gel begins to flow, the liquefaction time is thus recorded.

2.4 Rheological tests

Rheological experiments are carried out on a Thermo Haake with a plate geometry (10 mm diameter) at 25 °C with the gap fixed at 3 mm. As for liquefaction kinetics measurements, the storage modulus (G') and loss modulus (G'') of freshly prepared SAcr gel are tested as a function of time at a strain of 1% and frequency of 1 Hz. The frequency sweep from 0.1 to 20 Hz is conducted at a constant strain of 1%. The strain sweep from 0.1 to 50% is conducted at a constant frequency of 1 Hz.

2.5 NMR kinetics tests

All ^{1}H NMR experiments were carried out by Bruker DRX-400 spectrometer under the same parameters. Chemical shifts and integrals were referenced to the residual solvent peak of D_2O (4.80 ppm). Stock solutions for mPEG-SH and mPEG Acr were freshly prepared in D_2O. The mixture was vortexed and pipetted into the NMR tube, followed by adding the sodium carbonate solution in D_2O. The final concentrations of the solutes included 8 mM mPEG-SH and mPEG Acr, as well as 20 mM of sodium carbonate.

2.6 Compression tests

The compression tests are conducted on a universal tensile machine (3365 Instron, USA). SAcr gels are prepared according to the method above in cylindroid molds (10 mm in diameter and 4 mm in height). The rate of compression tests is set at 3 mm/min until the sample is fractured (n=3). The nominal stress (σ) is calculated by dividing the force (F) by the cross-sectional area.

2.7 Torsion experiments

The torsion experiments were to investigate the adherence of the SAcr hydrogels to the porcine skin. Briefly, the SAcr hydrogels were synthesized *in situ* on the surface of the porcine skin and stained with Rhodamine B. Thus, torsion stress was applied on the porcine skin to test the adherence performance on the skin.

2.8 Adhesive strength

To measure the adhesive strength, porcine skin with an adhesion area of width 2 cm and length 2 cm were prepared and tested by a universal tensile machine (3365 Instron, USA). Then, 100 μL of freshly mixed precursor solution was piped on the porcine strip in the adhesion area. All tests were conducted with a constant tensile speed of 50 mm min-1. Adhesive strength was determined by dividing the maximum force by the adhesion area. The commercially available fibrin glue was involved as control.

2.9 SEM morphology

Scanning electron microscopy (SEM) images are performed at an acceleration voltage of 5 kV on a JSM-6700F microscopy (JEOL, Japan). The freeze-dried hydrogel samples are sputter-coated with a thin layer of Pt for 120 s to make the samples conductive before testing**.**

2.10 Sealing experiment of rubber bladder

A 2-mm gap was cut on the surface of the rubber bladder, and 15 wt% SAcr gel was prepared *in situ* at the gap. After waiting for 5 minutes, the bladder was slowly filled with N_2 to observe the gas tightness of the gap.

2.11 *In vitro* cytotoxicity studies

The cytotoxicity of the degradation product of SAcr gel is evaluated by CCK-8 assay using mouse fibroblast cells (NIH-3T3, provided by China Infrastructure of Cell Line Resource). The NIH-3T3 cells are incubated at 37 °C in DMEM culture media containing 10% fetal bovine serum, in a 5% CO_2 incubator. The cells are seeded onto 96-well plates at a density of 10,000 cells per well and cultured for 24 h. Then the medium in each well is replaced with 200 μL fresh medium and is added by 20 μL degradation liquids to yield a final concentration of 0.01–1 mg/mL. After incubation for 24 h, the culture medium is removed and 100 μL of fresh medium and 10 μL of CCK-8 is added into each well, followed by incubation for another 3 h (37 °C, 5% CO_2). The absorbance of the medium of each well was measured at 450 nm with a microplate reader. The cell viability was gained by comparing the absorbance with that of the control groups with untreated cells. The viability NIH3T3-E1 cells is further investigated by Live-Dead staining using Calcein-AM/PI (Propidium Iodine) detection

kit at 2 days after seeding. The morphology of cells was observed by the inverted fluorescence microscope.

2.12 *In vivo* biocompatible experiments

The *in vivo* biocompatibility of SAcr gel was verified by subcutaneous implantation in the dorsum of Sprague-Dawley rats (Beijing Vital River Laboratory Animal Technology Co., Ltd.). The hydrogels were made into a cylinder of 8 mm in diameter and 5 mm in height and implanted subcutaneously in the back of the rats. At specific times (12 h, 1, 3, 5, 7 and 14 days), the rats were executed according to the animal use protocol of the Chinese PLA General Hospital, and the wounds and surrounding tissues were collected, and then the tissues were fixed in formalin for 3 days. After fixation, the samples were dehydrated using alcohol gradients (70%, 80%, 95% and 100%) for 30 min each. Afterwards, they were immersed in xylene for 40 min, embedded in paraffin, cut into 3-5 μm and then stained with H&E and Masson and anti-CD48. All sections were observed on an Olympus microscope.

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of General Hospital of Chinese People's Liberation Army and approved by the Animal Ethics Committee of General Hospital of Chinese People's Liberation Army.

2.13 *In vivo* wound closure and regeneration

All animal experiments were performed following the guidelines of the Council for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Public Health, China. Female Sprague-Dawley rats (Beijing Vital River Laboratory Animal Technology Co., Ltd), aged 4 weeks old and weighing 150 g, were used as animal models. Rats were anesthetized with 2 wt % pentobarbital saline before surgery. The back of the rats was shaved and sterilized with betadine. Two 2 cm incisions were made on the left and right sides of the back using a scalpel and surgical scissors. Freshly prepared 1 mL solution of Tetra PEG Acr (15 wt%) with sodium carbonate (10 mM) were inhaled into one syringe and the other syringe was filled with 1 mL solution of Tetra PEG-SH (15 wt%). A dual-channel syringe was used to apply the adhesive hydrogel at the incisions (one incisions for around 0.6 mL hydrogel). The wound sites

were closed and gently pressed for 10 s. Traditional medical sutures were utilized as a control. The rats were executed at days 3, 7, and 14, respectively, and the incision tissues were removed and fixed in formalin for 3 days, and after fixation, the samples were dehydrated using alcohol gradients (70%, 80%, 95% and 100%) for 30 min each. Afterward, they were immersed in xylene for 40 min, embedded in paraffin, cut into 3-5 μm, then stained with H&E and photographed with an Olympus microscope.

The incisional tissues of mice in the suture and adhesive groups healed for 14 days were cut in a rectangular (7 cm × 2 cm) shape centered on the incision, and healing performance was examined by tensile testing at a rate of 50 mm/min (n=3).

3. Supporting Results

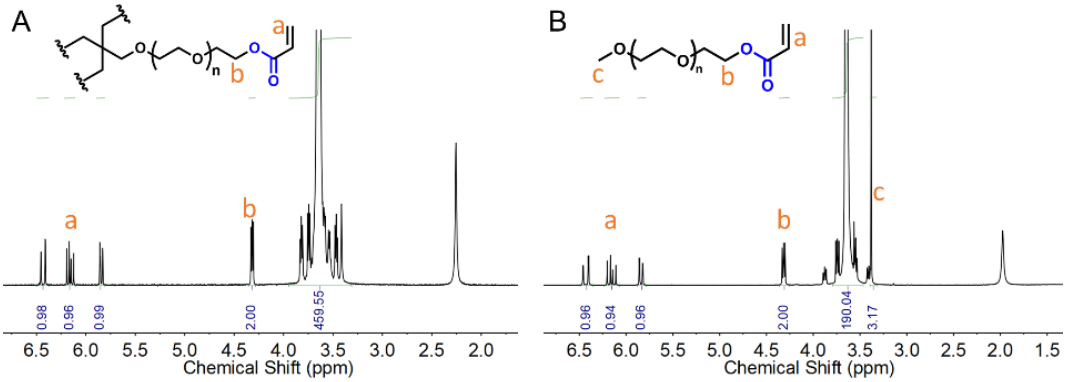

Fig. S1. ^{1}H-NMR spectra of (A) Tetra PEG Acr and (B) mPEG Acr ($CDCl_3$).

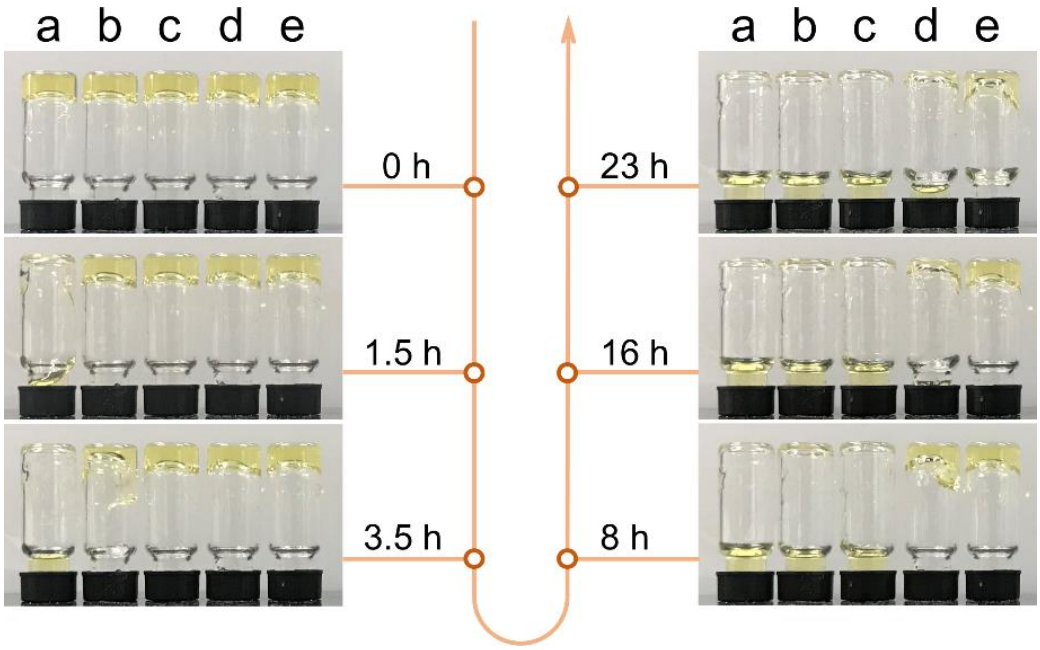

Fig. S2. *In-situ* liquefaction photos of the 15 wt% SAcr gels with concentrations of sodium carbonate at (a) 33, (b) 20, (c) 12, (d) 10, and (e) 7 mM.

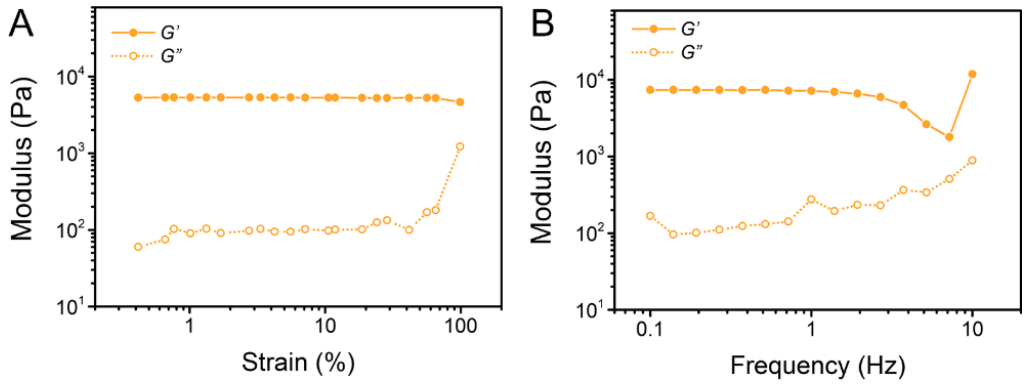

Fig. S3. (A) Frequency sweep of the 15 wt% SAcr gel, f = 1 Hz. (B) Strain sweep of the 15 wt% SAcr gel, γ = 1%.

Table S1. Changes in the peak areas of NMR spectra of the products generated by the model reaction of mPEG-SH and mPEG Acr

Time (min)	Integral value of peak			Calculated concentration of the addition product* (mM)
	a	a′	b′	
0	2.00	0	0	8.00
7	0.24	1.77	1.67	7.08
12	0.10	1.66	1.68	6.64
20	0.07	1.55	1.59	6.20
29	0	1.48	1.47	5.80
39	0	1.40	1.40	5.60
63	0	1.21	1.22	4.84
94	0	1.03	1.09	4.12
166	0	0.80	0.80	3.20

*The concentration was calculated from the integral value of peak a′.

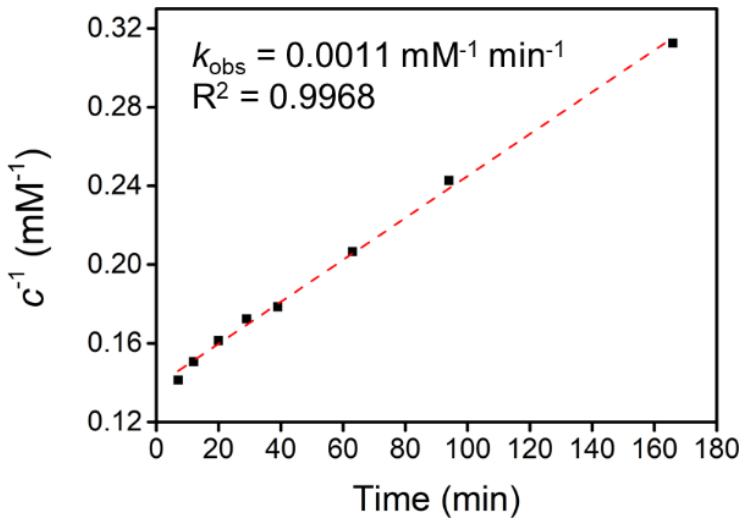

Fig. S4. ^{1}H-NMR kinetics of the hydrolysis process of the thioether ester, calculated from the integrals of the peak at 4.19 ppm at different time.

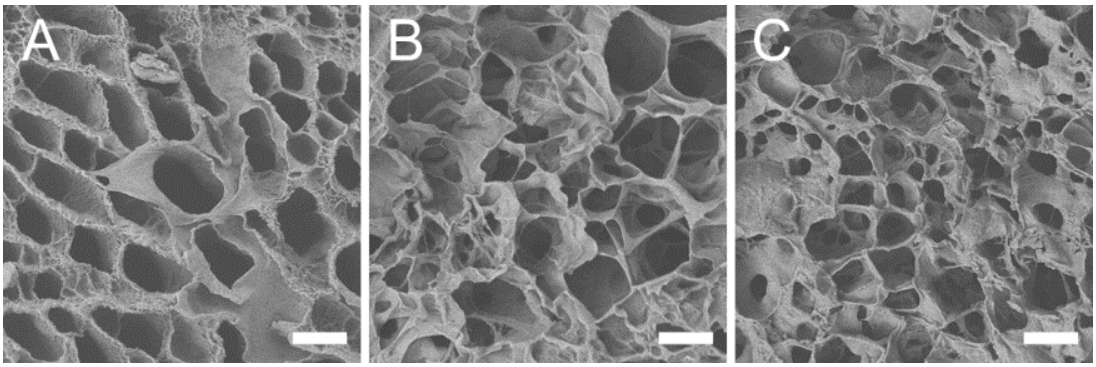

Fig. S5. SEM images of the SAcr gel at (A) 10 wt%, (B) 12 wt% and (C) 15 wt%. Scale bar = 10 μm.

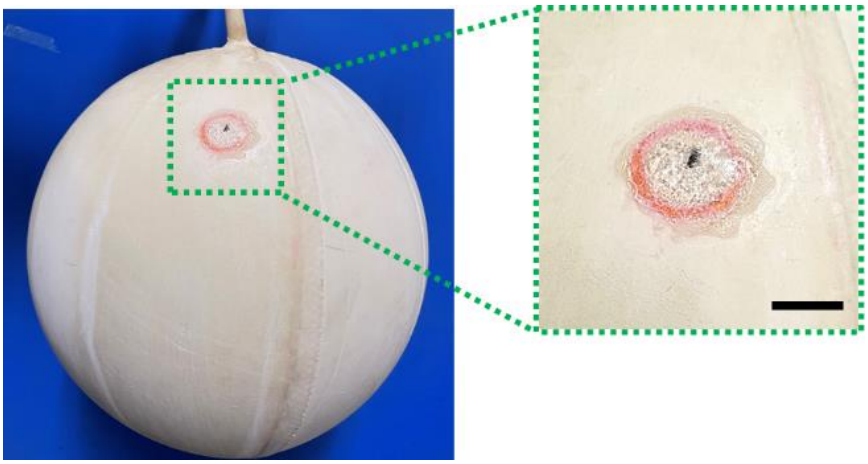

Fig. S6. Sealing performance of the 15 wt% SAcr gel covering the hole on rubber bladder. Scale bar = 1 cm.

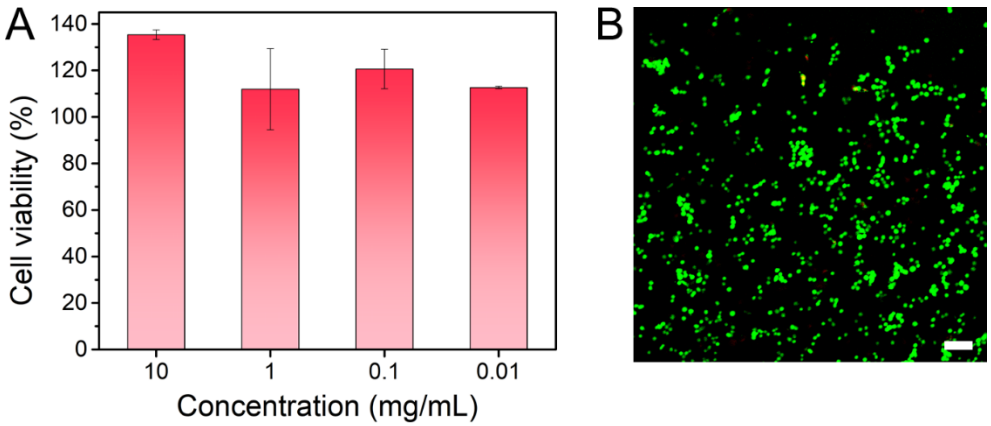

Fig. S7. (A) CCK8 assay for the proliferation of NIH-3T3 cell co-cultured with the degradation product of SAcr gels at various concentration for 24 h. (B) The Live/Dead staining of NIH-3T3 cell co-cultured with the degradation product of SAcr gels at 10 mg/mL for 24 h. Scale bar = 100 μm.