

## A Dynamic-Coupling-Reaction-Based Hydrogel with Ultra-High Autonomous Self-Healing, Stretching and Adhesion Properties and Good Biocompatibility

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**Abstract:** Hydrogels with autonomous self-healing property and excellent mechanical and adhesion properties have wide application in many fields. However, to achieve them is a big challenge. We explored a novel strategy to do that by using a dynamic coupling reaction of tyrosine hydrochloride. The compressive strength of 15 MPa and the elongation of over 4000%, the highest adhesion strength (to pigskin) of 453 KPa and the autonomous self-healing ratio of 99% in mild conditions were achieved for the as-obtained hydrogels. The excellent mechanical and adhesion properties and super-high self-healing properties of the as-prepared hydrogel provide a good prospect for their applications in tissue engineering, wound dressings, surgical sticky bandages, soft robotics and machinery, etc. Meanwhile, this strategy that employs this kind of coupling reaction under mild conditions to construct the hydrogel network provides a new idea for the design and preparation of autonomous self-healing materials.

## Experimental Procedures

### Materials

$\gamma$ -PGA (weight-average molecular weight 1000KDa) was obtained from Xi'an Bella Biotechnology Co., Ltd. (Xi'an, China), Tyramine hydrochloride (Tyr-HCl) was purchased from Zhengzhou Keyulong Chemical Products Co., Ltd. (Zhengzhou, China), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC-HCl,  $\geq 98.0\%$ ) and 1-hydroxypyrrolidine (NHS,  $\geq 98.0\%$ ) were purchased from Shanghai Dibo Biotechnology Co., Ltd. (Shanghai, China). All the above materials were used without further purification. Ultra-pure water was used throughout the experiment.

### Preparation of hydrogels

Hydrogels were synthesized using a one-pot method. PGA (1.4 g) was added to Ultra-pure water (10 mL) and stirred at room temperature (25 °C) until it dissolved completely to form a homogeneous solution. Then it was mixed with Tyr-HCl with various weight and stirred until it dissolved completely, EDC-HCl with various weight was added into the mixed solution, after stirring of about 15 min, NHS with various weight was added into the mixed solution. The weight (g) of Tyr-HCl, EDC-HCl and NHS were listed in Table S1. After few minutes, a hydrogel was formed at room temperature, which was denoted as the PGA-Tyr hydrogel in the later section.

The contrast sample was prepared similar to the Hydrogel-5. briefly, 1.4 g of PGA and 0.6 g of Tyr-HCl were dissolved in 10ml of distilled water under room temperature to form a solution and then 0.6 g of EDC and 0.6 g of NHS were mixed with the solution under vigorous stirring. The whole process was carried out in a nitrogen atmosphere.

**Table S1.** The varied weights (g) of Tyr-HCl, EDC-HCl and NHS in hydrogels.

	Tyr-HCl (g)	EDC-HCl (g)	NHS (g)
Hydrogel-1	0.4	0.4	0.2
Hydrogel-2	0.4	0.6	0.4
Hydrogel-3	0.4	0.8	0.6
Hydrogel-4	0.6	0.4	0.4
Hydrogel-5	0.6	0.6	0.6
Hydrogel-6	0.6	0.8	0.2
Hydrogel-7	0.8	0.4	0.6
Hydrogel-8	0.8	0.6	0.2
Hydrogel-9	0.8	0.8	0.4

### FT-IR and $^1\text{H}$ NMR characterization of hydrogels

The analysis of the material composition was performed using a FT-IR spectrometer (Spectrum One, Perkin Elmer, Norwalk, USA). 2 mg of each tested sample (Hydrogel-5 and the contrast sample) was carefully mixed with 200 mg of KBr (infrared grade), ground and then pressed into discs for the measurements. The spectra were recorded from 400 to 4000  $\text{cm}^{-1}$  wavenumber at a 4  $\text{cm}^{-1}$  resolution, averaging 64 scans.

$^1\text{H}$  NMR (400 MHz) spectra were recorded using a Bruker Avance QNP 400. Chemical shifts were recorded in ppm (d) in  $\text{D}_2\text{O}$  with the internal reference set to d 7 ppm. Hydrogel-5 was used for testing.

### Thermodynamic characterization of hydrogels

The thermal properties of the hydrogel and contrast samples were evaluated by DSC/DTA-TG using a STA 449 F3 Jupiter (NETZSCH-Gerätebau GmbH, Germany), measurement was carried out under nitrogen with increasing temperature (10°C/min, from room temperature to 800°C). The test used hydrogel-5.

### Rheological measurement

Hydrogel-5 was used for testing. Rheological measurement was carried out using a DHR-1 (TA Instruments, USA) rheometer with a parallel plate geometry (25 mm diameter acrylic plate). Shear storage and shear loss moduli ( $G'$  and  $G''$ , respectively) were obtained at constant deformation (10%) and temperature (25°C) with increasing frequency (from 0.1 to 100 rad/s).  $G'$  and  $G''$  were also obtained at constant deformation (10%), temperature (25°C) and frequency (1 Hz) with increasing time (from 0 s to 1200 s), hydrogel was synthesized and applied to the bottom plate of the rheometer immediately.  $G'$  and  $G''$  were also obtained at temperature (25°C) and frequency (1 Hz) with increasing strain (from 1% to 1000 %).

### **Mechanical characterization of hydrogels**

The compressive stress-strain measurement of the PGA-Tyr hydrogels was performed using an Instron-5967 universal testing system at a loading velocity of 15 mm min<sup>-1</sup> in air. During testing, the tested samples with cylinder shape (~8 mm in diameter and ~10 mm in initial thickness) were placed on a metal plate. Elastic property was also investigated via the compressive stress-strain measurement to the maximal strain of 95% and repeated 6 times for each sample.

The tensile stress-strain measurement of the PGA-Tyr was conducted on the same universal testing system at a deformation rate of 60 mm min<sup>-1</sup> in air. The test was carried out on dumbbell-shaped samples with the standard JIS-K6251-7 size (25mm (L) × 2-3mm (d) × 5 mm (w)). The work of extension at fracture  $W_b$  (J/m<sup>2</sup>), a parameter that characterizes the work required to fracture the sample per unit volume, was calculated from the area below the tensile stress-strain curve until fracture. For the cyclic tensile test, the samples were stretched at a velocity of 60 mm min<sup>-1</sup> close to a fixed extension ratio (300%, 500%, and 1000%, respectively) at room temperature, and then were moved back to the initial displacement after one minute at the same velocity of stretching. After a minute's rest, the hydrogels were stretched for second tensile test to study their self-recovery behavior. Totally five cycles were conducted. The cyclic tensile test used hydrogel-5.

### **Adhesiveness of hydrogels**

The tissue adhesiveness of the hydrogels was characterized by a tensile-adhesion test using porcine skin, glass, stainless sheet steel, and Teflon sheet, respectively, to mimic the natural tissue on a universal mechanical testing machine (Instron 5567). Briefly, porcine skin (glass, stainless sheet steel, and Teflon sheet, respectively) was used as the represented skin tissue. The hydrogel samples were adhered to the porcine skins (glass, stainless sheet steel, and Teflon sheet, respectively) with a bonded area of 25 mm × 20 mm. Then the samples were immediately pulled to failure by a universal testing machine with a crosshead speed of 5 mm/min. Lab-shear strength tests were performed with different substrates [glasses, stainless steel, polytetrafluoroethylene (PTFE) and pigskin]. The specimens were prepared according to Zadeh's work by gluing two identical plates (length × width × thickness = 75 × 20 × 1 mm<sup>3</sup>) with a junction contact area of 4 cm<sup>2</sup> using 0.2 g hydrogels. The lap joint was compressed under a 300 g weight for 10 min, and then the two ends of the substrates were mechanically clamped to prevent undesired changes of the junction contact area. Subsequently, the adhesion test was immediately conducted at a crosshead speed of 2 mm/min until the occurrence of separation. The adhesion strength was calculated by the maximum load divided by the initial bonded area. Each sample was tested a minimum of 3 times and averaged.<sup>1,2</sup> The adhesion strength was calculated by the measured maximum strength divided by the bonded area. The above tests all used hydrogel-5.

### **Self-healing characterization of hydrogels**

In order to investigate the self-healing ability of the samples clearly, the hydrogels were stained in red using edible pigment, which has no influence on self-healing. All the hydrogels were cut into half pieces and then one red half piece and another half piece without dye joined together end to end into a whole one. The self-healing process was investigated at 25°C for 4, 12 and 24 h, respectively. The self-healing ability of the hydrogels was characterized by using the ratio of the tensile strength of the hydrogels after self-healing to the original tensile strength. The above tests all used hydrogel-5.

### **Electron paramagnetic resonance spectroscopy (EPR)**

The radical formation during the preparation and healing of hydrogels was determined directly by Electron Paramagnetic Resonance (EPR) spectroscopy. EPR detection was carried out at 20 °C with an ER-200D-SRC-10/12 (Germany) using the following settings: Modulation amplitude of 1.000 G; center field 3500.00 G, sweep width 150.00 G, and a sweep time of 30 s. PGA (1.4 g) was added to Ultra-pure water (10 mL) and stirred at room temperature (25 °C) until it dissolved completely to form a homogeneous solution. Then it was mixed with 0.6 g Tyr·HCl and stirred until it dissolved completely, 0.6 g EDC·HCl was added into the mixed solution, after stirring of about 15 min, 0.6 g NHS with various weight was added into the mixed solution, after few minutes, adding spin trapping agent DMPO, then the samples were withdrawn for ESR spectrometry (sample 1). The hydrogel-5 was ground into powder after freeze-drying, then added 10 ml of Ultra-pure water to the powder, stirring for a moment, and spin trapping agent DMPO was added before the solution reformed hydrogel for ESR spectrometry (sample 2).

### **Biocompatibility of hydrogels**

The biocompatibility of hydrogels was evaluated by fibroblast cells from rats. The blank glass discs and degradation solution (hydrogel-5, after degradation for one week) were sterilized under high pressure steam, and then blank glass discs were put in 24-well plates and used as the control groups while those adding 1ml degradation solution were used as the degradation groups. The investigated hydrogels were synthesized and coated on glass discs using a spinning coater immediately. And then they were immersed in 75% ethanol for 2 h for 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<sub>2</sub> atmosphere with medium replacement every 2 days. The investigated time points were days 1, 3, 5 and 7, respectively. Cell proliferation was determined using an MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazoliumbromide) assay. After 4 hours of adding 20 ml of MTT, excess medium was removed and dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystal. And then 100 ml of DMSO was added to each well and the absorbance was measured at 570 nm with an enzyme-linked immunosorbent assay reader. The results were expressed as percentages relative to the data obtained with the blank control.

Dual-fluorescence viability, using acridine orange (AO) and propidium iodide (PI), was employed for the accurate viability analysis of the primary cells in our study. AO is permeable to both live and dead cells and stains all nucleated cells to generate a green fluorescence, whilst PI enters dead cells with compromised membranes and stains all dead nucleated cells to generate a red fluorescence. As a result, all live nucleated cells fluoresce green whilst all dead nucleated cell ones red. After cell culture for 5 days, the cells were incubated with AO and PI (both 5 mM) at 37 for 10 min. The supernatant medium and excess dye were removed by washing three times with PBS, and then the cells were photographed using an Olympus fluorescence microscope equipped with a digital camera.

After cell culture for 5 days, cells were examined by F-actin/DAPI staining. In brief, cells were rinsed twice with PBS and fixed in 4% paraformaldehyde for 20 min at room temperature and then rinsed three times with the PBS. After that, these cells were incubated with phalloidin for 40 min at 37°C in the dark and then rinsed three times with the PBS. The nuclei were counterstained with DAPI (4'-6-diamidino-2-phenylindole) for visualization. Microscopy observation was performed using an Olympus fluorescence microscope equipped with a digital camera and Image Proplus. The above tests all used hydrogel-5.

### 3-Dimensional (3D) cell culture using self-healing hydrogels

In order to collect adherent chondrocytes (C28/I2 cell) and osteoblasts (NHOC cell), the cell culture medium was removed first, and then adherent cells were washed with PBS several times and digested with Trypsin-EDTA, and finally PBS was added again to make cell suspension. The cell suspension was centrifuged (4 minutes, 1200 rpm) and the supernatant was removed and then serum-free medium was added and were fully mixed with the cells to form cell suspension again. Calcein-AM and Hoechst dyes were used to stain chondrocytes and osteoblasts to label them, respectively. They were added to cell suspensions of chondrocytes and osteoblasts, respectively, and incubated for 30 minutes at 37°C. The supernatant was removed by centrifugation (4 minutes, 1200 rpm). The stained cells were cleaned by adding PBS buffer and centrifuged. Finally, the number of stained cells was adjusted to  $10^6$ /ml by adding complete culture medium. 200  $\mu$ l stained cell suspension was added to the hydrogel precursor fluid to form a cell-loaded hydrogel. One piece of hydrogel loaded with labeled chondrocytes and another piece of hydrogel loaded with labeled osteoblasts were put together in a laser confocal Petri dish and then placed in a cell incubator for culture without using any liquid culture medium under sterile conditions. 32 hours later, a laser scanning confocal microscope was used to investigate the cells in the hydrogels that were loaded with chondrocytes and osteoblasts, respectively, and have self-healed. 490 and 460 nm wavelengths were used to stimulate the dyes in the chondrocytes and the osteoblasts, respectively.

## Results and Discussion

In the IR spectrum (figure S1 a), peaks at 1238 and 1189  $\text{cm}^{-1}$  can be ascribed to the asymmetric vibrations of the C-O-C linkage and to the C-OH vibration, respectively, and a peak at 1161  $\text{cm}^{-1}$  represents the symmetric vibration of the ether bond.<sup>3</sup> These data indicate that polymerization reaction consisted of a mixture of phenylene and oxyphenylene units;<sup>4</sup> it is preliminarily proved that the coupling reaction occurs only the products of the experimental group with  $\text{O}_2$ . In order to further confirm this conjecture, the products were detected by  $^1\text{H}$ NMR, the results showed that the positions of  $\text{H}_{1,2}$  and  $\text{H}_{3,4}$  in the products of the experimental group with  $\text{O}_2$  moved, and the peaks were split. Phenolic hydroxyl groups cause the hydrogen peaks connected to the  $\text{C}_\alpha$  to move toward the low field. The substituents of C-O-C also attract electrons to move the hydrogen peaks connected to the  $\text{C}_\alpha$  toward the low field, thus shifting the positions of  $\text{H}_{1,2}$  and  $\text{H}_{3,4}$  toward the low field and splitting the peaks. It is due to the coupling that makes the type of H change, and the new type H occurs.<sup>5</sup>

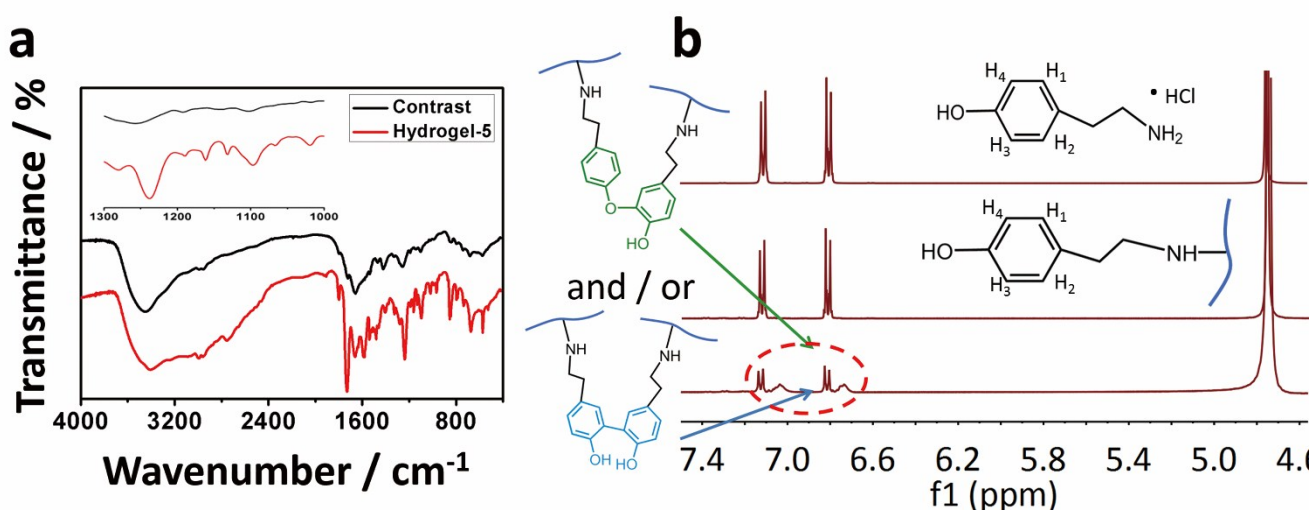
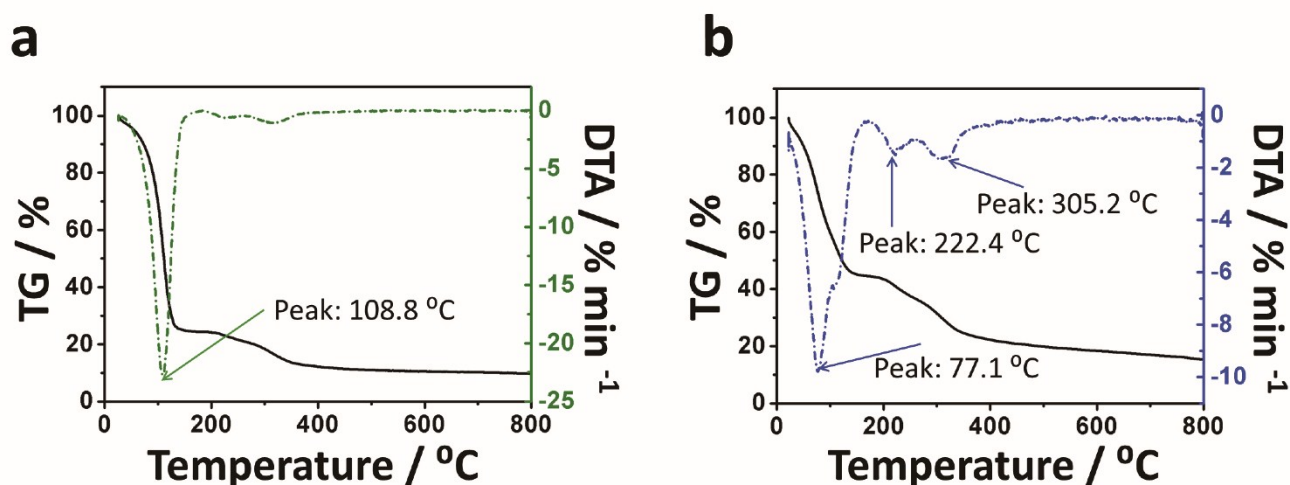


Figure S1 a) FI-IR spectra of hydrogel-5 and contrast sample. b)  $^1\text{H}$  NMR spectrum of pure Tyr-HCl, hydrogel and contrast sample.

Figure S2 shows the DTA-TG curves of the hydrogels. It can be seen that the products for each experimental group had large endothermic peaks at about 100°C. Among them, the peak areas of the experimental group with  $\text{O}_2$  were significantly smaller than those of the experimental group without  $\text{O}_2$ . In addition, the experimental groups with  $\text{O}_2$  had endothermic peaks at 222°C and 305 °C, which couldn't be detected in the experimental group without  $\text{O}_2$ . We speculate that the endothermic peak of all products at about 100°C is due to the decomposition of hydrogen

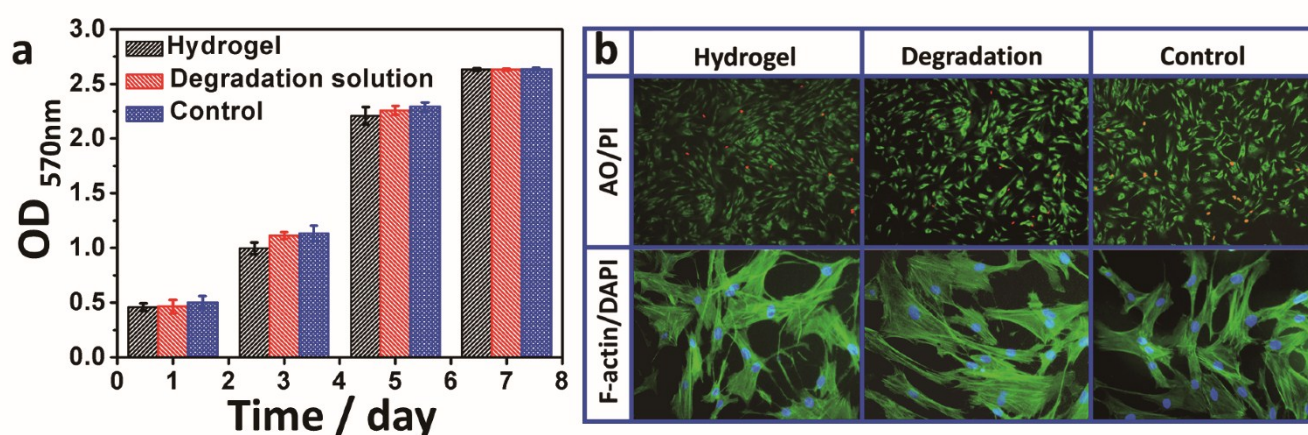
bonds at this temperature while the endothermic peak at 240°C is attributed to the decomposition of reversible chemical bonds at this temperature.<sup>6,7</sup> This indicates that there are both hydrogen bonds and reversible chemical bonds in the products of the experimental group with O<sub>2</sub>. The reversible chemical reaction leads to the reduction in the number of hydrogen bonds, thus making the area of the endothermic peak of



**Figure S2** DTA-TG curves of a) contrast sample b) and hydrogel.

hydrogen bond smaller than the experimental group without O<sub>2</sub>. Furthermore, we observed that the experimental group without O<sub>2</sub> could not form hydrogels and that the as-obtained products were only viscous solution while only the products of the experimental group with O<sub>2</sub> could form gel. It is deduced that the hydrogen bonds between the existing substrates are not enough to form gel. This is because hydrogen bonds have smaller bond energies and require larger reversible chemical bonds to form condensate networks.

To evaluate the biocompatibility of the synthesized PGA-Tyr hydrogel, the cell viability was measured via fluorescent microscope and MTT assays. From Figure S3, it can be seen that fewer dead cells existed all the groups, cells were evenly distributed and their density was high. There were no significant differences in cell morphology, structure and density between the cells cultured in hydrogel group, the degradation product group and the blank group, and all the cells were in good condition, consistent with MTT assays. Moreover, from the F-actin/DAPI staining images (Figure S3 b), it can be seen that the cell structure was intact and the cell surface was clear, showing that the hydrogel did not cause damage to the cells and other negative effects, and thus demonstrating that the obtained hydrogels have good biocompatibility.



**Figure S3** a) Cell proliferation determined by means of the MTT assay at days 1, 3, 5 and 7. The results were presented as the mean  $\pm$ SD, and the experiments were performed in triplicate. b) Images of the dual-fluorescence viability of the cells (after cell culture for 5 days) stained using AO/PI (all live nucleated cells fluoresce green and all dead nucleated cell ones red.) and F-actin/DAPI.

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