Supplementary Information

Naturally green polymer hydrogels with inherent antioxidative capability for efficient wound healing and spinal cord injury treatment

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1. Materials and methods

1.1 Materials

α-Lipoic acid (LA) was purchased from Aladdin (Shanghai, China). Sodium bicarbonate was obtained from Adamas-beta (Shanghai, China). Fetal bovine serum (FBS) and 4', 6-diamidino-2-phenylindole (DAPI) were supplied by Thermo Fisher Scientific. Cell Count Kit-8 (CCK-8) and reactive oxygen species assay kit were purchased from Beyotime Biotechnology, Shanghai, China. The commercial ELISA kits for tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were sourced from R&D Systems (Minneapolis, MN, USA). All the chemical reagents were used without further purification.

1.2. Animals used in spinal cord injury experiments

Six-week-old adult female Sprague Dawley (SD) rats with the weight between 170 and 220 g were selected in this experiment. All SD rats were housed in a 10 h light and 14 h dark cycle in a specific-pathogen-free facility with controlled temperature and humidity. All experimental animals are provided by the Experimental Animal Center of Xi'an Jiaotong University and are treated in strict accordance with the relevant standards issued by the Experimental Animal Committee of Xi'an Jiaotong University. All experimental rats were randomly divided into 3 groups: PLAS-3 hydrogel treated group (PLAS-3 group, n=15), control group (Control, n=15), and sham operation group (Sham, n=3).

1.3. Extraction and primary culture of neural stem cells (NSCs)

The NSCs we used were obtained from 5-day-old rats. All rats were provided by Experimental Animal Center of Xi'an Jiaotong University. The specific steps are as followed: the rat was disinfected with 75% alcohol, after which the head was quickly cut off with scissors. Then the cerebral cortex was isolated by cutting along the median line of the skull from back to front, and cut into pieces with ophthalmic scissors. Then we put it in a centrifuge tube with a pre-added culture medium and blew it until it turned turbid. The precipitated tissue blocks were added with 1 mL Accutase and digested at 37 ^{o}C for 15 min. Then we centrifuged it at 1000

rpm for 5 min, discarded the supernatant, and added medium to resuscitate the cells. The Petri dishes were labeled and placed in the incubator for suspension culture at 37 °C, saturated humidity and 5% CO_2 for subsequent use.

1.4. Characterization

^1H NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz). D_2O was used as the solvent. UV-vis absorption spectra were conducted by a UV-vis spectrometer (Lambda 35, PerkinElmer, America). The FT-IR spectra were performed on a Perkin Elmer 100 serial FT-IR spectrophotometer. The PLAS hydrogels were quickly immersed into liquid nitrogen and lyophilized for 48 h. Subsequently, the surfaces and cross-sections were sprayed with a gold layer, and the microstructures were visually characterized by scanning electron microscope (SEM, Gemini SEM 500). The acceleration voltage was 20 kV.

1.5. Rheological test

The rheological properties of the PLAS hydrogels were evaluated by using a TA rheometer (DHR-2). Before the collection of the data, 500 μL of the PLAS hydrogels was placed between 20 mm parallel plates with a gap of 1000 μm and the periphery was sealed by silicone oil to prevent the evaporation of water. Oscillation-frequency test with 1% constant strain and angular frequency varied from 0.1 to 100 rad/s at 37 °C was used to evaluate the stiffness of the PLAS hydrogels. Temperature sweep tests with 1% strain and 10 rad/s angular frequency were conducted to study the thermo-stability of PLAS hydrogels. The temperature was increased from 25 to 80 °C by cooling/heating speed of 2 °C/min. The shear-thinning test with the shear rate varied from 1 to 1000 1/s at 25 °C was performed to study the effect of shear rate on the viscosity of PLAS hydrogels.

1.6. Self-healing properties of PLAS hydrogels

PLAS-3 hydrogel with a diameter of 12 mm was prepared. The hydrogel cylinders were cut into two sections. Macroscopic self-healing performance of PLAS-3 hydrogel was evaluated by incubation at room temperature after contact. For the self-healing property test, the PLAS-3

hydrogel samples with diameter of 12 mm and thickness of 2 mm were first prepared. The strain amplitude sweep test (γ = 0.1%-2000%) with constant frequency of 10 rad/s at 37 ^{o}C was performed to detect the critical strain point. The alternate step strain sweep test was performed at a fixed angular frequency (10 rad/s) at 37 ^{o}C. Amplitude oscillatory strains were switched from small strain (γ = 1.0%) to subsequent large strain (γ = 1000%) during three cycles.

1.7. Adhesive strength evaluation

The adhesive strength of the PLAS hydrogels was evaluated using porcine skin or glass slide via a lap shear test. A piece of hydrogel (10 mm × 10 mm × 1 mm) was sandwiched between two dry porcine skins. The crosshead speed was set at 50 mm/min. The adhesion performance of the PLAS hydrogels was determined using CMT-1503 electromechanical tester (SUST Inc., China) at ambient conditions. At least five specimens were tested for each hydrogel sample.

1.8. In Vitro Cytotoxicity

The cell viability was examined by quantitative Cell Counting Kit-8 (CCK-8) cytotoxicity assay. NSCs were seeded into 96-well microculture plates and incubated for 24 h at 37 °C in a 5% CO_2 humidified incubator. PLAS-3 (10 mg) hydrogel was immersed in cell culture medium (10 mL) for 24 h to get the extracts. Then, the extracts were used to incubate the NSCs for 1, 3 and 6 d. The cells cultured on tissue culture plate were used as control. The extracts were exchanged into fresh medium containing CCK-8 (10 mg/mL) for 1 h. Finally, the absorbance of the sample medium was detected at 450 nm. The relative cell viability was calculated as the ratio between the mean absorbance of the PLAS-3 and that of the control group.

1.9. Live/Dead staining

Briefly, Live/Dead staining was conducted with NSCs which were seeded into a 24-well plate with a density of 1×10^5 per well and the cells were expanded as neurospheres in serum-free DF-12 medium supplemented with 100 mg/mL penicillin-streptomycin, 20 ng/mL epidermal growth factor (EGF), 10 ng/mL basic fibroblast growth factor (bFGF), B27 supplement and N_2 supplement under standard cell culture condition (37 ^{o}C and 5% CO_2) for 12 hours. Hydrogel

was immersed in cell culture medium (10 mL) for 24 h to get the extracts. After incubation with PLAS-3 hydrogel extract for different periods (1, 3 and 6 d), the medium was removed and cells were centrifuged and washed twice with PBS. The cells (5×10^5/mL) were treated with staining solution (prepared by 4 mmol/L ethidium homodimer-1 and 2 mmol/L calcein-AM in 0.01 mol/L PBS) and incubated at 37 °C for 15 min. The staining solution was then discarded and the samples were centrifuged and washed twice with PBS. The live cells were stained with green fluorescence and dead cells showed red fluorescence.

1.10. Hemolytic activity of the PLAS hydrogels

Erythrocytes were separated by centrifugation (1,000 rpm) from the mice blood for 10 minutes at 4 °C. After that, the serum was removed and an equal volume of freshly prepared 0.9% saline solution was added. The set was repeated three times to completely remove the remnant serum. After removing the saline solution at the final washing procedure, red blood cells (RBC) were suspended with 0.1 M PBS and diluted 25 times to acquire 1.11×10^8 mL^{-1} RBC suspension. PLAS hydrogels (100 mg) mixed with erythrocytes stock (500 µL) were added to a 24-well microplate, then shaken in an incubator at 37 °C for 1 h with a shaking speed of 100 rpm. 0.1% Triton X-100 was used as the positive control while PBS buffer was used as the negative control. After that, the microplate was centrifuged (1,000 rpm) for 10 minutes and the supernatant (100 µL) was then introduced into a 96-well microplate. The absorbance of the solution was read at 541 nm by SpectraMax microplate reader. The hemolysis percentage was calculated from the relation: Hemolysis rate% = $(A_s - A_c/A_t) \times 100\%$, Where A_s, A_c, and A_t represents the absorbance at 541 nm for the sample group, saline solution group, and the Triton X-100 group, respectively.

1.11. In vitro degradation of PLAS-3 hydrogel

The hydrogels (10 mm in diameter and 10 mm in height) were weighed (W_0) and put into the centrifuge tube with 10 mL of PBS at 37 ^oC with shaking speed of 100 rpm. At predetermined time intervals, hydrogel samples were taken out and dried with filter paper. The weight was

recorded as W_t. The weight loss ratio was calculated from the relation: Weight remaining ratio (%) = $W_t / W_0 \times 100\%$.

1.12. In vivo degradation of PLAS-3 hydrogel

Subcutaneous implantation of PLAS-3 hydrogel was performed on mice to study the in vivo degradation (Experimental Animal Center of Xi'an Jiaotong University). Briefly, round-shaped PLAS-3 hydrogel (8 mm in diameter and 2 mm in thickness) was implanted subcutaneously in mice. The degradation behavior of the hydrogel was observed at predetermined periods (1, 3, 5 and 8 d). Histological sections were stained with H&E. All mice were sacrificed according to Experimental Animal Committee of Xi'an Jiaotong University. All the sections were visualized on microscope (DMi8, Leica, Germany).

1.13. Antioxidant activity of PLAS hydrogels

The antioxidant capacity of the hydrogels was evaluated by testing their capability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH·) free radicals. The PLAS hydrogels were lyophilized and ground into powder. Afterward, 100 μM DPPH and desired amount of PLAS-2, PLAS-3 and PLAS-4 hydrogels (30 mg) were dispersed in 3.0 mL ethanol. The mixture was stirred and incubated in a dark place at 37 °C for half an hour. Then, the UV absorption at 517 nm was detected. The degradation of DPPH was calculated by the following formula:

$$DPPH\ scavenging\ \% = \frac{A_B - A_H}{A_B} \times 100$$

where A_B and A_H were the absorption of the blank (DPPH + ethanol) and the absorption of the hydrogel (DPPH + ethanol + PLAS hydrogels), respectively.

Next, the intracellular ROS scavenging ability of PLAS-3 hydrogel was measured by reactive oxygen species assay kit. The NSCs were seeded at 5×10^5 cells/mL density into poly-L-lysine-coated (Sigma-Aldrich, USA) 96-well plates and incubated for 12 h. NSCs were first treated with DCFH-DA (100 μL, 1 μmol/L) in serum-free medium and incubated for 30 min. The cells were washed three times with serum-free cell culture medium to fully remove DCFH-DA. The cells were disposed with special medium solutions containing PLAS-3 hydrogel (25 mg/mL)

with Rosup (1 μg/mL) for 30 min. NSCs with only Rosup treatment was used as the positive control, and NSCs with no treatment were used as negative control. The ROS levels were detected by microscope (DMi8, Leica, Germany).

1.14. In vivo hemostatic ability test

Hemorrhaging liver in SD rats was employed to evaluate the hemostatic potential of the PLAS-3 hydrogel. Briefly, by injecting chloral hydrate (10 wt%), the rat was anesthetized and then fixed on a surgical corkboard. The liver of the SD rat was exposed by abdominal incision, and the tissue fluid around the liver was carefully removed to prevent inaccuracies of blood weight obtained from the samples. The livers were placed above the pre-weighed filter paper (W_0). A scalpel was used to induce liver bleeding, and the PLAS-3 hydrogel was immediately applied to the bleeding site. The hemorrhaging site of the liver was photographed (15, 30, 45 and 60 s). The weight of the filter paper (W_1) with absorbed blood was measured and compared with control group (no treatment after pricking the liver) after 60 s. The increase in the weight of the filter paper (W_1-W_0) was equal to the weight of the blood loss of the SD rats. All animal experiments were carried out following current guidelines for the care of laboratory animals and were approved by the proper committee of Xi'an Jiaotong University.

1.15. In vivo wound healing

A full-thickness skin incision model was carried out to evaluate the in vivo wound healing performance and female Kunming mice (30-40 g, 5-6-week age) were employed to conduct the experiments. All animal experiments were approved by the animal research committee of Xi'an Jiaotong University. All mice were randomly divided into 3 groups (3 mice per group) including the suture treated group, PLAS-3 hydrogel treated group and the untreated group. All mice were acclimatized for 1 week before surgery. For the surgery, all procedures were performed under aseptic conditions. After standard anesthesia procedure with intraperitoneal injection of chloral hydrate (0.3 mg/kg body weight), the dorsal region of mice above the tail and below the back was shaved for surgery. A 10 mm full-thickness mice dorsal skin wound was created for the in

vivo wound closure test. The skin wound was treated by the suture and PLAS-3 hydrogel, and the untreated wound was used for comparison. After 7 days of recovery, the photographs of the wound site were taken and H&E staining was used to assess the wound closure.

1.16. Spinal cord injury model

The female Sprague Dawley rats (body weight: 170-220 g) were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.35 mL/kg) and a 4-5 cm incision was made centered on T10 (ribs counting). Afterward, a 3 mm semi-incision on the left side of the spinal cord at the T10 level was created followed by suturing layer by layer. In the PLAS-3 hydrogel-treated group, 5 μL of the hydrogel was injected at the injured site, and the control group was treated with the same amount of PBS solution. In the sham group, T10 lamina was only exposed followed by PBS injection. After operation, the rats were put on the electric blanket for resuscitation to avoid hypothermia shock. Penicillin was used within 7 days after the surgery to prevent infection. Bladder massage was performed twice a day for two weeks after surgery to assist urination and prevent urinary tract infection until the spontaneous urination reflex function was restored.

1.17. Motor function assessment

The recovery of motor function was evaluated by recording the BBB score at predetermied periods after surgery as well as the footprints and motor images of rats at the time of 0 and 28 days after surgery. In the BBB ratings, the rats were placed and allowed to move freely on an open field individually. Two experienced readers assessed the performance of each rat according to the BBB scale while being blinded to the groups. The BBB score ranges from 0 (no limb movement or weight support) to 21 (normal locomotion). Functional evaluations were performed blindly at 1, 2 and 3 days and subsequently once a week for 4 weeks. For footprint analysis, the hindlimbs of the rats were dipped in black ink. Subsequently, the rats ran along a narrow corridor lined with white paper. Gait and motor coordination parameters, including stride length and rotation angle, were measured from the middle of the runway. The angle of paw rotation was defined as the angle (in degrees) of the hindlimb axis relative to the runway

axis. An increase in rotation angle indicated external rotation of the hindlimbs. Values from both hindlimbs were averaged to calculate the stride length and limb angle values.

1.18. Serum extraction and tissue processing

The rat's hearts were exposed after deep anesthesia with chloral hydrate. 1.2 mL of blood was collected using a 5 mL syringe by stabbing into the heart under direct vision. The fresh blood was stationary in a 37 ^{o}C water bath for 60 minutes, centrifuged at the speed of 3000 rpm for 10 minutes, after which the supernatant was extracted and refrigerated at -80 ^{o}C. The right atrial appendage was cut and the heart was perfused rapidly with physiological saline. When the liver was whitened, 4% paraformaldehyde solution was used. After the rats were hardened, a section of spinal cord tissue, which was about 1 cm in length, was removed from the injured site and fixed in 4% paraformaldehyde solution. The fixed spinal cord tissue was washed with PBS for 3 times, dehydrated with 30% sucrose solution, embedded in OCT and then sectioned longitudinally in a cryostat frozen slicer. The slice with a thickness of about 15 μm in the middle position was selected for analysis. At 28 days after surgery, 3 rats were randomly selected from each group for H&E staining and LFB staining.

Results

Movie S1. Adhesiveness of PLAS-3 hydrogel with human skin

Movie S2. The walking gait of SD rats in the control group 0 day postsurgery

Movie S3. The walking gait of SD rats in the PLAS-3 treated group 0 day postsurgery

Movie S4. The walking gait of SD rats in the control group 28 days postsurgery

Movie S5. The walking gait of SD rats in the PLAS-3 treated group 28 days postsurgery

Table S1. Gelation behavior of PLAS aqueous solution with the lower concentration of monomers.

Group	Monomer Concentration (mol/L)	LA/SL (molar ratio)	Gelation Behavior
PLAS-6	0.87	1:0.475	Gelation
PLAS-7	0.78	1:0.475	Gelation
PLAS-8	0.68	1:0.475	Gelation
PLAS-9	0.58	1:0.475	Precipitation
PLAS-10	0.49	1:0.475	Precipitation
PLAS-11	0.39	1:0.475	Precipitation

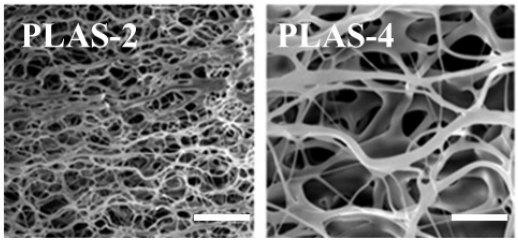

Fig. S1 SEM image of PLAS-2 and PLAS-4 hydrogels. Scale bar: 30 μm.

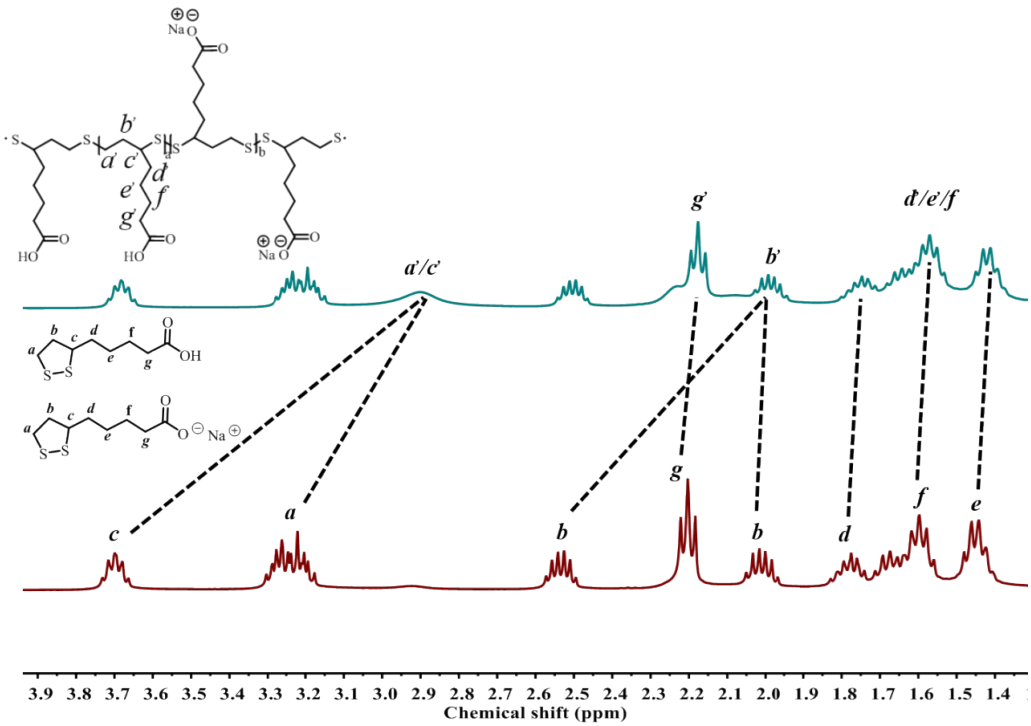

Fig. S2 ^{1}H NMR spectra of PLAS-3 and monomers.

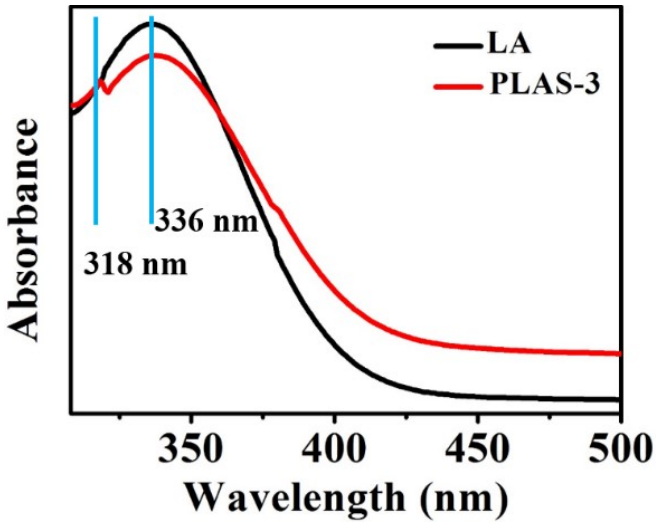

Fig. S3 UV-vis absorption spectra of LA and PLAS-3.

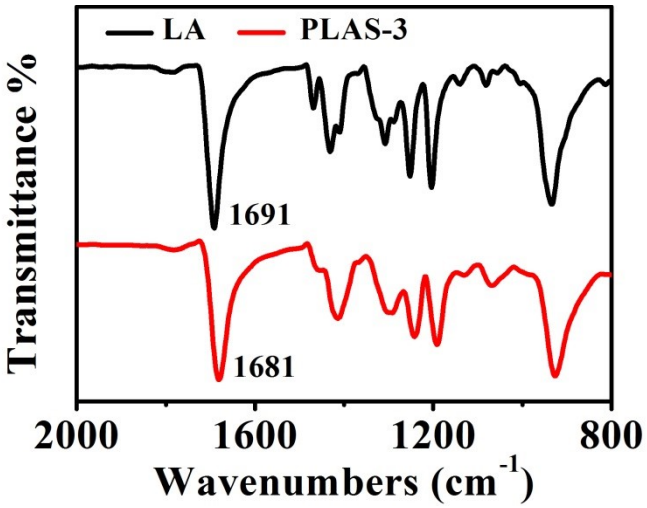

Fig. S4 FT-IR spectra of LA powder and PLAS-3 hydrogel.

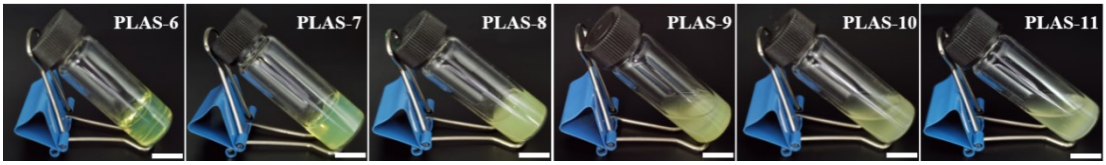

Fig. S5 Photographs of gelation behaviors of PLAS with different monomer concentrations.

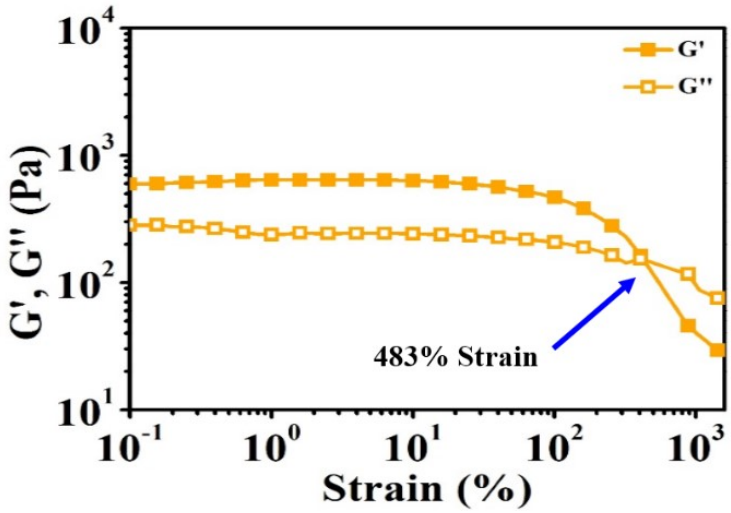

Fig. S6 Strain amplitude sweep test of PLAS-3 hydrogel with constant frequency of 10 rad/s and varied strain (0.1% to 2000%).

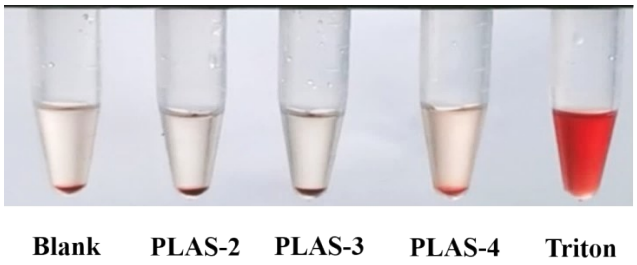

Fig. S7 Photographs of hemolytic activity of the PLAS hydrogels.

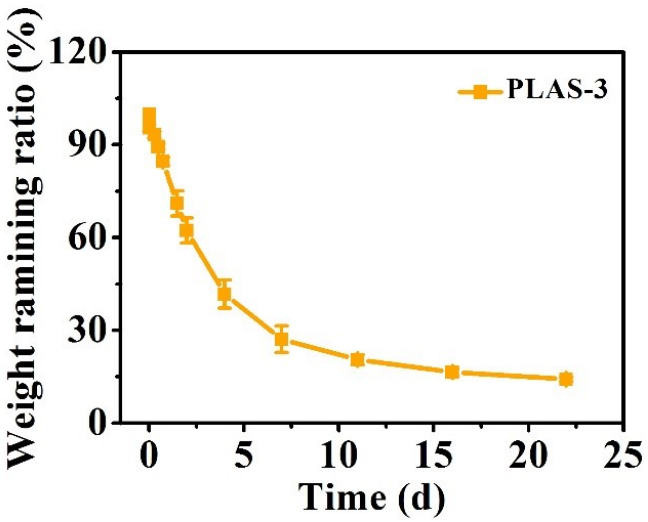

Fig. S8 In vitro weight loss of the PLAS-3 hydrogel.

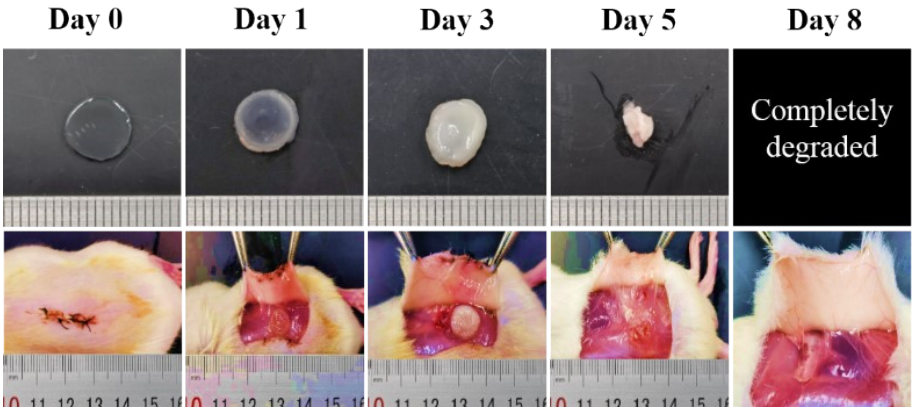

Fig. S9 In vivo degradation behaviors of PLAS-3 hydrogel.

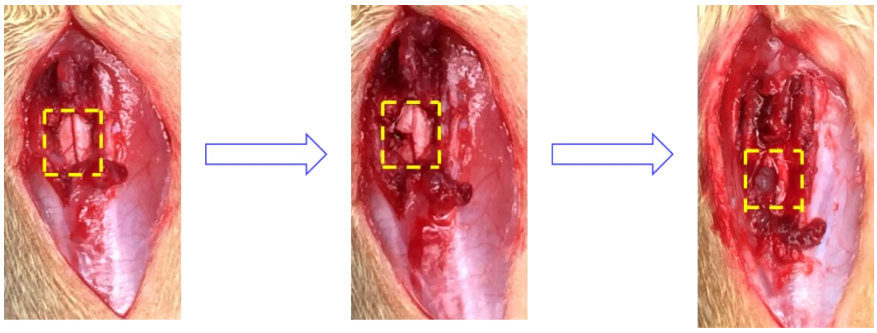

Fig. S10 Surgical procedure of spinal cord hemisection and implantation of the PLAS-3 hydrogel.

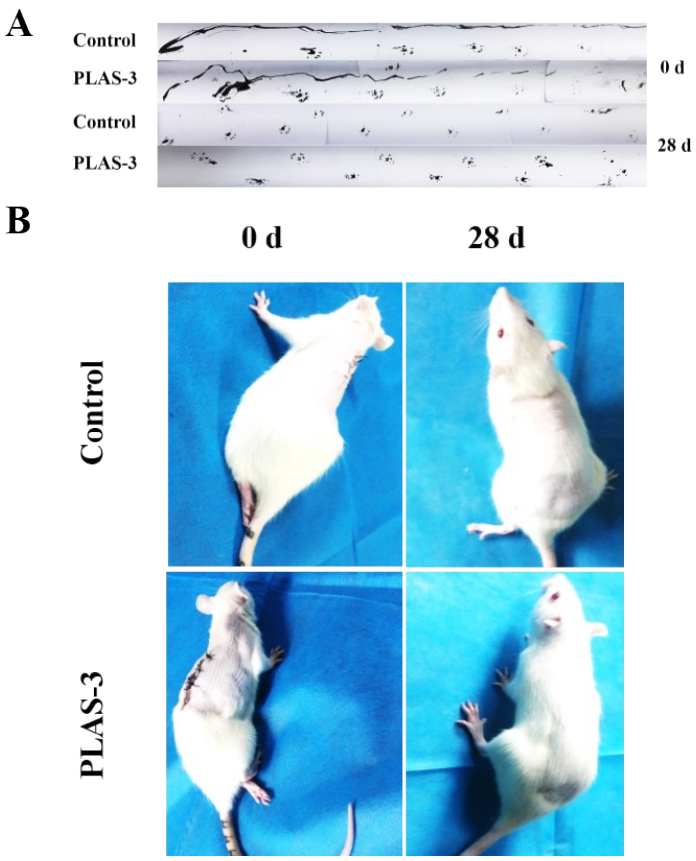

Fig. S11 A) Representative footprints of the SD rats in the control and PLAS-3 hydrogel treated groups. B) Typical records of walking gaits 28 days post-surgery.

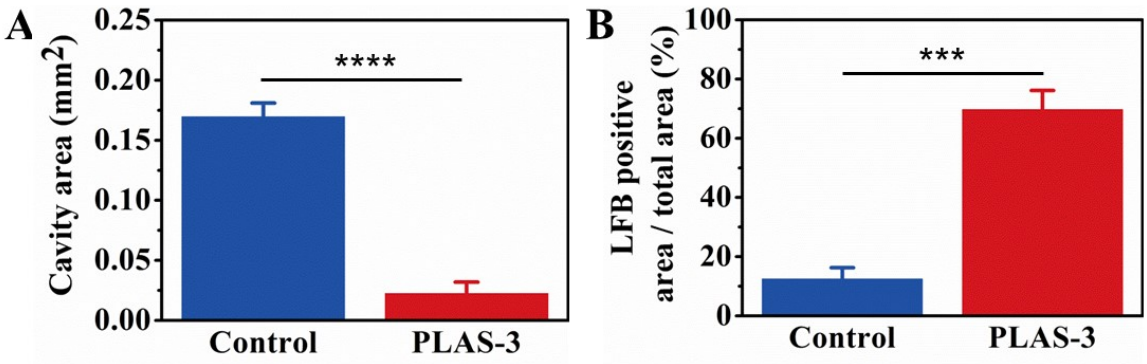

Fig. S12 A) Statistical analysis of cavity area based on H&E staining. **B)** Histological analyses of demyelinated area post-SCI showing LFB-positive area to total area ratio at the indicated lesion sites in different groups. Data are shown as mean ± SD (n = 3). ****$p < 0.0001$.

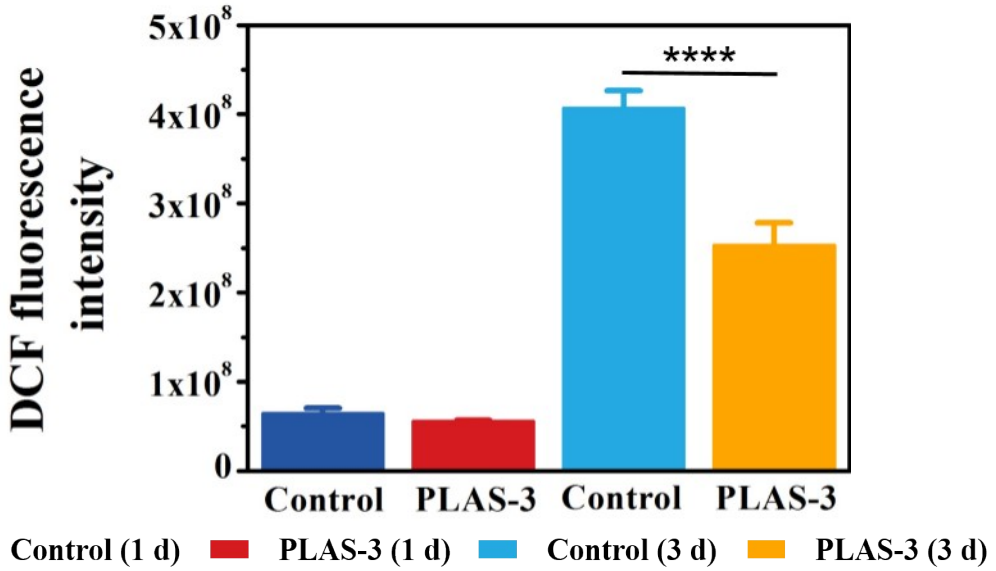

Fig. S13 DCF fluorescence intensity in different groups at 1 and 3 days. Data are shown as mean ± SD (n = 3). ****$p < 0.0001$.