Supporting Information

Mussel-inspired cryogels for promoting wound regeneration through photobiostimulation, modulating inflammatory response and suppressing bacterial invasion

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1. Experimental Section

1.1 Materials

Dopamine hydrochloride and chitosan powder (CS, deacetylation degree >90%; viscosity = 50~800 mpa.s) were purchased from Sigma-Aldrich (USA). Raw mulberry silk was purchased from commercial market. Ethanol, ammonium hydroxide ($NH_3 \cdot H_2O$), lithium bromide (LiBr), sodium carbonate (Na_2CO_3), hydrogen peroxide (H_2O_2, 30 wt.%), and glutaraldehyde were purchased from KESHI, Chengdu, China. Epidermal growth factor (EGF) was purchased from Shanghai Primegene Bio-Tech, China. Live/dead cell assay (ELISA) kits were purchased from KeyGEN BioTECH, China. The EGF assay kit was purchased from Wuhan ColorfulGene Biological Technology Co., Ltd, China. The ROS assay kit (DCFH-DA) was purchased from Beyotime (Haimen, China).

1.2 Preparation of CS and SF cryogels:

The pure CS cryogel was synthesized according to the following procedure. Firstly, chitosan (CS) powder was dissolved in an acetic acid solution (2 vol.%) to obtain a 3 wt.% CS solution under stirring. Then, 300 μl of a glutaraldehyde solution was added into the CS solution (10 ml) under ultrasonic agitation. After 5 min, the mixed solution was poured into a mold and then the mold was stored in a freezer at -20 °C for 24 hours. Finally, the mold with samples was freeze-dried to form the pure CS cryogels. The synthesis of the pure SF cryogel followed the above procedure by replacing CS solution with SF solution.

1.3 NIR assisted antibacterial activity

The antibacterial activity of the cryogels was tested by using Staphylococcus aureus (*S. aureus,* ATCC 25923) and Escherichia coli (*E. coli*, ATCC 25922) as the representing gram-positive bacteria and gram-negative bacteria. Five types of cryogels (with a diameter of 10 mm and thickness of about 1 mm), including PDA-NPs-CS/SF cryogels containing 0, 1.6, 3.2, 6.4, and 12.8 wt.% of PDA-NPs were used for the antibacterial tests. The cryogels were firstly sterilized in 75% alcohol and purified in sterilized PBS for one day to remove residue alcohol. A volume of 400 μl of bacterial suspension (10^5 CFU/ml) was added onto the surfaces of each sample in a 48-well culture plate. Then the cryogels were randomly divided into two groups, including the group with NIR irradiation and the group without NIR irradiation. For the irradiation groups, the cryogels were exposed to the NIR laser light (808 nm, 2 $W \cdot cm^{-2}$) for 15 min. After allowing all the groups incubated with bacteria for 12 h, 600 μl of in Luria-Bertani broth was added into each well to re-suspend the bacterial, and then the optical density of the bacterial resuspension were measured at 600 nm after incubation for another 12 h at 37 °C.

1.4 *Antioxidant efficiency of cryogels:*

(1) The antioxidant efficiency of cryogels was tested by measuring their capacity to scavenge the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to previous study.[29] Five types of cryogels, including PDA-NPs-CS/SF cryogels containing 0, 1.6, 3.2, 6.4, and 12.8 wt.% of PDA-NPs were used. Briefly, the cryogels were incubated with 200 μl 0.1 mM DPPH solution in 95% ethanol different time intervals. The adsorption of the products was

analyzed by an UV−vis spectrometer. The absorbance at 520 nm, the typical adsorption peak of DPPH was recorded as A_t. The absorbance of DPPH solution without cryogels was recorded as A_0. The free radical scavenging activity of the cryogels was calculated as follows after recating for 120 min:

$$Scavenging\ Ratio\ (\%) = A_t / (A_0 - At) \times 100\%$$

(2) Intracellular ROS scavenging: Raw 264.7 cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, USA) supplemented with 10% of fetal bovine serum (HyClone), and 1% of penicillin-streptomycin in 24-well plates at a density of about 5 × 10^5 cells per culture dish. Cells were cultured at 37 °C with 5% CO_2 for 4 h. After 4 h of incubation, cells were incubated in fresh medium with different concentrations of PDA (0.2, 0.5, 1 mg/ml) for another 12 h. After 12 h of incubation, the culture medium was replaced with medium containing DCFH-DA followed by incubation for 2 h at 37 °C in dark. Wells were then rinsed with medium and 100 μM H_2O_2 was added to each well for 20 min. Then each well was washed three times with PBS to remove uncreated DCFH-DA. Group without any treatment was defined as control. The fluorescence imaging of the cells in culture plates were observed under confocal laser microscopy (CLSM TCSSP5, Leica, Germany) with excitation wavelength at 488 nm. To quantify the fluorescent intensity, the cells were collected by centrifuge and flow cytometry was conducted with a Flow Cytometry (BD FACS Caliber, U.S.A.).

(3) In vivo antioxidant activity: To investigate the *in vivo* antioxidant activity of the cryogels at wound site, the full-thickness skin defects were created in a rat model. 6 male Sprague-Dawley (SD) rats weighing 250-300 g each were used. Two circular wounds with diameter of

10 mm were created on the upper back of each mouse by a biopsy punch. Then the CS/SF cryogels and PDA-NPs-CS/SF cryogels were randomly implanted into the wound sites. The blank wounds without any treatments were used as control groups. At 3 days of post-wounding the rats were sacrificed for immunofluorescence staining of CD68 and *in situ* detecting ROS generation by using an oxygen radical sensitive fluorescent probe dihydroethidium (DHE).

1.5 *In vitro* cell activity evaluation

In vitro cell culture was performed by seeding fibroblasts (NIH3T3, Stem Cell Bank, Chinese Academy of Sciences, SCSP-515) on the different cryogels. Five types of cryogels, including PDA-NPs-CS/SF cryogels containing 0, 1.6, 3.2, 6.4, and 12.8 wt.% of PDA-NPs were used to evaluate cell behaviors. Before cell culture, samples were sterilized in 75% alcohol and purified in sterilized PBS for one day to remove residue alcohol. Fibroblasts at passage 2-5 were seeded on the cryogels with density of 5×10^4 cells/sample. The cell-seeded cryogels were cultured in DMEM supplemented with 10% of fetal bovine serum and 1% of penicillin-streptomycin solution (HyClone) in a CO_2 incubator at 37 ºC. The culture medium was replaced twice a week. The proliferation of the cells seeded on the cryogels was evaluated by MTT assay after 3 and 5 days of culture. The morphology and spreading of cells seeded on the cryogels were examined by CLSM (TCSSP5, Germany) after the cells were stained by Calcein AM (A017, GeneCopoeia Inc. USA) on day 3. The morphology cells seeded on the cryogels were also observed by SEM after the cell seeded samples rinsed by PBS buffer, fixed with 2.5% glutaraldehyde for 4 h, washed with PBS buffer twice, dried, and coated by

gold sputtering. For NIR irradiation groups, the cells seeded on cryogels were examined after irradiation with an infrared lamp (0.02~0.03 $W \cdot cm^{-2}$, 10 min).

1.6 *In vivo* wound repairing experiments

The cryogels were implanted *in vivo* to repair full-thickness skin defect in a rat model. 8 male Sprague-Dawley (SD) rats weighing 250-300 g each were used. Briefly, the rats were firstly anesthetized and then hair on the dorsal skin was shaved. Then four circular wounds with diameter of 10 mm were created on the upper back of each mouse by a biopsy punch. The CS/SF cryogel, PDA-NPs-CS/SF cryogels (1.6 wt.% PDA-NPs), and EGF loaded PDA-NPs-CS/SF cryogels were implanted into the wound defects (Φ 10 × 0.5 mm). The blank wounds without any treatment were used as control groups. The implantation procedure is shown in Figure S9. After surgery, the rats were randomly divided into two groups with four rats in each group, including irradiation groups for photothermal therapy and non-irradiation groups for comparison. The rats in irradiation groups were irradiated by an infrared lamp (0.2~0.3 $W \cdot cm^{-2}$) for 30 min each day. The wound area of the rats was recorded at 7, 14, and 21 days. At day 21, the rats were euthanized by cervical dislocation, and the skin was collected and fixed in 10% formalin. Then the skin surface tissues were cut into 4-μm thick vertical slices for cross-sectional observation. Histological observations were performed after hematoxylin-eosin (H&E) staining. The experiments were performed in accordance with protocols approved by the local ethical committee and laboratory animal administration rules of China.

1.7 Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey multiple-comparison post hoc test to determine any statistical significance of observed differences between the test groups. The level of statistical significance was set at $p < 0.05$.

Table S1 The composition of various cryogels.

Cryogels	CS (g)	SF (g)	PDA-NPs/(CS+SF) (wt.%)	glutaraldehyde/(CS+SF) (wt.%)
CS	3	0	0	0.95
SF	0	3	0	0.95
CS/SF	2	1	0	0.95
	1	2	0	0.95
PDA-NPs-CS/SF	2	1	1.6	0.95
	2	1	3.2	0.95
	2	1	6.4	0.95
	2	1	12.8	0.95

2. Results

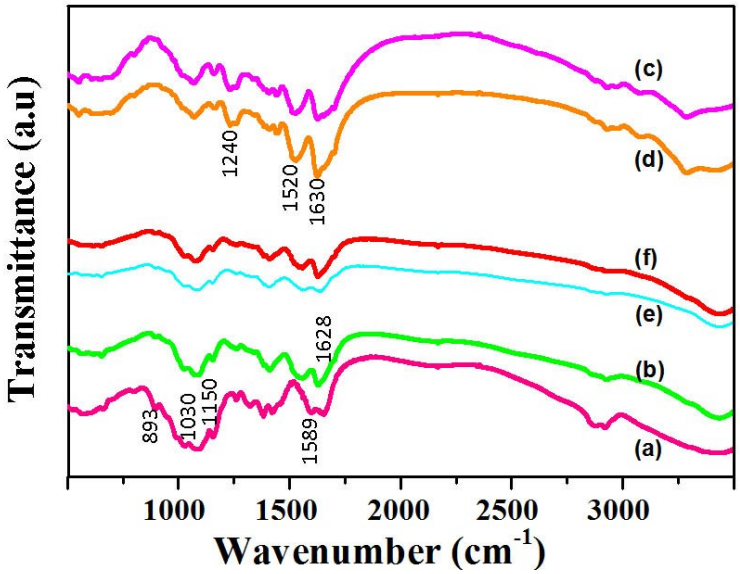

Figure S1. The FTIR spectra of (a) pure CS, (b) pure CS cryogel crosslinked by glutaraldehyde, (c) pure silk fibroin, (d) pure SF cryogel crosslinked by glutaraldehyde, (e) CS/SF cryogels, and (f) PDA-NPs-CS/SF cryogel.

The chemical structure of the cryogel was characterized by using Fourier transform-infrared spectroscopy (FTIR; Nicolet 5700, Thermo, USA). As shown in Figure S1, the spectrum of the pure chitosan (a) displayed peaks of assigned saccharine structure at 1150 cm^{-1}, 1030 cm^{-1}, and 893 cm^{-1}. It also showed the distinctive peaks of NH_2 at 1589 cm^{-1}. For the pure CS cryogel crosslinked by glutaraldehyde (b), a new peak at 1628 cm^{-1} appeared that belonged to imine binding, which confirmed the crosslinking of glutaraldehyde. The spectrum of pure silk fibroins (c) exhibited the characteristic bands of protein, which included the amide I band at 1630 cm^{-1}, amide II band at 1520 cm^{-1} and amide III band at 1240 cm^{-1}. The peaks of the pure SF cryogel crosslinked by glutaraldehyde (d) were similar to that of pure silk fibroin (c). The absorption peaks of the saccharine and protein appeared in the spectrum of CS/SF cryogels (e). The peaks of amide I band of CS/SF cryogels were similar to these of pure silk fibroin in peak position and shape. The amide II band of CS/SF cryogels exhibited a border band between 1520 and 1550 cm^{-1}, while that of pure silk fibroin only had one peak at 1520 cm^{-1}, and the intensity of amide III band of the CS/SF cryogels decreased. The incorporation of PDA-NPs showed negligible influence on the spectrum (f).

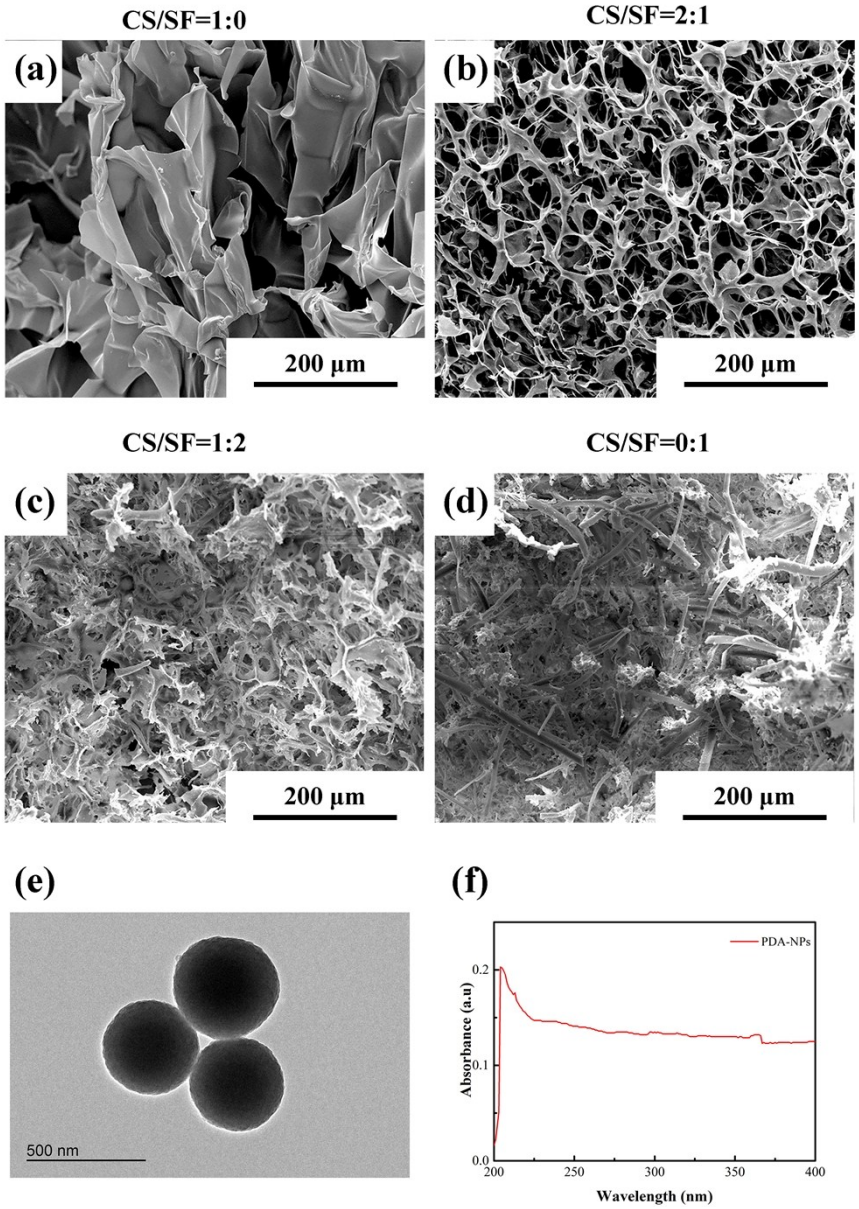

Figure S2. The SEM images of (a) pure CS cryogels, (b) SF cryogels, (c-d) CS/SF crogles with different CS/SF mass ratios. (e) TEM image and (f) UV-visible spectroscopy of PDA-NPs.

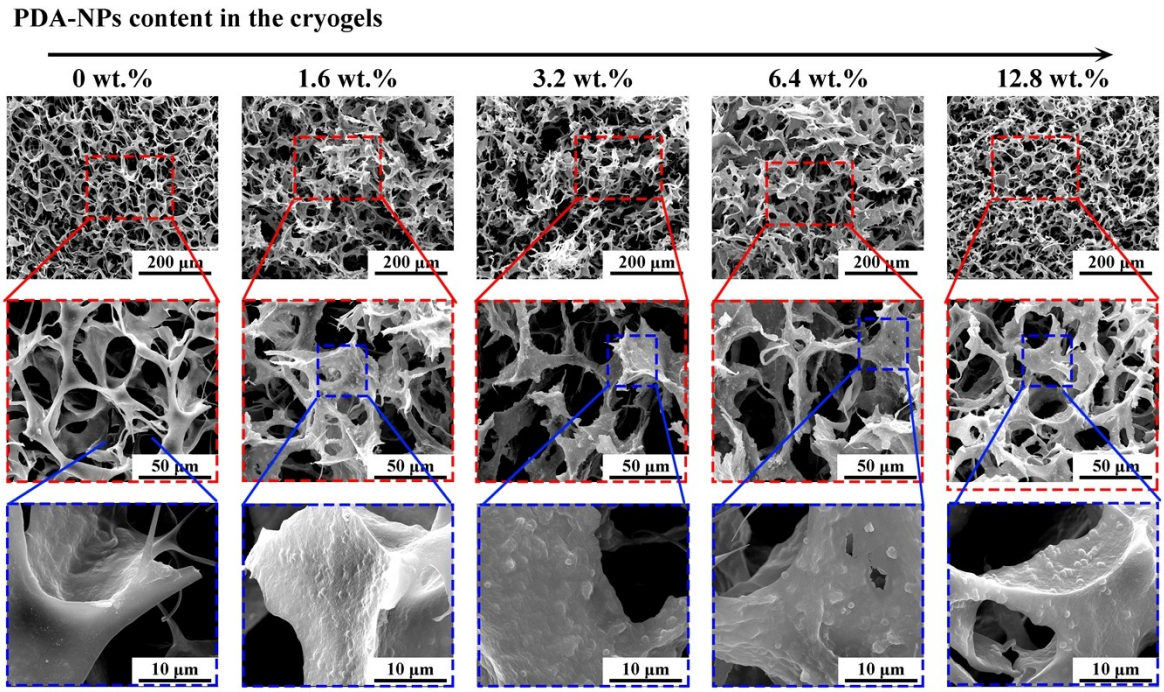

Figure S3. The SEM images of PDA-NPs-CS/SF cryogels with different contents of PDA-NPs.

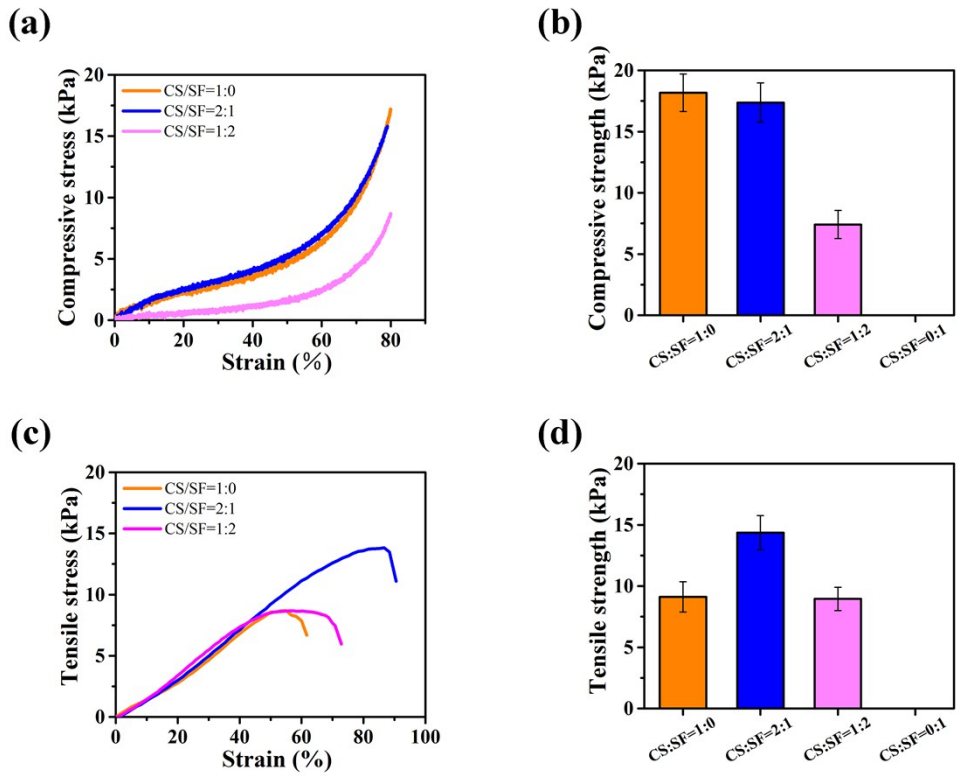

Figure S4. The mechanical properties of CS/SF cryogels with different CS/SF mass ratios. (a) Typical compressive stress-strain curves of the CS/SF cryogels. (b) Compressive strength of the CS/SF cryogels. (b) Typical tensile stress-strain curves of the CS/SF cryogels. (d) Tensile strength of the CS/SF cryogels.

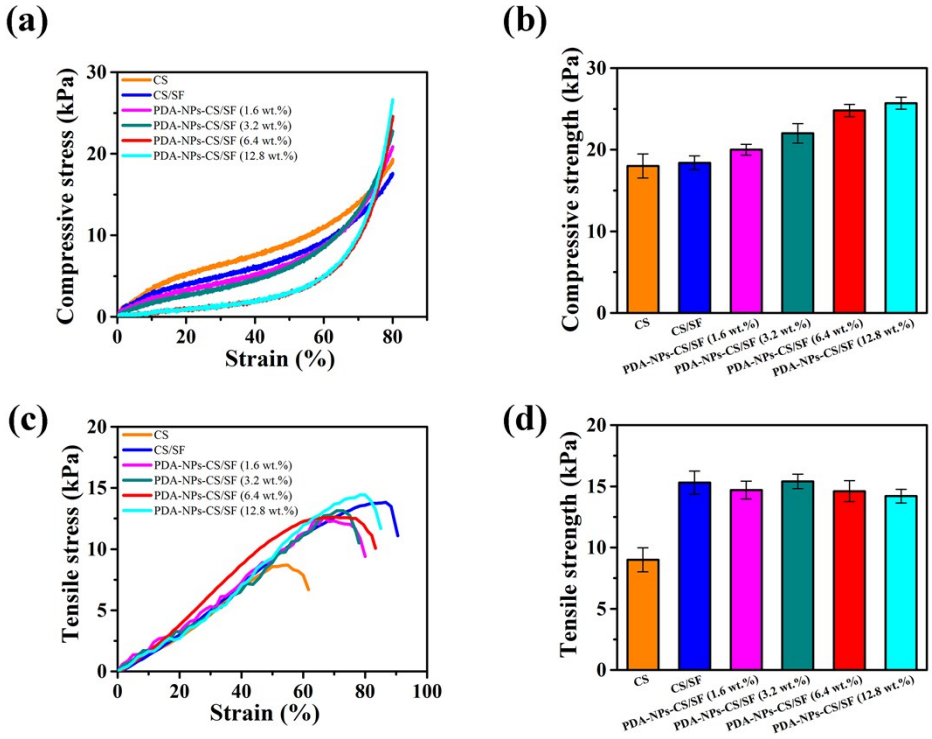

Figure S5. The mechanical properties of PDA-NPs-CS/SF cryogels with different contents of PDA-NPs. (a) Typical compressive stress-strain curves of the cryogels. (b) compressive strength of the cryogels. (b) Typical tensile stress-strain curves of the cryogels. (d) Tensile strength of the cryogels.

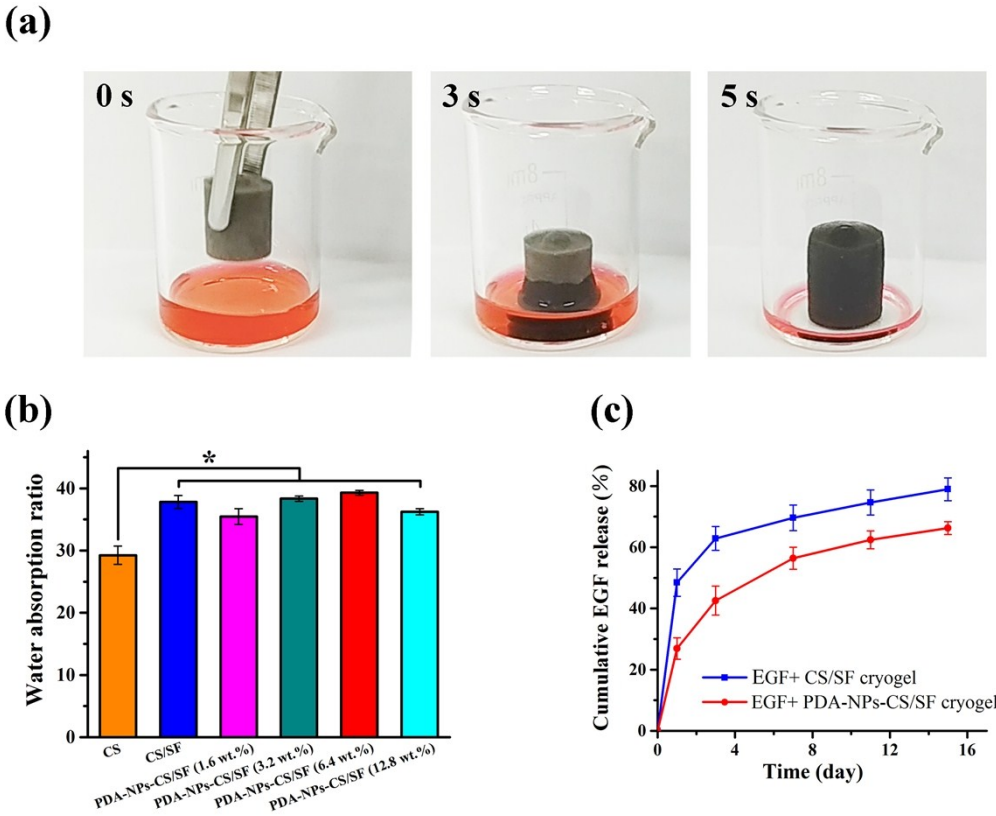

Figure S6. (a) The cryogels ((6.4 wt.% PDA-NPs; CS/SF=2:1; 30 mg) fully absorbed a bottle of red ink (1.2 g). (b) Water adsorption ability of CS/SF cryogel and PDA-NPs-CS/SF cryogel. (c) EGF release profiles from the CS/SF cryogel (CS/SF=2:1) and PDA-NPs-CS/SF cryogel (6.4 wt.% PDA-NPs, CS/SF=2:1). (*) denotes significant di□erences ($p < 0.05$). Error bars represent the standard deviation from the mean of three samples.

Table S2 EGF release kinetics parameters obtained from the Peppas model.

sample	k	n	R^2
PDA-NPs-CS/SF cryogels	29.08	0.31	98.88

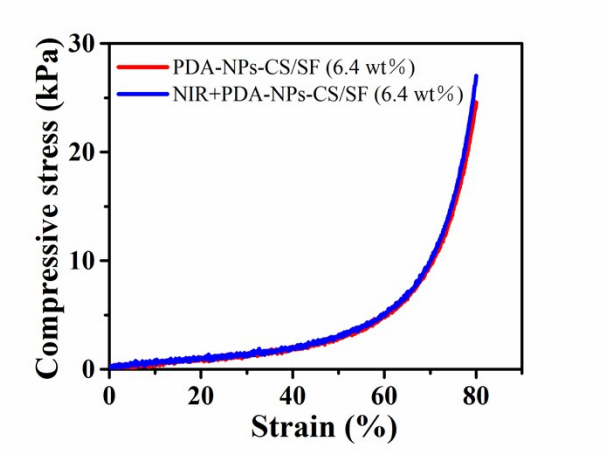

Figure S7. The compressive behavior of the PDA-NPs-CS/SF cryogels before and after 5 cycles of NIR irradiation, which indicated the thermal stability of the cryogels.

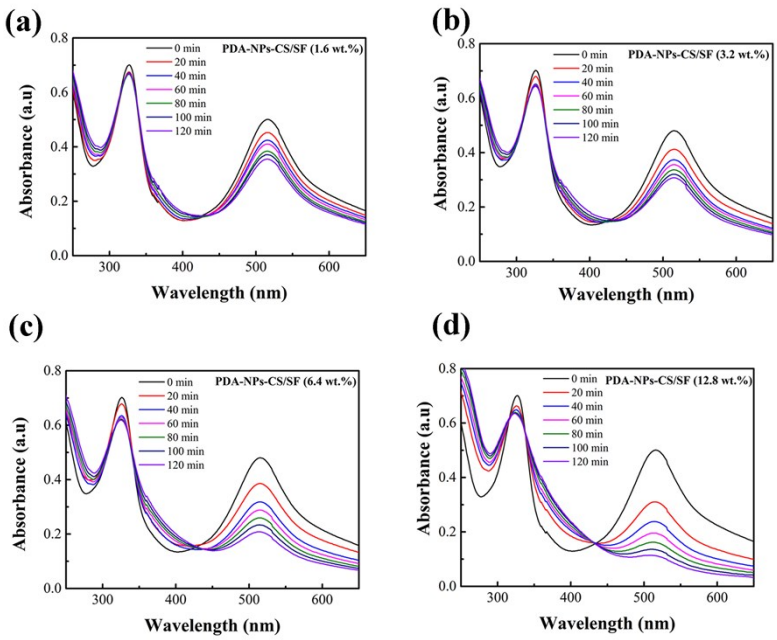

Figure S8. Time-dependent UV−vis spectra of DPPH after reacting with PDA-NPs-CS/SF cryogels incorporating with different contents of PDA-NPs.

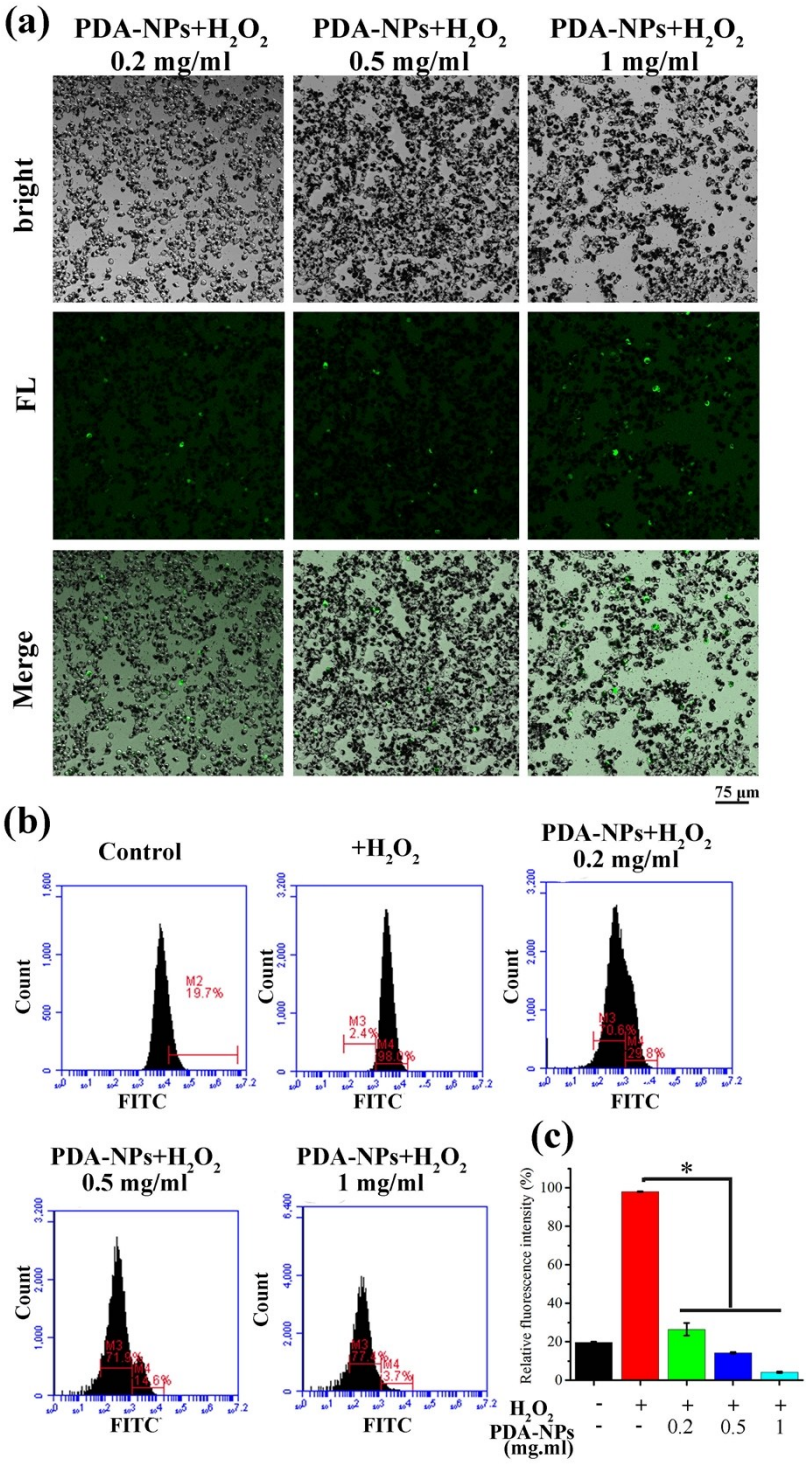

Figure S9. In vitro ROS scavenging ability of the PDA-NPs. (a) Fluorescence images of H_2O_2 -treated Raw 264.7 macrophages with or without PDA-NPs treatment using DCFH-DA as the ROS probe. (b) Intracellular ROS scavenging by PDA-NPs in H_2O_2-treated Raw 264.7 cells as detected with a flow cytometer. (c) Mean fluorescence intensity calculated based on the flow cytometer data in (b). (*) denotes significant difference ($p < 0.05$). Error bars represent the standard deviation from the mean of three samples.

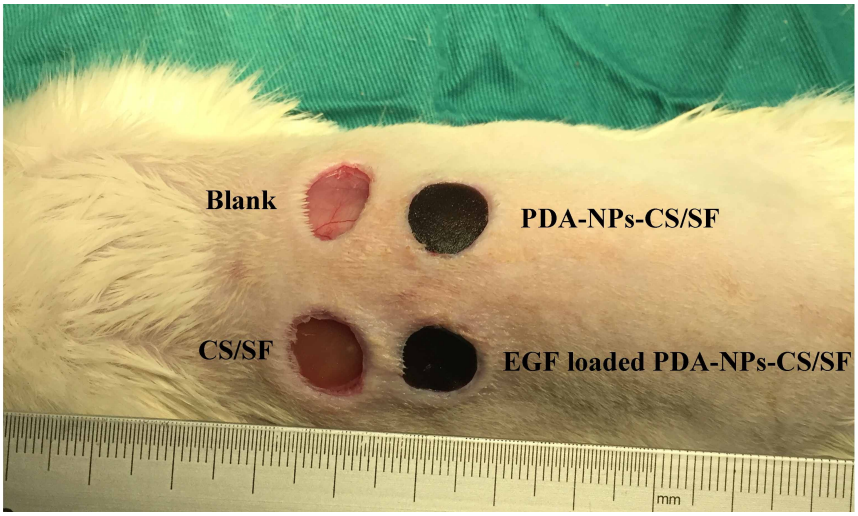

Figure S10. The various cryogel were implanted *in vivo* to repair full-thickness skin defect in a rat model so as to evaluate the chemo-photothermal effect of the cryogels on wound healing.

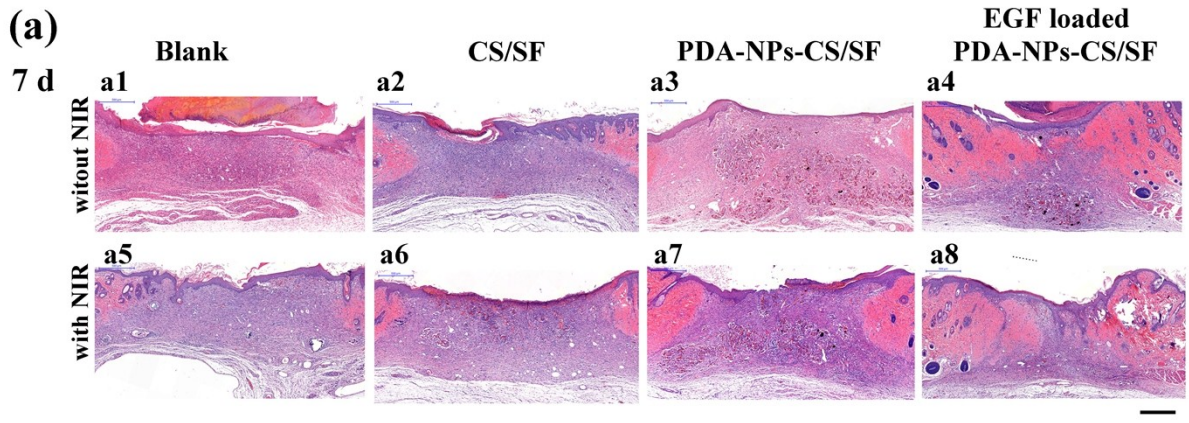

Figure S11. Histological analysis for skin healing. H&E staining of wounds after 7 days of post-healing treated with CS/SF, PDA-NPs-CS/SF (6.4 wt.% PDA-NPs), and EGF-loaded PDA-NPs-CS/SF cryogels (6.4 wt.% PDA-NPs); Blank defect without treatment was used as control. The black arrows show the location of granulation tissue. Scale bar is 500 μm. C: cryogel, G: granulation tissue.

Supplementary video S1

The video shows that the “heart” like cryogel was pre-folded, and then recovered to its original shape immediately after immersion in water.

Supplementary video S2

The video shows that the cylinder cryogel can withstand 85% of compressive strain with the water squeezed out and quickly recover to its initial shape by re-absorbing water after the stress was released.