Supporting Information

Facile fabrication of chitin/ZnO composite hydrogels for infected wound healing

Hongli Zhang[a,1], Xu Zhang[b,1], Qi Cao[a], Shuangquan Wu[c], Xiao-Qiang Wang[a], Na Peng[a], Danlin Zeng[a], Jinfeng Liao[b,*], Huan Xu[a,*]

[a] Key Laboratory of Coal Conversion and New Carbon Materials of Hubei Province School of Chemistry and Chemical Engineering, Wuhan University of Science and Technology, Wuhan 430081, China

[b] State Key Laboratory of Oral Diseases, National Clinical Research Centre for Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, China

[c] Zhongnan Hospital of Wuhan University, Institute of Hepatobiliary Diseases of Wuhan University, Transplant Center of Wuhan University, Hubei Key Laboratory of Medical Technology on Transplantation, Wuhan 430071, China

[1] These authors contributed equally to this work

Experimental Section

Materials

The raw chitin powder from shrimp shell was purchased from Golden-Shell Biochemical Co. Ltd. (Zhejiang, China). The raw chitin was purified with 1 M aqueous NaOH at ambient temperature overnight and combined with 3% (w/w) aqueous H_2O_2 at 80 °C for 1.5 h. Washing with deionized water was performed after each step to remove any residual proteins and chemical regents. The purified chitin was finally stoving, and kept in a desiccator until use. ZnO, KOH, urea, and other reagents were of analytical grade and purchased from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China), and used without further purification. Agar, yeast extract, and tryptone were purchased from Biofroxx, Germany. Chloral hydrate was obtained from Beijing bailingwei Technology Co., Ltd. And paraformaldehyde was acquired from Lanjieke Technology Co., Ltd. SD rats (male, 6-8 weeks old, body weight = 160 -180 g) were purchased from Chengdu Dashuo Animal Experiment Co. Ltd. The animal investigations were authorized by the animal committee of Sichuan University.

Fabrication of the chitin hydrogels (CG) and chitin/ZnO hydrogels (CZG)

Chitin (5 wt%) was dispersed in aqueous 20 wt% KOH and 4 wt% urea solution with different ZnO mass fraction of 0 wt%, 0.5 wt%, 1 wt%, and 1.5 wt%. The mixtures were stirred at -30 °C for about 1 h to obtain the solutions. Then 1.8 g of epichlorohydrin (ECH) was added to the chitin solutions, and the mixture was stirred at -10 °C for 30 min. The blended chitin/ZnO solutions were subjected to centrifugation at 1500 rpm for 10min at -10°C to remove the bubbles. The chitin pre-gel solution was poured into mold and kept at 4 °C for 30 h to form a "raw" chemical hydrogel. Finally, the "raw" chemical hydrogel was purified by immersion into 20% (w/w) ethanol solution for 3 days to remove the residual alkali and urea. Thus, the chitin hydrogel and a series of chitin/ZnO composite hydrogels were fabricated, labeled as CG, CZG-0.5, CZG-1 and CZG-1.5.

Characterization

Before measurements, all the samples were freeze-dried in a conventional freezer dryer (Scan Vac Coolsafe55-4, Denmark), and then were cut into powders. Fourier-transform infrared spectroscopy was obtained using a FTIR spectrometer (VERTEX70 FTIR Spectrometer, Bruker, Germany) in the region of 4000–400 cm^{-1}, the test specimens were prepared by the KBr-disk method. X-ray diffraction (XRD) in reflection mode was performed on a diffractometer (ZSX Primus IV, Neo-Confucianism, Japan). The XRD patterns with Cu$K\alpha$ radiation (λ = 0.15406 nm) at 40 kV and 30 mA were recorded in the region of 2θ from 5° to 80° at a scanning speed of 3°/min. X-ray photoelectron spectra (XPS) were recorded using the machine (AXIS SUPRA+, Kratos, Japan). The binding energy was corrected to 284.6 eV for C 1s. Thermogravimetric analysis (TGA) was carried out using a TA Q500 instrument (TA Instruments, USA), the sample was placed in a platinum pan and heated from 80 °C to 800 °C at a rate of 10 °C/min under an air atmosphere. The compressive strength and cyclic compressive properties of the wet hydrogels were determined on a universal testing machine (Model 6503 UTM Instrument, China) equipped with a 500 N load cell. The compressive test was performed using a crosshead speed of 5 mm/min, and the modulus was calculated from the initial linear region of the stress–strain curves. Cyclic compressive tests were performed at a compressive strain of 60% for five cycles. Scanning electron micrograph (SEM) images of hydrogels were obtained on a BAOER microscope (Q Series, USA) using the back scattered electron imaging (BSE) mode and an accelerating voltage at 5 kV. The wet hydrogels were frozen in liquid nitrogen, immediately snapped, and freeze-dried, then the surface and cross-section of the samples were sputtered with gold for SEM measurements.

The water content of the samples was tested by the gravimetric method. After weighted (W_b), the wet hydrogels were freeze-dried and weighted as W_a. Each sample was tested three times. The water content was determined using the following equation:

$$\text{Water content (\%)} = (W_b - W_a) / W_b \times 100$$

Antibacterial property of the CG and CZG samples

The antibacterial activity of the CG and CZG, against the Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) and Gram-negative bacterium *Escherichia coli* (*E. coli*), was examined using the disc diffusion method, colony-forming count method, antibacterial kinetic curve and SEM observation for qualitative and quantitative analyses, respectively.

For disc diffusion method, single colonies of *S. aureus* and *E. coli* were cultured separately in 20 mL LB liquid culture medium in an incubator at 37 °C. The log-phase bacteria were diluted 3 times with PBS, followed by uniformly spreading 100 μL of the bacterial suspension on an agar plate. Wafer type CG/CZG (circular disks 16 mm in diameter) was placed on the agar plates and cultured at 37 °C for 16 h, then the inhibition zone of the hydrogels was recorded.

For colony-forming count method, after *S. aureus* or *E. coli* were cultured on LB agar plates at 37 °C for 24 h, a single colony was selected, inoculated into LB broth, and incubated at 37 °C under shaking at 250 rpm overnight to reach the mid-log phase. Then, *S. aureus* and *E. coli* were diluted to 1.0×10^5 and 1.0×10^4 CFU/mL in fresh broth, respectively. After a cylindrical hydrogel with a diameter of 16 mm and a height of 2 mm was added to a 24-well plate, 180 μL of sterile water and 20 μL of *S. aureus* or *E. coli* suspension were added, and the mixture was incubated at 37 °C under shaking at 150 rpm for 14 h. Next, 100 μL of the mixture was diluted 7 times, then 100 μL of the diluent mixture was spread onto solid agar plates. The plates were incubated at 37 °C for 24 h, and the number of viable cells was counted. *S. aureus* or *E. coli* suspensions with CG hydrogels were used as control.

For SEM observation, *E. coli* and *S. aureus* were treated with CG/CZG at 37 °C for 24 h, untreated and treated bacteria were centrifuged at 8000 rpm for 1 min, washed three times with phosphate-buffered saline (PBS) and fixed with 2.5% glutaraldehyde for 5 h, dehydrated with a graded alcohol series (10, 20, 30, 50, 70, 60, 90, and 100%), and then freeze-dried. Lyophilized powders were sputtered with gold for SEM measurements.

Biocompatibility test

Cytocompatibility studies were assessed using CCK-8 assay and carried out using embryonic mouse fibroblast cell line (NIH-3T3 cells). The cytotoxicity test started with liquid extracts of hydrogels. Each hydrogel with diameter of 12 mm and height of 1 mm was immersed in 1 mL cell growth media at 37 °C for 72 h, the cell growth media (negative) were treated with the same condition. 200 μL NIH-3T3 was seeded in 96-well plates with 4×10^3 cells per well and cultured to adhere at 37 °C for 24 h in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin. 60 μL culture media was replaced with 60 μL treated cell growth media (negative) or 60 μL liquid extracts of hydrogels (experiment) labelled as 30% (v/v) extract containing complete growth media. Next, they were incubated for an additional 24 and 72 h to expose the cells to the extract. To evaluate the viability, the media was replaced by 200 μL of 10% (Cell Counting Kit-8, Dojindo Laboratories, Japan) (CCK-8) solution and further incubated for 4 h at 37 °C. 150 μL supernatant liquid incubated media were transferred to fresh 96-well plates for colorimetric assessment using a microplate reader at 450 nm. Cytocompatibility study of CZG-1 to human umbilical vein endothelial cells (HUVEC) for 24 h was conducted as the above procedure. Cell viability was calculated using the flowing formula:

$$\text{Cell viability (\%)} = (A_{\text{test}} - A_0) / (A_{\text{control}} - A_0) \times 100$$

Where the A_{test} and the A_{control} were the absorbance of the experimental groups and the control group, A_0 was the absorbance of CCK-8 in culture medium without cells. The data of sextuplicate samples are expressed as mean ± the standard deviation.

Additionally, the cell biocompatibility was evaluated by the Calcein-AM/ PI staining. At first, 1 mL fresh NIH-3T3 cells suspension with 2.5×10^4 cells/mL were seeded in 24-well plates, then 300 μL of the liquid extracts treated with sterilized hydrogels were placed in the 24-well plates and cultured with NIH-3T3 cells at 37 °C for 24 and 72 h, with the culture medium itself without samples as a control. After washing by PBS, 200 μL Calcein-AM

(green)/ PI (red) solution was added and followed by incubation at 37 ℃ for 20 min. Then they were washed with PBS to remove unstained fluorescence dye and immediately observed by a fluorescence microscope at the corresponding excitation wavelength (490 ± 10 nm).

For hemolysis assay, CG, CZG-0.5, CZG-1, and CZG-1.5 with diameter of 12 mm and height of 1 mm were incubated with red blood cells (RBCs) from rabbits and the hemolysis ratio was calculated. The prewashed (5% v/v in PBS) RBCs (0.1 mL) was incubated with CG, CZG-0.5, CZG-1, and CZG-1.5 at 37 °C for 4 h. Then 150 μL of the supernatant was transferred to a 96-well plate, and the absorbance was measured at 545 nm. PBS was used as negative control group and 0.1% Triton x-100 as positive control, respectively. The hemolysis ratio was calculated as follows:

$$\text{Hemolysis ratio (\%)} = (A_{test} - A_0) / (A_{100} - A_0) \times 100$$

Where A_{test}, A_0 and A_{100} represent the absorbance of the experimental groups, negative and positive controls, respectively.

Evaluation of CZG-1 hydrogels in infected wound healing

All animal procedural methods in this experiment were in accordance with the protocols and regulations of Sichuan University. Surgical operation was applied in the left and right backsides of rats to build a 10 mm × 10 mm round wound. *S. aureus* suspension (50 μL) with 3×10^8 CFU/mL was added to each wound area. After 24 h of bacteria inoculation, the rats were randomly divided into the following four groups: control group, mupirocin-treated group, CG hydrogels-treated group, and CZG-1 hydrogels-treated group.

To demonstrate the ability of CZG-1 in antibacterial therapy *in vivo,* the wounds were treated with CZG-1 hydrogels for 3 days. At this time point, the wound skin was thoroughly disinfected with iodophor, and the full-thickness skin was completely removed and immersed in PBS for 4 h. Subsequently, the surface of samples was washed to obtained bacterial suspensions. Bacterial suspensions were spread on LB agar plates after gradient dilution. After incubation for 24 h at 37 °C, the number of bacteria in each group was counted. On the 3rd, 7th, and 14th day of

accepting antibacterial therapy and wound healing, a digital camera was employed to record the wound images and further calculate the wound closure rate. Image J was employed to measure the wound closure rate after magnification four times to obtain precise wound margins. The irregular pattern drawing of the wound was followed by measurements. During calibration and drawing, the images were enlarged x4 to obtain accurate edges. The relative wound closure rate was deduced from the drawing area.

For histological evaluation, on the 7th and 14th day, rats were sacrificed to collect full-thickness skin samples (n = 3). These samples were paraffin-embedded and sectioned after fixation in paraformaldehyde for 3 days. Besides, hematoxylin and eosin (H&E) and Masson staining were performed to histologically evaluate wound healing. And the average thickness of neo-epithelium and neo-granulation tissue was semi-quantitatively analyzed using the software Image pro plus.

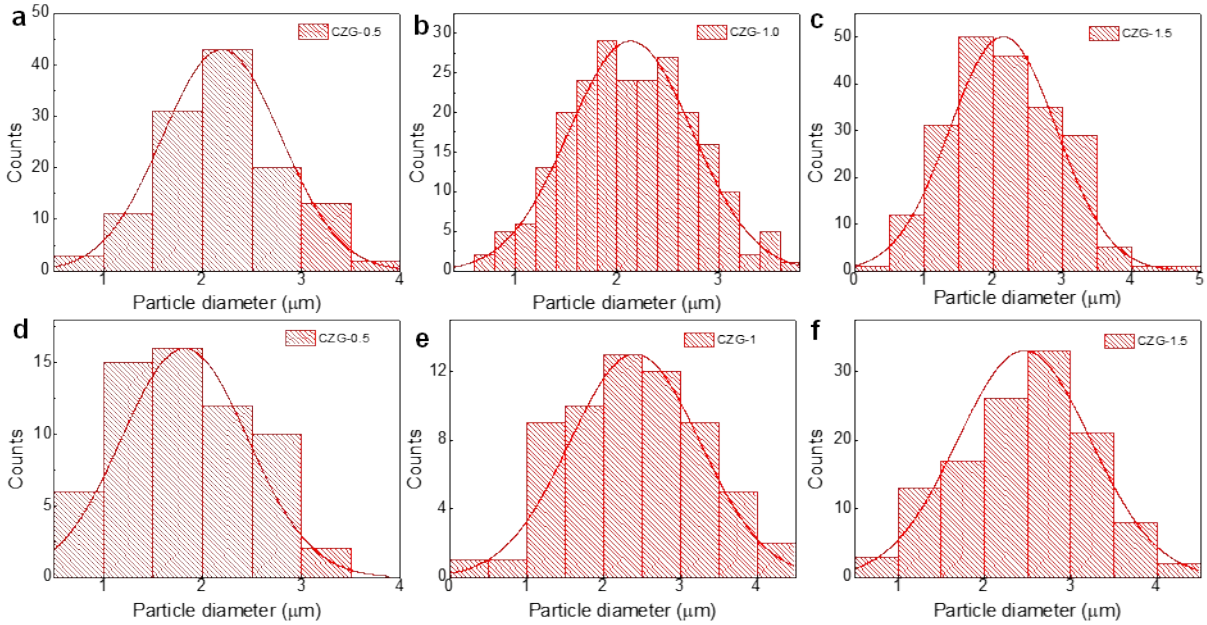

Fig. S1 Size distribution of the ZnO particles on the surface (a-c) and cross-section (d-f) of CZG-0.5 (a, d), CZG-1 (b, e), and CZG-1.5 (c, f)

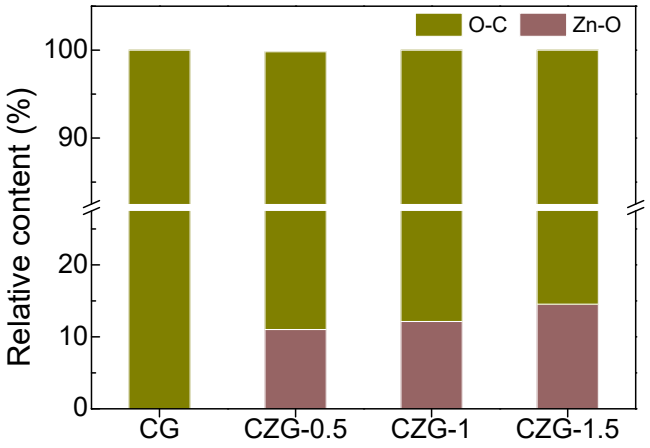

Fig. S2 Relative Zn-O content in CG, CZG-0.5, CZG-1, and CZG-1.5 calculated according to high-resolution XPS spectra of O 1s.

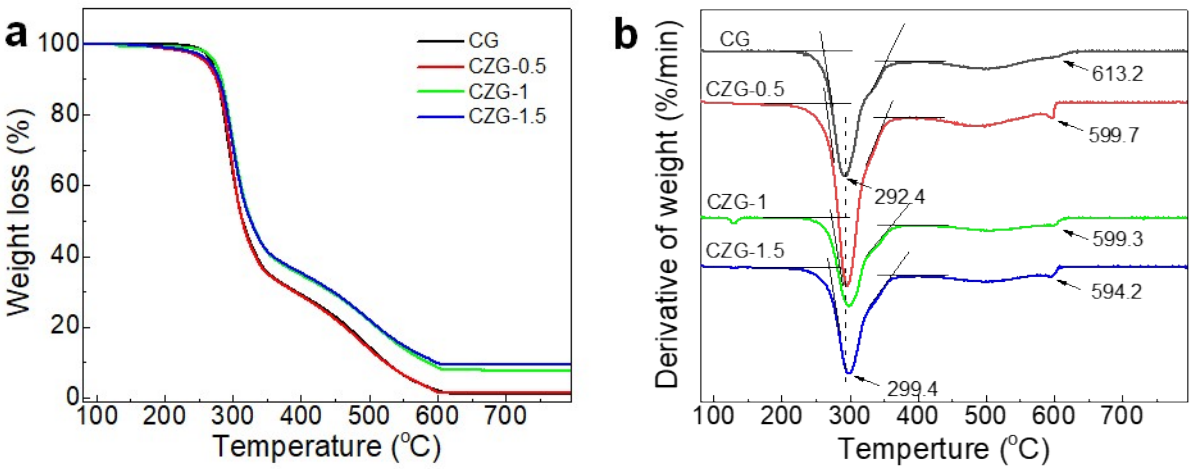

Fig. S3 Thermal properties. TGA (a) and DTG curves (b) of CG, CZG-0.5, CZG-1, and CZG-1.5

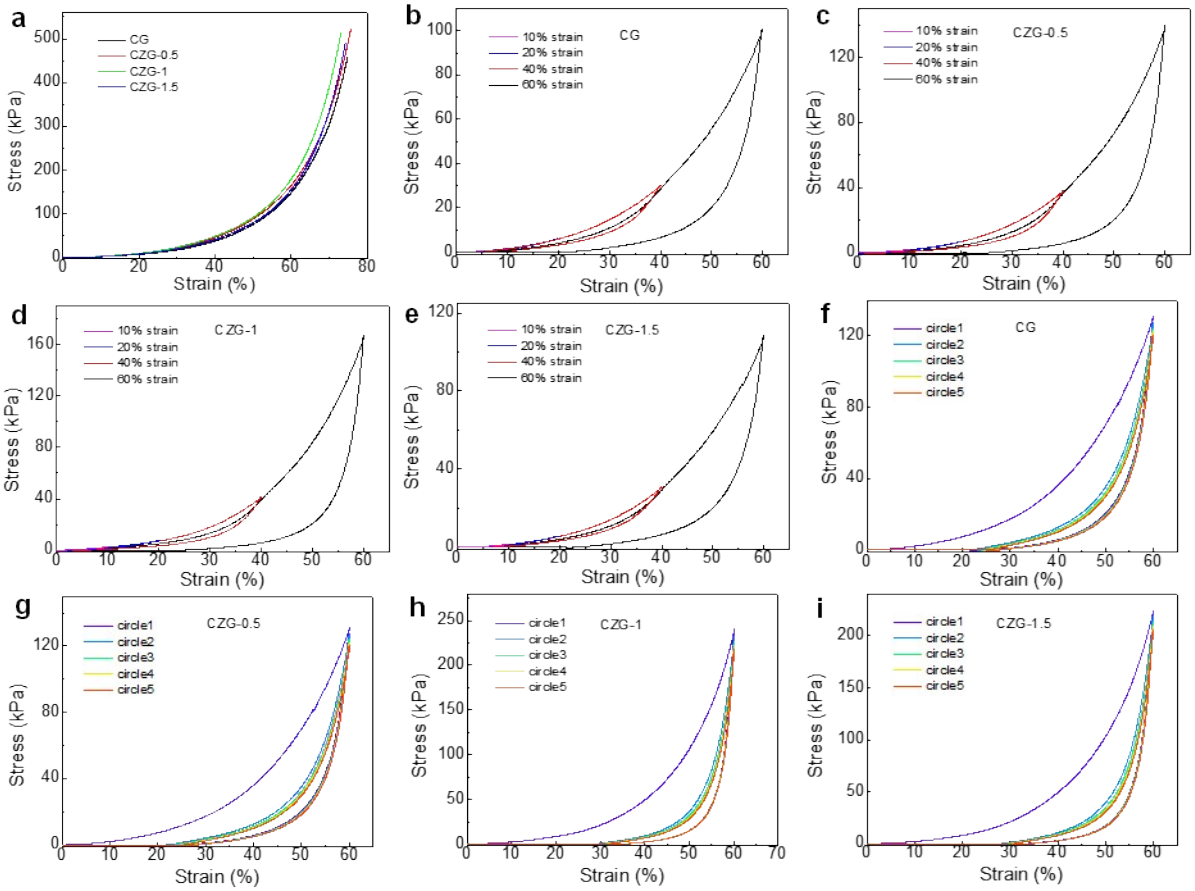

Fig. S4 Mechanical properties. (a) The compressive stress–strain curves of the CG, CZG-0.5, CZG-1, and CZG-1.5 hydrogels. (b-e) Compressive stress-strain curves of the CG (b), CZG-0.5 (c), CZG-1 (d), and CZG-1.5 hydrogels (e) at strains from 10% to 60%. (f-i) Fatigue resistance of the CG (f), CZG-0.5 (g), CZG-1 (h), and CZG-1.5 hydrogels (i) at 60% strain.

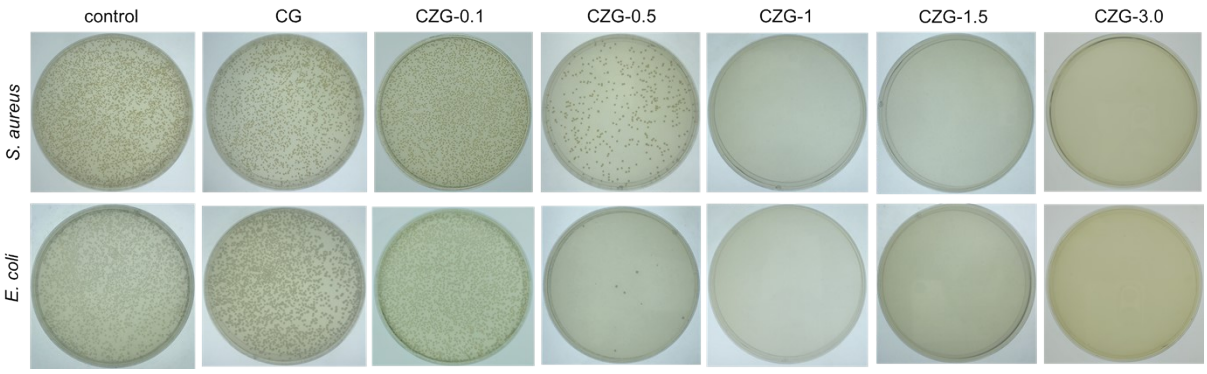

Fig. S5 The images of *S. aureus* and *E. coli* colonies on the agar plates.

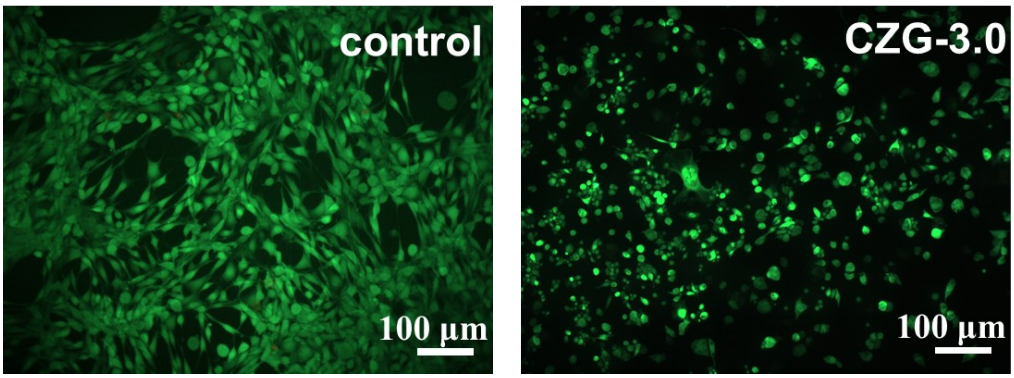

Fig. S6 Live/dead cell viability assay of NIH-3T3 cells in the control (cell growth media) and CZG-3.0 hydrogel extracting medium for 72 h.

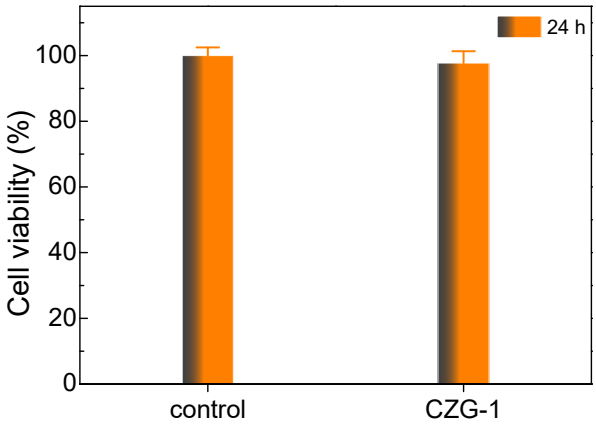

Fig. S7 Cell viability of HUVEC cells after co-incubated with CZG-1 hydrogel extracting mediums for 24 h.