Supporting Information

Near-infrared light-responsive multifunctional nanocomposite hydrogel for efficient and synergistic antibacterial wound therapy and healing promotion

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A: Experimental section

Reagents

PEG (MW 2000, Energy Chemical Reagent, 96%), chitosan (CS, China National Medicines Co., Ltd., degree of deacetylation: 85%, Mw: 6×10^5), sodium cyanoborohydride (Macklin Chemistry Co., Ltd., 95%). 4-formylbenzoic acid (98%), dodecyl aldehyde (95%), *N,N'*-dicyclohexylcarbodiimide (DCC, 99%), ciprofloxacin hydrochloride (CPFX, 99%), oleic acid (85%), oleylamine (80-90%), 4-(dimethylamino) pyridine (DMAP, 99%), tungsten hexachloride (WCl_6, 99.9%), carbon disulfide (anhydrous, 99%), 1,1-diphenyl-2-picrylhydrazyl (DPPH, 99%) and hexamethyldisilazane (HMDS, 98%) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Fetal bovine serum (FBS), RPMI 1640 medium and trypsin-EDTA were purchased from Gibco Life Technologies. CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (MTS) was purchased from Promega Biotech Co., Ltd. All other chemicals used in this study were analytical reagent grade and

used directly. Millipore deionized (DI) water (18.2 MΩ/cm, 25 °C) was used to prepare all the solutions.

Instruments

H-600 electron microscope (TEM, Hitachi, Japan) was used to characterize the morphology of the WS_2 NSs. Cary 50 Bio Spectrophotometer (Varian Inc., CA, USA) was used to record the UV–vis absorption spectra using a 1 cm cuvette. Fluorescence emission spectra were collected using Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon Inc., USA). Nicolet 520 FTIR spectrometer was used to record the Fourier transform infrared (FTIR) spectra. Confocal laser scanning microscope (CLSM) images were obtained using Nikon ECLIPSE Ti microscope (Japan). X-ray photoelectron spectra (XPS) were measured through ESCALab 220i-XL electron spectrometer from VG Scientific using 300 W Al Kα radiations.

Synthesis of the WS_2 nanosheets

WS_2 nanosheets were synthesized by the modified Morrison method.[S1] Briefly, 15 mL of oleylamine was heated to 65 °C and degassed under vacuum in a 100 mL flask for 1 h. Then the liquid was heated to 320 °C in the argon protection environment. 500 μL HMDS was then added. Meanwhile, 50 mg WCl_6 was fully dissolved in 300 μL oleic acid. Then 5 mL oleylamine was introduced and the color of the solution changed from dark brown to yellow. Next, 240 μL CS_2 was introduced promptly, causing the color of the solution changed to orange. This precursor solution was injected into the prepared HMDS/oleylamine sample solution using a syringe pump at a rate of 0.15 mL/min. After cooling down to room temperature, 10 mL hexane and 10 mL isopropanol were added to precipitate the WS_2 nanosheets followed by centrifugation. The nanosheets were finally dried in vacuum at 50 °C.

Surface modification of the WS_2 nanosheets (WS_2 NSs)

10 mL isopropanol was used to disperse 10 mg WS_2 nanosheets, then 5 mL isopropanol solution containing 100 mg L-cysteine hydrochloride was added into the mixture. After dealt with sonication treatment for 1 h (500 W, 4 s on and 4 s off), the resulting modified nanosheets were collected by centrifugation at 10,000 rpm for 5 min. The precipitate was then washed with a solution mixture of isopropanol and water (v / v = 1 : 1) three times. Finally, the precipitate (WS_2 NSs) was dried in vacuum at 50 °C.

Preparation and characterization of the CPFX-loaded nanosheets (WS_2 NS-CPFX)

50 mg CPFX was incubated with 15 mg WS_2 NSs in 50 mL DI water on the rotator for 24 h. Then the unabsorbed CPFX were removed by centrifugation of the sample suspension at 10,000 rpm for 5 min. The obtained WS_2 NS-CPFX was washed 10 times with DI water. The content of CPFX in WS_2 NS-CPFX was quantified by the characteristic band of CPFX at 447 nm in the fluorescence emission spectrum (Ex: 275 nm), the loading capacity (LC) were calculated based on the following equation:

$$LC = W_L / (W_L + W_N) \times 100\% \qquad (1)$$

where W_L and W_N represented the weight of the loaded CPFX and weight of the WS_2 NSs.

Synthesis of the functionalized CS (FCS)

1 g chitosan was first dissolved in 0.2 M ethanoic acid (50 mL), followed by the addition of 40 mL of ethanol. The pH was adjusted to 5.1 after complete dissolution. Then adequate dodecyl aldehyde was introduced. After reacting for 20 min, an excess of $NaCNBH_3$ (5 moles per aldehyde mole) was added to reduce the imine bonds. The solution was further stirred for 18 h and 50 mL ethanol was added to precipitate the FCS, followed by adjusting pH to 7.0. The sediment was washed with ethanol 3 times, and the solid was obtained by lyophilization.

Synthesis of the dialdehyde-functionalized PEG (PEG-CHO)

0.50 g of 4-formylbenzoic acid, 1.60 g of PEG 2000 and 0.025 g of DMAP were mixed in 50 mL of THF, then 0.84 g of DCC was introduced into the solution mixture and further reacted at room temperature overnight. The white solid produced was filtered and the PEG-CHO was purified by dissolution with THF and sedimentation with ether 3 times. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 10.11 (s, 2H), 8.22 (d, J = 8.3 Hz, 4H), 7.95 (d, J = 8.3 Hz, 4H), 4.53-4.50 (m, 4H), 3.86-3.84 (m, 4H), 3.70-3.60 (m, 172-176H), (Figure S1).

Swelling test

The wet hydrogel HG1 was put in a vial, 10 mL PBS (0.01 M, pH 7.4) was added into the vial, and the gel solution was incubated at 37 °C. When arriving at the pre-set time point, HG1 was taken out from the solution and weighted after removing the superficial water. When the weight of HG1 kept constant, the test was completed.

Swelling ratio (SR) was calculated following the relation:

$$SR = (m_t - m_0) / m_0 \times 100\% \qquad (2)$$

where m_0 and m_t represented the initial mass of the wet hydrogel and the mass of HG1 after swelling, respectively.

Morphology characterization of HG1

Scanning electron microscopy (SEM) was used to characterize the morphology of HG1. Particularly, HG1 was formed on silicon pellet and then freeze-dried. After platinum coating for 60 s, the sample was analyzed using SEM.

Injectability of HG1

A piece of HG1 stained with methylene blue was added into 1 mL syringe with needle. HG1 was then injected onto the surface of a glass slide forming the written word “gel”.

Self-healing performance of HG1

(1) Two discs of HG1 were fabricated. Trace amounts of rhodamine B and methylene blue were used to give the two gels different color. The two discs were then each cut in half. One blue half and one red half were put together to form a new united disc. A hole (diameter: 0.8 cm) was created at the center of the new hydrogel, and the self-healing performance of HG1 were photographed.

(2) Rheology analyses were performed to further evaluate the recovery ability of HG1, using the strain amplitude sweep method (γ from 1% to 1000%, each continuous test lasting for 200 s), and the values of G' and G" were recorded (frequency, 1.0 Hz).

Self-adapting performance of HG1

A capillary tube (diameter: 0.5 mm) was inserted into 2 mL of FCS-1% solution, HG1 and agarose hydrogel, respectively. Photographs were taken to record the liquid or gel levels in capillary.[S2]

Antioxidant efficiency of HG1 and HG1-WS_2

To evaluate the antioxidant efficiency of HG1, HG1-WS_2 and HG1-CW, the stable free radical DPPH was used. The gels were cut into small pieces, and followed by adding desired amount of the gels (50 mg HG1, and 50 mg HG1-WS_2 containing 0.5 mg WS_2 NSs) to the 100 μM DPPH ethanol solution (3 mL). The mixture was incubated in a dark place for predetermined time interval. Next, the absorption of DPPH was measured by a UV-vis spectrophotometer. The degradation of DPPH was confirmed by the following formula:

$$\text{DPPH scavenging\%} = (A_B - A_H) / A_B \times 100\% \qquad (3)$$

where A_B, A_H was the absorption of the control (DPPH solution only) and the absorption of the sample (hydrogel + DPPH solution), respectively.[S3]

In vitro hemostatic capability

Heparinized human whole blood was used to test the hemostasis capability of the chitosan solution, FCS-1% solution and HG1. The blood (100 μL) was added into centrifuge tubes containing CS, FCS-1% solution (300 μL, 3%, in 0.2 M acetic acid), or HG1 (300 μL), respectively. Finally, the tubes were inverted to observe the fluidity of blood after different treatments.

Adhesive strength of HG1

Fresh porcine skin was used to test the adhesion property of HG1 to the host tissue.[S4] Typically, the porcine skin was cut into 1 cm × 3 cm pieces and immersed into fresh PBS buffer before use. One piece of the skin tissue was covered with 100 μL HG1, and another piece was put onto the HG1 coated skin tissue. The adhesive area was 1 cm × 1 cm. After aging at 25 °C for 2 h, the adhesive strength was evaluated using the Instron 1121 testing machine (Canton, Massachusetts, USA). Lap shear test mode with 50 N load cell at a rate of 2 mm/min was applied to conduct the test. Three parallel tests were conducted.

Photothermal activity and stability of the WS_2 NSs

WS_2 NSs solutions of different concentration (0, 0.1, 0.2, 0.3, 0.5, 1.0 mg mL^{-1}) were irradiated by an 808 nm laser (0.5 W cm^{-2}) for 10 min. And the real-time temperature changes were recorded with a thermocouple every 10 s.

To estimate thermal stability of the WS_2 NSs, a WS_2 NSs sample solution (1 mg mL^{-1}) was irradiated with the 808 nm laser (0.5 W cm^{-2}) for 10 min, and the temperature was lowered by natural cooling for 10 min. The same procedures were repeated five times, and the real-time temperature variations were recorded.

NIR light-triggered CPFX release from HG1-CW

The drug release behavior of our nanocomposite dressing was measured in PBS buffer (pH 7.4). 0.5 mL of PBS was placed in a quartz cell containing 0.5 mL of HG1-CW. An 808 nm laser was used to trigger the CPFX release. The sample solution was first irradiated at 0.5 W/cm^2 from the top for 10 min, then left without irradiation for 20 min. The procedures were repeated three times. A fluorescence spectrometer was employed to monitor the amount of released CPFX. The HG1-CW PBS sample solution without irradiation was used as control.

Cell Culture

L929 cells were cultured in RPMI 1640 medium with 10% FBS, 1% penicillin/streptomycin, and 5 % CO_2 at 37 °C.

Cytotoxicity of HG1 and HG1-CW

L929 cells were seeded in a 96-well plate with 1 × 10^4 cells per well for 24 h before the experiment. 100 μL of the medium (RPMI-1640 medium) in each well was removed, and replaced with 100 μL extract solutions of different concentrations of HG1 (0, 1, 3, 5, 10 and 20 mg/mL), and the samples were incubated for 24 h. The cytotoxicity of HG1-CW containing different dosages of WS_2-CPFX (0, 0.25, 0.5, 1, 1.5, 2 mg) was also studied. Cell viability was determined by the MTS assay. Each test was repeated three times.

Cell live/dead fluorescence staining

Briefly, 1 × 10^5 L929 cells per well were cultured in a 6-well dish overnight to allow the attachment of cells. Then the medium was replaced with 24 h aged extract solution of HG1 (20 mg/mL). After incubation for 24 h, acridine orange (AO) and propidium iodide (PI) were used to stain and visualize the viability of the cells. Fluorescence images were collected on a CLSM system.

In vitro anti-inflammation assay

RAW 264.7 macrophages were seeded in 24-well plates (3×10^5 cell/well) at 37 °C and 5% CO_2 for 24 h. Cells were then separated into different groups: (a) blank control (PBS treated); (b) negative control (LPS treated only, inflamed cells); (c) experimental groups, cells treated with HG1, HG1-WS_2, HG1-CW, respectively. After incubation with the hydrogels (100 μg mL^{-1}), the cells were stimulated with LPS (1 μg mL^{-1}) for 24 h, and the supernatant was collected. The nitrite/nitrate (NOx) production and TNF-α secretion were monitored by the Griess method and ELISA, respectively.

Cell migration experiment

L929 cells with a density of 10^5 cells per well were cultured in a 6-well plate overnight. Then artificial scratches were created on cells by a 200 μL pipette tip. Cells were washed twice with PBS solution (10 mM, pH 7.4) and incubated with fresh medium, and the extract solutions of HG1 and HG1-CW (20 mg/mL) for 0, 24, 48, and 72 h. Cell migration was quantitatively visualized via a Nikon Ti-U optical microscope system (Japan).

Hemolysis activity of the hydrogel

Hemolysis activity of the gels was explored following literature reference.[S5] Whole blood from the rabbit was collected, and the blood was centrifuged at 1000 rpm for 10 min to separate erythrocytes. The erythrocytes were washed with saline 3 times and then diluted to 5% concentration (v/v) by saline. 50 mg HG1, HG1-WS_2, or HG1-CW was mixed with 800 μL erythrocytes sample solution in centrifuge tubes. The samples were rotated at 37 °C with a speed of 99 rpm for 1 h. 0.1% Triton X-100 treated sample was set as the positive control, while saline treated sample was the negative control (the blank). After that, all the samples were centrifuged at 1000 rpm for 10 min, and photographs were taken. The supernatants (100

μL) were collected and added into a 96-well microplate. The absorbance of the supernatants at 540 nm was recorded by a microplate reader (Biotech Co., Ltd.).

The hemolysis percentage was calculated according to following equation:

$$\text{Hemolysis (\%)} = [(A_p - A_b) / (A_t - A_b)] \times 100\% \quad (4)$$

where A_p was the absorbance value of the hydrogel solution. A_t was the absorbance value of the Triton X-100 treated sample, and A_b was the absorbance value of PBS.

Bacteria live/dead fluorescence staining

Live/Dead BacLight Bacterial Viability Kit (Thermo Fisher Scientific, L13152) was used to evaluate the bacterial viability. Typically, *S. aureus* sample solutions under different treatments were centrifugated at 6000 rpm for 5 min. After re-suspended in saline solution, the bacteria samples were co-stained with SYTO 9 and PI for 30 min. A Nikon Ti-U optical microscope system (Japan) was used to collect the fluorescence staining images. The same protocol was carried out to stain the *E. coli* cells.

Morphological characterization of bacteria

The bacterial samples under different treatments (1×10^8 CFU/mL, 1 mL) were fixed with 2.5% glutaraldehyde solution at 4 °C overnight. Then the bacteria were dehydrated sequentially with 30, 50, 70, 90, and 100% ethanol for 20 min. After gold sputter coating, the samples were observed by SEM.

B: Supplementary figures

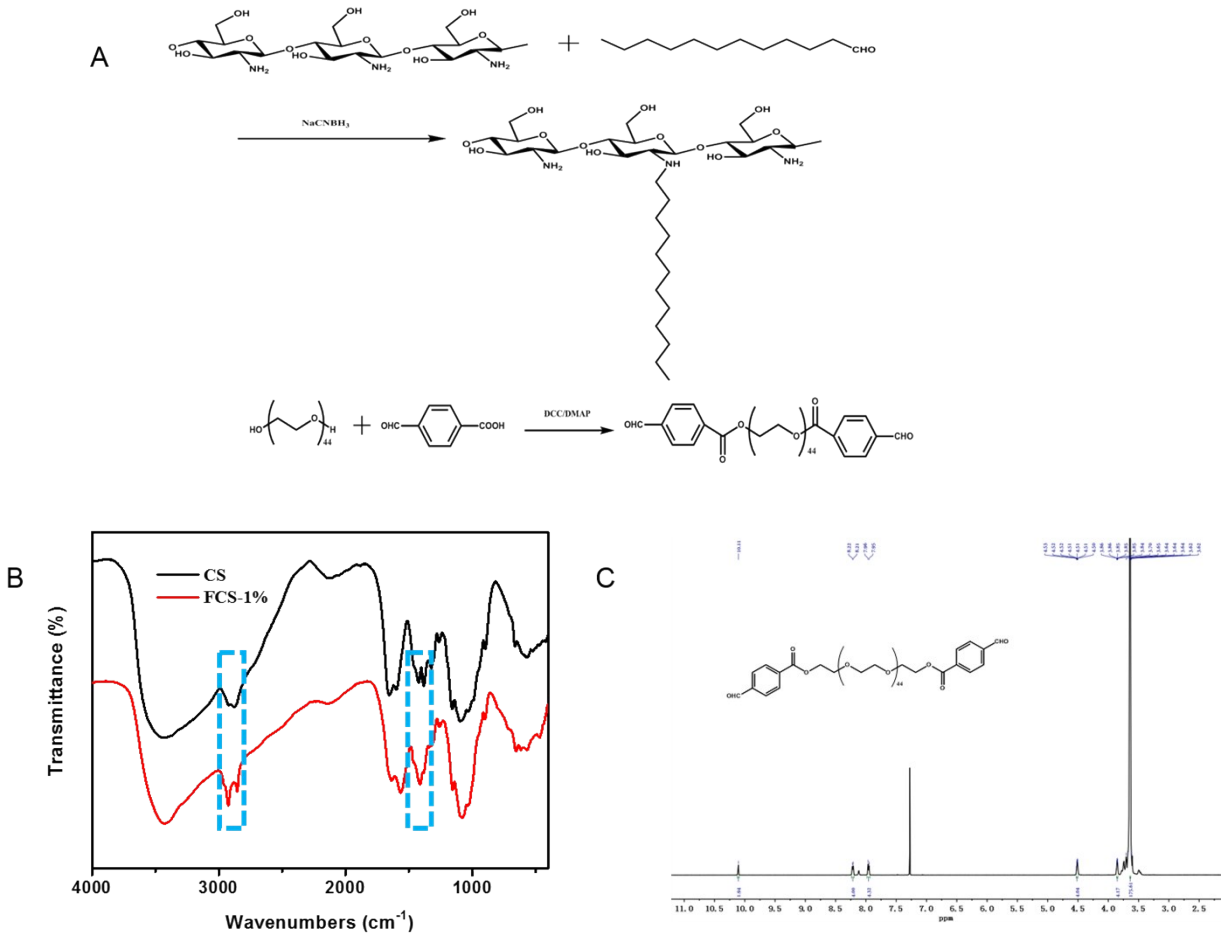

Figure S1. (A) Synthesis of FCS and PEG-CHO, (B) FTIR spectra of CS and FCS-1%, and (C) ^{1}H NMR spectrum of PEG-CHO.

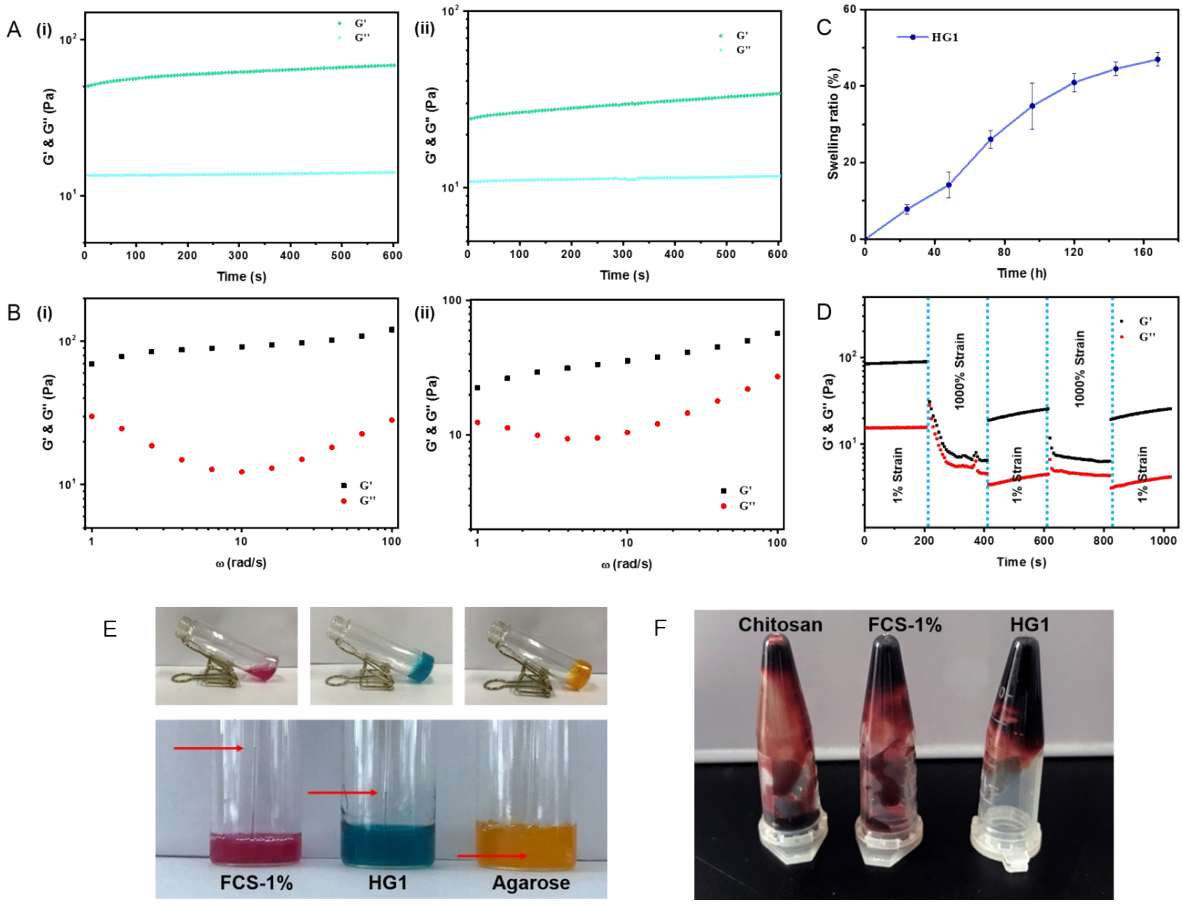

Figure S2. (A) Gelation behavior of the hydrogels (i) FCS-1% (ii) FCS-3%. (B) Frequency-dependent oscillatory shear rheology of the hydrogels (i) FCS-1%, (ii) FCS-3%. (C) Swelling test of the gel in PBS (pH 7.4). (D) Rheology analysis of the self-healing property of HG1. (E) Capillary experiment, FCS-1% solution (purple), HG1 (blue), and agarose hydrogel (orange). (F) In vitro hemostasis performance of chitosan, FCS-1% and HG1.

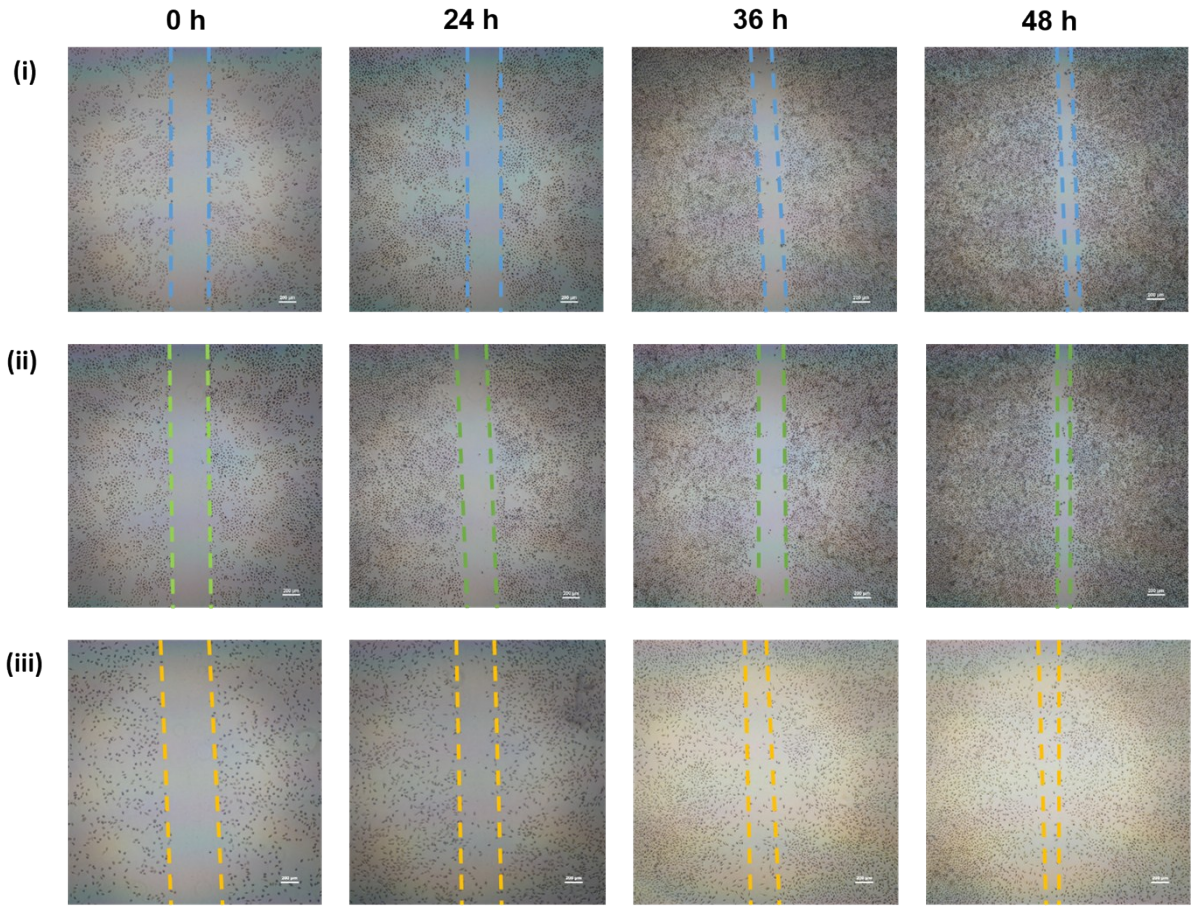

Figure S3. The effect of the gel on cell migration (i) control without HG1, (ii) with HG1, and (iii) with HG1-CW. Scale bar: 200 μm.

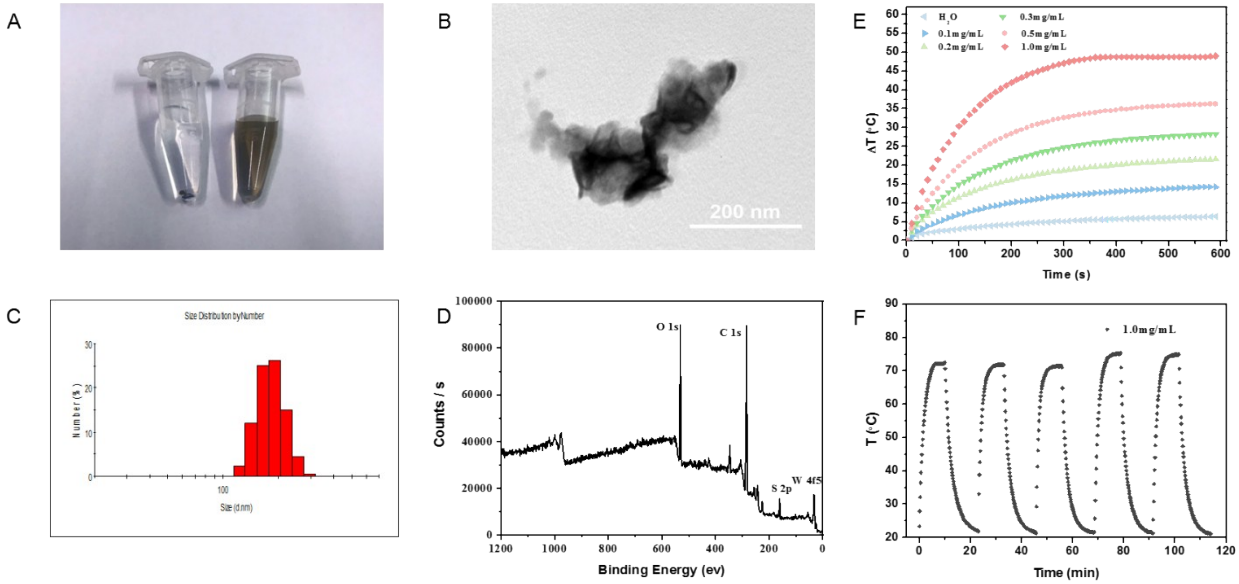

Figure S4. (A) Dispersity of the WS_2-NSs without (left) and with (right) L-cysteine modification. (B) TEM image of the WS_2 NSs. (C) The hydrodynamic size distribution histogram of the WS_2-NSs. (D) XPS analysis of the 2H-WS_2 NSs. (E) Temperature enhancement of samples containing different concentrations of WS_2 NSs. Samples were irradiated with an 808 nm NIR laser at 0.5 W cm^{-2} and the temperature changes with irradiation time were recorded. (F) Photo-thermal stability of the WS_2 NSs.

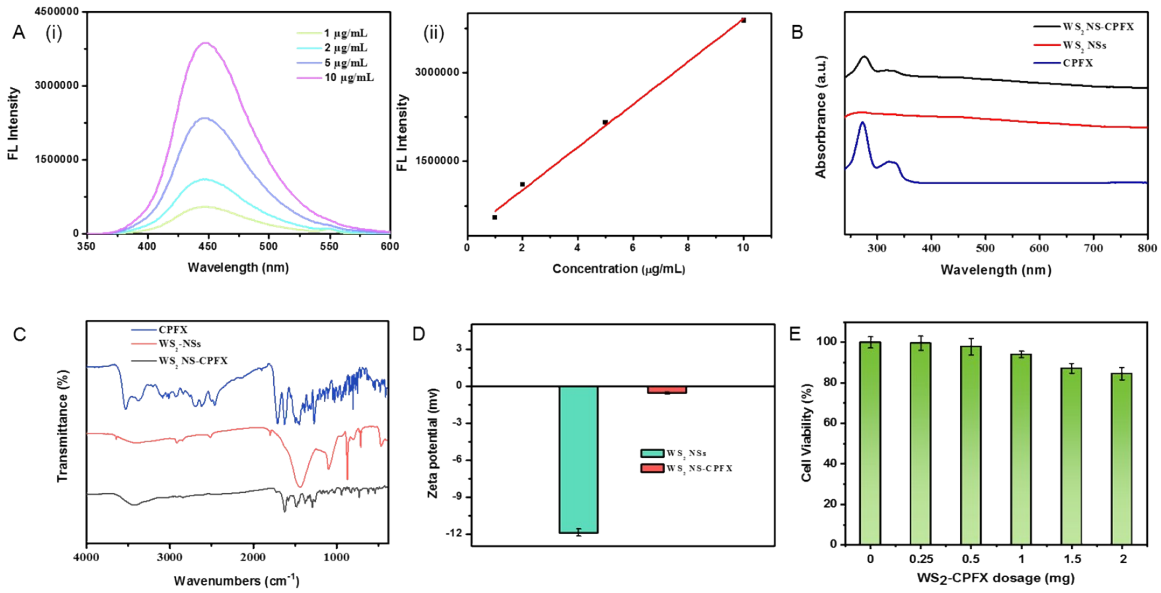

Figure S5. (A) (i) CPFX fluorescence intensity changes versus concentration, (ii) The linear calibration curve of CPFX. (B) UV−vis absorption spectra of CPFX, WS_2 NSs and WS_2 NS-CPFX. (C) FTIR spectra of CPFX, WS_2 NSs and WS_2 NS-CPFX. (D) Zeta potential of WS_2 NSs and WS_2 NS-CPFX. (E) Biocompability of HG1-CW, HG1 containing different dosage of WS_2-CPFX was tested.

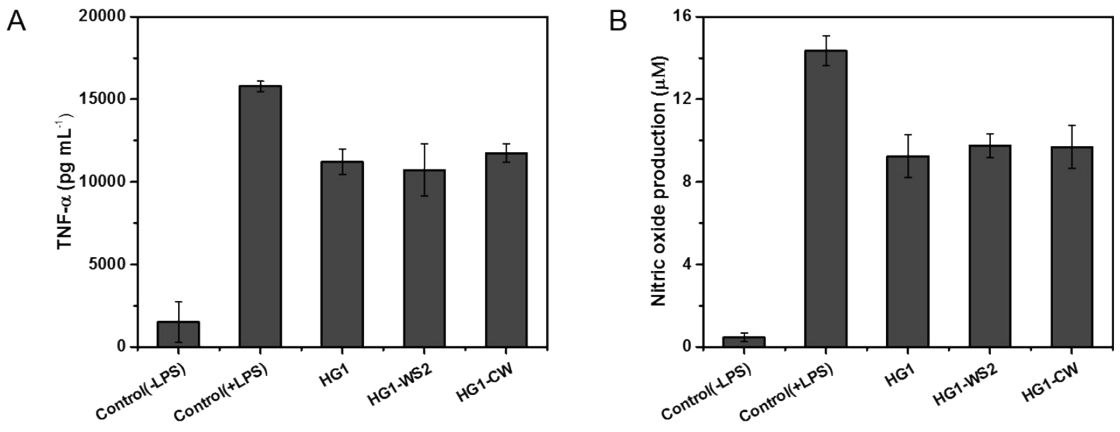

Figure S6. The changes in (A) TNF-α and (B) NO levels in the culture medium after the cells were treated with HG1, HG1-WS_2 and HG1-CW. Cells without treatment and cells only treated with LPS were used as control.

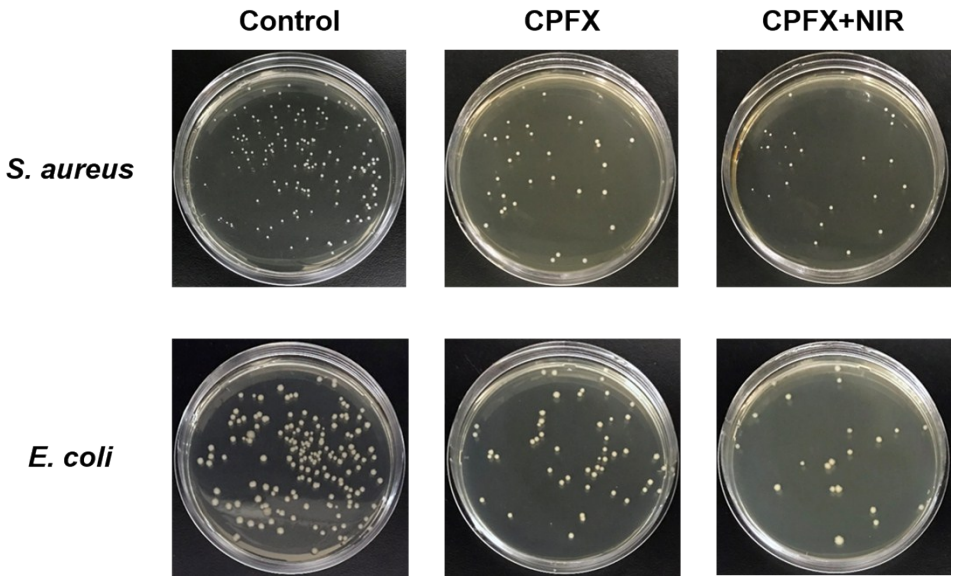

Figure S7. The antibacterial effect of CPFX.

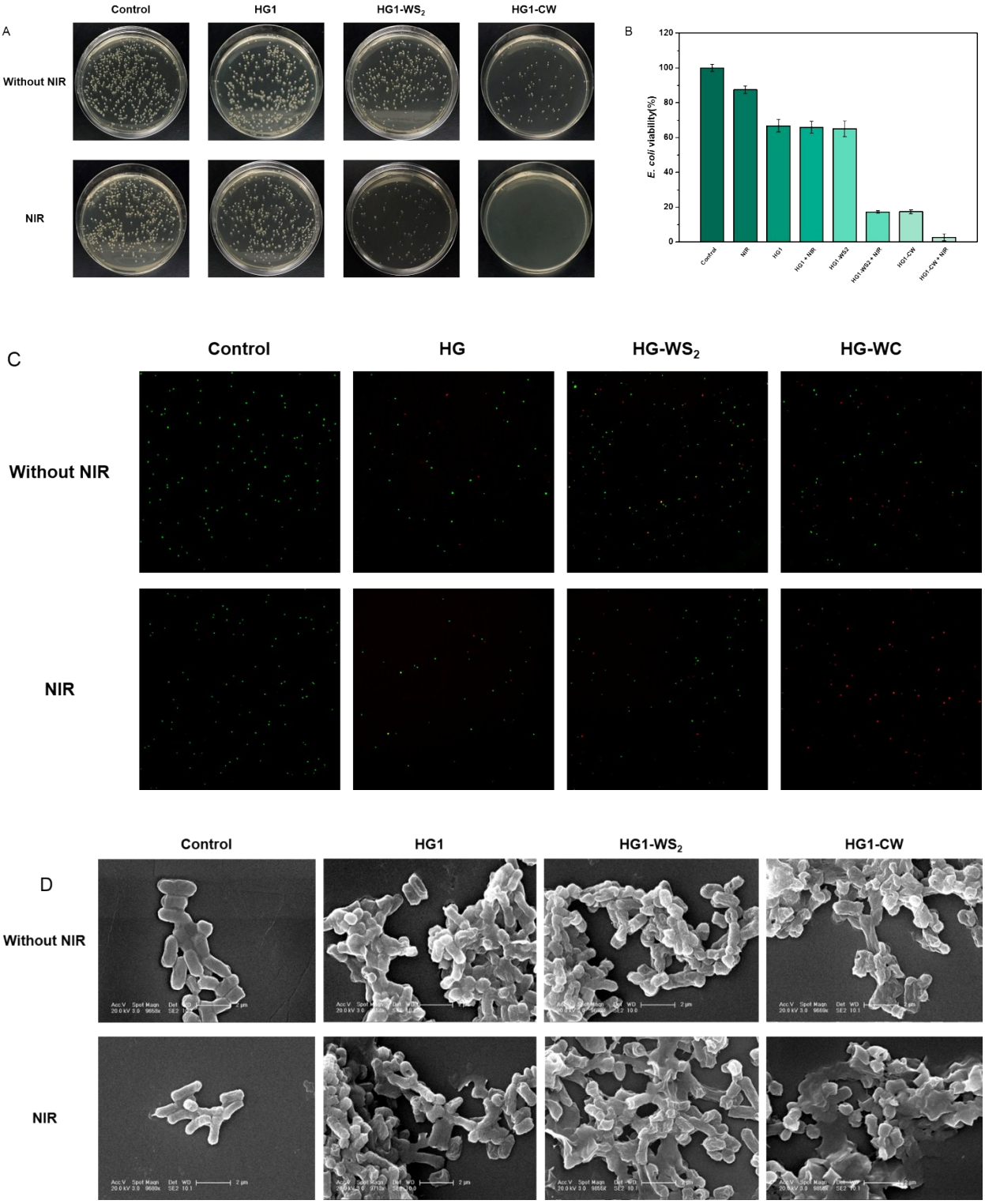

Figure S8. (A) Antibacterial effect of the dressing material against *E. coli.* (B) Viability of *E. coli* cells after different treatments. (C) Live/dead images of *E. coli* stained with SYTO 9/PI dyes after different treatments. (D) SEM images of *E. coli* cells under different treatments.

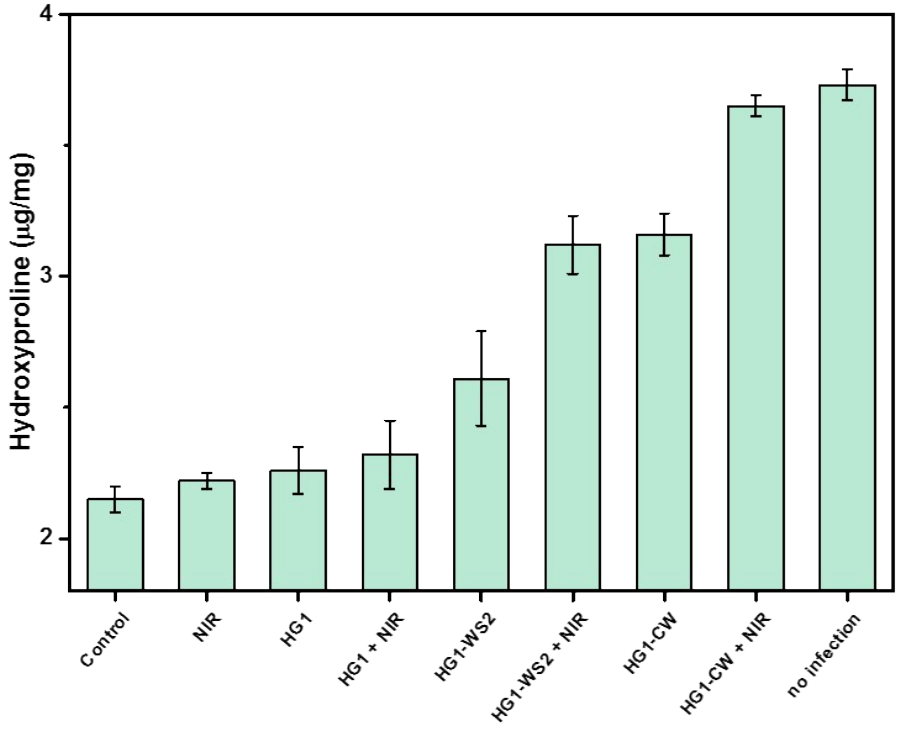

Figure S9. Quantification of the collagen content in different treatment groups via the measurement of the hydroxyproline content.

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