Supporting Information

Hydrogel Functionalized Silver Nanocluster for Bacterial-Infected Wound Healing

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Materials

Silver nitrate (99.8%), $NaBH_4$ (98%), sodium hydroxide (NaOH, 98%), L-glutathione (GSH, 98%) were purchased from Energy Chemical Co., Ltd (Shanghai, China). Thiazolyl blue tetrazolium bromide (MTT, 99%) was purchased from Macklin Biochemical Co. Ltd (Shanghai, China). All the fluorescence probes (SYTO 9 and PI) were purchased from Thermo Fisher Scientific. Pseudomonas aeruginosa (*P. aeruginosa*, ATCC 27853), Escherichia coli (*E. coli*, ATCC 25922), Staphylococcus aureus (*S. aureus*, ATCC 25923) and Methicillin-resistant Staphylococcus aureus (*MRSA*, ATCC 43300) were obtained from ATCC (USA) and reconstituted according to the suggested protocols. The materials used in bacterial culture were purchased from Sangon Biotech Co., Ltd (Shanghai, China). Millipore deionized water (18.2 MΩ cm) was used in all experiments. All chemicals were used as received and without further purification.

Characterization

UV−vis absorption spectra were recorded using a UV-6000PC instrument, and the solution samples were prepared using ultrapure water as the solvent. Transmission electron microscopy (TEM) images were obtained from a JEMF200 microscope. X-ray photoelectron spectroscopy (XPS) measurements were performed on a ESCALAB 250 high-performance electron spectrometer with monochromatic Al Kα radiation as the excitation source. The silver content in the mice was measured using inductively coupled plasma mass spectrometry (ICP-MS) on an Agilent 7800 instrument.

Synthesis of Ag NCs.

Firstly, 60 mg silver nitrate was dissolved in 10 mL of ethanol, and then 150 mg

triphenylphosphine was mixed with $AgNO_3$ solution at room temperature. After stirring for 1h, 80 mg of sodium borohydride was quickly added into the reaction solution for 3 h. Then, 200 mg of GSH dissolved in 2 mL H_2O. 12 h later, the aqueous solution was washed with 30 mL methanol three times. Finally, the product was dried at room temperature to obtain the crude GS-protected Ag NCs.

Native polyacrylamide gel electrophoresis (PAGE) was carried out using discontinuous gels (1.5 mm × 80 mm × 70 mm). Resolving and stacking gels were prepared from 30 and 4 wt.% acrylamide monomers. 200 μL of crude products was mixed with 20 μL of 5 vol% glycerol and then electrophoresis was run for 2 h at a constant voltage of 150 V at 4 ^{o}C. After electrophoresis, the band was cut from the gels and soaked in ultrapure water overnight at 4 ^{o}C to obtain the pure Ag NCs with monodisperse.

Synthesis of Hydrogels.

Briefly, the hygrogel was prepared by dispersing Carbopol 940 in distilled water with constant stirring (3000 rpm). ~12 h later, the pH reaction system was adjusted up to 5.5–6.5 by adding triethanolamine. Finally, the prepared Ag NCs were added to the Carbopol hydrogel while stirring to obtain the Ag NCs-Gel.

Determination of minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC)

Pseudomonas aeruginosa (*P. aeruginosa*, ATCC 27853), Escherichia coli (*E. coli*, ATCC 25922), Staphylococcus aureus, (*S. aureus*, ATCC 25923), and Methicillin-resistant Staphylococcus aureus (*MRSA*, ATCC 43300) were used for antibacterial assay. 50 μL of bacterial stored in glycerin at -80 ℃ was transferred to 50 mL fresh LB broth medium for incubation at 37 °C within a shaking incubator under 120 rpm rotation overnight. The bacterial solution was then added to the 96-well plate with different concentrations of Ag NCs, and then incubated at 37 °C for 10 h in the shaking incubator. The MIC value was taken as the minimum concentration of antibacterial samples where no visual turbidity of bacteria. To determine the MBC, 100 μL of the treated bacterial cultures that did not show visual turbidity were delivered onto the surface of LB agar plates. After incubating at 37 °C for 24 h, the bacterial suspensions were swabbed on Luria-Bertani (LB) agar plates. The lowest dose at which bacteria failed to grow was recorded as MBC.

The antibacterial activity of Ag NCs and Ag NCs-Gel.

Pseudomonas aeruginosa (*P. aeruginosa*, ATCC 27853), Escherichia coli (*E. coli*, ATCC 25922), Staphylococcus aureus (*S. aureus*, ATCC 25923), and Methicillin-resistant Staphylococcus aureus (*MRSA*, ATCC 43300) were used as the bacterial suspension assay. Inoculating a single colony into 3 mL of LB medium and incubating it at 37°C overnight to obtain a logarithmic phase bacterial suspension. Dilute the suspension with LB medium to an optical density (OD) of 0.164 at 600 nm (OD600). Next, add the bacterial solution to a 96-well plate containing different concentrations of Ag nanoclusters. Measure the OD value of the material continuously for 24 hours using a microplate reader to create a growth curve. To quantify the number of bacteria, continuously dilute the bacterial suspension using LB

medium. Obtain a 1000-fold dilution of the bacterial suspension and evenly spread 100 μL of the diluted bacterial suspension on an agar plate. Culture the plate in a 37 °C incubator for 12 hours and take photos of the bacterial plate. Calculate the number of colonies formed. In addition, the antibacterial activity of Ag NCs-Gel was also evaluated by determining the diameter of the inhibition zone. Repeat all experiments three times.

Fluorescent-based bacteria live/dead test

Bacterial mortality was observed by an inverted fluorescence microscope. To clearly observe the bacteria's live/dead status intuitively, the fluorescence-based cell live/dead assay was carried out. Bacterial solution containing Ag nanoclusters and bacteria (1×10^6 CFU per mL) were incubated at 37 °C. The bacterial solution without Ag nanoclusters was set as the control group. Then, the bacteria were collected by centrifugation and stained with SYTO 9 and propidium iodide (PI) (SYTO 9: PI = 1:1, SYTO 9 marked live bacteria as green, PI marked dead bacteria as red) for 30 min simultaneously, then rinsed with PBS twice. After that, bacterial mortality was observed by an inverted fluorescence microscope.

Cell cytotoxicity assay of Ag NCs and Ag NCs-Gel.

In detail, the cells 3T3 were incubated in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (FBS, 10%) at 37 ℃ under 5% CO_2. The cells were seeded onto 96-well plates (5.0×10^3 cells/well) and incubated in complete DMEM medium (200 μL) for 24 h. Then the medium was replaced with fresh complete DMEM medium containing Ag NCs with different concentrations. After incubation for 24 h, medium containing materials was extracted thoroughly and the cell samples were treated with 100 μL MTT (1 mg/mL) for another 4 h at 37 °C. 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals, followed by shaking for 15 min at room temperature. Finally, the liquid was taken out and measured with a microplate reader (wavelengths of 570 nm) to obtain the absorbance. Results were obtained as the mean values by three measurements.

The cells 3T3 were cultured in high-glucose DMEM containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin. First, freeze-dry the hydrogel and place it in a biosafety cabinet for 30 minutes of ultraviolet radiation. Then allow the hydrogel to air dry completely in the cabinet. Place the dried hydrogel into a 6-well plate and add high-glucose DMEM culture medium to immerse it. Place the plate in a 37 °C incubator. After 72 hours, remove the hydrogel from the plate. Filter the obtained extract using a 0.22 μm filter membrane. Then, add 10% fetal bovine serum and 1% double antibody to prepare a complete culture medium. The cells were seeded onto 96-well plates (5.0×10^3 cells/well). After culturing for 24 h, an extract of the Ag NC-Gel or the Blank-Gel was added to the complete growth medium. The cell viability of Ag NCs-Gel was evaluated by MTT assay after culturing for 24 h.

The antibacterial activity in the wound healing model

The Balb/c Female mice (8 weeks) were obtained from the Laboratory Animal Center at Anhui Medical University (Hefei, Anhui Province, China), and all the animal experiments were conducted following the guidance and approval of the Ethical Committee of Anhui Medical University (approval number: LLSC20231653). All animal experimental procedures

were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China. All surgical procedures were performed under aseptic conditions to ensure the well-being of the animals. Prior to the experiments, female Balb/c mice (8 weeks old, weighing between 16-20 g) were acclimatized for one week. To assess the antibacterial activity of the hydrogel in vitro, an artificial wound with a diameter of approximately 10 mm was created on the shaved back of the anesthetized mice. The mice were then infected with 30 μL of bacteria (*MRSA* or *E. coli*) in the logarithmic growth phase (10^8 CFU mL^{-1}). After 24 hours, the wounds became infected, and the mice were divided into three groups: (1) PBS, (2) Gel, and (3) Ag NCs-Gel. Each group consisted of seven mice. Subsequently, 50 μL of PBS, blank-gel, or Ag NCs-Gel was applied to the infected wounds. The wound diameter and body weight of the mice were recorded on days 0, 2, 4, 6, and 8. Daily photographs of the wounds were taken, and the weights of the mice were recorded. The mice were treated for a total of 8 days before being released. On the 8th day, all the wound area tissues were collected and stored at -80 °C for further analysis. To monitor the wound area, measurements were taken on days 0, 2, 4, 6, and 8 by tracing the wound boundaries on plotting papers or other suitable measurement tools. Wound contraction (%) was calculated using the following formula:

$$\text{Wound contraction} = (\text{Area}_{(0\ \text{day})} - \text{Area}_{(n\ \text{day})}) / (\text{Area}_{(0\ \text{day})}) \times 100\%$$

where "n" represents the day, like 0th, 2th, 4th, 6th, 8th day.

Metabolism Study

The major organs hearts, livers, spleens, lungs, and kidneys) of mice and of mice after 8 days of treatment with Ag NCs-Hydrogel and then homogenized and treated with aqua regia. The Ag element distributions in different tissues were determined by ICP-MS.

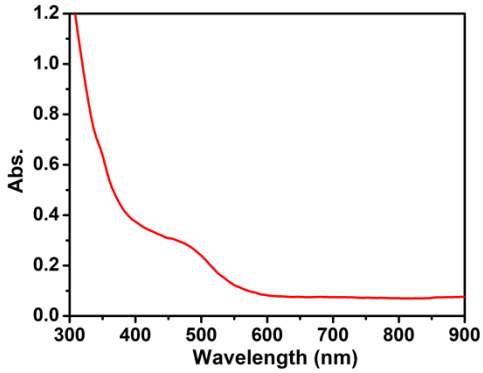

Fig. S1 UV-vis absorption spectrum of the crude product.

Fig. S2 Digital photo of the PAGE bands.

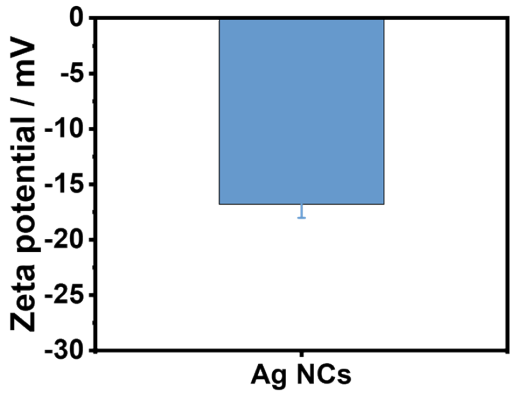

Fig. S3 Zeta potentials of Ag NCs in PBS.

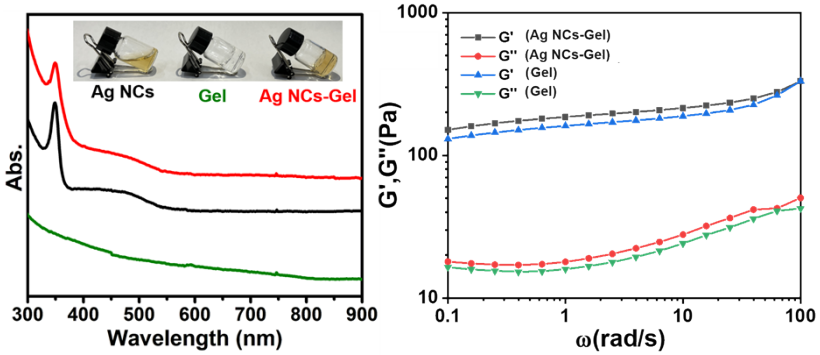

Fig. S4 (a) the corresponding UV-vis absorption spectra and photographs of Ag NCs, Gel and Ag NCs-Gel. (b) Frequency dependence of storage G′ and loss G′′ moduli of Gel and Ag NCs-Gel.

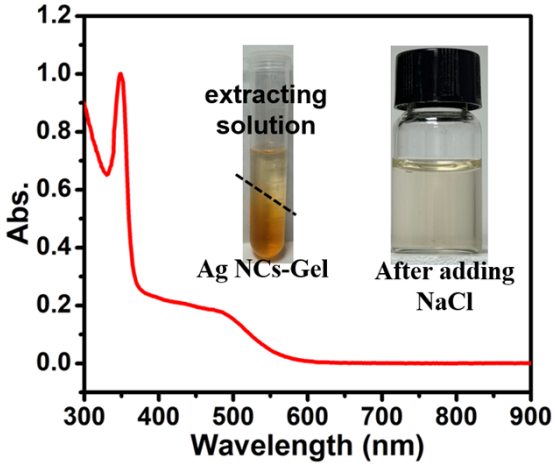

Fig. S5 The UV-vis absorption spectrum and photographs of the extracting solution of Ag NCs-Gel.

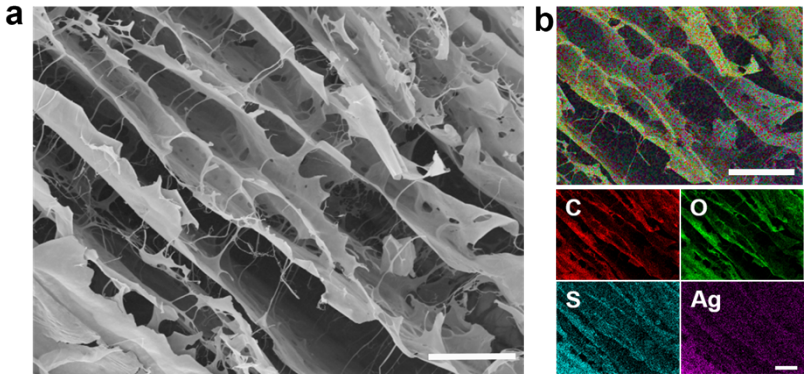

Fig. S6 (a) SEM and (b) element mapping of the Ag NCs-Gel (Scale bar is 100 μm).

Fig. S7 Determination of MIC for the antibacterial ability of Ag NCs against *E. coli*, *MRSA*, *P. aeruginosa* and *S. aureus* with the 96-well plate.

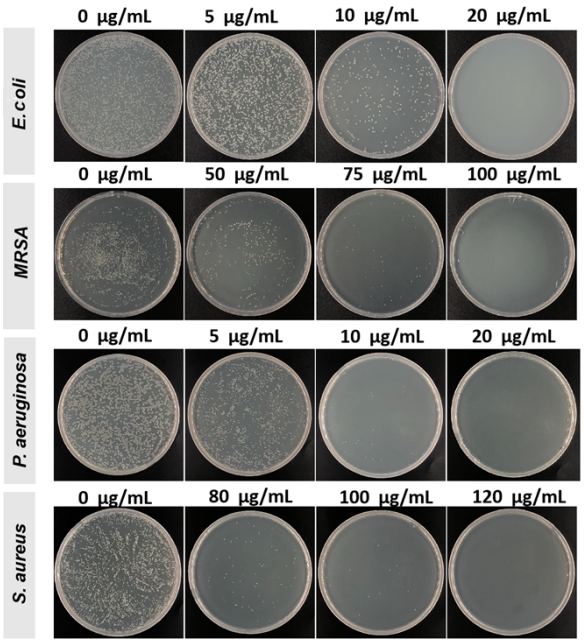

Fig. S8 The photos of the bactericidal experiments of Ag NCs with different concentration.

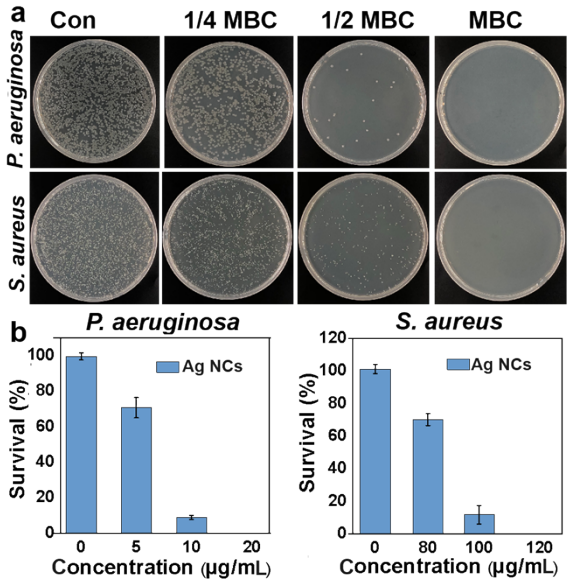

Fig. S9 (a) Photographs of bacteria colonize of *P. aeruginosa* and *S. aureus* after adding the different concentrations of Ag NCs. (b) The number of bacterial colonies formed by *P. aeruginosa* and *S. aureus*. The values are shown as the mean ± SD (n=3).

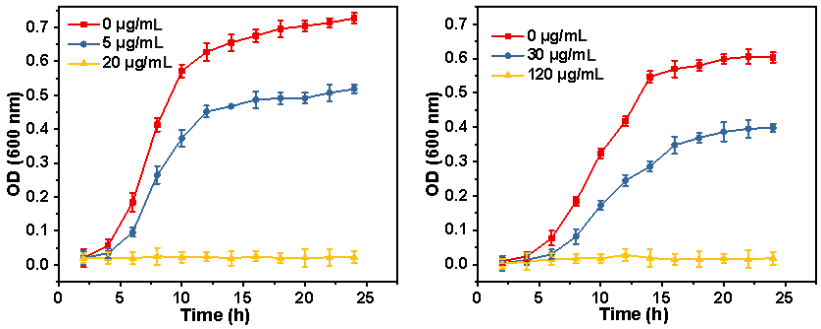

Fig. S10 Growth curves of *P. aeruginosa* (left) and *S. aureus* (right) bacterial suspensions after 24 h co-incubation with silver nanocluster.

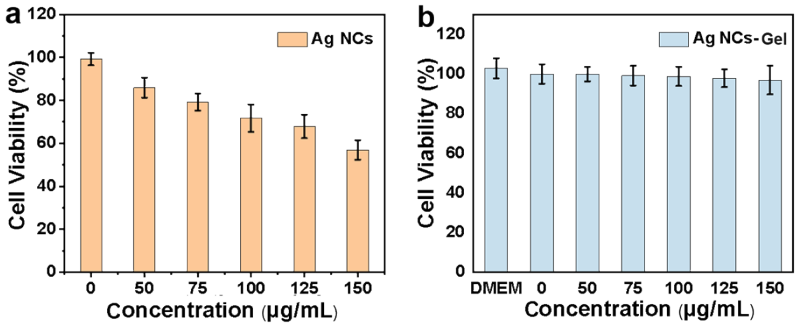

Fig. S11 The cytotoxicity of (a) Ag NCs and (b) Ag NCs-Gel in 3T3 cells.

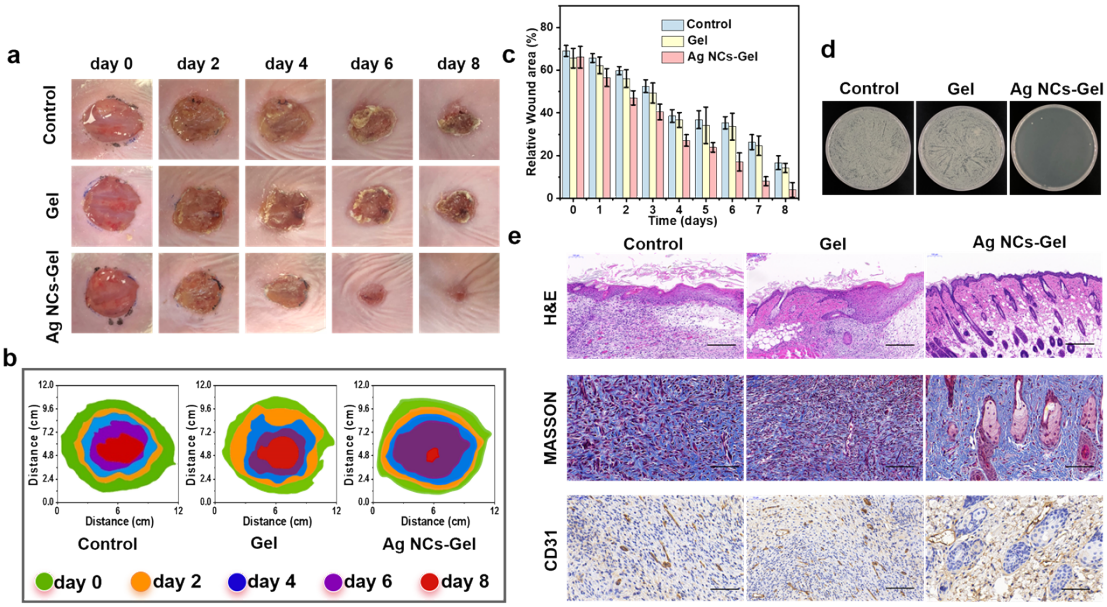

Fig. S12 The effect of Ag NCs-Gel on the *E. coli*-infected wounds healing. (a) Infected wound pictures in each group. (b) Wound area traces of each group and (c) the corresponding histogram. (d) Bacterial density of *E. coli* of the infected wound on 8th day. (e) H&E staining, Masson's trichrome staining and CD31 staining images of *E. coli*-infected skin wounds with the corresponding scale bar of 200, 100 and 100 μm.

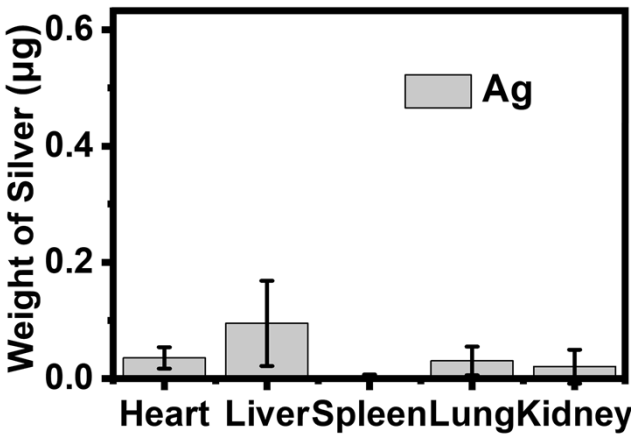

Fig. S13 Biodistribution of Ag^{+} in heart, liver, spleen, lung, and kidney on the 8^{th} day treated with Ag NCs-Gel (n = 3).