

Supplementary Information for

**In situ synthesis of silver nanoclusters inside bacterial
cellulose hydrogel for antibacterial application**

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Experimental

1. Materials

Silver nitrate (AgNO_3), (R)-Alpha lipoic acid (R- α -LA), sodium borohydride (NaBH_4), sodium hydroxide (NaOH) were purchased from Sinopharm Chemical Reagent Co., Ltd. Bacterial cellulose (BC) was kindly provided by Hainan Yeguo Foods Co., Ltd. Standard strains of *Staphylococcus aureus* ATCC 6538 (*S. aureus*), *Bacillus subtilis* (*B. subtilis*), *Escherichia coli* ATCC 25922 (*E. coli*), *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*) and Hela cells were purchased from China General Microbiological Culture Collection Center. Luria-Bertani (LB), phosphate-buffered saline (PBS) and Luria Bertani agar were purchased from Qingdao Hope Biol Co., Ltd. 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) dye was purchased from Sigma-Aldrich LLC. LIVE/DEAD viability/cytotoxicity kits for mammalian cells were purchased from Beijing Solarbio Science & Technology Co., Ltd. All the reagents were used as received without further purification.

2. Fabrication of Ag nanoclusters (NCs) inside BC hydrogel (Ag NCs@BC)

The Ag NCs@BC was prepared through an in-situ NC synthesis procedure. Briefly, the BC hydrogel was firstly washed repeatedly with ultrapure water till the pH is around 6.5. Then five BC hydrogels were immersed in AgNO_3 solutions with different concentrations (4 mM, 6 mM, 8 mM, 10 mM, and 12 mM) for 20 hrs. Afterwards, 1, 1.5, 2, 2.5 and 3 mL of 200 mM R- α -LA solutions were added into those reaction solutions with stirring of 15 min, followed by adding 0.6, 0.9, 1.2, 1.5 and 1.8 mL of freshly prepared 200 mM NaBH_4

solution into each reaction system. After gentle stirring of 24 hrs, the Ag NCs@BC samples were obtained for later use. Meanwhile, the composite of Ag NCs and hydrogel (Ag NCs/BC) was also prepared through directly immersing the pristine BC hydrogel into 10 mM Ag NCs solution for 48 hrs.

3. Characterization

The surface morphology and structure of the Ag NCs and Ag NCs@BC were examined by field emission scanning electron microscopy (FESEM, JEOL JSM-LV5610), energy dispersive X-ray elemental mapping (EDS, Oxford), and transmission electron microscopy (TEM, JEOL 2000 FX). The optical properties of samples, and optical density at 600 nm (OD_{600}) of bacterial cells were analysed on a Shimadzu UV-1800 spectrophotometer and/or a PerkinElmer FL8500 fluorescence spectrometer. The size of Ag NCs was measured by electrospray ionization mass spectrometry (ESI-MS, Bruker Impact II). The decoration amount of Ag NCs on BC hydrogel and the releasing property of the Ag NCs@BC were investigated by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7700 ICP-MS system). Prior to controlled release test, the Ag NCs@BC was soaked in ultrapure water under magnetic stirring condition at 37 °C, and the concentrations of the released Ag species in the solutions were measured by ICP-MS at predetermined time intervals (30 min, 1 hr, 2 hrs, 4 hrs, 8 hrs, 12 hrs, 18 hrs, 24 hrs, 36 hrs, and 48 hrs). Fluorescence microscopy images of bacteria were captured using an Olympus IX73 fluorescence microscope (Olympus, Japan).

4. Antibacterial experiments

4.1 Bacteria cells culture

The *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* were cultured in LB medium in an orbital shaker at 37 °C, 150 rpm and maintained by growing the cell on LB agar.

4.2 Agar diffusion assay

The cultured *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* were diluted to $OD_{600} = 0.1$ ($\sim 1.5 \times 10^8$ CFU/mL), while 100 μ L dilution of such cells was plated on the LB agar. Then the Ag NCs@BC fabricated in different concentrations of Ag precursor solutions and the plain BC were placed on the center of the agar plates, and further incubated for 12 hrs at 37 °C. The antibacterial experiment of the Ag NCs/BC was conducted under the same condition for comparing purpose.

4.3 Long-acting antibacterial test

In order to quantifiably investigate the antibacterial durability of the Ag NCs@BC, the bacterial suspension ($OD_{600} = 0.1$, 2 mL) and Ag NCs@BC (10 mM) were added into a sterilized glass tube, and then cultured in an orbital shaker at 150 rpm under 37 °C for 12 hrs. Serial dilutions (100 μ L) from the bacterial suspension (10^{-1}) were cultured in duplicate on LB agar after 12 hrs incubation. Pristine Ag NCs solution with the same Ag content was also tested under the same condition for comparing purpose.

4.4 Reactive oxygen species (ROS) detection

The intracellular ROS levels were measured with the help of DCFH-DA dye. The bacterial cells were incubated with Ag NCs@BC and plain BC for 2 hrs, and then 10 μ L DCFH-DA (200 μ M) was introduced into the bacterial solutions and incubated for another 15 min at 37 °C and 150 rpm. After that, the bacterial suspension (1 mL) was centrifuged (7000 rpm) and re-suspended in PBS to the original volume. The concentration of the as-resulted dichloro

fluorescein (DCF) was measured by a microplate reader (Tecan Infinite M200 Pro) at excitation wavelength of 480 nm, since the amount of the generated ROS could be determined by the luminescence intensity of DCF. Fluorescence microscopy images of viable cells were captured using the fluorescence microscope. The intracellular ROS levels were also measured under the dark condition for comparing purpose.

4.5 Cytotoxicity evaluation

Cytotoxic effects of Ag NCs@BC and plain BC on HeLa cell lines were assessed using LIVE/DEAD viability/cytotoxicity kit for mammalian cells. HeLa cells were grown on 96-well plates and treated for 12 hrs. Dual fluorescence staining solution (150 μ L) consisting of calcein acetoxymethyl ester (Calcein-AM) and ethidium homodimer (EthD-1) was used to stain the cells. Excitation and emission wavelengths of both fluoresceins were set at 494/517 nm for calcein acetoxymethyl ester and 528/617 nm for ethidium homodimer. Samples images were captured using a fluorescence microscope under 100x magnification for further analysis by the cellSens data analysis software. HeLa cells treated with ultrapure water were also tested under the same condition for comparing purpose.

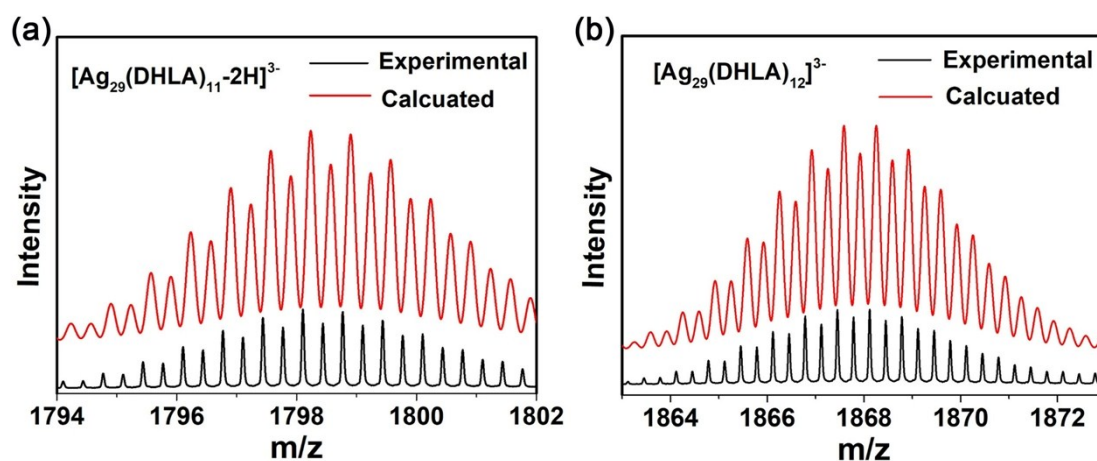


Fig. S1 Isotope patterns of DHLA-protected Ag_{29} NCs acquired experimentally (black curve) and theoretically (red curve).

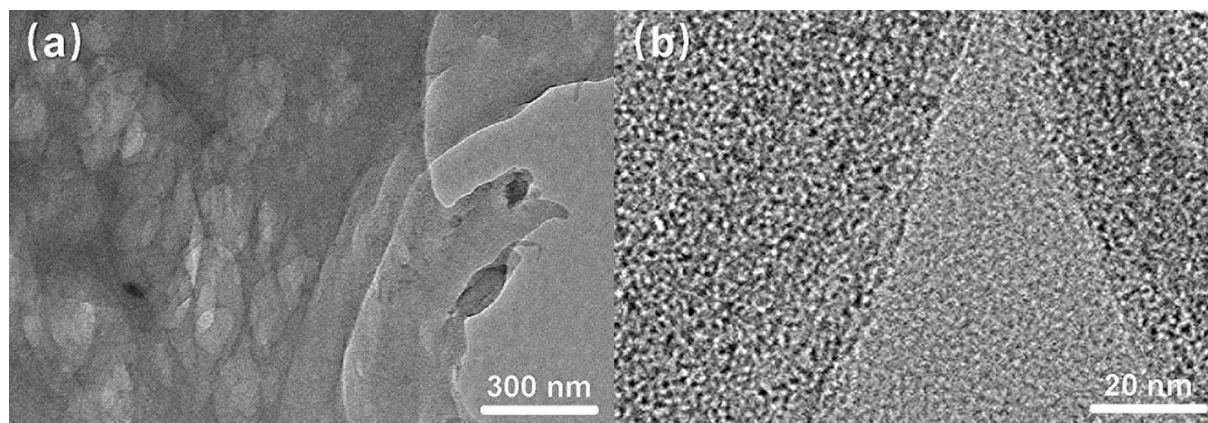


Fig. S2 TEM images of the Ag NCs@BC with different magnifications.

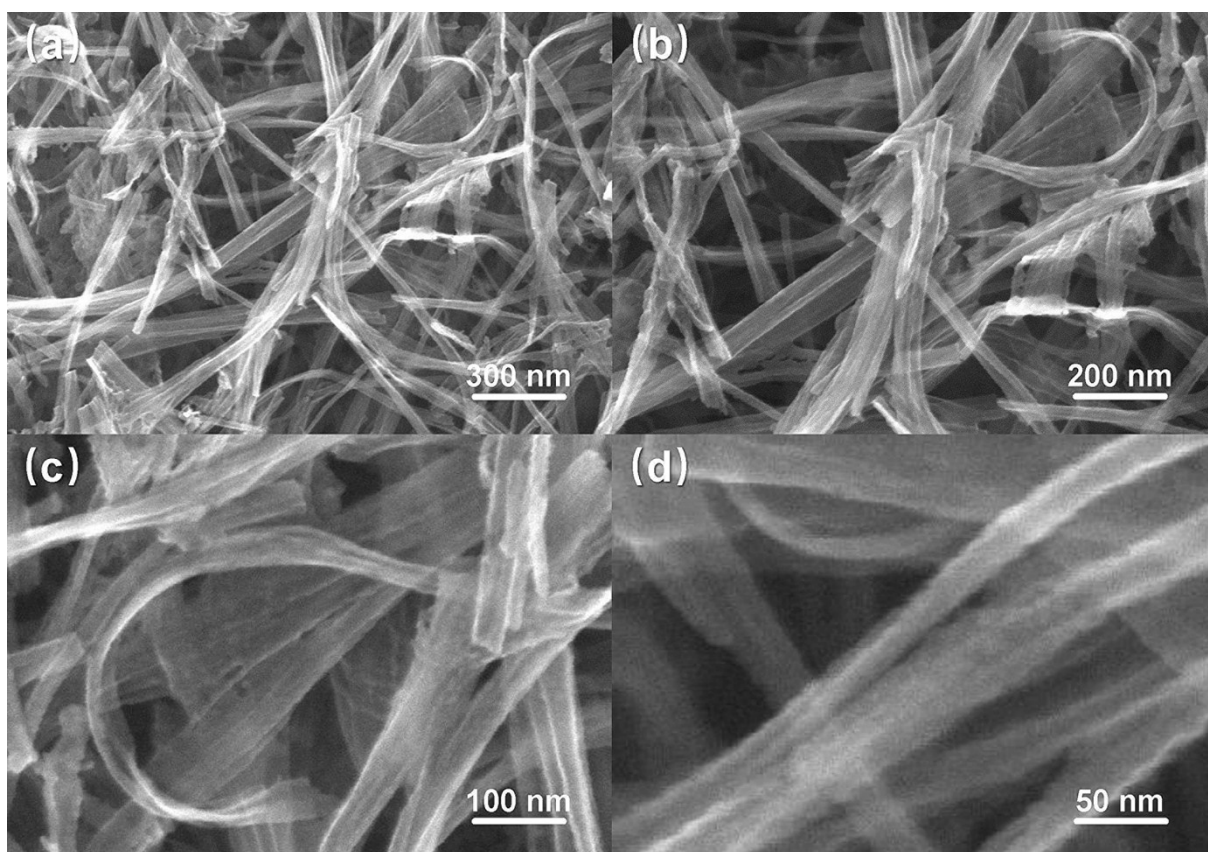


Fig. S3 FESEM images of the Ag NCs@BC with different magnifications.

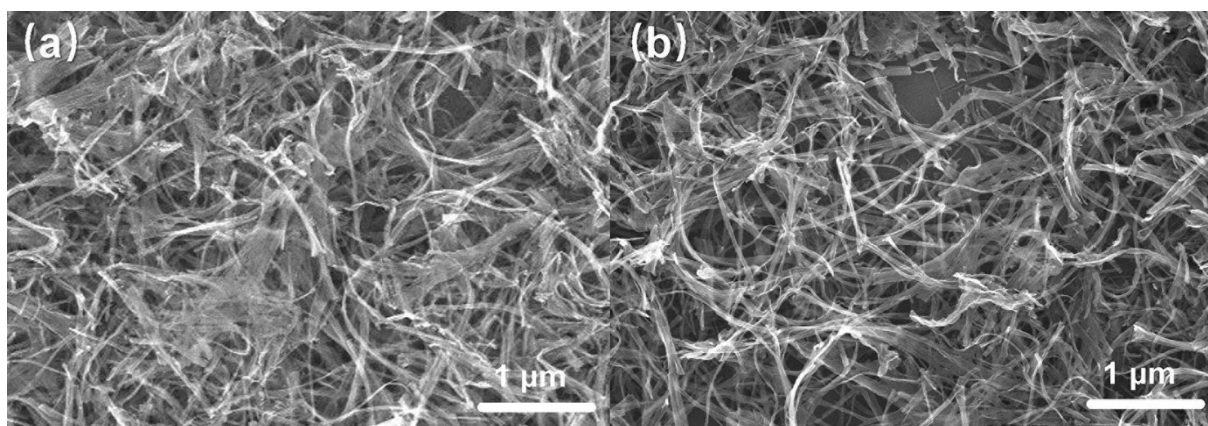


Fig. S4 Representative FESEM images of the Ag NCs@BC (a) and the pristine BC (b).

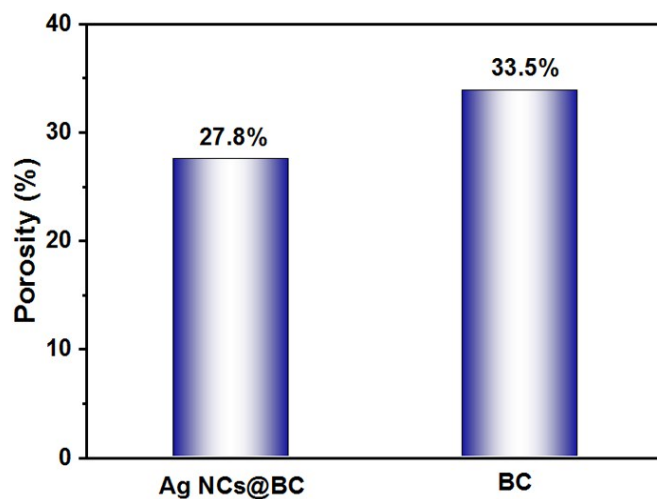


Fig. S5 The porosity comparison between Ag NCs@BC and plain BC.

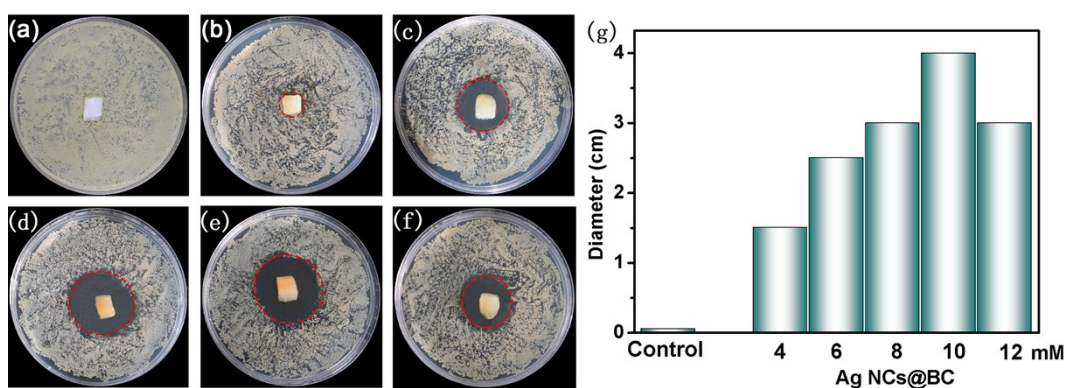


Fig. S6 Antibacterial activities of (a) plain BC as control and the Ag NCs@BC prepared in different concentrations of AgNO₃ solutions: (b) 4 mM, (c) 6 mM, (d) 8 mM, (e) 10 mM, and (f) 12 mM. (g) The diameters of the inhibition zones on agars treated with such Ag NCs@BC samples and plain BC (as control).

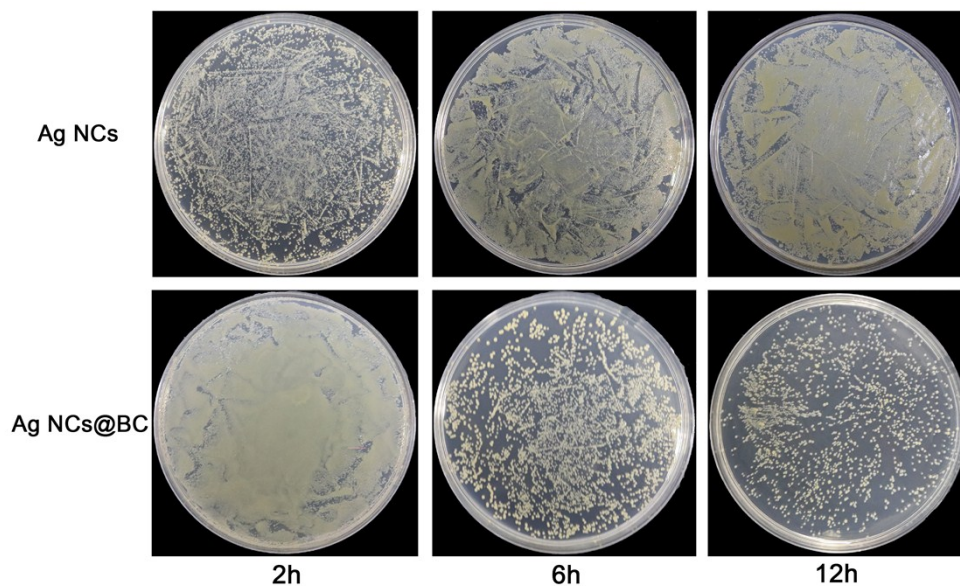


Fig. S7 Long-acting antibacterial results of Ag NCs@BC and Ag NCs solution after incubation with 1.5×10^8 bacteria for 12 h (The Ag content of Ag NCs@BC and Ag NCs solution is approximately 0.5 mg/mL). As shown, the Ag NCs@BC shows good antibacterial activity in a longer time, while Ag NCs could lose antibacterial efficacy quickly.

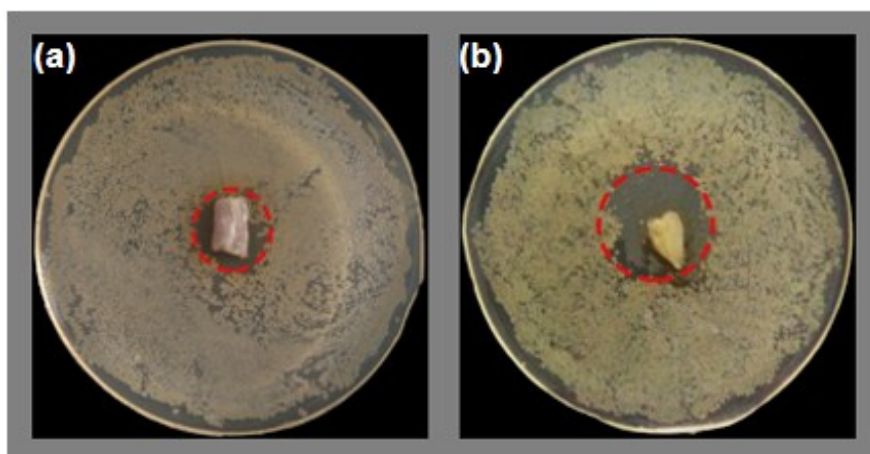


Fig. S8 Comparison in the antibacterial activities of (a) Ag NCs/BC and (b) Ag NCs@BC.

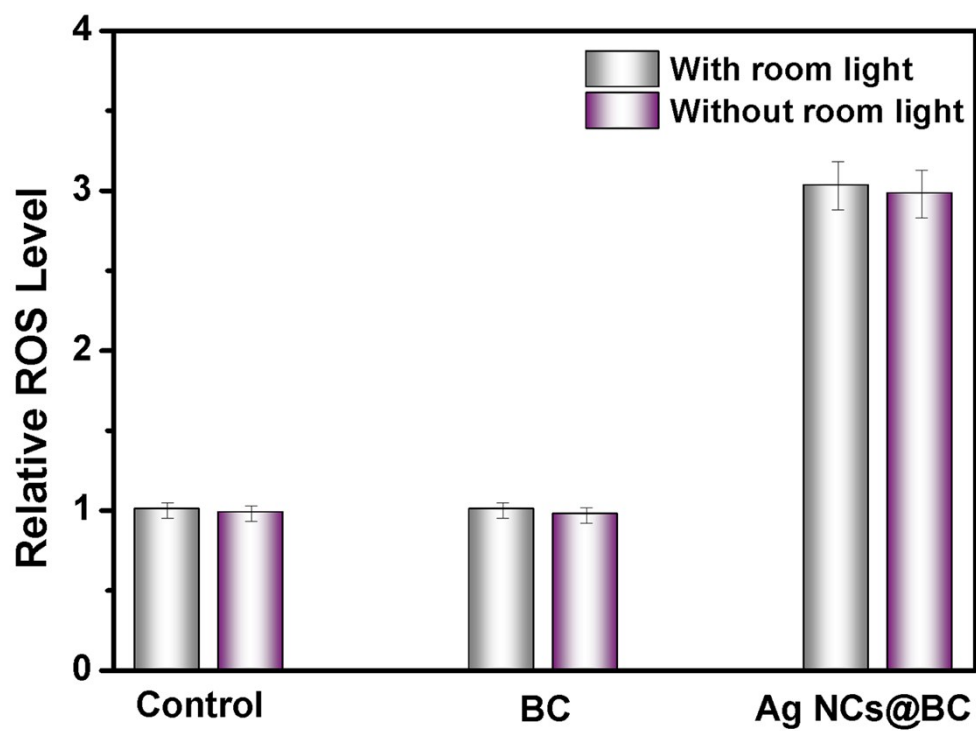


Fig. S9 The ROS level of the bacteria treated with Ag NCs@BC, plain BC, and ultrapure water used as control under room light irradiation or dark conditions.