Supporting Information

Facile fabrication of two-dimensional iodine nanosheets for antibacterial therapy

Yingmin Ye,[a, b] Yanmin Wang,[a, b] Kai Zhang,[a, b]* Wei Guo,[a, b] Tianyu Kong,[a, b] Xiaokang Ding,[a, b] Nana Zhao[a, b]* and Fujian Xu[a, b]*

1 Key Laboratory of Biomedical Materials of Natural Macromolecules (Beijing University of Chemical Technology, Ministry of Education), Beijing Laboratory of Biomedical Materials, Beijing University of Chemical Technology, Beijing, 100029, China

2 College of Materials Science and Engineering, Beijing University of Chemical Technology, Beijing, 100029, China

*To whom correspondence should be addressed

Email: zzhangkai@sina.com (K. Zhang); zhaonn@mail.buct.edu.cn (N. Zhao); xufj@mail.buct.edu.cn (F. J. Xu)

Material and reagents

Iodine powder was purchased from Acros (Belgium). Triethylamine (TEA) and ethanol (>99.7%) were provided by Beijing Chemical Works (China). The strains of *S. aureus* (CMCC (B) 26003) was obtained from Promega (Madison, USA). The Live/Dead^{TM} BacLight^{TM} bacterial viability kit (L7012) was bought from Invitrogen (LifeTechnologies, USA). A TIANamp bacteria DNA kit (DP302) was provided by TIANGEN Biotech (China). Cell counting kit-8 assay was obtained from Sangon Biotech Co., Ltd (Shanghai, China). The lysogeny broth (LB) medium in all bacterial cultures was pre-treated by autoclaving at 120 ^{o}C for 20 min before use.

Characterization

Scanning electron microscope (SEM) images were obtained from field emission SEM (Zeiss Supra 55) after platinum coating for 60 s. Transmission electron microscopy (TEM) measurements were observed by TEM (FEI Tecnai G2, USA and HT7800, Japan). For the preparation of the TEM specimen, the samples were dropped onto a formvar-covered copper grid, followed by drying naturally. Confocal laser scanning microscope (CLSM) images were conducted using an oil immersed 100× objective lens (Leica, SP8). Atomic force microscopy (AFM) images were obtained using an AFM system (Bruker, Dimension Icon). For preparing the AFM specimen, the samples were spin-coated on the substrate of a microscope cover slide (Assistant, Germany), followed by drying naturally.

Preparation of iodine nanosheets (NSs)

2D iodine NSs was prepared by ultrasonically exfoliating iodine bulk in an aqueous solvent[1]. Briefly, 40 mg of iodine bulk was dispersed in 8 mL of deionized water and subsequently sonicated in an ice bath for 4 h. Then, the sonicated mixed solution was centrifuged at 3000 rpm for 7 min to remove the precipitate and the supernatant was collected. The centrifugation was repeated three times to remove the bottom precipitate and finally obtain the upper suspension of 2D iodine NSs.

Preparation of bacterial suspensions

S. aureus (Gram-positive bacteria) was seeded in Luria-Bertani (LB) medium and incubated overnight at 37 ^{o}C with shaking (150 rpm) until the bacterial cultures grew to an optical density

(OD 600) ranging from 0.6 to 0.8. The bacterial cultures were collected by centrifugation at 4000 rpm for 5 min. Then, the bacteria were resuspended in phosphate buffer saline (PBS, 10 mM, pH=7.4) for further application.

In vitro antibacterial activity

The antibacterial activity of the iodine NSs was evaluated by the Cell Counting Kit-8 (CCK-8) assay. *S. aureus* was diluted with PBS to the final concentration of 2×10^6 CFU/mL. Then, 45 μL of iodine NSs solutions at different concentrations were mixed with 45 μL of bacterial suspensions, and the mixture was incubated into 96-well plates for 2 h. The final concentrations of iodine NSs were 0, 4, 8, 16, 32, 64, 128, and 256 μg/mL, respectively. After that, 10 μL of CCK-8 was added to each well and incubated for 4 h, and a cell imaging microplate detector was used to determine the bacterial viability of different groups.

Live/dead staining analysis

The bacterial viability with or without the treatment of iodine NSs was investigated by the LIVE/DEAD™ BacLight™ Bacterial Viability Kit. The suspensions of *S. aureus* (10^8 CFU/mL) were collected and incubated with PBS (as control) and iodine NSs for 6 h respectively. The final concentration of iodine NSs was 512 μg/mL. The co-interacted bacterial suspensions were then washed by centrifugation and collected. Then, *S. aureus* was double-stained with the mixture of SYTO 9 and propidium iodide (PI) in the dark for 15 min. Finally, the bacteria were imaged and observed through a CLSM (Leica, SP8) using an oil immersion 100× objective.

Morphology observation of bacteria

SEM imaging was employed to observe the morphology of bacteria after the treatment of iodine NSs. First, the suspensions of *S. aureus* (10^8 CFU/mL) were collected and co-cultured with or without the iodine NSs. Then, the mixtures were centrifuged at 4000 rpm for 5 min, and the precipitates were collected, washed, and resuspended in PBS buffer solution. Subsequently, the samples were fixed overnight at 4 °C with 2.5% glutaraldehyde. The samples were dropped onto silicon wafers, dried, dehydrated, and separately treated with 30, 50, 70, 90, and 100% ethanol in sequence for 5 min. Finally, the morphology of bacteria was observed through SEM.

Degradation of genomic DNA

S. aureus respectively received different treatments: (1) PBS (10 mM, pH=7.4) and (2) iodine NSs (512 μg/mL). After co-cultivation for 2 h, bacterial genomic DNA was extracted and collected using the TIANamp Bacterial DNA Kit (Tiangen, DP302) according to the protocol. Bacterial genomic DNA was measured by NanoDrop One (Thermo Scientific) and diluted to 5 μg/mL. Using SolarRed (Beijing, Solarbio) as a staining dye, the genomic DNA of different groups was evaluated by agarose gel electrophoresis (0.8% agarose gel, 110 V, 30 min) and imaged using the BioDoc-It imaging system.

Antibacterial activity *in vivo*

To investigate the antibacterial performance of iodine NSs in vivo, all animal experiments were approved by the Ethical Committee of Chinese Academy of Medical Sciences and Peking Union Medical College and performed under legal protocols. A subcutaneous model was established to assess in vivo antimicrobial performance. Briefly, female mice (BALB/c, 6 weeks) were anesthetized with isoflurane, and their right legs were shaved and disinfected with 75% ethanol. 100 μL of bioluminescent bacteria suspensions (10^8 CFU/mL) was injected subcutaneously into the shaved and disinfected leg areas of the mice. After 1 day, the mice were randomly divided into 2 groups (n=3) and received different treatments: (1) PBS (10 mM, pH 7.4), and (2) iodine NSs. For the iodine NSs-treated mice, the iodine NSs solutions were injected in situ into the infected area on days 0 and 2, and the bioluminescence intensity was recorded using a bio-optical imaging system on days 0, 2, 4, and 6, respectively. After 6 days of treatment, the mice were euthanized. The tissues from the abscess sites were collected and transferred to sterile PBS, and the bacteria were completely suspended by tissue grinding. Finally, the suspensions were serially diluted and spread on LB-agar plates for colony counting.

Toxicity determination

Blood was collected from the retro-orbital plexus of the mice received different treatments, and the serum was obtained after centrifugation at 3000 rpm for 15 min to analyze the serum urea nitrogen (BUN) and alanine aminotransferase (ALT). Hematological analysis was carried out by blood samples containing anticoagulants.

Cell viability analysis

The cytotoxicity of iodine NSs was assessed with L929 cells (mouse fibroblasts) using the CCK-8 assay. Briefly, L929 cells were seeded in 96-well plates at a cell density of 1×10^4 per well and incubated at 37 °C for 24 h. Then, the medium was moved and 100 μL of fresh medium containing iodine NSs at different concentrations (3.125, 6.25, 12.5, 25, 50, 100, 150, and 200 μg/mL) was added to each well and further incubated for another 24 h. Then, the culture medium containing CCK-8 (10 μL) was added to each well and incubated for 1 h, and the absorbance of water-soluble formazan at 450 nm was recorded using a cell imaging microplate detector (Bio Tek Instruments, Cytation3).

Hemolysis Analysis

The hemolytic activity of the iodine NSs was estimated by fresh mouse red blood cells (RBCs). The whole blood from mice was centrifuged at 2000 rpm for 15 min at 4 °C to obtain RBCs. The RBCs were washed with PBS to make the supernatant colorless and were diluted to 4% (v/v) with PBS. Erythrocyte suspensions (500 μL, 4% v/v in PBS) and iodine NSs solution at different concentrations were mixed to reach final concentrations of 3.125, 6.25, 12.5, 25, 50, 100, 150, and 200 μg/mL, respectively. The PBS was used as a negative control, while deionized water was used as a positive control. The mixture was incubated at 37 °C for 3 h and then centrifuged at 2000 rpm for 15 min. The supernatant was transferred to a 96-well plate and the absorbance values at 540 nm were recorded using a cell imaging microplate detector (Bio Tek Instruments, Cytation3). The percentage of hemolysis is calculated as follows:

$$Hemolysis\ (\%) = \frac{OD_{I_2 540} - OD_{negative540}}{OD_{positive540} - OD_{negative540}} \times 100\%$$

Fig. S1. Photograph of the bulk iodine.

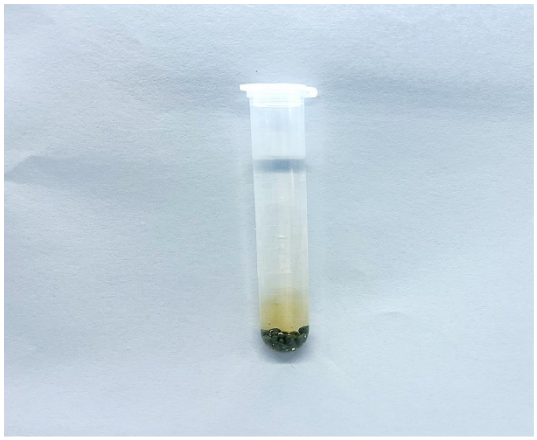

Fig. S2. Photograph of bulk iodine precipitated at the bottom of an aqueous solution.

Fig. S3. Photograph of a dispersion of iodine NSs.

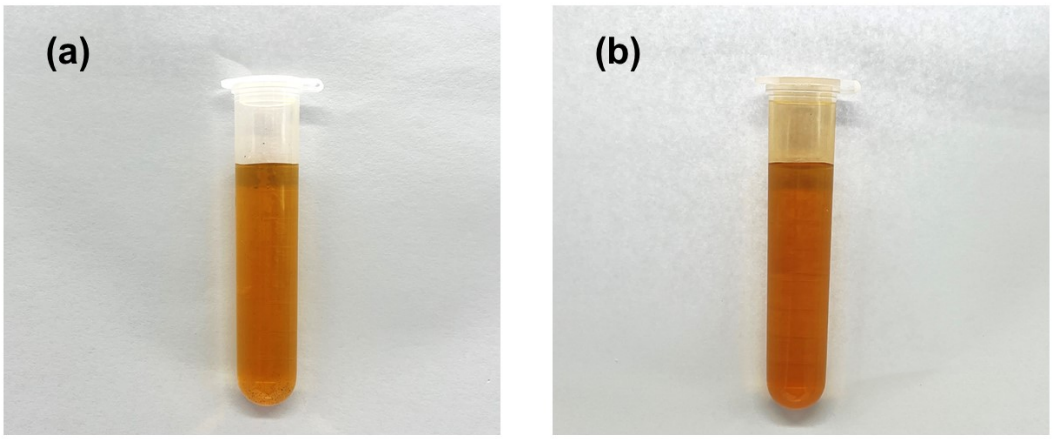

Fig. S4. Photograph of iodine NSs before (a) and after (b) 3 days.

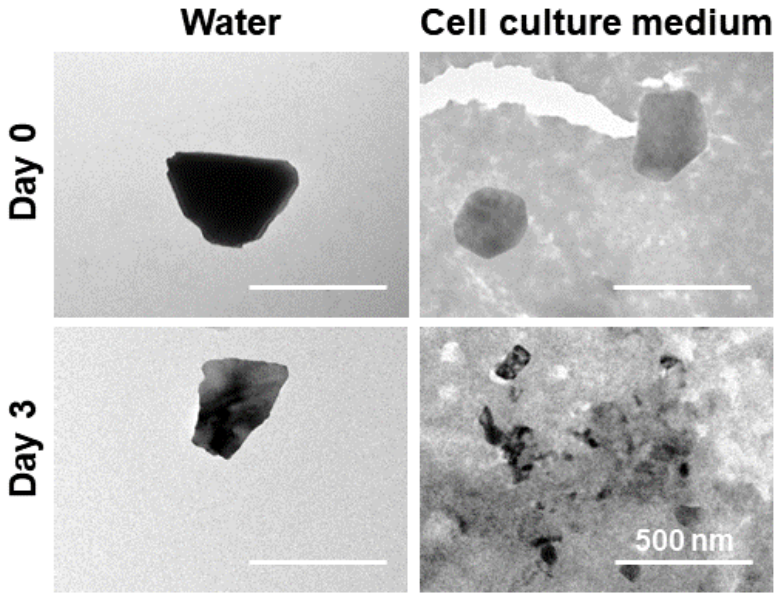

Fig. S5. TEM images of iodine NSs before and after 3 days in water and cell culture medium environment.

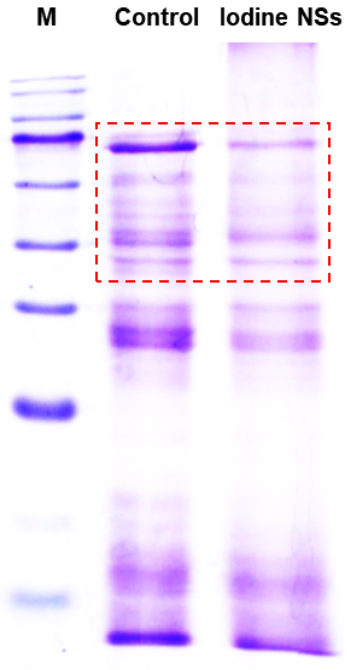

Fig. S6. Protein images of *S. aureus* treated without or with iodine NSs.

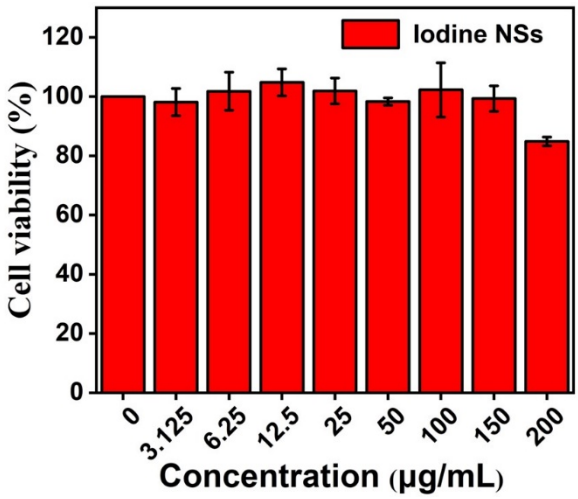

Fig. S7. The cytotoxicity analysis of iodine NSs at different concentrations.

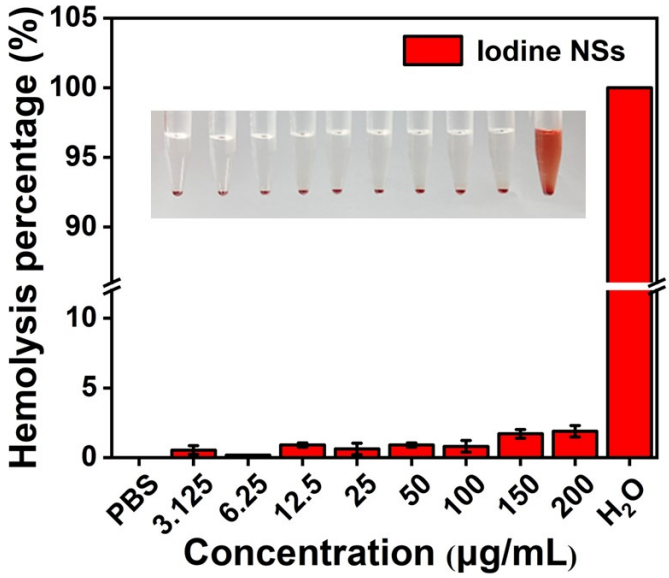

Fig. S8. The relative hemolysis ratio of iodine NSs at different concentrations.

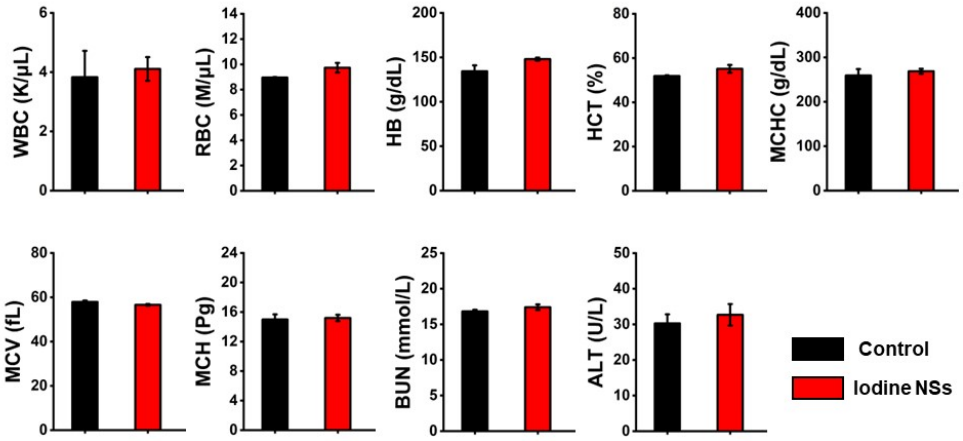

Fig. S9. The blood physiological indexes of mice with or without the treatment of iodine NSs.

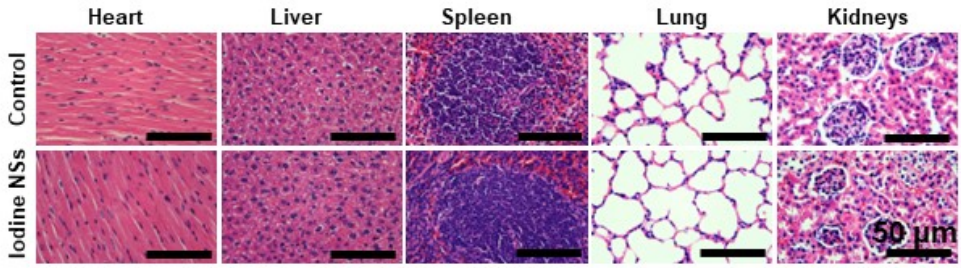

Fig. S10. The H&E images of the major organs from the mice with or without the treatment of iodine NSs.

References:

[1] M. Qian, Z. Xu, Z. Wang, B. Wei, H. Wang, S. Hu, L.-M. Liu and L. Guo, *Adv. Mater.*, 2020, **32**, 2004835.