Supporting Information

Multifunctional Two Dimensional Bi$_2$Se$_3$ Nanodiscs for Combined Antibacterial and Anti-Inflammatory Therapy of Bacterial Infections

Jiang Ouyang, a Mei Wen, a Wansong Chen,*, a,b Yanni Tan, Zhenjun Liu, a Qunfang Xu, a Ke Zeng, a Liu Deng, a and You-Nian Liu*, a,b

a College of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan 410083, P. R. China. E-mail: chenws@csu.edu.cn; liuyounian@csu.edu.cn
b State Key Laboratory for Powder Metallurgy, Central South University, Changsha, Hunan 410083, P. R. China.

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1. Experimental Procedures

1.1 Materials. Sodium selenite (Na$_2$SeO$_3$), bismuth nitrate pentahydrate (Bi(NO$_3$)$_3$·5H$_2$O), sodium borohydride (NaBH$_4$), poly(vinylpyrrolidone) (PVP, Mw = 55000), and bulk Bi$_2$Se$_3$ were purchased from Alfa Aesar. Ethylene glycol and hydrazine hydrate (N$_2$H$_4$·H$_2$O) were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). L929, HEK 293T and 4T1 cells were obtained from Xiangya Hospital of Central South University (Changsha, China). 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) and 1,3-diphenylisobenzofuran (DPBF) were purchased from Heowns Biochemical Technology (Tianjin, China). Reduced glutathione (GSH), DMEM culture medium, fetal bovine serum (FBS), penicillin-streptomycin and trypsin were purchased from Thermo Fisher Scientific (Beijing, China). Propidium iodide (PI), 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) and phosphate buffered saline (PBS) were purchased from Beyotime Biotechnology (Shanghai, China). Bacterial culture media were provided by Dingguo Changsheng Biotechnology (Beijing, China). All the chemicals were analytical reagent grade and used without further purification.

1.2 Synthesis of the Bi$_2$Se$_3$ nanodiscs. PVP (Mw ≈ 55 000, 0.5 g) and Na$_2$SeO$_3$ (0.121 g) were dissolved in 24 mL of ethylene glycol. Then Bi(NO$_3$)$_3$·5H$_2$O (0.226 g) in 30 mL of ethylene glycol was added into the solution under magnetic stirring. The mixture solution was gradually heated to 180 °C under nitrogen atmosphere. During the reaction, the mixture solution became milky white and then turned faint yellow. Afterwards, 10 mL of ethylene glycol containing N$_2$H$_4$·H$_2$O (1 mL) was rapidly injected into the reaction solution and the reaction was proceeded for another 30 min. After cooling to room temperature, Bi$_2$Se$_3$ nanodiscs were centrifuged at 12000rpm for 20 min, and washed with acetone/water (5:1, v/v) for three times.

1.3 Bacterial culture. E.coli (ATCC25922), P. aeruginosa (ATCC27853), P. vulgaris (CMCC49027), S. aureus (ATCC43300), E. faecalis (ATCC29212) and B. subtilis (ATCC6633) were obtained from Xiangya Hospital of Central South University (Changsha, China). E.coli, P. aeruginosa and P. vulgaris were cultured in Luria–Bertani (LB) medium (tryptone 0.5%, yeast extract 0.25%, NaCl 1%, pH 7.4). S. aureus, E. faecalis and B. subtilis were cultured in tryptic soy broth (TSB) culture medium (pancreatic digest of casein 0.85%, papain digest of soybean 0.15%, glucose monohydrate 0.125%, NaCl 0.5% and K$_2$HPO$_4$ 0.25%, pH 7.4). All the bacteria were culture at 37 °C on a shaking incubator. Bacterial density was recorded on a turbidimeter (WGZ-XT, Qiwei Instrument Co., LTD, China).
1.4 Antibacterial activity. To 0.1 mL of bacteria suspension (2 × 10^6 CFU mL⁻¹) in fresh culture medium, 0.1 mL of Bi₂Se₃ nanodiscs (0, 10, 20, 50, 100 or 200 μg mL⁻¹) in culture medium were added. After 12 h of incubation, bacterial suspension was diluted and cultured on agar plates for colony counting. Bacterial viability was calculated according to equation (1).

\[
\text{Bacterial viability (\%)} = \frac{N_T}{N_C} \times 100\%
\]

where \(N_T\) and \(N_C\) represent the colony numbers with and without the treatment of Bi₂Se₃ nanodiscs. To compare the antibacterial activity of PVP, bulk Bi₂Se₃, Bi(NO₃)₃, Na₂Se and Bi₂Se₃ nanodiscs, the experiment was performed following the above mentioned procedure. The concentrations of PVP, bulk Bi₂Se₃, Bi(NO₃)₃, Na₂Se and Bi₂Se₃ nanodiscs were 200, 50, 60, 29, and 50 μg mL⁻¹, respectively. The antibacterial activity of Bi₂Se₃ nanodiscs against P. aeruginosa, P. vulgaris, E. faecalis and B. subtilis was measured following the similar procedure, and the concentration of Bi₂Se₃ nanodiscs was 50 μg mL⁻¹.

1.5 Morphological characterizations of bacteria. S. aureus bacteria suspension (~1 × 10^6 CFU mL⁻¹) was treated with Bi₂Se₃ nanodiscs (50 μg mL⁻¹) at 37 °C for 12 h. Then S. aureus bacteria were collected by centrifugation at 10000 rpm for 5 min. After washing with PBS for twice, S. aureus bacteria were fixed by paraformaldehyde for 2 h, and dehydrated with ethanol at different concentrations (10%, 25%, 50%, 75%, 90% and 100%). Bacterial morphology was observed under a scanning electron microscope (Nova NanoSEM230, FEI, USA). For TEM imaging, dehydrated bacteria were embedded in epoxy resin, sliced and stained with 3% uranyl acetate. TEM images were taken on a transmission electron microscope (Tecnai G2 Spirit, FEI, USA).

1.6 Cytotoxicity and hemolysis studies. Cells (L929, HEK 293T, 4T1, HUVEC and L-02) were provided by Xiangya Hospital of Central South University and cultured in DMEM medium at 37 °C under 5% CO₂ atmosphere. Cells in logarithmic phase were collected and seeded in 96-well plate (1 × 10^4 per well). Bi₂Se₃ nanodiscs in cell culture medium at different concentrations (0, 10, 25, 50 and 100 μg mL⁻¹) were cultured with cells for 24 h. All the cells were washed with PBS, and the viability was measured using CCK-8 cell viability assay kit (Beyotime Biotechnology, China). For hemolysis study, red blood cells were separated from mouse whole blood and washed with PBS for three times before usage. Then 0.25 mL of 4% red blood cells were mixed with 0.25 mL of Bi₂Se₃ nanodiscs (0, 10, 20, 50 and 100 μg mL⁻¹) in PBS, and incubated at 37 °C for 8 h. Afterwards, the mixture solution was centrifuged to remove red blood cells and Bi₂Se₃.
nanodiscs. The absorption of released hemoglobin at 540 nm (OD_{540nm}) was measured on UV-vis spectrophotometer. Hemolysis rates were calculated according to equation (2).

\[
\text{Hemolysis (\%) = } \frac{OD_{540nm}}{OD_T} \times 100\% \tag{2}
\]

where OD_T is the absorption of red blood cells after complete hemolysis in pure water.

1.7 LDH release assay. S. aureus bacteria (1 \times 10^6 CFU mL\(^{-1}\)) were treated with Bi\(_2\)Se\(_3\) nanodiscs (0, 10, 25 and 50 \(\mu\)g mL\(^{-1}\)) at 37 °C for 12 h. Afterwards, the mixture solution was centrifuged at 10000 rpm for 10 min, and the supernatants were collected for lactase dehydrogenase measurement using LDH assay kit (Beyotime Biotechnology, China). LDH leakage rate was calculated according to equation (3).

\[
\text{LDH leakage (\%) = } \frac{C - C_0}{C_T - C_0} \times 100\% \tag{3}
\]

C_0 and C represent LDH leakage from untreated bacteria and bacteria treated by Bi\(_2\)Se\(_3\) nanodiscs, respectively. C_T is the total LDH leakage from bacteria treated with 1% Triton X-100.

1.8 Antibacterial mechanism studies. The binding capacity of Bi\(_2\)Se\(_3\) nanodiscs to lipoteichoic acid or lipopolysaccharide was measured. In detail, 0.1 mL of Bi\(_2\)Se\(_3\) nanodiscs (200 \(\mu\)g mL\(^{-1}\)) in water were mixed with 0.1 mL of lipoteichoic acid or lipopolysaccharide aqueous solution (1 mg mL\(^{-1}\)). After 2 h of incubation, the mixture solution was centrifuged (12000 rpm, 10 min). The left lipoteichoic acid or lipopolysaccharide in the supernatant was quantified using colorimetric phosphate assay kit (AAT Bioquest Inc., USA). The binding capacity of Bi\(_2\)Se\(_3\) nanodiscs to lipoteichoic acid or lipopolysaccharide was calculated according to equation (4).

\[
\text{Binding capacity} = \frac{W_T - W_S}{W_B} \tag{4}
\]

where W_T was the total weight of lipoteichoic acid or lipopolysaccharide, W_S was the weight of lipoteichoic acid or lipopolysaccharide in the supernatant, and W_B was the weight of Bi\(_2\)Se\(_3\) nanodiscs. Antibacterial activity of Bi\(_2\)Se\(_3\) nanodiscs in the presence of lipoteichoic acid or lipopolysaccharide was investigated. Briefly, 0.1 mL of Bi\(_2\)Se\(_3\) nanodiscs (50 \(\mu\)g mL\(^{-1}\)) in TSB culture medium were mixed with lipoteichoic acid or lipopolysaccharide (0.2, 0.4 and 1.0 mg mL\(^{-1}\)). Then 0.1 mL of S. aureus bacteria (2 \times 10^6 CFU mL\(^{-1}\)) in TSB culture medium were added into the mixture solution and cultured for 12 h. Then the bacterial viability of S. aureus bacteria was calculated according to equation (1).

1.9 Antibacterial activity against bacteria in biofilms
S. aureus bacteria in stationary phase were seeded in 96-well plate and cultured in TSB medium. After 24 h of incubation, the medium in each well was removed, and the obtained biofilms were carefully washed with PBS for twice to remove unbound bacteria. Then, 0.1 mL of TSB medium was then added into each well, followed by the addition Bi₂Se₃ nanodiscs solution in medium (0.1 mL, 100 μg mL⁻¹). After 12 h of incubation, the supernatants were discarded and the biofilms were stained by Hoechst 33342 and PI. The fluorescent images were taken on a confocal microscopy (LSM800, ZEISS, Germany). Bacterial viability was detected on agar plates.

To study the formation of biofilms in the presence of Bi₂Se₃ nanodiscs, S. aureus bacteria were cultured with or without Bi₂Se₃ nanodiscs in 24 well plate for 24 h. Afterwards, biofilms on the bottom of plate were washed with PBS twice and stained with crystal violet. Crystal violet on biofilms were dissolved in ethanol and quantified on microplate reader (OD 590nm). Relative biofilm mass was calculated according to equation (5):

\[
Biofilm\ mass\ (\%) = \frac{m_0}{m_T} \times 100\%
\]

where \(m_T\) and \(m_0\) represent OD 590nm values without or with Bi₂Se₃ treatment, respectively.

1.10 GSH consumption assay. GSH (0.2 mL, 2 mM) was mixed with 0.2 mL of Bi₂Se₃ nanodiscs in water (20, 50, 100 μg mL⁻¹), and incubated at 37 °C for 1 h. Then 10 μL of mixture solution was added into 150 μL of DTNB (0.32 mM) in PBS. After another 0.5 h, the absorption of the reacting solution at 405 nm was measured on a UV-vis spectrometer. The relative GSH content was calculated following equation (6).

\[
GSH\ (% ) = \frac{C_0}{C_T} \times 100\%
\]

where, \(C_T\) and \(C_0\) represent GSH content before and after Bi₂Se₃ treatment, respectively.

1.11 Drug resistance assay. To induce drug resistance, S. aureus bacteria were incubated with Bi₂Se₃ nanodiscs (15 μg mL⁻¹) or doxycycline (5 μg mL⁻¹) for 10 passages. The antibacterial activity of Bi₂Se₃ nanodiscs or doxycycline against S. aureus at different passages (passage 1, 3, 6 and 10) was measured following the above mentioned procedures.

1.12 Cyclic voltammetry. Bi₂Se₃ nanodiscs (25 μg mL⁻¹) were dispersed in 0.1 M KNO₃. Glassy carbon disk with 3 mm diameter was used as the working electrode, platinum wire was taken as auxiliary electrode, and Ag/AgCl electrode was employed as reference electrode. The cyclic voltammetry was conducted on an
electrochemical workstation (5200F, Zhengzhou Shiruisi Instrument Co., Ltd, China) at a scan rate of 50 mV s⁻¹.

1.13 In vitro ROS scavenging and anti-inflammation studies. Bi₂Se₃ nanodiscs (0, 10, 25, 50 μg mL⁻¹) in water was incubated with H₂O₂ (100 μM) or NaClO (100 μM) aqueous solution for 2 h. H₂O₂ concentration was measured using H₂O₂ quantitative assay kit (Sangon Biotech., China). DPBF was employed as a colorimetric probe to quantify the concentration of NaClO. Mice were provided by Hunan SJA Laboratory Animal Co. Ltd (Changsha, China). Animal experiments were carried out in compliance with the regulation approved by the Laboratory Animal Center of Central South University (protocol number 201803587). Male BALB/c mice (6 weeks old) were euthanized, and peritoneal macrophages were collected. Then peritoneal macrophages were plated into 96 well plates at the density of 2 × 10⁴ per well and cultured overnight. Afterwards, macrophages were pretreated with Bi₂Se₃ nanodiscs (50 μg mL⁻¹) for 5 h. Meanwhile, macrophages without the pretreatment of Bi₂Se₃ nanodiscs were used for comparison. The cell culture medium was then removed, and macrophages were stimulated by lipoteichoic acid (500 μg mL⁻¹) in culture medium for 12 h. For in vitro ROS scavenging study, macrophages were stained by DCFH-DA (20 μM) for 30 min, and the fluorescent images were taken under an inverted fluorescence microscope (IX 83, Olympus, Japan). For anti-inflammation studies, macrophages were pretreated with Bi₂Se₃ nanodiscs (10, 25 and 50 μg mL⁻¹) for 5 h. Then lipoteichoic acid (500 μg mL⁻¹) in culture medium was used to stimulate macrophages for another 12 h. Afterwards, the supernatants were collected, and the contents of inflammation markers (IL-6 and TNF-α) were detected using mouse IL-6 ELISA kit and mouse TNF-α ELISA kit (NeoBioscience, China), respectively. Macrophages were lysed by cell lysis buffer (Beyotime Biotechnology, China), and the intracellular NO was measured using total NO assay kit (Beyotime Biotechnology, China).

1.14 In vivo studies. Male BALB/c mice (6 weeks) were provided by Hunan SJA Laboratory Animal Co. Ltd (Changsha, China). Mice were randomly divided into 3 groups (n = 6 in each group): (1) blank control; (2) bacteria; (3) bacteria + Bi₂Se₃ nanodiscs. The experiments were carried out in compliance with the regulation approved by the Laboratory Animal Center of Central South University (protocol number 201803587). To construct in vivo bacterial infection model, 50 μL of S. aureus bacteria (~1 × 10⁸ CFU mL⁻¹) was subcutaneously injected into BALB/c mice. After 6 h post injection, the infected mice were subcutaneously injected with 50 μL of Bi₂Se₃ nanodiscs (2 mg mL⁻¹) in saline. The bacterial infection areas were monitored every two days. After 11 days, mice were sacrificed and blood was collected. The
concentrations of cytokines in serum were measured using CBA assay kit (BD Biosciences, USA). The infected skins were excised for H&E staining. Neutrophils were stained with PE labeled anti-mouse Ly-6G/Ly-6C (Gr-1) antibody (108407, Biolegend, USA), while the nuclei were stained with Hoechst 33342. Besides, the infected skins were homogenized in sterile PBS, and the bacterial density was quantified through agar plate assay.

1.15 Biosafety study. Mice were randomly divided into 3 groups (n = 6 in each group): (1) blank control; (2) mice injected with Bi₂Se₃ nanodiscs and sacrificed on day 5; (3) mice injected with Bi₂Se₃ nanodiscs and sacrificed on day 11. To study the in vivo biosafety, mice in group (2) and (3) were received intravenous injection of Bi₂Se₃ nanodiscs (at the dosage of 4 mg kg⁻¹). On day 5, mice in group (2) were sacrificed for blood collection. On day 11, mice in group (1) and (3) were sacrificed for blood collection. Blood cell number and blood biochemistry were analyzed on an auto haematology analyzer (BC-2800 Vet, Mindray, China) and Pointcare V2 blood chemistry analyzer (MNCHIP, China). Main organs (heart, liver, spleen, lung and kidney) were also collected for H&E staining.
2. Supplementary Figures and Table

![Fig. S1](image1.png)  
**Fig. S1** a) EDS analysis and b) XRD spectrum of Bi$_2$Se$_3$ nanodiscs.

![Fig. S2](image2.png)  
**Fig. S2** a) AFM image of Bi$_2$Se$_3$ nanodiscs, and b) the height profile along the white line.

![Fig. S3](image3.png)  
**Fig. S3** a) Full-scan XPS spectrum of Bi$_2$Se$_3$ nanodiscs and b) individual spectra of Bi and Se.
**Fig. S4** Raman spectrum of bulk Bi$_2$Se$_3$ nanodiscs.

**Fig. S5** Size distributions of Bi$_2$Se$_3$ nanodiscs in PBS, DMEM cell culture medium with or without 10% fetal bovine serum (FBS).
Fig. S6 Size stability over time of Bi$_2$Se$_3$ nanodiscs dispersed in water. Error bars indicate standard deviations (n = 3).

Fig. S7 Antibacterial activity of PVP, bulk Bi$_2$Se$_3$ (B-Bi$_2$Se$_3$), Bi(NO$_3$)$_3$, Na$_2$Se and Bi$_2$Se$_3$ nanodiscs against *E.coli*. Error bars indicate standard deviations (n = 3).

Fig. S8 Cytotoxicity of Bi$_2$Se$_3$ nanodiscs to 4T1, HEK 293T, L929, HUVEC and L-02 cells. Error bars indicate standard deviations (n = 3).
Fig. S9 Hemolysis percentages of red blood cells in the presence of Bi$_2$Se$_3$ nanodiscs at different concentrations. Error bars indicate standard deviations (n = 3).

Fig. S10 Antibacterial profiles of Bi$_2$Se$_3$ nanodiscs and doxycycline against S. aureus after repetitive treatments at sub-lethal concentrations. Error bars indicate standard deviations (n = 3) and statistics were determined by one way analysis of variance (** p < 0.01).
Fig. S11 Chemical structure of (a) lipoteichoic acid$^{1-3}$ and (b) lipopolysaccharide$^4$.

Fig. S12 Antibacterial activity of Bi$_2$Se$_3$ nanodiscs (25 μg mL$^{-1}$) against S. aureus in the presence of lipopolysaccharide (LPS). Error bars indicate standard deviations (n = 3) and statistics were determined by one way analysis of variance (n.s. means no significant difference).
**Fig. S13** a) Bactericidal activity of Bi$_2$Se$_3$ nanodiscs (50 μg mL$^{-1}$) against *S. aureus* embedded in biofilms: (i) bright filed, (ii) fluorescence imaging with Hochest 33342 and PI, (iii) agar plate assay (scale bars = 50 μm). b) Biofilm formation in the presence of Bi$_2$Se$_3$ nanodiscs at different concentrations. Error bars indicate standard deviations (n = 3) and statistics were determined by one way analysis of variance (**p < 0.01).

From the bright field images in **Fig. S13a**, amounts of Bi$_2$Se$_3$ nanodiscs with brown color were attached on *S. aureus* biofilms, which could be ascribed to the strong interactions between Bi$_2$Se$_3$ nanodiscs and lipoteichoic acid on biofilms. The viability of bacteria embedded in biofilms were checked through fluorescent staining. All the bacteria were stained by Hochest 33342 with blue fluorescence; meanwhile, propidium iodide (PI) as membrane impermeable dye only stains the dead bacteria with red fluorescence. According to the results in **Fig. S13a**, most bacteria were dead with red fluorescence when treated with Bi$_2$Se$_3$ nanodiscs. Agar plate assay further confirmed that almost all *S. aureus* bacteria lost viability after the treatment.
Because both Bi$_2$Se$_3$ nanodiscs and biofilms present negative charge (Table S1), the statistic interactions between Bi$_2$Se$_3$ nanodiscs and biofilms can be ruled out. It is reported that biofilm matrices are rich in micrometer size channels (0.4 to 1 μm). Therefore, Bi$_2$Se$_3$ nanodiscs with average size around 90 nm are believed to be able to penetrate into biofilms. As lipoteichoic acid is the component of biofilms, the specific interactions of Bi$_2$Se$_3$ nanodiscs with lipoteichoic acid could facilitate their penetration into biofilms and then interact with S. aureus. The formation of biofilms in the presence of Bi$_2$Se$_3$ nanodiscs was also investigated using crystal violet staining assay. The result in Fig. S13b shows that Bi$_2$Se$_3$ nanodiscs (50 μg mL$^{-1}$) remarkably reduce biofilm formation by 84%.

**Fig. S14** GSH consumption by Bi$_2$Se$_3$ nanodiscs at different concentrations. Error bars indicate standard deviations (n = 3) and statistics were determined by one way analysis of variance (n.s. means no significant difference).
**Fig. S15** Live/dead staining of macrophages with or without the treatment of Bi$_2$Se$_3$ nanodiscs (50 μg mL$^{-1}$). The live and dead macrophages were stained by calcein-AM and PI with green fluorescence and red fluorescence, respectively (scale bar = 200 μm). The images clearly show that almost all the macrophages present green fluorescence, indicating their high cell viability.
Fig. S16 The excellent biocompatibility of Bi$_2$Se$_3$ nanodiscs in vivo. (a) H&E staining of main organs after injection of Bi$_2$Se$_3$ nanodiscs (scale bar = 100 μm). (b-i) Blood cell counts and biochemistry analysis of mice after injection of Bi$_2$Se$_3$ nanodiscs. Red blood cells (RBC), white blood cells (WBC), platelets (PLT) and haemoglobin (HGB) were measured. Markers of liver function include glutamic oxaloacetic transaminase (AST) and total bilirubin (T-Bil). Markers of kidney function include blood urea nitrogen (BUN) and creatinine (Cr). The yellow areas indicate the normal ranges. Error bars indicate standard deviations (n = 6).
Table S1. Zeta potential measurements.

<table>
<thead>
<tr>
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<th>Bi$_2$Se$_3$</th>
<th>S. aureus</th>
<th>E. coli</th>
</tr>
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<tbody>
<tr>
<td>Zeta potential</td>
<td>$-5.2 \pm 0.5$</td>
<td>$-13.3 \pm 0.9$</td>
<td>$-12.5 \pm 0.7$</td>
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$^a$ Results are presented as mean ± S.D. (n = 3).

References