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Metabolic Labeling Mediated Visualization, Capture, and Inactivation of Gram-Positive Bacteria though Biotin-Streptavidin Interaction

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Materials and methods

Materials. BD, BDN, and BPDN were synthesized by Bankpeptide Biological Technology Co., Ltd. and were used without further purification. *S. aureus* ATCC 6538 and *E. coli* BL21 were obtained from China Center of Industrial Culture Collection (Beijing, China). *E. faecalis* 29212 was purchased from Shanghai Microbiological Culture Center Collection. Streptavidin-modified FITC (SA-FITC) was purchased from APEBIO Biotechnology Co., Ltd. (USA). Streptavidin-coated magnetic beads were purchased from Thermo Fisher Scientific. Streptavidin-modified Cy3 (SA-Cy3) was obtained from Beijing Biosynthesis Biotechnology Co., Ltd. Streptavidin-coated gold nanocages (SA-AuNCs) were acquired from Beijing Zhongke Keyou Technology Co., Ltd. D-Proline, glutaraldehyde, proteinase K, lysozyme, and sodium chloride were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. Polyethylene octylphenyl ether (Triton X-100) was purchased from Beijing Solarbio Science & Technology Co.,Ltd. Sodium phosphate buffer solution and defibrinated sheep blood were purchased from Shanghai yuanye Bio-Technology Co., Ltd. Sodium dodecyl sulfate (SDS) was purchased from Sinopharm Chemical Reagent Co., Ltd. Phosphate-buffered saline (PBS), DMEM and fetal bovine serum (FBS) were purchased from Shanghai Adamas Reagent Co., Ltd.

Bacteria culture. Bacterial cells were initially stored at -80°C. They were taken out, inoculated into LB medium, and incubated overnight at 37°C. Subsequently, 1 mL of the activated bacterial culture was diluted in LB medium to an optical density at 600 nm (OD_{600}) of approximately 0.1 for use in subsequent experiments.

Confirmation of optimal labeling time. To determine the optimal labeling time, 0.4 mM BDN was added to bacterial cultures and incubated with bacteria for various durations (1 h, 2 h, 4 h, 6 h). Following centrifugation and removal of the supernatant, the bacteria were washed three times with sterile PBS and resuspended in PBS. Subsequently, a solution of 0.1 mM SA-FITC was added, and the mixture was incubated for an additional 2 h. Afterward, the samples were washed three times with PBS, resuspended in PBS, and analyzed using flow cytometry.

Confirmation of optimal staining time. To determine the optimal staining time, bacterial cultures labeled with BDN were treated with a 0.2 mM solution of SA-FITC or a 50 μM solution of SA-Cy3. The mixtures were incubated for various durations (0.5 h, 1 h, 2 h, 4 h). Following incubation, the samples were washed three times with PBS, resuspended in PBS, and analyzed using flow cytometry.

Concentration dependence of labeling. *S. aureus* treated with different concentrations of BDN (50, 100, 200, 400, and 800 μM), followed by staining with 100 μM SA-FITC. After washing three times with PBS, the samples were analyzed using flow cytometry.

Competition experiment. *S. aureus* and *E. faecalis* with an OD_{600} of approximately 0.1

were treated with 0.4 mM labeling probe or 0.2 mM labeling probe and 0.2 mM D-Ala. After incubation for 4 h (2 h for *E. faecalis*), the samples were washed three times with PBS. Subsequently, a 0.2 mM solution of SA-FITC or a 50 μM solution of SA-Cy3 was added, and the mixtures were incubated for an additional 2 h. Following washing three times with PBS, the samples were analyzed using flow cytometry.

Peptidoglycan isolation. We followed the protocol described in previous studies[30]. Briefly, in a 50 mL culture of *S. aureus* with an OD_{600} of approximately 0.1, solution of BDN were added to a final concentration of 0.4 mM and incubated with bacteria for 4 hours, followed by centrifugation to discard the supernatant. The bacterial cells were then washed three times with 50 mL PBS and resuspended in PBS. Subsequently, the samples were boiled for 7 minutes, followed by centrifugation at 14,000g for 8 minutes at 4 °C. The bacterial pellets were treated with 25 mL of 5% sodium dodecyl sulfate (SDS), boiled for 25 minutes, and then centrifuged. After centrifugation, another 25 mL of 4% SDS was added to the bacteria and the mixture was boiled for 15 minutes, and washed five times with deionized water at 60°C to remove all SDS. The bacteria were then incubated in a solution containing 50 mM Tris HCl and 2 mg/mL proteinase K at 60°C for 1 h, followed by three washes with deionized water. The samples were then collected and digested in a 25 mM sodium phosphate buffer (pH=5.6) containing 250 μg/mL lysozyme at 37°C for 15 h. The digestion was stopped by boiling for 3 minutes, followed by centrifugation at 14,000g for 8 minutes. The supernatant was collected, freeze-dried, and analyzed using MALDI-TOF MS.

Confocal microscopy imaging. *S. aureus* labeled with BDN was incubated with 0.2 mM SA-FITC or 50 μM SA-Cy3 for 2 h. After incubation, the samples were washed three times with PBS buffer and fixed with 4% paraformaldehyde at 4°C for 1 h. Subsequently, the samples were washed three times with PBS and resuspended in PBS buffer. The obtained samples were characterized using confocal microscopy.

Bacteria capture. Bacteria labeled with BDN were incubated with streptavidin-coated magnetic beads for 2 hours. Subsequently, magnetic attraction on the sidewall of the centrifuge tube was used to facilitate bead separation. The samples were washed three times with PBS through repeated magnetically assisted washing. Finally, the samples were analyzed using an inverted microscope.

Antibacterial experiment. Bacteria labeled with BDN were diluted in PBS to a concentration of approximately 10^6 CFU/mL. Then, different concentrations of streptavidin-coated gold nanocages and bacterial solution were mixed and co-incubated for 1 h. Subsequently, the mixture was subjected to laser irradiation at 660 nm with a power density of 1.5 W/cm^2 for various irradiation durations. After irradiation, the bacterial suspension was diluted 5 times in PBS. A volume of 100 μL of the diluted bacterial solution was spread on solid LB agar plates and incubated at 37 °C for 24 hours for colony counting. Additionally, the irradiated bacteria were fixed in 2.5% paraformaldehyde overnight at 4°C. Finally, the bacteria were dehydrated with a gradient of ethanol concentrations (50, 70, 80,

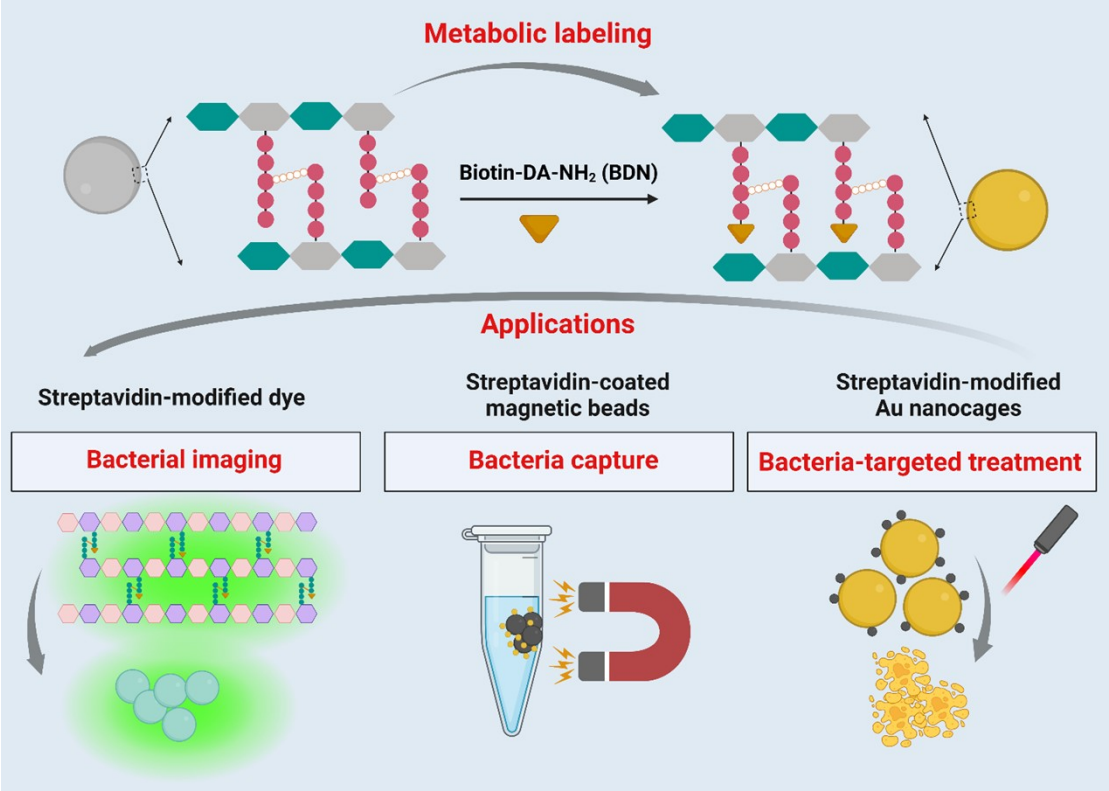
90, and 100%) and characterized by using scanning electron microscopy (Phenomenon LE, Phenom-World, Netherlands).

Hemolytic activity. Sheep blood was centrifuged (3000 rpm, 5 min, 4 °C) to remove plasma, white blood cells, and platelets. The remaining red blood cells were washed five times with PBS and diluted with PBS to 4%. Different concentrations of BDN were mixed with the 4% red blood cell suspension and the mixture were incubated at 37°C for 4 h. After centrifugation, the supernatant was transferred to a 96-well plate, and the absorbance at 540 nm was measured using a microplate reader (SYNERGY-LX, BioTek, USA) to determine hemolytic activity. Red blood cell suspensions in PBS and Triton X-100 were used as blank and positive controls, respectively. The hemolysis rate was calculated using the formula:

Hemolysis Rate (%) = $(A - A_0) / (A_t - A_0) \times 100\%$

where A_0 is the blank control and A_t is the positive control.

Cytotoxicity. The Cell Counting Kit-8 (CCK-8) assay was used to evaluate the cytotoxicity of BDN. 293T cells were seeded into a 96-well plate and cultured for 24 hours. After removing the supernatant, different concentrations of BD were added, and the cells were further incubated for 24 h. After washing the cells three times with PBS, 10 μL of CCK-8 solution was added to each well, followed by a 2-hour incubation at 37°C. Finally, the absorbance at 450 nm was measured using a multiplate reader (SYNERGY-LX, BioTek, USA).



Scheme S1. Schematic illustration of metabolic labeling of bacterial PG based on biotin-modified D-amino acid for various applications (including bacterial imaging, bacteria capture, and bacteria-targeted treatment)

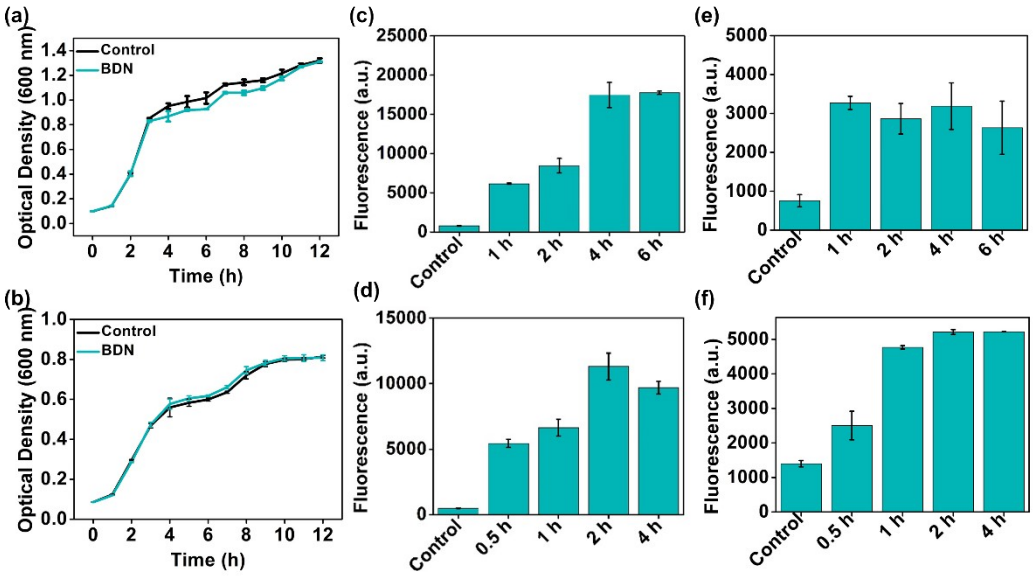


Figure S1. (a-b) The effect of BDN (0.4 mM) on the growth of *S. aureus* (a) and *E. faecalis* (b). (c-d) Flow cytometric analysis of metabolic labeling of *S. aureus* at different labeling times (c) and different staining times (d). (e-f) Flow cytometric analysis of metabolic labeling of *E. faecalis* at different labeling times (e) and different staining times (f).

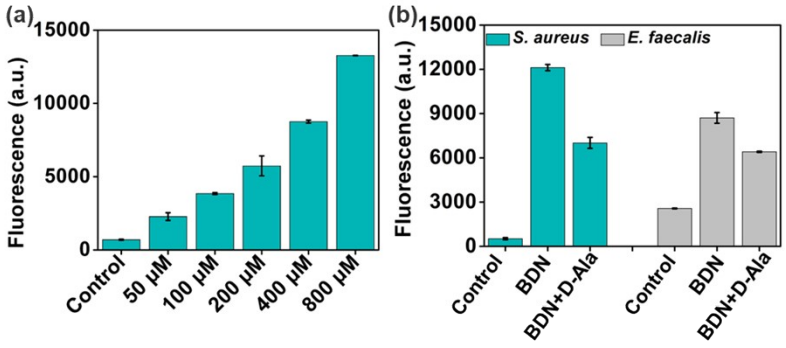


Figure S2. (a) The labeling intensity of *S. aureus* treated with different concentrations of BDN (50, 100, 200, 400, and 800 μM), followed by staining with 100 μM SA-FITC. (b) Competition experiment results. The addition of D-Ala during the metabolic labeling process of bacteria with BDN, followed by staining with SA-FITC, resulted in a significant decrease in fluorescence intensity for both *S. aureus* and *E. faecalis*.

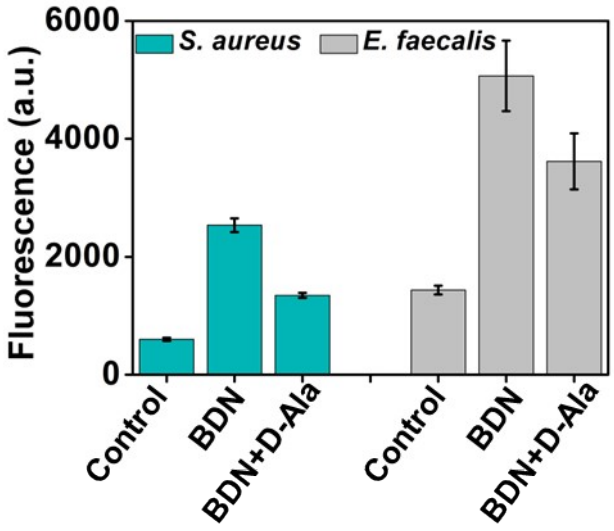


Figure S3. Competition experiment results. The addition of D-Ala during the metabolic labeling process of bacteria with BDN, followed by staining with SA-Cy3, resulted in a significant decrease in fluorescence intensity for both *S. aureus* and *E. faecalis*.

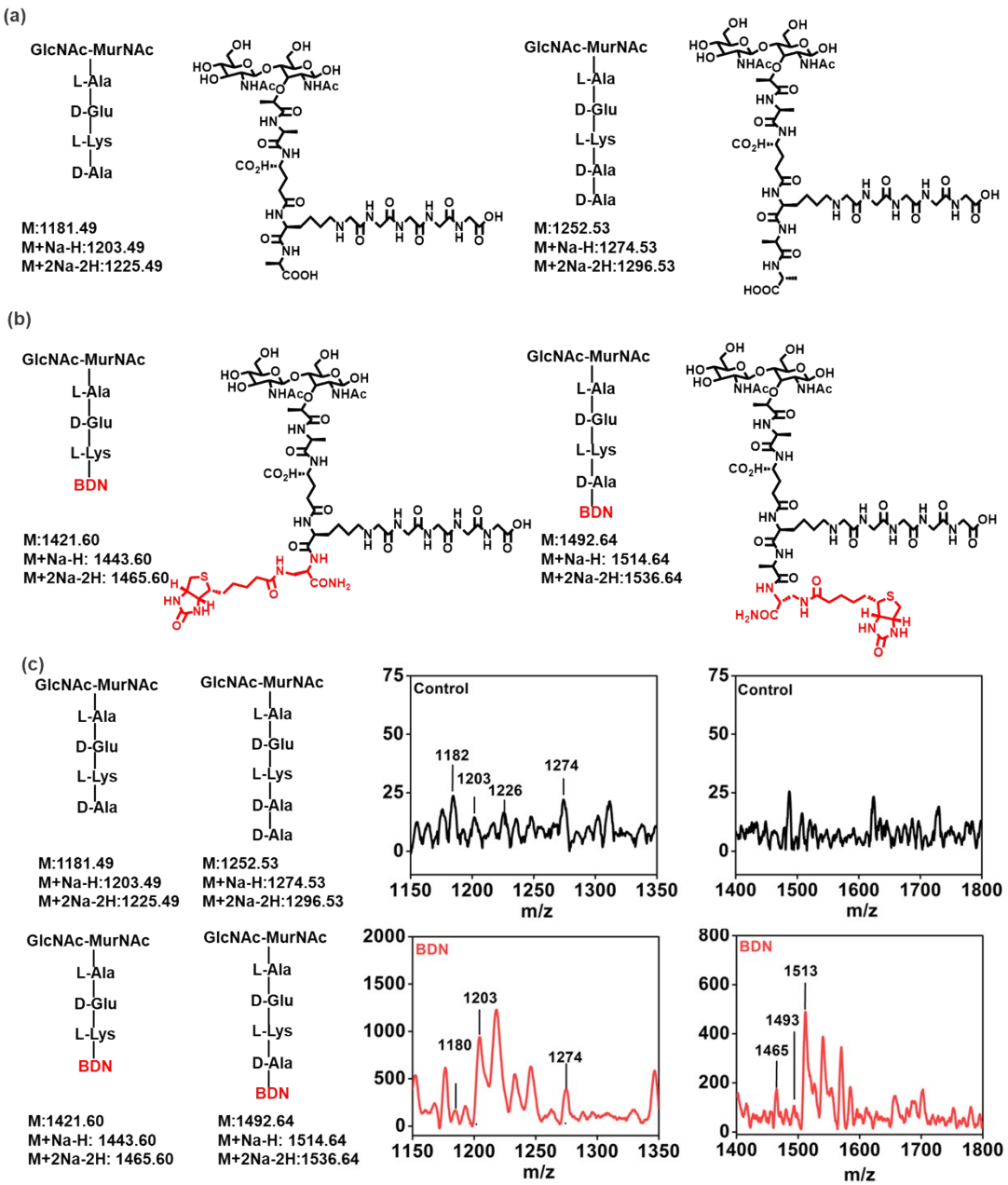


Figure S4. Chemical structures of PG repeating unit of original *S. aureus* (a) and BDN-labeled *S. aureus* (b). (c) MALDI-TOF MS analysis of PG repeating unit of original and BDN-labeled S. aureus.

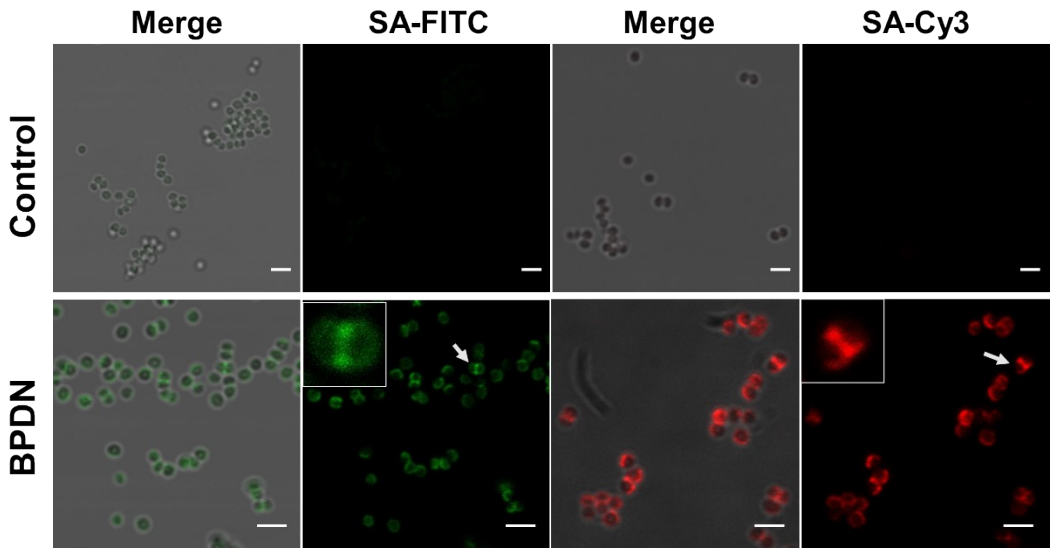


Figure S5. Confocal laser scanning microscopy (CLSM) and merged images of *S. aureus* treated with BPDN, followed by staining with SA-TITC or SA-Cy3. The insert and white arrows mark the labeled septal planes of dividing bacteria. Scale bars, 2 μm.

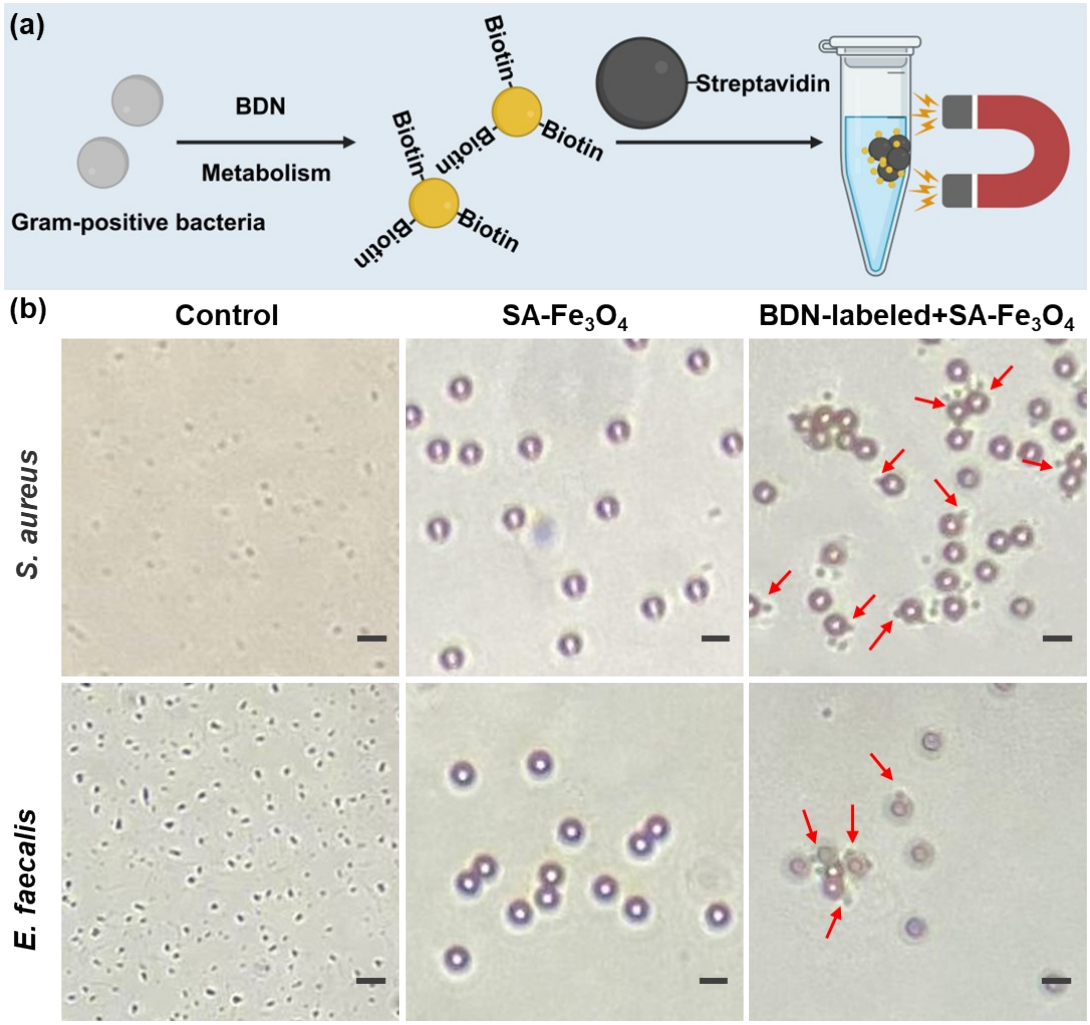
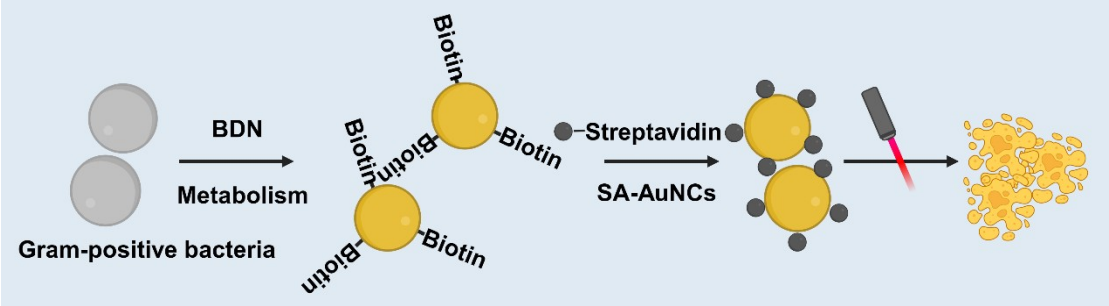


Figure S6. (a) Schematic illustration of metabolic labeling of bacterial PG based on biotin-

modified D-amino acid for capturing Gram-positive bacteria. (b) Photographs of unlabeled and BDN-labeled *S. aureus* and *E. faecalis* treated with magnetic streptavidin-coated beads (SA-Fe_3O_4). Control: the untreated bacteria. SA-Fe_3O_4: unlabeled bacteria treated with SA-Fe_3O_4. BDN-labeled+SA-Fe_3O_4: BDN-labeled bacteria treated with SA-Fe_3O_4. Scale bars, 2 μm



Scheme S2. Schematic illustration of metabolic labeling of bacterial PG based on biotin-modified D-amino acid for bacteria-targeted treatment.

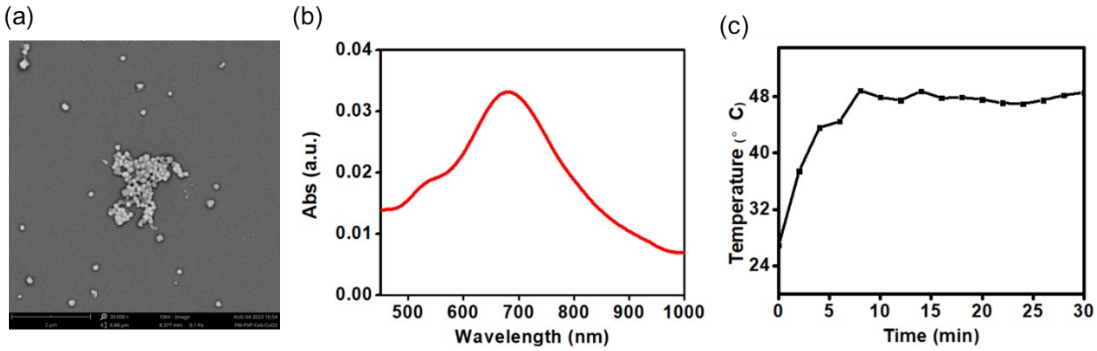


Figure S7. (a-c) SEM image (a), absorbance spectrum (b), and photothermal effects (c) of SA-AuNCs.

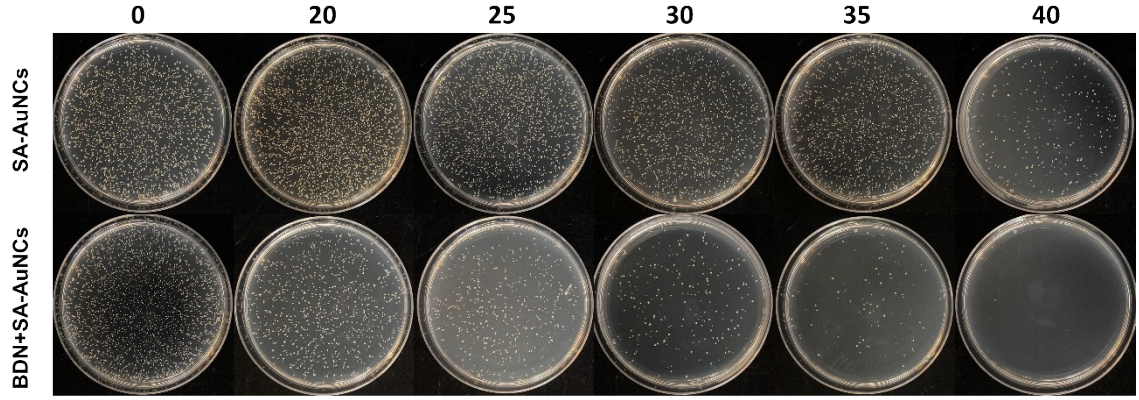


Figure S8. Photographs of plates of unlabeled *S. aureus* and BDN-labeled *S. aureus* treated with various concentration of SA-AuNCs after 10 minutes of illumination.

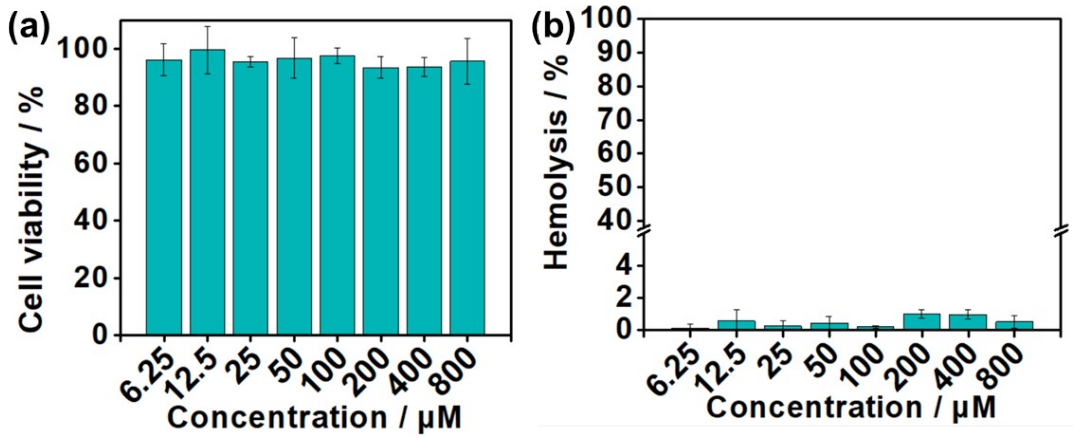
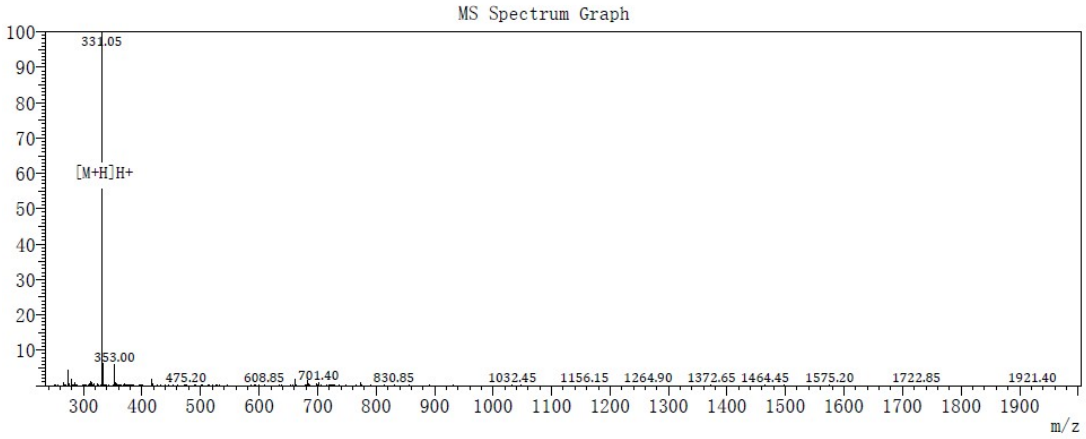
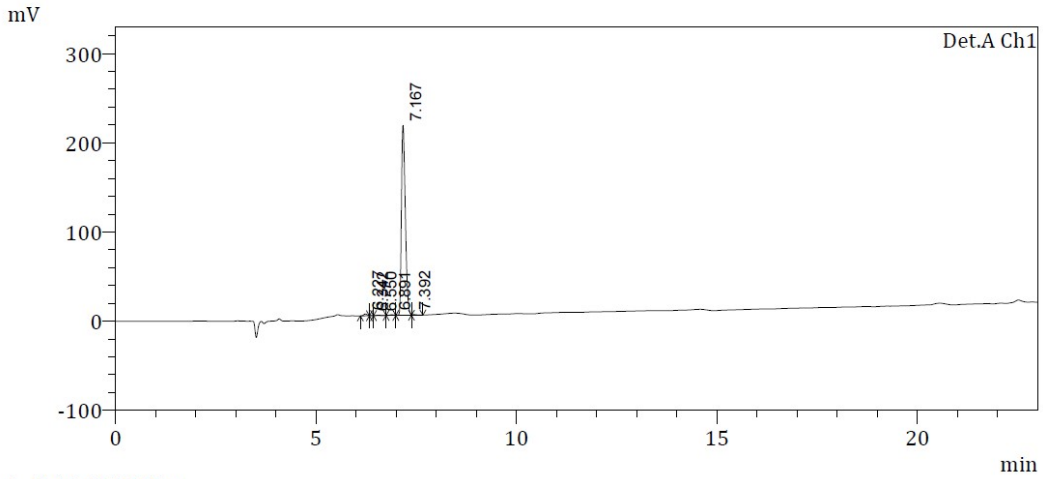


Figure S9. Cytotoxicity (a) and hemolytic activity (b) of BDN.

Mass spectrometry (MS) and high performance liquid chromatography (HPLC) data for these compounds are shown below.

1. BD





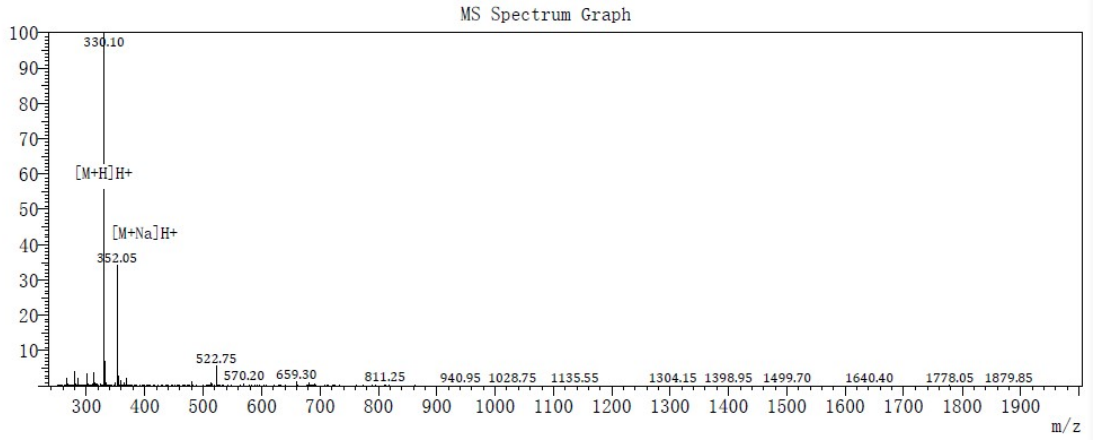
1 Det.A Ch1/220nm

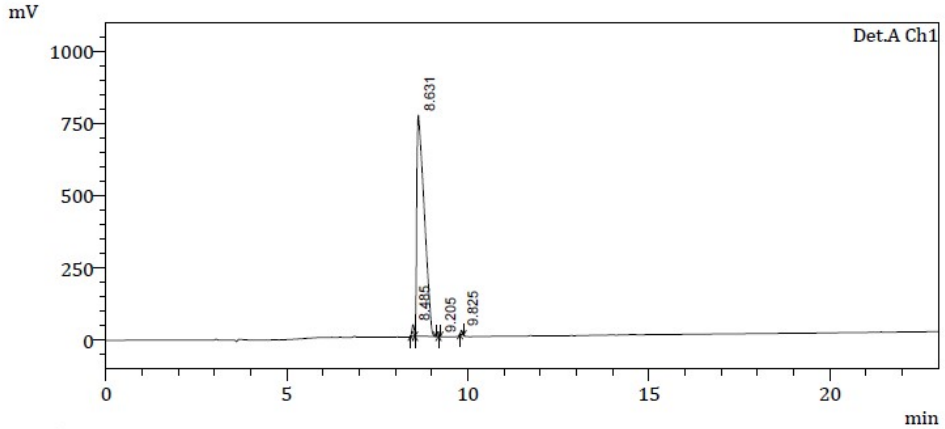
PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	6.227	12781	1989	0.875	0.916
2	6.342	1521	334	0.104	0.154
3	6.550	3547	355	0.243	0.163
4	6.891	4738	726	0.324	0.334
5	7.167	1431793	212752	98.032	97.994
6	7.392	6154	951	0.421	0.438
Total		1460533	217107	100.000	100.000

2. BDN





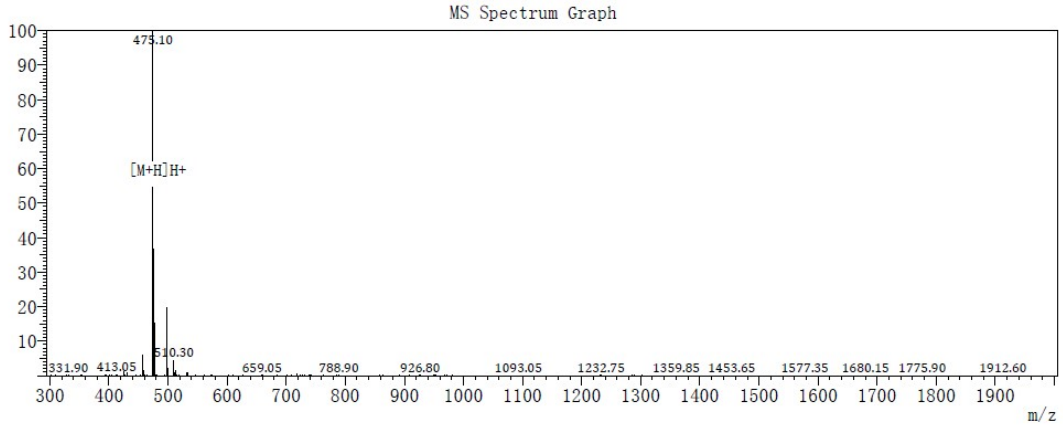
1 Det.A Ch1/220nm

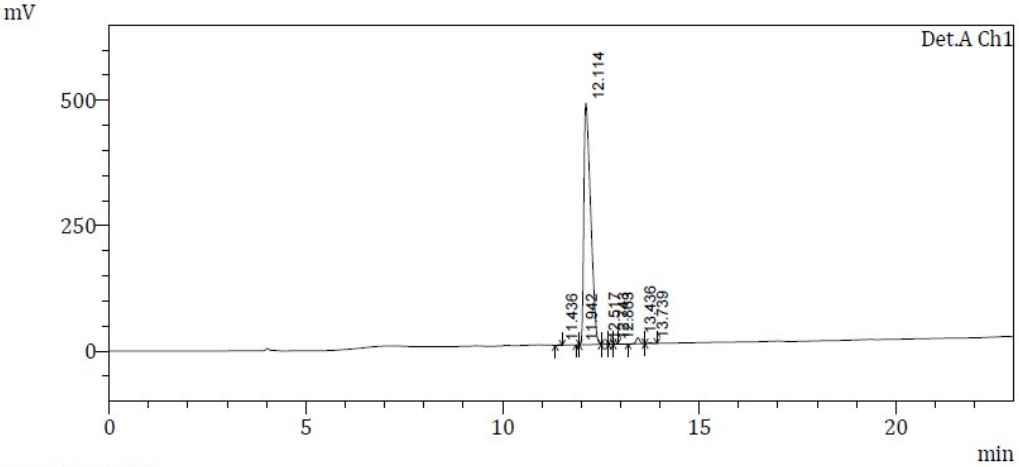
PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	8.485	170416	39402	1.518	4.810
2	8.631	11005563	765490	98.039	93.449
3	9.205	574	287	0.005	0.035
4	9.825	49202	13971	0.438	1.706
Total		11225755	819150	100.000	100.000

3. BPDN





1 Det.A Ch1/220nm

PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	11.436	5382	1090	0.092	0.218
2	11.942	221	86	0.004	0.017
3	12.114	5763758	481798	98.035	96.408
4	12.517	13148	2006	0.224	0.401
5	12.743	3312	516	0.056	0.103
6	12.863	1510	320	0.026	0.064
7	13.436	79937	12125	1.360	2.426
8	13.739	12001	1806	0.204	0.361
Total		5879269	499747	100.000	100.000