Supplementary Information

Nanozyme-Based Guanidine polypeptide Mediate Surface Reactive Oxygen Species for Multidrug Resistance Bacterial Infections Management

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Experimental section:

1. Materials

All the reagents were used as received without further purification. Iridium trichloride was obtained from Chemical Technology (Shandong) Co., Ltd. Sipunculus nudus Linnaeus Peptides (SNLP) was purchased from ChinaPeptides Co., Ltd. BS-12 was purchased from Maya Reagent. Vancomycin, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), N-hydroxysulfosuccinimide (Sulfo-NHS), 2-(N-morpholino) ethanesulfonic acid (MES), 3, 3′, 5, 5′-tetramethylbenzidine (TMB) and terephthalic acid were purchased from Aladdin (China). Luria-Bertani (LB), Luria Bertani agar were acquired from guangdong huankai microbial scl&tech Co., Ltd. Sodium hydroxide (NaOH), phosphate-buffered saline (PBS), MTT, DCFH-DA were obtained from Sigma-Aldrich (St.Louis, MO, USA). LIVE/DEAD viability/cytotoxicity kits for mammalian cells were purchased from Beijing Solarbio Science &Technology Co., Ltd. Standard strains of Methicillin-resistant Staphylococcus aureus ATCC 33591 (MRSA) and Escherichia coli ATCC 8739 (*E. coli*) were obtained from the Chongqing Boer Biotech. NIH/3T3 and HUVEC cells were obtained from National Biomedical Experimental Cell Repository. ICR mice were obtained from SPF (Beijing) Biotechnology Co., Ltd.

2. Fabrication of IrO_x NPs, SNLP/BS-12@IrO_x NPs (SBI NPs) and other reagents

2.1 Fabrication of IrO_x NPs

Iridium trichloride ($IrCl_3 \cdot xH_2O$, 0.15 g) were dissolved in 50 mL doubly distilled water and the solution of $IrCl_3$ was filtered by 0.22 μm syringe filter. After that, NaOH solution (1.0 M) was added and adjust the pH to 12.0 and the mixture was allowed to be further stirred at room temperature for ~2.5 h. Then, the mixture was allowed to react for 6 h at 80 ℃ under vigorous stirring. Finally, the product was collected and further washed by water for three times through being centrifuged at 10000 rpm for 10 min and collected freeze-drying.[1]

2.2 Synthesis of SNLP/BS-12

BS-12 (100 mg) was activated with EDC·HCl and sulfo-NHS in MES buffer solution (0.1 M, pH= 6.0). The molar ratio of BS-12: EDC·HCl: sulfo-NHS was 1:4:4. After activation for 12 h, the mixture was added to 130 μL of SNLP solution (10 mg mL^{-1}) in PBS (0.1 M, pH = 7.4) for 24 h. The molar ratio of SNLP: BS-12 was 1:1. At the end of the reaction, the solution was dialyzed (MWCO 500) overnight and collected freeze-drying.

2.3 Fabrication of SBI NPs

Different volumes (0.2 mL, 1 mL, 3 mL) of SNLP/BS-12 aqueous solution (15 mg mL^{-1}) was dropwise added to 5 mL of $IrCl_3$ xH_2O (3 mg mL^{-1}) aqueous solution and adjust the pH to 12.0. The mixture was allowed to be stirred for another 6 h at 80 ℃. The product was collected and washed by water for three times through being centrifuged at 10000 rpm for 10 min and collected freeze-drying.

2.4 Preparation of Rho B-SBI NPs

Rho B is activated in the same way as SNLP. Afterwards, an equal mass of SBI NPs was added and the reaction continued for 12 h. More than 10 times washing with pure water were used to remove the excess Rho B.

3. Characterization

The prepared nanozymes were characterized by using various methods. TEM measurements were carried out on a TECNAI G2 equipped with EDS at 200 kV. The EDX mapping images and atomic structure images were characterized using a JEM ARM-200 (JEOL, Tokyo, Japan) transmission electron microscope operated at 200 kV. X-ray photoelectron spectroscopy (XPS) analysis was conducted by a Thermo-VG Scientifific ESCALAB 250 spectrometer. Ultraviolet–visible (UV−vis) absorption spectra were recorded with a UV-2600 spectrophotometer (Shimadzu). The infrared spectra were collected with a Thermo Scientific Nicolet iS50 Fourier transform infrared (FT-IR) spectrometer.

4. Peroxidase (POD)-like activity assay

4.1 POD activity at different pH values

Firstly, SBI NPs (2 μL, 10 mg mL^{-1}), TMB (4 μL, 10 mM) and H_2O_2(2 μL, 2.0 M) were added to a 96-well plate, followed by 192 μL of HAc-NaAc buffers of different pHs (4.5, 5.5, 6.5 and 7.4). After 5 min of reaction, the absorption curves were scanned with a UV-vis spectrophotometer (n=3). The background solution was HAc-NaAc buffers for SBI NPs.

4.2 POD activity at different temperatures

SBI NPs (2 μL, 10 mg mL^{-1}), TMB (4 μL, 10 mM) and H_2O_2(2 μL, 2.0 M) were added to a 96-well plate, followed by 192 μL of pH 4.5 HAc-NaAc buffers. After 5 min of reaction at different temperatures, the absorption curves were scanned with a UV-vis spectrophotometer (n=3). The background solution was HAc-NaAc buffers for SBI NPs.

4.3 Calculation of Michaelis-Burk constant (K_m)

SBI NPs (2 μL, 10 mg mL^{-1}), TMB (4 μL, 10 mM) and H_2O_2(2 μL, 2.0 M, 1.0 M, 0.5 M, 0.25 M, 0.125 M, 0.063 M, 0.031 M, 0.016 M, 0.008 M) were added to a 96-well plate, followed by 192 μL of pH 4.5 HAc-NaAc buffers. After 5 min of reaction, the absorption curves were scanned with a UV-vis spectrophotometer (n=3). The background solution was HAc-NaAc buffers for SBI NPs.

Different concentrations of TMB (4 μL, 50 mM, 25 mM, 12.5 mM, 6.25 mM, 3.13 mM, 1.56 mM, 0.78 mM, 0.39 mM) were oprated as above.

The Michaelis-Burk constant was calculated by the Lineweaver-Burk Plot:

$$1/V=K_m/V_{max}(1/[S]+1/K_m)$$

V represents the initial velocity, V_{max} represents the maximal reaction velocity and [S] indicates substrate concentration.

4.4 TA method for the detection of hydroxyl radicals (·OH)

3 mL of pH 4.5 HAc-NaAc buffers containing TA, TA+H_2O_2, TA+IrO_x NPs, SBI NPs, TA+SBI NPs, TA+H_2O_2+IrO_x NPs and TA+H_2O_2+SBI NPs, respectively. After

20 min of reaction, the emission curves were measured by fluorescence spectrophotometer from 350 to 550 nm at 315 nm excitation wavelength.

4.5 Recoverability and stability of nanomaterials

A total volume of 1 mL pH 4.5 HAc-NaAc buffers was added to a 1.5 mL Eppendorf Tubes with a final concentration of 100 μg mL^{-1} IrO_x NPs, 20 mM H_2O_2 and 0.2 mM TMB. After 5 min of reaction, the absorption curve was scanned with a UV-vis spectrophotometer. The recovered nanomaterials were separated from the reaction mixture and washed three times with pure water. The recovered nanomaterial was mixed with the fresh reaction mixture and the absorption curve was measured using the same procedure. A total of 5 times were tested. SBI NPs were operated as above.

5. Antibacterial experiments

5.1 Bacteria cells culture

The MRSA and *E. coli* were cultured in LB medium in an orbital shaker at 37 ℃, 180 rpm and maintained by growing the cell on LB agar.

5.2 Measurement of Minimum Inhibitory Concentration (MIC)

Each strain was extracted from an exponentially growing LB medium and washed twice with 0.9% NaCl solution. Two-fold serial dilution gradients of LB medium (100 μL) containing different fractions were added to 96-well plates, followed by an equal volume of medium containing bacteria to a final concentration of 5×10^5 CFU mL^{-1} and incubation at 37 ℃ for 24 h with a shaker speed of 180 rpm. Absorbance values at 600 nm (OD600) were measured and growth was observed. Treatment concentrations at which no microbial growth was observed were identified as MIC values.

5.3 plate counting method

200 μL of LB medium containing different concentrations of IrO_x NPs (125 μg mL^{-1}, 62.5 μg mL^{-1}, 31.25 μg mL^{-1}) and 5×10^5 CFU mL^{-1} of bacteria (n=3) were added to 96-well plates and OD600 were measured at 0 h, 6 h, 9 h, 10 h, 11 h and 12 h. After 12 h of incubation, the suspensions were diluted 1000 times and 50 μL was applied to agar plates and incubated at 37 ℃ for 18 h and colonies were counted. SBI NPs were tested as above.

5.4 Fractional inhibitory concentration (FIC) measurement

The synergy between the SNLP and BS-12, IrO_x NPs and SNLP/BS-12 was assessed by checkerboard assays. Briefly, the two components were mixed in a 96-well plate with serial two-dimensional dilutions. The wells of the plates were inoculated with MRSA and OD600 was measured 24 h later for assessing the growth of the bacteria. The synergy effects were evaluated by calculating the FIC index according to the formula below:

$$\mathrm{FIC} = \frac{\mathrm{MIC_A^{Comb}}}{\mathrm{MIC_A}} + \frac{\mathrm{MIC_B^{Comb}}}{\mathrm{MIC_B^{Comb}}}$$

$\mathrm{MIC_A^{Comb}}$ and $\mathrm{MIC_B^{Comb}}$ indicate the MICs of the two components in combination. The interactions between the two components are defined according to standard criteria of considering FIC ≤ 0.5 as synergistic, 0.5 < FIC ≤ 1 as additive, 1 < FIC ≤ 4 as indifference, and FIC > 4 as antagonism.

5.5 Bacterial live/dead staining

After treatment 1 × 10^7 CFU mL^{-1} MRSA with different components (PBS, IrO_x NPs, SNLP/BS-12, SBI NPs, vancomycin, 1/2 × MIC) for 2 h, the bacteria were stained with SYTO9/PI for 30 minutes and then washed three times with PBS. Live (green fluorescent) and dead (red fluorescent) bacteria were observed using an inverted fluorescent microscope.

6. Anti MRSA biofilm in vitro

6.1 Biofilm formation inhibition test

Biofilm formation was determined by crystalline violet staining. MRSA (2 × 10^7 CFU mL^{-1}, 250 μL) was added to a 12-well plate, followed by equal volumes of different components (PBS, 62.5 μg mL^{-1} IrO_x NPs, 31.25 μg mL^{-1} SNLP BS-12, 62.5 μg mL^{-1} SBI NPs, 1.25 μg mL^{-1} vancomycin) in LB medium. After mixing, incubating at 37 ℃, discarding supernatant and suspended bacteria at 24 h intervals and re-dose. The culture medium was discarded after 48 h and 72 h, respectively, and washed three times with PBS. After the 12-well plates had dried, the biofilms were stained with crystalline violet (0.1%, 500 μL) for 30 min. Excess crystalline violet dye was removed, washed several times with PBS until the washing solution was clarified.

Afterwards, acetic acid solution (33%, 500 μL) was added to dissolve the crystalline violet dye and the OD600 was measured. SBI NPs were tested as above.

6.2 3D microscopic imaging of biofilms

Biofilms were formed in confocal dishes in a similar way as described above. MRSA biofilms were treated with different components (1 × MIC), stained with SYTO9 dye for 30 min, washed 3 times with PBS and the 3D images of the biofilms were observed with an inverted fluorescence microscope.

7. Detection of ROS content

The amount of ROS in MRSA can be observed by a DCFH-DA probe, at an excitation wavelength of 488 nm, using an inverted fluorescence microscope. Briefly, different fractions (1 × MIC) were added to 1 × 10^7 CFU mL^{-1} MRSA separately, incubated for 2 h, centrifuged at 8000 rpm for 2 min and the supernatant discarded. The MRSA was resuspend with 0.9% NaCl solution, adding DCFH-DA and incubating for 30 min. After washing 3 times, photograph and observe by inverted fluorescence microscopy.

8. SBI NPs and bacteria colocalization

125 μg mL^{-1} Rho B-SBI NPs were incubated with 1 × 10^8 CFU mL^{-1} MRSA in saline for 2 h. After 3 washes, the fluorescence imaging was observed by inverted fluorescence microscopy.

9. Construction of an acute lung infection model

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Guangdong Medical University and approved by the Animal Ethics Committee of Guangdong Medical University (SYXK（粤）2019-0213). The model of acute lung infection was constructed by intranasal drip MRSA. Briefly, 150 μL of 1×10^8 CFU mL^{-1} MRSA was administered intranasally to 6-8 weeks ICR mice. On the second day, tail vein administrations (PBS, 3 mg kg^{-1} SBI NPs and 3 mg kg^{-1} *vancomycin*) were performed in each group (n=5). On the third day, mice were executed by cervical dislocation, body weighed and lungs were removed and photographed. The lung tissues were also embedded, sectioned, H&E stained, Masson stained and Sirius red stained to observe the alveolar structure.

10. Diabetic wound healing

ICR mice were given 150 mg kg^{-1} streptozotocin (STZ) on an empty stomach, every other day, for a total of 4 injections. Mice with blood glucose values ⩾11.1 mmol L^{-1} were randomly divided into 3 groups (n=5) and each group was administered 50 μL PBS, 1 mg mL^{-1} SBI NPs and 1 mg mL^{-1} *vancomycin*. Two doses were administered in wounds. The mice were weighed and photographed on alternate days, and after day 9, skin tissues were removed from the injured areas for H&E staining to observe morphology.

11. Biosafety evaluation

11.1 Cell viability

Mouse embryonic fibroblasts cells NIH/3T3 and human umbilical vein endothelial cells HUVEC were used to assay cell viability after SBI NPs treatment. Briefly, 100 μL of culture medium containing 5×10^3 cells was added to a 96-well plate. After overnight incubation, the old culture medium was removed and fresh cultures containing different concentrations of SBI NPs were added and incubated for 24 h. After removal of the culture medium, these cells were washed three times with PBS and incubated with 100 μL of MTT (0.5 mg mL^{-1}) for 4 h. After removal of the MTT solution, the resulting purple methanamine was dissolved in 100 μL of DMSO for 10 min. Finally, the absorbance value at 490 nm was measured indicating cell viability.

11.2 Hemolysis assay

The hemolysis assay was performed using 4% mouse red blood cells (RBCs). Fresh RBCs were washed 3 times with PBS (3500 rpm, 5 min) and PBS solutions containing different concentrations of SBI NPs were added. After 4 h incubation, the supernatants were centrifuged and the absorbance value at 540 nm was used to determine the hemolysis rate. Triton X-100 (1% in PBS) which was able to lyse RBCs completely was used as a positive control, while PBS was used as a negative control. The hemolysis ratio of RBCs was calculated according to the following formula:

$$\text{Hemolysis ratio}(\%) = \frac{\mathrm{A}_{treat} - \mathrm{A}_{PBS}}{\mathrm{A}_{PC} - \mathrm{A}_{NC}} \times 100\%$$

where A_{treat}, A_{NC}, A_{PC} and A_{PBS} were denoted as the absorbance value after addition of SBI NPs, PBS, Triton X-100 and PBS alone, respectively.

11.3 Assessment of biosafety in *vivo*

Healthy ICR mice were injected with 5 mg kg^{-1} SBI NPs in the tail vein and 24 h later, the major organs (heart, liver, spleen, lung and kidney) were collected for H&E staining. The sections were observed under a microscope to assess biosafety in *vivo*.

Error Analysis

Statistical analysis was performed using Origin Pro 2018 and GraphPad Prism 8.0. All data are presented as mean ± standard deviation (SD). Significant differences were considered *p < 0.05; **p < 0.01; ***p < 0.001.

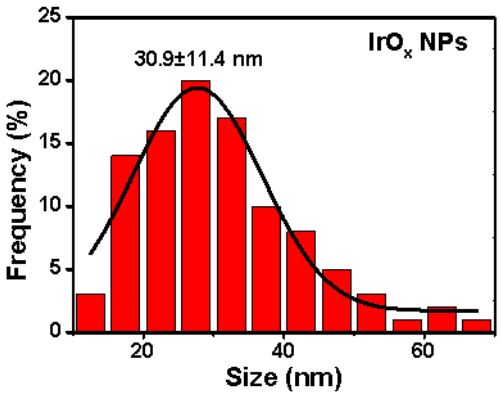

Figure. S1 Particle size distributions of IrO_x NPs.

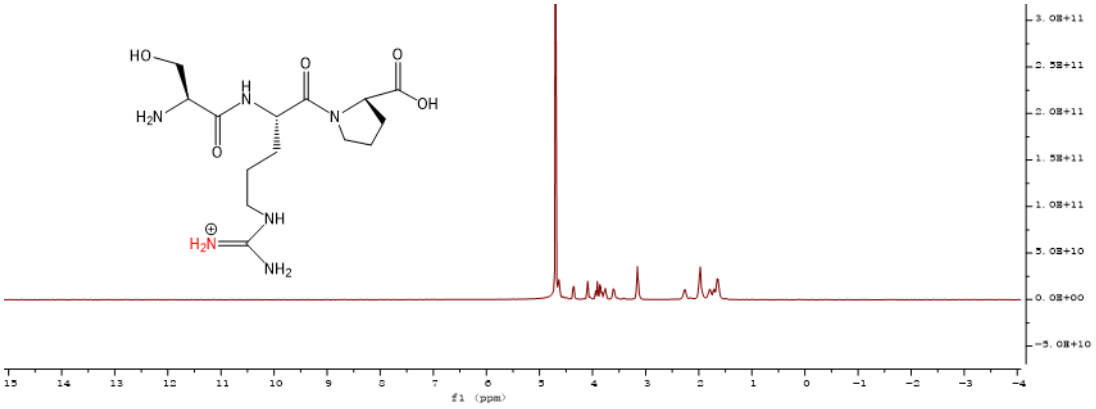

Figure. S2 ^{1}H NMR spectrum of SNLP (400 MHz, D_2O, 298 K).

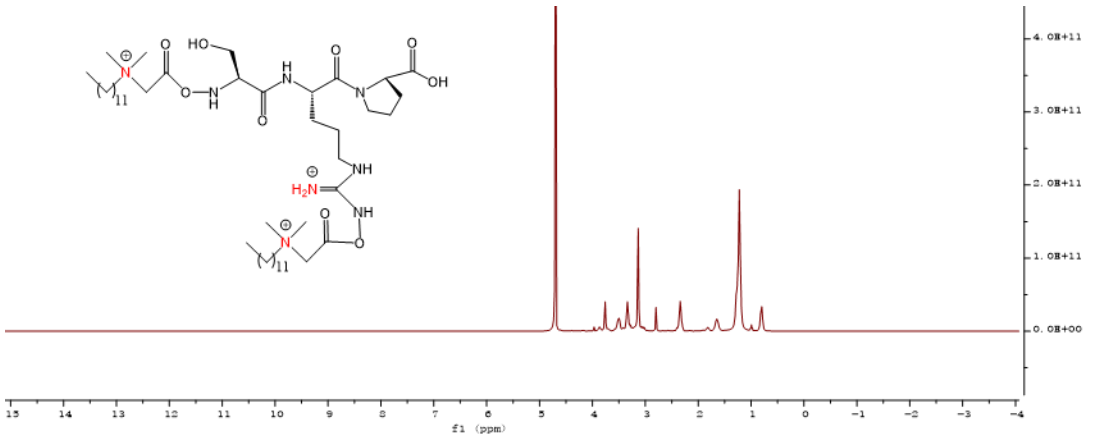

Figure. S3 ^{1}H NMR spectrum of SNLP/BS-12 (400 MHz, D_2O, 298 K).

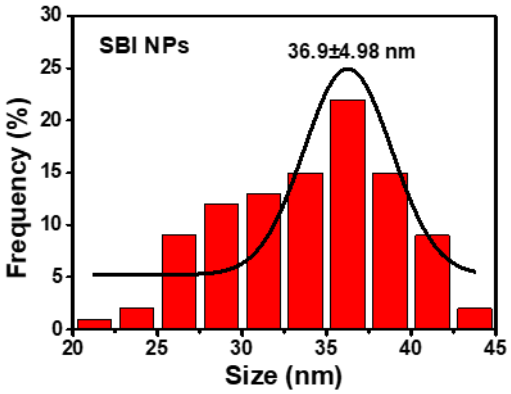

Figure. S4 Particle size distributions of SBI NPs.

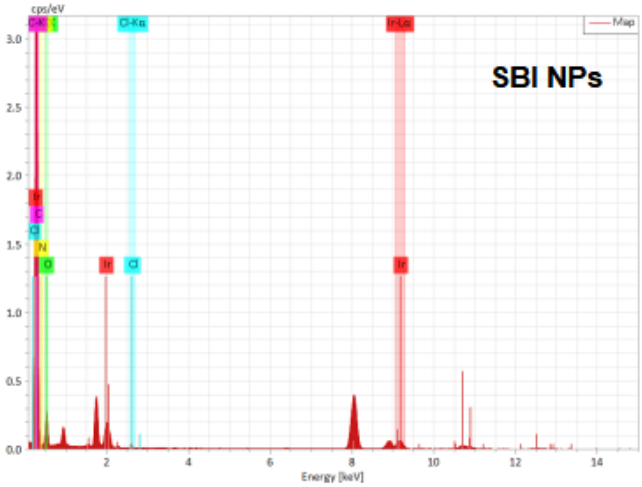

Figure. S5 EDS spectrum of SBI NPs.

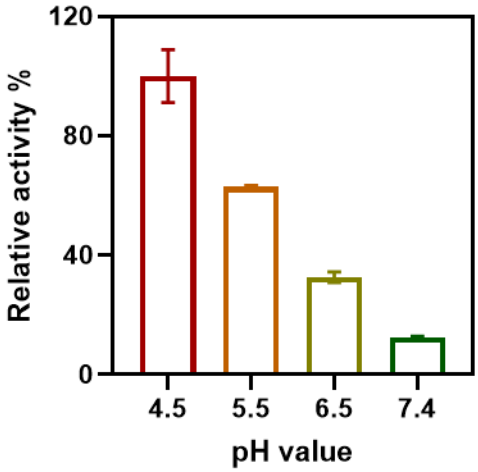

Figure. S6 The catalytic ability of SBI NPs at different pH.

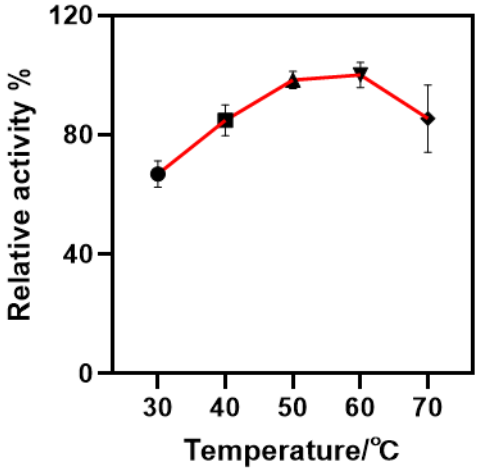

Figure. S7 The catalytic ability of SBI NPs at different temperature.

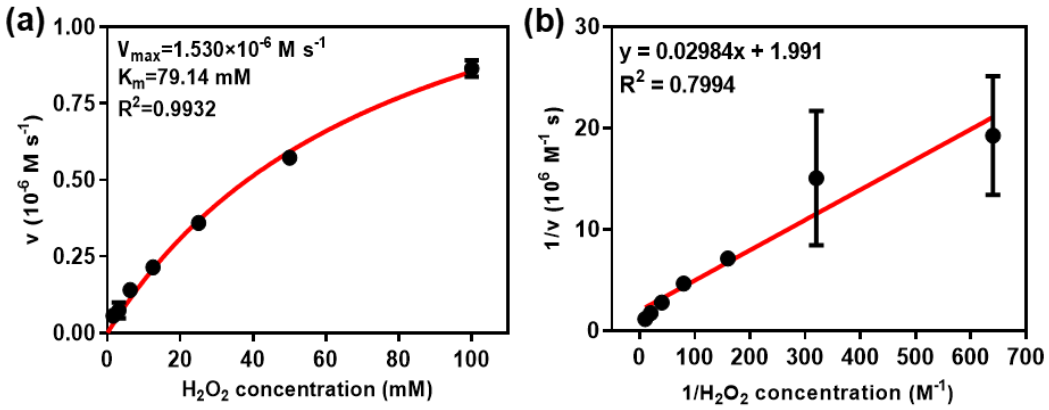

Figure. S8 Kinetic determination of peroxidase activity of SBI NPs under different concentrations of H_2O_2.

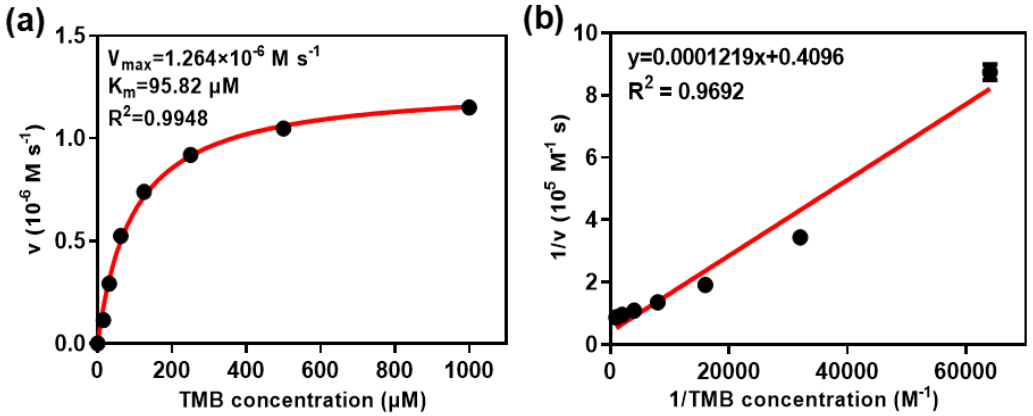

Figure. S9 Kinetic determination of peroxidase activity of SBI NPs under different concentrations of TMB.

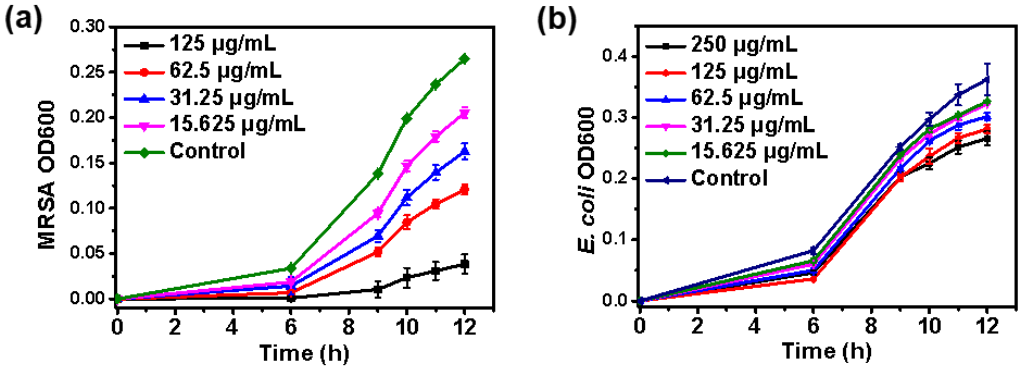

Figure. S10 Growth curves of (a) MRSA and (b) *E. coli* exposed to different concentrations of IrO_x NPs for 12 h.

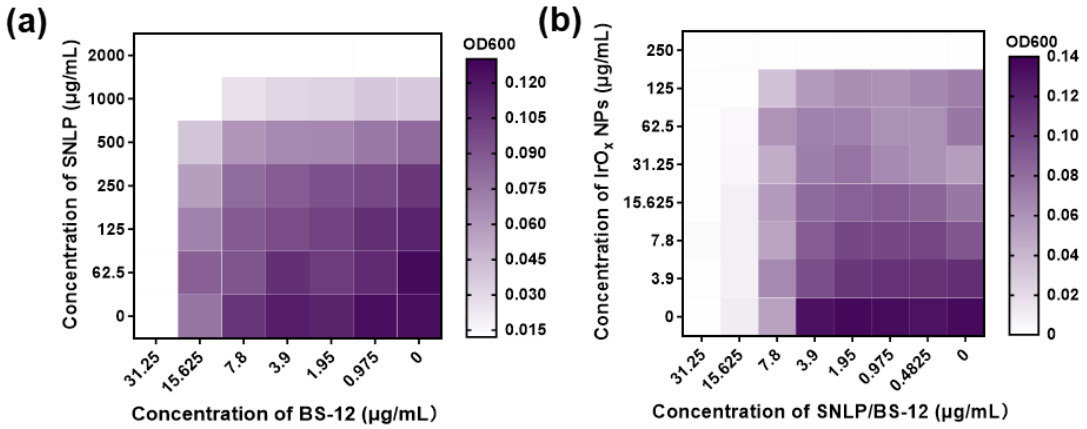

Figure. S11 Checkerboard dilution assays performed on MRSA, which was used to evaluate the synergy between (a) SNLP and BS-12, (b) IrO_x NPs and SNLP/BS-12.

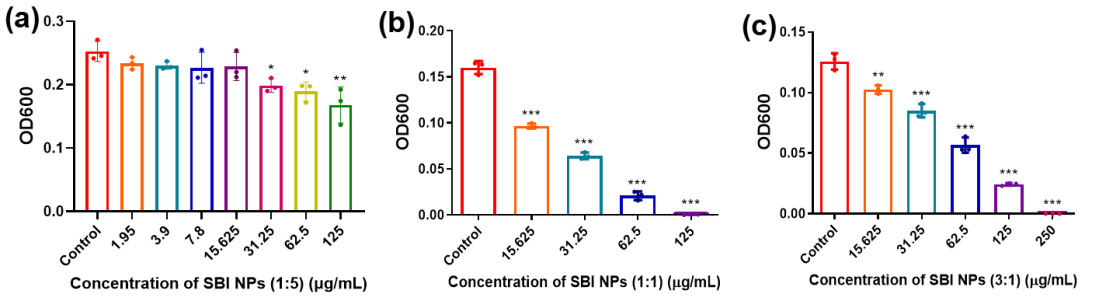

Figure. S12 Different concentrations of (a) SBI NPs (1:5), (b) SBI NPs (1:1) and (c) SBI NPs (3:1) on MRSA for 24 h. *p < 0.05; **p < 0.01; ***p < 0.001 and n.s. no significant.

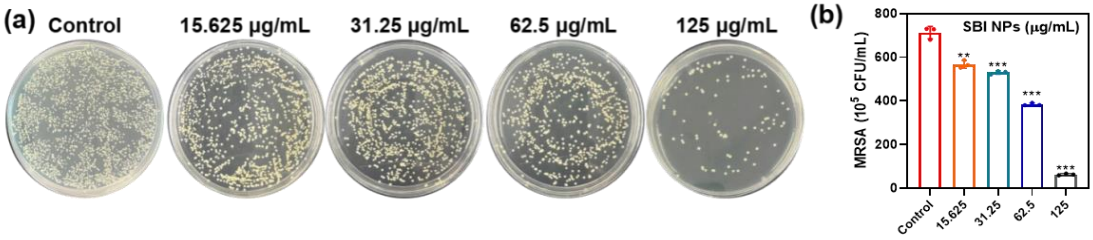

Figure. S13 (a) The agar plate method to evaluate inhibition of bacterial growth by SBI NPs at different concentrations and (b) quantitative analysis. *p < 0.05; **p < 0.01; ***p < 0.001 and n.s. no significant.

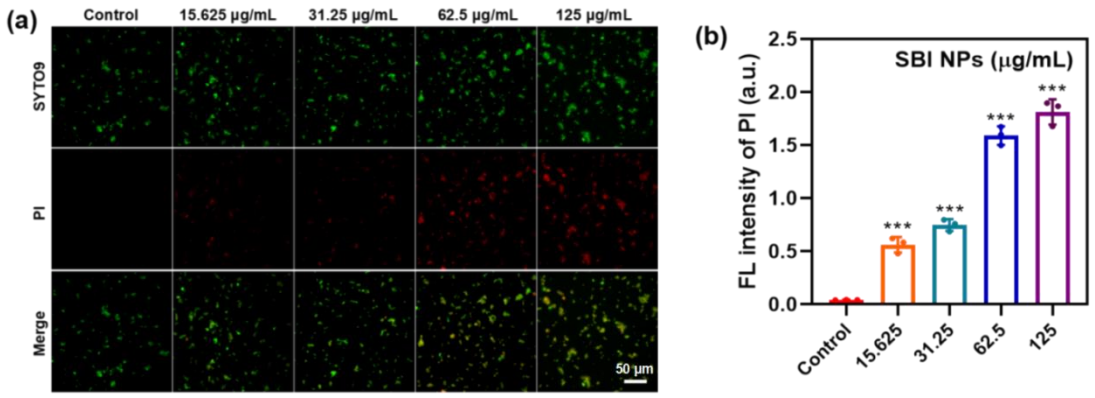

Figure. S14 (a) Live/dead fluorescence staining images of MRSA treatment different concentrations of SBI NPs and (b) quantitative analysis. Scale bar: 50 μm. *p < 0.05; **p < 0.01; ***p < 0.001 and n.s. no significant.

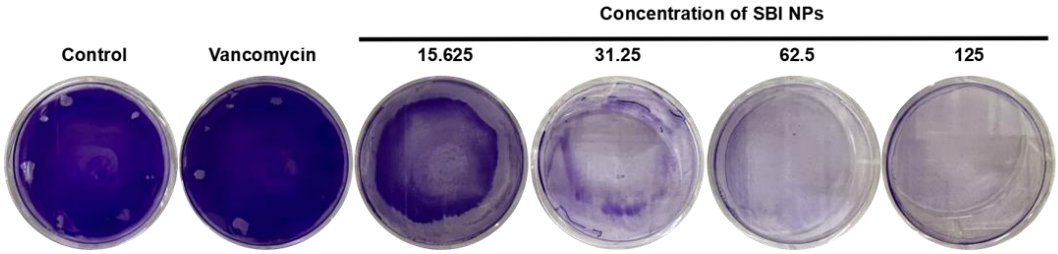

Figure. S15 Macroscopic MRSA biofilm formation images incubation with *vancomycin* and different concentrations SBI NPs for 24 h.

Table. S1 The Michaelis-Menton constant (K_m) and maximum reaction rate (V_{max}) of as prepared NPs with H_2O_2 as the substrate for peroxidase-like catalysis.

Catalysts	K_m (10^{-3} M)	V_{max} (10^{-8} M s^{-1})	References
Horse radish peroxidase (HRP)	3.70	8.71	2
Fe_3O_4	37.99	0.53	3
Ni-MOF snanosheets	2.49	130	4
Co_3O_4 NPs	5.9322	9.85	5
Au/CeO_2 NPs	44.69	2.23	6
CuO	400	16.1	7
GO-COOH	3.99	3.85	8
IrO_x NPs	19.4	179.6	This work
SBI NPs	79.14	153	This work

K_m: Michaelis constant

V_{max}: The maximal reaction velocity

Table. S2 Minimum inhibitory concentration (MIC) of different components for MRSA (μg mL^{-1}).

SNLP	BS-12	SNLP/BS-12	IrO_x NPs	SBI NPs (1:5)	SBI NPs (1:1)	SBI NPs (3:1)	Vancomycin
2000	31.25	31.25	> 125	> 125	125	250	1.25

Table. S3 Anti-bacterial activity of reported nanozymes.

Nanozyme	Type	Bacteria	Concentration (μg mL^{-1})	References
Au NCs	POD	MRSA	20	9
PtCu NPs	POD	*S. aureus*, *E. coli*	258	10
Au/C_3N_4	POD	*S. aureus*, *E. coli*	20	11
CuPt-GOx-CaP	POD, GOx	*S. aureus*, *E. coli*	200	12
MnO_x/HNCS	OXD	*S. aureus*, *P. aeruginosa*	100	13
Pd@Ir octahedra	OXD	*S. aureus*, *E. coli*	100	14
PtCo@Graphene	OXD	*H. pylori*	70	15
SPB NCPs	OXD	*S. aureus*, *P. aeruginosa*	10	16
$AgPd_{0.38}$	OXD	*S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*	16	17
SBI NPs	POD	MRSA	125	This work

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