Supporting Information

Enhanced Antibacterial Activity of Surface re-engineering Lysozyme against Gram-Negative Bacteria without accumulated Resistance

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Experimental section

Amination process of lysozyme.

In order to maintain the stability and alter the surface chemical environment of lysozyme, 1,6-diaminohexane (1.5 g) was firstly dissolved in 10 mL of deionized water, and the solution was adjusted to pH 6.0 using 3M HCl. Meanwhile a solution of lysozyme (200 mg) was stirred, and added with the solution above by drops. Then, the mixture was adjusted to pH 6.0, and the coupling process was initiated

by adding N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC,100 mg). 100 mg of EDAC need to be added again after 2 hours. The pH value was maintained at 6.0 by using 3M HCl, and the reaction was continuously stirred for totally 5 hours. The purification process was achieved by dialyzing the mixture with a 5000 Mw dialysis bag for 2 days, and the product Lyz-NH_2 was collected through freeze drying.

Conjugation process of Lyz-Gua.

Aiming to re-engineer the surface of lysozyme, glycocyamine was set up as medium of the guanidine functional group. 20 mg of glycocyamine was firstly dissolved in 10 mL of water and the solution was adjusted to pH 6.0. Meanwhile, Lyz-NH_2 (20 mg) was dissolved in 10 mL of deionized water, and was added to the solution above, which made the molecular ratio at nearly 100:1. The same initial process was applied to this reaction as above. The purification process is achieved by dialyzing the mixture with a 5000 Mw dialysis bag for 2 days, and the product Lyz-Gua was collected through freeze drying.

Determination of viability of bacteria co-cultured with Lyz-Gua.

Three typical Gram-negative bacteria (ATCC 25922, DH-5α, and MR-1) were cultured in LB medium at 37 ℃ and 220 rpm for 16 hours. The bacteria were then harvested by centrifugation and washed with PBS buffer for 3 times. The centrifuged bacteria were redistributed to fresh LB medium and diluted to a concentration of 2×10^6 cfu/mL, for which was measured at the value at 0.20 in optical density by UV-Vis spectrum. The concentration of bactericide was diluted in half fold per well, ranging from 2048 to 2 μg/mL, locating in a 96 wells plate. Afterwards, bacteria were added into the plate well respectively, and the co-culture was placed in 37 ℃ atmosphere for 3 hours. The samples were tested by UV-Vis spectrum to gain optical density result.

Determination of viability of NIH-3T3 stem cell co-cultured with Lyz-Gua.

NIH-3T3 mouse-sourced stem cell sample was performed from ProCell, Shanghai. A total of 20000 NIH-3T3 cells were cultured in Dulbecco's modified Eagle medium with 10% bovine calf serum and 1% antibiotics at 37°C in a humidified atmosphere of 5% CO_2. Cells were firstly cultured for 24 h to reach a confluent monolayer. Afterward, seeding solutions 108 cells/mL were inoculated in buffered DMEM. Old medium was removed from 3T3 cells followed by addition of 100 μL of seeding solution. The co-cultures were then stored in a box humidified with damp paper towels at 37 °C overnight without shaking. Lyz-Gua was diluted in DMEM media prior to use to obtain the desired testing concentrations. Old media was removed from co-culture again, replaced with freshly prepared testing solutions, and incubated for 3 h at 37 °C. To determine cell viability in co-cultures, the testing solutions were removed and co-cultures were washed with PBS. The samples were stained with Thiazolyl Blue and tested by UV-Vis spectrum to gain the results.

Determination of hemolysis of Lyz-Gua.

We used the previously established protocol to conduct hemolysis assays on Red Blood Cells. Heparin-

stabilized rabbit whole blood (pooled, mixed gender) was purchased from BioChannel LLC, NY and processed as soon as received, and all the experiments were prudently handled according to Tests for hemolysis of medical devices—Part 1: Material-induced hemolysis assay (YY/T 1651.1-2019). 10 mL of phosphate-buffered saline (PBS) was added to the blood and centrifuged at 3000 rpm for 5 min. The supernatant was carefully discarded and the red blood cells (RBCs) were distributed in 10 mL of Normal Saline. This step was repeated at least 3 times. The purified RBCs were re-distributed in 10 mL of Normal Saline. 0.1 mL of RBC solution was added to 0.4 mL of Lyz-Gua solution in NS in a 2 mL tube and mixed by shaking gently. RBCs incubated with NS and water was used as negative and positive controls, respectively. The mixture was incubated at 37 ˚C for 1 hours while shaking at 120 rpm. After incubation period, the solution was centrifuged at 3000 rpm for 5 mins and 100 μL of supernatant was transferred to a 96-well plate. The absorbance value of the supernatant was measured at 570 nm using a UV-Vis spectrum.

Determination of Bacterial Resistance Development.

E. coli (ATCC 25922) was inoculated in LB medium with 2/3 of MIC of different bactericides at 37 ˚C and 220 rpm for 24 h (~ 96 bacterial generations for 1 serial passage), and was monitored to gain bacterial growth curve. The co-culture was then harvested and tested for MIC by the method described above.

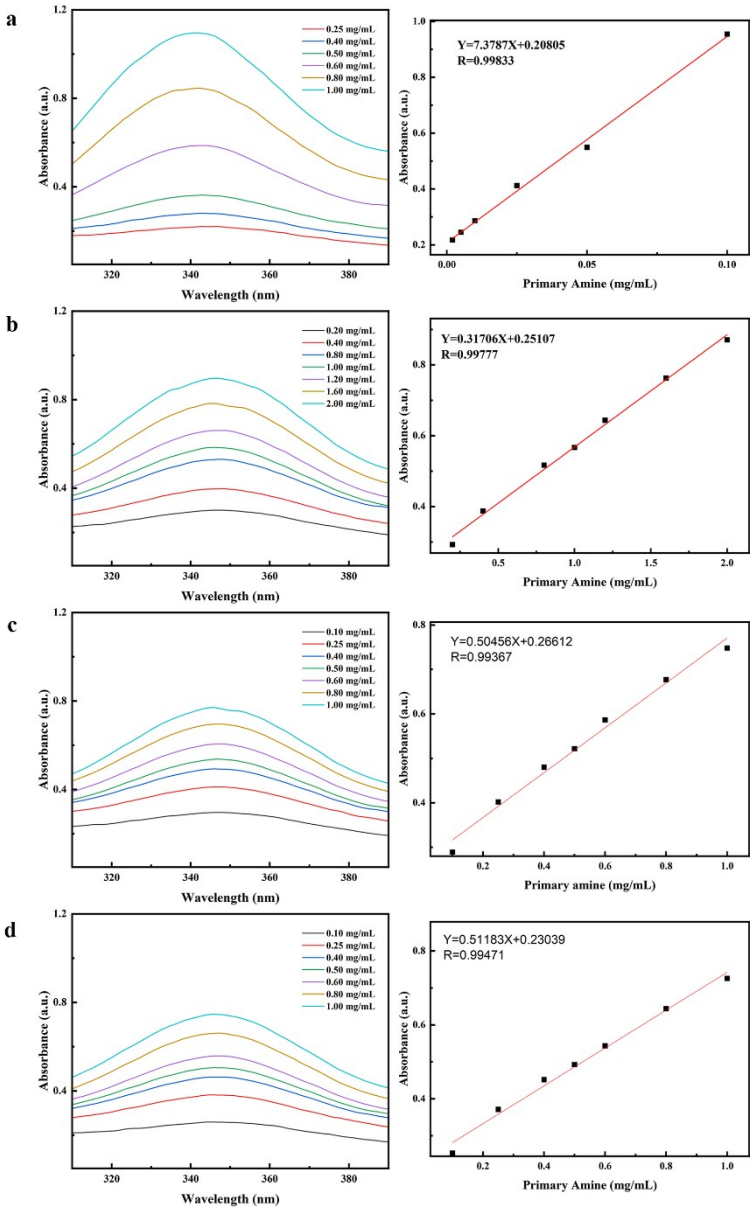

Figure S1. Determination of reactive amino groups amount for Glycine(a), Lysozyme(b), Lyz-NH2(c) and Lyz-Gua(d).

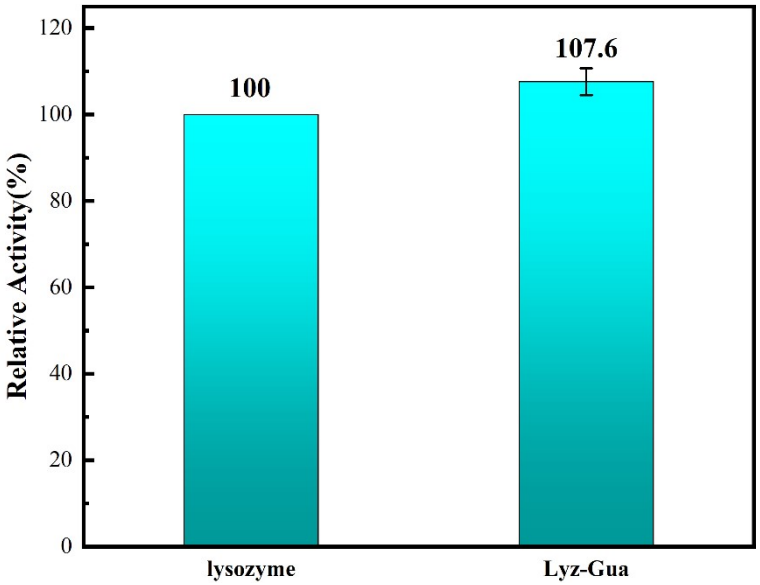

Figure S2. Relative activity of native lysozyme and Lyz-Gua.

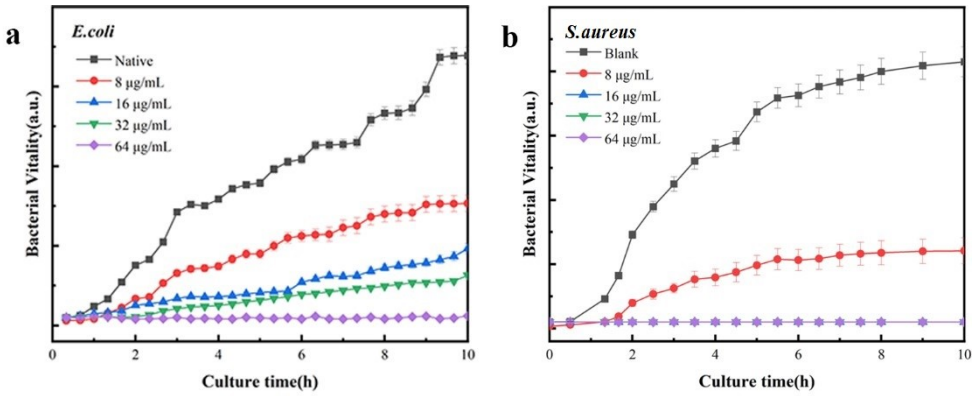

Figure S3. Determination of bacterial growth curve of *E.coli*(ATCC 25922, a) and *S.aureus* (ATCC 25925, b) treated by different concentrations of Lyz-Gua

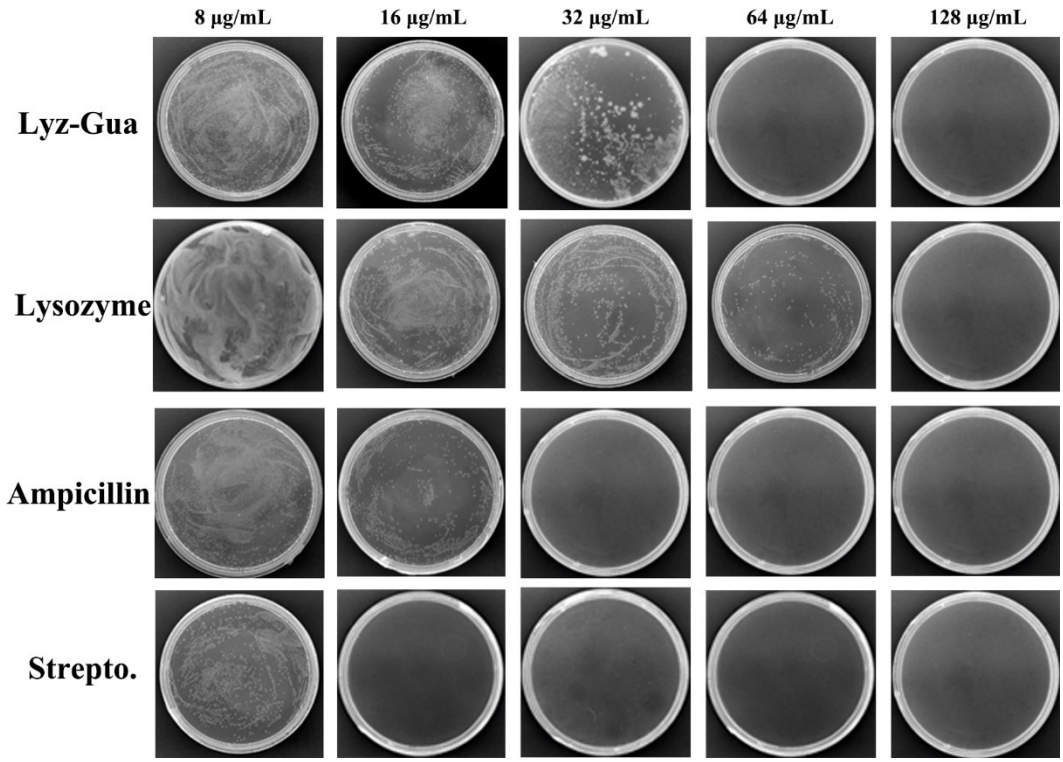

Figure S4. Agar Plate culture of ATCC 25922 after overnight co-culture with Lyz-Gua, lysozyme, Ampicillin and Streptomycin.

Table S1. Minimum inhibitory concentrations of Lyz-Gua, lysozyme, ampicillin and Streptomycin against multiple pathogenic bacteria species.

Species	Bactericide	MIC (μg/mL)	MIC (μM)	MBC (μg/mL)	MBC (μM)
ATCC 25922	Lyz-Gua	32	2.29	64	4.57
	lysozyme	64	4.57	128	9.14
	Ampicillin	16	43.08	32	86.16
	Streptomycin	8	5.51	16	11.02
DH-5α	Lyz-Gua	64	4.57	128	9.14
	lysozyme	128	9.14	256	18.28
	Ampicillin	128	344.64	128	344.64
	Streptomycin	64	44.08	128	88.16
MR-1	Lyz-Gua	16	1.14	16	1.14
	lysozyme	32	2.29	32	2.29
	Ampicillin	4	10.77	4	10.77
	Streptomycin	4	5.51	4	5.51
ATCC 25925	Lyz-Gua	32	2.29	64	4.57
	lysozyme	64	4.57	128	9.14
	Ampicillin	16	43.08	32	86.16
	Streptomycin	8	11.02	8	11.02

Table S2. Minimum inhibitory concentrations of Lyz-Gua, lysozyme, ampicillin and Streptomycin against multiple pathogenic bacteria species.

Bacteria Species	Bactericide	Diameter (mm)
ATCC 25922	Lyz-Gua	6.51
	lysozyme	4.22
	Ampicillin	6.71
	Streptomycin	3.98
DH-5α	Lyz-Gua	7.21
	lysozyme	2.72
	Ampicillin	7.19
	Streptomycin	2.58
Shewanella	Lyz-Gua	12.51
	lysozyme	9.72
	Ampicillin	7.71
	Streptomycin	7.58
ATCC 25925	Lyz-Gua	3.2
	lysozyme	1.1
	Ampicillin	3.5
	Streptomycin	2.1

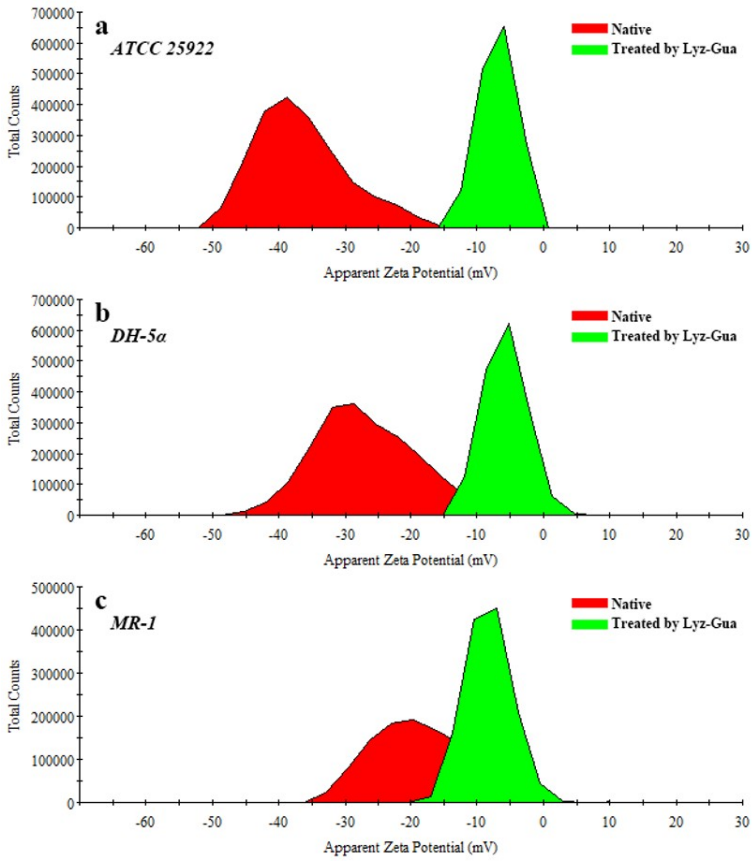

Figure S5. The measurement of Zeta potential of bacteria surface (ATCC 25922, a; DH-5α, b；MR-1, c) before and after treatment by Lyz-Gua

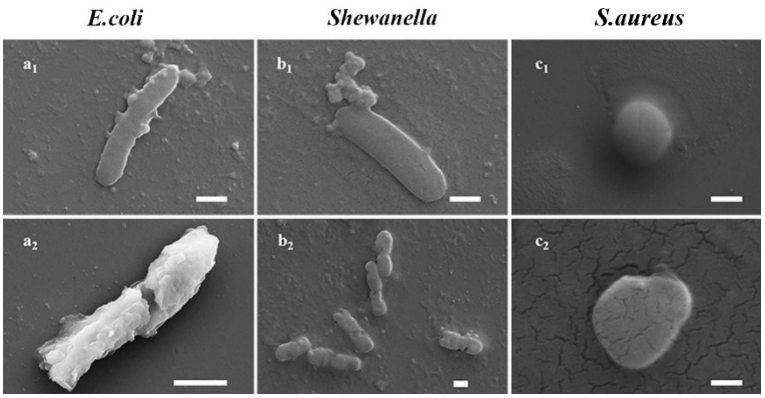

Figure S6. SEM images for E.coli (DH-5α, a), Shewanella (b) and S.aureus (ATCC 25925, c) before (1) and after (2) treatment by Lyz-Gua (mg/mL). The scale bar is 500 nm.

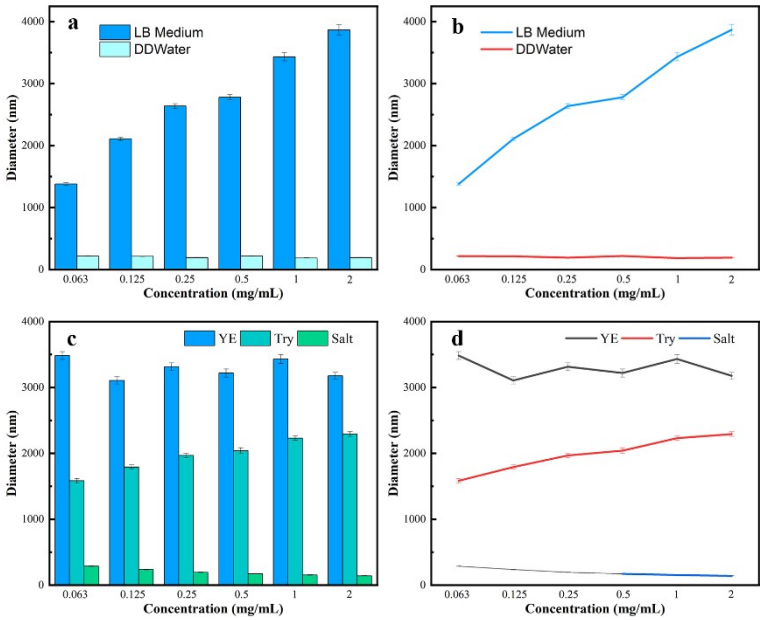

Figure S7. The diameter of Lyz-Gua assembly particles distributed in LB medium and double distilled water (a, b), and the solution of each component of LB medium (c, d).

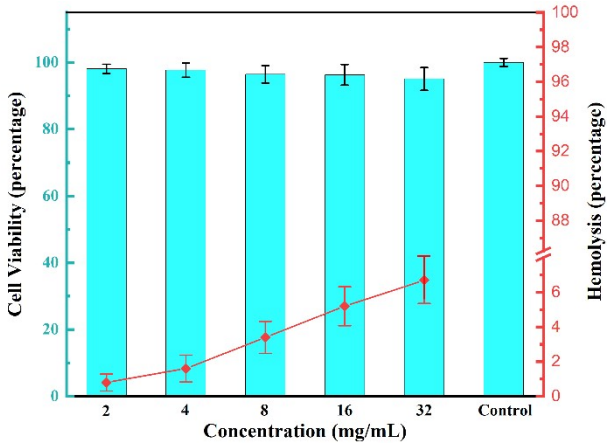

Figure S8. Viability of NIH/3T3 stem cells cultured with Lyz-Gua and hemolytic activity of Lyz-Gua at different concentrations indicates their excellent biocompatibility and non-hemolytic behaviors at relevant therapeutic concentrations.

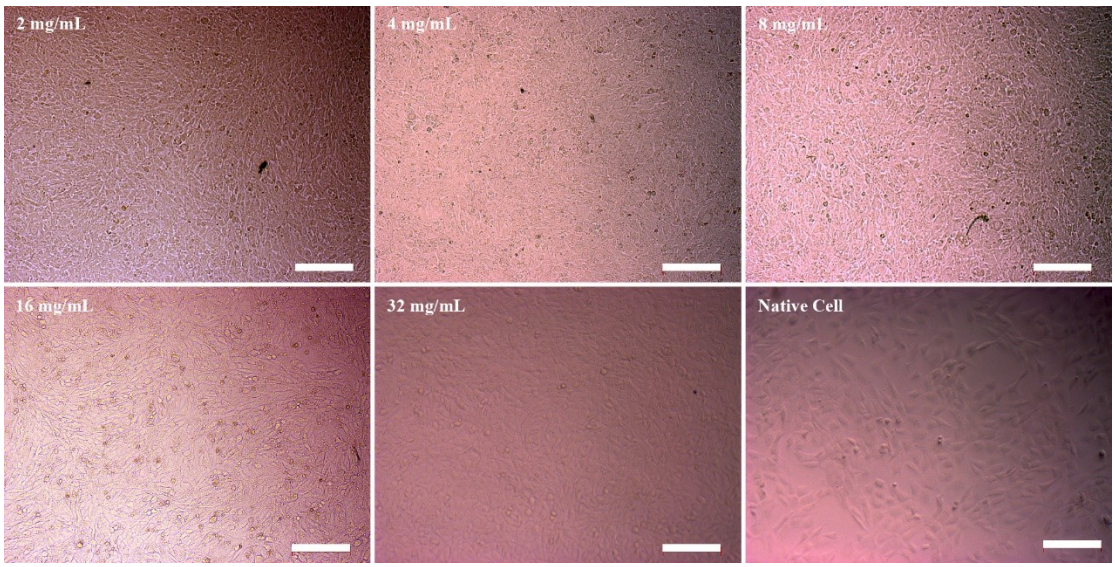

Figure S9. The microscope photos of NIH/3T3 fibroblast cells cultured with Lyz-Gua at different concentrations