Supplementary Information (SI)

Liposome-Based Delivery of DNA Aptamers to Inhibit Erythromycin Methyltransferase-Mediated Antibiotic Resistance

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TABLE OF CONTENTS

Section	Content	Page no.
1	Materials	S2
2	Methods	S2-S6
2.1	Preparation of Liposomes and DNA-aptamer–Loaded Liposomes (Fig. S1, Fig. S2)	S2-S3
2.2	Dynamic Light Scattering and Zeta Potential Measurements	S3
2.3	Cryogenic Transmission Electron Microscopy (Cryo-TEM)	S3
2.4	Encapsulation Efficiency	S3-S4
2.5	Culture of Resistant Bacterial Strains	S4
2.6	Delivery of Liposomes into Bacterial Strain	S4
2.7	Statistical Analysis	S4
2.8	Delivery of DNA Aptamer Using Liposomes into Bacterial Cells	S4-S5
2.9	Cell Viability Assay	S5
3	Characterization of unloaded liposomes and DNA-aptamer-loaded liposomes (Table: S1)	S6
4	Flow Cytometric Evaluation of Bacterial Cell Killing (Table S2)	S6
5	Interaction Between Rhodamine-Labeled Liposomes (Rh-LS) and Bacterial Cells (Fig. S3 and Fig. S4)	S7-S8
6	Delivery of DNA Aptamer Using Liposomes into Wild-type *S. aureus* (Fig. S5)	S8
7	Flow Cytometric Evaluation of Bacterial Cell Killing (Fig. S6, Fig. S7, Fig. S8)	S9-S10

1. Materials

(2,3-Dioleoyloxy-propyl)-trimethylammonium-chloride (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N (methoxy(polyethyleneglycol)-2000) (DSPE-PEG, sodium salt) were purchased from MedChemExpress. Chloroform, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Triton X-100, and 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI), and propidium iodide (PI) were purchased from Sigma-Aldrich. Lissamine™ Rhodamine B 1,2-Dihexadecanoyl-sn-glycero-3-phosphoethanolamine, Triethylammonium Salt (rhodamine DHPE) was purchased from Thermo Fisher.

2. Methods

2.1. Preparation of Liposomes and DNA-aptamer–Loaded Liposomes

Liposomes were formulated using DOTAP, DOPE, and DSPE-PEG (Fig. S1b) at a molar ratio of 49.5:49.5:1. The lipids were dissolved in chloroform and transferred to a round-bottom flask, followed by solvent evaporation under reduced pressure using a rotary evaporator maintained at 40 °C to obtain a thin lipid film. The dried film was subsequently hydrated with 20 mM HEPES buffer (pH 7.4) to achieve a final lipid concentration of 1mM and 5 mM. The resulting mixture was sonicated for 30 min using a probe sonicator to produce small, uniform vesicles. The hydrodynamic diameter and zeta potential of the liposomes were routinely measured by dynamic light scattering (Litesizer 500, Austria). DNA-aptamer-loaded liposomes were prepared by incubating the preformed liposomes with the DNA solution at room temperature to obtain final aptamer concentrations of 1 μM and 2 μM in the PEGylated formulations. For the preparation of Rh-labelled liposomes (Rh-LS), Rh-DHPE was added to the lipid mixture, and the formulation process was carried out following the same procedure as described above. The final Rh-DHPE concentration was 2 μM in the liposome formulations.

(a)

DNA Sequence: CGAAATGATGGCTATTTCCACGCACCGGTAAAAGGGTTGTTGTTGTTGGTAC (52 mer)

(b)

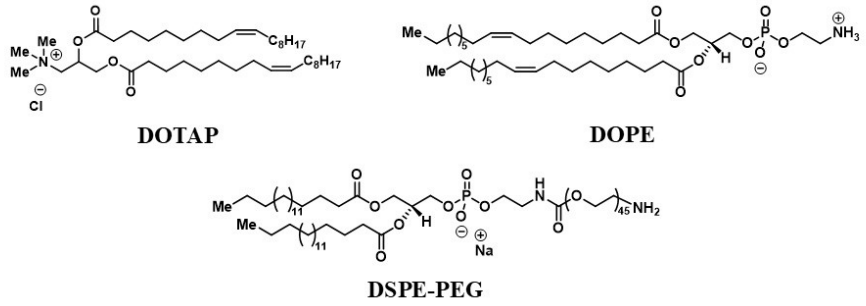

Fig. S1 (a) Sequence of the DNA-aptamer targeting Erm42, (b) Structures of DOTAP, DOPE, and DSPE-PEG lipids.

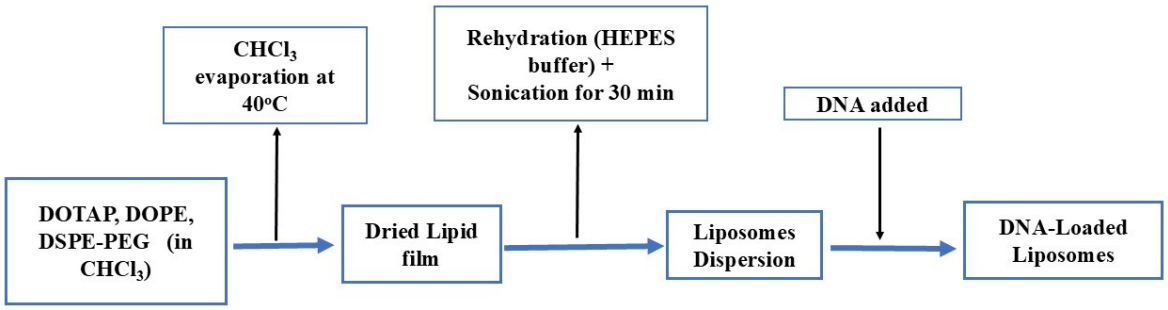

Fig. S2 Schematic representation of liposomes preparation.

2.2. Dynamic Light Scattering and Zeta Potential Measurements

The particle size distribution and surface charge of the liposomes were determined using an Anton Paar Litesizer instrument (Litesizer 500, Austria). Dynamic light scattering (DLS) was employed to measure the mean hydrodynamic diameter (z-average) and the polydispersity index (PDI), which indicates the uniformity of particle size distribution. The zeta potential of the liposomal dispersion was also determined using the same instrument to assess surface charge and colloidal stability. For all measurements, the liposome formulations were diluted 10 times with Milli-Q water. Diluted samples were transferred to cuvettes for size analysis and zeta potential measurements, ensuring that no air bubbles were present before analysis.

2.3. Cryogenic Transmission Electron Microscopy (Cryo-TEM)

For cryogenic transmission electron microscopy (cryo-TEM) analysis, 3 µL of the liposome formulation was applied onto glow-discharged Quantifoil holey carbon grids (R1.2/1.3, 200 mesh). The grids were glow-discharged for 120 s at 20 mA. After sample loading, the grids were blotted for 5 s, with a wait time of 3 s and blot force of 2, under 100% relative humidity at 4 °C using a Vitrobot Mark IV (FEI) and immediately plunge-frozen in liquid ethane. Imaging was performed on ThermoFisher Scientific's Titan Krios G4 operated at an accelerating voltage of 300 kV, equipped with a Gatan K3 Biocontinuum direct electron detector. Particles were visualized in the micrograph acquired at a nominal magnification of 105,000× with a defocus value of –3.0 µm.

2.4. Encapsulation Efficiency

The encapsulation efficiency (EE%) of the DNA aptamer within liposomes was determined using UV–visible spectroscopy. DNA-loaded liposomes were centrifuged at 12,000 rpm for 15 min at room temperature to separate unencapsulated DNA. The supernatant containing free DNA was collected, and its concentration was measured spectrophotometrically at 260 nm. The amount of encapsulated DNA was calculated by subtracting the unencapsulated fraction from the total initial DNA added (equation 1).

$$\% \text{ EE} = \frac{(\text{Initial DNA concentration} - \text{DNA concentration in supernatant})}{\text{Initial DNA concentration}} \times 100 \quad \text{eq. (1)}$$

To confirm encapsulation, the liposomal pellet was disrupted using a chloroform/methanol (2:1, v/v) mixture. Briefly, 400 µL of the solvent mixture was added to the pellet and vortexed until complete lipid dissolution, followed by the addition of 100 µL of distilled water. The sample was centrifuged for 10 min, and the aqueous phase was collected for quantification of the released DNA at 260 nm.

2.5. Culture of Resistant Bacterial Strains

Gram-positive resistant *Staphylococcus aureus* (isolated from a local Hospital) and wild-type *Staphylococcus aureus* strain obtained from MTCC96 was used in the study. The bacteria were grown in Luria Bertini (LB) Agar for 12-14 hours. In each assay, the bacteria were harvested from agar plates to inoculate Tryptic Soy Broth (TSB) for incubation at 37 °C for 12-14 hours. The secondary culture was inoculated further and grown until the optical density of the culture at 600 nm (OD_{600}) reached 0.1.

2.6. Delivery of Liposomes into Bacterial Strain

Freshly grown bacteria (1 mL) were centrifuged at 5000 rpm and then resuspended in 100 µL Rhodamine-labelled liposomes (1 mM and 5 mM), and incubated at 37 °C for 2 hours. The cells were then pelleted at 5000 rpm and washed twice with a wash buffer (15 mM NaCl, 0.1% (v/v) Triton X-100, and 5 mM Tris Base, pH 7.4) to remove any free liposomes that might have adhered to the bacterial membrane. Further, 4% paraformaldehyde (PFA) was added to fix the cells, and they were incubated for 10 minutes at room temperature, followed by another wash with the wash buffer. For slide preparation, the cells were resuspended in 10 µl of the buffer. Images were then collected in Z-stacking mode using a laser scanning confocal microscope (LSM 780, Carl Zeiss) in the red (568-600) channel. For Flow cytometry, data were recorded using PE filters to measure % stained cells. Flow cytometry data were analyzed using FlowJo software and confocal images were processed and analyzed using ZEN (Zeiss) imaging software. Statistical analysis was performed.

2.7. Statistical Analysis

Using GraphPad Prism 8.0.1, a Dunnett's multiple comparison test (ANOVA one-way) was used to establish statistical significance. Mean values in each column do not differ significantly at $p< 0.05$. Values less than 0.05 were used to calculate the statistical significance. The ns symbol showed that the difference was not significant, whereas the different * sign [p=<0.0001(****), p=<0.001(***), p=<0.01(**), p=<0.1(*)] indicated a significant difference.

2.8. Delivery of DNA Aptamer Using Liposomes into Bacterial Cells

Freshly grown bacteria (1 mL) were centrifuged at 5000 rpm and then resuspended with 100 µL liposome-DNA (1 mM and 5 mM liposomes, 1 µM and 2 µM DNA-loaded liposomes) complex for further incubation at 37 °C for 2 h. The cells were then pelleted at 5000 rpm and washed twice with wash buffer (15 mM NaCl, 0.1% (v/v) Triton X-100, and 5 mM Tris Base, pH 7.4) to remove any free liposomes that might have adhered to the bacterial

membrane. Further, 4% paraformaldehyde (PFA) was added to fix the cells, and they were incubated for 10 minutes at room temperature, followed by another wash with the wash buffer. After that, the cells were stained with 0.01% (w/v) DAPI (4',6-Diamidino-2-phenylindole) for 10 minutes at 37 °C. For slide preparation, the cells were resuspended in 10 µl of the buffer. Images were then collected in Z-stacking mode using a laser scanning confocal microscope (LSM 780, Carl Zeiss) in both the green (500-525 nm) and blue (420-500 nm) channels to capture FAM-labeled DNA and DAPI-stained cells. Confocal images were processed using ZEN (Zeiss) imaging software.

Additionally, the DNA delivery using liposomes was assessed through flow cytometry (BD FACS Aria SORP). Freshly grown bacteria (1 mL) were centrifuged at 5000 rpm and then resuspended with 100 µL liposomes and liposome-DNA complex for incubation at 37 °C for 2 h. The cells were then pelleted at 5000 rpm and washed twice with wash buffer (15 mM NaCl, 0.1% (v/v) Triton X-100, and 5 mM Tris Base, pH 7.4) to remove any free liposomes and then resuspended in 20 mM HEPES at pH 7.4. Data were collected using a FITC filter to analyze the FAM-labeled DNA liposomes present in the samples. Flow cytometry data were analyzed using FlowJo software.

2.9. Cell Viability Assay

The effect of erythromycin in the presence of DNA aptamer on macrolide resistance was assessed using flow cytometry and confocal microscopy, where propidium iodide (PI) dye was used to quantify dead cells in the system. 1 mL of freshly grown bacterial cells was pelleted and resuspended in a 100 µL liposome-DNA complex for incubation at 37°C for 1 hour, followed by treatment with 512 µg/ml erythromycin and an additional 1-hour incubation. The cells were then washed twice with the wash buffer (15 mM NaCl, 0.1% (v/v) Triton X-100, 5 mM Tris Base, pH 7.4) Further, for confocal microscopy, 4% paraformaldehyde (PFA) was added to fix the cells, and they were incubated for 10 minutes at room temperature, followed by another wash with the wash buffer. After that, the cells were stained with PI (5 µg/ml) for 10 minutes. For slide preparation, the cells were resuspended in 10 µl of the buffer. Images were then collected in Z-stacking mode using a laser scanning confocal microscope (LSM 780, Carl Zeiss) in the red (568-600) channels to capture PI-stained cells. For Flow cytometry, they were stained with PI (5 µg/ml) 10 minutes before data acquisition. Data were recorded using PE filters to quantify dead cells. Statistical analysis was performed as described above. Flow cytometry data were analyzed using FlowJo software and confocal images were processed using ZEN (Zeiss) imaging software.

3. Characterization of unloaded liposomes and DNA-aptamer-loaded liposomes

Table S1. Comparison of hydrodynamic diameter (d_h), polydispersity index (PDI), and zeta potential of unloaded liposomes (LP), DNA-aptamer–loaded liposomes prepared at 5 mM total lipid concentration (DOTAP/DOPE) containing 1 μM (LP5/N1) and 2 μM (LP5/N2) of DNA aptamer, and unloaded PEGylated rhodamine-labelled liposomes (Rh-LP) formulated at 1 mM and 5 mM total lipid concentration with 2 μM Rh-DHPE. Entrapment efficiency (EE%) values are also presented for DNA-loaded liposomes prepared at 1 mM and 5 mM total lipid concentration containing 1 μM and 2 μM of DNA aptamer. The results are shown as the mean ± SD (n=3).

Liposomes designation	Formulation	d_h (nm)	PDI	ζ-Potential (mV)	EE (%)
LP1	DOTAP + DOPE + DSPE-PEG	160 ± 20	0.23 ± 0.02	+ 16 ± 3	-
LP1/N1	DOTAP + DOPE + DSPE-PEG + DNA	165 ± 25	0.22 ± 0.04	+ 14 ± 2	24 ± 5
LP/N2	DOTAP + DOPE + DSPE-PEG + DNA	164 ± 30	0.21 ± 0.02	+ 13 ± 4	21 ± 4
LP5	DOTAP + DOPE + DSPE-PEG	170 ± 20	0.21 ± 0.03	+ 17 ± 3	-
LP5/N1	DOTAP + DOPE + DSPE-PEG + DNA	178 ± 20	0.20 ± 0.03	+ 15 ± 2	51 ± 8
LP5/N2	DOTAP + DOPE + DSPE-PEG + DNA	175 ± 15	0.21 ± 0.04	+ 14 ± 3	55 ± 7
Rh-LP1	DOTAP + DOPE + DSPE-PEG + Rh-DHPE	160 ± 20	0.23 ± 0.03	+ 14 ± 4	-
Rh-LP5	DOTAP + DOPE + DSPE-PEG + Rh-DHPE	161 ± 25	0.20 ± 0.04	+ 15 ± 3	-

4. Flow Cytometric Evaluation of Bacterial Cell Killing

Table S2. Cell death percentages for LP5 and aptamer-loaded LP5 formulations, the results are shown as the mean ± SD (n=3).

Formulations	% Cell death	Formulations	% Cell death
(-) Ctrl	0.32 ± 0.2	LP5/ScN1+Ery	24.2 ± 1.9
(+) Ctrl	99.3 ± 0.4	LP5/ScN2+Ery	26.3 ± 2.6
LP5	10.6 ± 1.5	LP5/N0.5+Ery	48.3 ± 2.9
Ery	9.19 ± 1.7	LP5/N1+Ery	83.8 ± 4.6
LP5+Ery	8.8 ± 1.4	LP5/N2+Ery	90.2 ± 3.4

Values are expressed as mean ± standard deviation from three independent experiments. (−) Ctrl and (+) Ctrl represent untreated and positive cell-death controls, respectively. LP5 denotes blank liposomes, Ery indicates erythromycin alone. LP5/ScN1 and LP5/ScN2 correspond to scrambled DNA loaded liposomes, while N0.5, N1, and N2 indicate increasing concentrations of the specific DNA aptamer. All erythromycin-containing formulations were tested at identical drug concentrations.

5. Interaction Between Rhodamine-Labeled Liposomes (Rh-LS) and Bacterial Cells

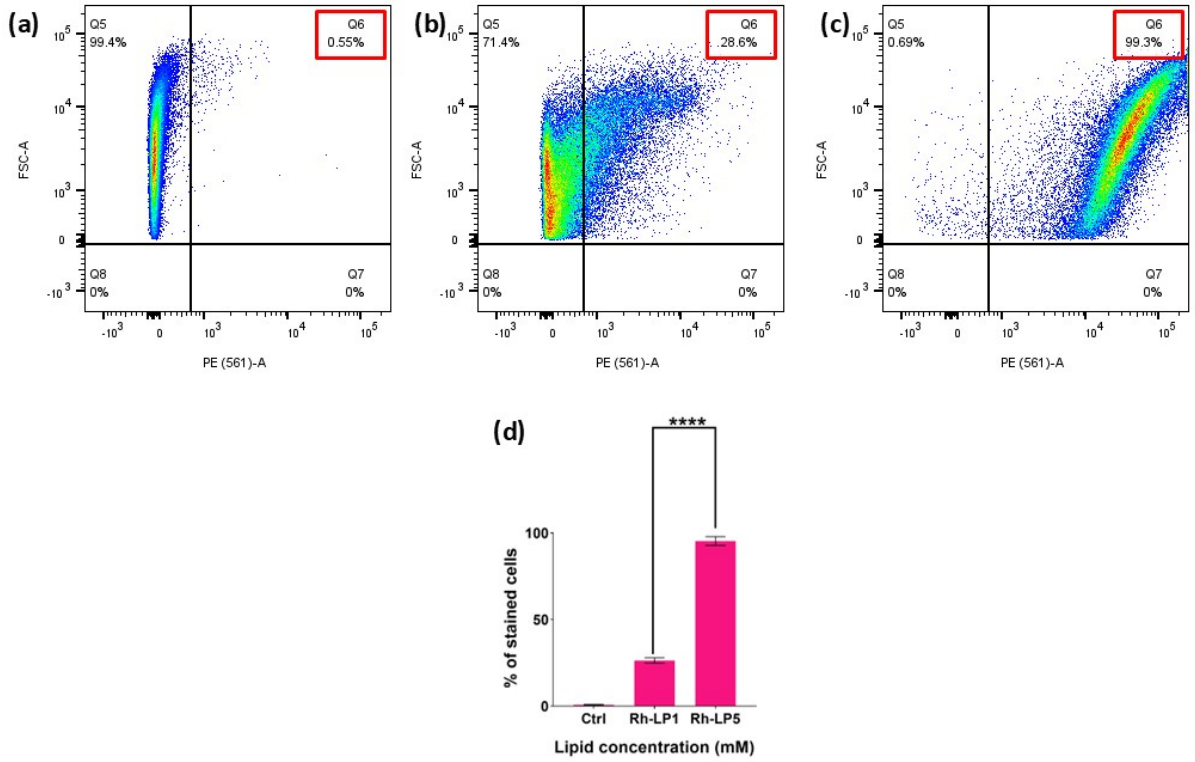

Fig. S3 Quantitative analysis of liposome-bacterial cell interactions in resistant *S. aureus*. Flow cytometry plots showing the percentage of rhodamine-positive *S. aureus* cells after incubation with (a) control (untreated), (b) Rh-LP1, and (c) Rh-LP5 liposomes. The gated population in Q6 (red box) indicates rhodamine-positive cells. (d) The percentage of fluorescently stained bacterial cells was quantified by flow cytometry and normalized to untreated control bacteria incubated in HEPES buffer. Data represent the mean ± standard deviation (SD) of three independent experiments. Statistical significance is indicated as follows: ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.1$; ns denotes not significant.

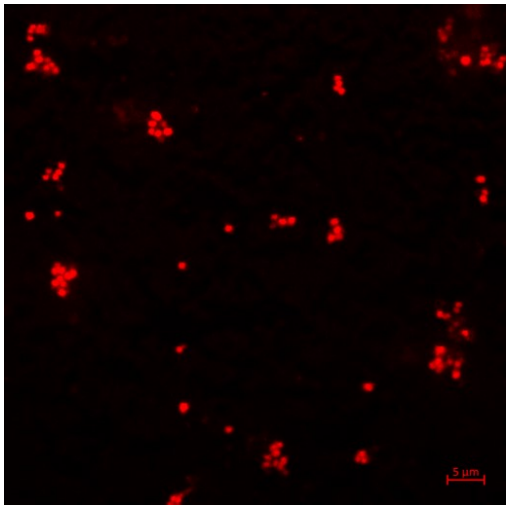

Fig. S4 Confocal laser scanning microscopy (CLSM) image showing the membrane localization of Rhodamine-PE following fusion of Rh-LP5 liposomes with resistant *S. aureus*. Small bright red spots correspond to bacterial cells exhibiting incorporated Rh-PE within their cell envelopes, indicating successful liposomal fusion and fluorescent lipid transfer. The uniform membrane-associated red signal across multiple bacterial clusters confirms efficient interaction of Rh-LP5 with the bacterial surface. Scale bar: 5 µm.

6. Delivery of DNA Aptamer Using Liposomes into Wild-type *S. aureus*

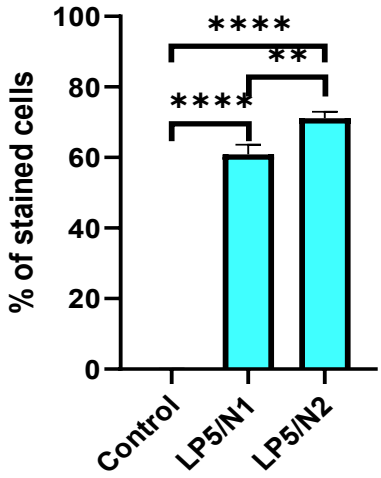

Fig. S5 Cellular internalization of DNA-loaded liposome in wild-type *S. aureus*. (A) Quantitative analysis of FAM-labeled DNA aptamer uptake by flow cytometry showing a dose-dependent increase in the percentage of stained cells with increasing lipid and DNA concentrations (LP5/N1 = 5 mM lipid + 1 µM DNA; LP5/N2 = 5 mM lipid + 2 µM DNA) after 2h incubation. A Dunnett's multiple comparison test (ANOVA one-way) was used to establish statistical significance. Sign [$p \leq 0.0001$(****), $p \leq 0.001$(***), $p \leq 0.01$(**), $p \leq 0.1$(*)] indicates a significant difference, and ns means not a significant difference. (B) Flow cytometry of untreated control cells showing negligible fluorescence (0.03%), (C) Cells treated with 5 mM lipid and 1 µM FAM-DNA liposomes (LP5/N1) show strong fluorescence, indicating 61% internalization, (D) Cells treated with 5 mM lipid and 2 µM FAM-DNA liposomes (LP5/N2) show strong fluorescence, indicating 72% internalization.

7. Flow Cytometric Evaluation of Bacterial Cell Killing

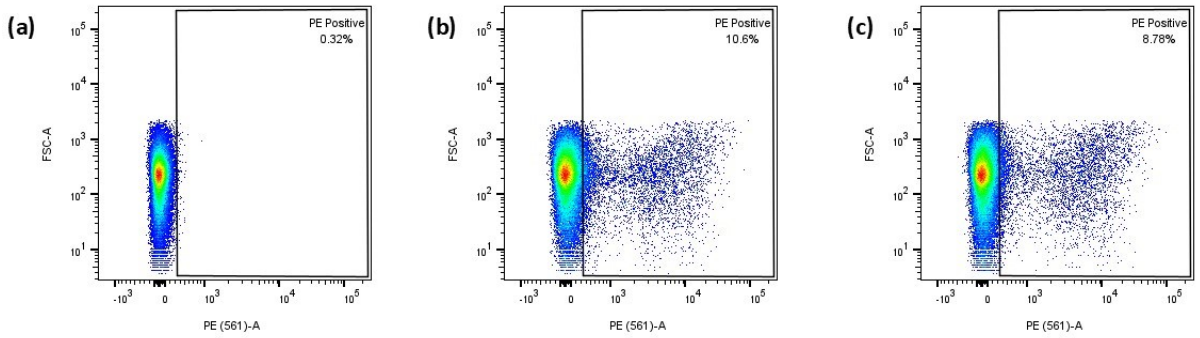

Fig. S6 Flow cytometry plots showing PI-positive (dead) *S. aureus* cells after 1-hour incubation with liposomes followed by erythromycin treatment for an additional hour. (a) The negative control shows minimal PI staining (0.3%). (b) LP5-treated cells display increased PI positivity (11%). (c) Combined LP5 + erythromycin treatment results in bacterial killing (9%).

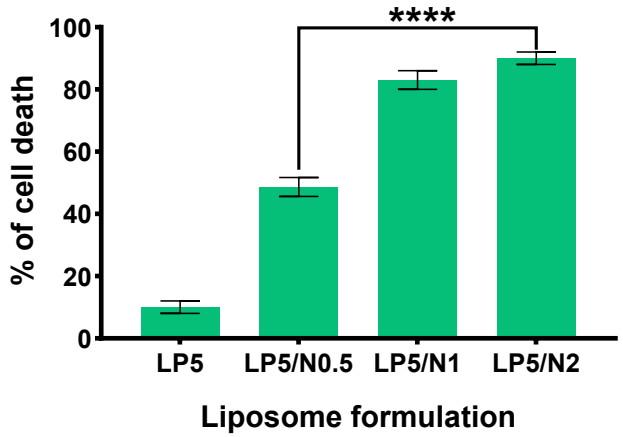

Fig. S7: Dose-dependent bacterial killing induced by aptamer-loaded liposomes. Increasing concentrations of DNA aptamer in LP5 formulations (LP5/N0.5, LP5/N1, and LP5/N2) resulted in a progressive increase in bacterial cell death, whereas the liposome-only control (LP5) showed minimal cytotoxicity.

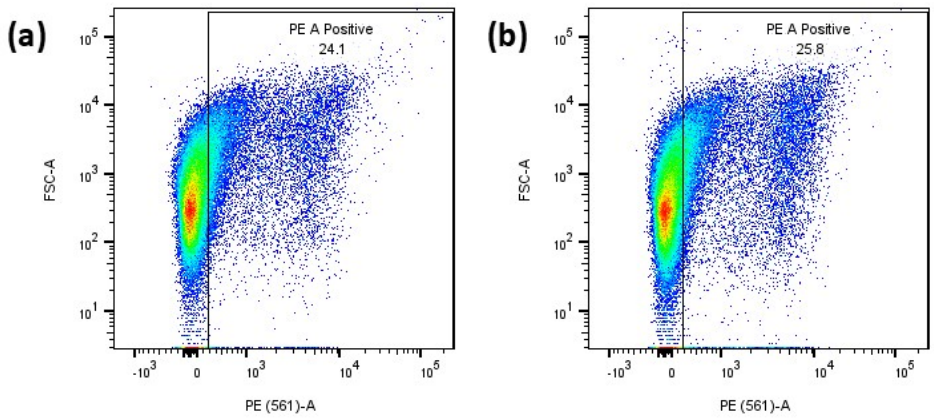

Fig. S8 Evaluation of bactericidal activity of scrambled aptamer-loaded liposomes against resistant *S. aureus*. (a-b) Representative flow cytometry plots showing the percentage of dead cells for (a) LP5/ScN1+Ery, (b) LP5/ScN2+Ery.