An aluminum lining to the dark cloud of silver resistance: Harnessing the power of potent antimicrobial activity of γ-alumina nanoparticles

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Characterization

Colony forming units (CFU/mL) assay

An isolated colony was cultured in 5 mL of Tryptic soy broth (TSB) medium at 37 °C and 180 rpm overnight in incushaker. Then, 50 μL of the cultured media was transferred into 5 mL of fresh TSB media and incubated for 4 hrs at 37 °C and 180 rpm. The resulting bacterial suspension contained ca. 1×10^9 colony forming units (CFU). Then 50 μL of above suspension was added to 5 mL of fresh TSB media (ca. 10^7 CFU/mL).

CFU counts: 20-μL of nanoparticle solutions with different concentrations was added to 980 μL TSB medium containing bacterial suspension from the above culture. After 24-hour incubation, 10 μL of this solution was taken out and diluted 10^6 times with TSB medium. Then 50 μL of the diluted suspension was spread on agar plates using glass spreaders. The plate was incubated for 24 hours at 37 °C and the number of colonies were counted in each plate and converted into CFU/ml values. All measurements were triplicated.

Optical density measurement

An isolated colony was cultured in 5 mL of Tryptic soy broth (TSB) medium at 37 °C and 180 rpm overnight in incushaker. Then, 50 µL of the cultured media was transferred into 5 mL of fresh TSB media and incubated for 4 hrs at 37 °C and 180 rpm. The resulting bacterial suspension contained ca. 1×10^9 colony forming units (CFU). Then 50 µL of above suspension was added to 5 mL of fresh TSB media (ca. 10^7 CFU/mL). Thereafter, 20-µL of nanoparticle solutions with different concentrations was added to 980 µL TSB medium containing bacterial suspension from the above culture. After 24 hours, 50 µL of each sample was taken and measured the optical density using the SpectraMax® M4 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, CA.

Minimum inhibitory concentration (MIC) assay

Nanoparticle solutions of different concentrations were inoculated with a chosen bacterial strain at a concentration of 10^5 CFU/mL in 96 well-plate. The bacteria were incubated at 37 °C for 24 hours. After incubation, the MIC of NPs was determined as the lowest concentration that inhibited visible growth of the tested microorganisms with unaided eyes.

Minimum bactericidal concentration (MBC) assay

Overnight culture of each bacterium was adjusted to 10^6 CFU/mL of suspension in broth. The bacteria were incubated at 37 °C overnight with varying concentrations of NPs in culture tubes. After overnight incubation subcultures were made by spreading 50 µL of each broth on agar plates from the tubes which showed no visible growth of bacteria. The plates were incubated for 24h, and the MBC value was defined as the lowest concentration of NPs which produced no colonies on the plate. The tests were repeated in triplicate.

Bacterial reactive oxygen species (ROS) detection

The activity of Al_2O_3-NPs on the intracellular ROS level toward MRSA cells were performed by DCFH-DA assay. First, 1 mL overnight cultured MRSA cells were collected by centrifugation (3750 rpm, 5 minutes) and re-suspended in 400 μL of fresh TSB medium. Then, the bacterial cells were incubated with different concentrations of Al_2O_3-NPs compared with DMSO as the control for 2 hours. Next, the cells of each group were harvested by centrifugation and washed twice with HBSS (Hank's Balanced Salt Solution,1X). The bacterial cells were incubated with 20 μM DCFH-DA dye in PBS at 37 °C with shaking for 30 min. In the meantime, CFU assay was performed before incubating with the dye in order to normalize the data. Finally, intracellular ROS levels were examined under a fluorescence microscope at the excitation and emission wavelengths of 497 nm and 529 nm, respectively.

Bacteria cell wall disrupting imaging

For high-resolution scanning electron microscope (HR-SEM) imaging, samples were prepared using the Protocol from M. Arakha et al.,[1] with some slight modifications. In brief, 1 mL of bacterial cultures were taken from the stationary phase of growth kinetics and centrifuged at 3750 rpm for 7 minutes at 4 ºC. Then the resulting pellet was collected, washed twice, and re-suspended in fresh 1X PBS. Bacterial cells were then fixed by incubating overnight in 2.5% glutaraldehyde (prepared in 1X PBS, 1mL). Fixed cells were then centrifuged and 500uL of glutaraldehyde was removed, suspended in 1% tannic acid (500uL) for 10-20 minutes, and washed with PBS (2 times), followed by dehydration using increasing concentrations of ethanol (25%, 30%, 50%, 75%, and 95%). Finally, cells were fixed, washed, and dehydrated bacterial cells were coated with gold for SEM.

MTT assays

Cytotoxicity of Al_2O_3-NPs toward mammalian cells was determined using an MTT viability assay. Mammalian cells (RAW cells and Human fibroblast cells) were seeded in a 96-well plate at a density of 4×10^5cells per well with DMEM medium and incubated for 24 hours at 37 °C in an atmosphere of 5 % CO_2 and 95 % air to allow cells to attach to the surface. Cells in each well were then treated with 100 µL of fresh medium containing varying concentrations of the Al_2O_3-NPs and then incubated for 24-72 hours. Control wells contained the same medium without NPs. After that, the cells were incubated with 10 µL of MTT reagent for 2 hours at 37 °C. Then, 100 µL of detergent reagent was added to all wells and the plate left with cover in the dark for 2 hours at 37 °C. The absorbance was measured at 570 nm using a microplate reader. The assay was run in triplicate, and the results were presented as the percentage of viable cells.

TEM protocol

Transmission electron microscopy (TEM) images were obtained on a FEI Tecnai G2 F20 microscope. Prior to TEM analysis, the samples were dispersed in ethanol by moderate sonication at concentrations of ~5-10 wt. %, followed by deposition of these samples on a Lacey carbon coated, 200-mesh, copper TEM grid by dipping into the sample suspension followed by drying under vacuum at 100 ºC for 1 h.

SEM protocol

Scanning electron microscopy (SEM) images were obtained using SEM-Quanta-450 scanning electron microscope. Prior to SEM analysis all samples were dispersed in ethanol followed by sonication at concentrations of ~5-10 wt. %. Sonicated samples were deposited on SEM Hitachi specimen holder and dried under vacuum at 100 ºC for 1h.

The LIVE/DEAD assay

The solution of fluorescent dyes was prepared by mixing 3 μL of SYTO 9 stain and 3 μL of propidium iodide stain to 1 mL of filter-sterilized water and used for cell staining in the same day to ensure freshness. After bacterial cells were treated with 5-μM Al_2O_3-NPs-30 and incubated at 37 °C and 180 rpm for 24 hours, the upper bacterial suspension and the lower substrate were separated. The lower substrate was washed with PBS three times. The upper suspension was centrifuged, and the substrate was washed with PBS three times. Then, 200 μL of the staining solution was added into 1 mL of the upper and lower PBS solutions, respectively. After covering the staining Eppendorf tubes and incubating the samples for 20-30 min at room temperature in the absence of light, both the sample and control groups were rinsing with PBS, the fluorescence images were collected using a Leica TCS SP5 II confocal microscope.

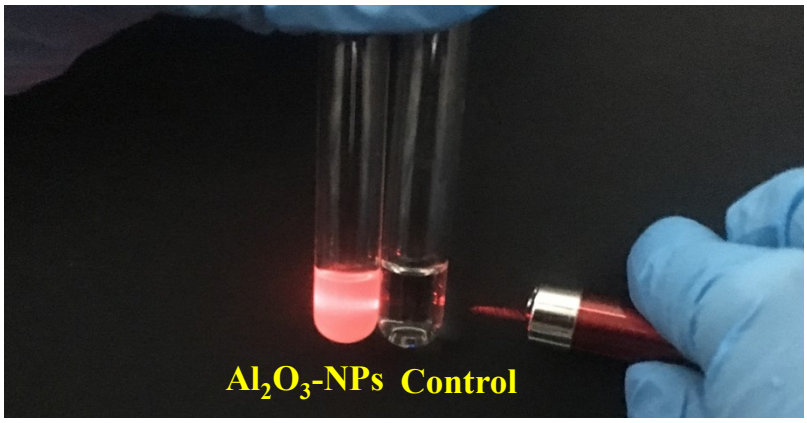

Figure S1. Al_2O_3-NPs showing Tyndall effect under the irradiation of LASER light

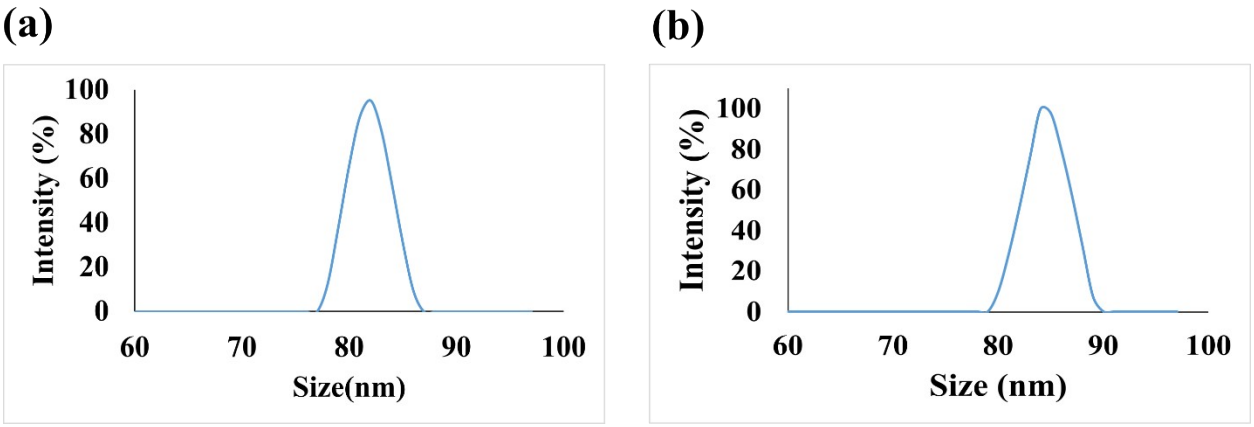

Figure S2. DLS curves of the as-synthesized (**a**) and the stored (**b**) sample of PEG-coated Al_2O_3-NPs-30 samples.

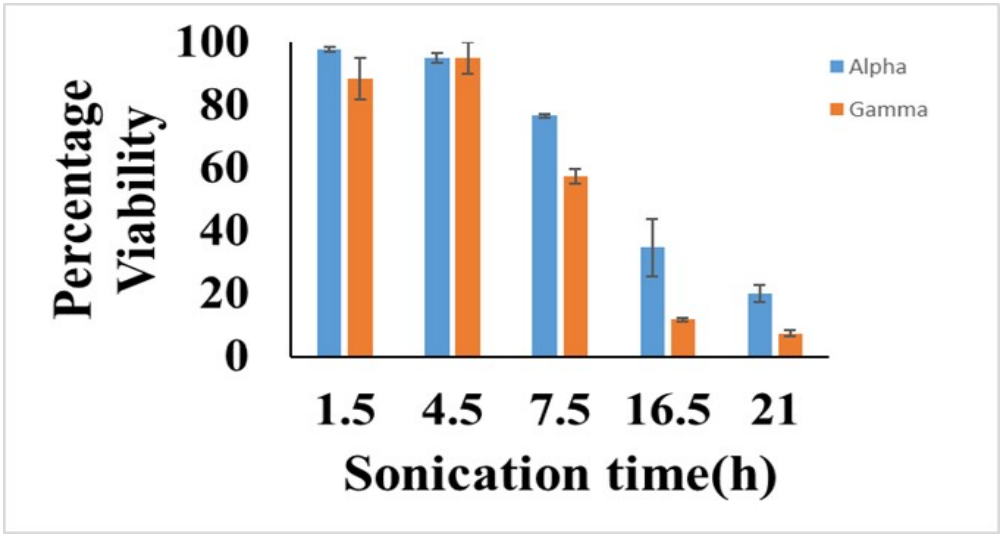

Figure S3. Growth inhibitory effect of α- and γ-Al_2O_3-NPs obtained with different sonication times on *S. aureus*

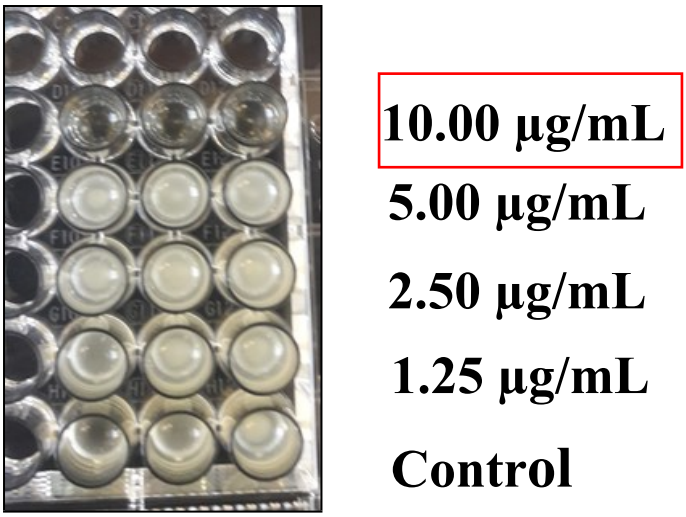

Figure S4. MIC assay performed on *S. aureus* in 96-well plates under different Al_2O_3-NPs-30 concentrations. (The red square indicates MIC value)

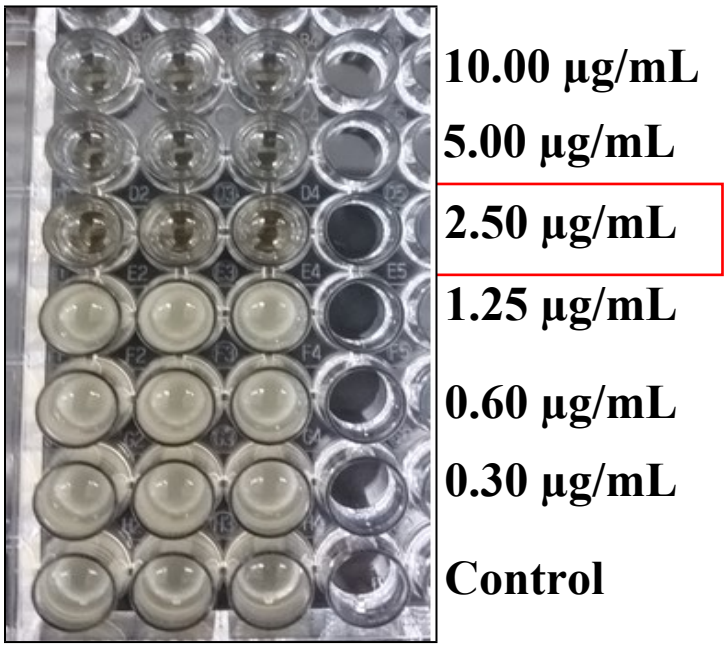

Figure S5. MIC assay performed on *P. aeruginosa* in 96-well plates under different Al_2O_3-NPs-30 concentrations. (The red square indicates MIC value)

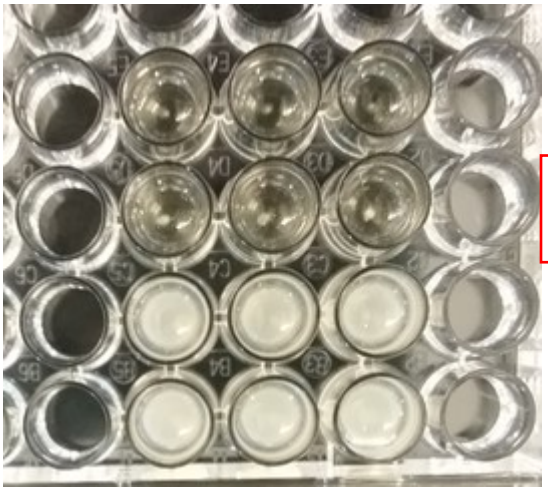

Figure S6. MIC assay performed on Methicillin-resistant *S. aureus (MRSA)* in 96-well plates under different Al_2O_3-NPs-30 concentrations. (The red square indicates MIC value)

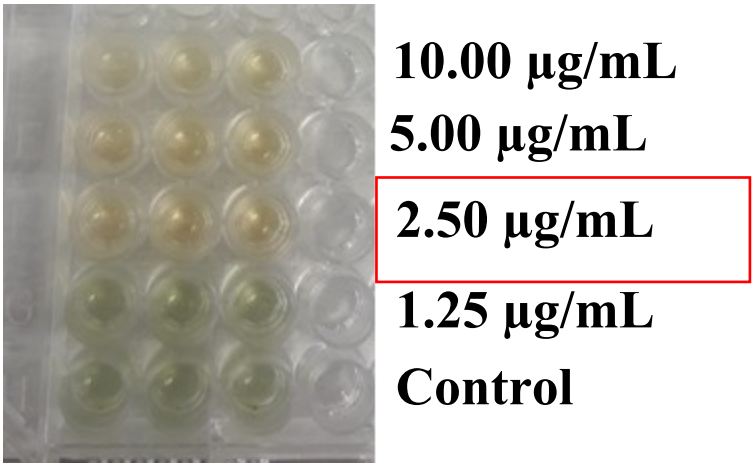

Figure S7. MIC assay performed on Drug-resistant *P. aeruginosa (DRPA)* in 96-well plates under different Al_2O_3-NPs-30 concentrations. (The red square indicates MIC value)

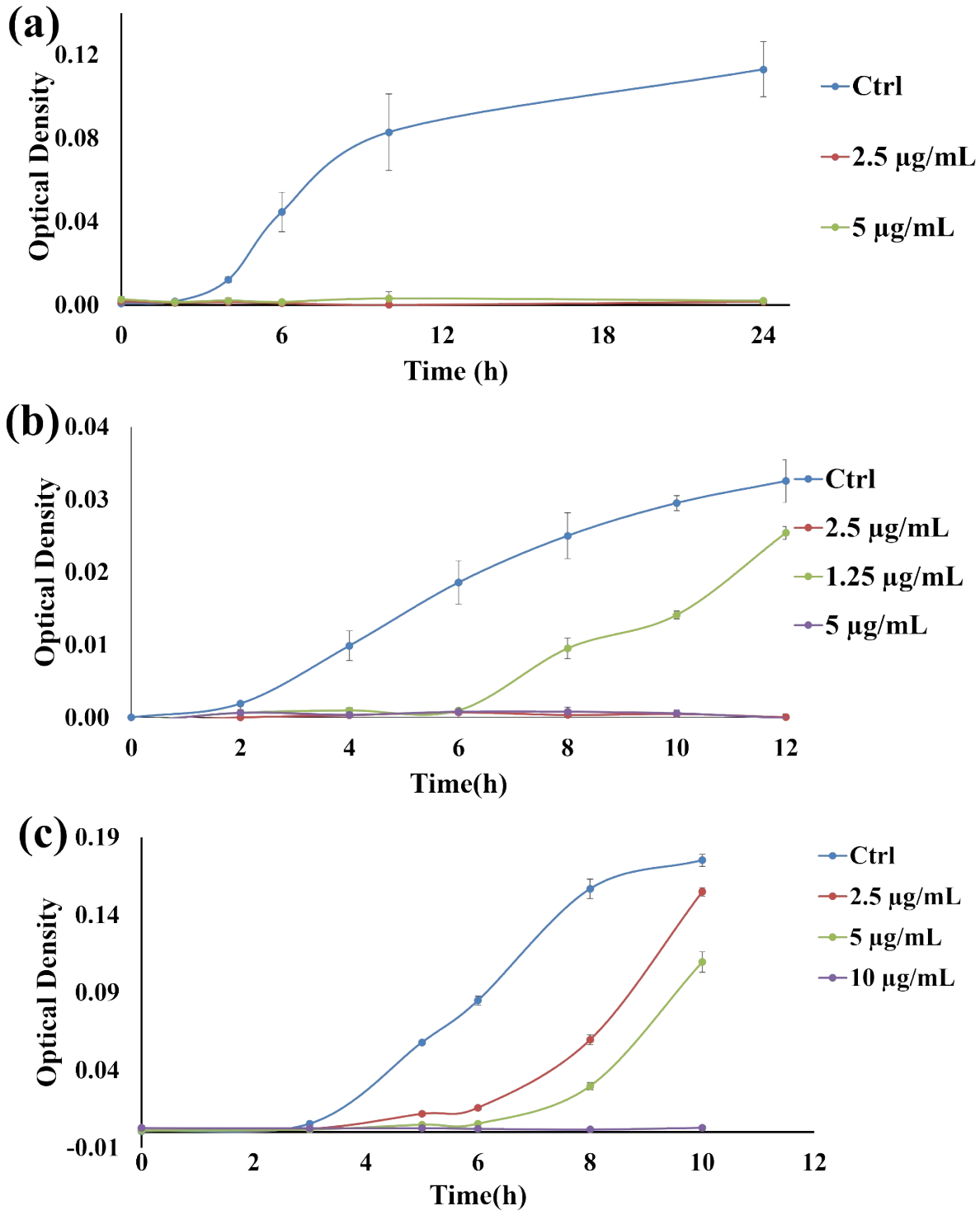

Figure S8. Time dependent Al_2O_3-NPs growth inhibition of *P. aeruginosa* (a), Drug resistant *P. aeruginosa* (b), and Methicillin-resistant *S. aureus* (c)

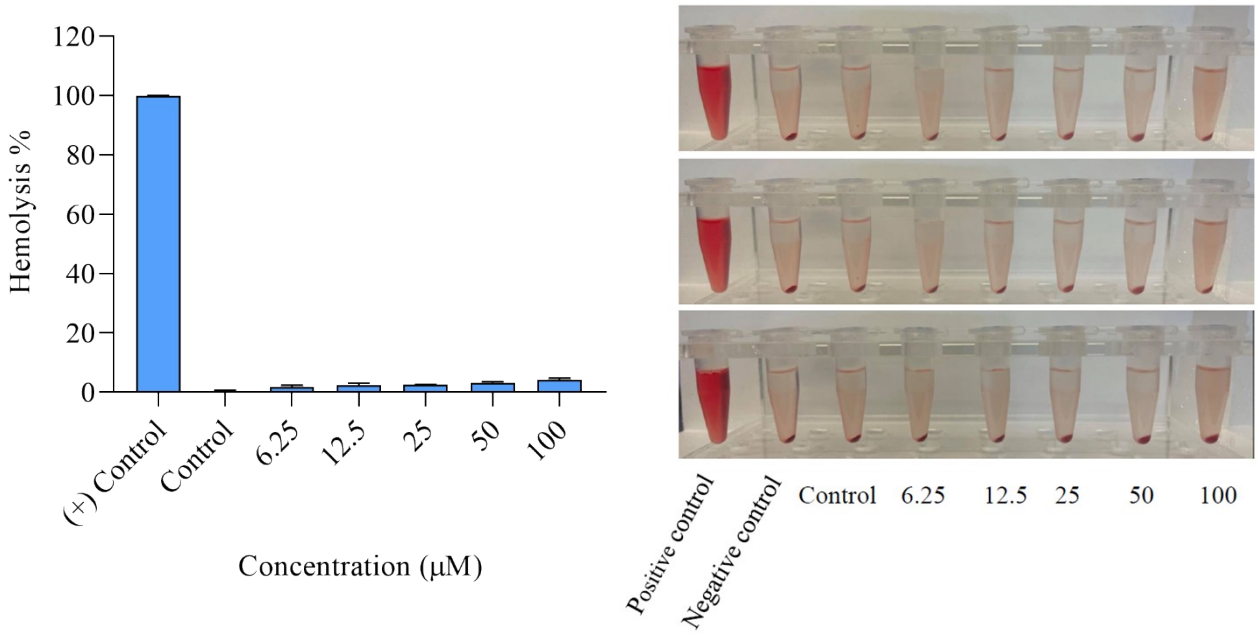

Figure S9. Percent hemolysis of Al_2O_3-NPs at different concentrations (left) and representative images of hRBCs hemolysis in response to Al_2O_3-NPs with the corresponding concentrations (right)(mean ± s.d, n =3 replicates).

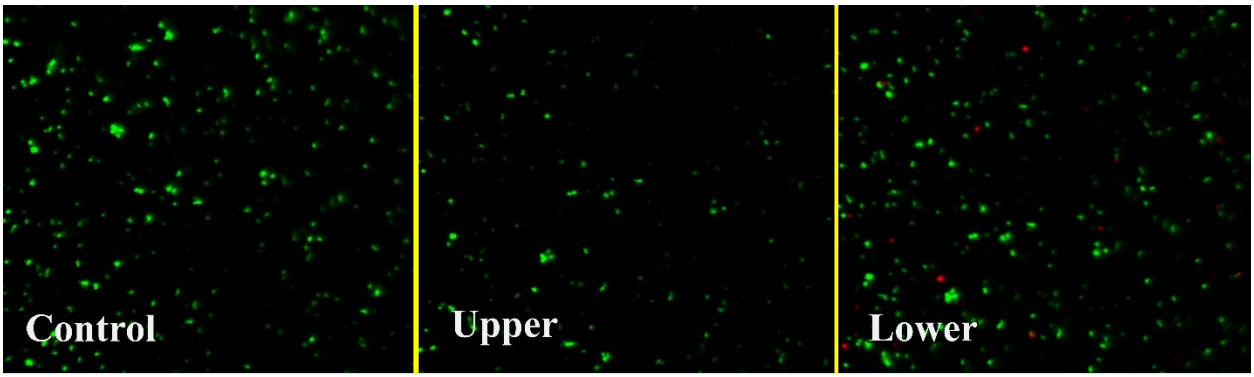

Figure S10. The fluorescence images of S. aureus bacterial cells treated with Al_2O_3-NPs-30 and stained with the LIVE/DEAD assay kit consisting of SYTO 9 and propidium iodide stains for monitoring the membrane integrity of both live (upper) and dead (lower) cells.

Table S1. Minimum bactericidal concentration (MBC) for different bacterial strains

Strain	MBC μg/mL
MSSA (ATCC 6538)	40
MRSA (ATCC BAA-44)	40
DSPA (ATCC 15692)	10
DRPA (ATCC BAA-2108)	10