AAC Accepted Manuscript Posted Online 24 August 2015
Antimicrob. Agents Chemother. doi:10.1128/AAC.01440-15
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Antibiofilm and membrane damaging potential of cuprous oxide nanoparticles against *Staphylococcus aureus* with reduced susceptibility to vancomycin

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Running title: Synthesis and antibiofilm behaviour of Cu_2O-NPs

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Abstract

Antimicrobial effects of copper ions and salts are well known, but the effects of cuprous oxide nanoparticles (Cu_2O-NPs) on staphylococcal biofilm have not yet been clearly revealed. The present study evaluated Cu_2O-NPs for antibacterial and antibiofilm activities against heterogeneous vancomycin intermediate *Staphylococcus aureus* (hVISA) and vancomycin intermediate *S. aureus* (VISA). Nanoscaled cuprous oxide (Cu_2O), generated by solution phase technology, contained Cu_2O octahedral nanoparticles. Field emission electron microscopy demonstrated particles size ranging from 100-150 nm. Cu_2O-NPs inhibited the growth of *S. aureus*, and showed antibiofilm activity. The minimum inhibitory concentrations and minimum biofilm inhibitory concentrations ranged from 625 µg/mL to 5000 µg/mL and 2500 µg/ml to 10,000 µg/ml, respectively. Exposure of *S. aureus* to Cu_2O-NPs caused leakage of cellular constituents and increased uptake of ethidium bromide and propidium iodide. Exposure also caused significant reduction in overall vancomycin-bodipy (dipyrromethene boron difluoride [4,4-difluoro-4-bora-3a,4a-diazas- indacene] fluorescent dye) binding, and decrease in viable cell counts in presence of 7.5% sodium chloride. Cu_2O-NPs toxicity assessment by haemolysis assay showed no cytotoxicity at 625 - 10000 µg/ml concentrations. The results suggest that Cu_2O-NP_S exert their action by disruption of bacterial cell membrane and can be used as effective antistaphylococcal and antibiofilm agents in diverse medical devices.

Key words: hVISA, VISA, Cu_2O, Nanoparticles, Biofilm.

Introduction

Biofilm formation, one of the defence mechanisms of *Staphylococcus aureus* represents a structural community of bacterial cells embedded in a self produced polymeric matrix

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adherent to artificial surface (1). Biofilms can be associated with a variety of complications, the worst being the risk of bacterial and fungal infections in surgical implanted devices. Bacteria embedded in biofilms are hard to eradicate with standard antibiotics and are intrinsically resistant to host immune response (2). *S. aureus* with reduced susceptibility to vancomycin like heterogeneous vancomycin intermediate *Staphylococcus aureus* (hVISA) and vancomycin intermediate *S. aureus* (VISA) are being increasingly reported worldwide (3). Emergence of such strains is attributed to excess or irrational use of vancomycin and poor tissue penetration (4). Biofilm formation also plays an important role in pathogenesis of staphylococcal infections, especially with prosthetic materials (5). It is presumed to be a significant initial step in the pathway to develop vancomycin resistance (6, 7). Biofilm infections are difficult to treat due to their inherent antibiotic resistance (8). Only limited numbers of antibiotics like daptomycin, quinupristin/dalfopristin, linezolid and tigecycline are active against the vancomycin non-susceptible *S. aureus* strains (9). Interestingly, daptomycin non-susceptibility is also reported in some hVISA and VISA isolates (10, 11) . Despite antimicrobial therapy, morbidity and mortality associated with these bacterial infections remain high (12). As resistance in bacteria to available antibiotics is increasing, new strategies are therefore, warranted to identify and develop the next generation of drugs or agents to treat such infections. Several nonomaterials are flourishing in medical science and technological areas while related research and applications are exploring potentials in biosensors, biomaterials, tissue engineering, DNA modification and drug-delivery systems (13). Recent advances in the field of nanotechnology, particularly the ability to prepare highly ordered nanoparticles of any size and shape, have led to the development of new biocidal agents. Previous studies indicated that nanoparticulates formulations could be used as effective bactericidal materials (14). It was reported that metal nanoparticles (Ag, Cu,

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CuO, Au) exhibited wide spectra of antimicrobial activities against different microorganisms, including fungi, Gram-positive and Gram-negative bacteria (15).

Among all the metal oxides, copper oxide nanomaterials have attracted more attention due to their unique properties. Cuprous oxide (Cu_2O) is a p-type semiconductor with a direct band gap of 2.17 eV. In recent years, there is a growing interest to synthesize Cu_2O-nanoparticles (Cu_2O-NPs) as antibacterial agent against Gram positive bacteria due to their rapid availability and properties similar to other expensive noble metals, including silver and gold (16). Recently, Huang and others (17) demonstrated that Cu_2O nanoparticles exhibit excellent biocidal action against *S. aureus*. Morever antibacterial activity against *Escherichia coli* has also been reported (4, 18).

Several techniques like thermal reduction, capping agent method, sonochemical reduction, metal vapour synthesis, micro-emulsion technique, laser irradiation, and induced radiation can be used to prepare copper nanoparticles (15). Most of these techniques require organic solvents, high temperature and involve multistep sample preparation process (19). However, solution phase technique is cheaper and it does not require any organic compound, surfactant or high temperature for Cu_2O-NP synthesis (20). Chemically and physically Cu_2O-NPs can be prepared with extremely high surface areas and unusual crystal morphologies, which act as a predominant valuable antistaphylococcal agent (2). Previously, it was observed that copper (Cu) containing nanoparticles (Cu metal, Cu, CuO) caused cell membrane damage depending on both chemical composition (metal vs. oxide) and surface area (21). Their activity against hVISA and VISA isolates has not been reported so far. Therefore, the present study was designed to investigate the antibiofilm activity and possible mode of action of Cu_2O-NPs synthesized by solution phase method against hVISA and VISA strains.

Methodology

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Bacterial strains:

Five bacterial strains, *S. aureus* (ATCC 29213), Mu3 (hVISA), Mu50 (VISA), and two clinical isolates of hVISA (St1745, B10760) were used in this study. The cultures were stored at -80°C in brain heart infusion broth (BHIB; Hi-media, Mumbai India) containing 20% glycerol (vol/vol) till further use.

Synthesis of Cu_2O-NPs:

Cu_2O-NPs were synthesized by solution phase method as follows: 0.5 g of $CuSO_4.5H_2O$ (2 mmol) and 0.361 g of D-glucose (2 mmol) were completely dissolved in 100 mL of double distilled water in a 250 mL round bottom flask fitted with constant argon gas flow and magnetic stirring. After 15-20 minutes (min) of stirring, 2 mL of 10 M NaOH was added in a drop wise manner with the help of dropping funnel into the solution and a blue coloured solution of Cu $(OH)_2$ was soon produced. After stirring for 30 min, 3 mL of 2 M hydrazine hydarate ($N_2H_4.H_2O$) solution was drop by drop into this solution; colour of the solution gradually changed from blue to brick red. The solution was stirred until the cuprous hydroxide [Cu $(OH)_2$] precipitates were completely reduced by the hydrazine hydrate to brick red. The brick red precipitates were collected, washed with methanol (HPLC grade) several times, centrifuged and dried in vacuum oven at 60^0 C for 3 hours.

Antimicrobial susceptibility assay:

Determination of minimum inhibitory concentration (MIC): MIC was determined for all the tested bacterial strains as per the guidelines of Clinical and Laboratory Standards Institute (22). Briefly, the bacterial suspensions were prepared by suspending 18 hour grown bacterial culture in sterile normal saline (0.85% NaCl wt/vol; Himedia, Mumbai India). The turbidity of the bacterial suspension was adjusted to 0.5 McFarland standards [equivalent to 1.5×10^8 colony forming units (CFU)/ml]. Cu_2O-NPs (A1518) and bulk copper stock solutions were prepared in milliQ water and 2-fold serial dilutions were prepared in Mueller Hinton broth

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(MHB; Hi-media, Mumbai India) in 100 µl volume in 96-well U bottom microtitre plates (Tarson, Mumbai, India). The above mentioned bacterial suspension was further diluted in the MHB and 100 µl volume of this diluted inoculum was added to each well of the plate resulting in the final inoculum of 5×10^5 CFU/ml in the well and the final concentration of Cu_2O-NPs ranged from 10 to 10000 µg/ml. The plates were incubated at 37°C for 18 hour and were visually read for the absence or presence of turbidity. Similarly, MICs of vancomycin and daptomycin were evaluated. MIC of vancomycin-bodipy (dipyrromethene boron difluoride [4,4-difluoro-4-bora-3a,4a-diazas-indacene] fluorescent dye; Invitrogen, Carisbad, USA) was evaluated only for St 1745 (hVISA) strain. MIC was defined as the minimum concentration of the compound that showed no bacterial turbidity.

Biofilm susceptibility assay:

The biofilms of selected *S. aureus* isolates (Mu3, Mu50, ATCC 29213 and clinical isolates (St1745, B10760) were prepared in 96-well flat-bottom polystyrene microtiter plates (Tarson, Mumbai, India), using a previously described method (23) . Wells with and without culture were used as positive and negative controls, respectively. The bacterial suspensions were prepared from the overnight grown culture and the turbidity of the suspension was adjusted to 0.7 O.D.610 (≈1×10^9 CFU/ml). Two fold serial dilutions of Cu_2O-NPs (A1518) were prepared in 100 µl volume in BHIB in the wells of 96-well flat bottom microtiter plate. Forty microliters of fresh BHIB was added to each well, followed by the addition of 60 µl of above bacterial suspension. This resulted in the final inoculum of 6×10^7 CFU/ml in each well; the final concentrations of the compounds ranged from 78 to 10000 µg/ml. The plate was incubated for 18 hours at 37°C. After incubation, the planktonic cells were removed from each well by washing with phosphate buffer saline (Hi-media, Mumbai, India). The biofilms were fixed with methanol for 15-30 minutes, stained with 0.1% (wt/vol) crystal violet (Sigma Chemical Co., St Louis, MO, USA) for 10 min and rinsed thoroughly with water until the

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142 negative control wells appeared colourless. Biofilm formation was quantified by addition of
143 200 µl of 95% ethanol to the crystal violet stained wells and the absorbance was recorded at
144 595 nm (A 595) using a micro plate reader (Spectra easy microplate reader, Inverness
145 medicals, India)

146 **Studies on mechanism of action of Cu_2O-NPs:**

147 **Leakage assay and protein estimation:** Leakage of 260 and 280 nm absorbing compounds
148 was determined spectrophotometrically (24). Briefly, the bacterial cells (all tested strains)
149 were grown overnight in 100 ml of MHB at 37°C, washed and re-suspended in 50-mmol/l
150 sodium phosphate buffer, pH 7·1. The turbidity of the suspension adjusted to 0.7 O.D.610
151 (≈1 ×10^9cfu/ml). Cu_2O-NPs (A1518) was added at 5000 µg/ml to the bacterial suspension (≈1
152 × 10^9 CFU/ml) and incubated for 120 min at 37°C. For complete release of 260 and 280 nm
153 absorbing compounds, the bacterial suspension was treated with cetyl trimethylammonium
154 bromide (CTAB; 10µg/ml) at 37°C for 120 min, followed by sonication that served as
155 positive control. Cell supernatants were obtained by centrifugation (10,000 g for 10 min). The
156 absorbance of cell supernatant at 260 and 280 nm was determined using spectrophotometer
157 (Specgene, model no FSPECGD made in U.K.). Background leakage was determined in
158 bacterial suspension without nanoparticles (negative control). The extent of leakage of 260
159 and 280 nm absorbing compounds was expressed as percentage of positive control
160 (suspension treated with CTAB) measured in supernatants. Protein estimation of leaked
161 supernatants was done by Lowry method (25) .

162 **Propidium iodide uptake assay (PI uptake assay):** PI uptake was assayed by flow
163 cytometric analysis following previously published protocol (26). Briefly, a clinical isolate of
164 hVISA (St1745) was cultured in BHIB with aeration at 37°C for 24 hours of incubation.
165 Cu_2O-NPs at the concentration of 2 MIC were added to the cells and incubated for 0, 8 and

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24 hours. Untreated bacterial cultures were used as control. The cells were harvested by centrifugation at 8,000 rpm for 10 min at 25 °C, washed twice with phosphate-buffered saline (PBS buffer 50 mM; pH 7.0) and subsequently re-suspended in PBS to obtain a final concentration of 10^8 CFU/ml. Bacterial suspensions were then incubated with 50 µg/ml of PI (Sigma Chemical Company, St. Louis, MO, USA) in the dark for 15 min. Further, the samples were analysed at the FL-1 channel (488 nm; blue argon laser) on flow cytometer (Canto II, Becton Dickinson, San Jose, CA, USA) using Flowjo software.

Ethidium bromide uptake assay (EtBr uptake assay): The disruptive effect of Cu_2O-NPs was assessed on clinical isolate of hVISA (St1745) by using Cu_2O-mediated EtBr uptake. One ml each of 1.0 X 10^6 CFU/ml cell suspension of hVISA (St1745) in PBS was incubated with 0.5, 1 and 2 MIC Cu_2O-NPs at 37°C for 2 hours with shaking. EtBr (100 µM of a 1 mg/ml solution) was added to all different concentrations of Cu_2O-NPs treated bacterial cells and incubated at room temperature for 15 min. Cells with EtBr and no Cu_2O-NPs served as negative controls. Cells were washed and re-suspended in PBS, and a drop of each suspension was examined under fluorescent microscope at 100 X (Olympus, Tokyo, Japan) for red fluorescence.

Salt tolerance assay: The ability of bacterial cells (St 1745) treated with mixtures of Cu_2O-NPs and sodium chloride to grow on MHA (Hi-Media, Mumbai, India) was investigated according to previously published protocol (27), with slight modifications. Suspensions of bacteria (10^6 CFU/ml) were prepared in MHB (Hi-Media, Mumbai, India) and treated with 0.5 MIC, MIC and 2 MIC concentrations of Cu_2O-NPs and 1% DMSO. In other groups, each concentration was combined with 7.5 % NaCl and the control was set up without Cu_2O NPs. The test tubes were incubated in a shaking incubator at 37 °C and samples were removed at intervals, serially diluted and plated on MHA. After 24 hours of incubation, the numbers of colonies (CFU/ml) were counted on plates and compared with the control.

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Binding of fluorescently labelled vancomycin to *S. aureus* St1745 biofilm:

Biofilms were formed on a 96-well flat-bottomed polystyrene plate as described above. After two washes with PBS, the biofilms were incubated with 50 µl of 40 mM vancomycin-bodipy dissolved in PBS for 0, 5, 15, 30, and 60 min at room temperature. After two washes with PBS, the fluorescence derived from vancomycin-bodipy was measured with a microplate reader (Biotek synergy HT; USA) at excitation and emission wave lengths of 485 and 535 nm, respectively. After 60 min incubation, the biofilms were washed with PBS to remove unbound vancomycin-bodipy and re-suspended. The localization of vancomycin-bodipy was analyzed by fluorescence microscopy using a green fluorescent protein filter (Olympus, Tokyo, Japan)

Toxicity assessment:

Toxicity of Cu_2O-NPs was determined by haemolysis assay as described previously (28) with few modifications. The modifications were as follows: we used different concentrations of nanoparticles, and red blood cells (RBCs) instead of whole blood. Briefly, blood sample was collected from healthy volunteer and stored in ethylene diamine tetra acetic acid (EDTA) vacutainer. Whole blood (5 ml) was added to 10 mL of PBS and centrifuged at 10016 g for 10 min to separate RBCs. The RBCs were then washed five times with 10 mL of PBS and diluted to 50 mL with PBS. To test the haemolytic activity of Cu_2O-NP_S, 0.2 mL of diluted RBC suspension was added to 0.8 mL of nanoparticle solution at different concentrations ranging from 625 to10000 µg/mL. Triton X 100 was used as positive control and sample without treatment with nanoparticle as negative control. All the samples were placed on a rocking shaker at 37 ^{o}C for 3 hours. After incubation, the samples were centrifuged at 10016 g for 3 min. The haemoglobin absorbance in the supernatant was measured at 570 nm.

Statistical analysis

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All the experiments were carried out in triplicates at different occasions. The data were analyzed by Mann Whitney test and one-way ANOVA for comparison of multiple means followed by Tukey's test using Graph Pad Prism 5 (Graph Pad software Inc. San Diego CA). The chosen level of significance for all statistical tests was $p < 0.05$.

Results:

Structural & morphological characterizations:

The powder x-ray diffraction (PXRD) pattern of the Cu_2O-NPs is given in Figure 1. There were four clear peaks. All of them could be perfectly indexed to a single phase of crystalline Cu_2O (JCPDS No. 78-2076), not only in peak position, but also to their relative intensity and the results presented here coincided well with the literature values. The peaks with 2θ values were 37.7402, 43.6758, 62.8232 and 75.0440 corresponding to the crystal planes of (111), (200), (220) and (311) of crystalline Cu_2O, respectively. From the PXRD data, the average crystallite size was calculated between 21-30 nm for different samples using the Scherrer's equation. The morphology and size of the product was studied by electron microscopy. The FESEM images (Figure 2) clearly showed the general morphology of the synthesized Cu_2O-NPs used in this work. Majority of the Cu_2O-NPs had octahedral structure with size ranging from 100-150 nm. Since the average crystallite size of the Cu_2O was between 21-30 nm, they fell under the category of nanoparticles.

Minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentration

Cu_2O-NPs (A1518) was found active against hVISA/VISA. MICs of A1518 (Cu_2O-NPs) were 1250, 2500, 625 and 1250, 1250 µg/ml against hVISA (Mu 3, standard strain), VISA (Mu 50, standard strain), *S. aureus* 29213 and two clinical isolates of hVISA respectively (table 1). A1518 (Cu_2O-NPs) effectively inhibited the formation of hVISA/VISA biofilms

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with 80% biofilm inhibition concentration (MBICs) ranging from 2500 µg/ml to 10000 µg/ml (Figure 3). Detailed MIC profiles of vancomycin and daptomycin against all tested strains are given in table 1. Furthermore, MIC of vancomycin-bodipy was performed against daptomycin non-susceptible strain of hVISA (St 1745) and the MIC (3 µg/ml) remained unchanged. In the present study St1745 (hVISA) was selected for further studies on activities of Cu_2O-NPs (A1518).

Studies on mechanism of action of Cu_2O-NPs

Leakage assay:

The membrane leakage assay illustrated the cytoplasmic membrane damage of all tested strains of hVISA. The amount of 260 and 280 nm absorbing material in *S. aureus* supernatants exposed to A1518 at 90 min and 120 min were observed. Bacterial cells with exposure to CTAB (100% cell lysis) and without exposure either to A1518 or CTAB served as positive and negative controls, respectively. Leakage of proteins from bacterial cells was 69.9% both at 90 min and 120 min when exposed to A1518 Cu_2O-NP and 39.8% in negative controls compared to positive controls. Leakage of proteins from cell membrane was higher in CTAB (positive control) and A1518 Cu_2O-NP treated strain Mu3 and clinical isolates of hVISA compared to Mu50 (VISA) strain ($p < 0.001$) (Figure 4).

PI uptake assay: hVISA cells (St1745) treated with 2 MIC of A1518 (Cu_2O-NPs) for 0, 8 and 24 hours resulted in the identification of two subpopulations (PI-positive and PI-negative cells) by flow cytometry analysis. PI-positive and PI-negative cells represented dead and viable cells, respectively. The effect of Cu_2O-NPs against hVISA cells was observed to be time-dependent. After 4 hours of incubation, the rate of dead cells was 92.6±2.4 % and this increased to 94.1 % within 8 hours in case of clinical hVISA isolate (Figure 5; $p=0.0001$)

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Loss of Salt tolerance: Reduction in the cell viability of hVISA (St1745) on agar medium with 7.5 % NaCl in the presence of A 1518 (Cu_2O-NPs) at MIC and 2 MIC was observed. This treatment reduced the bacterial ability of salt tolerance by about 3.6 folds in the clinical isolate of hVISA after both 12 and 24 hour treatment (Figure 6).

EtBr uptake: Non-Cu_2O NPs treated clinical isolates of hVISA (St1745) cells stained with EtBr did not show fluorescence except for very few. However, Cu_2O-NPs treated cells exhibited increased uptake of EtBr, which was proportional to Cu_2O NPs concentration. Cu_2O-NPs at 2 MIC resulted in complete and homogeneous staining of cells (Figure 7).

Binding of fluorescently labelled vancomycin to hVISA (St1745) biofilm:

We analyzed the localization of vancomycin-bodipy (fluorescently labelled vancomycin) in hVISA and VISA biofilms. Bodipy labelling seemed to have no effect on the activity of vancomycin, since the MIC of hVISA strain (St1745) remained unchanged (3 µg/ml) (table 1). After treatment of biofilm cells with Cu_2O-NPs (2 MIC) the fluorescence derived from vancomycin-bodipy was measured at different incubation times. A significant ($p=0.0001$) reduction in fluorescence was observed in treated *S. aureus* strains ATCC 29213, Mu3, Mu50, St1745 and B10760 isolates compared to respective untreated strains at 60 minutes and the reductions were 39.1%, 21.38%, 38.77%, 28.3% and 25.9%, respectively (Figure 8). The biofilm was suspended in PBS and observed under fluorescent microscope. Fluorescence was observed at the surface of virtually every cell. It was also observed at the level septa in few cells (Figure 9).

Toxicity assessment: The concentration of haemoglobin present in the samples was calculated from the absorbance values. No cytotoxicity was observed at 625 – 10000 µg/ml concentration of Cu_2O-NPs (Figure 10).

Discussion

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In the present study, Cu_2O-NPs were synthesized according to the solution phase method. Structural and morphological characterization of Cu_2O-NPs and their antibiofilm behaviour were investigated. Cu_2O-NPs showed bacterial membrane damaging potential and appeared to be the main cause of bactericidal activity against *S. aureus* strains with reduced vancomycin susceptibility. Identification of surface morphology, particle size and the defined elemental composition of nanoparticulate Cu_2O are required for its potential application as antimicrobial agent. FESEM image clearly showed the general morphology of the synthesized Cu_2O-NPs and majority of the Cu_2O-NPs were in the range of 100-150 nm. Therefore, it was estimated that most of the nanoparticulates generated by solution phase technology were indeed Cu_2O.

Previously, it had been reported that copper (Cu) containing nanoparticles (Cu metal, Cu, CuO) caused cell membrane damage depending on both chemical composition (metal vs. oxide) and surface area (21). Cu_2O-NPs are considered effective in killing a range of bacterial pathogens involved in hospital-acquired infections. However, higher concentrations of Cu_2O-NPs are required compared to silver nanoparticles (Ag-NPs) and copper nanoparticles (Cu-NPs) to achieve a bactericidal effect (29)**.** It was suggested that the reduced amount (between 3- and 20-folds) of negatively charged peptidoglycans would make Gram-negative bacteria less susceptible to positively charged antimicrobials (30). Previous work on nanoparticles revealed that Ag and Cu nanoparticles released Ag+ and Cu^{2+} ions having antiviral capabilities by altering local pH and conductivity along with the liberation of metal ions that had the ability to inactivate or kill viruses (29) .

In the present study, *in vitro* experiment with our laboratory synthesized A1518 Cu_2O-NPs revealed that these nanoparticles had significant antibacterial activity against hVISA compared to bulk copper. Our finding is concordant with a previous study (29). Staphylococci are responsible for a large percentage of catheter related infections, and like

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other pathogens, they tend to form a multilayered community of sessile bacterial cells known as a biofilm on medical implants and damaged tissues rather than living as free planktonic cells (31). However, reports are available where Cu_2O was shown to effectively inhibit the staphylococcal bacterial pathogens (32). But, antibiofilm activity of Cu_2O-NPs against hVISA/VISA type resistant strains has not yet been reported. In addition, patients infected with hVISA/VISA face a major therapeutic challenge, because of vancomycin treatment failure (33). Such infections are difficult to treat due to their inherent antibiotic resistance (8). In the present study, MBIC of A1518 Cu_2O-NPs was 2-fold higher than MIC for *S. aureus*. However, less antibacterial activity of A1518 was found against Mu50 (VISA) bacteria. This might be attributed to the presence of thick cell wall of Mu50 (VISA) to compared Mu 3 (hVISA)(3). Studies using atomic force microscopy and transmission electron microscopy with 'aerogel'- generated nano-magnesium oxide against *E. coli* showed that the cell wall of this bacterium was extensively damaged, allowing the contents to leak out and nanoparticles to gain entry (14, 34). Likewise, Ag-NPs had been shown to attach to the microbial cell surface and penetrate inside, where intracellular targets, including respiratory enzymes, were disrupted (35).

However, the exact mechanism of action of Cu_2O-NPs against hVISA/VISA isolates has not been reported so far. We investigated potential membrane damage due to Cu_2O-NPs by measuring the leakage of bacterial cell contents, EtBr uptake, salt tolerance assay and finally the PI uptake assay by flow cytometry. Leakage of cytosolic constituents (260 and 280 nm absorbing materials) from *S. aureus* cells in presence of 50 mg/l Cu_2O-NPs over a period of 2 hours was significantly higher than background levels (Figure 4). Leakage of cytosolic constituents indicated that A 1518 Cu_2O altered the cell membrane structure, resulting in the disruption of the permeability barrier of microbial membrane structures which is in concordance with the previous study (14) .

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Further, we examined the membrane integrity by salt tolerance assay. *S. aureus* is known for its ability to survive in presence of high salt concentration due to accumulation of osmo-protectants, such as choline, L-proline (36, 37). Cu_2O-NPs were found to reduce the tolerance of hVISA/VISA to low osmotic pressure (Figure 6). MIC and 2 MIC ($p=0.001$) of Cu_2O may alter the permeability and affect the ability of the membrane to osmoregulate cells adequately or to exclude toxic material. A previous study reported that essential oils of tea tree, and lichen *Usnea subfloridana* derived origanum vulgare and usnic acid caused significant loss of salt tolerance through membrane damage in bacteria (38) .

In the present study, a significant ($p = 0.0001$) increase in PI uptake was observed by Cu_2O-NPs treated hVISA (St1745) strain within 4 hour (92.6%), which further increased to 94.1% within 8 hour (Figure 5). This rapid increase in PI uptake confirms that antibiofilm activity of Cu_2O-NPs was due to cell membrane disruption. Because of its large size and negative charge, PI cannot pass through the intact membrane of bacteria (39) .

Bacterial cells damaged by Cu_2O-NPs were further visualized by EtBr uptake under florescent microscope. It is reported that EtBr enter and intercalate within DNA of compromised bacterial cells with high fluorescence (40). Therefore, the present experiment was designed to assess Cu_2O-NPs mediated uptake of EtBr in cells exposed to Cu_2O as a measurement of cell integrity. Microscopic images revealed an enhanced uptake of EtBr by Cu_2O-exposed cells that was proportional to the Cu_2O-NPs concentration (Figure 7). Increase in the penetration of cells by EtBr just minutes after the addition of Cu_2O indicates that this compound damages the bacterial cell membrane rapidly. Further, reduction in vancomycin-bodipy fluorescence was observed in Cu_2O-NPs treated cells compared to that in untreated cells (Figure 8), suggesting that Cu_2O-NPs might also affect the cell wall synthesis. Moreover, binding of vancomycin-bodipy with septum of bacterium within 15 minutes of vancomycin-bodipy exposure indicates penetration of vancomycin towards septum (Figure

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9A, 9B, 9C, 9D). Similar findings were reported with commercially purchased nisin A and lacticin Q isolated from *Lactococcus lactis* against MRSA (41). It is most likely that Cu_2O-NPs exposure also caused a significant reduction in cell wall thickness, as evidenced by reduction in florescence by vancomycin-bodipy. A similar relationship was reported earlier between cell wall thickness and exposure to nafcillin, and vancomycin and ceftroline combination against hVISA/VISA (42) . Our observations have led us to hypothesize that disruption of cell membrane and reduction in vancomycin-bodipy florescence is due to cell wall thinning that prevent vancomycin sequestration and improve vancomycin penetration into the septa of dividing cells. The division of the septum is the active site of cell wall biosynthesis in *S. aureus* and any antimicrobial agent that inhibits the late steps of cell wall biosynthesis must have access to lipid II at the septum (43). However, further studies are required to explore the exact mechanisms of cell wall thinning by Cu_2O-NPs in hVISA. The toxicity assessment of Cu_2O-NP_S by haemolysis assay showed no cytotoxicity at different concentrations (Figure 10). However, to substantiate this observation, in-vivo toxicity assessment is warranted.

Our results indicate that A1518 Cu_2O-NPs effectively inhibited the growth of hVISA and VISA strains and also reduced their ability to form biofilms. To our knowledge, this is the first report to provide the evidence that Cu_2O-NPs have membrane damaging potential in *S. aureus* and can be used as an effective anti-biofilm agent against *S. aureus*. However, the limitations of the study are- other alternative mechanism(s) of bacterial cell deaths like interactions of Cu_2O-NPs with biological macromolecules such as enzymes and DNA, and toxicity study on human cell lines and in animal models were not carried out.

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Conclusion: It is noteworthy that synthesis of Cu_2O-NPs by one pot solution phase method is simple, economical and safe. The probable mode of action of Cu_2O-NPs is by disruption of bacterial cell membrane. These nano-particles can be used as chemical biocide for destroying drug resistant staphyloccocci and their biofilms. Further, Cu_2O-NPs have the potential to be used in medical and household devices as effective antibiofilm agent since they showed no toxicity by in-vitro haemolysis assay. However, further in vitro and in vivo experiments are required to draw final conclusions.

Acknowledgement: Author is thankful to Prof. Hiramatsu for providing the strains of Mu3 (hVISA) and Mu50 (VISA). We acknowledge the financial support provided by SGPGIMS through intramural grants no 2013-30-IMP-EXP/177. Avinash Singh and Sonali Khanduja acknowledge the financial assistance received from Indian Council of Medical Research (ICMR), Government of India, New Delhi through Senior Research fellowship grant no. 80/675/10-ECD-I and 80/829/2003-ECD-I respectively. Satyendra K. Singh also acknowledges the CSIR, Govt. of India through junior research fellowship grant no. CSIR JRF- 09/590(0147)/2010-EMR-1.

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Figure legends:

1. Figure 1: The powder x-ray diffraction (XRD) pattern of the Cu_2O nanoparticles.

2. Figure 2: Field emission scanning electron microscopy (FESEM) images of the synthesized Cu_2O nanoparticles.

3. Figure 3: [A] Biofilm formation by *S. aureus* strains, [B, C, D, E and F]. Biofilm inhibitory concentration of A1518 Cu_2O-NPs against *Staphylococcus aureus* strains Mu3, Mu50, ATCC 29213, St 1745 and B 10760 respectively.

4. Figure 4: Percent leakage of protein estimated by Lowery method in Mu3 (hVISA) and Mu50 (VISA) strains treated with A1518 Cu_2O-NPs; CTAB treated cells served as positive control.

5. Figure 5: Mean florescence intensity of propidium iodide treated with 2 MIC concentrations of Cu_2O-NPs in St1745 (hVISA).

6. Figure 6: The ability of St1745 (hVISA) to form colonies on Mueller Hinton agar (MHA) supplemented with 7.5 % NaCl after treatment with 0.5 MIC, MIC and 2 MIC of Cu_2O-NPs.

7. Figure 7: Ethidium bromide uptake following exposure to 0, 0.5, 1 and 2 MIC of Cu2O-NPs as assessed by fluorescent microscopy at 100X (Olympus, Tokyo, Japan)

8. Figure 8: Relative vancomycin binding to hVISA strain St 1745 (mean± standard deviation) in the presence and absence of 2 MIC Cu_2O-NPs.

9. Figure 9: (A) hVISA (strain St1745) biofilms treated with 2 MIC concentration of Cu_2O-NPs for 1 h and then treated with 3 mg/l vancomycin-bodipy for 15 min (florescent microscopy at 40 X. (B) Localization analysis of vancomycin-bodipy after a 15-min incubation; the biofilms re-suspended in PBS were observed by fluorescence microscopy at 100 X. Arrows, division septa of the cells (Inset- enlarged of boxed area. (C) 3D (3 Dimensional) view of vancomycin binding to septum of hVISA during cell division. (D) 3D view of vancomycin binding to only cell wall of *S. aureus* during cell division.

10. Figure 10: Percent haemolysis value of A1518 Cu_2O-NPs at 625-10000 mg/l on human red blood cells (RBC), PC; Positive control (Triton X 100 treated RBC).

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Table 1: Minimum inhibitory concentration of Cu_2O nanoparticles (A1518), vancomycin and daptomycin (μg/ml) against *Staphylococcus aureus*.

S.N.	**Microorganism**	A1518	Bulk copper	Van	Dapto	Vanco Bodipy
1	Mu3 (hVISA)	1250	2500	2	1	ND
2	Mu50 (VISA)	2500	5000	8	1	ND
3	Clinical isolates of hVISA (n=2)					
	a. St 1745	1250	2500	3	2 (NS)	3
	b. B 10760	1250	2500	3	1	ND
4	*S. aureus* ATCC (29213)	625	2500	1.5	0.5	ND

Van :vancomycin, Dapto: daptomycin, Vanco Bodipy: vancomycin-bodipy (dipyrromethene boron difluoride [4,4-difluoro-4-bora-3a,4a-diazas- indacene] fluorescent dye), hVISA: heterogeneous vancomycin intermediate *S. aureus* ,VISA: Vancomycin intermediate *S. aureus*, NS: non-Susceptible, .ND: not done

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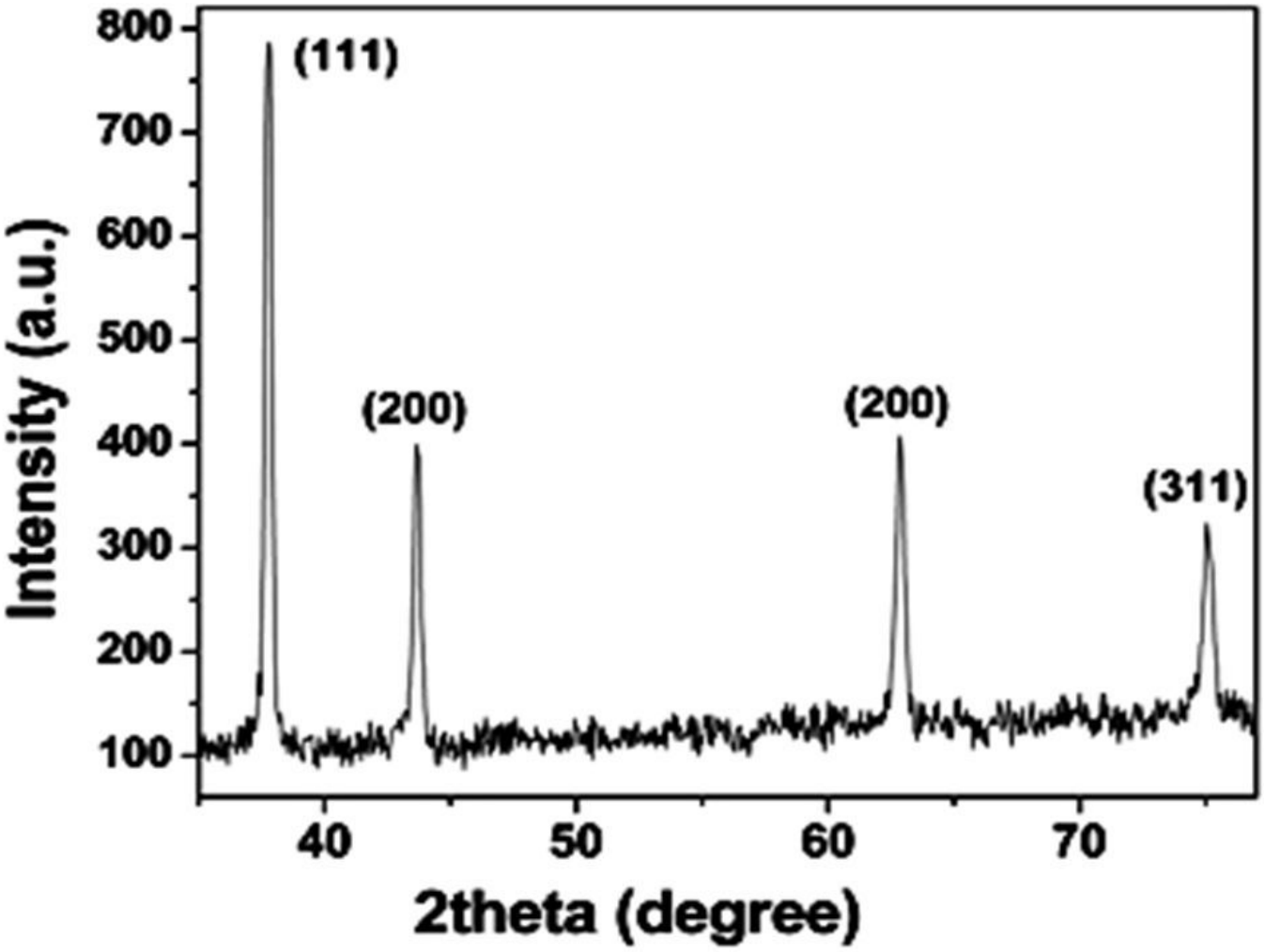

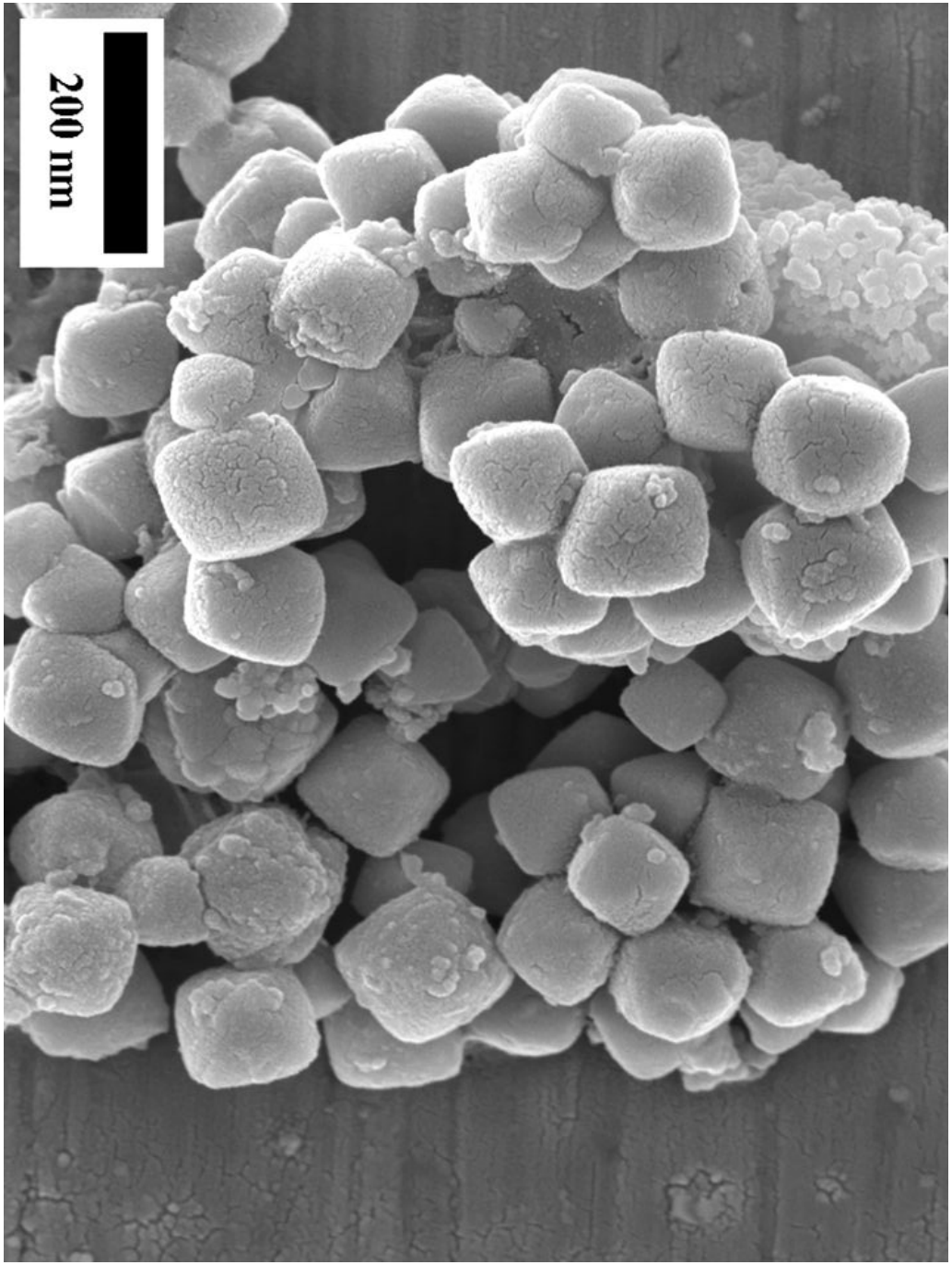
200 nm

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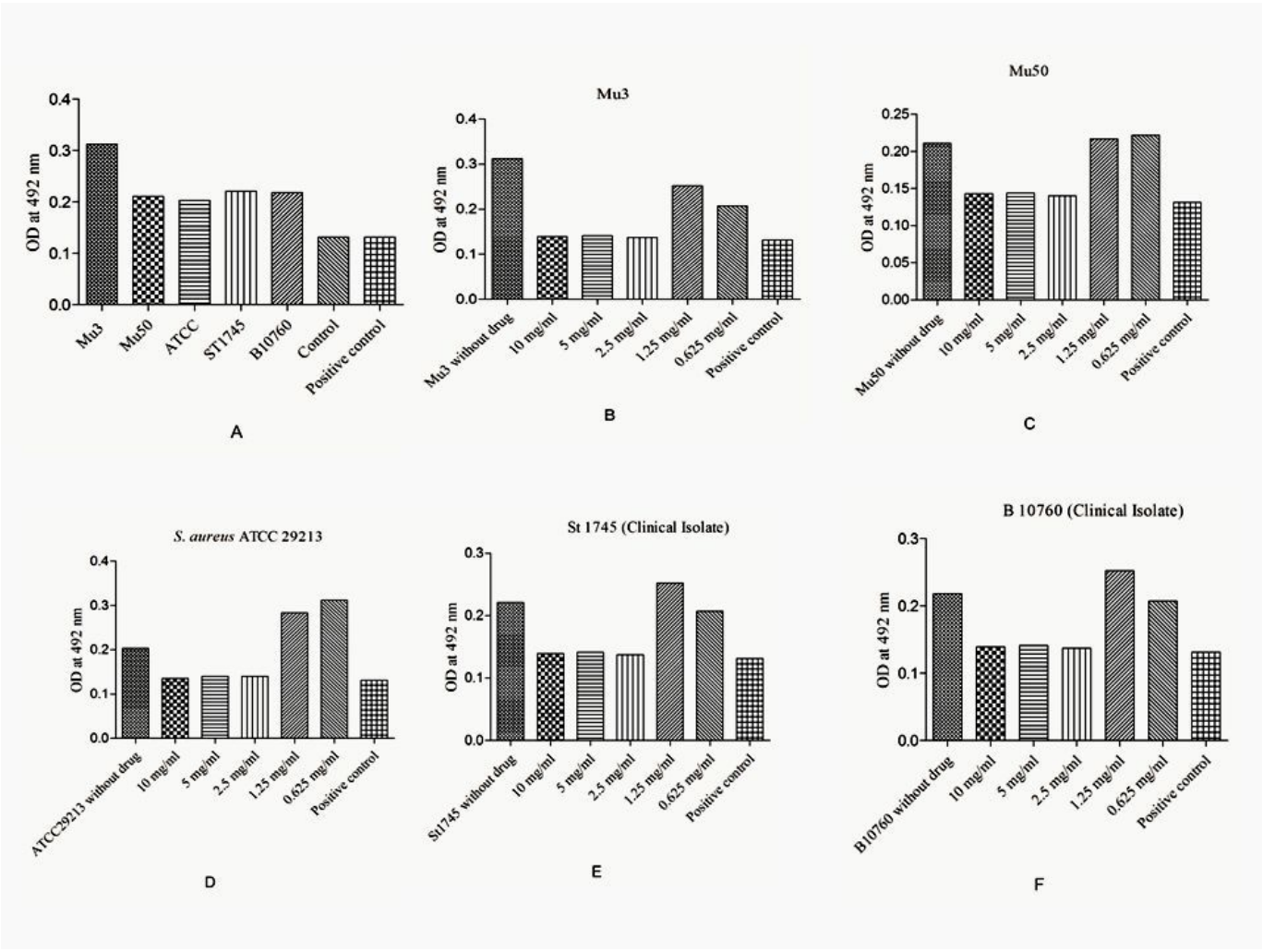
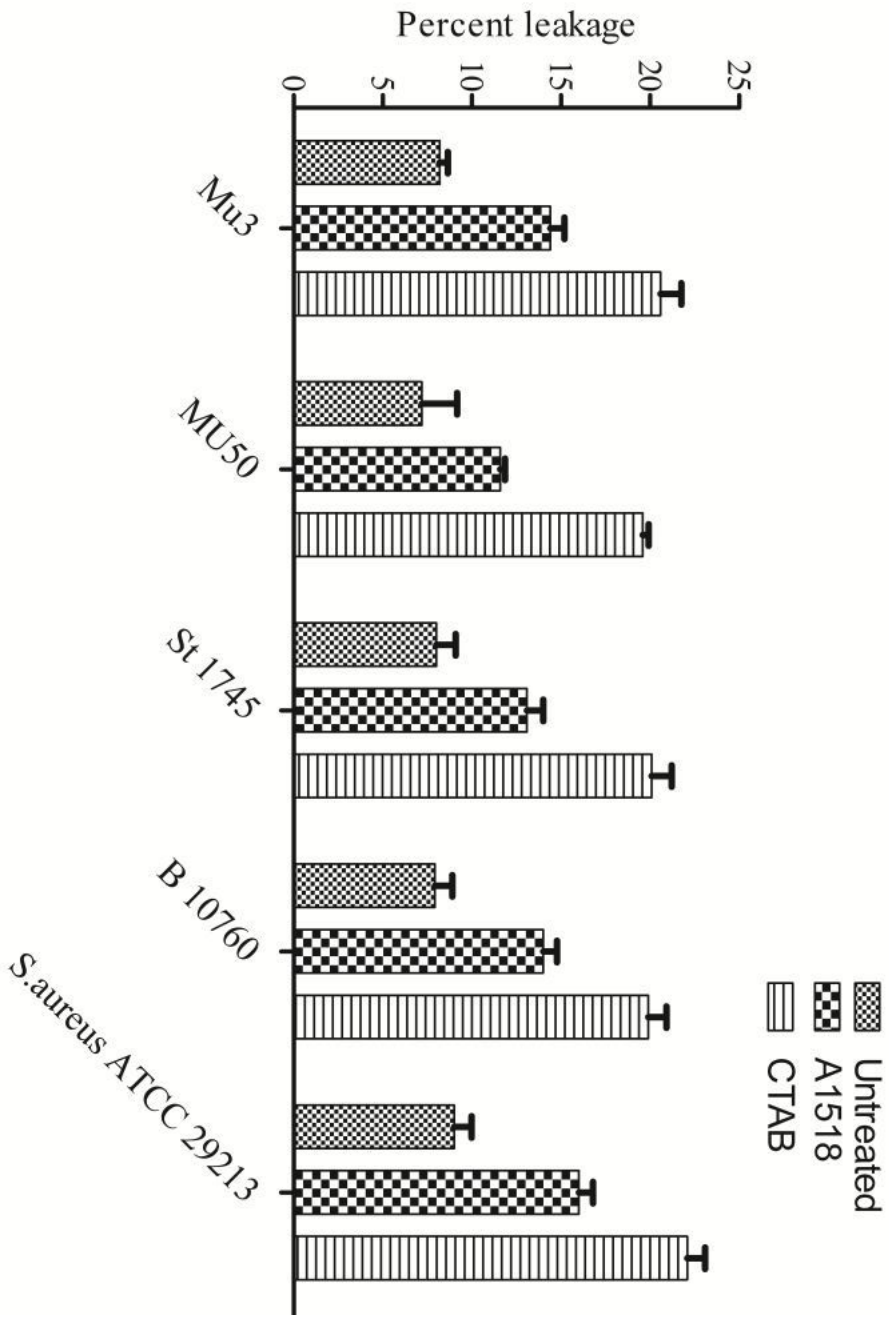
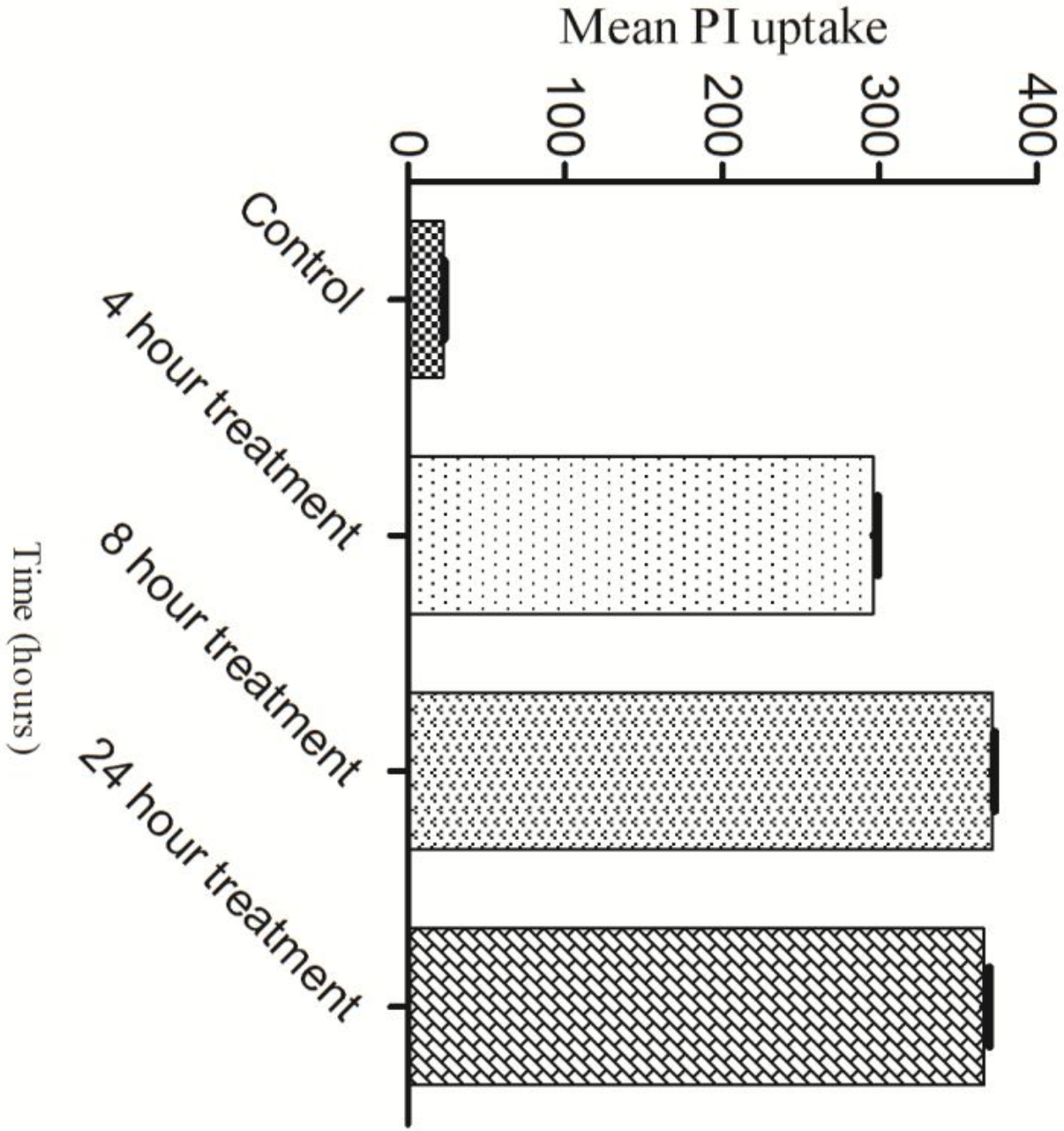



Percent leakage
0
5
10
15
20
25
Mu3
MU50
St 1745
B 10760
S.aureus ATCC 29213
Untreated
A1518
CTAB


Mean PI uptake
0
100
200
300
400
Control
4 hour treatment
8 hour treatment
24 hour treatment
Time (hours)

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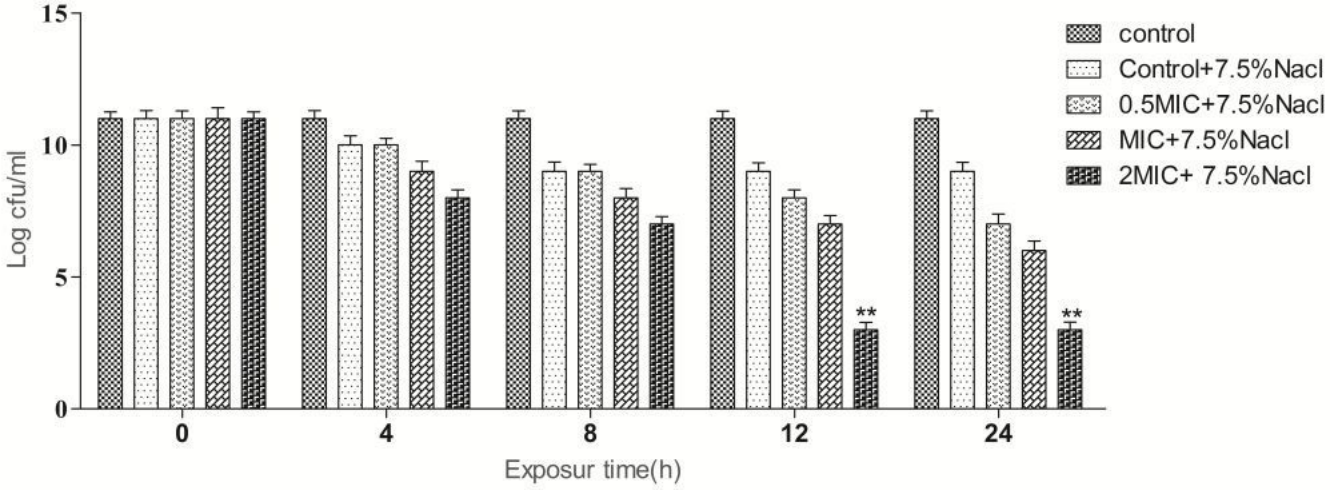
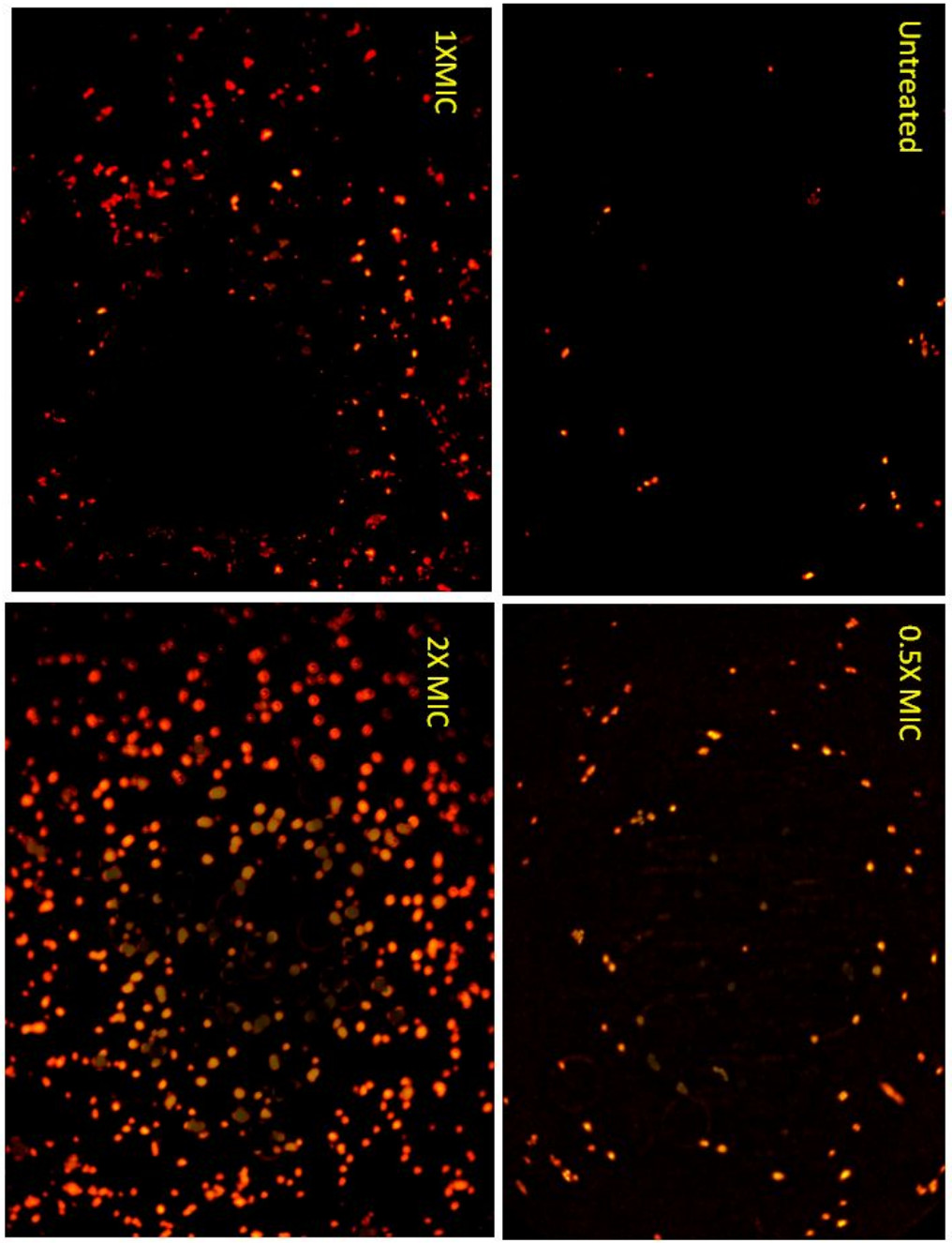
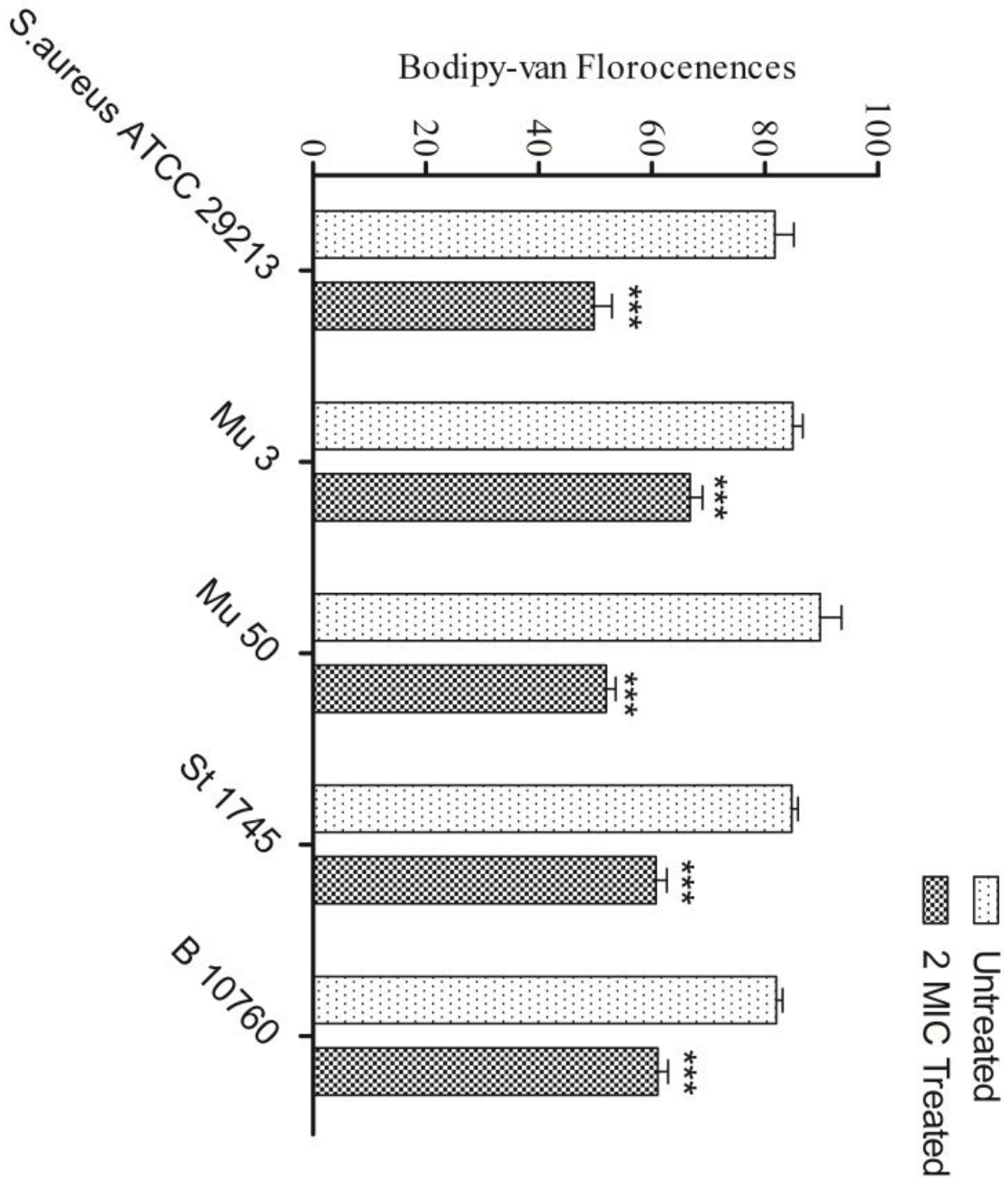
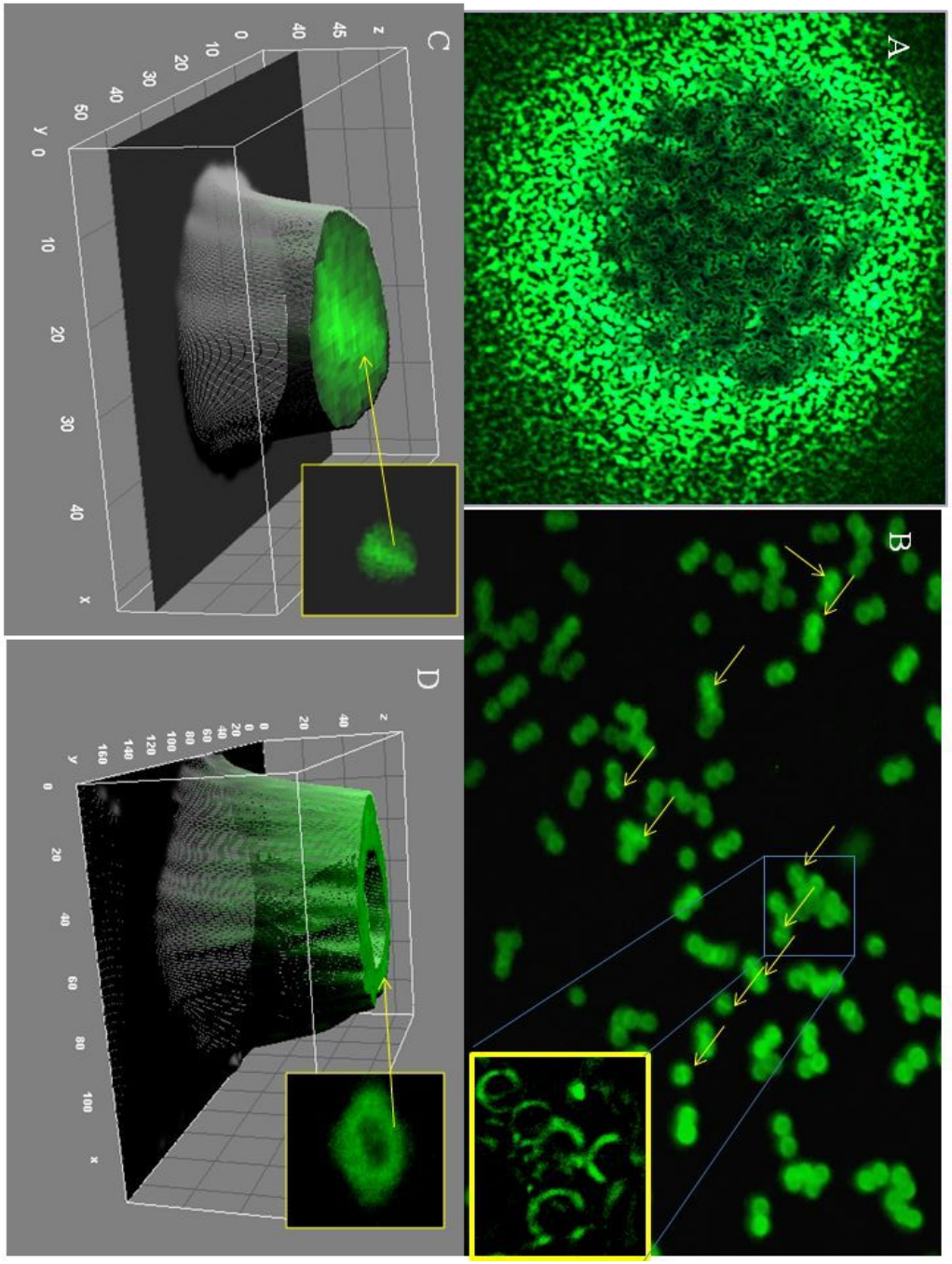
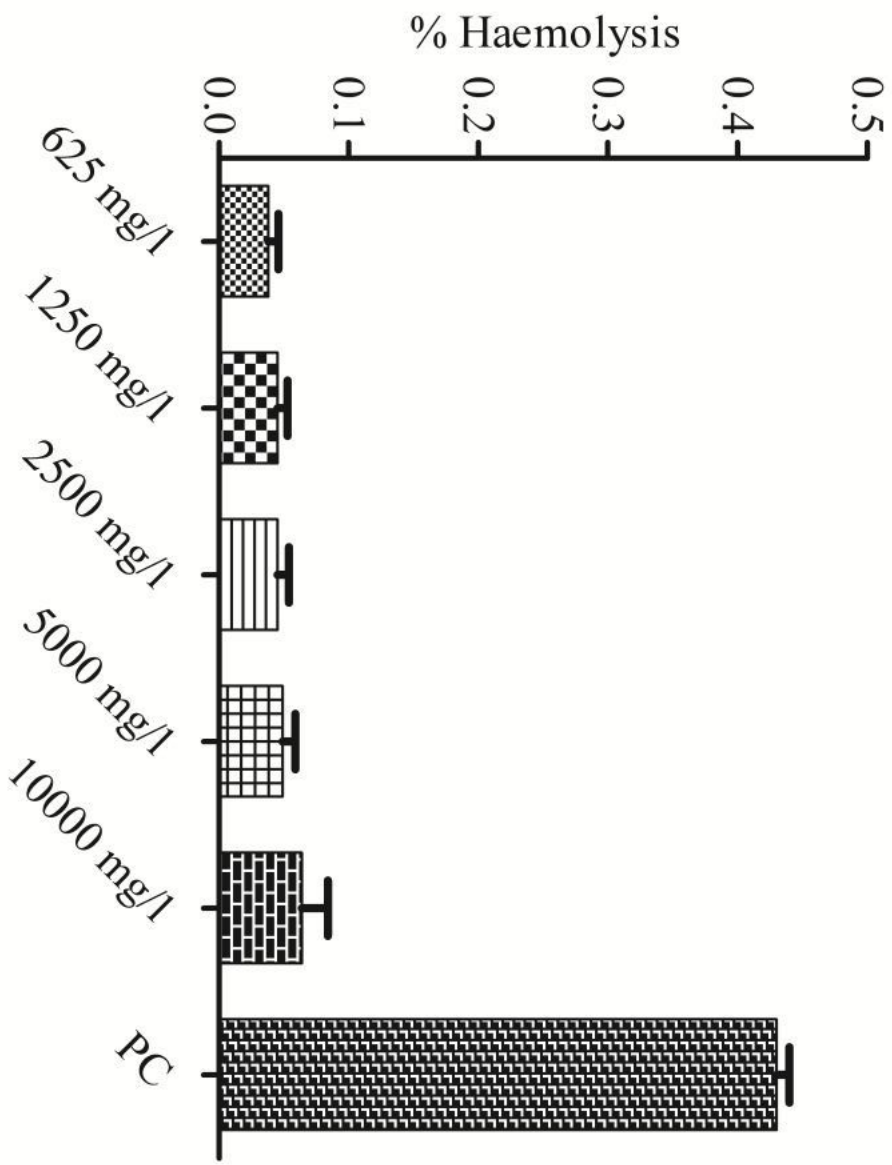



Untreated
1XMIC
0.5X MIC
2X MIC


Bodipy-van Florocenences
0
20
40
60
80
100
S.aureus ATCC 29213
Mu 3
Mu 50
St 1745
B 10760
***
Untreated
2 MIC Treated


A
B
C
D


% Haemolysis
0.0
0.1
0.2
0.3
0.4
0.5
625 mg/l
1250 mg/l
2500 mg/l
5000 mg/l
10000 mg/l
PC