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ARTICLE

Copper Nanoparticles as Efflux Pump Inhibitor to Tackle Drug Resistant Bacteria

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We explored if CuNPs can function as efflux pump inhibitor and as anti-biofilm agent. Our results showed that at 0.5 X (0.065mM) Minimum Inhibitory Concentration (MIC) concentration, CuNPs exhibited remarkable efflux inhibitory effect in wild type strains of both *Staphylococcus aureus* and *Pseudomonas aeruginosa* and relatively less but significant efflux inhibitory effect against MRSA and drug resistant mutant strains of *Staphylococcus aureus*. At 1X MIC (0.13mM) concentration, it significantly inhibited monoculture biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Membrane permeability, membrane integrity and confocal imaging provided insight into mechanism of antibacterial effect of CuNPs. Efflux inhibition and antibacterial effect of CuNPs was partly mediated by particle effect and partly by ion effect. A novel efflux pump inhibitory role for CuNPs is reported in our study. Proof of concept experiment revealed that CuNPs could reverse the MIC of the mutant *Staphylococci aureus* strain for ciprofloxacin by 4 fold (from 64 µg/ml to 16µg/ml) hence; our study reveals that CuNPs could be potentially employed as bacterial adjuvant to curtail Multi Drug Resistant (MDR) bacteria.

A Introduction

Numerous studies have reported antibacterial effect of various metal nanoparticles (NPs) viz., Au, Ag, Zn, Cu, TiO₂ and Pd¹⁻⁶. Among metal NPs, gold and silver has been widely exploited as antibacterial agents⁷⁻¹¹. Of late more reports on antibacterial effect of ZnO nanoparticles are forthcoming since they are relatively less toxic and more widely used¹²⁻¹⁴. ZnO NPs were found to exert bactericidal effect against *S.aureus*¹⁵ food borne pathogens like *Campylobacter jejuni*¹⁶, *E.coli* 0157: H7¹⁷. A very recent study reported mono

dispersed Zn O NPs effective as an antiinfective agent against *E.coli* and *S.aureus* in both *invitro* and in *invivo* experiments¹⁸. Although reports are available for antibacterial effect of chemically synthesized CuNPs¹⁹⁻²¹ on a comparative basis, copper is less exploited. Moreover, the mechanism of bactericidal activity of metal nanoparticles remains enigmatic. The antimicrobial property of metal NPs is primarily attributed to the increased surface area which enables NPs to interact with bacterial cell wall/ cell membrane and induce damage. NPs also lead to the formation of reactive oxygen species (ROS) which disrupts biological macromolecules;

they are also known to deplete intracellular ATP that aborts DNA replication. In addition, reports of NPs interfering with activity of respiratory enzymes of microbes are also prevalent^{1, 2, 9, 22-24}. A recent study showed that CuNPs disrupted the membrane potential of *E.coli*, which in turn led to filament formation and ROS mediated damage, both of which could account for its bactericidal effect⁴. The problem with metal NPs is that although, they are highly effective as bactericidal agents, they are toxic to non-target organisms like crustaceans, algae, fish and even higher eukaryotic cells beyond the threshold concentration, which overlaps with the determined toxic range of NPs for the target organisms²⁵. Moreover, dissolution of NPs lead to the formation of metal ions which are cytotoxic, apart from generalized toxic effects, individual specific and group specific toxic effects of NPs were also observed²⁶. Thus, NPs that are relatively non-toxic by virtue of its stability (stably coated NPs, diamond NPs, silica NPs) and or those which work effectively as antibacterial agents at much lower concentrations are preferred.^{6, 27}

Multidrug resistant bacteria like methicillin resistant *Staphylococcus aureus* (MRSA), *Enterococcus sp*, *Enterobacteriaceae*, *Pseudomonas aeruginosa* etc., pose increased threat to global public health, since they cannot be curtailed by standard treatments²⁸. MDR strains can withstand lethal doses of structurally diverse drugs and this ability is partly attributed to increased extrusion through efflux pumps^{29, 30}. Efflux pumps not only make the antimicrobial agents ineffective, by expelling the drug it also leads to accumulation of mutation, by exposing bacteria to substantially lower concentration of the antimicrobial agent²⁹. One viable approach to tackle drug resistant bacteria is to use efflux pump inhibitors^{29, 31}. Search for novel efflux inhibitors has been on the rise in the recent past^{32, 33}. Recently, our collaborator group reported casein stabilized CuNPs with significant antibacterial effect against both gram positive and gram negative bacteria and even against Methicillin Resistant *Staphylococcus aureus* (MRSA)³⁴. In

this work, we explored the antibiofilm effect of casein capped CuNPs against classical colonizers *Pseudomonas aeruginosa* and *Staphylococcus aureus*, as bacteria in biofilm mode of growth display enhanced resistance to conventional antimicrobial agents. In addition, we also tested efflux inhibitory ability of CuNPs against both *Pseudomonas* and *Staphylococcus*, apart from examining its membrane permeability and membrane integrity effect to understand the mechanism by which CuNPs exerted antibacterial effect. Finally, we tested whether the antimicrobial and efflux inhibitory effect was due to particle effect or due to the effect of ions released from the CuNPs. Our rationale was that, if CuNPs inhibits efflux activity, they can be used at much lower concentrations as bacterial adjuvants rather than as antibacterial agents and at such lower concentrations they would be non-toxic.

B Results

A MIC and MBC determination

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were discerned using standard protocols. Casein capped CuNPs exerted an MIC of 0.13mM against both *Pseudomonas aeruginosa* and *Staphylococcus aureus* and the MBC was observed to be 0.26 mM against both bacteria.

B Time Kill curve analysis

Bactericidal effect of CuNPs were evaluated by time kill studies and the results (Fig. 1a, b) revealed that at its 1X MIC (0.13mM) for *Pseudomonas aeruginosa* and at 2X MIC (0.26mM) concentration for *Staphylococcus aureus*, CuNPs caused significant reduction in plate counts for both *Pseudomonas aeruginosa* and *Staphylococcus aureus*. When tested at 4X MIC concentrations, no growth was observed for both groups of bacteria. The killing effect was more prominent with *Staphylococcus* where >2 log reduction were observed in 2 h post treatment, whereas *Pseudomonas* exhibited slightly < 2 log reduction in cell density at the same

time. By 4h post treatment, *Staphylococcus* exhibited significant 5 log reductions in cell density and *Pseudomonas* displayed 3 log reductions in cell density. Thus CuNPs exerted bactericidal effect against *Staphylococcus aureus* at 2X MIC, but it had significant bactericidal effect against *Pseudomonas aeruginosa* even at 1X MIC concentration.

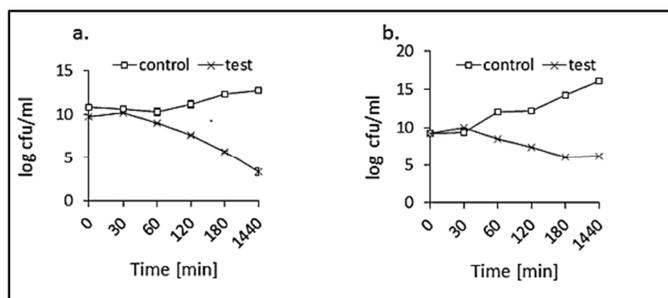


Fig. 1 CuNPs exerts bactericidal effect against both *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Time course of bacterial killing affected by CuNPs was followed from 0-24h by determining plate count which is represented as log₁₀ CFU/ml (a) kill curve for *S. aureus* challenged with 2X MIC of CuNPs (b) Kill curve for *P. aeruginosa* treated with 1X MIC of CuNPs. The error bars represents standard error of the mean from three independent experiments.

C Biofilm inhibition

We explored whether antibacterial effect of CuNPs was limited to planktonic cells or it was also observed against biofilms. Biofilms are known to display high resistance to toxic doses of antimicrobial agents, which usually eradicates planktonic bacteria³⁵. When tested at 1X MIC concentration, CuNPs caused significant inhibition of both *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilm biomass (Fig. 2). Relative to the untreated control, 88.6 % reduction in biofilm biomass was observed for *Pseudomonas aeruginosa* whereas; *Staphylococcus aureus* displayed 90.4 % decreases in biofilm biomass. In case of *Pseudomonas aeruginosa* at 2X, 4X and 8X MIC concentrations of CuNPs, biofilm inhibition was comparable to that observed for 1X MIC concentration (0.13 mM). With *Staphylococcus*, at 4X MIC concentrations significant biofilm inhibition was observed, which was greater than the inhibition noted at 1X MIC

concentration, rest of the concentrations displayed biofilm inhibition comparable to that observed at 1X MIC concentration. At concentrations lower than 1X MIC, only *P. aeruginosa* biofilms was significantly impaired whereas *S. aureus* exhibited significant increases in biofilm's biomass.

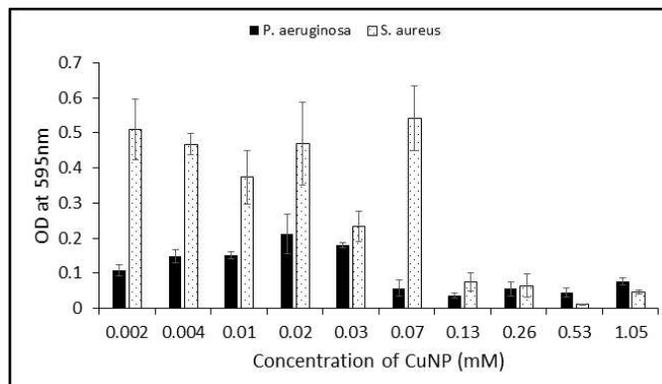


Fig. 2 CuNPs inhibits biofilm formed by monocultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Biofilm biomass of both gram positive and gram negative bacteria, following treatment with various concentration of CuNPs, was indirectly evaluated by washing twice to remove unbound cells, staining with 1 % crystal violet followed by de staining with acetic acid and determining crystal violet's absorbance at 595 nm. The error bars represents standard error of the mean from three independent experiments.

D Cartwheel assay

Cartwheel assay is a simple method that relies on the ability of bacteria to expel EtBr, which is a substrate for many efflux pumps³⁶. Efflux Pump inhibitors (EPI) cause accumulation of EtBr within the cells and induce fluorescence at a much lower concentration of EtBr. To evaluate the efflux inhibitory effect of CuNPs, we performed cartwheel assay for *Pseudomonas aeruginosa*, *Staphylococcus aureus*, MRSA and mutant strains of *Staphylococcus aureus* by incorporating 0.25 X (0.032 mM) and 0.5 X (0.065 mM) MIC concentrations of CuNPs in the agar media and evaluating the fluorescence exhibited by EtBr in all the bacteria tested. Treatment without CuNPs was maintained as control (Fig. 3a, b, c, d, e, f, g, h, i). Interestingly, at 0.25X MIC concentration of CuNPs only the

efflux pump of *Staphylococcus aureus* was inhibited, whereas at 0.5X MIC concentration, the efflux pump of wild type strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were highly inhibited and efflux pump of MRSA and mutant strains experienced relatively less but significant efflux inhibition (Fig. 3d, h, i). When CuCl_2 (which is used to form CuNPs) was tested at the same molar concentration it is present in CuNPs, 50 % of efflux inhibition as that displayed by CuNPs was observed (Fig. 3e). When CuNPs were mixed with EDTA to chelate the released Cu (II) ions, and then tested for efflux inhibition, partial (~50 %) efflux inhibition as that seen with 0.5 X CuNPs was observed (Fig. 3f) implying that the enhanced efflux inhibitory effect of CuNPs is partially contributed by CuNPs and partially by the Cu (II) ions released from the NPs. Whether efflux inhibition is directly related to the bactericidal effect of Cu NPs remains to be ascertained.

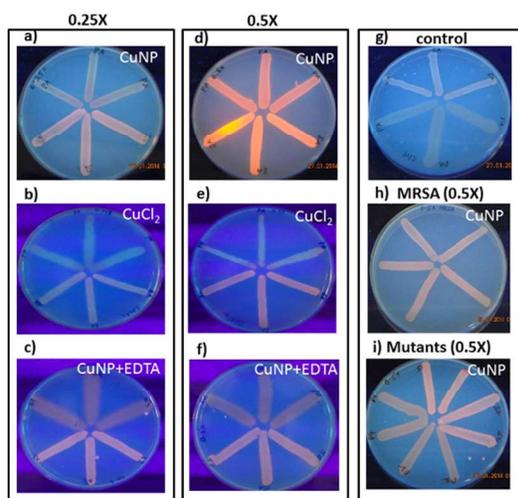


Fig. 3 CuNPs inhibit efflux activity in *Staphylococcus aureus*, *Pseudomonas aeruginosa*, MRSA and mutant strains of *Staphylococcus aureus*.

TSA agar plates containing EtBr (0.05 $\mu\text{g}/\text{ml}$) was swabbed with either *Staphylococcus aureus* and *Pseudomonas aeruginosa* or with MRSA/ mutant strains of *Staphylococcus* and efflux inhibitory activity of 0.25 X MIC concentrations of (a) CuNPs, (b) CuCl_2 and (c) CuNPs +EDTA, and EPI activity of 0.5 X MIC concentrations of (d) CuNPs, (e) CuCl_2 and (f) CuNPs +EDTA and (g) untreated control, treatments (a) to (g) were against wild type strains of both

Staphylococcus and *Pseudomonas* swabbed in a cartwheel pattern on the same plate (h) MRSA with 0.5 X MIC of CuNPs and (i) mutant strains of *Staphylococcus aureus* with 0.5 X MIC of CuNPs all treatments were evaluated after overnight incubation for growth followed by determining residual fluorescence in a UV transilluminator.

E Real time Efflux

Since we observed significant efflux inhibition by cartwheel assay, we quantitated the efflux inhibition by real time efflux studies in the presence and absence of CuNPs. Commercially available EPI inhibitor verapamil was used as a positive control. In case of *Staphylococcus aureus* cells loaded with EtBr displayed stable residual fluorescence which was similar to the fluorescence displayed by EPI inhibitor verapamil (Fig. 4a). With *Pseudomonas*, although residual fluorescence was not completely stable, it was much higher than the fluorescence exhibited by the positive control verapamil (Fig. 4b), which implies that efflux pumps are indeed inhibited by CuNPs in both *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Since the multidrug efflux pump of *Pseudomonas aeruginosa* belongs to RND class, whereas that of *S.aureus* belongs to MFS class, which are both structurally and functionally quite different, we observe a differential effect of verapamil on efflux pumps of *S.aureus* and *P.aeruginosa*

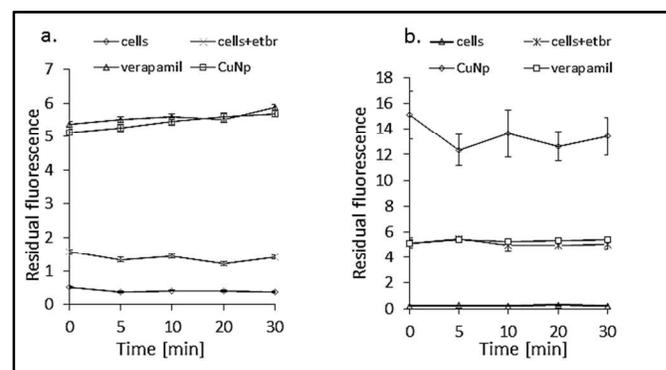


Fig. 4 Real time Efflux in *Staphylococcus aureus* and *Pseudomonas aeruginosa* is inhibited by CuNPs.

Residual fluorescence of EtBr in (a) *S. aureus* and (b) *P. aeruginosa*, over the time course of 0-30 min after 1h treatment with

CuNPs. The error bars represents standard error of the mean from three independent experiments.

F Membrane Permeability Studies

A *Staphylococcus aureus* – PI based Permeability Index

For *Staphylococcus aureus*, membrane permeability was assessed by permeability index studies using propidium iodide as reported earlier³⁷. Propidium iodide cannot permeate into intact cells; hence the accumulation of propidium iodide is indicative of loss of membrane integrity. Membrane perturbing agents like CTAB leads to increased accumulation of PI within cells. Ratio of accumulation of PI in CTAB untreated cells to that in CTAB treated cells is expressed as permeability index and is expressed in percent. Our results (Fig. 5a) show that CuNPs treatment led to increased accumulation of PI within the *Staphylococcus aureus* cells, relative to untreated cells, as revealed by increased permeability index of 38% and 33% displayed by these cells after 1 and 2h respectively.

B Gram negative bacteria- NPN assay

We tested to see if the CuNPs altered membrane permeability of outer membrane (OM) of *Pseudomonas aeruginosa* by NPN assay as reported earlier³⁸. The OM of gram negative bacteria is impermeable to hydrophobic molecules and hence increase in fluorescence due to retention of NPN, a lipophilic fluorescent probe will indicate that the OM is permeabilized. Our results (Fig. 5b) showed that LPS containing outer membrane of the *Pseudomonas aeruginosa* is compromised by treatment with CuNPs in a dose dependent manner until 1X MIC concentration. Statistically significant variation in NPN uptake could not be observed between 1X and 2X MIC concentrations ($p=0.153$) and at 1X MIC, the NPN uptake factor was 5.63 (Table 1), which implies significant permeability in OM of *Pseudomonas aeruginosa*, whether this translates to loss of LPS remains to be determined. Thus CuNPs are able to permeabilize both *Pseudomonas*

aeruginosa and *Staphylococcus aureus* which could partially account for the anti-bacterial effect of CuNPs.

Table 1. NPN uptake factor depicting Outer membrane permeability of *Pseudomonas aeruginosa*

Sample	NPN	Fluorescence	Fluorescence value after background subtraction	NPN uptake factor
Buffer	-	0.69		
Buffer	+	4.12±0.2		
Buffer+cell	-	8.15±0.01		
Buffer+cell	+	85.28±0.6	77.13	
Buffer+cell +CuNP(0.1 25X)	+	107.37±6.4	99.22	2.71
Buffer+cell +CuNP(0.2 5X)	+	113.59±3.2	105.44	3.47
Buffer+cell +CuNP(0.5 X)	+	117.73±3.8	109.58	3.98
Buffer+cell +CuNP(1X)	+	131.2±2.1	123.05	5.63
Buffer+cell +CuNP(2X)	+	136.03±1.3	127.88	6.23

Since membrane of both *Pseudomonas aeruginosa* and *Staphylococcus aureus* were permeabilized by CuNPs, we evaluated whether CuNPs compromised membrane integrity by causing leakage of biological macromolecules. Treatment of *Staphylococcus* with CuNPs caused 54 % and 38.5 % leakage of nucleic acids by 2h and 3h respectively and a protein loss of 21 % by 2h and 16.5 % by 3h, relative to treatment with triton x 100. Treatment of *Pseudomonas* with CuNPs did not induce significant leakage of biological macromolecules relative to challenge with triton x 100. Significant leakage of biological macromolecules only from *Staphylococcus* could possibly account for the enhanced

susceptibility of *Staphylococcus aureus* for CuNPs relative to *Pseudomonas aeruginosa*.

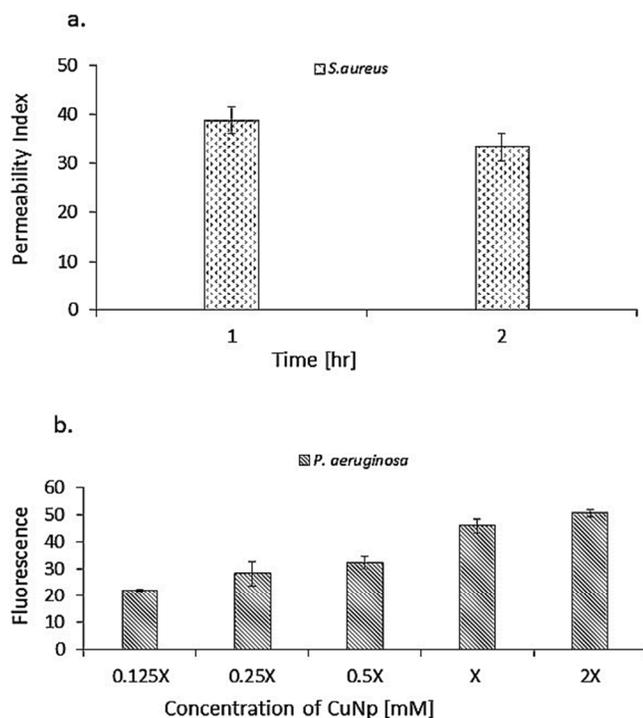


Fig. 5 CuNPs enhances membrane permeability in *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

(a) Cell membrane permeability discerned using propidium iodide uptake studies in *S. aureus*. (b) In *P. aeruginosa*, membrane permeability assessed using 1-*N*-phenyl-naphthylamine (NPN) uptake studies. The error bars represent standard error of the mean from three independent experiments.

G Confocal imaging

Live dead staining of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in the presence and absence of CuNPs revealed that in *Pseudomonas aeruginosa* CuNPs treatment induced significant loss of viability as evidenced by red fluorescence observed in treated samples (Fig 6). Interestingly, when *Staphylococcus aureus* was challenged with CuNPs, instead of observing an increased proportion of dead cells, significant loss in its colonization ability was observed, since only few patches of cells were found attached to the coverslip, after treatment with CuNPs (Fig 6 Right Panel bottom left image). Based on the time kill studies, we

can postulate that *Staphylococci* are killed effectively in a shorter span of time and lysed cells could not remain attached to the glass slide, which probably accounts for the drastically reduced number of colonies seen in confocal imaging studies.

We also explored viability of cells exposed to sub-inhibitory concentrations of CuNPs which is presented as Supplementary Fig 1. Thus in consonance with time kill studies, CuNPs are more effective in eradicating *S. aureus*.

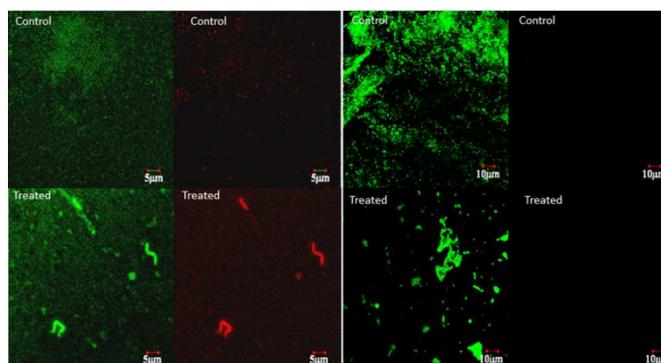


Fig. 6 Live dead imaging shows that CuNPs decreases viability in *Pseudomonas aeruginosa* and retards colonization in *Staphylococcus aureus*.

Confocal imaging of acridine orange and propidium iodide stained *Pseudomonas aeruginosa* (left panel) and *Staphylococcus aureus* (right Panel) with and without CuNPs treatment. Control - represents bacterial biomass grown for 3 days on glass surface in media without copper nanoparticles. Treated represents bacterial biomass grown for 3 days on glass surface in media containing (1X MIC) of CuNPs.

H Particle or Ion effect

In order to quantify whether the antibacterial effect is due to Cu (II) ions released from the CuNP or it is due to nanoparticle itself, alizarin red S conjugation test was performed as reported earlier³⁹. Alizarin red exhibits intense red colour upon complexing Cu²⁺ ions, which can be quantitated by measuring the absorbance at 510 nm. Our studies (Fig. 7) revealed that relative to solution containing CuNPs alone, cells along with CuNPs in nutrient media released more Cu (II) ions by 1h. Infact, CuNPs incubated with *Staphylococcus aureus* released more copper ions relative to CuNPs incubated with *Pseudomonas aeruginosa* in nutrient media, which could probably explain the enhanced

bactericidal effect displayed by Cu NPs against *Staphylococcus aureus*.

If Cu (II) ions from the CuNPs were indeed responsible for the antibacterial effect, growth inhibitory effect of CuNPs would be reversed by EDTA due to the chelation of Cu (II) ions. Hence, we tested for the effect of different concentrations of EDTA with and without CuNP on the growth of bacteria. Since EDTA itself displayed growth inhibitory effect on both *Pseudomonas aeruginosa* and *Staphylococcus aureus*, we could not clearly discern whether the bactericidal nature of CuNPs is due to particle or ion effect. But the trend (Fig. 8a) showed that with *Staphylococcus*, when EDTA was used at 0.16 g/l, growth inhibitory effect of CuNPs was considerably reversed and with *Pseudomonas* at 0.04g/l of CuNPs, similar reversal in growth inhibitory effect was observed (Fig.8b). As we observed significant but not complete reversal of growth in the presence of EDTA, we can surmise that the antibacterial effect is partially mediated by copper ions.

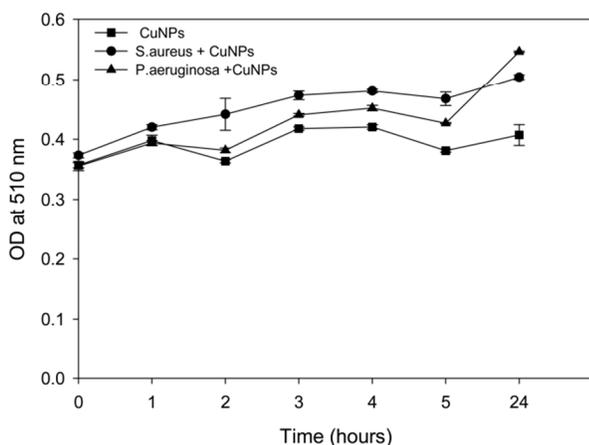


Fig. 7 Alizarin red conjugation test depicting Release of Cu(II) ions from CuNPs.

Time course of Cu (II) ions released from nutrient media with and without cells, the released Cu (II) ions upon conjugation with alizarin red S exhibits intense red color which is measured at 510 nm. CuNPs + *S.aureus* and CuNPs + *P.aeruginosa* indicates treatment of *Staphylococcus aureus* in nutrient media with Copper nanoparticles and treatment of *Pseudomonas aeruginosa* in nutrient

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media with copper nanoparticles respectively for quantifying copper ion release. The error bars represents standard error of the mean from three independent experiments.

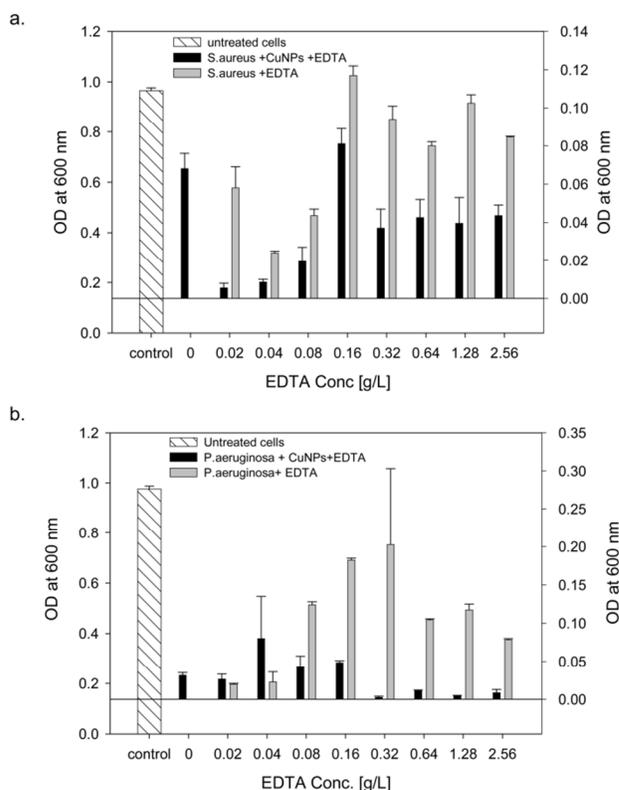


Fig. 8 EDTA treatment partially reverses growth inhibitory effect of CuNPs.

Bacterial cells a) *Staphylococcus aureus* b) *Pseudomonas aeruginosa* inoculated into MH broth were treated with 1X MIC concentration of CuNPs followed by incubation with increasing concentrations of EDTA and the growth attained at the end of 24h was quantified by measuring the absorbance at 600 nm. The error bars represents standard error of the mean from three independent experiments.

H Resensitization of Drug resistant Bacteria

As a proof of concept experiment, we tested the ability of CuNPs functioning as EPI to resensitize drug resistant mutant strain of *Staphylococcus* to ciprofloxacin, for which the mutants display resistance. Laboratory evolved mutant strains of *Staphylococcus aureus* were obtained by repeated sub culturing onto plates containing higher concentration of ciprofloxacin. The mutant strains displayed an MIC of 64

$\mu\text{g/ml}$ for ciprofloxacin, whereas the wild type strain displayed an MIC of 0.5 $\mu\text{g/ml}$. When CuNPs at 0.25 X MIC (0.032 mM) concentration was employed as efflux pump inhibitor, a significant 4 fold reduction (from 64 $\mu\text{g/ml}$ to 16 $\mu\text{g/ml}$) in MIC for ciprofloxacin was observed in 2 out of 3 mutants strains tested, the other mutant displayed a two-fold reduction (32 $\mu\text{g/ml}$) in MIC (Table 2). Even though a complete resensitization of mutants similar to that of the wild type was not achievable, reduction in MIC by 4 fold in 2 out of 3 mutant strains proved that CuNPs functioned effectively as EPI (bacterial adjuvant) and could enhance the susceptibility of drug resistant mutants to conventional antibiotics.

Table 2. CuNPs as bacterial adjuvant reverses MIC of mutant strains of *Staphylococcus aureus*

Organism	Ciprofloxacin MIC [$\mu\text{g/ml}$]	Ciprofloxacin+ CuNP * MIC [$\mu\text{g/ml}$]
SCIP1	64	32
SCIP2	64	16
SCIP3	64	16

*((CuNPs were used at 0.25 X MIC concentration (0.032mM))

C Discussion

MDR strains are known to cause severe infections like osteomyelitis and endocarditis^{40, 41} typically, through enhanced efflux activity. In fact, enhanced efflux activity, especially if the efflux pump involved extrudes diverse classes of drugs, leads to MDR phenotype^{42, 43}. A very good way to tackle the drug resistant bacteria is to use efflux pump inhibitors (EPIs) which resensitize the drug resistant bacteria to the antibiotic, by enhancing the concentration of antibiotic within the bacteria^{29, 31}. In the present study, we tested the efflux inhibitory action of CuNPs based on its antibacterial effect on MRSA (an MDR strain that overexpresses efflux pumps)³⁴. Being classical colonizers, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were the natural choice in our study to represent gram negative and gram positive groups respectively. In addition, the clinical isolates

of these two bacteria typically exhibit drug resistant phenotype^{44, 45}. Earlier reports have indicated the loss of proton motive force (PMF) occurs due to the use of metal NPs^{4, 46}. It is likely that metal nanoparticles dissipate PMF by interfering with bacterial respiration Choi et al.,⁴⁷ showed that Ag nanoparticles, Ag ions and Ag colloids cause different degree of respiratory impairment on *E.coli* of which maximum respiratory inhibition of 86 % was caused by Ag nanoparticles and this could possibly lead to loss of PMF. As PMF is essential for the normal functioning of many bacterial efflux pumps, we deduced that if metal NPs can disrupt PMF, they are more likely to inhibit the activity of efflux pumps. The experimental results tabulated above prove the aforesaid deduction. Metal NPs toxicity⁴⁸ can be easily alleviated if they are deployed as bacterial adjuvants, where a much lower concentration of metal NPs is necessary and sufficient than the effective concentration of antibacterial agents. One of the predominant classes of bacterial adjuvants is EPIs⁴⁹. Our experimental results and observations point to the finding that CuNPs, when used at 0.5 X MIC, functioned effectively as efflux pump inhibitor in *Pseudomonas aeruginosa* (gram negative) and *Staphylococcus aureus* (gram positive bacteria) (Fig. 3). At lower concentration of 0.25 X MIC, only wild type strain of *Staphylococcus aureus* was observed to be more susceptible to the efflux inhibitory effect of CuNPs (Fig. 3). Nor A being a predominant efflux pump in *Staphylococcus aureus* that contributes to MDR phenotype, by causing the extrusion of structurally diverse molecules^{50, 51}, it is likely that CuNPs inhibit the activity of Nor A. It remains to be investigated further if this inhibitory action of CuNPs on Nor A is a direct effect due to interaction of CuNPs with efflux pump or an indirect effect (due to loss of PMF).

Relative to *Pseudomonas aeruginosa*, CuNPs exerted significant efflux inhibition at a lower concentration against *Staphylococcus aureus* (Fig 3). Keeping in mind toxicity of CuNPs at higher concentrations, we chose to perform resensitization studies (reversal of MIC in drug resistant strains). Results of resensitization (proof of concept)

experiment performed with laboratory evolved ciprofloxacin resistant mutant strain of *Staphylococcus aureus* revealed that CuNPs functioning as EPI could restore activity of ciprofloxacin against the mutant strain (Table 2). Our choice of the drug resistant mutant for resensitization study can be justified by an earlier study which has shown that the MDR phenotype with enhanced efflux activity (AcrAB/AcrEF) can be selected in *E.coli* harbouring a single mutation in QDR region using fluoroquinolones⁵². Interestingly, CuNPs treatment could not completely reverse the MIC of the mutant strain as observed in wild type strain (Table 2). The reasons for the above observation could be attributed to the fact that resistance to ciprofloxacin is primarily associated with chromosomally encoded Quinolone Resistance Determinant Region (QRDR) and hence contribution of efflux pumps alone to quinolone resistance appears to be secondary⁵³. Moreover, at 0.5 X MIC, the EPI effect of CuNPs on mutant strains of *Staphylococcus aureus* was relatively lower than the EPI effect observed with wild type as evidenced by the varying fluorescence intensity in the cartwheel assay (Fig. 3). Though complete reversal of MICs in the drug resistant mutant strains was not possible, efflux inhibition caused by CuNPs increased intracellular concentration of ciprofloxacin and decreased MIC of the mutant strain by 4 fold. The fact that CuNPs caused a drastic 4 fold reduction in MIC in 2 out of 3 mutant strains very well supports the claim that it can efficiently restore the susceptibility of antibiotic in drug resistant mutants and can be successfully employed to tackle MDR bacteria. By virtue of the fact that use of CuNPs as EPI requires a very low concentration 0.25 X MIC (0.032 mM), concerns regarding toxicity of CuNPs against non-target organisms would be greatly alleviated. Thus our study shows that CuNPs can function as a safe and effective EPI (bacterial adjuvant) against drug resistant bacteria.

Earlier report exist showing that Ag NPs could be employed as probes to discern the efflux kinetics of MDR pump MexAB-OprM in *Pseudomonas aeruginosa* wherein size dependent extrusion of NPs occurs⁵⁴ and a recent study

uncovering the crystal structure of heavy metal efflux pump Cus CBA proposed a plausible mechanism of extrusion of toxic Cu (I)/ Ag(I) metal ions either from the cytoplasm or periplasm of *E.coli*, which involves use of methionine pairs as metal ion carriers⁵⁵. While this manuscript was in review, a recent study showed that iron oxide nanoparticles coated with poly acrylic acid was effective in inhibiting efflux of rifampicin and other anti TB drugs in *M. smegmatis*⁵⁶. Reports on metal nanoparticles altering the efflux activity are relatively recent and scarce⁵⁷ and our study attributed a novel efflux inhibitory role for capped CuNPs.

A Effect against biofilm mode of growth

Biofilms are highly recalcitrant and antimicrobial agents effective against planktonic state of growth were often observed to be ineffective against biofilm mode of growth predominantly due to slow growth phenotypes and heterogeneity observed in biofilm populations^{48, 58}. Hence, the hunt for novel classes of antibiofilm agents continues unabated. Of late reports on antibiofilm effect of metal NPs are forthcoming. A recent study showed that Ag Nps (20 µg/mL) inhibited the biofilm formation in sensitive and resistant strain of *Pseudomonas* by 65 % and 56 % respectively⁵⁹. Another study reported a 4 log reduction in *Pseudomonas* biofilms formed under flow conditions when challenged with Ag NPs at a concentration of 100 mg/ml⁶⁰. By using pullulan both as a reducing and stabilizing agent, Ag NPs were generated, which displayed significant antibiofilm effect against *E.coli* and *Pseudomonas* at 28 and 52 µg/ml respectively⁶¹. Reports on anti-biofilm effect of CuNPs are limited. An earlier study showed that ZnO and CuO NPs synthesized by sonochemical irradiation effectively inhibited biofilms formed by *Streptococcus mutants* in a teeth biofilm model⁶². In our study we observed that at 1X MIC concentration CuNPs caused significant biofilm inhibition in both *Staphylococcus* (90.4%) and *Pseudomonas* (88.6%), relative to the untreated control (Fig. 2). When tested at concentration lower than MIC, *Pseudomonas aeruginosa*

appeared to be more susceptible. Although the reasons for differential susceptibility of biofilms at lower concentrations are unclear, it was recently proved that multiple antibiotic response regulator (Mar R) is a copper sensor, and copper (II) ions released from membrane bound enzymes due to antibiotic treatment oxidizes cysteine residues in Mar R, which leads to tetramerization and dissociation of Mar R from DNA, this in turn activates antibiotic resistance genes in *E.coli*⁶³. Since biofilm formation and enhanced antibiotic resistance are quite related and the other sets of genes regulated by Mar R is not unravelled yet, we postulate that at lower concentrations, Cu (II) ions released from CuNPs might act as signalling molecule in *Staphylococcus aureus* and alter the gene expression of EPS molecules such that enhanced biofilm formation is observed.

Interestingly and importantly, a number of EPI inhibitors when used in combination with antibiofilm agents, reduced biofilm formation significantly and could even abolish biofilms completely in MFS and RND pumps expressing strains^{64, 65}. Since our study also showed that CuNPs exerted efflux inhibitory effect apart from significant biofilm inhibitory effect, it is tempting to speculate that its biofilm prevention effect and its efflux inhibitory effect could complement each other.

B Particle or Ion effect

Many studies have reported that anti-microbial effect of Ag is due to the released Ag(II) ions and not due to the particle effect⁶⁵. The issue of whether the antibacterial effect of Ag NPs was due to particle effect/ ion effect was critically explored by another study, in which the authors performed the experiment under strictly anaerobic conditions, wherein Ag(O) is prevented and silver ion release does not occur and by this study, the authors proved that antimicrobial effect is solely caused by ion effect and particle does not have any biological role⁶⁶. A recent comprehensive study elucidated that contact of AgNPs with the bacterial surface, led to dissolution and release of Ag (II) ions from the membrane

bound NPs, which along with the bacterial environment, induced dissolution of Ag (II) ions and accounted for the toxicity of nanosilver⁶⁷. Recently, it was also shown that enhanced release of metal ions from the NPs occurs mainly in the acidic environment of lysosomes, which contributes to the cytotoxic effect of AuNPs. If NPs directly enter the cell, they are less toxic than when they resort to endosomal route²⁷. Our study exploring time course of release of copper ions, using alizarin red conjugation test, showed that the presence of nutrient media and bacterial type dictated the amount of copper ion released from CuNPs. Infact, more Cu (II) ions were released from CuNPs in *Staphylococci* containing nutrient media than in *Pseudomonas* containing nutrient media (Fig. 7). In addition, our studies on efflux inhibition of CuNPs along with EDTA revealed that only a partial reversal in efflux inhibition was observed with EDTA for both groups of bacteria (Fig. 3c, f). Similarly when the ability of EDTA to reverse the growth inhibitory effect of CuNPs was tested for *Pseudomonas aeruginosa* and *Staphylococcus aureus*, only a partial growth reversal was observed (Fig. 8) implying that antibacterial effect and efflux inhibitory effect is partly mediated by CuNPs and partly by Cu (II) ions and hence our results are in consonance with recent study which claims that “particle/ion effect are two sides of the same coin”⁶⁷. In summary our study showed that CuNPs exhibited novel efflux inhibitory effect which is partially mediated by particle effect and partially by ion effect.

We are reporting that CuNPs can cause efflux inhibition in wild type strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and mutant strains of *Staphylococcus aureus*. The efflux inhibitory effect of CuNPs was partly mediated by CuNPs rather than solely by Cu (II) ions released from NPs. CuNPs functioning as EPI (at a very low concentration) reduced the MIC of ciprofloxacin in mutant strains of *Staphylococcus* by 4 fold. With CuNPs being toxic beyond certain concentrations, they could be effectively used as EPI's at lower concentrations where they are non-toxic as bacterial adjuvants, instead of using them solely as antibacterial agents

(at higher concentrations). Thus based on our studies we propose that CuNPs could be effectively employed as bacterial adjuvants (EPI) to tackle drug resistant bacteria.

D Materials and Methods

Copper- nanoparticles (CuNP) were synthesized at 2.1 mM concentration with casein as the stabilizing agent and ascorbic acid as the antioxidant as reported earlier³⁴. The casein stabilized CuNPs (total size including casein cap is 110 nm; Size range of CuNPs alone is 35-89 nm) were chosen for all the experiments. Gram negative bacterium *Pseudomonas aeruginosa* (MTCC 1688) and gram positive bacterium *Staphylococcus aureus* (MTCC 3160) were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. *Pseudomonas aeruginosa* was routinely subcultured from frozen glycerol stocks and maintained on Luria Bertani agar (LBA) and *Staphylococcus aureus* was subcultured from glycerol stocks were maintained on Tryptic Soy agar (TSA).

A Antimicrobial studies MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) were discerned by broth microdilution method⁶⁸. Briefly, CuNP solution was serially diluted till 10^{-10} and inoculated with test organisms in Mueller-Hinton broth and incubated at 37°C for 18-24h. The lowest concentration of CuNP, which prevented growth (visible turbidity), was assumed to be MIC. From the same experiment 10 μ l samples were withdrawn (from the samples that exhibited no turbidity) and were spotted onto TSA plates. The lowest concentration which gave rise to 3 or fewer colonies was considered as the MBC.

B Time- kill studies Bactericidal effect of CuNP over the time course (0-24h) is discerned using time-kill experiments and the viability of the cells were evaluated by determining plate count and expressed in terms of colony forming units/ml (CFU/mL). Planktonic cells in the early-log phase (0.1-0.2 OD) were challenged with either 1X / 2X MIC of CuNPs and

sampled at various time intervals from 0-24h, followed by plating onto TSA, plate counts were determined after incubation at 37°C for 24h⁶⁹. Decrease in cell count was expressed in log₁₀ CFU/ml and any reduction $\geq 3\log_{10}$ CFU, relative to the untreated control was considered to be significant.

C Retardation of biofilm formation Inhibition of biofilm formation in the presence of CuNPs was determined and quantified by crystal violet staining⁷⁰. Overnight grown cells of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were diluted (1:100) and inoculated into micro titre plates containing increasing concentrations of CuNPs in dilute (1:100) Tryptic Soy Broth. After 18-24 h, the micro titre plates were washed with PBS to remove unbound cells, dried and stained with 0.1% crystal violet for 15-20min. The plates were washed thoroughly in PBS and dried at 60°C. Crystal violet was extracted using 30% acetic acid for 15-20min and absorbance recorded at 595nm using a microplate reader.

D Efflux inhibition evaluation - EtBr cartwheel assay The effect of CuNPs on bacterial efflux pumps was evaluated by Ethidium Bromide agar-based cartwheel assay as reported earlier³⁶. A series of TSA plates were prepared with varying concentrations of EtBr (0.1-0.5 μ g/ml) and CuNPs were added to these plates at either 0.25X or 0.5X MIC concentrations. An overnight grown culture of *Pseudomonas aeruginosa* and *Staphylococcus aureus* was diluted to 0.5 OD and swabbed in cartwheel pattern on the agar surface. After 24 h of incubation, the plates were observed under UV transilluminator to check for the residual fluorescence displayed by each organism.

E Real time efflux study EtBr uptake and release was quantified as reported earlier⁷¹. *Pseudomonas aeruginosa* and *Staphylococcus aureus* were grown till mid-log phase and cells were pelleted at 1540 rcf for 10 min washed in uptake buffer, followed by resuspension in an equal volume of uptake buffer, CuNPs and EtBr were added at 0.13mM and

0.2 µg/ml respectively. After incubating for 1 h at 37°C (for EtBr uptake), the cells were again pelleted and washed twice with the uptake buffer and resuspended in the same buffer. The suspension was immediately quantified for the loss of fluorescence (Ex 530nm, Em 600nm), over a period of 30min, using JASCO FP-8200 spectrofluorimeter (JASCO, Tokyo, Japan).

F Cell-permeability assay:

A NPN-assay The extent of outer membrane damage in *Pseudomonas aeruginosa* (gram negative bacteria) caused by CuNPs was assessed by 1-*N*-phenyl-naphthylamine (NPN) uptake assay as reported earlier³⁸. NPN exhibits enhanced fluorescence in phospholipid environment. Since the Outer Membrane (OM) of gram negative bacteria prevents the access for hydrophobic molecules as it contains LPS, increased fluorescence indicates enhanced OM permeability. Briefly, cells were grown to mid-log phase collected and washed with 5mM HEPES buffer containing 0.2% glucose at pH 7.5 and resuspended in an equal volume of the same buffer. NPN was added at a concentration of 0.5 mM, this was immediately followed by addition of CuNPs in increasing concentrations. Fluorescence due to NPN was measured (Ex350 and Em 420 nm). Appropriate controls were maintained.

B Permeability Index Membrane Permeability of *Staphylococcus aureus* (gram positive bacteria) was assessed as reported earlier by Niven and Mullholland³⁷. Briefly, mid-log cells were pelleted, washed twice with PBS buffer and resuspended in an equal volume of PBS subsequently, CTAB and propidium iodide were added to a final concentration of 0.2mmoles/l and 30µmoles/l respectively, along with the CuNPs. Suitable controls were maintained. Fluorescence was recorded using JASCO spectrofluorometer FP-8200 after 1-2 h of incubation at 37°C. Fluorescence was normalized by deducting fluorescence of the untreated cells from that of the treated cells and Permeability Index was expressed as ratio of

fluorescence caused by treated cells in the absence of CTAB to the fluorescence produced in the presence of CTAB and is expressed in percent.

G Membrane integrity assay In order to discern membrane damage, cell membrane integrity was studied in the presence or absence of CuNPs as reported earlier⁷². Briefly, cells after treatment were collected at 0, 1, 2, 3 and 4 h, pelleted at 13,250 rcf for 5 min. The release of DNA and proteins due to cell damage caused by CuNPs were quantified at 260nm and 280nm respectively. 0.5 % triton X 100 treated cells was used as control.

H Estimation of Copper ion-release The amount of copper ions released from CuNPs in the presence and absence of test organisms were estimated using Alizarin red S conjugation test. This test is based on observation that Alizarin red S displays intense red colour when complexed with copper ions relative to its uncomplexed state, which can be quantified by measuring the absorbance at 510 nm³⁹. Cells grown till an OD of 0.2-0.3 were challenged with CuNPs at 1X MIC; a positive control with Copper chloride and negative untreated control (without copper ions) were maintained. Treated cells were sampled and pelleted at different time intervals (0-5 h) and resuspended in 1ml of 0.2M sodium acetate buffer pH 5.0. 200µl of Alizarin red S (4x10⁻³M) was added to these samples and absorbance was recorded after 5 min at 510nm.

I EDTA effect In order to discern whether the antibacterial effect caused by copper nanoparticles were due to particle or ion effect, growth curve inhibition and efflux inhibition based on cartwheel assay were tested in the presence and absence of varying concentrations of EDTA. Suitable controls (CuNP only, EDTA only and untreated) were maintained and fluorescence exhibited by the bacterial cells when illuminated with UV light was photographed.

J Confocal imaging Confocal imaging was performed on cells grown in the form of biofilms on the surface of coverslips placed inside sterile six-well plates containing 0.1X TSB media with/without CuNPs. Every 18 h, spent media was replaced with fresh sterile media. For sampling, slides were removed on Day 4, washed with sterile PBS to remove the non-adherent cells and stained with a mixture of acridine orange (0.2mg/ml) and propidium iodide (0.33mg/ml) and imaged using Olympus FV 1000 confocal microscope with 10X objective at a numerical aperture 0.3. For live dead staining using acridine orange, excitation was done using Multi Argon LASER and for detecting fluorescence due to propidium iodide excitation was performed using Helium Neon LASER. Acridine orange stains the nucleic acids of live cells, which when excited emits green fluorescence whereas, propidium iodide stains only membrane compromised or dead cells. Upon binding to DNA and subsequent excitation, propidium iodide emits red fluorescence. Thus all viable cells would appear green and all dead cells would appear red.

K Resensitization of drug resistant strain Since CuNPs were more effective against *Staphylococcus aureus*, mutant strains of the same were generated in the laboratory by consecutive exposure to increasing concentration of ciprofloxacin. It was confirmed by repeated observations that the mutant displayed increased MIC for ciprofloxacin. To test whether efflux inhibitory effect of CuNPs could resensitize the mutant to ciprofloxacin, MIC of both mutant and wild type for ciprofloxacin was evaluated in the presence and absence of 0.25 X and 0.5 X MIC of CuNPs by standard protocols.

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here] Suppl Fig 1 Live/dead status of cells exposed to various concentrations of CuNPs. See DOI: 10.1039/b000000x/

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