CHAPTER 8. CuNPs INHIBIT THE QUORUM SENSING REGULATED TRAITS IN PSEUDOMONAS AERUGINOSA STRAINS

8.1 Introduction

With an increasing spread of antibiotic resistance among bacteria and the difficulties which come in the way of treating the bacterial infections, there is an immediate need of an alternative way which can break this antibiotic resistance. The discovery of bacterial QS has picked the attention of the researchers towards the development of new therapeutic agents. The regulation of various pathogenic mechanisms in several bacteria including the formation of biofilm is controlled by QS system. *P. aeruginosa* is one among those bacteria which regulates its pathogenicity through QS system [123, 124]. *P. aeruginosa*, a ubiquitous microorganism and an opportunistic pathogen, generally causes infections in immunocompromised patients and is counted among the major causes of nosocomial infections. This microorganism is able to produce certain extracellular compounds including elastase, alkaline protease, pyocyanin, etc. which are regulated by QS system [125, 126]. QS system in *P. aeruginosa* regulates the formation of biofilm as well as gives protection. Hence, in order to reduce the infections caused by drug-resistant bacteria, inactivation of the QS system can be of great help [127].

This study was primarily focused on investigating the inhibitory effects of CuNPs on two important QS-regulated traits i.e. biofilm formation and pyocyanin production in an ATCC and a clinical strain *P. aeruginosa*. Furthermore, the effect of CuNPs was evaluated on extracellular polysaccharide (EPS) production, efflux-pump inhibition and the time-dependent effect on *P. aeruginosa*.

8.2 Methodology

As discussed in chapter 3, antibiotic susceptibility was performed to know about the resistance pattern of *P. aeruginosa* towards ceftazidime (CPM), ampicillin
(AMP), vancomycin (VA), azithromycin (AZM) and cefazolin (CZ). The antibacterial effect of CuNPs on P. aeruginosa was investigated through MIC and MBC and biofilm inhibition. MIC and MBC were performed through serial microdilution and surface drop method respectively. CuNPs were also utilized for their time-kill effect on P. aeruginosa. Biofilm inhibition assay was performed through serial microdilution followed by spectrophotometric measurements. The synergistic assay was performed to examine the combined effect of CuNPs and the antibiotic cefepime. CuNPs were also utilized to investigate their effect on the pyocyanin and EPS production by P. aeruginosa and finally, EtBr cartwheel method was used to test the efficacy of CuNPs on P. aeruginosa efflux pumps.

8.3 Results

8.3.1 Antibiotic susceptibility testing

![Antibiotic susceptibility testing graph](image)

**Figure 8.1:** Antibiotic susceptibility testing. The picture represents the resistance pattern of a clinical strain of P. aeruginosa towards 5 different antibiotics. R in the picture denotes the resistance of the strain towards that particular antibiotic.
ATCC strain of *P. aeruginosa* was sensitive towards all the antibiotics used in this study. For a clinical strain of *P. aeruginosa*, antibiotic susceptibility testing through disk diffusion test using five different antibiotics was performed. The following antibiotics were used: cefepime (CPM), ampicillin (AMP), vancomycin (VA), azithromycin (AZM), cefazolin (CZ) on sterile TPA plates and it was observed that the strain was resistant towards AMP, VA and CZ but was sensitive towards CPM and AZM (Figure 8.1).

8.3.2 MIC, MBC and tolerance level determination

MIC of CuNPs against *P. aeruginosa* strains was determined through serial microdilution using a 96-well microplate and it was observed that the MIC values for ATCC and clinical strain of *P. aeruginosa* were 0.625 and 1.25 mg/ml respectively. MBC value of 2.5 mg/ml was the same for both the strains. Tolerance level (MBC/MIC) for both the strains was calculated in order to find the bacteriostatic or bactericidal effect of CuNPs. For ATCC strain, the tolerance level was 4 which means that CuNPs were bacteriostatic towards this strain whereas for clinical strain, a tolerance level of 2 indicated a bactericidal effect of CuNPs on this strain.

8.3.3 Time-kill assay

Both the strains of *P. aeruginosa* were treated with their MICs to observe the effect at different time intervals using a 96-well microplate. The absorbance was recorded at 600 nm at different time intervals. From figure 8.2 it is quite evident that CuNPs efficiently controlled the growth of both the strains of *P. aeruginosa* when compared to the antibiotic cefazidime. The slope of the graph showed a decline until it reached a straight line in both the strains treated with CuNPs. While as the antibiotic was not that effective in case of the clinical strain, but for an ATCC strain the graph showed a decline with each passing time. It can thus be concluded that CuNPs were effective in controlling the growth of both the strains of *P. aeruginosa* compared to the antibiotic.
Figure 8.2: Growth curve analysis of an (a) ATCC and a (b) clinical strain of *P. aeruginosa* at different time intervals
8.3.4 Biofilm inhibition

Both the strains of *P. aeruginosa* were treated with different concentrations of CuNPs (0.039 mg/ml to 2.5 mg/ml) using a 96-well microplate in order to observe the effect on biofilm. As shown in figure 8.3, CuNPs inhibited biofilm in *P. aeruginosa* and the effect was dose-dependent. ATCC strain of *P. aeruginosa* was a slow biofilm producer while as the clinical strain produced luxuriant biofilm. For both the strains of *P. aeruginosa*, 2.5 mg/ml of CuNPs reduced the biofilm formation equal to or less than half of the control.

![Biofilm inhibition graph](image)

**Figure 8.3:** Biofilm inhibition in an (a) ATCC and a (b) clinical strain of *P. aeruginosa* in presence of CuNPs
8.3.5 Synergistic assay

Synergistic assay performed through disk diffusion method gave an information about the collective effect of CuNPs and the antibiotic (cefepime). As both the strains of *P. aeruginosa* were sensitive towards cefepime, there was no increase in the zone of inhibition in the combined disk of antibiotic + CuNPs. The zone of inhibition displayed by the antibiotic alone was similar to the zone displayed by the combined disk of antibiotic and CuNPs (Figure 8.4). Hence, CuNPs combined with the antibiotic did not show any synergistic effect.

![Figure 8.4](image)

**Figure 8.4:** No synergistic effect displayed by CuNPs in combination with the antibiotic cefepime

8.3.6 Pyocyanin production

ATCC strain of *P. aeruginosa* had the ability to produce more pyocyanin compared to the clinical strain. After treating both the strains with CuNPs, it was observed that there was a decrease in the production of pyocyanin in both the strains. Though the decrease was a little less in the clinical strain, the pyocyanin production was reduced to almost half in an ATCC strain of *P. aeruginosa* (Figure 8.5).
**Figure 8.5:** Pyocyanin production in control and treated strains of *P. aeruginosa*

### 8.3.7 EPS production

EPS production in both the strains of *P. aeruginosa* after treatment with CuNPs reduced when compared to the control strain (Figure 8.6). Though the reduction in EPS production was not that high, still CuNPs were able to reduce the production of EPS in both the strains.

**Figure 8.6:** EPS production in control and treated strains of *P. aeruginosa*  

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8.3.8 CuNPs as efflux-pump inhibitors

CuNPs were utilized to evaluate their effect on efflux pumps through the EtBr cartwheel method. This assay is based on the capability of bacteria to expel EtBr and the inhibition of efflux pumps is detected by the fluorescence emitted under UV-transilluminator. Both the strains of *P. aeruginosa* treated with CuNPs at their MIC concentration emitted fluorescence when visualized under UV-transilluminator meaning that the efflux pumps in the bacteria have been inhibited. Control *P. aeruginosa* strains did not show any emission of fluorescence (Figure 8.7) and hence it can be concluded that CuNPs acted as efflux pump inhibitors in both the strains of *P. aeruginosa*.

![Cartwheel method for efflux analysis](image)

**Figure 8.7**: Cartwheel method for efflux analysis showing the fluorescence emitted in the treated strains of *P. aeruginosa* (C: Control strain, T: Strain treated with CuNPs, RES: Resistant strain of *P. aeruginosa*)
8.3.9 Discussion

The increased resistance of bacteria towards antibiotics has focussed the attention of researchers in finding an alternative route to cope up with this resistance. As the bacteria have developed new means of avoiding the antibacterial agents, one such mechanism is the quorum sensing (QS) system which provides a kind of defence to the bacteria to avoid the damage which antibacterial agents can cause. Targeting bacterial QS system can provide new ways in treating and managing bacterial infections. Different QS inhibitors have been used since the past 15 years which include the furanone derivatives, different heterocycles, macrolide and non-macrolide antibiotics, salicylic acid, etc. [120]. Some plant extracts have also been reported to possess the anti QS activity in Gram-negative bacteria [121]. In the present study, CuNPs were utilized in testing their ability against two important QS-regulated traits i.e. biofilm and pyocyanin production in *P. aeruginosa* strains and CuNPs were able to inhibit both these QS-regulated traits. Biofilm inhibition by CuNPs against *P. aeruginosa* has been reported and it has been stated CuNPs can be coated on medical devices and implants to prevent biofilm [181]. Other nanoparticles such as silver and gold have been found to exhibit efficient anti QS activities in *P. aeruginosa*. Anti-biofilm activity of silver nanoparticles (50 nm diameter) in *P. aeruginosa* has been reported and silver nanoparticles were effective in killing the bacteria even in established biofilm structure [182]. Gold nanoparticles inhibited biofilm formation and the incursion of dental tissue stem cells in a recently conducted study [183].

The present study also showed that CuNPs are able to inhibit the EPS production in *P. aeruginosa* strains. EPS is important in the formation and maturation of biofilm which in-turn poses a hurdle in clinical practice by preventing the entry of antimicrobial agents. The inhibition of EPS production at very low concentrations of CuNPs has been reported [181]. Apart from EPS, CuNPs in the present study were also utilized as efflux pump inhibitors by observing the fluorescence emitted by the
strains treated with CuNPs. A similar method has been reported previously where CuNPs were utilized as efflux pump inhibitors in *P. aeruginosa* and *S. aureus* [184].

Taking all the results into consideration, CuNPs in the present study acted as efficient QS inhibitors as well as performed well in inhibiting the EPS production and efflux pumps in *P. aeruginosa*.

### 8.3.10 Conclusion

This study presented an approach towards the important factors that are very essential for *P. aeruginosa* in causing infections. Two QS-regulated traits were studied and the effect of CuNPs was evaluated. It was observed that both the traits were diminished in presence of CuNPs meaning that CuNPs acted as QS inhibitors. CuNPs were further utilized for their effect on EPS production and efflux pump inhibition in *P. aeruginosa* strains. The results suggested that CuNPs were able to reduce the production of EPS compared to control (untreated) strains. The efflux assay through the cartwheel method showed the presence of fluorescence in the strains treated with CuNPs thus depicting the role of CuNPs as efflux pump inhibitors.