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In vitro and *in vivo* antibiofilm effect of copper nanoparticles against aquaculture pathogens

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ABSTRACT

The emergence of multidrug resistance biofilms and inactive conventional antibiotics has encouraged a novel strategy to overcome biofilm infections in aquaculture industries. In the pioneer study the well-known copper nanoparticles (CuNPs) synthesized through one pot synthesis were explored in a new venue called antibiofilm agent against *Vibrio alginolyticus* (ATCC 17749), *Vibrio parahaemolyticus* (ATCC 17802) and *Aeromonas hydrophila* (ATCC 7966) both *in vitro* and *in vivo* analysis. In the *in vitro* analysis the CuNPs showed more than 60% biofilm inhibition in all the test pathogens at the biofilm inhibitory concentration of 100 ng/ml of D·H_2O. Further the CuNPs tremendously reduced 95% of the cell surface hydrophobicity (CSH) and 85% of extracellular polysaccharide (EPS) production, which are the major factors influencing biofilm formation in *V. alginolyticus*. In the *in vivo* analysis also CuNPs showed no toxic effect but higher survival rate in *Artemia salina*. The toxicological tests and *in vivo* studies explicate the potential of CuNPs as a novel candidate to overcome the biofilm problem in aquaculture industries.

1. Introduction

Biofilms are complex structures produced by microbes to resist against various environmental stress, antibiotics and immune system. Biofilm formations occur in both biotic and abiotic surface and a well-established microbial lifestyle in aquatic environments (Van Houdt et al., 2004). Microbes within these complex biofilm structures are unfeasible to eradicate and leads to a wide range of antibiotic resistance. Recent researches revealed the structural, physiological and genetic bases of biofilm formation to a great extend (Haussler and Fuqua, 2013; Karatan and Watnick, 2009). Biofilm formation remains a serious threat in aquaculture industries. Aquaculture is an important sector which fulfills the need for high animal protein supplement for human consumption. A total of one-third of the total food fish supply is captured from aquaculture while the remaining two-third is obtained from marine and inland water resources (Chatterjee and Haldar, 2012). The growing demand for sea food has increased the productivity rate of seafood in the recent years. Over the past 2 decades aquaculture industries has grown enormously and accounting for about 42% of global seafood production in 2012 (Lafferty et al., 2015).

Vibrio spp have been reported as an important pathogens which causes high rate of shrimp and prawn mortality in the aquaculture industries worldwide (Karunasagar et al., 1994). Further *Vibrio* spp are known to be a reason for severe mortalities occurs in commercially important marine organisms like pearl oysters (*Pinctada maxima*), fish (*Solea senegalensis*), *Hippocampus* sp. and lobsters (*Panulirus homarus*) (Chari et al., 2014). *Aeromonas hydrophila* has also been considered as a dominant pathogen in fish-bacterial-septicemia occurs in freshwater culture cyprinid fishes like crucian carp *Carassius carassius*, Wuchang bream *Megalobrama amblycephala* and silver carp *Hypophthalmichthys molitrix* (Qian et al., 1997). Conventional antibiotics are hopeless against these infections in aquaculture industries due to their delayed penetration; consequently there is a need for alternative strategies to overcome the biofilm infections (Defoirdt et al., 2011). An alternatives to this problem are inhibiting the formation of biofilm, reducing the cell surface hydrophobicity (CSH) and the extracellular polymeric sub-

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stances (EPS) collectively known as 'antibiofilm agents', is a novel strategy to overcome antibiotic resistance through biofilm formation in aquaculture industries (Nithya et al., 2010).

Using nanotechnological approach (Nanoparticles) is an ideal move towards combating the biofilm infections. Nanotechnology is a fast growing interdisciplinary field that connects the physics, chemistry, biology, material science and medicine (Pratik et al., 2012). Abundant up to date reports are available to explain the crucial role of nanoparticles in antimicrobial strategy. The abilities of nanoparticles like improved permeability through the cell membrane and target multiple sites in an organism makes them more effective against drug-resistant pathogens than conventional antibiotics (Haussler and Fuqua, 2013). Though copper nanoparticles (CuNPs) are well known for their antibiotic application against wide range of pathogens (Cho et al., 2005; Jain and Pradeep, 2005), they are very scantily explored as a potential antibiofilm agent (Agarwala et al., 2014; Eshed et al., 2014; MubarakAli et al., 2015). In a recent report, CuNPs has been used as a coating material over glass and steel surface to prevent the biofilm of *Pseudomonas aeruginosa* and *Listeria monocytogenes* (Ghasemian et al., 2015).

Therefore application of CuNPs as a coating agents in aquaculture industries and fish tank will prevent the growth of pathogenic biofilm forming bacteria contaminating sea food, further utilizing CuNPs will also reduce the exposure of pathogens towards aquaculture products. With this backdrop, the present study aimed to explore the antibiofilm efficiency of one pot synthesized nano particles against aquaculture pathogens, *Vibrio alginolyticus* (ATCC 17749), *Vibrio parahaemolyticus* (ATCC 17802) and *A. hydrophila* (ATCC 7966) both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

All the chemicals and reagents used in this study were purchased from Sigma-Aldrich, USA. Luria Bertani Broth and Zobell Marine Broth 2216 were purchased from HiMedia Laboratories, India. *Artemia salina,* a brine shrimp, was gifted as dormant eggs (cyst) from Department of Marine Sciences, Bharathidasan University, India. CuNPs used in this study were reported elsewhere (LewisOscar et al., 2015b; MubarakAli et al., 2015).

2.2. Bacterial strains and culture conditions

Reference strains used were: *V. alginolyticus* (ATCC 17749), *V. parahaemolyticus* (ATCC 17802) and *A. hydrophila* (ATCC 7966). All the *Vibrio* spp. were grown in Zobell Marine Broth 2216 (ZMB) at 28 °C and *A. hydrophila* was grown in Luria Bertani Broth at 28 °C.

2.3. Biofilm inhibitory activity of CuNPs

To determine the biofilm inhibitory activity of CuNP (100 ng −1000 ng/ml), the biofilm quantification assay was performed in 24 well polyvinyl chloride microtiter (NEST, Torson, India) plate to assess the antibiofilm effect of CuNPs against *Vibrio alginolyticus* (ATCC 17749), *Vibrio parahaemolyticus* (ATCC 17802) and *A. hydrophila* (ATCC 7966) (You et al., 2007). 1% of bacterial inoculum (10^7 CFU/ml) of reference strains were used as an inoculum for LB/ZMB medium supplemented with and without CuNPs. After incubation (24 h), planktonic cells were discarded and the wells were rinsed thoroughly with distilled water. Then stained with 0.4% crystal violet solution (w/v) and incubated at room temperature for 10 min. After incubation, excess dye was removed by washing twice with distilled water and distained with 1 ml of absolute ethanol for 30 min and the optical density was measured at 570 nm (LewisOscar et al., 2015b). Wells with LB/ZMB medium alone served as blank and wells without CuNPs served as controls. The percentage of biofilm inhibition was determined using the formula (1):

$$\text{Biofilm inhibition (\%)} = ([\text{Control OD}_{570\,nm} - \text{Test OD}_{570\,nm}]/\text{Control OD}_{570\,nm}) \times 100 \ldots\ldots \quad (1)$$

2.4. Light microscopic visualization of biofilms

The antibiofilm potential of CuNPs (100 ng/ml) was tested against the biofilm formation of *V. alginolyticus* and *V. parahaemolyticus* and *A. hydrophila* on glass surfaces as per LewisOscar et al. (2015a). The biofilm images were visualized under bright field microscope (Micros, Austria) at 40X magnification and documented using digital camera attached to the microscope.

2.5. Antibacterial activity of CuNPs

To check whether CuNPs possess any antibacterial effect against the reference strains, the treated (100 ng/ml) and control samples of *V. alginolyticus*, *V. parahaemolyticus* and *A. hydrophila*, were analyzed spectrophotometrically at 600 nm (LewisOscar et al., 2015b). To 1 ml of LB media 1% of bacterial inoculum was added from overnight culture (107 CFU/ml) and tested with CuNPs at a concentration of 100 ng ml-1. After 24 h of incubation at 37 °C, the free floating planktonic cells were taken and analyzed at 600 nm in UV Vis spectrophotometer (Agilent).

2.6. Bacterial Adhesion to hydrocarbons (BATH) assay

BATH assay was performed to determine the effect of CuNPs on the cell surface hydrophobicity (CSH) of *V. alginolyticus* and *V. parahaemolyticus* and *A. hydrophila* at 100 ng/ml concentration. Briefly, 1 ml (OD 600 nm = 0.8) of bacterial culture with and without CuNPs were mixed with 1 ml of toluene and vortexed for 2 min. OD of the aqueous phase was taken after vortexing. Percentage oh hydrophobicity was calculated by the formula (2) (Sethupathy et al., 2015).

$$\text{Hydrophobicity (HI) (\%)} = [1 - (\text{OD600 after vortexing/OD600 before vortexing})] \times 100 \ldots\ldots \quad (2)$$

2.7. Effect of CuNPs on extracellular polymeric substances (EPS)

The EPS quantification was done as follows. 10 ml of *V. alginolyticus* and *V. parahaemolyticus* and *A. hydrophila* treated (100 ng/ml) and untreated cultures (24 h) were centrifuged and the supernatants were collected to which 10% of Trichloro acetic acid and equal volume of acetone was added. After incubation at 4 °C for 8 h, the contents were centrifuged at 10,000 rpm for 10 min and the obtained pellet was weighed (Vicente-García et al., 2004).

2.8. In-vivo analysis of CuNPs using Brine shrimp

Around 3.2 g of cyst was suspended in distilled water (166 ml) mixed with sea salt and incubated for 24–36 h at 28–30 °C with aeration. After incubation, the cyst hatched and grew into nauplii which were used for further experiment. The nauplii of *Artemia salina* were cultivated in sea water (30 ppt salinity).

Ten nauplii of brine shrimp were transferred into a 6 well polysterene microtitre plate containing 5 ml of autoclaved seawater. To this 1% of bacterial inoculum from 10^7 CFU/ml culture of *A. hydrophila*, *V. alginolyticus* and *V. parahaemolyticus* was added along with 100 ng/ml of CuNPs. Well without CuNPs was served as control and nauplii of *Artemia salina* alone was taken as blank. The survival rate of the nauplii of *Artemia salina* was checked for 24 h and maintained at 28 °C. The survival rate of Nauplii was scored after every 24 h after inoculating the bacterial culture (Brackman et al., 2008; Lee et al.,