SYNTHESIS OF JACALIN FUNCTIONALIZED COPPER SULFIDE NANOPARTICLES AND EVALUATION OF *IN VIVO* THERAPEUTIC EFFECT IN BACTERIA INFECTED ZEBRAFISH
1. Summary

Metal nanoparticles are now extensively investigated to combat bacterial infection, but many study stopped at the level of *in vitro* investigation. *In vivo* testing in an animal model like rodent was limited by several factors which include high cost and ethical issues. In this chapter, a convenient method using an adult zebrafish animal model was described to study the antibacterial activity of N-lauryltyramine (NLTA) capped copper sulfide nanoparticles (CuS NPs) and jacalin functionalized NLTA capped copper sulfide nanoparticles (JCuS NPs). Transmission electron microscopy image showed that the CuS NPs are spherical in shape with the size range from 10 to 25 nm. FTIR spectra revealed that the CuS NPs were stabilized by NLTA. CuS NPs exhibit very good antibacterial against Gram-negative and Gram-positive bacteria and the minimum inhibitory concentration (MIC) were determined as 12.5 μM. Upon functionalization with jacalin, the MIC of CuS NPs decreased two folds to 6.25 μM. This is significant in terms using the drug at low concentration to eradicate the bacterial infection. CuS NPs kills the bacteria through damaging the cell membrane and producing reactive oxygen species. Zebrafish infected with bacteria survived when treated with medicated bath of CuS NPs. Bacteria colony count assay revealed that the medicated bath of CuS NPs depleted the infectious bacteria from the fish body within 24 h. Strikingly, CuS NPs of 10 times the MIC does not show toxicity in the liver and brain of zebrafish. Moreover, the NPs exhibit good hemocompatibility with human red blood cells. Our study for the first time demonstrated that the CuS NPs is a safer antibacterial agent and zebrafish in medicated bath is a hassle-free model for testing the antibacterial activity of nanoparticles.
5.2. Introduction

The growing records on the identification of multi drug-resistant (MDR) bacterial strains are the major concerns in modern medicine (Thabit et al., 2015; Lushniak, 2014). MDR cannot be curtailed with standard drug treatments, which required high-dose administration of multiple drugs that often leads to undesirable side-effects (Kaye et al., 2015; Fair et al., 2014). As an alternate to conventional antibacterial therapy, metal nanoparticles such as Gold, Silver, Copper, Platinum and Palladium are explored as antibacterial agents (Lemire et al., 2013, Miller et al., 2015). The precursor to synthesize copper nanoparticles is readily available when compared to other metal nanoparticles, therefore, developing an antibacterial agent with CuNPs would be cost-effective (Behlol et al., 2016). Although CuNPs exhibit good antibacterial activity, it is highly toxic when tested in zebrafish animal model (Dharsana et al., 2015). At the same time, at a lower concentration, it acts as an efflux pump inhibitor and prevents the biofilm formation (Christena et al., 2015; Megarajan et al., 2016). The beneficial nature of the CuNPs can be exploited by minimizing the toxicity and maximizing their therapeutic window through proper surface modification. For example, we recently showed that the sulfidation of CuNPs decreases the toxicity (Dharsana et al., 2015).

Copper sulfide (CuS), a P-type semiconductor have received attention recently due to their potential application in various filed, including thermoelectric, solar cells, ion-storage, batteries, photovoltaic, photocatalytic, chemical sensing, and so forth (Cheng et al., 2010; Lee et al., 2007; Zhao et al., 2012; Cai et al., 2012; Setkus et al., 2001). Besides semiconductor based application CuS NPs have been emerging as a
promising platform for photothermal cancer therapy, biomolecule sensing, and molecular imaging (Goel et al., 2014; Ku et al., 2012; Huang et al., 2015). In this study the antibacterial activity of CuS NPs against Gram positive and Gram negative pathogenic bacterium was demonstrated. CuS NPs kills the bacteria through the production of reactive oxygen species (ROS) and by damaging the bacterial membranes.

There is a wealth of reports about the antibacterial activity of various metal NPs including CuS NPs, (Wang et al., 2015; Yu et al., 2015) but most of them are basic evaluation and rarely studied their efficacy in an infected animal model. Another bottleneck in the usage of NPs is their toxicity in biological systems, which delays the advancement of these materials for clinical applications (Ai et al., 2011. Linkov et al., 2008). The paucity was addressed in this work through rescuing bacteria infected zebrafish using CuS NPs. Zebrafish is an important animal model in biomedical research because it shares many common biological pathways with humans and is 80% genetically identical to humans (MacRae et al., 2015; Barrisuo et al., 2015; Wills et al., 2016; Jain et el., 2016). CuS NPs halt the bacterial proliferation in zebrafish and cure from the microbial infection. The CuS NPs that can kill the bacteria also exhibit low toxicity and maintain good haemocompatibility. Noteworthy, the CuS NPs not only cure the infection but also disinfect the pathogens present in the water.
5.3. Material and methods

5.3.1. Materials

Copper chloride, hydrazine, ammonium hydroxide and sodium sulfide were obtained from Merck, India. Tyramine, laurylechloride, resazurin, dichlorofluorescein diacetate, acridine orange, ethidium bromide, 5,5’ dithio-bis(2-nitrobenzoic acid), acetylcholine iodide, naphthyl ethylenediamine hydrochloride were purchased from Sigma, India. All microbiological media were obtained from Himedia, India. E. coli (MTCC723), A. hydrophila (MTCC1739T), S. aureus (MTCC3160) and B. subtilis (MTCC441) were obtained from Institute of microbial technology, India. The organisms were preserved at 4°C and sub-cultured at regular intervals of 30 days. All other chemicals and reagents were of the highest analytical grade and commercial available.

5.3.2. Synthesis of copper sulfide nanoparticles

First, N-lauryltyramine (NLTA) capped copper nanoparticles were prepared. In brief 1 mg of NLTA dissolved in 100 μL of ethanol was added to 50 mL of 2.5 mM NaOH solution. Then, 1 mM of copper chloride and 200 μL of ammonium hydroxide was added to the solution. After 5 min stirring at 600 rpm, 400 μL of hydrazine hydride was added to the solution after which the blue solution changed colour and turned to reddish brown in 6 h. The obtained solution showed characteristic CuNPs surface plasmon maximum at 580 nm. As obtained CuNPs was directly allowed to react with 1 mM sodium sulfide for 3 h until a green solution was obtained, which suggests the formation of CuS NPs. The shape, size, crystallinity and elemental composition of the CuS NPs were investigated by transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDX). For TEM, an aqueous solution of CuS NPs was drop casted on graphite grid and air-dried prior to examine in TEM (JEOL JEM-
2100F), operating at an accelerating voltage of 200 kV. EDX was measured on a FEI Quanta FEG 200-high resolution scanning electron microscope. For FTIR, NLTA and NLTA-CuS NPs were freeze dried and ground with KBr to form a pellet and measured in PerkinElmer FTIR spectrometer with 1 cm⁻¹ resolution.

5.3.3. Interaction of CuS NPs with jacalin

A fixed volume of jacalin solution (3.0 ml, 5 μM) was titrated by adding small aliquots of the CuS NPs from a concentrated stock solution and the fluorescence intensity was recorded after an equilibration period of 2 min. All binding experiments were performed in 10 mM phosphate buffer, containing 0.15 M NaCl, pH 7.4 (PBS). All titrations were repeated at least three times to arrive at average values.

5.3.4. In vitro antibacterial activity of CuS NPs

Gram-negative (E. coli, A. hydrophila) and Gram-positive (S. aureus, B. subtilis) strains were used for the evaluation of antibacterial activity of CuS NPs. Bacterial cells were cultured aerobically for 12 h at 37°C in Luria-Bertani (LB) medium. The cultures were maintained by streaking on LB agar plates and being incubated at 37°C for 24 h. Then, the pure single colonies were isolated and sub-cultured every fortnight in a shaker incubator at 37°C until an optical density at 660 nm (OD₆₆₀) of 0.5-0.6 was reached.

To determine the minimum inhibitory concentration (MIC) of CuS NPs, 100 μL of CuS NPs (100 μM) was added to the 96-well plate and serial diluted and the final volume was made up to 100 μL using LB medium. Then, 100 μL of 1 × 10⁵ cfu/mL bacterial cells were seeded into each well and cultured under shaking for 24 h at 37°C. The OD₆₆₀ at each well was monitored in microtiter plate reader (Thermo Scientific Multiscan EX). Further, the cell viability was determined by adding 30 μL
of resazurin solution (0.01% wt/vol) to each well and cultured for 2 h at 37°C. (Sarker et al., 2004). The wells with viable cells showed colour change from blue to pink and exhibit strong fluorescence. The fluorescence intensity in each well was monitored in a fluorescence microplate reader (Biotech, synergy H1, Japan), setting the excitation and emission at 530 nm and 580 nm, respectively. The efficacy of CuS NPs was further judged by the zone of inhibition (ZOI) assay. Log phase bacteria cells were swabbed uniformly on LB agar plates using sterile cotton swabs. Wells of 10 mm diameter were made on the plates using gel puncture. Using micropipette, 50 μL of 5 μM, 10 μM and 100 μM CuS NPs were added to the respective well and incubated at 37°C for 24 h. The formation of the zone around the well confirms the antibacterial activity of CuS NPs.

Bacterial colony count assay was performed in LB agar plate. Typically, agar in LB medium was autoclaved and cooled to around 60°C. To this mixture, CuS NPs of defined concentration was added and poured into Petri plates and allowed to solidify at room temperature. After solidification, 0.1 OD$_{660}$ A. hydrophila culture was swabbed on the LB-agar plate containing CuS NPs. The plates were incubated for 24 h at 37°C. Control experiment was performed without CuS NPs. The experiment was performed in triplicates and the colonies were counted and reported in colony forming unit (cfu).

$$\text{cfu} = \frac{\text{No. of colonies} \times 100}{\text{Dilution factor} \times \text{Volume of culture spreaded}}$$  \hspace{1cm} -(1)

5.3.5. Evaluation of therapeutic efficacy of CuS NPs in zebrafish

To determine the optimal bacterial dosage for exposure, intramuscular infection of zebrafish with $E. \ coli$ was performed essentially following Neeley et al., with slight
modifications (Neely et al., 2002). Typically, zebrafish were injected intramuscularly using a 3/10-cc U-100 insulin syringe with a 0.5-in.-long, 29-gauge needle. The anesthetized fish were made to lie down prostrate on a wet sponge in a tank containing water and the needle of the insulin syringe was inserted cephalad at a 45° angle to the spine into a position immediately lateral to the dorsal fin. The needle was inserted up to bevel, and 10 µl of bacterial suspension was slowly injected, and the needle was held in place for a few seconds to make sure the material stayed in before being slowly withdrawn. Similarly, control fish were injected with equal volume of sterile buffer.

Table 5.1: Exposure of CuS NPs and mortality of fish in 48 h

<table>
<thead>
<tr>
<th>Group</th>
<th>Optimizing bacterial concentration</th>
<th>Optimizing CuS NPs concentration</th>
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<tbody>
<tr>
<td></td>
<td>Bacteria OD&lt;sub&gt;660&lt;/sub&gt;</td>
<td>Total number of fish</td>
</tr>
<tr>
<td>A</td>
<td>0.01</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>0.05</td>
<td>10</td>
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<tr>
<td>C</td>
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<td>D</td>
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<td>E</td>
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The fish were first exposed to bacterial strain of different optical density (OD<sub>660</sub> – 0.01, 0.05, 0.1, 0.3, and 0.5) by injecting 10 µL of the freshly prepared bacterial cells. Ten fishes were used for each group and the mortality was followed
for 24 h. Based on the mortality, 0.1 \( \text{OD}_{660} \) cells were used for further studies. The optimal CuS NPs dosage for exposure was determined by subjecting infected zebrafish in a medibath containing CuS NPs of varying concentration ranging 0.1, 1, 5, 7, 10 \( \mu \text{M} \) (Table 5.1). Based on the mortality in 48 h, 10 \( \mu \text{M} \) of CuS NPs was fixed for further studies.

To test the therapeutic efficacy, healthy zebrafish was infected with bacteria and then treated with CuS NPs. Zebrafish were maintained in aerated glass tanks containing tap water at 25 ± 2°C and the infection was initiated through injecting bacterial cells into the fish body (Neely et al., 2002). The optimized bacterial dosage for intramuscular infection was 0.1 \( \text{OD}_{660} \). About 10 adult zebrafish were intramuscularly injected with 10 \( \mu \text{L} \) of freshly prepared bacterial culture (0.1 \( \text{OD}_{660} \)) and allowed to survive with the infection for next 3 h. Then, the fish were divided into two groups, each group contains five fish. Group A fish were labelled as infected control and Group B was treated by injecting 10 \( \mu \text{L} \) of CuS NPs (10 \( \mu \text{M} \)). In another method of treatment, Group B fish were treated by exposing the intramuscular infected zebrafish to a water bath contains 10 \( \mu \text{M} \) CuS NPs. Mortality of the fish was monitored carefully for 48 h.

The infection in the fish tissue was analyzed by colony forming unit (cfu) counting. Typically, fish were sacrificed (anesthetized by 150 mM MS-222 and euthanized by decapitation) at particular time interval and the muscle tissue was dissected and homogenized in 1 mL of PBS buffer (10 mM phosphate, 150 mM NaCl, pH -7.4). The homogenate was diluted 10^4 times and plated in triplicate on sterile LB-agar plates and incubated at 37°C for 24 h and the bacterial colonies formed were counted and reported.
5.3.6. Scanning electron microscopy imaging

The changes in the bacteria morphology of the control (buffer treated) and CuS NPs treated cells was analyzed by scanning electron microscopy (SEM) (Tescan Vega 3). For SEM sample preparation, bacteria (1 × 10^5 cfu/mL) incubated with 10 μM CuS NPs for 12 h were collected by centrifugation at 6000 rpm for 3 min and fixed with 2.5 % glutaraldehyde. After three times washing with PBS, the bacterial cells were dehydrated through sequential treatment with different percentage of ethanol for 15 min. Then, cells were stained with gold sputtering for 35 s and imaged by SEM.

5.3.7. Membrane integrity study

The membrane integrity was evaluated by acridine orange (AO)/ethidium bromide (EB) double staining test (Liu et al., 2015). Briefly, bacteria (1 × 10^5 cfu/mL) incubated with 10 μM CuS NPs for 12 h were collected by centrifugation at 6000 rpm for 3 min at 4°C and dual stained with AO/EB for 2 h in dark. After that, 10 μL of the samples was placed on a glass slide with a coverslip and imaged under fluorescence microscope (Nikon Eclipse). Green and red filter were used for AO and EB, respectively.

5.3.8. Reactive oxygen species (ROS) test

The generation of ROS was quantitatively estimated using dichlorofluorescein diacetate (DCFH₂-DA) assay (Kalyanaraman et al., 2012). Typically, bacteria (1 × 10^5 cfu/mL) incubated with different concentration of CuS NPs for 12 h were collected by centrifugation at 6000 for 15 min at 4°C and washed three times with PBS buffer. The pellet was re-suspended in 1 mL PBS buffer and incubated with 100 μM DCFH₂-DA for 30 min. After that, the cells were lysed with an alkaline solution and the supernatant was collected by centrifugation at 6000 rpm for 10 min. The fluorescence
intensity of the supernatant was measured in a fluorescence spectrophotometer using a 485 nm excitation wavelength and a 525 nm emission wavelength (JASCO spectrofluorometer FP-8200). Bacterial cells treated with 10 μM hydrogen peroxide were served as positive control and untreated cells were considered negative control. The percent changes in fluorescence are expressed by:

\[ \% = \left(\frac{\text{sample} - \text{negative control}}{\text{positive control} - \text{negative control}}\right) \times 100\] - (2)

The generation of ROS was qualitatively imaged under fluorescence microscope. First, *E. coli* (Gram-negative) and *B. subtilis* (Gram-positive) (1 × 10⁵ cfu/mL) were incubated with 10 μM CuS NPs at 37°C for 12 h. Then, the bacteria solutions were mixed with 100 μM DCFH₂-DA and incubated for 30 min in dark. After that, 10 μL of the samples was placed on a glass slide with a cover-slip and observed under a fluorescence microscopy using a green filter.

5.3.9. Lipid peroxidation assay

The production of ROS might damage the membranes and generate malondialdehyde (MDA). Thiobarbituric acid (TBA) assay was used to determine the MDA in the culture media (Garcia et al., 2005). Briefly, an aliquot of 1 mL of bacteria culture treated with CuS NPs was collected and mixed with 10 % trichloroacetic acid. Then, 0.67 % of TBA was added and incubated at 95°C for 1 h. The reaction mixture was cool down to room temperature and subjected to centrifugation at 6000 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm. Untreated cells and cells treated with 10 μM hydrogen peroxide were served as negative and positive control, respectively. The percent of MDA production was determined according to the equation 2.
5.3.10. Reduced glutathione (GSH) assay.

The excess generation of ROS may reduce the intracellular concentration of GSH. The GSH levels in the cells after treatment with CuS NPs (0-10 μM) was estimated by 5-5′-dithiobis [2-nitrobenzoic acid] (DTNB) assay (Cribb et al., 1989). Briefly, the cells were collected after NPs treatment and lysed with 10 % TCA solution for 15 min on ice. About 200 μL of cell lysate was then mixed with 1800 μL of Tris buffer (30 mM, pH 8.3) and 100 μL of 0.1% DTNB solution and incubated for 90 min in dark at room temperature. The absorbance was monitored at 412 nm. Untreated cells and cells treated with 10 μM hydrogen peroxide were served as negative and positive control, respectively. The total GSH level was estimated according to the equation 1.

5.3.11. Evaluation of toxicity.

In vivo toxicity of CuS NPs was evaluated in a zebrafish model. Liver enzyme (carboxylesterase) and brain enzyme (acetylcholinesterase, AchE) were assayed (Dharsana et al., 2015). In a typical procedure, adult zebrafish was injected with 10 μL of 10 μM and 100 μM CuS NPs and the mortality if any was followed for a period of 48 h. Fish injected with PBS was served as control. After 48 h, fish from control and NPs treated group was sacrificed and dissected carefully to collect liver and brain. Then, the tissues were immediately homogenized in 1 mL of ice-cold PBS buffer and centrifuged at 10,000 rpm, 10 min, 4°C. The obtained supernatant was used in further studies.

Acetylcholinesterase assay was performed according to Edman degradation protocol (Benabent et al., 2014). Briefly, 100 μL of brain homogenate was diluted with 900 μL of PBS buffer and mixed with 50 μL of 10 mM DTNB solution. Then,
50 μL of 12.5 mM of acetylthiocholine iodide was added and incubated at 30°C. After 5 min, the reaction mixture turned a yellow colour and the absorbance at 400 nm was measured in ELISA plate reader (Thermo Scientific Multiscan EX). The activity was expressed as acetylcholine hydrolyzed per min.

The activity of α-carboxylesterase and β-carboxylesterase was determined according to previously reported procedure (Dharsana et al., 2015). Briefly, 200 μL of liver homogenate was diluted with 800 μL of PBS buffer and mixed with 5 μL of 250 mM of α-napthylacetate or β-napthylacetate and incubated at room temperature. After 30 min, 250 μL of freshly prepared 0.3 % Fast blue B in 3.3 % SDS was added to the reaction mixture and followed the development of dark blue colour. After 30 min, the absorbance of the blue colour solution was measured at 430 nm and 588 nm for α- and β-carboxylesterase, respectively. The amount of α- and β-carboxylesterase was determined using the standard values and was reported as mM of α- and β-napthol released per min. All toxicity assays were performed in duplicate.

5.3.12. Haemocompatibility test.
Biocompatibility of CuS NPs was investigated on red blood cells (RBC). In this work, the human whole blood sample was collected by the trained doctor from the volunteers in compliance with institutional guidelines. Informed consent was obtained from the volunteer to use RBC in our experiments. Citrate stabilized blood samples were centrifuged at 6000 rpm for 3 min and washed with PBS buffer. The collected RBC was resuspended in 10 mL of PBS buffer and used in further studies. About 100 μL of RBC was mixed with 100 μM CuS NPs and incubated at 37°C, 200 rpm for 30 min. The morphological changes in RBC were evaluated by placing 10 μL of the cells on a non-grease glass slide and visualized under a microscope. In addition, the treated
RBC and controls were centrifuged at 3000 rpm and the absorbance of the supernatant was noted. RBC mixed with 100 μM NH₄Cl and PBS served as positive and negative control, respectively. All samples are prepared in triplicate at one time.

5.4. Results and discussion

5.4.1. Synthesis of copper sulfide nanoparticles

The copper sulfide nanoparticles were prepared from N-lauryltyramine capped copper nanoparticles (NLTA-CuNPs). The freshly prepared NLTA-CuNPs on reaction with sodium sulfide produces a colour change from brown to olive-green, indicating the formation of CuS NPs (Fig. 5.1). The quantitative chemical composition of copper and sulphur in the preparation was estimated by energy dispersive X-ray spectroscopy (EDX). The EDX analysis was carried out only for Cu and S, the average atomic percentage ratio of Cu:S was 42:58, showing that the CuS NPs was in a good stoichiometric ratio (Fig. 5B). The size and morphology of the CuS NPs were measured by TEM analysis, which clearly revealed that the NPs are spherical in shape with the size range from 10 to 25 nm (Fig. 5.2A). High-resolution TEM showed a metallic fringe pattern (Fig. 5.2B). The selected area electron diffraction (SAED) pattern showed the ring patterns, suggesting the crystalline nature of NPs (Fig. 5.2C).
Fig. 5.1. (A) UV-visible spectra of (a) NLTA-CuNPs and (b) NLTA-CuS NPs. Inset corresponds to the photographs of (a) CuNPs appears brown colour and (b) CuS NPs appears olive green colour. The disappearance of CuNPs SPR maximum at 580 nm and the appearance of peak above 1000 nm indicate the formation of CuS NPs, which was supported by EDAX analysis (B).

Fig. 5.2: (A) TEM micrograph, (B) High resolution TEM image, and (C) SAED pattern confirms the crystalline nature of CuS NPs. (D) FTIR spectra of NLTA (thin line) and NLTA-capped CuS NPs (thick line).
The size of the NPs was further verified by particle size analyzer (PSA) and observed that the size obtained from PSA (70.6 nm) differed to that of TEM. The difference in the size can be attributed to the presence of N-lauryltyramine capping agent on the NPs surface because TEM measures the size of the individual NPs, whereas PSA takes into account the hydrodynamic ligand shell also. To validate the claim, FTIR spectra of NLTA and CuS NPs were measured (Fig. 5.2D). The IR spectra of NLTA showed a sharp band at 3305 cm\(^{-1}\) for –NH and –OH stretching vibrations of NLTA. The peak at 2920 and 2852 cm\(^{-1}\) is attributed to –CH symmetric and asymmetric stretching, respectively. The characteristic peaks of amide linkage were observed at 1638 cm\(^{-1}\) (amide I) and 1548 cm\(^{-1}\) (amide II). Methylene bending and rocking bands were observed near 1463 cm\(^{-1}\) and 715 cm\(^{-1}\). The IR spectra of CuS NPs showed peaks at 3470, 2918, 2851, 1637, 1548, 1466 and 720 cm\(^{-1}\), which corresponds to the asymmetric and symmetric stretching vibration of NLTA molecules. The broadening and shifting in –OH/–NH stretching vibrations (3470 cm\(^{-1}\)) were recorded in the CuS NPs, suggesting that the NLTA molecules were successfully coated on CuS NPs, leading to the good solubility and provide sufficient stability to the NPs. Zeta potential estimates for NLTA stabilized CuS NPs were -25.3 mV, suggesting that the NPs boundaries are well-separated to prevent agglomeration.

5.4.2. Preparation of jacalin functionalized CuS NPs

For preparing jacalin functionalized CuS NPs, first the interaction between jacalin and CuS NPs was evaluated by fluorescence spectroscopy. Fig. 5.3 shows the fluorescence spectra of jacalin in the absence as well as in the
presence of different concentration of CuS NPs. Addition of the CuS NPs neither changed the shape nor the maximum of jacalin fluorescence emission spectra, indicating that under the given experimental conditions no conformational changes are involved in drug binding to jacalin. However, the fluorescence emission intensity at 330 nm gradually decreased after addition of successive aliquots of CuS NPs, indicating the interaction between jacalin and CuS NPs (Fig. 5.3).

**Fig. 5.3:** Interaction of jacalin with Tyr- CuS NPs. Jacalin fluorescence emission spectra monitored after addition of increasing concentrations of Tyr-CuS NPs (0 – 50 µM). The upper spectrum in each panel corresponds to free jacalin and the remaining spectra with decreasing fluorescence emission were recorded in the presence of increasing concentrations of Tyr-CuS NPs.
In order obtain the binding constant ($K_a$), the fluorescence quenching data were analyzed as described in chapter 2. The $K_a$ characterizing for the interaction of CuS NPs to jacalin was obtained as $2.65 \times 10^5 \text{ M}^{-1}$ at 25°C. The obtained binding constant are comparable to those observed generally for lectin-mono saccharide complexes as well as those obtained for the interaction of jacalin with ruthenium complex, AgNPs and shikonin derivatives. These results suggest that jacalin form stable complex with CuS NPs, which can be explored in further application.

5.4.3. Antibacterial activity of CuS NPs

*In vitro* antibacterial susceptibility of CuS NPs was evaluated against two Gram-negative bacteria (*E. coli* and *A. hydrophila*) and two Gram-positive bacteria (*B. subtilis* and *S. aureus*). Resazurin microplate assay (REMA) was adopted for determining the minimum inhibitory concentration (MIC), the lowest concentration of the antibacterial agent to prevent the growth of a microorganism. Live bacterial cells irreversibly reduce resazurin to resorufin and display pink colour with strong fluorescence at 580 nm, whereas dead cells show blue colour with weak fluorescence. Results shown in Fig. 5.4A reveal that the bacterial cells exposed upto 12.5 µM of CuS NPs showed weak fluorescence and thereafter increases, indicating the existence of viable cells and conclude that the MIC is 12.5 µM (Fig. 5.4B). The MIC obtained from REMA assay for JCuS NPs is 6.25 µM. MIC determined by conventional turbidometry is consistent with REMA (Fig. 5.4C). The antibacterial activity of CuS NPs was also tested by the conventional zone of inhibition (ZOI) method. ZOI measures the killing efficiency of the NPs against the tested bacteria.
Results demonstrated that 10 µM of CuS NPs is required to induce ZOI, whereas 5 µM of JCuS NPs is sufficient to kill the bacteria, however, killing efficiency increased with the high concentration of CuS NPs (Fig. 5.5). In addition to ZOI, bacterial colonies formed at various concentrations of CuS NPs were also evaluated. As noted from Fig. 5.6, the number of bacterial colonies is lesser in plate containing JCuS NPs as compared to CuS NPs, indicating that the jacalin functionalization improves the antibacterial efficiency of the CuS NPs at lower concentration.

Fig. 5.4: Determination of MIC. (A) Digital photograph of REMA assay, (B) Representative REMA assay. Antibacterial activity of CuS NPs against pathogenic bacteria, E.coli. (C) Turbidometry assay. In all tested bacterium, optical density was observed below 12.5 µM and 6.25 µM for CuS NPs (solid circle) and JCuS NPs (open circle), respectively, indicating MIC.
**Fig. 5.5:** ZOI of (A) CuS NPs and (B) JCuS NPs. It is clear that 5 μM of JCuS NPs is sufficient to induce of ZOI. A representative bacterium is *A. hydrophila*.

**Fig. 5.6:** (A) Bacterial colonies formed on LB agar plates contain CuS NPs at defined concentration (B) Number of viable bacteria remaining in the 24 h incubation samples. Data were counted from the cfu results in (A). A representative bacterium is *A. hydrophila*.

5.4.4. *Antibacterial mechanism of CuS NPs*: To understand the antibacterial mechanism of CuS NPs, the morphology changes of bacteria was elucidated by
scanning electron microscopy. About 0.1 OD$_{660}$ of *E. coli* were incubated with 10 µM of CuS NPs for 12 h and imaged in SEM. The cell walls of the bacteria treated with CuS NPs are wrinkled and damaged, while the untreated cells are smooth, rod-shaped with intact cell wall (Fig. 5.7A and Fig. 5.7B). In addition, the integrity of bacteria membrane was also examined by dual acridine orange/ethidium bromide staining (AO/EB). AO is a membrane permeable dye stains live cells and display green fluorescence, whereas EB can only pass through the damaged membrane of dead cells and stain the nucleic acid to display red fluorescence. After incubation with 10 µM of CuS NPs for 12 h, the cells were stained with AO/EB mixture and examined by fluorescence microscopy. As shown in Fig. 5.7, the untreated cells rarely showed red fluorescence, but display green fluorescent, indicating that the cells are alive. In contrast, all the cells treated with NPs were stained by EB, as evident from red fluorescent, confirming cell death and the membrane are damaged (Fig. 5.7D).

**Fig. 5.7:** Scanning electron micrograph of *E. coli*, (A) untreated and (B) treated with 10 µM CuS NPs. AO/EB staining of *E. coli*, (C) untreated and (D) treated with 10 µM CuS NPs.
Oxidative stress is one of the proposed antibacterial mechanisms of metal nanoparticles (Kim et al., 2007; Chernousova et al., 2013; Carlson et al., 2008). The involvement of oxidative stress in the antibacterial activity of CuS NPs was examined through the measurement of intracellular reactive oxygen species (ROS) production by DCFH$_2$-DA assay. In presence of ROS, the non-fluorescent DCFH$_2$-FDA is oxidized and switches to green fluorescent dichlorofluorescein (DCF). For this study, H$_2$O$_2$ was chosen as a positive control and the untreated cells serve as negative control. As shown in Fig. 5.8A and Fig. 5.8B, weak ROS was generated in control cells, but CuS NPs and H$_2$O$_2$ triggers more production of ROS as evidence from strong green fluorescent. Fig. 5.9 further showed the concentration-dependent increase in the ROS production, suggesting the ROS-mediated cell death. At the same time, in comparison to positive control (100 %), ROS production level in Gram-negative and Gram-positive varied from 61 to 65 % when treated with 10 µM CuS NPs. This implied that, despite ROS production, membrane damage also responsible for killing the bacteria.

Fig. 5.8: Fluorescence microscopic images of (A) E. coli and (B) B. subtilis before and after treatment with CuS NPs and followed by staining with DCFH$_2$-DA.
Fig. 5.9: Effect of CuS NPs on the formation of ROS in Gram negative and Gram positive bacteria. Dichlorofluorescein diacetate (DCFH$_2$-DA) was used as fluorescence probe for detecting ROS. Hydrogen peroxide (PC) and untreated cells (NC) was used as positive and negative control, respectively.

The ROS production was further verified by measuring the production of malondialdehyde (MDA). The unsaturated fatty acids of cell membranes are highly vulnerable to ROS attack (Paradies et al., 2002). The excess production of ROS will react with cell membranes and produce lipid peroxide radical, which on rearrangements to form MDA (Cheng et al., 2011). The amount of MDA formed in the cells treated with CuS NPs was estimated using thiobarbituric acid assay (Wang et al., 2009). Fig. 5.10A showed the concentration-dependent increase in the MDA production in the NPs treated cells, supporting the generation of ROS. The intracellular ROS production was further investigated by measuring reduced glutathione concentration (GSH). GSH, a non-protein tripeptide maintains the intracellular redox environment and protects the cells from oxidative damage by scavenging ROS (Masip et al., 2006). Thus, the maintenance of GSH oxidative
defense is vital for cell survival. However, the generation of excess ROS oxidizes the GSH to disulphide (Vecitis et al., 2010). The amount of GSH present in the cells treated with CuS NPs was determined by (5,5-dithio- bis-(2-nitrobenzoic acid) assay (Cribb et al., 1989). It was found that GSH concentration depleted in the treated cells with subsequent increase in CuS NPs concentration (Fig. 5.10B). These results suggest that the interaction of CuS NPs with Gram-negative and Gram-positive bacteria triggered the intracellular ROS-mediated oxidative damage over the antioxidant defense and damage the cell membrane leading to cell death. A similar result was reported in *E. coli* and *P. aeruginosa*, when it was treated with biosynthesized silver nanoparticles (Ramalingam et al., 2016).

**Fig. 5.10:** Effect of CuS NPs (A) on MDA level and (B) intracellular GSH level. Excess ROS damages GSH level and oxidize the cell membrane and produce MDA. NC and PC are negative (untreated) and positive control (treated with 10 μM H2O2), respectively. Experiments are performed in triplicate.
5.4.5. *In vivo antibacterial activity.*

Having observed promising antibacterial activity against Gram-positive and Gram-negative microorganism, next the therapeutic efficacy of CuS NPs was investigated in an animal model. To this end, zebrafish was infected intramuscularly with an optimized concentration of microbes and initiated treatment with CuS NPs (see materials and methods). Two treatment methods were adopted, i) intramuscular injection treatment and ii) medicated bath treatment.

![Fig. 5.11: Injection Method. Bacterial colonies formed on LB-agar plate. Muscle tissues of the fish, (A) infected with *E. coli* and (B) treated with injection of CuS NPs. (C) Viable cells collected from the infected fish. NA indicates that the fish are dead at that time point. (D) Viable cells remained in the muscle after treatment with CuS NPs. Muscle tissues obtained by sacrificing fish at definite time point was homogenized, diluted 10^{-4} times and plated (50 μL) on LB-agar plate.](image)
For injection method, fish are divided into two groups and each group contains five healthy fish. Group A and Group B were infected intramuscularly with 10 μL of microbes (0.1 OD<sub>660</sub>) and allowed to live in the normal tap water. After three hours of infection, only Group B fish were injected with 10 μL of CuS NPs (10 μM). Group A fish infected with microbes slow down in 6 h and finally succumbed to infection in 8-10 h. Whereas Group B fish treated with CuS NPs remain alive and all its behaviour was like that of an uninfected fish. To understand how CuS NPs rescue the fish from infection, the fishes were sacrificed from both groups at regular time interval and collected the muscle tissues through dissection. The dissected tissues were homogenized and diluted appropriately in PBS buffer and plated on an agar plate. The plates are incubated for 24 h at 37°C and estimated the number of bacterial colonies present in each group. Representative plate corresponds to *E. coli* infection and treatment with CuS NPs was shown in Fig. 5.11A and Fig. 5.11B, respectively. As noted from Fig. 5.11, both Gram-negative and Gram-positive strain without NPs treatment exhibits typically a high number of bacterial colonies, while Group B showed that bacterial colonies were depleted considerably in 24 h. This indicates that CuS NPs kills the microbes and save the zebrafish from infection.

Although injection method is good, it is also painful and required a skillfull technician to administrate the drug. Moreover, it is difficult to identify individual infected fish from a big pool. Thus, it is imperative to develop an antibacterial agent to treat the infected fish in the whole pool without affecting the uninfected fish.
**Fig. 5.12**: Bacterial colony count assay for medicated bath treatment. Muscle tissues of the fish, infected with bacteria and treated with medicated bath of 10 μM CuS NPs was obtained by sacrificing fish at definite time point and then homogenized, diluted 10^{-4} times and plated (50 μL) on LB-agar plate. After 24 h, fishes treated with CuS NPs are nearly free from bacterial colonies, as a result fishes survived.

To meet the demand, we initiated the antibacterial study with water contains CuS NPs (called as medicated bath or medibath). Fish are divided into two groups and infected intramuscularly with 10 μL of microbes \((0.1 \text{ OD}_{660})\). Group A fish were
allowed to live in normal tap water and Group B fish were allowed to live in tap water containing 10 μM of CuS NPs. Group A fish died in 8-10 h due to the severity of infection. Interestingly, Group B fish survive the infection and started to live like uninfected fish. In order to understand the antibacterial activity of CuS NPs, the muscle tissues of the fish were dissected and analyzed for the bacterial colonies. As noted from Fig. 5.12, the medicated bath of CuS NPs halt the proliferation of Gram-negative and Gram-positive strains and rescues the zebrafish.

5.4.6. Role of jacalin in improving the antibacterial efficacy of CuS NPs:
The significance of jacalin in enhancing the antibacterial activity of CuS NPs was evaluated using A. hydrophila infected zebrafish model. The fishes were divided into three groups and each group consisted of five fishes. The antibacterial studies were initiated by infecting the fishes with A. hydrophila (10 μL of 0.1 OD₆₆₀). Treatment was started by transferring the infected fish to a water bath containing 5 μM of CuS NPs (Group B) and 5 μM of JCuS NPs (Group C), respectively and monitored carefully. As a control, fishes were infected only with bacteria (Group A). Fishes infected with A. hydrophila succumbed to infection in 8 h, similarly, infected fish treated with 5 μM of CuS NPs (Group B) was also died in 8 h. 5 μM of CuS NPs is inadequate to save the fish because the MIC of CuS NPs is 12.5 μM, however, fish treated with 10 μM CuS NPs recover from the infection. While the fishes infected with bacteria and treated with 5 μM of JCuS NPs completely recover from the infection and live like a normal fish for more than 2 months. The dead fish from Group A and Group B were collected immediately for estimating the number of bacterial colonies. Group C fish was collected after 18 h and sacrificed. The muscle
tissues were dissected and plate on LB agar plate and cultured for 12 h at 37°C. As noted from Fig. 5.13, the number of bacterial colonies decreased significantly from $6.54 \times 10^7$ to $0.1 \times 10^7$ cfu/mL when *A. hydrophila* infected fish were treated with JCuS NPs, suggesting that the JCuS NPs kills the bacteria and rescue the fish from infection. Whereas, the presence of more bacteria ($6.25 \times 10^7$) after the treatment with CuS NPs may be the reason for the observed mortality (Fig. 5.13). These results suggest the jacobian functionalization increases the therapeutic potential of CuS NPs at lower dose, which could be very important to nullify the development of drug resistance caused by the overuse of any drug.

![Fig. 5.13:](image)

*Fig. 5.13:* Zebra fish infected with *A. hydrophila* was treated with medicated bath of (A) infected control-no treatment, (B) water contains 5 μM CuS NPs and (C) water contains 5 μM of JCuS NPs. Fishes from (A) and (B) died in 8 h, the muscle tissue were collected, homoginzed and plate on LB agar plate. Fish from (C) sacrificed after 18 h and plated on LB agar plate. It is clear that 5 μM JCuS NPs stops the bacterial proliferation in zebrafish and rescue completely.
5.4.7. Evaluation of CuS NPs toxicity

The above studies demonstrated that the NLTA-CuS NPs has good therapeutic potential as an antibacterial agent. However, to meet the clinical standard, NPs should be less or non-toxic. To this end, the toxicology parameters was evaluated in zebrafish through assaying liver carboxylesterases (CES) and brain acetylcholinesterase (AchE) activity.

![Graphs showing liver carboxylesterase activity in zebrafish.](A) α-Carboxylesterase and (B) β-carboxylesterase. Black bar denotes CuS NPs, grey bar denotes CuNPs.](image)

Liver is an important organ for xenobiotic detoxification. Carboxylesterases, an enzyme activated during xenobiotic stress and involved in the hydrolysis of carboxylic ester (Christoph et al., 1974). AchE catalyzes the breakdown of acetylcholine and other choline ester to facilitate function as a neurotransmitter, therefore, it can serve as a marker for neuronal toxicity (Akassoglou et al., 2004). Strikingly, the treatment of zebrafish with CuS NPs at 100 µM (8 times the MIC) concentration had a negligible change in the liver
carboxylesterases and brain acetylcholinesterase activity (Fig. 5.14). Noteworthy, the fish exposed to 10 μM copper nanoparticles died in 2 h, and there is a considerable reduction in the CES and AchE activity (Fig. 5.14). This result clearly suggests that CuNPs is highly toxic, whereas CuS NPs is non-toxic.

Fig. 5.15: Microscopy image of human RBC (A) untreated-negative control, (B) treated with 100 μM CuS NPs and (C) treated with 100 μM NH₄Cl-positive control. Only (C) showed wrinkled RBC. Inset corresponds to the photographs of hemolytic study. Haemoglobin was only released in the cells treated with NH₄Cl; confirm the biocompatibilities of CuS NPs. Experiments are performed in triplicate.
Further, the haemocompatibility of the CuS NPs was studied through exposing the NPs against human red blood cells (RBC). As shown in Fig. 5.15, CuS NPs (100 μM) did not induce any adverse change in RBC morphology (Fig. 5.15B) and the membrane was intact as that of the control cells (Fig. 5.15A). However, RBC treated with NH₄Cl was wrinkled (Fig. 5.15C) and undergoes lysis and released the haemoglobin, indicating that the membranes are damaged (inset in Fig. 5.15C). At the same time, no haemoglobin was released from the cells treated with CuS NPs (Fig. 5.15B), demonstrating the intrinsic biocompatibility with human RBC. These results suggest that the NLTA-CuS NPs is quite safe with excellent antibacterial activity against Gram-positive and Gram-negative bacteria.

**Fig. 5.16:** Viable bacterial colonies present in water (A) untreated and (B) treated with 10 μM CuS NPs. (C) Bacterial colonies present at the mentioned time point.
Diluted water (10⁻⁴ times) was used for LB-agar plating. Experiments are performed in triplicate.

5.4.8. CuS NPs as water disinfectant.

Having demonstrated the therapeutic efficacy of CuS NPs, it is surmised that CuS NPs can also work as a general disinfectant. To test this hypothesis, two groups of fish were employed. *A. hydrophila* was chosen for this study because it is a fish specific pathogen. Group A fish were exposed to water containing 0.1 OD₆₆₀ of *A. hydrophila*. On contrary to the intramuscular infection, Group A fish succumbed to infection in 33 h, which suggests that the inherent defence mechanism present in the healthy fish delayed the waterborne bacterial infection. Group B fish exposed to water contains 0.1 OD₆₆₀ *A. hydrophila* and 10 μM CuS NPs were survived by the infection.

At the regular interval, water from both the group was collected and plated on LB-agar plate for counting the bacterial colonies. Strikingly, the water collected from Group A showed high number of bacterial colonies, which keep proliferating as evidence from the formation of uncountable bacterial mate on LB-agar plate after 6 h (Fig. 5.16A). Whereas the water containing CuS NPs showed a time-dependent decrement in the bacterial colonies, indicating that CuS NPs kills the bacteria and prevent their proliferation (Fig. 5.16B and Fig. 5.16C). Unlike intramuscular infection, the fish exposed to water containing *E. coli, B. subtilis* and *S. aureus* are not infected. This can be attributed to the non-specific nature of the pathogen. However, the addition of CuS NPs kills the bacteria present in the water, as evidenced from the bacterial colony count assay (Fig. 5.16C). These results suggest that CuS NPs may serve as water disinfectant and prevent us from waterborne diseases.