Synthesis of copper sulfide nanoparticles and evaluation of *in vitro* antibacterial activity and *in vivo* therapeutic effect in bacteria infected zebrafish

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Supporting Information

Zebrafish study

Adult zebrafish (*Danio rerio*) irrespective of sex, measuring 4 to 5 cm in length, weighing approx. 300 mg, were purchased from a local aquarium and used in the following experiments. They were maintained in aerated glass tanks containing tap water at $25 \pm 2^{\circ}C$. Fish were fed ad libitum with commercial fish diet and the tanks were cleaned, sterilized and water replaced periodically. Water quality was monitored regularly and water from the same source after filtering was always allowed to stand for 24 h prior to use. The fish were allowed to acclimatize for a week before initiation of nanoparticle exposure. Water parameters were: pH - 7.45; Total Hardness (as $CaCO_3$) – 160 ± 4.6 mg/L; chlorides - 72 ± 3 mg/L; Total dissolved solid - 284 ± 5 mg/L. Water temperature was $25 \pm 2^{\circ}C$. All fish experiments were conducted as per institutional protocol.

The optimal bacterial dosage for the study was determined by intramuscular infection of zebrafish with *A. hydrophila*. 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 $10 \mu l$ of bacterial suspension ($OD_{660nm} – 0.01, 0.05, 0.1, 0.3,$ and 0.5) 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injected, and the needle was held in place for a few seconds to make sure the material stayed in before being slowly withdrawn.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 PBS buffer. Five fish were used for this study and the mortality was followed for 24 h. The fishes exposed to 0.3 and 0.5 OD_{660nm} died in in less than 8 h, whereas the fish injected with 0.1 OD_{660nm} culture could survive for $8 – 10$ h and then succumbed to bacterial infection. In the case of 0.05 and 0.01 OD_{660nm}, the survival of fish was better but the fish were unable to survive beyond 24 h. Based on the mortality study, 0.1 OD cells were used in this work.
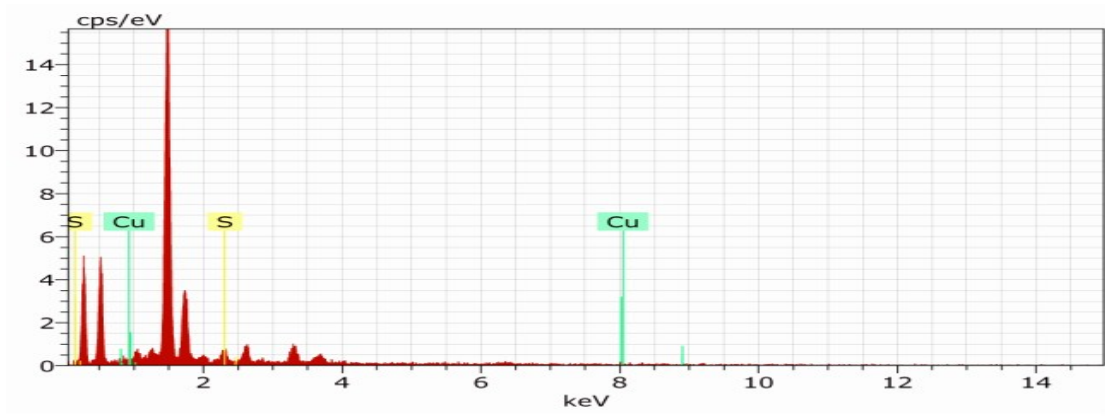
Fig. S1: EDAX spectrum of NLTA-CuS NPs. The presence of elemental Cu and S in the preparation confirms the formation of CuS NPs.

Results

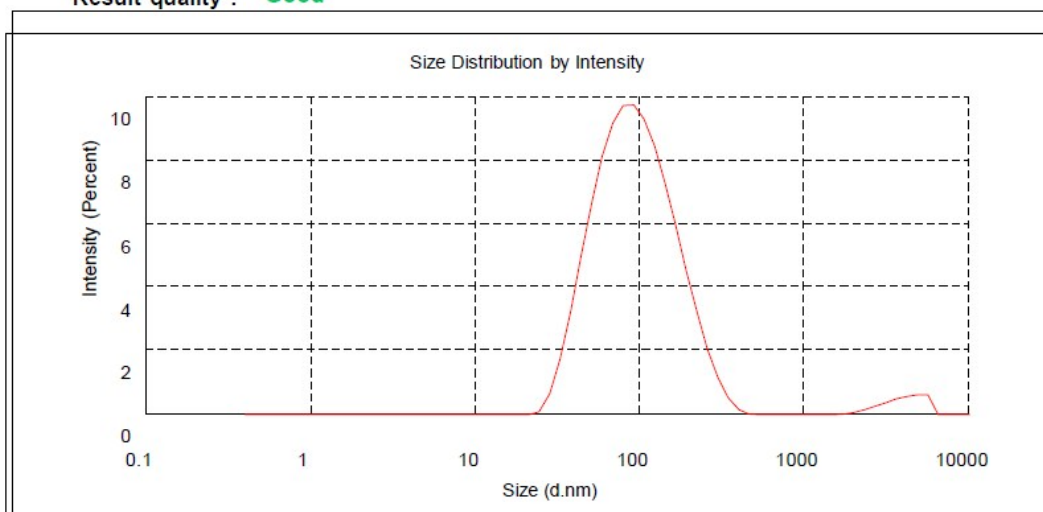|		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 70.63	Peak 1:	105.8	94.8	61.02
PdI: 0.340	Peak 2:	414.2	3.2	1000
Intercept: 0.816	Peak 3:	0.000	0.0	0.000
Result quality : Good				

Size Distribution by Intensity

Fig. S2: Particle size analysis of CuS NPs. PSA was measured in Malvern Zetasizer version 6.20. The sample was analyzed in quartz cuvettes, and the refractive index and the viscosity values used were that of pure water. Experiments were performed in triplicate. For each measurement data was acquired for 40 s at wavelength = 633 nm. Temperature = 25°C.

Results

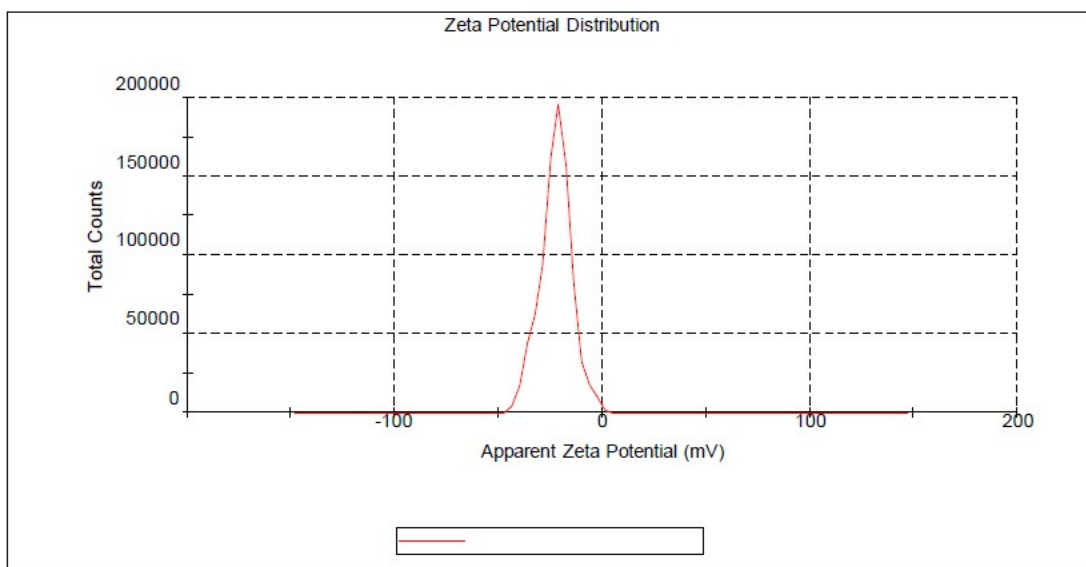|			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-25.3	Peak 1: -25.3		100.0	7.27
Zeta Deviation (mV):	7.27	Peak 2: 0.00		0.0	0.00
Conductivity (mS/cm):	0.700	Peak 3: 0.00		0.0	0.00
Result quality :	Good				

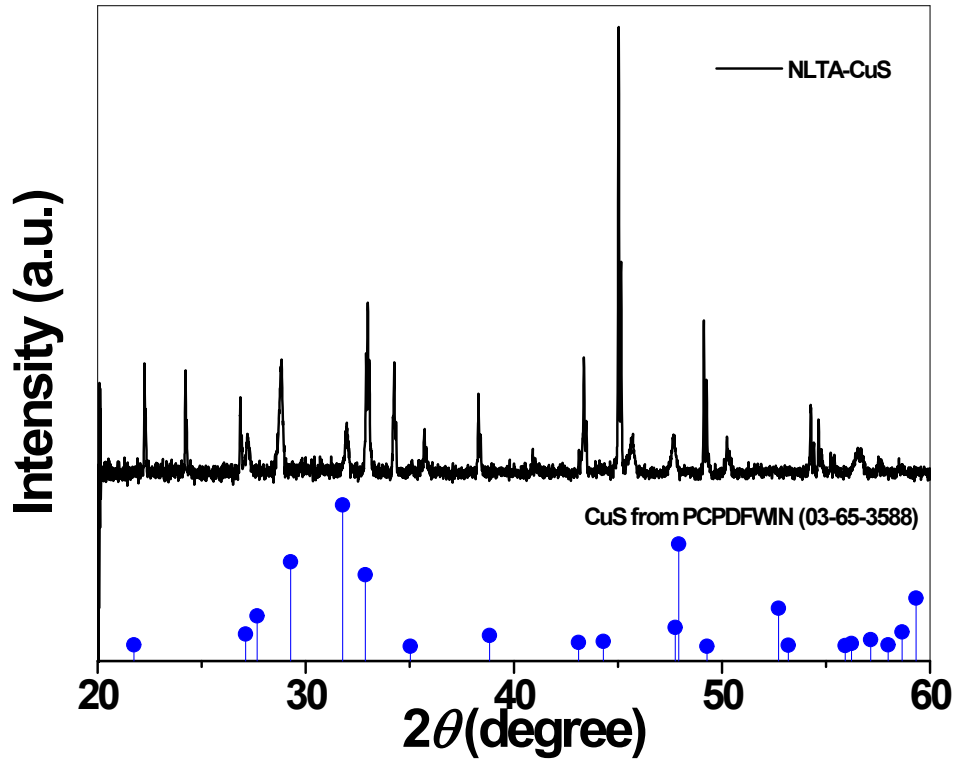Fig. S3: Zeta potential analysis of CuS NPs

Fig. S4: X-ray diffraction of NLTA capped copper sulfide nanoparticles. The appearance of sharp diffraction patterns confirms the NPs are crystalline nature. The diffraction peaks are compared with reference library using PCPDFWIN software, which validate the presence of CuS. Some mismatching peaks are observed in the diffraction pattern, which might be due to the coating of NLTA on CuS NPs.
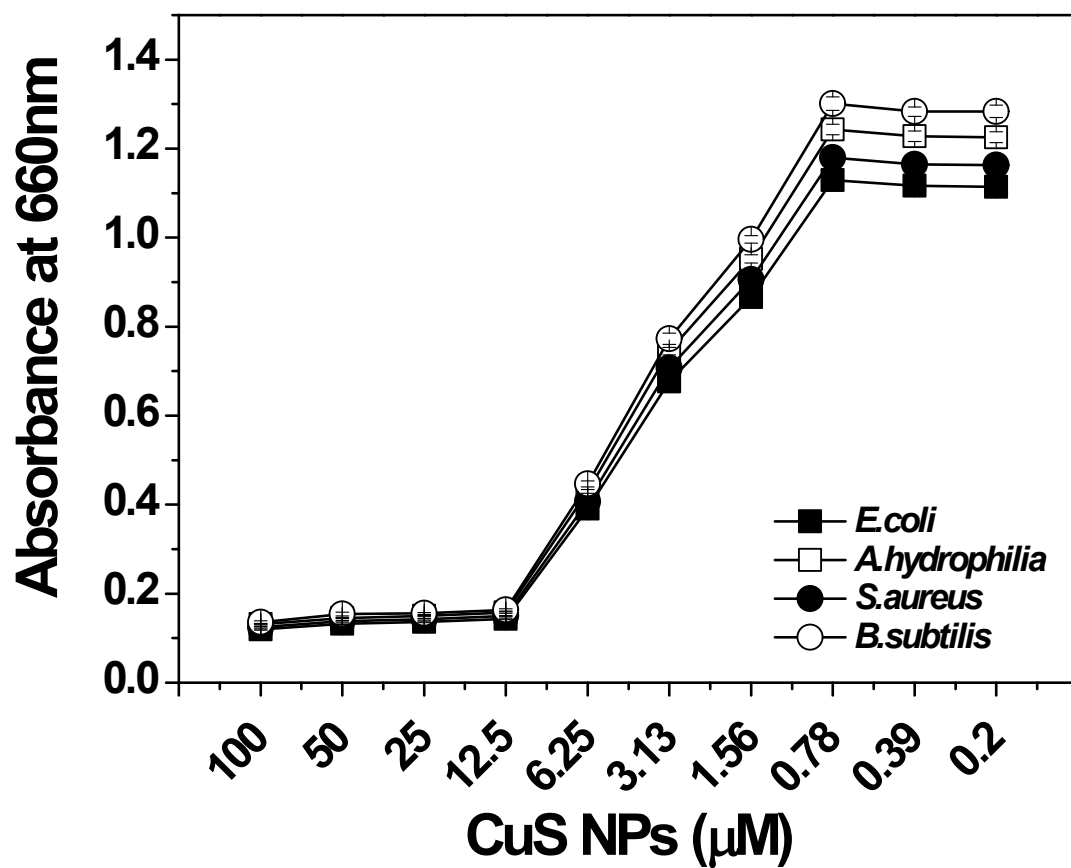Ref: H.T. Evans and J.A. Konnet, Am. Mineral, 61, 996 (1976).

Fig. S5: Turbiditimetry assay. The optical density was measured at 660 nm. In all tested bacterium, optical density was observed below 12.5 μM, indicating the minimum inhibitory concentration.

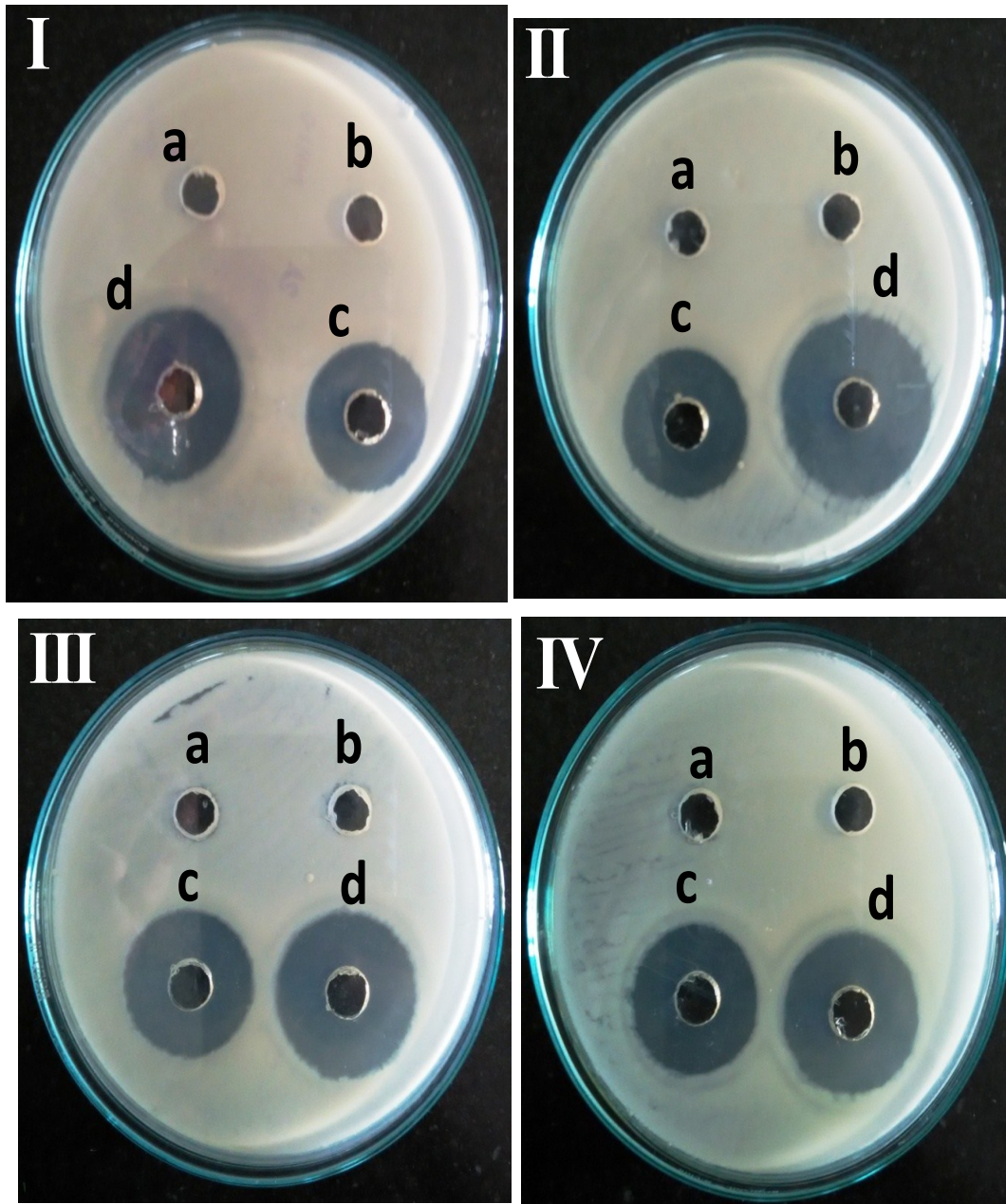Fig. S6: Zone of inhibition. As noted, NLTA (a) does not show ZOI, similarly 5 µM of CuS NPs also not shown ZOI (b), whereas 10 µM (c) and 100 µM (d) of CuS NPs showed ZOI. I). *E.coli*, II). *A. hydrophila*, III). *B. subtilis* and IV). *S. auerus*.
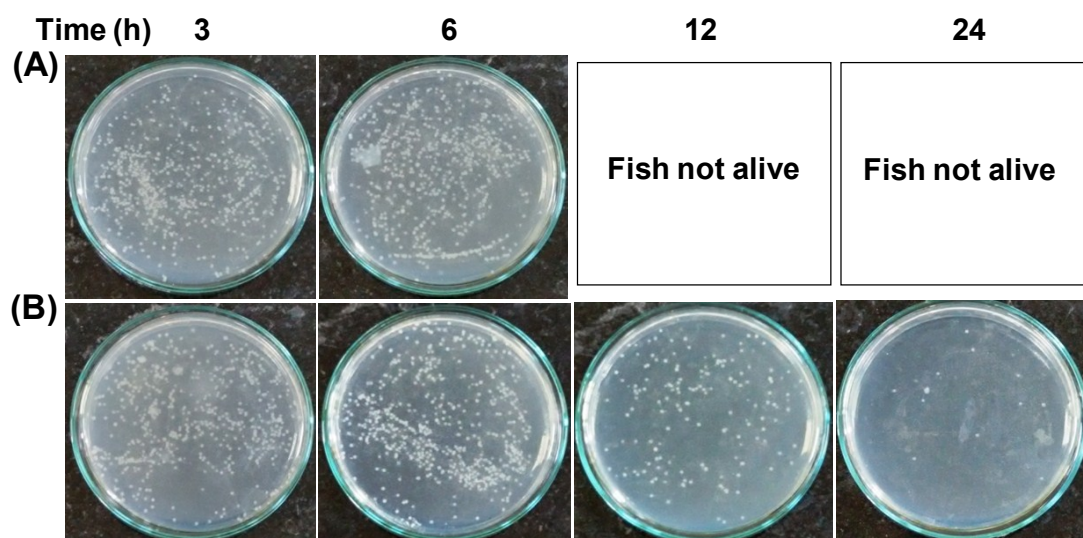
Fig. S7: Injection Method. Bacterial colonies are formed on LB-agar plate. Muscle tissues of the fish, (A) infected with *E. coli* and (B) treated with injection of CuS NPs. Muscle tissues obtained by sacrificing fish at definite time point was homogenized, diluted 10^{-4} times and plated (50 μL) on LB-agar plate. After 24 h, fish treated with CuS NPs are nearly free from bacterial colonies, as a result fish survived.

Time (h)		3	6	12	24
E. coli	untreated			Fish not alive	Fish not alive
	treated				
A. hydrophilia	untreated			Fish not alive	Fish not alive
	treated				
S. aureus	untreated			Fish not alive	Fish not alive
	treated				
B. subtilis	untreated			Fish not alive	Fish not alive
	treated				

Fig. S8: Bacterial colony count assay. Muscle tissues of the fish, infected with bacteria and treated with injection of 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. After 24 h, fishes treated with CuS NPs are nearly free from bacterial colonies, as a result fishes survived.
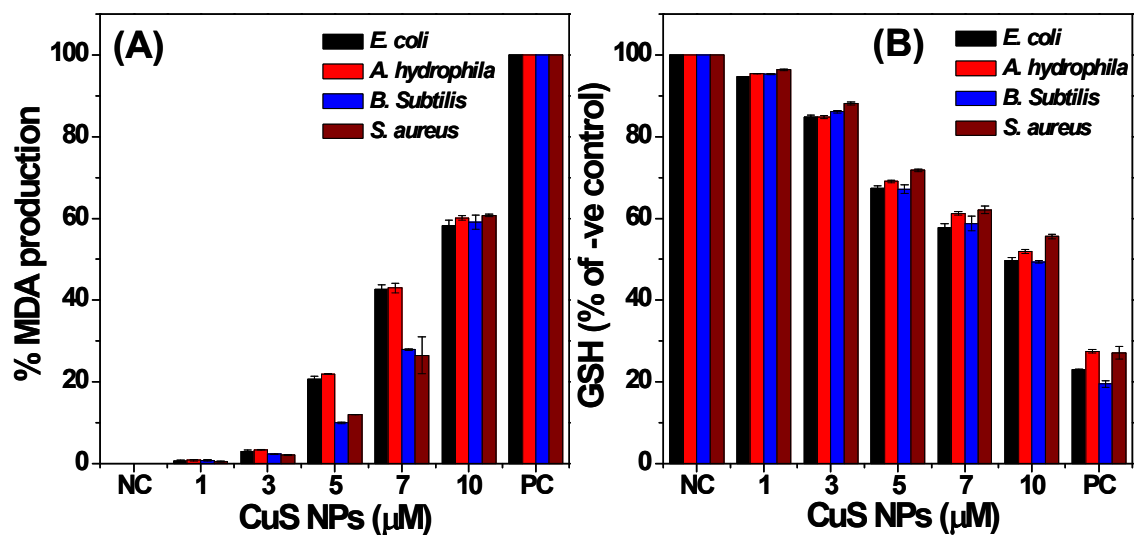
Fig. S9: Effect of CuS NPs (A) on MDA level and (B) intracellular GSH level. Excess ROS damages GSH level and oxidize the cell membrane and produce MDA. NC and PC are negative (untreated) and positive control (treated with 10 μM H_2O_2), respectively. Experiments are performed in triplicate.
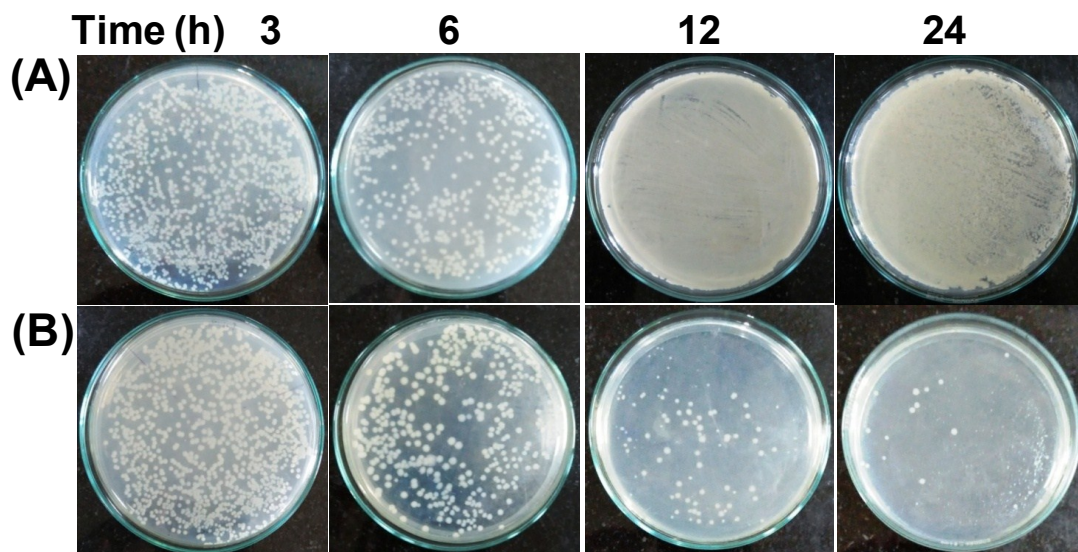
Fig. S10: Viable bacterial colonies present in water (A) untreated and (B) treated with 10 µM CuS NPs. Diluted water (10^{-4} times) was used for LB-agar plating.

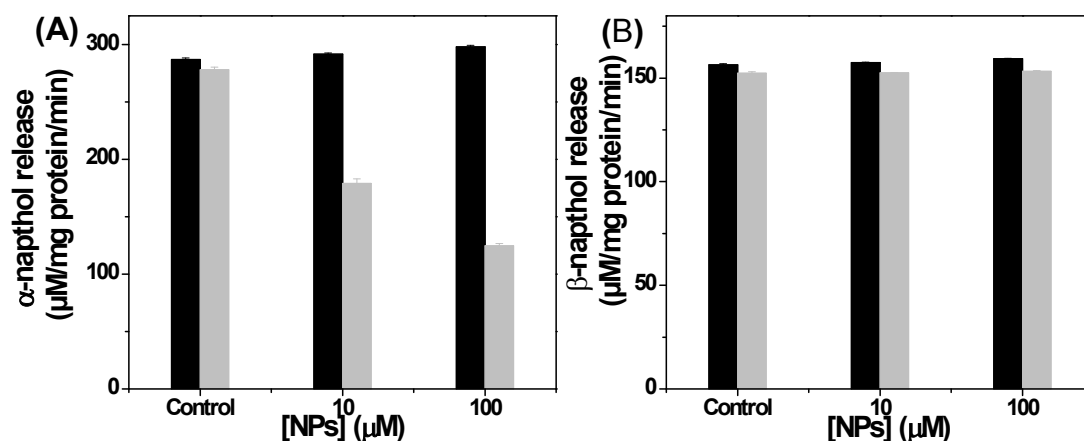Fig. S11: Liver carboxylesterase activity in zebrafish. (A) α-Carboxylesterase and (B) β-carboxylesterase. For toxicity assessment, fish were intramuscularly injected with 10 µL CuS NPs/CuNPs. Fish exposed to CuNPs died in 120 min, therefore fish were scarified in 90 min and liver were dissected and tested for carboxylesterase activity. Fish exposed to CuS NPs was living like control, therefore fish scarified after 48 h and liver were dissected and tested for carboxylesterase activity. Fish exposed with 10 µL of PBS buffer severed as a control. Values are expressed as mean±SD of 5 determinations using liver pooled from two fish of the same treatment group. Black bar – NLTA-CuS NPs, Gray bar – NLTA-CuNPs.

As noted from Fig. S11, α-Carboxylesterase activity was significantly reduced when exposed to CuNPs, indicating the damage of liver. Whereas CuS NPs does not influence the activity of α-Carboxylesterase and β-carboxylesterase, indicating that sulfidation of Cu NPs prevents xenobiotic stress.
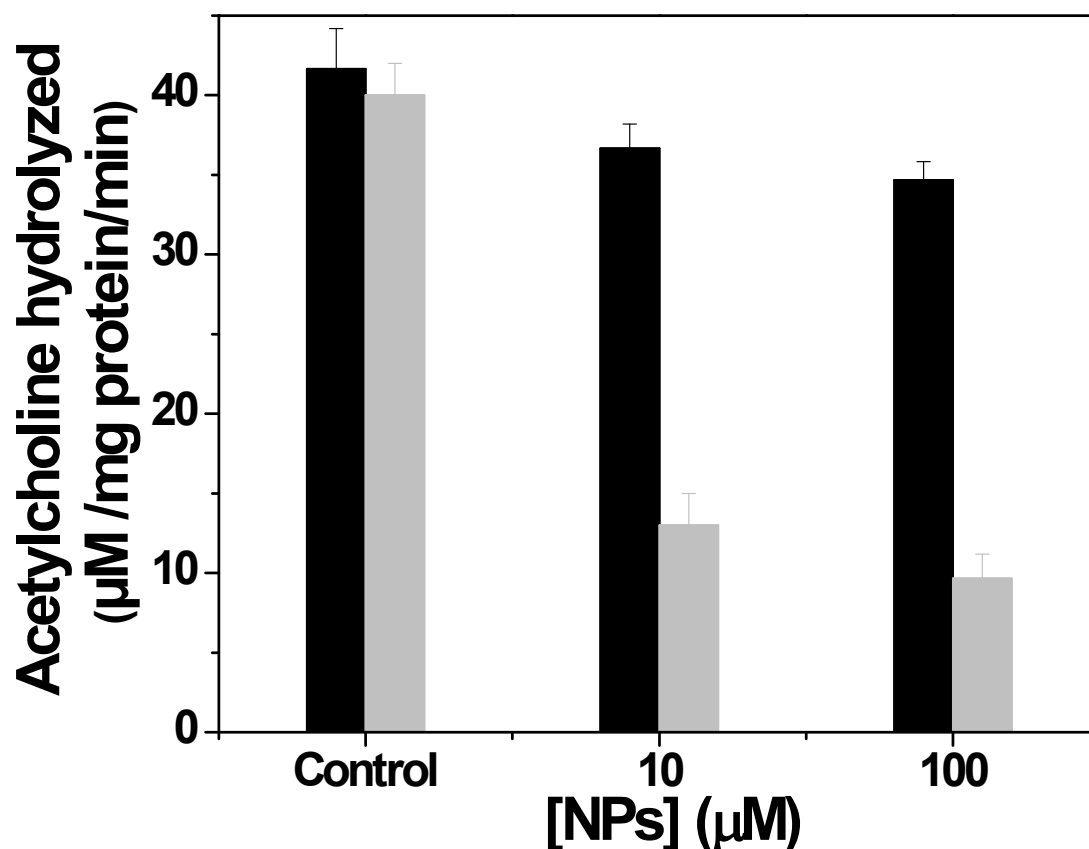
Fig. S12: Brain acetylcholinesterase activity in zebrafish. For toxicity assessment, fish were intramuscularly injected with 10 µL CuS NPs/CuNPs. Fish exposed to CuNPs died in 120 min, therefore fish were scarified in 90 min and liver were dissected and tested for carboxylesterase activity. Fish exposed to CuS NPs was living like control, therefore fish scarified after 48 h and brain were dissected and tested for acetylcholinesterase activity. Fish exposed with 10 µL of PBS buffer severed as a control. Values are expressed as mean±SD of 5 determinations using liver pooled from two fish of the same treatment group. Black bar – NLTA-CuS NPs, Gray bar – NLTA-CuNPs.

As noted from Fig. S12, acetylcholinesterase activity in zebrafish activity was significantly reduced when exposed to CuNPs, suggest that the CuNPs affects brain function. Whereas CuS NPs does not influence the activity of acetylcholinesterase, indicating that CuS NPs is safer and does not have neuronal toxicity at the concentration (8 times) used in the antibacterial study.
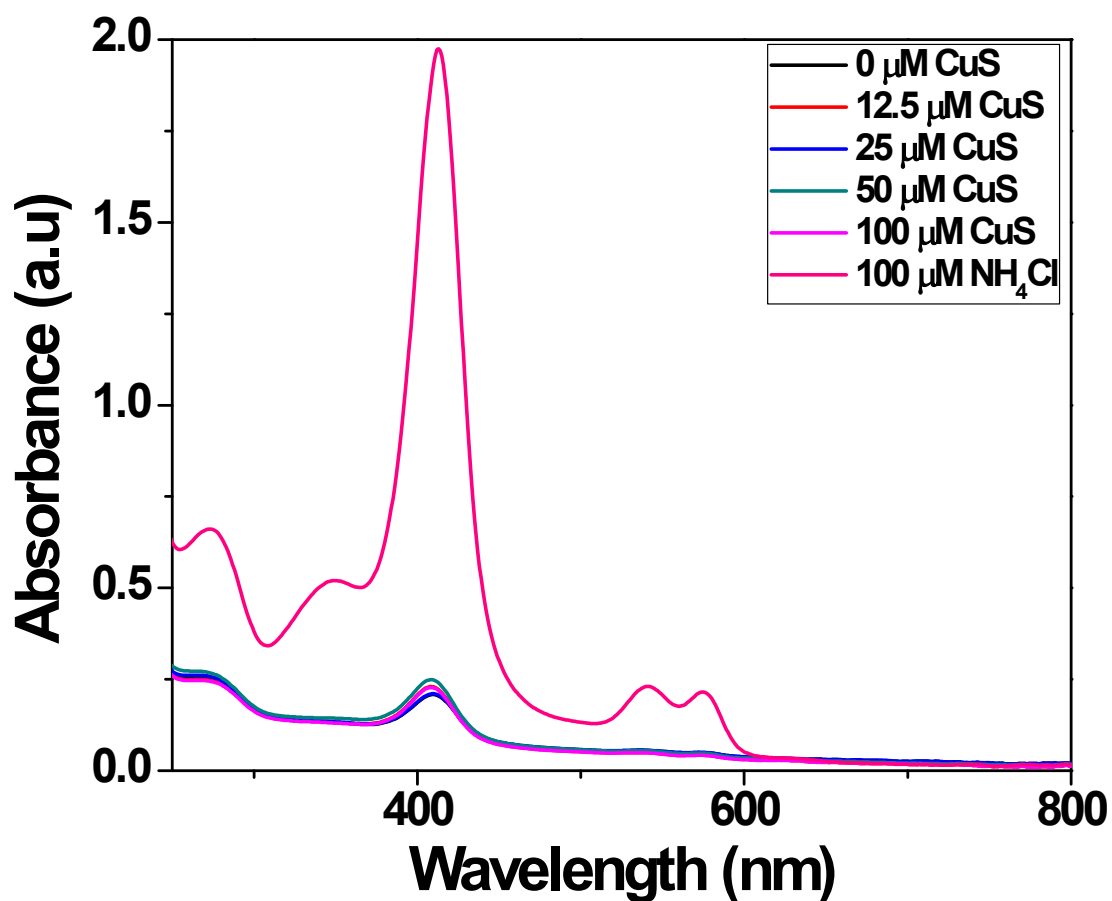
Fig. S13: Hemolytic study. Addition of CuS NPs to RBC does not induce hemolysis, whereas NH$_4$Cl damages the RBC as evidenced from the strong hemoglobin peaks. Peaks corresponds to hemoglobin was observed at 412 nm, 540 nm and 575 nm. RBC were treated with the materials and kept in shaker incubator (200 rpm) at 37°C. After 30 min, the reaction mixture was centrifuged and the supernatant absorbance was measured.