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Understanding the pathway of antibacterial activity of Copper Oxides Nanoparticles

Prachi Kabra[#1], Surapaneni Meghana[#1], Swati Chakraborty[2] and N.Padmavathy[3*]

[1]School of Bioscience and Biotechnology, [2]Jadavpur University, Kolkata,

[3]Centre for Nanomaterials, School of Advanced Sciences, VIT University,

Vellore – 632014, Tamilnadu, India

Methods

Reagents

Copper nitrate, sodium hydroxide, Hydrazine and L-Tryptophan were purchased from Sd-fine chemicals Pvt. Limited and other reagents were procured from Sigma Aldrich. Double distilled water was used throughout the experiments and the reagents were used as such unless specified.

Synthesis of tryptophan capped cupric oxide (Trp-CuO)

The precipitation method has been employed in the synthesis of Trp-capped CuO nanoparticles. For the synthesis, copper nitrate and sodium hydroxide in 1:2 molar ratio was used. 0.1mM copper nitrate dissolved in 50mL distilled water and 0. 2mM sodium hydroxide dissolved in 50mL distilled water was mixed together. The copper nitrate solution was placed on the magnetic stirrer, and the sodium hydroxide solution was added drop wise while stirring. The amino acid L-tryptophan which acts as a capping agent was added separately to the solution. 0.03mM L-tryptophan (0. 061g) was mixed with 10ml distilled water and heated at 70°C to allow the amino acid to dissolve completely. It was then added into the solution. The mixture was heated at 80°C - 90°C for 2-3 hours under constant stirring until the complete reduction of metallic salts was achieved. A black precipitate of Trp capped CuO was obtained and then purified. Precaution was taken to avoid the solvent from drying up during the process.

Synthesis of tryptophan capped cuprous oxide (Trp-Cu_2O)

In the typical synthesis process, aqueous solutions of 0.1 mM copper nitrate and 0.2 mM NaOH (drop wise added) is stirred together followed by the addition of 0.03 mM of the amino acid L-tryptophan. A deep-blue color colloidal $Cu(OH)_2$ is soon produced with continuous stirring for another 10 minutes. After that, 5 ml of 2M hydrazine (N_2H_4) solution was added at a

rate of 1mL/minute into the above stirring solution. The change in color from blue to reddish brown was noted. The precipitate is then dried under vacuum and washed with acetone several times and then used as such for further studies.

Enzyme Assays

To prepare extracts, cells were washed twice and resuspended in anaerobic buffer. Lysis and the removal of cell debris by centrifugation were also performed in the anaerobic chamber. All enzyme reactions were monitored at room temperature (25 °C). Fumarase[30] and Sulfite reductase [31] activities were measured according to the cited methods.

Characterization Techniques

Phase purity was determined from X- ray diffraction (XRD) recorded on the BURKER X-ray diffractometer. Absorption spectra were recorded using a Shimadzu UV-3600 spectrophotometer. The morphology and size of the particle were determined from HRTEM using JEM 3010 JEOL electron microscope at an accelerating voltage of 200eV. Photoluminescence (PL) spectra were recorded on a Perkin-Elmer LS 55 spectrophotometer using Xenon laser with an output power of 450W andexcitation source at 300K. Atomic Absorption Spectrophotometer (AAS) was used to measure intracellular copper content using copper lamp.

Culture and Analysis of Bacteria

Escherichia coli, a gram-negative bacterium was used in this study. Pure ATCC strain 25922 was obtained from the SBST stores of VIT University. This strain has been well characterized and is being used as a control organism for many years.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Agar-Broth dilution method was used to determine the MIC and MBC of Trp-CuO and Trp-Cu_2O on *Escherichia coli* (ATCC strain EC 25922). MIC values are used to determine susceptibilities of bacteria to the antimicrobial agent.

Bactericidal experiments were conducted with 50 mL of *E.coli* (0.9 optical density) in nutrient Hi-Veg broth (Hi Medioa Labs, India) initiated by adding an aliquot of wide-range concentrations (0.01mM to 1mM) of Trp-CuO and Trp-Cu_2O suspensions. Inactivation of *E.coli* was examined under aerobic conditions to determine the minimum concentration to inhibit / kill the bacterial cells. After the exposure, one mL of *E.coli* suspension exposed to copper oxides was withdrawn during the incubation period. The number of viable cells was quantified by plate counting method. L-tryptophan was used as the positive control and was checked separately since it was employed as a capping agent. After 24 hours, *E.Coli* colonies (Figure S2) present on the respective plates were counted and recorded.

Oxidative Stress Markers

Oxidative stress is proposed to be one of the key mechanisms responsible for the antimicrobial activity of the synthesized Trp-CuO and Trp-Cu_2O nanoparticles. To check the effect of these nanoparticles on the membrane integrity of the cell and to check on the production of reactive oxygen species caused by the nanoparticles, the following tests were done.

Cell Integrity study

The cell disruption caused by the nanoparticles is studied by finding the release of cellular components in the cell integrity study. If the cell membrane has been affected by the nanoparticles then the cellular components will be released and can be detected by UV

absorbance. In our work, study was performed at 260 nm to study the release of proteins as well as other cellular components as the characteristic absorbance of proteins is at 260 nm.

The conical flasks containing 25 ml of LB broth along with the MBC concentration of Trp-CuO (20mg), Trp-Cu_2O (14. 3mg) and a control without nanoparticle was sterilized and inoculated with 100µL of overnight grown *E.coli* culture. After 15 minutes interval 1.5mL of aliquots were pipetted out and centrifuged to pellet out bacterial cells. After centrifugation, absorbance of the supernatant was checked immediately at 260 nm and recorded.

Superoxide anion (O^{2-}) production test using NBT-light system

The superoxide scavenging assay involves the riboflavin-light-NBT system to detect the antioxidant activity. This method is based on the principle of reduction of nitroblue-tetrazolium (NBT) to blue coloured formazan, which is directly proportional to the concentration of superoxide anion in the system in the presence of riboflavin-light-NBT system. Photochemical reduction of flavins generates superoxide, which reduces NBT, resulting in the formation of blue formazan [21]. In this study, we quantified the formation of superoxide anions facilitated by the copper oxide nanoparticle. The reduction of NBT confirms the formation of superoxide anions.

Sample solutions were made by adding MBC concentration of Trp-CuO (20mg) and Trp-Cu_2O (14.3mg) in 25mL distilled water. The reaction mixture included 1mL phosphate buffer (50mM), 200µL EDTA (12mM), 1mL sample solution and 1mL NBT (1%). Two sets of samples were made where in one was kept in the dark serving as blank and one was illuminated for 60 s. Control was the reaction mixture without any nanoparticle. Using riboflavin as a standard, the results were compared. After illumination, absorbance readings were taken at regular intervals of 60 s – 180 s and recorded at 590 nm.

Hydroxyl ion (OH^-) production test using deoxyribose

Deoxyribose method is used for determining the hydroxyl radicals. This method is employed with certain modifications to determine the formation of hydroxyl radicals by Trp-CuO and Trp-Cu_2O nanoparticles.

Sample solutions were made by adding different concentration of Trp-CuO and Trp-Cu_2O in 25mL distilled water. The sample mixture was made by adding 500µL of phosphate buffer (28mM), 300µL EDTA (0.1mM), 500µL ascorbic acid (0.1mM), 1mL sample solution and 500µL deoxyribose (Figure S4). Control was the reaction mixture without any nanoparticle. These sample mixtures were incubated in a water bath maintained at 37°C for one hour. Then 1mL of 2% Trichloroacetic acid (TCA) and 1mL of 1% Thiobarbituric acid (TBA) were added, and the mixture was heated at 100°C in a water bath for 20 min. Once cooled, absorbance was measured at 532nm (Table S5). Using H_2O_2 as a standard, hydroxyl ion concentration was quantified using absorption coefficient. (Figure S5).

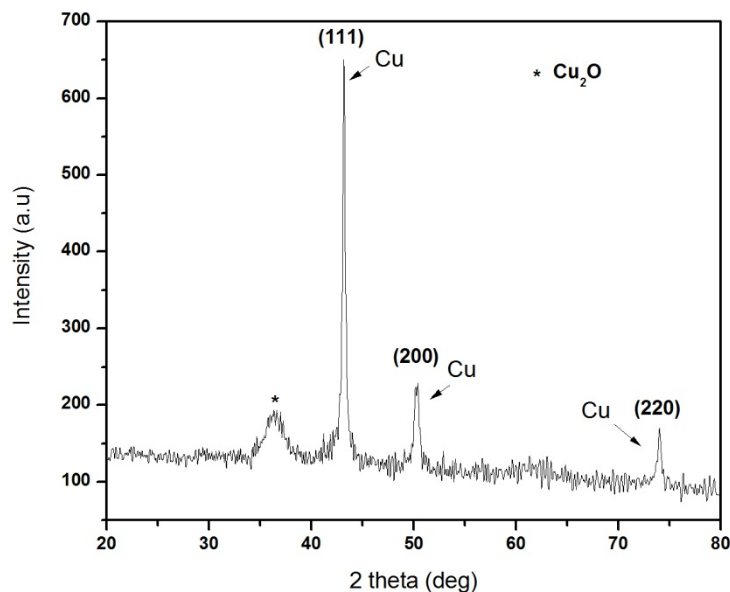


Figure S1. XRD pattern of Cu_2O with Cu using $NaBH_4$ as a reducing agent

Table S1. Concentrations of Trp-CuO towards antibacterial susceptibility test

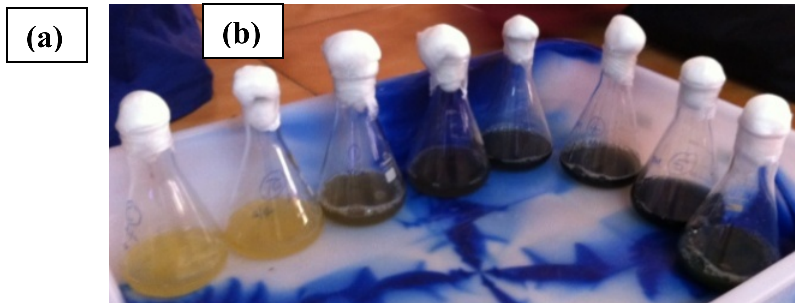
S.No	**Concentration of Trp-CuO (mM)**	**Weight of Trp-CuO (mg)**	**Weight of LB Broth added (mg)**	**Total volume (mL)**
Control	-	-	625	25
Trp-Control	0.03	-	625	25
1.	0.05	4	625	25
2.	0.1	8	625	25
3.	0.25	20	625	25
4.	0.5	40	625	25
5.	0.75	60	625	25
6.	1	80	625	25

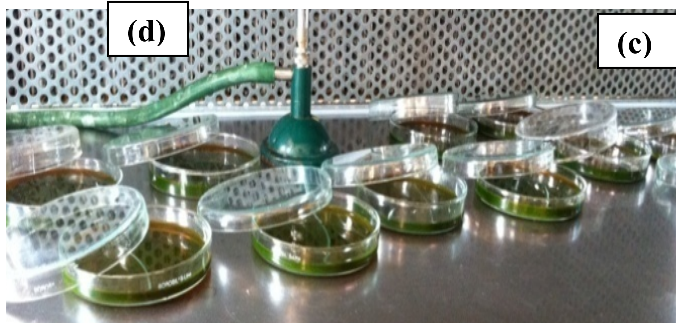
Table S2. Concentrations of Trp-Cu_2O towards antibacterial susceptibility test

S.No	Concentration of Trp-Cu_2O (mM)	Weight of Trp-Cu_2O (mg)	Weight of LB Broth added (mg)	Total volume (mL)
Control	-	-	625	25
Trp-Control	0.03	-	625	25
1.	0.025	3.6	625	25
2.	0.05	7.1	625	25
3.	0.075	10.7	625	25
4.	0.1	14.3	625	25
5.	0.25	35.7	625	25
6.	0.5	71.5	625	25

Figure S2. *E.coli* ATCC starin 25922 on EMB Agar

(a)





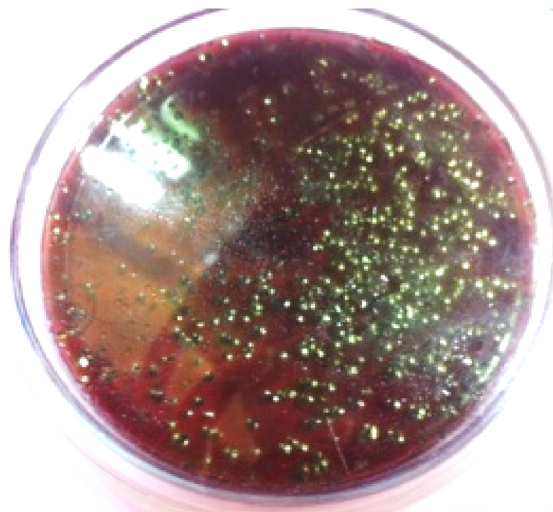


Fig S3. (a) Different concentration of nanoparticle in LB Broth. (b) After inoculation flasks kept on shaker overnight. (c) EMB agar plates. (d) Serial dilution of overnight culture and plating on EMB agar. (e) Formation of metallic green colonies after incubation of plates overnight.

Table S3. Absorbance values of different samples at specific intervals of time.

TIME	CONTROL	Trp-CuO Nanoparticles	Trp-Cu_2O Nanoparticles
Minutes	**Absorbance at 260nm**	**Absorbance at 260nm**	**Absorbance at 260nm**
0	0.632	0.861	0.980
15	0.744	1.057	1.250
30	0.830	1.025	1.123
45	0.866	1.156	1.261
60	0.850	1.095	1.340

Sample	S.No	Time (s)	Absorbance at 590nm
Trp- CuO MBC- 0.25mM	1.	0	0.268
	2.	60	0.255
	3.	120	0.247
	4.	180	0.241
Riboflavin (R1) 0.25mM	1.	0	0.129
	2.	60	0.138
	3.	120	0.144
	4.	180	0.158
Trp-Cu_2O MBC- 0.1mM	1.	0	0.091
	2.	60	0.088
	3.	120	0.087
	4.	180	0.085
Riboflavin(R2) 0.1mM	1.	0	0.015
	2.	60	0.022

	3.	120	0.037
	4.	180	0.043

Table S4. Absorbance at 590nm for the MBC concentration of Trp-CuO and Trp-Cu_2O – Superoxide anion production Test

Table S5.Hydroxyl ion production using H_2O_2 as standard

Sample	**S.No**	**Concentration of nanoparticle (mM)**	**Absorbance at 532nm**	**Concentration of OH^- ($x10^{-3}$ mM)**
Trp- CuO	1.	0.05	0.367	2.39
	2.	0.1	0.603	3.94
	3.	0.25	1.299	8.4
	4.	0.5	1.878	12
Trp- Cu_2O	1.	0.05	0.284	1.8
	2.	0.075	0.350	2.3
	3.	0.1	0.441	2.9
	4.	0.25	2.000	13
Standard- H_2O_2	1.	0.05	0.260	1.6
	2.	0.1	0.495	3.2
	3.	0.15	0.646	4.2
	4.	0.25	0.782	5.5
	5.	0.3	0.891	7
Control- L-trptophan	1.	0.03	0.025	0.16

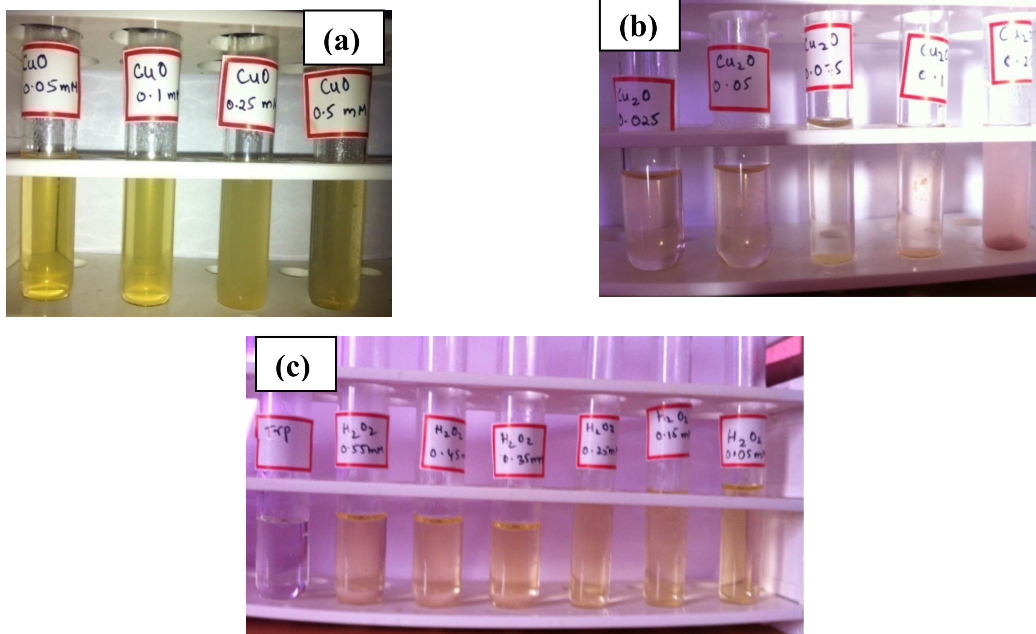


Figure S4.(a) Different concentrations of Trp-CuO after incubation with ThioBarbicuricAcid (TBA) and TriCholorAcetic acid(TCA). (b) Different concentrations of Trp-Cu_2O after incubation with TBA and TCA. (c) Standards of H_2O_2 and control with TRP after incubation.

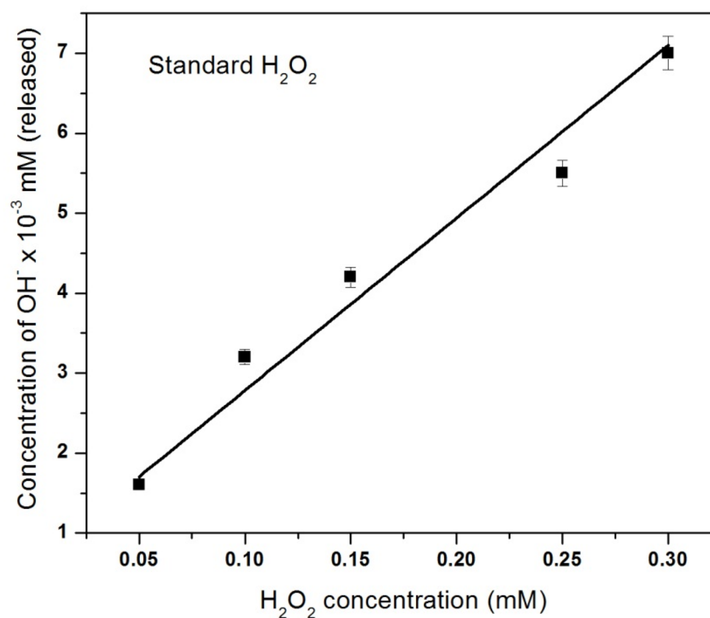


Figure S5. The standard graph of H_2O_2 at various concentrations

FT-IR

In the IR spectrum (Figure S6) the peak appearing in the range 3500-3300 cm^{-1} is due to N–H stretching. Bands observed at 613.36 and 495.71 cm^{-1} are characteristic of Cu–O vibrational modes. It is in good agreement with Cu-O stretching vibrational modes. The Peak in the range of 1400-1300$cm^{-1}$is similar to the peak in the IR of Tryptophan (Figure S7).

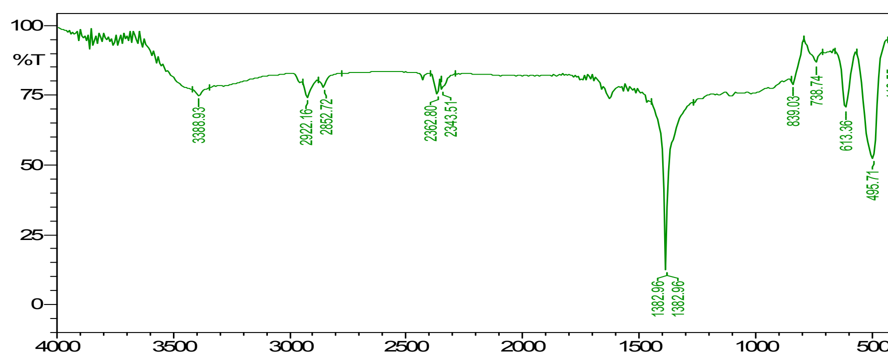


Figure S6. IR spectra of tryptophan capped CuO nanoparticles.

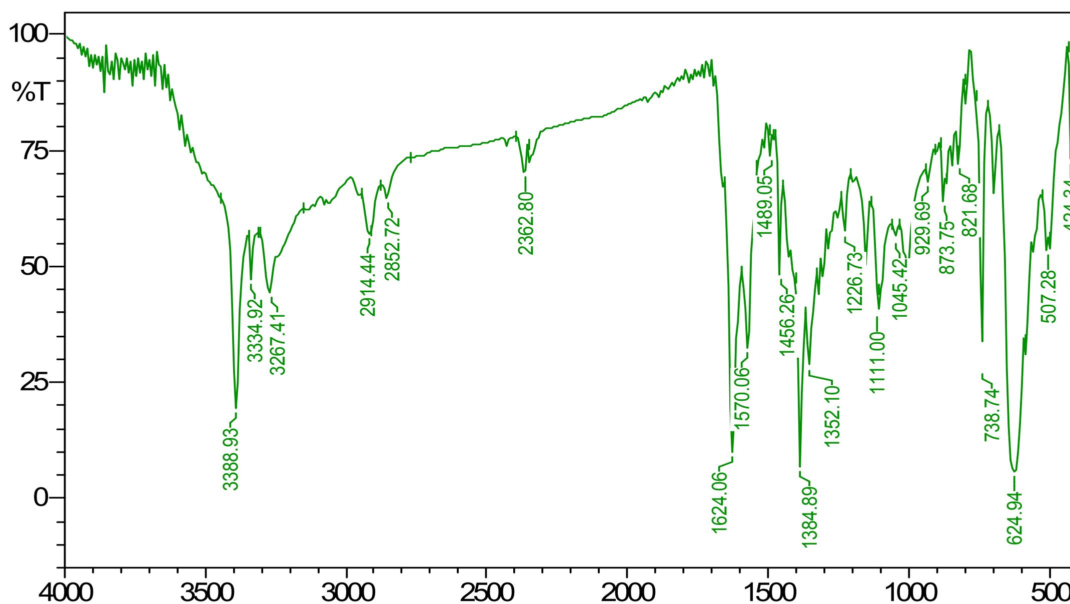


Figure S7. IR spectrum of Tryptophan.

In the IR spectrum (Figure S8) the strong absorption peak appearing at 625cm^{-1} indicates the stretching of copper (I)–O. The absorption peak appearing in the range 3500-3300cm-1 is due to the NH stretching's, which can also be seen in the spectrum of tryptophan (Figure S7).

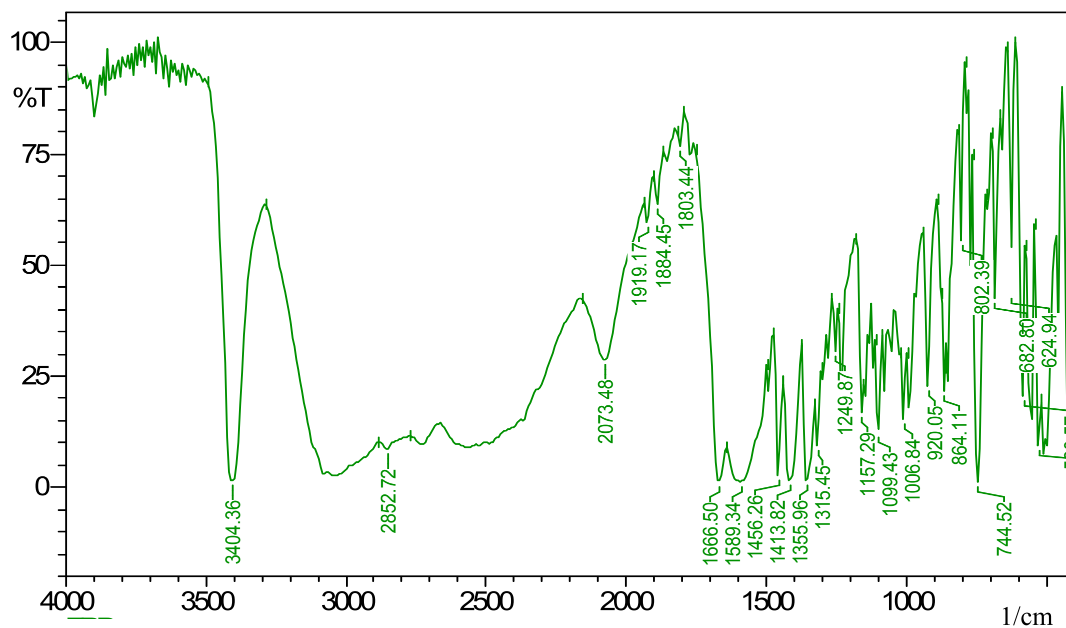


Figure S8. IR spectrum of Tryptophan capped Cu_2O nanoparticles