Chapter 2

Cuprous Oxide Nanoparticles: Synthesis and its Cytotoxicity Effects
2.1 INTRODUCTION

Nanoscience and nanotechnology have advanced greatly in recent years, and a large amount of research has focused on determining the properties and potential applications of nanomaterials.\(^1\) Metal oxide nanoparticles have been used to produce a wide range of products. Their industrial and commercial applications include catalysis, sensors, environmental remediation, personal care products, cosmetics, and they are showing great prospect in the field of medicine, including imaging and drug delivery.\(^2,3\) Among the different metal oxide nanoparticles, cuprous oxide (Cu\(_2\)O) nanoparticles are the subject of much current interest. It has been reported that excitons can propagate coherently through single crystalline.\(^4\) Cuprous oxide (Cu\(_2\)O) is a p-type semiconductor with a direct band gap of 2.17 eV.\(^5\) Cu\(_2\)O nanostructures have been demonstrated to be useful for gas sensing,\(^6,7\) photodegradation of dye molecules,\(^8\) CO oxidation,\(^9\) photoactivated water splitting into H\(_2\) and O\(_2\),\(^10\) and organic synthesis.\(^11\) A variety of Cu\(_2\)O nanostructures such as nanoplates,\(^5\) nanocubes,\(^12,13\) octahedra,\(^8,14,15\) spherical particles,\(^15\) nanocages,\(^16-19\) nanowires,\(^20-22\) and other highly symmetrical structures have been synthesized in recent years.\(^23,24\) Apart from great use in optoelectronics, proper tailoring of nanoparticles showed ample potentials of the particles to be used in medicine as a drug carrier\(^25,26\) and cancer-cell imaging.\(^27-29\) Nanoparticles were found to influence the cellular function of cells and activity of enzymes\(^30\) and in many cases it showed the strong toxic effect to living cells.\(^31-34\) Therefore, it requires target specificity and controlled toxicity to be useful in biomedical nanotechnologies.\(^35,36\) The study of the interaction of drug conjugated gold nanoparticles with bovine serum albumin has been reported.\(^37\) For control and specific use functionalization of gold nanoparticles with amino acids,\(^38-40\) proteins and DNA was reported.\(^41,42\) Studies also carried out to test how different metal oxide nano particles, for instances, ZnO and CuO nanoparticles could bind cellular component and the significance of the binding had been explained.\(^43\)

Presently a considerable interest has been focused on studying the interaction of inorganic nanoparticles with organic and biological molecules and it is an exciting field of basic and applied research.\(^44-48\) Modification of inorganic nanoparticles with biological molecules, such as nucleic acids, peptides and amino acids often found to modulate chemical and much optical behavior of nanoparticles.\(^49\) Evidences presented
that nanoparticles coated with ligand could induce the response of receptors bound to the cell membranes without entering in the cell.\textsuperscript{50,51} Engineered nanoparticles in suitable condition can also enter inside the cell after binding to a receptor.\textsuperscript{52,53} Transferrin coated gold nanoparticles proved to be internalized by HeLa cells into vesicles.\textsuperscript{54} Gold nanoparticles coated with alkaloids found to penetrate cell walls.\textsuperscript{55} Modification also affects the target specificity and toxic behavior of nanoparticles towards living cells.\textsuperscript{52,56}

In the present work we have demonstrated the synthesis and toxic behavior of cuprous oxide nanoparticles (CuNPs) and its conjugate with L-tryptophan to tap the potential of cuprous oxide nanoparticles and use it in control way in treatment of cancer and other diseases. Cuprous oxide nanoparticles may possess properties similar to azurite minerals which often used in natural Chinese medicine and showed potential antitumor properties.\textsuperscript{57} Cuprous oxide nanoparticles also bind to the hydrosulfide group, similar to arsenic trioxide which is used as anti tumor drugs and used to treat leukemia.\textsuperscript{58,59}

In Cuprous oxide, copper is in an unstable oxidation state (I) with d\textsuperscript{10} electronic configuration similar to ZnO. ZnO nanoparticles showed selective toxicity towards leukemia cells (HL60) and the toxic effect was much less of peripheral blood mononuclear cells.\textsuperscript{60,61} Due to the similar d\textsuperscript{10} electronic configuration of cuprous oxide and zinc oxide, CuNPs may have a similar cytotoxic effect. Nanostructure of cuprous oxide therefore may have potential application in medicinal chemistry. We successfully synthesized such nanoparticle. Further, we prepared the conjugate of the cuprous oxide nanoparticles with L-tryptophan to modulate the toxic behavior of the nanostructure. Tryptophan is an essential amino acid and often found in many proteins as an interesting aromatic amino acid residue. Its acceptance to cellular system may have less toxic effect. The toxic behavior of both the CuNPs and its biological conjugate with L-tryptophan was measured using different cancerous cell lines. X-ray diffraction and FT-IR (Fourier Transform Infrared) spectroscopic analysis was carried out to define the nano entities and binding of tryptophan to CuNPs. Along with XRD, TEM and AFM were used to characterize the topographic information of the produced particles. Optical properties of the materials were measured by UV-visible absorption spectroscopy. Binding analysis of the nanoparticles to BSA (bovine serum albumin),
HSA (human serum albumin) and Lysozyme (Lys) was further carried out using the intrinsic fluorescence behavior of the proteins to test the binding abilities of the proteins to the nanoparticle.

2.2 EXPERIMENTAL SECTION

2.2.1 MATERIALS and METHODS

Copper (II) sulphate pentahydrate salt, (CuSO$_4$. 5H$_2$O) (98% pure), sodium dodecyl sulphate (SDS) (98% pure) and NaOH (98% pure) were purchased from Merck. L-ascorbic acid (99% pure) and L-tryptophan (>98% pure) were purchased from Sigma-Aldrich. HPLC grade water and ethanol were purchased from Spectrochem (Mumbai, India). All of these chemicals were analytical grade and used as purchased without further purification.

2.2.2 SYNTHESIS

2.2.2.1 Synthesis of cuprous oxide nanoparticles (CuNPs)

A simple and effective method was established to synthesize cuprous oxide nanoparticles using SDS as a stabilizing agent and the preparation was done at room temperature. A concentrated copper (II) sulphate pentahydrate salt, CuSO$_4$.5H$_2$O solution was made with deionised water (Milli-Q Millipore purified) and to this solution SDS solution was added under stirring condition. The optimized pH was 9.0 and the mole equivalent ratio of CuSO$_4$. 5H$_2$O and SDS was established to be 1:6. The stirring continued for ~30 additional minutes until the solution color changed to pale blue from blue. In the next step aqueous solution of ascorbic acid was added to the reaction mixture and the color became pale yellow. The stirring was continued for 10 additional minutes. The final concentrations of the reactants were 0.04M, 0.24M and 0.16M, respectively of CuSO$_4$. 5H$_2$O, SDS and ascorbic acid. Finally, the pH of the solution was adjusted to 9.0 by slow addition of NaOH (0.5M) solution under continuous and rapid stirring. The color of the solution quickly changed to golden/deep yellow indicating the appearance of the CuNPs. Stirring continued for additional 30 min. The yellow solution was centrifuged at 8000 rpm to sediment CuNPs. The sedimented particles were dispersed in 99% ethanol followed by centrifugation at 10000 rpm for 10 minutes. This process was repeated thrice to remove excess SDS.
ascorbic acid and NaOH. The prepared nanoparticles appeared as golden yellow particles and the color was similar in the dry condition. Figure 2.1 shows the color photograph of CuNPs taken under fluorescent room light.

### 2.2.2.2 Synthesis of CuNPs - L-tryptophan conjugate

Conjugation of L-tryptophan to CuNPs was made to modify the surface property and activity of the nanoparticle. CuNPs conjugate with L-tryptophan was made by mixing CuNPs solution in ethanol with an aqueous solution of L-tryptophan. The optimized weight percentage of CuNPs to Trp was 1:3.5. Briefly, 3 mg of purified CuNPs was dispersed in 10 ml ethanol (HPLC grade) using a water bath sonicator. Sonication results clear yellow solution of CuNPs. Subsequently 10.5 mg L-tryptophan was dissolved in 20 ml water. These two solutions were mixed in a 50 ml round bottle flask and stirring continued for 1.5 hours. The solution color changes from yellow to sky blue. The unbound tryptophan was separated by centrifugation at 10,000 rpm for 10 min. A solid appeared and it was further washed three times with HPLC grade ethanol and dried under vacuum. Photograph of the conjugate material is shown in fig. 2.1 along with CuNPs.

![Figure 2.1: Color photograph of aqueous suspension of CuNPs and its conjugate with L-tryptophan under room light (fluorescence lamp light).](image)
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2.2.3 CHARACTERIZATION

2.2.3.1 Absorption

Absorption spectra of all the samples for this study were measured with a JASCO V-630 Spectrophotometer (JASCO International Co. Ltd, Japan) in the range of 230 to 800 nm wavelength. High quality quartz cuvette was used to record the spectra.

2.2.3.2 Transmission Electron Microscopy (TEM)

The size of the nanoparticles and their composite was studied using transmission electron microscope. For TEM imaging, a drop of aqueous sample solution was placed on carbon coated 300-mesh copper grid (Allied Scientific Product, USA) and dried in a dust free atmosphere. The bright field electron micrographs of the samples were taken on a Tecnai G2 Spirit Bio TWIN (Type: FP5018/40) at an acceleration voltage of 80 kV.

2.2.3.3 Atomic Force Microscopic (AFM)

AFM images were obtained on a Pico plus 5500 AFM (Agilent Technologies, Tempe, AZ USA) with a piezo scanner, range of 9 μm. For the AFM imaging of CuNPs samples, 10μl of the sample were deposited onto freshly cleaved Muscovite Ruby mica sheets (ASTM V1 Grade Ruby Mica from MICAFAB, Chennai) for 5–10 min. The samples were washed gently with water and dried by nitrogen gas prior to AFM imaging. Micro-fabricated silicon cantilevers of 225 mm in length with a nominal spring force constant of 21–98 Nm⁻¹ were used from Nanosensors. The cantilever oscillation frequency was tuned into the resonance frequency of 150- 300 kHz. The images (512 X 512 pixels) were captured with a scan size of between 0.5 and 5 mm at a scan speed rate of 0.5 rpm. Images were processed by flattening, using Pico view software (Molecular Imaging Corporation, USA).

2.2.3.4 Fluorescence

Intrinsic tryptophan fluorescence of BSA, HSA and lysozyme (Lys) was measured to investigate the binding of CuNPs to the protein molecules. All the fluorescence measurements were carried out using a Perkin Elmer LS-45 spectrofluorophotometer with a micromolar range protein concentration. Steady state
fluorescence spectra were recorded with an excitation wavelength of 295 nm to selectively excite tryptophan residues in the protein molecules. The selected emission range was 310-450 nm. Both the excitation and emission slit widths kept at 2.5 nm. Most of the experiment was carried out at room temperature (25 °C).

For fluorescence quenching measurements, the intrinsic fluorescence of tryptophan residue(s) in the protein was measured in the presence and in the absence of CuNPs. The fluorescence of the protein was found to quench in the presence of CuNPs. The quenching experiment was carried out simply by adding 5 µL aliquot (stock solution each time) of concentrated (500 µM) CuNPs solution to 2 mL solution containing an appropriate concentration of HSA/BSA/Lys (2 µM in 20 mM sodium phosphate buffer, pH 6.5) taken in 1 cm path length quartz cuvette. Small error due to dilution upon addition of the CuNPs was neglected. CuNPs showed negligible absorbance at the excitation wavelength (295 nm) compared to protein absorption at this wavelength. Fluorescence intensities at 340 nm were recorded as a function of CuNPs concentration. To derive the binding parameters, obtained data were analyzed using modified Stern–Volmer equation.62

2.2.3.5 Circular Dichroism (CD) analysis

The CD spectra were obtained at room temperature (25 °C) using a JASCO-810 spectropolarimeter under constant nitrogen flow condition. A 1 mm path length quartz cell was used for CD measurements and at room temperature. For all the measurement the protein concentration of 10 µM was used. The far-UV region was scanned between 200 and 250 nm using a bandwidth of 5 nm. Each represented spectra were average of five 5 individual scans.

2.2.3.6 FT-IR experiment

The FT-IR spectra of the samples were recorded on a JASCO FT/IR 4200 spectrometer using the KBr disc technique. Solid samples were mixed with KBr in a clean glass pestle and mortar and compressed to obtain a pellet. The spectra were recorded from 400–4000 cm⁻¹. Background spectra were obtained with a KBr pellet for each sample. JASCO software was used for data processing.
2.2.3.7 Thermogravimetric analysis

The thermal stability of the synthesized CuNP-Trp nanoparticles was monitored by thermogravimetric analysis (TGA) and differential scanning calorimetric (DSC) analysis and the thermogram is given in Fig. 2.6. The experiment was carried out in SDT (Simulated DSC and TGA) Q600 T.A. instrument. The tryptophan conjugated CuNP (CuNP-Trp) was subjected to heating from 40 °C to 700 °C with a heating rate of 10°C per minute.

2.3 RESULTS and DISCUSSIONS

2.3.1 Absorption spectroscopy

Fig. 2.4A displays the UV–visible spectra recorded from the solution of CuNPs dispersed in aqueous solution at room temperature. It showed a wide band at ~ 470 nm. The band was due to plasma resonance excitation from the surface of the nanoparticles. The band position indicated the presence of cuprous oxide nanoparticle in the solution.\(^{64}\)

![Figure 2.2](image-url)\(^{68}\)

**Figure 2.2:** (A) Optical spectra of CuNPs in deionised water. Deionised water was used as a reference. The time gap between the two readings was 75 min.; (B) UV-Visible spectrum of the 65μM L-tryptophan (blue) and 20μg/ml tryptophan coated CuNPs (CuNP-Trp) (pink) in water suspension. Some broadness in the absorption spectrum of the CuNP-Trp was due to colloidal nature of the solution; (C) Absorption Spectra of Cu_2O nanoparticles after 90 days kept in vacuum condition.
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The spectrum remained similar even after 90 min incubation at room temperature. It indicated the stability of the suspended particle. Under vacuum, the particles remained stable for months and the resuspended solution showed a similar spectrum. The CuNPs was golden yellow in color, however, the composite (CuNP-Trp) became sky blue in aqueous solution as shown in Fig. 2.1. This strongly indicated the formation of a composite with L-tryptophan. CuNP-Trp in water shows an absorption band similar to free tryptophan in aqueous solution (Fig. 2.2B). It confirmed the presence of L-tryptophan in the conjugate.

2.3.2 X-ray diffraction study

The results of a XRD diffraction pattern of the CuNPs was shown in Fig. 2.3A. The diffraction pattern of the particle was similar to cuprous oxide \(^{65}\) and confirmed the formation of cuprous oxide nanoparticle. No strong peak from impurities was detected; it indicated that the major amount of particles were pure cuprous oxide. The XRD patterns showed peaks at \(2\theta = 29.84, 36.6, 42.46, 61.71, 73.93\) and \(77.52\) which were indexed to (110), (111), (200), (220), (311) and (222) planes, respectively, of cubic cuprous CuNPs (JCPDS Card No. 05-0667). These XRD results matched well with cuprous oxide particles as reported by others.\(^{65-67}\) XRD of the composite showed a very strong peak from the planer aromatic ring along with the signature peaks of the nanoparticle and confirmed the conjugation with L-tryptophan with the CuNPs (Fig. 2.3B).

Figure 2.3: (A) Powdered X-ray diffraction pattern of the CuNPs. The peaks are labelled with the standard cuprite reflections. (B) Powder X-ray diffraction pattern of CuNP-Trp. CuNP-Trp showed a strong peak due to the presence of aromatic ring of L-tryptophan residue. An expanded region in the inset shows the diffraction patterns of the nanoparticle.
2.3.3 Thermogravimetric and Differential Scanning Calorimetric Analysis

The thermal stability of the synthesized CuNP-Trp nanoparticles was monitored by thermo gravimetric analysis (TGA) and differential scanning calorimetric (DSC) analysis and the thermogram is given in Fig. 2.4. The experiment was carried out in SDT (Simulated DSC and TGA) Q600 T.A. instrument. The tryptophan conjugated CuNP (CuNP-Trp) was subjected to heating from 40 °C to 700 °C with a heating rate of 10 °C per minute. TGA curve showed that the thermal decomposition process was represented by two step falls in weight over the temperature ranges from 250–295 °C and 310–475 °C. The weight loss between 250 and 295 °C was attributed to the dehydration of tryptophan whereas the rest was due to the decomposition of organic content. The residual mass at 650 °C in the sample was 36% and it indicated the high amount of a tryptophan contents in the tryptophan coated Cu₂O nanoparticles.

![Thermogravimetric analysis of L-tryptophan conjugated Cu₂O nanoparticles.](image)

Figure 2.4: Thermogravimetric analysis of L-tryptophan conjugated Cu₂O nanoparticles.

2.3.4 Transmission Electron Microscopy

Topographic information of the nanoparticles and its conjugate was obtained from the transmission electron microscope images taken at room temperature (24°C). Fig. 2.5 shows the TEM images of synthesized CuNPs and CuNP-Trp conjugate. The TEM pictures confirmed that the nanoparticles were well dispersed and the topography/morphology of the particles was spherical in nature. Some cases we observed particles in agglomerated state. Expanded image shows roughness of the
surfaces and may be composed of smaller particles. In the TEM images the CuNPs appeared as a rough and of nonuniform spheroidal shape, however, the CuNP-Trp was smoother and spheroidal in shape.

Right side panel of respective TEM pictures in Fig. 2.5 shows the size distribution histogram of the nanoparticles with normal distribution fit. Histogram plot was made by measuring 100 randomly selected particles in enlarged TEM images. To determine the average particle size, the data were fitted with normal distribution function. The particle size of the CuNPs varied between 40-110 nm and the average diameter obtained was ~ 70 nm. The average particle size of CuNP-Trp nanocomposite was ~85 nm.

![Figure 2.5: Transmission Electron Microscopic (TEM) images of (A) CuNPs and its conjugate, (C) CuNP-Trp. The size distribution histogram and the fitted normal distribution curve of (B) CuNPs and (D) CuNP-Trp are shown beside the TEM images of respective samples.](image)

### 2.3.5 Atomic Force Microscopy

AFM image (two dimensional plot) of cuprous oxide nanoparticles and their conjugate with tryptophan are shown in Fig. 2.6, upper panels. Bottom panels showed the 3D-plot of the images and indicated length, width and various height distributions.
CuNPs were nearly spherical with 65-80 nm diameters. Nanoparticles conjugated with L-tryptophan also showed the almost similar size distribution; however, density of larger particles were more than the numbers found in bare CuNPs. The X-ray diffraction analysis showed the relatively smaller size of the nanoparticles compared to the values obtained from dynamic light scattering (DLS), TEM and AFM measurements. The DLS analysis indicated average diameter of 95 nm and 127 nm, respectively, for the CuNPs and its conjugate. Nano-crystals are combined to form nanoparticles. DLS provides the hydrodynamic radius of these nanoparticles which are composed of nano-crystals. The particle size may be further increased due to agglomeration in solution state.

Figure 2.6: AFM images of the nanoparticles. (A) and (B), respectively are the topographic mode AFM images of CuNPs and CuNP-Trp. (C) and (D) shows 3D-AFM images of CuNPs and CuNP-Trp respectively. The area of each image is 8 μm × 8 μm. The particle size was estimated from the color density mapping.
2.3.6 FT-IR spectroscopy

The conjugation of CuNPs to L-tryptophan was confirmed by FT-IR spectroscopy; it also established the nature (oxidation state) of the copper oxide nanoparticle formed. FT-IR spectra taken with KBr pellets Trp, CuNPs and the conjugated particle are shown in Fig. 2.7. Being an aromatic amino acid, L-tryptophan showed strong FT-IR bands (Fig. 2.7a).\(^6\) N-H stretching band (due to the presence of 1\(^0\) and 2\(^0\) amine) was observed at 3402 cm\(^{-1}\). The band at 1453 and 1412 cm\(^{-1}\) are assigned to aromatic C=C stretching; other bands also closely matched with the reported values. The FT-IR spectrum of CuNPs (Fig. 2.7b) displays an absorption peak at ~624 cm\(^{-1}\) and attributed to the Cu (I)-O vibration.\(^6\),\(^7\) The spectrum also exhibited no strong FT-IR bands at ~588, ~534 and 480 cm\(^{-1}\) indicating that the compound was free of cupric oxide (Cu (II)-O) particles. FT-IR spectrum of CuNPs conjugated with L-tryptophan (Fig. 2.7c) showed many strong absorption bands due to incorporation of the tryptophan inside the nanoparticles.

![Figure 2.7: FT-IR spectra of (a) free tryptophan, (b) CuNPs, (c) CuNP-Trp in solid state.](image-url)
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Expanded region also showed that the marker band for Cu(I)O was present and confirmed that the particles was preserved cuprous state. Tryptophan could bind CuNPs via its electron rich carboxylate group and the primary and secondary nitrogen atoms of the amino group. We observed overlapping N-H stretching bands for primary and secondary amines in free tryptophan at 3402 cm\(^{-1}\). However, in the composite their bands appeared at 3388, 3334 and 3271 cm\(^{-1}\). It strongly suggested that the tryptophan nitrogen atoms are strongly interacting with CuNPs and bound to it. The binding is possible through electropositive cuprous state via nitrogen atoms in the tryptophan ring and the COOH group also may be involved in the bonding.

\subsection*{2.3.7 Binding study of the Cu\(_2\)O Nanoparticles with BSA/HSA/Lysozyme}

Serum albumin proteins are often used as drug carrier in the blood. Bovine serum albumin (BSA), human serum albumin (HSA) and lysozyme could bind many small molecules, including drug candidates and carries two different cellular organs. We examined the binding affinity of the Cu\(_2\)O nanoparticles with blood carrier proteins, bovine serum albumin (BSA), human serum albumin (HSA) and also with hen egg white lysozyme (Lys). We utilized the intrinsic tryptophan fluorescence of individual proteins to determine the interaction behavior of CuNPs in aqueous solution. Fig. 2.8 shows fluorescence spectra of individual proteins (BSA/HSA/Lys) in the absence and presence of different concentrations of CuNPs. The proteins showed a strong fluorescence with an emission peak at \(~340\) nm due to its tryptophan residue (s). The fluorescence intensity of proteins at \(~340\) nm decreased gradually with increasing peptide concentration, indicating effective fluorescence quenching of the protein fluorescence.

To determine the binding behaviour the fluorescence data obtained in the above experiments was analyzed using a modified Stern-volmer (S-V) equation (equation 1)\textsuperscript{62} (page no. 289). Fluorescence peak intensity values of the protein at different concentration of CuNPs (Q) were used to fit a modified S-V equation as given below:

\[ \frac{F_0}{\Delta F} = \frac{1}{fK[Q]} + \frac{1}{f} \]  

\( F_0 \) is the initial fluorescence intensity in the absence of CuNPs, \( \Delta F \) is the difference in fluorescence in the absence and presence of the CuNPs at concentration [\( Q \)], \( K \) is the Stern–Volmer quenching constant, and \( f \) is the fraction of the initial fluorescence which
is accessible to the quencher. The plots of $F_0/\Delta F$ versus $1/[Q]$ yields $f^{-1}$ as the intercept, and $(fK)^{-1}$ as the slope. Table 1 shows the result. The intercept on the y axis ($1/f$) indicated that ~100% of the total HSA fluorescence and ~90% of BSA, ~74% for Lys. Quenching constant ($K_{SV}$), was found to be in the micro molar range and the quenching constants for HSA, BSA and Lys quenching by CuNPs are given in Table 1. To derive the binding constant we have used Stern-Volmer quenching constants. 

Fluorescence quenching processes usually explained by two mechanisms: (i) dynamic fluorescence quenching and (ii) static quenching. Dynamic fluorescence quenching occurred either primarily via a collision process, and solvent viscosity, and size of the particles play important roles on the extent of quenching. In the static quenching process close association of the fluorophore and the quencher lead substantial decrease of fluorescence. Substantial fluorescence quenching of the protein by CuNPs indicated static fluorescence quenching and this static quenching arose from

Figure 2.8: Binding of CuNPs to BSA, HSA and Lys assessed by intrinsic tryptophan fluorescence quenching. Fluorescence emission spectrum of 2.1 μM HSA (A), 2.4 μM of BSA (B) and 2.5 μM of Lys (C) in the presence and absence of CuNPs. Protein samples were prepared sodium phosphate buffer (20 mM), pH 6.5. Quenching was carried out with micromolar range (1.25−12.5 μM) CuNPs. $\lambda_{ex} = 295$ nm.
the formation of a (dark) complex between protein and CuNPs.\textsuperscript{62,71} The Stern Volmer quenching constant as obtained from the modified S-V equation can be expressed as binding affinity constant, $K_a$.\textsuperscript{62,71,72} Reciprocal of this $K_a$ gives the dissociation constant, $K_d$ (Table 1).

![Figure 2.9: Stern–Volmer plots obtained using the fluorescence data obtained from Figure 2.10 A, B and C respectively.](image)

**Table 1. Binding parameters (binding dissociation constant, $K_d$, binding affinity constant/ Stern-Volmer quenching constant, $K_a$)**

<table>
<thead>
<tr>
<th>Type</th>
<th>$K_d$ (µM)</th>
<th>$K_a$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA-NP</td>
<td>22.00</td>
<td>$4.54 \times 10^4$</td>
</tr>
<tr>
<td>BSA-NP</td>
<td>19.60</td>
<td>$5.10 \times 10^4$</td>
</tr>
<tr>
<td>LYS-NP</td>
<td>29.95</td>
<td>$3.33 \times 10^4$</td>
</tr>
</tbody>
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**2.3.8 Circular Dichroism Spectroscopy**

To determine the effect on protein conformation of CuNPs binding we carried out the circular dichroism (CD) as it often provides the secondary structure information of proteins in solution state. Different panels in Fig.2.10 represent the CD spectra of BSA, HSA and lysozyme in the presence and in the absence of CuNPs. Two negative bands that appeared appearing at ~208 and ~222 nm are the characteristics of $\alpha$-helical conformation. The addition of the CuNPs into BSA, HSA and lysozyme did not cause huge perturbation of the CD spectrum indicating minor changes in the secondary structure of proteins when interacts with the Cu$_2$O nanoparticles.
2.3.9 Stability study of CuNP-Trp conjugate in Dulbecco's Modified Eagle Medium (DMEM)

2.3.9.1 Absorption spectroscopy study

Typically, 2 mg dry sample of the CuNP-Trp was dispersed in 10 ml double distilled water. 200 μl of the prepared solution was added to 2 ml of DMEM (low glucose, pyruvate, no glutamine, and no phenol red) and record the absorption spectra using DMEM as reference solvent. The absorption spectrum is shown in fig. 2.11. From the spectra it was shown that with the increase of time interval the absorbance increases at 278 nm because of the release of some tryptophan residues from the CuNPs surface. We have recorded the absorption up to 48 hours.
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2.3.9.2 X-ray diffraction study of the CuNPs-Trp

Typically, 50 mg of dry CuNPs-Trp sample was dispersed in Dulbecco's Modified Eagle Medium and incubated at 37°C for 96 hours. Subsequently the solution was centrifuged at 12000 rpm for 20 min and collected the sedimented particles. This was again dispersed in double distilled water and centrifuge and repeated twice. Collected particle was dried at 50 °C for 6 hours and XRD was taken from the dried sample, Fig. 2.12.

Figure 2.11: Time dependent stability study of CuNP-Trp in DMEM medium at room temperature.

Figure 2.12: (A) Powdered X-ray diffraction pattern of the CuNP-Trp after incubation in DMEM medium at 37 °C for 96 hours.
The diffraction pattern was similar to the original diffraction pattern (Fig. 2.3) except some attenuation of the peak originated due to the aromatic ring of the L-tryptophan. It confirmed that the CuNP-Trp in biological medium was not highly unstable and the release of tryptophan occurred slowly.

### 2.3.9.3 X-ray diffraction Line Broadening Analysis (LBA)

Crystal size of Cu$_2$O nanoparticles was determined using Scherrer’s equation:

$$d = \left(\frac{K \lambda}{\beta \cos \theta}\right) \times \left(\frac{180}{\pi}\right)$$

Where $d$: the thickness of crystallites in the direction perpendicular to the diffracting planes (hkl) i.e. mean size of the crystalline domains, it may be smaller or equal to the particle size, $K$ = Scherrer constant, typical value is 0.9, but it changes with the actual shape of the crystallites, $\lambda$: X-ray wavelength. Cu K$\alpha$ radiation source was used, $\lambda = 1.54060$ Å $\beta$: the line broadening at half the maximum intensity (FWHM), $\beta$ was measured by fitting the raw data (obtained by XRD) with Voigt function, OriginPro graph. Fig. 2.13 displays the fitted curve (red) and $\beta$ was 1.14397 radian at $2\theta \sim 36.709^\circ$. Using the obtained $\beta$ and Scherrer formula obtained $d$ value was 7.4049 nm.

![Figure 2.13](image.png)
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2.3.10 Dynamic Light Scattering

Solutions of the nanoparticles were prepared by the dispersing the particles in water (1mg/10 ml water). It was diluted to ~55 μg/ml to yield an optimum scattering intensity for DLS measurements on a Malvern Zetasizer Nano ZS90 (Malvern instruments Ltd., UK) instrument equipped with a He-Ne laser of 633nm. The experiment was carried out at 25 °C. The scattered light from the sample was detected by a photomultiplier tube placed at 90° to the incident laser beam. The measured diameter of the CuNPs was 95 nm and the value was 127 nm for the conjugate.

2.4 CYTOTOXICITY STUDY

2.4.1 Cell Culture Preparation

The cytotoxicity tests were carried out using three cell lines: (i) human breast cancer cell line MCF 7, (ii) kidney cancer cell line HEK 293 and (iii) hepatocellular carcinoma cell line Hep G2. These cells were cultured in 25 cm² culture flasks using Dulbecco’s modified Eagle’s medium (DMEM), Invitrogen, Life Technologies, USA) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, and antibiotics (penicillin/streptomycin and gentamicin). The culture was maintained at 37 °C (CO₂ incubators) in an atmosphere of 5% CO₂ and 95% relative humidity. The growth medium was changed every alternate day until the time of the experiment. Prior to each cytotoxicity test, the cells were harvested by using trypsin, ethylenediamine tetraacetic acid (EDTA)-phosphate buffered saline (PBS) solution and
diluted at a density of $6 \times 10^5$ cells / mL in MTT assay. The cell suspension was seeded in 96 well plate at 100 µL/well and incubated for about 24 h before the MTT test to reach the confluency.

2.4.2 The MTT Assay

The MTT assay determines the ability of metabolically active viable cells to reduce the yellow tetrazolium salt $[3-(4,5$-dimethylthiazol-2-yl)-2,5-$\text{diphenyltetrazolium bromide}]$ (MTT) to purple formazan crystals by mitochondrial dehydrogenase. The concentration of purple color formazan crystals were spectrophotometrically (in ELISA reader) determined when dissolved in an organic solvent, dimethyl sulfoxide (DMSO). Cultured primary MCF 7, HEK 293 and Hep G2 were treated with tryptophan, CuNPs, and tryptophan coated CuNPs separately (5 µM, 10 µM, 20 µM and 50 µM). The plates were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO$_2$. MTT was dissolved in PBS at a concentration of 5.0 mg/mL, filtered through a 0.22 µm filter to sterilize and remove insoluble residues, and then stored in the amber vials at 4 °C. After treatment the medium was removed and 50 µl of fresh medium was added along with 10 µl of MTT added in wells of the 96-well plates and incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO$_2$. At the end of the incubation period, the media were discarded using a suction pump. In a 96-well plates 1.4 ml of DMSO were added to solubilise the purple crystals in different wells. The absorbance was measured at a test wavelength of 590 nm in an Elisa plate reader (Dynex MRX Microplate reader). The absorbance obtained from treated cells were expressed as percentages of absorbance obtained from untreated cells and are reported as mean ± Sd (n = 3).

The in vitro cytotoxicity tests of the tryptophan, CuNPs nanoparticles and tryptophan coated CuNPs were performed with human breast cancer cell line MCF 7, kidney cancer cell line HEK 293 and hepatocellular carcinoma cell line Hep G2. The Fig. 2.15 shows the cell viability of these cancer cell lines after 24h incubation of the control and with different concentrations of tryptophan, CuNPs, and tryptophan coated CuNPs. It was observed that tryptophan did not induce any significant change in the proliferation with a concentration up to 50 µM with respect to the control, suggesting the absence of toxicity of the tryptophan. Subsequently the proliferation of MCF 7,
HEK 293 and Hep G2 cells were reduced significantly with CuNPs which indicated the cytotoxic nature of the CuNPs. It was also noted that in all the concentrations the tryptophan coated CuNPs; the cell viability was significantly increased and seemed to be less toxic towards MCF 7, HEK 293 and Hep G2 cell lines.

**Figure 2.15.** Cytotoxicity of CuNPs on different cancer cell lines: (A) MCF 7, HEK 293 (B), HEP G2 (C) cell lines respectively. Cells were incubated for 24 hours in presence of different samples.
This strongly indicated that incorporation/conjugation of Trp to CuNPs modified the chemical nature of the CuNPs and caused a reduction in toxicity. The conjugate also showed dispersion stability in biological media (DMEM) as discussed earlier.

The major aim of the current investigation was to test the cytotoxic effect of bare copper (I) oxide nanoparticles and its conjugate with L-tryptophan. Using different type of cultured cell lines we showed that the cytotoxic effect was reduced when it was capped with L-tryptophan. Adsorption of tryptophan on nanoparticles found to provide smoothness to the surface, chemical nature got changed and therefore the interaction with other molecules such as proteins and others. It also produced sky blue color instead of dark yellow in water suspension (Fig. 2.1). It clearly indicated substantial effect on the electronic properties of the particle and resulted different interaction with the cell lines. The capping may prohibit the direct reaction of the cell surface and reduced the cytotoxic effect. It indicated possible dose dependent utility in cancer therapy. Also smoother surface and the lesser toxic effect put the CuNP-Trp conjugate as a viable drug carrier. Intrinsic copper (I) is unstable, very reactive and toxic and Cu (II) ion was not as toxic as its nanoparticles\textsuperscript{73,74}. Due to the unstable nature of the ion, the cell viability test was not performed.

2.5 **Summary and perspectives**

We have reported here a successful synthesis of cuprous oxide nanoparticles at room temperature by reducing copper (II) sulphate with ascorbic acid in alkaline condition. SDS served as the capping reagent during the preparation. Organic composite of the cuprous oxide nanoparticles was made with natural amino acid, L-tryptophan. Conjugation resulted new color, smoothness and different texture and indicated its unique potential to be used in optoelectronics and material science. However, our interest oriented towards its possible application in medicinal science. The conjugated particles were less toxic towards cultured cells. The investigation also established that the CuNPs strongly bind to blood carrier protein molecules. These results indicated that CuNPs conjugated with L-tryptophan may have a potential role in medicinal chemistry and pathology. The dose dependent use of the conjugate alone or with certain percentage of CuNPs may be beneficial for treatment of diseases. Another possibility as indicated earlier that it (CuNP-Trp) could be used as a drug carrier because of reduction in cytotoxic effect. An additional tailoring (making specific to
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particular receptor molecules) of the composite may be required to make it very target specific.

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