Synthesis, characterization and analysis of the effect of copper oxide nanoparticles in biological systems

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Abstract

Objectives: In the present study, synthesis of copper oxide nanoparticles was prepared by chemical reduction method. This synthesized metal oxide nanoparticles were targeted to study the antibacterial activity against Escherichia coli and Staphylococcus aureus.

Methods: The antibacterial activity against Escherichia coli and Staphylococcus aureus was seen by resazurin dye reduction method and disc diffusion method. Further, the effects of nanoparticles on enzyme activity (β- glucosidase) were seen in Escherichia coli. The physical interaction between metal oxides nanoparticles with protein (Ovalbumin) were studied to interrupt the conjugate forming properties. Toxicity studies of the metal oxide nanoparticles on human pathogens open the door for a new range of antibacterial agents.

Results: There is a lot of interest among the researchers for developing various protocols for the fabrication of other metal nanoparticles. However, these have been a constant research regarding the use of biological systems, friendly and rapid techniques.

Conclusion: The potential application of the nanomaterials-protein conjugates are limited only to novel techniques such as therapeutics in cancer, as drug delivery vehicles, smart nanocomposites, in bioremediation of industrial effluents, biodegradation of effluents and for water purification.

Keywords: Copper nanoparticles , FTIR , XRD, Resaurin assay, protein nanoparticles conjugate.

1. Introduction

Nanotechnology involves the production, manipulation and use of materials ranging in the size from less than a micron to that of individual atoms (Mohanpuria *et al.,* 2007). A wide variety of physical, chemical and biological process results in the synthesis of nanoparticles, some of these are very useful and others are quite common (Sastry *et al.,* 2003).

Nano scale particles have emerged as novel antimicrobial agents owing to the high surface area to volume ratio, which is coming up as the current interest in the researchers due to the growing microbial resistances against metal ions, antibiotics and the development of resistant strain (Ho Chan *et al.,* 2008).

Bionanotechnology is the integration between biotechnology and nanotechnology for developing biosynthetic and environmental friendly technology for the synthesis of nanomaterials (Sobha *et al.,* 2010).

2. Copper nanoparticles

Copper is one of the most widely used materials in the world. It has a great significance in all industries, particularly in the electrical sector due to low cost. Copper nanoparticles have been synthesized and characterized by different methods. Stability and reactivity are the two important factors that impede the use and development of the metal cluster in a new generation of nanoelectronic device (Feldheim & Foss, 2002).

2.1 Importance of CuO nanoparticles

- It is used as a pigment on ceramics to produce blue, red and green and sometimes gray, pink or black glazes.
- It is used in chemotherapy for patients with AIDS.

2.2 Antibacterial activity of CuO nanoparticles

- For centuries, people have used copper for its antibacterial qualities. However, copper nanoparticles have shoed antibacterial activities more than copper. Minuscule amounts of copper nanoparticles can lend antimicrobial effects to hundreds of square meters of its host material.
- This is more effective and proved to have antibacterial activity in the formulation of microscale to nanoscale sized particles. Also Guanog Ren said that the copper nanoparticles have widespread antibacterial activity against *E. coli* and *S. aureus*. Copper

has potent biocidal properties. Copper ions, either alone or in copper complexes, have been used for centuries to disinfect liquids, solids and human tissue. This manuscript reviews the biocidal mechanisms of copper and the current usages of copper and copper compounds as antibacterial, antifungal and antiviral agents, with emphasis on novel health related applications.

2.3 Synthesis of copper oxide nanoparticles

2.3.1 Chemical reduction method (Kooti & Matouri, 2010)

Solution I: 6.9 g of copper sulphate pentahydarate was dissolved in 100 ml of distilled water.

Solution II: 34.6 g of sodium potassium taratate and 12 g of sodium hydroxide was dissolved in 100 ml of distilled water.

50ml of solution I and 50 ml of solution II was mixed together with vigorous stirring and 5 g of starch (reducing agent) was added and then the mixture was stirred vigorously was 10 mins and then keep in boiling water bath at 60°C for 10 mins. Then, the obtained mixture is centrifuged and washed with distilled water twice and with ethanol twice and it was air dried and the powdered substance was used for further analysis.

3. Characterization of copper oxide nanoparticles

3.1 UV-Visible spectrometry

The reduction of metal nanoparticle was monitored by measuring the UV-Visible spectrum of the reaction medium at 24hrs time interval and the absorbance was recorded at 200-800nm using Perkim Elmer lambda 25 spectrophotometer (Prema, 2010).

3.2 FTIR spectroscopy

Fourier transforms infrared spectra generated by the absorption of electromagnetic radiation in the frequency range 400 to 4000 $cm^{-1.}$ Different functional groups and structural features in the molecule absorb at characteristics frequencies. The frequency and intensity of absorption are the indication of the band structures and structural geometry of the molecule. FTIR spectra were taken using Perkin Elimer-spectrum RXI model

3.3 DLS particle size analyzer

A laser diffraction method with a multiple scattering technique has been used to determine the particle size distribution of the CuO nanoparticle powders. It was based on Mie-scattering theory. In order to find out the particles size distribution of the samples were dispersed in water by horn type ultrasonic. Then experiment was carried out in computer controlled particle size analyzer to find out the particles size distribution.

3.4 XRD analysis

XRD power diffraction was used to characterize the metal nanoparticles prepared by using chemical synthesis method. The size of the particles was determined using the X-ray diffraction technique. XRD patterns were calculated using X'per Rota flex diffraction meter using Cu K radiation and λ =1.5406 A°. Crystallite size is calculated using Scherrer equation (Prema, 2010).

$$CS = K\lambda / \beta \cos \theta$$

Where CS is the crystallite size
Constant (K) = 0.94
β is the full width at half maximum (FWHM)
Full width at half maximum in radius (β) = FWHM x π/180
$\lambda = 1.5406 \times 10^{-10}$, Cos θ = Bragg angle.

3.5 Scanning Electron Microscope (SEM)

The nanoparticles obtained were analyzed by SEM. Their image reveals the size and shape of nanoparticles (Prema, 2010). SEM image shows that the synthesized nanoparticles were more or less uniform in size and shape.

4. Antibacterial activity of the chemically synthesised CuO nanoparticles

4.1 Resazurin dye reduction method (Satyajit *et al.,* 2007)

4.1.1 Reagents required

- Ciprofloxacin antibiotic solution
- Resazurin dye

- Culture of *E.coli* and *Staphylococcus auerus* maintained in 10^6 cells
- Luria bertani broth and Nutrient broth

4.1.2 Dye preparation

The resazurin solution was prepared by dissolving a 270 mg tablet in 40 ml of sterile distilled water. A vortex mixer was used to ensure that it was a well-dissolved and homogenous solution.

4.1.3 Procedure

- Plates were prepared under aseptic conditions. A sterile 96 well plate was labeled.
- A volume of 100 µL of test material in 10% (v/v) sterile water (usually a stock concentration of 1 mg/ml for purified compounds, and 10 mg/ml for crude extracts) was pipetted into the first row of the plate.
- To all other wells 50 µL of nutrient broth or normal saline was added. Serial dilutions were performed using a multichannel pipette. Tips were discarded after use such that each well had 50 µL of the test material in serially descending concentrations.
- To each well 10 µL of resazurin indicator solution was added.
- Finally, 10 µL of bacterial suspension (5×10^6 cfu/ml) was added to each well to achieve a concentration of 5×10^5cfu/ml.
- Each plate was wrapped loosely with cling film to ensure that bacteria did not become dehydrated.
- Each plate had a set of controls: a column with a broad-spectrum antibiotic as positive control (usually ciprofloxacin in serial dilution), a column with all solutions with the exception of the test compound, and a column with all solutions with the exception of the bacterial solution adding 10 µL of nutrient broth and placed in an incubator at 37 °C for 18–24 h.
- The colour change was then assessed visually. Any colour changes from purple to pink or colourless were recorded as positive.

4.2 Disc Diffusion Method (Bauer *et al.*, 1966)

- Nutrient broth/agar
- 1g beef extract
- 1g peptone
- 0.5 g NaCl dissolved in 100 ml of double distilled water.

The media was autoclaved, cooled and kept for inoculation.

The Muller Hinton agar media was prepared and poured in the petri discs and kept for 30 mins for solidification. After 30 mins the fresh overnight cultures of inoculum (100 µl) of two different cultures were spread on to solidified Muller Hinton agar plates. Sterile paper discs made of Whatman filter paper, 5 mm diameter (dipped into various concentrations from 20µg to 100 µg/ml CuO nanoparticles) along with standard antibiotic containing discs were placed in each plate. The cultured agar plate were incubated at 37^0C (310K) for 24 h. After 24 h of incubation the zone of inhibition was investigated.

5. Effect of copper oxide nanoparticles on activity of enzymes in *E.coli*

5.1 Assay of β-glucosidase activity

250 ml of *E.coli* culture was grown at 25° C for 72 hrs and centrifuged at 4500 rpm for 10 minutes at 4°C.The pellet was washed with chilled PBS of 10 ml and the sample was sonicated. Sonicated sample was centrifuged at 10000rpm and 4^0C, the supernatant was separated.100µl of that was added in each flask along with 100 µl of citrate phosphate buffer (0.1M, pH5).This was followed by addition of 100µl of 0.55×10^{-2}M of *p*NPG and incubated at 37 ˚C for 30 minutes. After incubation, 1M sodium carbonate was added to stop the reaction allowing the yellow color of the *p*-nitrophenolate ion to develop and 2.0 ml of ethanol was added. This was followed by centrifugation at 10000 rpm for 15 minutes .The supernatant was taken and the optical density of each sample of measured to know the relative β- glycosidase activity.

In the presence of enzyme β-glycosidase enzyme, the substrate p-nitro phenyl–β-glycoside (pNPG) is converted into the products p-nitrophenol (PNP) and glucose. This reaction can be monitored by the change in color from colorless to yellow colored solution.

5.2 Formation of ovalbumin and copper oxide nanoparticles complex monitored by DLS particle size analyzer

Ovalbumin solution was prepared by dissolving the protein in 100ml in PBS to a concentration of 5mg/ml and it is added1000µl of the protein solution to pre-synthesized metal Nanoparticles solution. Here 935*µl* of CuO nanoparticles was added with 65 µl of protein to make up the volume to 1ml and this was incubated with 1mM copper oxide nanoparticles for 10mins. After the incubation formation of ovalbumin and copper nanoparticles binary complex was monitored by DLS particle size Analyzer.

5.3 Isolation and characterization of yeast cells

5.3.1 Sample collection

Yeast sample were collected from the sugar factory effluents.

5.3.2 Isolation of yeast

The samples were serially diluted using the sterile saline. The yeast species were isolated by streak plate technique using YPD agar plates. The samples were mixed thoroughly and streaked and incubated at 37 C for two days. Later the plates were observed for single colonies. A single colony was picked out from the plate and it was sub cultured in YPD broth for further studies.

5.3.3 Characterization of yeast by biochemical test

The prepared subculture was used for the following test

5.3.4 Simple staining

- The smear was produced and the heat fixed on to a microscopic slides.
- The crystal violet stain was added ton to the smear and kept for 1 minute.
- The slide was rinsed with water and after that it was air dried and observed under microscope.

5.3.5 Gram staining

- The smear was produced and the heat fixed on to a microscopic slides.
- The crystal violet stain was added ton to the smear and kept for 1 minute.
- Then the slide was rinsed with water and Gram's iodine was added and kept for 1 minute.
- Then the slide was rinsed with water and decolouriser (acetone) was flooded on to the slide.
- After rinsed with water the saffranin solutions were added and again the slide was rinsed with water, air dried and observed under the microscope.

5.3.6 Estimation of LPO in yeast cells

Yeast cells were obtained from YPD broth/ the broth was centrifuged at low speed to remove the supernatant(TBARS method).

5.3.7 Reagents required

- KCl (30mM)
- Ferrous sulphate (0.16mM)
- Ascorbate (0.06mM)
- SDS (10%)
- TBA (1%)
- Acetic acid
- n- propanol:pyridine (15:1)

5.3.8 Procedure

The reaction mixture containing 0.1ml of yeast cells ,0.1ml of potassium chloride , 0.1ml of Ferrous sulphate and 0.1 ml of ascorbate was incubated for one hour at 37°c in the presence and copper oxide nanoparticles.0.4ml of the reaction mixture was added with 0.2ml of SDS, 1.5mlof TBA and 1.5ml of acetic acid. The mixture was kept in a boiling water bath for 20mins. After cooling, 1.0ml distilled water and 5.0ml of n-propanol and pyridine was added. After centrifugation, the pink colored chromophore was measured at 532nm.

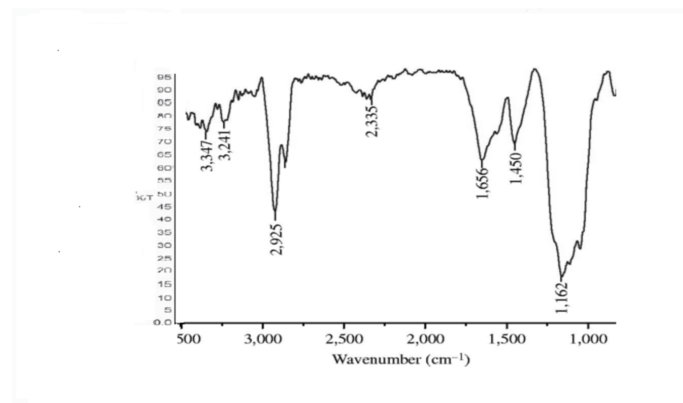
6. Results

The synthesized copper oxide nanoparticles were visually observed. The colour change was observed which confirmed the reduction of Cu^{2+} to Cu^{+} ions. The precipitate was washed with water and ethanol and kept for air dry (Plate 1).

Plate.1. *Copper oxide nanoparticles synthesis*

The FTIR spectra of copper nanoparticles are shown in the fig.1. The following peak were observed in spectrum 3,347 cm^{-1} assigned to O-H (s) stretch, 3,241cm^{-1} assigned to O-H (s) stretch, 2,925 cm^{-1} assigned to C-H alkane (s) stretch, 2,335 cm^{-1} assigned to S-H vibration stretch, 1,656cm^{-1} assigned to C=C (m, w) stretch, 1,450cm^{-1} assigned to CH_3 umbrella deformation (v) scissoring and bending and 1,162cm^{-1} assigned to C=O (s) stretch.

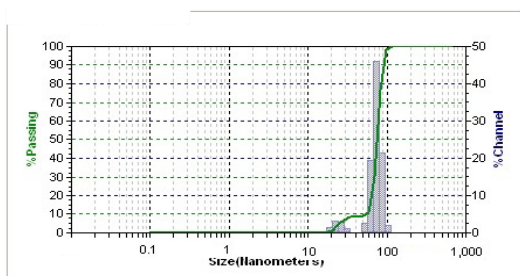
Fig.1. *FTIR spectrum of copper oxide nanoparticles*



The FTIR analysis of the copper oxide nanoparticles revealed the presence of various compounds.

The DLS particle size analyzer was used to analysis the range of the nanoparticles in figure 2 copper oxide nanoparticles. Size of Copper oxide nanoparticles are in the range of 20-100 nm.

Fig.2. *DLS particle size analyser for Copper oxide nanoparticles*



The X- ray diffraction studies reveal the characterization of the chemically synthesised metal oxide nanoparticle respectively. The 2 θ values ranges from 10-80 were observed (table 1: figure 3). According to this, the size of crystal was calculated and the

average size of the metal nanoparticles was observed. Copper oxide nanoparticle is 26.9 nm.

Fig.3. *XRD analysis of Copper oxide nanoparticles*

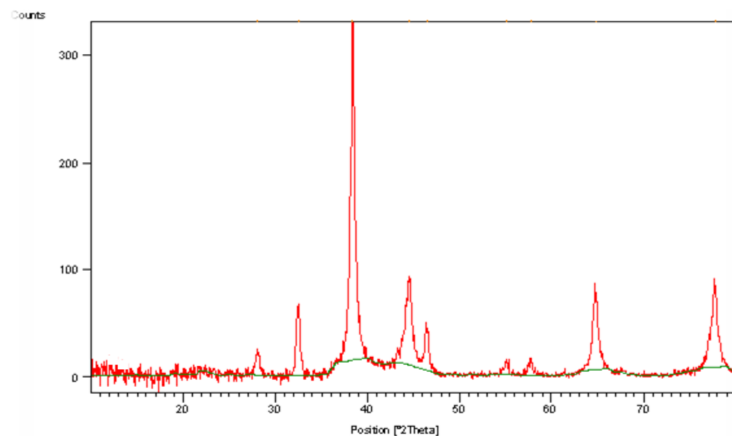
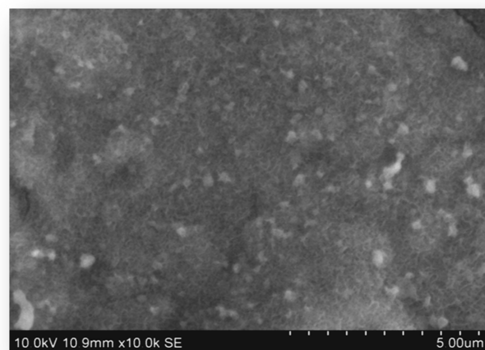


Table 1. *XRD table of copper nanoparticles*

Pos. [°2Th.]	Height [cts]	FWHM [°2Th.]	d-spacing [Å]	Rel. Int. [%]	Particle size in nm
25.0523	14.98	0.2004	3.10185	33.51	42.44
28.0437	12.93	0.2007	3.18185	44.71	42.63
32.4386	78.92	0.4015	2.76010	25.88	21.535
34.2248	101.61	0.3346	2.62003	33.32	25.92
46.4844	188.60	0.2175	1.95363	63.43	41.54
55.1135	49.99	0.3680	1.66643	16.81	25.44
57.6944	46.58	0.3346	1.59788	15.66	28.32
67.7475	14.42	0.8029	1.38318	4.85	12.454
74.7433	5.61	0.9792	1.26906	1.89	10.67
77.4916	79.89	0.5712	1.23077	26.20	18.64
Average size =26.9					

The scanning electron microscope was used to detect the size of metal nanoparticle was seen in various magnification (Plate 2). From the SEM micrographs it is revealed that the aggregations of chemically synthesised nanoparticles were observed.

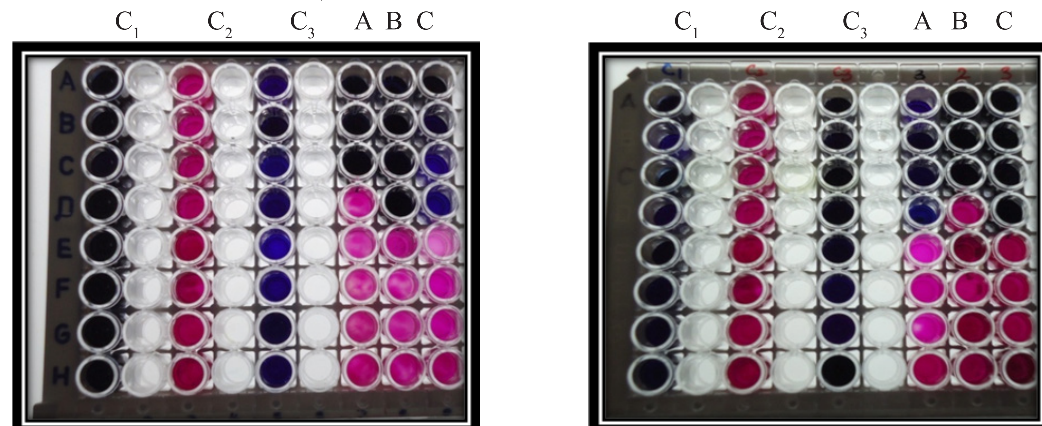
Plate.2. *SEM micrograph of Copper oxide nanoparticles*



Further the synthesised metal (CuO) nanoparticles were subjected to study the antibacterial activity against skin infection pathogens *E.coli* and *S.aureus* by Resazurin dye reduction method and disc diffusion method.

The antibacterial activity by resazurin dye reduction method where the resazurin dye is an oxidation- reduction indicator which evaluates the cell growth. The change in colour from dark blue to pink indicate no inhibition, dark blue remaining indicate inhibition of cell growth. In this method the observed results where the dark blue indicate the level of inhibition for various metal oxide nanoparticles against various microorganism (Plate 3).

Plate.3. *Antibacterial activity of copper oxide nanoparticles*

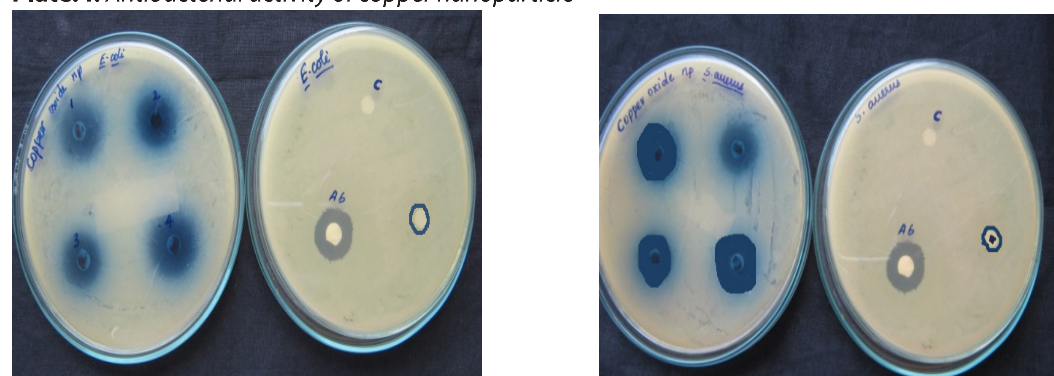


E.coli **Staphylococcus aureu**

C_1 - Dye control C_2 – ve control and C_{3+} ve control and A, B and C are the test samples at serial diluted

The antibacterial activity was done by disc diffusion method. The zone of inhibition of copper nanoparticles against *E.coli* was 11-16 mm and *S.aureus* was 12-17 mm. (Plate 4).

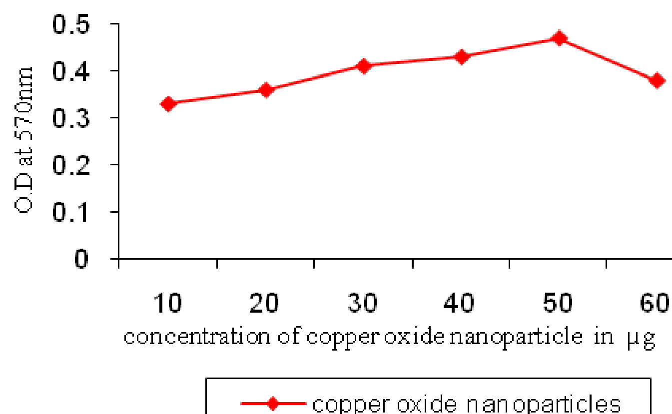
Plate.4. *Antibacterial activity of copper nanoparticle*



Escherichia coli **Staphylococcus aureus**

Fig.5. *Effect of copper oxide nanoparticles in glucosidase activity in E.coli cells*

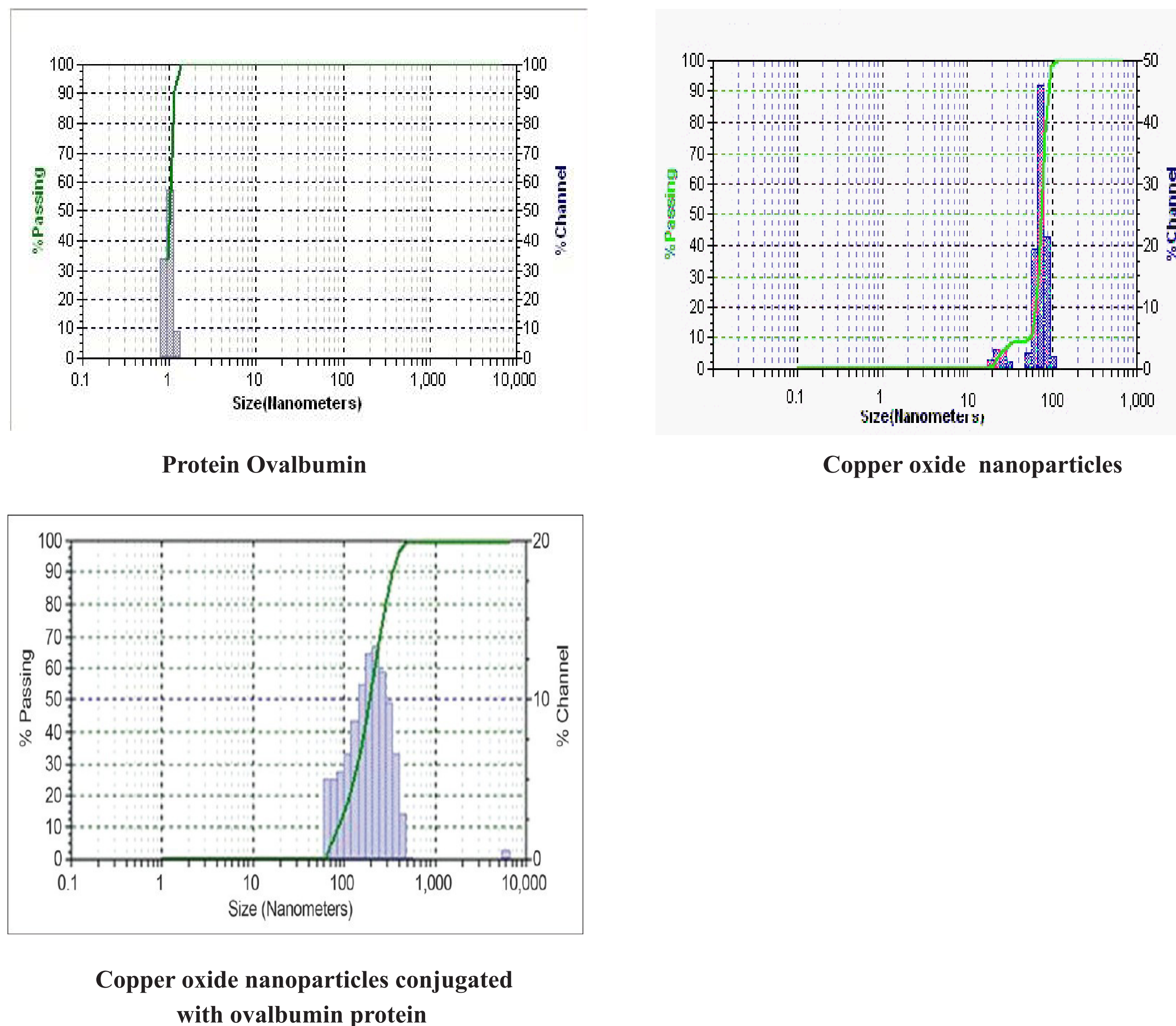
B-glucosidase activity in *E.coli* treated with copper oxide nanoparticles



The β-D glucosidase activity was analyzed using *E.coli* culture treated with various concentrations of metal nanoparticles. The substrate used here is OPNG which is chromogenic substrate. The action of the enzyme results in the colour change which was

absorbed at 570nm. The increase in silver nanoparticle concentration up to 30 µg/ml exhibited normal activity and after that the activity of the enzyme was reduced similarly for copper nanoparticles and zinc nanoparticles at 40 µg/ml concentration (Fig.6).

Fig.6. *Protein nanoparticles conjugate formation with DLS particle size analyser*



Protein Ovalbumin

Copper oxide nanoparticles

Copper oxide nanoparticles conjugated with ovalbumin protein

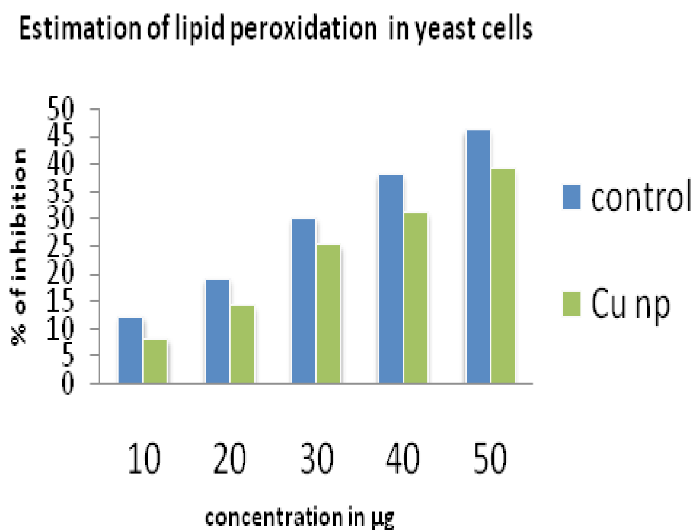
The metal nanoparticle (copper oxide) have the ability to form conjugate with protein and it is used in drug delivery and the proteins gets absorbed by hydrogen bonds or by other forces. From the DLS particle size analyzer the size of the protein is 0.8-1.2 nm , the size of the copper oxide nanoparticle 20-100 nm. The size of the metal nanoparticle conjugate is in the range of 60-400nm copper oxide nanoparticles.

Table 2. *Copper oxide nanoparticles conjugated with ovalbumin protein*

Protein size in nm	Avg. protein size in nm	Copper oxide nanoparticle size in nm	Avg. copper oxide nanoparticle size in nm	Protein nanoparticle Conjugate size in nm	Avg. Protein nanoparticle Conjugate size in nm
0.8-1.2	1.0	20-100	40	60-400	170

The nanoparticles can cause oxidative stress in the cells and there by the Enzymic and non Enzymic antioxidants level increases. The yeast cells were treated with various concentrations of copper oxide nanoparticles 100,200,300,400 and 500 mg/ ml. This culture was used to estimate lipid peroxidation in the cells. The Lipid peroxidation was studied by TBARS methods where the for copper oxide nanoparticles were effective at 400 mg/ml(fig. 7).

Fig.7. *Estimation of LPO in yeast cells*



7. Discussion

Metal oxide nanoparticles were prepared by using chemical reduction method and the respective protocols described in the methodology was followed. The characterization of nanoparticles was done by UV-Vis spectrophotometer, FTIR, XRD and SEM analysis.

The absorption spectrum between 540-600 nm is the characteristic of the copper nanoparticles where the shift from red or blue is observed (Anjali *et al.,* 2005). These results were in accordance to the observed results.

The FTIR spectrum of copper oxide spectrum were identified using the standard table where the nature and structure of the chemical compound was detected. The copper oxide nanoparticles spectrum found in Anjali et al. (2005) was similar to the spectrum observed in the result.

The scanning electron micrograph of silver nanoparticles is depicted and the micrograph of copper nanoparticles is depicted and the micrograph where the particles as powder and those embedded in were scanned which revealed them formation of fluffy agglomerates (Khanna et al., 2007).

The XRD analysis of the chemically synthesized metal oxide nanoparticles were analysed to find the average size of the crystal by using Debye Scherer formula (Prema, 2010). copper oxide nanoparticles average crystallite size was 20 nm were found to be accordance with the observed results .

The antibacterial activity was studied by resazurin dye reduction method. It is found that the change of colour of the dye is due to the oxidation- reduction reaction, where the resazurin is reduced to resorufin by the oxido reductase within the viable cells. Resorufin is further reduced to hydroresorufin. A resazurin reduction test has also been used for decades to demonstrate bacterial inhibition (Bigalke *et al.,* 1984). The reduction was observed by the characteristic colour change.

The antibacterial activity studied (disc diffusion method) by Bauer et al. 1966 method for the synthesized silver nanoparticles showed higher activity for gram +ve and gram –ve (Guzman et al., 2009).the results were in accordance to the observed results. These results clearly demonstrated the inhibition of growth and multiplication of the test bacteria.

The study of a protein nanoparticle conjugates has gained lot of interest as it have a wide application in detection and drug delivery. Connolly et al. (2001) have often observed the study of protein attachment protocol that minimizes nanoparticles aggregation. The sample with higher degree of aggregation showed decreased enzyme activity (Sergreev *et al.,* 2003). This is in accordance to the observed result where the concentration of nanoparticles increases the activity of the enzyme decreases.

The interaction of the metal oxide nanoparticles to the cells depends on not only the metal nanoparticles employed but also the microbial species tested (Hassen *et al.,* 1998).

8. Conclusion

Although new proteins are available for medical purposes, their administration as therapeutics still remains difficult. Nanosystems seem to be the optimal solution to improve protein bioavailability, bio distribution and safety. Moreover, the combination of nanoparticles with proteins could also be a valid system to achieve the design of efficient nanovectors for drug delivery. Indeed, nanoparticles can be properly tuned for specific applications and could be precisely designed to meet biological needs. However,

to fulfill this purpose, it is necessary to better clarify the nature of interaction between nanoparticles and biomolecules. The control of the protein denaturation is another important parameter that needs a deeper understanding. Further investigations should help to manage these hybrid nanosystems, opening new therapeutic and diagnostic perspectives as well as new challenges in the near future.

9. Reference

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