An investigation on the synergistic effect of Cu2O-Ag nanoparticle on its bactericidal and anticancerous properties

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Abstract
Copper and silver are known to have good antibacterial and anti-fungal properties at nanoscale. Cu2O-Ag synthesis was attempted using hydrazine hydrate reduction method. The formation of Cu2O-Ag nanoparticles was confirmed using powder x-ray diffraction technique. Further the morphology and elemental compositions were analyzed by FESEM, HRTEM and XRF techniques. The antibacterial activity of the formed nanoparticles exhibited an excellent zone of inhibition compared to the existing antibiotics. The microbial broth curve obtained against Pseudomonas aeruginosa further confirmed the remarkable inhibiting property of the nanoparticle in a short span of 6 h post treatment (HPT). The synthesized nanoparticle showed very good activity against HCT-15 colon cancer cells by inhibiting their viability up to 27% when treated at higher concentration. Thus the synthesized nanoparticles seem to be a good candidate for various biological applications.

1. Introduction

Food and water contaminations are major setbacks in recent times especially in developing countries as they result in majority of the parasitic diseases and infections [1]. Most pathogenic microorganisms which contaminate the water/food have to be treated prior to its use. Additionally, antimicrobial resistance (AMR) of the microorganisms seems to be an added challenge as the existing antibiotics are rendered incompetent and also their usage cause adverse side effects [2]. This pose great health challenges around the world as the multidrug resistance bacteria and biofilm formations evolve from time to time. Lately, substantial interests are shown in using nanotechnology for microbial treatments as they possess good biocidal effects.

The antibacterial effect of the nanomaterials on microorganisms is accredited to their shape, small sizes and high volume to surface area that allows them to interact effectively [3]. The metal nanoparticles also find its applications in dentistry, textile industries, wound healing, medicinal instruments and device coatings etc [4]. The mechanism of action of the nanomaterial is still debatable as they possess the ability to penetrate into or act on the bacterial cell surface leading to the death of the microorganism [5]. The effective bactericidal property of any nanomaterial depends on the concentration of the nanomaterial, type of bacterial strain and the initial concentration of the bacteria used. Some common techniques used in identifying their antibacterial effect include plating techniques like well and disk diffusion method and suspension techniques like microbial broth method [6].

Metal nanoparticles such as silver [7–10], cobalt [11], mercury, gold, copper [12] and zinc exhibit excellent antibacterial activity against a set of microbial strains. These nanoparticles are less stable because they are air sensitive leading to their futile effect on microorganisms. Surface modification/ stabilization are one solution to the problem but they pose complications in preparation and characterization [13]. For the effective use, alloying them is a desirable option as the synergistic effects can enhance their biological property.

Development of these alloys using simple wet chemical process is challenging because of the differences in redox potentials of the metals ions and the type of synthesis used [14]. Among the existing metals copper and
silver when combined exhibit enhanced antibacterial effects thus can be utilized for the aforementioned applications. Nazeruddin et al have successfully studied the synergistic bactericidal effects of Ag-Cu nanoalloy against B.subtilis microorganism which showed an inhibitory zone of 15mm for Ag nanoparticles, 18mm for Cu nanoparticles and 28mm for AgCu nanoalloy respectively [15]. Some commonly used solution based techniques involve co-reduction or successive reduction of metal complexes using chemical or biomolecule reducing agents in presence or absence of external radiations [3]. The synthesis approaches evolve continuously to make the process easier, cost effective and scalable thus making them useful for wide variety of applications [16]. Also by developing simple and novel synthesis techniques it is easier to produce alloy nanoparticles with desired properties.

The present manuscript addresses the synthesis of Cu2O-Ag heterostructured nanoparticles through chemical synthesis route by using hydrazine hydrate as the reducing agent. Further the viability of bacteria and colon cancer cells under the influence of the Cu2O-Ag nanoparticle are studied using well diffusion, microbial broth and MTT cytotoxicity assay inorder to understand their effects on living cells.

2. Materials and method

2.1. Materials
Silver Nitrate (99%, AR) was purchased from Emplura, Merck, India. Copper sulphate (extra pure AR, 99.5%) was purchased from SRL Chemicals, India and Hydrazine hydrate (80%, AR) was from Rankem Pvt. Ltd, India. Ethanol used for centrifugation purposes was procured from Haymann, India. The DI water was obtained from Merck Millipore MilliQ unit with a resistivity of 18.2 MΩ. Cm and all the reagents were used without further processing.

Muller Hilton agar (MHA) used for the antibacterial activity and microbial broth method was bought from Sigma Aldrich. DMEM media with 1X Antibiotic Antimycotic Solution and 10% fetal bovine serum used for cytotoxicity assay was procured from Himedia, India. MTT dye, PBS buffer solution and DMSO used for cytotoxicity testing were purchased from Sigma Aldrich. The bacterial strains used for studying the activity include B. subtilis (gram positive) and P.aeruginosa (gram negative) were procured from Microbial type culture collection (MTCC), Chandigarh, India and colon cancer cell line (HCT-15) was obtained from National centre for cell science (NCCS), Pune, India

2.2. Method
0.05 M (equimolar) of copper sulphate and silver nitrate in 50 ml DI water was magnetically stirred until they dissolved completely. The solution temperature was increased to 60 °C. On reaching, 5M hydrazine hydrate was added and the temperature was further raised to 90 °C. The precipitate obtained was centrifuged, washed using water and ethanol alternatively and dried in hot air oven overnight at 60 °C.

2.3. Antibacterial activity
Test pathogens were spread on Mueller-Hinton agar (MHA) plates having a well of 6 mm dia. The wells were made using sterile cork-borer in which the test compounds in required amount were loaded. The plates were then incubated for 24 h at 37 °C. The zone of inhibition (diameter measured in mm) for various quantity of nanoparticles were measured using a scale in mm. Positive control (Ciproflaxin drug) of 20 μg was loaded for comparison and all the measurements were carried out in triplicate.

2.4. Microbial broth method
The killing kinetics of the Cu2O-Ag heterostructured nanoparticles at various concentrations were investigated at 4x MIC (minimum inhibitory concentration). Cultures were allowed to grow to exponential phase (optical density @ 600 nm ~ 0.5) and were then diluted to 3.5–5.5 × 10⁶ CFU/ml in MH broth 2. They were then challenged with various concentrations of the nanoparticles and incubated at 37 °C. The optical density for each incubation period was recorded at 600 nm. All the measurements were done three times for standardisation.

2.5. Cytotoxicity assay
HCT-15 colon cancer cell lines were plated separately in 96 well plates with the concentration of 1 × 10⁴ cells/well in DMEM media with 10% fetal bovine serum and 1X Antibiotic Antimycotic Solution in CO₂ incubator at 37 °C with 5% CO₂. The cells were washed with 200 μl of 1X PBS. The cells were subjected to various concentrations (25, 50, 100, 250 and 500 μg ml⁻¹) of test compound within the serum free media and incubated for 24 h. The medium was aspirated from cells at the end of the treatment period. 0.5 mg ml⁻¹ MTT prepared in 1X PBS was added and incubated at 37 °C for 4 h in the CO₂ incubator. After incubation, the medium containing MTT was discarded and the cells were washed using 200 μl PBS. The formed crystals were
dissolved with 100 μl DMSO and mixed thoroughly. The absorbance at 570 nm was measured using microplate reader.

2.6. Characterization techniques

The crystallinity of the Cu2O-Ag nanoparticles was measured using Panalytical xpert pro instrument with a Cu Kα source (λ = 1.5406 Å) for 2θ ranging from 20° to 90°. The morphology, and size of the Cu2O-Ag nanoparticles were characterized using field emission scanning electron microscopy (FESEM, Model: Quanta 200) procured from FEI company, HRTEM (TEM 2200 Plus, JEOL) while Xray fluorescence procured from Panalytical (XRF, Model: Minipal 4 Benchtop) was used for confirming the elements present in the sample.

The optical density measurements carried out for microbial broth method and cytotoxicity assay were measured using ALERE (AM2100) microplate reader. The morphology of the cells for cytotoxicity studies was examined using phase contrast inverted fluorescence microscopy (images were not acquired under fluorescence) procured from Nikon instruments Inc., USA (Nikon ECLIPSE TE2000-U).

3. Results and discussion

The crystalline nature of the heterogeneous nanoparticle analyzed using XRD is shown in figure 1. Two separate phases namely copper oxide and silver peaks with hkl planes as marked in figure 1 matches well with JCPDS card number 077–0199 of copper oxide and 087–0720 of silver respectively confirming its heterogeneous nature formed due to the difference in the reduction potential of copper and silver. Also presence of a very small quantity of copper impurity in the sample was observed (peaks marked as*). From the xrd pattern the crystallite size was calculated using Debye- Scherer formula to be ∼15 nm. Also the ratio of Cu2O : Ag: Cu were analyzed and found to be in the ratio of 65:29:6.

Several factors influence the formation of the products some of which includes the type precursor salts and their concentrations used, pH and temperature of the reaction, time and the type and concentration of reducing agent used for the reduction of the metal salts [17]. Hydrazine hydrate, a strong reducing agent was employed here for the synthesis in order to avoid the influence of the difference in the reduction potentials of Ag (0.80 V) and Cu (Cu0+/Cu2+ = 0.34 V) during the co-reduction of both the metal ions [5]. However, copper due to its feasibility towards oxidation led to the formation of Cu2O-Ag nanoparticles. Also, copper being highly unstable in aqueous medium [14], the high temperature (almost 90 °C) that was used for hydrazine hydrate - metal ion complex reduction and the presence of silver ions in the solution facilitated the formation of Cu2O-Ag nanoparticle as confirmed through XRD.

The Cu2O-Ag was studied using FESEM and HRTEM and the images from both the analyses are shown in figure 2. The size and shape of the particles are in wide range due to the formation of a mixed phase nanoparticles which include copper oxide and silver as confirmed by XRD. The FESEM image shows particles of varied sizes but of two different morphologies i.e. faceted and irregular shaped particles (figure 2(a)). TEM analyses of the sample exhibit particles of different contrast (figure 2(b)). The d-spacings of the nanoparticles were calculated from the high resolution TEM images. The darker faceted particle (marked by circle 1 in figure 2(b)) has a d-spacing of 0.232 nm corresponding to the (111) plane of Ag. On the other hand the Cu2O particles are composed of many smaller particle of around 10–15 nm in sizes and are agglomerated together. The d-spacing of 0.247 nm obtained for these particles (marked by a circle 2) aligns well with the (111) plane of Cu2O. This is
further confirmed by EDS analysis. However due to the presence of Cu in the sample quantitative measurement was not possible from TEM analysis. Additionally, the presence of copper and silver was confirmed using XRF (figure 3).

The bactericidal property of the synthesized copper oxide silver nanoparticles was evaluated against *B. subtilis* gram positive and *P. aeruginosa* gram negative bacteria. Agar plates exhibiting the inhibition zone is seen in figure 4 and the measured values (in mm) of the zones is tabulated in table 1. The inhibition zone for gram negative bacteria is little higher than gram positive bacteria (figure 5) indicating that the nanoparticles could have ruptured the cell walls of the bacteria and penetrated inside leading towards cell death. Although profound difference in the inhibitory zone was not observed in this case between gram positive and negative bacteria there are several factors that could possibly lead towards cell death some of which includes the surface charge, size,

Figure 2. FESEM image (a) and HRTEM images of the of Cu$_2$O-Ag nanoparticles at different magnifications (b)–(d).

Figure 3. XRF spectrum of Cu$_2$O-Ag nanoparticle.
shapes and ion dissolution of the nanoparticles [18]. Based on these factors the nanoparticles either interact from the surface or penetrate into the cell leading towards DNA damage, membrane perforation, protein denaturation etc ultimately causing morbidity [19, 20]. The difference in the cell wall between gram positive and gram negative bacteria is one of the main cause for the difference in the inhibition zone [21] where gram negative bacteria has thin peptidoglycan layer and no teichoic acids compared to gram positive which has a thick peptidoglycan layer and many teichoic acids leading to easier penetration of the nanoparticle.

In general gram negative bacteria like \textit{P. aeruginosa} exhibit microbial resistance against the existing wide variety of antibiotics and the nanomaterials as they have the ability to form slime layers (biofilms) on any inanimate surfaces [20]. This biofilm formation entraps and diminishes the interaction of any antibacterial agent towards the bacterial membrane. In case of Cu$_2$O-Ag heterostructured nanoparticle, it could be inferred that they exhibit a very good antibacterial zone against \textit{P. aeruginosa} expressing their significant antimicrobial property which could possibly be attributed to the high dissolution of both copper and silver ions when

### Table 1. Showing the zone of inhibition formed when treated with different quantity of nanoparticle against the gram positive and gram negative bacteria.

| S.no | Strains  | 1 mg/ml | 500 μg/ml | 250 μg/ml | 125 μg/ml | Positive control |
|------|----------|----------|------------|------------|------------|-----------------|
| 1.   | \textit{B. subtilis} | 28 mm    | 27 mm      | 19 mm      | 17 mm      | 28 mm           |
| 2.   | \textit{P. aeruginosa} | 30 mm    | 28 mm      | 22 mm      | 19 mm      | 28 mm           |

### Figure 4. Agar plates exhibit the inhibitory zone formed due to the treatment of various volumes of Cu$_2$O-Ag heterostructured nanoparticles against gram positive and gram negative bacteria.

### Figure 5. A graph displaying the difference in zone of inhibition between gram positive and gram negative bacteria.
dispersed in liquid water medium further leading to their active interaction to the bacterial cell surface (bacteria has a negative cell surface).

Li et al synthesized copper oxide nanoparticles with different shapes and studied the antibacterial activity of these nanoparticles on 4 different strains where it revealed that the inhibition zone was obtained only when the plates were treated with higher volumes of nanoparticles (1 mg ml$^{-1}$ to 8 mg ml$^{-1}$) [22]. Below 1 mg ml$^{-1}$ concentration of copper oxide nanoparticle the zone was negligible whereas for Cu$_2$O-Ag nanoparticles the presence of both copper oxide and silver inhibits the cell multiplication at lower volumes indicating the synergistic effect of both the elements against the bacteria. Further detailed studies need to be carried out for the better understanding about the antibacterial mechanism of the Cu$_2$O-Ag heterostructured nanoalloy and also the exact cause of the bacterial death.

Due to the high virulent nature and antibacterial resistance of \textit{P. aeruginosa}, the bacteria was subjected to microbial broth dilution method and the effect of the Cu$_2$O-Ag nanoparticles was effectively studied for upto 6 h after incubation.

The bacterial growth curve was monitored in liquid media against \textit{P. aeruginosa} strain and the time dependent changes were plotted at a regular interval of 2 h upto 6 h as shown in figure 6. The OD measurements of the control and bacterial solutions treated with different quantity of Cu$_2$O-Ag nanoparticle (varying from 500 µg ml$^{-1}$ to 31.25 µg ml$^{-1}$) shows the viability of the bacteria over a period of time. In general, as bacteria grows the turbidity of the liquid medium increases leading to the increase in the absorption values. From the below graph its clearly visible that as the volumes of nanoparticle added increases the OD values decreases indicating the effect of the nanoparticles in delaying the cell growth. The OD values of the control bacteria is the highest whereas as the concentration of the nanoparticle increases the growth curve continuously decreases indicating the bactericidal effect of the nanoparticles. Also, it can be observed that for higher concentration the death phase has already started at 6 h implying the potential of the nanoparticles to act against the microorganism.

Pang et al in 2009 have studied the bacteriostatic effects of copper oxide against 4 different bacteria and have evaluated their minimum inhibitory concentrations (MIC) [23]. It was stated that MIC of a particular bacteria changes according to the morphology of the copper oxide nanoparticles they were subjected too. Here in this report the synergistic effect of both copper oxide and silver present helps in the inhibiting the bacterial growth at further lower concentrations. This indicates that the Cu$_2$O-Ag nanoparticles possess potential bactericidal properties which can be utilized for various biomedical, pharmaceutical, industrial applications and in water purification. Further studies needs to be carried out for better understanding of the effect and mechanism of these nanoparticles against microorganisms before they can be used for the aforementioned applications.

Copper oxide nanoparticles and silver nanoparticles have been assessed and analyzed separately for their anticancerous properties by various groups and the reports are documented thoroughly. The in vitro toxicity studies were carried out on different normal and anticancerous existing cell lines and the dose dependent nature of both the copper oxide and silver nanoparticles were highlighted [24, 25]. Sangliyandi gurnathan et al [26] and raga et al [27] biosynthesized silver nanoparticles and compared their dose dependent effects on human colon cancer cell lines (HCT15) thus indicating the anticancerous properties of silver. Similarly, Gopinath et al [28] and Nasim et al [29] have synthesized copper oxide using green synthesis and alcothermal method to study the anticancer nature on human adino carcinoma cell line (AGS) and glial cancer cell line (B92) in which the copper oxide exhibited dose dependent effects on cancer cell line while the normal cells were intact at the same concentration after 24 h. This clearly suggests the cytofriendly nature of these two nanoparticles towards normal cells while eradicating the cancer cells. Thus MTT assay was executed on the cancerous cell lines to know the effect of Cu$_2$O-Ag nanoparticles on them.
In vitro MTT cytotoxicity assay was carried out for 5 different concentrations of Cu$_2$O-Ag nanoparticles by treating them against colon cancer cell lines (HCT15) for 24 h and % the cell viability analyzed are shown in figure 7. When compared with control ones the % of cell viability decreases as the concentration of the nanoparticle increases. This confirms the dose dependent toxicity of the nanoparticles against cancerous cell lines and also the fact that they possess anti-cancerous properties. After 24 h, for the higher concentration of Cu$_2$O-Ag nanoparticles only 27% of the cells were viable for (500 μg ml$^{-1}$) indicating their potential to act as an anti-cancerous agent.

The morphology and viability of the cells studied under the microscope 24 h post treatment is shown in figure 8 which further confirms the dose dependent toxic nature of the nanoparticles when compared with control ones. The untreated control ones appeared normal whereas the nanoparticle treated cells appeared...
unhealthy and also clustered with cellular extensions at higher concentrations. This could be attributed to the disturbances in cytoskeletal function owing to the treatment of nanoparticles [30, 31].

4. Conclusion

Cu$_2$O-Ag nanoparticles synthesized were faceted and irregular with wide range of sizes due to the mixed phase formation. The mixed phase formation of copper oxide silver was confirmed using XRD while XRF confirmed the presence of copper and silver and absence of any impurities. Further Cu$_2$O-Ag nanoparticles showed and excellent antibacterial activity against B. subtilis and P. aeruginosa proving their biocidal nature. The bacterial growth curve obtained against P. aeruginosa strain depicts the effect of the nanoparticles at short span of time of 6 h due to the combinational action of both copper oxide and silver present in the nanoparticle. The Cu$_2$O-Ag nanoparticles also possess brilliant anti cancerous properties with increase in the nanoparticle concentrations. Thus the synergistic effect of Cu$_2$O-Ag nanoparticle makes it a potential candidate that can be exploited for various biomedical and other industrial applications.

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