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A simple, fast and cost-effective method of synthesis of cupric oxide nanoparticle with promising antibacterial potency: Unraveling the biological and chemical modes of action

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ABSTRACT

Background: Gradual attainment of bacterial resistance to antibiotics led us to develop a robust method of synthesis of stable, colloidal cupric oxide nanoparticle of physiological pH with potential antibacterial action.
Methods: Cu(II) oxide NP was synthesized by reduction–oxidation of $CuCl_2$, using polyvinyl alcohol as stabilizer. Characteristics and antibacterial activity of the particles were investigated by techniques like UV–Vis spectrophotometry, DLS, AFM, TEM, EDS, FTIR, AAS, agar plating, FACS, gel electrophoresis and XPS.
Results: The NPs were about 50 nm in size and cubic in shape with two surface plasmon peaks at 266 and 370 nm and had semi-conducting behavior with a band gap of 3.40 and 3.96 eV. About 80% of precursor $CuCl_2$ was converted to NP. The minimum inhibitory and the minimum bactericidal concentrations of CuO-NP were respectively 120 and 160 μg/mL for *Escherichia coli* and 180 and 195 μg/mL for *Staphylococcus aureus* in Luria–Bertani medium. In growth media, the NPs got modified by media organics with displacement of the stabilizer PVA molecules. This modified NP (around 240 nm) killed cells by generating ROS, which finally caused membrane lipid per-oxidation and chromosomal DNA degradation in NP-treated cells.
Conclusion: Reports indicate that we are among the few who had prepared CuO-NP in colloidal form. The antibacterial potency of our particle in growth media was much promising than other reports. Our findings demonstrated that 'particle-specific' effect, not 'ion-specific' one, was responsible for the NP action.
General significance: The NP may be used as a sterilizing agent in various bioprocesses and as substituent of antibiotics, after thorough toxicological study.

1. Introduction

Increasing resistance in bacteria towards antibiotics makes it imperative to research on new nanodrugs to combat a wide spectrum of infectious diseases. From this standpoint, metallic and metal oxide nanoparticles may be potential agents, from which antimicrobial drugs can be designed in the future. Of the different metals, copper is recognized as a good and less expensive antimicrobial agent and there is an age-old tradition of Indians to preserve drinking water in copper vessels for sterility, even before the existence of microorganism was acknowledged. Some recent reports on antimicrobial potency of metallic copper and copper oxide nanoparticles [1–8] suggest that the NPs may have wide applications in developing antibacterial drugs and in sterilizing foods, liquids, medical instruments and implants, human tissues, textiles, public areas etc. Compared to bulk copper salts, NPs of metallic copper or copper oxides are believed to have an enhanced antimicrobial activity because of their large surface to volume ratio and crystallographic surface structure.

Cupric oxide NP was prepared, mostly in precipitated solid form, by different methods like solid state reaction [9], electrochemical method [10], sonochemical process [11], microwave irradiation [12] and sol–gel technique [13], with various morphological structures such as nanoparticles, nanowires, nanotubes, nanorods, nanoflakes, nanosheets, nanoneedles, nanoribbons, nanorings, nanoleaves and nanoflowers. However, the method of synthesis of CuO-NPs in a suspended colloidal form is very rare and so far our knowledge goes, there are only two published reports on the preparation of colloidal CuO-NP [14,15]. The present study deals with the i) synthesis of stable colloidal suspension of Cu(II) oxide NP of physiological pH-7.5 by an innovative method, which was simple, fast and economic compared to the above referred methods, ii) characterization of the size, shape,

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composition and intrinsic properties (like optical and electrical) of the particles, iii) antibacterial potency and the biological mechanism of bacterial cell killing by the NPs and iv) chemical mechanism of the antibacterial action of CuO-NP.

2. Experimental

2.1. Preparation of Cu(II) oxide NP

Our method of synthesis of Cu(II) oxide NP was based on successive reduction–oxidation of $CuCl_2$ to CuO in presence of polyvinyl alcohol (PVA) and sodium borohydride ($NaBH_4$) under ambient condition. A solution of 5% PVA in Milli-Q water was first prepared by vigorous stirring for 2–3 h, until white foam was formed. To 3.0 mL of freshly prepared PVA, 1.5 mL of 100 mM $CuCl_2$ and 1.5 mL of Milli-Q water were added and vigorously stirred for about 2 h. Reduction of $CuCl_2$ to copper was first carried out by drop-wise addition of 9.0 mL of 33.72 mM $NaBH_4$ and pH of the reaction mixture was subsequently adjusted to 7.5 by adding 100 μl of 1(N) NaOH. Thereafter, the reduced copper was left for simple aerial oxidation by continuous stirring at 45 °C for 1–2 h, until a clear leafy green color appeared with the formation of CuO-NPs. Finally, pH of the solution was further adjusted to 7.5 with addition of 1–2 drops of 1.0(N) NaOH. The prepared colloidal suspension of Cu(II) oxide NP was stable for at least 2–3 months.

2.2. Characterization of the nanoparticles

2.2.1. Study of the optical property of the NP

The light absorption nature of the synthesized CuO-NP was investigated by scanning the absorbance of the 10 times diluted NP suspension in the wavelength region of 220–800 nm, using a UV–Vis spectrophotometer (Shimadzu, UV-1800); a solution of 10 times diluted 10 mM $CuCl_2$ in 1% PVA was taken in the reference cuvette.

2.2.2. Study of the size, shape and crystallinity of the NP

Hydrodynamic size of the NPs was regularly measured by a dynamic light scattering (DLS) instrument (Malvern, Nano-ZS). Moreover, to visualize the shape and to measure the size of core particles, the techniques of atomic force microscopy (AFM) and transmission electron microscopy (TEM) were used. For AFM study, dried NP film was prepared on a cleaned cover slip as described in [4] and scanned by an atomic force microscope (Veeco, di-Innova) in contact mode. For TEM study, NP suspension was placed on carbon-coated copper grid, dried in a vacuum desiccator, and analyzed by a transmission electron microscope (JEOL, JEM-2010) to obtain the size, shape and crystal planes [determined from the SAED (small angle electron diffraction) pattern] of the NPs.

2.2.3. Study of the composition of the NP and the trace of metal oxide bond in it

To analyze the elemental composition of CuO-NP, the NP suspension was taken on a cover-slip and dried in a vacuum desiccator. The cover-slip was then mounted using a carbon tape and the sample was coated with gold–paladium to study by an electron dispersion spectrometer (QUANTA-200).

Existence of metal oxide bond in Cu(II) oxide NP was traced by Fourier transformed infrared (FTIR) spectrometric study. NP suspension was first centrifuged at 55,000 rpm for 25 min in an ultracentrifuge machine (Sorvall, WX Ultra 90); the pellet was washed twice by Milli-Q water and then lyophilized in a lyophilizer (Heto, DW-3) to obtain fine dust of NPs, which was finally analyzed by a FTIR spectrometer (PerkinElmer L 120-000A), as described in [16].

2.2.4. Study of the conducting property of the NP

To investigate whether our CuO-NP had any conductive property, AFM study was performed using conductive probe-tip. NP sample was prepared on a silicon wafer and vacuum dried. The conductive mode AFM image was generated by measuring the current passing through the tip and the sample, when a DC bias was applied between them, using 'DLPCA-200 variable-gain, low-noise current amplifier' made by Femto, Germany.

2.2.5. Study of the extent of conversion of the precursor $CuCl_2$ to Cu(II) oxide NPs

The percentage of conversion of precursor $CuCl_2$ to Cu(II) oxide NP was determined by atomic absorption spectrometric (AAS) technique. For this study, the NP suspension was centrifuged at 55,000 rpm for 25 min; the supernatant was separated and the NP pellet was re-suspended in the same volume of Milli-Q water. The supernatant and the NP suspension (each of 1.0 mL) were acid digested and the copper content in each digested sample was analyzed using an atomic absorption spectrometer (PerkinElmer-AA200), as described in [4].

2.3. Analysis of antibacterial activity of CuO-NPs

This study was performed on Gram negative bacteria *Escherichia coli* K12 and Gram-positive bacteria *Staphylococcus aureus* 29213. For each bacterium, all the experiments were carried out with synchronized cells. The synchronization was done by the following steps: i) an inoculum of overnight grown cells was diluted 100 times in fresh Luria–Bertani (LB) medium [1.0 g bactotryptone, 0.5 g yeast extract and 1.0 g NaCl dissolved in 100 mL distilled water; pH adjusted to 7.5], ii) the inoculated cells were grown further at 37 °C in a gyratory shaker (Lab Companion, IS-971R) at 125 rpm, till the bacterial $(OD)_{600\ nm}$ reached the value 0.2 (corresponding to the exponentially grown cell concentration 10^8 cells/mL), iii) cells were then centrifuged, washed with and finally suspended in the same volume of starvation buffer (SB) [5.0 g KCl, 1.0 g NaCl, 1.2 g Tris, 0.1 g $MgSO_4$, 1.0 mL of 1.0 M $CaCl_2$ in 1000 mL distilled water; pH adjusted to 8.1], and iv) the cells were allowed to starve with shaking at 37 °C for 1 h for synchronization.

2.3.1. Study of the antibacterial potency of the NP

The strength of antibacterial action of CuO-NP was studied by determining its minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) on *E. coli* and *S. aureus* cells. For a particular bacterium, the MIC of an antibacterial agent is defined as such a concentration, which when present in growth medium causes complete inhibition of cell growth without cell killing even after overnight (18 h) incubation [8]. On the other hand, the MBC of an antimicrobial substance is defined as the concentration, the presence of which in the growth medium results 99.9% cell killing on overnight (18 h) incubation [8]. The following protocol was used to determine the MIC and MBC of Cu(II) oxide NP. Synchronized cells, made as described above, were diluted 100 times in fresh LB medium containing different concentrations of the NP ranging from 0 to 160 μg/mL (in terms of copper content, considering 80% conversion of $CuCl_2$ to CuO-NP, as observed from the AAS result). The diluted cultures were incubated at 37 °C with shaking at 125 rpm for 18 h. Cell aliquots of equal amount were withdrawn from each of the incubated cultures and each aliquot was serially diluted in SB. An amount of 0.1 mL cells from properly diluted samples was spread on LB agar plates [1.5% w/v agar in LB medium] with a glass-spreader and the plates were kept upside down in an incubator at 37 °C, until the colonies appeared. Viable cell counts were calculated by multiplying the number of colonies with the dilution factor. The culture that showed no change in the number of viable cells before and after incubation had contained the NP at MIC, and the culture which showed the number of viable cells after incubation to be 0.1% of that before incubation had contained the NP at MBC.