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Hydroxyethyl Cellulose Stabilized Copper Nanoparticles and its Antibacterial Activity

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ABSTRACT

In this study, we report the green synthesis of copper nanoparticles (CuNPs) by reduction of copper nitrate ($Cu(NO_3)_2$) using ascorbic acid as reducing agent and hydroxyethyl cellulose (HEC) dissolved in water which acts as stabilizing agent. The formation of copper nanoparticles was studied by optimizing the reaction condition using different parameters such as concentration of $CuNO_3$, concentration of HEC, temperature and reaction time. The presence of copper nanoparticles was assured by characterization of UV-vis spectroscopy. The complete reduction of copper ions to copper nanoparticles is revealed by the surface plasmon resonance peak at 550-600 nm. The synthesised copper nanoparticles showed good antibacterial activity. The antibacterial property against the bacteria such as *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus* were evaluated by agar well diffusion method and minimum inhibitory concentration method. This study presents a feasible green method to synthesize copper nanoparticles which had antibacterial activity and promising for various applications.

Keywords: Hydroxyethyl cellulose; aqueous medium; copper nanoparticles; antibacterial activity; environmentally friendly synthesis.

INTRODUCTION

The introduction of transition metallic nanoparticles has extensively spread throughout worldwide due to their application in chemical, biological and environmental science. It has a high surface to volume ratio with diameters in the range of 1-100 nm. The size and the shape are the key factors for their unique properties. Among all the nanoparticles, copper nanoparticles is the most widely used material in the world because copper is the one most important metal used in modern technology [12,7]. Among various metal particles, copper nanoparticles have attracted more attention because of their optical, electrical, catalytic, antibacterial and antifungal applications [11]. Copper nanoparticles is a semiconducting compound that has attracted particular attention in the industry due to its interesting properties and the cost. This nanoparticle are widely used in the field of electronics, solar energy transformations, catalysis, magnetic storage media, gas sensors, batteries and antimicrobial agent [6].

Copper nanoparticles can be synthesized using various routes, including hydrothermal and solvothermal methods, chemical reduction, electrolytic synthesis, sonochemical methods, sol-gel preparations, micro-emulsion techniques, wire explosion, and vacuum vapour deposition. Those synthesizing methods require a complicated reaction and involving expensive equipment. It also uses toxic chemicals and reducing agents like organic solvents. All these production methods, not only increases the cost of production, but also develop hazardous chemical synthesis and thus increase the toxicity issues as well as environmental issues [14] Hence, in this study green synthesis method was introduced to produce copper nanoparticles.

Green synthesis method is a process of synthesizing nanoparticles by using various plants, algae, bacteria, yeast, fungi and polysaccharides [3]. This method is an ecologically friendly, cost

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effective, and saves time. The usage of polysaccharides such as starch, chitosan, glucose, heparin, and cellulose has considerably increased the synthesis of nanoparticles. Cellulose is a naturally available material and environmentally friendly sustainable resource. This polysaccharide is rich in hydroxyl group and thus became a green renewable alternative way to synthesise metallic nanoparticles [2].

Hydroxyethyl cellulose (HEC) is a polysaccharide with biocompatibility and biodegradable property. HEC is widely used in several fields including food and cosmetics industries which functions as a thickeners and viscosity increasing agents, biotechnological and biophysical industries [4]. One of the most important methods for the synthesis of copper nanoparticles is the chemical reduction method. In this method a copper salt is reduced by various reducing agents such as polyols, sodium borohydride, Hydrazine, Ascorbic acid and hypophosphite.

Some noble nanoparticles like gold and silver are well known for their antibacterial activity, however, these nanoparticles are of high cost when compare to copper nanoparticles. CuNPs are relatively nontoxic to mammals, but toxic towards wide range of microorganisms. Thus, it was introduced as a good alternative material for antibacterial effect. The antimicrobial property of nanoparticles can be known by their specific attachment to the surface of microbes and the associated metabolism of nanomaterials inside the cell [13].

Silver and copper nanoparticles are found to have antibacterial activity [18,19]. The bactericidal property of nanoparticles is due to their small size and high surface to volume ratio which helps them to interact with membranes of microorganisms and it's merely not due to the release of metal ions in the solution [10]. The antibacterial activity of copper nanoparticles finds applications in various fields such as medical instruments and devices and water treatment and food processing [15].

In this study, we adopt a green chemistry approach to synthesize copper nanoparticles using Hydroxyethyl cellulose and ascorbic acid. Ascorbic acid, known as vitamin C was used as a reducing agent. In our study ascorbic acid is used as a reducing agent and HEC is used as a stabilising agent. By using ascorbic acid copper is reduced to copper nanoparticles but aggregated at the bottom. Without ascorbic acid Hydroxyethyl cellulose couldn't reduce the copper nanoparticles. The stability of the sample is tabulated in table 1. The schematic representation of copper nanoparticle synthesis is shown in figure 1. Hence in this study we have used both ascorbic acid and HEC.

Table 1: Showing the stability of nanoparticles.

Sample number	With Ascorbic acid	With HEC	Stability of the sample
1.	✓	✓	stable
2.	✓	X	Not stable
3.	X	✓	Sample aggregated and settled at the bottom

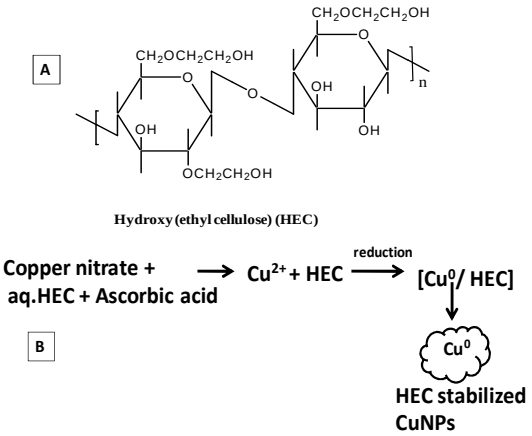


Fig. 1: Showing chemical structure of HEC and schematic representation of copper nanoparticle synthesis.

2. Experimental:

2.1. Materials and bacterial strains:

2-Hydroxyethyl cellulose (HEC) (average molecular weight Mw ~250,000) and ascorbic acid ($C_6H_8O_6$) (Mw~176.12 g/mol) were purchased from Sigma-Aldrich. Copper nitrate ($Cu(NO_3)_2.3H_2O$) with molar mass 241.60 g/mol were purchased from R&M Chemicals. All chemicals were of highest purity and used without further purification. All glass wares were thoroughly washed with detergent solution and copiously rinsed with millipore water. Bacterial strains such as *Escherichia coli* (ATCC 10536), *Staphylococcus aureus (*ATCC BAA1026), *Pseudomonas aeruginosa* (ATCC 15442),

*Enterococcus faecalis (*ATCC 14506) and *Bacillus subtilis* (ATCC 11774) were purchased from Fisher.

2.2 Preparation of copper nanoparticles:

3 ml of HEC with different wt. % (5, 7 and 10) were prepared by dissolving HEC in millipore water and keeping it under continuous stirring overnight to get a homogenous solution. A constant concentration of $Cu(NO_3)_2$ solution was prepared at 50 mM using millipore water for the synthesis of Cu-NPs. In a typical synthesis different volume of $Cu(NO_3)_2$ (100, 200, 400, 600 and 800) µl at 50 mM was added into 3 ml of 2-hydroxyethyl cellulose and 2 ml of deionized water under constant stirring. The reaction mixture was run under different temperature (70, 80 and 100) °C and at different time intervals (10, 15, 20, 25 and 30) min. All these reaction mixtures were tested under UV spectrophotometer analysis. The optimised condition for producing copper nanoparticles was used for studying antibacterial activity.

2.3. Characterization of synthesized copper nanoparticles:

The UV-visible absorption spectra were recorded using a Shimadzu 3101 PC spectrophotometer using a 1 cm quartz cell. The absorption wavelength was set up in the range of 280nm to 700 nm. A solution containing HEC alone was dissolved in deionised water and used as a blank.

2.4. *Antibacterial assay:*

2.4.1. Agar well diffusion method:

Agar well diffusion method was used to study the antibacterial activity of the synthesized copper nanoparticles. All the glassware and reagents used in this method were sterilized in an autoclave at 121˚C for 20 min. *Escherichia coli* (ATCC 10536), *Staphylococcus aureus (*ATCC BAA1026), *Pseudomonas aeruginosa* (ATCC 15442), *Enterococcus faecalis (*ATCC 14506) and *Bacillus subtilis* (ATCC 11774) were used as model test strains. The turbidity of bacterial suspension was adjusted to 0.5 McFarland Standards (Kora *et al*., 2009) and then the suspension was used to inoculate on agar plate. The 8 mm size wells were made into the centre of agar plate. Copper nanoparticles were filled into the well and the plates were incubated at 37 ˚C for 24 h. The inhibition zone was measured using a ruler up to 1 mm resolution. This experiment was done in triplicate to get the accurate inhibition zone measurement.

2.4.2. Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration:

Minimum inhibitory concentration (MIC) test was performed to determine the lowest concentration of antimicrobial agent which inhibits the growth of bacteria. The MIC was read by visual turbidity of bacterial growth before and after incubation. The tube of positive control contained nanoparticles and nutrient broth (devoid of inoculum) and a tube of negative control contained nutrient broth and inoculum (devoid of nanoparticles) was used in this experiment. A dilution series with 8 mL nutrient broth containing 2 mL of Cu-NPs were prepared. Each set was aseptically inoculated with 50 µl of respective bacterial suspension with a concentration of 10^6-10^8 CFU ml^{-1} (CFU = Colony forming units). All the tubes were incubated in an orbital shaker at 250 rpm and 37 ˚C for 18 h. The minimum bactericidal concentration (MBC) was determined by quantifying the number of colonies for lowest concentration of copper nanoparticles. To test MBC, 100 µl from the lowest concentration that inhibited the visible growth of bacteria was inoculated on nutrient agar plates and incubated at 37 ˚C for 16-48 h. This experiment was done in triplicates.

Results and Discussion

3.1. UV-vis spectroscopy analysis:

Figure 2 show the digital camera picture taken before and after reaction. The formation of copper nanoparticles could be followed by the visual colour change of reaction solution. The colour of the solution changed from light blue to brownish red within 3 minutes indicated the formation of copper nanoparticles.

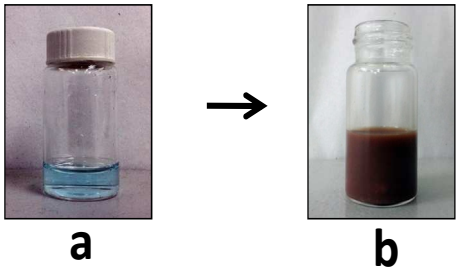


Fig. 2: Reaction mixture of copper nitrate solution, a) before heating (b) after heating.

The presence of absorption band of copper nanoparticles in UV-Vis at the range of 550-600 nm shows the successful preparation of the nanoparticles [17]. Fig.3 (a) shows the absorbance band obtained

from the synthesis of copper nanoparticles using five different volumes of $Cu(NO_3)_2$. The spectrum in UV-vis spectra shows that absorption band occurs at the wavelength around 550-600 nm for all the reactions. This is due to the presence of strong surface plasmon resonance (SPR) of Cu colloids, which confirms the formation of copper nanoparticles. The UV-Vis spectrum shows the effect of $Cu(NO_3)_2$ volume at the same concentration (0.05 M) on Copper nanoparticles with different volumes (100, 200, 400, 600 and 800) µL at 100 ˚C, with 10 % w/v of HEC in 3 min. According to the graph, the absorption intensity increases as the volume of copper nitrate increased.

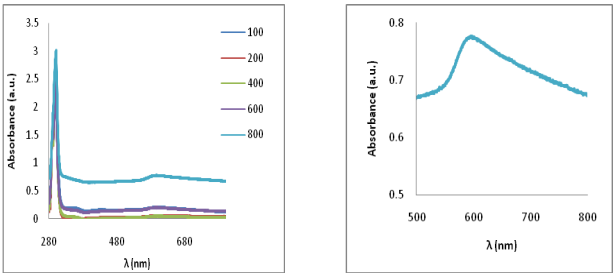


Fig. 3: (a).Absorption spectra of copper nanoparticles at 0.05 M of $Cu(NO_3)_2$ at different volume (µl); 3(b) The zoom absorption spectra of copper nanoparticles at 800 µl.

The UV spectra in fig. 4 (a) shows the effect of HEC concentration in wt % to produce copper nanoparticles. Three different wt % were used and thus the high concentration (as shown in fig. 4(b)) of HEC showing the high peak intensity in producing CuNPs. Hence, the optimum HEC concentration was at 10 wt % because the absorbance value is high and it will give a better conversion of copper ion to CuNPs. This is because the increase in concentration of HEC contributes to the increase in hydroxyl (OH) groups which is responsible for the reduction of copper ion to metallic copper in nanosize [9].

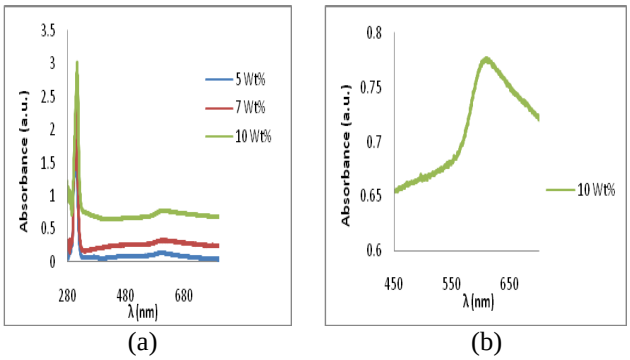


Fig. 4: (a) UV spectra of Copper nanoparticles at different wt % of HEC; 4(b) The zoom UV spectra at 10 wt. %.

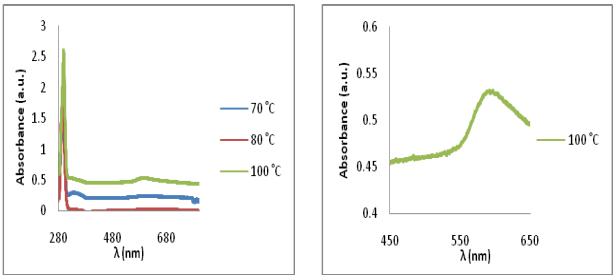
Figure 5(a) and (b) shows the effect of temperature on the synthesis of Cu-NPs. The Synthesis rate will increase as the temperature increased. The maximum temperature used was at 100 ˚C due to the boiling point of water which used as a solvent in this experiment. It was noticed that the low temperature at 70 ˚C takes a long time to synthesize copper nanoparticles. However, for the reaction at 100 ˚C, the rate of reduction and reaction time increased respectively. Moreover, at 100 ˚C, the absorption band increased and solution turns from colourless to brownish colour due to increase in number of nanoparticles formed. The colour of the reaction solution changed fast at the high temperature because it speeds up the synthesis rate.

3.2 Antibacterial activity:

3.2.1 Agar Diffusion Method:

The optimised conditions used to synthesise copper nanoparticles (800 µl of 0.05 M of $Cu(NO_3)_2$, 3 ml of 10 wt.% of HEC, 3 ml of ascorbic acid and 2 ml of H_2O at 100 ˚C for 3 min) were used for evaluating the antibacterial activity against both Gram positive and Gram negative organism. As shown in fig.6, CuNPs were placed at the centre of agar plates. The inhibition zone formed indicates that CuNPs have the antibacterial activity on the tested bacteria, due to the small size and high surface area of copper nanoparticles; it allows interacting with microbial membranes. It is also possible that copper ions penetrate inside the bacteria cell wall and

puncture it leading to cell death [1]. The diameters of the inhibition zone around the well were tabulated in table1. E.coli is more susceptible to Cu-NPs with a zone diameter of 16mm followed by Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus and Enterococcus faecalis. It is evident from this study that copper nanoparticles can act as antimicrobial agent to different bacterial strains. According to the results of inhibition zone in table 2, *Escherichia coli* show the highest inhibition zone. This is because; *Escherichia coli* is a Gram negative bacteria which has thin peptidoglycan layer around the cell membrane [1]. Hence, copper ions can easily penetrate inside the cell wall compare to Gram positive bacteria. Gram-positive bacteria possess a thick cell wall containing many layers of peptidoglycan. In contrast, gram-negative bacteria have a relatively thin cell wall consisting of less peptidoglycan layer. Surfaces of copper nanoparticles interact directly with the bacterial outer membrane, causing the membrane to rupture and killing bacteria.



(a) (b)

Fig. 5: (a).Absorption spectra of copper nanoparticles at varying temperature (˚C), 5(b) The zoom absorption spectra of copper nanoparticles at 100 ˚C.

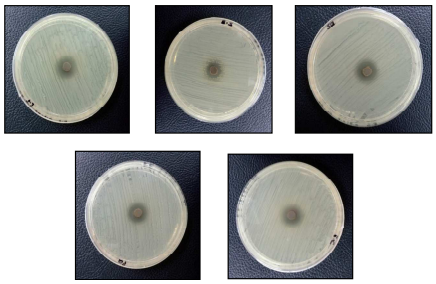


Fig. 6: Plates showing antibacterial activity of HEC stabilized copper nanoparticles (a) E. faecalis, (b) B. subtilis, (c) S. aureus, (d) P. aeruginosa and (e) E. coli.

Table. 2: Table showing the antibacterial activity of copper nanoparticles.

S.No	Test bacterial strains	Diameter of Inhibition zone in mm
1	Escherichia coli	16
2	Pseudomonas aeruginosa	15
3	Enterococcus faecalis	14
4	Bacillus subtilis	15
5	Staphylococcus aureus	15

3.2.2. Determination of Minimum inhibitory concentration and Minimum bactericidal concentration:

Copper nanoparticles inhibit the growth of both Gram negative and Gram positive bacteria. The MIC value for CuNPs was identified by visual bacterial growth. From the broth dilution method (fig. 7) the lowest concentration (MIC) that completely inhibited the bacterial growth were equal for all tested bacteria which were at 1:2 ratios. The MBC readings for this lowest concentration were shown in fig. 8. From the figure, it shows that the MBC results for all types of bacteria were similar where there is no colony observation at the lowest concentration. There are two types of bacteria growth can be described in MBC which are bactericidal and bacteriostatic. Bactericidal means no bacteria colony was observed when inoculated with lowest concentration of copper solution. The number of colonies also can be counted if the tested materials was bacteriostatic. Therefore, our study shows that the tested bacteria were bactericidal because almost 99.9 % of bacteria were killed by the copper nanoparticles. The green synthesis of copper nanoparticles in this study is proven active against the microorganism.

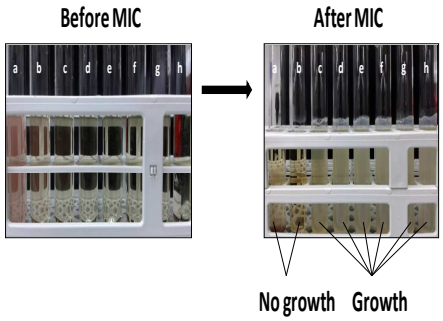


Fig. 7: Pictures showing the MIC results. The range of sample dilutions analysed were a) 1:1, b) 1:2, c) 1:4, d) 1:8, e) 1:16, f) 1:32, g) 1: 64 and h) 1:128.

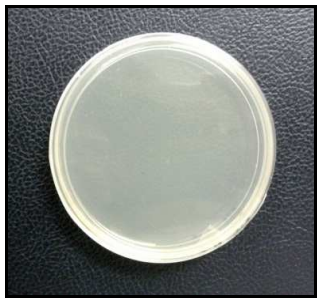


Fig. 8 Plate showing the MBC results. The MBC results for all types of bacteria were similar at 1:2 dilution.

Conclusion:

Synthesis of HEC-mediated copper nanoparticles was developed in a very simple and facile process, cost effective, safe and energy saving method. The study used green chemistry principle by using HEC as a stabilizing agent and ascorbic acid as a reducing agent. The synthesized copper nanoparticles were characterized using UV-visible spectroscopy at the range of 550-600 nm. Agar well diffusion method, MIC and MBC testing showed that aqueous solutions of HEC stabilised CuNPs to be antibacterial towards Gram positive and Gram negative bacteria. Because of the environment friendly condition, this study suggests that further study is needed to apply on copper nanoparticles for biomedical application and dentistry application.

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Authors' Contribution:

Sasikala and Kumaran carried out the sample preparation and experimental measurements, Prema Lakshmi drafted the manuscript, Fathima Shahitha Jahir Hussain designed the experiments and revised the manuscript, and Mashitah Mohd Yusoff provided the financial support. All the authors read and approved the final manuscript.

Financial Disclosure:

There is no conflict of interest.

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