Fabrication, characterization and evaluation of *in-vitro* antimicrobial properties of Cu nanoparticles towards mixed bacterial culture

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Abstract

Cu nanoparticles were synthesized using low temperature aqueous reduction method at pH 3, 5, 7, 9 and 11. The nanoparticles were characterized using transmission electron microscopy (TEM), scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS), BET surface analysis and X-ray diffraction (XRD) techniques. Results demonstrated a strong dependence of synthesis pH on the size, shape, chemical composition and structure of Cu nanoparticles. While lower pH conditions produced Cu^0, high pH levels (more than 7) led to the formation of Cu_2O/CuO nanoparticles. The results of *in-vitro* disk diffusion tests showed excellent antimicrobial activity Cu_2O/CuO nanoparticles against a mixture of bacterial strains (Staphylococcus *aureus*, Escherichia *coli* and Pseudomonas *aeruginosa*) indicating that the size as well as oxidation state of Cu contribute to the antibacterial efficacy.

1. Introduction

Noble metal nanoparticles continue to be the focus of research due to their distinct physico-chemical properties which are dramatically different from the bulk equivalents. They are increasingly used in applications such as optoelectronics, advanced devices, sensors and catalysis [1-4]. Many metal nanoparticles are also well known for antimicrobial activity

(e.g., Ag, Au, Cu, Zn etc.) due to their high surface to volume ratio [6], as well as the ability to release metal ions [5]. However their crystal structure and shape also play a role. For example, the bactericidal activity of Ag nanoparticles decreases with increase in particle size, while truncated triangular shaped particles are found to have greater bactericidal effect compared to spherical or rod shaped particles [7, 8].

While Ag nanoparticles are most studied and best known for offering strong antimicrobial activity against a wide variety of microbes, they are also expensive. . The investigation of Cu nanoparticles as an alternative is thus receiving much attention nowadays.. This interest has opened a wide range of possibilities for Cu nanoparticles in antimicrobial [9], antifungal, bacteriostatic [10] and anti-germ applications and surface coatings [11].

Cu nanoparticles can be prepared by different well known methods , such as thermal reduction [12], vapor deposition [13], microwave irradiation [14], sono-chemical reduction [15] and metal vapor synthesis [16] as well as chemical reduction [17]. Due to its simple process, suitability for small scale sample preparation and the ease of control of reaction parameters, chemical reduction is the most widely used technique for Cu nanoparticle synthesis. However, alterations in synthesis conditions such as water content, surfactant concentration, reactant concentration, anions, pH and temperature have significant effect on the reaction kinetics and thus the size and shape of the nanoparticles formed, and which in turn affect their antimicrobial properties [17]. Another challenge in the chemical synthesis of Cu nanoparticles is the tendency of the particles to oxidize. These oxide phases are thermodynamically more stable [18], with the (Cupric oxide) Cu^{+2} oxidation state being the most prevalent. In aqueous solutions this can be explained by the fact that the Cu^{2+} ion is smaller than cuprous oxide (Cu^{+}) and, having twice the charge, interacts much more strongly with solvent water. This outweighs the second ionization energy of copper and renders the Cu^{2+} more stable. Cuprous oxide (Cu_2O) is unstable in an aqueous solution thus it can be expected to rather form a mixture of Cu^{2+} and metallic copper (Cu^{0}). However with the addition of a complex forming agent, the stability of the copper oxides will improve, thus a higher percentage of Cu^{+} is expected [19]. The antimicrobial activity of zero valent Cu nanoparticles has been widely reported [20-23]. Usman et al. reported a high level of antimicrobial activity of Cu nanoparticles against several microorganisms which includes *S. aureus, B. subtilis, P. aeruginosa, Salmonella choleraesuis,* and *C. albicans* [24]. On the other hand, oxide forms of Cu such as Cu_2O, CuO also have been shown to exhibit antimicrobial effects [25-28]. Ren et al. reported antimicrobial efficacy of CuO nanoparticles

generated by thermal plasma technology that contain traces of pure Cu and Cu_2O nanoparticles towards a range of bacterial pathogens [29]. The suggest that changes in chemical composition and surface charges and shape of Cu nanoparticles play a crucial role in determining their antimicrobial activity, leading to the objectibe of this work: To synthesise Cu nanoparticles by the chemical reduction method and to study the effect of pH on their shape, size and chemical composition and antibacterial efficacy. To the best of our knowledge, such investigation has not been concluded to date.

The research question is thus: How is the stability of Cu nanoparticles affected by and its oxidation probability in presence of ascorbic acid (antioxidant) and polyvinylpyrrolidone (dispersant used to prevent colloidal aggregation) under different pH conditions. The antimicrobial properties of Cu synthesized nanoparticles were investigated against a mixture of bacterial strains consisting of S. *aureus,* P. *aeruginosa* and E. *coli* in order to provide a realistic and more challenging environment to the material. An attempt was also made to correlate the chemical and structural properties of the Cu nanoparticles with the observed bactericidal activities.

2. Experimental

2.1 Materials

All reagents used in the experiment were of analytical grade and obtained from Minema Chemicals, Randpark Ridge, Johannesburg, South Africa ($CuSO_4$ and ascorbic acid) and Sigma Aldrich, Centurion, South Africa (polyvinylpyrrolidone (PVP), H_2SO_4 and NaOH). All materials where used as received.

2.2 Synthesis of the Cu nanoparticles

$CuSO_4$ (0.2 mol/L) and ascorbic acid (1.0 mol/L) solutions were prepared and bubbled with Argon gas for 30 minutes. 3 g of Polyvinylpyrrolidone (PVP) was added to 100 ml of the $CuSO_4$ solution. The pH levels of individual solutions (PVP/$CuSO_4$ and ascorbic acid) were adjusted to the required levels using either H_2SO_4 or NaOH. 100 ml of PVP/$CuSO_4$ and 40 ml of ascorbic acid were mixed and stirred for 4 hours. The final pH of the reaction mixture was noted. The precipitate was collected through centrifugation, rinsed with distilled water and ethanol, and finally dried in an oven for 24 h at 70 °C [30].

2.3 Characterization

The crystallinity and phase composition of the Cu nanoparticles were investigated using a PANalytical XPERT-PRO diffractometer using Ni filtered CuKα radiation (λ = 1.5406 Å) with a fixed slit at 45 kV (voltage) and 40 mA (current) in the 2θ diffraction angle range of 4.5 to 90 °. The diffraction peaks were also used for the semi-quantitative determination of the different oxidation states of copper; Cu^0, Cu^+ and Cu^{2+} in the samples using Rietveld analysis (High-score 3+) indirectly. The XRD patterns were also compared and interpreted with JCPDS (Joint Committee for Powder Diffraction Data) standard data. Morphological analysis of the samples was carried out using a Zeiss Auriba field emission scanning electron microscope (SEM) at an operating voltage of 5 kV. Samples were sputter-coated with carbon to avoid charging prior to imaging. The morphological details of the particles weher investigate on a Jeol Jem 2100 transmission electron microscope (TEM) and the particle size distribution was obtained by Image J V.1.36B software. For TEM analysis, the powder samples were sonicated in ethanol for 2 minutes and dropped on a carbon coated Cu-grid and dried.

2.4 Antibacterial properties

Antimicrobial properties of the nanoparticles were tested on a mixture culture of bacteria using the disk diffusion method. A suspension containing Staphylococcus *aureus,* Escherichia *coli* and Pseudomonas *aeruginosa* was made up to a concentration of around 200 000 cfu/mL. A sterilized pour plate was prepared using the inoculum and allowed to stand for about 15 minutes. A well of 8 mm diameter was aseptically made at the center of each agar plate and0.1 g of powder placed in the well.. All treated plates including a control with only bacterial inoculation were incubated in an upright position for 48 hours at 35°C. After incubation, the diameter of the inhibition zones were measured and it was confirmed that a bacterial lawn did form on the control plates. The screening tests were performed in triplicate and the average results are reported here.

3. Results and Discussion

It was observed that the pH of PVP/$CuSO_4$ and ascorbic acid for each experiment decreased after the reaction had taken place. The individual solutions of pH 11, 9, 7, 5 and 3 changed to 6.5, 5.1, 4.4, 3.7 and 1.3 respectively after the reaction. This could be due to the reduction of $CuSO_4$ hydrate in an aqueous medium. While the Cu^{2+} ions form $CuOH^-$ which is acidic, the sulphate ions form bisulfate when in contact with water. This leads to the formation of an

excess of H^+ in the solution as the reaction proceeds, which could lower the pH of the mixture [31].

Figure 1 presents the XRD patterns of nanoparticles synthesized at different pH conditions. It can be seen that the diffraction patterns of nanoparticles prepared at pH 3 and 5 have narrow and well defined peaks which shows that all particles are predominantly crystalline in nature with very little or no amorphous phase. The sharp peaks at about 43.4 ° and 50.6 ° correspond to the (111) and (200) planes of crystalline metallic Cu with bigger crystallite size. (ref. pattern Cu-00-003-1018, cubic).

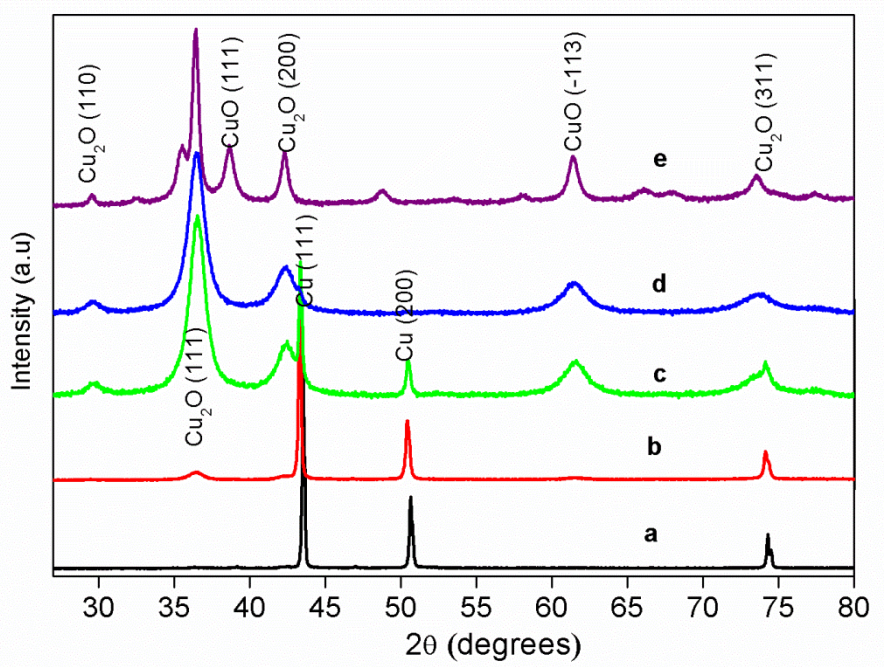

Figure 1 XRD patterns of the copper nanoparticles synthesized at different pH conditions a) pH 3, b) pH 5, c) pH 7, d) pH 9, e) pH 11

XRD patterns of samples synthesized at pH 7 or above show broader peaks at 29.9°, 36.4°, 42.3°and 74.1° which can be assigned to (110), (111) and (311) planes of Cu_2O (ref. pattern Cuprite-04-004-7864, cubic). The additional peaks in these samples seen at 38.8° and 61.8° match well with the (111) and (-113) planes of crystalline CuO (ref. pattern Tenorite -04-004-7864, monoclinic). Broadening of peaks is observed for samples prepared above 7 suggesting smaller crystallite sizes.

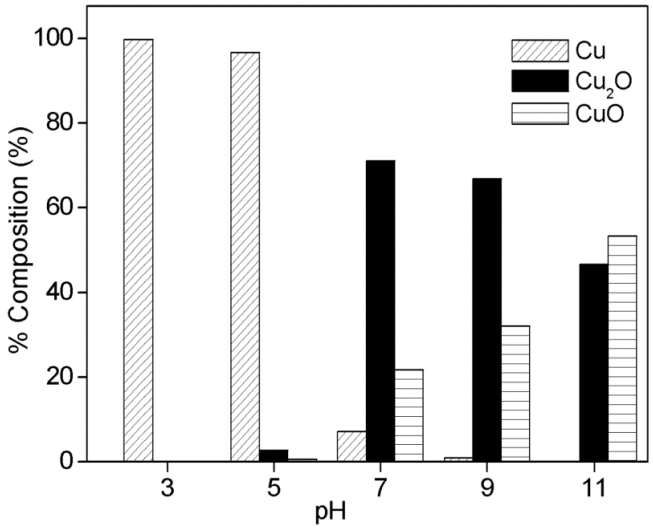

Figure 2 Variation of Cu phase composition against initial synthesis pH obtained from Reitveld analysis

Using Rietveld semi-quantitative analysis (High-score 3+), the percentile composition of Cu phases were determined per sample and the results are presented in Figure 2. It is observed that the crystalline phase composition of the samples vary with changes in pH condition and different Cu phases namely Cu, Cu_2O and CuO correspond to Cu^0, Cu^+ and Cu^{2+} oxidation states respectively. Samples prepared at a pH of 3 and 5 contain more of Cu whereas oxidized phases of Cu are observed from pH 7 onwards. From the data it is evident that the sample synthesized at pH 7 contains highest concentration of Cu_2O which decreases thereafter with further increase in pH. No Cu is detected at pH 11, however, increasing the pH from 7 to 11 is found to favour the formation of CuO. From Figure 2 it is further apparent that particles produced at lower pH levels of between 3 and 5 are constituted of Cu^0, while those produced at an initial pH of 7 and higher are made up Cu^+ , Cu^{2+}.

Taking these findings into consideration it can be deducted that reactions that involve oxidation reduction and exchange of H^+ and OH^- ions are dependent of the oxidation/reduction potential of the species and the pH. Under similar preparation conditions, in acidic conditions (pH 3 and 5) direct reduction of Cu^{2+} to Cu^0 is thermodynamically favoured due to the higher reduction potential of Cu^{2+} (E^0=+0.34) in comparison to H^+ (E^0=0) ions. Increase in pH to 7 however, firstly favours hydrolysis of Cu^+ and secondly Cu which eventually forms Cu_2O (Cu^+) and CuO (Cu^{2+}) as major forms. It is also noticeable that in highly alkaline solution of pH 11, the second hydrolysis is predominant resulting in higher concentration of thermodynamically more stable CuO (Cu^{2+}). This result also indicates that

ascorbic acid effectively reduces Cu ions to single phase Cu at pH 3 and 5 although minor quantities of Cu_2O and CuO are formed at pH 5. However, its reduction capacity seems to be diminished due to its instability and autoxidation in neutral alkaline conditions [33] which can explain the decreasing Cu concentration from pH 7 or above.

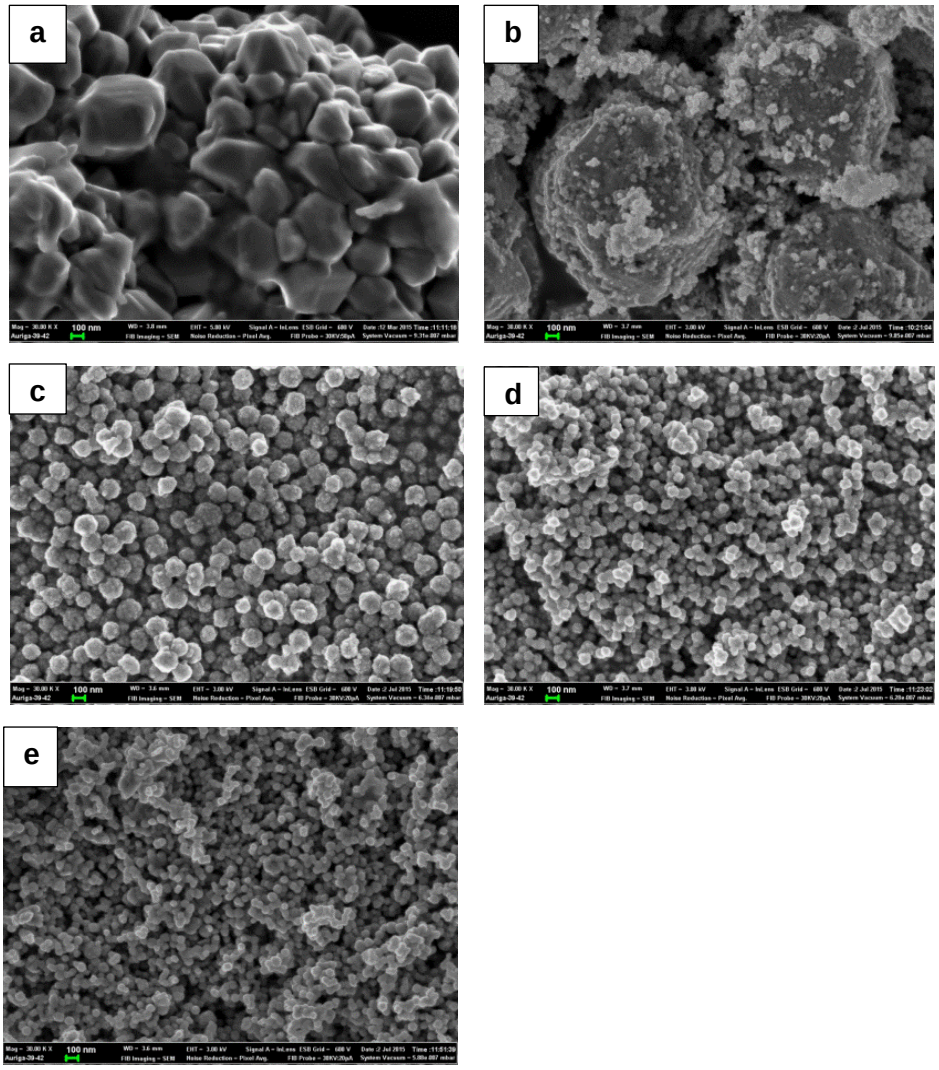

Figure 3 SEM images showing the representative mophology of Cu nanop[articles produced at different starting pH levels of a) pH 3, b) pH 5, c) pH 7, d) pH 9 and e) pH 11

Zayyoun et al. [32] recently demonstrated that by sol-gel method, both stable Cu_2O (pH below 6) and CuO nanoparticles (pH above 12) can be prepared by adjusting the acidity-basicity of the precursor solution. Nikam et al. [34] also reported that pH dependent formation of Cu_2O and CuO could be obtained from Cu (II) acetate as the metal precursor and using benzyl alcohol as the solvent, under the microwave condition. CuO nanoparticles were formed under basic conditions while phase pure Cu_2O was formed at a pH below 7. These results support our observation. Granata et al. [35] observed that mildly reducing D-

glucose was unable to reduce Cu ions in alkaline pH of 9 and 11 during chemical reduction where they obtained mixed oxidized phases of Cu/Cu_2O.

Scanning electron micrographs showing surface morphology of samples prepared under different pH conditions are presented in Figure 3. The image analysis shows that synthesis pH greatly influences the surface morphology and size of Cu nanoparticles. It is apparent that particles produced in acidic conditions (pH 3 and 5) are relatively bigger (biggest being pH 3) and clustered whereas, the formation of smaller and more isolated particles is observed at pH 7 and above. The degree of aggregation is higher for particles produced at pH 7 in comparison to the ones produced at alkaline conditions (pH 9 and 11). The reduction in particle size from pH 7 omwards is also in line with the corresponging XRD peak broadening proposing smaller crystallites. The slow nucleation of Cu nanoparticles in acidic pH leads to the formation of larger particles whereas at high pH, due to the presence of accessible –OH ions, faster nucleation occurs and hence smaller size particles are formed [36]. Chithra et al. [37] also reported a similar correlation between pH and size of biosynthesized Ag nanoparticles.

The size and shape of particles obtained under the different experimental conditions were analysed by transmission electron microscope (TEM) and Figure 5 illustrates the representative TEM micrographs (low- and high- resolution) of Cu nanoparticles produced at different pH levels. At highly acidic condition (pH 3), monodisperse polyhedral Cu particles between 200-500 nm size are formed. As the pH increases to 5, the particle sizes reduces to a 100-200 nm range. Some smaller particles are also observed in this case. According to XRD results, at pH 7 the predominent phase is Cu_2O along with CuO and small concentration of Cu. Correspdingly the sample appears to be aggregates of particles with relatively bigger cubic and smaller spherical particles (refer Figure 5 b') in the TEM image. The cube morphology can be ascribed to the cubic crystal structure of Cu_2O whereas the spherical particles can be those of CuO [34, 38]. Further reduction in aggregation is observed as the pH rises to 9 and 11. However, particles formed at pH 7 and above are the smallest in comparison to the ones formed at pH 9 and 11. The particles at higher pH are not isolated but rather fused together and seem capped by surfactant. The extent of capping is found to increase as the pH increases. Although it is difficult to acrurately determine the particle size due the presence of capping agent in these cases, it can be concluded that the average size is reduced to below 100 nm.

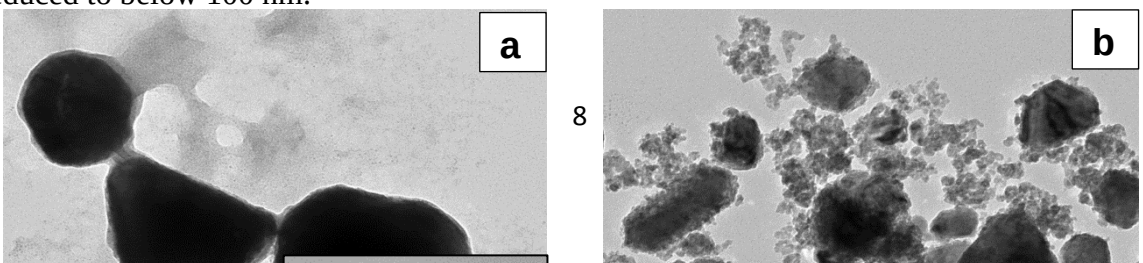

Figure 5 TEM micrographs of Cu nanoparticles produced at different pH levels a) pH 3, b) pH 5, c) pH 7, d) pH 9 and e) pH 11. Each insert is a higher magnification image of the same sample..

PVP, a polymeric surfactant is used as a dispersing agent during synthesis. Zang et al. [39] observed more aggregated Ag nanoparticles due to the lack of steric protection from PVP when used in lower concentration in a chemical reduction process. According to them if the PVP either adsorbed on the surface or chemically bonded to the nanoparticle, it is crucial in preventing particle aggregation. Granata et al. [35] on the other hand reported the high capping efficiency of PVP when used with mild (D-glucose) reducing agent in the chemical reduction process to produce Cu nanoparticles. In this work the effect of pH was not studied in detail.

In our case, the formation of significantly large particles of Cu at lower pH indicates the inefficiency of PVP to be absorbed on Cu^0 particles to prevent particle growth. This may be due to the fact the PVP forms positively charged complexes under acidic conditions which reduces the binding capacity to cations [40]. However at relatively higher pH (7 or above) PVP stabilizes Cu^+ and Cu^{2+} ions through co-ordinated (nitrogen and oxygen polar groups donates lone-pair electrons) interactions. Cu^+–PVP in the presence of sufficient concentration of –OH ions forms Cu_2O, whereas at high pH, an excess of –OH ions Cu^{2+}-PVP results in more stable CuO [40, 41].

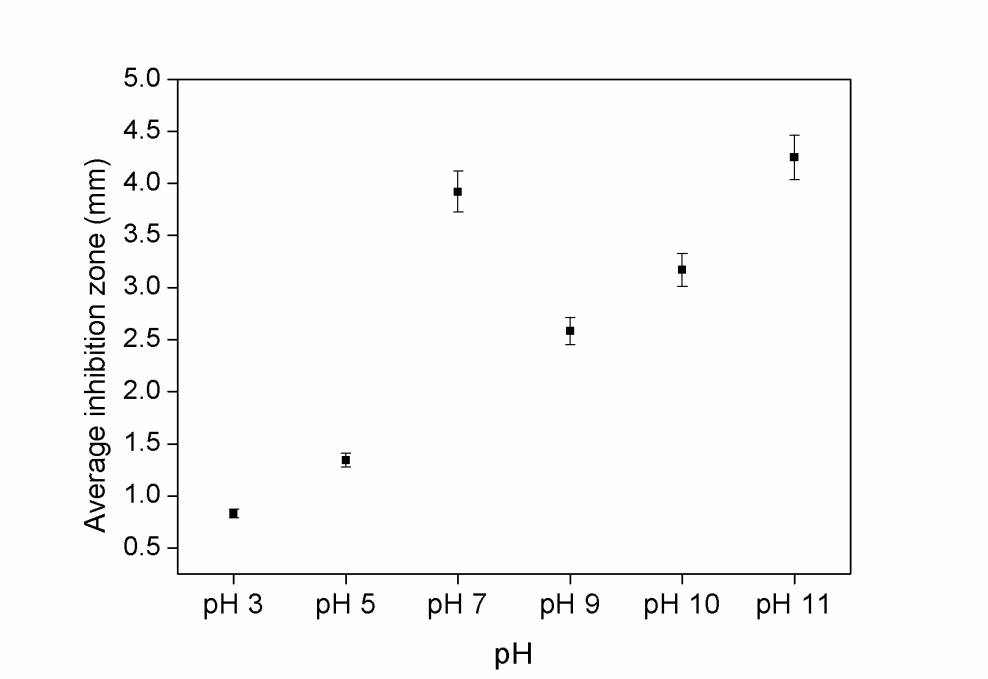

Figure 6 Average zone inhibition of the Cu nanoparticles produced at the different pH levels

The disk diffusion method was used to evaluate the antimicrobial efficiency of our Cu particles produced under different pH conditions and Figure 6 shows the average of triplicate tests performed on the two batches of nanoparticles prepared under similar conditions. Three bacterial strains namely Staphylococcus *aureus*, Escherichia *coli* and Pseudomonas *aeruginosa* were selected for the mixed bacterial culture. A culture with more than one strain of bacterial is one of the strategies to increase bacterial diversity to mimic a realistic situation where a synergistic interaction can impose a more challenging microbial environment [42].

From the results, it is apparent that there is a relatively linear increase in bacterial killing efficacy as the reaction pH increases. However, particles produced at pH 7 show relatively higher efficiency, very close to those produced at pH 11. For particles at pH lower than 7, the lower antimicrobial activity of thes Cu particles correlates with an increase in their size as supported by SEM and TEM results. According to the reported literature [43, 44], larger nanoparticles due to their bigger size cannot easily penetrate through the cell walls of microorganisms and cause cell destruction. The particles prepared at pH 7 or above show smaller particle sizes which explains their higher antimicrobial efficiency in general. Other factors that can influence the antimicrobial efficiency of nanoparticles are the possibility of metal ion leaching and generation of reactive oxygen species from nanoparticle surface [45, 46]. In this case, it is seen that cumulative content of oxidized phases of Cu increases with increase in synthesis pH. This can lead to increased Cu^{+} and Cu^{2+} release in the bacterial medium which facilitates their efficient binding to the negatively charged surface of the bacteria and eventually result in enhancement of bactericidal efficiency [47]. Conversely, the oxidative stress through reactive oxygen species induced by Cu_2O and CuO can also result in cell damage and apoptosis [48]. A combination of these effects of oxides along with smallest size of particles explains the significant contact killing efficiency of particles produced at pH 7 in comparison to those prepared at 9 and 11.

Azam et al as well as Ahamed and co-workers [44, 49] revealed significant size dependent antimicrobial activity of CuO nanoparticles against various bacterial strains (E. *coli*, P. *aeruginosa*, K. *pneumoniae*, E. *faecalis*, S. Flexneri, S. *typhimurium*, P. *vulgaris*, and S. *aureus*). Lee et al. reported superior antimicrobial properties of cubic Cu_2O in comparison to octahedral Cu_2O, and in recent work by Meghana et al [51] the mechanism of antimicrobial activity in Cu oxide nanoparticles in E. *Coli* Was investigated. According to them, after the initial cell damage caused on the cell wall, further damage is caused by two different pathways; Cu_2O forms Cu^{+}-peptide complex while CuO forms free radicals to cause cell

destruction. They also suggest that the oxidation state has a major role deciding the activity, where Cu_2O showed efficient activity and cell affinity. These reports are in agreement with our findings on size and oxidation state depended antimicrobial activity of Cu nanoparticles which in turn is directly dependent on the synthesis pH conditions.

4. Conclusions

Cu nanoparticles were synthesized at different pH levels using the aqueous reduction method and ascorbic acid and PVP. The nanoparticles produced varying in size morphology and are found at different levels of oxidation. Ascorbic acid, being a mild reducing agent, could not reduce Cu ions in alkaline conditions due to its instability. The particle size decreased with increasing synthesis pH which could be correlated to the efficiency of PVP in dispersing and capping the Cu ions in alkaline conditions. The bactericidal efficiency of the prepared samples was tested against a mixed bacterial culture consisting of Staphylococcus *aureus*, Escherichia *coli* and Pseudomonas *aeruginosa.* The results indicated that the contact killing efficacy of particles is dependent on size as well as the oxidation sate. Cu Particles produced at pH 7 showed higher killing efficiency compared to those produced at below pH 7 and close to that of pH 11. The significant contact killing efficiency of samples prepared at pH 7 and above could be due to the combined effect of size, leaching of ions and reactive oxygen generation. The nanoparticles synthesized in alkaline conditions thus offer good potential in antimicrobial packaging and coatings applications.

5. Acknowledgements

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