Green synthesis of silver and copper nanoparticles using ascorbic acid and chitosan for antimicrobial applications

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Abstract

Silver and copper nanoparticles were produced by chemical reduction of their respective nitrates by ascorbic acid in the presence of chitosan using microwave heating. Particle size was shown to increase by increasing the concentration of nitrate and reducing the chitosan concentration. Surface zeta potentials were positive for all nanoparticles produced and these varied from 27.8 to 33.8 mV. Antibacterial activities of Ag, Cu, mixtures of Ag and Cu, and Ag/Cu bimetallic nanoparticles were tested using *Bacillus subtilis* and *Escherichia coli*. Of the two, *B. subtilis* proved more susceptible under all conditions investigated. Silver nanoparticles displayed higher activity than copper nanoparticles and mixtures of nanoparticles of the same mean particle size. However when compared on an equal concentration basis Cu nanoparticles proved more lethal to the bacteria due to a higher surface area. The highest antibacterial activity was obtained with bimetallic Ag/Cu nanoparticles with minimum inhibitory concentrations (MIC) of 0.054 and 0.076 mg/L against *B. subtilis* and *E. coli* respectively.

Keywords: Bimetallic nanoparticles; Minimum inhibitory concentration; Minimum bactericidal concentration*; Bacillus subtilis*; *Escherichia. coli*

1. Introduction

Interest in metal nanoparticles as antimicrobial agents is now over a decade old (Dai & Bruening, 2002). The large number of particles that can be made and the high surface area to volume ratio allows nanoparticles to be effective in very small amounts (Sundaresan, Sivakumar, Vigneswaran, & Ramachandran, 2012). Ag nanoparticles have been extensively used for biomedical applications (Marambio-Jones & Hoek, 2010). Cu is relatively non-toxic to mammals, (Flemming & Trevors, 1989) but is toxic towards many micro-organisms and this offers new prospects for antimicrobial treatments (Hsiao, Chen, Shieh, & Yeh, 2006).

A number of methods for producing Ag and Cu nanoparticles have been developed using both physical and chemical approaches. The most frequently applied method for the preparation of Ag nanoparticles is the chemical reduction of silver salt solutions in water or an organic solvent to produce a colloidal suspension. The most common approach for synthesizing Cu nanoparticles is by creating microemulsions (Solanki, Sengupta, & Murthy, 2010). However, the microemulsion technique requires large amounts of surfactants and organic solvents which increases the cost of production (El-Nour, Eftaiha, Al-Warthan, & Ammar, 2010). Physical methods using laser ablation, radiolysis or aerosol techniques, although effective, require expensive instruments and consume large amounts of energy (Thakkar, Mhatre, & Parikh, 2010). These methods typically makes use of agents which are both toxic and environmentally polluting (Lim & Hudson, 2004).

Methods based on chemical reduction offer the best chance of being both low cost and environmentally friendly. Ag nanoparticles have been synthesized using water as solvent and starch as a capping agent and these were shown to have advantages over conventional methods involving chemical agents that are associated with

environmental toxicity (Sharma, Yngard, & Lin, 2009). The synthesis of Ag nanoparticles using chitosan as both a reducing and a capping agent has also been developed (Sanpui, Murugadoss, Prasad, Ghosh, & Chattopadhyay, 2008). In addition, Cu nanoparticles have been made using alginate as a stabilizing agent (Díaz-Visurraga et al., 2012).

More recently, nanoparticles involving alloys of two metals have been produced. Valodkar, Modi, Pal, & Thakore (2011) synthesized bimetallic Ag and Cu nanoparticles using starch and Said-Galiev et al. (2011) have synthesized Ag and Cu nanoparticles using chitosan. These had been treated with supercritical carbon dioxide followed by reduction of Ag and Cu organometallic complexes with hydrogen to form metal–chitosan nanoparticles. Bimetallic Ag and Cu nanoalloys have also been generated from silver nitrate and copper acetate solution with hydrazine hydrate as a reducing agent (Taner, Sayar, Yulug, & Suzer, 2011).

This work is an attempt to further develop the green synthesis of Ag and Cu nanoparticles, mixtures of Ag and Cu nanoparticles (denoted as “Ag + Cu”) and alloy nanoparticles of Ag and Cu (denoted as “Ag/Cu”) with chitosan as a stabilizing agent and using microwave heating. The attraction of using chitosan for this function compared to starch is that it possesses antimicrobial properties (No, Park, Lee, & Meyers, 2002), and can be readily solubilised using organic acids (Muzzarelli et al., 1984; Muzzarelli, 1985). Chitosan can form various chemical bonds with metals components thus enhancing the stability of the nanoparticles (Muzzarelli, 2011). It has low toxicity and it is therefore safe for human applications (Muzzarelli, 2010), although it is acknowledged that the metallic nanoparticles produced may well have some environmental toxicity (Li et al., 2010).

In this study nanoparticles were synthesized at different concentrations of chitosan. The synthesized nanoparticles were characterized by spectrophotometry and by the use of a zetasizer. Their antibacterial properties were tested using *Bacillus subtilis* and *Escherichia coli*.

2. Materials and methods

2.1 Reagents

Copper (II) nitrate ($Cu(NO_3)_2$. xH_2O) (Sigma Aldrich Chemie GmbH, Steinheim, Germany), silver nitrate ($AgNO_3$) (BDH Ltd, Poole, United Kingdom), L-ascorbic acid (Sigma Aldrich, Poole, United Kingdom), acetic acid (Fisher Scientific, Loughborough, United Kingdom) and chitosan (Sigma Aldrich, Poole, United Kingdom) were used for synthesis of the nanoparticles. The molecular weight of the chitosan was in the range 50 000 - 190 000 Da and was 75-85% deacetylated. All reagents were used without further purification.

2.2 Preparation of chemical solutions

The following solutions were all prepared using distilled water: silver nitrate (10, 30 and 50 mM), copper nitrate (10, 30 and 50 mM), ascorbic acid (10% w/v). Chitosan solutions (1, 2 and 3% w/v) were prepared by dissolving chitosan in 1% (v/v) acetic acid solution. These were then left for 3 days to allow the chitosan to completely dissolve (Wei, Sun, Qian, Ye, & Ma, 2009).

2.3 Preparation of nanoparticles

2.3.1 Ag and Cu nanoparticles

For the preparation of Ag or Cu nanoparticles solution, 40 mL of silver nitrate or copper nitrate solution (10, 30 or 50 mM) was mixed with 40 mL of chitosan solution (1, 2 or 3% w/v) and 4 mL of 10% (w/v) ascorbic acid solution. The reduction reaction was carried out by heating in a microwave oven (EM-SI067 UK, Sanyo, China) at full power of 800 W for 4 minutes (Valodkar, Modi, Pal, & Thakore, 2011).

2.3.2 Bimetallic Ag/Cu nanoparticles

For the preparation of bimetallic nanoparticles hereinafter referred to as “Ag/Cu” nanoparticles, 20 mL of silver nitrate solution and 20 mL of copper nitrate solution were mixed with 40 mL of 3% (w/v) chitosan and 4 mL of 10% (w/v) ascorbic acid solution.

The reaction was then carried out under microwave at full power of 800 W for 4 minutes (Valodkar et al., 2011).

2.3.3 Mixtures of Ag and Cu nanoparticles

For the preparation of simple mixtures of Ag and Cu nanoparticles, subsequently referred to as “Ag + Cu” nanoparticles, 40 mL of Ag nanoparticles and 40 mL of Cu nanoparticles were separately synthesized in 3% (w/v) of chitosan as described in section 2.3.1 and then mixed together (Valodkar et al., 2011).

2.4 Characterization of nanoparticles

2.4.1 Spectrophotometry

UV-vis absorption spectra of Ag, Cu, Ag/Cu and Ag + Cu nanoparticles solutions were taken over the wavelength range 200 to 800 nm using UV-vis spectrophotometer model UV mini-1240 (Shimadzu Corporation, Kyoto, Japan).

2.4.2 Particle size and zeta potential analysis

Particle sizes of Ag, Cu, Ag/Cu and Ag + Cu nanoparticle solutions were measured using a Zetasizer instrument (Model ZEM5002, Malvern Instruments Ltd, Malvern, UK) using UV Grade cuvettes after treatment in an ultrasonic water bath (Model FB11012, Fisherbrand, Loughborough, UK) for 30 min to break up any aggregates present (Ribeiro, Hussain, & Florence, 2005). The zeta potentials of each type of nanoparticle were measured using a Zetasizer model 3000HS (Malvern Instrument Ltd, Malvern, UK). All measurements were performed in triplicate.

2.5 Microbiological methods

2.5.1 Bacteria

The Gram-positive bacterium, *Bacillus subtilis* ATCC 6633 was obtained from the National Collections of Industrial, Food and Marine Bacteria (NCIMB), Aberdeen, Scotland and the Gram-negative bacterium, *E. coli* K12 was kindly donated by Dr Jon Hobman of Nottingham University, Nottingham, United Kingdom.

2.5.2 Cultivation of bacteria

Bacteria were taken from frozen (-80 ^{o}C) stock and streaked onto Tryptone Soy Agar (TSA) and incubated overnight at 37 ^{o}C. A single colony was then used to inoculate 100 mL of Tryptone Soy Broth (TSB) in a 500 mL Erlenmeyer flask which was then placed in a shaking incubator at 37 ^{o}C at a speed of 140 rpm for 12 hours. Following this, 100µL of this culture was used to inoculate 100 mL of fresh TSB incubated at the same conditions as described above until the mid-logarithmic phase had been attained. The culture at this point was suitably diluted in phosphate buffered saline (PBS) to yield a suspension containing 10^8 colony forming units (CFU) per mL. Identical procedures were followed for both *B. subtilis* and *E. coli*.

2.5.3 Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

A range of dilutions of nanoparticles in sterile distilled water were prepared and 4 mL of the dilution was added to 20 mL of TSB medium with 20 mL of bacteria of 10^8 CFU/mL and incubated in an incubator shaker (model CERTOMAT® BS-1, Sartorius, Göttingen, Germany) at 37 °C overnight (Cao *et al*., 2010). The MIC was determined by visual observation and confirmed by turbidity measurement using a UV-vis spectrophotometer (Jenway 6300, Bibby Scientific Ltd, Essex, UK) at 600 nm before and after incubation. Diluted aliquots (100µL) of those samples in which growth of the bacteria was not observed were spread plated using TSB to determine the minimum bactericidal concentration (MBC). The samples were incubated at 37 °C overnight and the colonies formed were observed. The MBC was determined as the lowest concentration that inhibited the visible growth of the bacteria tested (Wei et al., 2009).

2.6 Statistical analysis

The data from triplicate experiments are presented as the mean and the standard error of the mean. The statistical analysis of the results was performed using one-way ANOVA with Bonferroni multiple comparison post hoc test. All statistical analyses were performed using IBM SPSS Statistics 21.0 (SPSS UK Ltd., Surrey, United Kingdom).

3. Results and discussion

3.1 Physical characteristics

Images of the nanoparticle suspensions prepared according to the methods described above are presented in Fig. 1. The formation of colloidal suspensions of nanoparticles was evident from visual inspection of the reagent mixtures following microwave heating. Ag nanoparticles (Fig. 1a) were of a light yellow colour which became stronger as the chitosan concentration was increased. The formation of metal nanoparticles was confirmed by UV-vis spectrophotometry. Fig. 2a shows an absorbance spectrum with a peak at approximately 420 nm, indicating the formation of Ag nanoparticles (Ahmad et al., 2003). Ag nanoparticles have previously reported to be of yellowish colour in aqueous solution (Perera et al., 2013). The colour and absorbance spectra at 420 nm is due to the excitation of surface plasmon vibrations of Ag atoms (Twu, Chen, & Shih, 2008). Increasing the chitosan concentration was found to result in a slight downward shift in the peak absorbance coupled with an overall increase in absorbance. These results are consistent with those obtained by Huang, Yuan, & Yang (2004) who synthesized Ag nanoparticles in chitosan by reduction with sodium borohydride.

The Cu nanoparticles (Fig. 1b), varied in colour from light pink to deep red as the concentration of chitosan was increased. The UV spectrum (Fig. 2b) showed a peak at 550 nm, which confirms the presence of Cu nanoparticles. These have been reported to be in the range of 500 to 600 nm (Mallick, Witcomb, & Scurrell, 2006). Similarly, it has been reported that the color of the freshly synthesized Cu nanoparticle stabilized using a water soluble aminoclay matrix suspension was red which is characteristic of Cu nanoparticles (Datta, Kulkarni, & Eswaramoorthy, 2010).

Fig. 1c shows that mixtures of Ag and Cu nanoparticles are orange in color (as would be expected from a blend) but alloyed nanoparticles of Ag and Cu were dark brown. Taner et al. (2011) formed bimetallic Ag/Cu nanoparticles by bringing about the

194 reduction of metal salts in aqueous solution with hydrazine hydrate and also reported
195 that the colour of the suspension was dark brown. UV-vis spectra of Ag + Cu and Ag/Cu
196 nanoparticles are presented in Fig. 2c. The Ag + Cu spectra show two distinct peaks at
197 420 and 550 nm, indicating a physical mixture of Ag and Cu nanoparticles. The Ag/Cu
198 nanoparticles, on the other hand, showed a single absorbance peak at an intermediate
199 ratio confirming that a bimetallic alloy had been formed (Valodkar et al., 2011).

200 In this study, chitosan was solubilized using dilute acetic acid solution. The
201 chitosan reacts with H^+ from the acid solution to produce protonized chitosan with $–NH_3^+$
202 functional groups. The introduction of these functional groups into the chitosan
203 backbone improves its solubility in water. When silver and copper nitrate were added to
204 the chitosan solution, Ag^+ and Cu^{2+} ions would attach to chitosan macromolecules by
205 electrostatic interactions, because the electron-rich oxygen atoms of polar hydroxyl and
206 ether groups of chitosan are likely to interact with electropositive metal cations. A
207 reducing agent is required to provide the free electrons needed to reduce ions and to
208 form nanoparticles (Tolaymat et al., 2010). In this study, ascorbic acid was used as the
209 reducing agent. Ag and Cu nanoparticles thus were formed by the reduction of Ag^+ and
210 Cu^{2+} ions respectively, with an excess of ascorbic acid (for 100% conversion). The Ag
211 and Cu nanoparticles are stabilized by protonized chitosan to prevent aggregation and
212 to control the size of final nanoparticles produced. The same mechanism applies for the
213 synthesis of Ag/Cu nanoparticles.

214 **3.2 Mean particle size**

215 The changes in UV-vis absorbance referred to above indicate that the size of
216 nanoparticles formed altered with the concentration of chitosan, which operates as a
217 controller of nucleation as well as a stabilizer (Huang et al., 2004). Fig. 3 shows the
218 mean particle size of Ag and Cu nanoparticles synthesized using 10 mM salt solution at
219 different concentrations of chitosan. It was observed that the mean particle sizes of both
220 Ag and Cu nanoparticles decreased with increasing chitosan concentration and that for
221 the same chitosan concentration Ag nanoparticles were slightly larger than Cu
222 nanoparticles as presented in Fig. 3. This decrease is due to a protective action by

chitosan whereby chitosan may prevent the growth of the nanoparticles by adsorbing onto their surfaces (Esumi, Takei, & Yoshimura, 2003).

The effects of silver and copper nitrate concentration on the mean particle size of the nanoparticles are presented in Fig. 4. As the concentration of silver and copper nitrate increased from 10 to 50 mM, the size of nanoparticles increased almost proportionately. At the same concentration of chitosan, with increased concentration of metal salts, less protonized chitosan absorbed onto the pre-formed nanoparticles, and therefore larger nanoparticles were formed (Leung, Wong, & Xie, 2010). Statistical analysis showed that the mean particle sizes of the nanoparticles are significantly different and influenced by the concentrations of chitosan and metal salts. Therefore, the size of the nanoparticles may be controlled either by adjusting the concentration of metal ions or the concentration of chitosan during the synthesis process.

3.3 Zeta potential

The zeta potential is a crucial parameter for determining the stability of nanoparticle suspensions. For a physically stable nanoparticle suspension to be stabilized solely by electrostatic repulsion, a zeta potential of ±30 mV is required as a minimum (Singare et al., 2010). The mutual repulsion of nanoparticles depends on having either a large negative or positive zeta potential. Zeta potential measurements of the nanoparticles are presented in Fig. 5. These show that Ag nanoparticles have a positive surface charge which increases with chitosan concentration from +23.8 mV at 1% (w/v) to +32.1 mV at 3 % w/v chitosan solution. Cu nanoparticles had a slightly higher positive surface charge which ranged from +26.4 mV at 1% (w/v) to +33.9 mV at 3% (w/v), although statistically the zeta potentials of Ag and Cu nanoparticles were not found to be significantly different to each other. The zeta potential of the nanoparticles increases with chitosan concentration due to the greater availability of protonized $-NH^{3+}$ on the surface of nanoparticles formed. This will produce a greater electrostatic repulsion between the particles and therefore a lower incidence of agglomeration and resulting in a more stable nanoparticle dispersion.

Fig. 5b shows zeta potentials of Ag, Cu, Ag + Cu and Ag/Cu nanoparticles prepared from 50 mM metal salt solutions and 3% (w/v) chitosan solutions respectively. In this instance the Ag nanoparticles have a slightly higher surface charge (+37.8 mV) than Cu nanoparticles (+35.5 mV). Mixed (Ag + Cu) nanoparticles gave a value of +39.1 mV and Ag/Cu nanoparticles one of +35.2 mV. These differences were not statistically significant.

As there may be an effect of particle size on the zeta potentials of the four nanoparticle types (Ag, Cu, Ag + Cu and Ag/Cu), the zeta potentials were compared for selected samples which most closely corresponded to a mean particle size of 200 nm. These were synthesized in 3% (w/v) chitosan and corresponded to metal ion concentrations of 12.1, 32.0 and 18.2 mM for Ag, Cu and Ag/Cu respectively. Ag + Cu was made by mixing Ag prepared at 12.1 mM with Cu prepared at 32.0 mM. The corresponding zeta potentials are +33.8, 33.1, 32.6 and 33.3 mV for Ag, Cu, Ag+Cu and Ag/Cu respectively (Fig. 5c). These are very similar to those shown in Fig. 5b where all samples were prepared with a 50 mM solution (corresponding to mean particle sizes of 793, 292, 542 and 633 nm respectively). This finding reveals that the concentration of metal salts used in the preparation does not significantly affect the charge of the nanoparticles. The results obtained here are consistent with those of Xiong et al. (2013), in which it was reported that the zeta potential of Cu nanoparticles is +32 mV, suggesting that that value was high enough to maintain stable colloidal dispersions.

3.4 Antimicrobial properties

MIC and MBC tests were performed to assess the antibacterial activity of the nanoparticles towards *B. subtilis* and *E. coli*. The MIC is defined as the lowest concentration at which there is no visible growth whilst MBC is defined as the lowest concentration at which no colony is observed (Wei et al., 2009). MIC and MBC values towards *B. subtilis* and *E. coli* of Ag, Cu, Ag/Cu and Ag + Cu nanoparticles prepared from 50 mM metal salt solutions are shown in Table 1. A lower MIC or MBC corresponds to higher antibacterial effectiveness. As a control, to assess the possible antimicrobial action of ascorbic acid, separate experiments were performed in which samples were prepared according to the method in Section 2.3.1, but in the absence of chitosan and silver or copper nitrate and tested against both *E. coli* and *B. subtilis*.

These samples showed no bactericidal effect when undiluted, but an inhibitory effect at full strength and 1/2 dilution. However, under such conditions the ascorbic levels are typically a thousand times greater than those present in the nanoparticle suspensions detailed in Table 1, and therefore any antimicrobial effects directly due to the presence of ascorbic acid in our experiments can be neglected.

The results show that Ag nanoparticles have significantly higher MIC and MBC values for *B. subtilis* and *E*. *coli* than Cu nanoparticles at the same concentration of chitosan (3% w/v) and metal salts (50 mM). The results presented here demonstrated that at the same metal salts concentration, Cu nanoparticles have a characteristically smaller particle size than Ag nanoparticles. Smaller particle size tends to enhance antibacterial properties because as size decreases, there are a larger number of atoms on the surface available to interact with bacteria (Marambio-Jones & Hoek, 2010). A mixture of Ag and Cu nanoparticles at 50 mM showed intermediate behaviour although statistically this was similar to that of Ag nanoparticles for both type of bacteria. The 50 mM Ag/Cu alloy nanoparticles showed the highest antibacterial effectiveness of all.

All the nanoparticles showed very similar values for MIC and MBC which shows that the nanoparticles have a bactericidal rather than bacteriostatic effect on these two bacteria.

Valodkar *et al*., 2011 reported MIC and MBC values of 0.26 and 0.78 mg/L for 10 mM Ag nanoparticles and the MIC and MBC of 0.23 and 0.65 mg/L for 10 mM Ag/Cu alloy nanoparticles against lower bacterial concentrations (10^4 CFU/mL) of *E. coli* than were employed here (10^8 CFU/mL). Taner *et al*., 2011 reported an MIC value only of >150 mg/L for Ag nanoparticles and identical values for MIC and MBC of Ag?Cu nanoparticles of 0.5 mg/L against high concentrations (10^8 CFU/mL) of *E. coli*. The MIC/MBC values reported here are lower than those achieved by Valodkar *et al.*, 2011 and Taner *et al*., 2011 which indicated better antibacterial activity and suggested that chitosan, which serves as stabilizing agent is also contributing to the antimicrobial effect. The findings obtained here are not directly comparable with those of Said-Galiev et al. (2011) as they did not report their findings in terms of MIC or MBC. Huang et al, (2004) synthesized nanoparticles using chitosan but did not report on the antimicrobial

activity of their nanoparticles. The findings reported here reveal that MIC/MBC values of bimetallic alloy nanoparticles were substantially lower than that of pure i.e. single metal nanoparticles.

As particle size will have an effect on antimicrobial activity, due to differences in specific surface area, the samples are compared against each other in Table 2 keeping an approximately constant mean particle size across all samples of approximately 200 nm (as performed for the zeta potentials presented in Fig. 5c. On this basis the Ag nanoparticles showed significantly lower MIC and MBC values than Cu nanoparticles towards both types of bacteria. Thus it can be surmised that Ag has a greater intrinsic antimicrobial activity than Cu. The lowest MIC and MBC values were observed for Ag/Cu nanoparticles in contact with both *B. subtilis* and *E. coli* either at the same concentration of metal salts and chitosan or fixed nanoparticles size. Cu nanoparticles displayed statistically the same antimicrobial effect as was obtained with a mixture of Ag and Cu nanoparticles towards both *B. subtilis* and *E*. *coli*.

Bacteria bear a negative charge due to the excess number of carboxylic and other groups which make the cell surface negative (Stoimenov, Klinger, Marchin, & Klabunde, 2002). The nanoparticle suspensions produced here have positive charge as revealed by zeta potential measurements. Electrostatic forces between positively charged nanoparticles and negatively charged bacteria cells will enhance the effect of antibacterial activity. Adhesion of nanoparticles to the surface of a bacterium alters its membrane properties ultimately causing death (Li et al., 2008). The MIC and MBC of the synthesized nanoparticles against both *B. subtilis* and *E. coli* revealed that the Gram-positive bacteria were more susceptible than the Gram-negative bacteria to the nanoparticles. This is most likely due to differences in bacterial cell-wall structure of Gram-positive and Gram-negative with the Gram-negative cell wall having a structure which is more complex than the Gram-positive cell wall.

4. Conclusions

Ag and Cu nanoparticles were synthesized using ascorbic acid as a reducing agent in chitosan solutions using an efficient microwave heating method. Moreover, the

synthesis was fast, inexpensive, environmentally benign, energy efficient and does not produce toxic waste. It was shown that nanoparticle size could be controlled by manipulating the concentrations of chitosan and silver and copper nitrate used in their synthesis. Particle size could be increased either by decreasing the chitosan concentration or increasing the metal ion concentration. The nanoparticles produced had a positive surface charge and the chitosan used in their synthesis contributed to the stability of suspensions of such particles and prevented agglomeration. MIC and MBC testing showed a potent bactericidal effect with Ag nanoparticles showing a greater killing effect when compared to Cu nanoparticles at the same mean particle size. All the nanoparticles showed very similar values for MIC and MBC which shows that the nanoparticles have a bactericidal rather than bacteriostatic effect on these two bacteria. The greatest antimicrobial effect was seen when Ag and Cu are combined during synthesis to form alloyed particles. The use of the latter in medical applications is currently being investigated.

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472

LIST OF FIGURES

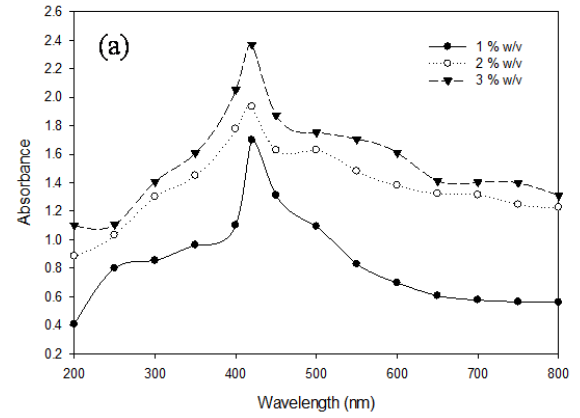
LIST OF TABLES

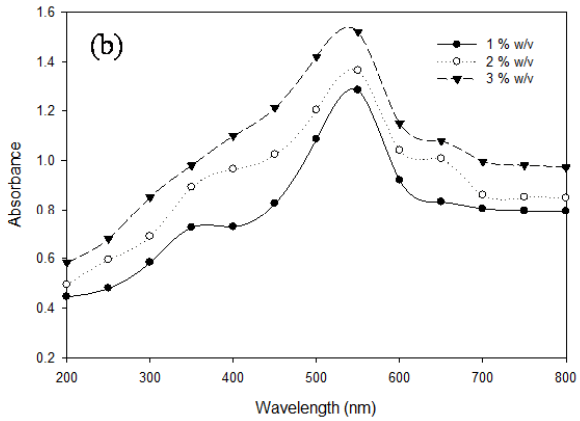
(a)

(b)

(c)

Fig. 1.


(a)
1 % w/v
2 % w/v
3 % w/v
Absorbance
Wavelength (nm)


(b)
1 % w/v
2 % w/v
3 % w/v
Absorbance
Wavelength (nm)

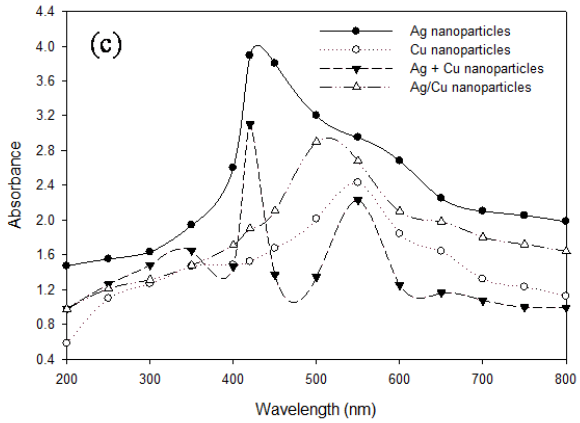
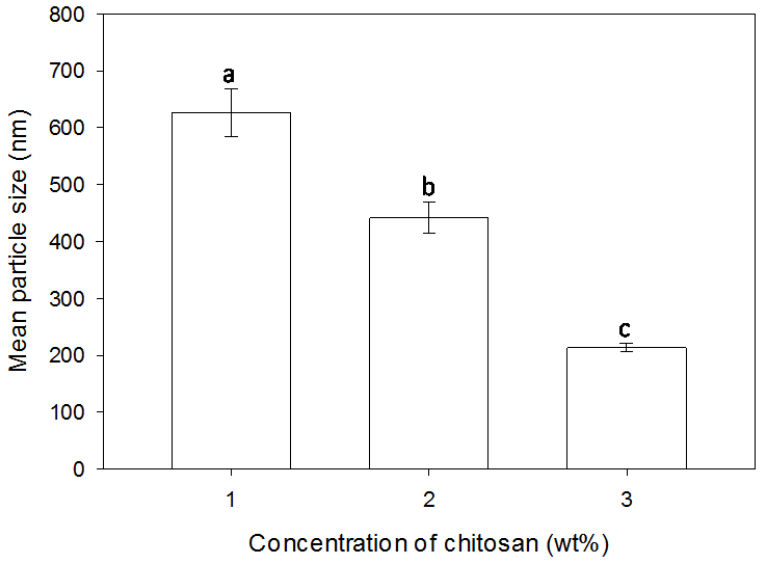
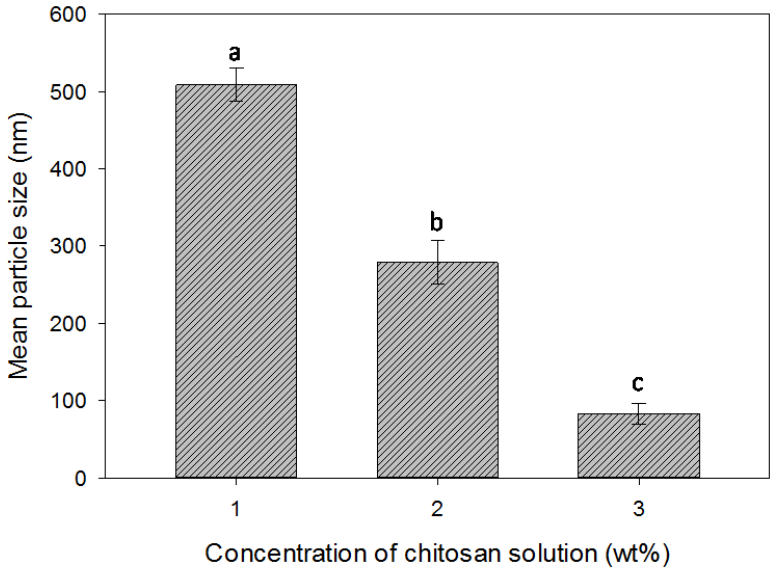

(c)
Ag nanoparticles
Cu nanoparticles
Ag + Cu nanoparticles
Ag/Cu nanoparticles
Absorbance
Wavelength (nm)

Fig. 2.


800
700
600
500
400
300
200
100
0
Mean particle size (nm)
a
b
c
1
2
3
Concentration of chitosan (wt%)

(a)


600
500
400
300
200
100
0
Mean particle size (nm)
a
b
c
1
2
3
Concentration of chitosan solution (wt%)

(b)

Fig. 3.

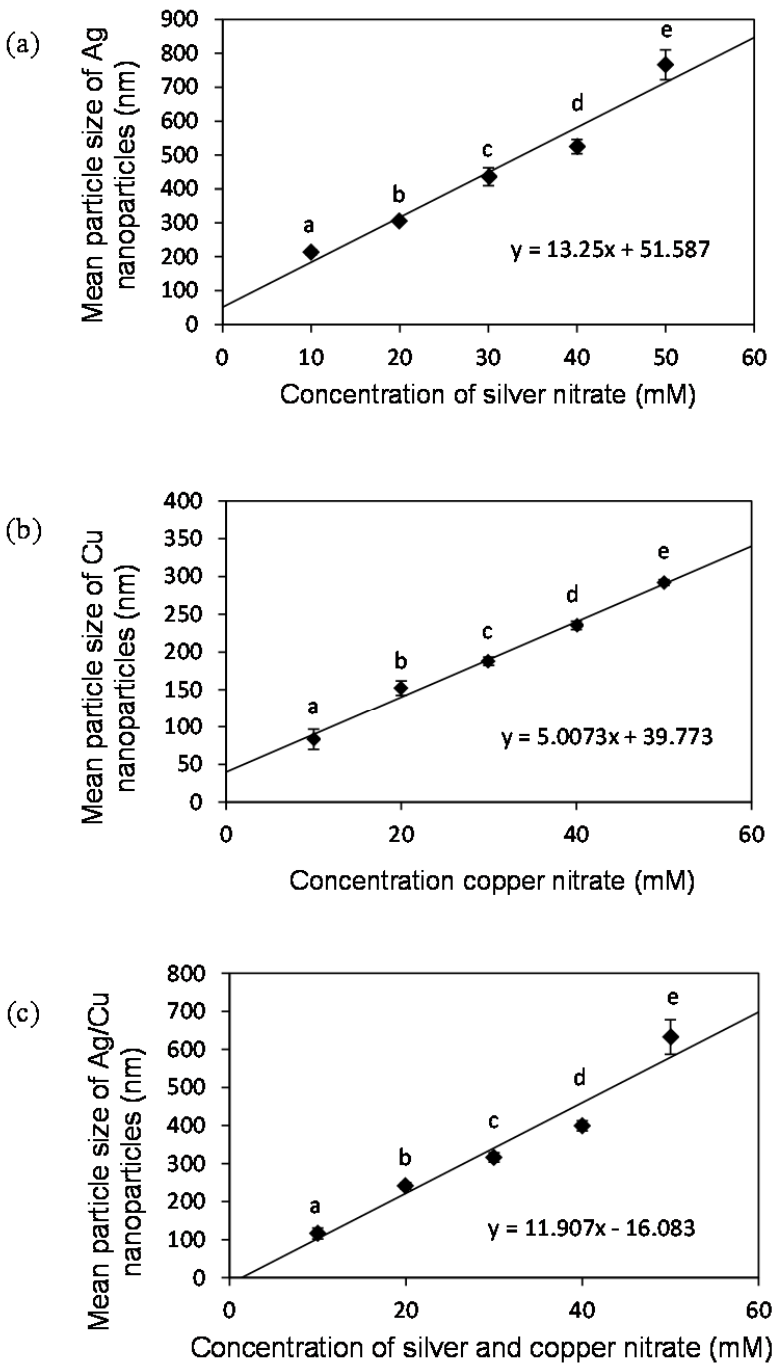

(a)
Mean particle size of Ag nanoparticles (nm)
900
800
700
600
500
400
300
200
100
0
a
b
c
d
e
y = 13.25x + 51.587
0
10
20
30
40
50
60
Concentration of silver nitrate (mM)
(b)
Mean particle size of Cu nanoparticles (nm)
400
350
300
250
200
150
100
50
0
a
b
c
d
e
y = 5.0073x + 39.773
0
20
40
60
Concentration copper nitrate (mM)
(c)
Mean particle size of Ag/Cu nanoparticles (nm)
800
700
600
500
400
300
200
100
0
a
b
c
d
e
y = 11.907x - 16.083
0
20
40
60
Concentration of silver and copper nitrate (mM)

Fig. 4.

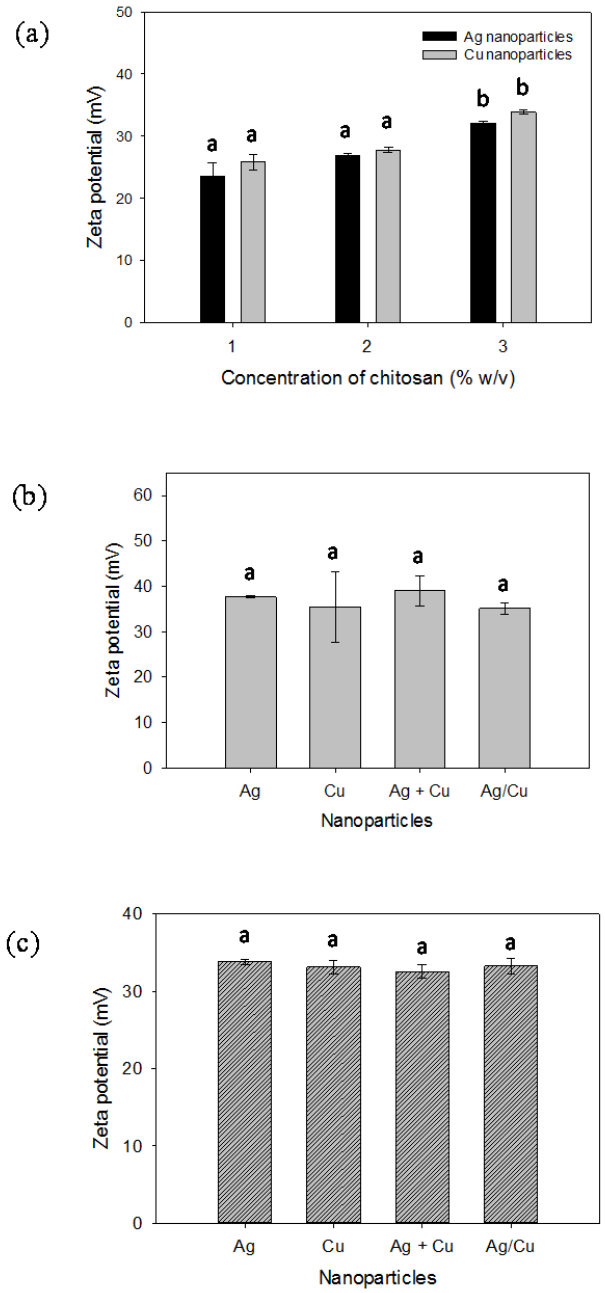

(a)
Ag nanoparticles
Cu nanoparticles
Zeta potential (mV)
Concentration of chitosan (% w/v)
(b)
Zeta potential (mV)
Ag
Cu
Ag + Cu
Ag/Cu
Nanoparticles
(c)
Zeta potential (mV)
Ag
Cu
Ag + Cu
Ag/Cu
Nanoparticles

Fig. 5.

Table 1:

Bacteria	MIC (mg/L)				MBC (mg/L)			
	Ag	Cu	Ag+Cu	Ag/Cu	Ag	Cu	Ag+Cu	Ag/Cu
B. subtilis	0.652^{A}_{a}	0.313^{B}_{a}	0.576^{A}_{a}	0.179^{C}_{a}	0.666^{A}_{a}	0.438^{B}_{a}	0.596^{A}_{a}	0.194^{C}_{a}
E.coli	0.793^{A}_{a}	0.469^{B}_{b}	0.670^{A}_{b}	0.273^{C}_{b}	0.746^{A}_{a}	0.531^{B}_{b}	0.700^{A}_{b}	0.298^{C}_{b}

Table 2:

Bacteria	MIC (mg/L)				MBC (mg/L)			
	Ag	Cu	Ag+Cu	Ag/Cu	Ag	Cu	Ag+Cu	Ag/Cu
B. subtilis	0.172^{A}_{a}	0.320^{B}_{a}	0.262^{B}_{a}	0.054^{C}_{a}	0.189^{A}_{a}	0.387^{B}_{a}	0.336^{B}_{a}	0.061^{C}_{a}
E.coli	0.199^{A}_{a}	0.433^{B}_{b}	0.327^{B}_{b}	0.076^{C}_{b}	0.199^{A}_{a}	0.470^{B}_{b}	0.379^{B}_{b}	0.081^{C}_{b}