Preparation of silver nanoparticles of enhanced antibacterial effect with benzalkonium bromide

W. T. YANG[a], H. LI[a], Y. GONG[a], W. Y. CHEN[a*], C. GAIDAU[b]
[a]*National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University, Chengdu 610065, Sichuan, P. R. China*
[b]*Leather and Footwear Research Institute, 93, Ion Minulescu St, sector 3, 031215, Bucharest, Romania*

In the study, a colloidal silver nanoparticles stabilized by a bactericide benzalkonium bromide was prepared by borohydride reduction of silver nitrate in water. A dosage of benzalkonium bromide could greatly influence the stability, the size and the dispersity of the prepared silver nanoparticles. When the concentration of benzalkonium bromide was 1×10^{-5}g·mL^{-1}, most of the silver nanoparticles could be coagulated and the solution had no obvious absorption in UV-vis spectra. When the concentration of benzalkonium bromide reached 10×10^{-5}g·mL^{-1}, a stable dispersion of silver nanoparticles without evident precipitation could be obtained. The results of dynamic light scattering (DLS) and transmission electron microscopy (TEM) showed that the average size of the nanoparticles was about 26 nm. Antibacterial tests proved that the silver nanoparticles capped with benzalkonium bromide exhibited a notable antibacterial effect against both Gram-positive and Gram-negative bacteria. The antibacterial effect of the material against *Escherichia coli* was four times higher than benzalkonium bromide and two times higher than only with the silver nanoparticles. Silver nanoparticles capped with benzalkonium bromide is a potential bactericide for different applications.

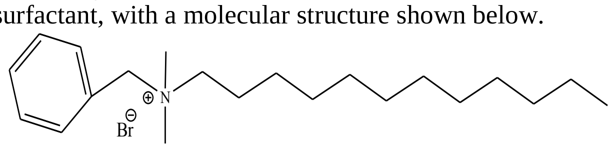
(Received January 28, 2011; accepted June 9, 2011)

Keywords: Colloid, Silver nanoparticle, Antibacterial, Benzalkonium bromide

1. Introduction

In recent years, the abuse of antibiotics has caused the appearance of new strains of resistant bacteria, which has become a serious problem in public healthcare [1, 2]. Over conventional antibiotics, silver nanoparticles may have an important advantage as they can kill all pathogenic microorganisms, and no organism has ever been reported to readily develop resistance to them [3]. Therefore, there is a rapid increase in the scientific publications devoted to the development of the preparation techniques of silver nanoparticles and further understanding of their properties [4-7]. Metal/polymer nanocomposites which are formed by silver nanoparticles with polymer matrix are widely utilized in medical applications [8]. Currently, it was reported that when silver nanoparticles are used along with traditional antibiotics, the antibacterial efficacy was increased due to the synergetic effect [9, 10]. From a fundamental point of view, as high level acquired resistance to conventional antibiotics is frequent, and to utilize silver nanoparticles effectively, it seems reasonable to use combination therapy in order to achieve bactericidal synergism.

Herein we report the preparation of colloidal silver nanoparticles using benzalkonium bromide as dispersant capping silver nanoparticles. Benzalkonium bromide is a quaternary ammonium bactericide and a cationic surfactant, with a molecular structure shown below.



This material has a strongly and fast bactericidal action and low toxicity, being non-irritating to skin and tissue. It is widely applied to surface disinfection of environment and body in hospitals [11-14]. In the study, colloidal silver nanoparticles were capped with benzalkonium bromide to improve its antibacterial effect. The influence of the concentration of benzalkonium bromide to the stability, the average particles size and the dispersion of the colloidal silver nanoparticles was studied for getting a stable solution without agglomeration. Finally, the antibacterial effect of the prepared colloidal silver nanoparticles was evaluated. The colloid was found exhibiting a notable antibacterial effect against the tested microorganisms.

2. Experimental

2.1. Materials

Silver nitrate, sodium borohydride and sodium citrate were of analytical grade. Benzalkonium bromide (5% solution in water) was purchased from Nanchang Baiyun Pharmaceutical Co. (China). All aqueous solutions were prepared with double distilled water.

2.2. Preparation of silver nanoparticles

7.5×10^{-3}g $AgNO_3$ were dissolved in 50mL distilled water and placed in water bath at 30 ℃ for 15 min. A 50mL benzalkonium bromide solution with various dosages was added dropwise to the $AgNO_3$ solution with intense stirring for 30min to form a combined solution at the concentration of benzalkonium bromide from $0g\cdot mL^{-1}$ to $100\times10^{-5}g\cdot mL^{-1}$. Subsequently, a 100mL of $3.7\times10^{-3}g$ $NaBH_4$ and the same concentration of benzalkonium bromide combined solution were added dropwise for 1.5h to get silver nanoparticles. Then, the prepared colloidal silver nanoparticles were conserved at 30 ℃.

2.3. Measurements

The generation of the silver nanoparticles was analyzed with a UV-vis Spectrophotometer (UV-2501PC, SHIMADZU, Japan). The Zeta-potential and particles size analyses of the silver nanoparticles were performed using a nanoparticle size and Zeta-potential analyzer (Zetasizer NanoS90, Malvern, England). TEM images were obtained by using transmission electron microscopy (JEM-100CXII, Japanese electronics company, Japan).

2.4. Antimicrobial activity

2.4.1. Preparation of beef extract peptone medium

The beef extract peptone medium was prepared with 5g beef extract, 10g peptone, 5g NaCl, 18 g agar and 1000mL water. The pH of the culture medium was adjusted to 7.0. Then, the culture medium was sterilized at 121℃ for 20 min.

2.4.2. Preparation of bacterial spore suspension

Various strains of bacteria including Gram-negative bacteria: *Escherichia coli, Enterobacter Hough, Acinetobacter*, Gram-positive bacteria: *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus* were used to evaluate the antibacterial activities of the prepared silver nanoparticles. Each species of bacteria was separately inoculated into the tubes with beef extract peptone medium and incubated in a Mould Incubation Chamber of 37℃ and 90% relative humidity for 7 days. Then, a sterile transfer loop was used to scrape a few loops of pure spores off the fresh cultures. The spores were well dispersed in a 50ml physiological saline solution (0.85%, w/w) after shaken in a vibrator for two hours. Through the physiological saline solution, the concentration of bacterial spores was arranged as 10^5 to $10^6 cfu\cdot mL^{-1}$ (colonies formed units per milliliter).

2.4.3. Preparation of culture dishes

Benzalkonium bromide, silver nanoparticles capped with sodium citrate which was prepared as described by G. K. Vertelov [15] , and sodium citrate were used as controls to compare antimicrobial effect with the silver nanoparticles capped with benzalkonium bromide. Firstly, a 10mL of the culture medium and a 1mL of the bacteria suspension were added in a culture dish. Then, the prepared antimicrobial agents were diluted in the culture dishes at a concentration of 0-200$\mu g\cdot mL^{-1}$. The culture dishes were incubated in a Mould Incubation Chamber at 37℃ and 90% relative humidity for 24h [16].

3. Results and discussion

3.1. Generation of silver nanoparticles

The absorption peak in 400-450nm range is the characteristic absorption peak of silver nanoparticles. As shown in Fig. 1, when the concentration of benzalkonium bromide was of $1\times10^{-5}g\cdot mL^{-1}$, there was no obvious absorption peak around 400nm, showing that most of the silver nanoparticles could be coagulated. When the concentration of benzalkonium bromide was of $2\times10^{-5}g\cdot mL^{-1}$, a weak absorption peak at 402nm was observed, indicating that only a few of silver nanoparticles were stabilized. As the benzalkonium bromide concentration increased from $2\times10^{-5}g\cdot mL^{-1}$ to $3\times10^{-5}g\cdot mL^{-1}$, a substantial rise of the absorbance showed that there were more silver nanoparticles in the solution.

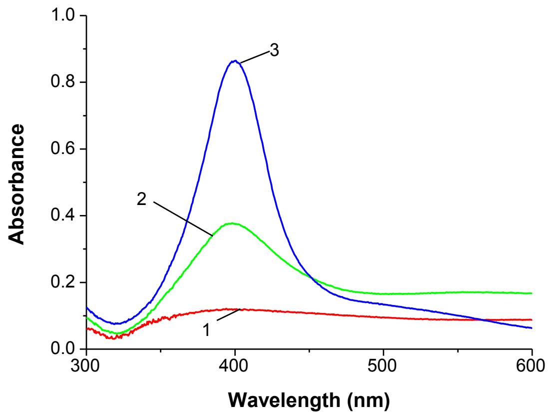


Fig. 1. UV-vis absorption spectroscopy at various benzalkonium bromide concentrations 1 - $1\times10^{-5}g\cdot mL^{-1}$ 2 - $2\times10^{-5}g\cdot mL^{-1}$, 3 - $3\times10^{-5}g\cdot mL^{-1}$

It was found that the UV-vis absorbance of the silver nanoparticles was not significantly changed upon the concentration of benzalkonium bromide of $3\times10^{-5}g\cdot mL^{-1}$. Interestingly, the absorption spectroscopy peak moved gradually to high wavelengths with a red shift as an increase in the amount of benzalkonium bromide. But there was no more correlation between the position of absorption peak and the size distribution of the silver nanoparticles, which was also mentioned by G. K. Vertelov [15]. The reason lies in the fact that the size of all the studied silver nanoparticles was much smaller than the light wavelengths, so the specific absorption was independent of the particles size in Mie theory [17].

3.2. Stability, dispersity and size analysis

The Zeta-potential of silver nanoparticles was of 6.73mV when the concentration of benzalkonium bromide was of $1\times10^{-5}g\cdot mL^{-1}$ (Fig. 2). Then the potential increased gradually with increasing benzalkonium bromide concentration. In a stable system, the theoretical absolute potential value is of 30mV. It means that a colloidal system would be stable if its potential value is greater than 30mV or less than -30mV [18]. The Zeta-potential values of the silver nanoparticles at the concentrations from $20\times10^{-5}g\cdot mL^{-1}$ to $100\times10^{-5}g\cdot mL^{-1}$ were all above 30mV, which would mean that the silver nanoparticles are stable. But some precipitations were observed in all of the silver nanoparticles after deposited three days except the concentrations between $8\times10^{-5}g\cdot mL^{-1}$ and $16\times10^{-5}g\cdot mL^{-1}$, which had no precipitations after one-week deposition. It could be suggested that the Zeta-potentials are not the only influence factor of the stability of the silver nanoparticles. To further clarify the stabilization mechanism and get a optimal preparation condition of the silver nanoparticles, the dispersity and the average particles size of the silver nanoparticles in the concentrations between $8\times10^{-5}g\cdot mL^{-1}$ and $16\times10^{-5}g\cdot mL^{-1}$ were analyzed next.

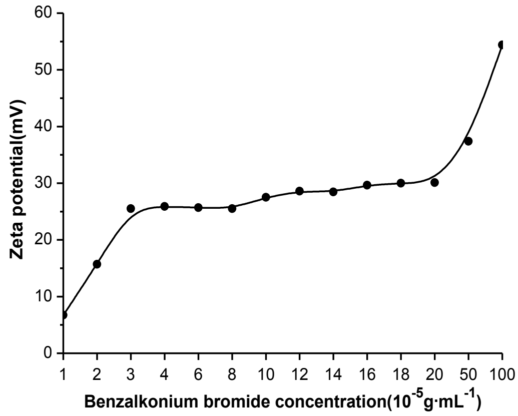


Fig. 2. Zeta-potential at various benzalkonium bromide concentrations

Fig. 3 shows electron micrographs for different benzalkonium bromide concentrations. At the lowest benzalkonium bromide concentration of $8\times10^{-5}g\cdot mL^{-1}$, the nanoparticles agglomerated seriously (Fig. 3a). The nanoparticles were dispersed well in small size in an intermediate range of the benzalkonium bromide concentration about $10\text{-}14\times10^{-5}g\cdot mL^{-1}$ (Fig. 3b-d). At high benzalkonium bromide concentration of $16\times10^{-5}g\cdot mL^{-1}$ (Fig. 3e), large particles with agglomeration was observed.

The average particles size distributions are accordant with the electron micrograph observations (see Fig. 4). The average particles size of the silver nanoparticles was the least at 26 nm when the intermediate benzalkonium bromide concentration was of $10\times10^{-5}g\cdot mL^{-1}$, where the polydispersity index (PDI) value was the most, indicating the silver nanoparticles were the best dispersed in the sol. Lower or higher than this concentration, enlargement of the silver nanoparticles was shown with a poorer dispersity.

So, the differences in the silver nanoparticles at various benzalkonium bromide concentrations indicated different stabilization mechanisms. At low benzalkonium bromide concentrations, the positive charge of silver nanoparticles was also low, which favored condensation of the silver nanoparticles to build up larger ones. In the intermediate range ($(10\text{-}14)\times10^{-5}g\cdot mL^{-1}$), the molecule amount of benzalkonium bromide was high enough to build up a strong repelling layer to stabilize the silver nanoparticles without further growth. As to high benzalkonium bromide concentrations, the benzalkonium bromide layer around silver nanoparticles was not effectively built-up. So the silver nanoparticles were not protected from further reaction with each other, which leaded to an enlargement and agglomeration [19].

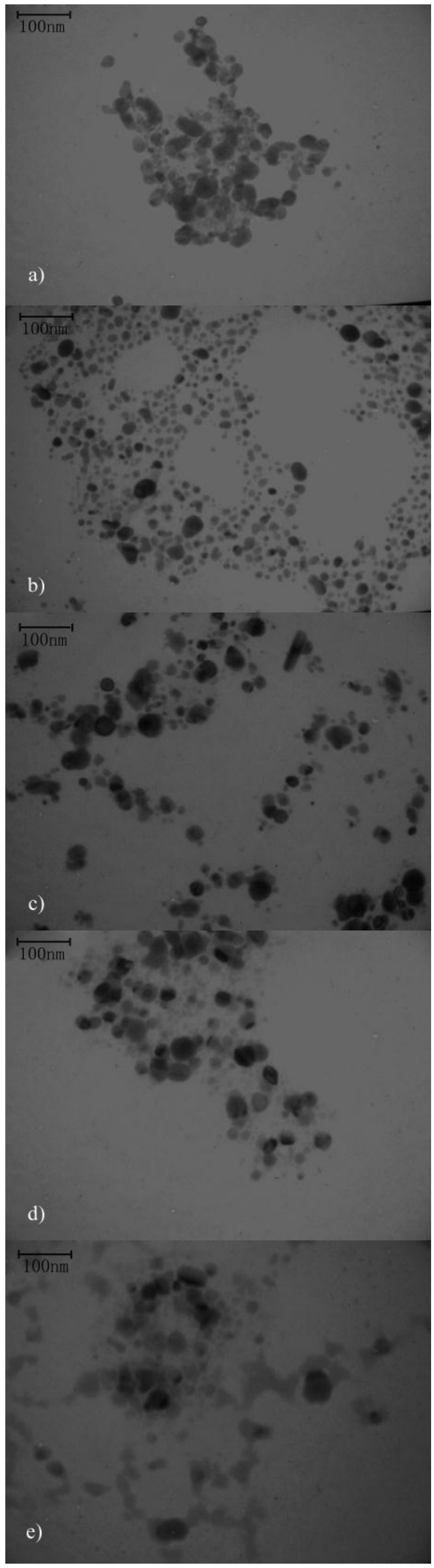


Fig. 3 TEM images at various benzalkonium bromide concentrations a) $8\times10^{-5}g\cdot mL^{-1}$, b) $10\times10^{-5}g\cdot mL^{-1}$, c) $12\times10^{-5}g\cdot mL^{-1}$, d) $14\times10^{-5}g\cdot mL^{-1}$, e) $16\times10^{-5}g\cdot mL^{-1}$

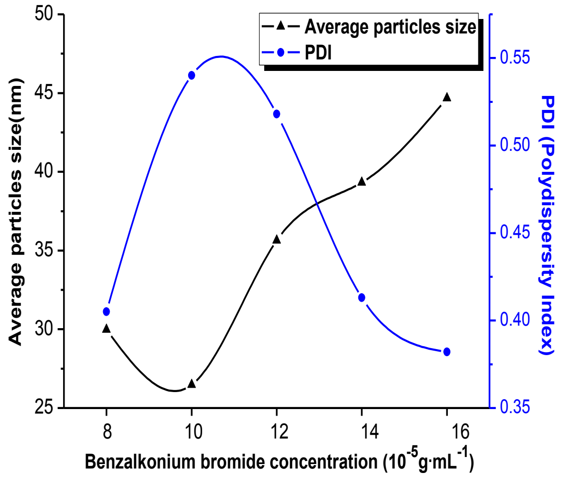


Fig. 4. Average particles size and PDI at various benzalkonium bromide concentrations

3.3. Antibacterial study

The dilution micromethod [16] was applied to study the antibacterial activity of the silver nanoparticles capped with benzalkonium bromide which was prepared at the benzalkonium bromide concentration of 10×10^{-5}g·mL^{-1}. Silver nanoparticles capped with sodium citrate [15], benzalkonium bromide and sodium citrate, were subjected to the antibacterial tests. Minimal inhibition concentrations (MICs) of the tested samples are summarized in table 1.

As can be seen in Table 1, the MICs of sodium citrate against the six bacteria strains are all more than 200μg·mL^{-1}, showing sodium citrate has no antibacterial effect. As against the Gram-negative bacteria and the Gram-positive bacteria, there is no evident difference among the antimicrobial effect of all the antimicrobial agents. But the antimicrobial effects of SNP-BKB against the six bacteria strains are all better than SNP-SC. Also the antibacterial effect of only benzalkonium bromide is not as well as SNP-BKB. The antibacterial tests show that the antibacterial effect of SNP-BKB against *Escherichia coli* is the best in comparison to the six bacteria strains. The antibacterial effect of the material is four times higher than benzalkonium bromide and two times higher than only with the silver nanoparticles.

The effective antibacterial activity of SNP-BKB may be owing to benzalkonium bromide react to the bacterial cell membrane and weaken it firstly, thus the silver nanoparticles can subsequently invade into the bacteria cell easily and fast and cause the bacteria death [20]. Due to the synergistic antibacterial effect of benzalkonium bromide and silver nanoparticles, the antimicrobial capacity of SNP-BKB was greatly strengthened, better than the benzalkonium bromide and only with the silver nanoparticles.

It is worth to be mentioned that the SNP-BKB at the benzalkonium bromide concentration of 10×10^{-5}g·mL^{-1} was applied to treat wool by soaking. The nanoparticles were found attaching on the surface of the wool from scanning electron microscopy (SEM) observations. The antibacterial effect of the modified wool was also studied by a shake flask method [21]. The results showed that SNP-BKB treated wool had a notable antibacterial effect against *Escherichia coli*, *Staphylococcus aureus*, *Acinetobacter* and *Bacillus subtili*, with the minimum inhibition rate above 88%.

Table 1. MICs（*μg·mL^{-1}*）*of different antimicrobial agents*

Description	Gram-negative bacteria			Gram-positive bacteria		
	Escherichia coli	*Enterobacter hough*	*Acinetobacter*	*Staphylococcus aureus*	*Bacillus subtilis*	*Bacillus cereus*
SC[a]	>200	>200	>200	>200	>200	>200
BKB[b]	20	20	60	20	40	20
SNP-SC[c]	10	40	40	30	40	50
SNP-BKB[d]	5	20	30	20	30	10

[a] sodium citrate, [b] benzalkonium bromide, [c] silver nanoparticles capped with sodium citrate, [d] silver nanoparticles capped with benzalkonium bromide.

4. Conclusion

A dosage of benzalkonium bromide could influence the stability, size and dispersity of the silver nanoparticles greatly. When the concentration of benzalkonium bromide is of 10×10^{-5}g·mL^{-1}, a stable dispersion of silver nanoparticles with average particles size about 26 nm could be obtained. The silver nanoparticles capped with benzalkonium bromide exhibit a notable antibacterial effect against both Gram-positive and Gram-negative bacteria. The antibacterial effect of the material is higher than the benzalkonium bromide and only with the silver nanoparticles. In summary, this new material exhibits strong antibacterial activity as a potential bactericide.

Acknowledgments

The authors wish to thank the Ministry of Science and Technology of China for the project of the Co-operation in Science and Technology between Romania and People’s Republic of China (item No. 2009DFA42850). We would also like to thank the Agency of Science and Technology of Sichuan Province for financial support (item No. 2009HH0004).

References

[1] J. D. Garrity, B. Bennett, M. W. Crowder, Biochemistry, **44**, 1078 (2005).

[2] S.V. Kyriacou, W. J. Brownlow, X. H. N. Xu, Biochemistry, **43**, 140 (2004).
[3] C. Bhupendra, K. V. Anjana, A. Nidhi, R. V. Upadhyay, R. V. Mehta, Nano. Res., **2**, 955 (2009).
[4] E. Shahriari, W. M. M. Yunus, J. Optoelectron. Adv. Mater. **12**(11), 2306 (2010).
[5] D. Kim, S. Jeong, J. Moon, Nanotechnology. **17**, 4019 (2006).
[6] K. J. Lee, B. H. Jun, J. Choi, Y. I. Lee, J. Joung, Y. S. Oh, Nanotechnology, **18**, 335601 (2007).
[7] A. Petica, N. Buruntea, C. Nistor, J. C. R. Ionescu, J. Optoelectron. Adv. Mater., **9**(11), 3435 (2007).
[8] W. K. Son, J. H. Youk, T. S. Lee, W. Park, Macromol. Rapid. Commun., **25**, 1632 (2004).
[9] A. D. Souza, D. Mehta, R. W. Leavitt, Current Science, **91**, 926 (2006).
[10] P. Li, C. Wu, Q. Wu, J. Li, Nanotechnology **16**, 1912 (2005).
[11] K. Kuca, M. Bielavska, J. Cabal, V. Dohnalb, Analytical Letters, **38**, 673 (2005).
[12] K. Kuca, J. Marek, P. Stodulka, K. Musilek, P. Hanusova, M. Hrabinova, D. Jun, Molecules **12**, 2341 (2007).
[13] F. Brill, P. G. Bermes, W. Sand, Int. J. Hyg. Environ. Health, **209**, 89 (2006).
[14] H. F. Rabenau, G. Kampf, J. Cinatl, H. W. Doerr, J. Hosp. Infect., **61**, 107 (2005).
[15] G. K. Vertelov, Y. A. Krutyakov, O. V. Efremenkova, A. Y. Olenin, G. V. Lisichkin, Nanotechnology **19**, 355707 (2008).
[16] L. Kvitek, A. Panacek, J. Soukupova, M. Kolar, R.Vecerova, R. Prucek, M. Holecova. R. Zboril, J. Phys. Chem. C., **112**, 5825 (2008).
[17] U. Kreibig, Vollmer, Optical Properties of Metal Clusters, Springer, 1995.
[18] ASTM D: 4187-82 (1985) - American Society for Testing and Materials (Zeta Potential of Colloids in Water and Waste Water).
[19] A. Henglein, M. Giersig, J. Physic and Chemistry B, **103**, 9533 (1999).
[20] Q. S. Wei, J. Ji, J. H. Fu, J. C. Chen, J. Science. China B, **50**, 418 (2007).
[21] H. B. Gu, W. Y. Chen, C. Q. Zhao, X. Hao, Journal of the American Leather Chemists Association, **103**(3), 114 (2008).

*Corresponding author: wuyong.chen@163.com