

Research Article

Preparation, Characterization and Antibacterial Properties of Silver-Chitosan Nanocomposites Using Different Molecular Weight Grades of Chitosan

S Honary^{1*}, K Ghajar¹, P Khazaeli² and P Shalchian²

¹ Mazandaran University of Medical Sciences, School of Pharmacy & Pharmaceutical Sciences Research Center, Sari, Iran; ² Kerman University of Medical Sciences, School of Pharmacy, Kerman, Iran

Abstract

Purpose: To study the effect of chitosan molecular weight on the physicochemical and antibacterial properties of silver-chitosan nanoparticles.

Methods: A series of silver-chitosan nanoparticles of different sizes were produced using various molecular weight (MW) grades of chitosan by an aqueous chemical reduction method. The nanoparticles were characterized by ultraviolet-visible absorption spectroscopy (UV-Vis), scanning electron microscopy (SEM), differential scanning calorimetry (DSC), dynamic light scattering (DLS) and laser Doppler electrophoresis (LDE). The antibacterial properties of the nanoparticles were also evaluated by agar diffusion method.

Results: The size of the silver-chitosan nanoparticles, ranging from 21.9 to 175.3 nm, was influenced by chitosan MW as well as by other process conditions. Although, the nanoparticles were not stable in liquid form, they however showed good stability in the solid state due to their low zeta potential. SEM images indicate that the nanoparticles were spherical. The antibacterial activity of the nanoparticles against *Staphylococcus aureus* increased with decrease in particle size owing to increase in surface area. The smallest particle size (21.9 nm) was obtained by using high chitosan MW at 4 °C and a stirring speed of 800 rpm.

Conclusion: Chitosan is an effective agent for the preparation of silver nanoparticles. The size of the nanoparticles can be modulated by varying both chitosan MW and process conditions such as temperature and stirring speed.

Keywords: Chitosan; Antibacterial activity; Silver nanoparticles; Molecular weight; Particle size; Dynamic light scattering; Laser Doppler electrophoresis

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*Corresponding author: **E-mail:** shonary@yahoo.com, SHonary@mazums.ac.ir; **Tel:** +98 151 3543084, 151 3246491; **Fax:** +98 151 2224521

INTRODUCTION

The antibacterial effects of silver (Ag) salts have been known for over 2000 years, but they have only been in common use as an antimicrobial since the 19th century [1]. Ag is currently used to control bacterial growth in a variety of applications, including dental work, catheters, and burn wounds [1]. Silver nitrate solution causes argyria (staining of the skin) and a burning sensation on application. Ag ions and Ag-based compounds are highly toxic to microorganisms. They show strong biocidal activity against as many as 12 species of bacteria including *E. coli* [2]. Today, other forms of silver are available which do not have the disadvantages of earlier solutions.

Modern dressings are capable of delivering silver through a sustained-release mechanism. This helps to avoid toxicity and yet ensures delivery of a therapeutic dose of silver to the wound [3]. It has been shown that reducing the particle size of materials is an efficient and reliable tool for improving their actions; hence, silver nanoparticles in some modern dressings, such as Acticoat[®], aids in burns management [4]. The antimicrobial properties of silver-containing wound dressings have been studied by a microcalorimetric technique. The results showed that this kind of dressing has the capacity to kill common wound pathogens - *Staphylococcus aureus* and *Pseudomonas aeruginosa* [5]. Different nano silver-coated barrier dressings were reported to exhibit antimicrobial activity and reduce infection in wounds [6]. The antibacterial activity of chitosan tripolyphosphate nanoparticles loaded with various metal ions was investigated by Du *et al* and the results showed that antibacterial activity was enhanced by the loaded metal ions [7]. An earlier work showed that accumulation of silver nanoparticles in bacterial membrane caused a significant increase in membrane permeability, resulting in the death of the cell [8].

Silver nanoparticles have been produced by different methods, one of which is the chemical reaction method [9]. In this method, Ag ions are reduced by UV or γ - irradiation, ultrasound, prolonged reflux, or chemicals to produce silver nanoparticles [10]. Two other methods used include nanoparticle colloid [11] and organic-inorganic composition film [12]. Hybridation of Ag nanoparticles with amphiphilic hyperbranched macromolecules has been shown to exhibit good antimicrobial properties [13].

Chitosan is the N-deacetylated derivative of chitin. The positive charge of chitosan affords the polymer numerous and unique physiological and biological properties. Several studies have demonstrated the effect of molecular weight and concentration of chitosan on antibacterial and antifungal activities [14]. The aim of this work was to formulate and evaluate the antibacterial activity of simple and cost-effective silver-chitosan nanocomposite formulations made with different molecular weight grades of chitosan, for possible use as dressings.

EXPERIMENTAL

Materials

Low, medium and high molecular weight (MW) grades of chitosan with MW of 100, 400 and 600 KD, respectively, were purchased from Fluka BioChemica, Japan. Their degree of deacetylation was 85 %. Silver nitrate (AgNO_3) and sodium borohydride (NaBH_4) were purchased from Merck, Germany.

Preparation of silver-chitosan nanocomposites

A solution of chitosan (1 - 3 mg/ml) in acetic acid solution (1 - 2 %) was first prepared. Due to the poor solubility of chitosan, the mixture was vortexed to achieve complete dissolution, and then kept overnight at room temperature. The solution was filtered through a 0.22 μm millipore syringe filter to remove any impurity before use. Silver-

chitosan nanocomposites were obtained by chemical reduction of the silver salt to yield the corresponding zero valent silver nanoparticles with NaBH₄. To ensure complete reduction, the concentration of NaBH₄ was 10 times that of the silver salt. Table 1 shows the composition of the various formulations prepared and the process conditions. The silver nanoparticles were separated by centrifugation at 15000 rpm and dried at 60 °C for 24 h on a Petri dish, yielding a thin layer.

Characterization of nanoparticles

Scanning electron microscope (model 2360, Leo Oxford, England) was used to evaluate the surface and shape characteristics of the particles after prior coating with gold.

UV-Vis absorption spectra of the samples were recorded in the wavelength range of 300 to 500 nm with Genesys 2 spectrophotometer, while size, polydispersity and zeta potential of the particles were determined using Zetasizer 3600 (Malvern Instruments, UK) at 25 °C and scattering angle of 90°. Differential scanning calorimetric studies (Perkin Elmer, Pyris 6, Germany) on the nanoparticles were carried out with the sample (≈10 mg) placed in a conventional aluminium pan with aluminium lid and heated from 50 to 250 °C at a rate of 10 °C/min under a nitrogen atmosphere. An empty, loosely covered aluminium pan served as the reference.

Antibacterial test

The antibacterial activity of the nanoparticles was evaluated against *Staphylococcus aureus* (PTCC 1112) by the agar diffusion method with soybean-casein digest agar (SCDA) as the medium. An aliquot of silver-nanoparticle dispersion (50 µl) was added into each of six wells in a plate, and then incubated for 24 h at 40 °C. Antibacterial activity was measured, in triplicate, as the diameter of the inhibitory zones in the plates; the mean of three measurements was taken. Gentamicin solution (50 µl, 1 mg/ml) was

used as reference standard. Control experiments with polymers and solvents were carried out and the control inhibitory zone was subtracted from inhibitory zone for the test sample to arrive at the actual value of the inhibitory zone [15].

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Student's t-test and one-way analysis of variance (ANOVA) were applied to ascertain significant differences in antibacterial activity and particle size for various formulations using Sigma Stat software, version 2. Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

Size and zeta potential of nanoparticles

Table 1 shows, amongst others the particle size of the formulations. It indicates that the size of silver nanoparticles depends on various process factors including chitosan MW, temperature, zeta potential and stirring rate. Alteration of these process variables had a major effect on the size of the nanoparticles.

In terms of chitosan molecular weight (MW), the rank order of particle size was medium MW > low MW > high MW. Nanoparticle size decreased significantly with increase in stirrer speed and also as temperature decreased from 25 to 4 °C ($p < 0.05$). The smallest size (21.9 nm) was achieved for F18 when high MW chitosan was used at 4 °C and 800 rev/min.

All the formulations showed low zeta potential (Table 1) and significant variation in particle size after storage at 4, 25 or 45 °C for 10 days in dispersion form ($p < 0.05$). There was a direct correlation between particle size and temperature of preparation. Although the nanoparticles showed aggregation and physical instability in liquid form, leading to the formation of larger particles, they nevertheless exhibited good physical stability in the solid state.

Table 1: Composition and characteristics of silver-nanoparticle formulations

Code	Chitosan MW	Chitosan (mg/ml)	AgNO ₃ (mg/ml)	SR (r/m)	Temp (°C)	Acetic acid (%)	Diameter (nm)	PDI	ZP (mV)
F1	L	2	20	400	25	1	70±5	0.042	-0.59
F2	M	2	20	400	25	1	76±4	0.031	0.01
F3	H	2	20	400	25	1	26±2	0.030	-4.17
F4	L	2	20	400	4	1	30±3	0.026	-4.25
F5	M	2	20	400	4	1	66±4	0.057	-5.86
F6	H	2	20	400	4	1	24±3	0.065	-4.37
F7	L	2	40	400	25	1	80±5	0.061	0.89
F8	M	2	40	400	25	1	97±6	0.049	1.77
F9	H	2	40	400	25	1	31±2	0.036	-5.70
F10	L	3	20	400	25	1	142±3	0.086	7.44
F11	M	3	20	400	25	1	134±4	0.047	6.19
F12	H	3	20	400	25	1	102±4	0.060	2.86
F13	L	2	20	400	25	2	114±4	0.057	4.89
F14	M	2	20	400	25	2	175±3	0.062	3.72
F15	H	2	20	400	25	2	102±3	0.035	9.10
F16	L	2	20	800	4	1	25±2	0.036	-5.51
F17	M	2	20	800	4	1	35±2	0.049	-5.13
F18	H	2	20	800	4	1	21 ±1	0.037	-7.33

L, M and H = low, medium and high MW chitosan, respectively; SR = stirring rate (rev/min); ZP = zeta potential; PDI = polydispersity index;

Spectral properties

Fig 1 shows the UV-Vis spectra of the silver-chitosan nanocomposites. All the spectra exhibited an absorption band at around 400 - 420 nm, which is a typical plasmon band, suggesting the formation of silver nanoparticles. The width of the absorption band for the nanoparticles containing medium molecular weight chitosan is greater than those containing low and high molecular weight chitosan. Furthermore, the center of absorption band also varied slightly with chitosan molecular weight, a factor that operates as a controller of nucleation as well as a stabilizer [16].

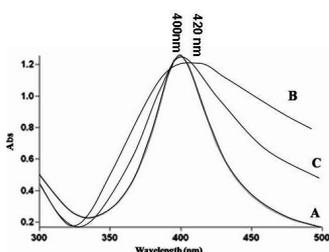


Figure 1: UV-Vis spectra of the silver-chitosan nanoparticles produced with low (A), medium (B) and high (C) molecular weight chitosan.

Scanning electron microscopy (SEM)

The results of SEM, shown in Fig 2, indicate that the silver-chitosan nanoparticles were spherical with particle size in the range of 20 - 120 nm.

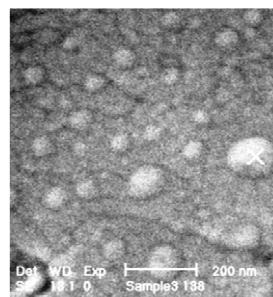


Figure 2: SEM photo of silver-chitosan nanoparticles (F12).

Differential scanning calorimetry (DSC)

Fig 3 indicates that the thermograms of the nanoparticles containing low, medium and high MW chitosan exhibited an endothermic peak below 100 °C, which is probably related to water loss, and a slight exothermic transition above 200 °C, which likely corresponds to chitosan decomposition.

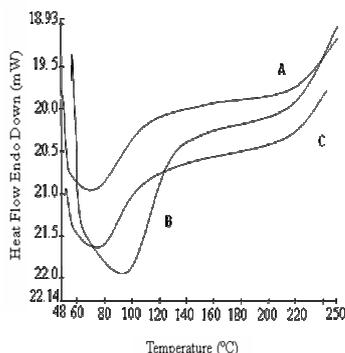


Fig 3: DSC thermograms of silver-chitosan nanoparticles containing low (A), medium (B), and high (C) molecular weight chitosan

Antimicrobial activity

Table 3 shows that the diameter of the zone of inhibition increased as the size of the particles decreased.

Table 3: Antibacterial activity of some silver-chitosan nanoparticles (mean±SD, n = 3)

Code	Zone of inhibition (mm)
F16	19.3±0.5
F17	18.3±0.4
F18	20.7±0.5
Gentamicin (1 mg/ml)	23.3±0.5

DISCUSSION

When AgNO₃ was mixed with chitosan solution, Ag⁺ ions probably bound to chitosan macromolecules via electrostatic interaction between the electron-rich oxygen atoms of the polar hydroxyl and ether groups of chitosan and the electropositive transition cations (Ag⁺). However, a study of the mechanism of the reaction process of silver nitrate with chitosan by Wei et al using FTIR [17] also showed possible interaction between silver salts and chitosan molecules, which may account for the reduction of Ag ions and stabilization of silver-chitosan nanoparticles. Thus the attachment of silver to the nitrogen atoms in chitosan reduced the

vibration intensity of the N-H bond due to increased molecule weight after silver binding. It has been shown that the stiffness and conformations of small molecular weight chitosan were greater and more extended, respectively, than higher molecular weight chitosan [18]. Furthermore, high molecular weight chitosan is more flexible [19]. Generally, high MW chitosan would be a more effective stabilizer for Ag-chitosan nanoparticles owing to its greater flexibility and viscosity than lower MW grades. Also, production of the nanoparticles at 4 °C would result in smaller particles than at 25 °C due probably to increase in medium viscosity.

The inhibitory effect of silver on microorganisms tested is effected via two possible mechanisms First, is the electrostatic attraction between the negatively charged cell membrane of the microorganisms and the positively charged Ag, and second, is the formation of ‘pits’ in the cell wall of bacteria related to Ag concentration [20]. In this study, since the zero valent metal nanoparticles were obtained by chemical reduction of metal salts, it seems the latter mechanism would have been mooted. A previous study showed that 25 nm diameter particles exhibited maximum antibacterial activity [18]; therefore, the method used in this study would be suitable for producing 25 nm silver-chitosan particles. The smaller the size of the nanoparticles the larger the surface area in contact with the bacterial cells and hence, the greater the interaction with the cells.

CONCLUSION

Although chitosan has often been used as a stabilizer in silver nanoparticles production process, there is no report, to the best of our knowledge, about the effect of its MW in this process. This study shows that the size of silver nanoparticles can be controlled by varying chitosan MW as well as process conditions such as temperature and stirring speed. One of the most important properties of silver nanoparticles is their antimicrobial

property. This study demonstrates that the antimicrobial effect of silver nanoparticles strongly depends on their size. Smaller particles showed greater antimicrobial activity than bigger one.

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