Silver Colloid Nanoparticles: Synthesis, Characterization, and Their Antibacterial Activity

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A one-step simple synthesis of silver colloid nanoparticles with controllable sizes is presented. In this synthesis, reduction of $[Ag(NH_3)_2]^+$ complex cation by four saccharides was performed. Four saccharides were used: two monosaccharides (glucose and galactose) and two disaccharides (maltose and lactose). The syntheses performed at various ammonia concentrations (0.005−0.20 mol L^{-1}) and pH conditions (11.5−13.0) produced a wide range of particle sizes (25−450 nm) with narrow size distributions, especially at the lowest ammonia concentrations. The average size, size distribution, morphology, and structure of particles were determined by dynamic light scattering (DLS), transmission electron microscopy (TEM), and UV/Visible absorption spectrophotometry. The influence of the saccharide structure (monosacharides versus disaccharides) on the size of silver particles is briefly discussed. The reduction of $[Ag(NH_3)_2]^+$ by maltose produced silver particles with a narrow size distribution with an average size of 25 nm, which showed high antimicrobial and bactericidal activity against Gram-positive and Gram-negative bacteria, including highly multiresistant strains such as methicillin-resistant *Staphylococcus aureus*. Antibacterial activity of silver nanoparticles was found to be dependent on the size of silver particles. A very low concentration of silver (as low as 1.69 μg/mL Ag) gave antibacterial performance.

Introduction

Silver has been known for antibacterial activity since the times of ancient Greece. Currently, the investigation of this phenomenon has regained importance due to the increase of bacterial resistance to antibiotics, caused by their overuse.[1] Recently, silver nanoparticles[2−4] as well as various silver-based compounds containing ionic silver (Ag^+)[5,6] or metallic silver (Ag^0)[7−11] exhibiting antimicrobial activity have been synthesized. Antibacterial activity of the silver-containing materials can be used, for example, in medicine to reduce infections in burn treatment[12,13] and arthroplasty,[14] as well as to prevent bacteria colonization on prostheses,[15] catheters,[16,17] vascular grafts,[18] dental materials,[19] stainless steel materials,[20] and human skin.[21,22] Silver-containing materials can be employed to eliminate microorganisms on textile fabrics,[23,24] or they can be used for water treatment.[25] Silver nanoparticles also exhibit a potent cytoprotective activity toward HIV infected cells.[26] Because of such wide range of applications, numerous synthetic methods have been developed.[27−32] Contrary to bactericide effects of ionic silver, the antimicrobial activity of colloid silver particles are influenced by the dimensions of the particles—the smaller the particles, the greater antimicrobial effect.[3,4] Therefore, in developing routes of synthesis, an emphasis was made to control the size of silver nanoparticles.

The most common synthesis of silver nanoparticles is the chemical reduction of a silver salt solution by a reducing agent such as $NaBH_4$, citrate, and ascorbate.[28−34] The use of a strong reductant such as borohydride, resulted in small particles that were somewhat monodisperse, but controlling the generation of the larger particles became difficult. The use of citrate, a weaker reductant, resulted in a slower reduction rate, but the size distribution was far from narrow. The controlled synthesis of silver colloid particles was attempted using a two-step reduction process in order to control the particle size. In this process, a strong reducing agent was first applied to produce small silver particles, which were then enlarged in a secondary step using a weaker reducing agent.[34,35] Though the enlargement of particles in the secondary step from about 20−45 nm to 120−170 nm has been reported, the initial sol was not reproducible, and the resulting particles were considerably polydisperse. Moreover, such reducing agents may be associated with environmental toxicity or biological hazards. It has been, therefore, of increasing interest to develop green synthesis of silver colloid nanoparticles.

In recent studies, a synthesis using a Tollens process was used to demonstrate the formation of silver particles with controlled size in a one-step process.[36,37] The basic reaction in this process involves the reduction of a silver ammoniacal solution using glucose. In this modified Tollens procedure, films with colloidal silver particles ranging from 50 to 200 nm, and silver hydrosols with particles in the order of 20−50 nm, were obtained. In a more recent study, the size of the particles in our laboratory was further controlled by using a difference in the redox potentials of the reducing agents and the silver ions

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TABLE 1: The Average Sizes *d* (nm) and Half Widths *hw* (nm) of Lognormal Size Distribution Obtained from DLS Measurements of Silver Colloid Nanoparticles Synthesized by the Reduction of $[Ag(NH_3)_2]^+$ Using Various Reducing Saccharides

	ammonia concentration (mol L^{-1})							
saccharide	0.005 d/hw	0.01 d/hw	0.02 d/hw	0.035 d/hw	0.05 d/hw	0.10 d/hw	0.20 d/hw	ref.
				monosaccharides				
glucose	44/13	52/18	52/20	304/140	318/138	394/165	453/246	this study
	57/24	63/28	87/49	−/−	270/133	302/156	336/150	*38*
galactose	50/15	50/16	53/19	54/22	58/26	314/170	343/181	this study
				disaccharides				
maltose	25/8	30/9	32/10	290/95	286/99	213/75	262/91	this study
	47/13	68/36	116/57	−/−	239/112	328/167	352/158	*38*
lactose	35/11	41/12	41/13	286/96	282/106	168/59	186/63	this study

undergoing reduction.[38] This difference was easily controllable by changing the concentration of complexing agent, ammonia, of the silver ion, and using a range of reducing saccharides (xylose, glucose, fructose, and maltose). This process gave silver colloid particles with controllable sizes ranging from 45 to 380 nm. This synthetic route is environmentally friendly because of the use of nontoxic chemicals.

In the present paper, we have achieved further reduction in the size of silver particles by varying the pH of the reaction system and using other reducing saccharides, galactose, and lactose. The smallest size particles ranging from 25 to 50 nm having nearly monodispersity could be obtained. These particles were then tested for their antibacterial and bactericidal activity against a broad spectrum of Gram-negative and Gram-positive bacteria including multiresistant strains.

Experimental Section

Materials. Silver nitrate (99.9%, Safina), ammonia (25% w/w aqueous solution), and sodium hydroxide (both of p.a. purity, Lachema) were used without any further purification. D-glucose (p.a. purity, Lachema) and D(+)-galactose (p.a. purity, Fluka) and D(+)-maltose monohydrate (p.a. purity, Riedel-de Haën), and D(+)-lactose (p.a. purity, Penta) were used without further purifications. A cultivating medium, Mueller-Hinton broth, used in the antibacterial assays was supplied by Difco (France). The following standard reference strains (labeling according to Collection of Samples, Masaryk University, Brno, Czech Republic) were used: *Staphylococcus aureus* CCM 3953, *Enterococcus faecalis* CCM 4224, *Escherichia coli* CCM 3954, and *Pseudomonas aeruginosa* CCM 3955. Further, the following bacterial strains isolated from human clinical material at the University Hospital, Olomouc, Czech Republic were used: *Pseudomonas aeruginosa*, methicillin-susceptible *Staphylococcus epidermidis*, methicillin-resistant *Staphylococcus epidermidis*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE), and ESBL-positive *Klebsiella pneumoniae*.

Synthesis Method. Colloidal silver particles were synthesized by the reduction of $[Ag(NH_3)_2]^+$ complex with glucose, galactose, maltose, and lactose. The initial concentrations of the reaction components were 10^{-3} mol L^{-1} and 0.01 mol L^{-1} for $AgNO_3$ and the reducing agent, respectively. The concentration of ammonia varied from 0.2 to 0.005 mol L^{-1}. Sodium hydroxide solution was added to the reaction system to initiate the reduction, as well as to achieve a reaction time of several minutes. The course of the reduction of silver ions was continuously monitored by measuring the turbidity of the colloid system as it formed. All measurements were performed at room temperature (~20 °C).

Characterization. The synthesized silver nanoparticles were characterized by a dynamic light scattering (DLS) using a Zeta Plus analyzer (Brookhaven). The average values of the particle size and polydispersity, defined as a relative width of the size distribution, were determined from the DLS measurements. Microscopic TEM observations of silver particles were performed with a CM12 TEM/STEM (Philips) transmission electron microscope. UV/VIS absorption spectra of the silver colloids were acquired by using a Helios α (Thermo Unicam) spectrophotometer.

Antimicrobial and Bactericidal Assays. Antimicrobial activities of the synthesized silver colloidal sols were assessed using the standard dilution micromethod, determining the minimum inhibitory concentration (MIC) leading to inhibition of bacterial growth (National Committee for Clinical Laboratory Standards. *Performance standards for antimicrobial susceptibility testing. Twelfth informational supplement.* NCCLS document M100-S12. NCCLS, Wayne, Pennsylvania, 2002). Disposable microtitration plates were used for the tests. The silver sols were diluted 2−128 times with 100 μL of Mueller−Hinton broth inoculated with the tested bacteria at a concentration of 10^5 to 10^6 CFU/mL. The minimum inhibitory concentration (MIC) was read after 24 h of incubation at 37 °C as the MIC of the tested substance that inhibited the growth of the bacterial strain.

The minimum bactericidal concentration (MBC) may be characterized as the minimum concentration of the sample required to achieve irreversible inhibition, i.e., killing the bacteria after a defined period of incubation. The MBC was examined by a modified imprint method. With an applicator, ~5 μL of the tested samples with defined concentrations were transferred from the microplate wells and imprinted on the surface of blood agar without antimicrobial agents. The MBC was determined as the lowest concentration that inhibited the visible growth of the used bacterium. The silver sols were used in the form in which they had been prepared. Therefore, control bactericidal tests of solutions containing all the reaction components with the exception of silver nitrate, and reducing agents were performed.

Results and Discussion

Synthesis of Silver Colloid Nanoparticles. The reduction of the $[Ag(NH_3)_2]^+$ complex cation was performed by adding saccharides into the reaction mixture. Saccharides studied were two monosaccharides: glucose and galactose and two disaccharides, maltose and lactose. The characteristic sigmoid shapes of the induction kinetic curves were obtained. Kinetic curves for galactose and maltose are shown in Figure S1 of the Supporting Information (SI). The length of the initial stage of slow product formation (i.e., induction period) was changed with ammonia concentration and pH values.

Silver colloid particles synthesized range in average sizes from 25 to 450 nm, depending on the ammonia concentration

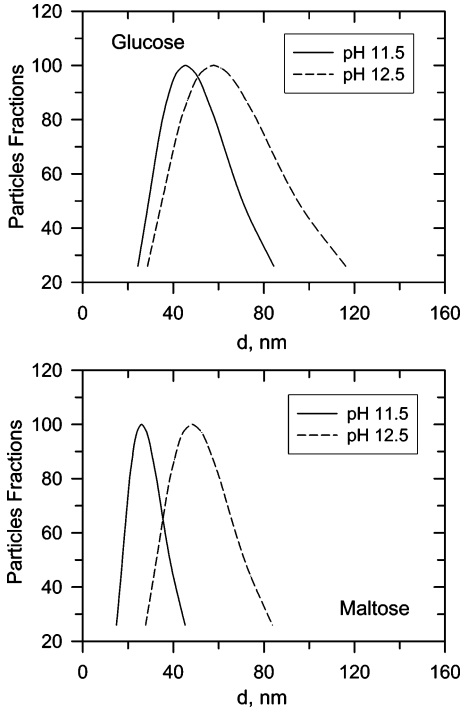

Figure 1. Log-normal size distribution of silver nanoparticles at different pH for glucose and maltose in 0.005 mol L^{-1} ammonia concentration.

and on the type of reducing agents in the reaction system (Table 1). The average size of silver particles increases with the increase in the ammonia concentration and reaches a maximum at the concentration of 0.035 mol L^{-1} for disaccharides and 0.20 mol L^{-1} for monosaccharides (Figure S2, SI). The ammonia concentration of 0.035 mol L^{-1} represents a critical value at which the size of the silver particles changes from tens to hundreds of nanometers (Figure S2, SI). Moreover, the polydispersity of the larger silver particles dramatically increases at a critical value of ammonia concentration (Figure S3, SI). Galactose displays a rather different effect, with the shift of the critical value of ammonia concentration toward a higher concentration of 0.1 mol L^{-1}. This difference in monosaccharides may be related to higher redox potential of galactose than that of glucose.[39]

The observed increase in the average size of the silver colloid particles with ammonia concentration is related to the increase of stability of the $[Ag(NH_3)]^+$ complex cation with increasing ammonia concentration. The Ag^+/Ag standard redox potential decreases from +0.80 for uncomplexed Ag^+/Ag to +0.38 V for $[Ag(NH_3)_2]^+$.[40] This causes decrease in the reduction rate of silver ions upon complexation. At the initial reduction stage, the decreased reaction rate causes a decrease in the rate of silver nuclei formation, and therefore, fewer stable nuclei are produced. In the subsequent reaction stages, the growth of the previously formed nuclei toward the final stable colloid particle occurs.[41] Thus the size of the synthesized particles is limited only by the available amount of silver ions. If the number of nuclei formed in the initial stage is low, the resulting particles will be larger.

At the highest ammonia concentration (0.2 mol L^{-1}), the reaction rates are slow, and therefore, the average particle sizes are largest if monosaccharides are used as the reducing agents (Table 1). The formation of silver colloid nanoparticles using disaccharides have the maximum particle sizes at lower concentration (0.035 mol L^{-1}) compared to monosaccharides

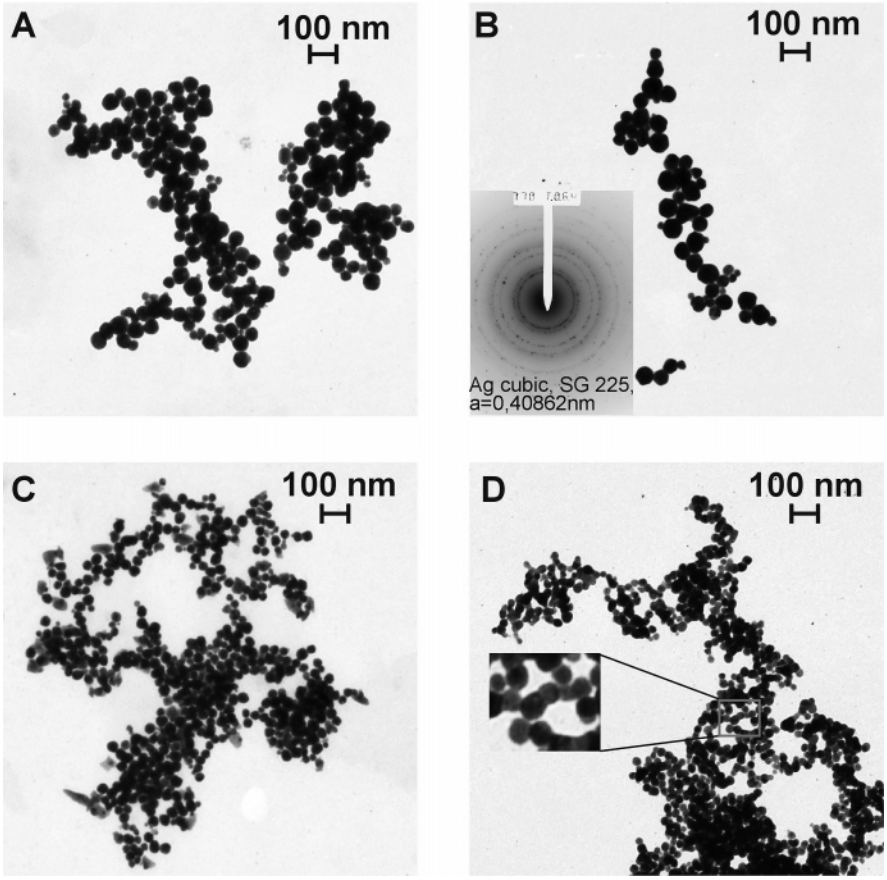

Figure 2. TEM images of silver colloid nanoparticles synthesized via reduction of $[Ag(NH_3)_2]^+$ by glucose (A), galactose (B), lactose (C), and maltose (D) ([Ammonia] = 0.005 mol L^{-1}).

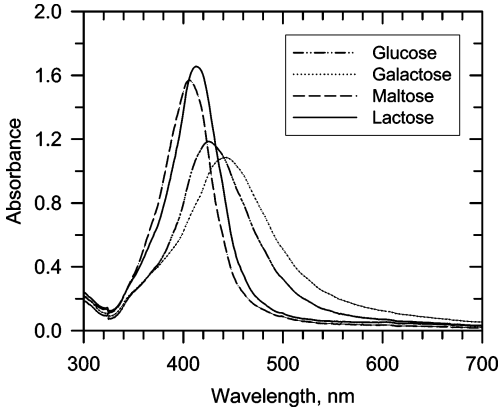

Figure 3. UV/VIS absorption spectra of the silver colloid particles prepared via reduction of $[Ag(NH_3)_2]^+$ using glucose, galactose, maltose, and lactose. ([Ammonia] = 0.005 mol L^{-1}).

and a slight decrease in particle size was observed with increasing ammonia concentrations. This observed slight decrease in average particle size suggests, in addition to the complexing agent, that particle formation is also affected by the strength of the reducing agent and its molecular structure. Therefore, electrochemical and structural properties of the reducing agent also determine the overall reduction rate of silver complex. Another factor which should be taken into account is the rapid hydrolysis of disaccharides to monosaccharides occurring in a strong alkaline medium; therefore, the real concentration of the reducing saccharides in the reaction system is higher compared to when monosaccharides are used. This results in a higher number of silver nuclei in the system, and consequently, it gives smaller particle sizes. Therefore, the particle size is generally smaller for disaccharides at any ammonia concentration.

Additionally, the particle sizes are also influenced by the pH of the reaction system. In a previous work, pH of the reaction system was approximately 12.5.[38] In the present study, we lowered the pH to 11.5 for 0.005 and 0.01 mol L^{-1} ammonia concentrations, and then slowly increased the pH to 13.0 for other concentrations. The final pH of the reaction for the two highest ammonia concentrations, 0.10 and 0.20 mol L^{-1}, was 13.0. Comparison between the two studies is made in Table 1. The variation of pH resulted in two observations about particle sizes. First, particles obtained at pH 11.5 were smaller than those at pH 12.5 (Figure 1). Additionally, the size distribution was also decreased by lowering the pH (Figure 1). In contrast, increase of pH to 13.0 gave larger size particles having broader size distribution for glucose (Table 1). Interestingly, variation in pH not only allowed us to synthesize smaller silver particles, but also resulted in a broader range in sizes than those obtained in a previous study. Obtaining the smaller particles under optimized pH conditions is very important for practical applications including antibacterial activity. This is demonstrated in the antibacterial study performed in this paper and is discussed later.

Characterization of Silver Particles. The smallest average sizes of the silver colloid particles were subjected to different analytical techniques to characterize them. TEM micrographs in Figure 2 demonstrate the symmetric shape and very narrow size distribution of all the samples. Generally, the sizes of the observed particles in TEM are in good agreement with the values obtained by DLS. Both TEM and DLS data confirm that the use of disaccharides (lactose and maltose) as reducing agents yields smaller particle sizes than using monosaccharides (glucose and galactose) at the same concentration of ammonia. TEM micrographs showed only Ag particles of the cubic structure.

In the UV/VIS absorption spectra (Figure 3) of 25−50 nm silver particles, synthesized using 0.005 mol L^{-1} ammonia concentration, narrow surface plasmon absorption peaks at 390−420 nm wavelengths were observed. This confirms the nanocrystalline character of the particles,[35] and the low degree of their polydispersity, which are in agreement with our TEM observations. Colloids containing particles larger than 100 nm, possess a uniform light absorption, which slightly increases at longer wavelengths; a characteristic feature of metal particles of such sizes.

Antibacterial Study. The smallest size silver nanoparticles having a very narrow size distribution obtained in this work were tested as antimicrobial agents. The average sizes, *d*, were 44, 50, 25, and 35 nm for silver particles, prepared by using glucose, galactose, maltose, and lactose, respectively. The size distributions of these particles were narrow (see Table 1). The standard dilution micromethod was applied in performing this study. Minimum inhibition concentrations of the tested silver particles and the controlling samples against Gram-positive and Gram-negative bacteria are summarized, as average values from 10 separate measurements, in Table 2. The used dilution micromethod enables testing of antibacterial activity of samples diluted two times up to 128 times. Total concentration of silver in the tested colloidal systems, and the control sample with ionic silver, were 108 μg/mL Ag after preparation. The final total concentrations of silver ranged from 54 to 0.84 μg/mL Ag, depending on the dilution.

As expected, a control sample containing all the initial reaction components except for the silver nitrate showed no

TABLE 2: Minimum Inhibition Concentrations and Minimum Bactericidal Concentrations of Silver Particles Prepared via Reduction of $[Ag(NH_3)_2]^+$ by Various Reducing Saccharides at an Ammonia Concentration of 0.005 Mol L^{-1}

	minimum inhibition and bactericidal concentrations (μg/mL)[f]					
bacteria	glucose	galactose	maltose	lactose	control[d]	control[e]
Enterococcus faecalis CCM 4224	−[c]	−	13.5	54.0	6.75	−[c]
Staphylococcus aureus CCM 3953	6.75	54.0	6.75	6.75	6.75	−[c]
Escherichia coli CCM 3954	27.0	−	3.38	27.0	1.69	−[c]
Pseudomonas aeruginosa CCM 3955	27.0	−	6.75	13.5	0.84	−[c]
Pseudomonas aeruginosa	13.5	27.0	3.38	13.5	0.84	−[c]
Staphylococcus epidermidis[a]	13.5	6.75	1.69	6.75	0.84	−[c]
Staphylococcus epidermidis[b]	6.75	54.0	1.69	6.75	1.69	−[c]
Staphylococcus aureus MRSA	27.0	54.0	6.75	27.0	6.75	−[c]
Enterococcus faecium (VRE)	−[c]	−[c]	13.5	54.0	3.38	−[c]
Klebsiella pneumoniae (ESBL-positive)	27.0	−[c]	6.75	54.0	3.38	−[c]

[a] Methicillin-susceptible. [b] Methicillin-resistant. [c] "−" growth inhibition of bacteria unsubstantiated. [d] Control sample containing all initial reaction components without reducing saccharides. [e] Control sample containing all initial reaction components without silver nitrate. [f] MIC and MBC of silver sols had same values.

antibacterial activity (Table 2). Table 2 provides evidence that silver particles synthesized using disaccharides have higher antibacterial activity than those synthesized using monosaccharides. This phenomenon is related to size of colloidal silver particles, which were lower for disaccharide than monosaccharide. Thus, the 25 nm-sized silver particles synthesized via reduction by maltose showed the highest activity, comparable even with ionic silver for certain strains. The lowest antimicrobial effect was observed for galactose, which is related not only to the largest particles, but also to the rapid formation of aggregates after the sample preparation (hours compared to weeks or even months in the case of other saccharides). The antimicrobial activity of a control sample containing all the initial reaction substances except the reducing agent was very high because of the presence of free silver ions, which are very toxic toward bacteria.

It is necessary to emphasize that the tested silver nanoparticles have bactericidal effects resulting not only in inhibition of bacterial growth but also in killing bacteria. This irreversible inhibition of bacterial growth is desirable to prevent bacterial colonization of silver-containing medical devices, such as catheters,[16,17] where bacteria-killing activity is required. Bactericidal activity was examined simultaneously with the antibacterial assays and was observed for all tested silver nanoparticles. The MBC of the silver particles against Gram-positive and Gram-negative bacteria were the same as MIC (see Table 2). Various concentrations required for growth inhibition or killing of bacteria were determined by the biological properties of individual bacterial species.

The mechanism of the bactericidal effect of silver colloid particles against bacteria is not very well-known. Silver nanoparticles may attach to the surface of the cell membrane and disturb its power function such as permeability and respiration. It is reasonable to state that the binding of the particles to the bacteria depends on the surface area available for interaction. Smaller particles having the larger surface area available for interaction will give more bactericidal effect than the larger particles. Experiments conducted using the scanning tunneling electron microscopy (STEM) and the X-ray energy dispersive spectrometer (EDS), showed silver nanoparticles not only at the surface of cell membrane, but also inside the bacteria.[42] This then suggests the possibility that the silver nanoparticles may also penetrate inside the bacteria and cause damage by interacting with phosphorus- and sulfur-containing compounds such as DNA. Silver tends to have a high affinity to react with such compounds.[43] One more possibility would be the release of silver ions from nanoparticles, which will have an additional contribution to the bactericidal properties of silver nanoparticles.

Currently, the increase of bacterial resistance to antimicrobial agents poses a serious problem in the treatment of infectious diseases as well as in epidemiological practice.[44] Increasingly new bacterial strains have emerged with dangerous levels of resistance, including both of Gram-positive and Gram-negative bacteria. Dealing bacterial resistance will require precautions that lead to prevention of the emergence and spreading of multiresistant bacterial strains, and the development of new antimicrobial substances. The results of this study clearly demonstrated that the colloidal silver nanoparticles inhibited the growth and multiplication of the tested bacteria, including highly multiresistant bacteria such as methicillin-resistant *Staphylococcus aureus*, methicillin-resistant coagulase-negative staphylococci (e.g., *Staphylococcus epidermidis*), vancomycin-resistant *Enterococcus faecium*, and ESBL-positive *Klebsiella pneumoniae*. Such high antibacterial activity was observed at very low total concentrations of silver below 2 μg/mL.

Conclusions

Size-controlled silver colloid nanoparticles were generated using a one-step modified Tollens process. The pH and type of reducing saccharide of the reaction system were found to influence the size of particles. A wide range of particle size, particularly 25−100 nm with narrow size distributions were obtained using four different saccharides. The bactericidal properties of these nanoparticles to many Gram-positive and Gram-negative bacteria including multiresistant strains were successfully demonstrated.

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Supporting Information Available: Three figures illustrating kinetics curves for galactose and maltose, average size of silver particles, and ammonia concentration. This material is available free of charge via the Internet at http://pubs.acs.org.

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