Silver nanoparticles: A novel antibacterial agent for control of *Cronobacter sakazakii*

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ABSTRACT

Silver nanoparticles (AgNP) have been widely applied because of their broad spectrum of antimicrobial activities against bacteria, fungi, and viruses. However, little research has been done to evaluate their effects on *Cronobacter sakazakii*, an opportunistic pathogen usually infecting infants and having a high fatality rate. The aims of this work were to investigate the antibacterial property of novel, synthesized, positively charged silver nanoparticles against *C. sakazakii* and to discuss the potential antibacterial mechanisms involved. In this study, the spherical and face-centered cubic silver nanoparticles had a mean particle size of 31.2 nm and were synthesized by reducing Ag⁺ using citrate and dispersed by glycerol and polyvinylpyrrolidone (PVP) under alkaline conditions. Minimum inhibitory concentrations (MIC) and inhibition zone tests showed that the AgNP exhibited strong antibacterial activity against 4 tested *C. sakazakii* strains with mean MIC of 62.5 to 125 mg/L and average inhibition zone diameters of 13.8 to 16.3 mm. Silver nanoparticles caused cell membrane injury accompanied by adsorption of AgNP onto the cell surface, as shown by changes in cell morphology, cell membrane hyperpolarization, and accelerated leakage of intracellular reducing sugars and proteins outward from the cytoplasm. In addition, dysfunction of the respiratory chain was induced after treatment with AgNP, which was supported by a decrease in intracellular ATP and inhibition of related dehydrogenases. This research indicates that AgNP could be a novel and efficient antibacterial agent to control *C. sakazakii* contamination in environments producing powdered infant formulas from milk.

Key words: silver nanoparticles, *Cronobacter sakazakii*, cell membrane, antibacterial mechanism

INTRODUCTION

Cronobacter sakazakii belongs to the *Cronobacter* genus of the *Enterobacteriaceae* family (Iversen et al., 2007); it is a life-threatening opportunistic pathogen that is gram-negative, motile, peritrichous, non-spore-forming, and facultatively anaerobic. The pathogen is widespread and has been isolated from various food substrates, including vegetables (mixed salad), meat and meat products, tea, dry ingredients, milk, and cheese (Beuchat et al., 2009). Particularly, powdered infant formula (PIF) is among the most commonly known sources of infection and can be contaminated by *C. sakazakii* from food processing environments (air, ground, and water), processing ingredients, accessories, or capping materials during PIF production (Kandhai et al., 2004). In clinical research, *C. sakazakii* is epidemiologically linked to neonatal and infant infections in immunocompromised individuals, especially preterm and low-birth-weight infants (Healy et al., 2010). Clinical symptoms of infection in infants include meningitis, bacteremia, and severe forms of necrotizing enterocolitis, with a mortality rate ranging from 40 to 80% (Friedemann, 2009; Endersen et al., 2017).

Cronobacter sakazakii has a strong ability to resist dry and osmotic stresses and it exhibits thermal and acid-base tolerance (Riedel and Lehner, 2007; Yemiş et al., 2011). These characteristics are thought to contribute to the survival of *C. sakazakii* in PIF products. To control contamination by *C. sakazakii* in the food industry, various interventions have been proposed and developed, including UV-near infrared radiation, photon hydroxylation technology, gaseous ozone, ultrasound with thermal treatment, and some natural antimicrobials such as heat-labile bacteriocins and syringic acid (Adekunte et al., 2010; Awaisheh et

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al., 2013; Torlak and Sert, 2013; Ha and Kang, 2014; Shi et al., 2016). However, these methods are not able to efficiently eliminate bacteria from food processing surfaces due to the strong biofilm-forming ability of many bacteria during propagation (Hartmann et al., 2010). With the development of nanomaterials, silver nanoparticles (**AgNP**) could be a novel antibacterial agent because of their broad spectrum of antimicrobial activities against bacteria, fungi, and viruses (Lara et al., 2011; You et al., 2012).

Silver nanoparticles are metal nanoparticles with a size distribution from 1 to 100 nm in 3-dimensional space, and AgNP have generated significant interest as effective antimicrobial agents in recent years (El-Naggar et al., 2017; Chai et al., 2018). Silver nanoparticles exhibit strong antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Eberthella typhi*, and *Bacillus subtilis* (Morones et al., 2005; Ruparelia et al., 2008; Ramalingam et al., 2016; Gomaa, 2017). The antibacterial property depends on the size distribution, crystal structure, and surface zeta potential of silver nanoparticles (Pal et al., 2007; Carlson et al., 2008). El-Naggar et al. (2017) hypothesized that Ag nanoparticles cause cell death due to a decrease in cellular ATP production and DNA damage.

Although silver nanoparticles have been evaluated against various microorganisms, their antibacterial activity against *C. sakazakii* is rarely reported. The mechanisms related to the role of cell membrane and the respiratory chain of *C. sakazakii* remain unclear and require further investigation. Understanding the mechanisms of antibacterial activity is important in developing intervention strategies for the control of *C. sakazakii* in the manufacturing environment. In this study, silver nanoparticles were synthesized based on the chemical reduction method, using citrate as reducing agent and glycerol and polyvinylpyrrolidone (**PVP**) as stabilizers. Characteristics of silver nanoparticles were determined by UV-visible (**UV-Vis**) absorption spectroscopy, transmission electron microscopy (**TEM**), X-ray diffraction (**XRD**), Fourier transform infrared spectroscopy (**FT-IR**), and dynamic light scattering (**DLS**) methods to obtain comprehensive information about surface topography, size distribution, crystal structure, connection of surface functional groups, and surface charge, all of which may contribute to the antibacterial activity of AgNP. Moreover, the antibacterial activity of AgNP against *C. sakazakii* strains was evaluated according to MIC and inhibition zone tests. Finally, a potential antibacterial mechanism was proposed based on changes in cell morphology, membrane integrity, and the disruption of respiratory chain activity by AgNP.

MATERIALS AND METHODS

Reagents, Bacterial Strains, and Culture Conditions

The silver to produce AgNP was from silver nitrate (AgNO_3 , Sinopharm Chemical Reagent Co. Ltd., Shanghai, China), using citrate sodium as the reducing agent (Fuchen Chemical Reagent Factory, Tianjin, China); glycerol and PVP (Sangon Biotech Co. Ltd., Shanghai, China) were used as stabilizers. All chemicals were of analytical grade. Four *C. sakazakii* strains: ATCC 29544^T, ATCC BAA894, ATCC 29004, and ATCC 12868, were purchased from American Type Culture Collection (ATCC, Manassas, VA) and preserved in our laboratory at -80°C . Each strain was inoculated into 20 mL of Luria-Bertani (**LB**) medium and incubated for 12 h at 37°C with shaking at 160 rpm.

Preparation and Purification of Silver Nanoparticles

Silver nanoparticles were prepared according to Dey et al. (2015) with some modifications. Briefly, 50 mL of 1 mmol/L AgNO_3 was added to an Erlenmeyer flask, followed by glycerol and PVP at 1% (vol/vol), respectively. The reaction system was adjusted to pH 8 by using ammonium hydroxide and the mixture boiled on a thermostatic magnetic stirrer (H01-3; Meiyongpu Instrument Manufacturing Co. Ltd., Shanghai, China). With the stirring speed controlled at 200 rpm, 2 mL of sodium citrate (1% wt/vol) was added drop-by-drop over 30 s. The color changed from colorless to yellow and finally to brownish black, which indicates the formation of silver nanoparticles. The nanosilver solution was cooled at 4°C immediately and stored overnight at room temperature for intraparticle ripening. Silver nanoparticles were obtained by centrifugation at $9,000 \times g$ for 15 min using a high-speed refrigerated centrifuge (3K15, Sigma, Steinheim, Germany) and purified with 5 washes of 75% (vol/vol) alcohol and deionized water.

For further characterization of AgNP, a freeze-dried powder was prepared by mixing the AgNP solution with 50 mM sucrose solution and freeze-drying using a freeze-dryer (Alpha 1-4 LSCplus, Martin Christ, Osterode am Harz, Germany). The process was as follows: samples were frozen at -75°C to -80°C for 2 h, and then freeze-dried below co-melting point temperature at about -45°C for 8 h and kept at 10°C until freeze-drying was completed. The solubility and dispersion of freeze-dried powdered silver nanoparticles were tested. All flasks were soaked in a mixture of concentrated HNO_3 (65–68 wt %) and HClO_4 (70–72 wt %) at a volume ratio of 9:1 for 12 h and rinsed with deionized water before use.

Characterization of AgNP

Analysis of UV-Vis Absorption Spectra. In UV-Vis analysis, the surface plasmon resonance (SPR) peak depends on the size and shape of nanoparticles, with the wavelengths for spherical nanoparticles usually being in the range of 405 to 425 nm, and the wavelength value becomes larger when particle size increases (Sikder et al., 2018). The freeze-dried powder of AgNP was dissolved in deionized water to prepare nanosilver suspensions at different concentrations. Aliquots of 1.5 mL were taken out to determine the absorption spectrum using a wavelength from 300 to 800 nm with a UV-visible spectrophotometer (UV-2600, Shimadzu, Tokyo, Japan). The characteristic wavelength at the maximum absorption peak and the full width at half maxima (FWHM) were recorded to characterize the shape and homogeneity of AgNP. The AgNO₃ solution was used as a blank control throughout this test.

TEM Analysis. Transmission electron microscopy provides direct information about the morphology, coagulation, and size distribution of AgNP. An ultra-high-resolution transmission electron microscope (H-7500 type, Hitachi, Tokyo, Japan) was used to capture images at the nanometer scale under an accelerating voltage of 100 kV. Samples were dropped onto carbon-coated copper grids and dried in darkness for 20 min to complete the physical adsorption process. Then, nanoparticles were observed at magnifications of 200,000 $\times$ and 100,000 $\times$.

Surface Charge and Size Distribution of AgNP. Surface charge is an important property for synthesized AgNP, influencing interactions with bacteria. Positively charged nanoparticles were showed to have the highest effectiveness against both gram-negative and gram-positive bacteria compared with neutral or negatively charged AgNP (Abbaszadegan et al., 2015). The surface zeta potential and size distribution were determined using DLS by using a laser particle sizer (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, UK) at 25°C with a detection angle of 90°.

X-Ray Diffraction Analysis. X-Ray diffraction analysis is a common method to evaluate the crystal structure and atomic distance of AgNP. An X-ray diffractometer (Empyrean, PANalytical, Almelo, the Netherlands) was applied in this experiment with a radiation source of CuK α ($k = 1.54 \text{ \AA}$; which is a characteristic fluorescent X-ray produced by high-energy electron bombardment of a copper target, with a specific wavelength of $1.54060 \text{ \AA}$) and a programmable divergence slit. Parameters were set as follows: the voltage and current were 40 kV and 20 mA, respectively; the scanning angle (2θ) was monitored from

10° to 90°, and the scanning speed was set as 2.0°/min in a consecutive manner. The freeze-dried powder of AgNP was placed onto a silica plate and the EasySAXS software (PANalytical B. V., Almelo, the Netherlands) was applied to analyze the composition of elements. By comparing with the standard powder diffraction file of Ag (No. 04-0783; Supplemental Material S1, <https://doi.org/10.3168/jds.2018-15258>), the possible crystal structure of AgNP was obtained.

FT-IR Spectroscopy Analysis. We used FT-IR to show the conjunction of surface functional groups, aiming to display interactions among chemical bonds during nucleation and growth of silver nanoparticles. The freeze-dried powder of AgNP was analyzed using an FT-IR spectrometer (Nicolet iS 50, Thermo Fisher Ltd., Waltham, MA), and the spectrum was collected at scanning scope from 4,500 to 450 cm⁻¹ with a resolution of 1 cm⁻¹. By deducting the background signal, the FT-IR spectrum was obtained.

Antibacterial Tests of AgNP on *C. sakazakii*

MIC Test. Five milliliters of sterilized LB liquid medium was aliquoted into sterile tubes (10 mL), followed by addition of 1 mL of each *C. sakazakii* strain adjusted to approximately 10⁸ cfu/mL. The AgNP were added to make final concentrations from 1,000 to 7.81 mg/L by 2-gradient dilutions for each tube. Tubes with tested strains alone were used as positive controls, and tubes without the addition of AgNP and tested strains were negative controls. After incubation at 37°C for 12 h with shaking at 160 rpm, the survival of organisms was observed by visual inspection. Experiments were performed in triplicate and MIC was measured as the minimum concentration of AgNP resulting in no visible growth of bacteria.

Inhibition Zone Test. The Oxford cup method was applied to test the antibacterial activity of AgNP against *C. sakazakii* strains. The solid culture medium consisted of 2 layers: a water-agar layer of about 1 to 2 mm at the bottom with 1.5% (wt/vol) agar and a nutrient-agar layer about 2 mm at the top with 1% (wt/vol) agar. For each strain, 100 μ L of bacterial culture ($\sim 10^8$ cfu/mL) was spread on the plate followed by addition of 100 μ L of nanosilver suspensions with different concentrations to each hole after the Oxford cup was removed. Holes spotted with 5 mM AgNO₃ solution and sterile water were used as positive and negative controls, respectively. After incubation at 37°C for 12 h, inhibition zones were examined and their diameters measured to evaluate the antibacterial activity of AgNP.

Clarification of Potential Mechanism of AgNP Against *C. sakazakii*

Observation of AgNP on the Cell Structure of *C. sakazakii*. A bacterial suspension of *C. sakazakii* ATCC 29544^T was prepared by centrifuging 1 mL of cell culture ($\sim 10^8$ cfu/mL) at $5,000 \times g$ for 5 min. The pellet was then rinsed and suspended in sterilized 0.85% NaCl. After treatment with AgNP at a final concentration of 200 mg/L, the mix was incubated at 37°C for 2 h with shaking at 160 rpm. The cultures were centrifuged at $5,000 \times g$ for 5 min at room temperature to separate the bacteria and then observed using scanning electron microscopy (Hitachi S-3400N, Shimadzu Co. Ltd.) with an acceleration voltage of 5.0 kV. For scanning electron microscopy evaluation, the cell pellet was fixed using 2.5% (vol/vol) glutaric dialdehyde for 2 h at 4°C, rinsed with 0.1 M PBS, pH 6.8, and subjected to gradient dehydration with 50, 70, 90, and 100% (vol/vol) alcohol, each for 15 min. Pure tertiary butanol was added into the final mixture for 15 min to replace the alcohol, and the mixtures underwent freeze drying (ES-2030, Hitachi) for about 4 h to obtain solid samples in powder. Before observation, samples were transferred to a conductive adhesive upon the sample stage and surface-coated with a layer of platinum film (100–150 Å) with a sputter coater (E-1010, Hitachi). Cells without AgNP treatment were used as the negative control. Meanwhile, an energy-dispersive X-ray (EDX) fluorescence spectrometer (EDX-7000, Shimadzu Co. Ltd.) was used to determine whether AgNP were adsorbed to the surface of bacteria by determining characteristic X-ray signals and comparing them with that of elemental Ag.

Cellular Membrane Integrity Test. The LIVE/DEAD BacLight Bacterial Viability Kit (L7007, Molecular Probes/Thermo Fisher Ltd.) was used to investigate changes in cell membrane integrity before and after addition of AgNP. Two nucleic acid stains, green-fluorescent SYTO 9 dye and red-fluorescent propidium iodide (PI), were dissolved into 2.5 mL of sterile distilled water separately and mixed in darkness. Using *C. sakazakii* ATCC 29544^T as the test strain, 1 mL of bacterial suspension ($\sim 10^8$ cfu/mL) was prepared. After incubation at 37°C for 2 h with shaking at 160 rpm, without or with the addition of AgNP at a final concentration of 200 mg/L, 100 μ L of each bacterial suspension was taken out and incubated with the prepared nucleic acid stains at a volume ratio of 1:1 for 10 min. Then, a laser scanning confocal microscope (LSCM, TCS SP2 AOBS, Leica, Wetzlar, Germany) was used to observe the fluorescence signal at excitation and emission wavelengths (Ex/Em = 480/500 nm for

STYO9 green stain; Ex/Em = 535/615 nm for the PI red stain).

Determination of Cellular Membrane Potentials. To detect membrane potentials, a sensitive slow-response membrane potential probe DiBAC4(3) [Bis-(1,3- dibutyl barbituric acid) trimethine oxonol; Sigma, St. Louis, MO] was used. One milliliter of bacterial suspension ($\sim 10^8$ cfu/mL) of each *C. sakazakii* strain was prepared in 0.85% NaCl. Then, AgNP were added to test groups at a final concentration of 200 mg/L and treated for 2 h; a control group had no AgNP added. Samples were collected by centrifugation at $6,000 \times g$ for 5 min and washed 3 times with 0.85% NaCl. One microliter per well of bacterial suspension was added to black poly-D-lysine 96-well plates, followed by addition of 100 μ L of 1 μ M DiBAC4(3) probe dissolved in dimethyl sulfoxide. Subsequently, the dye-loading plate was incubated in a cell incubator (250B type, Runhua Electric Appliance Co. Ltd., Jiangsu Sheng, China) at 37°C for 30 min. A membrane potential assay was performed by monitoring fluorescence intensity at Ex/Em = 493/516 nm by using a fluorescence microplate reader (SpectraMax iD3, Molecular Devices, San Jose, CA).

Determination of the Leakage of Reducing Sugars and Proteins. To detect the leakage of reducing sugars and proteins, 4 *C. sakazakii* strains (ATCC 29544^T, ATCC BAA894, ATCC 29004, and ATCC 12868) were added separately into 10 mL of sterile LB culture with AgNP and incubated at 37°C with shaking at 160 rpm. The final concentration of AgNP was 200 mg/L and the initial bacterial count of *C. sakazakii* was 10^8 cfu/mL. Cultures without AgNP were used as a control. One milliliter of each sample was collected after 0, 1, and 2 h and centrifuged at $12,000 \times g$ for 5 min. Supernatants were collected and stored at -20°C immediately for subsequent analysis. Determination of the contents of reducing sugars and proteins was according to the methods of Miller (1959) and Sedmak and Grossberg (1977).

Detection of Intracellular ATP Levels. The ATP levels of each of the *C. sakazakii* strains before and after treatment with AgNP were measured by using a firefly luciferase-based ATP assay kit (Beyotime Bioengineering Institute, Shanghai, China) according to the manufacturer's instructions. After treatment with AgNP at a final concentration of 200 mg/L for 0, 1, 2 and 4 h, cells were collected, lysed, and centrifuged at $12,000 \times g$ for 5 min. The initial bacterial number was about 10^8 cfu/mL. One hundred microliters of each supernatant was mixed with ATP detection working dilutions at a volume ratio of 1:1 in a black 96-well plate. The concentration of ATP was determined by

Figure 1. Visual appearance of (a) AgNO₃ solution at a concentration of 5 mM, and (b) synthesized silver nanoparticles by reducing Ag⁺; (c) UV-visible absorbance spectrum of silver nanoparticles. Color version available online.

using a luminometer (SpectraMax iD3, Molecular Devices) to determine relative light unit (RLU) values and calculated by generated standard curves. Assays were repeated in triplicate.

Assay for the Enzyme Activity of Respiratory Chain Dehydrogenases. To determine the enzyme activity of respiratory chain dehydrogenases before and after addition of AgNP, the iodonitrotetrazolium chloride (INT) method was used according to Li et al. (2010). Colorless INT can be reduced by dehydrogenases to a dark-red and water-insoluble iodonitrotetrazolium formazan (INF), which exhibits maximum absorbance at 490 nm (A_{490}). Thus, enzyme activity can be evaluated according to the spectrophotometric value of INF. One milliliter of bacterial suspension (~108 cfu/mL) was added to 1.5-mL sterile tubes, then divided into 3 experimental groups: (1) added 0.1 mL of 1,000 or 200 mg/L of nanosilver suspensions to the test group; (2) supplied with 0.1 mL of PBS at pH 7.0 to the positive control group; and (3) boiled for 20 min to inactivate dehydrogenases as the negative control group. Then all samples were co-incubated with 0.1 mL of 0.5% (wt/wt) INT solution at 37°C for 2 h. Subsequently, the INF was extracted with 1 mL of acetone/ethanol solution at a volume ratio of 1:1 and determined at wavelength of 490 nm. The active unit (U) for enzymes was defined as the production of 1 micromole of INF per gram of dry cell weight in 1 minute and expressed as $\mu\text{mol}/(\text{g}\cdot\text{min})$. The enzyme activity of dehydrogenases was calculated as follows:

$$\text{Enzyme activity} = C_{\text{INF}} \times \frac{V}{1,000} \times \frac{K}{Bt},$$

where C_{INF} is the molar concentration of INF ($\mu\text{mol}/\text{L}$) calculated from the generated standard curve of A_{490} versus C_{INF} ; V is the sample volume (mL) injected into each well of 96-well plate; K is the dilution factor of INF extracting solutions; B is the dry cell weight (g), and t is the reaction time (min).

Statistical Analysis

All experiments were conducted in triplicate; SPSS software (version 20.0; SPSS Inc./IBM Corp., Chicago, IL) was used to perform statistical analyses, and Origin software (version 2017; OriginLab Corp., Northampton, MA) was applied to construct curves. The data are reported as mean values $\pm$ standard deviations ($n = 3$).

RESULTS AND DISCUSSION

Characterization of AgNP

UV-Visible Spectral Analysis. After reducing Ag⁺ with citrate, the reaction mixture changed from colorless to a bright yellow color, indicating the formation of AgNP (Figure 1a, b). The SPR of silver nanoparticles showed an absorption peak at 405 nm (Figure 1c), which was within the specific absorption range of spherical silver nanoparticles. The results also revealed a narrow FWHM value of 42 nm (Figure 1c), showing that nanoparticles were uniform in size.

Morphology and Size Distribution Analysis of AgNP. The TEM results showed that the synthesized AgNP were spherical with a primary size distribution from 20 to 40 nm, which occupied over 70% of the total range (Figure 2a, b, and c). No aggregation of nanoparticles was observed. Meanwhile, the hydrodynamic diameter and dispersity of the AgNP were analyzed by the DLS method (Table 1). The DLS results showed the mean size of AgNP was 46.5 nm, slightly larger than the physical diameter of 31.2 nm as determined by TEM. As a weighted-average measurement, DLS is biased toward larger particle sizes and especially toward agglomerates and aggregates. The polydispersity index value was 0.292, suggesting a uniform dispersity of AgNP.

XRD Analysis. X-Ray diffraction of AgNP is shown in Figure 3, with 4 diffraction peaks (at 2θ degrees) of 38.071, 44.226, 64.445, and 77.348. According to the standard powder diffraction card of silver file no. 04-0783 published by the Joint Committee on Powder Diffraction Standards (JCPDS; Supplemental Material S1, <https://doi.org/10.3168/jds.2018-15258>), the 4 diffraction peaks correspond to the (111), (200), (220), and (311) planes of face-centered cubic (fcc) silver.

Figure 2. Transmission electron microscopy images of silver nanoparticles (AgNP) at (a) 200,000× (scale bar = 100 nm) and (b) 100,000× (scale bar = 200 nm); and (c) size distribution of AgNP by random statistics of 1,000 sample points from 10 visual fields, each field having 100 sample spots.

Thus, the crystal structure of silver nanoparticles was essentially in the fcc pattern and crystallized in nature.

FT-IR Spectrum of AgNP. The FT-IR spectrum of silver nanoparticles is displayed in Figure 4, showing 11 peaks at positions 3403.29, 2921.59, 2320.81, 1626.27, 1341.01, 1137.05, 1050.43, 995.95, 925.47, 867.74, and 591.66 cm^{-1} . The strong absorbance band at 3403.29 cm^{-1} is related to O–H stretching vibrations of glycerol and sucrose (Noh et al., 2012). The peak at 2921.59 cm^{-1} is assigned to C–H stretching vibration and CH_2 bending vibration (Devaraj et al., 2013). The band at 2320.81 cm^{-1} attributed to the conjugated double bond of $\text{O}=\text{C}-\text{C}=\text{O}$ originating from the oxidation of citrate. The characteristic amide $(\text{NH})-\text{C}=\text{O}$ and $\text{C}=\text{C}$

stretching vibration was observed at 1626.27 cm^{-1} , whereas N–H stretch was at wavenumber 1341.01 cm^{-1} (Noh et al., 2012). This result showed the involvement in AgNP of nitrogen atoms, which might have originated from the dispersant PVP used in the preparation process. The bands at 1137.05, 1050.43, and 995.95 cm^{-1} were assigned to the stretching vibrations of primary amine C–N and C–C (Jyoti et al., 2016; El-Naggar et al., 2017). These bands were shifted to a lower wavenumber at 925.47 cm^{-1} due to the spatial resistance of AgNP. The peaks at 867.74 cm^{-1} and 591.66 cm^{-1} were related to $\text{C}=\text{CH}_2$ and $-\text{C}-\text{CN}$ stretching vibrations, respectively (Dauthal and Mukhopadhyay, 2013). As a result, most functional groups belonging to citrate and

Table 1. Comparative analysis of the size and polydispersity of silver nanoparticles determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM)

Detection method	Temperature (°C)	Mean particle size (nm)	PDI ¹	Scanning angle (°)
DLS	25	46.5	0.292	90
TEM	37	31.2	—	—

¹Polydispersity index.

its degradation products were involved in the synthesis of AgNP and surrounded with a silver nucleus (Ag^0). Silver nanoparticles may conjunct with PVP to prevent agglomeration in the preparation process.

Surface Zeta Potential of AgNP. The surface zeta potential results are shown in Figure 5, revealing a mean zeta potential of +18.7 mV (SD: 3.18 mV) with a mean conductivity of 0.0153 mS/cm. Thus, the surface of AgNP was capped with a cation layer resulting in a positive charge, which may result from embedded $[\text{NH}_4]^+$ in the reaction system.

Antibacterial Activity of AgNP Against *C. sakazakii*

MIC of AgNP on *C. sakazakii*. Silver nanoparticles exhibited strain-specific variation in antibacterial activity against 4 *C. sakazakii* strains. For ATCC 29544^T and ATCC BAA894, the MIC was 125 mg/L AgNP, whereas for ATCC 20994 and ATCC 12868, the MIC was 62.5 mg/L (Table 2).

Diameters of Inhibition Zone. Silver nanoparticles can inhibit the growth of surrounding bacteria to form an aseptic area when added to LB agar medium. Mean diameters of inhibition zones for ATCC 12868, ATCC 29004, ATCC 29544, and ATCC BAA894 in the

AgNP treatment group were 16, 16.3, 14.3, and 13.8 mm (Figure 6), greater than that of Ag^+ group (mean diameters of 11.8, 12.8, 13.3 and 12 mm, respectively). Silver nanoparticles showed stronger antibacterial activity than Ag^+ against all 4 strains of *C. sakazakii*. The blank group showed no antibacterial activity, and the inhibition zone values were displayed as the external diameter of the Oxford cup.

Potential Antibacterial Mechanism of AgNP on *C. sakazakii*

Changes in Cell Structure by Scanning Electron Microscopy. Scanning electron micrographs showed that the morphology of *C. sakazakii* in a normal state was full, rod-shaped with a wrinkled superficial surface (Figure 7c), whereas strains treated with AgNP showed adsorption of particles (Figure 7a) and bacterial cell disruption (Figure 7b). To further confirm the elemental component of adsorbed particles, EDX analysis was applied and the spectrum is displayed in Figure 7d. The EDX spectrum showed a typical optical observation peak at near 3 keV due to SPR, confirming the presence of silver and adsorption of crystal AgNP.

Figure 3. X-Ray diffraction of silver nanoparticles synthesized by sodium citrate. 2 theta (2θ) = the diffraction angle of characteristic X-ray; WL = wavelength. Four characteristic peaks of 2θ at 38.071, 44.226, 64.445, and 77.348 were displayed, corresponding to the (111), (200), (220) and (311) planes, inserted on the peak numbers separately of crystal silver according to the standard powder diffraction file No. 04-0783.

Cellular Membrane Integrity Injury. In this test, green-fluorescent SYTO 9 dye (Ex/Em = 480/500 nm) can pass through intact cell membranes and fluoresce bright green, whereas bacteria with damaged membranes show a reduction in green fluorescence or fluoresce red because of the red-fluorescing PI (Ex/Em = 535/615 nm). Laser scanning confocal micrographs of untreated bacteria showed bright green fluorescence with no red signal (Figure 8a), indicating that the cellular membrane was intact. However, after treatment with AgNP for 2 h at a final concentration of 200 mg/L, strong red fluorescence was observed in partial bacterial cells (Figure 8b), indicating loss of cellular membrane integrity. The mechanism of cell membrane damage and subsequent cell damage requires further investigation.

Membrane Potential. Changes in plasma membrane potentials, shown in Figure 9, were determined by using the slow-response potential-sensitive probe DiBAC4(3). Bacterial cells of *C. sakazakii* strains treated with AgNP showed a noticeable decrease in fluorescence, demonstrating hyperpolarization of the cell membrane. This result suggests that AgNP may have damaged cell membrane integrity by inducing cell membrane hyperpolarization at an early stage.

Leakage of Intracellular Reducing Sugars and Proteins. The standard curves for determination of the contents of reducing sugars and proteins were $Y = 1.746X - 0.0054$ ($R^2 = 0.9996$, shown in Supplemental

Figure S1; <https://doi.org/10.3168/jds.2018-15258>) and $y = 6.9943x + 0.0031$ ($R^2 = 0.9991$, shown in Supplemental Figure S2; <https://doi.org/10.3168/jds.2018-15258>) respectively, where Y and y represented absorbance at 540 and 595 nm, respectively, and X and x represent the contents of standard solutions of glucose and BSA, respectively. Figure 10 shows the contents of reducing sugars and proteins in supernatants of bacterial cultures with or without AgNP treatment. For *C. sakazakii* ATCC 29544^T, the amounts of leaked reducing sugars and proteins increased from 11.4 (at 0 h) to 172.5 (at 2 h) $\mu\text{g}/\text{mg}$ of bacterial dry weight for sugars and 7.8 (at 0 h) to 14.6 (at 2 h) $\mu\text{g}/\text{mg}$ of bacterial dry weight for proteins (Figure 10a). Similar results were obtained for ATCC BAA894 (Figure 10b), ATCC 29004 (Figure 10c), and ATCC 12868 (Figure 10d), indicating that AgNP accelerated the leakage of reducing sugars and proteins from the cytoplasm through damage and disruption of the cell membrane. However, bacterial cells of the control group exhibited a slight increase in reducing sugars and proteins contents that were secreted by cells during incubation.

Intracellular ATP Concentrations. Adenosine triphosphate is an important energy resource that participates in various physiological activities of bacterial cells. Changes in intracellular ATP concentrations in bacterial cells of each of the *C. sakazakii* strains before and after addition of AgNP are shown in Figure 11. The ATP levels in untreated bacterial cells of ATCC

Figure 4. Fourier transform infrared spectroscopy of silver nanoparticles synthesized with sodium citrate. The x-axis indicates the wavenumbers (cm^{-1}) of applied continuously varying infrared in frequency, while the y-axis represents the transmittance (%) of infrared light. The absorption peaks reflect the atom or functional groups vibration in substance. Eleven absorption peaks appear at wavenumbers (cm^{-1}) of 3,403.29, 2,921.59, 2,320.81, 1,626.27, 1,341.01, 1,137.05, 1,050.43, 995.95, 925.47, 867.74, and 591.66, showing a visualized connection of functional groups among synthesized silver nanoparticles (AgNP). Color version available online.

Figure 5. Apparent zeta potential of silver nanoparticles with a positive zeta value synthesized by sodium citrate at pH 8. The inset figure shows a corresponding histogram of apparent zeta potential (mV) of silver nanoparticles (AgNP) versus total counts, which gives a direct description about primary zeta potential distribution around 10 to 25 mV.

29544^T, ATCC BAA894, ATCC 29004, and ATCC 12868 were 8.51, 4.11, 4.087, and 5.457 μM respectively, and these values dramatically decreased ($P < 0.05$) to 0.019, 0.37, 0.125, and 0.102 μM after 1 h of treatment with AgNP. Reduction of intracellular ATP levels usually occurs with associated impairment or dysfunction of respiratory chain enzymatic activity.

Enzyme Activity of Respiratory Chain Dehydrogenases. The effect of AgNP on the enzyme activity of respiratory chain dehydrogenases of the 4 *C.*

sakazakii strains is shown in Figure 12. For *C. sakazakii* ATCC 29544^T, the activity of dehydrogenases in the positive control bacterial cells fluctuated around 150 $\mu\text{mol}/(\text{g}\cdot\text{min})$, whereas dehydrogenases in the negative control cells (boiled for 20 min) showed nearly no activity (Figure 12a). Interestingly, the enzyme activity of bacterial cells increased temporarily during the first 15 min of treatment with the lower concentration of AgNP. A similar phenomenon was observed for ATCC BAA894 (Figure 12b). However, the activity

Table 2. Minimum inhibitory concentrations (mg/L) of silver nanoparticles (AgNP) and growth (negative or positive) of different strains of *Cronobacter sakazakii*

Dilution	AgNP (mg/L)	<i>C. sakazakii</i> strain			
		ATCC 29544 ^T	ATCC 29004	ATCC 12868	ATCC BAA894
1	1,000	—	—	—	—
2	500	—	—	—	—
3	250	—	—	—	—
4	125	—	—	—	—
5	62.5	+	—	—	+
6	31.25	+	+	+	+
7	15.63	+	+	+	+
8	7.81	+	+	+	+
Control (–) ¹		—	—	—	—
Control (+) ²		+	+	+	+

¹Control (–) = control group without addition of AgNP and tested *C. sakazakii* strains.

²Control (+) = control group with tested strains but no AgNP.

of dehydrogenases decreased rapidly with increasing incubating time after AgNP treatment of ATCC 29004 (Figure 12c) and ATCC 12868 (Figure 12d). The results showed that AgNP could efficiently inhibit the activity of respiratory chain dehydrogenases; a higher concentration of AgNP was associated with a stronger inhibitory activity.

General Discussion

In this study, AgNP were synthesized by the chemical reduction method using citrate as reducing reagent and glycerol and PVP as stabilizers. The chemical reducing method is usually used to prepare AgNP; reaction temperature, pH, and molar ratio of Ag^+ and reducer are 3 critical influencing factors (Lee et al., 2004; Dong et al., 2009). High temperature speeds up the reducing reaction, forming a certain amount of silver nuclei in a short period, and accelerates Brownian movement to enhance opportunities for conjunction with silver nanoparticles (Kreibig, 2001). The pH value is consistent with the pH-dependent reducing activity of citrate, with higher pH conditions of reaction systems showing a fast nucleation process (Ji et al., 2007). Dong et al. (2009) proposed a stepwise reduction method in which nucleation and growth processes were performed at high and low pH, respectively, to synthesize a spherical AgNP. The molar ratio of Ag^+ and reducer affects the amount of silver nuclei and subsequently controls the concentration and shape of prepared AgNP. Moreover, dispersants are essential for the reaction system of the chemical reduction method; PVP is commonly used as a non-ionic polymer by compounding with Ag to pro-

tect the AgNP from growing and agglomerating (Wang et al., 2005).

The antibacterial activity of AgNP depends on the size and shape of nanoparticles, with a smaller size and presence of the (111) plane enhancing biocidal property against *Escherichia coli* (Pal et al., 2007; Carlson et al., 2008; Raza et al., 2016). Meanwhile, the surface charge of silver nanoparticles is related to the action of adsorption to the cell membrane at early stages by electrostatic interaction (El Badawy et al., 2011). Thus, it is important to characterize AgNP in terms of their surface topography, size distribution, crystal structure, connection of surface functional group, and surface charge condition. In this study, the synthesized AgNP were spherical with a mean particle size of 31.2 nm, similar to the results of Ranoszek-Soliwoda et al. (2017) but with a narrower size distribution due to the reducing capacity of both citrate and glycerol in boiling systems. The zeta potential was +18.7 mV, carrying the opposite charge to certain silver nanoparticles prepared by bio-fabrication, for example, using a proteinaceous pigment phycocyanin extracted from *Nostoc linckia* as the reducing reagent (El-Naggar et al., 2017). Meanwhile, the AgNP in the current study proved to be stable in aqueous solution with such zeta potential due to sufficient electrostatic repulsion. In addition, after freeze-drying, we determined an fcc crystal structure of AgNP, and this crystal structure is common to spherical silver nanoparticle powders, as reported by Naik et al. (2002) and Majeed Khan et al. (2011), prepared by in vitro biosynthesis using silver-binding peptides and wet chemical solution method by reducing Ag^+ with glucose in alkaline conditions, respectively.

The cell envelope of *C. sakazakii*, a gram-negative bacterium, consists of 2 membranes, with the outer membrane providing the outermost cell barrier and the cytoplasmic membrane serving as an electrochemical barrier and maintaining metabolism and energy transduction (Schwechheimer and Kuehn, 2015). It is important for bacterial cells to maintain the integrity of their cell membrane to perform material transportation and energy metabolism. Damage of the cell membrane is usually accompanied by (1) increasing membrane permeability, causing leakage of intracellular substances such as reducing sugars and proteins; (2) changes in membrane potentials; and (3) decreases in intracellular ATP due to diminishing proton motive force. In previous studies, Raffi et al. (2008) reported that AgNP adhered to and penetrated bacterial cells after interaction with *Escherichia coli*; similar results published by Li et al. (2010) showed that AgNP caused damage and fragmentation of the cell membrane of *E. coli*. In the current study, adsorption of AgNP on the surface of *C. sakazakii* bacterial cell and subsequent cell membrane

Figure 6. Diameters of inhibition zone of Ag^+ and silver nanoparticles against *Cronobacter sakazakii* strains. The blank group represents the external diameter of the Oxford cup with no formation of inhibition zone. The Ag^+ or 200 mg/L group was treated by spotting 100 μL of 5 mM AgNO_3 solution or 200 mg/L of nanosilver suspensions separately into nutrient-agar plates, which were spread with 100 μL (~ 108 cfu/mL) of bacterial cultures of 4 different species in advance. The diameters of inhibition zone are displayed as mean $\pm$ SD of data collected from 3 repeated experiments.

damage was observed by scanning electron microscopy after treatment with AgNP. Moreover, accelerated leakage of intracellular reducing sugars and proteins existed in treated bacterial cells in the current study,

suggesting that AgNP either affected cell membrane integrity or increased membrane permeability to produce antibacterial activity against *C. sakazakii*. However, in the current study, we focused on the binding of

Figure 7. Scanning electron microscopy of *Cronobacter sakazakii* ATCC 29544^T treated with silver nanoparticles (a, b) and untreated (c). Panel a shows the adsorption of particles on the surface of bacteria, whereas panel b displays cell disruption of partial bacteria. The full, rod-shaped cell morphology with a wrinkled superficial surface was observed for untreated bacteria in panel c. The magnification is 35,000 $\times$ at a voltage of 5.0 kV. (d) Energy-dispersive X-ray spectrum of particles adhered to the surface of cell membrane, which proved the existence of elemental silver. Color version available online.

Figure 8. Images of *Cronobacter sakazakii* ATCC 29544^T before (a) and after (b) addition of silver nanoparticles determined by laser scanning confocal microscopy. Green fluorescence excited by SYTO 9 dye at a wavelength of 480 nm represents the bacteria with intact cell membrane, whereas red fluorescence generated by propidium iodide (PI) at a wavelength of 535 nm represents bacteria with damaged cell membrane integrity. The length of scale bars is 5 μ m. Color version available online.

nanoparticles onto cells and sugar and protein leakage and did not specifically measure membrane integrity or permeability. This is a limitation of the present study that could be rectified by using methods such as liquid chromatography (LC)-MS or LC-tandem MS (LC-MS-MS) based on van Breemen and Li (2005). In future studies, LC-MS and LC-MS-MS methods could provide a more sensitive and selective analysis of the concentrations of sugars and proteins on either side of the bacterial cell compared with the spectrophotometric method applied here, which was based on characteristic absorption of synthesized color compounds. Thus, we were

able to know, before and after treatment with AgNP, which components were acceleratedly effluxed out from cells due to changes of cell membrane in integrity or permeability. Additional studies should focus on components of intracellular and extracellular oligo- or polysaccharides such as trehalose, a disaccharide inside the cell that contributes to the desiccation adaptation of *Cronobacter* spp., and capsular polysaccharides located on the cell surface that play a pivotal role in biofilm formation (Lehner et al., 2005; Jaradat et al., 2014). In addition, it would be interesting to identify proteins unique to *C. sakazakii* strains, such as those involved in pilus assembly and toxin/antitoxin systems, or as a function of phosphotransferase, sialic acid transporter, and *N*-acetylneuraminase lyase (Kucerova et al., 2010).

To detect membrane potential, we used DiBAC₄(3), a sensitive slow-response probe that can pass through the cellular membrane freely to maintain a dynamic balance between the internal and external charges due to its lipophilic anionic properties. Membrane hyperpolarization resulted from a decrease in the membrane potential. Hyperpolarization of the membrane has been reported as a characteristic phenomenon of cell membrane damage according to research with *Pseudomonas huttienensis* strain D1 as described by Yuroff et al. (2003). Meanwhile, research on the mode of action of membrane hyperpolarization by Bot and Prodan (2009) found that increased K⁺ pumped from the cell membrane K⁺ channels decreased membrane potential in *E. coli*. Similar results were observed in *Staphylococcus aureus*, in which K⁺ was released from bacterial cells in a dose-dependent manner after treatment with shikimic

Figure 9. Changes in membrane potential of 4 *Cronobacter sakazakii* strains untreated (blank) or treated with 200 mg/L silver nanoparticles for 2 h. Cells of both the blank group and treatment group were collected separately by centrifugation at $6,000 \times g$ for 5 min and co-incubated with DiBAC₄(3) at 37°C for 30 min. The fluorescence intensity was determined by using a fluorescence microplate reader at wavelength of excitation/emission = 493/516 nm. Error bars display the SD of data collected from 3 repeated experiments.

acid, an important antibacterial organic acid. Increased K^+ efflux leads to the changes of membrane permeability and membrane damage; as a result, some contents of bacterial cells including ions, nucleotides, sugars, and proteins can leak (Bot and Prodan, 2009; Bai et al., 2015). In the present study, membrane potentials of all 4 *C. sakazakii* strains decreased following treatment with AgNP, suggesting that AgNP hyperpolarized the bacterial cell membrane and affected cellular homeostasis. However, further study is required to elucidate whether K^+ efflux occurred in *C. sakazakii* cells when treated with AgNP.

Adenosine triphosphate is an important energy resource, participating in various physiological activities of bacterial cells. Changes in intracellular ATP levels usually accompany dysfunction of the respiratory chain and cell death (Johler et al., 2010). Bragg and Rainnie (1974) reported that silver ions inhibited the oxidation

of endogenous substrates by reacting with the respiratory chain at 2 sites in *E. coli*: one was located between the *b*-cytochromes and cytochrome a_2 , and the other in the NADH and succinate dehydrogenase regions. Another report showed that low concentrations of Ag^+ induced massive proton leakage through the membrane of *Vibrio cholerae*, causing a dramatic reduction in ATP levels and resulting in cell death (Dibrov et al., 2002). Li et al. (2011) reported on the antibacterial activity of silver nanoparticles on *Staphylococcus aureus* ATCC 6538P and the results showed that AgNP reduced the enzymatic activity of respiratory chain dehydrogenases and decreased intracellular ATP levels. In this study, reduction of ATP levels and inhibition of respiratory chain dehydrogenases were observed after treatment with AgNP. Interestingly, a temporary increase in enzymatic activity of dehydrogenases was detected in *C. sakazakii* ATCC 29544^T and ATCC BAA894, which

Figure 10. Leakage of reducing sugars (bars) and proteins (—●—) for 4 *Cronobacter sakazakii* strains without (0 mg/L) or with treatment of silver nanoparticles at a final concentration of 200 mg/L: (a) ATCC 29544^T, (b) ATCC BAA894, (c) ATCC 29004, and (d) ATCC 12868. Error bars represent the SD of data from 3 repetitive experiments.

could imply resistance to stress with treatment at a lower concentrations of AgNP. However, this hypothesis should be verified in further experiments by combined transcriptomic and proteomic analysis to identify genes and proteins that might be involved in this finding.

To date, nano-Ag is used frequently and is found in 25% of nano-enabled products (Vance et al., 2015). The major application is as protection against fungi, virus, and bacteria, especially some pathogenic microbes in clinical and food industry settings. Contributed to the specific antibacterial mechanisms, eukaryotic cells could be similarly damaged by silver nanoparticles, which caused concerns to human health (Laux et al., 2018; Zheng et al., 2018). In recent years, a growing body of literature reports public concerns about the cytotoxicity, genotoxicity, and ecotoxicity of silver nanomaterials, factors that mainly depend on size, shape, surface property, dosimetry, and stability (e.g., the aggregation, dissolution, and release of ions; Asha-Rani et al., 2009; Becker et al., 2011; DeLoid et al., 2015; Mitrano et al., 2015). In this regard, the European Food Safety Agency (EFSA) has issued guidelines for the risk assessment of nano-materials to determine their suitability for (partial) application in agriculture, food, and feed (EFSA Scientific Committee, 2011). These guidelines are involved in common physico-chemical methods and constantly developing analytical methods, for instance, inductively coupled plasma mass spectrometry based on laser ablation (LA-ICP-MS) for localization and quantification of metallic nanopar-

ticles in single cells (Drescher et al., 2012). Combined with the results obtained in this study and discussions above, we propose 2 plans for potential application of the synthesized AgNP to control *C. sakazakii* within the PIF production environment. First, antimicrobial films composed of low-density polyethylene and synthesized silver nanoparticles (LDPE-AgNP) could be prepared through high-energy ball milling under cryogenic conditions to achieve a well-blended powder of both and then hot-pressed to films, according to Olmos et al. (2018). In fact, it has been shown that nanoparticles >3 to 4 nm in diameter cannot migrate from LDPE due to its high diffusion properties (Maia et al., 2016). In the current study, the mean size of synthesized nanoparticles was 31.2 nm, feasible to prepare an LDPE-AgNP film with no concerning transference issues of silver nanoparticles to food. Thus, it supplies an acceptable solution to efficiently control *C. sakazakii* by covering the films upon the surface of equipment or facilities for PIF production. Second, an antimicrobial ecological water-borne paint could be formulated with synthesized silver nanoparticles at concentrations between 0.15 and 1% (wt/wt), according to the report of Tornero et al. (2018). This paint could be applied in food industry environments; for example, painted on the walls, to prevent potential food contamination by *C. sakazakii* due to direct contact with bacteria in the air or exhibit a certain inhibition zone to disrupt the reproduction of bacteria. For homogeneous distribution of AgNP in paint matrix, nanoparticles could be pre-dispersed by ultrasonic sonication in water and then added into the paint formulation. Furthermore, because of potential contact with water during production of PIF, dissolution of silver nanoparticles into ions could occur and the release of Ag⁺ ions should be monitored before practical use; dissolved Ag⁺ can re-form nanoparticles under slightly reductive conditions, causing an environmental hazard, even ecotoxicity, with efflux into the ecosystem (Störmer et al., 2017). In summary, nanomaterials such as AgNP have potential as an efficient means to control *C. sakazakii* in the food industry; however, caution is warranted due to potential safety problems. A long-term risk assessment of toxicity, including inhalation and oral toxicity, and ecotoxicity testing is needed.

CONCLUSIONS

The chemical reduction method is a practical way to synthesize silver nanoparticles. In the present study, spherical, positively charged, and face-centered cubic silver nanoparticles with a mean particle size of 31.2 nm were synthesized by reducing Ag⁺ using citrate and dispersed by glycerol and PVP in alkaline conditions. These silver nanoparticles had efficient antibacterial

Figure 11. Changes of intracellular ATP concentrations in bacterial cells of the 4 *Cronobacter sakazakii* strains before (0 h) and after addition of silver nanoparticles at a final concentration of 200 mg/L.

activity against *C. sakazakii* strains according to the MIC and inhibition zone results. Moreover, we proposed a possible antibacterial mechanism as follows: AgNP adhere to the surface of bacteria, damaging the integrity of the cellular membrane, leading to leakage of

reducing sugars and proteins from the cell. In addition, dysfunction of respiratory chain was caused through a reduction of intracellular ATP levels and inhibition of enzymatic activity of dehydrogenases, eventually causing cell death.

Figure 12. Changes of enzymatic activity of respiratory chain dehydrogenases for 4 *Cronobacter sakazakii* strains over time. The control (+) group shows bacteria treated with 0.1 mL of PBS at pH 7.0, the control (–) group represents bacteria boiled for 20 min to totally inactivate dehydrogenases, whereas the 200 and 1,000 mg/L groups show bacteria treated with corresponding concentrations of AgNP for a certain time individually.

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