Research Article

Preparation of Size-Controlled Silver Nanoparticles and Chitin-Based Composites and Their Antimicrobial Activities

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A simple method for the preparation of size-controlled spherical silver nanoparticles (Ag NPs) was reported for their generation by autoclaving a mixture of silver-containing glass powder and glucose. The particle size is regulated by the glucose concentration, with concentrations of 0.25, 1.0, and 4.0 wt% glucose providing small (3.48 ± 1.83 nm in diameter), medium (6.53 ± 1.78 nm), and large (12.9 ± 2.5 nm) particles, respectively. In this study, Ag NP/chitin composites were synthesized by mixing each of these three Ag NP suspensions with a <5% deacetylated (DAc) chitin powder (pH 7.0) at room temperature. The Ag NPs were homogenously dispersed and stably adsorbed onto the chitin. The Ag NP/chitin composites were obtained as yellow or brown powders. Approximately 5, 15, and 20 μg of the small, medium, and large Ag NPs, respectively, were estimated to maximally adsorb onto 1 mg of chitin. The bactericidal and antifungal activities of the Ag NP/chitin composites increased as the amount of Ag NPs in the chitin increased. Furthermore, smaller Ag NPs (per weight) in the chitin composites provided higher bactericidal and anti-fungal activities.

1. Introduction

In recent years, silver nanoparticles (Ag NPs) have been the subject of many studies due to their potential in various applications such as catalysts, photonic devices, biosensors, antimicrobials, and drug delivery systems [1–4], since their chemical properties are significantly different from those of the bulk material. Their special and unique properties can be attributed to their smaller size and larger specific surface area, and many preparation processes have been proposed for controlling the physical and/or chemical characteristics of Ag NPs [5–9].

Environmentally friendly processes using harmless materials are often used to prepare Ag NPs, since complicated purification is then not required for biomedical and environmental applications. Raveendran et al. produced Ag NPs with diameters of less than 10 nm using a process that employs D-glucose as the reducing agent and soluble starch as the stabilizing agent [10]. The particle sizes of Ag NPs are usually controlled by modifying reaction system parameters such as pH, temperature, and reactant concentrations. The choice of stabilizing agent is an important factor for controlling the particle size of Ag NPs since Ag⁺ is reduced within the nanoscopic templates of the stabilizing agent [11].
The antimicrobial activity of zerovalent silver is strictly dependent on the surface development of the solid phase. When the solid phase is in a nanoparticle form, the resulting antimicrobial activity can be significantly increased, and smaller Ag NPs may be several orders of magnitude more active than the corresponding bulk solid. Therefore, Ag NPs adsorbed onto surfaces of various biomaterials are a potentially great choice when fabricating materials with antimicrobial properties [12, 13].

The particle size of Ag NPs is one of the most fundamental parameters that affects their optical [14], antimicrobial [15–17], and antiviral properties [18, 19]. Sondi et al. reported that the antimicrobial activity of Ag NPs towards Gram-negative bacteria is dependent on the concentration of Ag NPs, and that the Ag NPs form “pits” in the cell wall of bacteria [13]. Sondi et al. speculated that a similar mechanism may cause the degradation of the membrane structure of *Escherichia coli* during treatment with Ag NPs [13]. Ag NPs also exhibit potent antifungal effects, probably through the destruction of membrane integrity [20].

However, there are some concerns about the biological and environmental risks of Ag NPs. It is known that Ag NPs have adverse effects on some aquatic organisms, for example, cytotoxicity and genotoxicity to fish [21] and the inhibition of photosynthesis in algae [22]. In mammals, a significant decline in mouse spermatogonial stem cells has been observed following dosing with Ag NPs [23]. Therefore, methods for preventing the diffusion of Ag NPs into the environment and their uptake by living organisms are required before designed antimicrobial materials containing Ag NPs can be widely used [21–23]. In a previous study, we developed an environmentally friendly process for tightly controlling the size distribution of Ag NPs [24]. This process uses only three materials: AgNO₃-containing glass powder, glucose, and water. The AgNO₃-containing glass powder is commonly used in environmental, osteal, and dental applications as an antimicrobial agent since it releases silver ions (Ag⁺) into aqueous environments in a sustained manner. Glucose has the advantages of being environmentally friendly and a mild reducing agent, which enables the reaction kinetics to be easily controlled. The synthesis of the Ag NPs was performed in an aqueous medium using an autoclave at 121°C and 200 kPa for 20 min. Caramel, which is formed from glucose during autoclaving, in turn functions as the stabilizing agent for Ag NPs in this system [24]. However, it is difficult to remove the caramel from the generated Ag NPs suspension without agglomeration and precipitation of the Ag NPs.

Chitin/chitosan is the collective name for a family of de-N-acetylated chitin with different degrees of deacetylation [25, 26]. In general, when the number of *N*-acetylglucosamine units exceeds 50%, the biopolymer is termed chitin, whereas the term “chitosan” is used to describe the polymer when the *N*-acyethylglucosamine content is less than 50%. Chitin/chitosan has been studied as a natural cationic biopolymer because of its excellent biocompatibility, biodegradability, nontoxicity, antimicrobial capability, and stimulation of wound healing [25, 26]. These properties of chitin/chitosan are dependent on the molecular weight and degree of deacetylation (DAc) [25, 26]. The DAc affects the solubility, hydrophobicity, and ability of chitosan to interact electrostatically with polyamions via its protonated amino groups. In fact, our previous study showed that chitosan with 83% DAc interacts with Ag NPs, which are negatively charged due to halogenation and oxidation [24].

In the present work, we added chitin with <5% DAc as a stabilizer to Ag NP suspensions to remove the generated caramel and to prevent agglomeration and precipitation of the Ag NPs. The Ag NPs adsorbed onto chitin powder were substantially stabilized compared to those in the absence of chitin. The size-controlled Ag NP/chitin composites were evaluated for their bactericidal (against *E. coli* (strain DH5α)) and antifungal (against *Aspergillus* (A. niger)) activities.

2. Materials and Methods

2.1. Materials. Silver-containing glass powder (BSP21, silver content: 1 wt%, average grain size: 10 μm) was obtained from Kankyo Science (Kyoto, Japan). Chitin with <5% DAc was purchased from Seikagaku Corp. (Tokyo, Japan). D-glucose was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All chemicals were used as received.

2.2. Preparation of Ag NPs. A suspension of size-controlled Ag NPs was prepared as previously described [24]. Briefly, 0.50 g of silver-containing glass powder was dispersed in 50 mL of an aqueous solution of 0.25, 1, or 4.0 wt% glucose in a 100 mL glass vial. The mixture was autoclaved using an Ikemoto IMC-30L autoclave (Ikemoto Inc., Tokyo, Japan) at 121°C and 200 kPa for 20 min. The mixture was then gradually cooled to room temperature and centrifuged at 3000 rpm for 10 min. The supernatant containing the Ag NP suspension was removed and stored in the dark at 4°C. The average diameters of Ag NPs prepared with 0.25, 1, and 4 wt% glucose were 3.48 ± 1.83, 6.53 ± 1.78, and 12.9 ± 2.50 nm, respectively [24].

2.3. Preparation of Ag NP/Chitin Composites. In this study, 10 mg of chitin (<5% DAc) was added to 1 mL of each Ag NPs suspension (about 60 μg/mL). The mixture was mixed well (at pH 7.0) on a shaker (MildMixer PR-36; TAITEC, Tokyo, Japan) for 30 min. The insoluble Ag NP/chitin composites were centrifuged at 6000 rpm for 10 min. The supernatant was analyzed using UV-visible spectrometer (Jasco V-630, Tokyo, Japan) to measure the amount of unreacted Ag NPs. The centrifuged composites were washed twice with distilled water by centrifugation at 6000 rpm for 10 min. The washed composites were dried up at 70°C on a block heater (EYELA/MG-2200, Rikakikai Co., Ltd., Tokyo, Japan) for 2 h and used in bactericidal or antifungal assays the same day.

The Ag NPs were homogenously dispersed and stably immobilized in the chitin matrices. TEM specimens were prepared by casting 5 μL of a suspension of Ag NPs onto a carbon-coated copper grid; excess solution was then removed using filter paper, and the specimens were dried at room temperature. TEM images were obtained using a JEOL JEM-1010 microscope (Nihon Electronics Inc., Tokyo, Japan) at
80 kV. The size distribution of each Ag NPs was determined using an image analysis software [27] in each TEM image.

2.4. Bactericidal Activity of the Ag NP/Chitin Composites. A culture of Escherichia coli (E. coli: strain DH5α, Takara Co., Kyoto, Japan) was stored in Luria-Bertani (LB) broth medium containing 50% sterile glycerol at −80°C. Overnight cultures were prepared by growing a single E. coli colony in 5 mL LB medium at 37°C. On the next day, 200 μL of cultured E. coli was inoculated in 2 mL of LB medium and grown at 37°C for 6 h until optical density at 600 nm (OD₆₀₀) reached 0.260 to ensure obtaining good quality of E. coli cultures. The cultured E. coli was diluted fourfold with LB broth, and then 50 μL of the diluted E. coli suspension was added to 1.5 mL sterile ClickFit polypropylene microcentrifuge tubes (TreffLab AG, Degersheim, Switzerland) containing dried Ag NP/chitin composites with the indicated amount of each Ag NPs (2, 1, 0.5, and 0μg) adsorbed onto 10 mg chitin as Ag NP/chitin composites, followed by incubation at 37°C for 18 h. After incubation, 1 mL of LB medium was added to the E. coli suspensions and mixed well. The suspensions were stood for 3 min to precipitate the Ag NP/chitin composites. Viable cells were enumerated by plating the 50 μL of tenfold serial dilutions of the suspensions onto LB agar (ForMedium Ltd., Hunstanton, England) in a Petri dish (90 × 15 mm) followed by incubation at 37°C for 24 h.

2.5. Antifungal Activity of the Ag NP/Chitin Composites. A. niger (NBRC105649) (Japan Collection of Microorganisms; Wako, Saitama, Japan) was maintained in molten potato dextrose agar (PDA) medium (Difco, Becton Dickinson & Co., Sparks, MD, USA). Twenty μL of A. niger spore suspension (6.35 × 10⁴ spores/mL) was inoculated into each well of an agar plate (24-multiwell plate; well diameter: 17 mm (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) containing 20, 10, 5, 2.5, and 1.25 μg of each Ag NP suspension adsorbed onto 4 mg/mL chitin in 1 mL of PDA medium as Ag NP/chitin composites. The plates were incubated in the dark at 25°C for 3 days, then the A. niger spores were recovered in 500 μL of 0.3% sterile Tween 80 solution using a platinum loop. The absorbance of each spore suspension after vortexing was measured at 550 nm with a Jasco V-630 spectrophotometer [28].

2.6. Statistical Analysis. Statistical analyses were carried out using StatMate III, Macintosh version (ATMS Co., Tokyo, Japan). Statistical significance was assumed when P < 0.01.

3. Results and Discussion

3.1. Characterization of Ag NP/Chitin Composites. In this work, chitin with <5% DAc was added as a stabilizer to Ag NP suspensions to remove the produced caramel and prevent agglomeration and precipitation of the Ag NPs. Ag NP/chitin composites were synthesized by mixing chitin and Ag NP suspensions at pH 7.0 for 30 min. The generated Ag NP/chitin composites were washed twice with distilled water by centrifugation to remove the produced caramel. Typical TEM micrographs of small (a), medium (b), and large (c) Ag NPs adsorbed onto 1 mg chitin were shown in Figure 1. The particle sizes and shapes of the Ag NPs adsorbed onto chitin were identical to those of the original Ag NPs used for synthesizing the composites. The color of the composites was yellow or brown; a darker composite was obtained when larger amounts of Ag NPs adsorbed onto chitin.

We estimated that approximately 5, 15, and 20 μg of the small, medium, and large Ag NPs, respectively, maximally adsorbed onto 1 mg of chitin. Figure 2 shows the UV-Vis spectra of the three sizes of the original Ag NPs in suspension and the spectra of the supernatants of the postreaction mixtures in which various amounts of chitin were reacted with the Ag NPs. The peak at 390.5 nm is representative of the spherical Ag NPs used in this work [12, 24]. There is a relationship between the absorbance at 390.5 nm and the concentration of each size of Ag NPs in the suspension (Figure 3). The amount of Ag NPs remaining in the supernatant of the postreaction mixture decreased as the concentration of chitin in the reaction mix increased (Figure 2). Thus, it appears that Ag NPs selectively reacted with chitin instead of caramel, and the two components precipitated together upon centrifugation.
Figure 2: UV-Vis spectra of small (a), medium (b), and large (c) original Ag NPs in suspensions (original) and supernatants from the postreaction mixture in which various amounts of chitin were reacted with the Ag NPs. Excess Ag NPs in the supernatant of the postreaction mixture decreased as the amount of chitin added increased.

Figure 3: The relationship between the peak at 390.5 nm arising from small (a), medium (b), and large (c) spherical Ag NPs and the concentration of each size of Ag NPs.
3.2. Antimicrobial Activity of Ag NP/Chitin Composites. Ag NPs have been found to exhibit interesting antimicrobial activities [13–15, 18, 20], and this phenomenon has gained importance due to the increase of resistance to antibiotics caused by their overuse. The size-controlled Ag NP/chitin composites and chitin alone were evaluated for their bactericidal (against \textit{E. coli}) activities in LB medium. Chitin alone exhibited only weak bactericidal activity (data not shown). The composites with various amounts of small, medium, and large Ag NPs in 10 mg chitin/mL showed bactericidal activity in a concentration-dependent manner for each composite type (Figure 4). Furthermore, the result showed that smaller Ag NPs had higher bactericidal activity.

3.3. Antifungal Activity of the Ag NP/Chitin Composites. Size-controlled Ag NP/chitin composites, chitin alone, and Ag/NPs alone were also evaluated for their antifungal (against \textit{A. niger}) activities. The fungi were incubated in molten potato dextrose agar (PDA) with the test materials. Chitin alone had no antifungal activity up to 10 mg chitin/mL (data not shown). Small, medium, and large Ag NPs had concentration-dependent antifungal activity with half-growth inhibitions of about 10, 30, and 40 \(\mu\text{g/mL}\), respectively (data not shown). When composites with various amounts of small, medium, and large Ag NPs in 4 mg chitin/mL were added to the fungal cultures, the composites showed antifungal activity in a concentration-dependent manner for each composite type with half-growth inhibitions of about 5, 10, and 20 \(\mu\text{g/mL}\), respectively (Figure 5). Thus, smaller Ag NPs had higher antifungal activity. Furthermore, the Ag NP/chitin composites exhibited higher antifungal activity than Ag NPs or chitin alone.

For all three sizes of Ag NPs used in this work, the antimicrobial activities of Ag NP/chitin composites increased as the amount of adsorbed Ag NPs increased. On a %weight basis, stronger antimicrobial activity was generally evident with Ag NP/chitin composites containing smaller Ag NPs. We surmise that smaller Ag NPs have enhanced antimicrobial activities for two main reasons; (i) the fraction of surface zerovalent silver atoms and silver ions increases with decreasing particle size, and (ii) a greater fraction of surface silver atoms and ions is weakly bonded to the surface of Ag NPs such that they can be easily released into the surrounding medium or can efficiently interact with microorganisms.

The mechanism of the bactericidal action of silver ions is closely related to their interaction with proteins, particularly at thiol groups (sulphydryl, \(-\text{SH}\)), which is believed to bind protein molecules together by forming bridges between them. Since these proteins are often enzymes, cellular metabolism is inhibited and the microorganisms die [13, 14]. However, little work has been conducted on the mechanism by which Ag NPs act against bacteria and fungi. It has been reported...
that the antimicrobial activity of Ag NPs on Gram-negative bacteria is dependent on the concentration of Ag NPs and is tied to the formation of “pits” in the cell walls of bacteria [13]. The authors of that report speculated that a similar mechanism may degrade the membrane structure of E. coli during treatment with Ag NPs [13]. The bactericidal activity of Ag NPs (against E. coli) is likely due to their direct binding to microbial envelope glycoproteins, thereby destroying membrane integrity. Ag NPs also exhibit potent antifungal effects (against A. niger), probably by destroying membrane integrity [20].

The effect of the size of Ag NPs on their bactericidal and antifungal activities was investigated in the present study. It appears that the binding of Ag NPs to the microorganisms tested depends on the surface area available for the interaction. Smaller Ag NPs have a larger surface area available for the interaction, thus providing stronger antimicrobial activity than larger Ag NPs. Furthermore, an increased number of small Ag NPs are present per unit weight compared to large Ag NPs. However, the present study showed that a lower amount of small Ag NPs could be maximally adsorbed onto chitin powder (5 μg/1 mg chitin) compared to large (20 μg/1 mg chitin) and medium (15 μg/1 mg chitin) Ag NPs.

For Ag NP/chitin composites, spatial restriction due to the chitin was expected to prevent or weaken the interaction between microorganisms and Ag NPs. However, the present study indicates that the antifungal activity of the composites is twofold higher than those of Ag NPs alone. When Ag NPs adsorbed onto chitin particles interact with microorganisms, the interaction may increase with the increasing number of Ag NPs in the composites. This is supported by the present experimental results showing a relative relationship between antimicrobial activity and the amount of Ag NPs added to the cultures as Ag NP/chitin composites.

4. Conclusion

In this work, we added chitin as a stabilizer to Ag NP suspensions to remove the generated caramel and to prevent agglomeration and precipitation of the Ag NPs. The Ag NPs adsorbed onto the chitin powder were substantially stabilized compared to those in the absence of chitin and were homogenously dispersed and stably adsorbed. The bactericidal and antifungal activities of the Ag NP/chitin composites increased as the amount of Ag NPs in the chitin increased. Furthermore, smaller Ag NPs (per weight) in the chitin composites provided higher bactericidal and antifungal activities. These results show the potential for chitin as a novel stabilizer and carrier for Ag NPs. Furthermore, the Ag NP/chitin composites might be directly applied as useful antimicrobial materials.

Conflict of Interests

The authors state no conflict of interests. This study was carried out using commercially available materials and equipment.

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References


