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Silver/chitosan-based Janus particles: Synthesis, characterization, and assessment of antimicrobial activity in vivo and vitro

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ABSTRACT

Janus particles containing chitosan and silver were synthesized in an eco-friendly manner and were confirmed using transmission electron microscopy. Based on the data of the antimicrobial activity assessment, this material exhibited a higher antimicrobial activity than virgin chitosan with long–lasting antibacterial effectiveness against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Salmonella choleraesuis* bacteria, as well as *Botrytis cinerea* fungi. The results showed that the Janus polymer could completely suppress the growth and germination of *B. cinerea* at a concentration of 0.02 mg/mL in vitro and in vivo. This Janus polymer is an advanced functional material that combines the suitable properties of both components and could be an alternative new antimicrobial agent due to its unique chemical properties and pronounced antimicrobial activity. This material is a potential candidate for use in the food industry to prevent microbial contamination and to inhibit the growth of microorganisms, enhancing product quality and, extend shelf-life of fresh and processed agri-food products.

1. Introduction

Silver and its compounds have a broad spectrum of antimicrobial activity for approximately 650 types of disease-causing microorganisms that can be traced back to antiquity, in which silver pottery and cutlery were used for food-related storage applications (Klasen, 2000; Martinez-Abad, Sanchez, Lagaron, & Ocio, 2013; Punitha, Ramesh, & Geetha, 2015). Currently, silver nanoparticles are known to be toxic to microorganisms and are effective against a broad range of microorganisms by binding to the microbial DNA, preventing bacterial replication and causing the inactivation of bacterial functions (Mohamed & Sabaa, 2014). Normally, nano-composites can be synthesized by the incorporation of metallic particles into easily-processable polymer matrices, offering a pathway for improved exploitation of their characteristic properties (Bajpai et al., 2007). Methods based on chemical reduction offer the best opportunity for both low cost and environmentally friendly materials. Silver nanoparticles have been synthesized using water as the solvent and starch as the capping agent, and these have been shown to be advantageous over conventional methods involving chemical agents that are associated with environmental toxicity (Sharma, Yngard, & Lin, 2009).

Chitosan is a cationic amino polysaccharide attracting more attention in the medical field due to its bioadhesivity, biodegradability, biocompatibility, safety and non-toxicity, as well as the promotion of drug absorption (Muzzarelli et al., 1990). The presence of chitosan is important for the stabilization of the formed nanoparticles because it prevents the silver clusters from aggregating at the macroscopic level due to the ion–dipole intermolecular forces (Shameli et al., 2010), and it also acts as a reducing agent for the incorporation of silver nanoparticles into the chitosan matrices (Murugadoss & Chattopadhyay, 2008).

The synthesis of silver nanoparticles with chitosan as both the reducing and the capping agent has also been well developed (Sanpui, Murugadoss, Prasad, Ghosh, & Chattopadhyay, 2008). Jena and coworkers demonstrated that chitosan-based AgNPs exhibited potent antibacterial activity against different human pathogens and also impeded bacterial biofilm formation without harming the host cells (Jena, Mohanty, Mallick, Jacob, & Sonawane, 2012). Silver nanosized particles were synthesized and shown to have a strong antifungal activity against the wood staining fungi *Ophiostoma flexuosum*, *Ophiostoma tetropii*, *Ophiostoma polonicum* and *Ophiostoma ips* by Velmurugan, Kumar, Han, Nahm, and Lee (2009). A hybrid hydrogel composed of chitosan and silver nanoparticles is the antibacterial most commonly used against *Streptococcus mutans* (Sámano-Valencia et al., 2013). Silver nanoparticles have been synthesized using commercially available carboxymethyl chitosan and sunlight (Long et al., 2013; Rodríguez-

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Argüelles, Sieiro, Cao, & Nasi, 2011; Yang & Li, 2014; Mohamed & Sabaa, 2014). The antibacterial activity was also investigated, and β-cyclodextrin-coated Ag-Cts NPs showed promising antibacterial activity against the Gram negative *Escherichia coli* (*E. coli*) and the Gram positive *Staphylococcus aureus* (*S. aureus*) micro-organism (Punitha et al., 2015).

Sanpui et al. and Rhim et al. have reported the preparation of different chitosan–Ag composites (Rhim, Hong, Park, & Ng, 2006). However, the method in which the chitosan and silver nanoparticles were combined is different from the present method because they prepared nanoparticle suspensions (silver nanoparticles covered with chitosan molecules) or films, which are not as manageable as a gel for possible oral or skin treatment applications (Rhim et al., 2006). Antimicrobial films consisting of chitosan and silver nanoparticles homogeneously distributed throughout the polymer matrix were developed by López-Carballo, Higueras, Gavara, and Hernández-Muñoz (2013).

However, in the present study, a chitosan-capped silver material was obtained using the reaction of silver nitrate with chitosan in an alkaline medium in which the low-molecular-weight chitosan degradation products (e.g., glucosamide) supply the electrons and function as the reducing agent. Ultraviolet–visible spectrophotometry (UV–vis), Fourier transform infrared spectroscopy (FTIR spectra) and transmission electron microscopy (TEM) were performed for prepared material. Additionally, the increasing prevalence of antibiotic resistance and the broad-spectrum bactericidal activities of silver nanoparticles, the antimicrobial activities of resulting Janus nanoparticles were further assessed.

2. Materials and methods

2.1. Materials

Chitosan with a centipoise viscosity of 20 mPa·s (1%, 20 °C) and a degree of substitution deacetylation value of 96.1% was obtained from Ningbo Zhenhai Haixin Biological Products Co., Ltd. (Zhejiang, China). Other reagents and solvents were purchased from Aladin Reagent Co., Ltd. (Shanghai, China) and were used directly without further purification. *E. coli* (CICC 21524), *Salmonella choleraesuis* (CICC 21493), *S. aureus* (CICC 10384), and vegetative cells of *Bacillus subtilis* (CGMCC 1.1377) were used in this study and grown in LB broth (Hopebiol, China). They were then agitated at 200 rpm and 37 °C for 18–24 h. *Botrytis cinerea* (*B. cinerea*) was isolated from spoiled blueberries in our laboratory.

2.2. Methods

2.2.1. Synthesis and characteristics of silver/chitosan Janus nanoparticles

Silver/chitosan Janus nanoparticles were synthesized according to our previous report (Chen, Jiang, Ye, Li, & Huang, 2014). Different amounts (mL) of a fresh 10 mmol/L $AgNO_3$ solution were added dropwise to a 50 mL (0.2% (*w*/*v*) chitosan solution with vigorous stirring at 95 °C. Afterwards, the pH of the reaction solution was adjusted to 10 with 0.3 mol/L aqueous NaOH. The color of the solution gradually turned from colorless to light yellow, indicating the formation of silver/chitosan composites. Subsequently, the suspension was dialyzed (dialysis tube, 8000–14,000 Mw, HuiPu Chemical Instrument Co. LTD., China), centrifuged (10,000 ×*g*, 10 min) (3–30 K, Sigma), filtrated, washed with water and dried in a freeze–dryer to produce the silver/chitosan composites.

The FTIR spectra were recorded using a Nicolet Nexus 870 spectrometer (Thermo, USA) with KBr pellets. The UV–visible spectra of the silver/chitosan Janus nanoparticles in 1% (*v*/*v*) acetic acid were obtained using a UV–vis spectrophotometer (UV-2550, Shimadzu, Japan).

The TEM samples were prepared by placing a drop of the aqueous suspension of silver/chitosan Janus nanoparticles on a carbon-coated copper grid under ambient conditions and allowing the solvent to evaporate at room temperature. They were then analyzed by transmission electron microscopy (TEM) on a Hitachi H-7650 microscope (Japan) at an acceleration voltage of 80 kV.

2.2.2. Antimicrobial properties of silver/chitosan Janus nanoparticles against E. coli, S. choleraesuis, S. aureus, and B. subtilis

The minimal inhibitory concentration (MIC) was read by the visual turbidity of the tubes noted before and after incubation according to previous reports (Mallick et al., 2012; Geng, Yang, Huang, Zhang, & Wang, 2013; Huang et al., 2013). The Janus nanoparticles were dissolved in a nutrient broth to prepare different solutions with varied concentrations at pH 6.3. After autoclaving at 121 °C for 15 min, the stock solutions were inoculated with 100 μL of a bacterial suspension (approximately 10^5 CFU/mL) and then cultured for 1 day in a 37 °C incubator and immediately used in standard MIC assays. The growth of the bacteria was determined using the optical density measured at 600 nm using a microplate reader (M200, TECAN, Switzerland). The lowest concentration of samples that inhibited the growth of the bacteria was considered to be the MIC. A total of 3 replicates were carried out for each treatment and the corresponding controls.

The MBC was determined according to the previous report (Qi, Xu, Jiang, Hu, & Zou, 2004). After the MIC determination of the Janus nanoparticles tested, an aliquot of 1 mL from each test tube in which no visible bacterial growth was observed was inoculated on Plate Count Agar (PCA, Hangzhou Microbial Reagent Co. Ltd., China) and then incubated overnight at 37 °C. MBC is defined as the lowest concentration of antimicrobial agent that kills >99.9% of the initial bacterial population, at which there is the lowest concentration of antimicrobial agent that on the PCA have no bacterial growth. Each bacterium was assayed at least twice.

2.2.3. Time-kill analysis

The time sensitivity assay was used according to our previous report (Huang et al., 2013). The tested sample solutions, including distilled water and LB broth (1:1, *v*/*v*) with the final concentrations ranging from 1× MIC to 4× MIC, were inoculated with bacterial suspensions in triplicate at 37 °C for 24 h. The solution containing no copolymer sample was simultaneously carried out as a control. The number of viable cells was determined at 1, 3, 6, and 24 h after plating aliquots of undiluted and 10-fold serial dilutions of each sample onto the PCA medium. Subsequently, plates were incubated for 24 h at 37 °C, and the colonies were counted. Data from the triplicate runs were averaged and plotted as log CFU/mL versus time (hours) for each point.

2.2.4. Transmission electron microscopy examination

Cultures of *S. aureus* and *S. choleraesuis* were treated with silver/chitosan Janus nanoparticles (4 × MIC) and incubated at 37 °C, while the untreated ones served as controls. After 24 h, they were harvested (4000 ×*g*, 5 min, 4 °C), fixed with 2.5% glutaraldehyde in phosphate buffer, dehydrated using a series of different concentrations of ethanol solutions, stained with 2% uranyl acetate, and separately analyzed using transmission electron microscopy (H-7650, Hitachi, Japan) at an accelerating voltage of 80 kV.

2.2.5. Antifungal ability assays

Antifungal assays were performed based on the method of Jasso de Rodrígueza et al. (2011). The silver/chitosan Janus nanoparticles were dissolved in 0.5% acetic acid, and the pH was adjusted to 5.6 ± 0.1 with a 1 mol/L NaOH solution. Potato dextrose agar (PDA) media including 0, 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, and 0.4 mg/mL silver/chitosan Janus nanoparticle solutions and PDA media including 0, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, and 2.0 mg/mL chitosan solutions were sterilized at 121 °C for 20 min and then poured onto sterile petri dishes (9-cm diameter). The plates were inoculated with 3-mm-diameter plugs taken from the margins of 7-day-old colonies on the PDA. Control plates were