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Preparation and characterization of uniform-sized chitosan/silver microspheres with antibacterial activities

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ABSTRACT

The chitosan/silver microspheres (CAgMs), which possess effective inhibitory on microorganisms, were prepared by an inverse-emulsification cross-linking method using CS/Ag sol as dispersed phase, whiteruss as continuous phase, and glutaraldehyde as crosslinking agent. The size and shape of CAgMs, greatly affecting their antibacterial activities, were controlled by varying the concentrations of cross-linking agent, emulsifier and CS/Ag colloid. The preparation conditions for obtaining uniform-sized microspheres were optimized. The morphology of CAgMs was characterized by scanning electron microscopy (SEM) and laser particle size analysis. The spherical CAgMs with smooth surface in the mean size of ca. 5 μm exhibited a narrow particle size distribution. Energy Dispersive X-ray spectroscopy (EDX) revealed the elemental composition of the microspheres. Transmission electron micrographs (TEM) and Fourier transform infrared spectroscopy (FTIR) of the microspheres confirmed the formation of silver nanoparticles (AgNPs). The X-ray diffraction (XRD) patterns and UV–Visible diffuse reflectance spectroscopy (UV–vis DRS) of the sample showed that AgNPs with the diameter no more than 20 nm were face-centered cubic crystallites. X-ray photoelectron spectroscopy (XPS) proved that Ag–O bond existed in the microspheres. Thermogravimetric analysis (TGA) showed that the starting decomposition temperature of CAgMs (ca. 260 °C) was much higher than that of CS (ca. 160 °C), suggesting that the as-prepared CAgMs possessed better thermal stability than original CS did. Antimicrobial assays were performed using typical Gram bacteria and fungi. The inhibitory effect indicated that the as-prepared microspheres exerted a stronger antibacterial activity as the concentration of the AgNPs is increasing, and the microspheres in smaller size had much better antibacterial activity than those in the larger size. The antimicrobial mechanism of CAgMs was discussed.

1. Introduction

Chitosan [b-(1-4)-2-amino-2-deoxy-D-glucose, CS], a linear polycationic heteropolysaccharide derivate from chitin by partial deacetylation [1], demonstrates excellent biocompatibility, physical stability and processability. The reactive hydroxyl and amino groups in CS molecules could provide capability to chelate with noble metals [2]. Moreover, CS is in conjunction with antitumoral activity, immunoadjuvant activity, acceleration of wound healing and antimicrobial activity due to the high percentage of amino groups (6.89%), which make CS attract much attention in biomedical fields [3,4].

The antimicrobial activity of CS against a wide variety of microorganisms including Gram bacteria, fungi and algae has been proposed to be due to its positively charged properties [5–7]. One of the important mechanisms indicates that the positive charge of the amino groups ($-NH_3^+$) at the C-2 position in the glucose unit can interact with the anionic compounds of bacterium cell's walls in a weak acid environment [8,9]. This interaction can alter bacterial surface morphology, increase membrane permeability and disrupt their normal structure of microorganisms in the end [10,11]. However, the antibacterial functions of CS are limited because amino groups on CS molecule backbone can only be protonated as relatively weak positive charge centers, which cannot bind strongly to negatively charged surface of bacterium cell walls. To improve the antibacterial activity of CS, it is reasonable to enhance the strength of positive charges on the CS molecules by adding more positively charged groups, such as metallic ion.

Silver nanoparticles (AgNPs) have been widely applied in many biomedical fields due to their highly inhibitory and bactericidal effects at a low concentration [12,13]. In principle, AgNPs could bind to DNA after penetrating through cell membranes of microorganisms with compromised permeability [14]. Many efforts, therefore, have been made to synthesize some new antimicrobial composites by combining AgNPs with CS [15,16]. Madhuchanda et al. reported synergy in antimicrobial activity of a chitosan–silver nanoparticle (CS–AgNP) composite in the presence of molecular iodine. They suggested that molecular iodine on the surface of AgNPs possibly changed into iodine atom which can contribute toward free radical induced oxidative stress, whereas CS and AgNPs facilitated the process of cell killing, leading to collectively enhanced potency of antimicrobial effect at the lowest concentrations of

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individual components [17]. Li et al. prepared CS/Ag/ZnO blend films via a sol–cast transformation method. They revealed that ZnO and AgNPs with spherical and granular morphology had uniform distribution within CS polymer. The product had excellent antimicrobial activities against the typical Gram bacteria and fungi [18]. Krishna Rao et al. prepared chitosan nanocomposites doped with silver nanoparticles by desolvation and followed by crosslinking with poly(ethylene glycol-di-aldehyde). The developed nanocomposites had antibacterial activity toward *Escherichia coli* [19]. Thus, the organic-friendly antimicrobial activity of CS and Ag complex has begun to attract attention. However, the antimicrobial properties of CS/Ag composites are generally limited since CS will lose its cationic nature and generate a strong hydrogen bond at circum-neutral pH, which leads to poor solubility and poor moldability of the composites.

It is well known that CS microspheres (CMs) show excellent mucoadhesive and permeation enhancing effect across the biological surfaces [20]. In general, CMs have important applications in controlled release of protein and peptide drug [21]. It was reported that CMs were synthesized by several methods such as precipitation, emulsion crosslinking, spray-drying, emulsion-droplet coalescence and reverse micellar methods [22]. Biró et al. produced CMs with mean particle size by water-in-oil (w/o) emulsion crosslinking method. They investigated and elucidated the influence of process parameters on the microsphere's structure [23].

However, few authors have applied the chitosan microspheres to the antibacterial field. This study focused on the formation and inhibitory action on undesirable microorganisms of the CS-based nanocomposite microspheres containing AgNPs. Firstly, novel CAgMs with AgNPs dispersed uniformly were successfully prepared via an emulsification cross-linking technology. Secondly, the as-prepared CAgMs were characterized by transmission electron microscopy (TEM), scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), UV–Visible diffuse reflectance spectroscopy (UV–vis DRS), X-ray photoelectron spectroscopy (XPS), and thermogravimetric analysis (TGA). Finally, the antimicrobial activity of CAgMs against different species of microorganisms was systematically investigated.

2. Experimental

2.1. Materials

CS (deacetylation degree 92%, $\overline{M}_w = 130{,}000$) was purchased from Fluka and used as received. Acetic acid, silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$) and glutaraldehyde (50%) were obtained from Shanghai Sanpu Chemical Co., Ltd. (China). Sorbitan monooleate (Span-80), alkyl phenol polyethylene glycol ether (OP-10) and whiteruss were supplied by Beijing Fuxing Chemical Reagent Factory (China). The pure Ag nanoparticles (average diameter <20 nm) were purchased from Wuhan Chemical Co., Ltd., (China). The AgNPs purchased were used as reference sample in the structural characterization and antimicrobial assay. Other chemicals were of analytical grade and used as received without further purification. Acetic acid was diluted to a 2% (w/v) aqueous solution before use. *Escherichia. coli* (ATCC 44752), *Staphylococcus aureus* (ATCC 26003), *Rhizopus* and *Mucor* were purchased from Beijing Center for Disease Prevention and Control.

2.2. Preparation of CS/Ag colloid

CS/Ag colloid was prepared by chemical reduction with $AgNO_3$ as Ag precursor, CS as stabilizer and $NaBH_4$ as reductant in the acetic acid solution. Firstly, CS was dissolved in a 2% (w/v) acetic acid solution to form a clear solution with CS concentration of 4% (w/v). Secondly, 1 mL of $AgNO_3$ aqueous solution with the concentration of 2 mol/L was added into the above CS solution under stirring at 30 °C. Thirdly, the freshly prepared $NaBH_4$ solution was added quickly into the above mixture. In order to complete the chemical reduction, the amount of $NaBH_4$ was used in double times of that of $AgNO_3$. The resultant CS/Ag colloid was kept at room temperature for characterization and further use.

2.3. Preparation of CAgMs

The w/o emulsification cross-linking reaction was employed to prepare CAgMs. Firstly, 6.3 mL of Span-80 and 2.7 mL of OP-10 were poured into 100 mL of whiteruss and the mixture was stirred for 30 min with nitrogen continuously bubbling. Then 10 mL of CS/Ag colloids was dropped into the above mixture to form an emulsion. The emulsion system was strongly stirred for 12 h at room temperature in order to enable monomer disperse into micro-droplets as soon as possible. After that, 5 mL of glutaraldehyde was dropped into the emulsion and the cross-linking reaction was kept at 40 °C for 4 h, then the temperature of the crosslinking system was increased to 65 °C for another 1 h. Finally, the crude product was demulsified from the above emulsion by acetone, washed with petroleum ether, ethanol and distilled water, respectively. After dried at 60 °C under vacuum to constant mass, the CAgMs were obtained.

2.4. Characterizations of CAgMs

The surface morphology and EDX of CAgMs were observed by a Philips XL-30 scanning electron microscope. The size and shape of AgNPs in the CAgMs were examined by a TEM (Philips G2 F20), operating at 100 kV. Samples were prepared by placing a drop of CAgMs suspension onto a carbon–copper grid at room temperature. Size distribution and number-average particle diameters were obtained via a laser particle size distribution instrument (3000HSA, Malvern Instr Co., USA).

FTIR spectra of the samples were recorded on a spectrometer (Nicolet Impact NEXUS-670) in the range of 400–4000 cm^{-1} with the transmission mode in spectroscopic grade KBr pellets for all the powders.

XRD patterns were recorded at 40 kV and 100 mA on a Rigaku D/MAX-2500 diffractometer using Cu-K_α radiation ($\lambda = 0.15406$ nm) with the diffraction angle range $2\theta = 10$–$90°$.

A Varian Cary 100 Scan UV–Visible system equipped with an integrating sphere (USRS-99-010) attachment was used to obtain UV–vis spectroscopy of Ag nanoparticles over a range of 300–500 nm. A UV–vis spectrophotometer (SP-723, Shanghai Spectrum Instruments Co., Ltd., China) was used to determine the optical densities of the cultures at 460 nm (O.D. 460) during aerobic incubation.

XPS measurement was conducted with a PHI 5000C ESCA spectrometer (Perkin-Elmer, USA) with an Al-K_α radiation as the X-ray source, the C, N, O, F and Ag contents on the sample surface were determined by the instrument.

TGA curves of CS and CAgMs were recorded using TA instruments' sequential thermal analyzer (Model-SDTQ600, USA). Analysis of the samples was performed at heating rate of 1 °C/min under a nitrogen atmosphere at a purging rate of 100 mL/min.

2.5. Assay for antimicrobial activities of as-prepared CAgMs against microorganisms

Microbial tests were performed to examine the antimicrobial properties of the CS/Ag colloids and CAgMs against the Gram-negative bacterium of *E. coli*, the Gram-positive bacterium of *S. aureus*, and the fungi of *Rhizopus* and *Mucor*. The quantitative growth inhibition was measured by disk diffusion and LB broth method. Nutrient agar medium was prepared by adding agar (15.0 g) into the solution (peptone 5.0 g, beef extract 3.0 g, sodium chloride 5.0 g and distilled water 1000 mL) with pH of 7.0. The agar medium was sterilized in a conical flask at a pressure of 15 lb for 30 min. This medium was transferred into sterilized Petridishes in a laminar air flan. After solidification of the medium, the