Scientific Journal for Damietta Faculty of Science 9 (1) 2019, 1-6

ISSN Print 2314-8594
ISSN Online 2314-8616

Synthesis, characterization and antimicrobial activity of chitosan/Ag nanocomposite using *Escherichia coli* D8

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Received: 22-02-2019 /Accepted: 06-04-2019
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Abstract

The present study provided a green approach for chitosan/silver nanocomposite (CS/AgNC) synthesis. CS/AgNC were synthesized by a biological method using the supernatant of *Escherichia coli* D8 (MF062579) strain in the presence of sun light. The absorbance spectra of particle solutions have been characterized by UV-Vis spectrophotometry, Fourier transform-infrared (FT-IR) spectroscopy and transmission electron microscopy (TEM). The obtained CS/AgNC showed absorption peak at 446 nm with an average particle size of ~21-31 nm. Moreover, CS/AgNC showed a potent antimicrobial activity against *Candida albicans*, *Staphylococcus aureus* and *E. coli*.

Keywords: chitosan, silver, nanocomposite, Escherichia coli, antimicrobial.

Introduction

Nanobiotechnology has created as a basic field of current researches as front-line innovation, interdisciplinary with physics, chemistry, biology **(**Rehan et al., 2018; Kafshgari et al., 2019). Nanomaterials showed a potent antimicrobial potential against human, animal and plant pathogens during the last decade. Researchers refer this potential to their unique optical, electronic, or mechanical properties due to the quantum and surface boundary effects compared with their bulk materials (Luo and Stutzenberger, 2008).

Nanocomposites (NC) are classified into ceramic based NC, metallic based NC and polymer-based NC (Akca and Gursel, 2015). Polymer nanocomposites (PNC) consist of a polymer having nano-fillers (nanoparticles (NPs), fibers or clusters) embedded in the polymer matrix (Sun et al., 2018). Among NC, PNC are represented more easily processing and cheaper. Moreover, polymers give lower modulus and quality with lower temperature application (Qiu et al., 2018).

NC are commonly prepared using two strategies: bottom-up or top-down (Fendler, 2008). Top-down methods based on breaking down the bulk materials gradually to nano-scale materials whereas in bottom-up methods, particles are collected to molecular structures in the nanoscale. Usually, chemical and biological synthesis of nanoparticles use the bottom-up approach (Narayanan and Sakthivel, 2010). At present, many studies carried out on the biological synthesis of NPs and NC using different varieties

of microorganisms such as algae, fungi, yeasts, actinobacteria, bacteria and viruses (Keat et al., 2015). Microorganisms can produce enzymes and/or some inorganic materials intracellularly or extracellularly that act as a reducing agent. This green synthesis does not include an addition of any toxic chemicals or using of any instrument which made the microbial biosynthesis of NPs and NC the best safe and eco-friendly method (Patil et al., 2019).

Chitosan (CS) is a natural polymer relatively cheap and extremely abundant with excellent biodegradability, biocompatibility and low toxicity. It has a significant importance due to its biological applications such as antimicrobial and antitumor activities. CS has functional groups which enable surface binding to NPs (Nate et al., 2018). Silver nanoparticles (AgNPs) have chosen for binding to CS in medical applications due to their antimicrobial potential and exhibiting high performance even at a very low concentration. In addition, AgNPs have been identified to possess good anticancer potential (An et al., 2018; Fahimirad et al., 2019).

The very low concentration of chitosan/silver nanocomposite (CS/AgNC) possessed a potent antibacterial activity against Gram-negative bacteria (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) (Shah et al., 2018).

The present study provided a green method for CS/AgNC synthesis using a low concentration of the supernatant of *E. coli* D8 (MF062579). Moreover, the synthesized CS/AgNC showed antimicrobial activity against *Candida albicans*, *S. aureus* and *E. coli*.

Materials and methods

Materials

Media and chemicals were purchased from Oxoid Ltd., England. Silver nitrate was purchased from Panreac Quimica S. L.U, Barcelona, Spain. All microbial strains were obtained from the culture collection of Microbiology Laboratory, Faculty of Science, Damietta University, Egypt. Fluconazole (Diflucan) and Penicillin G (Pfizerpen) were purchased from Pfizer Inc., New York, NY.

Methods

Preparation of the supernatant of Escherichia coli D8

The *E. coli* D8 strain slant was sub-cultured and streaked on sterile nutrient agar plates and incubated at 37 ℃ for 48 hrs. After incubation, about 5 single colonies were transferred aseptically and grown aerobically in sterile nutrient broth tubes. The inoculated tubes were incubated at 37 ℃ for 48 hrs at 150 rpm. After incubation, the culture supernatants were obtained by centrifugation at 4000 rpm for 20 min under aseptic conditions.

Preparation of chitosan/silver nanocomposite

CS/AgNC were synthesized by a biological method using the *E. coli* D8 supernatant in the presence of sun light as a reducing agent. CS (0.25g) was dissolved in 2 % acetic acid, while magnetic stirring followed by sonication for 15 min. The mixture was then filtered to obtain a clear solution. The amine sites of CS were activated by making the solution slightly alkaline at pH 8 using ammonia. Using of the optimized conditions for AgNPs biosynthesis according to El-Zahed et al. (2017), 1 mL of $AgNO_3$ (1.5 mM) was added to 40 mL of CS solution. After that, 1 mL of the bacterial supernatant was quickly added and stirred at 70 ℃ for 90 min. The solution was then centrifuged at 10000 rpm for 15 min and the residue was washed with distilled water to remove excess solution, and then re-dispersed in distilled water.

Characterization of chitosan/silver nanocomposite

Formation of CS/AgNC were monitored using UV-Vis spectrophotometry (Beckman DU-40, Chemistry Department, Damietta University, Egypt) and Transmission Electron Microscope (TEM) (Transmission Electron Microscope JEOL JEM-2100, Japan, Mansoura University, Electron microscope Unit, Egypt). Fourier transforms infrared spectroscopy (FTIR) analysis was carried using FT/IR-4100typeA in the diffuse reflectance mode operating by scanning the spectrum in the range 400-4000 cm^{-1} at resolution of 4 cm^{-1}.

Antimicrobial activity of chitosan/Ag nanocomposite

Antimicrobial potential of $AgNO_3$, AgNPs, CS and CS/AgNC were tested against human pathogenic bacterial strains such as *E. coli* (Gram negative bacteria) and *S. aureus* (Gram positive bacteria) in addition to *C. albicans* as a pathogenic yeast. Penicillin G and Fluconazole were used as positive controls against bacteria and yeast, respectively. The tested bacteria and yeast were refreshed by sub-culturing aseptically on the nutrient agar and yeast extract peptone dextrose (YEPD) agar plates, respectively. The Mueller-Hinton agar (MHA) and YEPD agar were prepared, autoclaved and the tested microorganisms (10^6 CFU.mL^{-1}) were added to the cold melted agar medium at the time of pouring of the plates in triplicate. Once the plates are solidified, equal volumes (100 µL) and same concentrations (1.5 mM) of the tested colloidal solutions were prepared and added into punched holes (5 mm). Bacterial and yeast plates were incubated at 37 °C and 30 °C for 48 hrs., respectively. After the incubation period, the plates were examined and zones of inhibition (mm) of bacterial and yeast growths were observed.

Transmission Electron Microscopic (TEM) study

The selected pathogenic bacterial strain, *S. aureus*, was subjected to CS/AgNC (150 µg/mL) for 2 hours at 37°C. Normal bacteria was included as a control. The cell culture was centrifuged (5000 rpm, 20 minutes), collected and washed 3 times with cacodylate buffer. The primary fixative solution (2.5% glutraldehyde in 0.1 M cacodylate buffer at pH 7) was added and left for 20 minutes at room temperature, post-fixed in 1% osmium tetroxide and dehydrated with a graded series of ethanol. The specimens were embedded in a plastic resin and sectioned on ultramicrotome. The sections were placed on carbon-coated copper grids and stained with uranyl acetate followed by lead citrate and examined using TEM (JEOL JEM-2100, Japan) at TEM Unit, Mansoura University, Mansoura, Egypt.

Statistical analysis

The data were statistically analyzed using SPSS software version 18. All values in the experiments were expressed as the mean ± standard deviation (SD) and were analyzed with one-way Analysis of Variance (ANOVA). The significant level was set at $p < 0.001$.

Results

The first signature for CS/AgNC formation was the colour change from colourless into brown. The UV-Vis spectra of CS/AgNC showed absorption peaks at 446 nm as shown in **Fig. 1**.

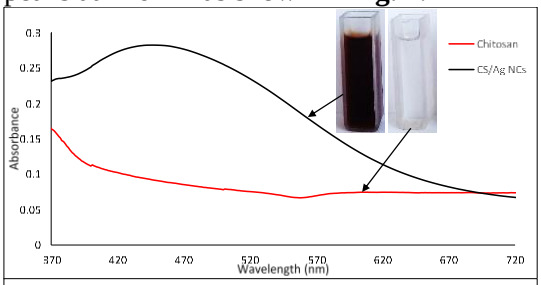

Fig. 1 UV-Vis absorption spectra of chitosan and CS/AgNC.

TEM images showed spherical shaped and well-dispersed AgNPs embedded in CS nanopolymer **(Fig. 2)**. The particle size distribution analysis in the present study showed a mean size of 21-31 nm of spherical shaped AgNPs.

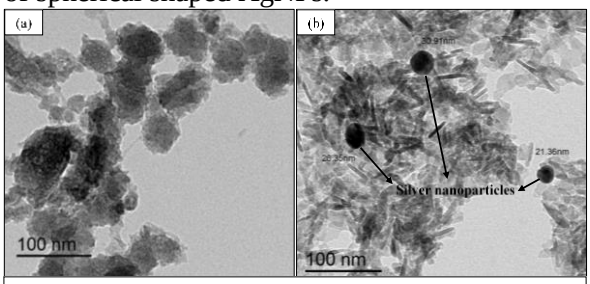

Fig. 2 TEM micrograph of chitosan; (a) and CS/AgNC; (b) (scale bar = 100 nm)

The FTIR spectrum of CS and CS/AgNC are showed in **Fig. 3**.

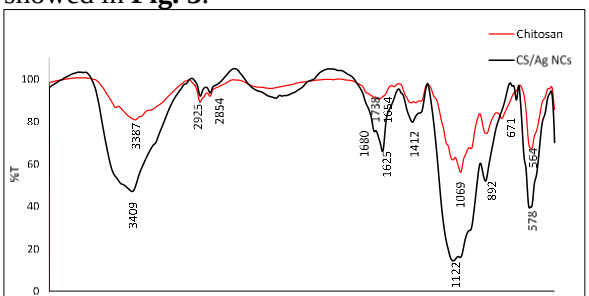

Fig. 3 FTIR spectrum of chitosan and CS/AgNC.

AgNPs and CS/AgNC revealed a good antimicrobial activity as shown in **Table 1** and **Fig. 4 and Fig. 5**. There are significant differences in antimicrobial effects between the samples with and without CS/AgNC treatment. Highly significant (P <0.001) was observed between the microbial strains; *E. coli*, *S. aureus C. albicans* and the diameter of inhibition zone.

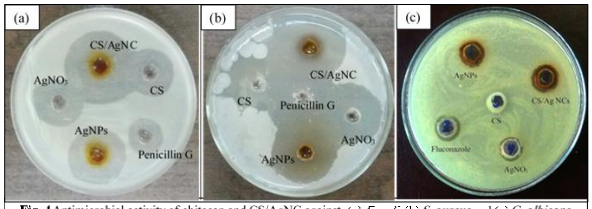

Fig. 4 Antimicrobial activity of chitosan and CS/AgNC against; (a) *E. coli*; (b) *S. aureus* and (c) *C. albicans*.

The examination of bacterial cells with TEM revealed that the untreated ones showed normal cell divisions and DNA replication. On the other hand, the treated bacterial cells with CS/AgNC at 150 µg/mL showed a complete cell lysis and encapsulated cells. The separation that occur between the bacterial cell wall and cell membrane was also detected.

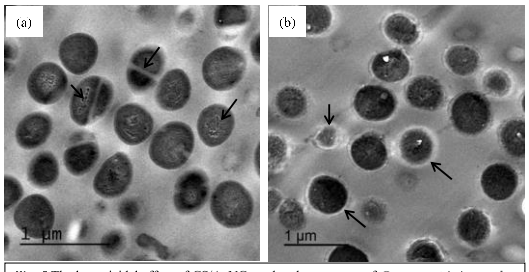

Fig. 5 The bactericidal effect of CS/AgNC on the ultrastructure of *S. aureus*. (a) A negative control (without CS/AgNC). Note normal cell division (arrows) and DNA replication. (b) A treated sample (at 150 µg/mL), there is no cell division observed. Note the complete cell lysis and encapsulated cells (arrows). Note also the separation that occur between the bacterial cell wall and cell membrane.

Table 1. Antimicrobial activity of chitosan and CS/AgNC against *C. albicans*, *S. aureus* and *E. coli* (Highly significant = $^{**}p<0.001$; $n = 3$).

a) Antibacterial activity (Inhibition zone, mm ±SD)		
Substance	*E. coli*	*S. aureus*
$AgNO_3$	$30 \pm 0^{**}$	$25 \pm 0.03^{**}$
CS	$25 \pm 0^{**}$	$19 \pm 0^{**}$
AgNPs	$35 \pm 0.06^{**}$	$30 \pm 0^{**}$
CS/AgNC	$42 \pm 0^{**}$	$35 \pm 0.06^{**}$
Penicillin G	$32 \pm 0^{**}$	$22 \pm 0.14^{**}$

b) Anticandidal activity (Inhibition zone, mm ±SD)	
Substance	*C. albicans*
$AgNO_3$	$11 \pm 0.03^{**}$
CS	-ve
AgNPs	$15 \pm 0^{**}$
CS/AgNC	$17 \pm 0.03^{**}$
Fluconazole	$12 \pm 0.03^{**}$

Discussion

Bacteria are considered as an excellent source for the extracellular biosynthesis of nanomaterials. There is a bigwig whack to discover novel bacterial strains having motivated biological potential (Patil et al., 2019). The current study used the crude metabolite of *E. coli* D8 in the extracellular biosynthesis of CS/AgNC. The presence of free ions in the *E. coli* D8 supernatant have accelerated the polyol synthesis of CS/AgNC (Youssef et al., 2014). We noticed the progress of the NPs synthesis throughout the colour changes from colourless to red-brown due to the increase in silver ions and CS reduction rates (Tran et al., 2017). TEM was used to study the morphology and size of CS/AgNC. The image showed that the NC are well-dispersed, uniform and spherical in shape (Phuoc et al., 2007). The chitosan FTIR spectrum confirmed the presence of O-H stretching at ~3387-3409 cm^{-1}, C-N and C-H stretching vibration at ~2925-2854 cm^{-1}, N-H bends at 1680-1738 cm^{-1} and the band at 1069 cm^{-1} referred to C-O-C stretching (Saraswathy et al., 2001). The FTIR spectrum of CS/AgNC showed the shifting of the CS peaks which could be owing to Ag and CS interaction in the formation of the NC (1654 cm^{-1} shifted to ~1625 cm^{-1}). In addition, the reduction in the hydroxyl (-OH) peak intensity and the increase in the C-O stretching, which is occurring when the presence of AgNPs in the CS matrix and formed the CS/AgNC (Ali et al., 2011). Kumar (2000) reported that AgNPs is strongly coordinated and coupled with hydroxyl groups of CS producing polymeric chains network. This strong bond prevents nanoparticles agglomeration and increases its stability. It was notable that the efficiency of AgNPs improved by the combination with CS due to its antimicrobial properties (An et al., 2018). CS/AgNC exhibit a superior microbicidal effect against *C. albicans*, *S. aureus* and *E. coli* compared with the antimicrobial activities that reported by Venkatesan et al. (2017) and Rahimi et al. (2019). The chemical produced CS/AgNC by Abdelkrim et al. (2020) showed a similar activity against *S. aureus* but were not effective against *C. albicans* and *E. coli*. On the other hand, *Bacillus subtilis* synthesized CS/AgNC with a higher activity against *C. albicans* in comparing to this study but it was less than our results against *S. aureus* and *E. coli* (Youssef et al., 2014).

The ultrastructure of *S. aureus* changed in response to the treatment with CS/AgNC. The changes included the inhibition of cell division in addition to the complete lysis of the bacterial cell or its encapsulation. Some treated cells showed a separation between the bacterial cell wall and cell membrane. The mode of action of CS/AgNC against microbial pathogens is not fully understood. It was reported that the integrity of cell membrane may lost due to cationic imbalance.

This imbalance increases the osmotic pressure and alters the cellular membranes within the microbial cell which may lead to cell burst (Selvaraj et al., 2014; Abdelkrim et al., 2020).

Conclusion

E. coli D8 (MF062579) biosynthesized CS/AgNC extracellularly in a green and cost-effective method. This method provides CS/AgNC and AgNPs have competitive size, shape and antimicrobial action. The present study demonstrated that using *E. coli* D8 crude metabolite for the synthesis of CS/AgNC have brought many biomedical applications throughout protecting human health (non-toxic to humans in minute concentrations) in addition to opto-electronics in the future. Further study will be designed to elucidate the mode of action of CS/AgNC as an antimicrobial agent.

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الملخص العربي

عنوان البحث: تخليق وتوصيف والنشاط المضاد للميكروبات لمتراكب الفضة النانومترية مع الكيتوزان باستخدام إشريشيا كولاي D8

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الملخص العربي

قدمت هذه الدراسة نهجا آمنا لتصنيع متراكب الفضة النانومترية مع الكيتوزان. تم تصنيع هذا المتراكب بطريقة بيولوجية باستخدام المستخلصات البكتيرية لسلالة إشريشيا كولاي D8 (MF062579) و في وجود ضوء الشمس. تم توصيف المتراكب الناتج من خلال قياس منحني الامتصاص باستخدام الطيف المرئي والأشعة الفوق بنفسجية، طيف فورييه للأشعة تحت الحمراء (FT-IR) وتحليل الميكروسكوب الالكتروني النافذ (TEM). وقد أظهر المتراكب الناتج قمة الامتصاص عند ٤٤٦ نانومتر مع متوسط حجم يتراوح ما بين ٢١ حتى ٣١ نانومتر. علاوة على ذلك، أظهر المتراكب نشاطًا فعالاً مضادًا للميكروبات ضد كانديدا البيكانس، ستافيلوكوكس اوريس و إشريشيا كولاي.

الكلمات الدالة: الكيتوزان، الفضة، المتراكب، إشريشيا كولاي، مضادات الميكروبات.