

GREEN SYNTHESIS OF ANTIBACTERIAL CHITOSAN FILMS LOADED WITH SILVER NANOPARTICLES

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Abstract An eco-friendly chemical reduction method was successfully used for the preparation of chitosan (CTS) composite films loaded with silver nanoparticles (AgNPs) by self assembly method using poly(ethylene glycol) as both reducing and stabilizing agent. UV-Vis spectra of the prepared chitosan loaded silver nanoparticles (CTSLAg) films reveal that full reduction of silver ions to silver nanoparticles takes place at 90 °C. The effect of reaction conditions on the silver nanoparticles formation was investigated using UV-Vis spectrophotometer. The morphology of the films was tested by scanning electron microscopy (SEM). The DSC curves showed that the CTSLAg film had a favorable compatibility and heat stability. AgNPs were confirmed by XRD and UV-Vis spectroscopy. The TEM findings revealed that the silver nanoparticles synthesized were spherical in shape with uniform dispersal, and by increasing CTS:PEG ratio larger silver nanoparticles could be obtained. The results of antibacterial study reveal that the prepared nanocomposite films exhibited potential inhibition.

Keywords: Chitosan; Silver nanoparticles; Antibacterial activity.

INTRODUCTION

In recent years noble metal nanoparticles have been the subjects of focused researches due to their unique electronic, optical, mechanical, magnetic and chemical properties that are significantly different from those of bulk materials^[1]. The preparation of colloidal AgNPs is of great interest for their unusual chemical and physical properties and their applications^[2]. Silver nanoparticles (AgNPs) are well known for strong antibacterial properties and no harm to human cells^[3]. The main advantage of AgNPs is that even nano molar concentrations are effective than micro molar concentration of silver ions^[4]. In addition, AgNPs were found to be relatively nontoxic to human cells^[5]. Silver nanoparticles have been produced using different methods: electrochemical method^[6], thermal decomposition^[7], laser ablation^[8], microwave irradiation and sonochemical synthesis^[9]. The simplest and the most commonly used bulk-solution synthetic method for metal nanoparticles is the chemical reduction of metal salts^[10, 11]. In fact, production of nanosized metal silver particles with different morphologies and sizes using chemical reduction of silver salts has been reported, this synthetic method involves reduction of an ionic salt in an appropriate medium in the presence of surfactant using various reducing agents^[12–14].

Polymer blending technology is an effective way to obtain new polymeric materials with optimized properties. The advantages of this technology include versatility, simplicity and inexpensiveness^[15, 16]. Numerous

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polymers have been employed to prepare polymer-silver nanocomposites and have been successfully used for the synthesis of metal nanoparticles^[17, 18]. Polymer stabilizes the metal nanoparticle-polymer composite, and hence, the nanoparticles attached to the polymer chains will disperse in the solution when the composite dissolves^[19]. Therefore, the synthesis protocols often involve a stabilization process by reducing the silver ions in the presence of surfactant, polymers and hydrogels^[17].

Among the various biopolymers, poly-cationic chitosan (CTS), which is composed of polymeric 1-4 linked 2-amino-2-deoxy- β -D-glucose units is considered as a potential antimicrobial active material which has been reported to prepare and stabilize the metal nanoparticles^[20]. It has interesting features such as low toxicity, good biocompatibility, biodegradability with enormous metal complexing capacity as the presence of largely free amino and hydroxyl groups in chitosan chains offer its unique physico-chemical properties including the polycationic, chelating, and film-forming characteristics^[21]. Because of these advantages of chitosan, it can be used in the fields of wastewater treatment, food processing, cosmetics, pharmaceuticals, biomaterials and agriculture^[22, 23]. Blending poly(ethylene glycol) (PEG) with chitosan has gained considerable attention because PEG is a polymer that has been approved by Food and Drug Administration (US FDA). Besides, PEG-CTS blend exhibited well physico-chemical properties comparable to chitosan^[24]. In our previous work, we studied the adsorptive removal of iron and manganese ions from aqueous solutions with micro porous chitosan/polyethylene glycol microporous blend membranes^[25].

The present study reported a facile synthesis of chitosan films loaded with silver nanoparticles through reduction of silver nitrate using poly(ethylene glycol) as both reducing and stabilizing agent. The developed nanocomposites were characterized using UV-Vis, DSC, SEM, XRD and TEM to understand their physico-chemical properties. The effect of reaction conditions on the silver nanoparticles formation in the CTSLAg films were studied and optimized for full conversion of silver ions to silver nanoparticles. The developed silver nanocomposite films were evaluated for their antibacterial applications.

EXPERIMENTAL

Materials

Chitosan powder (CTS) (high MW, > 75% deacetylated) was purchased from Sigma Aldrich, Acetic acid (glacial, 99%–100%), different molecular weights of poly(ethylene glycols) (PEG) (200, 400, 2000 and 6000), and glutaraldehyde were obtained from Merck (Mumbai, India). Silver nitrate (AgNO_3) was purchased from Acros Organics (New Gersy, USA). The water used for experiments was obtained by double distillation of de-ionized water.

Preparation of Chitosan Blend (CTSB) Films

Chitosan dissolved in 2% acetic acid and the counterpart polymer (PEG 6000) dissolved in water with different mass ratios (CTS/PEG = 1:1, 2:1 and 4:1) were mixed thoroughly and stirred for 1 h. Then, 1 mL of 2% glutaraldehyde solution (cross-linker) was added under stirring at room temperature (27 °C). The solution was transferred immediately into a Teflon covered glass plate (100 mm Dia.) and dried at 90 °C in an electric oven (TK 3108, EHRET, Germany) for 4 h. After drying, the formed cross-linked CTS-PEG blend film was neutralized with 2% aqueous NaOH solution for 30 min. Afterwards, the film was washed thoroughly with water to remove the remaining NaOH. Finally, the film was kept in water with bath temperature 80–90 °C for more than 10 h to dissolve the PEG component and to generate porous structure. The wet film was wiped with a filter paper to remove the excess water present on the surface of the film, then framed on a glass to prevent shrinkage along the surface and allowed to dry^[25].

Preparation of Chitosan Loaded Silver Nanoparticles (CTSLAg) Films

100 mg of silver nitrate (AgNO_3) was added separately in three beakers to 10 g/L PEG 6000 solution at 90 °C. The solutions were stirred at this temperature for 1 h to generate AgNPs. Chitosan was dissolved in 2% acetic acid with different CTS:PEG mass ratio's (1:1, 2:1 and 4:1). The two solutions were mixed and stirred for 1 h. Then, 1 mL of 2% glutaraldehyde (cross-linker) was added with mechanical stirring at room temperature

(27 °C). The solutions were poured immediately into Teflon covered glass plates and dried in an oven at 90 °C. Finally, chitosan-silver nanocomposite films were immersed in hot distilled water (80 °C) for more than 1 h. This process involved the extraction of most of PEG into distilled water since PEG is highly soluble in water at this temperature and forming porous structure^[26].

The photos of CTSB films and CTSLAg films with different compositions of CTS:PEG (1:1), (2:1) and (4:1) are shown in Fig. 1.

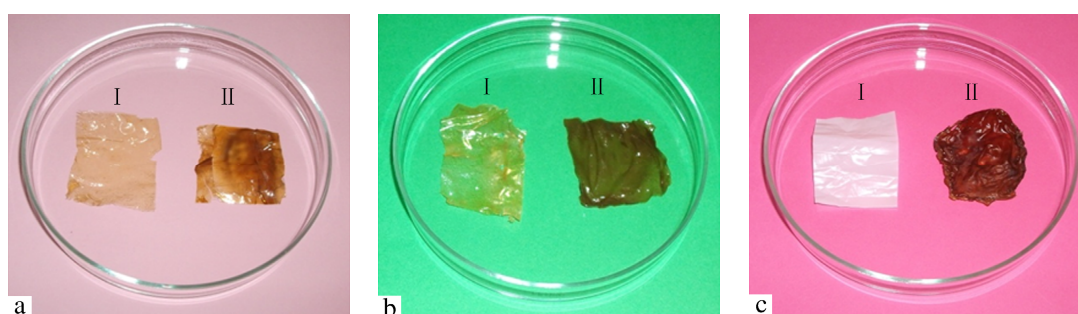


Fig. 1 Photos of (I) CTSB film, (II) CTSLAg film; (a–c) different compositions of CTS:PEG: (a) (1:1), (b) (2:1) and (c) (4:1)

Effect of Reaction Conditions on AgNPs Formation

The effect of reaction conditions on the reduction capacity of silver ions into AgNPs in the synthesized composite films was studied and optimized for full conversion of silver ions to silver nanoparticles by varying the reaction temperature, molecular weight, composition of PEGs and composition of glutaraldehyde (cross-linking agent).

Swelling Study

Film samples (2 cm × 2 cm) were cut from the CTSB and CTSLAg films. The thickness of the films was found to be in the range 200–250 μm as measured by a digital micrometer. The swelling behavior was tested by immersing the pre-weighed film samples in 250 mL of phosphate buffer (pH 7.4) at 27 °C for predetermined intervals. The sample weights were then determined as function of time by an analytical balance (AP250D, OHAUS Company, Switzerland) after blotting the film with a tissue paper to remove surface water. The swelling ratio (SR) of the membranes was calculated using Eq. (1)

$$SR = \frac{W_s}{W_d} \quad (1)$$

where, W_s (g) is the weight of the swollen film, and W_d (g) is the weight of the dry film.

Characterization

Spectroscopic measurements were carried out using spectrometer (Shimadzu 3600 NIR UV, Kyoto, Japan) from 200 nm to 1000 nm. The structures and morphologies of the films were observed through scanning electron microscopy (Inspect S, FEI Ltd., Holland) after gold coating. The fractured cross-sections of the films were achieved by breaking the samples deeply cooled in liquid nitrogen. X-ray diffraction (XRD) patterns of the films were recorded (X Pert Bro, Panalytical, Holland) at room temperature, using Nickel-filtered Cu K α radiation generated at 45 kV, and 50 mA. The diffraction patterns were determined over a diffraction angle range of $2\theta = 5^\circ$ – 80° . Thermal studies of the films were measured using a differential scanning calorimeter (DSC-H50, Shimadzu, Japan) at heating and cooling rates of ± 10 K/min. under nitrogen atmosphere at flow rate 10 mL/min. from room temperature to 400 °C. Transmission electron microscopy (TEM) (Tecnai F12, FEI Ltd., Japan) at an accelerating voltage 120 kV was used to measure the size and morphology of AgNPs. The samples were prepared by placing few drops of the NPs suspension on carbon coated copper grids, followed by allowing the

solvent to slowly evaporate overnight at room temperature and under vacuum before recording the TEM images.

Antimicrobial Activity

The effect of the developed CTSB film, and CTSLAg film on gram-negative bacteria *Escherichia coli* (*E. coli*) and gram positive bacteria *bacillus* was measured qualitatively and quantitatively by disc diffusion and viable cell count methods. For disc diffusion, the films were cut into a disc shape with 5 mm diameter, sterilized by autoclaving for 30 min at 120 °C, and placed on different agar plates. The plates were incubated for 2 days at 37 °C in an incubation chamber maintaining with 5% CO₂ flow and the inhibition zone was then measured. In the quantitative analysis method, the viable cell count method, suspensions containing approx. 10⁸ CFU (colony forming unit) of the bacterial species *E.coli* and *bacillus* were treated overnight with the chitosan blend discs, and chitosan loaded silver nanoparticles discs, respectively, these suspensions were then cultured on nutrient agar plates and incubated for 24 h. The colonies of both bacterial species were then counted.

RESULTS AND DISCUSSION

Optimization of AgNPs Formation in CTSLAg Films

The effects of reaction conditions affecting the silver nanoparticles formation in the CTSLAg films were investigated to evaluate the optimum conditions for silver nanoparticles formation in the CTSLAg composite films that result in the complete conversion of Ag⁰ to AgNPs, which can be used as an effective antibacterial product. For this, the reaction takes place by varying the reaction temperature, molecular weight of PEG, composition of PEG and composition of glutaraldehyde.

First, the formation of AgNPs was monitored by UV-Vis spectroscopy at different reaction temperatures. The formation of AgNPs can be observed by change in color from pale pink to dark brown. It can be observed that at temperature as low as 40 °C, surface plasmon resonance (SPR) barely appeared, indicating that silver reduction rate under these experimental conditions is very slow (Fig. 2a). By increasing the temperature to 60 °C a broad surface plasmon resonance (SPR) absorption peak around 438 nm appears, belonging to the dipole resonance of conducting electron on the surface of silver nanoparticles. When temperature increased beyond 90 °C, the intensity of the surface plasmon resonance peak shift towards lower wavelength to 420 nm, the shape of the plasmon band is symmetrical and quite narrow, suggesting that AgNPs are spherical and mono disperse^[2]. Therefore, an optimum temperature 90 °C was fixed for the synthesis of AgNPs in the foregoing experiments.

Afterwards, Keeping the temperature constant at 90 °C, using 10 g/L of different molecular weights of PEG, *i.e.*, PEG-200, PEG-400, PEG-2000 and PEG-6000 (Fig. 2b). The results showed that higher molecular weights were efficient in reducing the silver ions into AgNPs. A small intense SPR peak that observed in the UV-Vis spectra of PEG-2000 indicates a slightly improved efficiency in the reduction of silver ions, in spite of the related peak correlates to the silver ion still exist in the UV-Vis spectra. In the case of PEG-6000, it is observed that there is no silver ions peak and the appearance of a broad and strong SPR absorption peak around 420 nm, which revealed that this molecular weight of PEG results in complete reduction of silver ions to AgNPs.

In the next step, experiments were conducting by varying the composition of the PEG-6000 (3–20 g/L) in the reaction mixture at reaction temperature 90 °C for the synthesis of silver nanoparticles (Fig. 2c). By increasing the concentration of PEG-6000, the intensity of the absorption band increases gradually, which indicate the improvement in reduction capacity of PEG-6000 by increasing the concentration of PEG-6000.

Finally, experiments were conducting by varying the composition of the glutaraldehyde in the range of (3–20 g/L) at reaction temperature 90 °C (Fig. 2d). It is observed that by increasing the concentration of glutaraldehyde, the intensity of the absorption band increases, which indicates the increase in the reduction capacity of PEG-6000 by increasing the concentration of glutaraldehyde.

From the above experiments, the optimum reaction conditions which result in the formation of uniform AgNPs were: reaction temperature 90 °C; molecular weight of PEG = 6000; concentration of PEG = 20 g/L and concentration of glutaraldehyde = 2%.

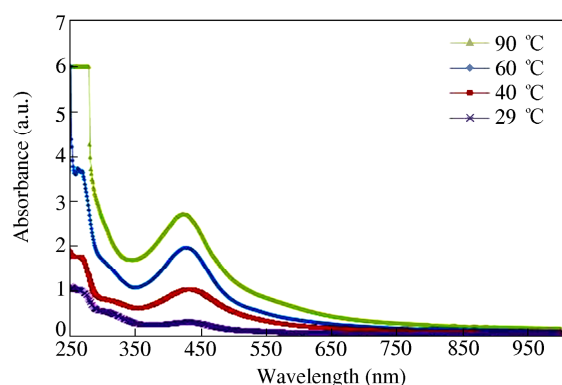


Fig. 2a Effect of reaction temperature on the formation of silver nanoparticles

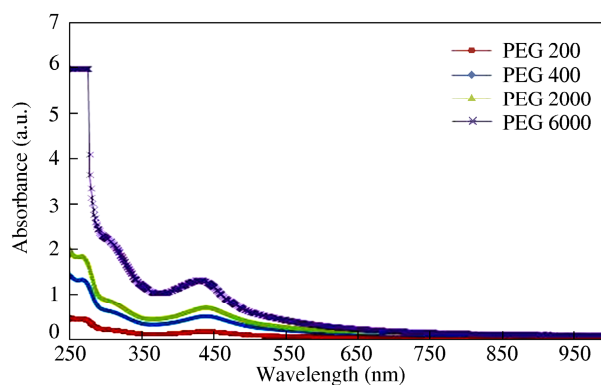


Fig. 2b Effect of molecular weight of PEG on the formation of silver nanoparticles.

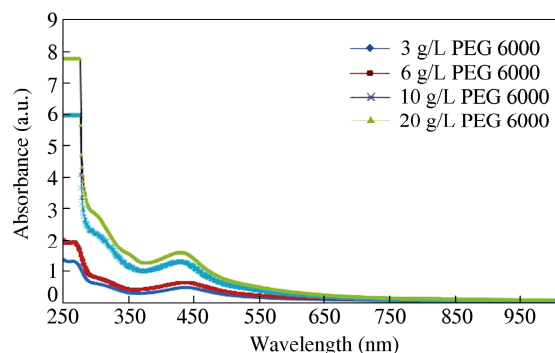


Fig. 2c Effect of composition of PEG-6000 on the formation of silver nanoparticles

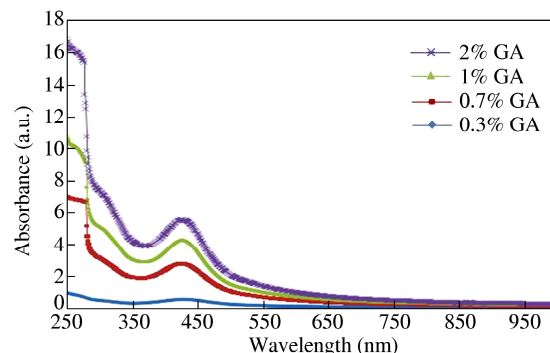


Fig. 2d Effect of composition of glutaraldehyde on the formation of silver nanoparticles

Swelling Capacity

The swelling capacity of the films has a great effect in the antibacterial activity. Figure 3 shows the swelling capacity of CTSB and CTSLAg films. It is observed that the swelling capacity of the films follows the order as, CTSLAg > CTSB film, thus, the CTSB film show lower swelling ratio (2.8 g/g) than the CTSLAg film (4.2 g/g). It is observed that porous CTSLAg films show higher swelling capacity due to the presence of a lot of pores in their network structure which allow more water to enter inside the film.

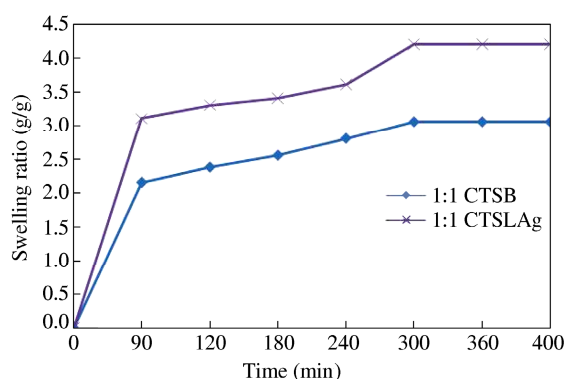


Fig. 3 Swelling rate of CTSB film and CTSLAg film with CTS:PEG ratio (1:1)

Membrane Characterization

DSC measurements

Differential scanning calorimetry (DSC) analysis was carried out to determine the thermal properties of the composite films. DSC curves of CTS powder, CTSB film, and CTSLAg film are collected in Fig. 4. In the CTS curve, the main feature is a broad endothermic peak at 180 °C. As for CTSB film, its melting peak is affected remarkably by chitosan content, as the melting point decreases with the increase of chitosan content. This is in agreement with the results obtained by Zhao *et al.*^[27] who found that T_m (melting point) of PEG decreased with the increase in CTS content up to 50%. In case of CTSLAg film, the degradation starts earlier than that of CTSB film because of moisture present due to nanoparticles incorporation process leading to porosity. The results indicate that the structure of chitosan chains has been changed due to the introduction of acid moieties and the reduced ability of crystallization. Therefore, it can be inferred that CTSLAg film has over all higher stability than CTSB film.

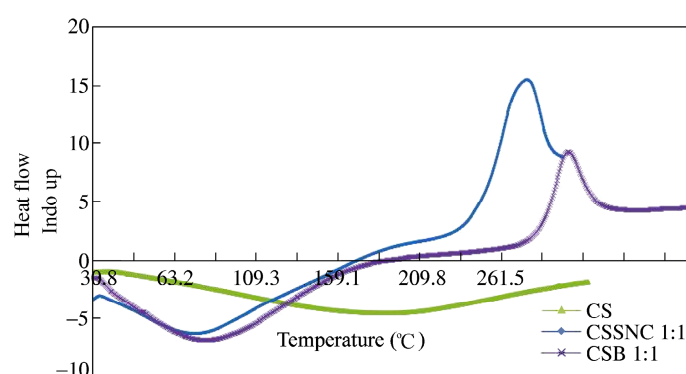


Fig. 4 DSC curves of CTS powder; CTSB and CTSLAg films with CTS:PEG ratio (1:1).

XRD analysis

The XRD patterns of CTS powder, and CTS/synthetic polymer blend film and CTSLAg film with CTS:PEG ratio (1:1) are shown in Fig. 5. Crystalline peaks for CTS appear at around $2\theta = 9^\circ$, 15.8° , 20° . For CTSB film with CTS:PEG ratio (1:1), its reflection pattern at $2\theta = 9^\circ$, 15.8° are almost the same as those of CTS and correspond to the hydrates crystalline structure, while the broaden beak observed at $2\theta = 20^\circ$ indicates the existence of an amorphous structures^[28], but its reflection pattern at $2\theta = 20^\circ$ becomes broader and stronger than CTS. This means in the CTS/PEG blends the crystalline structure of each component increased upon blending. The XRD pattern of CTSLAg film with CTS:PEG ratio (1:1) is illustrated in Fig. 5(c). It shows a peak at $2\theta = 20^\circ$, which corresponds to the characteristic peak for chitosan chains^[29], thus showing its high degree of crystallinity. In addition, we also observed a sharp peak at 32.1° and two relatively smoother peaks at 38.2° and 46.1° , thus indicating the reflections through (111), (200), and (311) planes of the face centered cubic structure of silver nanoparticles. Therefore, this gives a clear evidence for the presence of silver nanoparticles in the CTSLAg film.

Scanning electron microscopy (SEM) results

The scanning electron microscopy was used to collect information regarding morphology and cross-sectional structures of the films. The SEM micrographs are presented in Fig. 6. In general, CTSB film exhibited a dense and uniform microstructure, whereas CTSLAg film showed the presence of spherical silver nanoparticles and hundreds of pores within the CTSLAg film. It is worth mentioned that SEM micrographs confirmed the results of the swelling studies.

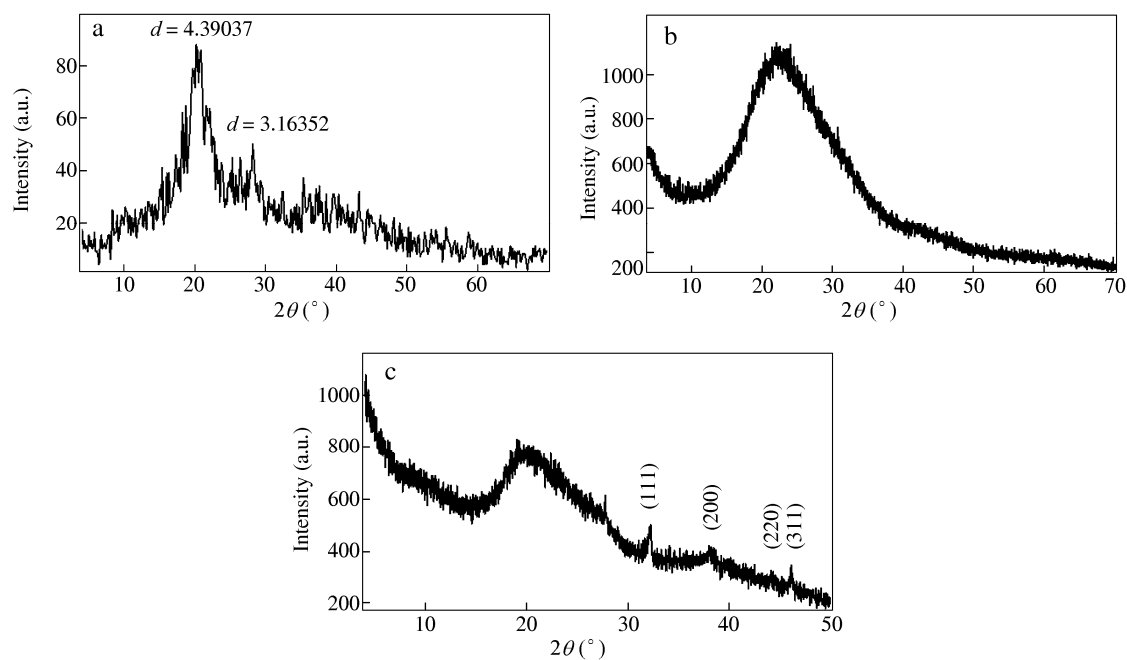


Fig. 5 XRD curves of (a) CTS powder, (b) CTSB film with CTS:PEG ratio (1:1) and (c) CTSLAg film with CTS:PEG ratio (1:1)

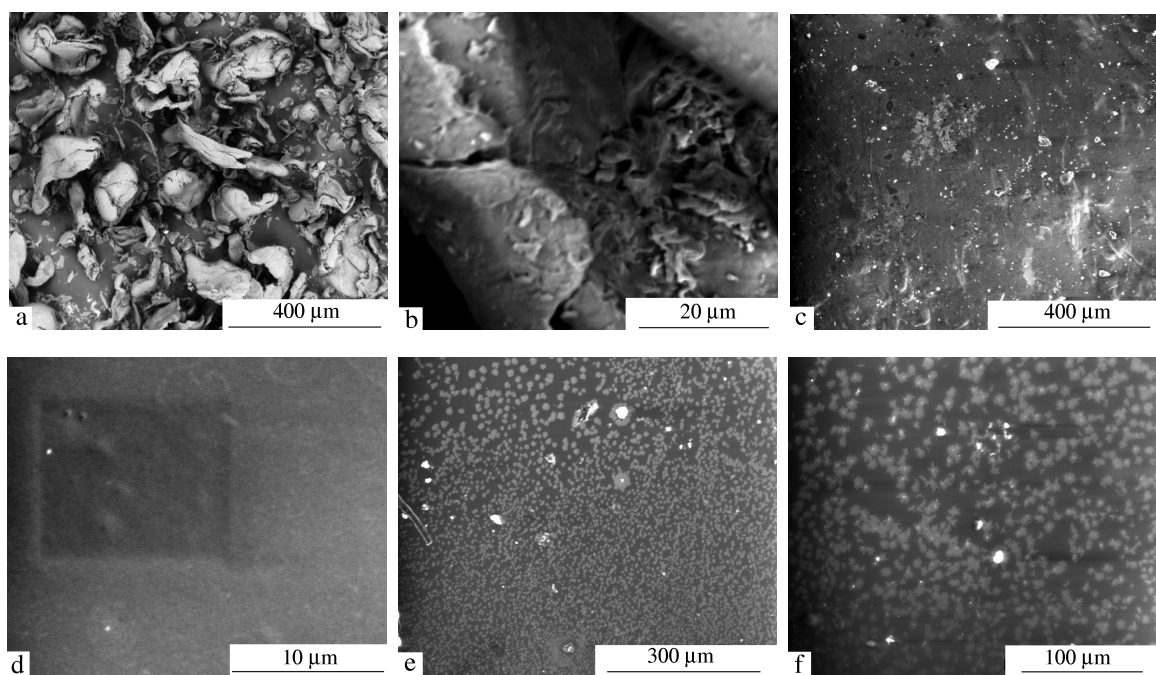


Fig. 6 SEM micrographs of CTS powder: (a) surface, (b) cross-section; CTSB film with CTS:PEG ratio (1:1): (c) surface, (d) cross-section and CTSLAg film with CTS:PEG ratio (1:1): (e) surface, (f) cross-section

TEM studies

TEM studies determined the exact size and morphology of silver nanoparticles loaded in CTSLAg films. The TEM results are depicted in Fig. 7. It is observed that the particles have well spherical geometry with relatively smaller size, which is essential for their use in antibacterial applications. The particle size distribution curve for

CTSLAg film with CTS:PEG ratio 1:1 is shown in Fig. 7(a), it reveals that nearly 75% particles have diameter in the range of 11–16 nm. The results showed that by increasing the CTS:PEG ratio to 2:1, larger silver nanoparticles were obtained as shown in Fig. 7(b), the particle size distribution curve showed that nearly 80% particles have diameter in the range of 48–55 nm. It is observed that the nanoparticles formed in the chitosan nanosilver loaded films are within the useful range for antibacterial applications.

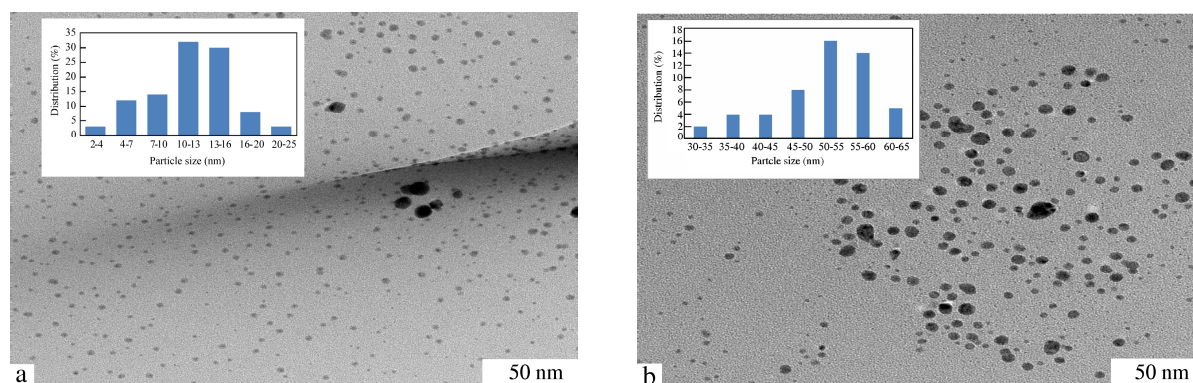


Fig. 7 TEM image and the size distribution curve of silver nanoparticles in CTSLAg films with (a) CTS:PEG ratio (1:1) and (b) CTS:PEG ratio (2:1)

Antibacterial Activity

Many synthetic nanocomposite polymers have been used as surgical and wound dressing due to their superior biomedical activity, but often it causes skin irritation due to leaching of harmful chemicals which cause side effects to human^[30, 31]. Therefore, synthesis of antimicrobial films using natural renewable biopolymers could be a better choice and thereby reduce the potential infection risk. In a promising study a dermal equivalent having a trilayered structure was designed by combining a silver nanoparticles incorporated chitosan film with a bilayer collagen-chitosan/silicon membrane dermal equivalent (BDE), the results of *in vitro* antibacterial test and *in vivo* animal test indicate that the incorporation of silver nanoparticles can restrict the growth of bacteria, thus may be potentially applied to a broader field in skin repair such as full thickness defect and burn^[32].

Anti-bacteria nano silver particles have an extremely large relative surface area, nano silver antibacterial residual killing. The antibacterial activity of developed CTSB and CTSLAg films was determined qualitatively using disc diffusion method for *E. coli*, and *bacillus*, as shown in Fig. 8. It was found that the CTSLAg film exhibited an inhibition zone while CTSB film does not involve in the inhibition zone for both types of bacteria. The antibacterial activity may be due to the electrostatic attraction between positively charged AgNps and negatively charged bacterial cells. The excellent antibacterial activity using CTSLAg film might be due to the presence of porous structure that adsorb large quantity of water which results in the release of silver nanoparticles efficiently into the media. The viable cell count method showed that the numbers of CFU (colony forming unit) of *E. coli* and *bacillus* in the nutrient agar plate with the suspensions treated with chitosan blend film discs are much more than those formed with silver-nanoparticles-treated chitosan film discs. Figure 9 shows the number of CFU of *E.coli* bacteria on the nutrient agar plate treated with a suspension containing chitosan blend discs (75 CFU/cm²) is much more than the number of CFU on the plates treated with suspension of silver-nanoparticles-loaded chitosan discs (15 CFU/cm²), similarly, the number of CFU of *bacillus* bacteria on the nutrient agar plate treated with a suspension containing chitosan blend discs (55 CFU/cm²) is much more than the number of CFU on the plate treated with the suspension of silver-nanoparticles-loaded chitosan discs (10 CFU/cm²). This observed decrease in the number of colonies of *E.coli* and *bacillus* in the plates treated with suspensions of silver-nanoparticles loaded chitosan films clearly indicates that the silver nanoparticles in the film are quite capable of killing the *E. coli* and *bacillus* cells and that the synthesized silver nanoparticles-loaded films possess efficient antibacterial properties. The various antibacterial tests done above prove the excellent antimicrobial activity of the prepared chitosan loaded silver nanoparticles films.

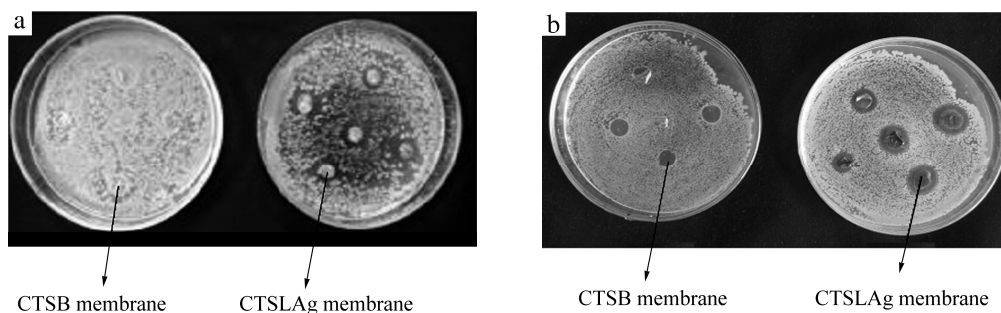


Fig. 8 The antibacterial activity of CTSB films and CTSLAg films against (a) *E. coli* and (b) *Bacillus* by disc diffusion method

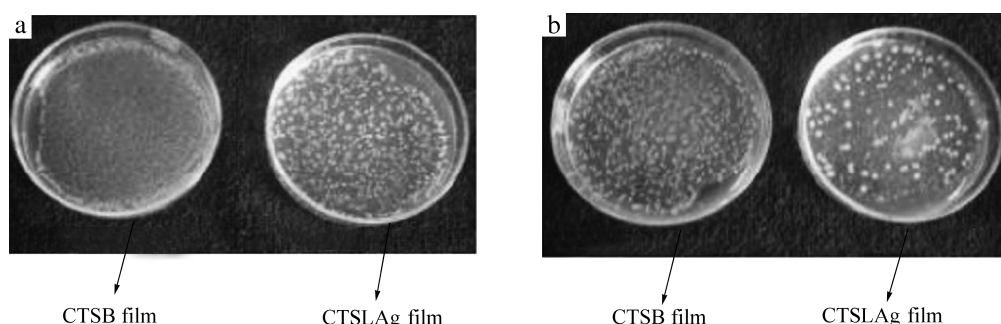


Fig. 9 Colony forming units (CFUs) on agar plates treated with suspensions of (a) *E. coli* and (b) *Bacillus*

CONCLUSIONS

The above study presents a novel, *in situ* and simple method to prepare chitosan loaded silver nanoparticles (CTSLAg) films by chemical reduction method. Chitosan used here acts as a very good chelating and stabilizing agent; and poly(ethylene glycol) (PEG) acts as reducing agent/pore-generator. The variables affecting the silver nanoparticles formation in the CTSLAg films were studied and optimized for full conversion of silver ions to silver nanoparticles. Optimum conditions for conversion of silver ions to silver nanoparticles are: reaction temperature 90 °C, 20 g/L PEG6000 and 2% glutaraldehyde. The developed CTSLAg films were characterized by UV-Vis spectroscopy, XRD and DSC analysis. The morphology and size of silver nanoparticles in the films were tested by transmission electron microscopy (TEM). The antibacterial efficiency of the developed nanocomposite films was studied. It is proved that this film has sustainable antibacterial activity and is safe in use.

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