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Preparation and characterization of novel β-chitin/nanosilver composite scaffolds for wound dressing applications

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ABSTRACT

We developed novel β-chitin/nanosilver composite scaffolds for wound healing applications using β-chitin hydrogel with silver nanoparticles. The prepared nanosilver particles and nanocomposite scaffolds were characterized using SEM, FT-IR, XRD and TGA studies. The antibacterial, blood-clotting, swelling, cell attachment and cytotoxicity studies of the prepared composite scaffolds were evaluated. The prepared β-chitin/nanosilver composite scaffolds were bactericidal against *Escherichia coli* and *Staphylococcus aureus* and it showed good blood-clotting ability as well. Cell attachment studies using vero (epithelial) cells showed that the cells were well attached on the scaffolds. These results suggested that β-chitin/nanosilver composite scaffold could be a promising candidate for wound dressing applications.

1. Introduction

Wounds are generally classified as, wounds without tissue loss, wounds with tissue loss, acute and chronic (Paul & Sharma, 2004). Wound infection is one of the main problems of today's wound care management. Bacterial infection of wound can impede the healing process and may lead to life threatening complications. Generally, in severe burns or extensive skin loss, a large amount of fluid loss and bacterial infection would lead to serious consequences. In addition, exudates between the wound and the dressing could trigger an infection. Acute inflammatory cells keep the wound from healing. One of the approaches for treating the wound infection is the use of wound dressings with antibacterial agents having a broad-spectrum of activity and high kill rate (Lee et al., 2000).

An ideal wound dressing material should maintain a moist environment at the wound interface, allow gaseous exchange, act as a barrier to microorganisms and remove excess exudates. It should also be non-toxic, non-allergenic, nonadherent in the sense that it can be easily removed without trauma, and it should be made from a readily available biomaterial that requires minimal processing, possesses antimicrobial properties and promotes wound healing (Shuangyun, Wenjuan, & Gu, 2008). Currently there are a variety of interactive products that control the microenvironment on the wound surface and some bioactive products that stimulate some part of the healing cascade (Stashak, Farstvedt, & Othic, 2004). Many polymeric materials have been investigated for the purpose of wound dressing. These include synthetic polymers like polyurethane, polyethylene, polycaprolactone, poly(lactic acid), polyacrylonitrile, poly(amino acid), silicone rubber; and natural polymers such as alginate, chitin, chitosan, gelatin, collagen, etc. (Dagalakis, Flink, Stasikelis, Burke, & Yannas, 1980; Davies, 1983; Jayakumar, Nwe, Tokura, & Tamura, 2007; Jayakumar, Prabaharan, Nair, & Tamura, 2010; Jayakumar, Prabaharan, Reis, & Mano, 2005; Mi et al., 2003; Yang, Yang, Lin, Wu, & Chen, 2008).

Chitin is one of the most abundant organic materials in nature. It can be easily prepared from the shells of crab, shrimp and squid pens. Because of its availability, biodegradability as well as biocompatibility, chitin and its derivatives have been used for a variety of applications such as water treatment, textile, paper, cosmetic, food and health supplements, agriculture, wound dressings and in biotechnology (Goosen, 1997, chap. 1 & chap. 2). The outer skeleton of crab consists of α-chitin and squid pen consists of β-chitin. α-Chitin has been proposed to form a much tighter crystalline structure than β-chitin (Nagahama, Higuchi, Jayakumar, Furuike, & Tamura, 2008). In biomedical area, it was found that chitin possess high activity as a wound healing accelerator (Usami, Okamoto, Takayama, Shigemasa, & Minami, 1998). As a result of the molecular packing, intermolecular interactions in β-chitin are weaker than those in α-chitin, making β-chitin susceptible to dissolution in a number of solvents. This finally results in β-chitin being more reactive and versatile (Lavall, Assis, & Filho, 2007;

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Lee, Kim, Sang, & Lee, 2004; Madhumathi, Binulal, et al., 2009; Maeda, Jayakumar, Nagahama, Furuike, & Tamura, 2008; Saito, Okano, Gaill, Chanzy, & Putaux, 2000; Yang et al., 2008).

A number of studies have been reported where chitin films and membranes were used to treat patients with deep burns, orthopedic injury, etc. (Kojima, Okamoto, Miyatake, & Kitamura, 1998; Muzzarelli, 1994; Senda, He, & Inoue, 2001; Su et al., 1997; Xu, Ma, Shi, Gao, & Han, 2007). These membranes and films have less mechanical strength and low flexibility. Our aim is to develop β-chitin/nanosilver composite scaffolds from the β-chitin hydrogel with good mechanical strength, flexibility, tear resistance and excellent antimicrobial properties. The advantages of using scaffolds are; for retention of good moisture environment, permeation of gas, absorption of wound exudates and easy removal without trauma to the wound. The wound healing ability and antibacterial activity of β-chitin can be further enhanced by the addition of silver nanoparticles (Demling & DeSanti, 2001). Thus, the β-chitin/nanosilver composite scaffolds will function as ideal wound dressings.

2. Experimental

2.1. Materials

β-Chitin (degree of acetylation – 72.4%) was purchased from Koyo chemical Co., Ltd., Japan, $CaCl_2$ and methanol were purchased from Qualigens, India. Silver nitrate and sodium citrate were obtained from Merck. *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) strains were obtained from Microbiology Lab of Amrita Institute of Medical Sciences, Kochi, India. The vero cell line was provided by National Center for Cell Sciences, Pune, India. The chemicals were used without further purification.

2.2. Preparation of β-chitin hydrogel

β-Chitin hydrogel was prepared according to literature (Tamura, Nagahama, & Tokura, 2006). Five grams of β-chitin was added to 1 liter of saturated $CaCl_2$/methanol solvent and stirred vigorously for 48 h at room temperature. After getting a transparent β-chitin solution, the solution was filtered to remove the undissolved traces. To this solution excess water was added and stirred for 2 h vigorously to obtain β-chitin hydrogel. After keeping the hydrogel for 24 h, it was filtered using Whatman filter paper number 1. The obtained hydrogel was dialyzed against distilled water for 2 days to get pure β-chitin hydrogel.

2.3. Preparation of nanosilver solution

Nanosilver solution was prepared by the well-known Turkevich method as described in the literature (Katrin et al., 1997). This method is based on the reduction of silver nitrate to metallic silver nanoparticles using sodium citrate as the reducing agent. The reduction of silver ions occurs at high temperature and silver nanoparticle formation is observable by a visible color change of the solution to pale yellow.

2.4. Preparation of β-chitin/nanosilver composite scaffolds

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of silver nanoparticles are in the range of 1.56–6.25 μg/ml and 12.5 μg/ml, respectively (Jaya et al., 2009). β-Chitin/nanosilver composite scaffolds were prepared by adding 0.001, 0.003 and 0.006% of nanosilver to β-chitin hydrogel and stirred well for 15 min. All the samples were lyophilized for 48 h to obtain β-chitin/nanosilver composite scaffolds.

2.5. Swelling studies

Each preweighed scaffolds were immersed in phosphate buffered saline (PBS) and distilled water. After immersing for 1, 2, 3 and 7 days at 37 °C, the swollen scaffolds were removed from the water and PBS, gently blotted with filter paper to remove surface liquid and immediately weighed (W_{wet}).

$$DS = [(W_w - W_d)/W_d] \times 100$$

where DS is the degree of swelling and W_w and W_d are wet and dry weight of the scaffolds, respectively (Chen et al., 2009).

2.6. Antibacterial activity studies

Escherichia coli and *S. aureus* were used to test the antibacterial potential of composite scaffolds. The antibacterial activity was determined by Kirby–Bauer disc diffusion method (Heggers & Robson, 1978). These bacteria were grown overnight in tryptic soy broth. The disc diffusion method was performed on Mueller–Hinton agar (Himedia, India) and zone of inhibition was measured after 24 h of incubation.

2.7. Whole-blood-clotting studies

The blood-clotting studies were done based on reported literature (Ong, Wu, Moochhala, Tan, & Lu, 2008). Blood-clotting ability of β-chitin/nanosilver composite scaffolds were analyzed and compared with commercially available wound dressing Kaltostat™. Blood was drawn from human ulnar vein using 10 ml BD discardit™ II sterile syringe and mixed with anticoagulant agent acid citrate dextrose at a ratio of 9:1 Triplicate samples were used for this study and blank in a 25 ml plastic Petri dish without scaffold was used as control. 100 μl of this blood sample was added to each scaffold and placed in a 25 ml plastic Petri dish, which was followed by the addition of 10 μl of 0.2 M $CaCl_2$ solutions to initiate blood-clotting. Then these scaffolds were incubated at 37 °C for 10 min. 15 ml of distilled water was then added drop wise without disturbing the clot. Subsequently, 10 ml of solution was taken from the dishes and was centrifuged at 1000 rpm for 1 min. The supernatant was collected for each sample and kept at 37 °C for 1 h. 200 μl of this solution was added to each well of a 96-well plate. The optical density was measured at 540 nm using a plate reader (BioTek PowerWave XS).

2.8. Cell viability study

Cell viability of the prepared β-chitin/nanosilver composite scaffolds was evaluated by indirect cytotoxicity test using Alamar blue. The cytotoxicity test of composite scaffolds was done according to ISO 10993-5. The cytotoxic effect of β-chitin and β-chitin with different concentrations of nanosilver was evaluated on vero cells. Scaffolds were sterilized overnight using UV and extraction ratio was 4 g/ml (sample/medium). Vero cells were cultured in MEM supplemented with 10% FBS, 50 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin (Invitrogen, CA, USA). Vero cells were seeded into 96-well plates at a seeding density 1×10^4 cells/well and incubated for overnight. Triplicates of each sample were taken and sterilized overnight following which they were incubated in serum containing media for 24 h at 37 °C. 100 μl of the media from each sample was taken and transferred into each well. The cells were then incubated for 24 h and Alamar blue assay (Invitrogen, USA) was performed. The optical density measured at 570 nm with 620 nm set as the reference wavelength using a microplate spectrophotometer (BioTek PowerWave XS, USA).

2.9. Cell attachment studies

The scaffolds were seeded with vero cells in a 24-well plate at a concentration of 1×10^5 cells/well. Vero cells are epithelial cells and hence were selected for this study. After 24 h incubation the scaffold were washed with PBS and fixed with 2.5% glutaraldehyde for 1 h. After thoroughly washed with PBS the samples were sequentially dehydrated in a graded ethanol series, then air-dried, platinum sputtered in vacuum and examined with a scanning electron microscope.

2.10. Characterization

The nanosilver solution prepared was characterized using ultraviolet–visible spectroscopy (UV-1700 pharmaspec, Shimadzu), the size of the nanoparticles were determined by dynamic light scattering measurements ((DLS-ZP/Particle Sizer Nicomp™ 380 ZLS, particle sizing system). The β-chitin/nanosilver composite scaffolds were characterized using scanning electron microscopy (JEOL, Ltd., JEOLJSM-6490LA), X-ray diffraction (PANalytical X'Pert PRO), Thermogravimetric analysis (SII TG-DTA6200) and FT-IR spectroscopy (Perkin-Elmer Co., SPECTRUM RX1, FT-IR) instruments.

3. Results and discussion

3.1. Characterization of nanosilver particles

The prepared nanosilver particles were characterized by UV–visible spectroscopy. Fig. 1A depicts the UV spectrum of silver nanoparticles. The absorption spectrum of silver nanoparticles showed a characteristic surface plasmon absorption band 435 nm, which indicates the presence of silver nanoparticles in the solution (Kong & Jang, 2006). The particle size distribution of silver nanoparticles is also shown in Fig. 1B, which showed a size range of 4–8 nm.

3.2. SEM analysis

Fig. 2A shows the images of the prepared composite scaffolds and as the concentration of silver nanoparticle was increased, the color of the scaffolds were changed from white to dark. This was due to the presence of silver nanoparticles. Fig. 2B and C depicts the surface morphology of β-chitin/nanosilver composite scaffolds. Since the prepared silver nanoparticles are very small (4–8 nm), they are not visible in the SEM. The scaffold possesses a very good porous structure with smooth surface morphology. In the case of β-chitin control, porosity is less compared to composite scaffolds. This is due to the fact that, when the nanosilver solution was mixed with β-chitin hydrogel, it got incorporated into the hydrogel. This was then freeze-dried. The water in the silver colloid, which got incorporated into hydrogel, gets evaporated and the vacant space is left as pores in the hydrogel scaffold, ultimately resulting in highly porous composite scaffold.

3.3. FT-IR studies

Fig. 3A shows the FT-IR spectrum of β-chitin control (spectrum a) and β-chitin/nanosilver (spectrum b). The two spectrums are almost similar and there is no shift in any of the peaks. It indicates that nanosilver particles were physically entrapped onto the scaffold by van der Waal's force (Madhumathi, Sudheesh, et al., 2009).

FT-IR (KBr): 3423 cm^{-1} (O—H stretching vibration), 1644 cm^{-1} (amideI), 1376 cm^{-1} (C—CH_3 bending).

3.4. XRD studies

Fig. 3B shows the XRD spectrum of β-chitin/nanosilver composite scaffolds. The peaks at $2\theta = 10.4°$ and 20.1° are the characteristic peaks of β-chitin, (Nagahama, Kashiki, et al., 2008) in addition, the peaks at 39.8° and 44.5° can be attributed to (1 1 1) and (2 0 0) crystalline planes of silver (Chen et al., 2008).

3.5. Thermogravimetric analysis

Fig. 4 shows the thermogram of the composite scaffolds. The samples were measured at a linear rate of 10 °C/min from 30 to 500 °C in a nitrogen atmosphere. In the thermogram, all the composite scaffolds showed decomposition in the range of 50–100 °C. This was due to the water evaporation. In the case of β-chitin control, it showed a second decomposition in the range of 270–350 °C and all other scaffolds showed in the range of 270–370 °C and it can be attributed to the decomposition of polysaccharide structure of β-chitin (Jayakumar & Tamura, 2008). The decomposition rates of the composite scaffolds were decreased slightly, as the concentration of nanosilver increased. This was due to the incorporation of silver nanoparticle into the scaffold.

3.6. Swelling studies

Fig. 5A and B shows the swelling behavior of β-chitin control and β-chitin/nanosilver composite scaffolds in water and PBS, respectively. Nanosilver particles incorporated scaffolds showed higher swelling compared to control and also degree of swelling was higher in PBS compared to distilled water. This is due to the scaffolds with nanosilver have more porosity compared to the control. The swelling was increased with time and it reached around 1000 and 1200 wt.% after 1 week in water and PBS, respectively. The increased swelling in the case of PBS compared to water may be due to the presence of ions present in PBS. These results showed

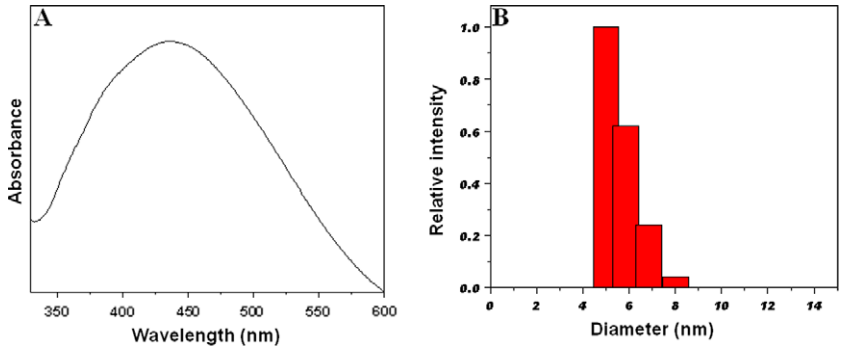

Fig. 1. (A) UV–vis spectrum of silver nanoparticles. (B) Particle size distribution of silver nanoparticles.

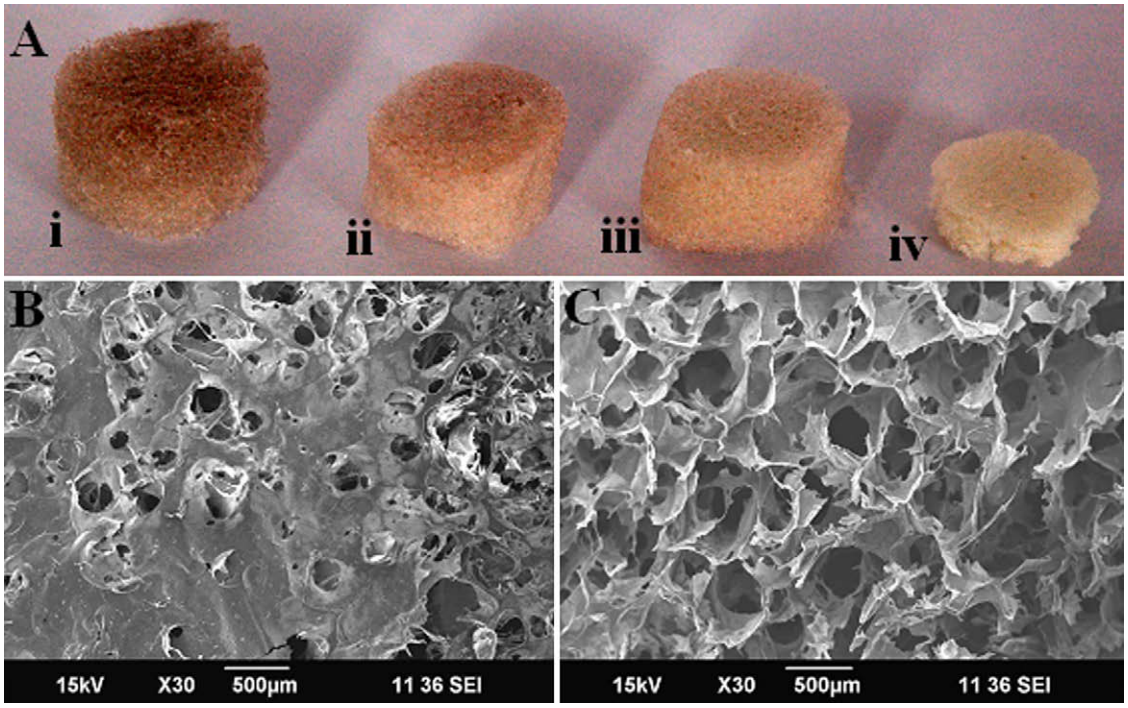

Fig. 2. (A) Images of composite scaffolds. (i) β-Chitin + 0.006% nanosilver; (ii) β-chitin + 0.003% nanosilver; (iii) β-chitin + 0.001% nanosilver; (iv) β-chitin control. (B) SEM image of β-chitin control scaffold (C) SEM image of β-chitin with nanosilver (0.006%).

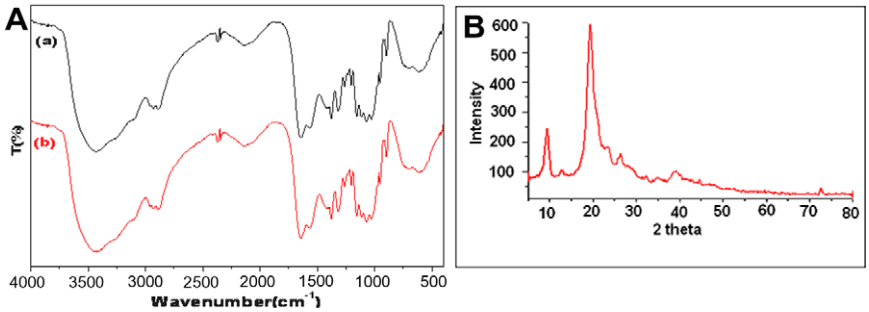

Fig. 3. (A) FT-IR spectra of composite scaffolds: (a) β-chitin/nanosilver and (b) β-chitin control. (B) XRD spectrum of the composite scaffold.

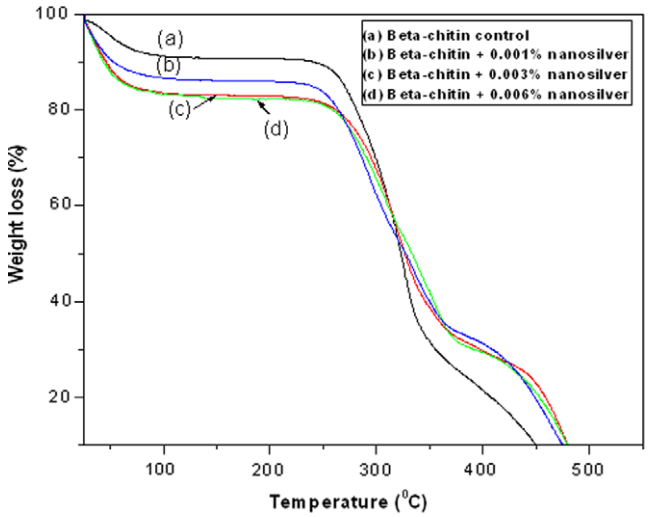

Fig. 4. TG profiles of composite scaffolds.

that, this material can do better absorption in case of exudating wounds ($n = 3$).

3.7. Antibacterial studies

Fig. 6 shows the antibacterial activity studies of the composite scaffolds. Fig. 6A shows the bactericidal efficiency of β-chitin/nanosilver composite scaffolds against Gram-positive *S. aureus* while Fig. 6B shows activity against Gram-negative *E. coli*. It can be seen that the inhibition zone is higher in *E. coli* than in *S. aureus* indicating higher susceptibility of Gram-negative bacteria to nanosilver. This was due to that Gram-positive bacteria are protected by thick peptidoglycan wall, which limits the penetration of silver nanoparticles (Siddhartha et al., 2007). It can also be seen that as the nanosilver concentration was increased, the zone of inhibition also increased. These results revealed that the antibacterial activity was due to the presence of nanosilver particles in chitin scaffolds. The interaction between chitin and nanosilver will be physical adsorption of silver nanoparticles and the release of silver will be

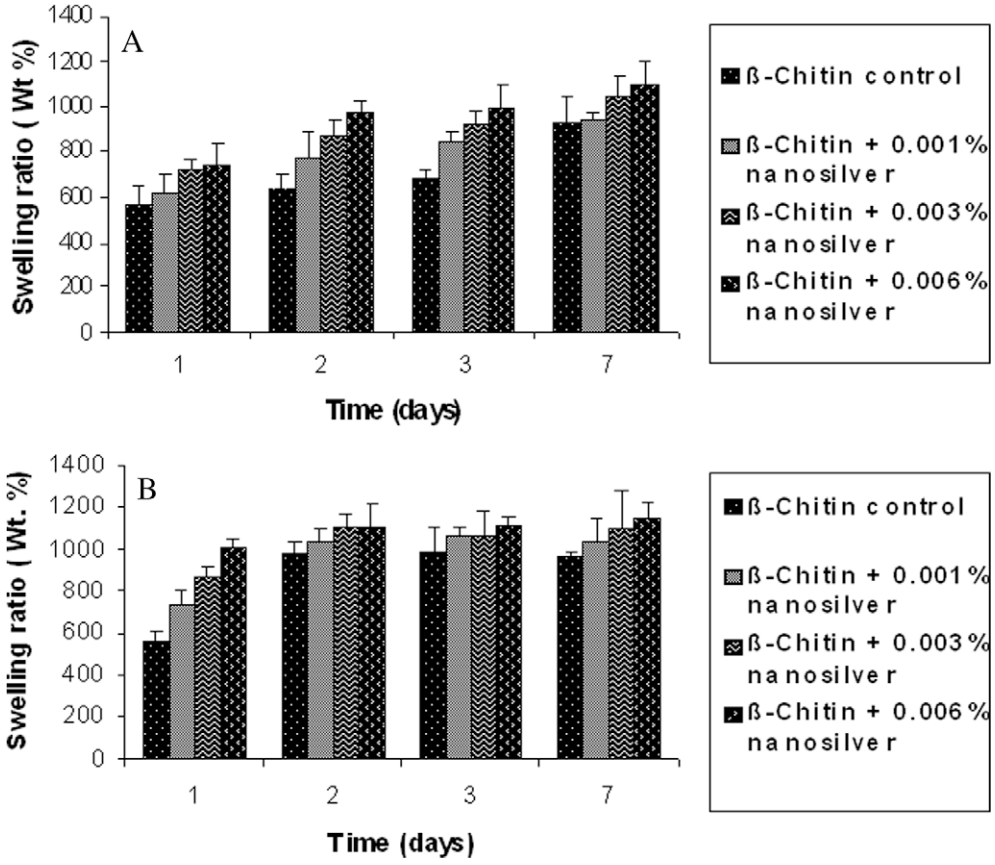

Fig. 5. (A) Swelling studies of composite scaffolds in water. (B) Swelling studies of composite scaffolds in PBS.

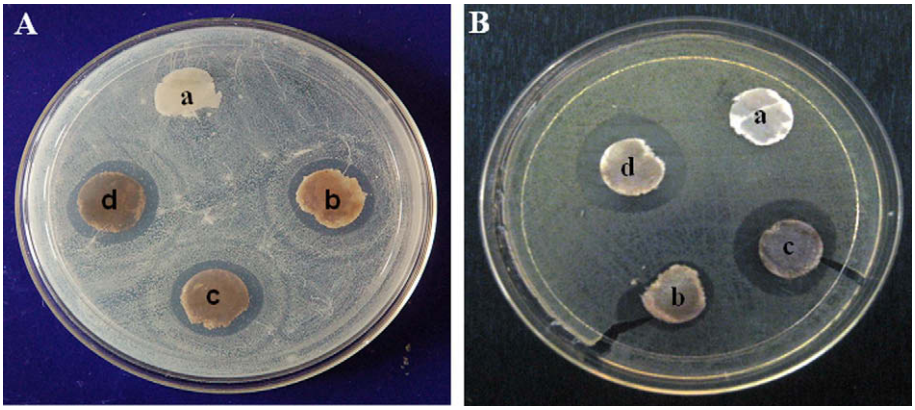

Fig. 6. (A) Antimicrobial studies of β-chitin/nanosilver composite scaffolds against *S. aureus*. (a) β-chitin control, (b) β-chitin + 0.001% nanosilver, (c) β-chitin + 0.003% nanosilver and (d) β-chitin + 0.006% nanosilver. (B) Antimicrobial studies of β-chitin/silver nanoparticle scaffolds against *E. coli* (a) β-chitin control, (b) β-chitin + 0.001% nanosilver, (c) β-chitin + 0.003% nanosilver and (d) β-chitin + 0.006% nanosilver.

Table 1
Antimicrobial activity of β-chitin/silver nanoparticle incorporated scaffolds against *S. aureus* and *E. coli* expressed in terms of the diameter of the zone of inhibition.

Samples	Zone of inhibition diameter (mm)	
	S. aureus	*E. coli*
β-Chitin control	–	–
β-Chitin + 0.001% nanosilver	18 ± 2	17 ± 2
β-Chitin + 0.003% nanosilver	19 ± 1	23 ± 1
β-Chitin + 0.006% nanosilver	21 ± 2	24 ± 1

controlled. Table 1 gives the values of zone of inhibition of antibacterial studies ($n = 3$ at each time point).

3.8. Whole-blood-clotting studies

Fig. 7A shows the whole-blood-clotting studies of the composite scaffolds. The scaffolds were contacted with whole blood for 10 min at 37 °C prior to hemolyzing red blood cells (RBC) that were not trapped in the clot that formed on the scaffolds surface. A high-

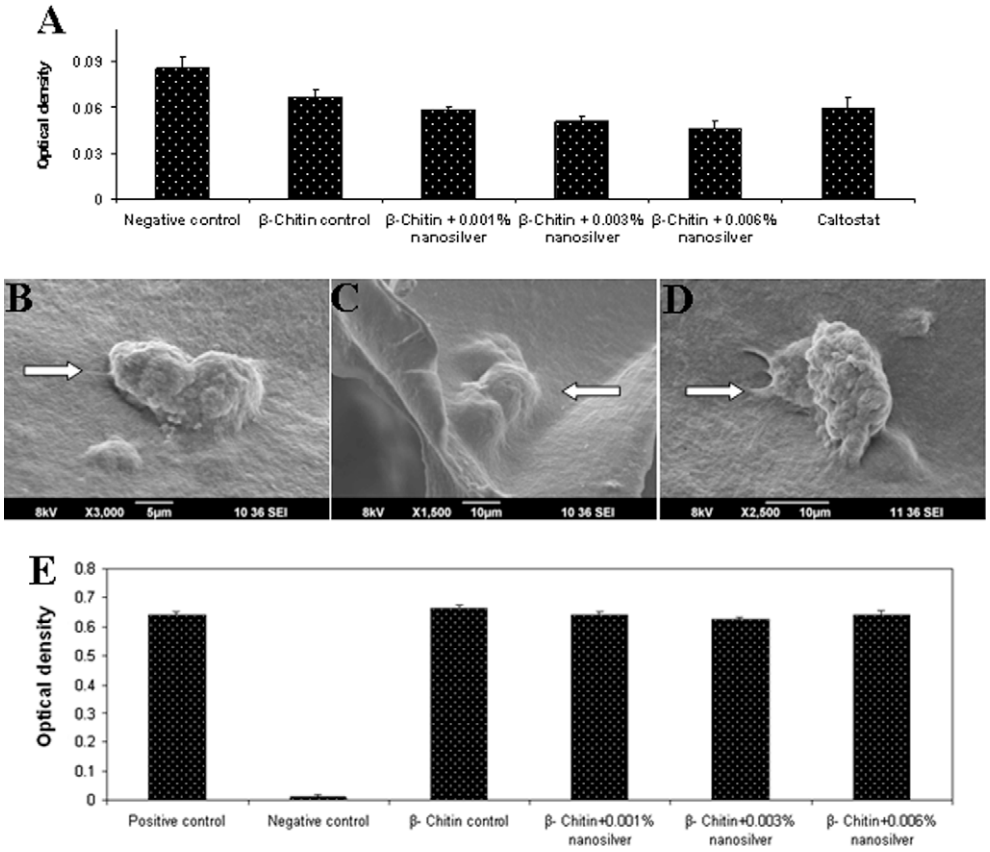

Fig. 7. (A) Whole-blood-clotting studies of β-chitin/nanosilver composite scaffolds, (B–D) SEM images of cell attachment on the β-chitin (control) composite scaffold, β-chitin + 0.001% nanosilver and β-chitin + 0.003% nanosilver; respectively. (E) Cell viability studies of β-chitin/nanosilver composite scaffolds.

er absorbance value of the hemoglobin solution thus indicates a slower clotting rate. Whole-blood-clotting ability of the β-chitin/nanosilver composite scaffolds was higher than β-chitin scaffold and Kaltostat™ dressing. From the Fig. 7A, it was clear that β-chitin with 0.006% nanosilver shows least optical density; it means that it showed more blood-clotting capability. β-Chitin is hemostatic, as being a cationic polymer it tends to aggregate negatively charged red blood cells (Muzzarelli, 2009). This blood-clotting capability of β-chitin scaffolds was further enhanced by the incorporation of silver nanoparticles because silver denatures the anticoagulant proteins and affects the intrinsic pathway of blood coagulation by producing a shortened clotting time (Kapadia, Kristol, & Spillert, 2005) ($n = 3$).

3.9. Cell attachment studies

The attachments of vero cells on the scaffolds were analyzed using scanning electron microscope. Fig. 7B–D shows that the cells were attached on the surface of the composite scaffolds (white arrows show cells). Cell attachment studies showed that the cells were attached on the scaffolds with a globular morphology after 24 h of incubation. The globular morphology indicates moderate or weak attachment, which would be preferred for the case of wound dressings. Hence we propose that these composite scaffolds are ideal materials that could be used for wound dressing applications from the preliminary studies of cell interactions.

3.10. Cell viability study

Antibacterial materials for wound dressing have to fulfill several major preconditions. The important thing is that the scaffolds must not have intolerable toxic effects in human body. Fig. 7E shows the Cell viability studies of the composite scaffolds. Indirect cytotoxicity test was done using Alamar blue assay showed that the scaffold with different concentration of silver was not toxic to vero cells. In prokaryotes, nanosilver targets the exteriorly located essential protein complex of the bacterial electron transport chains. But in eukaryotes same structures are found intracellularly in the mitochondrial organ and, therefore, higher silver concentrations are required to achieve comparable toxic effects than for bacterial cells. Jain et al. reported that silver nanoparticle is non-toxic *in vitro* and *in vivo* at low doses (Jaya et al., 2009).

4. Conclusions

Novel β-chitin/nanosilver composite scaffold was prepared and characterized by FT-IR, UV, XRD, TGA and SEM. The SEM studies showed that the composite scaffolds having porous morphology. The XRD studies confirmed the incorporation of silver nanoparticles in the β-chitin scaffolds. The prepared composite scaffolds showed controlled swelling in water and PBS compared to control scaffold. The TGA studies indicated that the incorporation of silver nanoparticles decreasing the decomposition temperature of the composite scaffold. The blood-clotting studies indicated that the scaffold with nanosilver, clots the blood quickly as compared to the control scaffold without nanosilver and Kaltostat™. The composite scaffold showed inhibitory effects on the bacterial growth signifying its role as an antibacterial agent. Cytotoxicity studies on vero cell line proved that the composite scaffold was non-toxic. The cell attachment studies showed that the vero cells were attached on the composite scaffolds. These results indicated that the β-chitin/nanosilver composite scaffold could be used as a potential wound dressing material.

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