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Preparation and characterization of chitosan-hydroxybenzotriazole/polyvinyl alcohol blend nanofibers by the electrospinning technique

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ABSTRACT

Nanofibers of a chitosan-hydroxybenzotriazole (CS-HOBt)/polyvinyl alcohol (PVA) blend were successfully prepared using electrospinning techniques. In this study, nanofibers were fabricated without the use of standard organic solvents or organic acids. CS was dissolved with HOBt in distilled water, and this solution of CS-HOBt (2 wt%) with PVA (10 wt%), blended in different weight ratios, was electrospun to obtain nanofibers. The morphology, diameter, and structure of the electrospun nanofibers were investigated. SEM images showed that the morphology and diameter of the nanofibers were mainly affected by the weight ratio of the blend. Nanofibers were observed when the CS content was less than 50 wt%. The average diameter of the nanofibers was 190–282 nm, and this average diameter gradually decreased with increasing CS content. Cytotoxicity tests of the nanofiber mats showed that the fibers were non-toxic to human fibroblast cells. Hence, these biodegradable nanofibers may be suitable for drug delivery or tissue engineering applications.

1. Introduction

In recent years, the development of electrospinning techniques has been increasingly investigated. Electrospinning is a simple and easy way to control the morphology of ultrafine fibers. The fibers produced by this method have shown amazing characteristics, such as a very large surface-to-volume ratio and a high porosity with a small pore size (Deitzel, Kleinmeyer, Harris, & Beck Tan, 2001; Huang, Zhang, Kotaki, & Ramakrishna, 2003). In the electrospinning process, high voltage is applied to a capillary containing a polymer solution or the molten polymer precursor. A droplet of the polymer solution then forms at the tip of the capillary, creating a point known as the "Taylor cone." As electrostatic forces overcome the surface tension of the polymer solution, the solution is ejected from the apex of Taylor cone. The charged jets of polymer solution move towards a collector, solvent rapid evaporates and non-woven fiber mat was collected on the collector (Sill & von Recum, 2008).

Many varieties of polymer nanofibers have been prepared by electrospinning techniques, such as polyvinyl alcohol (PVA), poly L-lactic acid (PLL), collagen, DNA, etc. Because of this flexibility, electrospun nanofibers have been applied to many fields, including biomedical sciences, filtration, optical sensor fields, and many more. In biomedical applications, these particles can serve as a tissue engineering scaffold and are also valuable in drug delivery and wound dressing. For these applications, the polymer must be biocompatible and of low toxicity (Agarwal, Wendorff, & Greiner, 2008).

Chitosan (CS) is a copolymer of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) that is produced by alkaline deacetylation of chitin. CS is a weak base, in which the pKa value of the D-glucosamine residue is approximately 6.2–7.0. As a result of this basicity, CS is insoluble at neutral and alkaline pH values but is soluble in acidic media. CS is biodegradable, biocompatible, and non-toxic; therefore, it has been proposed as a safer material for use in biomedical applications (Kim et al., 2008; Rinaudo, 2006). Recently, CS nanofibers have been successfully generated from the electrospinning of homogeneous CS or CS derivatives, such as carboxymethyl CS (Shalumon et al., 2009), carboxyethyl CS (Zhou et al., 2008), quaternized CS (Alipour, Nouri, Mokhtari, & Bahrami, 2009; Ignatova, Manolova, & Rashkov, 2007; Ignatova, Starbova, Markova, Manolova, & Rashkov, 2006) and hexanoyl CS (Neamnark, Rujiravanit, & Supaphol, 2006; Neamnark, Sanchavanakit, Pavasant, Rujiravanit, & Supaphol, 2008). However, some organic solvents or organic acids, such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Min et al., 2004), chloroform (Neamnark et al., 2006), trifluoroacetic acid (TFA) (Sangsanoh & Supaphol, 2006), acrylic acid (Zhou, Yang, & Nie, 2006), and acetic acid (Geng, Kwon, & Jang, 2005), must be employed in the fabrication of these

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homogeneous chitosan nanofibers or derivatives. Traces of these toxic organic solvents or acids in electrospun products are harmful when applied to wounded human skin or tissue. Chitosan-based nanofibers have also been successfully generated by the electrospinning of chitosan solutions blended with poly(ethylene oxide) (Bhattarai, Edmondson, Veiseh, Matsen, & Zhang, 2005; Martinova & Lubasova, 2008; Zhang, Su, Ramakrishna, & Lim, 2008), PVA (Alipour et al., 2009; Ignatova et al., 2006; Jia et al., 2007; Li & Hsieh, 2006; Shalumon et al., 2009; Zhang et al., 2007; Zhou et al., 2006, 2008), collagen (Chen, Chang, & Chen, 2008; Chen, Mo, & Qing, 2007), silk fibroin (Park, Jeong, Yoo, & Hudson, 2004), poly(L-lactic acid) (Xu et al., 2009), poly(caprolactone) (Shalumon et al., 2010) and agarose (Teng, Wang, & Kim, 2009).

Hydroxybenzotriazole (HOBt) is an organic compound that is often used as a racemization suppressor and is popular for its ability to improve yields in peptide synthesis. HOBt-monohydrate contains about 11.8% water. An aqueous CS-HOBt solution is prepared by simply mixing the chitosan and the HOBt in water without the need for any organic solvent, acid or heat (Fangkangwanwong, Akashi, Kida, & Chirachanchai, 2006). Due to the hydroxy groups present in HOBt, the molecule can form a salt with the amine groups of CS, thus improving CS water solubility and allowing CS to be dissolved in water.

In this study, we used electrospinning to prepare nanofiber mats from a CS-HOBt solution. Our aim was to reduce the toxicity of these fibers, which is usually caused by traces of organic solvents and acids remaining in the final product. In a preliminary study, we attempted to use electrospinning to prepare a nanofiber mat from a 100% CS-HOBt solution with no success. Therefore, we added poly(vinyl alcohol) (PVA) to the CS-HOBt solution as a guest polymer in the different weight ratio blends. PVA is a water-soluble, non-toxic, biodegradable, and biocompatible synthetic polymer with enhanced fiber-forming ability, and it is therefore well-suited as a guest polymer in blends with CS (Alipour et al., 2009; Ignatova et al., 2006; Jia et al., 2007; Li & Hsieh, 2006; Shalumon et al., 2009; Zhang et al., 2007; Zhou et al., 2006, 2008). The morphology and structure of nanofiber mats were characterized, and the effects of the viscosity and conductivity of the electrospun solution on the morphology of CS-HOBt/PVA nanofiber mats were evaluated. The composite of the CS-HOBt/PVA nanofiber mats was characterized by Fourier transform infrared (FT-IR) spectroscopy and differential scanning calorimetry (DSC). The cytotoxicity tests for the CS-HOBt/PVA nanofiber mats were performed with MTT assays using human fibroblast cells.

2. Experimental

2.1. Materials

Chitosan (degree of deacetylation 0.85, MW 110 kDa) and hydroxybenzotriazole monohydrate ($HOBt{\cdot}H_2O$) were purchased from Sigma-Aldrich Chemical Company, USA. Polyvinyl alcohol (PVA) (degree of polymerization ≈ 1600, degree of hydrolysis ≈ 97.5–99.5 mol%) was purchased from Fluka, Switzerland. Normal human foreskin fibroblast (NHF) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Dimethyl sulfoxide (DMSO) was obtained from BDH Laboratories, UK. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Trypsin-EDTA, and penicillin-streptomycin were purchased from Gibco BRL (Rockville, MD, USA). All other reagents and solvents were commercially available and were of analytical grade.

2.2. Preparation and properties of spinning solutions

The 2% (w/v) CS solution was prepared by dissolving CS and HOBt in distilled water at a weight ratio of 1:1. The 10% (w/v) PVA solution was prepared by dissolving PVA in distilled water at 80 °C and then allowing the solution to stir for 4 h. The 2% CS-HOBt solution was mixed with a 10% PVA solution at weight ratios of 10/90, 20/80, 30/70, 40/60, 50/50, 60/40, 70/30, 80/20 and 90/10. The solution viscosity was measured using a Brookfield viscometer (Model DV-III ultra, Brookfield Engineering Laboratories, Inc., Massachusetts, USA). The conductivity of the electrospinning solution was measured using a EUTECH ECtestr11$^+$ conductivity meter (Eutech Instruments Pte Ltd, Singapore).

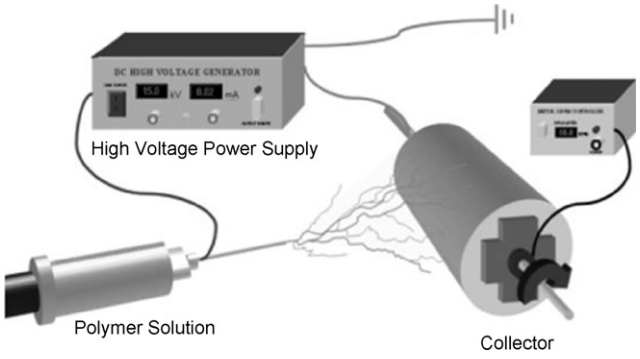

Fig. 1. Schematic of the electrospinning setup.

2.3. Electrospinning process

The electrospinning setup is illustrated in Fig. 1. The polymer solution was taken up in a 5-mL glass syringe equipped with a 20-gauge, stainless steel needle (diameter = 0.9 mm) at the nozzle. The needle was connected to the emitting electrode of positive polarity of a Gamma High Voltage Research device. The electric potential was fixed at 15 kV. The nanofibers were collected as-spun on an aluminum sheet that was wrapped on a rotating collector. The solution was electrospun at room temperature, and the collection distance was fixed at approximately 15 cm. The solution feed was driven by gravity and the electrostatic force that was generated during spinning.

2.4. Characterization of composite nanofibers

The morphology and diameter of nanofiber mats were determined using scanning electron microscopy (SEM; Camscan Mx2000, England). For this process, a small section of the electrospun fiber mats was sputtered with a thin layer of gold prior to SEM observation.

The thermal behavior of the nanofiber mats was evaluated by Differential Scanning Calorimeter (DSC, Pyris Sapphire DSC, PerkinElmer instrument, USA) under an atmosphere of nitrogen. DSC traces were recorded from 100 to 250 °C at a heating rate of 5 °C/min.

The chemical structure of the nanofiber mats was characterized using a Fourier Transform Infrared Spectrophotometer (FT-IR, Nicolet 4700, Becthai, USA) with a wave number range of 400–4000 cm^{-1}.

2.5. Indirect cytotoxicity evaluation

The cytotoxicity of the nanofiber mats was evaluated based on a procedure adapted from the ISO10993-5 standard test method (indirect contact) (Chen et al., 2008). The nanofiber mats of CS-HOBt/PVA at the with weight ratios of 10/90, 30/70, 50/50, and 7% CS in acetic acid were sterilized by UV radiation for 1 h. The mats were then immersed in a serum-free medium (SFM; containing DMEM, 1% L-glutamine, 1% lactalbumin and 1% antibiotic and

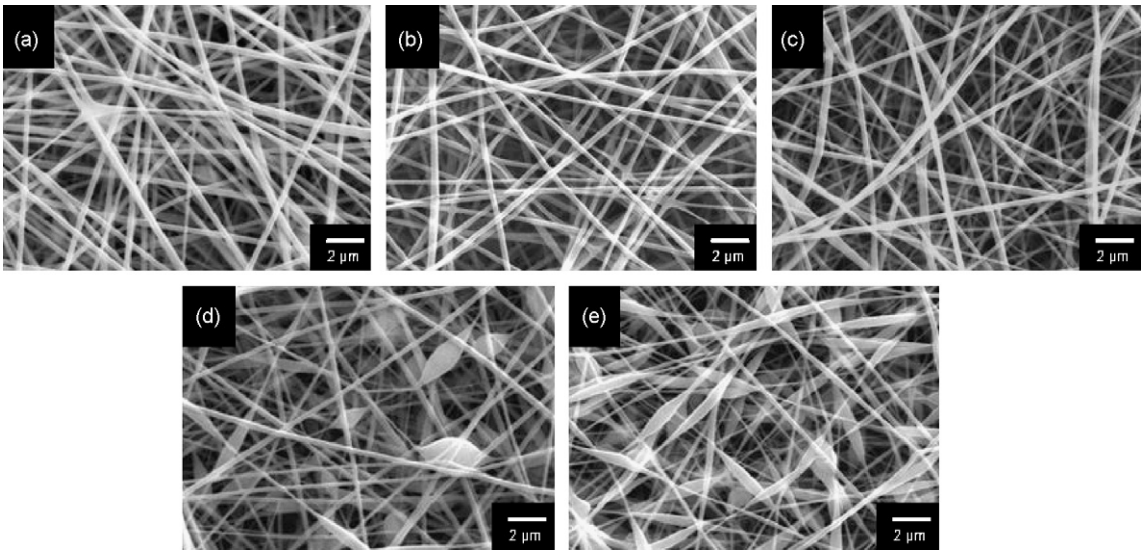

Fig. 2. The scanning electron microscopy (SEM) images of nanofiber mats with different weight ratios of CS-HOBt/PVA: (a) 10/90, (b) 20/80, (c) 30/70, (d) 40/60 and (e) 50/50.

antimycotic formulation) in an incubator for 24 h to produce extraction media of varying concentrations (10, 7.5, 5, 2.5 and 1 mg/mL). Normal human foreskin fibroblast (NHF) cells were plated in 90 μL of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, at a density of 8000 cells/well in 96-well plates. When the cultures reached confluency (typically 48 h after plating), the tested extraction media at varying concentrations were replaced and the cells were re-incubated for 24 h. After treatment, the tested extraction solutions were removed. Finally, the cells were incubated with 100 μL of a MTT-containing medium (1 mg/mL) for 4 h. The medium was removed, the cells were rinsed with PBS (pH 7.4), and the formazan crystals formed in living cells were dissolved in 100 μL DMSO per well. Relative viability (%) was calculated based on the absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). Viability of non-treated control cells was arbitrarily defined as 100%.

2.6. Statistical analysis

Data were collected from triplicate samples and are depicted as the mean ± standard deviation (SD). Statistically significance differences in cell viability were examined using the Student's *t*-test. The significance level was set at $p < 0.05$.

3. Results and discussion

3.1. Electrospinning

It was difficult to obtain the desired fibers from electrospinning using the 100% CS-HOBt solution; instead of yielding a fibrous structure, pure CS-HOBt gave globular, drop-like deposition on the collecting target. Zong et al. (2002) reported that the addition of a small amount of salt increased the charge density in the ejected jets. The addition of salt was found to greatly change the morphology of the electrospun fibers in our experiments as well, changing the fibers from a bead-like structure to a uniform structure. Moreover, the diameter of the nanofibers also decreased with the addition of salt. Son, Youk, Lee, and Park (2005) reported that the addition of cationic and anionic polyelectrolytes increased the conductivity of polymer solutions and resulted in a thinner fiber diameter. Jia et al. (2007) prepared the nanofibrous membrane of a polyvinyl alcohol (PVA)/CS blend using a solution of acetic acid-water as a spinning solvent. When the CS content was greater than 30%, nanofibers hardly formed. CS is a cationic polysaccharide with amino groups at the C2 position that are ionizable under acidic or neutral pH conditions. Therefore, the morphology and diameter of electrospun fibers were expected to be seriously affected by the weight ratio of CS/PVA. In this study, we prepared the nanofibrous membrane of the CS-HOBt/PVA blends in various weight ratios in aqueous solution without using any acids or organic solvents. The results showed that nanofibrous membranes barely formed, when the CS content was more than 50 wt%. This indicated that the aqueous solution of CS in HOBt was more effective than the CS solution in acetic acid with the same blend of PVA, as previously reported (Jia et al., 2007).

Fig. 2 shows the SEM images of nanofiber mats with different CS-HOBt/PVA weight ratios, including the 10/90, 20/80, 30/70, 40/60 and 50/50 solutions. When the amount of CS-HOBt was increased from 10 to 50 wt%, the average diameter of the nanofibers decreased from 282 ± 49 to 190 ± 52 nm. The diameter distribution of the nanofiber mats is shown in Fig. 3. This result is similar to previous studies of PVA/CS blend nanofiber membranes (Jia et al., 2007); the diameter gradually decreased with increasing CS content in the blend, and more beads were obtained in the composite. CS is an ionic polyelectrolyte, causing a higher charge density on the surface of the ejected jet that is formed during electrospinning. As the charges carried by the jet increase, higher elongation forces are imposed on the jet under the electrical field. It is known that the overall tension in the fibers depends on the self-repulsion of the excess charges on the jet. Thus, as the charge density increases, the diameter of the final fibers becomes smaller. The beads in mats occurred when the content of CS-HOBt in the blended solution increased to 40 wt% (Fig. 2d). The fibers in mats decreased and more beads were formed when the CS-HOBt ratio in the blended solution increased to 50 wt% (Fig. 2e). When the CS-HOBt content was more than 60 wt%, fibers could not form a jet during the electrospinning process. A similar result was previously reported by Jia et al. (2007) and Shalumon et al. (2009). This indicates that the repulsive forces between ionic groups in the CS backbone obstructed the formation of continuous fiber during electrospinning (Park et al., 2004).

3.2. Viscosity and conductivity measurements

The relation between viscosity and conductivity of the compositions of the CS-HOBt/PVA mixtures is shown in Fig. 4. The viscosities of the CS-HOBt/PVA blend solutions slightly decreased

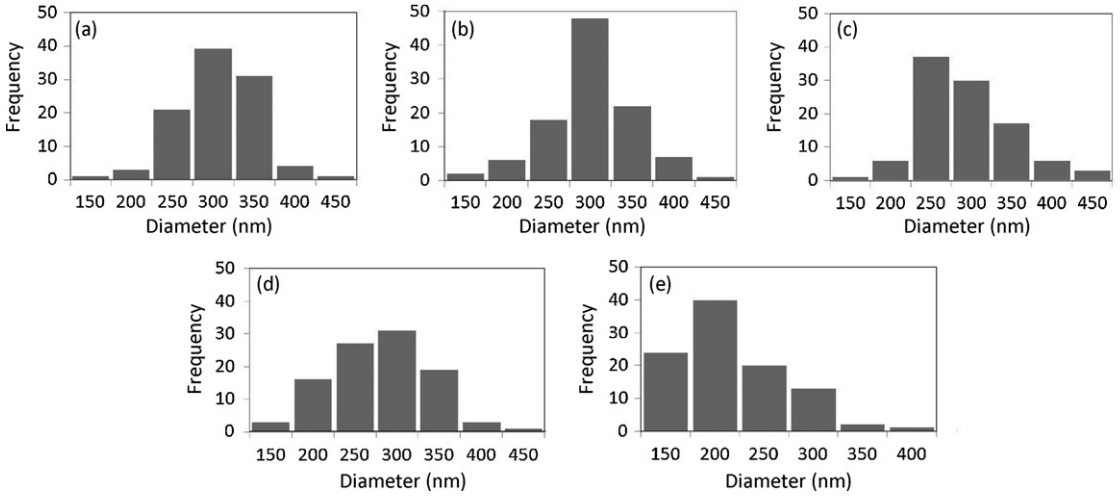

Fig. 3. Diameter distribution of nanofiber mats with different weight ratios of CS-HOBt/PVA: (a) 10/90, (b) 20/80, (c) 30/70, (d) 40/60 and (e) 50/50.

from 790.8 ± 1.5 to 612.1 ± 1.3 cP when the CS-HOBt content was increased from 10 to 50 wt% (Fig. 4a). This affected the morphology and diameter of the nanofiber mats: when the viscosity of the solution decreased, the formation of beads in mats and the diameter of the nanofiber mats decreased. Because CS-HOBt is a polyelectrolyte while PVA is a nonionic polymer, increasing the ratio of the CS-HOBt content in the blend solution leads to an increase in solution conductivity. The conductivities of the CS-HOBt/PVA blend solutions increased from 1036.7 ± 0.6 to 1500.3 ± 1.5 μs when increasing the CS-HOBt content of the blend from 10 to 50 wt% (Fig. 4b). When the blend composition increased from 0% to 50% CS-HOBt, the conductivity values of the corresponding solutions were also increased. Therefore, when the content of CS-HOBt in the solution blend was above 50 wt%, it could not be electrospun because of the higher conductivity (Zhou et al., 2008). This indicated that the repulsive force between ionic groups within the CS backbone inhibits the formation of continuous fibers during electrospinning. Thus, both the viscosity and the conductivity of the solution influenced the morphology of the nanofiber mats and electrospinnability. Even when the viscosity value of the CS-HOBt solution was optimal for the formation of nanofibers, the conductivity value was unacceptably high. Shalumon et al. (2009) revealed that the optimum viscosity and conductivity of carboxymethyl chitin/PVA solution range approximately between 500 and 700 cP and 1000 and 4000 μs, respectively (Shalumon et al., 2009).

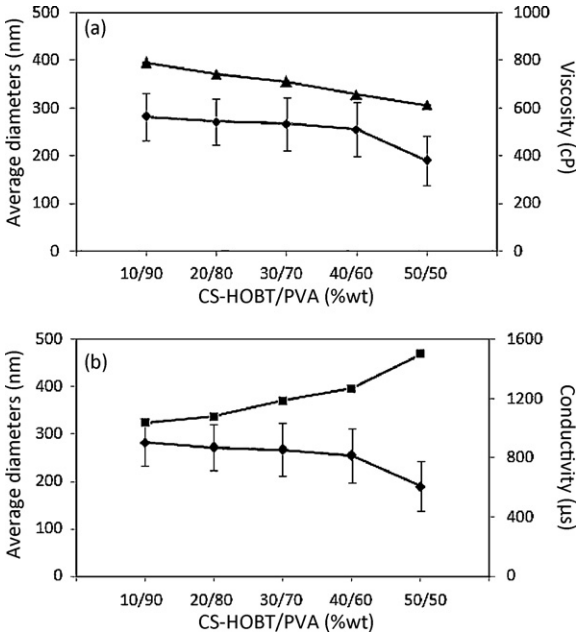

Fig. 4. (a) viscosity (▲) and (b) conductivity (■) with respect to the combination of CS-HOBt and PVA solutions against average diameters (♦) of nanofiber mats.

3.3. FT-IR spectra

Fig. 5 shows the FT-IR spectra of nanofiber mats of CS-HOBt/PVA blended in different weight ratios with PVA nanofiber mats and pure chitosan powder. The PVA nanofiber mats' spectrum showed the dominant absorption peaks at 3360, 2940, 1430 and 1095 cm^{-1}, which were attributed to the ν(O–H), $\nu_s(CH_2)$, δ(CH–O–H) and ν(C–O), respectively (Jia et al., 2007). All of the different weight ratios of CS-HOBt/PVA nanofiber mats also exhibited the same peaks as those found in the PVA nanofiber mats. However, when the amount of CS-HOBt in the blend increased, the peak at 1655 cm^{-1}, the ν(C=O) of a primary amide (Ignatova et al., 2006), was found to be the same as that in the CS powder spectrum. The absorption peak at 1430 cm^{-1} of the PVA nanofiber mats also gradually decreased. These data suggest the formation of hydrogen bond between the CS-HOBt and PVA molecule. The results illustrate that CS-HOBt and PVA were molecularly dispersed in nanofiber mats (Jia et al., 2007).

3.4. DSC analysis

Fig. 6 shows the DSC thermograms of the nanofiber mats of different weight ratio blends of CS-HOBt/PVA. The endothermic curves of all of the nanofiber mats were broad and obtuse, indicating that the crystalline structure could not be developed in mats. Table 1 shows the thermal properties of the nanofiber mats. The results show that when the amount of CS-HOBt in the blend increased, the T_m shifted to lower temperatures, from approximately 207 to 201.4 °C, and the ΔH_m values also decreased from 35.5 to 29.6 J/g. This indicated that the CS-HOBt content in the blend resulted in less favorable conditions for the crystallization of the nanofiber mats (Jia et al., 2007).

3.5. Indirect cytotoxicity evaluation

Various chitosans in acid solutions and chitosan polymer blends have been reported to prepare electrospun fiber mats. However, the toxicity of those chitosan fiber mats was dependent on the acid

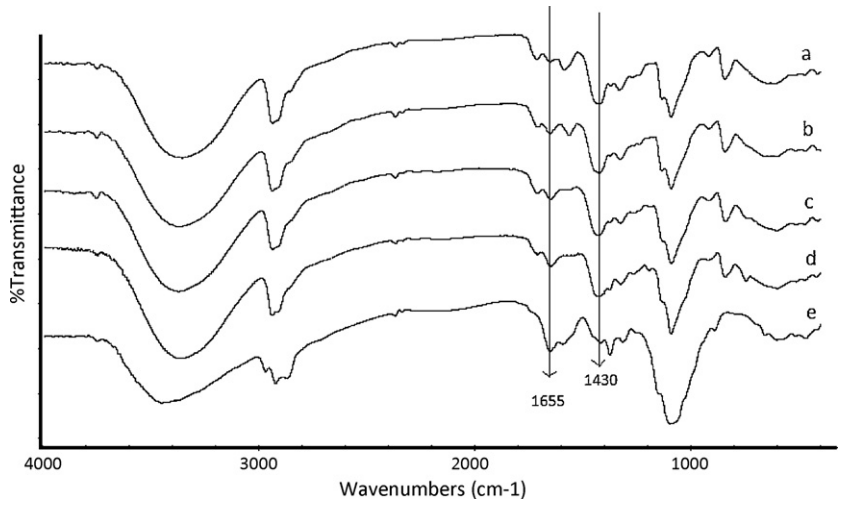

Fig. 5. FT-IR spectra of the nanofiber mats with different weight ratios of CS-HOBt/PVA: (a) 0/100, (b) 10/90, (c) 30/70, (d) 50/50, and (e) chitosan powders.

solutions and polymer blends studied. To test the cytotoxicity of the fibers that we have produced, a study of the CS-HOBt/PVA nanofiber mats was performed in human fibroblast cells (NHF cells). The cytotoxicity of various concentrations of the extract medium from the nanofiber mats of CS-HOBt/PVA blends in ratios of 10/90, 30/70, and 50/50 are shown in Fig. 7. There was a significant decrease in cell viability when the NHF cells were incubated with various concentrations of the extraction media of nanofiber mats of 7% CS in acetic acid when compared with the control ($p < 0.05$). The average cell viability was decreased when the concentration of the extract increased. However, the viability was not significantly different in any of the concentrations of extract medium of CS-HOBt/PVA nanofiber mats when compared with the control. Therefore, from these data the CS-HOBt/PVA nanofiber mats are clearly proven to be safe and have the potential to be developed

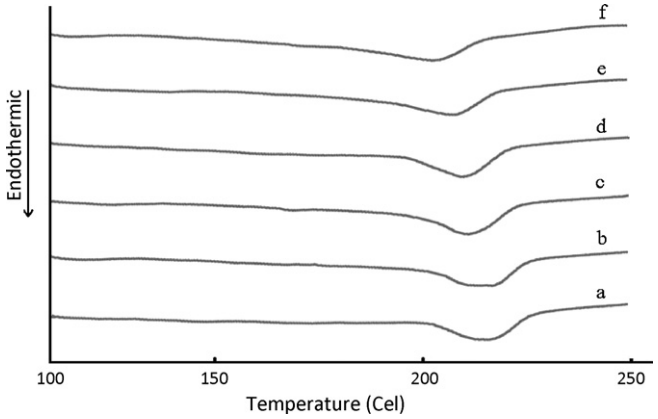

Fig. 6. The Differential Scanning Calorimeter (DSC) thermograms of the nanofiber mats with different weight ratios of CS-HOBt/PVA: (a) 0/100, (b) 10/90, (c) 20/80, (d) 30/70, (e) 40/60 and (f) 50/50.

Table 1
DSC data from the nanofiber mats with difference weight ratio of CS-HOBt/PVA..

CS-HOBt/PVA weight ratio	T_m (°C)	ΔH_m (J/g)
0/100	207.2	35.5
10/90	207.5	32.6
20/80	207.7	31.6
30/70	207.0	30.8
40/60	204.0	30.1
50/50	201.4	29.6

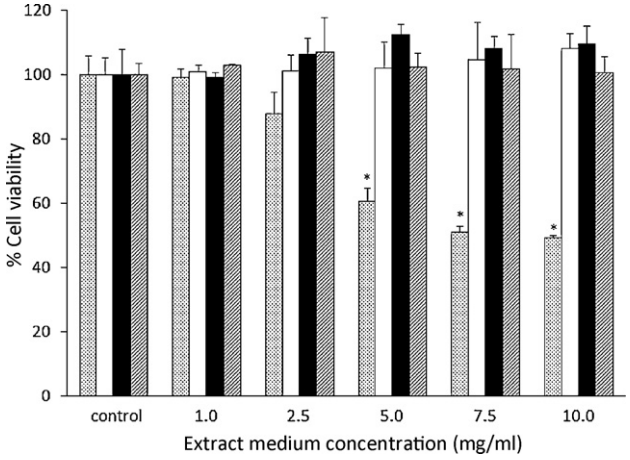

Fig. 7. Cell viability of the extract of nanofiber mats (▨) 7% CS in acetic acid and CS-HOBT/PVA with different weight ratio (□)10/90, (■)30/70 and (▨)50/50 at various concentrations in NHF cells. Each value represents the mean ± SD of six wells. Difference values (*) were statistically significant ($p < 0.05$).

as transdermal drug delivery carriers or skin tissue engineering scaffolds.

4. Conclusion

Electrospun CS nanofiber mats have been successfully prepared without organic solvent or organic acids by blending CS with PVA. The weight ratio in this blend affects the viscosity and conductivity of the solution. The morphology of fibers and their diameters were strongly influenced by the composition of the solution. Cytotoxicity tests showed that CS-HOBt/PVA nanofiber mats were non-toxic to fibroblast cells. This approach could have potential application in the field of drug delivery and in tissue engineering scaffolds.

Acknowledgements

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