Electrospun chitosan/polyvinyl alcohol nanofibre mats for wound healing

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Abstract
Chitosan (CS) aqueous salt blended with polyvinyl alcohol (PVA) nanofibre mats was prepared by electrospinning. CS was dissolved with hydroxybenzotriazole (HOBt), thiamine pyrophosphate (TPP) and ethylenediaminetetraacetic acid (EDTA) in distilled water without the use of toxic or hazardous solvents. The CS aqueous salts were blended with PVA at different weight ratios, and the effect of the solution ratios was investigated. The morphologies and mechanical and swelling properties of the generated fibres were analysed. Indirect cytotoxicity studies indicated that the CS/PVA nanofibre mats were non-toxic to normal human fibroblast cells. The CS-HOBt/PVA and CS-EDTA/PVA nanofibre mats demonstrated satisfactory antibacterial activity against both gram-positive and gram-negative bacteria, and an in vivo wound healing test showed that the CS-EDTA/PVA nanofibre mats performed better than gauze in decreasing acute wound size during the first week after tissue damage. In conclusion, the biodegradable, biocompatible and antibacterial CS-EDTA/PVA nanofibre mats have potential for use as wound dressing materials.

Key Messages
- CS-HOBt/PVA, CS-TPP/PVA and CS-EDTA/PVA electrospun nanofibre mats were prepared without use of toxic or harmful solvents
- these mats exhibited non toxic to normal human fibroblast cells and antibacterial activity against S.aureus and E. coli
- the wound healing activity of CS-EDTA/PVA electrospun nanofibre mats was better than gauze in reducing acute wound size during the 1st week after treatment

Introduction
Chitosan (CS) is a natural polysaccharide with biodegradable, biocompatible properties and non toxic effects; therefore, it has been proposed as a safer material for use in biomedical applications such as tissue engineering, delivery of pharmaceutical agent and wound dressing (1,2). CS has been widely investigated as wound dressing material because it can function as proliferation promoters, antibacterial agents and macrophage activator. CS gradually depolymerises to N-acetyl-d-glucosamine, which initiates fibroblast proliferation, aids in the ordered deposition of collagen and stimulates increased synthesis of natural hyaluronic acid at the wound site. CS is a haemostatic agent, which helps to promote natural blood clotting and blocks nerve endings to reduce pain. Recently, CS-based materials have been prepared as fibres, hydrogels, membranes, sponges and scaffolds for wound dressing applications (3,4).

Electrospun nanofibres are appropriate for use as a wound dressing material because of their useful properties, which include oxygen permeability, high porosity, variable pore size distribution and a high surface-to-volume ratio that can promote haemostasis and absorb wound exudates. Furthermore, the morphology of electrospun nanofibres is similar to that of the natural extracellular matrix (ECM) in the skin that promotes cell adhesion, migration and proliferation (3,5). The fabrication of CS nanofibres via electrospinning techniques has recently been actively investigated for the development of wound dressings. CS nanofibres have been created from the electrospinning of pure CS, CS derivatives and CS blended with other polymers. However, some organic solvents or toxic acids, such as trifluoroacetic acid (TFA) (6),...
chboroform (7) and acetic acid (8–10) may be leftover from the CS nanofibre fabrication process, and these trace residues may have undesirable effects on wound healing. The types of solvents used are often classified as toxic and hazardous and have potential long-term impacts on the environment and the health of the user. To eliminate toxicity from residual solvent exposure, water-soluble CS has been used to prepare nanofibres using quaternary CS (11) and carboxyethyl chitosan/polyvinyl alcohol (CS/PVA) (12) for wound dressing applications.

We recently prepared CS as aqueous salts to allow the formation of nanofibres without the use of organic solvents or toxic acids. Nanofibres were generated using CS-hydroxybenzotriazole (HOBt)/PVA (13) and CS-ethylenediaminetetraacetic acid (EDTA)/PVA (14) by blending the CS salts with PVA. Another salt was generated by dissolving CS with thiamine pyrophosphate (TPP) in distilled water. Owing to the phosphate groups of TPP, the molecule can form a salt with the amine groups of CS, improving the polymer’s aqueous solubility. The amine groups of TPP, and especially that of thiazolium can be deprotonated, and are always positive, even at physiological pH. Chitosan thiamine pyrophosphate (CS-TPP) was successfully prepared as a novel carrier for siRNA delivery (15).

In this study, CS-HOBt, CS-TPP and CS-EDTA blended in PVA solution were prepared as nanofibre mats via the electrospinning technique with different CS/PVA weight ratios. The morphology, structure, mechanical properties and swelling ability of the nanofibre mats were characterised, and the effect of the viscosity, conductivity and surface tension of the electrospinning solution on the morphology of these nanofibre mats was evaluated. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assays using normal human fibroblast cells were performed to study the cytotoxicity of the CS/PVA nanofibre mats. The antibacterial activities of the mats against gram-positive and gram-negative bacteria were investigated, and the in vivo healing activity of these nanofibre mats was evaluated using an animal model.

Experimental

Materials

CS (degree of deacetylation = 0.85, molecular weight = 110 kDa), hydroxybenzotriazole monohydrate (HOBt-H2O), TPP and EDTA were purchased from Sigma-Aldrich Chemical Company, Saint Louis, MO. PVA (degree of polymerisation \( \approx 1600 \), degree of hydrolysis \( \approx 97.5–99.5 \text{ mol}\% \) ) was purchased from Fluka, Buchs, Switzerland. Normal human foreskin fibroblast (NHF) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). All other reagents and solvents were commercially available and were of analytical grade.

Preparation of spinning solutions

CS solutions (2% w/v) were prepared by dissolving CS with HOBt, TPP and EDTA at weight ratios of 1:1, 1:1 and 2:1, respectively. Briefly, HOBt (2 g), TPP (2 g) or EDTA (1 g) was dissolved with CS (2 g) in 100 ml of distilled water, and the solutions were continuously stirred with a magnetic stirrer at ambient temperature until the solutions became clear. The PVA solution (10% w/v) 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, CS-TPP and CS-EDTA solutions were 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 viscosity, conductivity and surface tension of the solutions were measured using a Brookfield viscometer (Model DV–III ultra, Brookfield Engineering Laboratories Inc., Middleboro, MA), a EUTECH ECTestr11+ conductivity meter (Eutect Instruments Pte Ltd., Ayer Rajah Crescent, Singapore) and a Drop Shape Analyzer (FTA 100, First Ten Angstroms Inc, Portsmouth, VA), respectively.

Electrospinning process

The solutions were 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 and was electrospun at room temperature. The nanofibres were collected as-spun on an aluminium sheet that was wrapped on a rotating collector. The solution feed was driven by a syringe pump with a controlled flow rate of approximately 0.25 ml/h. The collection distance was fixed at approximately 20 cm. The process duration was fixed at 24 h for each CS/PVA weight ratio to provide mats with a 20–30 \( \mu \text{m} \) thickness.

Characterisation of nanofibre mats

The morphology of the nanofibre mats was observed under scanning electron microscopy (SEM; Camscan Mx2000, Obd-ucat Camscan Ltd, Cambridge, UK). Randomly selected areas of the fibres were cut into squares and coated with a thin layer of gold. The average diameter of the nanofibre mats was analysed by randomly measuring the diameters of the nanofibres at 100 different points from SEM images using the image analysis software (JMicrowVision V.1.2.7, University of Geneva, Geneva, Switzerland).

The tensile strength of the nanofibre mats was evaluated using a texture analyser (TA.XT plus, Stable Micro Systems, Godalming, UK) with a 5-kg load cell equipped with a tensile grip holder. The samples were cut into a rectangular shape (5 mm \( \times \) 25 mm\(^2\)). The thicknesses of these samples ranged from 20 to 30 \( \mu \text{m} \).

The degree of swelling of the nanofibre mats was investigated in phosphate buffer saline (PBS, pH 7-4) at room temperature for 1 h according to Equation (1):

\[ \text{Degree of swelling (\%)} = \frac{(M - M_d)}{M_d} \times 100 \]  

where \( M \) is the weight of each sample after submersion in the buffer solution for 1 h, and \( M_d \) is the initial weight of the sample in its dry state.

Indirect cytotoxicity of nanofibre mats

The cytotoxicity of the nanofibre mats was evaluated on the basis of a procedure adapted from the ISO10993-5 standard.
test method (indirect contact) (16). The nanofibre mats were sterilised by ultraviolet radiation for 1 h. The mats were then immersed in a serum-free medium [SFM; containing Dulbecco’s modified Eagle’s medium (DMEM), 1% v/v L-glutamine, 1% v/v lactalbumin and 1% v/v antibiotic and antimycotic formulation] in an incubator for 24 h to produce extraction media of different concentrations (10, 7.5, 5, 2.5 and 1 mg/ml). NHF cells were plated in 100 μl of DMEM, supplemented with 10% fetal bovine serum (FBS), at a density of 8000 cells/well in 96-well plates. When the cultures reached confluence (typically 48 h after plating), the cells were incubated with extraction media of varying concentrations for 24 h. After treatment, the tested extraction solutions were removed, and the cells were incubated with 100 μl of an MTT-containing medium (1 mg/ml) for 4 h. Then, the MTT 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 dimethylsulfoxide per well. The relative cell viability (%) was calculated on the basis of the absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and A153601, Packard BioScience, Meriden, CT). The viability of non-treated control cells was defined as 100%.

**Determination of antibacterial activity**

The antibacterial activity of nanofibre mats was tested against *Staphylococcus aureus* ATCC 6538P and *Escherichia coli* ATCC 10536. For the minimum inhibitory concentration (MIC) test, *S. aureus* and *E. coli* were cultivated in tryptone soy broth (TSB) in a shaking incubator at 37°C and 0.14 g for 24 h. The bacterial suspension was diluted until the bacterial concentration reached 1 × 10^6 colony-forming unit (CFU)/ml and was pipetted into a 24-well plate at 1 ml/well. Different amounts of the nanofibre mats (1–10 mg) were placed into wells containing bacterial suspension and were incubated at 37°C for 24 h. The MIC was defined as the minimum concentration of mats for which no growth was observed after a 24-h incubation. The optical density (OD) at 550 nm was measured using a microplate reader. For the determination of the minimum bactericidal concentration (MBC), the media mixtures from wells with no growth (100 μl) were spread onto agar plates. The MBC was defined as the minimum concentration of mats for which no colony growth was observed on agar plates after a 24-h incubation at 37°C. The MIC and MBC determinations were carried out in triplicate. Penicillin (1 mg/ml) was used as a positive control.

**Wound-healing activity of nanofibre mats**

Male Wistar rats (240–280 g) were used in this study. This study was approved by an Investigative Review Board (Animal Studies Ethics Committee, Faculty of Pharmacy, Silpakorn University, Approval No. 2-2553). After anaesthetisation, the neck area of the dorsal of each rat was shaved and wiped with 70% ethanol. Two wounds were created on the neck area of each rat using a skin biopsy punch (wound area of 0.8 cm²). The wound was treated by placing an equal size of nanofibre mat, gauze and commercial antibiotic gauze dressing (Sofra-tulle®, Sanofi Aventis, Guildford, UK) (n = 6) over it without removing the test material throughout the study period. The area of each wound was measured every day by the planimetry method until the wound completely healed. The percentage of wound healing is defined in Equation (2).

Wound area (%) = \( \frac{A_i - A_f}{A_i} \times 100 \) \hspace{1cm} (2)

where \( A_i \) is the initial wound area and \( A \) is the wound area after a fixed time interval.

**Statistical analysis**

All data for the experiment were collected from triplicate samples and are expressed as the mean ± standard deviation (SD). Statistically significant differences in cell viability and wound area were analysed using the Student’s t-test. The significance level was set at \( P < 0.05 \).

**Results and discussion**

**Morphology of nanofibres**

The SEM images of CS-HOBt/PVA, CS-TPP/PVA and CS-EDTA/PVA with different weight ratios are shown in Figure 1. When the CS/PVA weight ratio was increased to 30/70, the SEM micrographs demonstrated no beading. However, at CS/PVA 40/60, fibres with beads were observed. The maximum content of CS aqueous salt was 50/50 in the electrospinning mixture. When the CS content exceeded 50/50, nanofibres were unable to be formed; this may have been because of unsuitable solution parameters. Table 1 shows the solution parameters and average diameter of CS-HOBt/PVA, CS-TPP/PVA and CS-EDTA/PVA fibres generated at different CS/PVA weight ratios. When the content of CS salt was increased from 10/90 to 50/50, the average diameter of the CS-HOBt/PVA, CS-TPP/PVA and CS-EDTA/PVA fibres decreased from 228 ± 37 to 146 ± 33 nm, from 216 ± 34 to 100 ± 19 nm and from 221 ± 34 to 94 ± 20 nm. This decrease in the average fibre diameter was mainly ruled by solution viscosity and conductivity. The viscosity of the CS-HOBt/PVA, CS-TPP/PVA and CS-EDTA/PVA solutions decreased with increasing CS salt content (Table 1). The changes in the viscosity of the CS-TPP/PVA and CS-EDTA/PVA solutions were greater than those of the CS-HOBt/PVA solution, so the average diameters of the CS-TPP/PVA and CS-EDTA/PVA fibres were smaller than those of the CS-HOBt/PVA nanofibres. The reduced viscosity of solutions affected bead formation when the CS salt content was higher than 30/70. Increases in solution conductivity also contributed to decreases in nanofibre diameter. This result was in good accordance with our previous work (13). The surface tension increased slightly when the CS salt content was increased from 10/90 to 50/50. These differences could have led to bead and droplet formation during the electrospinning process (8). Nanofibres with CS/PVA weight ratios of 30/70 that did not contain beads were selected for further investigation of antibacterial and wound healing activity.
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The maximum CS/PVA weight ratio that provided nanofibre mats from the electrospinning process was 50/50. However, the tensile strength was only measured for the nanofibre mats that could be peeled off of the aluminium foil collector. The 10/90-30/70 CS/PVA nanofibre mats were easily peeled off. As the content of CS salt increased to 40/60, the mats became difficult to peel. Owing to the fibre structure of the 40/60 and 50/50 CS/PVA nanofibre mats, they contained many beads with very fragile fibres. Figure 2 shows the tensile strength of the CS/PVA nanofibre mats generated using different weight ratios. The tensile strength of all of the tested CS/PVA nanofibre mats ranged from 8.9 to 1.5 MPa, whereas the tensile strength of neat PVA nanofibre mats was 12.8 MPa. The results indicated that as the content of CS salt increased, the tensile strength decreased, the diameter of the nanofibres decreased and bead formation occurred in the mats. These findings demonstrated that these CS/PVA nanofibre mats possess tensile strength that is nearly equal to that of commercial microfibrous dressings, which have tensile strengths on the order of 10 MPa (17).

Swelling of nanofibre mats

The swelling of CS is the most important property that characterises its use for wound dressing applications. Figure 3 shows...
the degree of swelling of CS/PVA nanofibre mats with various weight ratios after immersion in a PBS (pH 7.4) at room temperature for 1 h. All the CS/PVA nanofibre mats demonstrated swelling over 100%, and this effect was slightly increased with increases in the CS content. The swelling of the 10/90 CS/PVA was approximately 100%, which increased to approximately 170% when the content of CS salt reached 50/50 in the blend. CS is a hydrophilic polymer, and water diffuses very rapidly through the material before CS degradation (18). The results indicated that increases in the degree of nanofibre mat swelling depended on the CS salt content. This observation is in agreement with Meng et al., who found that the swelling ratio of polylactic-co-glycolic acid (PLGA)/CS nanofibres was higher than that of neat PLGA nanofibres and increased with CS content (19).

**Indirect cytotoxicity of nanofibre mats**

The indirect cytotoxicity of various concentrations of medium extracts from CS/PVA nanofibre mats composed of 10/90, 30/70 and 50/50 weight ratios is shown in Figure 4. There was no significant decrease in cell viability when the NHF cells were incubated with various concentrations of the extraction media of CS-HOBr/PVA, CS-TPP/PVA and CS-EDTA/PVA nanofibre mats when compared with the control \((P < 0.05)\). The average cell viability decreased slightly when the concentration of the extract increased, but these changes were not statistically significant. From these data, the CS-HOBr/PVA, CS-TPP/PVA and CS-EDTA/PVA nanofibre mats appear to be non-toxic to NHF cells and have the potential to be developed as wound dressings.

**Determination of antibacterial activity**

The antibacterial mechanism of CS is generally attributed to the amino group at C-2 position of the glucosamine residue that is responsible for the cationic nature of CS in acidic conditions (20). When the CS/PVA nanofibre mats were immersed in a TSB solution containing a bacteria suspension, the CS was dissolved and showed a cationic charge in solution. Figure 5 shows the inhibition of bacterial growth by CS/PVA nanofibre mats, compared with neat PVA nanofibre mats, after a 24-h incubation with *S. aureus* and *E. coli*. The CS/PVA nanofibre mats exhibited concentration-dependent antibacterial activity against *S. aureus* and *E. coli*. The 30/70 CS-EDTA/PVA and 30/70 CS-HOBr/PVA nanofibre mats inhibited *S. aureus* growth at 5 and 7.5 mg/ml, respectively, and the 30/70 CS-TPP/PVA almost inhibited *S. aureus* growth at 10 mg/ml (Figure 5A). The 30/70 CS-EDTA/PVA and 30/70 CS-HOBr/PVA nanofibre mats also inhibited *E. coli* growth at 5 and 10 mg/ml, respectively, but 30/70 CS-TPP/PVA did not do so (Figure 5B). The neat PVA nanofibre mats did not show any effect on bacteria growth. The MIC and MBC of the 30/70 CS salt/PVA nanofibre mats are shown in Table 2. The results showed that the different antibacterial activities of the CS/PVA nanofibre mats were dependent on the type of CS salt used. The 30/70 CS-EDTA/PVA nanofibre mats showed the highest antibacterial activity at 5 mg/ml, likely because of the intrinsic antibacterial activity of EDTA. Several recent publications have indicated that EDTA (alone and in combination with antibiotics) was an effective antimicrobial agent, with a spectrum covering both gram-positive and gram-negative bacteria. The antimicrobial activity of an EDTA–CS acetic acid combination has been tested, and the study showed that while EDTA possessed a weaker bacteriostatic effect than CS acetic acid, the combination of CS acetic acid and EDTA elicited synergistic activity against *S. aureus* (21). All the CS/PVA nanofibre mats tested at 1–10 mg in this study showed higher antibacterial activity against gram-positive bacteria (*S. aureus*) than against gram-negative bacteria (*E. coli*). The mode of action of antimicrobial agents depends on the nature of the target microorganisms, and differences between the response of gram-positive and gram-negative bacteria to various antibiotics are mainly related to their cell wall structure and the arrangement of their outer membrane (20,22). Different theories have been proposed to explain CS’s antimicrobial mode of action. In one mechanism (23,24), positively charged CS molecules interfere with negatively charged residues on the bacterial surface. CS could interact with the membrane of the bacteria to alter cell permeability. Other studies (25) demonstrated that lower molecular weight CS may have intracellular targets. Dissociated CS molecules in solution could bind with DNA and inhibit the synthesis of mRNA and proteins. Xing et al. investigated the antimicrobial mode of oleoyl-CS nanoparticles against *E. coli* and *S. aureus* and showed that the particles achieve their antibacterial activity by damaging the structures of the cell membrane and by putative binding to extracellular targets such as phosphate groups or to intracellular targets such as DNA and RNA (26,27).

**Wound-healing testing of nanofibre mats**

In the wound-healing test, two full-thickness round wounds with surface areas of 0.8 cm² were created on the ventral neck of each rat. Figure 6 shows the images of wound appearances and the percentage wound areas at 1, 4, 7 and 10 days after treatment with 30/70 CS-HOBr/PVA, 30/70 CS-TPP/PVA,
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Figure 4 Cell viability in NHF cells at various concentrations of the extract of CS/PVA nanofibre mats (A) CS-HOBt/PVA, (B) CS-TPP/PVA and (C) CS-EDTA/PVA with different weight ratios; □ 10/90, ■ 30/70 and ▲ 50/50. The data are expressed as mean ± standard deviation (n = 5).

Figure 5 The growth of (a) S. aureus and (b) E. coli in the presence of (■) 30/70 CS-HOBt/PVA, (▲) 30/70 CS-TPP/PVA, (▲) 30/70 CS-EDTA/PVA and (●) PVA nanofibre mats, after 24-h incubation. The data are expressed as mean ± standard deviation (n = 3).

Table 2 Minimum inhibition concentrations (MIC) and minimum bactericidal concentrations (MBC) of 30/70 CS-HOBt/PVA, CS-TPP/PVA and CS-EDTA/PVA nanofibre mats against S. aureus and E. coli compared with PVA nanofibre mats

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<tr>
<th>Nanofibre mats</th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
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<tr>
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<td>S. aureus</td>
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<td>PVA</td>
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<td>30/70 CS-HOBt/PVA</td>
<td>7.5</td>
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<td>30/70 CS-TPP/PVA</td>
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CS, chitosan; EDTA, ethylenediaminetetraacetic acid; HOBt, hydroxybenzotriazole; PVA, polyvinyl alcohol; TPP, thiamine pyrophosphate.

30/70 CS-EDTA/PVA nanofibre mats, gauze (negative control) or commercial antibacterial gauze dressing (positive control). The wound areas decreased gradually and reached 2% of the original wound size after 10 days when treated with the five different wound dressings. Wound closure was achieved within 10 days of all treatments. However, at day 1 after the operation, the percentage wound area of wounds treated with CS-HOBt/PVA, CS-EDTA/PVA nanofibre mats and commercial antibacterial gauze dressing was smaller than those of the gauze treatment group (P < 0.05). This might be involved with the high surface area of the nanofibre mats provided high wound exudates absorption. The wounds treated with nanofibre mats were dry and smaller in wound size than the gauze treatment. At 4 days after the operation, wound healing with the CS-EDTA/PVA nanofibre mat dressing was the greatest (P < 0.05), and in the first week, the 30/70 CS-EDTA/PVA nanofibre mats exhibited excellent wound healing activity. This result was in accordance with the high antibacterial activity of the CS-EDTA/PVA nanofibre mats. N-acetyl-d-glucosamine from CS depolymerisation is reported to enhance...
These fibre mats were in the nanometre range and provided suitable tensile strength and swelling properties. The CS-EDTA/PVA nanofibre mats showed the greatest antibacterial and wound-healing activity during the first 4 days after wounds. The biodegradable, biocompatible and antibacterial CS-EDTA/PVA nanofibre mats have immense potential for use as wound dressing materials.

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