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INTRODUCTION

Chitosan has been used to fabricate the electrospun nanofiber mats for tissue engineering. However, the chitosan electrospun scaffold is very fragile, strongness not enough to support cell growth and differentiation. Poly ε-caprolactone (PCL) was selected as a blending polymer due to their compensatory characteristics such as slow degradation and good mechanical properties. The electrospun chitosan/ PCL nanofibres has recently been investigated[1-4]. These studies reported the potential of chitosan/PCL nanofibers for biomedical applications. However, the inflammatory response to chitosan/PCL nanofiber was limit studied. Chitosan has been reported the inhibitory effect on reactive oxygen species (ROS)[5] and inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6). Therefore, the aim of this study was to investigate the effect of chitosan/PCL aligned nanofiber scaffolds on inflammatory response to macrophage cells. The aligned nanofiber scaffolds were prepared by electrospinning of chitosan/PCL solutions with different volume ratios. The morphology and degree of alignment of nanofiber scaffolds were characterized using scanning electron microscope (SEM). The cell morphology was examined by SEM. Cell proliferation was determined by DNA assay using 3T3 fibroblast cells. The effects of chitosan/PCL aligned nanofiber scaffolds on inflammatory response were investigated by dichlorofluorecein assay using J774A.1 macrophages cells.

MATERIALS AND METHODS

Materials Chitosan (degree of deacethylation = 0.85, molecular weight = 110 kDa), poly(ε-caprolactone) (PCL) (Mn 80000), 3T3 swiss albino fibroblast cells were purchased from American type culture collection (ATCC). J774A.1 mouse BALB/c monocyte macrophage cells were obtained from European collection of cell cultures (ECACC). All other reagents and solvents were of analytical grade.

Preparation of Chitosan/PCL solution and electrospinning Chitosan (8% w/v) was dissolved in a mixture of trifluoroacetic acid (TFA) and dichloromethane (DCM) (75:25 v/v). PCL (30% w/v) was dissolved in trifluoroethanol (TFE). The 8% chitosan solution was mixed with 30% PCL solution at volume ratio of 25/75. The solutions were taken up in a 5 ml plastic syringe connected with a 21 gauge, stainless needle (diameter = 0.8 mm) at the nozzle. The needle was connected to the high voltage DC power supply of a Glassman High Voltage device. The electric potential was fixed at 15 kV. The nanofibers were collected on a rotating collector with rotation speed at 1200 rpm. The solution feed was driven by a syringe pump, and the rate was fixed at 0.25 ml/h during spinning. The collection distance was fixed at approximately 15 cm. The process duration was fixed at 2 h to provide fibrous meshes with the same thickness. Prior to cell seeding the electrospun mats were cut to fit into a sterile 24 well plate Scaffdex well insert (C00002S, Scaffdex®, Tampere, Finland) to hold its at the bottom of the well plate, and sterilized under UV light in laminar flow hood for 30 minutes on either side.

Fiber characterization The morphologies of chitosan/PCL nanofibre scaffolds were perform using scanning electron microscope (SEM; Phenom G2 pro, Eindhoven, Netherlands). Randomly selected areas of the fiber covered foil were cut into squares and mounted onto carbon coated stubs (Agar Scientific) and gold sputter-coated for a total of 2 min (Edwards Sputter Coater–S150B). Fiber diameter and angles for alignment were determined from the SEM images using the Fibermetric software (n=300, 100 fiber measurements from 3 different SEM images).

Determination of 3T3 cell proliferation For 3T3 cell viability and morphology studies cells were seeded at 25000 cells/ml. The 3T3 fibroblast cells viability was evaluated by DNA assay at 3, 5 and 7 days after seeding. DNA assay was performed to quantify the number of cells on the nanofibrous scaffold. At each time point, cells were washed in PBS, covered with distilled water and frozen at - 80 °C. Cells were frozen and thawed three times and aliquots of 50 µl (in triplicate) were moved into a clear flat bottom 96-

well plate. Fifty µl of TNE buffer (10 mM Tris, 2M NaCl, 1 mM EDTA, pH 7.4) and 100 µl of Hoechst stain solution (stock solution: 1mg/ml Hoechst 33258 in water; dilution 1:50 in TNE buffer) were added to each well. The number of cells was calculated base on the fluorescence at 355 nm excitation and 460 nm emission using a fluorescence plate reader. Data were expressed as cell number.

Determination of 3T3 cell morphology At day 7, cell seeded samples were fixed with 1.5% glutaraldehyde for 30 minutes at 4°C and rinsed in 0.1M phosphate buffer. Cellular samples were dehydrated using a series of increasing ethanol concentration (50, 70, 90 and 100% v/v) incubations followed by an overnight incubation in hexamethyldisilasane (HMDS). Samples were mounted on carbon tabs and gold sputter coated for 2 minutes. The morphology of cell attach on nanofibers scaffolds were observed under SEM.

Determination of murine macrophage response For dichlorofluorecein assay, J774A.1 macrophages cells were seeded at 50000 cells/ml. The inflammatory response to J774A.1 macrophages cells was evaluated at 2, 4, 8, 24 and 48 hours after seeding. Dichlorofluorecein assay was used to determine to peroxide released in medium when J774A.1 macrophages cells contract to different scaffolds. 2,7 Dichlorofluorescin diacetate was dissolved in fresh methanol at 2mM and incubated at room temperature for 20 min in the dark. The cells were seeded in dilute 2mM DCF 1:100 in Hank's Balanced Salt Solution. At each time point, transfer 100µl aliquots of cell media into the black well plates. The peroxide release (%) was calculated base on the fluorescence at 485 excitations and 530 emissions using fluorescence plate reader compare with control (copper discs).

Statistical analysis All experiment data were collected from triplicate samples and are expressed as the mean ± standard deviation (S.D.). Statistically significant differences were analysed using the Student's t-test. The significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

Electrospinning and morphology The SEM images of chitosan/PCL aligned nanofiber scaffolds with different volume ratios are shown in Figure 1. The average diameters of PCL and 25/75 chitosan/PCL were 519.65 ± 172.15 and 406.37 ± 98.71 nm. The diameter distribution and degree of aligment of chitosan/PCL aligned nanofiber scaffolds are also shown in Figure 1. The average diameter was in nanometer range and fibers were smooth. The diameters of fiber decrease when the chitosan was incorporated into nanofiber. Chitosan is an ionic polyelectrolyte, causing a higher charge density on the surface of the ejected jet that is formed during electrospinning. Thus, as the charge density increases, the diameter of the final fibers becomes smaller[6]. The alignment of pure PCL fiber was higher than the blended fiber. This result may be because the properties of blend polymer altered which effect to fiber formation during electrospinning process.

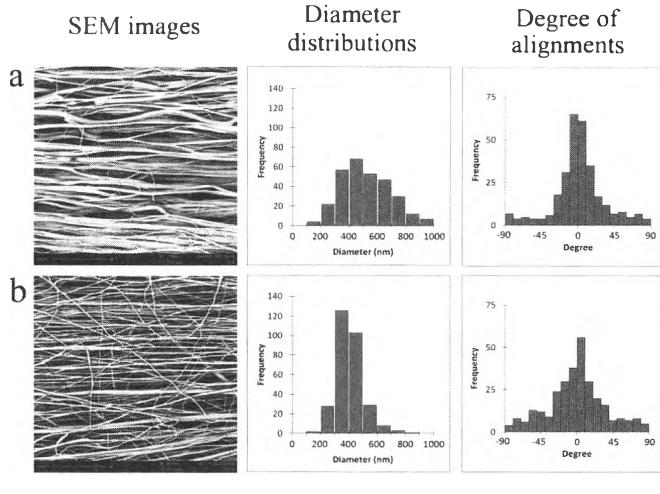

Figure 1 SEM images, diameter distributions and degree of alignments of aligned nanofiber scaffolds PCL (a) and 25/75 chitosan/PCL (b).

Cell morphology and proliferation 3T3 fibroblast cells cultured on the chitosan/PCL aligned nanofiber scaffolds for 7 days were examined with SEM. Figure 2 shows the 3T3 fibroblast cells grown on glass control, PCL and 25/75 chitosan/PCL scaffolds. The SEM images indicated that 3T3 fibroblast were well attached to both scaffold and showed that the cells on the glass exhibited a stellate-patterned phenotype

(Fig. 2a). Whereas, cells exhibited the fibroblastic phenotype on the chitosan/PCL aligned nanofibers, cells gradually more elongated, presenting a spindle-shaped morphology spreading parallel to the fibers (Fig. 2b and c). Figure 3 shows the cell number on scaffolds through DNA assay. After 5 days culture, the cell number of cells evaluated on PCL and 25/75 chitosan/PCL aligned nanafiber scaffold was higher compared to control ($P<0.05$). There were also no significant differences between both scaffolds in cell number. The results of cell proliferation and cell number were in the same way. These indicated that the chitosan/PCL was non toxic to 3T3 fibroblast cells and promote the cell growth.

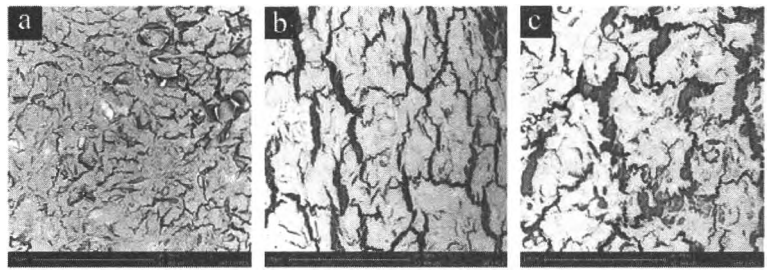

Figure 2 SEM images of 3T3 fibroblast cells on glass control (a), PCL and (b) 25/75 chitosan/PCL (c) aligned nanofiber scaffolds after 7 days of cell culture.

Response to macrophage cell Dichlorofluorecein assay was used to determine to peroxide released in medium when J774A.1 macrophages cells contract to different scaffolds. Figure 4 shows the peroxide release (%) at each time of PCL, 25/75 chitosan/PCL aligned nanofiber scaffolds and control from J774A.1 macrophages cells. This result shows the statistical different between peroxide level of the 25/75 chitosan/PCL with the PCL and control. The % peroxide release of the PCL and control was around 100% on the on other hand the 25/75 chitosan/PCL was around 40% and decrease to 20% in 2 day. Chitosan has been reported the inhibitory effect on reactive oxygen species (ROS)[5]. These result indicated that the chitosan in scaffolds can reduce peroxide release from J774A.1 macrophages cells.

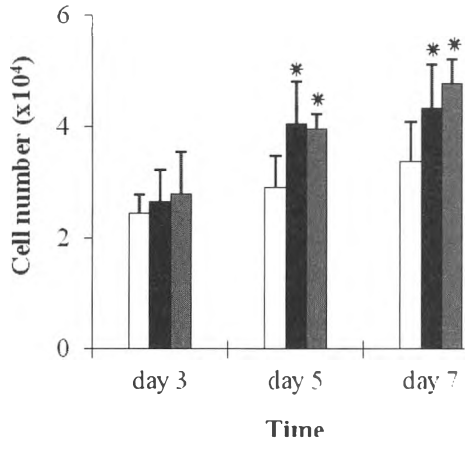

Figure 3 Cell number of 3T3 fibroblast cells on control (□), PCL (■) and 25/75 chitosan/PCL (■) aligned nanofiber scaffolds.*Statistically significant ($P<0.05$).

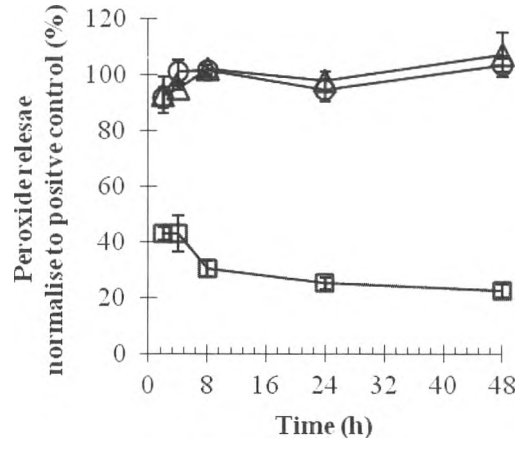

Figure 4 The percentage peroxide release from J774A.1 macrophage cells with control (△), PCL (○) and 25/75 chitosan/PCL (□) aligned nanofiber scaffolds.

CONCLUSION

The chitosan/PCL aligned nanofiber scaffolds were successfully prepared using the electrospinning process. These fibers were aligned and were in the nanometer range. The chitosan/PCL aligned nanofiber scaffolds showed well compatibility with 3T3 fibroblast cells. The scaffolds exhibited inhibitory effect on peroxide release from J774A.1 macrophages cells. These biodegradable, biocompatible and anti-inflammatory aligned nanofiber scaffolds have promising potential for tissue engineering application.

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