

## **Electronic Supplement for: Electrospinning of chitosan derivative nanofibers with structural stability in an aqueous environment**

### **Experimental Details**

Materials. Medium molecular weight chitosan (85% deacetylation and viscosity 200–800 centipoise), DL-lactic acid (LA), hexafluoroisopropanol (HFIP), trifluoroacetic acid (TFA) methylene chloride (MC), 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Chemical Co. (St. Louis, MO). Schwann cells (RT4-D6P2T) were obtained from American Type Culture Collection (Manassas, VA). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and phosphate buffered saline (PBS) were obtained from Invitrogen Co. (Carlsbad, CA). The CytoTox 96 Nonradioactive assay kit was obtained from Promega Co. (Madison, WI). The Alamar Blue assay kit was obtained from Biosource Co. (Sacramento, CA). The Effectene™ Transfection Reagent kit was obtained from Qiagen Co. (Valencia, CA).

Electrospinning of nanofibers and thermal treatment. Chitosan powder was dissolved in a dilute aqueous solution of LA at a 1: 3 mole ratio of chitosan to lactic acid. The mixture was stirred for 12 hrs at room temperature and lyophilized to produce chitosan lactic-acid salt. The powdered chitosan lactic-acid salt was dissolved in TFA/MC (80/20) to produce a chitosan solution of desired concentration (3–6 wt%). The electrospinning system used in this study was similar to that reported previously.<sup>1</sup> A DC voltage, 15–22 kV (High DC power supply, Del Electronics Corp.) was applied between the solution injection nozzle and a grounded cylindrical collector. The typical distance between the nozzle and the collector was 18–22 cm. The solution feed was driven by gravity and the feed speed was controlled by the tilt angle of the solution reservoir.

The polymer solution was positively charged with a platinum wire placed in the solution. The deposited chitosan nanofibers were incubated in an oven at 70°C for 12 hrs to remove the residual solvent, followed by thermal treatment at 100°C for 3 hrs under vacuum. Chitosan films were also prepared from chitosan powder as a control by casting and air drying.

Characterization of Nanofibers. The polarized FTIR spectra of nanofibers (200 scans at 4 cm<sup>-1</sup> resolution) were acquired using a Nicolet 5DX spectrometer equipped with a DTGS detector and a solid transmission sample compartment. Spectrum analysis and display were performed using Nicolet and Microcal Origin software. Chitosan-LA powder was washed for 24 hrs in chloroform and methanol and dried at room temperature under vacuum. Washed chitosan-LA was dissolved in D<sub>2</sub>O containing 0.5 M DCl. <sup>1</sup>H NMR was performed on a Bruker AVance spectrometer at 300 MHz and 50°C. The degree of substitution of LA on chitosan was determined using the integration values of peaks associated with chitosan and LA as outlined in Fig. 2. The crystallinity of the nanofibers was assessed using a Bruker D8a diffractometer. Thermogravimetric analysis (TGA) was performed with a Perkin Elmer TGA 7. All analyses were performed with a 10 mg sample placed in an aluminum pan under a nitrogen atmosphere at 50–500°C. The weight loss profile was acquired using Pyris V.06 software. The experiments were run at a scanning rate of 10°C per min.

The stability of the nanofibers in an aqueous environment was assessed by incubation in PBS (pH 7.4) at room temperature for 48 hrs. Samples were collected, washed three times with DI water, and dried under vacuum. The samples were then sputter-coated with Au-Pd, and examined by a scanning electron microscope (SEM, FEI Sirion XL30) operated at an accelerating voltage of 10 kV. TEM (Phillips CM 100, operating at 100 kV) was used to observe detailed

morphological features of the nanofibers. A thin nanofibrous membrane was sandwiched in a PELCO® folding grid and the image was acquired at the membrane edge.

Preparation of nanofibers for cell culture. Aligned chitosan-LA nanofibers were produced by electrospinning directly on a 10 mm round glass cover slip placed between two parallel grounds attached to a non-conducting collector separated 4 cm apart. All other experimental conditions were identical to electrospinning non-aligned nanofibers described above. The collected nanofibers were wrapped on the cover slip and glued with a solution of poly(L-lactic acid) in hexafluoroisopropanol. Chitosan films were prepared as control substrates by spin-coating dilute chitosan solutions on a 10 mm round cover slip. Both nanofiber and film samples were subjected to the thermal treatment described previously and incubated in 100% ethanol prior to cell seeding.

Cytotoxicity and cell proliferation assessment. Schwann cells (SCs) were seeded on chitosan-LA nanofibers and cast chitosan films at a cell density of 12,500 cells/well in 24-well cell culture plates and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. For the cytotoxicity study, a CytoTox 96 Nonradioactive assay was used to measure the cytotoxic behavior by colorimetrically measuring the level of lactose dehydrogenase (LDH), which is released upon cell lysis. After 1, 3 and 5 days of incubation, the media was removed and assayed according to manufacturer's protocols. The cell proliferation was assessed using the Alamar Blue colorimetric assay. Material-cell samples were collected 1, 3 and 5 days after seeding, washed twice with PBS, and incubated for two hrs in DMEM containing 10 vol% Alamar Blue and 10% FBS. 300  $\mu$ L of the medium was transferred to a 96 well plate for absorbance measurements at the wavelengths of 570 and 600 nm, and the percent reduction of the Alamar Blue solution compared to control samples without cells were calculated using manufacturer's protocols.

Cell transfection and optical imaging. SCs were transfected with p-EGFP-N1 plasmid DNA (GFP+ SCs) using the Effectene Transfection Reagent kit, and maintained in DMEM media supplemented with 1 mg/mL G418 sulfate. For fluorescence imaging, SCs were cultured on chitosan-LA nanofibers and cast chitosan films on glass slides for three days and rinsed briefly with PBS, and fixed in 4% methanol-free formaldehyde for 15 min. The samples were then rinsed with PBS, counterstained with a 1:500 dilution of DAPI in PBS for one min, and imaged with a confocal microscope (Zeiss 510 Meta TE2000-5) using appropriate filters.

## **Reference**

1. Narayan Bhattarai, Dennis Edmondson, Omid Veisheh et al., *Biomaterials* **26** (31), 6176 (2005).