Stability Improvement of Electrospun Chitosan Nanofibrous Membranes in Neutral or Weak Basic Aqueous Solutions

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Communications

Stability Improvement of Electrospun Chitosan Nanofibrous Membranes in Neutral or Weak Basic Aqueous Solutions

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Further utilization of chitosan nanofibrous membranes that are electrospun from chitosan solutions in trifluoroacetic acid (TFA) with or without dichloromethane (DCM) as the modifying cosolvent is limited by the loss of the fibrous structure as soon as the membranes are in contact with neutral or weak basic aqueous solutions due to complete dissolution of the membranes. Dissolution occurs as a result of the high solubility in these aqueous media of $-NH_3^+CF_3COO^-$ salt residues that are formed when chitosan is dissolved in TFA. Traditional neutralization with a NaOH aqueous solution only maintained partial fibrous structure. Much improvement in the neutralization method was achieved with the saturated Na_2CO_3 aqueous solution with an excess amount of Na_2CO_3(s) in the solution. We showed that electrospun chitosan nanofibrous membranes, after neutralization in the Na_2CO_3 aqueous solution, could maintain its fibrous structure even after continuous submersion in phosphate buffer saline (pH = 7.4) or distilled water for 12 weeks.

1. Introduction

In recent years, much attention has been paid on the use of high electrostatic potentials in fabricating ultrafine fibers from materials of diverse origins with diameters in the submicrometer down to nanometer range by a process known as electrospinning.[1] This process involves the application of a strong electrostatic field across a conductive capillary attaching to a reservoir containing a polymer liquid and a screen collector. Upon increasing the electrostatic field strength up to a critical value, charges on the surface of a pendant drop destabilize its shape from partially spherical into conical. Beyond a critical value of the electrostatic field strength, a charged polymer jet is ejected from the apex of the cone. The ejected charged jet accelerates toward the collector by the electrostatic forces, during which the jet elongates and either dries out or solidifies to finally leave ultrafine fibers on the collector.

To mimick natural tissues in vitro, the electrospinning technique has been heavily explored, due mainly to its potential for fabricating highly porous fibrous membranes with the diameters of the individual fibers being in the range close to the fibrous collagen bundles of about 30−130 nm found in the natural extracellular matrix (ECM).[2] Because of the great expectations for utilizing electrospun fibers in biomedical applications, a number of natural and synthetic biodegradable polymers have been electrospun: they are, for examples, native collagens from calfskin and human placenta,[2] bovine fibrinogen,[3] *Bombyx mori* and *Samia cynthia ricini* silk fibroins,[4] dextran, methacrylated dextran, and dextran/poly(D,L-lactide-*co*-glycolide) (PLGA) hybrids,[5] poly(ester urethane)urea (PEUU)/bovine collagen type I hybrids,[6] PLGA/chitin and poly(glycolic acid) (PGA)/chitin hybrids,[7] and hyaluronic acid.[8]

Chitosan or poly(*N*-acetyl-D-glucosamine-*co*-D-glucosamine) is a partially *N*-deacetylated derivative of chitin or poly(*N*-acetyl-D-glucosamine), one of the most abundant polysaccharides. Even though chitin is structurally similar to glycosaminoglycans (GAGs), such as condroitin sulfate and hyaluronic acid in the ECM,[9] its utilization is limited by its poor solubility and its physical properties that are rigid and brittle. Chitosan has been explored as a suitable functional material for biomedical utilization, mainly due to its biocompatibility, biodegradability, and nontoxicity.[10] Electrospinning of chitosan has proven to be difficult; therefore, electrospinning of chitosan fibers has been in blends with another polymer, such as poly(ethylene oxide) (PEO) in an aqueous solution of acetic acid[11,12] or water,[13] silk fibroin in an aqueous solution of formic acid,[14] and poly(vinyl alcohol) (PVA) in an aqueous solution of formic acid[15] or acetic acid.[16]

Nevertheless, successful fabrication of pure chitosan nanofibers has been reported from the electrospinning of chitosan solutions in trifluoroacetic acid (TFA) or a cosolvent system of TFA and dichloromethane (DCM),[15] deacetylation of chitin nanofibers obtained from the electrospinning of chitin solutions in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP),[17] and the electrospinning of chitosan solutions in 90% aqueous acetic acid solution.[18] To further explore the use of electrospun chitosan fibrous membranes as tissue scaffolds, we unsuccessfully tried to electrospin the chitosan solutions in concentrated acetic acid solutions.[18] Since the molar mass of chitosan is a critical parameter determining whether uniform chitosan fibers can be obtained,[18] it is postulated that our failure was a result of the unsuitable molar mass of the chitosan sample used. Subse-

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quently, we had a success with a chitosan solution in both TFA and TFA/DCM solvent systems,[15] but the resulting chitosan membranes lost their fibrous structure as soon as they were in contact with phosphate buffer saline (PBS; pH = 7.4) or even 70% ethanol during sterilization. The loss in the fibrous structure of the chitosan membranes is thought to be a result of the dissolution of the chitosan trifluoroacetate salts that are formed when chitosan is dissolved in TFA.[19]

Since we found that the fabrication of pure chitosan nanofibrous membranes from chitosan solutions in TFA or TFA/DCM was relatively easy, a neutralization procedure is needed to further realize the actual usefulness of the membranes in areas that require a contact of the membranes with neutral or weak basic aqueous media. In the present contribution, we report an alternative method for neutralizing the electrospun chitosan nanofibrous membranes that were fabricated from a chitosan solution in TFA or TFA/DCM. The effect of the neutralization on morphology and stability in PBS of the as-spun chitosan membranes was also investigated.

2. Experimental Section

2.1. Materials. Chitosan powder [degree of deacethylation (DD) = 95%], trifluoroacetic acid (TFA, CF_3COOH; ~98% purity), and dichloromethane (DCM) were purchased from Sigma-Aldrich (USA). NaOH and Na_2CO_3, used as neutralizing agents, were purchased from Sigma-Aldrich (USA). Phosphate buffer saline (PBS; pH = 7.4), used as the medium for weight loss and swelling assessments, was purchased from Sigma-Aldrich (USA). Both the weight-average and the number-average molar masses of chitosan were determined using a Waters 600E size exclusion chromatograph (medium = 0.5 M acetate buffer, column: Ultrahydrogel linear, detector: refractive index, temperature = 30 °C, and software: PL LogiCal) to be about 570 000 and 70 000 $g \cdot mol^{-1}$, respectively.

2.2. Fabrication of Electrospun Chitosan Nanofibrous Membranes. Electrospun chitosan nanofibrous membranes were prepared in a manner similar to that reported previously by Ohkawa et al.[15] Briefly, 7% w/v chitosan solution was prepared by dissolving a measured amount of chitosan powder in a mixture of TFA and DCM (70:30 v/v). The as-prepared chitosan solution was continuously stirred for 12 h at room temperature and later fed into a 5-mL glass syringe fitted with a gauge 20 stainless steel needle (OD = 0.91 mm) used as the nozzle. Both the syringe and the needle were tilted 45° from a vertical baseline. The as-spun chitosan nanofibers were collected on an aluminum sheet wrapped around a homemade rotating cylinder (width and diameter ≈ 15 cm), placed at a fixed distance of 20 cm from the needle tip. The needle was connected to the emitting electrode of positive polarity of a Gamma High-Voltage Research ES30P-5W power supply. Both the electrical potential and the collection time were fixed at 25 kV and 24 h and the solution feed was driven mainly by the gravity and the electrostatic forces generated during spinning. The resulting chitosan nanofibrous membranes were dried in vacuo at room temperature prior to further investigation.

2.3. Neutralization Treatments. Neutralization of the as-spun chitosan nanofibrous membranes was carried out by immersing the membranes in either 5 M NaOH or 5 M Na_2CO_3 aqueous solution for 3 h at ambient condition. "5 M Na_2CO_3 aqueous solution" was used in a loose term: since the solubility limit of the Na_2CO_3 salt in water is about 33%, the as-prepared 5 M of the solution then comprised a saturated Na_2CO_3 aqueous solution and an excess amount of Na_2CO_3-(s). After the immersion, the membranes were repeatedly washed with distilled water until neutral pH was obtained, dried at ambient condition for 1 d, and further dried in an oven at 40 °C overnight prior to further characterization.

2.4. Characterization of Pre- and Post-Neutralized Chitosan Nanofibrous Membranes. To evaluate the effectiveness of the neutralization treatments, the morphology of the as-spun chitosan nanofibrous membranes was investigated by a JEOL JSM-5200 scanning electron microscope (SEM). Five samples for each neutralization treatment were coated with gold by a JEOL JFC-1100E sputtering device for 3 min prior to SEM observation. A Nicolet Nexus 671 Fourier transformed infrared spectroscope (FT-IR) was used to verify the chemical structure of both the pre- and the post-neutralized membranes. X-ray diffraction (XRD) was also used to observe the packing nature of chitosan both before and after the neutralization treatments. X-ray diffraction was carried out on a Rigaku Rint2000 X-ray diffractometer over the 2θ range of 5−90° at a scanning speed of 5°/min.

Additionally, the physical integrity in terms of weight loss and swelling of the post-neutralized chitosan nanofibrous membranes after submersion in PBS at various time intervals [i.e., up to 12 weeks (for the weight loss measurement) or 80 min (for the degree of swelling measurement)] was investigated. The results were also compared with those obtained from chitosan films having a similar thickness. The chitosan films were cast from a chitosan solution in 1% acetic acid aqueous solution in a glass Petri dish, dried at ambient condition for 2 d, neutralized with 5 M NaOH aqueous solution for 3 h, dried at ambient condition for 1 d, and finally dried in an oven at 40 °C overnight prior to further characterization. Both the nanofibrous membrane and the film samples were submerged in PBS for 24 h at ambient condition prior to official timing of both the weight loss and the swelling measurements.

The weight loss (%) of each sample (circular disk of about 2 cm in diameter) was calculated according to the following equation:

$$\text{weight loss (\%)} = \frac{(W_{di} - W_{dt})}{W_{di}} \times 100 \qquad (1)$$

where W_{di} denotes the initial weight of the sample in its dry state prior to submersion in PBS and W_{dt} denotes the weight of the sample in its dry state after submersion in PBS for an arbitrary time interval. The swelling behavior of each sample (circular disk of about 2 cm in diameter) was assessed by gravimetric method. Each sample, after submersion in PBS for an arbitrary time interval, was taken out and placed between two pieces of tissue paper. A flat metal sheet (300 g) was placed on top of the sample to remove excess PBS. The degree of swelling (%) of each sample was then calculated according to the following equation:

$$\text{degree of swelling (\%)} = \frac{(W_{st} - W_{dt})}{W_{dt}} \times 100 \qquad (2)$$

where W_{st} denotes the weight of the sample in its wet state after submersion in PBS for an arbitrary time interval.

3. Results and Discussion

Electrospinning of 7% w/v chitosan solution in 70:30 v/v trifluoroacetic acid/dichloromethane (TFA/DCM) was relatively easy. Ohkawa et al.[15] pointed out that the successful electrospinning of the chitosan solution in TFA was likely a result of the formation of salts between TFA and amino groups along the chitosan chain,[19] causing the rigid interaction between chitosan molecules to decrease, thus improving the electrospinnability of the solution. A selected SEM image of the as-spun chitosan fibrous membranes is shown in Figure 1a. Clearly, fibers with smooth and bead-free structure were obtained. Statistical analysis of the measured diameters showed the values of 130 ± 10 nm. After consecutive spinning for 24 h, the thickness of the obtained membranes was 20 ± 3 μm. In an attempt to assess the biological compatibility with mammalian cells, these chitosan nanofibrous membranes failed to maintain their fibrous structure due to the complete dissolution of the

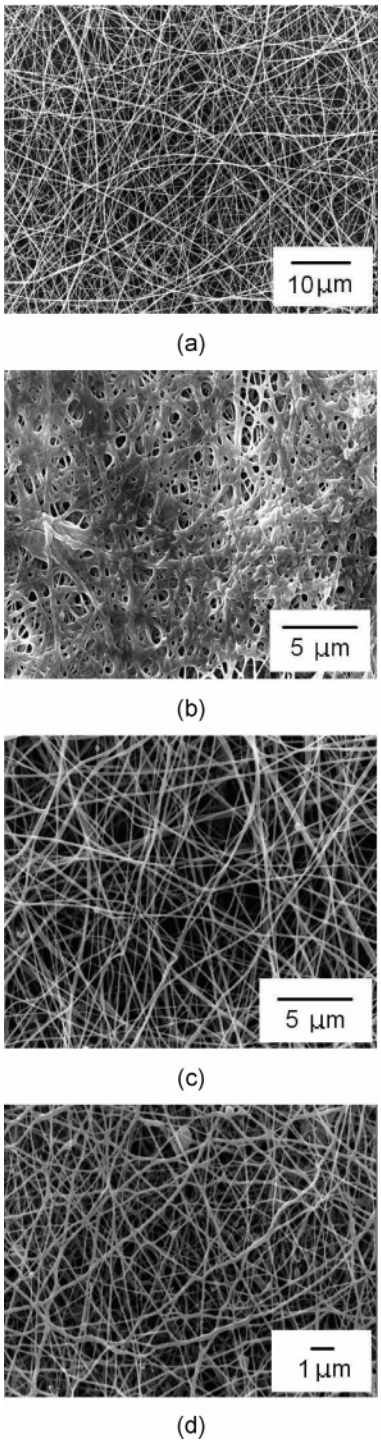


Figure 1. Selected SEM images of (a) pre-neutralized as-spun chitosan nanofibrous membrane from 7% chitosan solution in 70:30 v/v TFA/DCM, chitosan nanofibrous membrane after neutralization with (b) 5 M NaOH or (c) 5 M Na_2CO_3 aqueous solution, and (d) chitosan nanofibrous membrane in panel c after submersion in PBS for 12 weeks.

fibers when they came into contact with PBS or even the sterilized 70% ethanol solution (see additional experiment in the Supporting Information).

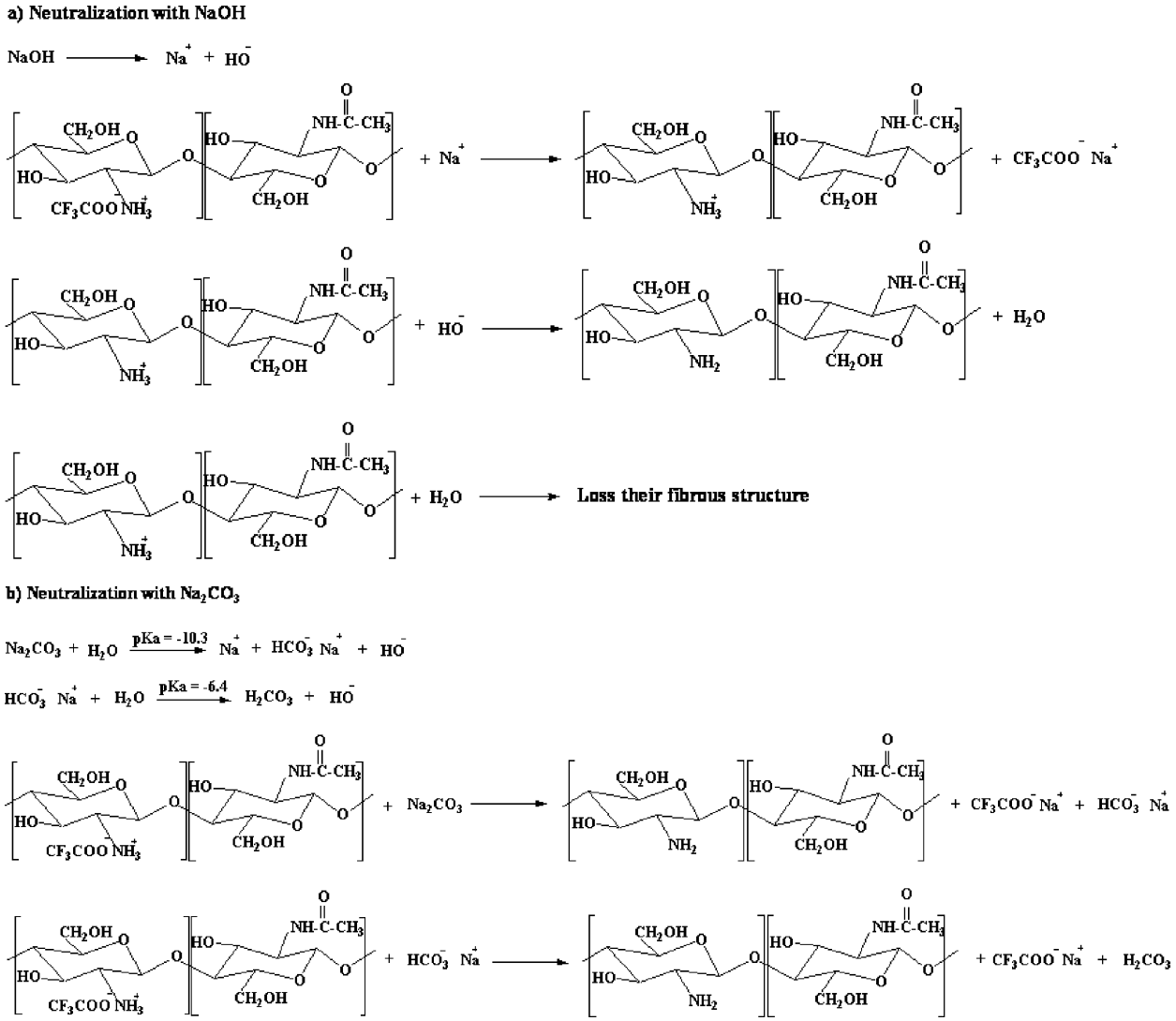
Upon the dissolution of chitosan in TFA, the formation of salts between TFA molecules and the amino groups of chitosan is thought to occur in two sequential steps: (1) protonation of the amino ($-NH_2$) groups along the chitosan chains and (2) ionic interaction between the protonated amino ($-NH_3^+$) groups and trifluoroacetate anions[19] and these salts are readily soluble in an aqueous medium. To fully utilize the as-spun chitosan nanofibrous membranes in applications that require a contact with an aqueous medium, it is necessary to explore a possibility to overcome the dissolution problem. The most logical way is through deprotonation of the amino groups through a treatment in an alkaline solution.[19]

Figure 1b shows a selected SEM image of a chitosan nanofibrous membrane that was treated in 5 M NaOH aqueous solution for 3 h. Evidently, even after the chitosan nanofibrous membrane was neutralized with the NaOH aqueous solution, its initial fibrous structure (see Figure 1a) was lost (see additional experiment in the Supporting Information). Scheme 1 delineates the reactions that might occur during the neutralization of $-NH_3^+CF_3COO^-$ salt residues along the chitosan chains with NaOH(aq). As soon as the chitosan nanofibers were in contact with NaOH(aq), the salt residues dissolved, leaving $-NH_3^+$ groups on the chitosan chains. Some of these groups would be deprotonized with $-OH$ ions to leave $-NH_2$ groups on the chitosan chains, whereas others would become hydrated. Based on this postulated mechanism, chitosan nanofibers would become either partially or completely dissolved after neutralization with a NaOH solution, depending mainly on %DD and molar mass of chitosan, concentration of the NaOH aqueous solution, and diameters of the as-spun chitosan nanofibers.

Further improvement in the neutralization treatment of the as-spun chitosan nanofibrous membranes was made by submerging the membranes in 5 M Na_2CO_3 aqueous solution for 3 h. Figure 1c shows a selected SEM image of such a membrane. Apparently, the nanofibrous structure of the membrane was intact after such a treatment (see additional experiment in the Supporting Information). The reactions that might occur during the neutralization of $-NH_3^+CF_3COO^-$ salt residues with Na_2CO_3(aq) on the chitosan chains are also described in Scheme 1. Similarly, as soon as the chitosan nanofibers were in contact with Na_2CO_3(aq), the salt residues dissolved to leave $-NH_3^+$ groups on the chitosan chains. Deprotonation of the $-NH_3^+$ groups would occur very rapidly such that the detached proton would react with CO_3^{2-} ions to become HCO_3^- ions. In addition, the detached proton can further react with HCO_3^- ions to finally obtain carbonic acid, H_2CO_3. Due to the excess amount of Na_2CO_3(s) in the as-prepared solution, neutralization of the salt residues can continue until no residues are available (i.e., complete neutralization). Indeed, the post-neutralized chitosan nanofibrous membranes still maintained their nanofibrous structure even after being submerged in PBS for 12 weeks (see Figure 1d). Though not shown, a similar result was also observed on the post-neutralized membranes that were submerged in distilled water for the same period.

Figure 2 shows the FT-IR spectra of the as-spun chitosan nanofibrous membranes before and after neutralization with the Na_2CO_3 aqueous solution in comparison with that of the as-received chitosan powder. The characteristic absorption peaks of the pre-neutralized chitosan membrane were observed at 1675 and 1530 cm^{-1}, corresponding to the stretching of the protonated amino ($-NH_3^+$) groups. Evidently, the presence of the large absorption peak at 1675 cm^{-1} and the three absorption peaks around 840−720 cm^{-1} are indicative of the presence of trifluoroacetic acid in chitosan nanofibers as amine salts.[19] On the other hand, the post-neutralized chitosan nanofibers and the as-received chitosan powder exhibited strong absorption peaks at 3300 and 3400 cm^{-1}, corresponding to the stretching of the amino ($-NH_2$) groups.[17] Evidently, FT-IR results confirmed the regeneration of the amino groups after the treatment. An improvement in the packing ability of chitosan molecules in

Scheme 1. Possible Chemical Reactions that Occur during Neutralization of As-Spun Chitosan Nanofibrous Membranes with (a) 5 M NaOH or (b) 5 M Na_2CO_3 Aqueous Solution



the fibers after neutralization was also supported by XRD analysis (see the Supporting Information), in which the regeneration of the amino groups after neutralization that resulted in the reestablishment of intermolecular hydrogen interaction between chitosan molecules improved the molecular packing.

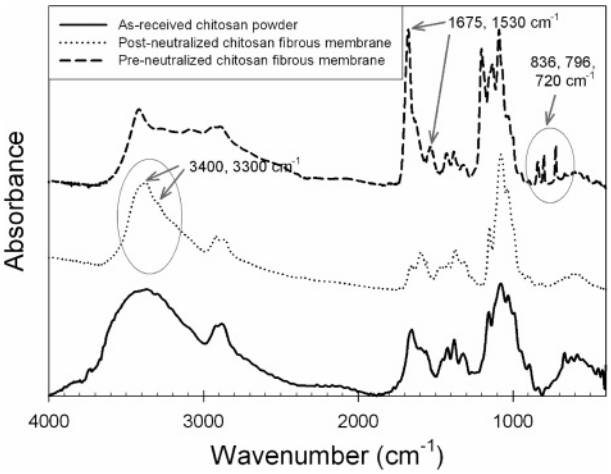


Figure 2. FT-IR spectra of as-spun chitosan nanofibrous membranes before and after neutralization with 5 M Na_2CO_3 aqueous solution for 3 h in comparison with that of as-received chitosan powder.

The effect of neutralization on both the weight loss and the swelling behavior of the as-spun chitosan nanofibrous membranes was also investigated (see Figures 3 and 4, respectively). Without the treatment with the Na_2CO_3 aqueous solution, the as-spun membranes dissolved completely in PBS and distilled water almost instantaneously. After the treatment, the loss in the weight of the fibrous membrane samples submerged in PBS increased very rapidly during the first three weeks and increased gradually until it leveled off after about 6 weeks. For comparison, the loss in the weight of the solution-cast film samples (thickness $= 21 \pm 2$ μm) also increased very rapidly during the first three weeks in submersion, but, after three weeks, no significant change was observed. After 12 weeks, the loss in the weight of the film samples was about half of that of the fibrous samples (i.e., 9 versus 16%, respectively), a result of the very much greater surface area of the fibrous membranes in comparison with that of the films. In analogy to the weight loss, the degree of swelling for both the fibrous membrane and the film samples increased initially with submersion period, but became unchanged after a certain submersion period. Obviously, the swelling of the fibrous membranes increased very rapidly during the first 20 min and started to level off only after 30 min, with the highest degree of swelling being about 100%. On the other hand, the swelling of the films increased monotonically, but less rapidly in comparison with that of the

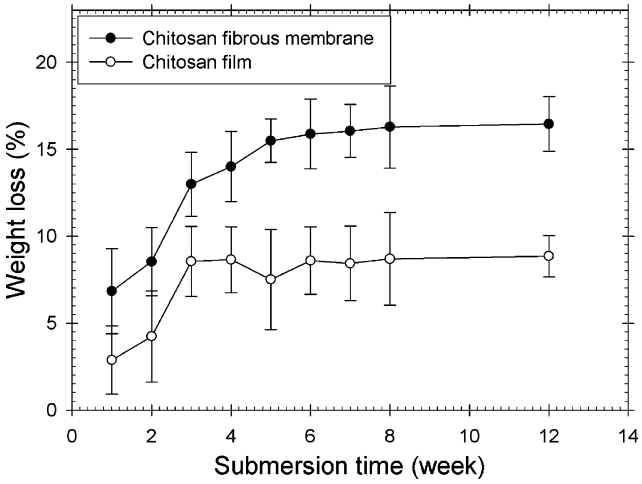


Figure 3. Weight loss in PBS as a function of submersion time for chitosan nanofibrous membranes after neutralization with 5 M Na_2CO_3 aqueous solution for 3 h and solution-cast chitosan films (neutralized with 5 M NaOH aqueous solution for 3 h).

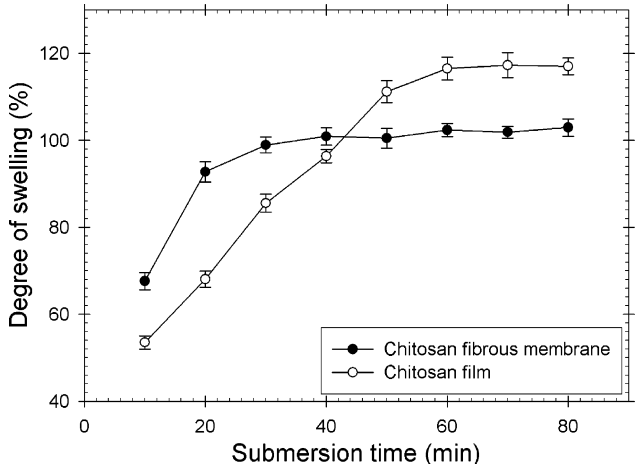


Figure 4. Degree of swelling in PBS as a function of submersion time for chitosan nanofibrous membranes after neutralization with 5 M Na_2CO_3 aqueous solution for 3 h and solution-cast chitosan films (neutralized with 5 M NaOH aqueous solution for 3 h).

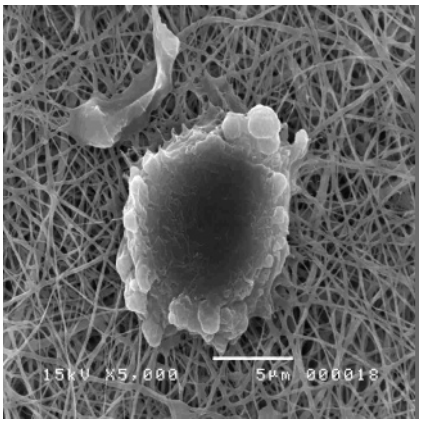


Figure 5. Selected SEM image of Schwann cell (RT4-D6P2T cell line) that was allowed to attach for 1 h on chitosan nanofibrous membrane after neutralization with 5 M Na_2CO_3 aqueous solution for 3 h.

fibrous membranes, during the first 50 min and started to level off after about 60 min, with the highest degree of swelling being about 115%.

As an actual example, Figure 5 shows a selected SEM image of a Schwann cell (RT4-D6P2T cell line) that was allowed to attach on a post-neutralized chitosan nanofibrous membrane for 1 h. Clearly, the fibrous structure of the membrane was maintained after sterilization with 70% ethanol, multiple washing with PBS, and submersion in the culture medium. This confirms the necessity of the neutralization to further explore the usefulness of the electrospun chitosan nanofibrous membranes in biomedical and other applications.

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Supporting Information Available. Additional experiment including results in Figure I and Table I. This material is available free of charge via the Internet at http://pubs.acs.org.

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