In vitro and in vivo evaluations of a novel post-electrospinning treatment to improve the fibrous structure of chitosan membranes for guided bone regeneration

To cite this article: Hengjie Su et al 2017 Biomed. Mater. 12 015003

View the article online for updates and enhancements.

Related content
- Mechanically stable surface-hydrophilized chitosan nanofibrous barrier membranes for guided bone regeneration
  Chaoxi Wu, Hengjie Su, Anastasios Karydis et al.
- Biocompatibility, resorption and biofunctionality of a new synthetic biodegradable membrane for guided bone regeneration
  Alain Hoornaert, Cyril d’Arros, Marie-Francoise Heymann et al.
- Electrospun polycaprolactone/chitosan scaffolds for nerve tissue engineering: physicochemical characterization and Schwann cell biocompatibility
  Ena Bolaina-Lorenzo, Cristina Martinez-Ramos, Manuel Monleon-Pradas et al.
**Biomedical Materials**

**PAPER**

**In vitro and in vivo evaluations of a novel post-electrospinning treatment to improve the fibrous structure of chitosan membranes for guided bone regeneration**

Hengjie Su\(^1\), Kwei-Yu Liu\(^2\), Anastasios Karydis\(^3\), Daniel G Abebe\(^4\), Chaoxi Wu\(^5\), Kenneth M Anderson\(^1\), Najib Ghadri\(^3\), Pradeep Adatrow\(^5\), Tomoko Fujiwara\(^2\), Joel D Bumgardner\(^1\)

\(^1\) Department of Biomedical Engineering, University of Memphis, Memphis, TN, USA
\(^2\) Department of Chemistry, University of Memphis, Memphis, TN, USA
\(^3\) University of Tennessee College of Dentistry, Memphis, TN, USA
\(^4\) Jinan University, Guangzhou, People’s Republic of China

E-mail: hjsu@memphis.edu

**Keywords:** chitosan, electrospinning, nanofibrous structure, rat critical sized calvarial defect, guided bone regeneration membrane/guided tissue regeneration membranes (GBR/GTR)

**Abstract**

Electrospun chitosan membranes have been investigated for guided bone regeneration but are susceptible to swelling, dissolution, and loss of biomimetic nanofiber structure due to residual acid salts. A novel process was investigated for acidic salt removal from chitosan electrospun in 70% trifluoroacetic acid (TFA) by treating with triethylamine (TEA)/acetone and di-tert-butyl dicarbonate (tBOC) instead of the common Na\(_2\)CO\(_3\) treatment. TFA salt removal and nanofiber structure stabilization were confirmed by EDS, FTIR, \(^{19}\)F NMR and electron microscopy before and after soaking in water. Membrane degradation after 4 weeks in PBS with 100 \(\mu\)g ml\(^{-1}\) lysozyme and osteoblastic proliferation were similar between TEA/tBOC-treated and Na\(_2\)CO\(_3\)-treated membranes. A simulated surgical tear test using surgical tacks showed that the peak tensile tear strength of the TEA/tBOC-treated chitosan membranes (62.1 ± 1.9 N mm\(^{-1}\)) was significantly greater than a commercial polylactic acid (PLA) membrane (13.4 ± 0.4 N mm\(^{-1}\)), similar to one commercial collagen membrane (55.3 ± 7.5 N mm\(^{-1}\)) but lower than another commercial collagen membrane (133.9 ± 21.5 N mm\(^{-1}\)). Rat 8 mm critical-sized calvarial defects covered with TEA/tBOC-treated chitosan membranes prevented soft tissue infiltration and supported new bone growth (15.76 ± 10.28%) similar to a commercial collagen membrane (16.08 ± 10.69%) at 12 weeks based on microCT analyses. Hence our novel TEA/tBOC process significantly improved nanofiber structure and mechanical strengths of electrospun chitosan membranes as compared to Na\(_2\)CO\(_3\)-treated membranes, without affecting in vitro degradation or cytocompatibility, improved membrane mechanical properties to be greater than a commercial PLA membrane and to be in range of commercial collagen membranes and supported calvarial bone defect healing similar to collagen. Thus TEA/tBOC-treated chitosan membranes exhibit many characteristics and properties that strongly support their potential for use in guided bone regeneration.

**Introduction**

Guided bone regeneration (GBR) membranes are widely used to support the formation of new bone in periodontal, bone grafting and dental implant procedures [1]. Because of the faster growth rate of soft gingival tissues than that of bone, GBR membranes are placed between the soft tissue and regenerating bone to prevent the gingival tissues from intruding into the alveolar bone site [1, 2]. Current GBR membranes are made of expanded polytetrafluoroethylene (ePTFE), collagen or poly-lactic acid (PLA). Short-comings of these membranes include need for a second surgical procedure for the removal of ePTFE membranes, premature and unpredictable degradation of collagen membranes and acidic degradation products of PLA membranes, all of which can lead to reduced bone regeneration [3–7].
Ideally, GBR membranes should be biocompatible, biodegradable with non-toxic degradation products to avoid a second surgery for removal, have high interconnected porosity in the nanosize range to be cell occlusive, have nanofiber structure to mimic natural nanofiber structures in the extracellular matrix, and have adequate mechanical strength [8]. One way to make GBR membranes is by electrospinning, a process which applies high voltage to a polymer solution to extrude or spin the polymer into fibers. Chitosan is a natural polysaccharide used in electrospinning the GBR nanofiber membranes because it is biocompatible, biodegradable, non-toxic and has been widely used in tissue scaffolds [9–12]. While electrospun chitosan nanofiber membranes in general have good biocompatibility and biodegradation rates over clinically relevant time frames and provide support for newly forming bone, the mechanical properties and handleability are inferior compared with other commercial membranes [2, 9–15]. In some reports, the low mechanical properties/handleability of chitosan membranes were attributed in part to the thinness of the electrospun membranes as compared to thicker commercial membranes [13–15].

Trifluoroacetic acid (TFA) is widely used as the solvent for electrospinning chitosan solutions since it provides better viscosity for electrospinning than other solvents such as acetic acid [10, 16]. TFA salts are generated with the chitosan polymer during the electrospinning process, rendering the chitosan fibers extremely hydrophilic and easily inducing swelling and dissolution in aqueous environments. Previous studies have employed saturated Na₂CO₃ and NaOH solutions to neutralize and remove the TFA salts [17, 18]. Crosslinkers such as glutaraldehyde and genipin also have been used to stabilize the membranes in aqueous environments [19–22]. However, these strategies have met with limited success in retaining the nanofiber structure and preventing fiber swelling when exposed to physiological solutions, in addition to having limited handleability or mechanical strengths [12, 13, 17].

To improve and maintain the nanofibrous structure of the electrospun membranes, a new post-electrospinning chemical treatment was proposed, consisting of a triethylamine (TEA) in an acetone wash step coupled with the blocking of chitosan amino groups by di-tert-butyl dicarbonate (tBOC) in a tetrahydrofuran (THF) solution. The TEA/acetone solution is used to remove residual acid salts from the as-spun membranes due to the strong affinity of the TEA to the TFA salts. The tBOC is used to protect amino groups and prevent swelling of the fibers to maintain the nanofibrous and nanoporous structure of the membranes when exposed to aqueous solutions.

The goal of this research was to evaluate and compare the new TEA/tBOC post-electrospinning process with a typical 5 M Na₂CO₃ (sodium carbonate) treatment for retention of nanofiber structure. In addition, membrane thickness was increased compared to electrospun membranes evaluated by Norowski et al. in order to increase general handleability and mechanical strength. Fiber morphology of the TEA/tBOC and carbonate treated membranes was determined by scanning electron microscopy (SEM) and the chemical/crystal structure evaluated by energy dispersive x-ray spectroscopy (EDS), Fourier transform infrared spectroscopy (FTIR), ¹⁹fluorine nuclear magnetic resonance spectroscopy (¹⁹F NMR) and x-ray diffraction (XRD). Mechanical strength of the TEA/tBOC treated membranes, using a mock clinical surgical tack-tear test, was measured and compared to commercial collagen and PLA membranes. Degradation of the membranes over 4 weeks was measured in vitro using a lysozyme solution. The attachment/growth of cultured osteoblastic cells to the membranes over 5 d was done to evaluate the cytocompatibility. In addition, TEA/tBOC treated chitosan GBR membranes were evaluated over 12 weeks in a preliminary rat 8 mm critical sized calvarial defect to evaluate the in vivo biocompatibility, biodegradability, barrier function and the ability of the membranes to support new bone growth compared to a commercial collagen membrane.

Materials and methods

Electrospinning

Electrospinning of the chitosan membranes was based on the method previously reported [9, 13]. Before the electrospinning, chitosan solution was prepared by mixing 5.50% (w/v) chitosan (degree of deacetylation (DDA) = 71%, molecular weight (MW) = 311.5 KDa) in 70% (v/v) trifluoroacetic acid and 30% (v/v) dichloromethane overnight. A 10 ml syringe with a 20 gauge, 3.81 cm blunt needle was filled with the solution and then placed on a syringe pump set to 15 µl min⁻¹. The needle tip was positioned a distance of 15 cm, from the collection plate. The collection plate was covered by nonstick aluminum foil and the fibers collected on the surface while rotating the plate at speed of 8.4 rpm to ensure random orientation. A power supply (Gamma High Voltage Research, FL) was used to apply a 26 kV voltage between the needle and collection plate. To increase the thickness of the membranes, three 10 ml volumes of the chitosan spinning solution were spun consecutively to produce an as-spun membrane approximately 0.7 ± 0.1 mm thick and approximately 15 cm in diameter. The electrospinning process was shielded by ventilated plexiglass box and operated at room temperature and 40%–60% moisture level.

Post-electrospinning treatment

As-spun chitosan membranes were immersed in 10% (v/v) TEA/acetone solution for 24 h under mild magnetic stirring to completely remove trifluoroacetate ions and then rinsed in pure aceton for 2 h (figure 1) The rinsing procedure was repeated two times to remove excess TEA. The salt free chitosan membranes were then soaked in a THF solution containing 0.1 g ml⁻¹ tBOC for 48 h under mild magnetic stirring at 65 °C. Membranes were rinsed with aceton for 2 h three times to fully remove unreacted tBOC and then dried between two pieces of nylon mesh in air to keep the membranes flat.
For the control group, electrospun membranes were treated by saturated Na2CO3 solution. Chitosan membranes were soaked in 5 M Na2CO3 solution for 3 h [12]. Then the Na2CO3 treated membranes were rinsed 3–4 times with distilled (DI) water. The membranes were also dried in air between two pieces of nylon mesh.

**Scanning electron microscopy (SEM)/energy dispersive x-ray spectroscopy (EDS)**

The membranes were examined before and after TEA/tBOC and Na2CO3 treatments to determine effects on size, morphology and composition of the electrospun chitosan fibers using an EVO HD15 (Carl Zeiss AG, Germany) scanning electron microscope with EDS attachment (X-MaxN, Oxford Instruments, UK). For evaluating fiber size and morphology, disc-shaped specimens (~1 cm diameter) of the chitosan membranes were attached to an SEM stub and coated with 8 nm gold-palladium. Three samples of each membrane from three different electrospun membranes were examined from 2500X to 6500X. In each sample, more than 20 fiber diameters were measured using the SEM image analysis software. EDS spectra were collected from triplicate (~1 cm diameter) samples of each membrane without gold-palladium coating to determine presence of TFA salts via the F peak in the as-spun, TEA, and TEA/tBOC treated chitosan membranes and TEA/tBOC membranes after a 12 h in water using KBr platform. Four samples of each membrane were scanned from 500 cm⁻¹ to 4000 cm⁻¹ for 32 times.

**19Fluorine nuclear magnetic resonance spectroscopy (19F NMR)**

19F NMR spectroscopy also was used to evaluate and measure the amount of TFA salt in the membranes before and after TEA/tBOC and bicarbonate treatments. 19F NMR spectra were obtained using Varian 500 MHz (470 MHz for 19F) instrument with DCI-D2O (20% DCI) as a solvent. A solution of 1% (v/v) 2, 2, 2-trifluorothanol (TFE) in the DCI-D2O solvent was used as an internal reference. A calibration curve was prepared by using the integration ratios of TFA/TFE peaks of the standard solutions containing known amount of TFA between 1 µl and 6 µl in 0.7 ml of 1% TFE/DCI-D2O. The calibration curve passed through zero with $R^2$ value of 0.999. To determine the amount of TFA in the membranes, 10 mg of each chitosan membrane was weighed, placed in a glass vial with 0.7 ml of 1% TFE/DCI-D2O solvent and cycled between 15 min sonication and 15 min heating at 60 °C for 7 h. The amount of residual TFA in the membranes was calculated using the calibration curve.

**Fourier transform infrared spectroscopy (FTIR)**

FTIR spectra were collected to evaluate the extent of TFA salt removal by the TEA treatment and the attachment of the tBOC molecule to the chitosan fibers. FTIR spectra were collected using a Nicolet 380 FTIR spectrometer (Thermo Electron Corporation). Spectra were collected for the as-spun, TEA, and TEA/tBOC treated chitosan membranes and TEA/tBOC membranes after a 12 h in water using KBr platform. Four samples of each membrane were scanned from 500 cm⁻¹ to 4000 cm⁻¹ for 32 times.

**X-ray diffraction (XRD)**

XRD was used to examine the crystallinity of the chitosan in the membrane fibers. TEA/tBOC, Na2CO3, and non-treated (as-spun) membranes were immersed
in liquid nitrogen and ground into fine powders by a mortar and pestle. Original chitosan powder was also examined. The samples were scanned in the grazing angle reflection mode of the Bruker D8 Advance (Bruker Inc., MA, USA) x-ray diffractometer. Data was collected from $2\Theta = 4^\circ$–$30^\circ$. Quadruplicate ($n = 4$) samples of each type of membrane and of chitosan powders were examined.

**Contact angle measurement**

Water contact angle measurements were used to measure the hydrophobic characteristics of the TEA/tBOC treated membranes and carbonate treated membranes. Contact angle of the water drop contacting the membrane surface was recorded by VCA optima measurement machine (AST products, INC, USA). Four samples of each Na$_2$CO$_3$ treated and TEA/tBOC treated membranes were evaluated.

**Mock surgical membrane screw-tear test**

Since GBR membranes are generally secured to adjacent bone tissues using small tacks or screws [23, 24], a mock surgical screw-tear test was used to evaluate the mechanical properties of the TEA/tBOC treated chitosan membranes, as an indicator of clinical handle-ability and compared to two commercial collagen membranes (Bio-Gide, Geistlich, USA and BioMend Extend, Zimmer Dental, USA) and a commercial PLA membrane (GUIDOR, Sunstar, USA). For the test, $10 \times 30$ mm specimen were tacked to a $7.5 \times 7.5 \times 0.5$ cm white oak board as a bone analogue [24], using the AutoTack system kit, (Biohorizons, Birmingham, AL, USA). Specimen were tacked to the wood board at a position 5 mm from the top and in the center of each specimen. The wood with the sample was positioned in the lower clamp of an Instron™ Model 4456 mechanical test frame, and the free end of the membrane was positioned in the upper clamp. The load cell used was 50 N and the extension rate was 1 mm min$^{-1}$. Maximum load was recorded in Newton (N) and then normalized to membrane thickness (TEA/tBOC membrane thickness = 0.15 mm; Bio-Gide thickness = 0.1 mm; BioMend Extend thickness = 0.3 mm; GUIDOR thickness = 0.49 mm). Triplicate samples of each type of membrane were tested. In preliminary mechanical tests using sutures, the carbonate membranes exhibited total loss of fiber structure, so were not further evaluated in the mock surgical screw-tear tests.

**Degradation**

The degradation of membranes (based on mass loss) was evaluated in PBS solution at 37°C. Membranes were cut into nine (3/time point) 3 cm$^2$ squares and soaked in the PBS solution containing 100 µg ml$^{-1}$ lysozyme, 500 I.U. ml$^{-1}$ penicillin, 500 µg ml$^{-1}$ streptomycin, and 25 µg ml$^{-1}$ Amphotericin-B. The solution was changed every 2 d. At 1, 2, and 4 weeks time points in the PBS-lysozyme solution, membranes were retrieved, rinsed in deionized water, dried for 48 h at 60°C and then weighed (g) to record the change in mass. Membranes were then returned to the PBS-lysozyme solution and incubation time periods resumed. Compared with the lysozyme level in human plasma, which is $3-8$ µg ml$^{-1}$ [25], high level was used in the experiment to accelerate degradation and magnify potential differences over the course of the experiment [9].

**In vitro cell viability and proliferation**

Ethylene oxide gas sterilized disc-shaped chitosan membrane specimens (diameter = 1 cm) were inserted in 24 well-plates for the evaluation of osteoblast growth on the membranes over 5 d. Each plate contained both the experimental (TEA/tBOC treated) and control (Na$_2$CO$_3$ treated) chitosan membranes. Membranes were rinsed in culture media three times, and then seeded with Saos-2 human osteoblastic cells (Cat. No. HTB-85, ATCC, Manassas, VA, USA) at $1.5 \times 10^5$ cells/well. Cells were grown in McCoy’s 5 A medium (Modified) mixed with 10% FBS and 500 I. U. ml$^{-1}$ penicillin, 500 µg ml$^{-1}$ streptomycin, and 25 µg ml$^{-1}$ amphotericin-B. Cell growth was measured using the Cell Titer Glo™ luminescent cell viability assay (Promega, Madison, WI, USA). The assay was based on luciferin-luciferase reaction to measure the amount of ATP, which is proportional to cell number ($n = 4$ sample d$^{-1}$). Data were reported in relative luminescent units (rlu). Cell viability and morphology were qualitatively observed by fluorescent microscopy using Live-Dead® stain (Molecular Probes, Eugene, OR, USA).

**Experimental animal model**

A rat critical sized (8 mm) calvarial defect model was used to evaluate biocompatibility, degradability, barrier function and the ability of the TEA/tBOC treated membranes to support bone growth/healing as compared to a commercial collagen membrane (Biomend Extend, lot # CDMEN13M5, Zimmer Dental, USA). The in vivo study protocol was reviewed and approved by the University of Memphis IACUC committee (protocol #0732). In this study, 20 male Sprague Dawley rats were used with half of the animals treated with TEA/tBOC treated chitosan membranes and half with the collagen membranes. All chitosan membranes (diameter = 14 mm) were ethylene oxide sterilized for 24 h at ambient temperature (Anprolene AN 74i, Anderson Products, USA). An additional 2 h venting cycle after gas sterilization was applied for degassing process. Collagen membranes (diameter = 14 mm) were cut under aseptic conditions in a biological safety cabinet (1300 series, type A2 biological safety cabinet packages, ThermoFisher scientific, USA).

For surgery, animals were first anaesthetized with isoflurane gas via a mask. Then the surgical site was shaved and cleaned with betadine and 70% ethanol. A cut around 20 mm was made along the midline over the calvarial skin from the middle of the nasal bones to the posterior nuchal line. The underlying soft tissue and periosteum was incised and pulled back to expose the calvaria. An 8 mm circular defect was made in the
Center of the exposed calvaria using a custom trephine burr and a low speed dental drill with sterile saline irrigation to limit heating. After the cranium disk was carefully dissected, one TEA/tBOC treated chitosan membrane or a commercial collagen membrane was randomly selected and placed to cover the circular defect. Then the soft tissue and periosteum were closed using polyglycolic acid (PGA) suture (Butler Schein). Radiographs were taken after the surgery. Half of the animals in each group were euthanized by CO₂ inhalation at 3 weeks and the other half at 12 weeks post implantation. Radiographs were taken again after euthanasia.

**Tissue processing, microCT and histologic evaluation**

After euthanasia, the defect site and surrounding bone were carefully dissected and placed in 10% formalin and formalin solution was completely refreshed every 24 h for 3 d. After fixing, all specimens were scanned at 15 µm on a Scanco µCT40 scanner (Scanco Medical, Bassersdorf, Switzerland) and data were reconstructed using Scanco Imaging processing software. Dataviewer software (Bruker AXS Inc.) was used to reorient the reconstructed µCT graphs so that the principal axes of the dataset were the same as the principal axes of the calvarial defect along the centraxial direction of the cylinder [26, 27]. Analyses for percent new bone volume to defect volume, new bone density and new bone surface area were carried out with Mimics Research 18.0.0.525 (Materialise NV, Leuven, Belgium).

Samples were then decalcified with hydrochloric acid/formic acid bone decalcifier (IMEB Inc.) for 72 h and cut in the sagittal direction. The sections were stained by a hematoxylin and eosin (H&E) stain. All the histology slides were graded by a blinded pathologist using a 4-point system (0 = absent, 1 = mild, 2 = moderate, and 3 = severe inflammation). A score of 0 was considered as a total absence of neutrophils, lymphocytes and macrophages. A score of 1 showed as primarily lymphocytes with very few neutrophils, as well as some minor macrophages and focal foreign body reaction. A score of 2 was characterized by greater presence of macrophages, lymphocytes and foreign body reaction, especially the foreign body response. A score 3 was typically determined by even greater numbers of macrophages, lymphocytes and foreign body reaction, especially the foreign body response. A score 3 was typically determined by even greater numbers of lymphocytes, macrophages and significant foreign body reaction with heavy abscess formation. Histology slides were observed by Nikon Eclipse TE300, and were analyzed for percent new bone and degradation of membranes by Bioquant Osteo II software (Bioquant Image Analysis Corporation).

**Statistical analysis**

Analysis of variance (ANOVA) at the 0.05 level of significance was used in the statistical analysis of fiber diameters, contact angle and membrane screw-tear strengths. Two-way ANOVA was used to analyze the degradation, cell proliferation, histological and microCT results. As appropriate, Tukey’s post-hoc tests were used to distinguish significantly different
groups. Ninety-five percent confidence intervals were calculated and used to compare inflammatory response scores.

Results

SEM/EDS

SEM images of the TEA/tBOC and Na₂CO₃ treated membranes are shown in figure 2. TEA/tBOC treated membranes exhibited fibers with diameter 330 ± 130 nm. After soaking in distilled water for 7 d, the fiber of the TEA/tBOC treated membranes showed a small but not statistically significant increase in diameter, reaching 388 ± 132 nm (p = 0.1). In comparison, the Na₂CO₃ treated membrane lost all the fibrous structure with the Na₂CO₃ treatment even without soaking in distilled water (figure 2 (d)). It is noted that after 4 weeks in water, some evidence of fiber swelling was observed with the TEA/tBOC treated membranes (figure 2 (c)).

The results from EDS analysis are shown in figure 3. A strong F peak, indicating the presence of TFA, was seen in spectra for the as-spun non-treated chitosan membranes. After TEA and tBOC treatments and after the Na₂CO₃ treatment, the peak for F was no longer detected indicating that TFA salts were removed from the spun chitosan fibers.

FTIR

The results of the FTIR analyses of the different treated membranes are shown in figure 4. The FTIR spectra revealed that the three transmittance peaks related to TFA salts at 720, 802 and 837 cm⁻¹ (C–F) disappeared after the TEA treatment. After the tBOC treatment, the peak at 1529 cm⁻¹ for the CO–NH bend between the tBOC group and the amine group on the chitosan polymer appeared in the spectra. The other peaks that appeared in the spectrum; at 1370 cm⁻¹ for the C–H bend, at 1688 cm⁻¹ for the C=O stretch of tBOC group, and at 2980 cm⁻¹ for the C–H stretch were attributed to the tBOC group.

¹⁹F NMR

¹⁹F NMR spectra are shown in figure 5. The TFA peak at −77.66 ppm was easily observed in spectra for non-treated chitosan membranes, but was barely observed in TEA, TEA/tBOC, and Na₂CO₃ treated membranes. Using the calibration curve and the mass of the membranes, it was determined that non-treated as-spun membranes contained 39.56% TFA, the TEA-treated membranes contained 1% TFA and the TEA/tBOC- and Na₂CO₃- treated membranes contained 0.41% and 0.29% TFA respectively. It is noted that amount of TFA detected in TEA-treated, TEA/tBOC-treated, and Na₂CO₃-treated membranes were near zero of the calibration and outside of calibration range.

XRD

Figure 6 shows the XRD spectra of chitosan powder and electrospun chitosan membranes before treatment, after Na₂CO₃ treatment and after TEA/tBOC treatment. In the XRD spectra, only chitosan powder showed typical crystalline peaks at 2θ = 12° and 2θ = 20° which are typical of the anhydrous and hydrated crystalline forms of partially crystalline chitosan [28]. After electrospinning, regardless of post-electrospun treatments, the peak at 2θ = 20° disappeared and only the peak at 2θ = 12° was visible, which indicated that the electrospinning caused loss in the hydrated crystalline structure of the chitosan. The loss of the hydrated crystalline form is likely due to disruption of hydrogen bonding and formation of salts between the chitosan amine and the TFA solvent during the electrospinning process. The broad anhydrous peak at 2θ = 12° remained after electrospinning and post-spining processes.
Contact angle measurement
Water contact angles on the TEA/tBOC treated membrane surface were 119.4 ± 14°. Water contact angles on the Na₂CO₃ treated membrane were 95.9 ± 10.8°. The TEA/tBOC treatment resulted in a statistically significantly greater contact angle as compared to the Na₂CO₃ treated membranes \((p = 6.7 \times 10^{-5})\).

Mock surgical membrane screw-tear test
The results of the mock surgical screw-tear test were shown in figure 7 in which the maximum tear loads were normalized to membrane thickness. Analysis of variance indicated there were significant differences in the tear strengths of the membranes \((p = 0.0001)\). Tukey’s post hoc analysis indicated that the Bio-Gide collagen membrane had the highest tensile tear strengths \((133.9 \pm 21.5 \text{ N mm}^{-1})\), followed by the TEA/tBOC-treated chitosan \((62.1 \pm 1.9 \text{ N mm}^{-1})\) and BioMend Extend collagen \((55.3 \pm 7.5 \text{ N mm}^{-1})\) membranes which were not statistically different from each other, and then the PLA membrane \((13.4 \pm 0.4 \text{ N mm}^{-1})\) which exhibited the lowest tear strengths.

Degradation
There was no significant difference between degradation of the TEA/tBOC and Na₂CO₃ treated membranes as measured by percent change in mass \((p = 0.8)\). Membrane mass was reduced significantly over time from baseline to 4 weeks for both membrane types tested \((p < 0.001)\).
In vitro cell viability and proliferation

Osteoblast growth on the TEA/tBOC treated and the Na₂CO₃ treated membranes are shown in figure 9(a). ANOVA analysis indicated that there were significant differences in the number of cells over time \( (p = 0.0001) \) and between the types of membranes (TEA/tBOC versus Na₂CO₃) \( (p = 0.02) \), but no interaction between type of membrane and test
condition \((p = 0.8)\). Post-hoc analyses based on rlu, indicated cell numbers significantly increased from day 1 to 3 and day 3 to 5 on both types of membranes. There was a statistically lower number of cells, based on rlu measurements, on the TEA/tBOC treated membrane as compared to the Na₂CO₃ treated membranes on day 1 and day 3 \((p = 10^{-6}\) at day 1 and \(p = 4 \times 10^{-5}\) at day 3). However, by day 5, there was no statistical difference in number of cells between the two types of membranes \((p = 0.1)\). Viability staining revealed high cellular viability of osteoblasts for both membrane types (figure 9(b)).
Histology evaluation
The inflammatory tissue response scores indicated that in general, there were mild to moderate inflammatory responses (scores = 1–2) to the test TEA/tBOC and collagen control membranes at both 3 and 12 weeks test periods (table 1). It was noticed that at 3 weeks, two of the collagen implants received high inflammatory scores (score = 3), though there was no evidence of infection or other complications. There was no significant difference based on confidence intervals in inflammation scores between the two groups at either time point.

At 3 weeks, both membranes exhibited typical healing response with new bone beginning to form at edge of defect (figure 10). By 12 weeks’ radiographs revealed some increase in defect radiopacity and histological sections revealed new bone forming along the membranes to bridge the defect with both types of membranes (figures 11(a) and (b)). Data from histomorphometric and microCT measurements are shown in table 2. Both histomorphometric and microCT analyses indicated there was significant increase in percent new bone volume in defect space from 3 to 12 weeks for both types of membranes but there was no difference between the membranes. While there are differences in values between histomorphometric measurements and microCT which are due to differences in measuring methodologies, both are in agreement regarding the increase in bone in defects over time. Histomorphometric analyses indicated that the percent of the membranes in the defect area decreased significantly from 3 to 12 weeks suggesting degradation/resorption of the membranes but no statistical differences were detected. The ratio of the surface area to new bone volume decreased significantly from 3 to 12 weeks for both types of membranes but there were no differences between the membrane types. There were no differences over time or between groups for the microCT density values.

Discussion
The objective of this work was to evaluate a novel post-spinning treatment for removing acidic salts from electrospun chitosan GBR membranes compared to a typical carbonate treatment. Comparison was based on nanofiber structure, degradation, mechanical properties and cytocompatibility and in vivo tissue
responses. The in vivo rodent critical sized calvarial defect model was also performed to evaluate the biocompatibility and barrier function of the treated chitosan membrane as compared to a commercially available collagen GBR membrane.

Chitosan membranes were made by electrosprining three 10 ml volumes of chitosan solution to make thicker membranes as compared to those previously made by Norowski et al [9]. While membranes may have been produced by electrosprining a single 30 ml chitosan solution directly, the electrosprining process of chitosan requires close monitoring to ensure constant spinning process and the larger volumes would have required an undue extended time for monitoring. While sequential spinning to build up the membrane in layers added time to the overall process, the ability to monitor the spinning process in shorter time intervals was judged as an advantage. The SEM examination of torn edges of the sequentially spun TEA/tBOC treated membranes from the membrane screw-tear test did not exhibit any layering effect and the fibers throughout the membrane thickness were observed to be of similar nanometer diameter size. This indicated that electrosprining a thicker membrane using three sequential solutions did not result in distinct layers nor altered inner fibrous structure of the membrane.

In this work, TEA was used to remove TFA salts and prevent loss of the nanofiber structure of the electrospun chitosan fibers in physiological environments. Since chitosan is not dissolved in organic solutions, the treatment was undertaken in organic solvents (acetone/THF) to prevent swelling by water. Removal of the TFA salts formed with chitosan polymer during electrosprining requires a strong base. TEA was chosen because TEA has a pKa around 11, which is greater than that of the amino groups of chitosan (pKa ~ 6.5) and thus forms a stronger salt with the TFA than chitosan. Since the TEA-TFA salts are soluble in acetone, the TFA was leached out of the chitosan and bound to the TEA before rinsing with acetone. This was demonstrated by the disappearance of the TFA peaks at 720, 802 and 837 cm⁻¹ in the FTIR spectrum and the disappearance of the F peaks in the EDS spectra after TEA treatment. However, NMR analyses which is more sensitive in detecting lower concentrations of compounds than FTIR and EDS, indicated that about 1% TFA still remained in the TEA-treated chitosan membranes. Even though a very low amount of TFA was detected in the membranes by NMR, the results of all three techniques are in agreement indicating that essentially almost all of the TFA was removed by the TEA treatment. Even though 99% of the TFA was removed by the TEA treatment, swelling and loss of nanofiber structure still occurred in aqueous solutions. This is because after removal of the TFA, the chitosan amines are essentially free amines (i.e. base) and will become immediately protonated and cause the fibers to swell in aqueous environments.

In contrast, Chen et al demonstrated the disappearance of the TFA peaks from a series of electrosprun silk fibroin–chitosan composite fibers via FTIR and XPS after treatment in a 7% (v/v) ammonia solution/75% (v/v) ethanol solution, and retention of the fine fibrous structure in aqueous environments [29]. In this case though, the ammonia–ethanol solution is deprotonating the chitosan amines and inducing strong hydrogen bonding in the chitosan which prevents protonation of the amines by water. Chen et al also used a high DDA chitosan (98%) which also contributed to the spun fibers having significant crystallinity as indicated by large peaks at 2θ = 20° in their XRD spectra [29]. The highly crystallinity coupled with the extensive deprotonization/hydrogen bonding of chitosan amines would limit swelling of the fibers. Chitosan with very high DDA also show very slow degradation rates which may be too slow for GBR applications [30]. In fact, Chen reported no loss of fiber or membrane structure over 54 d in cell culture medium, suggesting very little degradation of the membranes [29]. Instead of using high DDA

---

### Table 2. Histomorphometry and microCT results for bone healing with membranes in 8 mm rat calvarial defects at 3 weeks and 12 weeks. (n = 5/group/time point).

<table>
<thead>
<tr>
<th></th>
<th>Collagen membranes (BioMend Extend)</th>
<th>Chitosan membranes (TEA/tBOC treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>12 weeks</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>12 weeks</td>
</tr>
<tr>
<td><strong>Histomorphometry</strong></td>
<td>% new bone volume/defect volume</td>
<td>% membrane/defect area</td>
</tr>
<tr>
<td></td>
<td>4.0 ± 5.3**</td>
<td>24.8 ± 6.5**</td>
</tr>
<tr>
<td></td>
<td>14.1 ± 14.1**</td>
<td>36.6 ± 16.2**</td>
</tr>
<tr>
<td></td>
<td>% membrane/defect area</td>
<td>42.4 ± 33.1**</td>
</tr>
<tr>
<td></td>
<td>15.4 ± 12.1**</td>
<td>30.8 ± 30.1**</td>
</tr>
<tr>
<td></td>
<td>9.3 ± 12.7**</td>
<td></td>
</tr>
<tr>
<td><strong>MicroCT analysis</strong></td>
<td>% new bone volume/defect volume</td>
<td>% membrane/defect area</td>
</tr>
<tr>
<td></td>
<td>5.0 ± 2.8</td>
<td>5.3 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>16.1 ± 10.7</td>
<td>15.8 ± 10.3</td>
</tr>
<tr>
<td></td>
<td>Density (g ml⁻¹)</td>
<td>1.6 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>1.6 ± 0.04</td>
<td>1.6 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>New bone surface area/new bone volume (mm⁻¹)</td>
<td>33.3 ± 13.8</td>
</tr>
<tr>
<td></td>
<td>17.1 ± 2.6</td>
<td>20.2 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>15.1 ± 2.7</td>
<td></td>
</tr>
</tbody>
</table>

*Note:* letter super scripts were used to indicate statistical difference (α = 0.05) over time for each membrane. (e.g. ‘a’ and ‘b’ indicate significant differences in new bone volume between 3 and 12 weeks for BioMend Extend membrane. Similarly, ‘a’ and ‘b’ indicate significant differences in new bone volume between 3 and 12 weeks for the TEA/tBOC treated membrane.)

Symbol super scripts were used to indicate statistical difference (α = 0.05) between membranes at each time point. (e.g. ‘x’ indicates no significant differences in new bone volume between the collagen membrane and the TEA/tBOC treated membrane at 3 weeks. Similarly, ‘□’ indicates no significant differences in new bone volume between 3 and 12 weeks for the TEA/tBOC treated membrane.)
chitosan, a relatively low DDA chitosan (71%) was used in the current work, which has less crystallinity and increased degradation rates as compared to typical 98% DDA chitosan [9]. Thus, generation of free amines coupled with low crystallinity of the chitosan fibers explain why, the TEA treatment alone did not prevent swelling and loss of nanofiber structure. Changing the TEA wash periodically and or increasing TEA wash time may be investigated as additional strategies to further reduce the amount of TFA remaining in the membranes.

Since tBOC is commonly used in organic reactions to protect amino groups [31–33], tBOC protection of chitosan amino groups after TEA treatments, was used to reduce the overall free-amines and form a hydrophobic wrap on the surface of the chitosan to prevent swelling. The protected amino groups of TEA/tBOC treated chitosan membranes prevented swelling of the fibers up to 4 weeks in water. Additionally, NMR analyses also indicated a further reduction of TFA to a trace level of 0.4% with tBOC reaction step. This reduction is likely due to the additional washing steps used perform the tBOC reaction. Moreover, since tBOC contains large butyl groups which are hydrophobic, their presence on the chitosan fibers resulted in increased water contact angle and a more hydrophobic surface. The increase of the hydrophobicity due to the tBOC on the chitosan provided an additional mechanism for inhibiting fiber swelling in aqueous environments. The appearance of peaks at 1529, 1370 and 1688 cm$^{-1}$ in the FTIR spectrum is characteristic of the N-tert-butyloxycarbonylation reaction and the tBOC protection of the chitosan amines [34]. These peaks are at similar positions to those reported by Ju et al where tBOC was used to protect amine groups of 3-amino-1-propanol [34]. Effectiveness of the TEA/tBOC process is also supported by SEM examinations of membranes pre and post treatments, which showed retention of nanofiber morphology, up to 4 weeks in water. By comparison, the typical Na$_2$CO$_3$ treatment, which is typically used to remove TFA, showed complete lack of nanofiber morphology after treatment even though TFA was found only at a trace level [9]. This is most likely due to the presence of water that is used in the bicarbonate process since the removal of the TFA and deprotonization of the chitosan amines by the base may not occur fast enough to prevent swelling. Thus the tBOC process is necessary since simply removing TFA salts using TEA was insufficient to prevent swelling and loss of nanofiber morphology. TEA/tBOC treated membranes were shown to be cytocompatible and degradable in in vitro cell culture, and degradation tests. These combined results strongly suggest that the TEA/tBOC treatment may be an effective means for retaining nanofibrous structure of chitosan electrospun membranes for GBR applications without affecting the general cytocompatibility and degradation properties of chitosan. Different methodologies have been used by various groups for the prevention of fiber swelling and the retention of microfibrous structure. In our experiments, TEA/tBOC treatment resulted in limited swelling and fusion of the fibers after soaking in water for up to four weeks, as evidenced by SEM imaging. This might be due in part to some hydrolysis of the tBOC from fiber surfaces. The FTIR spectra showed that the tBOC remained on the chitosan fibers for at least 12 h in water. Additional investigation is needed to evaluate the degradation of the tBOC-chitosan bond and swelling of the fibers. Since the fiber diameters of the extra cellular matrix range from 80 to 500 nm, and thinner fibers have been linked to increased biocompatibility [35], the fibrous structure we achieved was consistent with mimicking the extracellular matrix and holds promise for improving barrier membrane properties. In contrast, Norowski et al failed to achieve fiber stability after treatment with genipin in conjunction with Na$_2$CO$_3$ [9]. Volpato et al used a different approach, using NH$_4$OH to neutralize membranes and remove the TFA salts, but without success since the fibers still swelled to 1 μm after the treatment [36]. Austeró et al also tried to add several crosslinkers (genipin, hexamethylene-1,6-diaminocarboxysulphonate and epichlorohydrin) to the chitosan solution before the electrospinning in order to increase the water stability, but the nanofibrous structure was still lost after treating with 1 M NaOH to remove electrospinning salts [20]. Chen et al achieved success in removing TFA and stabilizing fibers in aqueous environments using an ammonia–ethanol solution, but the high extent of deprotonization and induced crystallinity may also limit degradation to unacceptably slow rates [29]. Wu et al successfully used fatty acid anhydrides to form hydrophobic groups on the outside of electrospun fibers to stabilize and retain nanofibrous structure during washing steps to remove the TFA salts [37]. Both the fatty acid treatment used by Wu et al and TEA/tBOC treatments in this study resulted in maintenance of the nanofibrous structure in aqueous condition and have advantages over other methods.

The XRD diffractograms of original chitosan powder exhibited typical crystalline peaks at approximately 20 = 12° and 20°. These peaks are associated with the hydrated and anhydrous crystal structures of the chitosan polymer [28]. The anhydrous crystal peak was lost in the electrospinning process indicating a significant disruption in polymer crystallinity, attributable to the strong TFA acid used to disrupt chain packing and make chains highly charged to facilitate the electrospinning process. Zhang et al reported a similar loss of the 20 = 20 after electrospinning [38]. Zhou et al also observed similar patterns with our data, as shown by the XRD diffractograms of the electrospun chitosan membrane [39]. In both the Zhang et al and Zhou et al studies, swelling and loss of fiber structure of the as-spun materials in aqueous solutions was a problem [38, 39]. Decreased crystallinity is associated with more open molecular structure, resulting in increased degradation, less resistance to swelling and reduced mechanical properties strength [9]. Though we observed a similar loss in crystallinity of the electrospun membranes,
the TEA/tBOC process overall prevented swelling by increasing hydrophobic properties.

Clinically, surgical tacks are a common method used to secure GBR membranes to bone during treatment [23, 24]. The mock screw-tear test using surgical tacks provided a means to evaluate handle-ability of the membranes, based on tearing strengths under simulated clinical procedures. The tearing strength of the TEA/tBOC treated membranes was comparable and not significantly different from the commercial collagen membrane BioMend Extend, but was lower than that of the commercial collagen membrane Bio-Gide, but well above those of the commercial PLA membrane. These results indicate that the TEA/tBOC treated chitosan membranes have mechanical properties well within the range other of commercial membranes. The increased strength of the Bio-Gide as compared to the other membranes may be due to its bi-layered structure which involves a dense collagen layer [40], though it is not clear if this high strength is really necessary for GBR applications. The TEA/tBOC process resulted in a nanofiber structure of the chitosan membranes that is more similar to the fibrous structure of the BioMend Extend membrane1 and may be why they exhibited similar tear strengths. In contrast, the tearing strength of the PLA GUIDOR membrane was approximately 22% of that of the TEA/tBOC and BioMend Extend and 10% of the Bio-Gide. The lower strength of the PLA membrane may be due to its more open net-like structure. Nevertheless, these mechanical tear results suggest that the TEA/tBOC treated membranes would function favorably in providing a secure barrier for bone graft materials by anchorage on bone with surgical tacks.

Previous studies have used sutures instead of surgical tacks in order to assess tearing strength. The tearing strength of Na$_2$CO$_3$ treated membranes electrospun from 10 ml chitosan was less than 10 N mm$^{-1}$ [13]. The effect of increasing the thickness of Na$_2$CO$_3$ treated membrane by spinning from 30 ml of electrospinning solution (as used in this study) was also assessed by a pilot study using the same suture tear test method, and resulted in approximately 8-fold increase in suture tear strength. This result indicated a significant benefit of increasing membrane thickness for membrane mechanical properties and handle-ability improvement. Additionally, the suture tearing strength of TEA/tBOC treated membranes was compared to Na$_2$CO$_3$ treated membranes. For these thicker membranes, there was no difference between their suture tear strengths (TEA/tBOC = 19.6 ± 3.4 N mm$^{-1}$; Na$_2$CO$_3$ = 20.1 ± 7.3 N mm$^{-1}$). Since the increase in suture tearing strength of Na$_2$CO$_3$ treated membranes also resulted in a formation of a more solid film and loss of the critical fibrous structure, Na$_2$CO$_3$ treated membranes were not included in subsequent evaluations by screw-tear testing.


The in vitro cell growth study using an osteoblastic cell line indicated that the TEA/tBOC treatment had little to no effect on the cytocompatibility of the electrospun chitosan membranes as compared to the Na$_2$CO$_3$ treated membrane controls. The lower growth of the osteoblastic cells at days 1 and 3 on the TEA/tBOC treated membranes is likely due to the increased hydrophobic character as compared to the carbonate treated materials as indicated by water contact angle measurements and not to any residual TFA since both types of membranes contained similar trace levels TFA. Though the 0.4% TFA residue in the TEA/tBOC treated membrane seems to be not responsible for initial low cell growth, further investigation may be needed to confirm that these trace levels of TFA are do not affect cell growth. The increased hydrophobic character may at least initially result in a slower rate of cell attachment to the membranes as compared to the carbonate treated membranes, since cells grow better on hydrophilic than hydrophobic surfaces. Interestingly, there was no difference in cell attachment between membranes by day 5. A possible explanation that required further investigation is that after a period of time, proteins from the culture media eventually absorbed on to the chitosan fibers, resulting in improved cell attachment and growth on the tBOC treated membranes. The high cell viability and normal morphology were confirmed visually via the live/dead stain (figure 9(b)). It is noted though that the actual growth of cells on these membranes may not necessarily be critical to their success since the main purpose of the membranes is to provide a barrier to prevent gingival cells from invading into the regenerating-bone space.

It has been suggested that GBR membranes degrade slowly over 4–6 months to provide protection to the regenerating bone [9]. The in vitro degradation of both the TEA/tBOC treated and the Na$_2$CO$_3$ treated membranes were not different, and exhibited between 43–53% mass lost over 4 weeks. This reduction in mass is similar to a previous in vitro study using one layer Na$_2$CO$_3$ treated membranes in which membranes exhibited degradation profile predictive of the 4–6 months time frame [9]. Thus these results indicate that the TEA/tBOC treatment did not have a significant effect on the chitosan membrane degradation rates. This slow degradation rate was also observed in the rat calvarial model with the TEA/tBOC membranes still present and intact at 12 weeks and without being penetrated by soft tissues as observed histologically. While the collagen membrane as also still present and intact at 12 weeks, concerns have been raised over the unpredictable and premature degradation of the collagen membranes [7, 12]. The faster degradation of the collagen membranes was observed in an in vivo rat subcutaneous model, in which the collagen membranes (BioMend Extend) were completely resorbed within 12–16 weeks as compared to Na$_2$CO$_3$ treated membranes which lasted over 20 weeks [12]. These results suggest that the TEA/tBOC treated membranes have
to the Na₂CO₃ treated membrane. For a pilot animal porous structure and similar other properties compared to the Na₂CO₃ treated membrane. For a pilot animal study, using the BioMend Extend membrane as a control group after it was applied for the same purpose in an earlier rat subcutaneously implant study [12], was in order to evaluate the GBR application potential of the TEA/tBOC membrane in the available condition. From table 1, the collagen membrane and TEA/tBOC treated membranes caused only mild or moderate inflammation during the healing process, demonstrating that the membranes were biocompatible. Table 2 showed that both histomorphometry and microCT results had significant new bone growth after 12 weeks. There was a trend showing more new bone growth with TEA/tBOC treated membranes than with collagen membranes in the histomorphometry analyses, although there was no difference on microCT analysis. While there was no statistically significant difference between the membranes with either methodology, histomorphometry may be more sensitive in detecting immature partially mineralized bone, while microCT detects mature mineralized bone tissue. After 12 weeks, new and old bone integrated together making it difficult to identify the edge of the original defect. Trichrome staining was used on several samples for enhanced visualization (figure 11(c)). The histology photomicrographs with the trichrome stain also showed that new bone and old bone integrated together and obscured the original defect edge after 12 weeks. The percentage of new bone surface area to new bone volume which is an indicator of osteoid activity, significantly decreased from 3 to 12 weeks for both membranes, also indicating that bone formation was occurring. Jung et al conducted a similar animal study using an 8 mm diameter calvarial rat model and a chitosan film membrane [41]. After euthanizing the rats at 2 and 8 weeks, the defect margin and the new bone area were measured with the results showed approximately 3 mm of defect closure after both 2 and 8 weeks, which is approximately 37.5% of the new bone to the defect in linear distance, indicating that there was no significant increase in the bone healing from 2 weeks to 8 weeks. Comparison using these results is difficult since it is unclear what the type of chitosan was used (e.g. DDA and MW) or how their membranes were made. Even so, TEA/tBOC treated membranes showed a marked increase in bone volume/filling between 3 and 12 weeks, which suggest an advantage of nanofibrous structure over the non-fibrous membranes. Another similar animal study was done by Park et al with collagen membranes (OSSIX™, OSSIX PLUS) and 8 mm diameter calvarial defect [42]. Results of Park et al showed that the bone regeneration was around 50% after 12 weeks in rats with collagen membranes. The reason that TEA/tBOC treated membranes did not show more bone growth may be due to differences in surgical technique, which appears to be supported by the large standard deviation of the new bone growth results. Some rats had new bone growth nearly filling the defect area, while some only generated little new bone even after 12 weeks. Nevertheless, these initial in vivo results support the potential of the TEA/tBOC membranes for use in GBR applications and further study.

**Conclusion**

The use of a novel TEA/tBOC treatment of electrospun chitosan fiber membranes improved the retention of the membrane nanofiber morphology in aqueous environments. The significance of this is that the new post-electrospinning treatment did not affect the in vitro compatibility and degradation rate of the electrospun membranes as compared to typical carbonate treated membranes but because of the improved nanofiber morphology, did significantly improve the tearing strengths over previous electrospun membranes. Moreover, the improved nanofiber morphology increased tearing strength to be comparable or significantly superior to other commercially available collagen and PLA membranes. The pilot animal study confirmed the biocompatibility, barrier function, and biodegradability of the TEA/tBOC treated membranes and the ability to support bone formation. The results of this study indicate that the TEA/tBOC treated membranes have much potential for use in GBR applications and in overcoming shortcomings of current GBR devices.

**Acknowledgments**

This project was funded by the Biomaterials Applications of Memphis (BAM) Laboratories in the Biomedical Engineering Department at the University of Memphis and by grants from FedEx Institute of Technology, Memphis, TN USA and NSF (Major Research Instrumentation CBET-1337676). The authors would like to thank Dr John L Williams for providing assistance in using the M Imics software system for performing the microCT analyses and Dr Karyl K Buddington, DVM for performing the animal surgery.

**References**


dimensional poly-lactic-glycolic acid (PLGA) scaffold


[18] Sanganos P and Sapoval P 2006 Stability improvement of electrospun chitosan nanofibrous membranes in neutral or weak basic aqueous solutions Biomacromolecules 7 2710–14


[35] Gang K and Bowlin G L 2011 Electrospinning jets and nanofibrous structures Biomicrofluidics 5 013403


[38] Wu C, Su H, Tang S and Bumgardner J D 2014 The stabilization of electrospun chitosan nanofibers by reversible acylation Cellulose 21 2549–56


[40] Zhou Y, Yang D, Chen X, Xu Q, Fu F and Nie J 2007 Electrospun water-soluble carboxyl chitosan/poly (vinyl alcohol) nanofibrous membrane as potential wound dressing for skin regeneration Biomacromolecules 9 439–54

