Electrospun Chitosan–Alginate Nanofibers with In Situ Polyelectrolyte Complexation for Use as Tissue Engineering Scaffolds

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Introduction

Polysaccharides are of great interest in the field of tissue engineering because they are naturally derived biomaterials with a chemical structure that mimics that of the glycosaminoglycans found in the extracellular matrix (ECM).1 Alginate and chitosan are two such polysaccharides that have been widely examined for their use as biomaterial scaffolds in tissue regeneration. Alginate is a linear, negatively charged, water-soluble polysaccharide comprised of α-L-guluronic acid and β-D mannuronic acid, which forms a hydrogel upon ionic crosslinking with divalent cations such as calcium.2 It has shown promise for a variety of tissue engineering applications such as skin,3,4 cartilage,3,5 bone,6–8 and nerve.5,10 Chitosan is positively charged, composed of repeating units of (1,4)-linked β-D-glucosamine and is generally soluble in solutions with a pH below 6.11 Chitosan has received much attention in tissue engineering due to its antibacterial and cell adhesivity properties.12 When blended together, alginate and chitosan can form a hydrogel due to complexation of the polyelectrolytes.13 This polyelectrolyte complex formation is dependent on parameters of the two polysaccharides and their solutions, such as pH, molecular weight (Mw), and the ratio of alginate to chitosan.14–18 Alginate is naturally nonadhesive to cells; to promote cell adhesion the material must be modified, for example by the covalent coupling of cell adhesive peptides to the polysaccharide backbone.19 Alternatively, alginate–chitosan complexed scaffolds may be utilized, which exhibit greater cell adhesivity compared to alginate-alone,20 likely due to the ability of the positively charged chitosan to adsorb serum proteins.21

Electrospinning is a technology that can create nanofibrous biopolymer scaffolds. A polymer solution is ejected...
through a charged needle toward a grounded collector. As the polymer jet is drawn to the collector, the solvent evaporates and polymer nanofibers are deposited onto the collector. Electrospinning has gained much attention in the tissue engineering field, as the resultant three-dimensional nanofibrous structures can be used as biomaterial scaffolds to support cell growth and/or to deliver bioactive agents at a site of interest. The use of nanofibrous scaffolds is intriguing, as the ECM in which cells naturally reside in vivo is also of nanofibrous structure. Additionally, electrospinning materials that are able to form polymeric complexes offer the benefit of not requiring an additional crosslinking step, which can sometimes alter the nanofiber morphology or require the use of toxic solvents or high temperatures which are often damaging to incorporated bioactive factors and cells. To our knowledge, there are very few reported instances of electrospinning polyionic complexes. Poly(acrylic acid) and poly(allylamine hydrochloride) were electrospun to form nanofibers consisting of two weak polyelectrolytes, but the resultant scaffolds had to be crosslinked at high temperatures overnight and subsequently immersed in a sodium hydroxide solution. Another group examined the electrospinning of chitosan with either poly(acrylic acid) or poly(2-acrylamido-2-methylpropanesulfonic acid), but the nanofibers were only insoluble in the pH range of 3–6, which would not allow for subsequent cell culture on these scaffolds.

Despite the promise of chitosan–alginate polyionic complexes as biomaterial scaffolds and the potential of electrospinning as a unique technique to fabricate scaffolds with defined nanoscale architecture, co-electrospinning of alginate and chitosan has yet to be investigated. Several groups have examined the use of chitosan–alginate fibrous scaffolds, but the fibers were micron-sized, which means that they have less surface area than nanofibers, and cells (which are also micron-sized) seeded on these scaffolds may not experience the same level of contact guidance as those cultured on nanofibrous structures. These micron-sized chitosan–alginate polyelectrolyte complexed fibers have been examined for gene delivery, mesenchymal stem cell encapsulation, and annulus fibrosus cell culture. Another group demonstrated the ability to form chitosan–alginate microfibers by spraying a chitosan solution into a stirred alginate solution. Alginate and chitosan crosslink very rapidly, which made our initial electrospinning attempts technically challenging, as they tended to crosslink into a hydrogel at the tip of the needle before being drawn out into nanofiber form. Further, neither alginate nor chitosan can be electrospun on their own due to lack of chain entanglements and high surface tension, respectively, but require an added high Mw polymer such as poly(ethylene oxide) (PEO) or poly(vinyl alcohol). These inert polymers have been shown to aid in the electrospinning process by decreasing the conductivity of charged polysaccharide solutions and increasing polymer chain entanglements.

In this work, we demonstrate for the first time the ability to electrospin alginate/chitosan polyelectrolyte complexed nanofibers that crosslink in situ during the electrospinning process. The nanofibers contain PEO to aid in the electrospinning process, but the PEO is subsequently easily leached from the fibers by incubation in water to leave only the complexed chitosan/alginate. The resultant scaffolds are thoroughly characterized, shown to promote both cell adhesion and proliferation, and could be useful for many tissue engineering, wound healing, and drug delivery applications.

Materials and Methods

Alginic preparation and modification

Sodium alginate powder (product name of 20/40; FMC Biopolymers) with a Mw of 196,000 g/mol as determined by SEC-MALS was lyophilized until dry. The percentage of guluronic (G) and mannuronic (M) acid in the alginate was 66% G-content and 34% M-content as determined by NMR (FMC Biopolymers). The peptide glycine–arginine–glycine–aspartic acid–serine–proline (GRGDS; Commonwealth Biotechnologies), which contains the cell-binding sequence RGD, was covalently coupled to the alginate as described previously, at a peptide density of 10 mg GRGDS per gram of alginate. Both the adhesion ligand-modified alginate and unmodified alginate were purified by dialysis for 4 days (Spectra/Per 3500 MWCO; Spectrum Laboratories), subjected to activated charcoal treatment by mixing with 0.5 g activated charcoal (50–200 mesh; Fisher Scientific) per 100 mL of 1% alginate solution for 30 min, sterilized through a 0.2 μm filter, and lyophilized until dry.

Preparation of chitosan–alginate–PEO nanofibrous scaffold

The polyionic nanofibrous scaffolds were fabricated using electrospinning from the blend of chitosan–PEO (CP) and alginate–PEO (AP) solutions; 2% (w/v) alginate and 4% (w/v) PEO (Mw 900 kDa; Sigma) were dissolved separately in ultrapure deionized water (diH2O) for 24 h at room temperature (RT). Chitosan (viscosity 20–300 cP, Mw 50–190 kDa, 75%–85% deacetylation, all as specified by the supplier; Sigma) was dissolved in 1 M acetic acid at 5% w/v in a 25 mL glass vial for 24 h at RT. Both CP and AP blend solutions with different volume ratios (20/80, 30/70, 40/60, 50/50, and 80/20) were prepared by mixing each solution with vortexing at low speed for 1 h and then rotating for 1 day at 37°C using a rotating hybridization incubator (Medel 400; Robbins Scientific).

For the polyionic nanofibrous scaffolds using water-soluble chitosan salts, the chitosan (product name of Protosan UP CL 213, viscosity 106 cP, 83.8% deacetylation, as specified by the supplier) was obtained from FMC Biopolymers; 2% (w/v) chitosan and 4% (w/v) PEO, and separately 2% (w/v) alginate (either adhesion ligand-modified alginate or unmodified alginate) and 4% (w/v) PEO were blended by vortexing at low speed for 1 h and then rotating for 1 day at 37°C using a rotating hybridization incubator. The final volume ratio of chitosan:alginate:PEO in these scaffolds after electrospinning was 15:15:70.

For electrospinning, the resulting solutions were loaded into 5 mL plastic syringes (BD Biosciences) and attached to a blunt, 5.1-cm-long, 20 gauge dual applicator tip needle (Fibrijet, SA-3615). The two 5 mL plastic syringes and dual applicator tip needle were then placed in a syringe pump (Model 22; Harvard Apparatus Inc.) and the needle was connected to the positive output of a high-voltage power supply (AU 60PO; Matsusada, Inc.). The cylindrical collector (outer diameter, 10 cm; length, 25 cm; NanoNC, Inc.) was
wrapped with aluminum foil, grounded, and located at a fixed distance of 10 cm from the needle. The flow rate of the solution, applied voltage, and spinning time were fixed to 0.02 mL/min, 13 kV, and 8 h at RT, respectively. Prepared nanofibers on the foil were dried overnight at RT. The resultant nanofibers were removed from the foil and cut into individual scaffold disks 1.2 cm in diameter using a stainless steel punch (McMaster Carr). In the case of CP or AP nanofibers, the same setup was used but only one syringe was attached to the dual applicator tip needle and the other port remained empty. For AP nanofibers that were used for testing the swelling ratio and cell proliferation, the nanofibers were crosslinked by immersion in 100 mL of 2% (w/v) CaCl₂ in a solution of water and 100% ethanol at a ratio of 1:5, respectively, for 10 s with slow shaking. The crosslinked scaffolds were rinsed with diH₂O three times to remove any residual chemicals, frozen, and lyophilized.

For PEO extraction from the chitosan–alginate–PEO (CAP) and crosslinked AP nanofibers, the fibers were incubated in 5 mL diH₂O in a 15 mL conical tube at 37°C for 5 days with gentle shaking. Each day during the extraction, the diH₂O was changed. After 5 days the nanofibers were rinsed gently twice with diH₂O, frozen at −70°C for 1 day, and lyophilized for 3 days.

Measurement of the Mw of chitosan

The average Mw and polydispersity of the water-soluble chitosan salt (Protasan UP CL 213) were determined using a Waters GPC with Alliance 2695 HPLC and 2996 photodiode array detector. The mobile phase was filtered water containing 0.075 M acetic acid (Sigma) and 0.25 M sodium acetate (Sigma). Chitosan was dissolved into the mobile phase at 1 g/mL and filtered with a 0.2 μm filter before analysis. Nine pullulan standards (Shodex Corporation) with Mws ranging from 5.8 to 1600 kDa were used to calibrate the GPC equipped with a series of two GPC columns (PL Aquagel; Polymer Labs). The instrument was maintained at 35°C and the sample flow rate was 1 mL/min. The weight average Mw was found to be 476 kDa, the number average Mw was found to be 197 kDa, and the polydispersity was 2.42. However, the use of GPC with pullulan standards may lead to an overestimation in the chitosan Mw, as previously reported.33

Scanning electron microscopy

The morphologies of the AP, CP, and CAP nanofibers were examined using a scanning electron microscope (SEM) (S-4500; Hitachi). Scaffold sections were mounted onto sample holders and coated with gold using a sputter-coater (E-1030; Hitachi). Three representative SEM pictures from each sample were taken and used to digitally measure the fiber diameters of 40 fibers from each image (120 fibers total) using an image analyzer (Image-Pro Plus 4.5).

Rheometry

Before electrospinning, the viscosity and conductivity of the solutions were characterized. Steady-state shear experiments were performed with a stress-controlled rheometer (AR2000; TA Instruments) using a 4 cm, 2° cone, and plate geometry, with the plate temperature maintained at 25°C. Shear stresses were applied from 0.05 to 200 Pa, with 10 measurements per decade. Zero shear viscosity values were obtained by averaging the first 10 points from the Newtonian regions of the viscosity profiles. Each measurement was performed at least two times to ensure repeatability within 10% error. The ionic conductivity of the solutions was evaluated with a potentiostat (Gamry Instruments), with potassium chloride (SpectroPure) as a standard.

To examine the gelling properties of the CP and AP blends, dynamic rheological measurements were performed using a stainless steel cone and plate geometry with 4° cone angle and 20 mm cone diameter (AR-2000ex; TA Instruments), again with the plate temperature maintained at 25°C. Chitosan–PEO and AP solutions were prepared as described above. Each solution was separately loaded into a 1 mL plastic syringe (BD Biosciences), and the two syringes were attached to a blending connector (Fibrijet; SA-3670). Immediately before testing, the two solutions passed through the blending connector and then through a 21 gauge needle (BD Biosciences) to be deposited onto the bottom plate of the rheometer. The total sample volume was 700 μL. Both the shear elastic storage (G’ ) and viscous loss (G″) moduli were measured every 6.5 s for the first 5 min of crosslinking. These measurements were taken at a loading frequency of 1 Hz and maximum strain of 0.002. Three trials were performed for each sample group.

Attenuated total reflectance Fourier transform infrared spectroscopy

To confirm the functional groups on the surface of CAP nanofiber mats, attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (Excalibur FTS 3000; Bio-Rad/Digilab) was used. ATR spectra were recorded at 32 scans with a resolution of 4 cm⁻¹ between 3800 and 600 cm⁻¹.

Swelling studies of polyion complexed chitosan–alginate nanofibers

The swelling ratio of lyophilized, PEO-extracted chitosan–alginate, and alginate nanofibers was obtained after incubation in diH₂O for 1 day at 37°C. After excess diH₂O was removed from the surface of the nanofiber scaffolds by gently dabbing with a KimWipe, the weights of the swollen chitosan–alginate samples were measured. The samples were then placed at −70°C for 1 day and then lyophilized for 3 days. The mass of the dried samples was then measured. The swelling ratio was calculated using the following formula:

\[
\text{Swelling ratio } (\%) = \frac{W_s - W_d}{W_d} \times 100,
\]

where \( W_s \) and \( W_d \) are the weight of samples in the swollen and dry state, respectively.

Adhesion and proliferation of MC3T3 cells on the nanofiber scaffolds

Lyophilized, PEO-extracted chitosan–alginate nanofibrous scaffolds (1.2 cm in diameter) were immersed in 70% ethanol for 1 h to sterilize, washed three times with diH₂O, and subsequently seeded with 20 μL of mouse calvarial pre-osteoblasts (MC3T3-E1 Subclone 4 [ATCC #CRL-2593] cells;
American Type Culture Collection) in α-MEM + 10% FBS, 1% penicillin/streptomycin (HyClone) at a density of 1.0 × 10^4 cells/mL on scaffolds (N = 5) placed in 24-well tissue culture plates. The cells were cultured in a 37°C humidified incubator with 5% CO₂. After 30 min, 980 μL additional medium was added. The scaffolds with cells were incubated for 4 h, gently washed with phosphate-buffered saline (PBS; HyClone) three times to remove any nonadherent cells, and then placed into a new 24-well plate with 1 mL fresh medium.

After 24, 72, and 120 h of culturing, the samples were stained using a Live/Dead staining solution freshly prepared by mixing 1 mL fluorescein diacetate (Sigma) solution (1.5 mg/mL in dimethyl sulfoxide) and 0.5 mL of ethidium bromide (Sigma) solution (1 mg/mL in PBS) with 0.3 mL of PBS. About 50 μL of Live/Dead assay staining solution was added to the cultured scaffolds and incubated at RT for 5 min. Fluorescent photomicrograph images of stained cells on the nanofibers were acquired using a fluorescence microscope (ECLIPSE TE 300; Nikon) equipped with a digital camera (Retiga-SRV; Qimaging).

For quantification of cell adhesion and proliferation, after 5, 24, 72, and 150 h of culturing the samples were transferred to a new 24-well plate, and 1 mL of a 20% CellTiter 96 Aqueous One Solution (Promega) containing 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution (1 mg/mL in PBS) with 0.3 mL of PBS. About 60 μL of Live/Dead assay staining solution was added to the cultured scaffolds and incubated at RT for 5 min. Fluorescent photomicrograph images of stained cells on the nanofibers were acquired using a fluorescence microscope (ECLIPSE TE 300; Nikon) equipped with a digital camera (Retiga-SRV; Qimaging).

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**Results**

When blended together, alginate (Fig. 1a) and chitosan (Fig. 1b) form a polyionic complex due to the interaction between the amine groups in chitosan with the carboxyl groups in alginate. This polysaccharide ionic complex is insoluble in aqueous solutions. In this study, the ability to electrospin chitosan and alginate to form polyionic complexes with nanofibrous morphology was examined. The chitosan and alginate complex with one another very rapidly, so a special dual needle was used in the electrospinning process (Fig. 1c). With this needle, the CP and AP solutions were exposed to one another immediately at the tip of the charged needle as they were ejected toward the collector drum. The majority of the solution was electrospun into nanofibers, but a portion of it gelled at the needle tip (Fig. 1c, d). However, uniform nanofibrous scaffolds composed of the ionically complexed chitosan and alginate were formed, as seen in the inset in Figure 1d. The various blending ratios of chitosan, alginate, and PEO examined are shown in Table 1.

The morphologies of the resultant nanofibers were examined using SEM. First, the morphologies of nanofibers formed using only CP or AP blends were observed in order to determine an optimal PEO concentration range that could produce uniform electrospun nanofibers with each of these
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<th>PEO concentration (wt%) (Mw = 900 kDa)</th>
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*Chitosan was dissolved in 1M acetic acid.

*Alginate and PEO were dissolved in dH2O.

Mw, molecular weight; PEO, poly(ethylene oxide).
polysaccharides (Fig. 2). For all blending ratios shown, uniform nanofibers could be obtained. If the volume ratio of PEO was decreased below 50%, it was found that all the nanofibers contained bead structures (data not shown); thus, the PEO volume ratio was maintained at 50% or higher for this study. The viscosity and conductivity of these solutions were measured to characterize the solution properties that allowed for optimal nanofiber formation. Uniform nanofibers were formed when the volume ratio of the PEO was between 50%–80%. These solutions had viscosities approximately between 0.15 and 0.7 Pa·s (Fig. 3a), indicating that this was an ideal range for electrospinning. The addition of PEO increased the viscosity of the alginate solutions but decreased the viscosity of the chitosan solutions. Additionally, it was found that solutions with more PEO exhibited lower conductivity, which resulted in the formation of uniform nanofibers during the electrospinning process (Fig. 3b).

Once it was determined that the ideal condition for electrospinning CP and AP was to maintain a PEO volume ratio >50%, alginate–chitosan polyelectrolyte complexed nanofibers were then fabricated and their morphologies examined. Chitosan and alginate were both blended with PEO, and various ratios of these solutions were used for electrospinning (Fig. 4). In two conditions, completely uniform nanofibers were obtained (Fig. 4d, e). Two others exhibited primarily uniform nanofibers with only a few beaded structures (Fig. 4b, f). In the two mixtures where the volume ratio of chitosan and alginate were not the same (CAP 104050 and CAP 401050), bead-containing nanofibers were obtained (Fig. 4a, c).

Although the PEO was required for the electrospinning, this polymer did not contribute functionality to the formed nanofibers, and thus it was desired to remove it from the fibers. To obtain nanofibers containing only alginate and chitosan without PEO, the nanofiber scaffolds were incubated in diH₂O at 37°C for 5 days to extract the water-soluble PEO. To ensure that the nanofibrous structure was still intact, the scaffolds were again examined using SEM (Fig. 5). For all blending ratios, it was apparent that the nanofibrous structure was indeed intact. The scaffolds that previously contained beaded structures within the nanofibers no longer had the beads present.

To examine the gel properties of the chitosan–alginate polyelectrolyte complexes at the various ratios used in electrospinning, their storage and loss moduli were measured over time with a rheometer (Fig. 6). In all blends, the storage modulus was always greater than the loss modulus, indicating that the polyelectrolytes complex and form a hydrogel immediately upon mixing,¹¹ CAP 104050 and CAP 401050, respectively.

FIG. 2. Scanning electron photomicrographs of electrospun CP (a–d) and AP (e–h) nanofibers. (a) CP 2080, (b) CP 3070, (c) CP 4060, (d) CP 5050, (e) AP 2080, (f) AP 3070, (g) AP 4060, and (h) AP 5050. Scale bars represent 3 μm. CP, chitosan–PEO; AP, alginate–PEO.

FIG. 3. (a) Viscosity and (b) conductivity of the CP and AP solutions at varying volume ratios of PEO in the blend. Values represent mean ± standard deviation.

FIG. 4. Alginate–chitosan polyelectrolyte complexed nanofibers were fabricated for various PEO volume ratios (a–d) CP 2080/50, (e–h) AP 2080/50. (a, e) 20/80, (b, f) 30/70, (c, g) 40/60, and (d, h) 50/50. Scale bars represent 3 μm. CP, chitosan–PEO; AP, alginate–PEO.
The two mixtures that formed bead-containing nanofibers upon electrospinning, both exhibited the highest storage moduli of any of the blends (Fig. 6a, c). The two mixtures that formed the uniform nanofibers without beads, CAP 202060 and CAP 151570, had the lowest storage moduli (Fig. 6d, e).

The average fiber diameter of each of the electrospun scaffold compositions was measured using the SEM images (Fig. 7). In all cases, the fiber diameters were larger before extraction of the PEO compared to after extraction. Additionally, in the scaffolds that did not contain beaded structures before PEO extraction (CAP 202060 and CAP 151570), the fiber diameter before extraction increased as the amount of PEO in the blend increased. The CAP 101080 only slightly increased compared to the CAP 252550. Also, although not shown in Figure 7, all of the fibers after PEO extraction had statistically significant differences in their fiber diameters, but they are all on the order of 100 nm still.

To confirm the chemical composition of the electrospun fibers, ATR FT-IR was used. The spectra of the pure components (chitosan, alginate, and PEO) were compared to those of the electrospun chitosan–PEO–alginate fibers both before and after PEO extraction. The peaks that indicate the amide and carboxyl groups of the chitosan and alginate are highlighted in Figure 8. The chitosan showed peaks at 3365 and 3302 cm⁻¹ due to the O–H and N–H stretch, and at 1654 and 1593 cm⁻¹ due to the amide bonds (Fig. 8a). The alginate showed a peak at 3327 cm⁻¹ from the O–H stretch, another at 1603 cm⁻¹ due to the antisymmetric carboxyl stretch and one at 1413 cm⁻¹ due to the symmetric carboxyl stretch (Fig. 8b). The spectra of the electrospun fibers before PEO extraction (Fig. 8d) exhibited peaks that were also present in the pure PEO sample (Fig. 8c). However, after the PEO extraction these peaks were no longer present (Fig. 8e), indicating that the PEO has indeed been successfully extracted from these nanofibers leaving only peaks corresponding to the complexed chitosan and alginate. Further, the spectra of the fibers after PEO extraction show a broad peak at 1596 cm⁻¹ and a peak at 1416 cm⁻¹ (Fig. 8e). These result from the carboxyl groups of the alginate overlapping with the signal from the chitosan, indicating the presence of both compounds.

The chitosan used in this study was not a water-soluble salt. Thus, it is expected that the nanofibers containing...
chitosan and alginate would have less swelling in an aqueous environment compared to those composed only of water-soluble alginate. The swelling ratio did decrease as the amount of chitosan increased (Fig. 9). The scaffolds containing 10% chitosan, 40% alginate, and 50% PEO by volume showed a higher degree of swelling than the other chitosan–alginate scaffolds, likely due to the higher amount of hydrophilic alginate present in these fibers.

FIG. 7. Average fiber diameter of chitosan–alginate nanofibers before and after PEO extraction in deionized water at 37°C for 5 days. Values represent mean ± standard deviation.

For use of these nanofibers in tissue engineering applications, it is important to examine how cells interact with the nanofibrous scaffolds. Alginate is naturally nonadhesive to cells. The addition of the chitosan was expected to promote cell adhesion since it is a polycation that is able to adsorb serum proteins, which can subsequently allow cells to adhere to the material. Mouse preosteoblast cells (MC3T3s) were seeded onto the surface of alginate-only nanofibers, chitosan–alginate nanofibers, and tissue culture plastic as a positive control. The cells were stained with a Live/Dead stain to assess their viability on these surfaces (Fig. 10a–i). On each of the surfaces, all of the cells...
Discussion

Electrospinning is an attractive option for the fabrication of nanofibrous scaffolds that could be used for tissue regeneration strategies. The nanofibrous structure closely mimics the structure of the native ECM in which cells normally reside in the body. The electrospinning of two naturally derived polysaccharides, alginate and chitosan, into polyelectrolyte complexed nanofibers is demonstrated. Chitosan and alginate complex very rapidly, and this rapid complexation makes electrospinning difficult as the solutions tend to gel at the tip of the needle before they form nanofibers. The system used here consists of a needle that is separated into two channels, keeping the alginate and chitosan separated until they reach the very tip of the needle as they are charged and ejected toward the grounded collecting drum. These polysaccharides must be electrospun with PEO in the solution blend, and it was found that PEO volume ratios of 50% or greater were required for uniform nanofiber formation. Additionally, the viscosity and conductivity are important parameters that influence the electrospinning, and in this system the optimal viscosity was found to be in the range of 0.15–0.7 Pa s. The opposite effect of PEO on the viscosity of these two polysaccharide solutions is interesting. The zero shear viscosity of the chitosan blend decreases with the addition of PEO in water. Since the chitosan used through most of this study was not a water-soluble salt, the solvent quality for the chitosan was reduced as greater amounts of the PEO–water solution were added. In turn, this caused the radius gyration of chitosan to decrease, reducing the entanglements in solution. For the alginate, we hypothesize that the increased viscosity with the higher amounts of PEO may be due to increased hydrogen bonding. The increased interaction of these two polymers would lead to increased viscosity. The optimal conductivity was below 4 mS/cm for chitosan–PEO and below 2.2 mS/cm for AP; solutions that had higher conductivities could not be electrospun. As expected, the addition of the uncharged PEO decreases the conductivity for both of these charged polysaccharide solutions.

The blending ratios of the chitosan, alginate, and PEO influenced the morphology of the resultant nanofibers. Only solutions with equal volume ratios of chitosan and alginate formed nanofibers without significant beaded structures throughout. Even among these solutions there were only two that electrospun entirely without bead formation (CAP 202060 and CAP 151570) and two that had few beads in the fibers (CAP 252550 and CAP 101080). This highlights the importance of examining a variety of blending ratios to optimize the nanofiber formation based on the electrospinning of these ionically complexed polysaccharides. The rheological properties of the hydrogels indicated that mechanical properties of the resultant hydrogels may strongly influence the fiber formation, as the solutions that formed hydrogels with greater storage moduli were the same ones that contained extensive bead-like structures in the electrospun nanofibers. This indicates that the electric field forces were likely unable to overcome the solid-like nature of these particular hydrogels. Regardless, after extraction of the PEO, none of the scaffold nanofibers contained any beaded structures.

Although PEO was required for electrospinning, pure chitosan–alginate nanofibers could be obtained simply by soaking the nanofibers in diH2O for several days. The chitosan and alginate formed a polyeionic complex that was insoluble in water, and the chitosan and alginate were the only components remaining after this PEO extraction, as evidenced by the FT-IR data. As a result, the nanofibers did shrink in diameter after PEO extraction, but still remained a similar size to when they were spun, on the order of 100 nm. As discussed earlier, the alginate and chitosan are of great interest as biomaterials in tissue engineering scaffolds, whereas the PEO is biologically inert. Thus, the ability to obtain nanofibers comprised of only the two polysaccharides was important.

Alginate is a polysaccharide that is soluble at neutral pH, but the chitosan employed throughout the majority of these
FIG. 10. Live/Dead staining of MC3T3 preosteoblast cells cultured for 24, 72, and 120 h on (a–c) TCP, (d–f) PEO-extracted AP 5050, and (g–i) PEO-extracted CAP 252550 nanofibers (scale bars represent 40 μm). (j) Cell proliferation of MC3T3s cultured on TCP, AP 5050, and CAP 252550 for up to 120 h, as determined by an MTS assay, which measures the cell metabolic activity. *p < 0.05. Values represent mean ± standard deviation. TCP, tissue culture plastic. Color images available online at www.liebertonline.com/ten.

FIG. 11. Scanning electron micrographs of CAP nanofibers fabricated with water-soluble chitosan salt and (a) cell adhesion ligand (GRGDSP)-modified alginate or (b) unmodified alginate. Scale bars represent 3 μm.
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studies was only soluble in an acidic solution. Since this chitosan was not a water-soluble salt, it limited the swelling of the scaffolds in aqueous solutions. As an alternative to using chitosan that is only soluble at lower pH, it was demonstrated that a water-soluble chitosan salt could instead be used. This may be useful for applications where it is desirable to encapsulate biologically active factors or cells in the nanofibers during the electrospinning. Additionally, it was demonstrated that the alginate could be modified with a peptide containing the cell-adhesive RGD sequence. Our future work will include examining whether the addition of this cell adhesion ligand and others could further promote and control the adhesion, spreading, proliferation, migration, and differentiation of cells on these scaffolds. Further, this work examined only the ability of two different chitosans to form polyionic complexes with alginate during electrosprining; in future, it will be informative to examine additional chitosans, especially with differing degrees of deacetylation, as using chitosans with different properties may affect the electrospinning process.

Even without the use of alginate containing a cell-adhesive peptide sequence, the nanofibers obtained in this study showed great potential as biomaterial scaffolds capable of supporting cell adhesion and proliferation. When pre-osteoblast cells were seeded on the nanofibrous scaffolds, those seeded on the chitosan–alginate scaffolds exhibited both greater adhesion after 5h and greater proliferation over the course of 120h compared to cells seeded on pure alginate scaffolds. This was due to the presence of chitosan, which allowed for protein adsorption and subsequent attachment and spreading of cells. Alginate is naturally nonadhesive to cells, and so they are unable to adhere or proliferate very well on this material by itself. Thus, the use of the polyelectrolyte complex enhances the utility of these scaffolds in tissue engineering applications.

Overall, this study demonstrates for the first time the ability to electrospin chitosan–alginate polyelectrolyte complexes. The optimal conditions for the electrospinning were determined, and the resultant nanofibers thoroughly characterized. These nanofibers were crosslinked in situ due to the polyionic complexation of the chitosan and alginate, and thus did not require any additional chemical crosslinking step. The nanofibrous scaffolds were able to promote the adhesion and proliferation of cells, and they offer great promise for use as scaffolds in tissue regeneration strategies. Future work will include optimization of conditions for electrospinning with water-soluble chitosan salts, the use of peptide-modified alginate to permit additional control over cell interactions with the scaffolds, and the incorporation of bioactive factors that can be delivered to guide cellular behavior for specific tissue engineering applications.

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Disclosure Statement

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