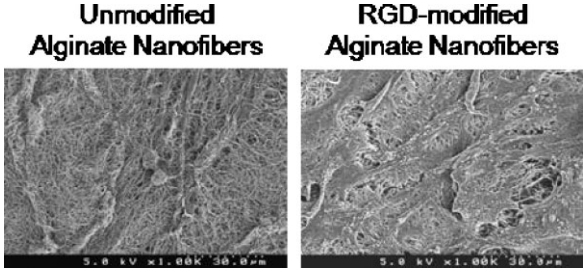
# Electrospun Alginate Nanofibers with Controlled Cell Adhesion for Tissue Engineering[a]

Sung In Jeong, Melissa D. Krebs, Christopher A. Bonino, Saad A. Khan, Eben Alsberg*

Alginate, a natural polysaccharide that has shown great potential as a cell scaffold for the regeneration of many tissues, has only been nominally explored as an electrospun biomaterial due to cytotoxic chemicals that have typically been used during nanofiber formation and crosslinking. Alginate cannot be electrospun by itself and is often co-spun with poly(ethylene oxide) (PEO). In this work, a cell adhesive peptide (GRGDSP) modified alginate (RA) and unmodified alginate (UA) were blended with PEO at different concentrations and blending ratios, and then electrospun to prepare uniform nanofibers. The ability of electrospun RA scaffolds to support human dermal fibroblast cell attachment, spreading, and subsequent proliferation was greatly enhanced on the adhesion ligand-modified nanofibers, demonstrating the promise of this electrospun polysaccharide material with defined nanoscale architecture and cell adhesive properties for tissue regeneration applications.




## Introduction

Electrospinning is a technique which produces polymer fibers with diameters on the nanoscale. This is accomplished by applying a high voltage to the polymer solution, which when ejected from a needle will extend toward a grounded collector. As the polymer jet travels to the grounded collector, the solvent evaporates and solid polymer fibers are deposited on the collector. Electrospun nanofibers are of interest in biomedical research, as they may closely mimic the nanofibrous structure of the native extracellular matrix.[1,2] Cell behaviors such as migration, orientation, and cytoskeletal arrangement can be greatly influenced by substrate topography alone without additional biochemical cues.[3] Furthermore, the scaffolds produced by electrospinning offer a high surface area to volume ratio, which would allow for increased cellular interaction with the material.[4] A variety of biomaterials have been examined using electrospinning for tissue engineering applications including synthetic and natural polymers.[4] Natural polymers have gained attention in the field of tissue engineering due to their beneficial properties such as hydrophilicity, low immunogenicity, non-toxicity, and their similarity to the extracellular matrix.[5] One

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natural biopolymer is alginate, which is derived from brown algae.[6]

Alginate is a naturally derived polysaccharide which is biocompatible and has been used extensively in a variety of tissue engineering applications, including bone,[7–9] cartilage,[9,10] skin,[11,12] and nerve regeneration.[13,14] It is a linear, unbranched polysaccharide composed of repeating units of α-L-guluronic acid and β-D-mannuronic acid.[6] Alginate can form a hydrogel upon ionic crosslinking with divalent cations such as calcium, as the cations cause the G-units on neighboring polysaccharide chains to interact.[6] This gentle method for crosslinking, combined with the hydrophilic nature of the alginate, allows cells to be encapsulated in the hydrogels with nominal loss of viability. However, alginate is not adhesive to cells, and does not readily adsorb serum proteins due to its hydrophilic nature. Therefore, a method for covalently coupling peptides with a cell adhesive sequence to alginate was previously developed.[15] Regulating how cells interact with alginate by modification with cell adhesion ligands can enhance tissue growth; for instance, it has been shown to increase the osteogenic response of pre-osteoblasts and osteoblasts cultured within peptide-modified alginate hydrogels both in vitro and in vivo.[8,9,15,16]

Despite the promise of alginate in tissue engineering, it has been only minimally investigated in electrospinning. Alginate cannot be electrospun by itself, likely due to a lack of chain entanglements.[17] However, by blending the material with other water-soluble biocompatible polymers, such as poly(ethylene oxide) (PEO) or poly(vinyl alcohol) (PVA), nanofibers can be obtained by electrospinning.[18–22] Previous work that blended PEO or PVA with alginate relied on the use of cytotoxic chemicals to crosslink the electrospun nanofibers. However, when considering the use of alginate nanofibers for tissue regeneration, it is important to minimize the use of cytotoxic chemicals during the processing. Furthermore, the only report to date that seeded cells on electrospun alginate scaffolds showed that the cells remained in a rounded morphology on the alginate and did not spread, likely due to the intrinsic lack of cell adhesivity of the material.[18] To utilize alginate nanofibers for tissue engineering applications, these limitations must be overcome.

In this work, alginate was blended with PEO and electrospun to form uniform nanofibrous scaffolds. A crosslinking method was utilized that requires only calcium. The alginate was crosslinked by the calcium while the PEO remained uncrosslinked; thus the PEO could be leached from the fibers by incubation in an aqueous solution to obtain pure alginate nanofibers. Furthermore, the alginate was covalently modified with a cell adhesive peptide to increase the number of cells that were able to adhere, spread, and proliferate on the scaffolds. These nanofibrous, cell adhesive alginate scaffolds are an exciting new technology for use in regenerative medicine.

## Materials and Methods

### Alginate Preparation and Modification

Sodium alginate powder (product name of 20/40, FMC Biopolymers, Princeton, NJ) with a molecular weight ($\overline{M}_w$) of 196 000 $g \cdot mol^{-1}$ 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 (GRGDSP; Commonwealth Biotechnologies, Richmond, VA), which contains the cell-binding sequence RGD, was covalently coupled to the alginate as described previously,[15,16] at a theoretical peptide density of 10 mg GRGDSP per gram of alginate. Both the adhesion ligand-modified alginate (RA) and unmodified alginate (UA) were purified by dialysis for 4 d (Spectra/Por 3500 MWCO, Spectrum Laboratories, Rancho Dominguez, CA), 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 0.22 μm filter, and lyophilized until dry.

### Solution Preparation and the Electrospinning Process

RA and UA were dissolved in ultrapure deionized water (di$H_2O$) at concentrations from 0.25 to 2.0% w/v. PEO (900 kDa, Sigma, St. Louis, MO) was dissolved in di$H_2O$ at a concentration of 4.0% w/v. RA/PEO (RAP) and UA/PEO (UAP) blend solutions with different volume ratios (alginate/PEO: 20:80, 40:60, 50:50, 60:40, 80:20) were vortexed at low speed for one hour and then rotated for 1 d at room temperature (RT) using a rotating hybridization incubator (Medel 400; Robbins Scientific, Sunnyvale, CA). A list of solutions examined is presented in Table 1.

The electrospinning system consisted of a high-voltage power supply (AU 60PO; Matsusada, Inc., Kusatsu, Japan), an infusion pump (Model 22; Harvard Apparatus, Inc., Holliston, MA), a stainless-steel blunt-ended needle (20 G, NanoNC, Inc., Seoul, Korea), a 5 ml plastic syringe (Henke Sass Wolf, Tuttlingen, Germany), and a custom-made rotating collecting drum (outer diameter: 10 cm, length: 25 cm; NanoNC, Inc.). Each blend solution was placed within a plastic syringe fitted with a stainless-steel needle. The collector was wrapped with aluminum foil and positioned at a fixed distance of 15 cm from the needle. The infusion pump was utilized to keep the feeding rate of the blend solution constant at 0.01 $ml \cdot min^{-1}$. A fixed voltage of 11 kV was continuously applied between the needle and the collector, and the blend solutions were then electrospun onto the aluminum foil affixed to the rotating collector. The resulting samples were crosslinked by

*Table 1.* Electrospinning conditions of alginate/PEO nanofibrous scaffolds.

| Sample code | Alginate Concentration (wt-%) | PEO Concentration (wt-%) | Final Alginate: PEO Volume Ratio | Final wt-% Alginate: Final wt-% PEO |
|---|---|---|---|---|
| RAP 55–0.25 | 0.25 | 4.0 | 50:50 | 0.125:2.00 |
| RAP 55–0.5 | 0.5 | 4.0 | 50:50 | 0.250:2.00 |
| RAP 55–1.0 | 1.0 | 4.0 | 50:50 | 0.500:2.00 |
| RAP 28 | 2.0 | 4.0 | 20:80 | 0.40:3.20 |
| RAP 46 | 2.0 | 4.0 | 40:60 | 0.80:2.40 |
| RAP 55 | 2.0 | 4.0 | 50:50 | 1.00:2.00 |
| RAP 64 | 2.0 | 4.0 | 60:40 | 1.20:1.60 |
| RAP 82 | 2.0 | 4.0 | 80:20 | 1.60:0.80 |
| UAP 55 | 2.0 | 4.0 | 50:50 | 1.00:2.00 |

immersion in 100 ml of 2% w/v $CaCl_2$ 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 di$H_2O$ three times to remove any residual chemicals, frozen, and lyophilized.

## Characterization of Alginate/PEO Nanofibers

### Scanning Electron Microscopy

The morphology of the electrospun alginate/PEO scaffolds was examined using a scanning electron microscope (SEM; S-4500, Hitachi, Tokyo, Japan) at an operating voltage of 5.0 kV. Scaffold sections were mounted onto sample holders and coated with gold using a sputter-coater (E-1030, Hitachi). One representative photomicrograph of each sample was taken and used to digitally measure the fiber diameters of 30 fibers using an image analyzer (Image-Pro Plus 4.5, Bethesda, MD).

### Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy

For the characterization of scaffold surface properties, attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy was performed (FTS 3000, Digilab, Randolph, MA). Transmission and ATR spectra were recorded with 32 scans and a resolution of 4 $cm^{-1}$ between 4 000 and 600 $cm^{-1}$. For positive controls, PEO powder and RGD-modified alginate powder were used to obtain the spectra for these materials by themselves. The electrospun RAP nanofibers before and after PEO extraction were then analyzed.

### Mass Loss

The mass loss behavior of the RAP55 nanofibers was investigated under static conditions. Briefly, the crosslinked and lyophilized nanofiber scaffolds (1 cm diameter, $N = 5$) were placed in closed 50-ml poly(propylene) conical tubes containing 30 ml of di$H_2O$ at 37 °C. The di$H_2O$ was changed weekly. At varying time points, samples were then washed with di$H_2O$ three times, lyophilized until dry, and weighed. Additionally, samples taken for the first 5 d were analyzed using ATR-FTIR. Samples taken at 1, 2, 3, and 4 weeks were imaged using SEM to examine scaffold morphology changes over time.

## In vitro Cell Culture Studies

Primary human dermal fibroblasts (HDFs, American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle medium with 4.5 $g \cdot L^{-1}$ glucose (DMEM-HG, HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS, HyClone) and 1% v/v penicillin/streptomycin (P/S; HyClone) at 37 °C in a humidified incubator with 5% $CO_2$. The HDFs were used at passages 5 and 6 for all experiments.

## Cytotoxicity of PEO Leached From Nanofiber Scaffolds

Crosslinked and lyophilized RGD-modified alginate/PEO nanofiber scaffolds (RAP28, RAP46, RAP55, and RAP64) were cut into disks 1.2 cm in diameter using a stainless steel punch (McMaster Carr, Elmhurst, IL). These scaffold disks ($N = 3$) were immersed in 70% ethanol for 1 h, washed three


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times with $diH_2O$ and then placed in 15 ml poly(propylene) tubes (BD Falcon, Franklin Lakes, NJ) and incubated in 5 ml DMEM-HG with 10% FBS and 1% P/S at 37 °C for 5 d. Controls had the same medium without a scaffold. The samples were then removed, leaving the PEO which had leached from the RAP scaffolds into the medium. HDFs were seeded in wells of tissue-culture treated 24-well plates (BD Falcon) at a density of $1 \times 10^4$ cells·well$^{-1}$. After culturing the cells for 24 h, the culture medium was removed and replaced with the PEO extraction medium or control medium, and the cells were cultured for an additional 24 h. Each well was rinsed with phosphate buffered saline (PBS, HyClone), and 1 ml of a 20% CellTiter 96 Aqueous One Solution (Promega, Madison, WI) which contains 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4-sulfophenyl]-2H-tetrazolium (MTS-tetrazolium) was added. The MTS-tetrazolium compound can be metabolized by mitochondria in living cells into a colored formazan product that is soluble in cell culture medium.[23] After incubating at 37 °C for 90 min, 100 μl of each solution was transferred to a 96-well plate (Fisher Scientific, Fairlawn, NJ) and the absorbance was measured at 490 nm using a 96-well plate reader (SAFIRE, Tecan, Durham, NC).

### In vitro Cell Adhesion, Spreading, Proliferation, and Viability on the Nanofiber Scaffolds

Crosslinked and lyophilized scaffolds (1.2 cm in diameter) were immersed in 70% ethanol for 1 h, washed three times with $diH_2O$, and seeded at $1.0 \times 10^5$ cells · ml$^{-1}$ to examine HDF adhesion or $1.0 \times 10^4$ cells · ml$^{-1}$ to study HDF proliferation on scaffolds ($N = 5$) placed in 24-well tissue culture plates. The nanofiber scaffolds were held down in the bottom of the tissue culture dishes with autoclaved stainless steel rings that were also 1.2 cm in diameter.

To investigate cell spreading and morphology, samples were fixed in 3.7% formaldehyde in PBS for 10 min and then permeabilized in cytoskeleton (CKS) buffer ($50 \times 10^{-3}$ M NaCl, $150 \times 10^{-3}$ M sucrose, $3 \times 10^{-3}$ M $MgCl_2$, $50 \times 10^{-3}$ M Tris base, 0.5% Triton X-100) for 5 min at 4 °C. The samples were incubated in blocking buffer (BB) (1% BSA, 0.1% Tween-20, 0.02% sodium azide in PBS) for 60 min at 37 °C. Following a wash in PBS, the samples were incubated for 60 min at 37 °C in BB containing rhodamine–phalloidin (1:200) (Invitrogen, Carlsbad, CA), a specific stain for F-actin, and Hoechst 33 258 (1:10 000) (Invitrogen), a nuclear stain. Following another gentle PBS wash, samples were mounted on glass slides. Fluorescent photomicrographs of stained cells on alginate nanofibers were acquired using a fluorescence microscope (ECLIPSE TE 300, Nikon, Tokyo, Japan) equipped with a digital camera (Retiga-SRV, Qimaging, Burnaby, BC, Canada).

For the cell viability studies, the samples were stained using a Live/Dead staining solution freshly prepared by mixing 1 ml fluorescein diacetate (Sigma) solution (1.5 mg · ml$^{-1}$ in dimethyl sulfoxide) and 0.5 ml of ethidium bromide (Sigma) solution (1 mg · ml$^{-1}$ in PBS) with 0.3 ml of PBS. 50 μl of Live/Dead assay staining solution was added to the cultured scaffolds in medium and incubated at RT for 5 min. Fluorescent photomicrographs were taken as described above.

To further examine the morphology of the cells on the nanofibers using SEM, samples were fixed using 2.5% glutaraldehyde in $diH_2O$ at RT for 1 h. After rinsing three times with $diH_2O$, the scaffolds were dehydrated using graded ethanol solutions of 70, 80, 90, and 100% for 30 min at each grading step, lyophilized until dry, sputter coated with gold and imaged by SEM.

For quantification of cell proliferation, the scaffolds seeded with cells were gently rinsed with PBS three times 4 h after cell seeding to remove non-adherent cells and then placed in fresh medium to continue culture. After 1, 3, and 7 d of culturing the samples were transferred to a new 24-well plate, 1 ml of 20% CellTiter 96 Aqueous One Solution was added to each sample, and after incubating at 37 °C for 90 min, 100 μl of each solution was transferred to a 96-well plate and the absorbance at 490 nm was determined.

### Statistical Analysis

All of the quantitative results were obtained from triplicate samples. Data are expressed as mean ± SD. Statistical analysis was carried out using a two-tailed unpaired Student's *t*-test (Excel, Microsoft), and a value of $p < 0.05$ was considered statistically significant.

## Results and Discussion

### Preparation and Characterization of Electrospun UAP and RAP Scaffolds

Nanofibrous scaffolds made of biodegradable, biocompatible polymers are of great interest in tissue engineering, as their structure is similar to that of the native extracellular matrix.[1,2] Alginate is a material that is widely used as it is biocompatible and allows for the regulation of cell adhesivity by the covalent coupling of adhesion ligands to the biopolymer backbone in a controlled manner.[15] However, alginate has been only nominally explored in electrospinning applications to date, and none of these prior electrospinning studies focused on tissue engineering strategies. This is likely due to the previous use of toxic chemicals during the fabrication and crosslinking of the nanofibers and the non-adhesive nature of the native alginate material to cells. Additionally, due to inadequate chain entanglements, alginate cannot be electrospun by itself.

In this work, both UA and RGD-modified alginate were electrospun with PEO to form uniform nanofibers. A few groups have examined the effect of polymer solution concentrations and blend ratios, solution flow rate, and voltage on the morphology, diameter, and mechanical properties of nanofibers electrospun from alginate with either PEO or PVA.[19–22] In this study, we investigated the influence of the blend ratio and concentrations of alginate and PEO solutions on nanofiber formation without the use of toxic chemicals. Table 1 presents the compositions of the RA/PEO and UA/PEO blend compositions examined in this work. Figure 1a–i shows SEM photomicrographs of the RAP and UAP nanofiber scaffolds. Solutions with 0.25, 0.5, and 1.0 wt.-% alginate were examined for electrospinning with the 4.0 wt.-% PEO. As shown in Figure 1a–c, all three conditions resulted in fibers exhibiting a beads-on-string morphology with a high bead density. As the RA wt.-% increased from 0.25 to 2.0, the bead density decreased and a uniform nanofibrous scaffold without bead formation was obtained at 2 wt.-% of RA (Figure 1d–g). Therefore, it was determined that the use of 2 wt.-% alginate blended with 4 wt.-% PEO was optimal for the fabrication of these nanofibrous scaffolds. Furthermore, the blending ratio of the alginate/PEO solutions was found to be important. At concentrations of 2 wt.-% alginate and 4 wt.-% PEO and volume blending ratios of alginate/PEO of 20:80, 40:60, 50:50, and 60:40, the alginate/PEO solutions were successfully electrospun to form nanofibrous structures (Figure 1d–g and i). However, a blending ratio of 80:20 produced fibers with a high bead density (Figure 1h). It is also apparent from the SEM photomicrographs that the pores formed between the nanoscaled fibers were interconnected. The RGD-modified and the UA nanofibers have similar morphologies, indicating that the covalently coupled peptide does not influence the electrospinning process (Figure 1f and i).

To confirm the compositions of the nanofibers, ATR-FTIR was used to examine pure PEO (Figure 2a), electrospun RAP nanofibrous scaffolds (Figure 2b–e), and pure RGD-modified alginate (Figure 2f). The peaks for PEO include one at

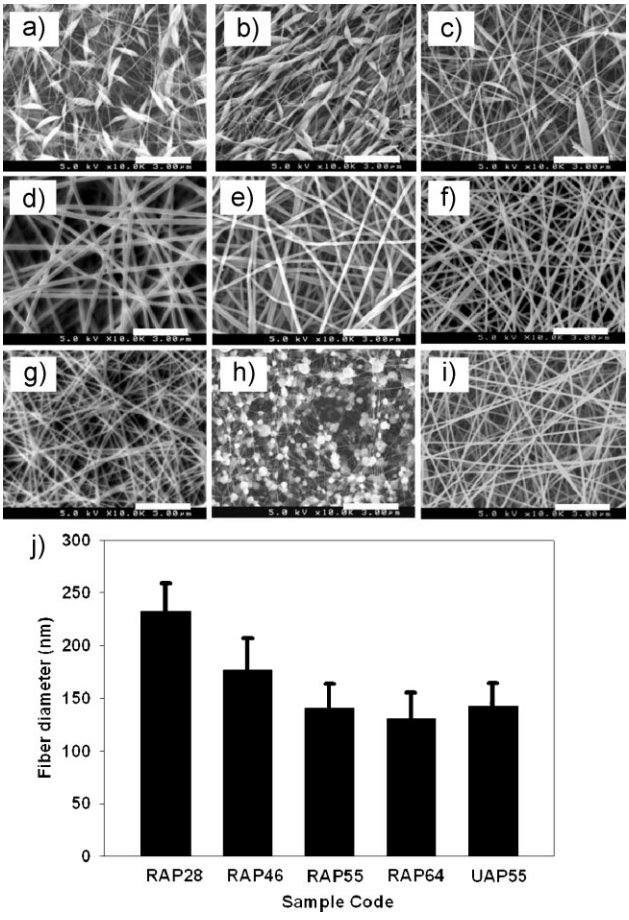


*Figure 1.* Scanning electron micrographs and fiber diameters of electrospun RGD-modified alginate and UA with PEO blend nanofibers. Scale bars are 3 μm. Images represent: (a) RAP 55–0.25, (b) RAP 55–0.5, (c) RAP 55–1.0, (d) RAP 28, (e) RAP 46, (f) RAP 55, (g) RAP 64, (h) RAP 82, and (i) UAP 55. (j) The mean fiber diameters of compositions that electrospun without bead formation.

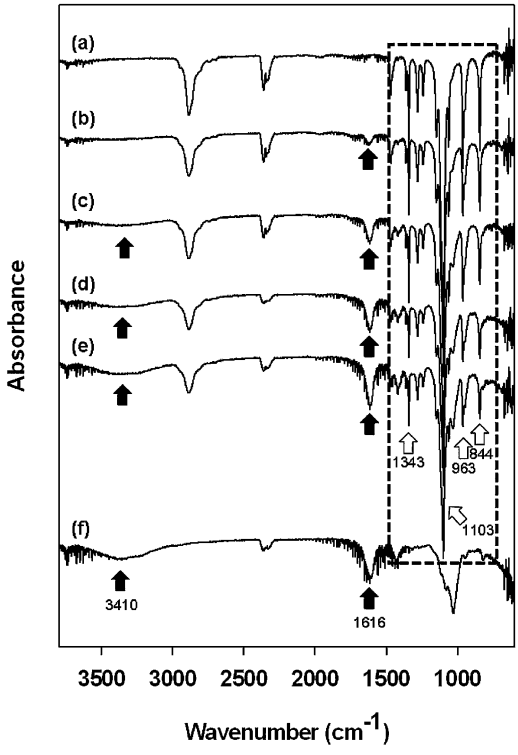


*Figure 2.* ATR-FTIR spectra of electrospun nanofibers composed of RGD-modified alginate blended with PEO at varying ratios. (a) PEO alone, (b) RAP 28, (c) RAP 46, (d) RAP 55, (e) RAP 64, and (f) RGD-modified alginate alone. Black arrows indicate characteristic peaks of alginate; white arrows indicate PEO peaks.


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844 $cm^{-1}$ indicative of C–O–C and C–C stretching, 963 $cm^{-1}$ for $-CH_2$ rocking and twisting, 1 103 $cm^{-1}$ for C–O–C stretching, and 1 343 $cm^{-1}$ for $-CH_2$ wagging.[24] As the concentration of PEO in the blend decreased, these peaks became smaller in magnitude. The alginate showed characteristic peaks at 3 410 $cm^{-1}$ corresponding to the hydroxyl group and 1 616 $cm^{-1}$ for the carboxylate group.[21] As the alginate concentration in the blends decreased, these peaks also became progressively smaller in magnitude.

Although PEO was required for the electrospinning of the alginate, pure alginate nanofibers were still obtained by crosslinking the fibers and subsequently leaching the water-soluble PEO from the fibers. The electrospun RAP and UAP scaffolds were crosslinked with $CaCl_2$ in a 5:1 ethanol/water solution and subsequently soaked in $diH_2O$ for 5 d to leach out the PEO. The calcium-containing crosslinking solution had a high amount of ethanol so that the alginate fibers would not immediately dissolve or aggregate upon exposure to water. Importantly, this crosslinking method was free from the use of highly toxic chemicals which could adversely affect cells. After crosslinking and PEO extraction, the morphological changes and mean fiber diameters of electrospun peptide-modified alginate scaffolds were examined. SEM photomicrographs demonstrated that the RA and UA scaffolds still maintained a nanofibrous structure after PEO extraction (Figure 3a–e). Fiber diameter distributions were quantitatively analyzed before and after PEO extraction. Prior to PEO extraction, average nanofiber diameters ranged from 131 to 232 nm (Figure 1j); after PEO extraction, the average nanofiber diameters ranged from 83 to 132 nm (Figure 3f). The RAP55 fibers were found to have a slightly smaller diameter than the UAP55 fibers ($p < 0.05$).

The rate of PEO extraction was examined by ATR-FTIR and measurement of the mass of the nanofiber scaffolds over time in order to determine how quickly scaffolds predominantly composed of alginate could be obtained. RAP55 scaffolds were crosslinked and then soaked in $diH_2O$ for 120 h. PEO was present in the FTIR spectrum at time zero, but the characteristic PEO peaks diminished greatly by 48 and 72 h, indicating the successful leaching of the PEO from the scaffolds (Figure 4a). Furthermore, the RA carboxylate and hydroxyl peaks became more pronounced as the PEO was leached from the scaffolds, and the final spectra at 72 h closely resembled that of pure alginate (Figure 2f). The change in mass of the RAP55 nanofiber scaffolds corroborated the FTIR data; after 1 h, the scaffold mass was $75 \pm 5\%$ of its initial mass, and after 2 d only $36 \pm 2\%$ of the initial mass remained, which is roughly the amount of alginate in the original blended solution (33.3%) (Figure 4b). There was no statistically significant difference between the mass of the scaffolds after 48, 72, and 120 h of extraction ($p > 0.5$). These results suggest that PEO in the RAP nanofibers was completely extracted after 48 h.

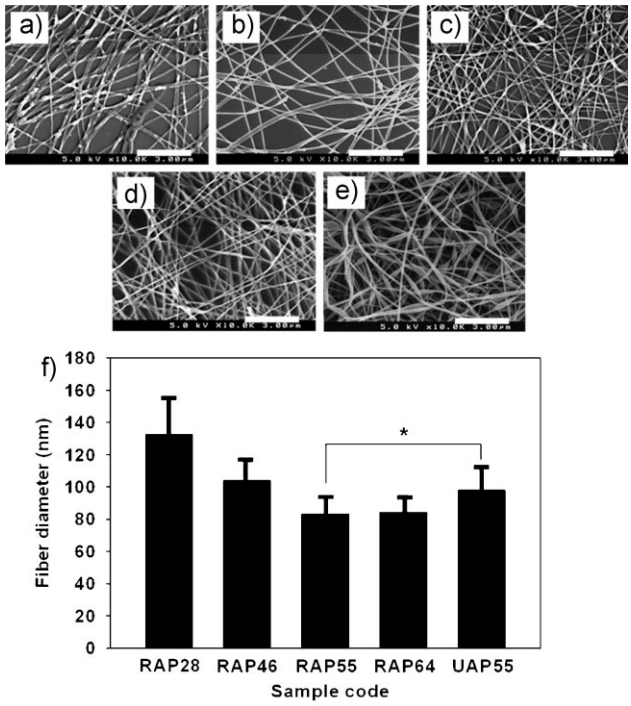


*Figure 3.* Scanning electron micrographs of electrospun RGD-modified and UA nanofibers following PEO extraction in $diH_2O$ at 37 °C for 5 d. Scale bars are 3 μm. Images represent: (a) RAP 28, (b) RAP 46, (c) RAP 55, (d) RAP 64, and (e) UAP 55. (f) The mean fiber diameters of compositions that electrospun without bead formation.

The stability of the nanofibers over time was studied by incubating the crosslinked alginate nanofibers in $diH_2O$ for 4 weeks. The morphology of these nanofibers was examined by SEM imaging, and minimal change was observed over time (Figure 5a–d). The only notable change is that the nanofibers began to associate more closely with each other after 3–4 weeks (Figure 5c and d). By measuring the mass of the nanofibrous scaffolds over the course of 4 weeks it was determined that there was no mass loss over this time period (Figure 5e). For some applications this high stability may be desirable; in other cases where degradation is desirable, as is the case for most tissue engineering applications, it should be possible to achieve increased degradation rates by use of alginate of lower molecular weight[8] or alginate that has been oxidized.[10]

### Cytotoxicity of PEO Leached From Electrospun RAP Scaffolds

Although the PEO can be extracted in only 48 h before scaffold use, in some instances it may be desirable to use the scaffold immediately and allow the PEO to diffuse out during culture with cells or in vivo. For example, if bioactive factors are incorporated into the electrospun fibers, immediate use of the scaffolds would ensure that no

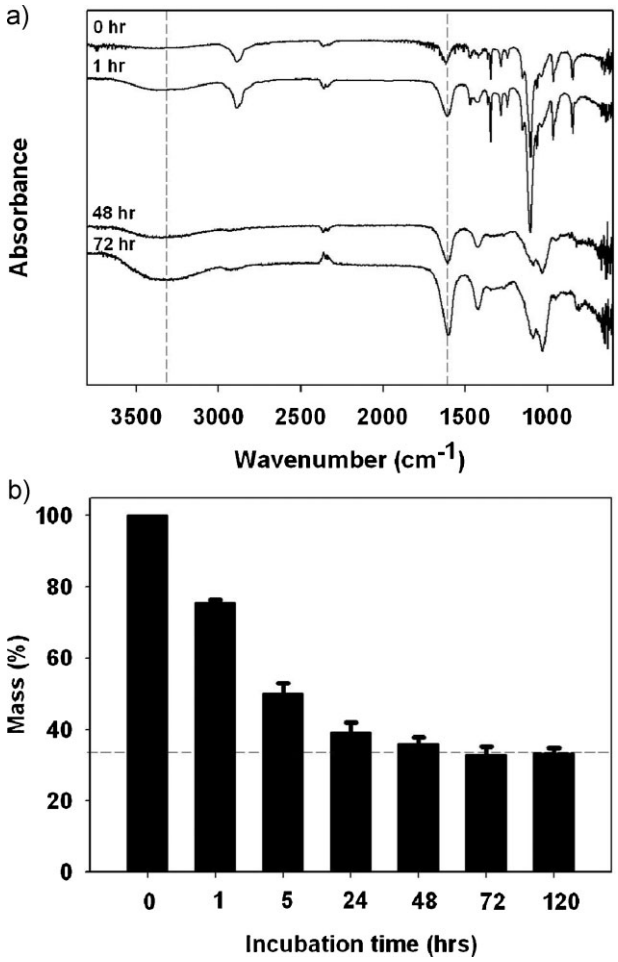


*Figure 4.* (a) ATR-FTIR spectra and (b) mass loss of PEO-extracted electrospun RGD-modified alginate (RAP55) nanofibers in $diH_2O$ at 37 °C over time.

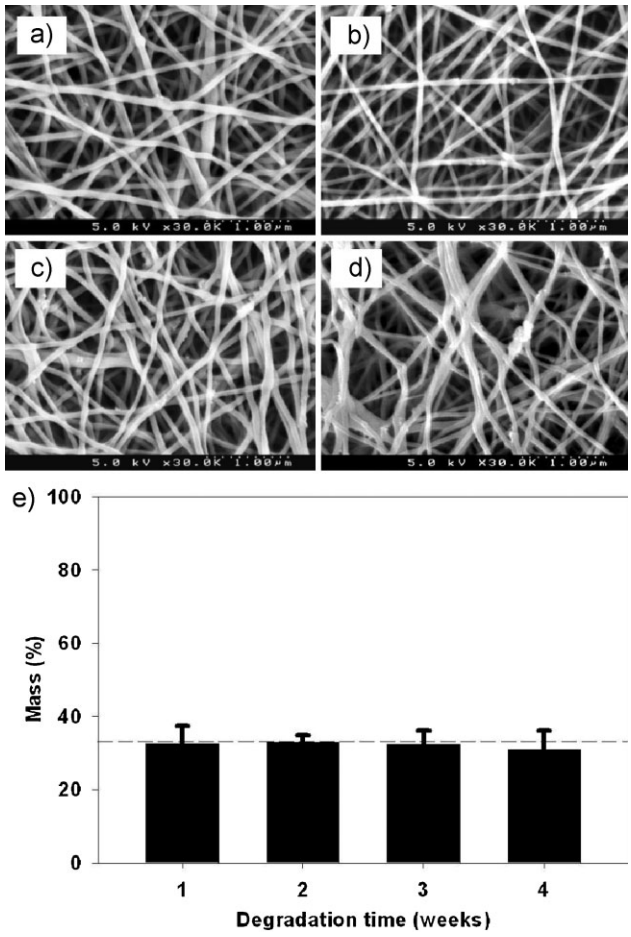


*Figure 5.* Scanning electron micrographs of electrospun RAP55 nanofibers after incubation in $diH_2O$ at 37 °C for (a) 1 week, (b) 2 weeks, (c) 3 weeks, and (d) 4 weeks. Scale bars are 1 μm. (e) The mass loss over time as a percent of the initial mass of the electrospun fibers prior to PEO leaching, indicating that the nanofibers do not degrade over the course of 4 weeks.

bioactive factor is lost during the PEO extraction process. In this case, it is important to examine possible cytotoxicity of the leached PEO. Various RAP scaffolds were incubated for 5 d at 37 °C to extract the PEO, and this extraction solution was applied to HDFs seeded on tissue culture polystyrene (TCP). After 1 d, the viability of the cells, as measured using an MTS assay, decreased minimally but significantly ($p < 0.05$) with absorbance readings at least 88% of control cells treated with serum-containing media alone (Figure 6). This indicates that there is low cytotoxicity associated with the extracted PEO, and the nanofibrous scaffolds could thus be used immediately if that was desired for a particular application.

## Cell Adhesion and Proliferation on Alginate Nanofibers

Alginate is a polysaccharide to which cells are unable to adhere since it is extremely hydrophilic and does not absorb serum proteins. Therefore, since cell adhesion is required for

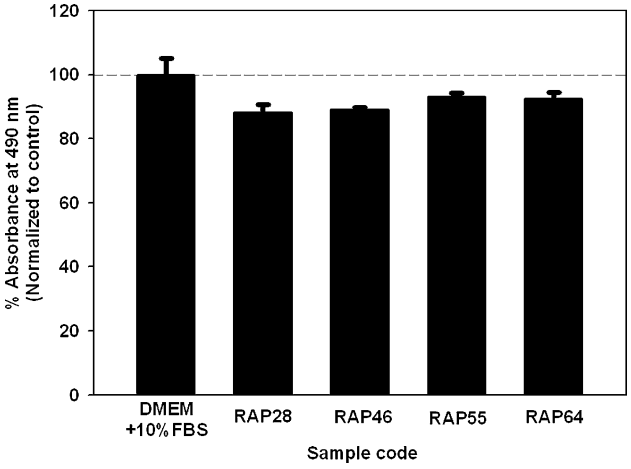


*Figure 6.* Cytotoxicity of PEO extracted from the nanofibers on HDFs as quantified by an MTS assay and normalized to cells cultured in control media.


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the survival of most mammalian cells types and has been shown to influence cell functions in both 2D and 3D microenvironments,[25] the alginate was covalently modified with a cell adhesive peptide sequence to allow for cellular adhesion and proliferation on the nanofibers. Specifically, the polymer backbone of the alginate comprising the nanofibers was chemically modified with an adhesion peptide (i.e., GRGDSP) containing the ubiquitous RGD amino acid sequence to promote integrin mediated cellular adhesion and proliferation on these scaffolds. To observe the ability of cells to adhere to the modified scaffolds, HDFs were seeded on unmodified and RGD-modified nanofiber scaffolds from which PEO had been extracted, and cell morphology was examined after 4 and 8 h by SEM (Figure 7). The few cells associated with the UA nanofibers had a round morphology and did not attach to or spread on the scaffolds, but rather appeared physically trapped beneath individual nanofibers of the scaffolds (Figure 7a and c). Due to the fragile nature of the electrospun nanofibers, only gentle washing was conducted, but it is anticipated that more rigorous washing would remove these non-adherent cells from the unmodified scaffolds. In contrast, cells on RGD-modified alginate exhibited spreading on the nanofibers as early as 4 h post-seeding (Figure 7b), which indicates that the cells were able to adhere to the scaffolds, presumably through integrin binding to the adhesion ligands presented by the alginate.

To further examine the morphologies of the cells on the nanofibers, after 12 h the cells were stained with rhodamine–phalloidin to observe F-actin organization and assembly in microfilaments within the CKSs and counterstained with Hoechst to visualize the nuclei. Cytoskeletal microfilaments

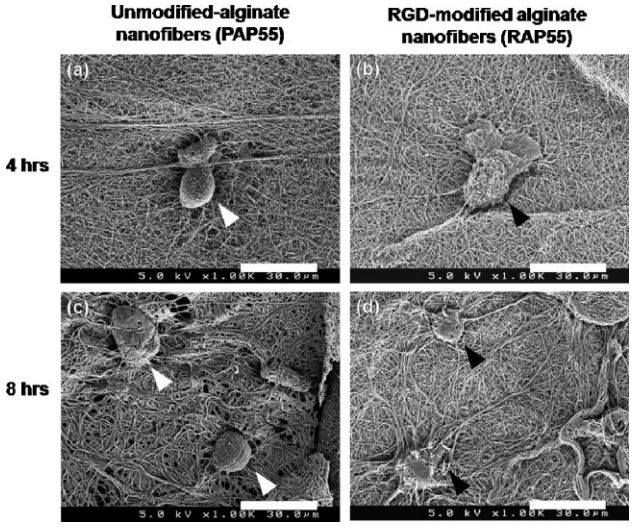


*Figure 7.* Scanning electron micrographs of HDFs cultured on alginate nanofibers for 4 and 8 h. (a and c) UAP55 and (b and d) RAP55 nanofibers. Arrows indicate cells associated with the nanofibers. Scale bars are 30 μm.

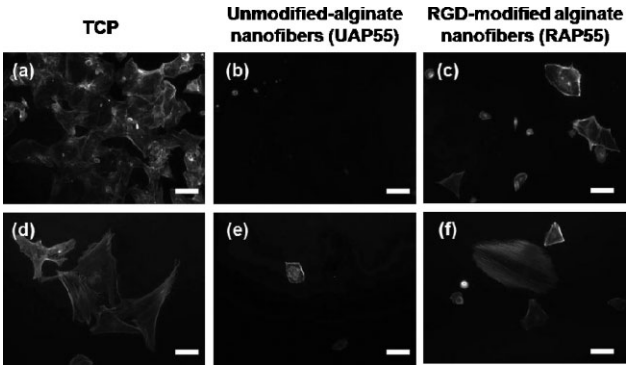


*Figure 8.* Fluorescence photomicrographs of phalloidin and DAPI stained HDFs cultured on (a and d) TCP, (b and e) UAP 55, and (c and f) RAP 55 for 12 h. Scale bars in (a–c) represent 100 μm; scale bars in (d–f) represent 30 μm.

were apparent in the HDFs seeded on the RGD-modified alginate nanofibers and on TCP (positive control) (Figure 8a, c, d, and f). These cells adhered to the substrates and spread considerably. In contrast, it is evident that the HDFs did not adhere well to the UA (Figure 8b and e).

The crosslinked unmodified and RGD-modified alginate nanofibers (UAP55 and RAP55) were also examined for cell adhesion and proliferation over time by visualization with SEM and fluorescent photomicrographs. The HDFs remained in a rounded morphology on the UA scaffolds after 1 and 7 d of culture and do not appear to have proliferated, as demonstrated by both SEM images and Live/Dead staining (Figure 9a, c, e, and g). In contrast, the cells seeded on the RGD-modified alginate nanofibers exhibited adhesion and spreading at both days 1 and 7, and furthermore showed evidence of extensive proliferation (Figure 9b, d, f, and h). In fact, the cells appeared to be confluent on the RA nanofibers at 7 d. In addition, the fluorescent photomicrographs indicated that all of the HDFs were viable, as there were no dead (red) cells present among the live (green) cells.

The MTS assay, which quantifies cell mitochondrial activity, was also used as a measure of cell proliferation on the unmodified and RGD-modified alginate nanofibers. The scaffolds for these assays were transferred to a new well prior to assaying for mictochondrial activity. Although they were rinsed three times after the initial 4 h of culturing, they were not rinsed again to remove any loosely adherent cells at the time of this assay due to the fragility of the matrices. Therefore the values shown represent all of the cells associated with the mat. Overall, the number of HDFs on RGD-modified nanofibers was 500% greater at 1 d, 470% greater at 3 d, and 450% greater at 7 d than on UA nanofibers (Figure 10). This indicates that the RGD modification promoted cell adhesion and allowed for an increased number of cells over time on these nanofibers compared to those that were unmodified. Furthermore, these results

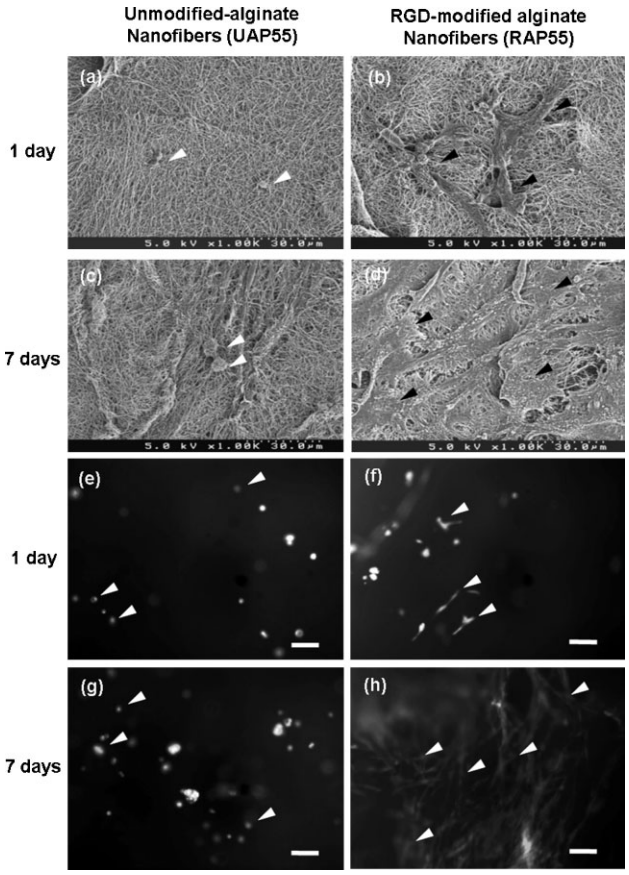


*Figure 9.* Scanning electron and fluorescence photomicrographs of HDFs cultured on PEO-extracted UAP55 and RAP55 nanofibers for 1 and 7 d. (a–d) SEM photomicrographs of cells on nanofibers. Scale bars are 30 μm. (e–h) Fluorescent photomicrographs of cells stained with a Live/Dead assay. Scale bars are 100 μm. Arrows indicate some of the cells associated with the nanofibers.

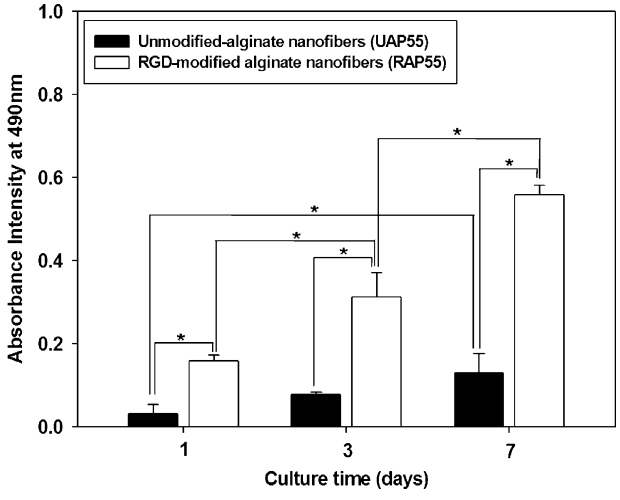


*Figure 10.* Cell proliferation of HDFs cultured on PEO-extracted UAP55 and RAP55 nanofibers for 7 d, as determined by an MTS assay which measures the cell metabolic activity. $^{*}p < 0.05$.

demonstrate that the cell adhesion peptide is bioactive after the electrospinning process. In future studies the cell adhesivity of these scaffolds can be specifically tailored by varying the peptide types and densities used. Furthermore, it may be possible to encourage cell migration and penetration into thicker nanofibrous structures with the peptide modification.

## Conclusion

In summary, uniform RGD-modified alginate/PEO and UA/PEO nanofiber scaffolds were prepared by electrospinning without the use of toxic solvents. SEM analysis of the scaffolds demonstrated that uniform nanofibers could be obtained at several blending ratios of alginate to PEO. The extracted PEO exhibited minimal cytotoxicity indicating that the PEO could be leached from the scaffolds either prior to implementation or after cell seeding on the scaffolds and/or implantation in vivo. Fibroblasts were shown to adhere to, spread, and proliferate on RGD-modified nanofibers. The ability to independently control the nanoscale architecture and the adhesive properties of these natural polysaccharide scaffolds may provide a powerful avenue to regulate cell behavior and tissue formation via these important signaling pathways.

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[1] N. Ashammakhi, A. Ndreu, L. Nikkola, I. Wimpenny, Y. Yang, *Regenerative Med.* **2008**, *3*, 547.
[2] P. R. LeDuc, D. N. Robinson, *Adv. Mater.* **2007**, *19*, 3761.
[3] R. G. Flemming, C. J. Murphy, G. A. Abrams, S. L. Goodman, P. F. Nealey, *Biomaterials* **1999**, *20*, 573.
[4] S. Y. Chew, Y. Wen, Y. Dzenis, K. W. Leong, *Curr. Pharm. Des.* **2006**, *12*, 4751.
[5] J. F. Mano, G. A. Silva, H. S. Azevedo, P. B. Malafaya, R. A. Sousa, S. S. Silva, L. F. Boesel, J. M. Oliveira, T. C. Santos, A. P. Marques, N. M. Neves, R. L. Reis, *J. R. Soc. Interface* **2007**, *4*, 999.
[6] A. D. Augst, H. J. Kong, D. J. Mooney, *Macromol. Biosci.* **2006**, *6*, 623.




DOI: 10.1002/mabi.201000046

[7] M. D. Krebs, E. Salter, E. Chen, K. A. Sutter, E. Alsberg, *J. Biomed. Mater. Res.* **2010**, *92*, 1131.
[8] E. Alsberg, H. J. Kong, Y. Hirano, M. K. Smith, A. Albeiruti, D. J. Mooney, *J. Dent. Res.* **2003**, *82*, 903.
[9] E. Alsberg, K. W. Anderson, A. Albeiruti, J. A. Rowley, D. J. Mooney, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 12025.
[10] K. H. Bouhadir, K. Y. Lee, E. Alsberg, K. L. Damm, K. W. Anderson, D. J. Mooney, *Biotechnol. Prog.* **2001**, *17*, 945.
[11] T. Hashimoto, Y. Suzuki, M. Tanihara, Y. Kakimaru, K. Suzuki, *Biomaterials* **2004**, *25*, 1407.
[12] W. R. Lee, J. H. Park, K. H. Kim, S. J. Kim, D. H. Park, M. H. Chae, S. H. Suh, S. W. Jeong, K. K. Park, *Wound Repair Regen* **2009**, *17*, 505.
[13] P. Prang, R. Muller, A. Eljaouhari, K. Heckmann, W. Kunz, T. Weber, C. Faber, M. Vroemen, U. Bogdahn, N. Weidner, *Biomaterials* **2006**, *27*, 3560.
[14] A. Mosahebi, M. Simon, M. Wiberg, G. Terenghi, *Tissue Eng.* **2001**, *7*, 525.
[15] J. A. Rowley, G. Madlambayan, D. J. Mooney, *Biomaterials* **1999**, *20*, 45.
[16] E. Alsberg, K. W. Anderson, A. Albeiruti, R. T. Franceschi, D. J. Mooney, *J. Dent. Res.* **2001**, *80*, 2025.
[17] H. Nie, A. He, J. Zheng, S. Xu, J. Li, C. C. Han, *Biomacromolecules* **2008**, *9*, 1362.
[18] N. Bhattarai, Z. Li, D. Edmondson, M. Zhang, *Adv. Mater.* **2006**, *18*, 1463.
[19] N. Bhattarai, M. Zhang, *Nanotechnology* **2007**, *18*, 455601.
[20] S. Moon, B. Ryu, J. Choi, B. Jo, R. J. Farris, *Polym. Eng. Sci.* **2009**, *49*, 52.
[21] J. Lu, Y. Zhu, Z. Guo, P. Hu, J. Yu, *Polymer* **2006**, *47*, 8026.
[22] S. Safi, M. Morshed, S. A. Hosseini Ravandi, M. Ghiaci, *J. Appl. Polym. Sci.* **2007**, *104*, 3245.
[23] A. H. Cory, T. C. Owen, J. A. Barltrop, J. G. Cory, *Cancer Commun.* **1991**, *3*, 207.
[24] Y. Ji, K. Ghosh, X. Zheng Shu, B. Li, J. C. Sokolov, G. D. Prestwich, R. A. F. Clark, M. H. Rafailovich, *Biomaterials* **2006**, *27*, 3782.
[25] M. A. Wozniak, K. Modzelewska, L. Kwong, P. J. Keely, *Biochim. Biophys. Acta* **2004**, *1692*, 103.