Materials Selection and Residual Solvent Retention in Biodegradable Electrospun Fibers

Jin Nam,[1] Yan Huang,[2] Sudha Agarwal,[2] John Lannutti[1]

[1]*Department of Materials Science and Engineering, The Ohio State University, Columbus, Ohio 43210*
[2]*Oral Biology, College of Dentistry, The Ohio State University, Columbus, Ohio 43210*

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ABSTRACT: Electrospun tissue engineering scaffolds provide mechanical support to seeded cells that populate the structure while depositing specific extracellular matrix components. The potent sterilizing agent 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) is often used in electrospinning investigations involving biologically-derived polymers. Surprisingly, there has been no study of solvent retention versus composition even though materials selection should influence organic solvent content. We developed a method quantifying HFIP retention following electrospinning of gelatin, polycaprolactone (PCL), and PCL-gelatin blends using electro-spray mass spectroscopy. The acetone content of acetone-spun PCL was also established. Pure gelatin fiber contained as much as 1600 ppm of HFIP. In contrast, little acetone or HFIP was detected in 100% PCL. Gelatin clearly has a greater affinity for HFIP than PCL and materials selection has a strong influence on the amount of retained solvent. Vacuum + heat treatment at 37 and 45°C reduced [HFIP] to 10 and 5.6 ppm, respectively, levels having no demonstrated effects on mammalian cell viability. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 107: 1547–1554, 2008

Key words: biodegradable; bioengineering; biological applications of polymers; biomaterials

INTRODUCTION

Tissue engineering is a rapidly expanding technical area that seeks to create specific human tissues and/or organs by combining cells and scaffolds formed typically using either synthetic or naturally-derived polymers. These tissue-engineered products will someday be able to deliver on the promise of functional replacement for diseased or failing organs. In pursuing these goals, our interactions with biologically-based collaborators has inevitably led to an interest in expanding upon or improving the ability of current tissue engineering scaffolds to promote or preserve the appropriate function of adherent mammalian cells. Chief among these is the native chemistry of the scaffold and how it influences cellular development.

A broad variety of techniques have been developed to fabricate such scaffolding. Within this range of options it is generally agreed that electrospinning has the potential to produce scaffolds possessing the appropriate strength, structure, economics, and biocompatibility. The technique enjoys wide usage.[1–10] Electrospinning often involves a solution of polymer in a toxic organic solvent.[11,12] Considerable effort has been directed toward establishing the effects of solvent on the spinning process and the morphology of the resulting fibers.[2,3,7,13–21] Dielectric constant has also been identified as an important governing factor.[17,20,22,23] Some of these solvents may be retained and could conceivably affect scaffold biological performance either beneficially or (more likely) adversely. However, no reports on the possible influence of residual solvent on biological activity exist. In a recent study Dalton et al.[24] state that residual solvent in electrospun polymers provides a potential source of cytotoxicity. Collagen is an example favored for use in a number of tissue engineering applications. Type I collagen can be dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and this has been used successfully to electrospin scaffolds for smooth muscle cells.[25] Natural collagen is relatively nonimmunogenic; however, dissolution of collagen in any organic solvent causes conformational changes and the processed collagen could conceivably retain the solvent to affect both biological and structural properties.

In addition, our knowledge of the microstructural-level behavior of these complex fiber arrangements during exposure to even small amounts of degradation is also limited. Historically, cells are typically

Correspondence to: John Lannutti (lannutti.1@osu.edu).
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cultured on the outside of electrospun fabrics; the viability of adherent mammalian cells could be influenced either beneficially[26–28] or negatively by microstructural changes in the scaffold resulting from environmental exposure.

These scaffolds are currently targeted to provide mechanical support while host-appropriate cells populate the structure and deposit specific extracellular matrix (ECM) components. While some efforts have occasionally been made to minimize the potential presence of residual solvents[14,29,30] there has been, to our knowledge, no systematic study of solvent retention or postprocessing steps that might minimize it. We undertook this investigation to shed light on this potential problem and to understand how the process of materials selection can influence surrounding biological environments.

Immediately after electrospinning from HFIP solution gelatin fibers can contain as much as 1600 ppm of residual solvent. The fluorinated analog of isopropanol, a common sterilizing agent, HFIP has the potential to negatively influence cellular proliferation both *in vitro* and *in vivo* and is used in a number of electrospinning investigations involving biologically-derived polymers.[31–38] Composite gelatin-PCL fibers that combine the biological functionality of gelatin with the relatively greater dissolution-resistance of polycaprolactone (PCL), showed that the residual HFIP closely scales with gelatin content. Subsequent treatment with vacuum and vacuum plus heating reduced HFIP levels to below 100 ppm. While much more easily removable solvents like acetone are widely used, the increasing need to electrospin scaffolds from relatively difficult-to-dissolve biological materials suggests that more detailed observations of the retention and biological effects of such residual solvents need to be made.

MATERIALS AND METHODS

Electrospinning

A 6.7 wt % solution of gelatin type A from porcine skin (~ 300 bloom, Sigma, St. Louis, MO) in HFIP (Aldrich, St. Louis, MO) was prepared and then electrospun using a high voltage DC power supply (Model FC50R2, Glassman high voltage, High Bridge, NJ) operated at 26 kV, a 20 cm tip-to-substrate distance and a flow rate of 15 mL/h. This 20 cm tip-to-substrate distance and 15 mL/h flow rate were used in all subsequent depositions. A 12 wt % solution of polycaprolactone (M_w 65,000, Aldrich, St. Louis, MO) in acetone (Mallinckroff Chemicals, Phillipsburg, NJ) was prepared and then electrospun at 23 kV. In addition, 6.7 wt % solutions of PCL and gelatin in HFIP were spun at 25, 50, and 75% final gelatin contents using 23, 23, and 26 kV, respectively. In all cases a $4 \times 4''$ (10.2×10.2 cm^2) sheet approximately 0.5 mm thick was deposited onto aluminum foil.

Postelectrospinning treatment

Electrospun fiber sheets were processed to generate the following categories of solvent content: (1) as-spun (no postprocessing); (2) vacuum (<30 mmHg) for 24 h; (3) vacuum (<30 mmHg) and 37°C for 24 h; (4) vacuum (<30 mmHg) and 45°C for 24 h. We were mindful of the relatively low melting point of PCL (~ 60°C).

Electro-spray ionization mass spectroscopy

For each of the above samples 0.10 g was submerged in 10.0 g of DI water in separate vials and held at 37°C for 24 h. For PCL electrospun from either HFIP or acetone, 0.10 g was digested in a 15% formic acid (Mallinckrodt Baker, Phillipsburg, NJ)–85% methanol (EM Science, Gibbstown, NJ) solution using a sonifier (Sonifier 450, Branson, Danbury, CT). The resultant solutions were then analyzed by a Bruker Esquire Ion Trap mass spectrometer (Esquire G1979A, Bruker, Billerica, MA) equipped with an orthogonal electrospray source operated in negative ion mode. The samples were infused into the electrospray source at a rate of 5–10 μL min^{-1}. The ESI conditions employed were a capillary voltage of 3500 V, a source temperature of 250°C and nitrogen drying gas. The ion trap was set to pass ions from m/z 50–2000 and 20–450 amu for HFIP and acetone, respectively. Data were acquired in continuum mode until acceptable averaged data was obtained. With the ability to achieve mass measurements that are within 0.01% of the calculated values,[39] Electro-spray ionization mass spectroscopy (ESI-MS) is capable of providing accurate analyses of solvent content. For comparison, 10, 50, 100, and 250 ppm standard solutions of HFIP in DI water were produced. In addition, 10, 50, and 100 ppm standard solutions of HFIP or acetone in the 15% formic acid–85% methanol solution were also produced to provide quantitative analysis of HFIP or acetone content in the digested PCL. All the measurements were calibrated with these known standards to compensate for detector drift.

SEM analysis

All the samples before and after the postspinning treatments were coated with 8 nm thick osmium using an osmium plasma coater (OPC-80T, SPI Supplies, West Chester, PA). The use of osmium plasma instead of Au or Au-Pd eliminated concerns regarding PCL melting during gold sputter coating and

allowed for higher resolution imaging of the fiber surface.

Toxicity test

Approximately 30,000 second passage chondrocytes from rat articular cartilage were exposed to 10, 50, 100, 250, 500, and 1000 ppm solutions of HFIP in media during seeding on 12-well tissue culture plates (Falcon, Franklin Lakes, NJ). The cell culture medium was composed of 10% fetal bovine serum (FBS) (Mediatech, Herndon, VA), 1% Penicillin/Streptomycin (Fisher scientific, Fair Lawn, NJ) and 1% L-glutamine (Mediatech, Herndon, VA) in Ham's F-12 medium (Mediatech, Herndon, VA). HFIP-free cell culture medium was used as a control. All samples were processed in triplicate and harvested at day 2.

For the determination of proliferated cell numbers by spectrophotometry, cells were fixed with 10% formalin (Richard-Allen Scientific, Kalamazoo, MI) for 30 min followed by four rinses with phosphate buffered saline (PBS) (Mediatech, Herndon, VA). Two wt % crystal violet (Sigma, St. Louis, MO) was dissolved in ethanol and the solution was further diluted by mixing 1 mL of the 2 wt % crystal violet solution with 25 mL of DI water. About 300 μl of the crystal violet reagent was added to each well for 10 min, and then the wells were washed four times with tap water. About 250 μL of 1% sodium dodecyl sulfate (SDS, National Diagnostics, Atlanta, GA) was added to each well, and the plate was shaken for 30 min. About 200 μL of the resultant crystal violet stain-dissolved SDS solutions was put into a 96-well plate (Falcon, Franklin Lakes, NJ) and the absorbance of each well was read at 560 nm using a Wallac Victor^{3TM} plate reader (Perkin Elmer, Wellesley, MA). Approximately 30,000, 60,000, 120,000, and 240,000 cells were stained with the crystal violet reagent using the same procedure and the absorbances read to produce a calibration curve.

The data of resulting cell numbers for the 7 HFIP concentration groups ($N = 3$) were analyzed by univariate analysis of variance (ANOVA) with SPSS 13.0 (Chicago, IL). Significance among each group was then determined by Tukey's Honest Significant Difference (HSD).

Cell culture

Approximately 30,000 second passage chondrocytes from rat articular cartilage were seeded on the 50 : 50 PCL-gelatin composite fiber mesh. The mesh was cut with an arch punch generating a round sample with 18 mm in diameter and 0.5 mm in thickness. The sample was treated at 45°C in vacuum for 24 h prior to the cell seeding. Adherent cells were then cultured for 5 days before morphological examination with SEM. The cell culture media was the same as that used for the toxicity test. These samples were fixed with 10% formalin followed by a graded ethanol series in DI water (50, 70, 85, 90, and 100% ethanol) for dehydration. The dehydration step was finalized using a graded ethanol-HMDS (hexamethyldisilazane, Electron Microscopy Sciences, Hatfield, PA) series (25, 50, 75, and 100% HMDS) followed by drying under a hood overnight. The dried sample was coated with 8 nm of osmium and examined with the SEM as before.

RESULTS

Fiber meshes of PCL (G0), 75 : 25 PCL-gelatin (G25), 50 : 50 PCL-gelatin (G50), 25 : 75 PCL-gelatin (G75) and gelatin (G100) could be electrospun as described in the materials and methods section. As gelatin content increases so does fiber diameter; the average diameters (± standard deviations of G0, G50, and G100 were (0.75 ± 0.49), (1.23 ± 0.45) and (1.42 ± 0.60) μm, respectively. The presence of gelatin produced a relatively flattened morphology (Fig. 1).

Exposure of the G100 fibers to 37°C water resulted in their immediate dissolution. In contrast, G0 fibers remained intact. The other fibers appeared to undergo partial dissolution in accordance with their gelatin contents showing that gelatin-containing fibers have a tendency to breakdown to produce cell-like structures even though no cells are present (Fig. 2). The ESI-MS was used to generate standard

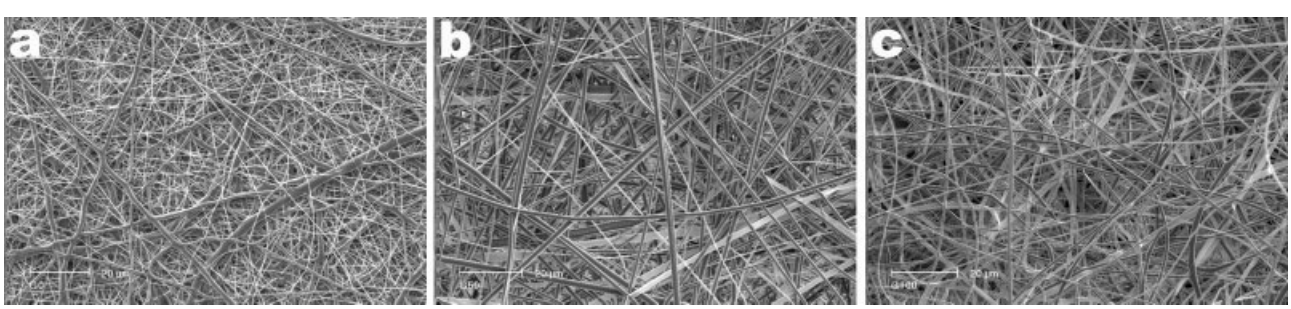

Figure 1 SEM images of as-spun (a) G0, (b) G50, and (c) G100.

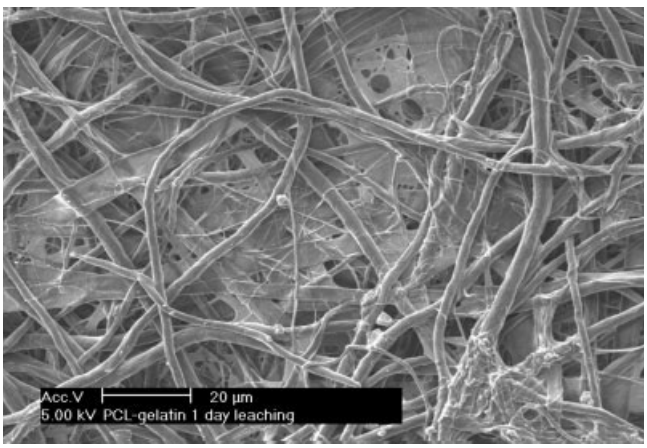

Figure 2 SEM image of G50 dissolved in DI water for 1 day at 37°C showing partial and nonuniform dissolution. Note that cell-like morphologies are produced by partial dissolution even in the absence of cells.

curve [Fig. 3(a)] providing a good least-square fit (R^2 = 0.9642) describing HFIP content. The HFIP peak in 100 ppm solution was detected at 167.2 *m/z* in ESI-MS spectrum [Fig. 3(b)]. The solutions resulting from the exposure of the fibers to DI water at 37°C were subjected to ESI-MS analysis and the intensities of the HFIP peak (*m/z* = 167.2) compared with the standard curve (Fig. 4). As little as 50 ppm was released by the G0 composition; as much as 1660 ppm was released by G100. The amount of HFIP detected scales with gelatin content according to $((\text{HFIP (ppm)}) = 14.552 \times (\text{gelatin (wt\%)}) + 23.76$ (R^2 = 0.8496).

Next, as-spun G0, G25, G50, G75, and G100 fiber meshes were stored under ambient conditions for 1 week prior to submergence in DI water at 37°C for 24 h followed by ESI-MS analysis of HFIP elution as described above. Figure 5 shows the HFIP content of the aqueous solution following exposure to these ''aged'' fibers. HFIP was not detectable in water exposed to 1-week-old PCL (G0), and only 314 ppm of HFIP was detected in the 1-week-old gelatin (G100). As before, HFIP scales with the gelatin content in this case according to $(\text{HFIP (ppm)}) = 2.5018 \times (\text{gelatin (wt\%)})$ (R^2 = 0.8244).

The effects of several different post-treatments on HFIP released from G50 fiber are presented in Figure 6. Vacuum for 24 h reduced the amount of HFIP released from 114 ppm (1-week-old G50) to 14 ppm. Vacuum combined with heating at 37 and 45°C further reduced the HFIP content to 10 and 5.6 ppm, respectively. The morphologies of treated G50 and other samples were examined by SEM to observe the effects of vacuum-based postprocessing and no microstructural changes were observed following treatment (data not shown). Heat treatment at 45°C in vacuum did not lead to any deterioration of surface of the gelatin fibers or change in the morphology of the PCL.

Complete digestion of G0 in 15 : 85 formic acid–methanol provided a measure of the total amount of HFIP, if any, remaining in pure PCL (Fig. 7). HFIP standards (10, 50, and 100 ppm) in 15 : 85 formic acid–methanol generated an excellent linear fit (R^2 = 0.9919). The extrapolated amount of HFIP present in 0.1 g of G0 sample was 0.3 ppm. For comparison's sake, PCL electrospun using acetone was also digested and the resultant solution was analyzed by ESI-MS. No acetone could be detected within the limits of ESI-MS (~ 1.7 ppm (1 μM)).

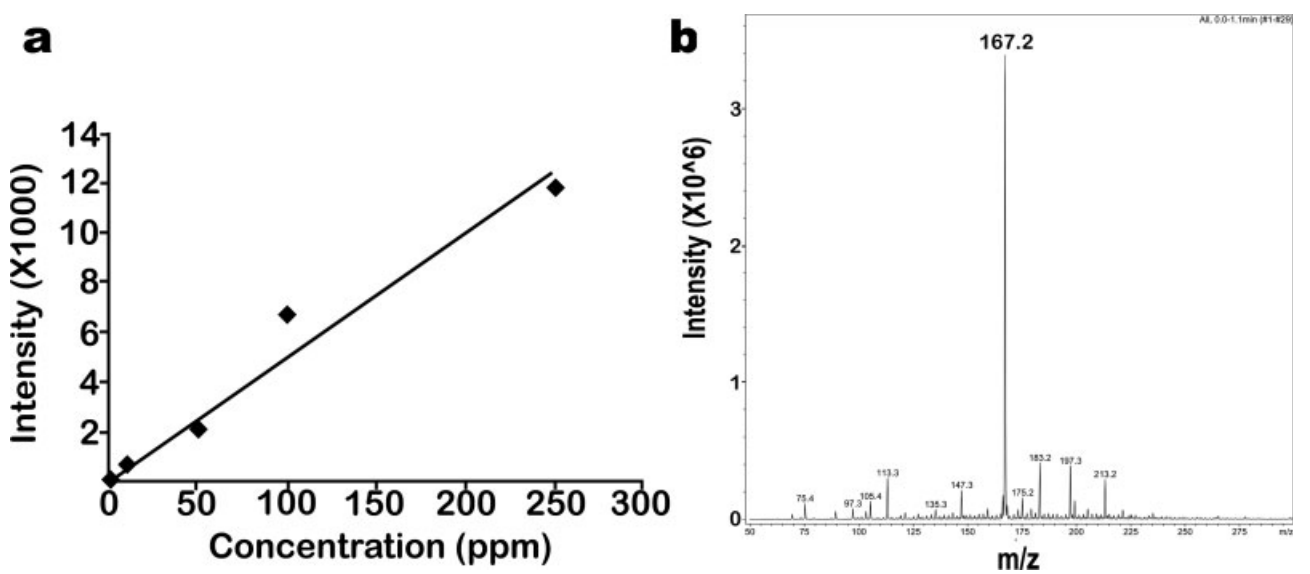

Figure 3 (a) The ESI-MS results from the 10, 50, 100, and 250 ppm HFIP standards in DI water and (b) a typical ESI-MS spectrum of the 100 ppm standard.

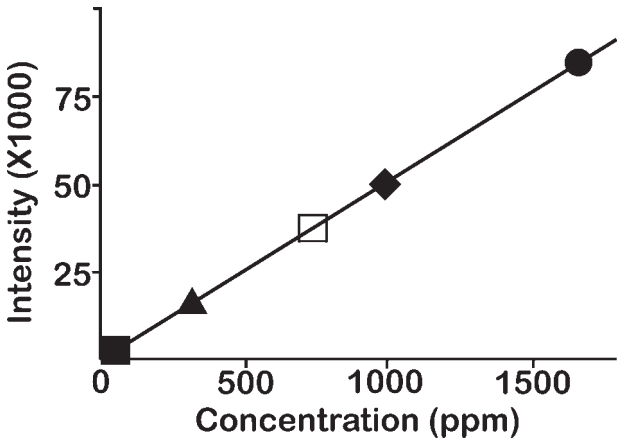

Figure 4 HFIP content in as-spun G0 (filled square), G25 (triangle), G50 (empty square), G75 (diamond), and G100 (circle).

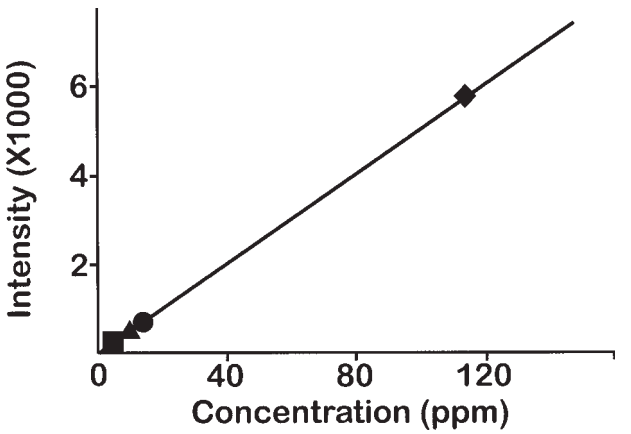

Figure 6 HFIP releases from the G50 composition following: 1 week under ambient conditions (diamond), vacuum (circle), vacuum and 37°C (triangle), vacuum and 45°C (square).

The inherent toxicity of HFIP was tested by culturing chondrocytes in 0, 10, 50, 100, 250, 500, and 1000 ppm HFIP in culture media. The cells were cultured for 2 days before harvesting and spectrophotometric counting (Fig. 8). The result of univariate ANOVA showed significantly reduced cell numbers ($p < 0.001$) in 500 and 1000 ppm compared with control.

Figure 9 shows SEM images of chondrocytes cultured at 45°C on vacuum-treated G50. The chondrocytes exhibit a well-spread morphology in which their filopodia extend across several fibers and also secrete extracellular matrices (likely glycosaminoglycan (GAG)) as evidenced by the crystalline deposits observed near the cells. These GAG deposit also enabled us to distinguish the cultured cells from the gelatin film observed in Figure 2.

DISCUSSION

Within the field of tissue engineering electrospinning has attracted great interest due to its simplicity and effectiveness in producing fine fibers that closely resemble the extracellular matrix. Hundreds of polymeric compositions have been spun but work involving natural polymers is limited. Biopolymers such as collagen,[36] gelatin,[30] elastin,[40] and hyaluronic acid[41] have been successfully electrospun. Blends of natural and synthetic polymers are desirable as they can provide precise control over degradation. The intelligent addition of slower degrading synthetics has been shown to successfully amend the relatively high solubility and rapid degradation characteristic of natural biopolymers.[30,42] These composites can slow the rapid loss of structural integrity expected from biopolymers in physiological condition. Therefore common solvents for both natural and synthetic polymers are needed to produce fibers that ideally combine the physical stability of synthetic polymers with the biological activity/acceptance of natural

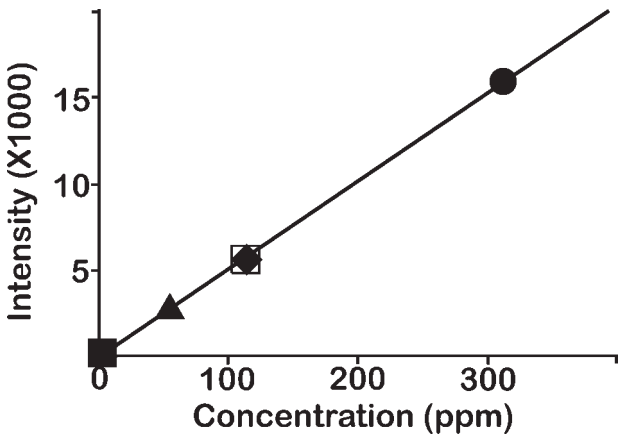

Figure 5 HFIP content of 37°C DI water exposed to 1 week old G0 (filled square), G25 (triangle), G50 (empty square), G75 (diamond), and G100 (circle) for 24 h.

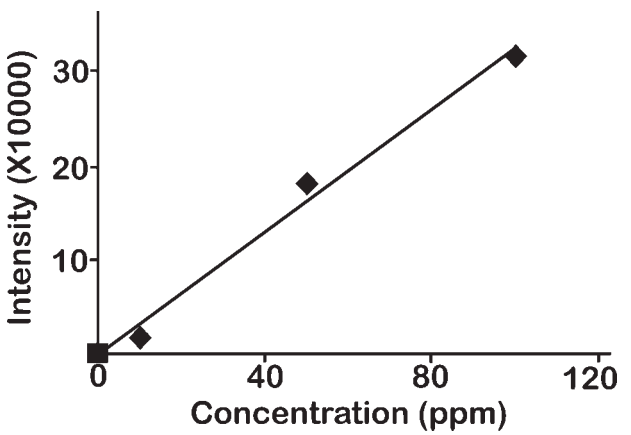

Figure 7 HFIP content of PCL completely dissolved in 15 : 85 formic acid-methanol (square: PCL, diamonds: standards (10, 50, and 100 ppm HFIP in 15 : 85 formic acid–methanol)).

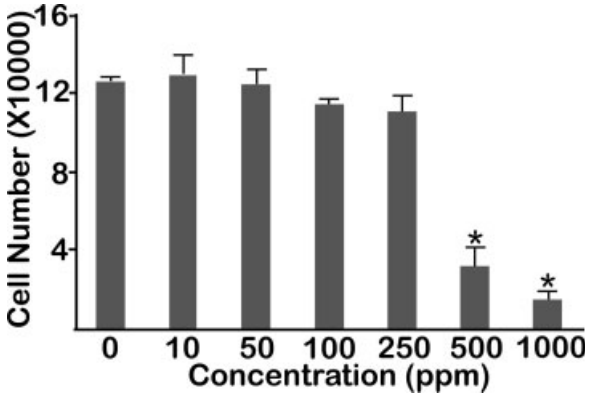

Figure 8 The number of chondrocytes on the 2nd day of culture in cell culture media containing the indicated HFIP concentrations. HFIP-free cell culture medium was used as a control. The asterisks indicate statistically significant reductions (relative to 0 ppm of HFIP) in cell number. P values for the 100 and 250 ppm contents are > 0.05.

polymers.[30,43,44] Fluorinated alcohols such as trifluoroethanol (TFE) and HFIP and variations are logical choices. However, there has been no systematic analysis directed toward establishing the existence of residual fluorinated alcohol following their use as electrospinning solvents. Furthermore, review of the literature provides no objective information on postspinning treatments to remove potentially trapped solvents.

Table I summarizes the amount of HFIP eluted per gram of electrospun gelatin, PCL and their blends. It is evident that HFIP has a much higher affinity for gelatin. Fluorinated alcohol has been used to denature proteins and stabilize structures in peptides due to its strong interaction with both polar and nonpolar amino acid side groups.[45] This interaction with both the hydrophilic and the hydrophobic groups within collagen[46] explains why residual HFIP scales directly with gelatin content.

TABLE I
HFIP Concentration Per Gram Either Eluted (in DI Water at 37°C) or Digested out of Electrospun Fibers

Condition/Sample	HFIP concentration (ppm) per g of sample
As-spun	
G0	500
G25	3190
G50	9910
G75	7370
G100	16600
1-Week-old	
G0	0
G25	550
G50	1140
G75	1130
G100	3140
Vacuum treated G50	140
37°C with vacuum treated G50	100
45°C with vacuum treated G50	60
Digested 1-week-old G0	3

Figures 5 and 6 simulate releases during typical rinsing, sterilization and *in vitro* exposures. No HFIP was detected in the elution experiment involving 1-week-old G0 and only a nominal amount (3 ppm/g) was detected in digested G0 (Table I). This implies that most of the HFIP is shed either during the electrospinning process or shortly thereafter. The high surface area of the fibers favors this in combination with the relatively lower polarity of the PCL. The fact that no acetone was detected in the PCL when acetone was used as a solvent supports the idea that electrospun PCL is usually relatively free of the effects of retained solvent. This is not meant to suggest that all synthetic polymers are immune to residual solvent retention during electrospinning. Kalayci et al. observed residual dimethylformamide when electrospinning polyacrylonitrile.[47]

Several different postprocessing treatments were utilized to reduce the amount of residual HFIP. All are effective in reducing HFIP content but a >99% reduction in the G50 sample could be obtained by treatment at 45°C and vacuum for 24 h (Table I). The toxicity test (Fig. 8) suggests that HFIP contents of 500 ppm or higher are significantly toxic for *in vitro* cell culture. This allows us to estimate that at least 4.2 g of treated G50 (for example) could provide a scaffold that does not elute toxic levels of residual solvent. When scaffold volume is considered, this equates to about 16.0 cm^3 based on the $\sim$ 80% relative porosity of the fiber mesh. However, if cells are seeded internally into such scaffolds (either by simple injection or via flow through configuration) instantaneous localized HFIP concentrations could conceivably be higher than those reported here. Cel-

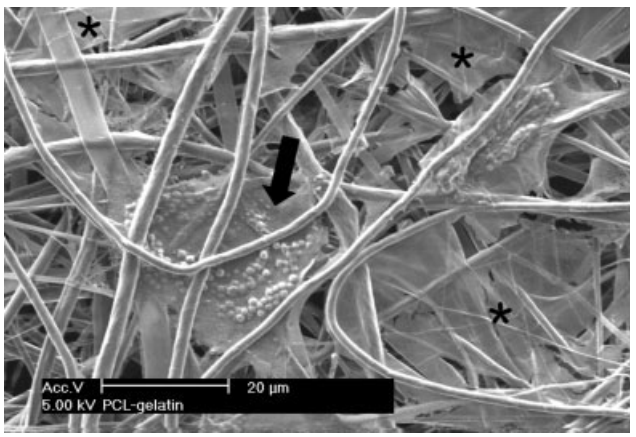

Figure 9 SEM image of chondrocytes (arrow) cultured on postprocessed (45°C in vacuum for 1 day) G50 for 5 days. Cell-like thin films generated from the breakdown of the gelatin fibers are also shown (*).

lular growth and proliferation might conceivably be delayed or limited until sufficient outward HFIP diffusion has taken place.

Figure 1 shows that flattened fibers can form and this is consistent with the formation of a polymer-rich fiber shell in combination with a solvent-rich fiber core[48] likely due to the high volatility of HFIP. Figure 2 shows that these blends can break down over a relatively brief time period and produce film-like structures that resemble the morphology of spread cells. We suspect that a gelatin-rich layer forms on specific fiber surfaces and that highly mobile films tend to originate and grow outwards from fiber–fiber intersections.

None of the vacuum treatments employed in this investigation resulted in obvious changes to fiber microstructure. This is somewhat anticipated as the uniform removal of a few ppm of a given solvent from an individual fiber is unlikely to cause obvious shrinkage or pitting.

Figure 9 shows that chondrocytes exhibit a well-spread morphology and other positive indicators on the 45°C vacuum-treated G50 specimens suggesting that this scaffold can successfully promote cell growth. This is in contrast to the results of Kwon et al.[31] who observed rounded or restricted-spread cells on electrospun collagen-PCL fibers fabricated using 30 and 50 wt % collagen. Highly elongated cells like those seen in Figure 9 were observed at lower collagen contents (5 or 10 wt %) where residual solvent levels might also be expected to be lower. Kwon et al. attributed this effect to the shrinkage of the scaffold during the culture period even though cell motility over many days should have been more than sufficient to allow for cell rearrangement.

CONCLUSIONS

To our knowledge, this study is the first to systematically analyze the residual solvent in fibers produced by electrospinning. Gelatin is clearly more prone to residual solvent retention than PCL. Other biopolymers likely have similar relative tendencies to retain residual solvents. Fortunately, a simple and effective procedure reducing residual solvent content was developed. The majority of the residual solvent could be reduced to a level that had no demonstrated effects on cell interactions with the scaffold. Electrospinning as a means of investigating tissue engineering needs to include consistent postprocessing treatments to avoid possible residual solvent release that could greatly complicate correct interpretation of biological activity on the resulting scaffolds.

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