Direct in Vitro Electrospinning with Polymer Melts

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The electrospinning of polymer melts can offer an advantage over solution electrospinning, in the development of layered tissue constructs for tissue engineering. Melt electrospinning does not require a solvent, of which many are cytotoxic in nature, and the use of nonwater soluble polymers allows the collection of fibers on water or onto cells. In this article, melt electrospinning of a blend of PEO-*block*-PCL with PCL was performed with in vitro cultured fibroblasts as the collection target. The significant parameters governing electrospinning polymer melts were determined before electrospinning directly onto fibroblasts. In general, a high electric field resulted in the most homogeneous and smallest fibers, although it is important that an optimal pump rate to the spinneret needs to be determined for different configurations. Many parameters governing melt electrospinning differ to those reported for solution electrospinning: the pump rate was a magnitude lower and the viscosity a magnitude higher than successful parameters for solution electrospinning. Cell vitality was maintained throughout the electrospinning process. Six days after electrospinning, fibroblasts adhered to the electrospun fibers and appeared to detach from the underlying flat substrate. The morphology of the fibroblasts changed from spread and flat, to long and spindle-shaped as adherence onto the fiber progressed. Therefore, an important step for producing layer-on-layer tissue constructs of cells and polymers in view of scaffold construction for tissue engineering was successfully demonstrated. The process of using cultured cells as the collection target was termed "direct in vitro electrospinning".

Introduction

Electrospinning has recently attracted interest as a method to make scaffolds for tissue engineering applications. The diameter of electrospun fibers is of similar magnitude to fibrils in extracellular matrix,[1] and electrospun fibers have demonstrated effectiveness as a substrate for cell growth. Where a cell may adhere to only one microfiber in a typical biomedical textile, one cell can adhere to multiple electrospun fibers. Cells may adopt significantly different morphologies on electrospun fabrics, when compared to flat surfaces.[2−7] Smooth muscle cells orient their bodies along the length of the fiber,[2] whereas electrospun fibers guide axons from neurons.[3] Cardiomyocytes organize depending on the scaffold morphology and can develop filopodia-like extensions along the electrospun fibers.[4] Fibroblasts are particularly motile and stretch their cell body into "spindle-like" morphologies.[5] Chondrocytes also adopt a spindle-shaped morphology when seeded on electrospun scaffolds.[6] However, the surface properties of electrospun fibers are important for cell adhesion and spreading, demonstrated by different spreading of keratinocytes and fibroblasts on electrospun fabrics treated with different adsorbed proteins.[7]

Although electrospun fibers of various polymers and different cell types have been brought into contact numerous times, the fibers are typically electrospun first and cells are typically seeded onto the material in a further step.[2−11] The limited literature on successful "direct in vitro electrospinning", the electrospinning of fibers directly onto cells, is likely due to two technical reasons. First, toxicity issues preclude the use of many nonaqueous solvents (which are typically volatile) commonly used in electrospinning; such solvents need to be fully removed prior to in vitro use.[7] Although the solvent toxicity aspect is not critical with initial in vitro experiments (as most of the solvent evaporates), such approaches will be subject to scrutiny when the tissue/scaffold construct is advanced to clinical trials.[12]

The use of aqueous polymer solutions bypasses this toxicity issue for direct in vitro electrospinning; however, the fibers must be cross-linked or redissolution results since the collection target is of aqueous origin.[13,14] Therefore, for any continuous process the cross-linking of the aqueous solution must take place during the airborne phase between spinneret and collector, typically a matter of milliseconds. It is likely that for these reasons electrospinning onto cells in culture (termed "direct in vitro electrospinning") or onto open wounds (direct in vivo electrospinning) is sparsely reported in the literature. In a recent article, promising tissue constructs of electrospun fibers were formed by the co-application of electrosprayed smooth muscle cells.[12]

A strategy for successful direct in vitro electrospinning, outlined in this article, is to electrospin a polymer melt, which contains no solvent. The airborne fiber/polymer jet must cool sufficiently between the heated spinneret and the collector to form a fiber. The stretching of a polymer jet at high elongations is important when developing the controlled collection of smaller diameter melt electrospun fibers, and therefore, the viscosity of the polymer melt is an important parameter in diameter formation. The polymer melts used for direct in vitro electrospinning in this article are polymer blends with high and low molecular weight components that demonstrate similar viscosities over a range of shear rates. The lower molecular weight polymer is additionally an amphiphilic block copolymer, to contribute the charge and so that a range of drug therapies can be included at a future date. Very few articles have been published on melt electrospinning,[15−19] and we aim to contribute to the knowledge of the necessary parameters for this process.

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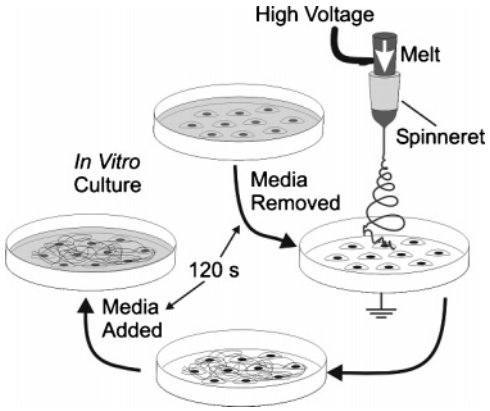

Figure 1. Schematic of direct in vitro electrospinning.

Table 1. Summary of Conditions for the Highest Quality Fibers Melt Electrospun onto Metallic Collectors

spinneret gauge (G)	spinneret diameter (mm)	optimum flow rate (mL/h)	optimum collection distance (cm)	electrospun fiber diameter (μm)
16	1.19	0.1	15	1.7 ± 0.3
20	0.58	0.05	10	1.5 ± 0.3
24	0.30	0.03	5	1.7 ± 0.3

In contrast to many former tissue-engineering experiments with electrospun fibers, electrospinning directly onto cells will be performed, as depicted in Figure 1. Fibroblasts are cultured onto a surface, the media is removed, and the electrospun fibers applied to the cells. The media is then replaced and the cell/electrospun fiber construct observed.

Materials and Methods

Materials and Copolymer Synthesis. All materials and solvents, unless otherwise stated, were purchased from Aldrich Chemicals (Milwaukee, WI). The high-molecular weight polymer blend component was 67 000 g/mol poly(ϵ-caprolactone) (PCL) and was used as received. The low molecular weight poly(ethylene oxide-*block*-ϵ-caprolactone) (PEO-*b*-PCL) amphiphilic block copolymer was synthesized by ring opening polymerization as previously described.[20] The molecular weight of the amphiphilic block copolymer (PEO_{47}-*b*-PCL_{120}) was determined to be 12 800 g/mol by ^{1}H NMR spectroscopy; the polydispersity index was 1.36 (GPC) measured in THF using PS standards; and a melting point of 55.9°C was determined by DSC. The polymer blend for melt electrospinning was prepared dissolving PCL and PEO_{47}-*b*-PCL_{120} (20:80 wt %) into a dichloromethane solution, followed by evaporation and removal of the solvent in a vacuum. After full solvent removal, the polymer blend was then ready for melt electrospinning.

Melt Electrospinning. The polymer blend was melted in a 1 mL syringe heated to 85 °C. To reduce air bubbles, the molten polymer was pushed backward and forward in the heated syringe. The syringe containing the polymer melt was inserted into a heated condenser at 85 °C and equilibrated for approximately 20 min. Blunt-ended stainless steel cannulas were used as spinnerets, with diameters of 16, 20, or 24 G (Table 1). An earthed aluminum stub for scanning electron microscopy (SEM) was covered with aluminum foil and used as a collector, and the distance between the spinneret and collector (collection distance) was variable from 5 to 30 cm. The polymer was pumped to the spinneret with a flow rate between 0.03 and 0.3 mL/h. For electrospinning, a negative voltage of 25 kV was applied to the spinneret, and the fibers were collected onto the aluminum SEM stubs. Depending on the polymer and the experiment, the collection times were between 10 s and 5 min. The shear viscosity of the polymer blend at 85 °C was determined over a range of shear rates ($0.01-100\ s^{-1}$) with a controlled stress rheometer (Rheometric Scientific DSR, USA).

Scanning Electron Microscopy (SEM). The electrospun material collected directly onto the stubs was gold-coated and imaged on a Cambridge S360 SEM (Leica, Germany). An electron beam of 15 kV and a collection distance of 10−15 mm were used to image the electrospun material. Representative images of the electrospun fibers are presented.

Modification of Petri Dishes. To use the cell layer as a target for electrospinning, the bottom of a 35 mm Petri dish was grounded with a metallic screw inserted through the bottom of the Petri dish which protruded into the well for approximately 1 or 1 mm. It was fixed and sealed with hot glue to the underside of the Petri dish, which was then filled with distilled water and left for 21 h, followed by 8 h in a solution of 30/70 ethanol/water. The Petri dish was then left in a vacuum for 48 h before final disinfection with 70/30 ethanol−water solution prior to use.

Cell Line. Preparation of the 9th and 11th passage of human fibroblasts of a 39 year-old woman was performed[21] and approximately 6×10^6 cells were plated within each modified Petri dish and filled with 4 mL of Dulbeccos Modified Eagle Medium (DMEM) with penicillin/streptamycin and 10% fetal calf serum (FCS). After 24 h, the cell condition was examined, and the media was exchanged. The fibroblasts were then ready for electrospinning 24 h later.

Direct in Vitro Electrospinning. The direct in vitro electrospinning was performed as schematically outlined in Figure 1. Prior to direct in vitro electrospinning, the electrospinning apparatus was placed in a disinfected flow hood and cleaned with a 70% ethanol−water solution two times. Immediately before electrospinning, the media was removed from the Petri dish with the cells. The fibers were collected onto the cells for approximately 60 s; immediately after electrospinning, fresh media was added, and the cell−fiber constructs were stored in the incubator. The media was removed from the Petri dish for a total of approximately 120 s. Controls of cultured fibroblasts with no exposure to the polymers or the electric field were used. All time points and controls were performed in triplicate. A 20 G spinneret, a temperature of 85 °C, and a collection distance of 10 cm were used, whereas a pump rate of 0.3 mL/h and applied voltage of 25 kV were selected from preliminary experiments with the grounded Petri dishes.

Staining. Staining with hämalaun gives an overview of the cell morphology. The media was removed, and 3 mL of formalin were added and incubated for 20−30 min before rinsing three times with distilled water. The cells were covered sparely with a freshly filtered solution of hämalaun for 30 min before rinsing again three times with distilled water. The samples were dried at a temperature of 35−40 °C and examined with a light microscope.

The Live/Dead Viability/Cytotoxicity Assay Kit (Molecular Probes, Germany) was used as per instructions. Briefly, a solution of 2 μM calcein AM and 4 μM EthD-1 in PBS was prepared. The fibroblasts/electrospun fibers were washed 3 times with PBS before 600 μL of the staining solution per Petri dish was added. The cells were incubated for 30−45 min and then imaged with a fluorescence microscope; red indicated cell death, and green demonstrated cell viability.

Staining for F-actin was performed with Alexa Fluor 568 phalloidin (Molecular Probes, Germany). Fibroblasts/electrospun fibers were washed twice with PBS and then fixed with formalin (3.7% formaldehyde in PBS) for 10 min at room temperature before rinsing three times with PBS. The cells were extracted with 1 mL of 0.1% Triton X-100 in PBS for 3−5 min at 20 °C before washing twice with PBS. Preincubation with PBS containing 1% bovine serum albumin (BSA) for 20−30 min at 37 °C was then performed. A solution of 15 μL of Alexa Fluor 568 phalloidin in 600 μL PBS and 1% BSA for each dish was then used to stain for F-actin. The cells were incubated for 20 min at room temperature, before washing the cells at least two times with PBS and air-drying. A fluorescence microscope with a filter set of ex/em = 578 nm/600 nm was used to visualize the F-actin filaments. A phase contrast image reveals predominantly the fibers and is digitally

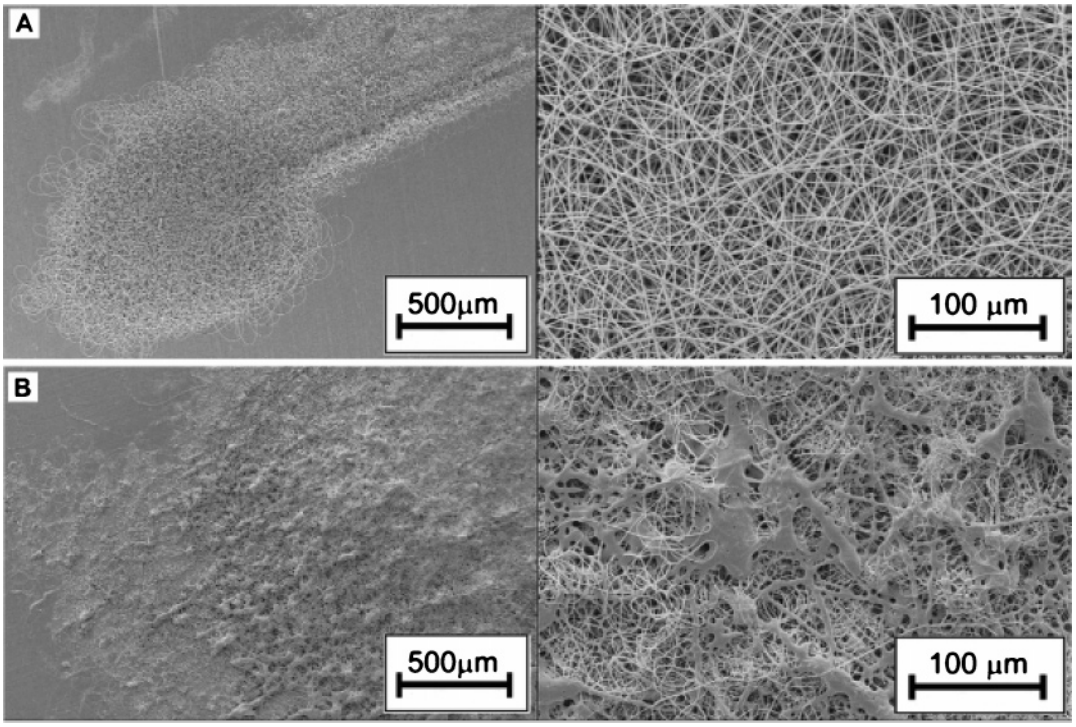

Figure 2. SEM images of electrospun fibers produced with a pump rate of (a) 0.03 mL/h and (b) 0.05 mL/h.

altered (Corel Paint 12) and overlaid to show the position of the fibers. The edge contours of the phase contrast images are found, and the image is inverted to allow a black background. The fiber outline image is then superimposed onto the cell-staining images with a degree of transparency. Live−dead percentages between groups were compared using 1-way ANOVA analysis (significance accepted if $p \leq 0.05$) using SPSS 14 Software (SPSS Inc., IL).

Results and Discussion

Electrospinning of Polymer Melts: Effect of the Experimental Configuration. Prior to electrospinning onto fibroblasts, the suitable parameters for collecting melt electrospun fibers onto aluminum SEM stubs were determined. The process of electrospinning is controlled by many parameters: some concerning the polymer melt/solutions[22,23] and others the electrospinning apparatus.[24,25] Some particularly important parameter differences between melt and solution electrospinning were observed.

The viscosity of the polymer melt (33 ± 1.76 Pa.S) is at least a magnitude greater than viscosities reported for solution electrospinning.[7,8,23] The flow rates required to obtain high quality electrospun fibers were 0.1 mL/h or lower, which is significantly lower than for solution electrospinning, where flow rates such as 5 mL/h are used.[26] Figure 2 shows a loss in quality of the electrospun material often observed when the pump rate is increased. This may be due to the increased charge density of the electrospun material, which has been shown, for polymeric solutions, to increase exponentially with lower flow rates.[22] The optimum flow rates for each electrospinning configuration are listed within Table 1.

Poor quality fibers that are not sufficiently cooled before reaching the collector are flattened onto the collector and are considered molten fibers; this describes their state when they land on the collector. Flow rates that are lower than this experimentally determined optimum level also result in fibers that are nonuniform in structure with both molten and high quality fiber diameters collected. At flow rates comparable to those used in solution electrospinning, the electrospun fibers are all molten, have a large diameter, and are of an extremely poor quality. The variation of the flow rate contributed the greatest influence on the quality, morphology, and diameters of the collected fibers.

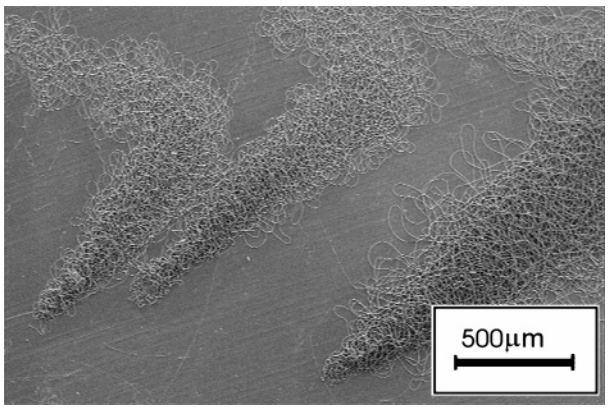

Figure 3. Electrospun fibers of the polymer blend, showing "islands" of fibers that are frequently collected.

While electrospinning with a 20 G spinneret and at 25 kV, the highest quality fibers were formed with a collection distance of 5 cm. The fibers were very focused in their deposition, and high quality fibers were almost exclusively formed into small coiling loops of fibers. The electrospun fibers formed with short collection distances were often deposited as "islands" of material as shown in Figure 3. In many instances, these "islands" were deposited in a similar direction and shape. It is possible that, in combination with focused deposition, the charges built up locally in a patch of electrospun fibres, until a point where deposition was moved to a nearby position. Additionally, the shielding that the deposited fibers present may result in a repositioning of the electrospun fibers. The formation of "islands" was quite a common phenomenon when melt electrospinning onto metallic collectors, and it is interesting to note that such formations did not occur in later in vitro investigations.

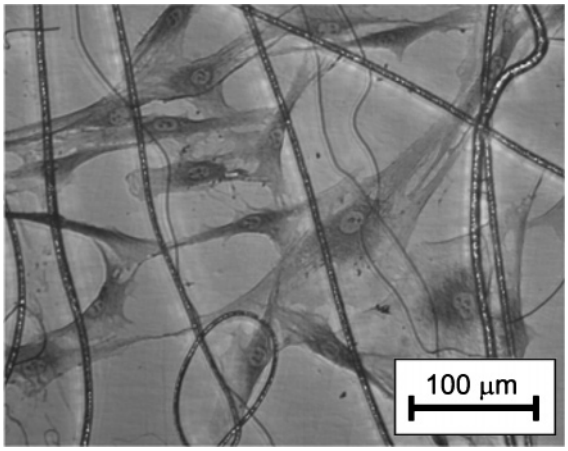

Figure 4. Electrospun fibers immediately after being deposited onto fibroblasts.

Irrespective of the conditions, only one Taylor cone was ever observed from the melt droplet, which was elongated toward the collector by up to 5 mm and was stable throughout experiments. This is another difference to solution electrospinning where it is very common for multiple jets to be erupted from the spinneret. As the spinneret diameter was increased, higher flow rates and collection distances produced the best quality electrospun fibers, as listed in Table 1. The 20 G spinneret resulted in the highest quality fibers, whereas electrospun material collected with the 16 or 24 G resulted in overall reduced fiber uniformity. Table 1 provides an overview of the various spinneret diameters that resulted in the highest quality for the electrospinning of the polymer blend onto the aluminum SEM stub. Overall short collection distances, in combination with low flow rates to the 20 G spinneret, produced the most consistent electrospun material with diameters of $1.5 \pm 0.3\ \mu$m.

Although electrospinning was performed from the melt, solvents were used to expedite the preparation procedure. An alternative, fully solvent-free approach, obtained by heating and mixing the PCL and PEO_{47}-*b*-PCL_{120} in a nitrogen atmosphere above 60°C, will be investigated.

Direct in Vitro Electrospinning. From preliminary studies with electrospinning polymer melts onto SEM stubs, it was decided that a 20 G spinneret, in conjunction with a 10 cm collection distance, would be used for direct in vitro electrospinning, and the flow rate would be varied to produce the highest quality fibers. When grounded Petri dishes were used in place of SEM stubs, however, the quality of the electrospun fibers was reduced and the range of fibers diameter varied considerably. The optimum flow rate for producing the highest quality fibers was 0.3 mL/h, which is 6-fold greater than for the SEM stubs. The difference was attributed to shielding that the bottom of the Petri-dish contributes to the configuration. One patch of fibres was electrospun onto the fibroblasts in each experiment and was mostly deposited in the Petri dish on the opposite side of the metal grounding.

Figure 4 demonstrates the mixed diameters of fibers that result from electrospinning in the current Petri dish configuration. Fibers of different sizes are apparent; however, the adherence of the fibers onto the surface is good, and the electrospun material does not readily float off the surface. Immediately after direct in vitro electrospinning, the fibroblasts remain spread onto the Petri dish, while all controls have similar fibroblast morphologies, suggesting that the electrospinning process did not immediately affect fibroblast morphology.

Cell Vitality. After 24 h, and then later at 72 h, the live−dead staining reveals no statistically significant differences ($p \leq 0.05$) between any of the controls or electrospun samples as determined with 1-way ANOVA analysis ($F = 0.286$; $p = 0.835$). There was a certain level of vitality loss since fibroblasts were grown to confluence, which results in a higher percentage of cell death. Furthermore, using different filter sets and observing individual cell vitality, there was no correlation with where the fibers were deposited. Even large, poor quality, molten fibers deposited upon fibroblasts did not result in localized cell death around the fiber (Figure 5).

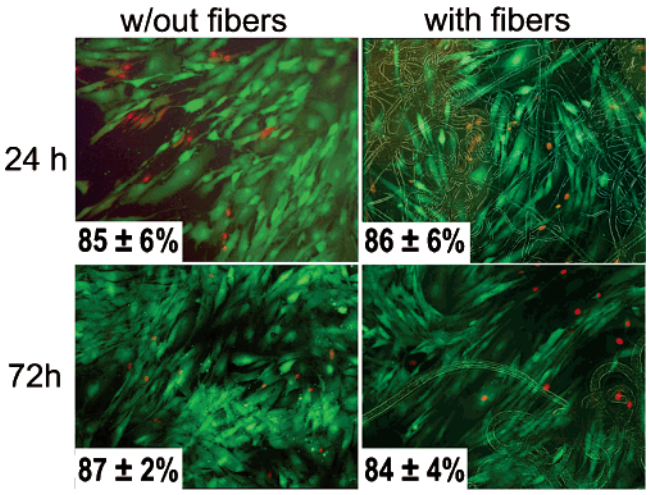

Figure 5. Live/dead images of fibroblasts at 24 and 72 h post-electrospinning, with inserts showing the live percentages. The fibers (enhanced by white highlighting the fiber edge) imaged from phase contrast are overlaid with fluorescence imaging for live cells (green) and dead cells (red) at the same position. The scale bar is 100 μm.

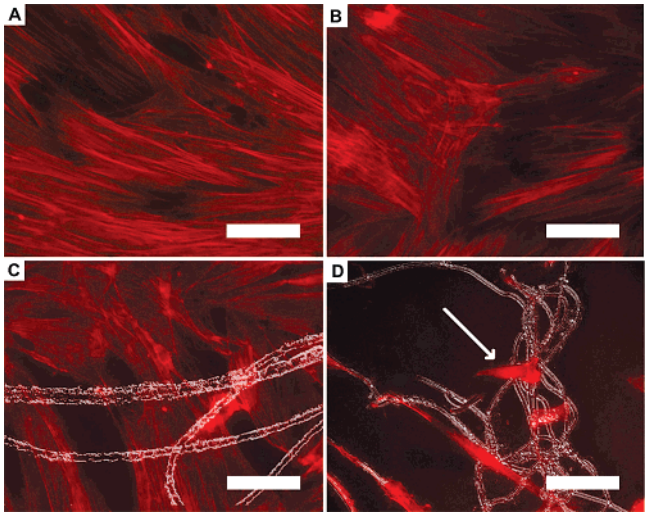

Figure 6. Fluorescent images of F-actin filaments stained at 72 h (a and b) and 6 d (c and d) post-electrospinning demonstrating how fibroblasts changed into a spindle like morphology, orienting their cell body with the fiber at 6 d. Panels c and d show overlaid images of fibers enhanced by white highlighting the fiber edge. Fibroblasts are often seen detaching from the surface of the Petri dish and adhering to the fibers (arrowed). The scale bar is 50 μm.

Actin Filament Staining. Actin filaments identify stress fibers that can pull on other cells or ECM via focal contacts. F-actin staining was done to observe if the cells adhere directly to the fibers, which might be seen in a change of orientation of the filaments after several days in contact with fibers. No qualitative differences in morphology between the sample and the control at the time-point of 72 h were observed (Figure 6a); however, fibroblasts attach and orient their cell bodies along the electrospun fiber at 6 d. This elongated, spindle-shaped

morphology of fibroblasts shown in Figure 6b has previously been reported with fibroblasts and electrospun fibers[5] and is significantly different to their "normal" flattened morphology when grown onto Petri dish surfaces. Some fluorescence of the fibers was also observed with the actin stain; however, such fibers could readily be differentiated with spindle-shaped fibroblasts. Fibroblasts were also often observed in the process of detaching from the tissue culture plastic substrate and adhering fully with the electrospun fibers as indicated in Figure 6b.

Summary

The parameters governing melt electrospinning differ from those reported in the literature for solution electrospinning. Spinneret flow rates are much lower, and the viscosity of the polymer is a magnitude greater for melt electrospinning systems. Only one polymer jet erupted from the spinneret, while the fibers were coiled and deposited in a focused manner, compared to solution electrospinning.

The first experiments with cells as the collection target for melt electrospinning demonstrates the difficulty in translating optimized conditions to biologically relevant systems. The quality of electrospun fibers collected onto Petri dishes was significantly poorer than those fibers collected on aluminum as the introduction of a polystyrene plate disrupts with the electric field. Therefore, further alterations with grounding the in vitro systems are necessary to produce high quality fibers for direct in vitro electrospinning.

The in vitro experiments themselves were successful and indicated that direct in vitro electrospinning with polymer melts did not result in cell death. Further direct in vitro electrospinning experiments with nonconfluent fibroblasts should determine exactly whether cell vitality is unaffected. After 6 days after direct in vitro electrospinning, F-actin staining showed a significant change in the orientation of the fibroblast along the fiber axis, forming a spindle-shaped morphology. Some fibroblasts appeared to be in the process of detaching from the lower culture surface and adhering to the electrospun fibers.

Conclusion

Electrospinning directly onto cells, or direct in vitro electrospinning, is possible via melt electrospinning. Cell vitality was maintained at the time points of 24 and 72 h post-electrospinning. After 6 d, many fibroblasts had detached from the initial culture surface and were orientated along the axis of the fiber. The conditions for producing high quality fibers for aluminum stubs are different for electrospinning into cell dishes, and future work will improve the quality of fibers electrospun onto cells.

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