Mammalian Cell Viability in Electrospun Composite Nanofiber Structures

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Incorporation of mammalian cells into nanofibers (cell electrospinning) and multilayered cell-nanofiber structures (cell layering) via electrospinning are promising techniques for tissue engineering applications. We investigate the viability of 3T3-L1 mouse fibroblasts after incorporation into poly(vinyl alcohol) nanofibers and multilayering with poly(caprolactone) nanofibers and analyze the possible factors that affect cell viability. We observe that cells do not survive cell electrospinning but survive cell layering. Assessing the factors involved in cell electrospinning, we find that dehydration and fiber stretching are the main causes of cell death. In cell layering, the choice of solvent is critical, as residual solvent in the electrospun fibers could be detrimental to the cells.

Introduction

Synthetic materials capable of supporting the proliferation, differentiation, and development of viable mammalian cells into a desired tissue in vitro are of considerable interest. Such materials, scaffolds, must provide structural support and interactions that direct cell growth. Ideally, the scaffold material would be biocompatible, porous with high surface area, fully biodegradable or absorbable, similar in mechanical properties to the target tissue, and sterilizable.\[1–15]\ Nanofibers are considered especially promising for tissue scaffolds because their size scale mimics the extracellular matrix.\[10,15–21]\ Electrospinning is a facile process used to generate nanofibers and nanofiber composites that involve the application of a high voltage (1–30 kV) to induce the formation of a liquid jet from a polymer solution or melt. The electrospinning setup consists of three major components: a high-voltage power supply, a capillary device, and a ground collector. The polymer solution or melt is fed out of the capillary by a positive displacement pump and a high-voltage electric field is applied which generates charges in the pendant drop of the polymer liquid at the nozzle. With increasing electrostatic forces, the droplet forms a conical shape (a Taylor cone) and a fine liquid jet ejects from its apex toward the collector. The liquid jet is stretched and whipped resulting in a long and thin fiber. As the jet is continuously extended and the solvent evaporates, the diameter of the jet is reduced from several hundred
micrometers to as low as tens of nanometers. The charged fiber is ultimately deposited onto the grounded collector plate as a non-woven mat of nanofibers.\[9,18–35\]

The use of electrospun nanofibers in tissue engineering provides a unique set of advantages that include process simplicity, inexpensive handling costs, versatility in material selection, and control of mat thickness over time.\[10–17\] In addition to mimicking the extracellular matrix, nanofibers possess high surface area to volume ratio, have pore interconnectivity and exhibit 3D fiber network structure.\[10,16–18\] Electrospun nanofibers have been studied as potential scaffolds for engineered cartilage, bone, arterial blood vessel, heart, nerve, and skin tissues. However, the electrospun scaffolds suffer from two major problems: insufficient thickness and small pore sizes, both of which can hamper cell migration and proliferation.\[10,36,37\]

We propose two different approaches toward developing polymer-based electrospun nanofiber structures to overcome some of the disadvantages inherent in tissue engineering including insufficient penetration of cells into the structure, inadequate thickness of the fibrous mat and unsatisfactory cell distribution throughout the 3D fibrous structure.\[38,39\] Our first proposed approach is direct incorporation of cells into nanofibers during electrospinning (cell electrospinning).\[40\] This would provide homogenous cell distribution within the nanofibrous mat structure and overcome pore size limitations of electrospun nanofibers. Our second approach entails sandwiching cells between two electrospun nanofibrous mats (cell layering). In this method, a nanofibrous mat layer is initially produced via electrospinning, a layer of cells is then put on top of the membrane, and a further layer of nanofibrous web is finally electrospun on top of the cell layer. Such an approach would increase the thickness of the final structure as well as provide cell penetration within the structure.\[41\] However, an understanding of how the viability of the cells is affected during these processes is missing, and needs to be addressed.

Cells (bacteria, virus, and mammalian cells) have been successfully incorporated into electrospun nanofibers using both single needle electrospinning and coaxial electrospinning.\[40,42–45\] However, previous reports indicate that a large fraction of cells are incapacitated during electrospinning;\[43\] yet the reason for cell death remains unconfirmed. In this study we electrospun polyvinyl alcohol (PVA) with 3T3-L1 mice fibroblast cells and systematically examined how different aspects of the electrospinning process, including the presence of the electric field, shear stresses during passage through the needle, stretching during fiber formation, and dehydration, affected cell viability. We used 3T3 mice fibroblasts as a model system because it is readily available and also has potential application in the reconstruction of adipose tissue.

Layered structures mimic the stratified, lattice like structure in tissues and have been successfully used to generate 3D structures with multiple cell types.\[46,47\] Layered structures constructed using electrospinning has also been recently reported.\[41,48\] In this study, we use poly (caprolactone), PCL, a biocompatible and biodegradable polymer, and electrospinning layers to entrap 3T3-L1 mouse fibroblasts for comparison to cell electrospinning. Additionally, we systematically determine how different aspects of the layering process affected cell viability. In this approach, factors that may affect cell viability include cell type, polymer system, application of the electric field and deposition of fibers when adding layers, and residual solvent. In particular, cell type has yet to be fully explored. Finally, studies directly comparing cell electrospinning and cell layering with respect to cell viability using the same cell type have yet to be performed.

### Experimental Section

#### Materials

- Polyvinyl alcohol (PVA) (average molecular weight 205 000 g · mol⁻¹, 88% hydrolyzed), PCL (average molecular weight 50 000), dimethyl formamide (DMF), chloroform, HFIP (1,1,1,3,3,3-Hexafluoro-2-propanol), Hank’s Balanced Salt Solution (HBSS), and Trypan Blue were purchased from Sigma Aldrich. Dulbeco’s Modified Eagle’s Medium (DMEM) with HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid), Thermo Scientific HyClone Fetal Bovine Serum (FBS), trypsin-EDTA, and 70% ethanol were purchased from Fisher Scientific.
- 1-Glutamine and Pen-Strep were purchased from Cellogram Biotechnology. 3T3-L1 fibroblasts were purchased from American Type Culture Collection (ATCC) Bioresource Center and stored in liquid nitrogen. The LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells was from Invitrogen Life Technologies. In this fluorescence-based assay, live cells are green and dead cells are red when viewed using fluorescent microscopy. All materials were used as received.

#### Cell Culture

Upon receipt of cells (cryopreserved) from ATCC, 3T3 – L1 fibroblasts were expanded according to ATCC instructions, and cryopreserved in multiple vials of approximately 250 000 cells per vial. A fresh vial of cells was thawed for each series of experiments. Upon thawing, for each vial, viability of cells was assessed with trypan blue prior to seeding at approximately 250 000 cells per 75 cm² flask. Cells were cultured at 37 ºC with 5% CO₂ in Dulbecco’s Modified Eagle’s Medium with 10% FBS using standard tissue culture protocols as specified by the supplier. The cells were harvested at 70–80% confluency, approximately 6–7 × 10⁶ cells · cm⁻², or 5 million cells per 75 cm² flask. For electrospinning experiments, cells were harvested using trypsin as recommended by supplier and washed once in growth medium by centrifugation at 550 rpm for 5 min. In experiments involving direct electrospinning of cells into nanofibers, cell suspension with polymer solution in the ratios...
stated were used. For cell layering experiments, we pipetted 20–25 μl cell suspension containing 25,000 cells between nanofiber mat layers.

**Electrospinning**

**Single Needle**

Polyvinyl alcohol (PVA) solutions were prepared by mixing PVA in deionized water at 60 °C until homogeneous. PCL (13 wt.-%) was dissolved in DMF/chloroform (30/70 w/w) or acetone by stirring at 60 °C until homogeneous. Similarly, PCL (10 wt.-%) was dissolved in HFIP by stirring at 50 °C until homogeneous.

To electrospin, the polymer solution or polymer/cell mixture was loaded into a syringe fitted with a stainless steel needle (0.508 mm ID) and attached to a power supply (Matsusada Precision Inc, NY), as previously described by Saquing et al. Flow rates were between 0.25 and 0.6 mL/h (New Era Pump Systems Inc, NY) with voltages between 3 and 20 kV and a set tip to collector distance of 15 cm. In a typical experiment of 8 wt.-% PVA with cell suspension, the flow rate was 0.5 mL/h and the voltage was 12 kV.

**Coaxial**

A coaxial electrospinning method was also used that had a suspension of 3T3-L1 fibroblasts in growth media in the core and PVA in the outer shell.[50, 51] The inner and outer needles (Hamilton Company, NV) were 22G (0.72 mm ID) and 16G (1.65 mm ID), respectively. In a typical experiment, we used a voltage of 15 kV, a tip to collector distance of 15 cm and flow rates of 0.8 and 0.3 mL·h⁻¹ for the shell and core solutions, respectively.

**Cell Electrospinning**

Polymer solution (PVA) and cell suspension were combined in appropriate ratios and stirred for 5 min immediately prior to electrospinning. Experiments were carried out systemically to determine how the various components of the electrospinning process affected cell viability. To isolate the effect of shear stress, cell viability was observed after a suspension of cells in growth media was passed through the needle at the appropriate flow rate, but in the absence of high voltage. To determine the effect of charging the electrospinning solution, this procedure was repeated, but a positive charge was applied to the needle while the cell suspension passed through the needle. In another experiment, the effect of high voltage was examined by applying positive voltages ranging from 1 to 20 kV to the cell suspension as it passed through the needle while grounding the collector plate.

We also examined cell viability in the presence of the polymer/solvent system: PVA in water. First, the viability of cells suspended in the polymer solution after stirring was tested after stirring for 5 min. The effect of voltage in the presence of the polymer was then observed. At low voltages, below the voltage required to successfully electrospin fibers, the mixture of cells and polymer was loaded into the syringe, and droplets of the polymer/cell mixture were collected from the tip of the syringe. At voltages above the voltage required to successfully electrospin fibers, the viability of the cells within the fibers and effect of fiber stretching during electrospinning could be evaluated. Although solvent evaporation before the fiber is deposited cannot be eliminated, in an effort to prevent a loss of viability due to additional dehydration that may occur after the fiber is deposited, we electrospun directly into growth media rather than onto foil as in previous experiments.

**Cell Layering**

For layering studies, PVA or PCL solutions were electrospun for 10 min using a voltage of 15 kV, a tip to collector distance of 15 cm and a flow rate of 0.5 mL·h⁻¹. Following electrospinning, a specified amount of cell suspension was put onto the nanofibrous membrane, a second layer of nanofibers was then electrospun for 10 min on top of the cells. The final multilayered structure was placed into growth medium for 10 to 30 min before testing for viability.

**Sample Characterization**

**Fiber Structure**

Fiber morphology with and without cells was examined by coating with approximately 10 nm of Au/Pd and observing under field emission scanning electron microscope (JEOL JSM-6400 F) at 5 kV. The average fiber size and standard deviation were determined by measuring the diameter of at least 100 fibers using Image J software.

![Figure 1. Optical microscopy images of 3T3-L1 fibroblast cells; 11.8 ± 2.9 μm, from measuring of 50 cells.](image-url)
Cell Viability

Following both cell electrospinning and cell layering processes, the resulting products were placed into growth medium and stained using the LIVE/DEAD Viability/Cytotoxicity Kit for Mammalian Cells from Invitrogen Life Technologies. The staining kit included two components, 4 mM Calcein AM, which is taken up by live cells and converted in the cytoplasm to a green fluorescent molecule, and 2 mM Ethidium Homodimer-1 (EthD-1), which stains the nuclei of dead cells red. We first combined PBS, EthD-1 stock solution and calcein AM stock solution in appropriate proportions. We then mixed the cell suspension and staining solution directly on a coverslip. Finally, the samples were observed under Leica fluorescent inverted microscope (Leica DM IL LED). In order to calculate the cell viability, representative images from eight areas under fluorescent microscope were captured by Q imaging software following staining in order to count a minimum of 250 cells. The viability was calculated from the ratio of live cells to total cells as previously described.[52,53]

Results and Discussion

Cell Electrospinning

Single Needle

Our first step entailed direct electrospinning of 3T3-L1 cells with PVA into nanofibrous structures. Figure 1 shows optical micrographs of these cells in their native state prior to electrospinning with average cell size of \( \approx 12 \mu \text{m} \). In order to verify cell incorporation into nanofibers, we examined the size and shape of nanofibers formed using optical and electron microscopy. PVA nanofibers with and without cells are shown in Figure 2. In the case of the PVA only (8 wt.-%), we observe uniform fibers with average fiber diameter of 142 \( \pm 37 \) nm. However, for samples electrospun with cells, we observe beaded nanofibers (Figure 2a, right). Since the cells are bigger than the fibers produced, it is possible that the cells are appearing as bead-like structures. In addition, a much broader fiber size distribution, almost bimodal in nature, is observed (Figure 2b, right). From the fiber size distribution, we observe the average fiber size of the beaded portion to be \( \approx 500 \) nm whereas the fiber structures have an average diameter \( \approx 150 \) nm, similar to the pure PVA nanofibers. This may indicate that cells have been incorporated into the nanofibers. Since the size of the bead-like structures is smaller than the expected size of the cells (11.8 \( \pm 2.9 \) \( \mu \text{m} \)), the electrospinning process may distort the cells. These results are similar to previous reports.[42,43] In a similar system in which we have electrospun PVA/yeast cell composites, we have performed elemental analysis on the structural
features thought to be cells. Using EDS with SEM, we observe the presence of nitrogen and other compounds due to the presence of the cell that cannot be attributed to PVA.\[54]\n
In order to determine the maximum amount of cells that can be incorporated into the polymer matrix and yet obtain electrospun nanofibers, we systematically increased the weight by weight ratio of cells to PVA in the solution while keeping the PVA concentration constant at 8 wt.-%. Figure 3 shows the results of electrospinning solutions containing 0 to 66% w/w cells. With increasing amount of cells, the number of beads within the fiber as well as defects in the membrane due to droplets increased, which could provide further indirect evidence that the beaded structures within the fibers encapsulate cells (Figure 3). At 66% cells by weight, the resulting material is mostly droplets. However, a PVA was still able to produce fibers with substantial cell loadings (up to 50% cell suspension by weight).

Coaxial

Cells could also be incorporated into electrospun nanofibers using a coaxial approach. In this case, the cell suspension (cells in growth medium) was introduced as the core material whereas the sheath solution was composed of PVA only. Coaxial electrospinning produced larger fibers, and the cells do not appear as bead-like structures since the fibers have an average diameter of $748 \pm 267$ nm (Figure 4).

Cell Viability

One of the critical issues in cell electrospinning is to examine the viability of the cells following electrospinning. Figure 5 shows results in this regard for cells exposed to single needle and coaxial electrospinning. In these experiments, the nanofibers collected were dissolved in growth media and a live/dead staining was used to examine the cells. Figure 5a and c show results for single needle electrospinning whereas Figure 5b and d reveal results obtained from coaxial electrospinning. In either case, we observe only red specs indicating dead cells. Figure 5a and b, which correspond to live cell images appear dark as there are no live cells present to show any green specs (for live cells). Interestingly, other work on mammalian cell electrospinning,\[46]\ authors reported cell viability...
of ≈30% which they attributed to polymer viscosity effects. However, different cells were used in their case, and electrospun into a Petri dish filled with growth media rather than on to aluminum foil. When we electrospin into a growth media, as discussed later, we obtain a maximum cell viability of 25%, similar to previous reports.\textsuperscript{[40]}

To better understand why the cells did not survive, we performed a series of experiments to systematically determine the effect of the different aspects of the electrospinning process on cell viability. We first examined the effect of the shear stress associated with passing the cells through the syringe by pumping the cell suspension containing no polymer through the electrospinning setup in the absence of the electric field. We found that the shear stress of being pumped through the syringe did not significantly affect the viability of the cells. This is illustrated in Figure 6 where the green and red specs correspond to live and dead cells, respectively. We found that only approximately 1% of the cells die during this process.

In the next step, we pumped the cell suspension through the syringe while applying a high voltage to charge the suspension. We charged the solution with 10 or 20 kV; however, charging the cell suspension even with up to 20 kV did not significantly affect cell viability. We call this the conductivity effect in Figure 6c which reveals minimal cell death. Finally, we subjected the cell suspension to the electric field associated with electrospinning by charging the solution with a high voltage and grounding the collector. At low voltages (below 5 kV), droplets were collected and at high voltages (above 6 kV), the electric field caused the suspension to spray (Table 1). The cell viability was over 95% at low voltages and over 90% when sprayed at higher voltages. Based on this set of experiments with the cell suspension, we surmise that the high strength electric fields required for electrospinning is not the main cause of cell death. This result is consistent with previous reports that cells survive exposure to high voltage electric fields since the current during electrospinning is low (on the order of 1 \( \mu A \)).\textsuperscript{[55]}

We next attempted to isolate cell viability during various parts of electrospinning process in the presence of polymer. Our control experiment included testing for viability of cells when mixed with a PVA solution; we call this the polymer compatibility (PC) test. As expected, we observed no effect on cell viability when the cell suspension was combined with an aqueous polymer solution (Figure 7, top). PVA solution with cells was then electrospun by systematically varying the applied electric field. At voltages below the threshold required to successfully electrospin (below 5 kV), we obtained droplets rather than fibers (Table 1). However, the majority of the cells survived despite the applied electric field and solvent evaporation, as shown in Table 1 and illustrated in Figure 7 for the 1 kV applied field case. At higher voltages when we obtained combinations of droplets and fibers or fibers, none of the cells in the collected fibers survived (Table 1). This is also shown for a representative case of 8 kV in Figure 7. We therefore observe

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\textbf{Figure 4.} Cell incorporated nanofibers by coaxial electrospinning with PVA in the shell and 3T3-L1 fibroblast cell suspension in the core (a) low magnification SEM micrograph, (b) fiber size distributions, (c) high magnification SEM micrograph.
two scenarios when a high voltage is applied. For the case of cell-only suspensions, only droplets are formed and the cells predominantly survive. In contrast, for the case of PVA/cell solution, droplets with fibers are formed and almost all cells are dead. Based on these results, we postulate that cell death occurs due to the stresses associated with fiber formation. During coaxial electrospinning, a substantial amount of solvent evaporates from the fiber after it is formed. It is possible that a portion of the cells survive the fiber formation during electrospinning, but die due to dehydration in the dry fiber. To assess if cell death is caused by dehydration of the fibers, we electrospun directly into growth media rather than onto the foil. Upon spinning into the growth media, we observe that 15–25% of the cells survive (Figure 7, bottom micrograph and bar graph) similar to a previous report by Klein and coworkers on Escherichia coli cells. This result suggests that the majority of cells die during fiber formation. Previous modeling work indicates that the extensional stress is significant during fiber formation, which likely ruptures the cell leading to death. This result is different from previously reported hypothesis that attributed E. coli cell death during electrospinning to dehydration or mammalian cell death to shear forces as they pass through the apparatus. Because coaxial electrospinning involves the same mechanisms of fiber formation (stretching), coaxial electrospinning of nanofibers should not provide a significant advantage over single needle electrospinning in terms of cell survival.

Cell Layering

As an alternative to cell electrospinning, we also explored entrapping the cells between nanofibrous mat layers using a layering approach. By placing cells between electrospun membranes, it may be possible to overcome the thickness limitations of electrospinning as well as provide penetration to the internal structure of the scaffold. In a related study, Wagner and coworkers have examined simultaneous electrospinning of polymer solution and electrospaying of cells. This is a clever approach, but it is neither cell electrospinning nor cell layering, with no control on where the cells are. The authors themselves cite non-uniformity as a challenge which they overcome by incorporating a translating, rotating, charged mandrel collector, a less than straightforward modification to the traditional electrospinning set-up. Cell layering offers a much more facile and elegant approach, with control over where the cells are. For this part of the study we examined both PCL and PVA as the polymers of interest. To investigate the cell viability, we electrospun a mat layer, added cell suspension onto the first layer, and electrospun a second layer. When we electrospun PCL in DMF/chloroform, we obtained over 60% cell viability initially, which fell to below 20% after 3 d. Further investigation into the cell death as a function of time revealed that the location of the cell relative to the nanofiber layer significantly affected cell viability. When the mat layers were pulled apart so as to test the cells, we found some cells attached to the membrane and others not attached. Samples of the unattached cells are taken from the fiber mat at different time intervals, put in growth media and checked for viability. For the attached cell, fiber samples with cells attached were put in the growth media at different time intervals and checked for viability. Figure 8 shows the cell viability studies for both populations of cells. We find that 80% of the unattached are alive on day one and there is a slight decrease in 3 d. The attached cells show approximately 60% viability initially which drops to 20% in 3 d. Such rapid deterioration in cell viability suggests that residual solvent in the electrospun fibers may affect the cells.

We explored the use of a different solvent to electrospin PCL and examined cell viability after seeding the cells on an initial electrospun layer. When we used acetone, we obtained 45% viability which was lower than 70% viability when using DMF and chloroform (Figure 9). We also

![Figure 5. Live/dead viability results (a) and (c) show single needle electrospinning, (b) and (d) show coaxial cell electrospinning; in either case, no viable cells were observed, thus (a) and (b) appear blank and all cells appear red (c) and (d).](image-url)
attempted to wash the fibers with ethanol (EtOH) for both solvent systems before adding the cell suspension; washing improved cell viability by 5 to 10% (Figure 9).

These results also indicate that residual solvent in the electrospun fibers was affecting cell viability. To probe this issue further, we identified solvents thought to minimally affect cell viability that could also be used to electrospin PCL, namely HFIP[41] and repeated the layering procedure. Using HFIP, 80% of the attached cells were alive (Figure 10) initially compared to 50% when using DMF/chloroform, both of which have previously documented mammalian cell cytotoxicity.[59,60] In addition, the magnitude and decay

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<th>Voltage [kV]</th>
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<td>6</td>
<td>Droplet and fiber</td>
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<td>&gt;6</td>
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of cell viability are analogous to that of unattached cells from the DMF/chloroform validating this to be a suitable solvent for cell layering.

For comparison purposes, we also performed cell layering with PVA although it is anticipated that water as a solvent in this would not affect cell viability. While PVA is a suitable
material for scaffolds using this technique, the addition of the cell suspension causes the polymer nanofiber to partially dissolve. So, in this case we do not have a true nanofibrous web, but our objective was to verify that cell viability was not affected by the layering process in the absence of a harsh solvent. Figure 10 shows that indeed cell viability is high and not affected within the time duration of our study. The results of this part of the work, taken together, suggest that while cell layering yields much higher cell viability when compared to cell electrospinning, the choice of solvent is critically important, as residual solvent in the electrospun fibers could be detrimental to the cells.

Conclusion

We studied two approaches to incorporate live mammalian cells into nanofibrous web, direct electrospinning and cell layering in which cells are sandwiched between two electrospun nanofibrous webs. While the cells were dead in the direct cell electrospinning case, a substantial fraction remained alive in the cell layering case. In order to understand the reason behind cell death during cell electrospinning process, we systematically examined parameters that we thought might have an effect on the viability of cells: polymer-cell suspension compatibility, shear stress, electrical conductivity, high voltage, dehydration, and fiber stretching. We found fiber stretching and dehydration to be the main factors in determining cell viability in cell electrospinning process. While it might be possible to find a solution for dehydration by introducing some growth medium onto stacked nanofibers containing cells in synchronization with electrospinning process, it is impossible to eliminate cell death due to fiber stretching. In the cell layering case, although the majority of cells were alive immediately after electrospinning, its viability was dependent on the polymer/solvent system used for electrospinning. We surmised that residual solution played a large role in this regard, and best results were obtained using the cell compatible HFIP solvent.

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