Characterization of a bioactive fiber scaffold with entrapped HUVECs in coaxial electrospun core-shell fiber

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DiI, 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; FDA, fluorescein diacetate; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; IL-8, interleukin-8; PCL, polycaprolactone; PEO, polyethylene oxide; PMT, photomultiplier tube; SEM, scanning electron microscopy

Human umbilical vein endothelial cells (HUVECs) were successfully entrapped in polyethylene oxide (PEO) core / polycaprolactone (PCL) shell electrospun fibers thus creating a “bioactive fiber.” The viability and release of biomolecules from the entrapped cells in the bioactive fibers were characterized. A key modification to the core solution was the inclusion of 50% fetal bovine serum (FBS), which improved cell viability substantially. The fluorescein diacetate (FDA) staining revealed that the entrapped cells were intact and viable immediately after the electrospinning process. A long-term cell viability assay using AlamarBlue® showed that cells were viable for over two weeks. Secreted Interleukin-8 (IL-8) was monitored as a candidate released protein, which can also act as an indicator of HUVEC stress. These results demonstrated that HUVECs could be entrapped within the electrospun scaffold with the potential of controllable cell deposition and the creation of a bioactive fibrous scaffold with extended functionality.

Introduction

The entrapment of cells holds promise for treating a wide variety of disorders such as diabetes mellitus, cancer and cardiovascular diseases.¹⁻³ Cells can release therapeutic proteins/ biomolecules to mitigate such diseases with delivery controlled by physiological cues via cell surface receptors.^{4,5} In such systems, non-autologous cells require entrapment by a semi-permeable membrane to shield them from the immune system. Therefore, the production of bioactive or biohybrid materials containing entrapped cells has been actively pursued.⁶⁻¹⁰

Electrospinning has been widely used to produce fibrous scaffolds that are three-dimensional and porous utilizing both natural and synthetic polymers. The diameters of the electrospun fibers range from tens of nano- to micrometers and can be formed as monolithic or coaxial produced by a reliable and up-scalable approach.^{11,12} Recently, there has been considerable interest in combining such fibers with biomolecules (e.g., growth factors) to produce a bioactive scaffold.¹³⁻¹⁵ The bioactive scaffolds can provide physical support for cellular growth concurrently with the release of biological factors to modulate tissue regeneration.¹⁶⁻¹⁸

The entrapment of eukaryotic cells has potential for use in areas such as the delivery of therapeutic molecules to diseased sites. The electrospinning of cells allows the formation of complex 3D cellular structures such as cardiac patches and blood vessels. Constructs containing the desired cell types and in the required numbers, allow direct generation of living architectures, without requiring additional cell seeding and distribution of cells throughout the scaffold.^{19,20} This technique will be useful in the production of functional scaffolds for tissue engineering and regenerative medicine.

Here, we entrap Human Umbilical Vein Endothelial Cells (HUVECs) within the fibers of a polycaprolactone electrospun scaffold via co-axial electrospinning to demonstrate the concept of direct endothelial cell deposition of a fibrous scaffold. Moreover, the optimization process involved will advance the development of electrospun bioactive scaffold, with the versatility to express countless different proteins by harnessing transgene expression by the entrapped cells.

Several groups have reported the entrapments of biological organisms/cells into electrospun fibers. Bacteria have been successfully entrapped in electrospun fibers for use in bioremediation and biofuel cells.²¹⁻²³ However, there is limited

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Figure 1. Production of porous core-shell fibers with PEO/FBS core. (A) 0, 5 and 50% FBS was included into 2% PEO solution and assessed for cytocompatibility on plated HUVECs. Cell viability assessed using AlamarBlue® assay and expressed as a percentage. (n = 4) (B) Camera image of a live coaxial electrospinning process, demonstrating the core solution entering the shell solution to form the core-shell fiber. (C) Cross sectional view of the electrospun fibers after the removal of PEO from the core, a hollow core-shell structure is observed. Scale bar = 10 μ m. (D) Core-shell fibers electrospun into water showed highly porous PCL surfaces. Scale bar = 20 μ m. The formation of pores on the PCL shell is evident when the fibers are collected in water.

clinical benefits to bacterial implantation. Thus, the entrapment of eukaryotic cells, such as HUVECs will expand the application of electrospinning toward regenerative and therapeutic medicine. Indeed, electrospun scaffold containing entrapped cells may act as a platform applicable to numerous conditions requiring therapeutic protein release.²⁴

Previous studies have shown the entrapment of cells using coaxial electrospinning. Jaysinghe and Townsend reported the electrospinning of a eukaryotic cell line (1321LN) while Shih et al. have published a study on the entrapment of PC12 cells (neural cells).^{25,26} Assessing the success of producing cell-bearing fibers can be a challenge as the electrospun fibers can produce artifacts when evaluating cellular integrity and viability. Here we apply several approaches at characterizing fiber-entrapped cells to avoid any misleading observations.

In this study, we have optimized a core for extended HUVEC viability within electrospun fibers, and successfully demonstrated their integrity using a membrane dye (DiI). We have visually determined cell viability using fluorescein diacetate, quantified extended viability using resazurin (AlamarBlue® assay) and

finally shown the release of a cytoactive protein expressed by the cell in the form of the chemokine Interleukin-8 (IL-8).

Results

Effect of FBS content in PEO solution on cell viability

It can be observed that the addition of FBS to the PEO solution improves HUVECs viability (Fig. 1A). The inclusion of 5% FBS dramatically increased the viability of cells immersed in PEO, moreover, with 50% FBS in the PEO, HUVECs exhibited a viability similar to PBS alone. These cells maintained greater than 90% viability whereas the cells exposed to PEO alone solution showed a rapid decline in viability in the first 30 min. Following two hour incubation, the viability of PEO exposed cells was less than 3%, compared with 97% of PEO/FBS sample.

Formation of porous core-shell fibers via coaxial electrospinning

The cell loaded in PEO/FBS solution had sufficient viscosity and immiscibility to form the central core to the PCL fiber since no detectable obvious mixing occurred as the fiber jetted emerged from the spinneret (Fig. 1B). Furthermore, the fibers

Figure 2. Fluorescence microscopic images of the entrapped HUVECs in core-shell fibers. **(A)** HUVECs stained with DiI are seen at numerous locations within the fibers. **(B)** The HUVECs were pre-stained with DiI only. The confocal image showed HUVECs with DiI before addition of DAPI. **(C)** After 24 h, DAPI was added to the media and the cells were allowed to incubate for 30 min before the image was taken. The result showed HUVECs exhibiting both DiI and DAPI. Scale bar = 50 μm for all images.

exhibit distinct hollow fiber structure as observed in the scanning electron microscopy (SEM) image of the fibers' cross-section (Fig. 1C) after dissolving the core polymeric solution.

Further SEM images of the coaxial electrospun fibers showed that the collection of electrospun fibers in water introduced a substantial presence of pores into the shell fiber surface. Image analysis revealed an average outer surface pore size of $0.7 \pm 0.4 \mu\text{m}$ (results not shown) on the PCL shell (Fig. 1D). The formation of this highly porous PCL/PEO co-electrospun fiber suggested that an exchange between the outside media and the entrapped environment could be made possible. This allowed oxygen and nutrients to reach the entrapped cells while removing waste.

Electrospinning of HUVECs

The cells suspended in the PEO/FBS solution were electrospun into the core of the porous PCL/PEO fibers. The set of fluorescence microscopic images (Fig. 2) showed the entrapment of HUVECs (stained with DiI or FDA) within the core of the fibers. Figure 2A showed that the cells are distributed along and entrapped within the electrospun fibers. For Figure 2B and C, the confocal images showed that the cells within the fibers were able to uptake the 4',6-diamidino-2-phenylindole (DAPI) stain, introduced post electrospinning. This demonstrated that the DAPI had transversed the PCL shell. The co-localization of

the two dyes indicated that there was an exchange between the outside media and the entrapped cells. This result demonstrates that primary cell such as HUVECs can be successfully entrapped by coaxial electrospinning into PCL polymer fibers.

Characterizing the viability of entrapped HUVECs

The entrapped cells must be shown to still have retained an intact plasma membrane and a functioning metabolism, both of which can be demonstrated by the activation of FDA fluorescence. Hence, the cells were observed to be viable one-hour post electrospinning (Fig. 3A) and continued to be viable (Fig. 3B–D) for up to at least two days within the fibers. This suggests that the cells were not just able to survive the electrospinning process but to also remain viable within their entrapped environment.

The growth profile of the entrapped HUVECs was studied using AlamarBlue® reagent. From the graph, it can be observed that the HUVECs were able to proliferate within the fibers (Fig. 4A). The cell number increased steadily after Day 4 and reached almost a 20-fold growth at the end of 16 d.

Interleukin-8 ELISA

Figure 4B shows the mean levels of IL-8 that was secreted into the culture media by the entrapped HUVECs after Day 1 and Day 4 respectively. The level of IL-8 is significantly higher than the control on Day 1 but not on Day 4. The IL-8 levels from

Figure 3. Fluorescence microscopic images of entrapped HUVECs after FDA assay. (A) HUVECs exhibiting the green FDA fluorescence one-hour post electrospinning, (B) HUVECs exhibiting the green FDA fluorescence one-day post electrospinning and (C and D) HUVECs exhibiting the green FDA fluorescence two days post electrospinning. All scale bar = 50 μ m. The entrapped HUVECs were incubated with FDA (added to media) for 45 min at 37 °C before the images were taken. For FDA assays, non-viable cells will appear dull or non-fluorescent while viable cells will appear bright.

scaffolds with entrapped HUVECs on Day 1 and Day 4 were, respectively 4.4 ± 0.7 -fold ($P < 0.002$) and 1.8 ± 0.8 -fold of those scaffolds without entrapped cells.

Discussion

HUVECs deposition and viability

HUVECs have been deposited with the core of electrospun fibers to create a cell-bearing scaffold. The in-depth characterization reported here has demonstrated that the HUVECs were able to endure the electrospinning process. This is because the cells were found to be deposited intact, displayed metabolic activity and extended viability for over 2 wk.

During cell electrospinning, there is the notable problem of cell lysis during the resuspension of the cell pellet into the core solution. Zussman et al.^{21,22} has previously employed 4% PEO in PCL core shell electrospinning and found uniform cell distribution difficult in such a viscous environment. It was found that entrapped cells were more readily electrospun into fibers that included FBS in the core solution (data not shown). The benefits of FBS were not just due to its nutrient qualities in serum-deprived conditions. In this context, FBS can both generate a suitable osmotic environment and act as a shear stress protectant (discussed further below).²⁷

Fiber shell porosity

To enhance the exchange of nutrients and waste products, substantial surface porosity was induced by collecting the fibers in water. The confocal results shown in Figure 2B and C proved that there is exchange between the media and the entrapped cells allowing DAPI entrance. Indeed, since the cells survive for

over 2 wk, it can be assumed they were able to access nutrients to support proliferation while entrapped. However, if need be, this porosity could be further increased by the inclusion of water soluble porogens such as polyethylene glycol into the shell solution before being electrospun.

The cell-expressed IL-8, an 8.4 kDa secretable protein, was detectable in the surrounding media; therefore the entrapped cells can influence the environment surrounding the scaffold in a paracrine fashion by such chemokines or growth factors.

Optimized core solution for shear stress and osmotic protection

The hydrodynamic stresses on a cell in the electrospinning jet can deform and induce shear stress on the HUVECs during this spinning process.²⁸ The requirement for a shear protectant in the core solution was not necessary when electrospinning prokaryotes due to their supportive bacterial cell wall and relative small size, unlike larger eukaryotes. Hence eukaryotic cells are more likely to undergo shear stress damage during electrospinning. Indeed, murine mesenchymal cells passed through low bore diameter needles at high flow rate, similar to that experienced during electrospinning, demonstrated increased production of caspase-3 protein; an early indicator of apoptosis. Moreover, FBS is also commonly applied as a shear protectant in dynamic hybridoma culture.^{29,30}

IL-8 as an indicator of shear stress

We found that the concentration of 2% PEO allowed for best resuspension of a cell pellet while also producing core shell fibers. The 2% PEO solution is hypotonic, with an osmotic pressure of 0.0082 atm (0.41 mOsm/kg) compared with physiological pressure of 6.8–7.3 atm (270–290 mOsm/kg).

Under these conditions, it is expected that the HUVECs will experience deleterious osmotic stress. Hartnett et al.³¹ demonstrated that as little as two minutes exposure of endothelial cells to 0.2atm (1–1.2 mOsm/kg) caused a significant decrease in viability of over 20% and a detectable increase in apoptosis. Hence the inclusion of the 50% FBS, reported here in this respect for the first time allows the core solution to become a more isotonic environment to improve cell survival.

The shear stress on the electrospun, entrapped HUVECs was demonstrated by increased expression of IL-8 24 h post electrospinning (Fig. 4B). IL-8 is a pro-inflammatory chemokine of the CXC family (Mw 8.4kDa). It is secreted constitutively by HUVECs, but also has been shown to be upregulated in endothelial cells under shear stress.^{32,33} We observed that 24 h following electrospinning there was a considerable increase in IL-8 protein secretion. The IL-8 gene expression level returns toward constitutive after four days. This decrease of IL-8 is not due a decrease in the cell viability as shown by the AlamarBlue® results (Fig. 4A). The electrospinning associated stimulation of IL8 secretion may allow us to monitor cell stress levels such that the process parameters may be optimized to minimize such stresses.

Summary

In summary we have developed a method to electrospin HUVECs into an entrapping cell-laden scaffold, with an optimized core to enhance cell viability. This method has the potential to be utilized as an effective scaffold for both bioengineering research and application to create a bioactive fibrous scaffold.

Materials and Methods

HUVECs culture

Human umbilical vein endothelial cells (HUVECs) (Lonza) were cultured in EGM-2 Complete Medium (Lonza) with 1% Antibiotic-Antimycotic (Gibco). Cells from passage 6–10 were utilized in this experiment. The culture media was replaced every two to three days.

Effect of FBS on cell viability

HUVECs (1×10^4 cells/cm²) were seeded onto 24-well plate and made to contact various potential core polymer solutions. 2% PEO (Sigma-Aldrich, Mw 600 kDa) with 0–50% FBS (Gibco) was used. After designated time points (30 to 120 min), the polymer solution was removed and wells were washed three times with Phosphate buffered saline (PBS, Gibco) before performing the AlamarBlue® assay.

The AlamarBlue® assay (Life technologies) was performed according to the manufacturer's instructions. To each well of the tissue culture plate, 1mL of 10% AlamarBlue® solution

Figure 4. Analysis of the entrapped HUVECs. (A) Growth profile of encapsulated HUVECs based on AlamarBlue® assay. The growth profile of the cells tracked up to 16 d showed increased cell numbers over time, suggesting proliferation. (B) Production of IL-8 by the encapsulated HUVECs displayed elevated secretion of the cytokine on Day 1 as compared with Day 4. Values are mean $\pm$ SD (n = 3). * $P < 0.002$ vs. controls without cells.

was added. The cells were then incubated in darkness at a 37 °C for four hours before analysis. After incubation, four 100 μ L replicates were taken from each well and transferred to a black 96-well plate for fluorescence reading by a Varioskan flash reader (Thermo Fisher Scientific) (excitation 540 nm, emission 585 nm). Cell viability was calculated from the fluorescence relative to the control.

Coaxial electrospinning of PCL/PEO with cells

Core-shell fibers were fabricated using PCL (Mw 70–90 kDa) and PEO (600 kDa) from Sigma-Aldrich. For the shell, a 12w/w% PCL solution was prepared in an organic solvent mixture of Chloroform and Dimethylformamide (60%:40% w/w).

After centrifugation, the cell pellets were resuspended in the 2% PEO/FBS solution (with 50% FBS) to reach a core solution concentration of 5×10^6 HUVECs/mL of solution. The shell and cell/core solutions were loaded into 10mL syringes (B.D) and added to the electrospinning machine (NanoBio-01-T, Nanoflux Pte Ltd).

The electrospinning conditions were 1.5 kV/cm at room temperature and the flow rates of the shell and core solutions were maintained at a ratio of 6:1 as suggested previously by Liao et al.³⁴ Porous PCL fibers were created by electrospinning the fibers directly into a distilled/deionized water bath in a method described by Seo et al.³⁵ After the collection of the core-shell fibers on the surface of the water bath for 15 min, the fibers were removed, washed thrice with PBS and incubated with EGM media at 37 °C. Core-shell fibers without cells were also fabricated under similar conditions and used as controls for this experiment.

Characterization of entrapped cells within core-shell fibers

The surface morphology and cross sections of the core-shell fibers were viewed under a scanning electron microscope (SEM) (JEOL JSM 6360LV) with an accelerated voltage of 3–5 kV. All samples are sputter coated with Platinum for 30 s prior to the imaging.

The entrapment of the HUVECs within the core-shell fibers was investigated visually using fluorescence microscopy (Nikon)

and confocal imaging (TCS SP5 II, Leica Microsystems). The Nikon fluorescence microscope was used to track the stained cells using the TRITC filter for DiI. Using the Leica confocal microscope, a 405 nm laser was used to excite DAPI and the photomultiplier tube (PMT) used to collect the emission was set to 470 ± 20 nm. For DiI, a 543 nm laser was employed and the PMT was set to 565 ± 20 nm.

Viability of entrapped cells was investigated visually using Fluorescein diacetate (Sigma-Aldrich) assay. FDA was added at a dilution of 1:100 to the electrospun fibers and incubated at 37°C for 45 min. The fibers were then washed thoroughly with PBS to remove any residual FDA. The exposure time of the microscope camera was calibrated before the addition of FDA to minimize the auto-fluorescence of the cells and fibers prior to the assay. These settings were then applied to further imaging. The cell viability was monitored by the fluorescence generated.

Quantitative viability was assessed using AlamarBlue® (Invitrogen) assay and the cell-loaded scaffolds were monitored for up to 16 d. The fibers containing entrapped HUVECs were incubated with 10% AlamarBlue® for 7 h at 37°C in the dark before the readings were taken. After each AlamarBlue® measurement, the fibers were washed with PBS to remove any excess reagent before fresh culture medium was added. AlamarBlue® assay was performed on core-shell fibers electrospun without cells as a control.

Interleukin-8 enzyme linked immunoassay (ELISA)

Interleukin-8 concentrations were measured in the media of the entrapped HUVECs using a commercially available IL-8 ELISA kit (Thermo Fisher Scientific). The significance levels of factors on FI for AlamarBlue® assay were assessed using one-way

ANOVA in Excel. All tests were two-sided and at a significance level of $P < 0.002$. Data are expressed as mean value $\pm$ standard deviation.

Conclusion

We have successfully produced and characterized electrospun fibers with entrapped cells. This provides a mechanism to fabricate a biomaterial scaffold with controllability to the distribution of cells throughout the scaffold. The bioactive scaffold can have improved functionality by the release of therapeutic proteins from the entrapped cells. The further advantage is that the porosity of the fiber shell can be optimized to produce an immuno-barrier for implantation, with porosity of approximately 70 kDa cut off.²¹ It is our belief that this system will act as the foundation of a functional bioactive fiber. The next stage of the development will involve the assessment of suitability for in vivo implantation and release studies of bioactive molecules.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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