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Nanofabrication of Electrospun Fibers for Controlled Release of Retinoic Acid

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ABSTRACT

The process of electrospinning has received a remarkable amount of attention as this technique can be used to encapsulate bioactive agents into polymer nanofibers while varying topographies. Encapsulation of bioactive agents inside electrospun nanofibers can be carried out by emulsion electrospinning where the specific agent is dissolved into the desired polymer solution. In this study, we demonstrate that retinoic acid (RA), a small molecule that regulates neural development, could be successfully encapsulated into electrospun poly (Ɛ-caprolactone) (PCL) nanofibers at different doses while varying fiber topography. Controlled release of RA was evaluated over a month from both randomly oriented and aligned PCL scaffolds. Finally, we show that these scaffolds support and direct murine CE3 embryonic stem cell (ESCs) proliferation and migration. Overall, this novel combination of RA releasing electrospun nanofibers and stem cell derived neural progenitors serves as a promising strategy for the repair of spinal cord injuries.

INTRODUCTION

Tissue engineering approaches can be used to develop therapies for reconstructing damaged tissues and organs through the use of biomaterial scaffolds that can mimic the microenvironment present in healthy tissue [1, 2]. The main role of a scaffold is to provide a structure which can support delivery of cells and specific drugs to the site of the injury site. In particular, neural tissue engineering employs biomaterial scaffolds to direct stem cell differentiation into neural phenotypes for nerve regeneration [3]. Thus, a tissue engineered three dimensional (3D) scaffold must be used as a replacement for healthy tissue, and deliver appropriate chemical cues for controlling stem cell differentiation into neural phenotypes [4]. In addition to using chemical cues, neural differentiation can also be achieved by using physical cues, such as nano and microscale topography [5, 6]. The aim of this study is to fabricate biocompatible, biologically active scaffolds that simultaneously present both chemical and physical cues. As our chemical cue, we have chosen to focus on the con trolled release of retinoic acid (RA), a small molecule that regulates neural development. RA can stimulate the differentiation of embryonic stem cells (ESCs) and induced pluripotent stem (iPS) cells into motor neurons [7].

In order to produce an effective controlled release drug delivery system, it is necessary to provide sustained dosage of incorporated agents over an extended time while reducing the initial burst release. Many strategies for loading bioactive agents into various biomaterials have been investigated [8]. Among many drug delivery systems, reservoir-based delivery systems are the most commonly used in neural tissue engineering applications. In reservoir-based delivery systems, a polymer structure surrounds the drug reservoir with drug release being modulated through the degradation rate of the polymer. In such systems, the drugs are noncovalently embedded in the porous polymer structure.

Synthetic polymers can be chemically and physically altered, influencing their biocompatibility and biodegradability [9]. Such flexibility makes these polymers attractive for drug delivery applications. We have chosen poly (ϵ-caprolactone) (PCL), a commonly used biodegradable polymer approved by U.S. Food and Drug Administration (FDA) for drug delivery applications. PCL is tailorable in its mechanical properties, rate of surface and bulk biodegradation, solubility and crystallinity, and structure topography [10].

Bioactive agents can be incorporated into electrospun nanofibers to enable them to serve as reservoir-based drug delivery systems [8]. The process of electrospinning uses a high voltage electric field to overcome the surface tension of a polymer solution and consequently a continuous and elastic polymer flow leads to nanofiber formation. Encapsulation of bioactive agents inside electrospun nanofibers can be achieved through emulsion electrospinning where the specific agent is dissolved into the desired polymer solution. Many groups have developed methods of encapsulating bioactive agents into electrospun nanofibers for tissue engineering applications [10-16]. For instance, Jiang et al. reported that by encapsulating 0.3 % (w) RA into PCL nanofibers, controlled release of RA was obtained for at least 14 days (60% released) [12]. The released RA enhanced the differentiation of mesenchymal stem cells into neural lineages, demonstrating the potential of encapsulated electrospun nanofibers as reservoir-based delivery systems for tissue engineering applications.

In this study, we successfully encapsulated RA into electrospun PCL fibers using a blending technique while varying

• corresponding author

topography (randomly oriented and aligned) and dosage as well. Encapsulation of 0.1, 0.2 and 0.3 % (w/w) of RA inside PCL nanofibers were carried out for both aforementioned topographies. The controlled release of RA from PCL-RA scaffolds containing 0.2 % (w/w) RA from both topographies is investigated over 1 month. Moreover, these scaffolds were able to support CE3 embryonic stem cell (ESC) attachment and proliferation.

MATERIALS AND METHODS

A. *FABRICATION OF BLANK AND ENCAPSULATED NANOFIBERS:*

Poly (Ɛ-caprolactone) (PCL), (Mn: 45,000) and all trans-Retinoic Acid (RA) were purchased from Sigma Aldrich Chemical Co, USA; Dichloromethane (DCM) from Fisher Scientific, Canada; Methanol (MeOH) from VWR International, Canada; Phosphate-Buffered Saline (PBS), (pH 7.4 from Gibco®. A mixture of DCM/MeOH at a volume ratio of 8:2 was prepared to dissolve PCL granules. A10% PCL solution (w/v) was thoroughly mixed using a bench top stirrer (Corning®) overnight at 1000 rpm at room temperature. To fabricate PCL-RA nanofibers, a stock solution of 2.5 mg/ml RA in MeOH was added into the 10% PCL (w/v) solution to obtain three different PCL-RA (0.1, 0.2 and 0.3% w/w) formulations. The PCL-RA solutions were also stirred overnight at 1000 rpm. These solutions were then electrospun into randomly-oriented and aligned nanofibers to determine the mechanism of release (surface and bulk erosion), how to modulate the controlled release of RA, and to finding the conditions that minimized burst release.

The electrospinning setup consists of a syringe pump (New Era Pump Systems Inc., USA), a dispensing needle (McMaster Co. USA), a machined water container, and a high voltage power supply (GAMMA High Voltage Research Inc., USA). The water container was filled with tap water and then placed in front of the nozzle tip to collect the random fibers by adjusting the distance between nozzle tip and water container (7.5cm). The PCL and all PCL-RA solutions were pumped at the constant flow rate of 2 ml/hr to electrospinning syringe in order to fabricate randomly-oriented plain PCL and RA-encapsulated PCL scaffolds respectively. The positive terminal of the high voltage power supply was connected to the water container while the ground terminal was connected to the nozzle tip. The voltage applied was 15 kV. Each scaffold (~10 mg total weight) was spun for 5 minutes and dried overnight.

In order to control the alignment of fibers, a drum fiber-collector machined Delrin with DC motor was placed between nozzle tip and the water container. All fibers were then spun on the drum that was charged at 10 kV. The collecting distance and speed of rotating drum were fixed at optimizing conditions as 5 cm and 4000 rpm respectively. The spinning time was 2 minutes for preliminary morphology characterizations and 1 h for the production of the scaffolds used for the cell culture assays. A summary of nanofabrication process is provided in Table 1.

B. *MORPHOLOGICAL CHARACTERIZATION USING SEM:*

Both randomly-oriented and aligned electrospun blank PCL and PCL-RA nanofibers were transferred to loading stubs before carbon coating. The Cressington 208 carbon coater was used to coat a 3nm thick carbon layer on the surface of nanofibers prior to scanning electron microscope (SEM) imaging. The samples were carbon-sputtered two times for 6 seconds at 10^{-4} mbar. The samples were loaded in a Hitachi S-4800 field emission SEM. High magnification images were obtained at 1 kV and 10 µA beam intensity. The average diameter of the electrospun nanofibers was determined using Quartz-PCI Image Management Systems®.

C. *IN VITRO RELEASE STUDIES AND ENCAPSULATION EFFICIENCY :*

Electrospun scaffolds (~10 mg, n = 3) were placed in 1.5 mL microfuge tubes for each replicate and suspended in 1 mL PBS. These tubes were then incubated at 37°C. Samples were taken at the predetermined time points (days 1, 2, 6, 10, and 14) for initial release studies. The absorbance of the samples and standards were read at 337 nm wavelength by using Ultrospec 3000 Pharmacia Biotech® spectrophotometer. In vitro release studies were carried out in triplicate and data is presented as means ± standard error of the mean (SE). The encapsulation efficiency was determined using an extraction method. A measured amount of dried scaffolds was placed in a 45 mL propylene conical tube. 500 µL of DCM and 2 mL of MeOH were added and vortexed for 1 min to dissolve the PCL. PBS was added and vortexed for 15 s to allow extraction of loaded agents into the PBS. The concentration of RA in the sample was determined by using the spectrophotometer. To calculate encapsulation efficiency, a ratio of actual encapsulated RA to theoretical RA was used.

D. *IN VITRO STEM CELLS CULTURE:*

We chose to use CE3 cells as they constantly express green fluorescent protein (GFP) under the β-actin promoter, enabling easy visualization of the cells when seeded upon electrospun scaffolds [17]. CE3 cells were maintained in an undifferentiated state through culture on mouse embryonic fibroblast feeder layers. To promote differentiation into neural progenitors, these cell lines were treated with RA as previously described as the 4-/4+ retinoic acid treatment protocol to produce EBs [18]. Sheets of PCL-RA nanofiber scaffolds were punched into 10 mm diameter circles and sterilized with Trans-UV 302 cleaner; GelDoc XR Bio-Rad®. Under aseptic conditions, the scaffolds were adhered to the bottom of each well in a 24-well polystyrene tissue culture plate coated with 0.1% ultra-pure gelatin solution (EmbryoMax®, Millipore), followed by the addition of 1 mL leukemia inhibitory factor (LIF) -free media. EBs were cultured on electrospun scaffolds for 10 days before analysis. Fluorescent images were acquired on a LEICA 3000B inverted microscope using an X-cite series 120Q fluorescent light source (Lumen Dynamics) coupled to a Retiga 2000R fast cooled mono 12-bit camera (Q-imaging). A schematic of single EB seeded on a PCL nanofiber scaffold is shown in Fig.1.

Table 1: Process parameters of nanofibers fabrication for both randomly oriented and aligned scaffolds.

Topography	Voltage	Collecting Distance (cm)	Drum Speed (rpm)
Randomly Oriented	15	7.5	N/A
Aligned	10	5	4000

RESULTS

A. *FABRICATION AND CHARACTERIZATION OF SCAFFOLDS:*

Fig. 2 illustrates SEM images of the randomly oriented and aligned PCL nanofibers when RA was not added to the polymer solution. By tuning the operational conditions such as collecting distance, and applied voltage, the topography showed a very porous structure with the absence of polymer beads. The aligned nanofibers were obtained using a collector drum. We have previously reported that a rotating drum could improve the degree of alignment of electrospun fibers significantly[19]. Due to PCL crystallinity, the polymer chains tend to be stretched uniaxially along the flow direction induced by the rotating drum at 4000 rpm speed.

For both topographies, the fiber diameter changed along an individual fiber so the fibers had non-uniform diameters. Controlling the uniform distribution of fiber size is very challenging and has been reported in some studies [8, 15, 20]. For instance, Yoshimoto et al. reported a broad fiber diameter distribution for PCL nanofibers (400nm ±200 nm) [20]. This variation in diameter may be due to the fast phase separation of PCL and the volatile solvent during electrospinning.

Fig. 3 shows the topography of the randomly oriented PCL-RA nanofiber scaffolds. Similar to the blank PCL nanofibers, the fiber diameter varied along an individual fiber. PCL-RA nanofibers have a larger nanofiber diameter compared to the blank PCL nanofibers with a larger fiber distribution observed as well. The phase separation may cause the RA to bead at certain locations in the PCL nanofiber. Similar results have been reported for the encapsulation of nerve growth factors (NGF) inside PCL nanofibers. In a study by Valmikinathan et al. it is hypothesized that this variation in diameter happened due to the phase separation of PCL and NGF [15].

Figure 4 shows the topography of the aligned PCL-RA nanofibers. The RA was contained completely inside PCL aligned nanofibers since bead-free nanofibers were obtained for all samples. The average fiber diameter for each type of scaffold (blank, encapsulated) for both types of topographies is given in Table 2.

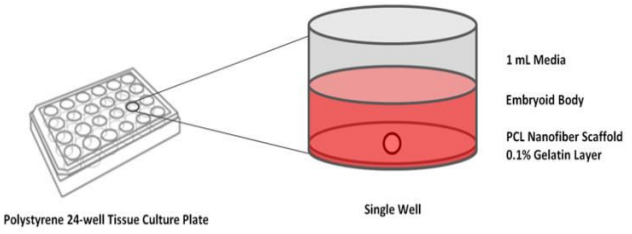

Fig. 1: A schematic of an individual embryoid body seeded on a poly (ε caprolactone) nanofiber scaffold

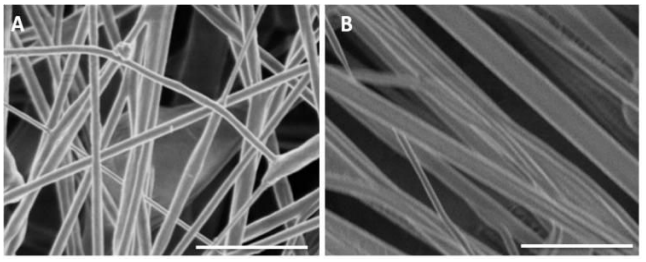

Fig. 2: Scanning electron microscopy images of blank poly (ε caprolactone) nanofiber scaffolds. (A) randomly oriented and (B) aligned poly (ε caprolactone) nanofibers. Scale bar is 4 μm.

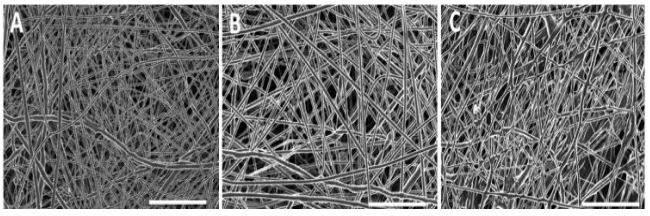

Fig. 3: Scanning electron microscopy images of randomly oriented encapsulated poly (ε caprolactone) nanofiber scaffolds. Scaffolds containing (A) 0.1% retinoic acid (w/w); (B) 0.2% retinoic acid (w/w) and (C) 0.3% retinoic acid (w/w). Scale bar is 5 μm.

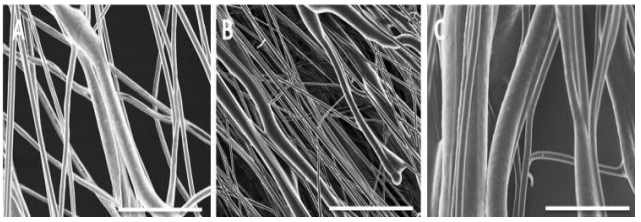

Fig. 4: Scanning electron microscopy images of aligned encapsulated poly (ε caprolactone) nanofiber scaffolds. Scaffolds containing (A) 0.1% retinoic acid (w/w); (B) 0.2% retinoic acid (w/w) and (C) 0.3% retinoic acid (w/w). Scale bar is 4 μm.

Table 2: Morphological properties of blank and encapsulated poly (ε caprolactone) nanofibers

Topography	Sample	RA Loading % (w/w)	Average Fiber Diameter (nm) ±SD (n=50)
Randomly Oriented	Blank PCL	0	103 nm ± 27 nm
	PCL-RA0.1	0.1	322 nm ± 150 nm
	PCL-RA0.2	0.2	517 nm ± 220 nm
	PCL-RA0.3	0.3	420 nm ± 103 nm
Aligned	Blank PCL	0	263 nm ± 97 nm
	PCL-RA0.1	0.1%	720 nm ± 150 nm
	PCL-RA0.2	0.2%	617 nm ± 401 nm
	PCL-RA0.3	0.3%	nm ± 200 nm

B. RETINOIC ACID RELEASE KINETICS

Figure 5 shows the release profiles of randomly oriented PCL-RA scaffolds containing 0.1%, 0.2% and 0.3% RA (w/w). Additionally, Figure 6 contains the release profiles of aligned PCL-RA scaffolds containing 0.1%, 0.2% and 0.3% RA (w/w). For initial studies, we assayed the release of RA from PCL nanofibers up to 14 days. No initial burst release was observed for the first two days then RA was released at a fairly constant rate of 0.98% and 0.85 % per day for 14 days for the randomly oriented and aligned PCL-RA scaffolds respectively. Table 3 shows a summary of release data for both topographies over two weeks. Controlled release of RA from PCL-RA scaffolds containing 0.2 % RA (w/w) with the highest encapsulation efficiency for both topographies was characterized for a 30 day time course (Fig.7). As shown in Fig.7 the aligned fibers showed 55% more release than the randomly oriented fibers. Both topographies have no burst release effect even for 5 days.

Figure 8 shows the SEM image of randomly oriented PCL-RA nanofibers containing 0.2 % RA (w/w) after 30 days of release studies. It is observed that nanofiber scaffolds remained morphologically undamaged during the period of release studies. Thus, diffusion is the controlling mechanism for the release of RA from PCL nanofibers. Average concentration of the released RA from each scaffolds for both topographies are also given in Table 3. Compared to a study by Jiang et al.[12] these concentrations are biologically reasonable since they are in the range between the concentration of human mesenchymal stem cell supplement media (9 μg/ml) and the release RA (1.1 μg/ml) [12].

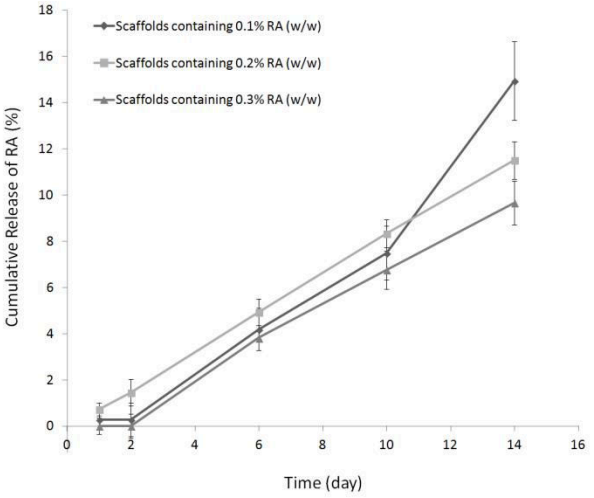

Fig. 5: Cumulative release of retinoic acid from randomly oriented poly (ε caprolactone) nanofiber scaffolds. (n=3, mean ± SE)

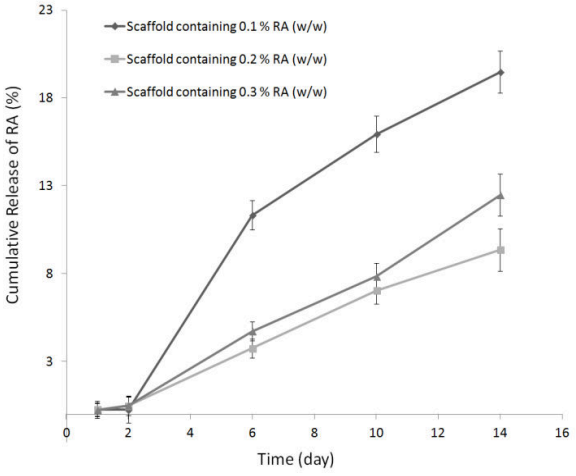

Fig. 6: Cumulative release of retinoic acid from aligned poly (ε caprolactone) nanofiber scaffolds. (n=3, mean ± SE)

Table 3: Controlled release data over 14 days for randomly oriented and aligned PCL-RA scaffolds.

Topography	RA % (w/w)	Encapsulation Efficiency (%)	Accumulative Release (%)	Average Concentration (μg/ml)
Randomly Oriented	0.1	42	19.4 ±1.2	1.94
	0.2	70	9.36 ±0.75	1.82
	0.3	30	12.47 ±1.2	3.74
Aligned	0.1%	25	14.94 ±1.7	1.49
	0.2%	40	11.05 ±0.8	2.21
	0.3%	20	9.66 ±0.95	2.89

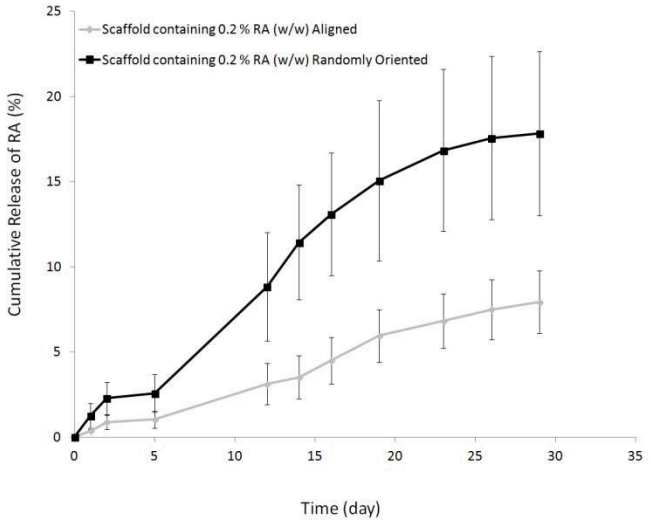

Fig. 7: Controlled release of retinoic acid from randomly oriented and aligned poly (ε caprolactone) nanofiber scaffolds containing 0.2% retinoic acid (w/w) over 30 days.

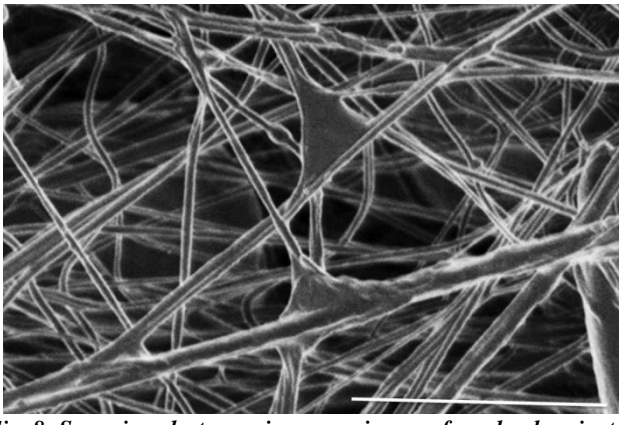

Fig. 8: Scanning electron microscopy image of randomly oriented poly (ε caprolactone) nanofibers containing 0.2 % retinoic acid (w/w) after 30 days of release studies. Scale bar is 4 µm.

C. IN VITRO TEST: CE3 CELL ATTACHMENT AND PROLIFERATION

The ability of the encapsulated PCL nanofibers to serve as a scaffold for neural tissue engineering was examined through CE3 cell seeding experiments. Cell viability and outgrowth were qualitatively assessed using fluorescent microscopy Live CE3 cells constantly express GFP under the β-actin promoter. Figure 9 shows the cell viability assay of CE3 4-/4+ embryoid body seeded on a randomly oriented PCL-RA scaffold containing 0.2% (w/w) RA after 10 days.

Cell seeding experiments showed undirected rapid proliferation and differentiation of the ESC-derived neural progenitors when plated in a controlled 3D environment. Interestingly, a more consistent cellular morphology was also observed in proliferating cells seeded on aligned PCL-RA nanofibers (Fig.10), suggesting that the topographical cues were controlling cell behavior. Overall, encapsulated PCL scaffolds were shown to support and direct CE3 cell proliferation, and

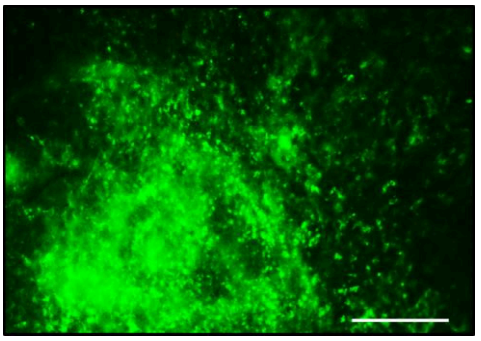

Fig. 9: Cell viability assay: CE3 4-/4+ embryoid body seeded on a randomly oriented poly (ε caprolactone) nanofiber scaffold containing 0.2% retinoic acid (w/w) after 10 days of culture. Scale bar is 5 µm.

can thus be seen as a suitable tissue engineering tool.

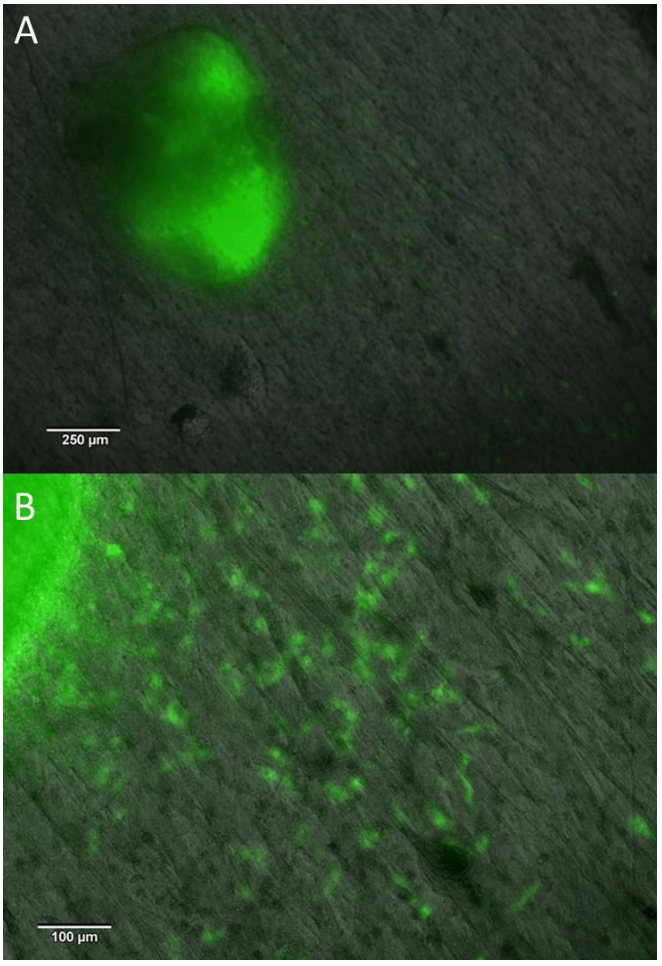

Fig. 10: Fluorescence microscopy images of CE3 4-/4+ embryoid body after seeding onto (A, B) aligned poly (ε caprolactone) nanofiber scaffold containing 0.2% retinoic acid (w/w) after 10 days of culture.

DISCUSSION

There are many methods to design and fabricate drug delivery systems constructed of biocompatible polymers. Solution electrospinning can be used to easily fabricate nanofibers along controlling the topography and porosity of scaffolds while altering polymer solution properties [19]. Moreover, it is possible to encapsulate small molecules and also macromolecules such as growth factors within electrospun nanofibers. Despite these advantages, solution electrospinning requires using toxic solvents. Also, controlling the uniform distribution of fiber size is still challenging as shown in this study. By using emulsion electrospinning, the encapsulation of RA inside PCL nanofibers was achieved and the release of RA from PCL nanofibers for both randomly oriented and aligned topography was obtained. In order to produce more uniform encapsulated nanofibers, using coaxial electrospinning could be used as an alternative strategy [21].

We also evaluated mouse ESC attachment; proliferation and viability on encapsulated scaffolds were evaluated. Directed outgrowth from EBs was also observed with the influence of topography serving as a physical cue for stem cell-based tissue engineering strategies. This work demonstrated the compatibility of these scaffolds fabricated via solution electrospinning with stem cells. One important property of fibrous scaffolds for neural tissue engineering applications is controlling the topography of scaffolds to mimic the extracellular matrix [2]. Our versatile technique to fabricate biocompatible nanofibers via solution electrospinning provided controlled release of RA with the aim of supporting stem cell growth, proliferation and viability. Our work combining stem cells and electrospun encapsulated PCL nanofibers demonstrates an attractive method of producing biomimetic scaffolds in regenerative medicine and novel drug delivery systems. On-going work is investigating the effects of aligned scaffolds and controlled delivery of larger molecules such as neural growth factors from such scaffolds to further enhance neural differentiation of iPS cells.

CONCLUSIONS

In this study, the encapsulation of RA into randomly-oriented and aligned PCL nanofibers was investigated. Due to the porous structure and small fiber diameters, encapsulated PCL electrospun nanofibers have a high surface area that enables construction of 3D scaffolds for neural tissue engineering applications. We were able to fabricate bead-free nanofibers while controlling scaffold morphology. Controlled release of RA from PCL nanofibers with both topographies was observed over 1 month. We can also successfully culture embryonic stem-derived neural progenitors on electrospun scaffolds. In our follow up studies, we will investigate the controlled release of proteins such neural growth factors from PCL nanofibers. A novel combination of the encapsulated nanofibers with stem cells would be introduced as a promising approach for neural tissue engineering applications.

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