Retinoic acid–loaded core-shell fibrous scaffold for neuronal differentiation of trabecular mesenchymal stem cells

Khatereh Asadi 1, Yousef Mortazavi 2, Samad Nadri 1,3,4*

1Department of Medical Nanotechnology, Zanjan University of Medical Sciences, Zanjan, Iran
2Department of Medical Biotechnology, Zanjan University of Medical Sciences, Zanjan, Iran
3Zanjan Metabolic Diseases Research Center, Zanjan University of Medical Sciences, Zanjan, Iran
4Zanjan Pharmaceutical Nanotechnology Research Center, Zanjan University of Medical Sciences, Zanjan, Iran

* Corresponding Author Email: Nadri_s@zums.ac.ir

Note. This manuscript was submitted on October 1, 2020; approved on December 15, 2020

How to cite this article
Asadi Kh, Mortazavi Y, Nadri S. Retinoic acid–loaded core-shell fibrous scaffold for neuronal differentiation of trabecular mesenchymal stem cells. Nanomed J. 2021; 8(1): 73-79. DOI: 10.22038/nmj.2021.08.08

ABSTRACT
Objective(s): Scientists believe that they can fabricate a biochemical scaffold and seed stem cells on it to create an extracellular matrix for tissue generation. This study sought to develop retinoic acid (RA)-loaded core-shell fibrous scaffolds (Poly-Caprolactone (PCL)/Polyethylene Oxide (PEO) based on electrospinning technique, to examine neural differentiation of trabecular mesenchymal stem cells (TM-MSCs).

Materials and Methods: PEO-PCL core-shell fibrous scaffold was fabricated using coaxial electrospinning and Fourier transform infrared (FTIR) used to evaluate the chemical bond structure, scanning electron microscopy (SEM) has been utilized to evaluate surface topography and fibrous diameter, and transient electron microscopy (TEM) to evaluate core-shell structure. The neural differentiation was evaluated using Real-Time PCR.

Results: The results of FTIR, SEM, and TEM confirm the fabrication of core-shell fibrous of PEO-PCL. The fabricated scaffold provides a suitable substrate for adhesion, cell proliferation, and differentiation. SEM images show changes in the morphology of TM-MSCs to neuronal cells. A sustained release of RA from the PEO/PCL scaffold was detected over 14 days. In addition, quantifying the expression of the gene indicates an increase in the gene expression of microtubule-associated protein 2 (MAP-2) gene.

Conclusion: The PEO/PCL core-shell fibrous scaffold containing a RA constructed using coaxial electrospinning technique was a suitable substrate for inducing neuronal differentiation of TM-MSCs cultivated on core-shell scaffold.

Keywords: Coaxial Electrospinning, Core-Shell Fibers, Nerve-Like Cells, Poly-Caprolactone, Polyethylene Oxide, Retinoic Acid

INTRODUCTION
Restoring neural tissue is a complex biological phenomenon. Nerve tissue regeneration and repair strategies has so far attracted a lot of attention all around the world. A bulk of studies have been conducted based on the use of mesenchymal stem cells on three-dimensional fibrous scaffolds, in which all attempts have been made to repair damages to the nervous system [1, 2]. Today, tissue engineering scaffolds are designed to accommodate the various types of cells, proteins, and biomolecules to provide a three-dimensional environment akin to the extracellular matrix for nerve tissue reconstruction [3].

An electrospinning method is one of the biomimetic constructing scaffolds technique in tissue engineering that creates similar fiber structures of the extracellular matrix [4, 5]. These nanofibrous scaffolds have improved mechanical properties and can be used in tissue engineering from a biocompatible polymer to a less biocompatible polymeric envelope [6-8]. Coaxial electrospinning is the cornerstone in to core-shell nanofibrous development. Core-shell fibrous scaffolds have a high capacity for loading biomolecules, exhibit higher stability in different environments, and protect core materials from
environmental damages. These properties make core-shell nanofibrous a suitable vehicle for biomolecule delivery and tissue engineering opportunities. Biomolecules play an important role in facilitating the restoration of the neural tissue; however, most of them are short-lived in the neural tissue and some active biomolecules around the damaged nerves can destroy them at any moment. The core-shell nanofibrous has a high capacity for loading biomolecules. Thus, the high efficiency of core-shell nanofibrous in loading biomolecules allows the creation of effective responses [8]. The retinoic acid (RA) is a key regulator molecule in the process of differentiation into neuronal cells and considered as a suitable neuropathic inducer for different source of stem cells.

Eye trabecular meshwork tissue has been recognized as a source of multipotent mesenchymal stem cells (MSCs; with spindle-shaped morphology, high proliferation and differentiation capacity), which can be acquired by a less invasive surgical procedure without any contamination with non-MSCs [9].

The purpose of this study was to construct a novel PEO-PCL core-shell fibrous scaffolds containing a RA induction molecule as a substrate based on coaxial electrospinning technique, for neural differentiation of trabecular mesenchymal stem cells (TM-MSCs).

MATERIALS AND METHODS

Fabrication of core-shell fibrous scaffolds

Polycaprolactone (PCL; 12% w/v; molecular weight of 80,000 kDa) was dissolved in organic solvent Dimethylformamide (DMF) and chloroform (Sigma, Steinheim, Germany). Also, polyethylene oxide (2% w/v; 900000 kDa molecular weight) and retinoic acid (RA; 0.2 mg/ml; Sigma) were dissolved in deionized water for fabrication of RA loaded PEO-PCL core shell scaffold. The PCL solution (shell) and the PEO solution (core) of the fibers were guided to coaxial needle through programmable syringe pumps. Positive high-voltage supply (22 kV), 13 cm distance were applied between the needle and the cylindrical collector. Also, the feed rates for solutions PCL and PEO with and without RA were 0.6 and 0.1 ml/h, respectively. Other conditions of the electrospinning device, such as the high speed rotating disk (2500 rpm), the temperature in range of 24° to 27° C degree, and the humidity of the environment between 35-37% were kept constant. Each scaffold was spun for 5 hours.

Electron microscopy imaging

The surface morphology of electrospun PEO-PCL core-shell fibrous scaffolds was studied with SEM device. The scaffolds containing the cell were fixed for 15 minutes in 2.5% glutaraldehyde solution. Then, the dehydration of scaffold was performed with the aid of distilled water diluted with 70, 80, 90, and 100 alcohol for 5 minutes. Then, using a sputter-coated device, a very thin layer, and a conductor of gold metal were put on the scaffolding and placed on a circular plate. Then, the plate was put on the SEM device (model AIS2100 made in the SERON TECHNOLOGY Company) for shooting the electron beam and viewing sample. The mean diameter of the electrospun fibrous was analyzed by Microstructure Measurement software. For TEM imaging, the copper grids suitable for sampling were placed on a fiber collector in the coaxial electrospinning device. After one minute of spinning the fibers, the grid of a number of fibers was removed from the collector and put in German ZIESS TEM machine, model EM10C for analysis.

Fourier transforms infrared spectroscopy (FTIR)

The Vector 22 Bruker Fourier transform (FTIR) was used to evaluate the spectral compatibility of PEO-PCL fibrous scaffold with pure powdered PCL and PEO polymers. The samples were placed on the device after powdering, and the resulting spectra was recorded in a range of 400 to 4000 cm⁻¹.

Isolation and differentiation into mesenchymal lineage cells

TM-MSCs were isolated according to a protocol from the study modified by Nadri et al. (10) in which 2–3 mm² of trabecular meshwork biopsy was obtained under sterile conditions. The biopsy was treated with 4 mg/ml collagenase for 1 h and cultured in low glucose DMEM (GIBCO-BRL) supplemented with 20% FBS (GIBCO), 200 ng/ml basic fibroblast growth factor (b-FGF) (Peprotech). After 2 weeks, the cells were trypsinized (0.25% Trypsin – GIBCO-) and expanded by two passages. To demonstrate the multipotent nature of the isolated cells, the cells were differentiated down three lineages as follows: osteogenic (DMEM including 50 mg/mL ascorbic acid 2-phosphate (Sigma), 10 mM β-glycerol phosphate (Sigma)), adipogenic (DMEM supplemented with 50 mg/mL indomethacine
(Sigma) and 100 nM dexamethasone (Sigma), and chondrogenic (DMEM supplemented with 10 ng/mL transforming growth factor- b3 (TGF-b3; Sigma), bone morphogenetic protein-6 (BMP-6), 107 M dexamethasone (Sigma), 50 mg/mL ascorbate-2-phosphate (Sigma), and 50 mg/mL insulin transferrin-selenium (ITS; GIBCO) medium [10].

Cell viability assay

In this study, cells (7103 cells) were cultured on PEO- PCL without RA core-shell scaffold and tissue culture polystyrene (TCPS) plate were maintained in culture medium (as the control group) and incubated at 37° C and 5% CO2 for 3, 5 and 7 days. Then, 300 ml of MTT solution and 5 mg/ml of DMEM solution without FBS were added into the samples and incubated for 3 hours at 37°C. Then, the supernatant was removed from the surface of the samples and a certain amount of dimethylsulphoxide (DMSO) for solubilizing the formazane crystal were added to each sample. The incubated suspension solution was poured into a 96-well plate after they were pipetted. Then, the samples with a wavelength of 570 nm were read by ELISA devise model ELX800; BioTeK, Winooski, VT. Finally, the results were compared between the scaffold group and TCPS on days 3, 5 and 7 after cell culture.

Neural differentiation of TM-MSCs on core-shell fibrous scaffold

First, the PEO- PCL core-shell nanofibrous scaffold containing RA and the PEO- PCL core-shell without RA were placed in 75% alcohol for 2 hours to be completely sterilized [11]. Then, they were completely washed three times with PBS solution. Scaffold (5cm * 5cm) was putted in Petri dishes and 3.5 × 105 cells were placed on each scaffold. The culture medium was then added to a sufficient amount. It was kept at the incubator (5% CO2 at 37°C) for 7 days. During this time, the cell culture medium was replaced every two days. At the end of these stages, the scaffolds of each group were evaluated for the degree of differentiation with the aid of quantitative real-time PCR, and the levels of expression of the genes were compared with the untreated cells.

Retinoic acid (RA) release study

The scaffold was punched into small squares (3 * 3 cm2), soaked in 10 ml of PBS (pH=7.4). Certain time intervals were set (30 min, 1 hr, 2 hr, 4 hr, 12 hr, 48 hr, 16 8hr, and 336 hr), 500μL of the PBS was collected and stored at -70oC. A standard curve of RA was obtained based on the min/max concentration stocks, and the optical absorbance was assessed wavelengths by UV-VIS spectroscopy. So, the drug cumulative release profile was reported. Then, the drug concentration determined by UV-VIS spectroscopy.

Table 1. Primer sequences for target and housekeeping genes employed in the study

<table>
<thead>
<tr>
<th>GENE</th>
<th>PRIMER SEQUENCE (5’ to 3’)</th>
<th>PRODUCT LENGTH</th>
<th>Accession Number</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP2F</td>
<td>AGT TCC AGC AGC GTG ATG</td>
<td>97 bp</td>
<td>NC_000002.12</td>
<td>(12)</td>
</tr>
<tr>
<td>MAP2R</td>
<td>CAT TCT CTC TTC AGC CTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TUBB3F</td>
<td>TGAGATGAGGAACAGGATG</td>
<td>76 bp</td>
<td>NC_00016.10</td>
<td>(12)</td>
</tr>
<tr>
<td>TUBB3R</td>
<td>GTGTGACGACAGATGATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTGAACCATGAGAAGTATGACA</td>
<td>123 bp</td>
<td>NC_00012.12</td>
<td>(12)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CATGAGTCCTTCACAGTAC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RNA of the whole samples was extracted with RNX-Plus solution (Sinaclo, Iran) and its quality and purity were measured using a spectrophotometer (Nanodrop 2000, Wilmington, USA). In the next step, cDNA was prepared by the cDNA synthesis kit PrimeScript 1st strand cDNA Synthesis Kit (Takara, Japan) according to the principles of the company. The real-time PCR tracking technique was performed using a High Rox RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) by Applied Biosystems StepOnePlus™ Real-Time PCR System (Life Technologies Corporation, USA) following to the standard protocol of the company. All genes assessed under real-time cycles 40 times. The real-time processing performed during 10 minutes at 95°C, then 40 cycles of 15 second (s) at 95°C and 30 s of annealing at 60°C, and finally a 30 s extension at 72°C. Also, the threshold cycle (Ct) and the melting curve of all samples were analyzed. The evaluated neural marker and their specific primers are given in table 1[12]. Finally, data were analyzed by Pfaffl equation developed.
by Michael W. Pfaffl using REST® software.

RESULTS

Fabrication of coaxial electrospun core-shell fibrous scaffolds

The PEO-PCL core-shell scaffold was fabricated using a coaxial electrospinning method. The co-axial electrospinning process is shown schematically in Fig 1. In this method, two polymeric solutions for the shell and core structure were used, where the shell solution was directed to the external coaxial nozzle and the core solution with RA small molecule to the internal nozzle.

Characterization of core-shell Fibrous Scaffolds

An electron microscope (TEM) was used to evaluate the core-shell structure of the fibrous scaffold. In the resulting image, the contrast of the electron beam passing through the various portions of the fiber structure shows dark and bright phases, the darker points representing the core and the lighter points representing the shell (Fig 2).

Fig 3B shows the distribution diameter of fibrous scaffold (200 nm - 4 micrometers. The highest frequency of nanofibers is from 1 - 2 micrometer. The spectra obtained by the FTIR test for PCL, PEO polymers, and the PEO-PCL core-shell nanofibrous scaffold is shown in Fig 4. PCL polymer spectra showed peaks in the specified range.

The peak of 1205 cm-1 belongs to the C-O-C symmetric stretch, 1730 cm-1 is for the stretching vibration of the functional group C=O. The peak 2850 cm-1 is related to the symmetric stretching vibration C-H and 2950 cm-1 stretching vibration is for asymmetric stretching vibration C-H. In the FTIR spectrum of the PEO polymer, the 890 cm-1 peak is for the H-C-H. A vibrational peak of 1090 cm-1 is related to the stretching vibration of the functional group C-O-C and the peak of 3440 cm-1 is related to the stretching vibration of the C-H group and the 3440 cm-1 is related to the functional group O-H.

In the spectra obtained from the FTIR test, the PEO-PCL core-shell nanofibrous scaffolds peaks at 1165 cm-1, 1205 cm-1, 1730 cm-1, 2850 cm-1, 2950 cm -1, and 3440 cm-1. It confirms the presence of both polymers in the structure of the scaffold.
Cell culture and mesenchymal differentiation of TM-MSCs

Isolated TM-MSCs have fibroblast-like cells with spindle-shaped morphology. To confirm their mesenchymal nature, the cells were treated with appropriate osteo-, chondro- and adipoinduction media, and their differentiation was confirmed via appropriate staining including alizarin red (for osteogenic differentiation), alcian blue (for chondrogenic differentiation) and oil red (for adipogenic differentiation) staining (Supplementary Fig 1 A-D).

MTT assay

The results of PEO-PCL core-shell nanofibrous scaffold (3D) and TCPS as a 2D culture group were compared on days 3, 5, and 7 after cell culturing (Fig 5). There was a significant difference between the growth rate of cells on the scaffold and the TCPS on days 5 and 7. In the whole study period (days 3, 5, and 7), the highest amount of cells was observed on TCPS. Also, the growth rate of cells on 3rd day was not significant between TCPS and scaffold.

RA release profiles from PEO-PCL scaffold

The release profile of RA-loaded PEO-PCL core-shell fibers is shown in Fig 6. After the fast release of RA (55%) at first 12h, on the 7th day (168 hours) and 14th day (336 hours), the release of RA extended 73% and 81% of the total RA from PEO-PCL fibers, respectively (Fig 6).

Neural gene expression

Seven days after TM-MSCs culture on a PEO-PCL core-shell scaffold with and without RA, the qPCR technique was used to evaluate the degree of neural differentiation. The analysis of the results indicated high expression of MAP-2 gene in the RA-loaded PEO-PCL scaffold compared to the PEO-PCL scaffold. The expression of the β-Tubulin III gene expression was not different between
the two groups (Fig 7). Furthermore, an imaging electron microscope SEM shows morphological changes in TM-MSCs cultured on PEO-PCL and RA-loaded PEO-PCL scaffolds (Fig 8).

DISCUSSION

Nanofibrous core-shell scaffolds have high efficiency in loading biomolecules and have a higher stability than conventional fibers [13]. They can better protect the encapsulated materials from damage on the part of the surrounding environment [14]. In the present study, we developed a PEO-PCL core-shell scaffold and incorporated retinoic acid (RA) in the core of fibrous, for neural differentiation of stem cells. Different techniques including FTIR, SEM and TEM analysis confirmed the construction of core-shell nanofibers. PEO-PCL core-shell nanofiber scaffold spectrum (FTIR) showed that all of the chemical bonds contained in the scaffold spectrum also existed in the PCL and PEO pure polymer range and there was no change in the peak location. The scaffolding spectrum was highly compatible with the spectrum from the pure PCL sample, which confirmed the structure of the core-shell nanofibers and the presence of PCL at the fiber surface [15, 16]. The holding stem cell viability is very vital in regenerative medicine and stem cell studies. In the present study, PEO-PCL scaffold fabricated and TM-MSCs cultivated on this. Although the cells on the scaffold have shown growth, the highest amount of cells was observed on TCPS. However it seems that the numerous of stem cells don’t adhered on scaffold and in MTT analyze the cell proliferation is lower in comparison to TCPS due to this fact that the hydrophobicity property of PCL scaffold hinder cell adherence on scaffold. The hydrophobic scaffold may made available less adhesion situations than the hydrophilic surface and may create a weaker and insufficient cell-adhesion signal [17, 18]. In this experiment, a greater part of stem cells adhered to TCPS as compared to electrosprun fibrous scaffold because, TCPS is treated by oxygenated gas plasma to create a more hydrophilic surface and gives a surface to encourage cell attachment[19].

Coaxial electrospinning is one of the best methods for fabrication of core-shell nanofibers and loading different biomolecules such as retinoic acid in their core structure. RA is a very strong neurodegenerative inducer which plays an important role in the neural differentiation of stem cells, but its high levels may lead to cytotoxicity and cell death [20, 21].

The final aim of the control release system is to achieve the induction factor burst release at first hours and a subsequent sustained release. In our study, RA was loaded in the core part of the scaffold, and its profile release was evaluated which indicated an initial burst release of RA in the first 12 hours and was likely attributed to RA, was located near the surface of the nanofibers which transfer from higher concentration (core) to the lower concentration part (shell) faster (burst amount (55%) then released at a fairly constant rate for at least 14 days. RA release was also affected by the shell layer polymer stability. Due to PCL slow degradation rate, diffusion appeared to be the main form of RA release [22]. This provides the concentration necessary for neural differentiation of stem cells which improves RA action and decreases RA side effects [23, 24].

In this study, RA was loaded into the core of the PEO-PCL scaffold and the characteristics of the induction of RA for neural differentiation of the TM-MSCs were assessed.

Seven days after TM-MSCs culture on the PEO-PCL core-shell scaffold that provided local concentration of RA, neural genes (β-Tubulin III and MAP-2) were expressed. Comparison of the results between RA-loaded PEO-PCL scaffold and PEO-PCL scaffold showed a significant increase in the expression of MAP-2 gene in RA-loaded PEO-PCL scaffold. MAP-2 organizes proteins related to self-assembly of tubules, which is one of the essential steps in neurogenesis, found exclusively on neuronal cells, and is an excellent marker for neural differentiation [25].

The expression of MAP-2 gene expression in TM-MSCs grown on scaffolds containing RA was significantly more than the cells grown on a scaffold without RA. The expression of the β-Tubulin III gene was up-regulated in early stage of neural tissue engineering and Low levels of that can be detected until 7 days [26].

Furthermore, SEM images were used to evaluate the interaction of stem cells with scaffolds, and the differentiation of mesenchymal cells into neuronal-like cells. Seven days after TM-MSCs culture on RA-loaded PEO-PCL scaffolds, SEM image showed the orientation, adhesion, bonding of the cells to the scaffold, and cellular morphology changing of the TM-MSCs into neuronal-like cells.
CONCLUSION
In this study, RA-loaded PEG/PCL core-shell scaffold was fabricated using the coaxial electrospinning technique and the release profile of RA was examined. Sustained release over a prolonged period while maintaining the bioactivity and functionality of RA were showed. However, when the material is loaded into the core structure of the shell nanofibers and slowly released, its undesirable effects decrease and cause stem cells to differentiate into nerve-like cells.

ACKNOWLEDGMENT
This work was supported by Zanjan University of Medical Sciences, Deputy of Research and Technology (Grant No: A-10-892-12, Ethical Code: ZUMS.REC.1395.67).

REFERENCES

ACKNOWLEDGMENT
This work was supported by Zanjan University of Medical Sciences, Deputy of Research and Technology (Grant No: A-10-892-12, Ethical Code: ZUMS.REC.1395.67).

REFERENCES