Controlled surface morphology and hydrophilicity of polycaprolactone towards selective differentiation of mesenchymal stem cells to neural like cells

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an ‘Accepted Article’, doi: 10.1002/jbm.a.35328
Abstract

Differentiation of mesenchymal stem cells (MSCs) into neuron cells has great potential in therapy of damaged nerve tissue. It has been shown that three dimensional (3D) biomaterials have great ability to up regulate the expression of neuronal proteins. In this study, O2 plasma technology was used to enhance hydrophilicity of poly (e-caprolactone) (PCL) towards selective differentiation of MSCs into neural cells. Random and aligned PCL nanofibers scaffolds were fabricated by electrospinning method and their physicochemical and mechanical properties were carried out by scanning electron microscope (SEM), contact angle, and tensile measurements. Contact angle studies of PCL and plasma treated PCL (p-PCL) nanofibers revealed significant change on the surface properties PCL nanofibers from the view point of hydrophilicity. Physiochemical studies revealed that p-PCL nanofibers were extremely hydrophilic compared with untreated PCL nanofibers which were highly hydrophobic and nonabsorbent to water. Differentiation of MSCs were carried out by inducing growth factors including basic fibroblast growth factor, nerve growth factor, and brain derived growth factor, NT3, IBMX in DMEM/F12 media. Differentiated MSCs on nanofibrous scaffold were examined by immunofluorescence assay and was found to express the neuronal proteins; β-tubulin III and Map2, on day 15 after cell culture. The RT-PCR analysis showed that p-PCL nanofibrous scaffold could upregulate expression of Map-2 and downregulate expression of Nestin genes in nerve cells differentiated from MSCs. This study indicates that mesenchymal stem cell cultured on nanofibrous scaffold have potential differentiation to neuronal cells on and could apply in nerve tissue repair.

Keywords: MSCs, Tissue Engineering, Hydrophilicity, Surface Modification, Neural Cells
Introduction

Several research studies have been recently applied for nerve regeneration such as autologous nerve graft although functional recovery in most of the studies is often poor [1-5]. Poly(ε-caprolactone) (PCL) is a biodegradable and biocompatible polymer that has been used as a biomaterial for nerve tissue engineering because of their suitable mechanical properties, however low hydrophilicity of PCL cause low cell attachment on scaffolds surface [6-9]. Research interest of MSCs in tissue engineering is mainly due to their potential in cell therapy for disorders such as degenerative and autoimmune disease [9-13]. There are few studies that have showed differentiation of MSCs into neural cell on electrospun nanofibers scaffolds [6, 9]. Over the years much has been attempted in generating tissue engineered nano-products [14-21]. Porous materials with various 3D structures have been investigated for the cell scaffold because they have larger surface for cell attachment and proliferation than 2D systems and are preferable to assist the formation of 3D cell constructs which may resemble the structure and function of body tissues [22-28]. Xie et al. has reported that the initial rate of cells grown was higher for the 2D culture, but once the cells reached confluent, their proliferation stopped [29]. Other reports have demonstrated that cell proliferation was superior in the 3D scaffold than the 2D one [30-34]. Ma et al. reported the effect of trophoblast differentiation on the non-woven fabrics with different porosities [35]. The cell morphology, such as cell aggregates, was essential to differentiate and different by the porosity of non-woven fabrics [36-38]. In comparison with conventional culture, cells maintained in 3D culture more closely resemble the in vivo situation with regard to cell shape that can influence the behavior of cells [39-43]. This study aims to investigate the differentiation potential of MSCs to nerve cell on p-PCL nanofibrous scaffolds as a 3D matrix to support cell proliferation and differentiation.
Materials and methods

Materials

Poly-e-caprolactone (molecular weight of 80 000), chloroform and dimethyl formaldehyde (DMF) purchased from Sigma-Aldrich (St Louis, MO, USA). MSCs obtained from stem cell technology Research Center (Tehran, Iran). Dulbecco’s Modified Eagle’s Medium (DMEM) obtained from Sigma; fetal bovine serum (FBS), antibiotics and trypsin-EDTA purchased from GIBCO Invitrogen (Carlsbad, CA, USA). Growth factors such as Neurotrophin3 (NT3), basic-fibroblast growth factor (bFGF), nerve growth factor (NGF), and brain derived neurotrophic factor (BDNF) purchased from Sigma.

Electrospinning of nanofibers

PCL (8 wt %) was dissolved in chloroform/DMF (9:1) for the electrospinning procedure. Polymer solution loaded into a 5 ml plastic syringe with a needle 21 G. Polymer solution fed to the needle tip using a syringe pump at a flow rate of 0.5 ml/hr. A positive voltage of 25 kV was used to the needle using a high voltage power supply. For collecting aligned nanofibers, a rotating disk with linear rate 1000 rpm was applied and rotating disk with 100 rpm in linear rate was applied for collecting random nanofibers. Collector was placed at a distance of 23 cm from the needle tip. Nanofibers collected on aluminum foil were dried overnight under vacuum before being used for experiments [44].

Surface modification of electrospun nanofibers

O$_2$ plasma treatment technology was applied to modify surface properties of electrospun PCL nanofibrous by Diener electronic plasma cleaner (Germany). Nanofibers were placed in the chamber of the plasma cleaner and plasma discharge applied for 3 min with radio frequency power set as 30 W under vacuum mode [44].

Physicochemical and mechanical characterization of nanofibers
nanofibrous scaffolds were coated with gold (Polaron SC 7620 sputter coater) and visualized under scanning electron microscope (LEO 1455VP, England) at an accelerating voltage of 10 kV. For determination of wet-ability of scaffolds, the water contact angle for PCL nanofibrous scaffolds before and after plasma treatment were measured by a Contact Angle measuring System G10 (KRüSS, Korea), mounted with a CCD camera. Scaffolds placed on the sample stage and a drop of distilled water dropped on the surface of each samples. Mechanical testing carried out using a tensile tester (SANTAM Stress machine STM20) at a speed rate of 50 mm/min.

**In vitro culture of mesenchymal stem cells**

MSCs were obtained from Stem Cell Technology Research Center (Tehran, Iran) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin-B. Cells were maintained in a humidified CO$_2$ incubator at 37°C until confluence and fed with fresh medium every 3 days. Before cell seeding, MSCs were separated from the cell culture flask with trypsin-EDTA and cells were counted using a neubauer lam.

**Neuronal differentiation of MSCs**

MSCs grown on electrospun nanofibers were exposed to a blend of induction factors containing BDNF (10ng/ml), bFGF (10ng/ml), NT3 (1ng/ml), NGF (10ng/ml), IBMX0.5mM in DMEM/F12 for a period of 15 days.

**Immunocytochemistry**

MSCs were grown on electrospun-PCL nanofibers by ‘nerve induction medium’ for 15 days and used for immunocytochemistry experiments. At first, MSCs fixed with 4% paraformaldehyde (Fluka, Sigma-Aldrich) at 4°C for 20 min. then, the cells rinsed with PBS and blocked with Triton-X100 for 5 min followed by5% goat serum for 45 min. The cells incubated with primary antibody; anti-Map2 (1:500, Bioscience) overnight at 4°C. Also,
similar incubation was carried out with another primary antibody, β-tubulin (2.5:200, Bioscience). Next day, the cultured cells washed with PBS and incubated with rabbit anti-mouse Fluorescein Isothiocyanate secondary antibody (FITC, 1:500, Sigma) for 3 hr and then nuclear staining carried out by 4,6-diamidino-2-phenylindole (DAPI 1:10000 in PBS, Invitrogen, USA) for 1 min at RT.

**Statistical analysis**

Data obtained in triplicate, averaged and expressed as mean ± standard deviation (SD). Statistical analysis carried out using one way analysis of variance (ANOVA). A value of $p \leq 0.05$ considered statistically significant.

**Results**

**Chemical and mechanical properties of electrospun nanofibers**

SEM micrographs of nanofibrous scaffolds showed porous and nanoscaled fibrous. Aligned PCL nanofibers revealed consistent thickness with fiber diameter of 400-1500 nm (Figure 1-b). Randomly PCL nanofibers are bead free nanofiber with fiber diameter between 400-1500 nm (Figure 1-a). Contact angle studies of PCL and p-PCL nanofibers showed significant change on the surface properties nanofibers from the viewpoint of hydrophilicity. On the other hand, p-PCL nanofibers were extremely hydrophilic in regard with their water contact angle less than 80° showing 100% wettability due to the presence of hydrophilic groups on the surface of p-PCL. A comparison between properties of aligned and random PCL with p-PCL nanofibers has been illustrated in Table 1. PCL and p-PCL nanofibers showed a typical non-linear stress–strain curve as showed in Figures 2-A and B. The random and aligned p-PCL nanofibers showed a reduction in mechanical strength. The elongation of p-PCL nanofibers was lower compared to PCL nanofibers. Table 2 demonstrates mechanical properties of random and aligned PCL and p-PCL nanofibrous scaffold with respect to tensile stress-strain.
**Morphological studies of mesenchymal stem cells**

SEM images observed for day 1 (Figure 3) of cell culture indicated normal form of MSCs on aligned and random p-PCL nanofibers. Cells on p-PCL aligned nanofibrous scaffolds indicated normal extensions and spindle-shaped morphology. Cells oriented along the direction of the fibers and clustered around the aligned fibers in a longitudinal fashion (Figure 3-a) while cultured cells on random fibers indicated different directions of the nanofibers (Figure 3-b).

**Gene expression study**

Studies of gene expression by real-time PCR were carried out to evaluate the differentiation of MSCs grown on p-PCL scaffolds. We evaluated gene expression level of nerve cells markers of Nestin and Map-2. We did not observe high level of gene expression of MSCs cultured in standard medium. However, by using neuronal induction medium Nestin showed a decreased gene expression in MSCs proliferated on both nanofibrous scaffold and tissue culture plate (Figure 4-A). Map-2 showed higher level of gene expression for MSCs cultured on p-PCL nanofibers, in particular on random nanofibers, as compared to tissue culture plate (Figure 4-B). Also, those cells cultured on nanofibrous scaffolds in standard medium indicated down regulation of expression of two nerve marker genes.

**Immunostaining of cultured scaffolds**

Immunocytochemistry of the differentiated MSCs on nanofibrous scaffolds yielded the following results. The presence of β-tubulin III and Map-2 could be demonstrated in differentiated MSCs on aligned and random p-PCL scaffolds. β-tubulin III and Map-2 could also be demonstrated in differentiated MSCs on tissue culture plate and could not be demonstrated in non-differentiated MSCs as a control group. Also, it can be seen that MSCs cultured on the aligned fibrous scaffolds showed contact guidance by growing parallel to the p-PCL nanofibers (Figure 5).
Discussion

Tissue engineering provided new therapy using biocompatible polymeric and appropriate cells [45-50]. In this study we selected plasma treated PCL (p-PCL) nanofibers for cell culture study because of its suitable hydrophilic properties. Therefore, our study was not involved in comparative differentiation efficacy testing on two different nanofibers (i.e; PCL and p-PCL). Proliferation of MSCs on p-PCL nanofibers was significantly higher than TCP (control). Also, proliferation of MSCs was increased in random nanofibers. A possible reason for this phenomenon is that random nanofibers have many conjunct pores and rough surfaces that cause at adhesion and proliferation of more number of cells. Topography and surface chemistry of materials significantly affect on cell adhesion, proliferation, and differentiation [51]. It has been showed that MSCs can be differentiated to neuronal cell under appropriate conditions [52-57]. RT-PCR analysis showed that MSCs naturally express neural cell markers before induction. Similar results have been reported by other groups [58-61]. Figure 4 showed that MSCs proliferated on aligned and random PCL nanofibers in the presence of nerve induction medium expressed a higher Map-2 gene compared with that on tissue culture plate. Our results showed that upregulation of Map-2 was observed on MSCs proliferated in 3D scaffolds. Our results showed that by using nerve induction medium, Nestin which is associated with progenitor cell; down regulation of gene expression of Nestin can be observed in MSCs proliferated on both electrospun nanofibers and tissue culture plate. We demonstrated that nanofibrous scaffold alone cannot induce the differentiation of MSCs into neuronal cell and neurotrophic growth factors are important in neural differentiation. Although it has been shown that nano-structured materials induced differentiation of stem cell without using growth factor [62]. To the best of our knowledge, there are no reports on the differentiation of MSCs on PCL nanofibrous scaffolds and molecular study of neuronal
marker expression. We showed protein expression of neuronal cells generated from MSCs on electrospun nanofibrous scaffolds.

**Conclusion**

Nanofiber scaffolds could be an ideal matrix for differentiation of MSCs for the treatment of nerve disorders. Our results clearly indicated that the possible enhancement in the differentiation of mesenchymal cells into mature neuron was achieved in contact with electrospun nanofibers in comparison with flat surface. While the exact mechanism of changes in cell morphology to gene expression remains unknown, electrospun scaffolds, particularly scaffolds with random nanofibers indicate to have potential in promoting the differentiation of mesenchymal cells.

**Acknowledgments**

This research supported by Stem Cell Technology Research Center, Tehran, Iran, and was performed through National Science Council (NSC) of Taiwan, and supported by the Research Grant of NSC 102-2221-E-011-010-.

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Table Title

**Table 1.** Comparison of nanofibers properties of PCL and $p$-PCL aligned and random nanofibers.

**Table 2.** Tensile properties of PCL nanofibers.

Figure Legends

**Figure 1.** SEM micrograph of nanofibers. (a) random $p$-PCL nanofiber; (b) aligned $p$-PCL nanofibers.

**Figure 2.** Stress-strain analysis of PCL and $p$-PCL nanofibers. (A) Random and (B) aligned nanofibers.

**Figure 3.** SEM micrograph of MSCs on $p$-PCL nanofibers scaffolds obtained after 1 day of cell culture. (a) aligned $p$-PCL nanofibers (b) random $p$-PCL nanofibers Arrow indicates MSCs along aligned fibers.

**Figure 4.** Real-time PCR gene expressions analysis of MSCs proliferated in nerve differentiation and standard medium on random and aligned $p$-PCL nanofibrous scaffolds. Relative expression of (A) Nestin and (B) Map-2. (A: aligned $p$-PCL nanofibers, R: random $p$-PCL nanofibers, S: scaffold, GF: growth factor). (+) with (–) without. *, indicate significant level of expression gene compared to MSCs at $\leq 0.05$.

**Figure 5.** Fluorescence microscopic micrographs of MSCs grown on aligned (a, e), random (b, f) $p$-PCL nanofibers, TCP (c, g) and Control (d, h) using the neuronal induction media for 15 days. MSCs were immunostained for Map-2 and $\beta$-tubulin.
Table 1. Comparison of physiochemical properties of aligned and random PCL with ρ-PCL nanofibers.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Aligned FCL</th>
<th>Aligned ρ-PCL</th>
<th>Random FCL</th>
<th>Random ρ-PCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porosity</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Contact angle (deg)</td>
<td>130</td>
<td>&lt; 80</td>
<td>134</td>
<td>&lt; 80</td>
</tr>
<tr>
<td>Wettability</td>
<td>Highly hydrophobic</td>
<td>Highly hydrophilic</td>
<td>Highly hydrophobic</td>
<td>Highly hydrophilic</td>
</tr>
</tbody>
</table>

254x190mm (96 x 96 DPI)
Table 2. Comparison of tensile properties of aligned and random PCL with p-PCL nanofibers.

<table>
<thead>
<tr>
<th>Nanofiber scaffold</th>
<th>Tensile stress</th>
<th>Tensile strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random PCL</td>
<td>1.85</td>
<td>363.79</td>
</tr>
<tr>
<td>Random p-PCL</td>
<td>1.68</td>
<td>247.39</td>
</tr>
<tr>
<td>Aligned PCL</td>
<td>2.41</td>
<td>51.37</td>
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<tr>
<td>Aligned p-PCL</td>
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<td>47.2</td>
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