Cell attachment studies on electrospun nanofibrous PLGA and freeze-dried porous PLGA

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ABSTRACT: Tissue engineering, which is a promising approach for treating mankind, aims to repair, replace, maintain, or improve the function of damaged tissue or organ. Electrospun nanofibers, providing a three-dimensional environment to mimic the natural extracellular matrix (ECM), play an essential role in tissue engineering as well as regenerative medicine. In this study we demonstrated the importance of scaffold fabrication method together with the effect of nanotopography to support the growth of cells. Two scaffolds were made from the biodegradable and biocompatible poly (lactic-co-glycolic acid) (PLGA) polymer by electrospinning and freeze-drying techniques respectively and the effectiveness in providing support for cell behavior was investigated. The scaffold structures were compared by scanning electron microscope (SEM). Fibroblast cell culture test was carried to investigate cell behaviors by inverted microscopic images. To investigate the effect of scaffolds on cell proliferation, we performed cell attachment experiments: 4',6-diamidino-2-phenylindole (DAPI) staining and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT). Our results showed that electrospun fibrous PLGA scaffolds provided more suitable surface for cell functioning compared with freeze-dried porous PLGA scaffolds, due to the feature similarity of the former to the native ECM. © Global Scientific Publishers 2013

KEYWORDS: Tissue engineering, Nanofiber, PLGA, Electrospinning, Freeze-drying.

1. Introduction

Tissue engineering, a recently most exciting area in biomedical engineering, has provided a cell-based therapy to repair, replace, maintain, and improve the function of damaged or defective tissues or organs [1-4]. In a native tissue, cells are surrounded by extracellular matrix (ECM), a complex network of pores, ridges, and fibers at nanometer scales [5, 6]. Being a three-dimensional nanostructure, ECM provides support and regulates intercellular communications. ECM can interact with the cell surface receptors involved in attachment such as integrins [7, 8]. The cell behavior is a result of the cell-matrix as well as subsequent cell-cell and cell-solution interactions [9, 10]. The cell attachment is the first and the most important step of culturing when cells are placed in close contact in the microenvironment [11, 12].

The key point for a successful tissue engineering technique is to mimic the structural and functional properties of a native tissue [13]. The scaffold structures have been designed for this purpose [14-16]. A scaffold is needed to serve as an adhesive substrate for cells and the physical support of them. Scaffolds, made by conventional scaffold fabrication techniques, are generally foams of natural and synthetic polymers. The synthetic polymers such as poly (glycolic acid) (PGA), poly (lactic acid) (PLA), and their copolymer of poly (lactic-co-glycolicacid) (PLGA), and the naturally derived polymers such as collagen and glycosaminoglycan are most commonly used for tissue engineering. Several techniques have been developed to process synthetic and natural scaffold materials into porous structures including gas foaming, fiber extrusion and bonding, three-dimensional printing, phase separation, emulsion freeze-drying, and porogen leaching [17]. Cells are highly sensitive to geometrical and structural feature of microenvironment. To achieve this goal, nanofibers can be employed to fabricate scaffolds and artificial ECM in nanoscale can structurally and biologically imitate the natural ECM [18-20]. Currently, there are three techniques available for the synthesis of nanofibers: electrospinning, self-assembly, and phase separation [21]. Of these, electrospinning is the most widely studied technique and also seems to exhibit the most promising results for tissue engineering applications. Electrospinning is a simple, inexpensive, and widely used method to produce nano-scale fibers in either random or aligned orientation [22].

The scaffolds made of electrospun nanofibers provide a high surface area-to-volume ratio, which promotes the cell-matrix interaction at the nanoscale level [23, 24]. The high interconnectivity of pores also improves cell growth and differentiation [25, 26]. PLGA has several biomed-
ical applications and widely used as scaffold in tissue engineering [27, 28]. The porosity and tensile properties of electrospun PLGA nanofibers have been studied [29-31]. Freeze-drying technique is applied to a number of different polymers including PGA, PLLA, PLGA and PLGA/PolyPhenylene Ether (PLGA/PPF) blends [32, 33]. Main advantage of this technique is that, it requires neither high temperature nor further leaching step [34].

In this study we made biodegradable PLGA scaffolds by electrospinning and freeze-drying techniques. The growth of fibroblast cells on the electrospun nanofibrous scaffolds was investigated and compared with freeze-dried porous scaffolds.

2. Method

L929 mouse fibroblast cell lines were obtained from the Cell Bank Department of Pasteur Institute of Iran. PLGA copolymer RG-504H was purchased from Boehringer, Germany, with a 50:50 ratio of lactic acid to glycolic acid. Tetrafluoroethanol (TFE) was provided from Merck, Germany, and was used without any further purification. And (4,5-dimethylazol-2-yl)-2,5-MTT was purchased from Sigma, Germany. Roswell Park Memorial Institute-1640 Medium (RPMI-1640), fetal bovine serum (FBS), and Trypsin-EDTA 0.25% solution were from Gibco, Canada. Phosphate buffered saline (PBS) were prepared using sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate (Na₂HPO₄), and monopotassium phosphate (KH₂PO₄).

Nanofibrous PLGA scaffolds were made using the electrospinning technique (CO881007NYI, Asian Nanostructures Technology Co.) under optimum certain conditions. The polymer solution with concentration of 20% (volume percentage) was prepared by dissolving PLGA into a mixture of chloroform and dimethylformamide (volume ratio 3:1). The solution was fed into a 12-mL plastic syringe, which was controlled by a syringe pump with a flow rate of 1 mL/h. A high voltage (20 kV) was applied to the needle tip, which was placed 15 cm away from the collector. We used a cylindrical collector (a drum with the width of 50 mm, covered with a sheet of aluminum foil). The rotating speed of drum was set at 2500 rpm to make the nanofibrous scaffolds.

The preparation procedures of porous PLGA scaffold by freeze-drying method were as the following: 12% of PLGA TFE solution was transferred to a plate covered with aluminum foil. The polymer solution was frozen at -30 °C and then was placed in a vacuum environment for 2-3 days (CHRIST freeze dryer, ALPHA 2-4LD). Then this porous scaffold had been stored at room temperature.

In order to study the morphology of the prepared nanofibrous and porous PLGA scaffolds, the samples were sputter-coated with gold and then examined using scanning electron spectroscopy (SEM) (Hitachi S3000N) at an accelerating voltage of 15 kV.

In vitro analysis of fibroblast cells was also performed. Although ethanol is usually used to sterilize the scaffolds, the UV radiation was used in this study to avoid the toxic effects of ethanol on cells. Both sides of the prepared scaffolds were exposed to UV radiation for 20 min. Cell culture was started from a frozen stock. After thawing rapidly in a 37°C water bath, the cells were cultured in a standard medium consisting of RPMI-1640 with 10% (vol/vol) fetal bovine serum (FBS) (RPMI-1640-10% FBS) and 1% (vol/vol) antibiotic-antimycotic in a 50-cm² flask and incubated for 48 h. The medium was then replaced with fresh RPMI -1640-10% FBS. The cells were maintained by sub-culturing them after arriving at an acceptable confluence. In order to ensure the accuracy of the test condi-
tions we did live/dead assay before each experiment, using Trypan blue staining and neubauer lam to determine the cell counts quantitatively.

The cell attachment experiments were conducted in a three-step test at 90 min, 24 h, and 72 h respectively. Cell attachment assay determined the fraction of the cells attaching to the matrix surface and being resistant to gentle washing [13, 35]. The bottoms of 6 wells of a 24-well cell culture plate were totally covered with scaffold. The bottom of the empty well was used as positive control. At the first step, at the selected scheduled time (passage No: 3), the cells were removed from the culture flasks by means of Trypsin 0.25% EDTA solution, re-suspended in RPMI-1640-10% FBS, counted, and transferred in each well at a concentration of 1 × 10^4 cells/mL. Cell suspension was carefully placed on the center of the prepared scaffolds and incubated for 2-3 h at 37 °C. After this time the medium containing 10% FBS was added slowly. Then the scaffolds/plates were washed gently with PBS at pH 7.4 and aspirated. Cell attachment was assessed in three replicates using cell counting quantitative method with Trypsin solution and neubauer lam. The second and third steps of the test were done at 24 h and 72 h. The viability of the cells was compared in test samples and controls.

Cells were seeded on two kinds of PLGA scaffolds. The morphology and distribution of the cells grown on the scaffolds at 1-3 days were visualized by inverted microscope (Bell, INV-100FL) and compared with the cells on the plate without scaffolds (Figure 1).

4′6-diamidino-2-phenylindole (DAPI) is a fluorescent DNA stain used for cell cycle studies, staining viable cells, and specifically for the qualitative or quantitative determination of the cells within a liquid culture [36]. The fibroblast cells were trypsinized, counted, and cultured on PLGA scaffolds (1 × 10^4 cells/mL) and incubated for 48 h. The scaffolds were washed with PBS at pH 7.4 for three times, transferred into a new 24-well plate and fixed with 3% paraformaldehyde solution in PBS (pH = 7.4) for 10 min, then washed with PBS for 2 min, and permeabilized with 0.1% triton X-100 in PBS for 2 min, aspirated with triton X-100, and washed with PBS for 2 min. DAPI stain was added and then incubated in dark for 5 min. The scaffolds were washed three times with PBS (2 min each) and maintained in the dark before the observation with fluorescent microscope (Bell, INV-100FL).

![Figure 2. Inverted microscopy images of cell proliferation besides the scaffolds after 24 h, 48 h and 72 h of culture. The dark areas of the images in (a) and (b) as shown by arrows are the scaffolds. (a) The cells grown on the electrospun nanofibrous scaffolds (b) The cells grown on the freeze-dried porous scaffold, (c) Monolayer culture without scaffold as control.](image-url)
In this study MTT assay also was employed to determine the viable cell numbers, based on the mitochondrial conversion of the tetrazolium salt, and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT). This assay was used to count the number of living cells on the scaffolds. The cells were trypsinized, re-suspended in RPMI-1640-10% FBS, counted, and carefully seeded at a concentration $1 \times 10^4$ cells/mL on the center of the scaffolds (1 cm $\times$ 1 cm), placed on the bottom of a 24-well cell culture plate. Cell suspension was incubated for 2-3 h at 37 $^\circ$C. Then the fresh RPMI-1640-10% FBS was added and incubated for 24 h, 48h and 72h respectively. The medium was then totally removed and MTT reagent (100k , 0.5 mg/mL) was added to each well in the 24-well plate and left to incubate for 4 h at 37 $^\circ$C. The MTT reagent was replaced with 100 k of DMSO to dissolve the formazan crystals. The absorbance was measured at 570 nm using an ELISA plate reader (BioTek ELx800). Also, the attachment tendency of the cells to the surface of the scaffold and the role of them in cell proliferation was investigated using inverted microscopy micrographs.

**Statistical analysis**

Data were evaluated by a Dunnet one way analysis of variance (ANOVA) using software SPSS version 16.0. The a priori alpha value was set at 0.05 with the level of significance for all statistical analyses $p < 0.05$.

3. Results and discussions

3.1 Morphology of two kinds of PLGAs

The morphology of electrospun nanofibrous PLGA scaffolds were compared with freeze-dried porous PLGA scaffolds in Figure 1. According to the SEM images, the electrospun scaffold has a fibrous structure in contrast with the porous structure of the freeze-dried PLGA.

![Figure 4.](image)

**Figure 4.** Fluorescent microscopic images from DAPI staining on the scaffolds at day 3. For study of the cell proliferation on the scaffolds we perform staining of cell nucleus. (a) Cell growth on electrospun nanofibrous PLGA scaffold, (b) Cell growth on freeze-dried porous PLGA scaffold.

![Figure 3.](image)

**Figure 3.** Cell interaction tendency to scaffolds at different times. This test was done with the number of cells attached to scaffold versus culturing time (1: 90 min, 2: 24 h, 3: 72 h). Number of cells are expressed as mean $\pm$ SD ($n = 3$). ES: electrospun, F.D. Freeze-dried, C.P. Control.

![Figure 5.](image)

**Figure 5.** Cell viability versus culturing time. Cell viability of the fibroblast cells was tested at 24 h, 48 h and 72 h from MTT assay. Data are expressed as mean $\pm$ SD ($n = 3$). Data are subject to Dunnet one way analysis of variance (ANOVA), * $p < 0.05$.
3.2 Cell attachment study results

The inverted microscopic images of the cell growth pattern in vitro on electrospun nanofibrous PLGA and freeze-dried porous PLGA scaffolds are shown in Figure 2. Cell attachment test showed biocompatibility of the nanofibrous and porous scaffolds to the cells (Figure 3). The comparison of the fluorescent micrographs of nucleus staining (Figure 4) from cell growth on the scaffolds revealed that electrospun nanofibrous scaffolds might provide a suitable microenvironment for cells during proliferation. Also, MTT assay results in this study showed that the cells proliferation gradually increased from 1 day to 3 days on electrospun nanofibrous scaffold. But the freeze-dried porous PLGA scaffold showed significantly higher cells proliferation (at p = 0.05) compared to electrospun nanofibers at 1 day. However, the cell viability attached to freeze-dried porous PLGA scaffolds was then decreased at 3 days (Figure 5). With the results of cell attachment test, both DAPI staining and MTT assay results showed that electrospun nanofibrous scaffolds were better for cell growth in vitro compared to the freeze-dried PLGA scaffolds.

The regulatory signals are related to ECM, adjacent cells, and soluble factors such as Fibroblast Growth Factors (FGFs). ECM as a major factor provides not only a cellular support, but also an immediate microenvironment that triggers regulatory signals to support cell attachment and cell proliferation [37-39]. Nanofibrous scaffolds can play a fundamental function in tissue engineering by facilitating cellular behavior [40-42]. Cell fate is a response to the combination of the different signaling events [43, 44].

The main goal of this study is to study the cell adhesion. We have focused on the synthesis of a biodegradable PLGA nanofibrous scaffolds by electrospinning method. But no comprehensive study has been carried out to compare of electrospinning with other methods. So, to investigate the importance of the scaffold preparation method and 3-dimensional nanostructures for cellular function, we compared with another PLGA scaffold fabricated by thermal induced phase separation freeze-drying technique. The electrospun nanofibers were completely different from the structure of the freeze-dried scaffolds. The morphological structures of PLGA nanofibers fabricated by electrospinning in this study (Figure 1) indicate that the produced scaffold has nanometer scale fibers without beads and is similar to those of natural ECM. Inverted microscopic images (Figure 2) showed that freeze-dried porous scaffolds had higher cell proliferation after 1 day compared with electrospun nanofibrous scaffolds, but decreased after 3 days. Electrospun nanofibrous scaffold and control showed increase in cell proliferation during growth from day-1 until day-3. These findings along with Trypan blue counting results for considering cell attachment indicated that the cell affinity for attachment to the surface of the scaffolds under the same conditions and at the same cell seeding density is better for the electrospun scaffold. The chief intention of the first day step of the test was to reflect on the attachment affinity of the cells to the surface of the scaffolds, and in the 24-h and 72-h steps, the role of the scaffolds composition in cell growth was investigated and compared with the control sample. Figure 4 showed that between electrospun nanofibrous scaffolds and freeze-dried scaffolds, there was specific difference in attachment affinity of cells to scaffold from control sample (plate without scaffold). The present data showed good biocompatibility for both types of scaffolds, in consistent with previous studies that investigated the biocompatibility of nanofibers [45-49].

These results suggested that electrospun nanofibers can be useful for tissue engineering in different tissues or organs. Trypan blue counting method only indicates that the cells are alive or dead and it cannot provide accurate result. To evaluate the function of cells, we applied DAPI staining to realize the activation of the cell nucleus and DNA content (Figure 3). From our data, it was found that the nano-structured PLGA scaffold acted as a positive factor to support cell growth. At day-3 the nanofibrous scaffold showed higher growth. These findings confirm the results of the adhesion test and all of them were in favor of the electrospun nanofiber. Additionally, cell proliferation and cell viability was measured using MTT assay that also showed that among these two kinds of scaffolds, the electrospun nanofibers had a better performance for incidence of suitable cell behavior (Figure 5).

These findings confirmed the crucial role of topography on cell behavior in consistence with the preceding work [50]. Topography not only strongly affects cell morphology, but also influences cell adhesion, proliferation, and gene expression [51,52]. For example, nanoscale topography has been applied in the development of neurogenic [53] and bone tissue [54-56]. The main goal of tissue engineering is to produce functional tissues. This study, therefore, continues the research of nanotopography effects on cell fate in tissue engineering.

4. Conclusions

In this study, we showed that nanofibrous PLGA scaffold fabricated by electrospinning method mimicked the natural extracellular matrix. These results suggested that the electrospun PLGA nanofiber was a potential scaffold in tissue engineering. It might be used in nerve tissue engineering because of its better cell adhesion and differentiation. In the future, we will study the effect of fiber orientation on the cell differentiation for nerve tissue engineering.

Abbreviations

ECM: extracellular matrix; PLGA: poly (Lactid-Co-Glicolid)
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