

Electrospun Poly (ϵ -Caprolactone) Nanofiber Mat as Extracellular Matrix

Laleh Ghasemi-Mobarakeh, M.Sc.¹, Mohammad Morshed, Ph.D.^{1*}, Khadije Karbalaie, M.Sc.², Mehrafarin Fesharaki, M.Sc.³, Mohammad Hossein Nasr-Esfahani, Ph.D.^{2*}, Hossein Baharvand, Ph.D.²

1. Textile Engineering Department, Isfahan University of Technology, Isfahan, Iran

2. Cell Sciences Research Center, Royan Institute, Stem Cell Department, ACECR, Tehran, Iran

3. Physiology Department, Faculty of Medical Sciences, Isfahan University, Isfahan, Iran

* Corresponding Addresses: Textile Engineering Department, Isfahan University of Technology, Isfahan, Iran

Email: morshed@cc.iut.ac.ir

P.O. Box: 19395-4644, Stem Cells Department, Cell Sciences Research Center,

Royan Institute, ACECR, Tehran, Iran

Email: mh.nasr-esfahani@royaninstitute.org

Abstract

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Objective: Investigation of the suitability of electrospun Poly (ϵ -caprolactone) (PCL) nanofiber scaffold for the Vero cell culture.

Materials and Methods: Electrospinning was used for production of PCL nanofiber scaffolds. Scanning electron microscopy (SEM), MTT assay, hematoxylin staining and histology analysis were used to investigate the cell morphology, viability, attachment and infiltration of the vero cells on the PCL nanofiber scaffolds.

Results: The results of the MTT assay, SEM images and hematoxylin staining showed that Vero cells attach and spread on PCL nanofiber scaffolds. The proliferation of Vero cells is as well as that of control group, but histological analysis showed the lack of cell infiltration into the scaffolds, which was found to be due to the small diameters of the pores of nanofibrous scaffold.

Conclusion: The result of this study show that PCL nanofiber scaffolds are suitable for cell culture, proliferation and attachment and Vero cells attach and proliferate on PCL nanofiber scaffolds.

Keywords: Poly (ϵ -caprolactone), Nanofiber, Electrospinning, Tissue Engineering

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Introduction

The current demands for transplant organs and tissues are more than the supply and this gap will continue to widen (1). Tissue engineering is a new approach to reconstruction and/or regeneration of lost or damaged tissue (2). It is a multidisciplinary field that applies the principles of engineering and the life science by combining synthetic and living components in appropriate configurations and environmental conditions (1, 3). There are three key parameters in tissue engineering: the cells, the scaffold and suitable condition for growing cells in three-dimensional scaffolds (1, 3, 4). In the living system, the extracellular (ECM) plays a pivotal role in controlling cell behavior (5). The scaffolds play an important role in tissue engineering by serving as a matrix for cellular ingrowth, proliferation and new tissue formation in the three dimensions (6). There are several requirements in the design of ideal scaffold in tissue engineering as follow:

1. High porosity and proper pore size. The pores of scaffolds are very important for cell growth. The cells adhere to the surface of the scaffolds, absorb nutrient and remove metabolite through the pore. The diameter of cells dictates the minimum pore size which varies from one cell type to another and must be controlled carefully. If the pores are too small the cells may not infiltrate and if they are too large the cells may not adhere (1, 3, 7-9).
2. High surface area that encourage cell adhesion, growth, migration and differentiation (3, 9).
3. The scaffold must be biodegradable. The degradation rate must match regeneration rate of neotissue.
4. Biocompatibility is a key parameter for scaffold. It should not be toxic to the cells both in bulk and degraded form (1, 3)
5. The scaffold should have sufficient mechanical properties to maintain neotissue.

The scaffolds should mimic the structure and biological function of native extracellular matrix as much as possible (10). Collagen is a major natural extracellular matrix component and has a fibrous structure with fiber bundles varying in diameter from 50-500nm (3, 6, 10). Therefore nanofiber scaffolds may serve as suitable environment for cell attachment, proliferation and function than traditional scaffolds (6, 11). The researches show that nanoscale dimension influences the cell behaviors. The cells attached and organized around the fibers with diameters smaller than those of the cells (10). Polymeric nanofibers can be processed by a number of techniques such as drawing, template synthesis, phase separation, self-assembly and electrospinning (3, 6, 12). Electrospinning is one of the most important techniques to manufacture nano-fibers. An attractive feature of electrospinning is the simplicity and inexpensive nature of the setup (13). Electrospinning has several advantages; it can produce continuous fibers; it can be applied to a wide range of polymers; the thickness of mat can be controlled by adjusting the collection time during the electrospinning; the dimensions and surface morphologies of the electrospun fibers can be varied by altering the solution properties and processing parameters (14). The theory of electrospinning is based on electrostatic force that acts on the polymer solution. In the electrospinning process a strong electrostatic field is applied to a polymer solution. When the voltage surpasses a threshold value, the electric forces overcome the surface tension of solution and a charged jet of solution is ejected toward a collecting material screen (15, 16). PCL is a biodegradable, biocompatible polymer that has been successfully electrospun; furthermore, it has been shown to be capable of supporting a wide variety of cell types (17- 21).

The goal of this study was to investigate the ability of electrospun Poly (ϵ -caprolactone) nanofiber scaffold for the Vero cell culture. It is the first time in Iran that electrospun Poly (ϵ -caprolactone) (PCL) nanofiber scaffold has been used for tissue engineering applications.

Materials and Methods

Materials

Poly (ϵ -caprolactone) with number average molecule weight (M_w) 80,000 was purchased from Aldrich. Methylene chloride (MC) and Dimethylformamide (DMF) were purchased from Merck Co.

Dulbecco. Modified Eagle Medium (DMEM), Fetal Calf Serum (FCS), Phosphate buffered saline (PBS), and Trypsin-EDTA were purchased from Gibco and the rest from Sigma.

Fabrication of PCL nanofiber scaffold

The polymer solution with concentration of 10wt% was prepared by dissolving PCL in a mixture of MC/DMF solvents with the ratio of 80/20 and stirred for 24hr at room temperature. The solution was electro-

spun from a 12ml syringe with a needle diameter of 0.6mm. Upon applying a high voltage (12kV), a fluid jet was ejected from the tip of the needle. As the jet accelerated toward a target which was placed 20cm from the syringe tip, the solvent evaporated and polymer nanofibers were collected on an aluminium foil. The polymer solutions were delivered via a syringe pump to control the mass flow rate. The mass flow rate of the solutions was 4ml/h. All electrospinnings were carried out at room temperature. Fig 1 shows schematic illustration of electrospinning set-up used in this research.

Nanofibers-coated coverslips

To observe cell response to electrospun structures under the light microscopy for cell attachment study, the polymer solutions were electrospun directly onto coverslips. Coverslips were cut into circle shapes with diameter of 1.2cm and placed upon the aluminium foil and electrospinning was performed as described above.

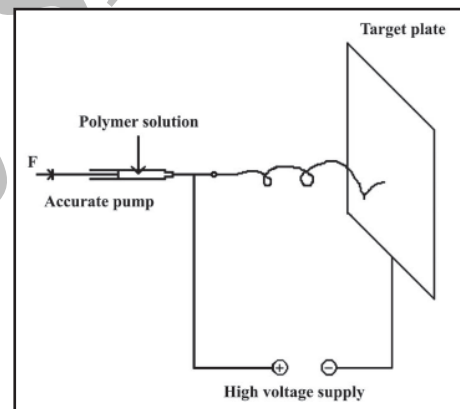


Fig 1: Schematic of electrospinning setup

Nanofiber morphology studies

The morphology of electrospun PCL nanofibers was observed by a scanning electron microscopy (SEM) (Philips XL30). Before the observation, the scaffolds were coated with gold using a sputter coating. The diameter of the fibers was measured from the SEM photographs.

Preparation of nanofiber scaffolds for cell culture

The nanofiber scaffolds were exposed to UV radiation for 30 min and then pre-wetted with 70% ethanol for a period of 60min. After that, they were soaked into Hank's Buffered Salt Solution (HBSS) for 24h.

Cell culture

Vero cells with a diameter of 14-18 μ m were cultured in DMEM supplemented with 15% fetal calf serum (FCS). After reaching sufficient confluence, the cells were detached by trypsin and counted. Then the cells were seeded onto tissue culture plate (TCP) as control and PCL scaffolds that, placed in a 24-well plate with

the density of 1×10^5 cell per well and cultured with DMEM containing 15% (FCS) supplement.

Cell morphology studies

The morphology of Vero cells on the PCL nanofiber scaffolds was observed by Scanning Electron Microscopy (SEM). After 7 days cell seeding, samples were fixed in 2.5% glutaraldehyde for 2h and then dehydrated with a graded concentration (50-100% v/v) of ethanol. After drying the samples, they were coated with gold using sputter coating for the observation of cell morphology.

MTT assay

Cell proliferation on PCL nanofiber scaffolds and tissue culture plate (TCP) was measured by 3-(4, 5-di-methyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) solution (5mg/ml). After 4 days of cell seeding in 24-well dish, the original medium was removed and 400 μ l fresh medium and 40 μ l MTT solution were added to each well. After 4-hour incubation at 37°C in 5% CO₂, MTT solution from each well was carefully removed and replaced by 250 μ l DMSO (Dimethyl Sulfoxide) for each well. Then the absorbance of solution was measured at 490nm.

Cell attachment study

Live cell morphology and cell-attachment was studied using staining with hematoxylin dye. After 4 days of cell seeding, samples were fixed in 4% paraformaldehyde for 2hrs at room temperature and then stained with hematoxylin for 5min. the samples were rinsed with phosphate buffered saline (PBS) and dehydrated with a graded concentration (50-100% v/v) of ethanol, then the scaffold were observed with an invert microscope.

Histology

In order to investigate the cellular infiltration of nanofiber scaffolds, the samples were fixed in 4% paraformaldehyde for 2hr at room temperature after 4 days of cell seeding and embedded in paraffin. The blocks were sectioned using microtome. After staining with hematoxylin and eosin the samples were mounted and images were obtained using a light microscopy.

Statistical analysis

All quantitative results were obtained from triplicate samples. Statistical analysis was carried out using single-factor analysis of variance (ANOVA). A value of $p < 0.05$ was considered to be statistically significant.

Results

Morphology of electrospun nanofiber

SEM micrograph of electrospun PCL nanofibers are shown in fig 2A. Average fiber diameter was estimated to be 418 ± 164 nm with diameter ranging from 200-1000nm (Fig 2B).

Figure 3 shows the SEM micrographs of the interaction between cells and electrospun nanofibrous after 7 days.

As can be seen, the cells have proliferated on the nanofibrous network and covered the scaffold (Fig 3A). Figure 3B reveals cell spreading on the nanofiber scaffold.

Cell attachment

Hematoxylin staining was used to investigate cell attachment on the nanofiber scaffold. Figures 4A-B show the phase contrast light microscopy (PCLM) pictures of vero cells on the nanofibers scaffolds and tissue-culture plate (TCP) stained with hematoxylin respectively. As can be seen from this figure, the attachment of cells on the nanofiber scaffold is similar to that of the tissue culture plate.

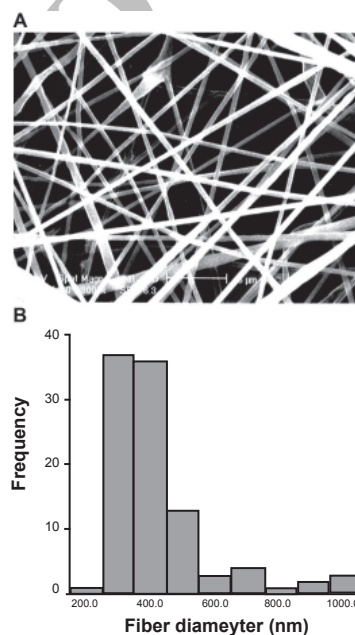


Fig 2: SEM image (A) Electrospun PCL nanofibers (magnification: 3000), (B) fiber diameter distribution Cell-matrix interaction

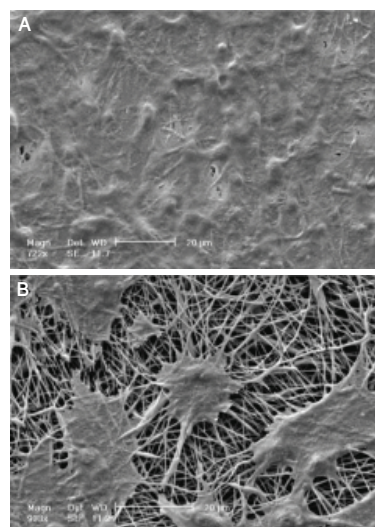


Fig 3: Vero cells on the electrospun nanofibrous after 7 days of culture

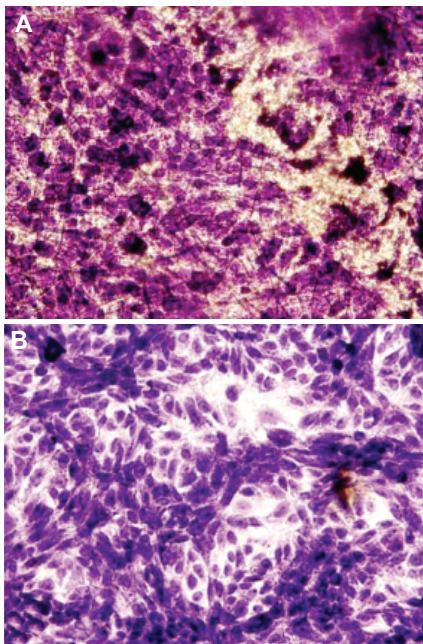


Fig 4: Hematoxylin staining of the Vero cells on the A) nanofiber scaffolds, B) Tissue culture plate as control, magnification: 200

Cell infiltration

Figure 5 shows the cross-section of scaffold seeded with Vero cells after 7 days. The cells can not penetrate into the scaffold.

MTT assay

MTT assay was used to compare the number of cells in the nanofiber scaffold and tissue culture plate (TCP). The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells. Figure 6 shows the viability graph of the Vero cells cultured on the nanofiber scaffold and tissue culture plate (TCP) as control.

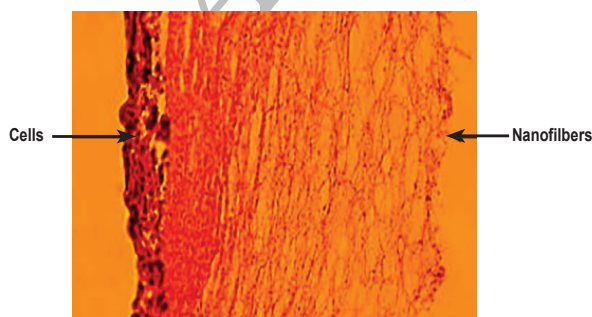


Fig 5: Light micrograph of Cross-section of PCL nanofiber scaffold cultured with Vero Cells 7 days after cell seeding, magnification: 200

Statistical analysis of data showed that the difference between viable cells on the tissue culture plate (TCP) and nanofiber scaffold is not significant.

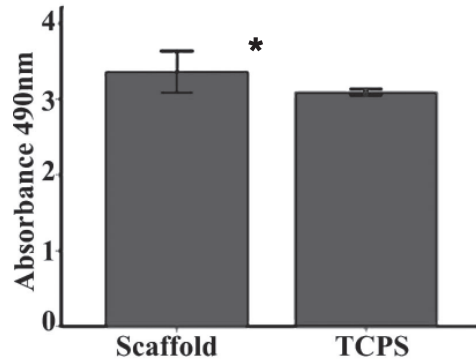


Fig 6: MTT results of Vero cells on the nanofiber scaffolds TCP as control (*: $p < 0.05$)

Discussion

Nanofibrous scaffolds have many advantages that make them well suited for tissue engineering applications. Besides mimicking the architecture of natural extracellular matrix, higher surface area to volume ratio of nanofibers leads to more cellular attachment in comparison to larger fibers. In the present study, a nanofibrous matrix of PCL was produced via electrospinning. Electrospinning has attracted great interest due to its simplicity and effectiveness in producing nanofibers. In addition, in this method the fiber diameter and alignment and the thickness of scaffold are controllable. It is predicted that cell proliferation on the nanofibers scaffolds must be more than that on the tissue culture plate due to nanoscale dimension and 3-dimension structure of nanofibrous scaffolds. But the results of MTT assay showed that the difference between proliferation of cells in tissue culture plate and nanofibrous scaffold is not statistically significant. Plasma treatment generally is done on the tissue culture plate (TCP) to create a more hydrophilic surface. In contrast, PCL nanofiber mat is hydrophobic with a water contact angle of 118°. Previous literature suggests that cellular adhesion improves with hydrophilicity (22), with regards to the improvement of cellular behavior on the hydrophilic surface, it is not surprising that the cell proliferation on the tissue culture plate (TCP) is comparable to nanofibrous scaffolds. The results of SEM images of Vero cells on the nanofibers scaffold and hematoxylin staining showed that cells tend to spread on the nanofibers scaffold and nanofibers scaffold supports cell adhesion and proliferation as well as tissue culture plate. However, as it is observed from the sectioning of cellular scaffolds, the Vero cells do not penetrate into scaffolds. Similar results were obtained with other cells such as CHO, Fibroblast and P19 (data not shown). A value of 10µm has been suggested as a necessary diameter for cellular infiltration into scaffolds (23) but the pore diameter of PCL nanofibrous scaffold is smaller. There-

fore, the cultured cells have difficulty penetrating into the scaffold. The results of other researches show that the cells can not penetrate onto the nanofiber scaffold due to small size of the pores of nanofiber scaffold (23-27). To overcome this problem, methods such as cell electrospinning and fiber leaching have been proposed (24, 25).

Therefore it is concluded that for applications that cells must remain on the surface of nanofibers mat, the prepared PCL nanofibers mat is a suitable candidate. For example, in tissue engineering vascular grafts, thrombus formation is a major problem. One of the effective approaches to prevent thrombus includes endothelial cells seeding onto synthetic materials to render the surface anti-thrombogenic because endothelial cells can release factors to control thrombogenesis or fibrinolysis (24). Therefore, it seems that in this case penetration of endothelial cells into the scaffolds is unnecessary. The results of SEM micrographs, MTT assay and hematoxylin staining suggest that electrospun PCL nanofibrous scaffold is a promising candidate for tissue engineering application. Previous works showed that PCL is capable to support a wide variety of cell types such as muscle cells, Mesenchymal stem cell, glia, chondrocyte, etc (17-21) and the results of this study confirm the suitability of PCL nanofiber scaffolds for tissue engineering application.

Conclusion

In this study, electrospinning was used for the production of PCL nanofibrous scaffold. PCL nanofiber scaffold was used for the Vero cell culture. The results of the MTT assay, SEM images and hematoxylin staining showed that electrospun PCL nanofibrous scaffold is suitable for Vero cell culture, but the drawback of nanofibrous scaffold is the lack of cell infiltration into the scaffolds. It is due to small pore diameter of nanofibrous scaffold. Current work is in progress to develop a technique in electrospinning for optimizing the pore size of nanofiber mat for tissue engineering application.

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