Biological behavior study of gelatin coated PCL nanofiberous electrospun scaffolds using fibroblasts

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ABSTRACT

Scaffold design has pivotal role in tissue engineering. In the present study, We modified the surface of electrospun poly(caprolactone) (PCL) nanofibers to improve their compatibility with living medium and to show the potential application of PCL nanofibers as a artificial extracellular matrix using in tissue-engineering. PCL nanofibers were fabricated by electrospinning method. To graft gelatin on the nanofiber surface, PCL scaffolds were first treated with air plasma to introduce carboxyl groups on the surface, followed by covalent grafting of gelatin molecules. The hydrophilicity of the electrospun PCL nanofibers was significantly increased by the gas plasma treatment, as confirmed by contact angle measurements. ATR-FTIR analysis demonstrated that the chemical composition of the PCL nanofiber surface was influenced by the gelatin coating, resulting in an increase in the number of amine groups. Our results show that the modified PCL nanofibers are suitable physical properties as polymeric artificial scaffold in tissue engineering application.

Keywords: Tissue engineering; Nanofibers; PCL; Biological behavior; Scaffolds

INTRODUCTION

Extracellular matrix (ECM) plays a fundamental role in controlling cell. Tissue engineering methods consist of three main components including scaffolds, cells and biomolecules. Nanofibrous scaffolds are a good candidate for treatments based on tissue engineering because of its suitable environment for cell attachment, and proliferation due to similarity to physical properties of natural ECM [1, 2]. With increasing interest in nanotechnology, development of nanofiberous scaffolds preparing by the technique of electrospinning is having a new momentum. Electrospinning is a very simple and inexpensive method which allows producing fibers with diameters varying from 3 nm to greater than 5 m [3].

Various natural and synthetic polymers can be used for nanofibers manufacturing, resulting in various degradation rates based on polymer choice. Synthetic polymers, such as polyethersulfone (PES), polylactic acid (PLA), and polycaprolactone (PCL), generally have slower degradation rates than natural compounds like collagen, gelatin and chitin [3, 4].

PCL is a semi crystalline linear hydrophobic polymer, FDA approved, and has a long history of safe use in humans. Though the electrospun PCL fibers mimic the identity of ECM in living tissues, its poor hydrophilicity caused a reduction in the ability of cell adhesion, proliferation, and differentiation [5]. Scaffolds coating is one of the most effective methods for providing new, desirable scaffolds for particular applications. For example, coating synthetic scaffolds with a natural polymer improves cell adhesion and the degradation rate of the system and can be modified depending on its application [6]. Gelatin is a natural biopolymer derived from collagen which is biodegradable, biocompatible and has been widely used in the pharmaceutical and medical fields [6, 7]. Therefore, gelatin can be coated on PCL nanofibers to obtain a scaffold with desired cell adhesion and degradation properties.

In this study, nanofibers prepared by electrospinning method and then were treated with plasma in the presence of oxygen gas and

coated with gelatin. Changes in the surface characteristics of plasma-treated PCL nanofibers, including surface hydrophilicity, chemical composition, and morphological changes, were investigated using contact angle method, ATR-FTIR spectroscopy, and mechanical properties and scanning electron microscopy. Biological behavior of this scaffold was investigated using cultured fibroblast cells and its proliferation rate was evaluated via MTT assay.

MATERIALS AND METHODS

Materials

PCL Ultrason E6020P with a weight average molecular weight of 58,000 Da was purchased from BASF (Germany). The solvent N,N-dimethylformamide was obtained from Merck (Germany). All other chemicals were purchased from Sigma (St Louis, Mo., USA) and used as received unless stated otherwise.

The cell culture medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were provided by Invitrogen (Invitrogen Co., Carlsbad, CA, USA). Fibroblast cell line was obtained from cell bank (Stem cell technology research center, Tehran, Iran). 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), was obtained from Roche Diagnostics GmbH (Germany).

Electrospun Nanofibers preparation

PCL nanofibers were produced by an electrospinning method in our laboratory using procedures reported earlier [8]. Briefly, a PCL solution (5% w/w) was prepared in chloroform and dimethylformamide (DMF) in 8.5:1.5 ratios. The polymer solutions fed into a blunted needle attached to a pump on a vertical mount. A rotating cylindrical drum was used as a collector and placed at a distance of 15 cm from the needle. The collecting rate was at 250 rpm. Voltage was 20 kV and flow rate was 0.5 mL/h.

Surface modifications

To increase the hydrophilicity of the nanofibers, surface modification of the PCL scaffold was performed by plasma treatment [8]. A low frequency plasma generator (40 kHz) with a cylindrical quartz reactor (Diener, Electronics, Ebhausen, Germany) was used. The pressure of pure oxygen gas was at a 0.4 mbar, and the glow discharge was ignited for 2.5 minutes. Plasma-treated sheets were punched with a device of 1 cm diameter.

Then to become more similar to natural ECM gelatin was coated on the surface of plasma-treated PCL. For gelatin coaeing, plasma-treated sheets were cut into 1.5-cm diameter punches and immersed in 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (Merck) solution (5mg/mL) for 12 h. After rinsing with distilled water, the scaffolds were immersed in 1mg/mL gelatin I solution (Nutacon BV) overnight. The total of this procedure depicted in figure 1. Air plasma treatment was used to introduce carboxyl groups onto the PCL nanofiber surface, followed by the covalent attachment of gelatin molecules.

Characterization of nanofibers

Morphology study:

The morphology of the PCL nanofibers befor and after plasma treatment and also after cell seeding was studied by scanning electron microscopy (SEM; XL30; Philips, Eindhoven, Netherlands).

Mechanical properties:

The tensile properties were performed on the nanofibrous webs using Galdabini testing equipment. Prepared scaffolds were cut into 10 mm × 60 mm × 0.11 mm specimens and tensile test was conducted at 50 mm/min crosshead speed at room temperature.

Contact angle measurement:

The hydrophilicity of the nanofibrous PCL scaffolds after surface modification was measured by contact angle goniometer (Krüss, Hamburg, Germany), at room temperature. A water droplet was dropped on surface of the nanofibers, and the contact angle was measured after 10 seconds.

ATR-FTIR spectroscopy:

Gelatin coating was investigated by FTIR-ATR. The spectra were recorded using an Equinox 55 spectrometer (Bruker Optics, Germany) equipped with a DTGS detector and a diamond ATR crystal.

Biocompatibility Evaluations

Cell culture:

Prior to cell seeding, scaffolds for sterilization were immersed overnight in the following solutions: (1) 70 % ethanol for sterilization, (2) penicillin, streptomycin, and amphotericin B to prevent from yeast growth, and (3) culture medium to ensure sterilization and enhance cell attachment after seeding.

Fibroblast cells were seeded on the surface of PCL scaffolds with a cells density of 10×10^3

cells per cm^2 and maintained in DMEM culture medium supplemented with 10% FBS, (all from Invitrogen Co., Carlsbad, CA, USA) and then the cells were fixed to SEM analysis after 10 days.

DAPI staining:

Cells were fixed with 4% paraformaldehyde for 20 minute at room temperature and then permeabilized with Triton X-100 (0.3%) for 20 minute. After washing with PBS, cells were incubated with DAPI (4′, 6-diamidino-2-phenylindole; 1:1000) for nuclear staining.

MTT assay:

MTT assay was performed to evaluation of biocompatibility and biological behavior of PCL scaffolds. Thus the proliferation rate of Fibroblast cells on PCL nanofibrous scaffolds was measured by MTT assay. PCL nanofibers after being sterile were placed in a 24-well culture plate and seeded with a cells density of 4×10^3 cells per cm^2 and incubated at 37 °C, 5% CO_2 under DMEM supplemented with 10% FBS, (all from Invitrogen Co., Carlsbad, CA, USA). After 1, 2, 3, 4 and 5 days of cell seeding, 50 µI of MTT solution (5 mg/ml in DMEM) was added to each well. For conversion of MTT to formazan crystals by mitochondrial dehydrogenases of living cells, the plates were incubated at 37 °C for 4 hours. Then, supernatant was removed and constant amount of an appropriate solvent was added. The optical density was read at a wavelength of 570 nm in a micro plate reader (BioTek Instruments, USA). The same procedure was performed for cultured cells in tissue culture polystyrene (TCPS) as control.

Statistical analysis

Each experiment was repeated independently at least three times *in vitro*. All data were reported as the mean ± SD. One-way analysis of variance (ANOVA) was used to compare the results. All analyses were performed by using SPSS 17.0 software (SPSS, Chicago, IL, USA). P-values of less than 0.05 were considered as statistically significant.

RESULTS

Nanofibers characterization

Electrospun nanofibrous PCL scaffolds were porous, beads free and had a uniform and smooth morphology (figure 2a,b). The diameter distribution was measured in the range of 450–800 nm with an average of 565 ± 30 nm. Surface modification of the PES nanofibers was performed by using plasma treatment. A scanning electron micrograph of the scaffolds after 10 days of cell culture is shown in figure 2c. The contact angle of PCL nanofibers decreased from 132° to 0° after surface treatment. The tensile strength of electrospun nanofibrous PCL scaffolds was 3.63 ± 0.55 MPa and elongation at break of 53.65 ± 1.28%.

ATR-FTIR

ATR-FTIR analysis was carried out for surface characterization of PCL and gelatin coated PCL nanofiberous scaffolds. Figure 3 shows the FTIR spectra of PCLand gelatin coated PCL nanofibers. Infrared spectra for PCL-related stretching modes wereobserved for PCL and gelatin coated PCL scaffolds.Theseinclude 2,940 cm^{-1} (asymmetric CH2 stretching), 1,724 cm^{-1} (carbonyl stretching).

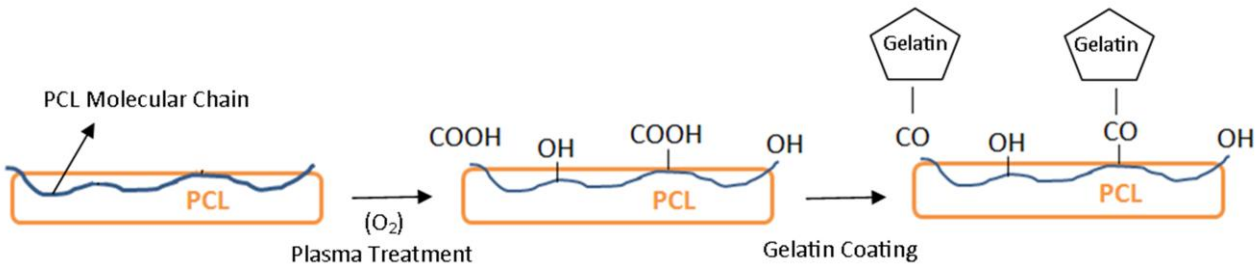

Figure 1. Reaction scheme of the surface modification process of PCL nanofibers. Air plasma treatment was used to introduce carboxyl groups onto the PCL nanofiber surface, followed by the covalent attachment of gelatin molecules.

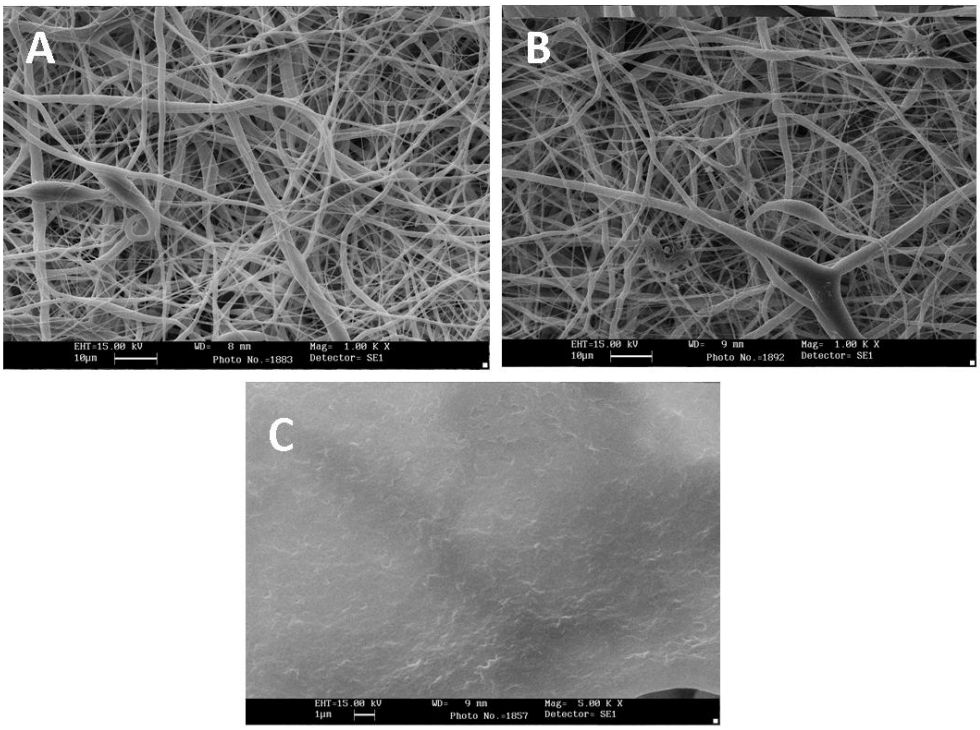

Figure 2. SEM images of nanofiberous PCL scaffolds. Untreated PCL scaffold (A), plasma treated PCL scaffold (B) and treated PCL scaffold with Fibroblast cells after 10 days (c).

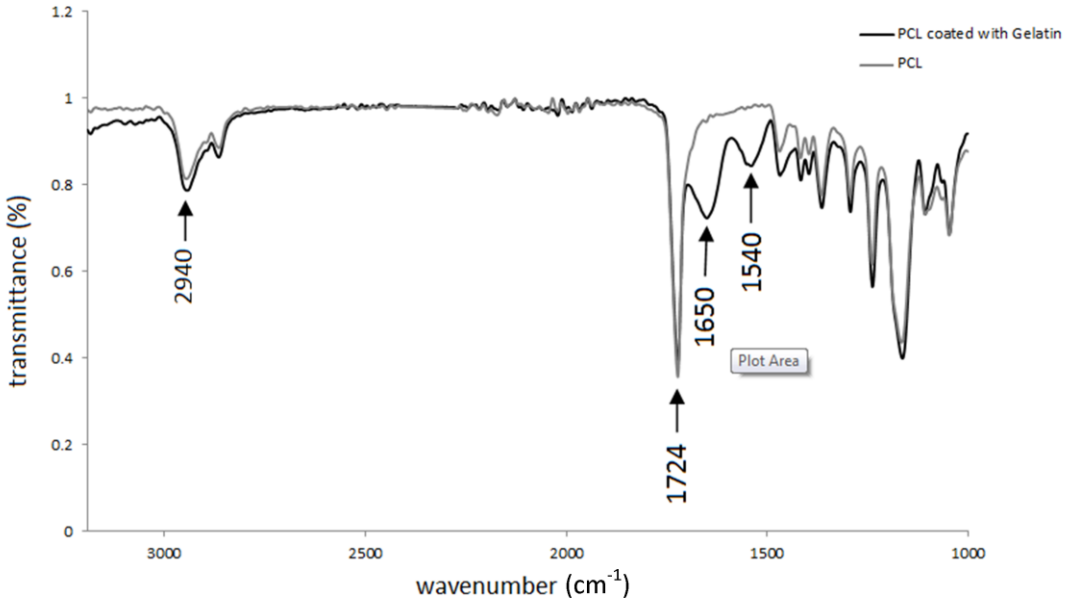

Figure 3. FTIR spectra of PCL and gelatin coated PCL nanofibers.

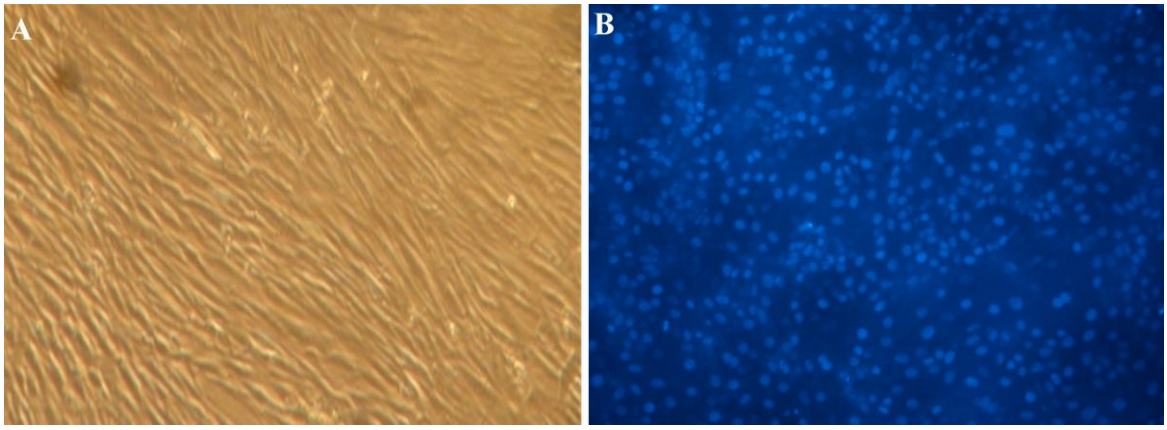

Figure 4. Optical micrographs of human fibroblast cells (a) and nuclei were stained with DAPI in the same cells cultured on the surface of PCL scaffolds (b) with magnification 10X.

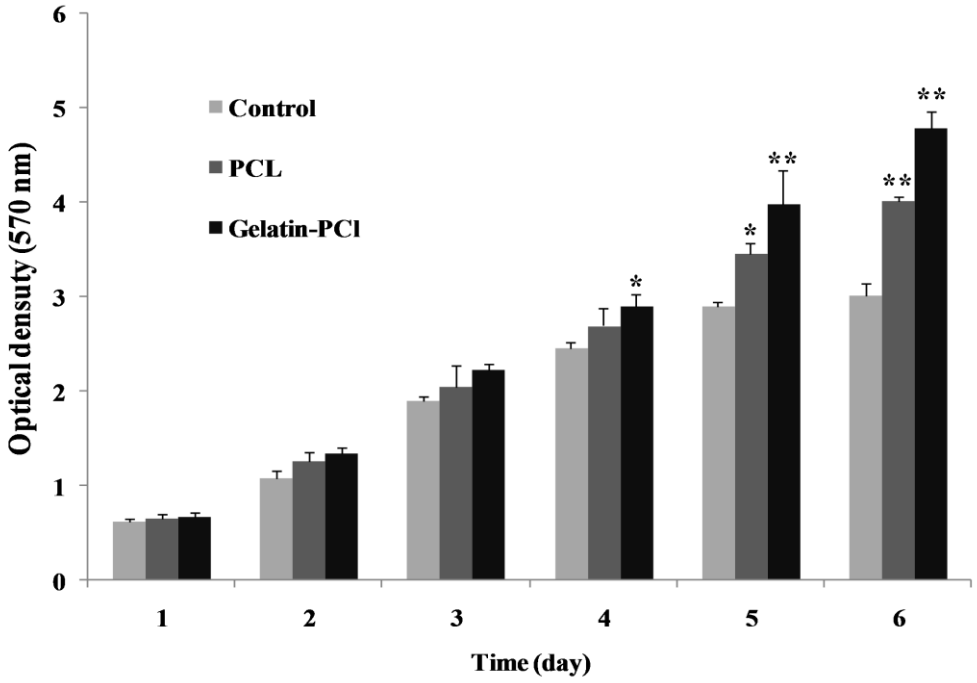

Figure 5. Proliferation rate of human fibroblast cells on nanofiberous PCL scaffolds during a 6 day cell culture period, asterisk shows significant difference with $p < 0.05$, two asterisks shows significant difference with $p < 0.01$.

Commonbands of protein appeared at approximately 1,650 cm^{-1} (amide I) and 1,540 cm^{-1} (amide II), corresponding to thestretching vibrations of C—O bond, and of N–H bond and stretching of C–N bonds, respectively [6]. The amide I band at 1650 cm^{-1} was attributable to both a random coil and α-helix conformation of gelatin [7].

Cell culture

Spindle like morphology of Fibroblast cells showed in Fig. 4a, the appropriate nuclear localization of these markers was confirmed after merging by DAPI (figure 4b).

Biocompatibility

Biocompatibility of the electrospun nanofibrous PCL scaffolds was investigated using MTT assay. Results of MTT assay have demonstrated the viability and proliferation rate of Fibroblast cells increased in PCL nanofibrous scaffold

compare with TCPS. As it shown in figure 5, also, significant differences were observed in proliferation rate of cells in surface modified PCL scaffolds compare with untreated PCL scaffolds.

DISSCUSSION

Many studies have been reported the important role of artificial scaffolds in tissue engineering to improvement of many disease treatments [9-11]. Nanofibers are increasingly being used for tissue engineering and have advantages over traditional scaffolds because of increased surface area-to-volume ratio, which increases cell to scaffold interactions [1, 12]. Nanofibrous PCL scaffolds, because of their native tissue like properties are widely used as the cell delivery carriers and supporting matrices for various tissue regeneration [13, 14].

Cell affinity towards synthetic polymers is generally poor as a consequence of their low hydrophilicity and lack of surface cell recognition sites. Improving the hydrophilic property and incorporation of cell-recognition domains such as ECM bioactive proteins like gelatin onto nanofibers are carried out to enhance cell–scaffold interactions [15, 16].

The results of contact angle measurement of PCL nanofibrous scaffolds showed the contact angle of 132° indicating that PCL nanofibrous scaffolds are hydrophobic. Generally, the hydrophilic/ hydrophobic characteristic of scaffold is important in tissue culture and can influence the initial cell adhesion and cell migration to a higher extent. According to previous literatures, hydrophobic surfaces lead to lower cell adhesion in the initial step of cell culture [17, 18]. PCL is normally treated by oxygenated gas plasma to create a more hydrophilic oxidized polymer surface and provides a surface chemistry that absorbs sufficient amounts of trace ECM proteins from serum-supplemented media to promote cell attachment.

The incorporation of gelatin as well as plasma treatment improves the hydrophilicity of PCL nanofibrous scaffolds. It was due to the lack of amine and carboxyl functional groups of gelatin in pristine PCL structure. The appearance of amide group in the FTIR spectra of gelatin coated PCL nanofibrous scaffolds indicates that the PCL chains were chemically bonded to gelatin molecules and it leads to the introduction of functional groups such as NH_2 and COOH on the surface of gelatin coated PCL scaffold [15, 16, 18].

In recent years, several approaches to modify surface of scaffolds with ECM proteins were reported. Van-Dijk et al. showed that use of laminin as ECM molecules during cell culture lead to higher rate of cell functions [19]. Wei et al. fabricated collagen coated biodegradable polymer nanofiber mesh and study its potential for endothelial cells growth (20). Salvey et al. and cronin et al. used extracellular matrix proteins such as collagen IV, fibronectin, gelatin and laminin coated scaffolds for the development of tissue engineering cell transplantation applications (21, 22).

CONCLUSION

In this study PCL nanofiberous scaffolds were prepared by elecrospinning technique. To improve mimicking ECM surface, scaffolds were modified by plasma treatment and coating gelatin on them surface. This proper supporting matrix can hold cells initially and further provide support for cell survival and functioning. Study of biological behavior of fibroblasts indicates that gelatin coated PCL scaffolds are a good candidate for using as artificial scaffolds in tissue engineering applications.

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