Biomaterials 29 (2008) 4532–4539

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Electrospun poly(ε-caprolactone)/gelatin nanofibrous scaffolds for nerve tissue engineering

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ARTICLE INFO

Article history:
Received 21 June 2008
Accepted 4 August 2008
Available online 30 August 2008

Keywords:
Nerve tissue engineering
Electrospinning
Nanofiber
PCL
Gelatin

ABSTRACT

Nerve tissue engineering is one of the most promising methods to restore nerve systems in human health care. Scaffold design has pivotal role in nerve tissue engineering. Polymer blending is one of the most effective methods for providing new, desirable biocomposites for tissue-engineering applications. Random and aligned PCL/gelatin biocomposite scaffolds were fabricated by varying the ratios of PCL and gelatin concentrations. Chemical and mechanical properties of PCL/gelatin nanofibrous scaffolds were measured by FTIR, porometry, contact angle and tensile measurements, while the *in vitro* biodegradability of the different nanofibrous scaffolds were evaluated too. PCL/gelatin 70:30 nanofiber was found to exhibit the most balanced properties to meet all the required specifications for nerve tissue and was used for *in vitro* culture of nerve stem cells (C17.2 cells). MTS assay and SEM results showed that the biocomposite of PCL/gelatin 70:30 nanofibrous scaffolds enhanced the nerve differentiation and proliferation compared to PCL nanofibrous scaffolds and acted as a positive cue to support neurite outgrowth. It was found that the direction of nerve cell elongation and neurite outgrowth on aligned nanofibrous scaffolds is parallel to the direction of fibers. PCL/gelatin 70:30 nanofibrous scaffolds proved to be a promising biomaterial suitable for nerve regeneration.

1. Introduction

Recently, tissue engineering provided new medical therapy as an alternative to conventional transplantation methods using polymeric biomaterials with or without living precursor cells. Nerve tissue repair is a precious treatment concept in human health care as it directly impacts on the quality of life [1–3]. The fundamental approach in neural tissue engineering involves the fabrication of polymeric scaffolds with nerve cells to produce a three-dimensional functional tissue suitable for implantation [4]. In living systems, extracellular matrix (ECM) plays a pivotal role in controlling cell behavior [5], while scaffold design plays a pivotal role in tissue engineering. Nanofibrous scaffolds serve as suitable environment for cell attachment, and proliferation due to similarity to physical dimension of natural extracellular matrix [5–8]. With increasing interest in nanotechnology, development of nanofibers by using the technique of electrospinning is having a new momentum [9]. This process involves using an electric field to convert polymer solution or melt into a fiber form [10–15]. Interest towards employing electrospinning for scaffold fabrication is mainly due to the mechanical, biological and kinetic properties of the scaffold being easily manipulated by altering the polymer composition and processing parameters [15]. The orientation of nanofibers is one of the important features of a perfect tissue scaffold, because the fiber orientation greatly influences cell growth and related functions in cells such as nerve, smooth muscle cells, etc. [5,16–20]. An established contact guidance theory illustrates that a cell has the maximum probability of migrating in preferred directions which are associated with chemical, structural and/or mechanical properties of the substratum [5,21]. Therefore, aligned nanofibers could provide better contact guidance effects towards neurite outgrowth [17,19,20].

Polymers can be used as scaffolds to promote cell adhesion, maintenance of differentiated, cell function and direct the growth of cells and help in the function of extracellular matrix. However, cell affinity towards synthetic polymers is generally poor as a consequence of their low hydrophilicity and lack of surface cell-recognition sites [22]. Improving the hydrophilic property and incorporation of cell-recognition domains such as RGD and ECM bioactive proteins onto nanofibers are carried out to enhance

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doi:10.1016/j.biomaterials.2008.08.007

cell–scaffold interactions [23]. Polymer blending is one of the most effective methods for providing new, desirable biocomposite for particular applications [24]. For example, blending synthetic and natural polymers improves cell adhesion and the degradation rate of the blended polymeric system can be modified depending on its application [15,17,22]. Poly(ε-caprolactone) or PCL is a semi crystalline linear hydrophobic polymer. Though the electrospun PCL mats mimic the identity of ECM in living tissues, its poor hydrophilicity caused a reduction in the ability of cell adhesion, migration, proliferation, and differentiation [25,26]. On the other hand, gelatin is a natural biopolymer derived from collagen by controlled hydrolysis. Because of its many merits, such as its biological origin, biodegradability, biocompatibility and commercial availability at relatively low cost, gelatin has been widely used in the pharmaceutical and medical fields [15,23]. Therefore, gelatin can be blended with PCL to obtain a scaffold with desired cell adhesion and degradation properties. In a previous study [15], PCL/gelatin nanofibrous scaffolds were used for dermal reconstitution. However, there are no reports utilizing PCL/gelatin nanofibrous scaffolds for nerve tissue engineering.

This study is aimed at investigating the properties of PCL/gelatin nanofibrous scaffolds by FTIR, porometry, contact angle and tensile measurements. Differentiation and proliferation of C17.2 cells on aligned and random PCL and PCL/gelatin nanofibrous scaffolds were also studied for understanding the effect of nanofiber alignment and gelatin incorporation towards nerve tissue engineering.

2. Materials and methods

2.1. Materials

PCL ($M_w = 80{,}000$), gelatin type A from porcine skin and hexafluoro-2-propanol (HFP) were all obtained from Sigma–Aldrich (St. Louis, MO). Hexamethyldisilazane (HMDS) was purchased from Fluka. Dulbecco Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin–EDTA and horse serum were purchased from Gibco, Singapore. CellTiter 96® AQueous One solution reagent (MTS), used in the cell-adhesion assay, was purchased from Promega.

2.2. Fabrication of aligned and random nanofiber scaffold

The polymer solution with concentration of 6 wt% was prepared by dissolving PCL and gelatin with a weight ratio of 50:50 and 70:30 in HFP and stirred for 24 h at room temperature. The solution was electrospun from a 5 ml syringe with a needle diameter of 0.4 mm and mass flow rate of 1 ml/h. A high voltage (12 kV) was applied to the tip of the needle attached to the syringe when a fluid jet was ejected. For collecting aligned nanofibers, a rotating disk was used, whereas a flat aluminum plate was used for collecting random nanofibers. The linear rate of the rotating disk was set to 1000 rpm. The resulting fibers were collected on 15-mm cover slips placed on respective collectors.

2.3. Characterization of scaffolds

The morphology of nanofibrous scaffolds was studied by scanning electron microscopy (SEM) (JSM 5600, JEOL, Japan) at an accelerating voltage of 15 kV. Before observation, the scaffolds were coated with gold using a sputter coater (JEOL JFC-1200 fine coater, Japan). The diameter of the fiber was measured from the SEM micrographs using image analysis software (Image J, National Institutes of Health, USA).

For determination of wettability (or hydrophilicity) of scaffolds, the contact angle of electrospun nonwoven materials was measured by a video contact angle system (VCA Optima, AST Products). The droplet size was set at 0.5 μl. Five samples were used for each test. The average value was reported with standard deviation (±SD).

Assessment of structural pore properties was done by Capillary Flow Porometry (1200-AEHXL capillary flow porometer, Porous Media Inc., Ithaca, NY). Capillary flow porometry provides a simple and non-destructive technique that allows rapid and accurate measurement of pore size and distribution. In a capillary flow porometry measurement, a non-reacting gas flows through a dry sample and then through the same sample after it has been wet with a liquid of known surface tension. The change in flow rate is measured as a function of pressure for both dry and wet processes. Because of the low pressure applied during the process, the porous structure of nanofiber membranes is not distorted [27]. The pore size was calculated by the software from Porous Media Inc (Ithaca, NY) using the equation:

$$D = \frac{4\gamma \cos\theta}{\Delta p} \quad (1)$$

Where D is the pore diameter, γ the surface tension of the wetting liquid, θ the contact angle of the wetting liquid, and Δp is the differential pressure.

Mechanical properties of different scaffolds were determined using a tabletop uniaxial testing machine (INSTRON 3345) using a 10-N load cell under a cross-head speed of 10 mm/min at ambient conditions. All samples were prepared in the form of rectangular shape with dimensions of 50×10 mm^2 from the electrospun fibrous membranes. At least five samples were tested for each type of electrospun fibrous membrane.

Chemical analysis of PCL and PCL/gelatin nanofibrous scaffolds were performed by ATR-FTIR spectroscopy over a range of 4000–400 cm^{-1}. ATR-FTIR spectra of PCL and PCL/gelatin nanofibrous scaffolds were obtained on a Nicolet spectrometer system with an Avatar Omni Sampler accessory.

Electrospun nanofibrous scaffolds were placed in 24-well plate containing 1 ml of a phosphate buffer solution (PBS; pH 7.4) in each well and were incubated *in vitro* at 37 °C for different periods of time (3, 7, 10, 14 days). After each degradation period, the samples were washed and subsequently dried in a vacuum oven at room temperature for 24 h. SEM of scaffolds were performed to understand the change in nanofibers morphology during this period.

2.4. Cell culture study

The nanofibrous scaffolds were exposed to UV radiation for 2 h, washed 3 times with PBS for 20 min each and incubated with DMEM/F12 1:1 mixture containing N2 supplement for 24 h before cell seeding. Neonatal mouse cerebellum C17.2 stem cells were cultured in DMEM supplemented with 10% FBS, 5% horse serum and 1% penicillin/streptomycin. After reaching 70% confluency, the cells were detached by trypsin–EDTA and viable cells were counted by trypan blue assay. Cells were further seeded onto nanofibrous scaffolds, placed in a 24-well plate and tissue culture polystyrene (TCP as control) at a density of 10×10^3 cells/well and cultured with DMEM/F12 1:1 mixture containing N2 supplement at 37 °C, 5% CO_2 and 95% humidity.

The morphology of C17.2 cells on PCL and PCL/gelatin nanofibrous scaffolds was observed by SEM. After 6 days of cell seeding, samples were fixed with 3% glutaraldehyde for 2 h. Specimens were rinsed in water and dehydrated with graded concentrations (50, 70, 90, 100% v/v) of ethanol. Subsequently the samples were treated with HMDS and kept in a fume hood for air drying. Finally the samples were coated with gold for the observation of cell morphology.

To study the cell proliferation on different substrates, viable cells were determined by using the colorimetric MTS assay. After 2, 4 and 6 days of cell seeding in 24-well plate, cells were washed with PBS and incubated with 20% of MTS reagent containing serum free medium. After 3 h of incubation at 37 °C in 5% CO_2, aliquots were pipetted into a 96-well plate. The absorbance of the content of each well was measured at 492 nm using a spectrophotometric plate reader (Fluostar Optima, BMG Lab Technologies, Germany).

2.5. Statistical analysis

All data presented are expressed as mean ± standard deviation (SD). Statistical analysis was carried out using single-factor analysis of variance (ANOVA). A value of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Morphology and properties of PCL and PCL/gelatin electrospun nanofibrous scaffolds

Fig. 1 shows the SEM micrographs of random and aligned electrospun PCL and PCL/gelatin 50:50 and 70:30, along with their diameter distribution. As can be seen from Fig. 1, by decreasing the gelatin content, a greater degree of alignment can be obtained. In the production of aligned nanofibers using electrospinning method, the polymer solution is continuously extruded from the tip of the needle while drawing the polymer solution towards the collector continues under the influence of the electric field. If the electrospinning jet is not interrupted, a long continuous fiber strand is produced as the solvent evaporates and higher alignment can be obtained [28]. A longer continuous fiber strand can be electrospun as the gelatin content is decreased. With increasing gelatin content, the probability of fiber breakage increases due to the decrease in solution viscosity (data not shown) and, therefore, the fiber alignment also decreased. Moreover, gelatin concentrations of more than 30% in the blend were found to be