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Bio-functionalized PCL nanofibrous scaffolds for nerve tissue engineering

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ABSTRACT

Surface properties of scaffolds such as hydrophilicity and the presence of functional groups on the surface of scaffolds play a key role in cell adhesion, proliferation and migration. Different modification methods for hydrophilicity improvement and introduction of functional groups on the surface of scaffolds have been carried out on synthetic biodegradable polymers, for tissue engineering applications. In this study, alkaline hydrolysis of poly (ε-caprolactone) (PCL) nanofibrous scaffolds was carried out for different time periods (1 h, 4 h and 12 h) to increase the hydrophilicity of the scaffolds. The formation of reactive groups resulting from alkaline hydrolysis provides opportunities for further surface functionalization of PCL nanofibrous scaffolds. Matrigel was attached covalently on the surface of an optimized 4 h hydrolyzed PCL nanofibrous scaffolds and additionally the fabrication of blended PCL/matrigel nanofibrous scaffolds was carried out. Chemical and mechanical characterization of nanofibrous scaffolds were evaluated using attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy, contact angle, scanning electron microscopy (SEM) and tensile measurement. *In vitro* cell adhesion and proliferation study was carried out after seeding nerve precursor cells (NPCs) on different scaffolds. Results of cell proliferation assay and SEM studies showed that the covalently functionalized PCL/matrigel nanofibrous scaffolds promote the proliferation and neurite outgrowth of NPCs compared to PCL and hydrolyzed PCL nanofibrous scaffolds, providing suitable substrates for nerve tissue engineering.

1. Introduction

Nerve tissue engineering is a rapidly expanding area of research, providing new and promising approach to nerve repair and regeneration [1]. Design and fabrication of scaffolds suitable for neural tissue engineering is critical for the success of tissue regeneration [2]. Scaffold surfaces play a vital role for the success of tissue engineering as the interaction between cells and scaffolds occur at the surface of the scaffolds [3]. Synthetic biodegradable polymers are now widely used in tissue engineering due to their mechanical properties along with adjustable degradation rates [4]. However, cell affinity towards synthetic hydrophobic polymers is poor as a consequence of their low hydrophilicity and lack of surface cell recognition sites [5]. Recently nano-structured materials are receiving considerable attention for application in tissue engineering, essentially because the body consists of nanoscale structures of extra cellular matrix (ECM) providing a natural web of intricate nanofibers supporting cells and provides an instructive background guiding their behavior [6]. Therefore improving the hydrophilicity of nano-structured scaffolds together with the incorporation of biological molecules on to the nanofibrous scaffolds could provide both physical and chemical signals required for cell growth.

Poly (ε-caprolactone) (PCL) has been used for the reconstruction of various tissues such as bone, skin, nerve, retina and has several advantages including its biocompatibility, low cost and easy processability. However, being a synthetic biomaterial, PCL has a hydrophobic surface, lacks functional groups and hence it is not a good substrate for cell adhesion. To improve the hydrophilicity and biological properties of PCL nanofibrous scaffolds various techniques have been applied. Previous studies mostly modified the PCL nanofibrous scaffolds by incorporation of biologically active polymers such as gelatin and collagen or the hydrophilicity of PCL scaffolds was improved by methods such as laser and plasma treatment [1,7–13]. However, there are very few reports available on the functionalization of PCL scaffolds using biomolecules for tissue engineering [10–13]. Previous studies have shown that laminin, collagen IV, fibronectin and heparin sulphate proteoglycans support neurite outgrowth [14].

Our study is aimed at investigating a simple procedure such as alkaline hydrolysis of PCL nanofibrous scaffolds for improving its hydrophilicity and further attaching matrigel on them, to produce

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scaffolds suitable for nerve tissue engineering. Matrigel is a soluble and sterile extract of basement membrane proteins derived from the Engelberth–Holm–Swarm (EHS) mouse sarcoma and it is rich in extracellular proteins such as laminin, collagen IV, fibronectin and heparin sulphate proteoglycans [14–17]. Matrigel being an important component of the nervous system, functionalization of matrigel on the surface of an already modified PCL nanofibrous scaffold was rapidly achievable. For comparative evaluation studies, matrigel was also mixed with PCL solution and the electrospun 'blended PCL/matrigel' nanofibrous scaffolds were fabricated. The effect of incorporation of matrigel into PCL nanofibrous scaffolds towards the proliferation and differentiation of NPCs was further investigated.

2. Materials and methods

2.1. Materials

PCL (Mw 80,000), 2-(N-morpholino)ethanesulfonic acid (MES), sodium hydroxide (NaOH), hydrochloric acid (HCL) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were obtained from Sigma-Aldrich (St. Louis, MO). Dimethyl formamide (DMF) and methylene chloride (MC) were purchased from Merck (Singapore) and hexamethyldisilazane (HMDS) was purchased from Fluka (USA). Dulbecco Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin-EDTA and horse serum (HS) were purchased from Gibco (Singapore). CellTiter 96® AQueous One solution reagent (MTS) used for cell viability measurement was purchased from Promega (Singapore) and matrigel was obtained from BD Biosciences (Singapore).

2.2. Fabrication of PCL nanofibrous scaffolds

PCL (11% w/v) was dissolved in MC/DMF at a ratio of 80:20 and stirred for a period of 24 h at room temperature. The solution was electrospun from a 5 mL syringe with needle diameter of 0.4 mm and mass flow rate of 1 mL/h. A high voltage (13 kV) was applied to the tip of the needle attached to the syringe. The resulting fibers were collected on 15 mm cover slips placed on a flat aluminum plate collector kept at a distance of 12 cm from the needle tip.

2.3. Modification of PCL nanofibrous scaffolds by alkaline hydrolysis

Electrospun PCL nanofibrous scaffolds were immersed in aqueous sodium hydroxide solution of 5% (w/v) for varying periods (1 h, 4 h and 12 h) at room temperature. After hydrolysis, the scaffolds were washed with de-ionized (DI) water repeatedly. For complete neutralization of NaOH, the samples were treated with 1 N HCL for 10 min at room temperature, washed with DI water and subsequently dried in a vacuum oven at room temperature for 24 h. The "Hydrolyzed PCL nanofibers" is termed as H-PCL, hereafter.

2.4. Modification of PCL nanofibrous scaffolds by matrigel

Matrigel was used for the modification of PCL nanofibrous scaffolds by two different methods: (i) matrigel was blended with PCL solution and the resultant solution was electrospun to obtain '*blended* PCL/matrigel' nanofibrous scaffolds termed as B-PCL and (ii) matrigel was covalently attached on the surface of H-PCL nanofibrous scaffolds to obtain '*functionalized* PCL/matrigel' scaffolds termed as F-PCL.

2.4.1. Electrospinning of 'blended PCL/matrigel' nanofibrous scaffolds

Electrospun PCL/matrigel nanofibers were fabricated by blending PCL solution with matrigel at a ratio of 1000:1 (wt/wt). In short, matrigel was thawed at 4 ° C, mixed with PCL solution and stirred for 24 h at room temperature. The solution was electrospun at a high voltage of 13 kV and nanofibers were collected.

2.4.2. Covalent attachment of matrigel on hydrolyzed PCL to obtain 'functionalized PCL/matrigel' scaffolds

Hydrolyzed PCL (H-PCL) was used for the covalent attachment of matrigel on their surface. For covalent attachment of matrigel on PCL nanofibers, the H-PCL scaffolds were immersed in 2-(N-morpholino) ethanesulfonic acid (MES) buffer solution (0.1 M, pH 5.0) of 5 mg/mL of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) for 1 h at room temperature. Scaffolds were further rinsed with DI water and incubated with 20 μL of matrigel for 24 h and subsequently washed with PBS. Fig. 1 shows schematic representation of covalent attachment of matrigel on the surface of PCL nanofibrous scaffolds.

2.5. Characterization of nanofibrous scaffolds

The morphology of electrospun PCL, H-PCL, B-PCL and F-PCL nanofibers 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 fibers 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 nanofibers were 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 and the average value was reported with standard deviation (±SD).

ATR-FTIR is a powerful technique to understand the surface chemistry of a modified surface. ATR-FTIR spectroscopy of PCL, H-PCL, B-PCL and F-PCL nanofibrous scaffolds were performed over a range of 4000–400 cm^{-1} at a resolution of 2 cm^{-1} using a Nicolet spectrometer system.

Mechanical properties of different scaffolds were determined using a table-top uniaxial testing machine (INSTRON 3345) using a 10-N load cell at a cross-head speed of 10 mm/min at ambient conditions. All samples were prepared in rectangular shapes with dimensions of 20×10 mm from the electrospun membranes. At least six samples were tested for each type of electrospun nanofibrous membrane and the average value was reported with standard deviation (±SD).

2.6. In vitro cell culture study

2.6.1. Neural precursor cell culture and seeding

In vitro cell culture studies were carried out using Neonatal mouse cerebellum C17.2 stem cells. These cells can be used as neuron precursors since they are involved in the normal development of cerebellum, embryonic neocortex and other structures upon implantation into mouse germinal zones [18]. Neural precursor cells (NPCs) were cultured in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5% horse serum (HS) and 1% penicillin/streptomycin. After reaching 70% confluency, the cells were detached by trypsin/EDTA and viable cells were counted on a hemocytometer by trypan blue assay. Electrospun nanofibrous scaffolds were exposed to UV radiation for 2 h, washed 3 times with PBS and incubated with DMEM/F12 (1:1) mixture containing N2 supplement for 24 h before cell seeding. Cells were further seeded on nanofibrous scaffolds placed in a 24-well plate at a density of 15×10^3 cells/well and cultured with DMEM/F12 (1:1) mixture containing 1% N2 supplement at 37 °C, 5% CO_2 and 95% humidity incubation conditions. Tissue cultured polystyrene cover glass (TCP) was used as the control.

2.6.2. Cell proliferation study

To study the cell proliferation on different substrates, cell proliferation was determined by the colorimetric MTS assay. MTS assay is based on the reduction of yellow tetrazolium salt in MTS to