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Multi-layered functional membranes for periodontal regeneration: Preparation and characterization

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ABSTRACT

Multi-layered biodegradable membranes (MLMs) were fabricated by combining electrospinning and solvent casting/particulate leaching methods. MLMs possess three layers of different functional properties; poly (caprolactone) (PCL)/nano-hydroxyapatite core layer and PCL/collagen, PCL/collagen-bone morphogenic protein 7 (BMP 7) outer layers on each side of the core layer. MC3T3-E1 cell culture tests showed that osteoblastic differentiation was enhanced on PCL/collagen BMP 7 layer. This facile method presents great potential for fabrication of multi-functional barrier membranes for periodontal regeneration as well as scaffolds possessing different properties to mimic complex extra cellular matrix structures with stable integrity.

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1. Introduction

Periodontitis is a major inflammatory disease that can lead to the destruction of the supporting tissues around teeth, which may eventually result in tooth loss [1]. Regenerative approaches use barrier membranes to repair the damaged periodontal tissue by repopulation of selective cell. These barrier membranes are designed as an interface-implant to enhance bone growth while preventing the connective tissue/epithelium infiltration into the defected site [1,2]. For this purpose, various functionally graded multi-layered membranes with tailored properties have recently been reported for periodontal regeneration [2,3].

Poly (caprolactone) (PCL) is a degradable polyester and used for several tissue engineering applications including bone repair [4]. Bioceramics and growth factors are often incorporated in PCL structure for increased biocompatibility and bioactivity. Among natural polymers, collagen is the most widely used material to mimic the natural extracellular matrix [5]. Hydroxyapatite is the predominant mineral phase in bone matrix and known as the key component for biointegration as well as mechanical integrity of bone tissue constructs [6]. Moreover, bone morphogenic proteins (BMPs) are reported to induce bone formation and osteoblastic differentiation. Among these, BMP 7 is the most widely used growth factor which is approved by FDA [7]. Augmentation of alveolar bone with grafting materials including BMPs, has been reported as an alternative approach in implant dentistry applications, as well [8].

In this study, it is aimed to fabricate multi-layered membranes with various structures and functions using a novel approach by combining electrospinning and solvent casting/particulate leaching methods. Structural and mechanical properties and osteoblastic cell differentiaton were tested.

2. Materials and methods

2.1. Materials

PCL (Mn: 70,000–90,000) and collagen (Type I from calf skin) were purchased from Sigma Aldrich. Recombinant Human BMP 7 was obtained from Millipore. All other chemicals were purchased from Sigma Aldrich and used without further purification.

2.2. Manufacturing of multi-layered membranes (MLMs)

MLMs consisted of a micro-porous core layer surrounded by two polymer/growth factor composite layers. The multi-layered scaffold fabrication approach is shown in Fig. 1.

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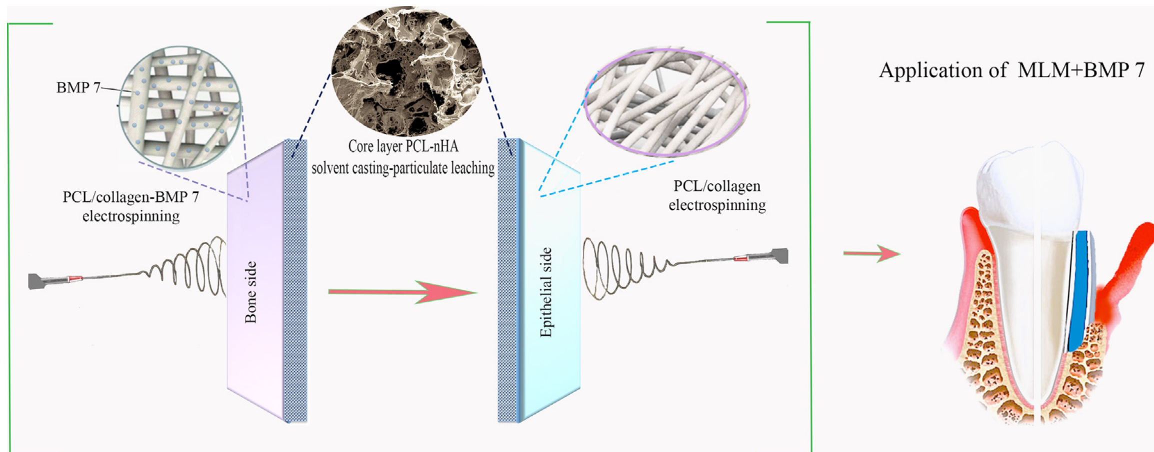

Fig. 1. Schematic illustration of the fabrication and application of multi-layered PCL based functional membranes (MLM+BMP 7: multi-layered membrane with BMP 7 incorporation).

The core layer composed of PCL-nHA was produced by solvent casting/particulate leaching technique. nHA particles were mixed (1%, w/v) with PCL solution (10% w/v in dichloromethane). NaCl particles (355–600 μm in size) were added to the mixture (PCL: NaCl=1:8, w/w) to generate a controlled porosity in the core layer. The mixture was cast into disc-shaped Teflon® molds (12 mm diameter). After evaporation of the solvent, the cylindrical structures were washed in excess distilled water to leach out NaCl and obtain the micro-porous core layer. The outer layers were produced by sequential electrospinning of PCL/collagen or PCL/collagen-BMP 7 solutions on either side of the core structure. Briefly, the PCL-nHA core was mounted on the collector and PCL/collagen solution (PCL: collagen=5.5:2.5, w/w in hexafluoroisopropanol) was electrospun with a 20 kV voltage and a 3 mL/h flow rate from 10 cm distance. Sequential electrospinning of PCL/collagen-BMP 7 was realized on the opposite side of the structure. Fifty μL of BMP 7 solution (1:20, w/v in ultra pure water) was added to 1 mL of PCL/collagen solution just prior to electrospinning. The MLM comprised three layers in the order: PCL/collagen electrospun layer followed by PCL-nHA micro-porous core layer and finally PCL/collagen-BMP 7 electrospun bioactive layer. MLMs without BMP 7 was prepared for comparison.

2.3. Characterizations

2.3.1. Scanning electron microscopy (SEM)

The structure of MLMs was characterized by a Quanta 400F Field Emission SEM for fibrous morphology of electrospun outer layers (PCL/collagen and PCL/collagen BMP 7) and micro-porous morphology of core layer (PCL-nHA). Samples were sputter coated with gold-palladium before imaging. Top view and cross sectional views were observed for outer layers and core layer, respectively. The average fiber diameter for outer layers and pore diameter for core layer were determined for each layer from SEM images and analyzed by Image J software.

2.3.2. Mechanical properties

Tensile strength and the elongating rate at break of the dry MLMs were tested at room temperature with the Zwick universal testing machine analyzer (Zwick/Roell Z250, Germany). The specimens were rectangular, 8 mm in width and 25 mm in length. The crosshead speed was set as 10 mm/min and applied until ultimate fracture of the specimen. All data were the average of three measured values.

2.3.3. Fourier transform infrared-attenuated total reflectance (FTIR-ATR)

To characterize the surface chemical composition of PCL/collagen outer layer, FTIR-ATR spectra were obtained for the electrospun fibers. The analysis was carried out over a wave number range between 4000 and 525 cm^{-1} at a resolution of 4 cm^{-1} and compared to the spectra obtained for neat PCL electrospun fibers.

2.3.4. Cell proliferation and differentiation

MC3T3-E1 preosteoblast cells were used to test the cellular response on MLMs. The scaffolds were sterilized with γ-irradiation with ^{60}Co source and conditioned in α-MEM containing 10% fetal bovine serum (FBS) for 1 h before cell seeding. Fifty μL cell suspension was injected on each MLM and placed in a humidifed incubator (37°C, 5% CO_2) for 60 min Osteogenic medium (α-MEM+10% (v/v) FBS, 1% (v/v) penicillin-streptomycin, 10 mM β-glycerol phosphate and 50 μg/mL ascorbic acid) was added to each well and refreshed every 3 days. Cell proliferation was quantitatively assessed by MTT up to 21 days. The morphological observation of cultured cells on MLMs was examined by SEM. Cells were fixed in 3% glutaraldehyde solution followed by dehydration in ethanol series. After treatment with HMDS, MLMs were mounted on aluminium stubs, coated with gold palladium and observed under SEM. The gene expressions of β-actin, RunX2, collagen type I (Col1), osteocalcin (OCN) and osteopontin (OPN) of the MC3T3-E1 pre-osteoblastic cells cultured on MLMs were evaluated using Reverse transcription polymerase chain reaction (RT–PCR) (Light cycler Nano, Roche, Switzerland). RT-PCR was performed in two steps by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and Hot FirePol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Estonia). At the first step, cDNA were synthesized with reverse transcription at 25 °C for 10 min, at 40 °C for 120 min and at 85 °C for 5 min At the second step, RT-PCR analysis was carried out with cDNA and forward-reverse primers. Following a RT-PCR initial activation step at 95 °C for 15 min, amplification was completed for 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s and extension at 72 °C for 20 s β-actin was used as housekeeping gene. Primers for each gene are: **β-actin Forward primer** 5′-GTGCTATGTTGCCCTAGAC TTCG-3′ **Reverse primer** 5′-GATGCCACAGGATTCCATACCC-3′ **Col1 Forward primer** 5′-CAAGAT GTGCCACTCTGACT-3′ **Reverse primer** 5′-TCTGACCTGTCTCCATGTTG-3′ **OCN Forward primer** 5′-CTTTCTGCTCACTCTGCTG-3′ **Reverse primer** 5′-TATTGCCCTCCTGCTTGG-3′ **OPN Forward primer** 5′-CACTTTCACTCCAATCGTCCCTAC-3′ **Reverse primer** 5′-ACTCCTTAGACTCACCGCTCTTC-3′ **RunX2 Forward primer** 5′-

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