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Nanostructured poly(ε-caprolactone)–silica xerogel fibrous membrane for guided bone regeneration

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ABSTRACT

A novel fibrous membrane was developed for guided bone regeneration (GBR) through electrospinning a uniform poly(ε-caprolactone) (PCL)–silica hybrid sol. The membrane was composed of fibers with a mean diameter of approximately 400 nm. The hybrid fibers were nano-sized with uniform patterns throughout the fibers, in contrast to the homogeneous structure of pure PCL fibers. The tensile strengths and elastic moduli of the membranes were significantly enhanced with increasing silica content up to 40%. The surfaces of the hybrid membranes were highly hydrophilic with a water contact angle of almost zero. The hybrid membranes possessed excellent in vitro cellular responses in terms of proliferation and differentiation of pre-osteoblast cells. The in vivo animal tests not only confirmed excellent biocompatibility but also revealed bioresorbability of the membranes. These mechanical and biomedical properties make the hybrid membranes very attractive as GBR applications.

1. Introduction

Poly(ε-caprolactone) (PCL) is a widely used biopolymer with good mechanical strength, flexibility, biocompatibility and biodegradability. In addition, good spinability of PCL makes it possible to fabricate them in a fibrous form [1–3]. Fibrous biomaterials are one of the most desirable forms in tissue engineering because of their high porosity, high surface to volume ratio and, more importantly, architectural analogy to the natural extracellular matrix (ECM) [4,5]. Actually, the good biological properties of fibrous materials for guided bone regeneration (GBR) have been demonstrated in many studies using in vitro cellular responses and in vivo tests [6–8]. Fibrous PCL has been fabricated in various forms, as single, dual, hollow and porous types [9–12]. However, limitations arise when applying PCL fibers to bone regeneration because of their low stiffness, hydrophobic nature and relatively low bioactivity [3,13].

The hybridization of PCL with inorganic substances is considered one of the most effective methods of overcoming these weaknesses of PCL [14,15]. The addition of bioactive ceramics improves the hydrophilicity and osteoconductivity of PCL. Furthermore, a uniform distribution of fine ceramics is often very effective in improving the stiffness of PCL [16,17]. Accordingly, significant efforts have been devoted to fabricating PCL-based hybrid fibers by incorporating CaP-based ceramics through electrospinning techniques [18,19]. However, obtaining hybrid fibers with a fine and uniform microstructure is rather difficult. In many cases ceramics such as CaP particles and polymers were hybridized only on the micro-scale with low uniformity, resulting in decreased strength [20]. To improve the uniformity and hybridization scale, preparation and maintenance of nano-sized inorganic substances is a prerequisite. In this sense, a sol–gel process is ideal for the generation of hybrid fibers, in which inorganic substances on the nano atomic or nano scale are formed in situ during the electrospinning.

Silica xerogel, which is produced at room temperature through a sol–gel process, is another strong candidate as an inorganic substance for generating polymer–ceramic hybrid fibers because of its biocompatibility and mesoporous structure [21–23]. In spite of many advantages, only a few reports have studied hybrid fibers of polymer–silica xerogel specifically for use in hard tissue applications. Therefore, the primary objective of this study was to develop a novel PCL–silica xerogel hybrid through electrospinning a silica sol containing dissolved PCL. The utilization of silica xerogel is advantageous for producing uniform nano-structured fibers because of its very fine and homogeneous microstructure [24]. The developed hybrids in the form of membrane were evaluated as potential materials for GBR. The effects of silica xerogel content on the morphology of PCL–silica hybrid fibers were estimated for various silica concentrations up to 60%. The mechanical properties of these fibers were examined using a tensile test. Finally, the in vitro

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cellular behavior and in vivo osteoconductivity of the hybrid fibrous membranes were compared with pure PCL membranes.

2. Materials and methods

2.1. Preparation of hybrid fibers via electrospinning

Unless otherwise specified, all of the reagents were purchased from Sigma–Aldrich Korea (Seoul, Korea). A solution of PCL (8%) was prepared by dissolving 8 g of PCL in 100 ml of 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) in order to obtain a precursor solution for the electrospinning process. The silica sols were synthesized at room temperature using one step acid-catalyzed hydrolysis of tetramethylorthosilane (TMOS). To make the silica sol 500 μl of TMOS, 100 μl of distilled water and 20 μl of 1 M HCl as a catalyst were mixed. After a single phase sol formed through stirring for 1 h, 400 μl of HFP was added to the sol to inhibit aggregation of the silica phase in the PCL solution. Subsequently, a calculated amount of the as prepared silica sol was mixed with a PCL (M_n = 80,000) solution in HFP. The final PCL concentration was fixed at 8% (w/v) for all of the precursors in order to ensure that the rheological properties of the precursor sols were suitable for the electrospinning process. The weight percentages of silica in the precursor sols were 20, 40 and 60 wt.%, based on the assumption that TMOS was completely transformed into silica through hydrolysis.

The precursor sols prepared as above were loaded into a glass syringe with a capacity of 5 ml and a needle diameter of 200 μm and electrospun at an applied voltage of 20 kV and a flow rate of 3.5 ml h^{-1}. After electrospinning the fibrous membrane was carefully removed from the mandrel and stored in a desiccator under vacuum for 2 days to remove any residual HFP. A pure PCL fibrous membrane was fabricated as a control.

2.2. Characterizations

The electrospun fibrous membranes were coated with gold and their morphology observed using field emission scanning electron microscopy (FESEM) (JSM-6330F, JEOL, Japan). The average diameter of the fibers in the membrane was obtained by measuring the diameter of 30 single fibers in the SEM image. The morphology of the fibers was observed more closely using transmission electron microscopy (TEM) (JEM-2000EXII, JEOL, Japan) after the sols were directly electrospun onto a copper grid coated with an amorphous carbon film. Energy dispersive X-ray spectroscopy (EDS) analysis was carried out during the TEM observations to confirm the existence of silica in the hybrid fibers. Additionally, Fourier transform infrared (FTIR) spectroscopy (Micro FT-IR 200, JASCO, Japan) and thermogravimetric analysis (TGA) (Q-5000, TA Instruments, USA) were carried out to determine the phase composition of the electrospun fibers. The wettability of the membranes was examined by measuring the contact angle of water droplets on the surface of the fiber mats using a Phoenix 300 contact angle analyzer (Surface Electro Optics, Korea). Five independent measurements were performed for each sample.

The mechanical properties of the fibrous membranes of various compositions were evaluated using a universal testing instrument (Instron 5565, USA) at a cross-head speed of 5 mm min^{-1}. Typically, the electrospun membranes were carefully cut into rectangular shapes (7 × 30 mm) and their precise thicknesses were assessed using a micrometer. Filter papers were pre-attached to both ends of the specimen to prevent slipping during the tests, which left a gauge length of 20 mm. The tensile strength and failure strain of the membranes were obtained from the stress–strain curves, and the elastic modulus was determined from the slope of the initial linear portion of the curves. At least five specimens were tested for each composition.

2.3. Biodegradability

The stability of the membranes was examined using the following procedure. The membranes (1.5 × 1.5 cm^2) were soaked in 0.01 M phosphate-buffered saline, pH 7.4 (PBS) at 37 °C. At predetermined times the medium was refreshed and the concentration of silicon released from the membranes was monitored using inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Optima-4300 DV, Perkin-Elmer, MA). After soaking in PBS for up to 14 days the membranes were washed with distilled water and freeze-dried, before being observed using FESEM.

2.4. In vitro cellular assays

In vitro osteoblastic responses to the fibrous membranes were evaluated in terms of the adhesion, viability and alkaline phosphatase (ALP) activity of MC3T3-E1pre-osteoblast cells (ATCC catalog No. CRL-2593). For the cell adhesion studies the cells were seeded at 5 × 10^4 ml^{-1} onto UV-sterilized membranes (1 × 1 cm^2) and cultured for 3 h in an osteogenic medium [α-minimal essential medium (Welgene) supplemented with 10% fetal bovine serum (GIBCO), 2 mM L-glutamine (GIBCO), 50 IU ml^{-1} penicillin (GIBCO), 50 μg ml^{-1} streptomycin (GIBCO), 50 μg ml^{-1} ascorbic acid (Sigma–Aldrich) and 10 mM β-glycerophosphate (Fluka)]. Then the cell morphology was examined using confocal laser scanning microscopy (CLSM) (LSM 510 NLO, Germany) and FESEM. Before CLSM examination the cultured cells were dyed with Alexa Fluor® 546 phalloidin (Eugene) and a ProLong® Gold antifade reagent with DAPI (Eugene). SEM observations were performed after the samples were fixed with 2.5% glutaraldehyde, dehydrated in graded ethanol, critical point dried and gold coated.

The cell viability and differentiation were assessed using MTS and ALP activity assays. Briefly, the cells were plated on sterilized membranes (1 × 1 cm^2) at a density of 3 × 10^4 ml^{-1} and cultured for 3 days. After culturing the viability of the cells on the membranes was measured using the MTS assay (CellTiter 96, Promega). For the ALP activity test 2 × 2 cm fibrous membranes were used at a seeding density of 1.5 × 10^4 ml^{-1}. After culture for 14 days the cells on the membranes were harvested and lysed using 0.1% Triton X-100. The absorbance of each aliquot of the cell lysates was obtained by Bradford protein assay (Bio-Rad) and the absorbance converted directly to total amount of protein using a bovine serum albumin standard curve in the range 0.2–1.2 mg ml^{-1}. After measuring the total protein concentration those aliquots with equal amounts of protein from each specimen were used to assess the ALP activity for equal amounts of protein. ALP activity was colorimetrically measured according to the manufacturer's protocol (Sigma ALP kit 104) using *p*-nitrophenyl phosphate as the substrate.

The MTS assay and ALP activity test were preformed on three duplicate samples (n = 3) for each condition. Statistical analysis of the data was carried out using a two-tailed, paired Student's *t*-test, where $P < 0.05$ and $P < 0.01$ were considered significant.

2.5. In vivo animal test

The in vivo test was performed on 11 male Sprague–Dawley albino rats (13 weeks old, average weight approximately 300 g) using a previously described method [25]. The PCL–40% silica xerogel hybrid membrane was examined because it had the highest ALP activity. A pure PCL membrane was also tested for comparison purposes.

Briefly, calvarial defects were generated on the bilateral sides of the midline in each animal using a trephine drill with a diameter of