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Icariin-loaded electrospun PCL/gelatin nanofiber membrane as potential artificial periosteum

Min Gong[a,b], Cheng Chi[a,b], Jingjing Ye[a,b], Meihong Liao[a,b], Wenqi Xie[a,b], Chengai Wu[c], Rui Shi[c,*], Liqun Zhang[a,b,**]

[a] Beijing Laboratory of Biomedical Materials, Beijing University of Chemical Technology, Beijing 100029, PR China
[b] State Key Laboratory of Organic-Inorganic Composites, Beijing University of Chemical Technology, Beijing 100029, PR China
[c] Institute of Traumatology and Orthopaedics, Beijing Jishuitan Hospital, Beijing 100035, PR China

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ABSTRACT

Due to the significant role of the periosteum in bone regeneration, we hypothesised that using a specially engineered artificial periosteum could lead to an enhancement in osteogenesis in bone grafts. Herein, we describe our work aimed at fabricating an electrospun fibrous membrane as an artificial periosteum that exhibits flexibility, permeability and osteoinduction. This membrane was designed to cover the complex surface of bone grafts to facilitate and accelerate bone regeneration. The traditional Chinese medicine icariin (ICA) was subsequently introduced into poly (ε-caprolactone) (PCL) /gelatin nanofibers to fabricate an artificial periosteum for the first time. The effects of ICA content on morphology, physical properties, drug release profile, *in vitro* degradability, biocompatibility and osteogenic differentiation properties were investigated. The ICA-loaded electrospun membranes showed significant improvement in hydrophilicity, high mechanical strength, appropriate degradation rates and excellent biocompatibility. Furthermore, clear enhancement in alkaline phosphatase (ALP) activity, as well as an increase in osteocalcin (OCN) and type collagen I (COL I) expression in MC3T3-E1 cells were detected. Furthermore, we observed clear calcium deposition content in MC3T3-E1 cells cultured on ICA-loaded fibrous matrix. The membrane loaded with 0.05 wt.% ICA displayed properties contributing to cell attachment, proliferation and differentiation. These results indicate the huge potential of this ICA-loaded PCL/gelatin electrospun membrane as a biomimetic artificial periosteum to accelerate bone regeneration.

1. Introduction

Large bone defects have been a long standing and challenging issue in orthopaedic surgery [1]. The gold standard for treatment of critical sized bone defects are bone allografts. Although routinely successful, these autogenous bone transplants have noted disadvantages including limited donor sources and donor site morbidity. A further concern with allogeneic transplants is unwanted immunological reactions. To combat this, the allogeneic transplants are decellularized to remove the periosteum, which can result in a greater than 60% decrease of new bone formation [2]. The periosteum is a membrane that covers the outer surface of all bones and contains two distinct layers: the outer layer which contains collagen fibers, fibroblasts and microvessels and an inner layer which contains osteoblasts and osteoprogenitor cells that contribute to bone growth, healing, and remodelling [3]. It has been widely recognized that the periosteum has remarkable regenerative capacity and it is essential in physiological bone formation and for pathological defect healing in conjunction with growth factors and mechanical stimulation [4–6]. Thus, *in vitro* design and construction of an artificial periosteum that mimics the fibrous microstructure and osteogenesis function of natural periosteum would therefore be a novel approach to better treat these large bone defects. In previous studies, an engineered periosteum from extracellular matrix membranes demonstrated enhanced osteogenic potential but the complexity in fabrication coupled with difficult storage requirements and high financial cost limited the application of these treatments [7].

It has previously been reported that the deficiency or low activity of growth factors in bone defects is a key aspect of bone regeneration [8]. Focal growth factor-loaded biomaterials can minimize systematic distribution of drugs and accomplish a sustained release by directly delivering its payload to the defect site with a therapeutically relevant dose. Since conventional growth factors such as bone morphogenetic proteins (BMPs), insulin-like growth factor (IGF) and fibroblast growth factor (FGF) have disadvantages including rapid degradation, high cost, and

* Corresponding author.
** Corresponding author at: Center of Advanced Elastomer Materials, Beijing University of Chemical Technology, Beijing 100029, PR China.
E-mail addresses: sharell@126.com (R. Shi), zhanglq@mail.buct.edu.cn (L. Zhang).

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deactivation issues, it is unsurprising that the clinical use of these growth factors has been limited [9,10]. It is of great importance to seek a replacement which works to correct these issues. Icariin (ICA), the main component of the traditional Chinese medicine *herba epimedium*, has been extensively studied in regenerative medicine [11]. Abundant studies have shown that ICA is an effective promoter in osteoblast proliferation and differentiation [12–14] as well as working as an inhibitor of osteoclast formation [15]. ICA has the potential to accelerate bone formation when loaded into different porous scaffolds using various physical methods such as entrapment or adsorption [11]. In this paper, we describe our work on the fabrication and utilisation of an ICA loaded artificial periosteum, which displays clear osteoinductive properties.

Electrospinning has gained widespread interest in the design and integration of high-performance and flexible membranes for healthcare. This is mainly due to the structural similarity of the electrospun polymers to the extracellular matrix. Electrospinning also facilitate tuneable surface morphologies, conformability to complex three dimensional (3D) profiles as well as being relatively low cost [16–18]. The inherently high surface to volume ratio and high draw ratios as well the inherently small diameter of the electrospun polymer fibers can provide excellent flexibility [19]. Additionally, these fibers can stimulate cell growth, enhance drug encapsulation, and accomplish controlled and sustained drug delivery [20–24]. The morphology, physical properties, drug release and degradation profiles of the fibrous membranes are all easily adjusted through judicious choice of fabrication parameters [25]. Therefore, we decided that electrospinning was an appropriate method of fabrication to achieve flexible, permeable and biocompatible membranes for this study. PCL/gelatin hybrid materials, combining the mechanical properties of PCL and good biocompatibility of gelatin, have been widely used for various tissue regeneration studies [26,27]. Additionally, the miscibility of PCL and gelatin can be effectively mediated by introduction of acetic acid to facilitate the formation of homogeneous nanofibers [28].

Herein, ICA-loaded PCL/gelatin nanofibrous membranes were fabricated *via* electrospinning. We speculate that the electrospun ICA-loaded PCL/gelatin membrane will provide excellent flexibility to cover the complex surface of bone grafts and biological properties to promote the proliferation and differentiation of osteoblasts during bone regeneration. This study aims to provide valuable information for the development of fiber-based artificial periosteum for clinical use.

2. Experimental

2.1. Materials

PCL (Mn = 80 kDa) was obtained from Sigma-Aldrich (USA). Type-B gelatin was obtained from Rousselot (France). Icariin was purchased from MANSITE BIO-TECHNOLOGY CO., LTD (China). Alpha minimum essential medium (α-MEM), fetal bovine serum (FBS), and phosphate buffer saline (PBS, pH = 7.4) were purchased from Hyclone (USA). Trifluoroethanol (TFE) and *N, N*-Dimethylformamide (DMF) were purchased from Aladdin (USA). MC3T3-E1 mouse preosteoblasts were kindly donated by Jishuitan Hospital.

2.2. Fabrication of nanofiber membranes

PCL/gelatin solution was prepared by dissolving PCL and gelatin in TFE. The total polymer concentration was 6 wt.% and the mass ratio of PCL to gelatin was 8:2. Acetic acid (0.2 v/v% TFE) was introduced into the polymer solution to afford a transparent PCL/gelatin solution. 100 μL ICA solutions (in DMF, 0 mg/mL, 1.2 mg/mL, 2.4 mg/mL, 12 mg/mL, 24 mg/mL, 120 mg/mL) was added to the PCL/gelatin solution to prepare the homogeneously solution suitable for electrospinning. The membranes with ICA contents of 0, 0.005, 0.01, 0.05, 0.1, and 0.5 wt.% were labeled as PGI0, PGI0.005, PGI0.01, PGI0.05, PGI0.1 and PGI0.5 respectively.

2.3. Morphology characterization of nanofiber membranes

Scanning electron microscopy (SEM) was used to characterize the morphology of the electrospun nanofibers. The membranes were coated with gold before being observed under the microscope (S4800, Hitachi, Japan) at a voltage of 5 KV. Image J software was used to measure the fiber diameter and pore size of the membrane on the SEM micrographs at 100 randomly selected positions [29]. The apparent density and porosity of the membranes were estimated as previously reported [30].

$$\text{Apparent density } (g/cm^3) = \frac{\text{Mass of membrane (g)}}{\text{Membrane Thickness (cm)} \times \text{Membrane area } (cm^2)} \quad (1)$$

$$\text{Porosity } (\%) = \left(1 - \frac{\text{Apparent density } (g/cm^3)}{\text{Bulk density}(g/cm^3)}\right) \quad (2)$$

2.4. Chemical, thermal and mechanical characterization of nanofiber membranes

Fourier transform infrared (FT-IR) (Bruker Tensor 27 spectrometer) spectra and differential scanning calorimetry (DSC) (Mettler-Toledo) were used to study the chemical and thermal properties of the electrospun membranes. X-ray diffraction (XRD) (Rigaku D/max-Ultima III X-ray diffractometer) was conducted between 5°–70° at a scan rate of 5°/min. BOSE ElectroForce 3200 test instrument with a 500 N load cell at 5 mm/min crosshead speed in 25℃ was used to evaluate the tensile strength and break elongation of the membrane [31].

2.5. Hydrophilicity characterization of nanofiber membranes

The contact angles of water droplets on the membranes were measured by a SL200 A Contact Angle Analyzer (China) to evaluate the hydrophilicity of the membrane. The membranes were cut into 2.0 cm × 2.0 cm × 0.26 mm (width × length × depth). 3.0 μL water droplets were vertically dropped onto the surface of the electrospun membranes. The water contact angle was measured as soon as the water droplets totally touched the membrane. Ten membranes for each sample were used to measure the average water contact angle (WCA) value.

2.6. Drug release profiles and in vitro biodegradation of nanofiber membranes

The ICA release profiles of the PGI membrane were determined by immersing the samples in triplicate in PBS (PH = 7.4) at 37 ℃. At predetermined time intervals (0.5 h, 1 h, 2 h, 3 h, 6 h, 12 h, and 1, 2, 4, 7, 10, 14, 21 days), the supernatant was collected and replaced with 5 mL fresh PBS. UV–vis spectrophotometer (UV-1700, Macy instrument, China) detection at 270 nm was used to determine the drug release profile as described previously [32]. As for the evaluation of *in vitro* degradation, the membrane was washed with deionized water for three times, dried at room temperature until no mass change and then weighed.

2.7. In vitro biocompatibility of the nanofiber membranes

The cytotoxicity of the membranes to MC3T3-E1 mouse preosteoblasts cells was evaluated using an MTT assay as described in previous study [24]. The viabilities of MC3T3-E1 cells proliferated onto PGI membranes were evaluated using a CCK-8 assay. Briefly, the membranes were firstly cut into 2.5 cm circles in diameter, sterilized, and then inserted in 24-well plate by Cell-Crown™. 100 μL MC3T3-E1

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