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Phosphoprotein/chitosan electrospun nanofibrous scaffold for biomineralization

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ABSTRACT

In this study, negatively charged phosvitin (PV) and positively charged chitosan (CS) were alternately deposited on negatively charged cellulose mats via layer-by-layer (LBL) self-assembly technique. Morphologies of the LBL films coating mats were observed by scanning electron microscope (SEM). Afterwards, in vitro biomimetic mineralization was carried out through incubation of the fibrous mats in a simulated body fluid (SBF) solution for different time. Scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS) and X-ray diffraction (XRD) were used to characterize the morphology and structure of the deposited mineral phase on the scaffolds. In addition, the cell culture experiment demonstrated that the scaffolds with the LBL structured films were of good cell compatibility for MC3T3-E1 cells. Moreover, the cell proliferation was affected by the number of deposition layers and the composition of outer-most layer. Confocal laser scanning microscopy (CLSM) and SEM imaging revealed a good performance of cell adhesion and spreading of MC3T3-E1 cells on the surface of biocomposite scaffold. So CS/PV nanofibrous mats were satisfactory for the composite to be used in bioapplications.

1. Introduction

Bone tissue engineering is an important strategy for bone repair and regeneration through the combination of scaffolds, implanted cells, and biologically active molecules [1–3]. The basic approach to bone tissue engineering involves the development of highly porous biodegradable three-dimensional (3D) scaffolds for cellular in-growth, facilitating nutrient and waste exchange by cells deep within the scaffold [4–7]. Several attempts have been made to develop the promising scaffolds including electrospinning [8,9], salt extraction [10], freeze-immersion [11], and others. Among these methods, electrospinning technique is a well-established method to fabricate porous scaffolds with micro- to nano-scale and controlled surface morphology [8,12].

Electrospun nanofibrous mates have a high porosity with interconnected pores and high surface-to-volume ratio, which benefit cell adhesion and proliferation [13,14]. As known, the physical and chemical nature of the biomaterial surface determined the manner in which a cell interacts with a biomaterial [15]. In order to further improve the functions for biomedical use, the surfaces of electropsun nanofibers have been decorated using some bioactive molecules after the electrospinning process using layer-by-layer (LBL) deposition technique [12]. Various deposition materials have been utilized to fabricate the functional LBL structured composite films including proteins, polysaccharides, metal irons, particles, etc [16]. Generally, electrostatic interaction, hydrogen bonding, hydrophobic interaction, and van der Waals interaction can be used as driving force for surface adsorption in the process of LBL assembly [17].

Cellulose nanofibrous mates have been widely used in both hard and soft tissue engineering including bone regeneration, tissue engineering, scaffolds for growing functional cardiac cell constructs in vitro, etc [18]. Many studies have reported that putting cellulose nanofibrous mates in simulated body fluid (SBF) was necessary to catalyze the calcium phosphate mineralization on the scaffold. In addition, the phosphorylation of cellulose fibers could effectively induce the growth of carbonate-containing Hydroxyapatite (HAP) to form a 3D nanocomposite network with appropriate mechanical performance [19,20]. HAP, a major inorganic component of natural bone, is a biomimetic material with good biocompatibility

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and bioactivity and it has been widely used in bone replacement systems [21,22]. So the phosphorylated decoration of cellulose nanofibers was implied to further enhancement on the biomineralization process.

In the current study, positively charged chitosan (CS) was first deposited on the surface of negatively charged cellulose nanofibrous mats. CS is an attractive natural material owing to its good properties of biocompatibility, biodegradability and non-toxicity, which is useful in many different applications [19,23–25]. Some researches reported that CS could regulate the mineralization process of HAP, and is always used as templates for the deposition of HAP [7,26]. Additionally, it can preferentially promote the attachment and proliferation of cells [27]. Egg yolk phosvitin (PV) negatively charged in aqueous media, was used as a second layer deposited on the nanofibrous mats via electrostatic LBL self-assembly technique. PV is a highly phosphorylated protein, which can initiate and regulate the formation of mineral crystals and may play a vital role in biological mineralization in human hard tissues [28]. Several researchers have shown that PV can promote the transformation process from metastable calcium phosphates to HAP [29,30]. Additionally, immobilized proteins on substrates can sometimes promote HAP nucleation [30]. Here, CS and PV were used to modify the surface of the cellulose mats. The crystal growth of HAP was induced on polymer membrane surfaces through soaking electrospun nanofibrous mats in 1.5 × SBF solution. The influences of outermost layer variation and the number of deposition bilayers for the scaffold material behavior were also investigated. The highly porous biodegradable nanofibrous mats prepared in this work may have potential applications in bone tissue engineering.

2. Materials and methods

2.1. Materials

Cellulose acetate (CA, *Mn* = 30,000) was purchased from Sigma-Aldrich Co., USA. Hen egg yolk phosivtin was isolated from the egg yolk [31]. Chitosan (CS, Mw = 2.0–4.0 × 10^5 Da) from shrimp shell with >90% deacetylation was provided by Qingdao Yunzhou Biotechnology Co., Ltd. The other reagents were analytical grade purchased from China National Pharmaceutical Group Industry Corporation Ltd. All aqueous solutions were prepared using purified water with a resistance of 18.2 MΩ cm. For cell culture, the mouse MC3T3-E1 preosteoblast cell line was obtained from the Chinese Academy of Medical Sciences (China).

2.2. Fabrication of template nanofibers

Nanofibrous CA mats were fabricated by modified Ding's method [32]. 2 g CA was dissolved into 8 g acetone-N,N-dimethyl acetamide (DMAc) (2: 1, w/w) mixed solvent and stirred to obtain homogeneous solution. Then it was loaded into a plastic syringe, which was driven by a syringe pump. The applied voltage was 16 kV and the tip-to-collector distance was 20 cm. The ambient temperature and relative humidity were maintained at 25 °C and 45%, respectively. The prepared fibrous mats were dried at 80 °C in vacuum for 24 h to remove the trace solvent. Hydrolysis of the CA mats was performed in alkaline aqueous solution at ambient temperature for 7 days following the previous report [33].

2.3. Formation of nanocomposite films on template nanofibers

The bilayer film was deposited by adding chitosan (1 mg/mL, pH 5.0) followed by PV (1 mg/mL, pH 6.0) each for 50 mL. Then, the solution was suction-filtered through the nanofibrous mats. Following each deposition step, the mats wash with 50 mL NaCl solution. The ionic strength of all dipping solution was regulated by the addition of NaCl at a concentration of 0.1 mg/mL. The water was suction-filtered through the nanofibrous mats as well. Here, $(CS/PV)_n$ was used as a formula to label the LbL structured films, where n was the number of the CS/PV bilayers. The outermost layer was CS composite when n equaled to 5.5 and 10.5. The LbL films coated fibrous mats were dried at 40 °C for 2 h under vacuum prior to further use.

2.4. Characterization of composite nanofibrous membranes

The surface morphology of the composite nanofibrous mats was performed using scanning electron microscopy (SEM)(S-4800, Hitachi Ltd., Japan). The cross-sections of the nanofibers were observed using a JEOL transmission electron microscope [5] (H-7650, Hitachi, Japan). X-ray photoelectron spectroscopy (XPS) was operated on an axis ultra DLD apparatus (Kratos, U.K.), to identify chemical compositions of the nanofibrous membranes. X-ray diffraction (XRD) was carried out using a diffract meter type D/max-rA (Rigaku Co., Japan) with Cu target and Ka radiation (λ = 0.154 nm).

2.5. Biomimetic growth of HAP on nanofibers

The 1.5 times simulated body fluid (1.5 × SBF) solution was prepared as reported [34]. The composite nanofibrous mats were incubated in 1.5 × SBF solution at 37 °C for various period. Fresh SBF solution was replaced once every other day. At desired time intervals, membrances were taken out from SBF solution, washed with deionized water and finally freeze-dried.

2.6. Cell culture and seeding

MC3T3-E1 cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C in humidified environment of 5% CO_2. For cell seeding, cellulose nanofibrous, $(CS/PV)_5$, $(CS/PV)_{5.5}$, $(CS/PV)_{10}$, and $(CS/PV)_{10.5}$ substrates were placed in a 24-well plate. Prior to seeding with cells, the samples were sterilized under Co 60 (γ) laser for 2 h.

2.7. Cell proliferation

Cell proliferation on the nanofibrous scaffolds was investigated by using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. MC3T3-E1 cells were seeded on the scaffolds and cultured at a density of 1 × 10^5 cells/cm^2 in 24 well culture plate for 1, 2 or 4 days. At prefixed culture time points, 100 μL of MTT solution (5 mg/mL in PBS) was added to each well, followed by incubation at 37 °C cell incubator for 4 h. The supernatant was then removed, and the formed formazan crystals were dissolved by dimethyl sulfoxide. After the incubation period, the samples were pipetted out into 96 well plates. The absorbance was determined at 490 nm using a microplate reader (RT-6000, Lei Du Life Science and Technology Co, Shenzhen, China).

2.8. Cell adhesion and morphology

For the confocal microscopy image analysis, MC3T3-E1 cells were seeded into a 35 mm petri dish at a density of 1 × 10^5 viable cells per well in 2 mL growth medium with the nanofibrous membranes in the bottom. After incubation at 37 °C and in 5% CO_2 atmosphere for 2 day, cells were fixed in 4% paraformaldehyde, stained with acridine orange for 30 min and were washed with PBS for three times to remove free dyes. Fluorescence images were