Materials Science and Engineering C 72 (2017) 341–351

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Laminated electrospun nHA/PHB-composite scaffolds mimicking bone extracellular matrix for bone tissue engineering

Zhuoyue Chen [a,b,1], Yue Song [a,1], Jing Zhang [a,b,c], Wei Liu [a], Jihong Cui [a,b,c,*], Hongmin Li [a,b,c], Fulin Chen [a,b,c]

[a] Lab of Tissue Engineering, Faculty of Life Science, Northwest University, 229 TaiBai North Road, Xi'an, Shaanxi Province 710069, PR China
[b] Provincial Key Laboratory of Biotechnology of Shaanxi, Northwest University, 229 TaiBai North Road, Xi'an, Shaanxi Province 710069, PR China
[c] Key Laboratory of Resource Biology and Modern Biotechnology in Western China, Ministry of Education, Northwest University, 229 TaiBai North Road, Xi'an, Shaanxi Province, 710069, PR China

ARTICLE INFO

Article history:
Received 11 July 2016
Received in revised form 8 November 2016
Accepted 21 November 2016
Available online 24 November 2016

Keywords:
Electrospinning
PHB
nHA
Laminated technology
Bone tissue engineering

ABSTRACT

Electrospinning is an effective means to generate nano- to micro-scale polymer fibers resembling native extracellular matrix for tissue engineering. However, a major problem of electrospun materials is that limited pore size and porosity may prevent adequate cellular infiltration and tissue ingrowth. In this study, we first prepared thin layers of hydroxyapatite nanoparticle (nHA)/poly-hydroxybutyrate (PHB) via electrospinning. We then laminated the nHA/PHB thin layers to obtain a scaffold for cell seeding and bone tissue engineering. The results demonstrated that the laminated scaffold possessed optimized cell-loading capacity. Bone marrow mesenchymal stem cells (MSCs) exhibited better adherence, proliferation and osteogenic phenotypes on nHA/PHB scaffolds than on PHB scaffolds. Thereafter, we seeded MSCs onto nHA/PHB scaffolds to fabricate bone grafts. Histological observation showed osteoid tissue formation throughout the scaffold, with most of the scaffold absorbed in the specimens 2 months after implantation, and blood vessels ingrowth into the graft could be observed in the graft. We concluded that electrospun and laminated nanoscaled biocomposite scaffolds hold great therapeutic potential for bone regeneration.

1. Introduction

The repair of bone defects resulting from trauma, inflammation, tumor resection and abnormal skeletal development remains a clinical challenge [1]. Currently, autologous bone graft transplantation is the most successful means for bone defect repair [1]. However, harvesting autologous bone graft requires a secondary operation and may cause severe donor site morbidity [1,2]. Allografts, though easy to obtain, risk eliciting immune responses and transferring pathogens [3]. Bone substitutes place no limits on amount and size and are ready for application in a predetermined shape. Yet limited bone repair capacity greatly impedes the application of bone substitutes.

Recent advances in bone tissue engineering provide a promising strategy for bone defect repair and regeneration through the seeding of osteogenic cells onto selected scaffold [4]. Various natural and synthetic materials have been studied and developed into cell-seeding scaffolds [5–8]. Aside from good biocompatibility, inter-connective porous structure and proper pore size, and a controlled degradation rate, scaffolds for bone tissue engineering should also facilitate osteogenic cells adhesion, proliferation, differentiation and bone matrix formation. However, these scaffolds are far from ideal for clinically applicable bone tissue engineering.

The geometry and surface morphology of the scaffold are critical factors for bone tissue engineering. Nanofibrous and nanocomposite scaffolds for tissue engineering are able to mimic bone extracellular matrix (ECM) due to their similarity to native ECM [9,10]. Palin et al. reported that surfaces that replicated nanoscale roughness enhanced osteoblast adhesion and proliferation [11]. Liao et al. reported that cells can attach and organize well around nanoscale materials, likely because nanomaterials have increased numbers of atoms and crystal grains at their surface [12]. Importantly, substratum topography is a nonbiological method of regulating cell function because textured surfaces serve simply as an extracellular physical milieu without involving growth factors [13].

Electrospinning is an effective means of generating nano- to micro-scale polymer fibers that resemble native extracellular matrix for tissue engineering. Electrospun nanofibers have been useful for developing a variety of artificial tissues and organs such as bone [14], skin [15–17], nerve [18], tendon [19], blood vessel [20] and cartilage [21]. However, electrospun scaffolds commonly exhibit dense morphologies and limited pore sizes, which may significantly influence cell-loading capacity and prevent tissue ingrowth. Park et al. employed a combination of direct polymer melt deposition (DPMD) and electrospinning to improve the scaffold pore size [22]. However, the obtained scaffold was

* Corresponding author at: Lab of Tissue Engineering, Faculty of Life Science, Northwest University, PR China.
E-mail address: cjh@nwu.edu.cn (J. Cui).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.msec.2016.11.070
0928-4931/

composed of micro- and nanoscaled fibers, and the fiber morphology differs greatly to native ECM [23].

In the current study, we prepared nHA/PHB-composite scaffolds via electrospinning and lamination and investigated the effect of the composite scaffolds on MSC loading, attachment, proliferation and osteogenic differentiation. Finally, tissue-engineered bone grafts with vascularization were successfully fabricated using the laminated nHA/PHB-composite scaffolds.

2. Experimental

2.1. Chemicals and materials

PHB was purchased from Sigma-Aldrich (USA). Synthetic nanoparticle hydroxyapatite (nHA, particle size <20 nm, Sigma-Aldrich) were of analytical-grade purity. All other reagents were of analytical grade and were used without further purification. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, trypsin and phosphate buffered saline (PBS) solutions were purchased from Sigma (USA).

2.2. Preparation of nHA/PHB composites

PHB solution was prepared at a concentration of 3% (*w*/*v*) in chloroform ($CHCl_3$). The solution was stirred with a magnetic stirrer at room temperature for 12 h. Nanophase HA was then added in the dispersion to form a 5% (*w*/*v*) solution. The resulting mixture was stirred again for 12 h and sonicated in the water bath for 1 h. The electrospun fibers were prepared as reported previously [24]. Briefly, the nHA/PHB blend solution held in a 5-ml syringe was delivered into a blunted medical needle spinneret (OD 1.2 mm, ID 0.84 mm) through teflon tubing by a syringe pump (KD-100, KD Scientific, Inc., USA). A polarity-reversible high-voltage power supply (RR50–1.25R/230/DDPM, Gamma High Voltage Research, USA) was used to charge the spinning dope of nHA/PHB by directly clamping one electrode to the metal needle spinneret and the other to aluminum foil wrapped on a lab rack. The separating distance between the needle tip and the aluminum foil was set to 20 cm. Other operating parameters in the chamber for producing the fibers are as follows: voltage 15 kV, flow rate 1 mL/h, ambient temperature 25 °C and humidity 55%. After 15 min, the nHA/PHB fibrous membranes were carefully obtained and then stored in a vacuum oven for 24 h to remove residual solvent. Finally, the nHA/PHB single-layer fibrous membranes were handling folded for three times to obtain scaffold with eight layers. Meanwhile, the nHA/PHB membranes (traditional scaffold) were obtained after electrospinning for 2 h. PHB scaffolds were also prepared as a control. Fig.1 shows a schematic diagraming the preparation of laminated scaffolds.

2.3. Characterization of composite scaffold

The thickness of each single-layer membrane was measured and the transmitted light through each single-layer membrane was registered by an ultraviolet and visible spectrophotometer (UV2700, Shimazu, Japan). The thin-layer membranes and laminated scaffolds were then observed on a digital microscope (VHX-5000, KEYENCE, Japan) at 20× magnification. After being sputter-coated with gold, the microstructures and surface morphologies of the PHB and nHA/PHB scaffolds were observed by scanning electronic microscopy (SEM, Hitachi S-4700, Japan).

Fiber diameters and pore sizes were quantified from SEM images of electrospun scaffolds. The pore diameters of the longest axes in each of 5 pores within an image for a total of 5 images per sample ($n = 6$ per treatment) were quantified using ImageJ. Scaffold porosity was quantified from hematoxylin and eosin (H&E)-stained scaffold cross-sections [25] with modifications. Briefly, the scaffolds were fixed in 10% (*w*/*v*) buffered formalin solution and embedded in paraffin. 5 μm sections were deparaffinized in xylene and stained with H&E. Section images were converted to binary format using ImageJ, where the fibers appeared black and pores white. The percentage of the white area relative to the total scaffold section surface area in each image was defined as the percent porosity.

The tensile behavior of single-layer membranes and the laminated scaffolds was investigated using a universal testing machine (3365, INSTRON, USA). The samples (each sample: $n = 6$) utilized for tensile tests were cut in the form of a rectangle (10×40 mm^2). Tensile testing was performed on the samples at a 0.5 mm/min strain rate in unconfined tension. The tensile strength was calculated by dividing the normal force by the sample cross-sectional area, and engineering strain was calculated as the change in the gap distance divided by the original gap distance.

2.4. In vitro assays of cell-scaffold interaction

2.4.1. Isolation and culture of rabbit MSCs

New Zealand rabbits (one month old) were obtained from the animal holding unit of Northwest University, and samples of bone marrow were harvested in accordance with Institutional Animal Care and Use Committee (IACUC) approval of Northwest University (ACUC2013015). Rabbit MSCs were isolated and cultured as reported previously [26]. Briefly, the obtained marrow was suspended and cultured in DMEM containing 10% FBS. The medium was refreshed after 24 h to remove non-adherent cells. On the 5th day, the culture medium was changed to osteogenic differentiation medium (DMEM containing 10% FBS, 10 mM β-glycerophosphate disodium, 10^{-7} M dexamethasone and 50 μg/mL L-ascorbic acid) to induce differentiation into marrow stromal osteoblasts. The media were then changed every 3 days. Before the cells formed a confluent monolayer, they were digested using trypsin 0.25%, and cells at passage 2 were used for experiments. The cell concentration was adjusted to 1×10^7 cells/mL with medium before cell seeding.

2.4.2. Attachment and proliferation of MSCs on scaffolds

Firstly, the scaffolds were prepared in the form of cuboid shape with 10 mm-length, 8 mm-width and 4 mm-height. The scaffolds were sterilized in 70% ethanol for 30 min, followed by rinsing twice with sterile PBS. The scaffolds were dried overnight and soaked for 3 h in culture medium prior to cell seeding. A total of 2×10^6 cells in 200 μL suspension were seeded into each scaffold. The cell-scaffold complexes were placed into a 6-well plate and moved to the incubator for 4 h to ensure cell adhesion to the scaffolds. Then, 700 μL of medium was carefully added around the complexes. Twelve hours later, the composites were rinsed with sterile PBS, stained with Hoechst 33342 according to the manufacturer's protocol (Sigma-Aldrich, St Louis, USA), and observed on a fluorescence microscope (TE2000-U, Nikon, Japan) to detect cell adhesion.

The proliferation of the MSCs on the material was determined using a MTT assay. The scaffolds were placed into a 6-well plate, and 4×10^4 MSCs in 200 μL medium were carefully seeded onto each scaffold and incubated for 4 h to ensure cell attachment. Then, an additional 700 μL of culture medium was added. After culturing for 1, 3, 5 and 7 days, the scaffolds were washed with PBS, after which 200 μL culture medium and 20 μL of MTT solution (5 mg/mL in PBS) were added to each well. After incubation for 4 h at 37 °C, the culture media was removed, and 150 μL of dimethyl sulfoxide (DMSO) was added. The absorbance of the supernatant was read on a spectrophotometer (VersaMax, Molecular Devices Corporation, USA) at 490 nm.

The flowchart of attachment and proliferation of MSCs on scaffolds assay was shown in Fig. 4A.