Materials Science and Engineering C 33 (2013) 3545–3552

Contents lists available at SciVerse ScienceDirect

Materials Science and Engineering C

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

Icariin delivery porous PHBV scaffolds for promoting osteoblast expansion *in vitro*

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ARTICLE INFO

Article history:
Received 7 August 2012
Received in revised form 9 April 2013
Accepted 24 April 2013
Available online 3 May 2013

Keywords:
Scaffold
Icariin
PHBV
Proliferation
Drug delivery

ABSTRACT

How cells could proliferate quickly and maintain their biological activity by using appropriate scaffolds remains a big challenge for tissue engineering. By integrating icariin, a bioactive ingredient of traditional Chinese medicine (TCM) Epimedii herba, with PHBV scaffolds, novel icariin delivery porous PHBV scaffolds (IDPPSs) were fabricated with a combination of the solvent casting and salt leaching techniques. IDPPSs displayed a high porosity, 88.80%, and appropriate mechanical properties which were required for 3-D cell culture. IDPPSs significantly promoted the proliferation of human osteoblast-like MG-63 cells and the pre-osteoblast MC3T3-E1 cells, while IDPPSs containing 0.1% icariin (wt.%) showed the highest effect compared with other samples. Although IDPPSs continuously released icariin to the solution in 28 days, cells attached to IDPPSs received an enhanced growth stimulation compared with which were not physically in contact with IDPPSs. Up-regulated transcription of growth factor genes and extracellular matrix genes, including *BMP2*, *BMP6*, *BMP7* and *BGN*, was observed in IDPPS-cultured MG-63 cells, illustrating that enhanced cellular proliferation results from alteration of gene transcription. These results implied the potential commercial use of IDPPSs in tissue engineering.

1. Introduction

The use of autologous bone graft, the current golden standard for the treatment of bone defects, is restricted by the resource from patients and the use of allogenous bone graft is limited by inadequate donor sources and immune responses as well. Therefore, a variety of synthetic bone graft materials (stainless steel, titanium, etc.) have been developed [1,2]. However, an ideal bone graft substitute can be eventually replaced with patients' native bone tissue after completely repair bone defects. According to the tissue engineering (TE) concept [3], the combination of artificial biodegradable materials with autologous cells is a feasible strategy to achieve this goal. Cells, expanding *in vitro* after being isolated from the patient, are seeded to an appropriate three-dimensional (3-D) scaffolds and cultured *in vitro*. Composite scaffolds with enough cells obtained by *in vitro* 3-D culture could be transplanted into patients and then native tissue could form with the degradation of the scaffold [4]. Finally, newly generated native tissue would replace the planted biomaterials completely to cure bone defects of the patient.

Efficient cell expansion *in vitro* plays a crucial role in tissue engineering [5–7] and its clinical applications [8], since it quickly generates a large number of cells and maintains their biological activity *in vitro* to shorten the time-consuming cell proliferation procedure and to provide active cells for tissue regeneration. Typically, cells are expanded *in vitro* in conventional monolayer culture, e.g., Petri dish and culture flask, by which cells are living in an artificial environment that is distinctly different from their living conditions *in vivo*. However, researchers have realized that cells interact with their surrounding microenvironment extensively during their growth and development process *in vitro* [9,10] and the behavior of the cells, such as attachment, proliferation and differentiation, could be regulated by these culture conditions [11,12]. To mimic the *in vivo* living environment of cells, a variety of 3-D porous scaffolds have been used as substitutes for the extracellular matrix (ECM) of tissues, which imitate the environment *in vivo* and provide a physical support matrix to enhance the cell–cell and cell–substrate interactions [13–15]. Therefore, this study is desired to prepare a new 3-D porous scaffold that have good biocompatibility, bioactivity, high porosity, and possess appropriate mechanical properties to promote cell expansion process *in vitro*.

Since growth factors, such as bone morphogenetic proteins (BMPs), insulin-like growth factor (IGF) and fibroblast growth factor(FGF), typically act as soluble signaling molecules that evidently affect cell proliferation and differentiation, they have been widely combined with scaffolds to regulate cell activity [16–18]. However, the high cost and rapid degradation (*in vivo* with a short half-life of 7–16 min) of BMPs and other protein drugs also limit their clinical use [19]. Undoubtedly, there is an urgent need to develop alternative bioactive molecules with higher stabilities and lower costs than BMPs and other proteins to enhance the cell proliferation *in vitro*.

Abbreviations: IDPPS, icariin delivery porous PHBV scaffold; TCM, traditional Chinese medicine.

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http://dx.doi.org/10.1016/j.msec.2013.04.050

Epimedii herba (Yinyanghuo) is one of the most frequently used traditional Chinese medicine (TCM) in formulas that are prescribed for the treatment of osteoporosis in China. Icariin, ($C_{33}H_{40}O_{15}$; molecular weight: 676.67) the main active flavonoid glucoside isolated from Epimedium herba, is insoluble in water. It has been recently revealed that this fat-soluble icariin displays therapeutic effects on a rat model of mandibular distraction osteogenesis and shortens the course of distraction osteogenesis [20]. Interestingly, icariin influences the transcription of BMP2 in primary rat osteoblast cells [21] and stimulates the proliferation of pre-osteoblast MC3T3-E1 cells [22] and MG-63 cells [23]. Hence, it is possible that icariin could replace the quick degradable growth factors in scaffolds and promote osteoblast expansion *in vitro*.

PHBV, a biodegradable biomedical material, displays well-established drug delivery properties and capacity of controllable drug release [24,25]. In this study, novel icariin delivery porous PHBV scaffolds (IDPPSs) were fabricated by combining the solvent casting and salt leaching techniques. The porosity, morphology, and mechanical properties of IDPPSs were detected to evaluate their properties as novel type scaffolds for 3-D cell culture. To examine whether IDPPSs stimulate cell expansion, the cell proliferation of human osteoblast-like MG-63 and pre-osteoblast MC3T3-E1 cells was detected by Alamar blue assay following 1, 3, 5 and 7 days culture with IDPPSs, respectively. The morphology of the MG-63 cells cultured on IDPPSs was monitored by SEM as well. In order to deeply understand properties of IDPPSs as the icariin carrier, the release curve of icariin from PHBV matrix and the way of IDPPSs stimulating cell growth were investigated. Finally, the mRNA level of growth factors and ECM genes were tested by employing reverse transcription-polymerase chain reaction (RT-PCR) assay to reveal molecular mechanism of cellular growth enhancement induced by IDPPSs.

2. Materials and methods

2.1. Materials

Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) with 3% HV in mole fraction (Mw of 400 kDa) was purchased from Tianan Biology, China. Icariin (purity > 98%), isolated from TCM Epimedii herba, was purchased from Zelang Medical Technology, China.

2.2. Preparation of IDPPSs and porous PHBV scaffolds

A combination of the solvent casting and salt leaching technique was applied to prepare porous PHBV scaffolds. Firstly, icariin was dissolved in DMSO (Genview, USA) to obtain a series of solutions, which contained 0 mg/ml, 12.5 mg/ml, 17.5 mg/ml, 25 mg/ml and 50 mg/ml icariin, respectively. 2.5 g sieved sodium chloride (NaCl, 200–300 μm, Damao Chemical reagents, China) was mixed with 0.25 g of PHBV powder manually. A series of samples, containing 0%, 0.05%, 0.07%, 0.1% and 0.2% icariin respectively, were obtained by sonicate 1 ml chloroform, uniformly mixed NaCl/PHBV and 10 μl of icariin solution (0 mg/ml, 12.5 mg/ml, 17.5 mg/ml, 25 mg/ml and 50 mg/ml respectively) at 65 °C in a sealed container. Then the solvent gradually evaporated over 24 h at room temperature. Solid specimens were immersed in ddH_2O for three days with gentle agitation in beakers. During this period, every 6 h ddH_2O was replaced until the solution was free of chloride ions. The prepared scaffolds were dried for 24 h and then stored for further use.

2.3. Porosity of IDPPSs and porous PHBV scaffolds

The apparent porosity of the scaffolds was measured by using the mass technique on the basis of Archimedes principle described in the reference [26] by using double distilled water as the displacement iquid. A scaffold (dry weight W_d) was immersed in double distilled water in evacuation–repressurization cycles to force the double distilled water into the pores of the scaffold until no air bubbles emerged. Then the weight of the water-impregnated scaffold in water was measured as W_a. After that, the water-impregnated scaffold was removed from the container quickly, and the weight of the water-impregnated scaffold was determined as W_b. The porosity (ε) of the scaffolds was calculated by the following equations, $\varepsilon = (W_b - W_d) / (W_b - W_a)$.

2.4. Morphology observation

The cross-section microstructure of IDPPSs and the morphology of cells on the scaffolds were observed by FEI QANTA 200 scanning electron microscope (SEM, FEI Company, USA). All samples were glued on metal stubs and sputter-coated with gold (SCD050, BAL-TEC, Switzerland) two times before observation.

2.5. Mechanical properties of IDPPSs and porous PHBV scaffolds

The mechanical properties of the scaffolds were detected by an electromechanical machine (China) equipped with a 1000 N load range with a speed of 1 mm/min at room temperature. The compressive tests were carried out by using column-shaped specimens (Φ25mm × 50 mm). The compressive load and extension curve were graphed, and the maximum load was determined as well as the maximum stress. The compressive modulus was defined by the slope of the linear section between 0 and 3% strain. Uniaxial tensile tests were carried out on dog-bone specimens (width 20 mm, length 70 mm, thickness 4 mm) following the ASTM D1708. The tensile modulus was evaluated between 0 and 1% strain. The tensile strength and tensile strain at break were calculated considering the nominal cross-sectional area of the tensile specimen. At least five specimens were tested for each data.

2.6. Cell culture

MG-63 and MC3T3-E1 cell lines were both purchased from the Center of Cell Resource, Chinese Academy of Science Shanghai Institute for Biological Science (China) and propagated in RPMI 1640 (Thermo) supplemented with 10% (v/v) fetal bovine serum (FBS, Thermo), 1% antibiotics(100 U/ml penicillin and 100 g/ml streptomycin). The cells were incubated in glass flasks that were kept in a humidified CO_2 incubator (BB15, Thermo) at 37 °C with an atmosphere of 5% CO_2. During subculture procedure, the cells were harvested and washed with phosphate buffered saline (PBS) and trypsinized with trypsin–PBS (0.25% trypsin) for 5 min at 37 °C to obtain a cell suspension for passage or inoculation.

2.7. Cell seeding on the IDPPSs and porous PHBV scaffolds

All samples were autoclaved at 120 °C for 30 min. Sterilized scaffolds (Φ 15 mm × 2 mm) were prewetted in 1 ml RPMI 1640 medium supplemented with 10% FBS and incubated for 24 h in a 24-well plate (Greiner). Then, 200 μl culture medium containing 5×10^4 MG-63 cells or MC3T3-E1 cells was loaded to each sample and all samples with cells were kept in the humidified CO_2 incubator at 37 °C with an atmosphere of 5% CO_2 for 4 h. After 4 h incubation, all samples were washed with PBS buffer at 37 °C twice to remove unseeded cells and transferred to a fresh 24-well plate for further culture. A parallel control experiment was carried out by culturing 5×10^4 cells in the same 24-well plate.

2.8. Alamar blue assay

Alamar blue assay kit was purchased from Invitrogen and the detailed protocol was described in the previous publication [7]. All quantitative data were collected from six samples and expressed as the means ± standard deviation (s.d.). Statistical analysis was performed by using an SPSS 10 software, and values were considered significant at