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Preparation, physicochemical properties and biocompatibility of PBLG/PLGA/bioglass composite scaffolds

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ABSTRACT

In this study, novel poly(γ-benzyl L-glutamate)/poly(lactic-*co*-glycolic acid)/bioglass (PBLG/PLGA/BG) composite scaffolds with different weight ratios were fabricated using a negative NaCl-templating method. The morphology, compression modulus and degradation kinetics of the scaffolds were characterized. The results showed that the PBLG/PLGA/BG composite scaffolds with a weight ratio of 5:5:1, namely PBLG5PLGA5BG composite scaffolds, displayed a pore size range of 50–500 μm, high compressive modulus (566.6 ± 8.8 kPa), suitable glass transition temperature (46.8 ± 0.2 °C) and low degradation rate (>8 weeks). The *in vitro* biocompatibility of the scaffolds was evaluated with MC3T3-E1 cells by live-dead staining, MTT and ALP activity assays. The obtained results indicated that the PBLG5PLGA5BG composite scaffolds were more conducive to the adhesion, proliferation and osteoblastic differentiation of MC3T3-E1 cells than PBLG and PBLG/PLGA composite scaffolds. The *in vivo* biocompatibility of the scaffolds was evaluated in both SD rat subcutaneous model and rabbit tibia defect model. The results of H&E, Masson's trichrome and CD34 staining assays demonstrated that the PBLG5PLGA5BG composite scaffolds allowed the ingrowth of tissue and microvessels more effectively than PBLG/PLGA composite scaffolds. The results of digital radiography confirmed that the PBLG5PLGA5BG composite scaffolds significantly improved *in vivo* osteogenesis. Collectively, the PBLG5PLGA5BG composite scaffolds could be a promising candidate for tissue engineering applications.

1. Introduction

To mimic the microstructural features of extracellular matrix of natural bone, a variety of methods have been developed to prepare porous bone scaffolds [1]. The most representative methods are electro-spinning [2] and salt-leaching [3]. Electro-spinning is widely used to prepare polymeric biomaterial into fibers with diameters from a few micrometers down to the tens of nanometers [4]. Some studies have confirmed that the nanofibrous structures could provide suitable microenvironments for cell growth and proliferation [5]. Unfortunately, it is still difficult for electro-spinning to prepare complex porous structures with a high porosity over 90% [6]. High porosity provides more structural space for cell accommodation and facilitates the exchange of nutrient and oxygen as well as the discharge of metabolic waste, enhancing *in vivo* osteogenic potential [7,8]. Salt-leaching is an efficient method to fabricate foam-like structures with a high porosity due to its easy preparation process. However, the residual NaCl particles often exist in the final scaffolds [9]. As an improved method of salt-leaching, sintered NaCl template method may overcome the above problem and fabricate highly connected foam-like structures [10].

In recent years, composite scaffolds composed of polymer and inorganic constituents have widely been prepared and used for tissue engineering applications due to their improved physicochemical properties as compared to single-component scaffolds [11–14]. Due to good biocompatibility, biodegradability, minimal inflammatory reactions and modifiable active side groups—CO, poly(γ-benzyl L-glutamate) (PBLG) has been used in drug and gene delivery systems [15–20]. Porous PBLG scaffolds have been confirmed, for the first time, to possess good biocompatibility and be suitable for bone tissue engineering in our previously reported study [21]. However, a challenge of fabricating PBLG-based scaffolds remains to improve their mechanical properties in order to meet various needs of different tissue regeneration applications [22]. Poly(lactic-*co*-glycolic acid) (PLGA), one of the most successfully developed biopolyesters, has attracted considerable attention due to its attractive merits of desirable biocompatibility and biodegradability, simple synthesis route and controllable mechanical property by adjusting molecular weight. The US FDA has approved the use of PLGA in drug delivery, diagnostics and other applications [23]. The mechanical properties of scaffolds play an important role in regulating cell behavior [24]. To develop scaffolds with bioactivity and tunable

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physicochemical properties, composite scaffolds have attracted special interest [25].

In this study, a composite of PBLG, PLGA and bioglass (PBLG/PLGA/BG) was fabricated into foam-like scaffolds by a negative NaCl templating method. In such a system, PLGA and BG were employed to improve the mechanical property and osteoinductivity of PBLG, respectively. PBLG and PBLG/PLGA scaffolds with the same porous structure were used as control. The physicochemical properties of all scaffolds were characterized. MC3T3-E1 cells were used to evaluate the *in vitro* biocompatibility of these scaffolds. The *in vivo* tissue compatibility of the scaffolds, including blood vessel ingrowth and tissue infiltration, was evaluated in SD rat subcutaneous model, and the *in vivo* osteogenesis was assessed in a rabbit tibia defect model. The obtained results demonstrated that the PBLG/PLGA/BG composite scaffolds with a weight ratio of 5:5:1, namely PBLG5PLGA5BG composite scaffolds, were a more favorable substrate for MC3T3-E1 cells than PBLG and PBLG/PLGA scaffolds and showed better cell and tissue compatibility and osteogenesis ability, rendering the great potential for bone tissue engineering application.

2. Materials and methods

2.1. Materials

PLGA (LA:GA = 75:25, Mw = 300 kDa) was purchased from Jinan Daigang Biomateiral Co., Ltd. (Jinan, China). PBLG and BG were synthesized according to our previously reported procedures [22,26]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Aldrich. The LIVE/DEAD viability/cytotoxicity kit for mammalian cells was purchased from Invitrogen (Carlsbad, CA, USA). Minimum essential medium α (MEMα) and fetal bovine serum (FBS) were purchased from Procell (Wuhan, China). Alkaline phosphatase (ALP) kit, hematoxylin and eosin (H&E) staining kit and Masson's trichrome staining kit were purchased from BOSTER (Wuhan, China). MC3T3-E1 cells, New Zealand rabbits and Sprague Dawley (SD) rats were supplied by Medical Center of Xi'an Jiaotong University (Xi'an, China). All other reagents were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Chloroform was dried by refluxing over CaH_2 and distilled before use.

2.2. Preparation of foam-like scaffolds

To prepare scaffolds, porous negative NaCl templates were first prepared by sintering NaCl particles with a size range of 100–400 μm in cylindrical alumina molds at 550 °C for 2 h. In a typical process of obtaining PBLG/PLGA/BG composite scaffolds, the negative NaCl templates were completely immersed into a mixture of PBLG, PLGA and BG with different weight ratios in anhydrous chloroform in a beaker. The beaker was kept under −0.08 MPa for 30 min and then the infiltrated templates were taken out, air-dried, desalted with distilled water and dried under vacuum. The weight ratios of PLGA and PBLG were 0:1, 3:7 and 5:5, respectively, and the corresponding scaffolds were referred to as PBLG, PBLG7PLGA3 and PBLG5PLGA5 composite scaffolds. When BG was doped into PBLG5PLGA5 composite, the weight ratio of BG and polymer was fixed at 10%. The obtained scaffolds were called as PBLG5PLGA5BG composite scaffold.

2.3. Characterization of scaffolds

Morphology of the scaffolds was observed by scanning electron microscopy (SEM, S-3400 N, Hitachi, Japan). Prior to this, the surface of dried scaffold samples was coated with gold. The pore diameters of scaffolds were measured using Image J software. The compressive properties of the scaffold samples were measured using Instron 5943 universal testing instrument (Instron, USA) at a constant loading rate of 1 mm/min. The compressive modulus was calculated as the slope of the linear region from 10% to 20% deformation of the stress-strain curve. All samples were measured in triplicates. The *in vitro* degradation behavior of the scaffolds was evaluated by incubating them in phosphate-buffered saline (PBS) at 37 °C for 4 and 8 weeks and recording weight changes after pre-determined time periods. PBS solutions were replaced every three days. To determine the glass transition temperature (T_g), DSC curves were recorded from −30 to 120 °C using a differential scanning calorimeter (Netzsch DSC200 F3, Germany) in nitrogen atmosphere with a gas flow of 40 ml/min. The porosity of the scaffolds was measured using the Archimedes' principle, using ethanol as liquid medium. The porosity was calculated *via* the following formula:

$$\text{Porosity}\ (\%) = (m_2 - m_1)/(m_2 - m_3) \times 100$$

where m_1 is the dry weight of the scaffolds, m_2 is the weight of scaffolds saturated with ethanol and m_3 is the weight of scaffolds suspended in ethanol. Three scaffold samples were tested to calculate the average value.

2.4. Cell culture and seeding in scaffolds

MC3T3-E1 cells were cultured in MEMα supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin (Invitrogen, Carlsbad, CA, USA). The medium was replaced every 3 days and the cultures were maintained in a humidified 5% CO_2 incubator at 37 °C.

Cells were seeded onto scaffold samples as described below. The porous disc scaffolds with a diameter of 15 mm and a thickness of 3 mm were placed in a 24-well plate, and then 1.8 ml of cell suspension was dropped onto each scaffold. After placed in incubator for 4 h, cells began to adhere onto the surface of scaffolds. The cell-seeded scaffolds were incubated in a humidified 5% CO_2 incubator at 37 °C for 1, 3 and 5 days. About 3×10^4 cells were seeded on each scaffold.

2.5. ALP activity assay

The ALP activity assay was performed on days 1, 3 and 5 to evaluate the early osteogenic differentiation of MC3T3-E1 cells cultured on the scaffolds. Briefly, after a pre-determined incubation time period, the cell/scaffold constructs were washed thrice with PBS to remove the medium. The MC3T3-E1 cells in each scaffold were lysed in 200 μl of 1% Triton X-100 (Aladdin Reagent Inc., China). Lysates were centrifuged at 14,000 rpm for 30 min at 4 °C. 50 μl of supernatants were mixed with 150 μl ALP assay working solution according to the manufacturer's protocol (Beyotime, China). The total protein content was measured by the bicinchoninic acid protein assay kit (Pierce, USA). The optical density (OD) was measured at 520 nm on a plate reader.

2.6. MTT measurement

The MTT assay was used to evaluate the metabolic activity of MC3T3-E1 cells on the scaffolds. After 1, 3 and 5 days of incubation, the culture medium was removed and 100 μl MTT solutions were added to each well in a 48-well plate. The scaffold/cell constructs were incubated for 4 h at 37 °C in humidified atmosphere with 5% of CO_2. Next, the culture medium was removed and 200 μl DMSO was added to dissolve the blue formazan products. The solution was then transferred to a 96-well plate and the optical absorbency of the solutions was measured at 490 nm by using a microplate reader (PerkinElmer, EnSpire, America). Non-seeded scaffolds were used as a negative control.

2.7. Live/dead staining

The viability of MC3T3-E1 cells cultivated on the scaffolds was evaluated using the live/dead viability/cytotoxicity assay. On days 1, 3 and 5, the scaffold/cell constructs were rinsed with PBS and stained with