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Structure design and fabrication of porous hydroxyapatite microspheres for cell delivery

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ABSTRACT

Porous microspheres fabricated from bioceramics have great potential for cell delivery in injectable tissue engineering application. The size and structure of pores in the microspheres are important for the effective protection and transportation of cells. In this study, porous hydroxyapatite microspheres are fabricated through the water-in-oil emulsion method followed by a calcination treatment at the high temperature. Both self-made resorcinol-formaldehyde (RF) composite spheres and camphene are used as pore-forming agents to produce big pores corresponding to the size of RF spheres and connected channel among big pores in hydroxyapatite matrix. The properties of the microspheres are characterized using X-ray diffraction, thermogravimetry analysis, universal material machine, field emission scanning electron microscopy. Cell assays are carried out to evaluate the cellular compatibility of the microspheres. The results showed that the hydroxyapatite microspheres with controllable pore structure and high porosity could be fabricated by this method, which have better strength to resist the compressive force. The microspheres are conducive to support adhesion, proliferation and differentiation of MC3T3-E1 cells. The results indicate that the obtained porous hydroxyapatite microspheres can be a permeable microenvironment for cell delivery in injectable tissue engineering.

1. Introduction

Bone defects and damage as serious health problems have attracted more and more attentions. As one of the most reliable alternatives, hydroxyapatite has been paid so many attentions due to their high bioactivity, biocompatibility, and chemical stability under physiological conditions [1]. Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA), Ca/P = 1.67, slightly soluble in water, freely soluble in acid, and insoluble in alkali. The crystal of hydroxyapatite is hexagonal system, and its crystal cell parameters are a = b = 0.043–0.938 nm, c = 0.686–0.688 nm [2]. The angle between a and b axis is 120°, and there are 10 Ca^{2+}, 2 OH^-, and 6 PO_4^{3-} in the unit cell. These structures determine the surface properties of hydroxyapatite. A lot of therapeutic approaches are proposed to solve the question. These previous efforts generally focused either on the overall mechanical strength of the scaffolds or on the function of the cells cultured within the scaffold, but not both [3]. One attractive approach is the implantation of cell-attached scaffolds to form new bone directly [4], in which the development of suitable scaffolds to support and induce the growth of cells is a key factor for successful implantation [5]. Considerable efforts have been made to develop three dimensional (3D) porous scaffolds for tissue engineering application [6]. The purpose of these efforts is the restoration of tissue with a structure similar to that of native tissue and functionally compatible with it [7]. 3D structure could serve as a template to guide cell spreading and new tissue regeneration, but also supply proper route for nutrients and oxygen transport [8]. 3D porous microsphere is designed as an effective scaffold to protect cells and permit cells preincubation in them in vitro. Compared to the pre-shaped 3D foam scaffolds, these microspheres can be easily adapted to defective sites via an injectable implantable system for minimally invasive maxillofacial and orthopedic surgery [9]. For this purpose, some interesting studies have been developed to prepare biopolymers microspheres for cell delivery utilizing a range of methodologies [10–13]. Fabrication technique for microparticles scaffolds with inorganic composition and open surface pore for effective cell intrusion, however, are scarce.

Compared with polymer carrier, inorganic carrier holds some

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additional advantages besides the delivery and protection of cells, and also includes the higher mechanical strength and better stability in vivo. But one major challenge associated with the fabrication of inorganic microsphere carriers is to modulate pore structure of particles, including high bulk porosities, open pores on the surface and interconnected channel among pores, which is very important for the sufficient supply of nutrients, the cells immigration and elimination of metabolic waste products. Many attempts have been applied for fabricating inorganic microcarriers with porous inner structure, including spray freeze drying technique, thermal decomposition and spray drying [14–17]. But these methods of obtaining pore structures generally have their own characteristics due to the limit of procedure, including low porosities, heterogeneous size and structure of pores, etc. Hence, how to fabricate the shape-integrity microsphere carriers with uniform pore structure and interconnected channels remains a problem.

Considering the existing problems, in this study, we fabricate porous hydroxyapatite microsphere through a conventional water-in-oil emulsion method. Self-made resorcinol-formaldehyde (RF) microparticles are used as sacrificial template to produce big pores corresponding to the diameter of RF particles. Camphene, is used to cause interconnected channels among big pores. It was found that the method could produce hydroxyapatite microspheres with a useful combination of better compressive strength, interconnected pores, and adjustable pore sizes. Therefore, it can be inferred this carrier has a good promotion for cell adhesion and penetration.

2. Experiments

2.1. Materials

Resorcinol, formaldehyde, camphene, Poly(vinyl alcohol) (PVA), and polyacrylic acid (PAA) (Shanghai Aladdin Reagent Company, China), hydroxyapatite nanoparticle (Nanjing Emperor Nano Material Company, China), fetal bovine serum (FBS, Gibco, USA), α-MEM, phosphate buffered saline (PBS), and trypsin-EDTA solution(Thermo Fisher Biochemical products(Beijing) co., LTD, China), Cell Counting Kit-8, Alkaline Phosphatase Assay Kit, Dio fluorescent dye (Beyotime Institute of Biotechnology, China) and Mouse Osteocalcin(BGP) ELISA Kit (Beijing Chenglin biological technology co., LTD, China), and the analytical grade chemicals (Hangzhou Mike Chemical Agents Company, China) were purchased for this experiment without further purification.

2.2. Preparation of resorcinol-formaldehyde (RF) microspheres

The RF composite microspheres were prepared via a sol–gel method within water-in-oil emulsions. In a typical synthesis, 4.85 g (0.044 mol) of resorcinol and 7.16 g of formalin (37 wt % formaldehyde(0.088 mol)) were dissolved in 15 ml deionized water containing 0.03 g(0.283 mmol) sodium carbonate. The solution was then diluted to 50 ml. After pre-polymerization at 45 °C for 20 min, the solution was transferred to a glass reactor containing a mixture of 300 ml paraffin oil and 1 ml sorbitan monooleate (Span 80). Then, the mixture was stirred with the speed of 200, 300, or 400 rpm respectively at 85 °C for 15 min and aged at 85 °C for 2 days. The as-made products were separated from the solvent by filtration and washed with ethanol and water [18]. The spheres were dried in an oven at 60 °C for 24 h to obtain RF composite microspheres. After drying, the particles were screened with a standard sieve set to get samples. The particle size distributions of RF composite microspheres were measured with a dynamic light scattering analyzer (DLS, Malvern Mastersizer 2000) covering a wide size range of 0.02 and 2000 μm. The DLS was operated under the following conditions: dispersant of alcohol, particle concentration of 0.065 vol%, temperature of 25 °C [16]. Fig. 1 illustrates the morphologies and the particle size distribution of RF composite microspheres after screening.

2.3. Preparation of hydroxyapatite (HA) microspheres

The porous HA microspheres were prepared though the water-in-oil emulsion method. Firstly, 2 mL of 0.2% tris (hydroxymethyl) aminomethane and 2 mL of 0.3% PAA were added into 90 mL of 10% gelatin solution containing 2% polyvinyl alcohol at 50 °C. Then, 0.5 g HA nanoparticle, 3.5 g camphene and 0.2 g RF microspheres were added sequentially into 4 mL of the above-mentioned gelatin solution and kept stirring for 4 h at 50 °C in a closed environment to achieve a uniform HA suspension. The suspensions were injected into the vegetable oil by a suitable speed separately in ice bath cooling to allow the rapid solidification of gelatin, accompanying with a magnetic stirring of 300 rpm for 10 min. The obtained microspheres were separated from the oil and rinsed with ice-cooled acetone followed by ethanol for three times. After lyophilization, the microspheres were screened with a standard sieve set to get products with a diameter of 400–700 μm. The sieved samples were calcined at 1200 °C for 4 h in the muffle furnace at a heating rate of 5 °C/min. In the process, a series of RF microspheres with the diameters of 30, 60 and 120 μm were used respectively to cause different pore diameters in HA microspheres. The obtained HA microspheres were coded as H_{30}, H_{60} and $H_{120,}$ corresponding to the used RF composite microspheres size respectively.

The calcination condition of the microspheres was determined based on the result of the thermogravimetric analysis (TG, Pyris Diamond, PE, USA). H_{60} was used as model of sample and heated from 25 °C to 800 °C at a rate of 10 °C/min under the atmosphere of nitrogen. The strength of the HA microspheres were detected by compression tests using electronic universal material testing machine. The morphology and the pore structure of microspheres were investigated by a field emission scanning electron microscopy (FE-SEM, S-4800, Hitachi, Japan). The porosity of HA microspheres was characterized by 3D X-ray microscope (μ-CT, nanoVoxel-2000). The sintered HA microspheres were crushed into fine powders for X-ray diffractometer (XRD, ARLX'TRA, USA) analysis at ambient temperature with a range from 10° to 60° (40 kV, 3 mA, Cu, Kα); the scanning rate was 4°/min with a scanning step of 0.02°. FTIR spectrum was acquired using a Nicolet 5700 (USA) spectrometer with a resolution of 4 cm^{-1} averaging 128 scans from 4000 to 600 cm^{-1}.

2.4. Cell assay

Pre-osteoblastic MC3T3-E1 cells were cultured in culture flasks (25 cm^2) for 2–3 days in α-MEM supplemented with 5% FBS, 100u/mL penicillin and 0.1 mg/mL streptomycin at 37 °C in a 5% CO_2 incubator [6]. When an adequate quantity of cells was available, they were trypsinized and resuspended at a density of 1×10^5/mL in the complete medium. The HA microspheres were sterilized in autoclave. Subsequently, the sterilized microspheres (5 mg) were placed into a well of a 96-well plate, rinsed 3 times with phosphate-buffered saline solution (PBS) for 15 min each time and 1 time with α-MEM (5% FBS) for 1 h in the incubator [3]. α-MEM was removed and 50 μL cell suspension (1×10^5 cells/mL) was added into the well to incubate for 6 h. After this, another 150 μL α-MEM was added [19]. The medium was replaced every two days.

After the coculture for 1, 3, 5 and 7 days according to the instruction of manufacturer, the Cell Count Kit-8 (CCK-8) was employed to quantitatively evaluate the cell proliferation on the microspheres. And cell samples were fixed by 4% paraformaldehyde, then dehydrated in a gradient ethanol and