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Fabrication and characterization of 3D complex hydroxyapatite scaffolds with hierarchical porosity of different features for optimal bioactive performance

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ABSTRACT

To improve the biological performance of hydroxyapatite scaffolds in bone tissue engineering, graphite was used as porogen to create additional micro/nanoporosity to macroporosity, resulting in hierarchical porosity. For maximum imitation of natural bone structures, scaffolds with different porosity features were fabricated using micron/nano-sized graphite. The sintering profile of graphite treated scaffolds was optimised to reduce the influence of shrinkage. To confirm the porosity features, the micro/nanostructures of scaffolds were characterised by scanning electron microscopy and Brunauer-Emmett-Teller method. Considering that hydroxyapatite is resistant to biodegradation *in vivo*, the degradation rate of scaffolds in modified simulated body fluid was examined. Furthermore, biological evaluations based on myoblasts were carried out to investigate the influence of porosity features on the essential performance such as adhesion, proliferation and differentiation. The results indicate that the scaffolds with dominant microporosity and little nanoporosity formed inside had high potential for clinical applications due to improved performance in bioactivity.

1. Introduction

Apart from high porosity, complex and three-dimensional structures [1], biological performance in terms of biodegradable ability and bioactivity is also essential for scaffolds to achieve satisfactory regeneration in bone tissue engineering [2]. Owing to its similarity to the natural bones in chemical composition, HA (hydroxyapatite, $Ca_{10}[PO_4]_6[OH]_2$) has become an excellent biomaterial as substitutes of natural bones [3–6]. However, the uses of HA for bone regenesis have encountered a number of technical challenges, including its resistance to biodegradation *in vivo* [5], low fracture toughness, and high crystallinity [3]. Therefore, attempts have been made, *e.g.* introducing conditioners, to modify its performance, thus to overcome certain limitation and satisfy clinical needs [4,7,8]. So far, the major improvements to HA are concentrated on the material composition, with a few considered from the angle of modifying structures to achieve high bio-imitability to natural bones. This work is primarily concerned with the potential approaches to fabrication of 3D complex structures with hierarchical porosity in HA scaffolds, consisting of nanoporosity, microporosity and macroporosity [9], which is expected to further enhance the biological performance.

The importance of porosity and pore structures on osteogenesis had been extensively investigated [10–12]. In general, the nanoporosity in scaffolds could significantly promote osteoinductivity during bone tissue engineering by enhancing osteogenic differentiation [13,14], as many natural tissues like bone can be regarded as materials with some nanostructures. It was also found that both macro and micro porosity could influence osteoinductive activity to certain extent [10,11]. Particularly, the microporosity in the scaffolds is able to make substantial contribution to bone regeneration [15,16]. Adequate microporosity in scaffolds is also necessary to allow in-growth of capillaries to promote osteogenesis [15]. As for macroporosity, it forms 3D complex structures, which encourages migration and proliferation of osteogenic cells, as well as reinforces mechanical connection with adjacent tissues [4,12,17]. Nevertheless, it has presented limitations in existing traditional technologies due to uncontrollable distribution

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and sizes of pores in scaffolds [18–20], and recently developed 3D printing techniques are not capable of producing microporosity in nano scale [21–23]. Hence it is almost impossible to fabricate scaffolds with refined structures consisting of porosity across macro, micron and nano scale (nm–μm–mm) through these methods.

In this study, a new fabrication method combining the extrusion deposition [20] with porogen foaming technique was developed to construct highly porous HA scaffolds with evident hierarchical porosity [24]. The process is controllable and reproducible; it is able to create a wide range and scale of porosities with adjustable distribution in HA scaffolds, meanwhile with suitable mechanical strength. However, the scaffolds may exhibit significant difference in features and characteristics of hierarchical porosity, *e.g.* the ratios between microporosity and nanoporosity, which mainly depends on the particle size distribution of porogen. Therefore, it is important to evaluate the influence of such difference on biological performance of scaffolds in order to optimise the structures through the control of particle sizes and distribution of porogen. In this paper, we present the process of graphite as pore former to generate in-rod porosity (additional porosity inside the rods of scaffolds), which can permit the adjustment of microporosity and nanoporosity with nano graphite (nG) or micron graphite (μG). It was highly anticipated that further optimisation is possible to control the distribution of porosity in scaffolds, so that acceptable and optimal solution for bone tissue engineering may be achieved to meet the needs of clinical applications.

2. Material and methods

2.1. Fabrication of scaffolds

Scaffolds were fabricated by MAM system [20] (motor assisted micro-syringe system; MAM-II, Fochif, China) with commercially available nano HA (MH-HAp, Emperor Nano Material, China) and graphite powder in micron (300 mesh, Sinopharm Chemical Reagent, China) / nano (~400 nm; XF011, XF-Nano, China) size if necessary. Scaffolds were then sintered in furnace (KSL-1700, Kejing Materials Technology, China) at high temperature (1200 °C) [8,25]. Other reagents used to get well dispersed slurry with favourable properties for deposition include glycerol (Sinopharm), ammonia water (Sinopharm), nitric acid (Sinopharm) and NH_4PAA (ammonium polyacrylate; Goodben, China), and dispersant for graphite (HT100, Goodben) [20].

On the basis of previous work [24], three kinds of scaffolds, *i.e.* normal HA for control (marked as HA-n-c), HA with 25 vol% micron graphite as pore former (short for HA-μG-25%) and HA with 25 vol% nano graphite (HA-nG-25%), were prepared for characterization and evaluation in terms of microstructures and biological performance. In addition, to reduce the influence of shrinkage on in-rod porosity, TGA/DSC (thermo-gravimetric analysis / differential scanning calorimetry; TGA7, Perkin Elmer, China) was employed to record the sintering behaviour of normal and graphite treated scaffolds (HA-G).

2.2. Characterization of morphologies

The particle size of graphite (nG and μG) was measured by dynamic light scattering (DLS) nano-particle size analyser (LB550, Horiba Scientific, Japan). Due to poor liquidity, graphite samples were dispersed in a solution with ultrasonic vibration. The distribution and size of porosity was measured by BET method (Brunauer-Emmett-Teller; ASAP2020, Micromeritics Instrument, USA). And SEM (scanning electron microscopy; Nova NanoSEM 450, FEI, Netherlands) was used to observe the morphologies of HA scaffolds before and 1 d after cell culture. Prior to SEM, the scaffolds after cell culture were washed with phosphate buffered saline (Sinopharm) and later critical-point dried [26].

2.3. Assessment of biodegradation

Modified SBF (simulated body fluid) was prepared according to reference [27]. The materials for SBF were listed as follows: sodium chloride (AR, 99.5%; Aladdin Bio-Chem, China), sodium bicarbonate (AR, ≥99.8%; Aladdin), potassium chloride (AR, 99.5%; Aladdin), potassium dihydrogen phosphate (AR, 99%; Aladdin), magnesium chloride hexahydrate (AR, 98.0%; Aladdin), calcium chloride (AR, 96.0%; Aladdin), sodium sulphate (AR, 99%; Aladdin), tris(hydroxymethyl)aminomethane (≥99.9%; Aladdin), hydrochloric acid (1 mol/L; Sinopharm) and standard pH solution (Sinopharm). Since HA degrades very slowly in neutral environment [5], the SBF used in this study was acidic (pH =3) to accelerate the degradation process. To make sure that the result could offer possible reference to *in-vivo* environment, the assessment was performed on the condition of water bath at 37 °C (SHA-C, Hongke Instrument, China). The actual pH and weight loss was measured twice a week during the degradation test. Moreover, once the pH of SBF under test was larger than 5, the SBF would be renewed to maintain the actual degradation rate. The number of scaffolds (HA-n-c, HA-μG-25% and HA-nG-25%) for test was 6 each.

2.4. Evaluation of bioactivity

To evaluate the effect of scaffolds on proliferation and cell morphology, C2C12s (myoblasts, a cell line with a visually determinable differentiation profile) [28] were incubated in growth media consisting of HG-DMEM (high-glucose Dulbecco's modified eagle medium; HyClone, GE Healthcare Life Sciences) supplemented with 20% FBS (fetal bovine serum; FBS Biotech, PAN Biotech) and 1% P/S (penicillin/streptomycin, 10,000 U/mL and 10,000 μg/mL; Gibco, Thermo Fisher Scientific). C2C12s were exposed to scaffolds until they were confluent (4 days). To test the influence on differentiation capacity, C2C12s were grown until confluent in normal growth media (4 days) and then incubated with differentiation media, *i.e.* HG-DMEM, 2% donor equine serum (HyClone) and 1 μM insulin (Sigma-Aldrich), for three days.

At the end of experiments, cells were lysed or fixed for further analysis in dH_2O or methanol and acetone (50% v/v; Sigma-Aldrich) respectively. C2C12s used in this study were in passage 3–6 and seeded at 4500cells/cm^2 onto tissue culture plastic (DNA/protein analysis) or gelatine-coated (0.2%) coverslips (for imaging) set within a 6-well plate. The samples with C2C12s were all incubated in a humidified CO_2 incubator (Heracell 150i, Thermo Fisher Scientific) at 37 °C during the whole process. The immucytochemical images were photographed by a fluorescence microscope (D71, Olympus, Japan).

3. Results and discussion

3.1. Particle size of porogen

Hierarchical porosity in this paper refers to macroporosity with considerable amount of microporosity and nanoporosity inside the rods of scaffolds. The classification of above mentioned porosity was based on literatures and dimensions: macroporosity (≥100 μm, the least required size for cell survivability [29,30]), microporosity (1–100 μm, allowing in-growth of human micro-vessels or capillaries [31]) and nanoporosity (< 1 μm, significantly promoting osteoinductivity through enhancing osteogenic differentiation [13,14]).

Based on our experience, the size and shape of chemically formed micropores or nanopores would be closely related to the original porogen; that is, after sintering, the in-rod micro-/nano-structures of scaffolds are mainly determined by the particle size and distribution of porogen. Therefore, it is of great significance to analyse the particle size and distribution of nano and micron graphite so as to predict the formation of hierarchical porosity. According to the DLS result shown in Fig. 1, the particle size of nG was mainly focused on the range of 241