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Effect of Mineralized Layer Topographies on Stem Cell Behavior in Microsphere Scaffold

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Modifying substrates through mineralization is a popular way to improve the osteogenic performance. Screening of the best mineralization characteristics on specific substrates for stem cells is meaningful but not fully studied. In this paper, poly(lactic-co-glycolic acid)/hydroxyapatite (PLGA/HA, PH) microsphere scaffolds with superficial pores were fabricated by a low-temperature fusion method. After the mineralization in the 5× stimulated body fluid (SBF) for 0, 7, 12 and 24 h, four mineralized scaffolds (MPH-0, MPH-7, MPH-12 and MPH-24) with different apatite topographies were obtained. It was found that the surface of MPH-7 was evenly decorated with abundant micro-pores, MPH-12 with dense and plain apatite layer, and MPH-24 with small spherical bumps. The responses of mouse bone mesenchymal stem cells (mBMSCs) to the four scaffolds were further studied. The results showed that MPH-7 and MPH-24 had more obvious effects on mBMSCs attachment, proliferation and differentiation than MPH-0 and MPH-12. This work indicated that to obtain the maximum improvement, the mineralization characteristics had to be carefully chosen. This was noteworthy in the chemical modification of surfaces to form the functionalized scaffolds for bone repair.

1. Introduction

In tissue engineering, artificial scaffolds are designed as temporal templates mimicking certain features of the natural extracellular matrix (ECM) so as to support cell growth and guide three dimension (3-D) tissue formation. During bone regeneration, many factors of physical and chemical properties affect cell behavior in vivo due to the match between biomaterials and host tissue. Among these, surface topography of a mineralized scaffold where cells attached is an ignored matter.

When cell interactions with their natural ECM or scaffolds take place, surface topography plays a vital and complex role in guiding cellular functions such as cell attachment, proliferation and differentiation[1]. Up to now, there are many researches published on effects of diverse surface topographies like groove-ridge[2,3], micropatterns[4] and pit-hills on cell behaviors. In particular, surface morphology with holes has its own advantages. Tailoring dimension of surface holes can regulate deposition of proteins[5], cell attachment[6] and tissue migration[7]. In addition, different categories of cells can be divided because of different responses to the same pore topography[8]. In our previous study, we found that density and size of pores could influence the proliferation and osteogenic differentiation of cells on microspheres[9].

Although polymers like PLGA have a good biocompatibility and degradability, the lack of osteoconductivity discounts its performance especially in bone regeneration. It is well known that minerals like hydroxyapatite and calcium carbonate can obviously enhance substrate osteoconductivity[10]. However, in our previous work, surface minerals were finally eliminated and only PLGA substrate was left behind. Mineralization is a prevalent approach to form apatite on a substrate in the stimulated body fluid (SBF)[11,12]. The osteoconductivity of scaffolds after mineralization can be significantly improved[13,14]. So far, the overwhelming majority of researches just deal with how the presence of the mineralized layer influences cell behavior. To the best of our knowledge, studies on how topographies of mineralized layer affect stem cell behaviors are rarely reported, especially on the multi-porous scaffolds[15].

Given the situation mentioned above, hybrid PH microsphere scaffolds with abundant of superficial pores were first fabricated via a low-temperature fusion method in this study. The morphology, composition and structure of the scaffolds were characterized by

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scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR) and micro-scanning tomography (micro-CT). Then oxygen plasma[16] and 5× SBF were used to quickly induce mineralization[17] to fabricate scaffolds with different apatite topographies. The morphology, proliferation and differentiation of mBMSCs on the MPH scaffolds were characterized to figure out how topographies of mineralized layer regulated the cell behaviors. The aim of this study was to elaborate functionalization of bone regeneration scaffolds by means of screening the best mineralization characteristics on specific substrates for stem cells.

2. Materials and Methods

2.1. Materials

PLGA (lactide/glycolide ratio = 85/15, M_w = 100 kDa, inherent viscosity = 0.71 dl g^{-1}) was purchased from Daigang Biomaterials (Jinan, China). Poly(vinyl alcohol) (PVA, 87.0%–89.0% hydrolyzed, M_w = 44.05 kDa) and D-(+)-Gluconic acid δ-lactone (GDL) were obtained from Aladdin Industrial Co. (Shanghai, China). Dichloromethane (DCM) was of analytical grade and bought from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China), as with some inorganic salts[18] which made preparation for SBF.

2.2. Preparation of PH microspheres and scaffolds

PH microspheres were prepared using a single emulsion solvent evaporation technique (s/o/w)[19]. In brief, PLGA was dissolved in DCM as the oil phase. Then HA (2.1 μm) was added into the oil phase, sonicated and stirred to mix uniformly. Subsequently, the slurry was put into a 0.6% (w/v) PVA aqueous solution with appropriate amount of GDL and stirred for 90 min. Finally, the microspheres were washed three times with deionized water and collected after screening (250–450 μm).

The scaffolds were fabricated by the low-temperature fusion technique[20]. In brief, wet PH microspheres were poured into Teflon cylindrical molds (6 mm × 5 mm) and then put in the incubator at 37 °C for 48 h.

Plasma treatment was utilized to change the physical and chemical condition of scaffold surface. In brief, scaffolds were put into plasma processor (Plasmatechnology, Germany) and vacuumized to 0.07 mbar after sealing. The pressure was maintained at 0.3 mbar when bubbling O_2. The gas was in radio frequency glow discharge at low temperature, the power was 80 W and the processing time was 5 min.

Mineralized scaffolds were fabricated by mineralization in the SBF. The 5× SBF was prepared according to Barrere's recipe[18]. Then scaffolds were soaked in the 5× SBF system and placed in the shaker (37 °C, 90 rpm) for different time (0, 7, 12 and 24 h). Subsequently, scaffolds were rinsed with deionized water, lyophilized and collected as MPH-0, MPH-7, MPH-12 and MPH-24, respectively.

2.3. Characterization of microspheres and scaffolds

The morphologies of microspheres and scaffolds were characterized by SEM (Merlin, Zeiss, Germany), the accelerating voltage of 5 kV. Scaffolds were fixed at the edge of the stage with electroconductive paste, and the samples were sputter-coated with platinum using coater (Quorum, England). Micro-CT (XTV160H, X-TEK, England) was used to obtain the porosity, size distribution and the average pore size of scaffolds. In brief, samples placed in the micro-CT system were scanned along the long axis to obtain sequential images with a resolution of 1024 × 1024 pixels. Then 3-D binary image was built and relative data on pores was calculated by threshold value according to Mimics software (Materialise, Belgium).

An energy dispersive X-ray spectrometer (EDX, 30XLFEG, Philips, The Netherlands) was used to analyze the element content of microsphere surface. The chemical composition of microspheres and mineralized layer was detected by FTIR (Vector 33, Bruker, Germany). The scan range was from 4000 to 400 cm^{-1} with a resolution 2 cm^{-1}. An X-ray diffractometer (XRD, Empyrean, PANalytical, Holland) was used to detect the inorganic constituent of microspheres using slit diffraction plug-in and the mineralized layer using thin film diffraction plug-in. The scan range was from 10° to 80° and scan step size was 0.013° s^{-1}. The roughness of mineralized layers was examined by atomic force microscopy (AFM, Asylum Research, USA). Scaffolds were fixed on the glass by double-sided tape and a 10 μm × 10 μm zone was scanned.

2.4. Cells seeding

mBMSCs (ATCC, CRL-12424) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Gibio) with high glucose supplemented with 10% fetal bovine serum (FBS, Life Technologies, Gibco, USA). The four groups of scaffolds were placed in 96-well plates (one scaffold/well) and sterilized with cobalt-60 gamma irradiation. After prewetted with culture medium for 12 h, 100 μL of mBMSCs suspension were seeded onto the scaffolds with a density of 1×10^4 cells/well and incubated in a humidified incubator with 5% CO_2 at 37 °C. For cell differentiation, mBMSCs were seeded on scaffolds and cultured in osteogenic medium for 14 d, the medium was supplemented with osteogenic factors (dexamethasone, vitamin C and β-sodium glycerophosphate).

2.5. Characterization of cell behaviors

SEM was used to observe mBMSC morphologies on four groups of scaffolds. Specifically, mBMSCs cultivated on MPH-0, MPH-7, MPH-12 and MPH-24 for 1 d were washed by PBS and fixed using with 2.5% glutaraldehyde for 30 min at 37 °C. Then cells were dehydrated through a series of graded ethanol and free dried before observation.

mBMSCs proliferation was assessed by Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan). The scaffolds were transferred to new plates at the prescribed time points (1, 3, 5 days). Then 150 μL of CCK-8 working solution were added and incubated at 37 °C for 1 h. Subsequently, the supernatant medium was extracted and the absorbance at 450 nm was measured by Thermo 3001 microplate reader (Thermo, USA) (n = 5).

Alkaline phosphatase was stained by ALP staining kit (BCIP/NBT Membrance Phosphatase Substrate System, KPL, USA), following the manufacturer's instruction. The cell-seeded scaffolds were first fixed in 3.7% formaldehyde for 30 min after washed twice with PBS. After staining, the scaffolds were washed twice with PBS again and observed by inverted fluorescence microscopy (Eclipsc Ti-V, Nikon, Japan).

After cultured in osteogenic medium for 14 d, the cell-seeded scaffolds were immersed in TRIzol reagent (Invitrogen, USA), and homogenized with a pipette tip. RNA of the homogenized samples was then extracted following the manufacturer's protocol. Total RNA concentrations were quantified using NanoDrop2000 (Thermo Scientific). Subsequently, first-strand cDNA was synthesized using oligo (dT)-adaptor primer and AMV reverse transcriptase (TaKaRa, Tokyo, Japan). Real-time polymerase chain reaction (RT-PCR) was achieved using the SYBR green system (GeneCopoeia, USA). Amplifications for cDNA samples were carried out at 50 °C for 2 min, 95 °C for 10 min,