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Fabrication of novel biodegradable porous bone scaffolds based on amphiphilic hydroxyapatite nanorods

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ABSTRACT

This paper describes a new synthetic strategy and biological application for novel amphiphilic hydroxyapatite (HAp) nanorods. The prepared HAp nanorods were able to be dispersed in water, ethyl alcohol and cyclohexane. The co-anchoring of the multidentate ligands of PEG 20000 and hydrophobic oleic acid (OA) on the rods' surfaces endowed them with excellent amphibious properties. Utilizing amphiphilic HAp nanorods with excellent biocompatibility as the inorganic phase, human-like collagen (HLC) as the organic phase and natural genipin as the cross-linker, optimal HLC/HAp porous scaffolds (HLC: HAp = 1:4, w/w) were fabricated. The compression stress and three-point bending strength of the scaffolds with pore diameters of 150 to 200 μm reached approximately 3.4 MPa and 5.4 MPa, respectively, and their porosity was 77.35 ± 3.75%. Cytological tests showed that HLC/HAp scaffolds could contribute to cell proliferation and differentiation. The results indicated that these novel amphiphilic HAp nanorods can be expected to become recognized as an excellent inorganic material for the porous scaffolds used in repairing bone and related applications.

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1. Introduction

Due to the similar chemical compositions and structures to the mineral phase of native bone, hydroxyapatite (HAp) often serves as an important component of bioactive, biocompatible, osteoconductive and osteoinductive ceramic materials, which plays important roles in tissue engineering and other fields [1–5]. With the rapid increase of interest in HAp over the past decades, many types of composites involving HAp have been reported, but clinical applications have been limited because of the difficulty of controlling their morphology, surface properties, mechanical properties, degradation rate, etc. [6,7].

Previous results showed that the performance (e.g., osteoconductivity, protein adsorption, cell adhesion, etc.) of the composites would be enhanced if synthetic HAp could be made to resemble inorganic nano-building blocks of natural bone in terms of shape, size, and composition, etc. [8–12]. HAp nanoparticles must have suitable surface properties and uniform sizes to facilitate their combination with other biomaterials. So far, a variety of hydrophilic and hydrophobic HAp nanocrystals and their derivatives have been obtained using different methods [13–19]. However, most of HAp particles were synthesized in aqueous phase, and the as-prepared particles were too large and/or prone to aggregation, limiting their effective application. To control the morphology of nanocrystals, we have synthesized monodisperse HAp nanorods, nanotubes, nanospindles, and nanowires using the Liquid–Solid–Solution (LSS) strategy. The results possess hydrophobic interfaces [20–24]. HAp nanocrystals with controllable shapes and sizes usually have hydrophobic surfaces, which are unsuitable for biological systems.

How can the size and the surface characteristics of HAp nanocrystals be controlled at the same time? To date, amphiphilic HAp nanocrystals resembling biological macromolecules have rarely been reported. If HAp nanocrystals had surface properties similar to those of biological macromolecules such as proteins with hydrophilic and/or hydrophobic groups, the bonds between the inorganic and organic materials would be enhanced, and the mechanical and biological properties of the end product would be improved. Then, composite materials could be biomimetically assembled, further optimizing their performance.

Based on the Scheme 1, we have developed a simple and effective hydrothermal method for preparing uniform HAp nanorods with amphiphilic surfaces. The resulting HAp nanorods could be dispersed in water, ethyl alcohol and cyclohexane. Utilizing amphiphilic HAp nanorods with excellent biocompatibility as the inorganic phase, human-like collagen (HLC) as the organic phase and natural genipin as the cross-linker, HLC/HAp porous scaffolds were fabricated. Cytological and mechanical experiments indicated that these HAp nanorods had

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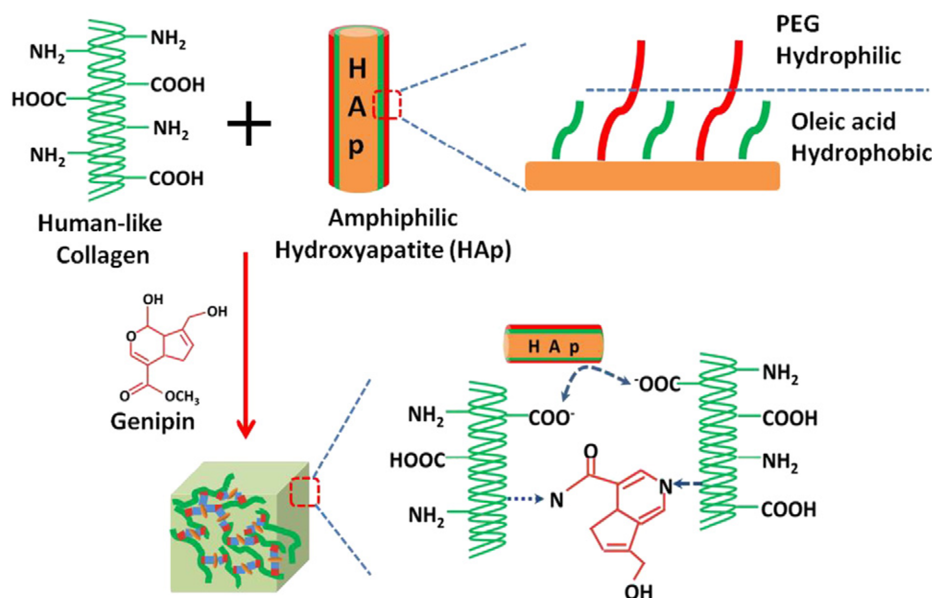

Scheme 1. Schematic representation of fabrication of novel biodegradable porous bone scaffolds by amphiphilic HAp nanorods, Human-like Collagen (HLC) and Genipin.

excellent biocompatibility and that the porous scaffolds based on them possessed better mechanical strength than those fabricated using commercially-available HAp (cHAp) nanorods with hydrophilic surfaces.

2. Materials and methods

2.1. Preparation of the amphiphilic HAp

HAp powder was prepared using the hydrothermal method. In a typical reaction, 4 mL of oleic acid, 16 mL of ethanol, and a varying amount of polyethylene glycol (PEG) were agitated in an autoclave with 40 mL of PTFE, and then 8 mL of 0.28 M $Ca(NO_3)_2$ solution was added and the mixture was stirred for approximately 10 min. Finally, 8 mL of 0.168 M Na_3PO_4 solution was added and the mixture was agitated violently for 10 min (the Ca/P molar ratio was 1.67, based on the standard stoichiometry for pure HAp). The mixture was sealed and hydrothermally treated at 100 °C for 10 h. After it had cooled to room temperature, the resulting deposits were washed twice with cyclohexane, anhydrous ethanol and distilled water, then dried at 80 °C for 10 h.

2.2. HAp characterization

The crystal structure and phase purities of the prepared samples were verified by means of X-ray diffraction (XRD) using a Rigaku D/max 2500PC diffractometer. Phases were identified by comparing the diffraction patterns of the HAp with International Center for Diffraction Data (ICDD) standards. The sizes and morphologies of the products were observed with a Hitachi H-1200 transmission electron microscope (TEM) and a JEOL JEM-2010F high-resolution transmission electron microscope (HRTEM). Fourier transform infrared spectroscopy (FTIR) was performed using a Nicolet 560 IR spectrometer with wave numbers between 400 and 4000 cm^{-1} to find the characteristic peaks of the HAp powder. An element analysis of the samples was performed using a JEOL JSM-6700F scanning electron microscope to perform X-ray energy dispersive spectroscopy (EDS).

2.3. Biocompatibility of the HAp

The cell viability of the uniform amphiphilic HAp nanorods was estimated by measuring the viability of MC3T3-E1 cells using the Cell Counting Kit-8 (CCK-8). MC3T3-E1 cells were cultured in α-MEM medium containing 10% FBS. To prepare different concentration HAp nanorods solutions, a stock solution of 320 g L^{-1} was pre-prepared by immersing HAp nanorods in α-MEM medium containing 10% FBS. After being incubated at 37 °C for 24 h, serially diluted HAp solutions (20, 40, 80, and 160 mg L^{-1}) were prepared by diluting the stock solution of 320 g L^{-1} with α-MEM medium containing 10% FBS. These solutions were filtered through 0.22 μm filter membranes in preparation for culturing. MC3T3-E1 cells were cultured on 96-well plates at a density of 3×10^3 cells/well in a CO_2 (5%) incubator at 37 °C for 24 h. Subsequently, the culture medium was replaced with prepared HAp solutions. The groups of cells incubated in the presence of HAp nanorods were used as the test groups, and the groups of cells incubated in the absence of HAp nanorods were used as the control groups, and the cell-free culture medium incubated at 37 °C was used as the blank groups. After being incubated for 24 h, the culture media were removed and 10 μL of CCK-8 plus 100 μL of the α-MEM medium containing 10% FBS were added to each well, and the cells were incubated at 37 °C for an additional 3 h. The optical density (OD) of samples was measured at 450 nm using a microplate reader (Power Wave XS2, Gene Company, USA). The OD of samples in the test, the control and the blank groups were labeled as OD_{test}, $OD_{control}$ and OD_{blank}, respectively. The cell viability was calculated using the following formula: Cell viability (100%) = $[OD_{test} - OD_{blank}] / [OD_{control} - OD_{blank}] \times 100\%$. All the samples were analyzed in quintuplicate and the results were averaged and presented as the mean ± standard deviation (SD).

The cell morphology was observed using the vital dye CFDA as an indicator. The 24-well plates were seeded with MC3T3-E1 cells at a density of 9×10^3 cells/well and incubated at 37 °C for 24 h. Next, HAp solutions were added to the wells. After incubation for 24 h, the supernatant was removed and 20 μL CFDA and 100 μL of α-MEM medium containing 10% FBS were added to each well to stain at 37 °C for 30 min. The cell morphology was visualized using an inverted microscope (Nikon Eclipse TE2000-U, Japan). The excitation/emission light was blue and green.

2.4. Scaffold preparation

An HLC solution (13.6%, w/v) was prepared by dissolving HLC in deionized distilled water at room temperature. HAp precipitates were added into HLC solution at ratios of 1:3, 1:4, or 1:5 (HLC:HAp, w/w) under ultrasonic treatment. After the mixture became homogeneous, it was transferred into a mold and frozen at −70 °C for 3 h, then

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