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Hydroxyapatite coatings with oriented nanoplate and nanorod arrays: Fabrication, morphology, cytocompatibility and osteogenic differentiation

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ABSTRACT

Hydroxyapatite (HA) crystals exhibit rod-like shape with *c*-axis orientation and plate-like shape with *a*(*b*)-axis orientation in vertebrate bones and tooth enamel surfaces, respectively. Herein, we report the synthesis of HA coatings with the oriented nanorod arrays (RHACs) and HA coatings with oriented nanoplate arrays (PHACs) by using bioglass coatings as sacrificial templates. After soaking in simulated body fluid (SBF) at 120 °C, the bioglass coatings are hydrothermally converted into the HA coatings via a dissolution-precipitation reaction. If the Ca/P ratios in SBF are 2.50 and 1.25, the HA crystals on the coatings are oriented nanorod arrays and oriented nanoplate arrays, respectively. Moreover, the bioglass coatings are treated with SBF at 37 °C, plate-like HA coatings with a low crystallinity (SHACs) are prepared. As compared with the Ti6Al4V and SHACs, the human bone marrow stromal cells (hBMSCs) on the RHACs and PHACs have better cell adhesion, spreading, proliferation and osteogenic differentiation because of their moderately hydrophilic surfaces and similar chemical composition, morphology and crystal orientation to human hard tissues. Notably, the morphologies of HA crystals have no obvious effects on cytocompatibility and osteogenic differentiation. Hence, the HA coatings with oriented nanoplate arrays or oriented nanorod arrays have a great potential for orthopedic applications.

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1. Introduction

Carbonated calcium-deficient apatite is the major inorganic constituent of natural bones, and corresponding synthetic hydroxyapatite (HA) has great potentials for reconstructions of skeletal defects [1–4]. HA coatings on medical metal substrates have great potentials for orthopedic and dental applications under load-bearing conditions because they combine the mechanical advantages of titanium alloys with the excellent biological properties of HA [5–7]. As we know, HA crystals exhibit rod-like shape with *c*-axis orientation in vertebrate bones and plate-like shape with *a*(*b*)-axis orientation in tooth enamel surfaces. However, the fabrication of HA coatings with specific orientation and morphology remains a great challenge.

Hexagonal HA crystals belong to $P6_3/m$ space group with crystal parameters $a = b = 0.9432$ nm, $c = 0.6881$ nm, $\alpha = \beta = 90^\circ$ and $\gamma = 120^\circ$ [8–11]. Two major crystal planes with different atomic arrangements are mainly present on the HA crystal, including *a*(*b*)-plane (*ac* and *bc* crystal faces) with rich positively charged calcium ions and *c*-plane (*ab* crystal face) with adequate negatively charged phosphate ions and hydroxyl groups [11–13]. HA crystals in vertebrate long bone surfaces have a *c*-axis orientation, leading to the development of the *a*(*b*)-plane; while they have an *a*(*b*)-axis orientation in tooth enamel surfaces, leading to the development of the *c*-plane [14–15]. The *a*(*b*)-plane and *c*-plane exhibit anisotropic characteristics, such as biomechanical property, protein adsorption and biocompatibility. Highly *c*-axis oriented HA coatings have higher hardness and Young's modulus values than the values of randomly oriented coatings [16]. Kandori et al. have reported that the large rod-like particles with flat surfaces are advantageous for the adsorption of negatively charged bovine serum albumin by the large number of positively charged adsorption sites on the exposed *ac* or *bc* crystal faces [17–18]. The adsorption of bovine serum albumin on the micrometer-sized HA particles exhibits a strong dependence on the particle length of the HA particles, while that of lysozyme on the HA particles shows a minor dependence [17–18]. Moreover, both well aligned, ordered RHACs and PHACs can promote cell initial adhesion and long-term growth [19–22]. Is the biocompatibility and osteogenic differentiation of the RHACs with *c*-axis orientation similar to the PHACs with *a*(*b*)-axis orientation? However, the above question is rarely addressed in previous reports.

Recently, HA coatings with specific orientations and morphologies have been fabricated by electrochemical method, pulsed laser deposition method, ZnO-seeded method and hydrothermal method [23–27]. The HA crystals on the above coatings exhibit preferred *c*-axis

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orientation or $a(b)$-axis orientation, but their chemical composition, crystallinity and morphology are still different from human hard tissues. Fortunately, research findings on the biomineralization mechanism of natural bones provide an important way to design and fabricate ideal HA coatings with specific orientations and morphologies. In bone minerals, an amorphous calcium phosphate (ACP) serves as a precursor to create a highly loaded mineral/organic composite with HA nanocrystals oriented in the [001] direction along the long axis of collagen fibers [28]. In our previous work, bioglass coatings ($CaO—SiO_2—P_2O_5$) resemble the ACP in natural bone, and SBF resemble the human plasma. The bioglass coatings are used as the precursors to convert to HA coatings with oriented nanorod arrays [19,20]. However, the fabrication of HA coatings with oriented nanoplate arrays is rarely reported up to now, and the effects of oriented HA nanorod arrays and oriented HA nanorod arrays on the biological properties still remain unknown. Herein, based on the different Ca and P amounts in the $a(b)$-plane and c-plane of HA crystals, we develop RHACs and PHACs by controlling the Ca/P ratios in SBF. The main aims are to study the formation mechanism of RHACs and PHACs, and to investigate the cytocompatibility and osteogenic differentiation of the HA coatings with specific crystal orientation and morphology.

2. Experimental

2.1. Preparation of bioglass coatings

Bioglass coatings ($CaO—SiO_2—P_2O_5$) were prepared according to the literature [20]. In brief, calcium nitrate (2.80 g), tetraethyl orthosilicate (TEOS, 12.30 mL), triethyl phosphate (0.70 mL), deionized water (3.00 mL) and ethanol (58.00 mL) were mixed, followed by addition of nitric acid solution (2.0 mL, 1.0 mol/L) as catalytic hydrolysis. The mixtures were stirred for 4 h at 40 °C, and then aged at 50 °C to form the sol. Titanium alloys (Ti6Al4V) substrates were dipped in the above sol, and were withdrawn in a rate of 1 mm/s followed by calcination at 400 °C for 1 h. The dipping and heating cycle was repeated three times.

2.2. Preparation of HA coatings

A simulated body fluid (SBF) with ion concentrations approximately equal to those of human blood plasma has been used widely to investigate the *in vitro* bioactivity of biomaterials (Table 1). SBFA with a similar ionic composition to human blood plasma was prepared by dissolving analytical reagent grade chemicals of NaCl, $NaHCO_3$, KCl, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2$, $(CH_2OH)_3CNH_2$ and Na_2SO_4 into deionized water, and buffering it at pH 7.40 with hydrochloric acid. In addition, the revised SBF (SBFB) was prepared under the same conditions but the change of P concentrations, as shown in Table 2. The bioglass coatings and 20 mL of SBF (SBFA or SBFB) were sealed in Teflon-lined stainless steel autoclaves, and reacted hydrothermally at 120 °C for 12 h ~ 2 days. Finally, the HA coatings with different morphologies were washed with deionized water and dried at room temperature. The HA coatings with oriented nanorod arrays (RHACs) were converted from the bioglass coatings in SBFA at 120 °C for 12 h, and the HA coatings with oriented nanoplate arrays (PHACs) were converted from the bioglass coatings in SBFB at 120 °C for 2 days. In addition, the bioglass coatings were soaked in 20 mL of SBFA at 37 °C for 24 h. The obtained HA coatings (SHACs) as control samples were washed with deionized water and dried at room temperature. The formation procedures of HA coatings with different morphologies are shown in Fig. 1.

Table 1
Ion concentration of human plasma and SBF ($\times 10^{-3}$ mol/L).

Ion concentration	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
Human plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5
SBF	142.0	5.0	1.5	2.5	147.8	4.2	1.0	0.5

Table 2
Order, amounts and purities of reagents for preparing 1000 mL of SBFA and SBFB.

Order	Reagent	SBFA	SBFB	Purity (%)
1	NaCl	8.035 g	8.035 g	99.5
2	$NaHCO_3$	0.355 g	0.355 g	99.5
3	KCl	0.225 g	0.225 g	99.5
4	$K_2HPO_4 \cdot 3H_2O$	0.231 g	0.462 g	99.0
5	$MgCl_2 \cdot 6H_2O$	0.311 g	0.311 g	98.0
6	1.0 M HCl	39 mL	39 mL	99.0
7	$CaCl_2$	0.292 g	0.292 g	95.0
8	Na_2SO_4	0.072 g	0.072 g	99.0
9	Tris	6.118 g	6.118 g	99.0
10	1.0 M HCl	0 ~ 5 mL	0 ~ 5 mL	99.0

2.3. Characterization

The morphologies of samples were investigated by using scanning electron microscopy (SEM, Hitachi S-4800, CamScan) with energy-dispersive spectrometry (EDS). Transmission electron microscopy (TEM) and electron diffraction (ED) were detected in CM200/FEG (Philips) at 200 kV. The HA samples were scrapped from the coatings and then embedded in epoxy resin. The crystalline phases of samples were examined with X-ray powder diffraction (XRD, D/max-II B, Japan) using CuKα radiation ($\lambda = 1.541874$ Å) within the scanning range of $2\theta = 10°$ to $60°$. Fourier transform infrared spectra (FTIR, VECTOR22, BRUKER) were collected to analysis the functional groups at room temperature by using the KBr pellet technique, working at the wavenumber range of 4000-400 cm^{-1}. Surface wettability of samples was determined by sessile drops of water on the coating surfaces via contact angle measurement.

2.4. Cell behaviour of hBMSCs

2.4.1. Culture of hBMSCs

The study was approved by the Ethic Committee of the Ninth People's Hospital of Shanghai Jiao Tong University. hBMSCs were isolated and expanded by using a modification of standard methods as described previously [29]. The donor was healthy without metabolic disease, inherited illnesses or other diseases that might affect the current study. Cells were grown in complete Alpha Minimum Essential Medium (α-MEM; GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Tauranga, New Zealand) and antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mL; Hyclone, Logan, UT, USA). The medium was replaced every 48 h, and the cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4.2. Morphology and viability of hBMSCs

The cell morphology and spreading of hBMSCs on samples were investigated by detecting cytoskeletal filamentous actin. The hBMSCs were cultured on HA coatings at a density of 5×10^4 cells/sample for 1 or 3 days in a 12-well plate at 37 °C. After 24 h of incubation, the samples were gently washed with PBS and maintained in 4% paraformaldehyde for 15 min, followed by immersing in 0.1% Triton X-100 solution for 15 min. Tetramethyl rhodamine-6-isothiocyanate (TRITC) phalloidin was used to stain the actin filaments of hBMSCs as red fluorescent light, and 4′,6-diamidino-2-phenylindole was used to stain the nucleus as blue fluorescent light. The cytoskeleton of hBMSCs was visualized with a laser scanning confocal microscope (LEICATCS-SP2). The SEM of hBMSCs was used to study cell morphology and cell-coating interaction. Briefly, the hBMSCs were cultured on samples at 5×10^4 cells/sample in a 12-well plate. After 1 day or 3 days, the medium was removed, and then the cells were fixed with 2.5% glutaraldehyde overnight at 37 °C. The fixed cells were dehydrated by increasing the concentration

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