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Influences of magnetized hydroxyapatite on the growth behaviors of osteoblasts and the mechanism from molecular dynamics simulation

Weihu Yang, Xingfeng Xi, Jiajia Fang, Peng Liu, Kaiyong Cai *

Key Laboratory of Biorheological Science and Technology, Ministry of Education College of Bioengineering, Chongqing University, Chongqing 400044, China

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ABSTRACT

To investigate the influence of magnetized hydroxyapatite on the growth and differentiation of osteoblasts, hydroxyapatite (HA) and magnetized hydroxyapatite (mHA) were synthesized and characterized. The cell viability, differentiation, and morphologies of osteoblasts were investigated in vitro, respectively. The results showed that compared to HA, cells cultured with mHA had better cell viability, and both HA and mHA were beneficial to the early differentiation of osteoblasts. Furthermore, the interaction mechanism between mHA and osteoblasts was elucidated using a molecular dynamics simulation. The simulation results indicated that when cultured with osteoblasts, HA adsorbed bovine serum protein onto its surface from the medium immediately, which was beneficial to the adhesion and proliferation of osteoblasts. The main driving force for the adsorption of bovine serum was the electronic properties of HA crystal faces. The (211) crystal face of HA had the highest electron density among its all crystal faces, thus mainly contributing to the protein adsorption of HA. Nevertheless, the (211) crystal face of mHA still had a relatively higher electron density than that of HA, thus possessing better protein adsorption than that of HA, and in turn promoting the biological functions of osteoblasts.

1. Introduction

For most cell types, including osteoblasts and mesenchymal stem cells (MSCs), their cellular responses are highly influenced by many factors such as physical stimulations [1], chemical signals [2], and biological factors [3]. Among them, magnetic stimulation presents itself as an efficient tool for the regulation of the biological functions of cells. Previous studies demonstrated that magnetic stimulations deriving from either a static or a pulsed electromagnetic field promoted the adhesion [4,5], proliferation [6] and differentiation [7] of osteoblasts. Thus, the magnetic stimulation has become to be one of the promising methods for accelerating bone repair.

Hydroxyapatite (HA) has been widely used in biomedical community, mainly because it has a structure very similar to the mineral component of natural bone. Previous studies demonstrated that it could not actively stimulate progenitor cells to differentiate into osteoblasts, although normal hydroxyapatite had good biocompatibility [8,9]. Furthermore, it had no cytological function itself [10]. Therefore, many efforts have been made to impose it with biological properties, such as the capability to induce the osteoblastic differentiation of mesenchymal stem cells (MSCs) or to accelerate the mineralization of osteoblasts [11–14]. Considering the positive effects of magnetic stimulation on osteoblasts, it is reasonable to develop magnetized HA (mHA) with magnetism property. Previously, Huang et al. [15] proved that the proliferation and differentiation of osteoblasts were promoted when cultured with HA coated Fe_3O_4 nanoparticles. Moreover, the mHA could also be used in other biomedical fields, such as cell labeling and separation, immunoassay, drug delivery, MRI contrast agents, and heavy metals removal, and so forth [16–18].

In previous studies [19,20], we have synthesized Fe_3O_4 magnetic particles with different sizes and morphologies, and produced mHA particles by co-precipitation method. Furthermore, the distribution of the magnetic particles within HA structure was also investigated [21]. However, few work documented the biological properties of mHA [22] and no study was found to investigate the interface between crystal faces of mHA and osteoblasts.

It is critically important to understand the interface between protein molecules and biomaterials for the development of biomaterial, since the adsorbed proteins actually act as the intermediate layer to contact cells and tissue once a biomaterial is implanted. The biomaterial surface would adsorb the proteins from blood (or serum) immediately, which effectively translates the structure and composition of the foreign surface into a “domestic” biological language. It is to this language that the cells respond, contributing to the ultimate outcomes in both implantation and tissue culture situations [23]. Among all interfaces, the organic–inorganic interface is of great importance since it provides the active sites for the regulation of biological functions, such as cell adhesion, proliferation and differentiation. Additionally, the interfaces between proteins and crystals are believed to be very important in terms of biomineralization. Such interfaces

* Corresponding author at: College of Bioengineering, Chongqing University, Chongqing 400044, China. Tel.: +86 23 65112619; fax: +86 23 65112877.
E-mail address: kaiyong_cai@cqu.edu.cn (K. Cai).

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were proposed to be responsible for the nucleation of crystals in mineralizing tissues, the inhibition of crystallization in soft tissues, formation of particular crystalline polymorphs, and biological control over the crystal orientation, growth, and morphology [24].

Despite its importance, there are still no experimental methods to investigate the interaction mechanism due to the complexity of cell–biomaterial interfaces. Nevertheless, it is possible to identify some important elements in this system by simulation method. Computational approaches could help integrate experimental data and create plausible structural models of proteins on solid surfaces. Several research groups [25–27] have simulated proteins and peptides on solid surfaces using detailed atomistic approaches such as molecular dynamics (MD) and Monte Carlo methods.

The objective of this study was to comparatively investigate the influences of HA and mHA on the growth behaviors of osteoblasts (i.e., cell viability, morphology and differentiation). In addition, we intended to clarify the interaction mechanism between HA/mHA and bovine serum protein with computation simulation method.

2. Experimental

2.1. Materials

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), alkaline phosphatase (ALP), and bicinchoninic acid (BCA) assay kit were purchased from Sigma Chemical Co. (Beijing, China). All other chemicals used were analytical reagent grade, which were supplied from Oriental Chemical Co. (Chongqing, China).

2.2. Nanoparticle synthesis and characterization

Magnetic iron oxide nanoparticles and magnetized HA were prepared according to previous studies [19,20], respectively. Briefly, magnetic nanoparticles were synthesized with a hydrothermal method. Meanwhile, HA and magnetized HA were prepared by a co-precipitation method. All samples were washed with excessive deionized water to neutral. The powder of magnetized HA was washed with 0.1 M HCl aqueous solution to eliminate the residual Fe_3O_4.

To investigate the crystalline content and crystal structure, X-ray diffraction (XRD, model DX-2600, Fangyuan, China) with a copper target was employed. The 2θ angle was varied from 10° to 90° with a step of 0.03°. The software of Jade 6.0 was employed to refine the data of XRD and to reveal the lattice parameters and cell volumes of the samples. Fourier transform infrared spectroscopy (FTIR, model 6300, Bio-Rad Co., USA) was performed with wavenumber in the range of 400–4000 cm^{-1}. All the samples were ground and autoclaved for sterilization before cell seeding.

2.3. Cell culture

Osteoblasts were isolated via a sequential collagenase digestion from neonatal rat calvaria according to an established protocol. Cells were cultured in Dulbecco's Modified Eagle medium (DMEM; Gibco) supplemented with 10% bovine serum (FBS; Gibco) under 5% CO_2 atmosphere at 37 °C. Media was changed every two days. After confluence, the cell monolayer was washed twice with phosphate-buffered saline (PBS) and incubated with trypsin–EDTA solution (0.25% trypsin, 1 mM EDTA; Gibco) to detach the cells. The effect of trypsin was then inhibited by adding the complete medium at room temperature. Then, the cells were re-suspended in complete medium for re-seeding and growing in new culture flasks. Osteoblasts at the third passage were used for the following cell experiments. The initial cell seeding density was 1×10^4 cells $well^{-1}$ (24-well plate) in this study.

2.4. Cell viability

Cell viability was determined by MTT assay, which is based on the mitochondrial conversion of MTT. In brief, osteoblasts were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in a 24-well plate at a volume of 700 μL/well and a seeding density of 1×10^4 cells/well with HA nanoparticles (200 μg/mL, according to Webster's work [28]), mHA nanoparticles (200 μg/mL) and without nanoparticles. Nanoparticles were sonicated and centrifuged before being added to the cells. The cells were incubated at 37 °C for 7 days. The medium was replaced every other day. Then, 100 μL of MTT (5 mg/mL) was added to each well and incubated at 37 °C for another 4 h. Finally, MTT-containing medium was removed, and 0.5 mL of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystal, and the solution was transferred to a new EP tube. After centrifugation, the suspension was used for the assay. The absorbance was measured by using a microplate reader (Bio-Rad 680) at the wavelength of 490 nm. Each treatment was performed six times. The mean value was used as the final result.

2.5. Total intracellular protein content assay

The assay was performed according to a previous study [29]. Osteoblasts cell were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in a 24-well plate at a volume of 700 μL/well and a seeding density of 1×10^4 cells/well with HA nanoparticles (200 μg/mL), mHA nanoparticles (200 μg/mL) and without nanoparticles. Nanoparticles were sonicated before being added to the cells. The cells were incubated at 37 °C for 7 days. The medium was replaced every other day. Next, osteoblasts were lysed by 1% Triton X-100 with three freeze–thaw cycles. Total protein content in the cell lysates was measured with a BCA assay kit. The absorbance was measured with a spectrophotometric microplate reader (Bio-Rad 680) at a wavelength of 570 nm. The total intracellular proteins (expressed as mg) produced by osteoblasts were determined from a standard absorbance curve versus known concentration of albumin run in the parallel experiment. Each treatment was performed six times. The mean value was used as the final result.

2.6. Alkaline phosphatase activity assay

The ALP activity assay was performed according to a previous study [30]. Cell lysate was prepared as mentioned above. Paranitrophenyl phosphate was employed as the reference substrate to determine the ALP activity of osteoblasts cultured on a 24-well plate with different nanoparticles. The absorbance was measured with a spectrophotometric microplate reader (Bio-Rad 680) at a wavelength of 405 nm. The ALP activity (expressed as μmol of converted p-nitro-phenol/min) was normalized by the total intracellular protein production. The ALP activity was thus expressed as μmol p-nitrophenol/min/mg protein. Each treatment was performed six times. The mean value was used as the final result.

2.7. Immunofluorescence staining

The assay was performed according to a previous study [31]. Osteoblasts were cultured with DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (P/S) in a confocal dish at a volume of 700 μL with an initial seeding density of 1×10^4 cells. Osteoblasts were co-cultured either with HA nanoparticles (200 μg/mL) or mHA nanoparticles (200 μg/mL). The sample of osteoblasts cultured without any nanoparticles was used as control. After 2 days of culture, cells were fixed with 2% glutaraldehyde at 4 °C for 20 min. Samples were then washed with PBS and permeabilized with 0.2% Triton X-100 at 4 °C for 2 min. Next, the treated samples were washed