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Silicon effect on the composition and structure of nanocalcium phosphates In vitro biocompatibility to human osteoblasts

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ABSTRACT

Nanostructured calcium phosphates, such as nanohydroxyapatite (HAP) and HAP with silicon content (HAP-Si) of 0.47 wt.% (1% SiO_2), 2.34 wt.% (5% SiO_2) and 4.67 wt.% (10% SiO_2) in the final product, were synthesized by aqueous precipitation, freeze dried and then calcined at 650, 950 and 1150 °C. The obtained materials were investigated by X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectrometry, transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) imaging. From the analysis of the XRD patterns, the HAP and β-tricalcium phosphate (β-TCP) phases were identified and their amounts in the samples were estimated. The size of HAP and β-TCP crystallites was estimated to be in the nanocrystalline domain. FTIR spectra showed the presence of characteristic vibrations for P–O, H–O and Si–O groups and their modification with Si content and calcination temperature. TEM, SEM and AFM images also revealed the morphology of the particles and of their aggregates. These materials have been used to manufacture scaffolds which were tested for their influence on adhesion and proliferation of cells, in human osteoblast culture, considering their further use in bone reconstruction. It was found that an appropriate addition of silicon in nanocalcium phosphate scaffolds leads to an enhanced adhesion and proliferation of cells in osteoblasts in vitro.

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1. Introduction

Presently most bone grafting materials are the autografts and allografts, while porous ceramic materials, such as calcium phosphates (e.g., hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, HAP) can be considered as an important class of future materials for bone reconstruction. Current research in this field is striving to exploit their osteoconductive, osteoinductive and osteogenic properties, but it is confronted with their unpredictable osteointegration [1]. In this respect, silicate substituted hydroxyapatites are a promising solution as bone grafting materials [1,2].

Tests with silicate substituted hydroxyapatites showed that their in vivo bioactivity was improved as compared to the activity of conventional HAP, leading to a better osteointegration [3–7] and to a promotion of bone remodeling at the bone/HAP interface [8]. Moreover, no cytotoxic behavior of silicon substituted hydroxyapatite bioactive coatings was evidenced [9,10].

There are different synthesis methods for silicon substituted hydroxyapatites (HAP-Si) and their results have been reviewed [11]. Most synthesis methods use the aqueous precipitation route and crystallization [6–8,12–17].

The synthesized powders were consolidated by different techniques, mostly by calcination [6–8,12,14,18], in order to improve their crystallinity and microstrain. This sintering was shown to modify the surface morphology, and to have different influences on the properties (such as, mechanical properties and porosity) according to the Si content of the sample [18]. It was also shown that silicate ions that dissolved from silica gels have the ability to form bonelike apatite layers [19].

For the characterization of calcium phosphate materials, the most used methods are X-ray diffraction (XRD) and X-ray photoelectron spectrometry (XPS) [7,14,17], FTIR and Raman spectrometry [12], high-resolution neutron powder diffraction [14], TEM [8] or SEM [7,14,17].

For a silicon content of 0.4%, only small structural changes in the lattice parameters of hydroxyapatite and a distortion of the phosphate tetrahedron were observed [14]. For this low silicon content, the hydroxyapatite structure is maintained even after sintering at 1200 °C

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[6], but for higher Si content (1.5–4.5%) in calcined powders at 800 °C a change to β-tricalcium phosphate (β-TCP) structure was observed [7].

Ab initio total energy calculations were used for the simulation of the structure of Si-doped hydroxyapatite and for the study of charge compensation when SiO_4^{4-} ion is substituting for the PO_4^{3-} ion. It was found that the presence of OH vacancies, oxygen vacancies or additional hydrogen (formation of $HSiO_4^{3-}$), depends on the degree of hydration [20].

Investigations by solid-state NMR spectroscopy on hydroxyapatites with 5.6% Si demonstrated that not all silicon atoms are incorporated in the hydroxyapatite lattice as SiO_4^{4-} ions, but a large amount of silicate units remain outside of this lattice as silica-gel units [21].

Similar investigations on Si and Zn substituted tricalcium phosphates also revealed that along with SiO_4^{4-} ions, substituting PO_4^{3-}, there are silicate nanoinclusions, containing most of Si and crystalline SiO_2 [22]. By ^{29}Si magic angle spinning NMR spectroscopy, $Si_2O_7^{6-}$ (Q^1) structures were found in calcium phosphate bioceramics [23].

Although, the research carried out on nanocalcium phosphates [24–27] is rather large, their physical and biological characterization as well as their application as bone substitutes is still under study [28].

Previously [29–31], we have synthesized and characterized nanohydroxyapatite (HAP). Functional porous layered scaffolds, made of HAP, chitosan and collagen, were used in cell culture. Our current purpose is to prepare silicon substituted nanocalcium phosphates, particularly Si modified hydroxyapatite (HAP-Si), to assess their composition and structure, as well as to estimate their biocompatibility to human osteoblasts in cell cultures. The XRD, FTIR, TEM, AFM and SEM-EDX have been used to characterize obtained nanomaterials in this work. Their in vitro biocompatibility and their influence on cell behavior have been evaluated by MTT test and by morphometric analysis of osteoblasts on scaffolds, made of nanocalcium phosphates.

2. Materials and methods

2.1. Materials

Chemical reagents for synthesis of calcium phosphates were of analytical grade and were purchased from various suppliers, like Merck and Sigma-Aldrich. The Dulbecco's modified Eagle's medium (DMEM) and various supplements for cell culture were purchased from Sigma-Aldrich. All other reagents, including MTT compound [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazol-3-ium bromide], Hank's isotonic balanced salt solution, trypsin-EDTA, trypan blue and phosphate buffered saline (PBS), were purchased from Sigma. High purity hydroxyapatite was purchased from Sigma-Aldrich to serve as an etalon for microcrystalline HAP, in XRD investigation. All chemicals were used as received. Deionized ultrapure water was used in all experiments.

2.2. Synthesis of nanostructured calcium phosphates

Nanostructured calcium phosphates were prepared from calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$, diammonium hydrogen phosphate, $(NH_4)_2HPO_4$, ammonium hydroxide (NH_4OH), sodium silicate in the molar ratio of $Na_2O{:}SiO_2 = 1{:}3.2$, and nonylphenol, by co-precipitation method in aqueous media as shown elsewhere [29–31]. For the synthesis of pure HAP, the volumes of the calcium nitrate solution and of diammonium hydrogen phosphate solution were monitored in order to assure the Ca/P ratio of 1.667, corresponding to stoichiometric hydroxyapatite. In the case of synthesis of hydroxyapatite modified with silicon, the sodium silicate solution was added in appropriate amounts to get silica, SiO_2, content 1, 5 and 10% respectively, corresponding to 0.47, 2.34, and 4.67% silicon (Si) content in the final product. The pH of the obtained colloidal dispersion was adjusted at a value between 9.5 and 11.5, with ammonium hydroxide. Then, the colloidal dispersion was sealed in a container and kept inside a water bath for 48 h at 80 °C for maturation. During the maturation process, the dispersion was vigorously and continuously stirred, to allow the calcium phosphate to nucleate and grow properly to give nanoHAP. In the presence of a surfactant, such as nonylphenol, the nucleation and the growth of HAP nuclei were controlled. The final suspension was filtered, and the precipitate was washed with deionized water until no nitrate ions were detected. To eliminate sodium, the wet precipitate was washed with a 5% oxalic acid solution, until the pH was between 6.5 and 7. Then, the precipitate was dried by lyophilization.

After that, each obtained powder [i.e., pure HAP, HAP-SiO_2 (1%), HAP-SiO_2 (5%) or HAP-SiO_2 (10%)] was calcined at three different temperatures, namely 650 °C, 950 °C and 1150 °C. The first temperature was chosen in order to assure the complete removal of all organic compounds used in the syntheses; 950 °C was selected as an intermediate temperature, to evaluate the structure modification below the temperature of 1150 °C, where the conversion of HAP to β-TCP is expected to take place. Thus, by heating the pure HAP powder at about 650 °C, an advanced nanoHAP powder of controlled stoichiometry, high crystallinity, with nanosized particles was obtained. Then, all the obtained powders were characterized.

2.3. Preparation of TEM and AFM samples

The synthesized calcium phosphate nanopowder was dispersed at a required concentration in deionized water [29] and the resulted colloidal dispersion further used for both TEM and AFM investigations. TEM and AFM samples were prepared by adsorption of nanophosphates on TEM grid and on glass, respectively. For AFM sample, the optically flat glass support was first cleaned with sulphochromic acid and washed with water. Then, it was treated with 5% HCl aqueous solution for 15 min, and afterward, rinsed in deionized water for three times. Next it was shortly immersed in 2% silicate solution for ten times. On the resulted hydrophilic activated glass support, the chosen calcium phosphate layer was applied by adsorption for 10 s from colloidal dispersions. The adsorbed layer on glass or on grid support was washed with pure deionized water and completely dried at room temperature before AFM and TEM investigations. Alternatively, samples were prepared for AFM investigation by spreading calcium phosphate powders directly on an adhesive plastic band, or on an adhesive graphitized tape for SEM samples.

2.4. Preparation of multilayered scaffolds

Self-assembled nanostructured scaffolds of HAP or HAP-Si were prepared by the layer by layer method, as previously described [29]. Briefly, a layer of a particular calcium phosphate, previously calcined at 650 °C, was obtained by its adsorption for 10 s from its aqueous dispersion on flat glass support, as also described for AFM sample preparation. The resulted adsorbed layer of calcium phosphate was further washed with aqueous solution of sodium silicate several times to assure strong interactions between adsorbed layers of chosen calcium phosphate. Then, another adsorbed layer of calcium phosphate was built. Finally, at least 10 adsorbed layers were periodically self-assembled as a whole scaffold, which was thoroughly washed in pure water and dried at room temperature. These scaffolds show good stability in cell culture, since a significant lost of scaffold material was not observed by dissolution in cell medium during one week of experiments.

2.5. Characterization of calcium phosphates

The synthesized calcium phosphates were characterized by XRD, FTIR, SEM-EDX, TEM and AFM.

The *XRD investigations* were made with a DRON-3 diffractometer, in Bragg–Brentano geometry, using cobalt target (K_α line, wavelength 1.79030 Å).

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