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Effects on insulin adsorption due to zinc and strontium substitution in hydroxyapatite

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ABSTRACT

Insulin-loaded calcium phosphate nanoparticles have been proposed as a potential drug delivery system for the oral treatment of diabetes and to stimulate bone cell proliferation and bone mineralization. The kinetics of insulin incorporation onto hydroxyapatite (HA) and Sr (SrHA)- and Zn (ZnHA)-substituted hydroxyapatite nanoparticles was investigated using X-ray photoelectron spectroscopy (XPS), Fourier transform infrared (FTIR) spectroscopy, zeta potential measurements and circular dichroism (CD) spectroscopy. The increase in insulin concentration on HA, SrHA and ZnHA was a typical physical adsorption process controlled by electrostatic forces and followed a Freundlich isotherm model. Zn substitution enhanced the capacity of the apatite surface to adsorb insulin, whereas Sr substitution inhibited insulin uptake. The surface stoichiometry and mesopore specific area induced by Zn and Sr substitution are proposed as the main causes of the difference in insulin adsorption. Despite the weak interaction between insulin and the apatite surface, the CD spectra revealed a decrease in the insulin ellipticity when the protein was adsorbed on the HA, SrHA and ZnHA nanoparticles. A reduction in alpha-helical structures and an increase in beta sheets were observed when insulin interacted with the HA surface. A less pronounced effect was found for ZnHA, for which a subtle decrease in alpha-helical structures was followed by an increase in turn structures. Interaction with the SrHA surface did not change the native insulin conformation. *In vitro* cell culture experiments lasting 24 h using F-OST stromal cells showed that the insulin loaded on HA and ZnHA did not affect cell proliferation but the insulin loaded on SrHA improved cell proliferation. These results suggest that the stability of the native protein conformation is an important factor to consider when cells interact with insulin adsorbed on metal-substituted HA surfaces.

1. Introduction

Demands for new biomaterials for tissue regeneration and therapeutics have motivated intense research on new formulations of biocompatible scaffolds and drug delivery carriers [1,2]. Nanostructured matrices and nanoparticles composed of metals, biopolymers, ceramics and composites have emerged as strong candidates for use in these applications due to their high specific surface areas and chemical activities in biological media. When associated with peptides, proteins or drugs, nanostructured biomaterials accelerate the repair of functional tissues that have suffered traumatic injury, chronic degeneration or illness [3].

Hydroxyapatite (HA) is widely used in orthopedics and dentistry to enhance bone regeneration and has been proposed as a delivery matrix for peptides, proteins, antibiotics, drugs and genes due to its nanostructured characteristics and chemical affinity for molecules, ions and metals [4,5]. The challenge of improving the biological efficiency of HA lies in understanding the mechanisms of the surface interaction with biomolecules and the constituents of the biological medium. Regarding the adsorption of proteins by ceramics and nanocrystalline HA, extensive experimental and theoretical studies have been performed in recent years involving this subject [6,7].

These investigations revealed that kinetic behavior and changes in protein conformational structure were highly dependent on the protein nature (structure, size and charge), medium properties (pH, composition of the medium and temperature) and material surface properties (adsorption sites, surface energy, stoichiometry and topography) [6,8,9].

Anionic and cationic substitutions in the HA structure may stimulate interactions with proteins existing in the biological medium and improve tissue regeneration [10,11]. In this regard, Zn- and Sr-substituted HA (ZnHA and SrHA) are candidates for improving new bone mineralization [12–14]. Despite numerous studies on this subject, few data are available concerning protein adsorption on ZnHA and SrHA surfaces [15,16].

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Insulin and insulin growth factors are hormones that are directly involved in lipid and carbohydrate metabolism. Recent studies have supported the idea that insulin and insulin growth factors play an important role in bone energy metabolism and osteoblast proliferation and differentiation [17–19]. The association of insulin with calcium phosphate nanoparticles has been proposed as a drug delivery system for the oral treatment of diabetes [20] and to stimulate bone cell proliferation [21] and bone mineralization [22]. Despite the potential of insulin delivery systems based on calcium phosphate, few studies have characterized the interaction of insulin with the HA surface in detail [23]. The purpose of this work was to investigate the insulin adsorption on HA, ZnHA and SrHA and evaluate the influence of the insulin coating on cell proliferation using F-OST stromal cells.

2. Materials and methods

2.1. Synthesis of HA, ZnHA and SrHA

HA ($Ca_{10}(PO_4)_6(OH)_2$) was synthesized by dropwise addition of an aqueous $(NH_4)_2HPO_4$ (0.12 M) solution to an aqueous solution of $Ca(NO_3)_2$ (0.2 M) with pH 9 at 90 °C. After the addition, the resulting suspension was stirred for 3 h. The solid was then filtered, washed several times and dried by lyophilization. The dried powder was manually ground. Particles <210 μm were separated by sieving.

Sr and Zn substituted HA, SrHA and ZnHA, ($Ca_{10-x}M_x(PO_4)_6(OH)_2$, M = Zn, Sr, x = 0.5,) were synthetized at 90 °C by adding aqueous solutions of $Ca(NO_3)_2.4H_2O$ and $Sr(NO_3)_2\cdot 6H_2O$ or $Zn(NO_3)_2\cdot 6H_2O$ to an aqueous $[(NH_4)_2HPO_4]$ solution. The pH was maintained at approximately 9.0 by adding ammonium hydroxide. After the addition of reactants, the suspensions were stirred for 3 h at 90 °C and pH 9. The solid was filtered, washed and dried using the same procedure as in HA preparation. The theoretical concentration of Zn and Sr in SrHA and ZnHA samples (x = 0.5) was compatible with the metal content in HA of previous works *in vitro* and *in vivo* studies of bone regeneration and antimicrobial action [13] [12] [10] [24].

2.2. Insulin adsorption on HA

A solution of human insulin (10 mg/mL) was purchased from Sigma-Aldrich (USA). Before adsorption, the HA, ZnHA and SrHA powders were immersed in 0.01 M Tris buffer (pH = 6.4) for 30 min for hydration, in order to establish an ionic equilibrium between samples and the buffer solution. Adsorption experiments were conducted in a batch system. Apatite powders (2 mg of HA, ZnHA and SrHA) were immersed in 0.01 M Tris buffer (pH 6.4) containing insulin (0 to 1.2 mg/mL) for 3 h (time in which the insulin adsorption achieved the equilibrium) under moderate stirring at 25 °C. After incubation, all the samples were centrifuged at 10,000 rpm and washed twice with Tris buffer and three times with deionized water to remove the insulin that was weakly adsorbed on the sample surfaces. The adsorption experiments were performed in triplicate.

The isotherms data fitting was performed using Langmuir, Freundlich and Langmuir–Freundlich equations through Origin 8.0 software. The Langmuir model can be described by the equation: $a = a_m K \cdot c_e / (1 + K \cdot c_e)$, where a ($mmol\cdot g^{-1}$) and c_e ($mmol\cdot L^{-1}$) are the equilibrium concentration of adsorbate on an adsorbent surface and the adsorbate concentration in solution, respectively. The constant K is the equilibrium constant that represents the affinity between adsorbate and adsorbent and a_m is the maximum amount adsorbed on surface ($mg.m^{-2}$). The Freundlich model can be expressed by the equation: $a = K \cdot c_e^{1/p}$ in which K is the equilibrium constant and p is a power parameter. The Langmuir-Freundlich equation is: $a = a_m(K \cdot c_e)^r / [1 + bK \cdot (c_e)^r]$, where c_e is the adsorbate concentration in equilibrium, K is the affinity constant that includes contribution from surface binding to monomer, monomer–dimer, and more highly associated forms of proteins. The coefficient r represents the cooperativity present in the binding interaction [25,26].

2.3. Sample characterization

The Ca, Zn and Sr contents of the precipitated powders were determined by quantitative chemical analysis using atomic absorption spectroscopy (AAS) on a Shimadzu 6800 instrument. The phosphate content was obtained by vanadomolybdate phosphoric acid colorimetric method, using a UV–Vis spectrophotometry (Shimadzu UV-2450) at 420 nm.

The crystallographic phases, lattice parameters and crystalline order were analyzed on an X-Pert Pro PANalytical diffractometer equipped with a proportional detector (Xe gas) and Cu-Kα radiation (λ = 1.542 Å) at 40 kV and 40 mA. Diffraction patterns were collected in a step of 0.02° at 50 s/step between 7° < 2θ < 60°. The mean crystallite size (Dv, nm) and lattice parameters were determined from the Scherrer equation and the interplanar distance (d_{hkl}) as a function of $a = b$ and c for a hexagonal lattice [27]:

$$D_v = K\lambda/\beta_{hkl} \cos\theta_{hkl}$$

$$1/d^2_{hkl} = 4/3 * \left(h^2 + hk + k^2\right)/a^2 + l^2/c^2$$

where k is the form factor (0.89), λ is the X-ray wavelength (λ = 1.542 Å), β_{hkl} is the linewidth at half-height and θ_{hkl} is the Bragg angle of the diffraction peak.

Fourier transform infrared spectroscopy (FTIR) was used to detect the vibration modes of HA, ZnHA and SrHA samples in three conditions: as-prepared samples, after incubation in Tris buffer and after insulin adsorption. The FTIR spectra were collected on a Shimadzu IR-Prestige-21/AIM-880 spectrophotometer over a range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} and 500 scans using KBr pellets with m^{KBr}/m^{sample} = 10 to observe more intense vibrational bands in the 1600–1400 cm^{-1} region, such as protein amide I and II bands.

The stoichiometry of the HA, ZnHA and SrHA samples surfaces before and after insulin adsorption was analyzed by X-ray photoelectron spectroscopy (XPS) using an SPECS PHOIBOS 100/150 spectrometer with a hemispheric analyzer operating at 1486.6 eV with Al Kα radiation. The spectra were collected from a high-resolution monochromatic X-ray source with an energy step of 0.02 eV. CasaXPS software was used to perform peak fitting analyses and determine the bindings energies of Ca, P, Zn, Sr, C and N. The element binding energies were corrected using the C1s carbon peak for surface contamination at 284.6 eV. The zeta potential (PZ) of the particle surfaces was determined on a Zetasizer Nano ZS90 instrument. The measurements were carried out with the HA, ZnHA and SrHA powders suspended in 0.001 M KCl at pH 6.0. The particle size distribution was determined by laser diffraction (SALD-2201, Shimadzu), and the specific surface area (BET) and mean pore size were determined using a physisorption technique (ASAP-2020, Micromeritics). Thermogravimetric analysis (TGA) was carried out on a Shimadzu DTG-60 instrument (5–15 mg, 25–800 °C, 10 K min^{-1}).

Circular dichroism (CD) spectroscopy was used to analyze possible changes in the conformation of insulin molecules when the protein was adsorbed on the HA, ZnHA and SrHA surfaces. CD spectra were collected on a Jasco J-810 spectropolarimeter operating at 190–260 nm using a 1 mm-thick quartz cuvette containing 400 μL of the sample dispersions (10 mg of each sample after insulin adsorption suspended in 1 mL of Milli-Q water). The far-UV spectra were analyzed by the open source software DichroWeb* using the CDSSTR analysis program [28] [29].

2.4. In vitro evaluation

F-OST stromal cells [30] were used to investigate the biological response in contact with HA, SrHA and ZnHA disks without or coated with 300 $\mu g\cdot mL^{-1}$ human insulin (HA I, SrHA I and ZnHA I). Cells were cultured in Dulbecco's modified essential medium (DMEM, Gibco) supplement with 10% fetal bovine serum (FBS, Gibco) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed at 2-