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Synthesis and characterization of xanthan–hydroxyapatite nanocomposites for cellular uptake

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ABSTRACT

In this work xanthan–nanohydroxyapatite (XnHAp) and its equivalent strontium substituted (XnHApSr) were synthesized by the precipitation of nanohydroxyapatite in xanthan aqueous solution, characterized and compared to conventional hydroxyapatite particles (HAp). XnHAp and XnHApSr were less crystalline than HAp, as revealed by X-ray diffraction. Xanthan chains enriched the surface of XnHAp and XnHApSr particles, increasing the zeta potential values from $-(7 \pm 1)$ mV, determined for HAp, to $-(17 \pm 3)$ mV and $-(25 \pm 3)$ mV, respectively. This effect led to high colloidal stability of XnHAp and XnHApSr dispersions and acicular particles (140 ± 10) nm long and (8 ± 2) nm wide, as determined by scanning electron microscopy and atomic force microscopy. XnHAp and XnHApSr particles were added to xanthan hydrogels to produce compatible nanocomposites (XCA/XnHAp and XCA/XnHApSr). Dried nanocomposites presented surface energy, Young's modulus and stress at break values comparable to those determined for bare xanthan matrix. Moreover, adding XnHAp or XnHApSr nanoparticles to xanthan hydrogel did not influence its porous morphology, gel content and swelling ratio. XCA/XnHAp and XCA/XnHApSr composites proved to be suitable for osteoblast growth and particularly XCA/XnHapSr composites induced higher alkaline phosphatase activity.

1. Introduction

Hydrogels are of great interest as scaffolds for cell culture, due to their similarity to extracellular matrix [1,2]. Particularly polysaccharide hydrogels are advantageous for biomedical applications, because they join biocompatibility, biodegradability, and abundance in nature with hydrogels similar to biological systems [3]. Some examples include the use of chitosan, alginates, and celluloses for drug delivery, protein immobilization, wound dressings, cell encapsulation and tissue engineering [4–10].

Hydroxyapatites (HAp) are the major inorganic component in skeletal tissue (about 50 wt.% of hard tissues is composed of calcium and phosphate ions) [11]. For this reason, there is an increasing interest on calcium phosphates in biological applications, which include bone cavity filling, osteologic implants and drug delivery, due to its bioactivity, biocompatibility and mechanical properties [12]. On the other hand, the benefits of strontium compounds, as for instance, strontium ranelate, against osteoporosis made them a well recommended therapy [13]. For this reason, HAp has been modified with strontium to enhance its biological activity. The replacement of calcium by strontium in the HAp structure has proven to be an efficient strategy for improving bone formation due to better osteoblast spreading and proliferation [14]. In comparison to poly(methyl methacrylate) bone cement, bioactive bone cements based on Sr-HAp present better adhesion, biocompatibility, bioactivity and osteoconductivity [15,16]. These issues motivate the search for new scaffolds for bone regeneration based on Sr-HAp.

Nevertheless, pure HAp is brittle and the devices prepared from this material can present poor mechanical performance [17,18]. To overcome these drawbacks, HAp composites or nanocomposites have been used; for instance, composites of collagen and HAp, in a 1:1 volumetric ratio have been successfully applied [19].

Hydroxyapatite and nanohydroxyapatites (nHAp) [12] can be combined with a large number of natural and synthetic polymers [20]. However, the lack of adhesion between the inorganic and the polymeric phase will result in failure at the interface causing deterioration of the mechanical properties [21]. Alternatives to enhance nHAp compatibilization and avoid nanoparticle agglomeration include its surface functionalization, for example with PLLA, alginate, chitosan and silanes [20,22–24], or *in situ* synthesis of nHAp in polymeric solutions [25,26]. Besides nHAp functionalization allows strong bond formation between nHAp and polymer matrices as an intermediate layer, their modified surface acts in modulating their colloid stability and in preventing dissolution in low pH and inflammatory response [27,28].

Nanocomposite hydrogels are soft materials consisting of a unique organic/inorganic network structure, with interesting characteristics, as mechanical, optical, and swelling/de-swelling properties [29–31].

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Compatibilization between nanohydroxyapatite and hydrogels is important for adequate and homogeneous hydroxyapatite distribution in hydrogel matrix, to obtain nanocomposite hydrogels. These materials have many applications on biomedical science, as drug delivery systems, scaffolds for cellular culture and bone cavity filling [32–34].

Xanthan gum is a high molecular weight polysaccharide with branched chains and acidic characteristics, produced by *Xanthomonas campestris* [35]. Their derivatives are used as thickener agent in food, cosmetics and drilling fluids [35,36]. Moreover, intra-articular injection of xanthan gum could protect joint cartilage and reduce osteoarthritis progression, being an effective therapeutic method of this disease [37]. O-acetyl and pyruvyl residues present at its structure are deprotonated at pH ≥ 4.5, enabling their physical crosslinking mediated by Ca^{2+} ions, to form physical hydrogels [38,39]. Xanthan can also be crosslinked by using citric acid as crosslinking agent to form xanthan-citric acid (XCA) chemical hydrogels [40], which carry and deliver proteins as bovine serum albumin and lysozyme [41]. Recently we have described a fast and simple method to prepare composite films of magnetite nanoparticles and XCA networks with coercivity of 27 ± 2 Oe at 300 K), which were successful on the development of scaffolds for the proliferation of fibroblast, particularly when an external magnetic field was applied [42].

This work describes the creation of compatible XCA/hydroxyapatite hydrogel nanocomposites, by using xanthan modified nanohydroxyapatite (XnHAp) and its equivalent strontium substituted (XnHApSr). The nanoparticles were obtained by precipitation in xanthan solution. Nanoparticle stability and the particles homogeneity distribution on hydrogel matrices were evaluated. The nanocomposite hydrogels made of xanthan modified hydroxyapatites and XCA were tested as scaffolds for osteoblast growth.

2. Experimental

2.1. Materials

Commercial xanthan (Kelzan®, CP Kelco, USA, degree of pyruvate = 0.38, degree of acetyl = 0.41, M_v ~ 1.0 10^6 g/mol, degree of polymerization ~ 1072) was used as received. Citric acid (Analitica Quimica, Brazil) was recrystallized twice from water prior to use. $Ca(OH)_2$, $Sr(CH_3COO)_2$ and H_3PO_4 were obtained from Givaudan, Sigma-Aldrich and Synth (Brazil) respectively. Deionized water was used in all experiments.

2.2. Methods

2.2.1. Nanoparticle synthesis

Xanthan-hydroxyapatite particles (XnHAp) were obtained as follows: 0.93 g of $Ca(OH)_2$ (0.013 mol) were added to 0.5 L of xanthan solution at 1 g L^{-1} at (50 ± 1) °C under vigorous stirring (Ika Turrax®, 18,000 rpm). After total homogenization 0.2 mol L^{-1} H_3PO_4 solution was added dropwise until medium achieved pH ~7.5, as schematically represented in the Supplementary Material Figure S1A. The resultant dispersion was dialyzed against deionized water until the conductivity of dialysis water reached 5 μS/cm. nHAp particles were obtained by the same procedure, but in the absence of xanthan. Xanthan-strontium-substituted hydroxyapatite particles (XnHApSr) were obtained as follows: 0.836 g of $Ca(OH)_2$ (0.011 mol) and 0.093 g of $Sr(CH_3COO)_2$ (0.0004 mol) were added to 0.5 L of xanthan solution at 1 g L^{-1} at (50 ± 1) °C under vigorous stirring (Ika Turrax®, 18,000 rpm). After total homogenization 0.2 mol L^{-1} H_3PO_4 solution was added dropwise until medium achieved pH ~7.5, as schematically represented in the Supplementary Material Figure S1B. The resultant dispersion was dialyzed against deionized water until the conductivity of dialysis water reached 5 μS/cm. nHAp particles were obtained by the same procedure, but in the absence of xanthan. Dispersions were freeze-dried to obtain nHAp, XnHAp and XnHApSr nanoparticle powders.

2.2.2. Nanoparticle characterization

Nanoparticle characterization was done by means of inductively coupled plasma atomic emission spectroscopy (ICP-AES), X-ray diffraction, thermogravimetric analysis (TGA), Fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), atomic force microscopy (AFM), dynamic light scattering (DLS) and zeta potential. ICP-AES was performed in a Spectro Cirus CCD apparatus. X-ray diffraction analyses were performed with a Rigaku Miniflex diffractometer (Cu, Kα; λ = 1.5418 Å, ~8 keV), with a goniometer θ:2θ based on Bragg–Brentano geometry. X-ray Scattering slit and receiving slit were 4.2° and 0.3 mm, respectively. Divergence slit was variable. Scan mode was continuous, scan speed was 0.100°/min and sampling width was 0.010°. Diffractograms were analyzed with Crystallographica Search-Match software. FTIR was performed with BOMEM MB 100 equipment using KBr pellets. SEM analysis was performed in a Jeol microscope FEG7401F equipped with a Field-Emission Gun, after coating the samples with a thin gold layer. Sizes obtained from SEM images were calculated using AxioVision V. 4.6.3.0 program, by measuring randomly >100 particles. For atomic force microscopy (AFM), diluted particles suspension was dropped onto Si/SiO_2 wafers and dried at 25 °C for 24 h. AFM was performed in air using a PicoSPM-LE Molecular Imaging system with cantilevers operating in the intermittent contact mode (AAC mode). DLS and zeta potential (ζ-potential) measurements were performed in a commercial instrument Zetasizer NanoZS (Malvern, UK). A He–Ne laser was used as a light source with wavelength λ = 633 nm. Concerning the DLS experiments, the intensity of light scattered was recorded at an angle of 90° with an avalanche photodiode detector. We used the Zetasizer Software 6.2 (provided by Malvern) to determine the particle size distribution, using the correlation function to obtain the distributions of the decay rates, and the apparent diffusion coefficients. Finally, the distributions of the hydrodynamic radius of the scattering particles in solution are estimated *via* Stokes–Einstein equation. DLS and ζ-potential measurements were performed for dispersions of nHAp, XnHAp and XnHApSr at 1 g L^{-1} after filtering through a 0.45 μm Millipore filter. Droplets of the filtered dispersions were deposited onto Si wafers and allowed to dry slowly at room temperature for AFM analyses in air, using a PicoSPM-LE Molecular Imaging system with cantilevers operating in the intermittent contact mode (AAC mode).

Colloidal stability of nHAp, XnHAp and XnHApSr nanoparticles dispersed in distilled water (pH ~ 6) at 1.0 g/L was monitored using a LUMiReader®414 Separation Analyzer (L.U.M. GmbH, Germany), by the SEP View 4.01 software, which registered the normalized integral light transmission as a function of time at (26 ± 2) °C. Glass cuvettes (10 mm diameter and 80 mm length) were used for the experiments. At the beginning, the dispersion is homogeneous and the transmitted light through the cuvette is very low. As time goes by, the dispersion starts to destabilize, mainly because the density of hydroxyapatite is 2.95 g/cm^3 [43], which is larger than the water density. For this reason, particles sediment, accumulating at the bottom of the cuvette and increasing the transmission of light through the upper liquid. Measurements were done in triplicate. The intensity of light transmitted through pure water was used as reference for 100% light transmittance.

2.2.3. Production and characterization of nanocomposite hydrogels

Xanthan based nanocomposite hydrogels were prepared as schematically represented in the Supplementary Material Figure S2A and S2B, by casting a 6 g L^{-1} xanthan aqueous solution containing nanoparticles (nHAp, XnHAp or XnHApSr) at 0.6 g L^{-1} (10% filler content) or 1.8 g L^{-1} (30% filler content) in the presence of citric acid at 0.3 g L^{-1}. Previously the dispersions were homogenized with an Ika Turrax® stirrer at 18,000 rpm for 3 minutes and submitted to centrifugation for 5 min at 3600 rpm to remove air bubbles. Crosslinking was achieved