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Dual responsive dysprosium-doped hydroxyapatite particles and toxicity reduction after functionalization with folic and glucuronic acids

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ABSTRACT

The development of probes for biomedical applications demands materials with low toxicity levels besides fluorescence or magnetic properties to be detected by confocal microscopes or MRI resonators. Several drug delivery systems or other biomedical materials prepared with hydroxyapatite have been proposed, however, toxicity effects might arise when the size of particles is nanometric. In this study, hydroxyapatite functionalized with glucuronic or folic acids presented lower oxidative stress, measured from lipoperoxides and nitric oxide indicators in rats than pure hydroxyapatite. In separated experiments, hydroxyapatite was doped with dysprosium cations by coprecipitation producing a single crystal phase with fluorescent properties easily visualized by confocal microscopy when excited at 488 nm. These particles also presented the ability to modify the proton relaxation time in T1 maps collected by magnetic resonance imaging. These modified hydroxyapatite nanoparticles could be candidates to design bimodal probes with low toxicity.

1. Introduction

Hydroxyapatite (HA) is one of the mineral components of human bones and belongs to the apatite family represented by $M_{10}(XO_4)_6Z_2$, where M is a divalent cation coordinated with XO_4^{3-} and Z anions [1]. The composition of HA is $Ca_{10}(PO_4)_6(OH)_2$ and the synthetic forms have been studied to develop materials for diverse biological or biomedical applications such as scaffolds for tissue engineering [2,3] delivery systems of anti-cancer drugs [4,5] or particles with the ability to recognize specific cells [5,6]. A key step in the material design to implement these applications is the functionalization of HA particles with one or more organic components depending on the desired final features. For instance, the HA functionalization with amino acids like arginine makes possible to bind and transport catalytic proteins to cleave mRNA [7]; the functionalization with polyethylene glycol converts HA particles into a system for slow-release of gentamicin sulfate antiobiotic [8]; the hydrophobic degree of polyethylene glycol also allows to retain liposoluble anti-cancer drugs like docetaxel [4], while a second functionalization with folic acid promotes the delivery to specific target cells [5]. In all these cases, the key modification was conducted on the HA surface with organic compounds.

Besides this, the crystalline structure of HA can be modified to increase the number of properties in the final material as it has occurred with the incrustation of Eu_2O_3 into HA particles to add luminescent properties [9], iron to introduce magnetism [10], strontium to improve implant adhesion and bone mineralization [11,12], as well as the simultaneous doping with europium and gadolinium [13] or terbium and gadolinium cations [14] to add magnetic and luminescent properties. Since these modifications are conducted in the internal structure with inorganic properties and the surface of such nanoparticles is available for functionalization reactions, the HA particles offer a potential base to design multifunctional materials.

On the other hand, the biological and biomedical applications of HA derivative materials are based on its biocompatibility and apparently null toxicity since no inflammatory or immunogenic effects are produced [4]. Slight toxicity effects have been detected in assays with cat fish [15] and guinea pigs only in concentrations above 600 μg mL^{-1} [16]. However, the toxicity of HA modified within its crystal structure is unknown, therefore biological assays are required to determine changes in biocompatibility and the effect of some functionalized forms. Therefore, considering that the insertion of lanthanide cations in HA is relevant to design luminescent or magnetic probes for biomedical applications [13], the assessment of toxicity of particles with any of these cations deserves to be investigated, as well as the effect of functionalization.

The objective of this work is to evaluate the toxicity of HA doped with dysprosium cations, as well as HA functionalized with either folic

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or glucuronic acids, which are of relevance to design nanoparticles for biomedical applications once nanoparticles functionalized with folic acid are capable of recognizing cancer cells [5,17], while the functionalization with glucuronic acid reduces the toxicity levels since this acid aids to excrete nanoparticles through urine [18].

2. Methodology

2.1. Synthesis of hydroxyapatite and derivatives

Reagents of analytical grade were purchased from Sigma-Aldrich and used as received. Deionized water with 18 MΩ cm^{-1} was used in the experiments. Hydroxyapatite was prepared by addition of ammonia to a solution with $CaCl_2$ and $(NH_4)_2HPO_4$ as previously reported in the literature [19,20]. The solution of both salts was prepared with 0.5526 g of $CaCl_2$ and 0.3944 g of $(NH_4)_2HPO_4$ in 50 mL of water, these masses correspond to a molar ratio PO_4^{3-}:Ca^{2+} equal to 6:10. The solution was stirred and an ammonia solution (14%) was added one drop at a time until pH = 9.7 was reached.

The functionalized samples were obtained with addition of 0.200 g of folic or glucuronic acid to the calcium/phosphate solution described above and thereafter precipitated with ammonia, whereas the sample doped with Dy^{3+} cations was prepared with the addition of 0.0914 g of $Dy(NO_3)_3$ to the calcium/phosphate solution.

The final pH of all precipitated samples was 9.7 and their suspensions were stirred for 24 h at room temperature, and then the powders were separated by decantation, washed three times with water and dried at 70 °C. The hydroxyapatite sample was labeled as HA and the folic, glucuronic and dysprosium derivatives as HA-F, HA-G and HA-Dy, respectively. In the present case, no further thermal treatments were conducted to avoid degradation of folic or glucuronic acids.

2.2. Characterization

Powder X-ray diffraction patterns were recorded on a STOE SEIFERT diffractometer using Cu Kα radiation at 30 kV and 20 mA. The goniometer was rotated at a step of 0.02° with a time of 0.5 s per step. Infrared spectra by attenuated total reflectance (ATR-IR) were acquired in a Thermo-Scientific spectrometer model Nicolet iS5, with 32 scans and a resolution of 2 cm^{-1}. A destructive analysis of HA-F and HA-G was conducted by UV-vis spectroscopy to confirm the presence of folic and glucuronic acids in the powder derivatives. For this, 0.01 g of the powder was mixed with 3 mL of 0.1 mol L^{-1} HCl solution. The resulting translucent solution was read in a Thermo-Sientific UV–vis spectrophotometer (Evolution series) using HCl solution as blank for calibration. The references were prepared with 0.01 g of folic or glucuronic acid in 3 mL of HCl solution. Thermogravimetric analysis was conducted in a TA instrument model SDT Q600 with a heating rate of 10 °C min^{-1} under air atmosphere. Fluorescence spectra were collected by mixing 0.01 g of powder sample with 2 mL of water in a test tube. The suspension obtained by stirring with Vortex was transferred to a quartz cell and then read with a Perkin-Elmer spectrofluorometer, model LS50B with a slit aperture of 10 nm. A laser scanning confocal microscope model Leica SP-2 was used to collect images of nanoparticles with green emission excited at 488 nm. T1 relaxation time response was measured in a Bruker Avance III 500 NMR spectrometer; solutions of HA-Dy particles were prepared in 5% H_2O 95% D_2O mixture. The solution of HA-Dy in 5% H_2O in standard 5 mm NMR sample tube was degassed with nitrogen for 2 h, then homogenized with ultrasound for 30 min and then T1 was measured using inversion-recovery sequence. After 4 h the T1 measurement was repeated to check if the sample precipitated. The obtained T1 value was averaged over 5 measurements. Magnetic resonance T1 images (MRI) were acquired in a General Electric platform model Optima MR360 using the following parameters: external field = 1.5 T, field of view = 25.6, field of view phase = 1, thickness = 1 cm, repetition time = 8.3 ms, and time to echo = 3.1 ms.

2.3. Animals and treatments

Animal experiments were carried out in accordance with the international guidelines on the ethical use of animals. Sprague Dawley rats were obtained from the animal facility of Centro de Investigación Biomédica de Occidente (CIBO, México). Rats weighing 350–400 g were housed in temperature and humidity controlled rooms with a 12-hour light–dark cycle at 25 ± 2 °C and fed with standard rat food and free access to tap water. Animals were randomly divided into control group (C) that received physiologic saline solution and four experimental groups receiving respectively, by way of intra-peritoneal injection, the following nanoparticles: 1) HA, 2) HA doped with dysprosium cations (HA-Dy), 3) HA functionalized with folic acid (HA-F), 4) and HA functionalized with glucoronic acid (HA-G). Animals received a single dose of 10 or 20 mg of nanoparticles. All animals were weighed at the beginning and end of each treatment. Four rats were used in each group. All rats were sacrificed by decapitation 7 days after treatment. After killing trunk blood was collected and the liver, kidney and lungs were excised and washed in saline solution (0.9% of NaCl) to remove excess blood.

2.4. Biochemical analysis

Tissues were homogenized in ice-cold 20 mM tris (hydroxymethyl) aminomethane buffer (pH 7.4) with a Potter-Elvehjem tissue grinder to produce 1:10 homogenates. After homogenization, samples were centrifuged at 760 ×*g* for 10 min to remove nuclei and unbroken cells. The resulting supernatant was centrifuged again at 3000 ×*g* for 20 min at 4 °C. The pellet and supernatant were collected and stored at −80 °C until use. Protein quantification was determined by a quantitative colorimetric process for proteins by Folin reaction based on the Lowry method [21].

Membrane fluidity was estimated from the excimer to monomer fluorescence intensity ratio (Ie/Im) of the fluorescent probe 1,3 dipyrenylpropane (DPyP) incorporated in submitochondrial particles. Briefly, 0.25 mg of protein and 0.1 nmol DPyP were mixed with 10 mM Tris–HCl buffer (pH 7.8). The mixtures were incubated in darkness at 4 °C for 3 h, in order to achieve maximal incorporation of the DPyP to the membranes. The fluorophore was excited at 329 nm and the monomer and excimer fluorescence intensities were read at 379 and 480 nm, respectively; from these readings, the excimer to monomer fluorescence intensity ratio (Ie/Im) was calculated. Membrane fluidity was expressed as fluorescence intensity ratio of excimer and monomer DPyP (Ie/Im ratio), and high Ie/Im ratio indicates a high membrane fluidity. Fluorescence was measured at 24 °C on a Perkin Elmer fluorescence spectrometer, LS50B, and for sample analysis the FL WinLab 3.0 Software was used. Fluorescence corrections obtained from readings of samples without DPyP were applied to all fluorescence values [22].

The level of end-products of lipid peroxidation (LPO) of mitochondrial membrane was measured as the result of malondialdehyde (MDA) plus 4-hydroxyalkenals (4-OHA) by a colorimetric method using a LPO Cuvette Based Assay Kit from Oxford Biomedical Research, Inc. (Oxford MI, USA). Each measurement was repeated four times. The Kit contains a chromogenic reagent (N-methyl-2-phenylindole) which reacts with the lipid peroxidation products MDA and 4-OHA at 45 °C yielding a stable chromophore with maximal absorbance at a wavelength of 586 nm. Lipoperoxides were measured using a Benchmark Plus Microplate Spectrophotometer System, 110/230 V by Bio-Rad, and sample analysis was made using the MPM 5.1 PC Software.

Nitrite and nitrate values were estimated as an index of nitric oxide production. The method for nitrite and nitrate levels was based on the Griess reaction and samples were initially deproteinized with sulfosalicylic acid [23].

Glutathione peroxidase activity was determined according to Lawrence and Burk [24] with minor modifications. Briefly, the activity was assayed by following the oxidation of NADPH at 340 nm for 5 min