Different hydroxyapatite magnetic nanoparticles for medical imaging: Its effects on hemostatic, hemolytic activity and cellular cytotoxicity

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A B S T R A C T

Magnetic nanoparticles (MNPs) should be highly biocompatible, stable and safely eliminated from the body, and can therefore be successfully used in modern medicine. Synthetic hydroxyapatite (HAP) has well established biocompatible and non-inflammatory properties, as well as a highly stable and flexible structure that allows for an easy incorporation of magnetic ions. This study characterized and compared the in vitro cytotoxicity and hemocompatibility of hydroxyapatite MNPs doped with different ions (Gd3+/Fe2+/Fe3+/Co2+). HAP doped with 10% of Gd and Fe(III) presented the highest magnetic moments. Our results showed that Gd doped HAP nanoparticles are non-cytotoxic, hemocompatible, non-hemolytic and non-thrombogenic, in contrast with Fe(III) doped HAP that can be considered thrombogenic. For these reasons we propose that, Gd doped HAP nanoparticles have the most potential for application as a MRI contrast agents. However, use of Fe (III) doped HAP as MRI contrast agents should be further investigated. © 2016 Elsevier B.V. All rights reserved.

1. Introduction

Over the last decade, numerous characteristics of nanoparticles (NPs) such as their size (from 1 up to 100 nm), administration route and biodistribution have been explored and channeled into delivery systems, targeting therapies and medical diagnoses [1–3]. Magnetic based nanoparticles, namely magnetic nanoparticles (MNPs), have several applications in modern medicine, from tumor imaging with magnetic resonance imaging (MRI) modality [4], to cancer hyperthermia therapy and even targeted drug/gene delivery systems [5,6].

Magnetic Resonance Imaging (MRI) is a clinical imaging technique that allows for noninvasive tomographic visualization of anatomic structures with high spatial resolution and soft tissue contrast. Contrast agents of MRI, composed of paramagnetic, superparamagnetic and ferromagnetic materials, can change the image contrast between normal and diseased tissue [6]. It is well accepted that nanosized magnetic contrast agents present distinct advantages over conventional contrast agent magnetic resonance imaging (MRI) modality. Chief among these advantages are their high surface area, their ability to be delivered to a specific cancer site by active targeting, and the possibility of engineering their blood circulation half-life [1,2].

Hydroxyapatite (Ca_{10}(PO_{4})_{6}(OH)_{2} (HAP)) is a bioceramic material with a calcium-to-phosphorus ratio similar to that of natural bone and teeth. There is therefore great clinical interest in its use, since it is biocompatible, bioactive and biodegradable. It is currently used for bone graft substitutes, such as porous granules, block scaffolds and coatings over metallic implants for bone regeneration [7]. Particular physicochemical properties of the apatite structure allow it to form many different compositions, therefore allowing
an easy incorporation of ions in the crystal lattice [8]. To date, several methods of preparing HAP nanoparticles have been developed, including sol-gel, biomimetic deposition, electrodeposition, ultrasonic spray freeze-drying, spray dry, combustion synthesis and the wet chemical route [9,10].

The most efficient method of administering magnetic nanoparticles to reach target tissues is intravenously [3,11], yet despite the importance of the intravenous method very few studies have addressed the hemostatic and thrombogenic effects on human blood of nanoparticles containing magnetic metal ions.

Magnetic contrast agents have been widely used in MRI diagnostics. The most clinically used ones are the gadolinium complexes that have seven unpaired electrons and a large magnetic moment [4,5]. Most of the gadolinium based MRI contrast agents available are non-specific for a particular organ. Although, there are organ-specific contrast agents (Gd-based and non Gd-based) designed to specifically accumulate in a given organ or tissue such as liver and lymph nodes or for MR angiography [12]. These contrast agents have an organic ligand with affinity to a specific type of cells. For example hepatobiliary contrast agents bind to a specific receptor site on the hepatocyte cell membrane [13]. On the other hand, superparamagnetic iron oxide (SPIO) particles for parenteral use are coated with various substances (such as albumin, a hydrophilic polymer, starch or dextran) to facilitate uptake by the reticuloendothelial system. SPIO particles are mainly T2-agents while the gadolinium and manganese-based products are mainly T1 agents. T1 and T2 relaxation times affect the signal intensities of tissues being imaged and are therefore crucial for creation of the final images. Signal in MR images can be high or low (bright or dark), depending on the pulse sequence used, and the type of tissue of interest [14,15].

Taking the above issues into account, the main goal of this study was to produce, characterize and compare hydroxyapatite nanoparticles doped with particular magnetic species (Gd, Fe(II), Fe(III) and Co) and evaluate their potential as MRI contrast agents with regard to their hemostatic, cytotoxic and hemolytic properties.

2. Methods

2.1. Hydroxyapatite-based nanoparticles preparation and characterization

The hydroxyapatite-based nanoparticles were prepared by the wet chemical precipitation method [16]. A control of pure hydroxyapatite nanoparticles was prepared using 40 mL of 0.5 M Ca(OH)2 (98% extra pure, Acros Organics), then heated to and maintained at 100 °C, to which 40 mL of 0.3 M H3PO4 (85 wt% solution in water, Acros Organics) was added at a rate of 500 μL/min. For the magnetic nanoparticles preparation, magnetic dopants such as: gadolinium chloride hexahydrate Cl3Gd·6H2O (99%, Sigma Aldrich), Cobalt nitrate hexahydrate Co(NO3)2·6H2O (98 %, Acros Organics), Iron nitrate nonahydrate Fe(NO3)3·9H2O (98 %, Acros Organics) and iron chloride tetrahydrate FeCl3·4H2O (99 %, Acros Organics) were added simultaneously with the phosphate precursor H3PO4 and dropwise to the Ca(OH)2 solution at the rate of 500 μL/min. The amount of dopant ions added depended on the required percentage of doping (2.5%, 5% and 10%) with respect to the atomic percentage of Ca [2]. All solutions were prepared with deionized water and the (Ca + doping ion)/P molar ratio was maintained at 1.67 in all procedures. Throughout the reaction, pH was maintained at ~7.4 using ammonium hydroxide (25% solution in water, Acros Organics). After the reaction, the mixture was kept in these conditions for 2 h and aged at RT overnight. Prior to the drying step at 60 °C, samples were centrifuged (5 min, ~2070g) and washed 3 times with hot deionized water. Finally, all nanoparticles were powderized using mortar and pestle and stored in a desiccator.

2.2. Size and morphology

To determine size and morphology of pure and doped HAP nanoparticles, transmission electron microscopy (TEM) analysis was performed in a JEOL 2100 F in bright-field mode and operated at 200 kV (JOEL, Tokyo, Japan). For the TEM sample preparation the as-received powder was first dispersed in ethanol and sonicated for fifteen minutes to reduce particle agglomeration. After sonication, particles were deposited onto carbon lacey TEM grids for subsequent observation. TEM images were then analyzed using Image

![Fig. 1] Bright-field TEM images of (A) non-doped and doped hydroxyapatite (HAP); (B) Gadolinium doped hydroxyapatite (Gd·HAP) and (C) iron (III) doped hydroxyapatite (Fe(III),HAP). (D) Longitudinal size distribution of HAP,Gd·HAP and Fe(III),HAP nanoparticles; Cross sectional size distribution of HAP,Gd·HAP and Fe(III),HAP nanoparticles.
Fig. 2. FTIR spectra of nanohydroxyapatite (HAP) samples at different percentages (2.5%, 5%, and 10%) of Gadolinium (Gd:HAP), Iron (II) (Fe(II)HAP), Iron (III) (Fe(III)HAP) and cobalt (Co:HAP) doping and non-doped HAP.
2.3. Chemical profile

The infrared spectra was recorded with a Fourier Transform Infrared (FTIR) spectrometer Perkin-Elmer 2000 with a 4 cm\(^{-1}\) spectral resolution and 100 scans per sample. In order to perform the analysis, \(~2\) mg of each powdered sample was mixed with \(~200\) mg of potassium bromide (KBr) to maintain low levels of humidity, and discs were produced in a uniaxial press (Grasedy Specac, Kent, England).

2.4. Crystal phase analysis

In order to investigate the crystallinity of the particles before and after doping, X-ray diffraction (XRD) measurements were performed. For this technique, samples were sintered to improve the signal obtained. The nanoHAP powders were heated up to 1000 °C at a rate of 4 °C/min as previously described [9].

The X-ray diffraction patterns were obtained in a PANalytical X’Pert PRO TCU 1000 diffractometer (PANalytical, Surrey, England), using monochromatic Cu radiation (\(\lambda = 1.541874\) Å). Data were acquired for 2\(\Theta\) values between 8° and 100° with steps of 0.008°.

2.5. Magnetic properties

Magnetic properties of doped and non-doped nanoparticles were studied by a superconducting quantum interference device (SQUID) (Quantum design Ref: MPMS-5S; San Diego, California, USA) in an applied magnetic field of \(\pm 50\) kOe at 300 K. The strength of magnetic moment is presented as a mass magnetization (M) – magnetic moment per total mass of each sample. For these measurements, sample powders were weighed and inserted in a see-through capsule and, in turn, the capsule was inserted in a see-through straw that was further introduced into the SQUID magnetometer. MR imaging experiments were performed with a 3T clinical scanner (Sigma HDXT 3T, GE Health Care, USA). T1 contrast images were recorded by pulse sequence technique with TR/TE = 4.8/1.3, FGR 30, EC 1/1 and band width 31.3 KHz. 4 mL of different samples dissolved in distilled water (pH \(\sim 7.4\)), at the concentration of 250 µg/ml, were taken in 3 different centrifuge tubes and imaged (Fig. 3).

2.6. Biological response

2.6.1. Cell culture

Human Dermal Microvascular Endothelial Cells (hDMECs) were purchased from ScienCellResearch Laboratories and cultured in Endothelial Cell Basal medium supplemented with 2% foetal bovine serum (FBS) and VEGF (EBM®,-2, Clonetics Lonza). The medium was renewed every 2–3 days until confluence was reached and cells were used until passage 7.

2.6.2. Cell metabolic activity

In order to estimate in vitro cell viability of doped HAP with the highest doping concentration (10%) of Gd, Fe(II), Fe(III) and Co, a resazurin (Sigma-Aldrich) assay was performed where fluorescence acts as a proportional response of cell metabolic activity [17].
Firstly, a 96-well plate was seeded with 5000 hDMEC cells per well. After 24 h of cell culture in TCPS alone, the same cells were incubated with a new medium containing autoclaved nanoparticles at a concentration of 500 μg/ml and incubated for the time period of 4 h, 24 h and 48 h at 37 °C. After each time-point, the medium was removed and resazurin was added with a fresh medium at a final concentration of 10% (v/v) and incubated for 4 h, at 37 °C. 100 μL of the medium was then extracted from each sample and fluorescence was measured at λex = 540 nm and λem = 590 nm in a microplate reader (BioTek Instruments, Winooski, Vermont, USA).

2.6.3. Cellular morphology
After 4 h and 24 h of culture, cell morphology was observed by staining F-actin with Alexa Fluor® 488 phallolidin (Invitrogen, Molecular Probes) and counterstaining nuclei with DAPI (4′,6-diamidino-2-phenylindole, Merck). Culture medium was removed from wells and samples were washed twice with phosphate buffered saline (PBS) and fixated with 4% formaldehyde at RT (room temperature) for 15 min. After washing, cells were permeabilized for 30 min with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) and washed

Fig. 4. Variation of magnetization with the applied field of ±50 kOe for synthesized doped and non-doped nanohydroxyapatite: (A) Gd doping; (B) Fe(II) doping; (C) Fe(III) doping; (D) Co doping; (E) highest doping percentage in contrast with non-doped nanoHAP and Gadoteric acid.

Fig. 5. Magnetic resonance image of Gadolinium doped hydroxyapatite (Gd,HAP), iron (III) doped hydroxyapatite (Fe(III),HAP) and Gadoteric acid.
again 3 times with PBS. Samples were then incubated with phal- 
loidein diluted 1:100 in PBS for 30 min in the dark at RT and washed 
3 times with PBS. To stain cells nuclei, samples were incubated 
with DAPI at a concentration of 0.1 μg/ml in PBS for 10 min and 
washed with PBS 3 times. Two drops of Fluoromount™ (Sigma- 
Aldrich) were added and samples were stored at 4 °C in the dark 
until observation under an inverted fluorescence microscope with 
proper light filters.

2.6.4. Hemolysis assay and hemostatic studies

To perform hemolysis assays and hemostatic studies, blood 
samples were collected by venipunction from 4 healthy human 
volunteers into tubes using sodium citrate 3.8% as an anticoagulant.

2.6.5. Hemolysis assays

The hemolysis assay was performed according to the Standard 
Practice for Assessment of Hemolytic Properties of Materials from 
the American Society for Testing and Materials (ASTMF756-00, 
2000). After blood collection, hemoglobin concentration was mea-
sured by using an automatic blood cell counter (Sysmex K10000; 
Sysmex, Hamburg, Germany). Subsequently, blood was diluted 
with PBS, pH7.4, to obtain a concentration of 8 g/dl of hemoglobin. 
Hemolysis tests were then performed in 5 different concentra-
tions (0.25; 0.5; 1; 2; 4 mg/ml) of samples HAP, Gd10HAP and 
Fe(III)10HAP. Positive and negative controls (tritonX-100 1% (w/v) 
and PBS, respectively) were also included. The samples and com-
ponents were placed in contact with blood for three hours. During incuba-
tion time, these were gently agitated every 60 min. Afterwards, 
they were centrifuged at 1600g for 10 min and supernatant was carefully 
removed for spectroscopic analysis. The absorbance of supernatant 
was measured at 540 nm and hemolysis percentage was calculated 
according to hemoglobin concentrations estimated by a calibration 
curve.

SEM analysis was performed to evaluate erythrocyte morphol-
ogy, where total blood was used to incubate HAP, Gd10HAP, 
Fe(III)10HAP and polypropylene (negative control) samples for 
3 h at 37 °C, at a concentration of 4 mg/ml. After incubation, 
biomaterials were rinsed with PBS, fixed with 1.5% glutaralde-
hyde in 0.14 M sodium cacodylate buffer for 10 min. After they 
were rinsed with PBS, they were fixed again with 4% formalde-
hyde for 30 min. They were then dehydrated in a graded series 
of alcohols and hexamethyldisilazane (HMDS, Ted Pella, USA). 
SEM evaluation was performed using a high resolution en-
vironmental scanning electron microscope with X-ray microanalysis 
and electron backscattered diffraction analysis (Quanta 400 FEG 
ESEM/EDAX Genesis X4 M, Hillsboro, Oregon, USA).

2.6.6. Platelet activation

For the platelet activation studies, blood was centrifuged at 200g 
for 10 min in order to obtain platelet-rich plasma (PRP). Samples 
HAP, Gd10HAP, Fe(III)10HAP and negative (polypropylene) con-
trol were incubated at 37 °C with PRP for 3 h at a concentration 
of 4 mg/ml. For SEM evaluation, samples were fixed and dehy-
drated using the same procedure referred to above to evaluate 
the interaction of biomaterial with blood. SEM evaluation was per-
formed using a high resolution environmental scanning electron 
microscope with X-ray microanalysis and electron backscattered 
diffraction analysis (Quanta 400 FEG ESEM/EDAX Genesis X4 M; 
Hillsboro, Oregon, USA), to evaluate platelet adhesion and activa-
tion.

2.6.7. Coagulation time (aPTT; PT) and whole blood kinetic time 
assays

To evaluate the effect of biomaterials on activated partial throm-
boplastin time (APTT) and prothrombin time (PT), blood samples 
were centrifuged in order to obtain platelet-poor plasma (PPP). 
Centrifugation was performed at 1500 g for 15 min at 4 °C. The HAP, 
Gd10HAP, Fe(III)10HAP and negative control (propylene) samples 
were incubated with PPP at 37 °C for 3 h in the following concen-
trations: 0; 25; 0.5; 1; 2; 4 mg/ml. After incubation, APTT and PT 
were determined by using commercial reagents (Diagnostica Stago, 
Almada, Portugal). Results are reported in seconds.

Thrombogenicity of HA doped-nanoparticles HAP, Gd10HAP 
and Fe(III)10HAP, was evaluated using a whole blood kinetic clot-
ing time method as previously described [13]. HAP, Gd10HAP and 
Fe(III)10HAP nanoparticle, as well as positive (glass) and negative 
(polypropylene) controls, were used per time point at a concentra-
tion of 4 mg/ml. Clotting reaction was activated with the addition 
of 100 μL CaCl2 (0.1 M) to each 1 mL of sodium citrate blood. A 100 μL 
volume of this activated blood was put in contact with controls and 
nanoparticles (HAP, Gd10HAP and Fe(III)10HAP), for 5, 15, 25, 35 
and 45 min. At the end of each time point, 3 mL of distilled water 
was added and incubated for 5 min. Supernatant absorbance was 
measured at 540 nm, in triplicate (200 μL each) in a 96 well plate 
reader (Bioteck Powerwave XS, Winooski, Vermont, USA). Red blood 
cells that were not trapped in a thrombus were lysed with addi-
tion of distilled water, thereby releasing hemoglobin into water for 
subsequent measurement. The clot size is inversely proportional to 
absorbance value. The same procedure was done for Fe(III)10HAP 
sample at the concentrations 0; 25; 0.5; 1; 2; 4 mg/ml.

2.6.8. Measurement of plasma recalcification profiles

Plasma recalcification times were determined by the method 
described by Motlagh D et al. [18] on HAP, Gd10HAP and 
Fe(III)10HAP. Controls consisted of PPP with and without CaCl2 
(respectively negative control (normal recalcification profile) and 
positive control (no clotting)). Following the addition of PPP, 100 μL 
of 0.025 M CaCl2 were added to each well (except for the positive 
control). The kinetics of the clotting process due to recalcification 
was monitored by measuring the absorbance at 405 nm (every 30 s 
for 45 min) at 37 °C. In calculating mean absorbance at each time 
point, six wells were averaged per sample. The slopes of the linear 
portion of each profile and clotting time to reach half maximum 
absorbance were calculated and analyzed.

2.6.9. Statistical analysis

Data from the resazurin assay were analyzed using a Scheffe 
test for multiple comparisons. Results were considered signifi-
cant when p<0.05. Calculations and descriptive statistics were 
performed using SPSS-Software for Windows® (version 17.0, IBM 
corporation, USA) and Microsoft Office Excel 2010® (Redmond, 
Washington, USA). All data are presented as mean± standard 
deviation. The Kolmogorov–Smirnov test was used to evaluate nor-
mality.

3. Results

3.1. Physicochemical characterization

3.1.1. Size and morphology

Both the size and the morphology of nanoparticles synthesized 
as MRI contrast agents influence not only their interaction with 
each other in a suspension, but also cell behavior. Fig. 1 shows 
bright-field TEM images of non-doped hydroxyapatite and doped 
nanoparticles and size distribution. Particles exhibit a rod-like 
shape, with an average longitudinal dimension between ~34 and 
~59 nm and cross sectional dimensions between ~15 and ~29 nm.

3.1.2. Chemical profile (FTIR)

In order to chemically characterize changes in nanoHAP chem-
ical profile, a FTIR analysis was performed (Fig. 2).
Considering all the represented species, FTIR spectra were characterized by lattice (~3573 cm\(^{-1}\)) and vibrational (~634 cm\(^{-1}\)) OH bands, phosphate bands (~v1 962 cm\(^{-1}\), ~v2 470 cm\(^{-1}\), ~v3 1090–1110 and ~1035 cm\(^{-1}\), ~v4 603 and ~565 cm\(^{-1}\)), residual carbonates C–O resulting from atmospheric CO\(_2\) adsorbed by apatite (~1457, ~1421 and ~876 cm\(^{-1}\)) and lattice water (3417–3495 cm\(^{-1}\) and ~1637 cm\(^{-1}\)) which are typical in a HAP spectra [19–21]. All species also presented a few weak peaks 2500–2000 cm\(^{-1}\) that might correspond to HPO\(_4^{2-}\) groups as previously reported in the literature [6,22]. Regarding doped-HAP nanoparticles, addition of Gd\(^{3+}\) increased the intensity of the band assigned to 2500–3500 cm\(^{-1}\) and also increased peak intensity assigned to hydroxyl groups (3570 cm\(^{-1}\)) but did not significantly affect the rest of the spectra (Fig. 2). Fe(II) doping also produced a similar effect in the water band and hydroxyl peak (Fig. 2). Fe(III) showed a major increase only in the intensity of the hydroxyl peak compared to pure HAP (Fig. 2). Cobalt insertion in the HAP matrix did not significantly alter the spectra compared to the nanoHAP one. A sharp peak was also found in Co10_HAP ~800 cm\(^{-1}\) which might correspond to the presence of some remaining nitrate from the used reagents.

### 3.1.4. Magnetic properties

Since the main objective of this work is to develop a robust and reproducible contrast agent system capable of enhancing tissue contrast, it is important to illustrate the magnetism of doped nanoHAP samples. The relationship of magnetization to the applied field (±50 Oe) at 300 K of nanoHAP with different doping ions is plotted in Fig. 4.

In all doped nanoparticles (Gd\(^{3+}\)HAP, Fe(II)\(^{2+}\)HAP, Fe(III)\(^{3+}\)HAP and Co\(^{2+}\)HAP), magnetization level increased with doping concentration of each ion, confirming their predominant role in making these nanoparticles magnetically functional.

When comparing nanoparticles that have the highest doping percentage with non-doped HAP (Fig. 4 E), it is clear that the pure hydroxyapatite remained diamagnetic. Also, it can be noted that the maximum magnetic moment of the nanoparticles varied with the doping ion, where Gd10_HAP had the highest and Co10_HAP the lowest maximum magnetic moment. MRI contrast image show us a brightness increment on images corresponding to Gd10_HAP when compared with sample Fe(III)10_HAP. Furthermore, Gd10_HAP image is very similar to Gadoteric acid image what suggest that nanohydroxyapatite doped with appropriate concentrations of Gd\(^{3+}\) is the best one to be used for MR imaging (Fig. 5).

### 3.1.5. Biological characterization

#### Nanotoxicity analysis

The cell viability of many recently developed nano-sized contrast agents is a major issue that presents a possible barrier to clinical use. To address this, one of the aims of this study was to detect toxic effects induced by doped NPs compared to non-doped HAP. For this purpose two parallel assays were performed analyzing the metabolic activity (Fig. 6B) and morphology (Fig. 6A) of...
hDMECs. As previously stated, higher ion doping corresponds to higher magnetic moment and, consequently, a higher potential as an MRI contrast agent. Therefore, nanoparticles with the highest value of ion doping were further investigated in this assay to assess their biocompatibility.

After 4 h of culture, there were no statistically relevant differences between the metabolic activity of the cells in different doped-HA nanoparticles. However, after 24 h of incubation, hDMECs cultured in TCPS had a statistically significant higher metabolic activity compared with those in other conditions, Fig. 6B. In order to reinforce results obtained previously, hDMECs were stained in parallel with cell metabolic activity assay to search for abnormalities in cells morphology and proliferation. The results are in accordance with the ones extracted from the cell metabolic activity assay. Interestingly, in each substrate we observed what resembles cells going through mitosis with double nuclei in the same cytoplasmic area (highlighted by a white square in Fig. 6C). Another interesting characteristic is the heterogenic morphology of hDMECs for both time points where there exist clear differences in cell size, suggesting that these cells are proliferating at different stages. Nonetheless, there does not seem to be a morphological change between time points in all conditions, which may suggest that these NPs were biocompatible for this cell type.

3.1.6. Hemolysis assays and hemostatic studies

Taking into account the previous results obtained in cytocompatibility and magnetic properties assays, only two different types of samples were chosen to continue the hemocompatibility tests. Gd10_HAP and Fe(III)10_HAP samples presented the best magnetic moments, very close to that of Gadoteric acid, making them the most suitable candidates for being used as MRI contrast agents.

The in vitro toxicity evaluation of Gd10_HAP, Fe(III)10_HAP and HAP nanoparticles to human erythrocytes was done according to ASTM (Standard practice for measurement of hemolitic properties of materials, American Society for Testing and Materials Designation: ASTM: F 756-00). If hemolysis percentage is below 2% the material is considered non-hemolytic, between 2% and 5% slightly hemolytic, and above 5% it is considered hemolytic. Fig. 7.1 shows that HAP samples presented a hemolysis percentage very similar to Gd10_HAP for all tested concentrations (without statistical differences). Fe(III)10_HAP presented a slightly lower percentage compared to the other samples. However, it is important to notice that all samples showed no hemolytic activity up to the concentration of 4 mg/ml, since all nanoparticles tested presented a hemolysis percentage below 2%, similar to the one found in negative controls (without polypropylene; 0.045%). It was also verified (by SEM analysis) that nanoparticles did not affect the morphology and membrane integrity of erythrocytes (Fig. 7.2).

3.1.7. Platelet adhesion and activation

As shown in Fig. 8, platelets that were in contact with the different samples of nanoparticles presented a round or dendritic shape with no aggregation. This flattened morphology with some pseudopodial extensions has been previously correlated with a low state of activation [23]. Furthermore, the same platelet morphology was found in the polypropylene surface used as a non-thrombogenic control material.

3.1.8. Coagulation time (aPTT;PT) and whole blood kinetic clotting time

In this study, nanoparticles effect on coagulation cascade activity was evaluated by measuring PT and aPTT in plasma samples incubated with different materials. No significant changes were found regarding PT results (Fig. 9.1); however, aPTT results showed that Fe(III)10_HAP nanoparticles decreased aPTT when compared to other materials and the negative control. The study proved that HAP and Gd10_HAP nanoparticles up to a concentration of 4 mg/ml did not interfere with the intrinsic or extrinsic pathways of coagulation. However, results suggested that the presence of ion ions activated the coagulation cascade and shortened aPTT.

Fig. 9.2A shows the kinetics of the clotting process for Gd10_HAP, Fe(III)10_HAP and HAP samples at the concentration of 4 mg/ml, where it can be seen that the Fe(III)10_HAP sample clearly had much lower absorbance levels than the positive control. This means that most of the red blood cells were trapped in a thrombus and erythrocytes were not lysed with the addition of distilled water and
hemoglobin was not released into the water. The clot size formed is inversely proportional to absorbance value. In Fig. 9.2B it is confirmed that even in lower concentrations, Fe(III)10HAP absorbance values were very near to the ones observed for the positive control.

3.1.9. Measurement of plasma recalcification profiles

Fig. 9.3A and 3B showed the effect of HAP, Gd10HAP and Fe(III)10HAP at a concentration of 4 mg/ml on the plasma recalcification profile. The slope of the linear portion and the clotting time to reach half maximum absorbance showed that with Fe(III)10HAP the plasma recalcification profile shortens significantly when compared to other nanoparticles and to the negative control, suggesting a prothrombotic effect of Fe(III)10HAP. In Fig. 9.3C and 3D it was confirmed that even in lower concentrations, the Fe(III)10HAP plasma recalcification profile was similar to that found in the positive control (glass).

4. Discussion

Our work proposed the use of magnetic hydroxyapatite nanoparticles as nanosized contrast agents for MRI. TEM observation of doped and non-doped HAP nanoparticles carried out in the study showed no significant differences regarding size and morphology after incorporation of the dopant ions. All nanoparticles exhibited rod-like shapes with similar sizes.

Samples were characterized as HAP single phase by XRD, except for Fe(II)10HAP and Co10HAP. For these samples, the secondary phase of β-tricalcium phosphate was identified, which could have been produced by Fe(II) and Cobalt content during heat-treatment. It is important to notice that this secondary phase only appeared when the quantity of doping ions was high (10%), confirming that metallic ions were incorporated in the hydroxyapatite lattice. Regarding FTIR analysis, results showed there was no great difference between pure hydroxyapatite nanoparticles and doped HAP nanoparticles.

In sum, FTIR and XRD results showed that hydroxyapatite structure did not change significantly after the addition of metal ions. These results are in accordance with the TEM observations, where no evident changes in nanoparticle morphology were observed. Chen et al. [24] have previously reported that Gd(OH)$_2$ spherical nanoparticles were frequently the final product of reactions between Gd(NO)$_3$ and NaOH. Because metallic ions were not incorporated into the HAP crystal structure, it was expected to find this oxide in the samples. Similarly, for gadolinium hydroxide, an excess of other metal oxides can precipitate during the reaction. None of these metallic oxides were detected on the FTIR or XRD spectrums from the samples analyzed, which indicates that they were included in the HA lattice.

Since the intent of this study was to produce a basic nanocontrast that could increase MRI sensitivity, the fact that Gd10HAP and Fe(III)10HAP presented the best magnetic properties is important. In order to have a positive control over the nanoparticles, a commercially available Gd-based MRI contrast agent composed of Gadoteric acid was also measured, and it was clear that Gd10HAP and Fe(III)10HAP present a magnetic moment very close to that of this positive control. However, Gd10HAP exhibited the highest relaxivity values compared to other magnetic-doped-HAP samples. These results are in accordance with the ones extracted from MRI images where Gd10HAP have the higher brightness when compared with Gadoteric acid.

Nanotoxicity in vitro assessment indicates that none of the synthesized nanoparticles was particularly toxic for the HDMEC primary cell line within the studied time frame. This was an important finding because in clinical medical applications primary endothelial cells are among the first cells to be in contact with nanoparticles. The biggest concern was that Gd$^{3+}$, present in hydroxyapatite structure, could be toxic for cells. It is well known that Gd$^{3+}$ ions present some adverse side effects such as renal failure, pancreatitis or local necrosis [25]. However, when chelated with ligands (usually organic compounds) Gd complexes are less toxic and can be used as a contrast agent for MRI applications [26]. It is therefore confirmed that Gadolinium incorporated into HAP structure is not toxic.

Most MRI contrasts agents are developed for intravenous administration, so they require a careful analysis with regard to blood compatibility. Hemolysis assays were performed in the present work for all doped nanoparticles (GdHAP, Fe(II)HAP, Fe(III)HAP and CoHAP) and non-doped HAP. No hemolytic activity was observed up to a concentration of 4 mg/ml with a hemolysis percentage below 2%, very close to the one found in negative controls (with polypropylene: 0.045%).

Platelet adhesion and activation and coagulation cascade activation assessments are very important to evaluate biomaterial blood compatibility, namely to evaluate the probability of thrombus formation. Therefore, HAP, Gd10HAP and Fe10HAP nanoparticles were incubated with platelets (platelet-rich-plasma) which adopted a round or dendritic morphology with no signs of aggregation. This flattened round morphology with some pseudopodial extensions has been previously correlated with a low activated state. Furthermore, the same platelet shape was found in the polypropylene surface used as a non-thrombogenic control material.

A blood clot is the final product of blood coagulation and it is not only composed of aggregated platelets, but also of a mesh of cross-linked fibrin protein. In this study, Hap and Gd10Hap nanoparticles did not interfere with intrinsic or extrinsic pathways of coagulation. Nevertheless, Fe(III)10Hap nanoparticles activated coagulation cascade shortening aPTT times, which is frequently related with an increased risk of thrombosis. In order to confirm the previous results from the coagulation time assay (aPTT;PT), another assay was performed: the whole blood kinetic clotting time. The main difference between these two coagulation assays is the fact that the first one is performed using only platelet-poor-plasma (PPP) and the last one is performed using all blood components. Once more, Fe(III)10Hap nanoparticles presented absorbance values that were clearly less than those observed for glass beads

Fig. 8. Evaluation of platelet adhesion and activation by SEM. Platelets in contact with: A) Gd10HAP; B) Fe(II)10HAP; C) HAP; D) negative control (Polypropylene) and E) positive control (Glass).
Fig. 9. Coagulation assays: 9.1-(A) aPTT and (B) PT coagulation time of PPP incubated with different concentrations of gadolinium doped HAP at 10% (Gd10 HAP) and iron doped HAP at 10% (Fe(III)10 HAP) and hydroxyapatite (HAP). * indicates a significant statistical difference between Fe(III)10 HAP and all the other groups (HAP, Gd10 HAP) at the same concentrations (p < 0.05; n = 5); 9.2 – Measurement of whole blood kinetic clotting time for HAP, Gd10 HAP and Fe(III)10 HAP samples at the concentration of 4 mg/ml (A) and Fe(III)10 HAP at the concentration of 0.25, 0.5, 1, 2, 4 mg/ml (B); 9.3 – Measurement of plasma recalcification profiles for HAP, Gd10 HAP and Fe(III)10 HAP at the concentration of 4 mg/ml, slope of the linear portion of each profile (A) and the clotting time to reach half maximum absorbance (B); Fe(III)10 HAP at the concentrations of
(the positive control), meaning erythrocytes were trapped in the thrombus formed during the assay, even when lower doses of Fe(III)10.HAp were tested. Regarding the profiles of Fe(III)10.HAp nanoparticles, the plasma recalcification profile shortened significantly when compared to other nanoparticles and to the negative control, suggesting a prothrombotic effect of Fe(III)10.HAp. Fig. 9.3C and 3D confirm that even in lower concentrations, Fe(III)10.HAp plasma recalcification profile was similar to that found in the positive control (glass). Results from the three coagulation in vitro tests reinforced the theory that iron doped HAp nanoparticles can be potentially thrombogenic materials, as opposed to gadolinium doped HAp nanoparticles. Also, previous work has shown that Fe3+ or its derivatives plays an important role in the coagulation process and can precipitate plasma proteins that might increase the risk of thrombosis while extending plasma clotting. Lipinski B et al. [27] showed that iron ions enhanced fibrin fibers formation and delayed fibrinolysis when in contact with human blood, which could be explained by a well-known correlation between thrombotic diseases and the increase of iron ions and/or hemoglobin derivatives in the blood [27–29]. There is a possibility that Fe3+ ions leached from nanoparticles were enough to interact with coagulation factors causing a decrease in aPfT values and improving clot size formed during whole blood kinetic clotting time assay.

Previous studies suggested that natural hydroxyapatite particles from human calcified tissue may interfere with platelet aggregation [30–32]. In the present study it was shown that doped and non-doped HAp nanoparticles did not interfere with the coagulation pathway up to a concentration of 250 mg/ml.

5. Conclusions

In this study, both hydroxyapatite and magnetic ion doped hydroxyapatite nanoparticles were successfully produced by a well-controlled wet chemical precipitation method. The magnetically doped particles obtained had a rod-like shape with sizes that vary between 30 and 60 nm that were not affected by the dopant percentage or the type of doping agent. It was shown that the dopant concentration to the Ca2+ atomic percentage ratio does not significantly alter the HAp structure although it has a predominant role in magnetic properties of the nanoparticles. 10% Gd doped HAp and 10% Fe doped HAp presented the highest magnetic moment levels compared to Fe(II) and Co doped HAp and the magnetic moment values closest to those of a commercially available Gd-based MRI contrast agent composed of Gadoteric acid.

Morphology and morphology of human dermal microvascular endothelial cells did not seem to be significantly altered after incubation with the synthesized materials.

Regarding the hemolytic assay and hemostatic studies, it was shown that HAp, Gd and Fe doped HAp showed no hemolytic activity up to the concentration of 4 mg/ml, with a hemolysis percentage less than 2%. Through SEM analysis, it was observed that nanoparticles did not activated platelets. The coagulation cascade activation assays and the whole blood kinetic clotting time showed that HAp and Gd10.HAp nanoparticles up to a concentration of 4 mg/ml did not interfere with the intrinsic or extrinsic coagulation pathways. However, results suggested that the presence of iron ion affected the coagulation cascade, showing that Fe(III) doped HAp nanoparticles are thrombogenic materials.

In sum, these findings suggest that when compared with the other doped HAPs, Gd doped HAp nanoparticles are a superior candidate for medical applications as a versatile and customizable paramagnetic T1 MRI contrast agent. Furthermore, the future clinical use of Fe(III) doped HAp should be approached with caution with regards to blood compatibility.

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