Research Article

Superparamagnetic Bifunctional Bisphosphonates Nanoparticles: A Potential MRI Contrast Agent for Osteoporosis Therapy and Diagnostic

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1. Introduction

Bisphosphonates exhibit a powerful binding affinity to bones and are routinely used for treatment in bone resorption and other bone disorders like Paget’s disease, osteoporosis, or tumor induced osteolysis [1]. The binding to bone mineral depends upon the P-C-P structure and is enhanced by including a hydroxyl group (hydroxy methylene bisphosphonate, called HMBP in the text). This was probably due to tridentate binding hydroxyl substituted bisphosphonates to calcium. In contrast, bisphosphonates lacking a hydroxyl group, that provide a bidendate binding to calcium crystals, had significantly lower binding affinities [2]. Hence HMBP molecules, such as Alendronate (4-amino-1-hydroxybutyliendene bisphosphonic acid), inhibit osteoclast-mediated bone resorption [3]. With the recent developments in magnetic resonance, in vivo studies showed that patients with, and without, osteoporotic fractures could better be separated with parameters of bone architecture obtained by MRI than BDM [4]. For molecular imaging, the use of nanoparticles emerge as very exiting nanoobjects in that many functionalities can be added to the surface of the particle. More specifically, superparamagnetic iron oxide [5] (SPIO, hydrodynamic diameter >50 nm) and ultrasmall superparamagnetic iron oxide (USPIO, hydrodynamic diameter <50 nm) particles have been introduced as an MRI contrast agent after the gadolinium chelates and appear to be currently a more relevant agent than Gd chelates due to the high MR signal per unit of metal. As these nanoparticles are made of thousands iron atoms, they defeat the inherent low contrast agent sensitivity of MRI and thus can be detected at micromolar concentration of iron. Moreover the iron ions are much less toxic than the gadolinium ones and can be reused or recycled by cells using normal...
biochemical pathways for iron metabolism [6, 7]. Our previous studies have shown that bisphosphonate such as 1-phenyl-1-hydroxymethylene-1,1-phosphonic acid (HMBP-COOH) [8] or 1-hydroxy-2-(imidazol-1-yl)ethylidene-1,1-bisphosphonic acid (zoledronate) [9] act as very efficient ligand for iron oxide nanoparticles. In the case of quaternary ammonium bisphosphonate coated iron oxide nanocrystals, it has been shown that this hybrid nanocrystals [10] presented adequate performance for blood remanence and weak liver capture. No significant desorption of the coating molecules was observed on steel plates. In recent work [11] it has been demonstrated that pretreatment of metal alloy surface with an aqueous polyallylamine bisphosphonate solution (BP...NH₂) result in the formation of a molecular bisphosphonate layer that permit the attachment via the amine terminated function of vector binding agent for therapeutic gene delivery. After 30 day incubation, the layer is not altered indicating that a mechanism of desorption reabsorption of BP molecules seems to be highly unlikely. In this article, an innovative approach is presented, leading to the optimization of the nanoparticle structure to achieve selective targeting for osteoporosis imaging and therapy. Superparamagnetic nanoparticle surface are passivated using a bifunctional passivating agent such as 1,5-dihydroxy-1,5,5-tris-phosphono-pentyl-phosphonic acid (call di-HMBP in the text, Scheme 1). One HMBP function complexes the nanocrystal surface and the other one at the outer surface allows bone targeting. A stable ferrofluid (γFe₂O₃@di-HMBP) is obtained on large concentration and pH range. The large numbers of HMBP functionalities on the magnetic core of the particle have a strong affinity for hydroxyapatite and can be used for bone targeting. The feasibility of such process is demonstrated by the complexation of the hybrid nanomaterial to calcium ions and hydroxyapatite and imaged using MRI.

2. Materials and Methods

2.1. Materials and Reagents. IR spectra were recorded on a Thermo Electron Corporation Nicolet 380 FTIR (KBr pellet). UV-visible spectra were recorded on a Varian Cary 50 Scan UV-Visible spectrophotometer. Transmission electron microscopy (TEM) measurements were carried out using a Philips CM10. 1H-NMR spectra were obtained on a Varian Gemini spectrometer at 200 MHz with chemical shifts being reported as ppm from trimethylsilane as internal standard. The size and the zeta potential of the nanocomplex were determined by dynamic laser light scattering (DLS) on a Nano-ZS (Red Badge) ZEN 3600 device (Malvern Instruments, Malvern, UK. Each sample was determined by transmission electron microscopy. Colloidal suspensions were deposited directly onto a carbon-coated copper grid. The size and the zeta potential of the nanocomplex was determined by dynamic laser light scattering (DLS) on a Nano-ZS (Red Badge) ZEN 3600 device (Malvern Instruments, Malvern, UK).

2.2. Synthesis of (1,5-Dihydroxy-1,5,5-Tris-Phosphono-Pentyl)-Phosphonic Acid [12] (Di-HMBPs). In a 50 mL round-bottom three-neck flask equipped with a thermometer, glutaryl chloride (18 mmol) was added dropwise, under argon, at −5°C, to tris(trimethylsilyl) phosphite (72 mmol). When addition was completed, reaction mixture was allowed to stand at room temperature for 1 hour. The evolution of the reaction was monitored by 31P{1H} NMR. Then, volatile fractions were evaporated under reduced pressure (0.1 Torr) before methanolysis (20 mL). After evaporation, crude products were precipitated in diethyl ether and lyophilized. The pure product was obtained in 95% yield. 31P NMR δ (1H) (161.9 MHz, D₂O) δ 19.3, δ 1H NMR (400.1 MHz, D₂O) δ 1.78–2.05 (m, 6H, (CH₂)₂-C(OH)-(CH₂)₂-C(OH)), δ 13CN M R (80.9 MHz): 19.17 ppm solution added with δ 19.3, 1H NMR (400.1 MHz, D 2O) δ 18.1, (δ -CH₂-CH₂-CH₂-δ), 34.0 (δ -CH₂-CH₂-CH₂-δ), 73.2 (t, 1Fp-δ = 143.7 Hz, P-C(OH)-P).

2.3. Synthesis of γFe₂O₃@Di-HMBP Nanocrystals. To prepare noncoated γFe₂O₃ particles, the first step is to add a solution of dimethylamine 40% in water ((CH₃)₂NH, 10.5 mL) to an aqueous micellar solution of ferrous dodecyl sulfate (Fe(DS)₂) (0.61 g, 10⁻² mol). The solution is stirred vigorously for 2 hours at 28.5°C and the resulting precipitate of uncoated nanocrystals is isolated from the supernatant by centrifugation. In the second step, this precipitate is washed with an acidic solution (HCl 10⁻¹ mol · L⁻¹) and a solution of di-HMBP molecules (n = 10⁻⁴ mol in 30 mL of water) is added. The solution is stirred for two hours at room temperature. The precipitate that appears is washed with an acidic solution (HCl 10⁻¹ mol · L⁻¹). Free HMBP are isolated from the coated particles thanks to a magnetic field and by centrifugation. The magnetic nanocrystals coated with di-HMBP molecules are dispersed in water. The initial pH is equal to 4 and then progressively increased to pH 7.4 by addition of sodium hydroxide NaOH (10⁻¹ mol · L⁻¹). The iron concentration is deduced from UV-vis absorption.

2.4. Nanocrystal Surface Characterization. FTIR spectroscopy is used to demonstrate nanocrystal surface complexation via phosphonate groups. The average number of molecules per nanocrystal is deduced with 31P NMR spectroscopy. A range of concentrations of free di-HMBP (NMR 31P{1H} (80.9 MHz): 19.17 ppm solution added with NaH₂PO₄ (in capillary, 10⁻¹ mol · L⁻¹; NMR 31P{1H} (80.9 MHz): 0 ppm) was prepared for calibration. The di-HMBP molecules are removed from magnetic γFe₂O₃ nanoparticles by addition of sodium hydroxide NaOH (1 mol · L⁻¹) in order to avoid shifting of the 31P NMR signal. The supernatant is analyzed with 31P NMR and the concentration (number of molecules per nanocrystal) of di-HMBP into the sample is deduced from this calibration plot.

2.5. Analysis of the Size and Surface Charges of the γFe₂O₃@Di-HMBP Nanocrystals. The mean particle size was determined by transmission electron microscopy. Colloidal suspensions were deposited directly onto a carbon-coated copper grid. The size and the zeta potential of the nanocomplex was determined by dynamic laser light scattering (DLS) on a Nano-ZS (Red Badge) ZEN 3600 device (Malvern Instruments, Malvern, UK). Each sample was
analyzed at room temperature with diluted ferrofluid ([Fe] = 5 $\cdot$ 10$^{-4}$ mol $\cdot$ L$^{-1}$) at pH = 7.4.

2.6. Calcium Complexometric Titration. Standard procedures with Eriochrome black T (EBT) was used to quantify the amount of calcium ions in solution. The EBT was mixed to $\gamma$Fe$_2$O$_3$@di-HMBP (or free di-HMBP) aqueous solution ([Fe] = 1, 47 $\cdot$ 10$^{-2}$ mol $\cdot$ L$^{-1}$) at pH 10. Then this solution is titrated with calcium solution ([Ca$^{2+}$] = 1, 44 $\cdot$ 10$^{-4}$ mol $\cdot$ L$^{-1}$) until the color solution change from blue to pink for free di-HMBP and from green to brown for $\gamma$Fe$_2$O$_3$@di-HMBP particles solution. The variation of color is due to the complexation between EBT and calcium ions. Then the amount of calcium ions complexed with the HMBP functionality is deduced.

2.7. Magnetic Properties and Magnetic Resonance Imaging. The magnetic behavior of the as-synthesized nanoparticles is characterized using the MIAplex$^R$ reader (Magnisense). The MIAplex reader [13] measures the nonlinear response of the magnetic labels when they are exposed to a multifrequency alternating magnetic field. This specific signature [14] is based on $d^2$B(H)/dH$^2$.

MR imaging of the test tubes was performed using a 4.7 T MR scanner (Bruker). For measurements of T1 relaxation times, axial spin echo (SE) sequences were obtained with TR values of 10,000 ms as well as TE of 16 ms at 4.7 T. For measurements of T2* relaxation times, axial T2*-weighted SE images were obtained with a TR of 800 ms and TE of 6.4 ms at 4.7 T.

2.8. In Vitro Hydroxyapatite Targeting. The lyophilized hydroxyapatite [15] with a ratio Ca/P equal to 1.64. HA (10 mg/mL) was suspended in a 5 millimeter $\gamma$Fe$_2$O$_3$@di-HMBP sol 0.4 mg/mL (Fe = 5 $\cdot$ 10$^{-3}$ M). Then nanoparticles are incubated and shaken with HA at 37°C during 24 hours. After filtration and water washing with a syringe filter with 0.45 μm pore size, HA is resuspended in sol and lyophilized for infrared spectroscopy. The concentration of nanoparticles remained in the water suspension was measured by UV/VIS spectrophotometer at 350 and 480 nm for the calculation of the amount bound to HA.

2.9. Cell Viability. Human osteosarcoma cells (MG63) line was cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% calf serum. MG63 osteoblast-like cells used in the present study were obtained from the American Type Culture Collection (ATCC N° CRL 1427).

Cell viability was evaluated using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay at day 1, day 3, and day 5. Cells were seeded at a density of 20 $\times$ 10$^5$ cells/well in 96-well flat-bottom plates (Falcon, Strasbourg, France) and incubated in complete culture medium for 1, 3, and 5 days. Then, medium was removed and replaced by 10% FCS-medium containing increasing concentrations $\gamma$Fe$_2$O$_3$@di-HMBP nanocrystals. After 1, 3, and 5 days of incubation, cells were washed with phosphate buffered saline (PBS, Invitrogen) and incubated with 0.1 mL of MTT (2 mg/mL, Sigma-Aldrich) for additional 4 hours at 37°C. The insoluble product was then dissolved by addition of DMSO (Sigma-Aldrich). Optical density was measured at 570 nm using a Labsystems Multiscan MS microplate reader. Each in vitro experiment was performed three times, with four wells per sample per experiment.

2.10. Cell Labelling. The labeling of living cells is evaluated using Prussian blue staining for $\gamma$Fe$_2$O$_3$@di-HMBP nanocrystals. The principle of Prussian blue staining is that the ferric iron (Fe$^{3+}$) in the presence of ferrocyanide ion is precipitated as the highly colored and highly water-insoluble complex, potassium ferric ferrocyanide, Prussian blue. The cells were cultivated for 24 hours in eight-well chamber slides in the presence or not of $\gamma$Fe$_2$O$_3$@di-HMBPs nanocrystals. The cells were then washed three times with PBS, fixed with acetone (10 minutes) and dried at room temperature for 20 mn. The attached cell monolayer was incubated with 5% potassium ferrocyanide (5 minutes), washed with PBS and then incubated again with solution containing 5% potassium ferrocyanide and 10% hydrochloric acid for 10 minutes and washed with distilled water three times. The iron particles in the cells were observed as blue dots using an optical microscope with phase contrast.

3. Results and Discussion

Nanoparticles functionalization plays a major role within nanotechnologies applications. Scheme 1 describes the procedure to design a new MRI nanoparticle for targeted drug delivery to bone. Small $\gamma$Fe$_2$O$_3$ nanocrystals were chosen for their superparamagnetic behavior and their high T2 contrast agent sensitivity for MRI. The 1,5-dihydroxy-1,5,5-tris-phosphono-pentyl-phosphonic acid (di-HMBPs) was chosen for the two HMBP functionalities: one HMBP moiety as anchoring agent for $\gamma$Fe$_2$O$_3$ surface and the second as targeting function due to strong affinity for bone. Our approach requires the two HMBP functions of
the molecule to be separated by a short spacer, to avoid
the nanoparticles anchoring with the two HMBP moieties,
leading to nanoparticles aggregation and lost of the specific
bone targeting.

Maghemite γFe2O3 nanocrystals were prepared as
described previously [16] by soft chemistry. At the end of
the synthesis, a solution of di-HMBP in water at pH 4
is added to the bare nanoparticle dispersion. The pH was
then progressively increased to pH 7.4 by the addition of
sodium hydroxide NaOH, thus achieving a stable dispersion
of nanoparticles.

After dialysis, the dispersed solution is lyophilized. The
powder is easily dispersed in water and the nanoparticles sols
are stable over a broad range of pH (4–12) and concentration
(over 40 wt%), in suitable ionic strength (<0.6 mol·L−1) and in
various biological buffers such as PBS and Hepes. The
TEM image (insert Figure 1) of deposited nanocrystals indic-
ates an average diameter and a polydispersity, respectively,
equal to 11 nm and 20%.

IR spectroscopy analysis (Figure 1) shows that the phos-
phonate groups are highly interaction with the nanoparticle
surface.

For the free HMBP-COOH molecules (blue curve),
within the P–O stretching region (1200–900 cm−1), the
spectrum exhibits two sharp peaks at 1172 and 900 cm−1,
assigned to P=O and P–OH, respectively [17]. The broad
band at 1071 cm−1 is characteristic for the vibrational mode
for the PO3 group [18].

Comparing the γFe2O3@di-HMBP nanocrystals (red
curve) with the free di-HMBP solution (blue curve), the
large changes observed within the P–O stretching region
(1200–900 cm−1) show that a strong interaction between the
phosphonate headgroup and the Fe2O3 surface is present.
These results are consistent with phosphonate binding to
the oxide surface [19] and we can suggest that the Fe atoms
within the particle surface are coordinated by oxygen atoms
from the phosphonate groups [20].

31P NMR titration is used in order to quantify the
average number of molecules per nanocrystal. An average
number of 2100 ± 100 di-HMBP molecules per nanoparticle
is obtained, corresponding to 0.1 equivalent per Fe ions
(around 0.3 per surface Fe ions).

Dynamic light scattering was used to characterize zeta
potential and hydrodynamic diameter. This measurement
is an indication of surface charge on a particulate species,
which plays an important role in determining solution
stability, susceptibility to aggregation and precipitation prob-
lems, as well as protein and cellular surface binding in vivo.
At physiological pH, the γFe2O3@di-HMBP particles exhibit
a negative zeta potential (−54 mV) and a hydrodynamic
diameter of 36 nm suggesting the presence of few aggregates
(mean crystalline core of 11 nm). The negative charge surface
suggests the presence of free HMBP functionalities on the
magnetic core of the particle (Scheme 1). To determine
the number of free HMBP, we used standard procedures
of colorimetric tests to deduce the number of calcium ions
complexed per γFe2O3@di-HMBP nanoparticles. For free di-
HMBP molecules, we found 3.8 calcium ions complexed
per molecule meaning that each HMBP functionality may
complex about 2 calcium ions. The amount of calcium
ions complexed per nanoparticle is found equal to 3100
± 200. Considering that each HMBP functionality may
complex 2 calcium ions, an amount of 1550 free HMBP per
nanoparticle is deduced. This result is consistent with NMR
measurements leading to 2100 HMBP per nanoparticle.
Hence, the free HMBP functionalities at the outer of the
nanoparticles surface should allow their bone targeting and
the increase of bone mineral density.

The magnetic properties of these nanoparticles have been
studied using a MIAplex® reader.

The second derivative of magnetization d²B(H)/dH²
(Figure 2), presents one maxima and one minima with no
hysteresis loop. This specific magnetic signature is charac-
teristic of superparamagnetic behavior of particles with low
dipolar interaction [21]. This superparamagnetic behavior
allows to use these particles as contrast agent for MRI.

To investigate the MR signal enhancement effects, the
aqueous as-prepared nanoparticles at different Fe concen-
trations were measured on a 4.7 T MRI scanner. As shown
in Figure 3(a), both T1 and T2* weighted images change drastically in signal intensity with an increasing amount of nanoparticles, indicating that as synthesized nanoparticles generated MR contrast on both longitudinal (T1) and transverse (T2*) proton relaxation times weighted sequences. Figure 3(b) shows the relaxation rates 1/T1 and 1/T2* as a function of the iron concentration. The relaxation rates varied linearly with the iron concentration, as expected. The longitudinal r1 and transverse r2* relaxivities (corresponding to the slopes of the lines) are found to be 1.40 Fe mM$^{-1}$ s$^{-1}$ and 295 Fe mM$^{-1}$ s$^{-1}$, respectively. Such values for r1 and r2* suggest that HMBP coated nanoparticles can act as both T1 and T2* contrast agents taking into account their small size, but seem to be more favourable as T2* contrast agents due to their much larger r2* value.

One of the factors that makes HMBP most potent BP drugs is its high skeletal uptake and retention, which is directly related to its affinity towards hydroxyapatite [22] (HA). To demonstrate the specific targeting of γFe$_2$O$_3$@di-HMBPs nanocrystals to bone, standard in vitro assay [12] were performed to demonstrate the strong affinity of those new MRI contrast agent with hydroxyapatite. A γFe$_2$O$_3$@diHMBP sol ([Fe] = 5 · 10$^{-3}$ M) have been incubated with HA at 37°C, and then separated and washed using a 0.45μm filter. The binding capacity of the as-synthesized nanocomplexes has been studied using UV-vis and infrared (Figure 4) spectroscopies. As shown insert Figure 4, the change of HA color from white to brown indicates γFe$_2$O$_3$@di-HMBPs binds HA with very high affinity due to the high amount of iron nanoparticles within HA. The concentration of nanoparticles remained in the water suspension was measured by UV-vis spectrophotometer at 350 and 480 nm for the calculation of the amount bound to HA. The deduced bound amount is equal to 0.05 ± 0.01 mg of nanoparticles per mg of HA (eq. 0.19 mM HMBP per mg HA). Figure 4 displays the IR spectrum of HA (blue curve) and incubated HA with γFe$_2$O$_3$@diHMBP nanoparticles (red curve).

The HA spectrum (red curve) exhibits different bands between 1250–600 cm$^{-1}$ that are characteristic of the P–O stretching region within HA [10]. For the HA nanocomplex, the analysis of the P–O stretching region is complicated due to strong background absorbance of the HA matrix (ν(PO4)) [23]. The HA incubated with γFe$_2$O$_3$@di-HMBP (blue curve), the P–O stretching region is broadened compared to initial HA. This is very difficult to clearly assign this effect. Obviously, more experiments are needed to elucidate the exact mechanism of nanoparticle surface bonding on HA.

The magnetite nanocrystals deduced from UV-vis spectroscopy and the brown color (insert Figure 4) of incubated HA with γFe$_2$O$_3$@di-HMBP nanocrystals are suggesting selective interaction of the nanocomplex with HA and then potential targeting to bone.

In order to assess cell viability we performed viability tests on osteosarcoma MG-63 cells, a cancer line, but a pertinent model to study efficiently the behavior of osteoblastic cell line [24]. The as-synthesized nanocrystals were incubated with MG63 osteosarcoma cells precultured for 24 hours, 3 days and 5 days for various extra cellular
iron concentrations up to 3 mmol · L⁻¹ (1 eq. mmol · L⁻¹ di-HMBP). The proliferation of MG63 cells was indicated by the MTT assay as shown in Figure 5).

For the three times of incubation, MTT proliferation assay showed normal growth of osteoblast cells. No cytotoxicity was observed. To determine the intracellular uptake of γFe₂O₃@di-HMBPs nanocrystals, blue prussian imaging was performed on human MG63 cells (Figures 5(a) and 5(b)). The iron particles into the cells were observed as blue dots using an optical microscope with phase contrast (Figure 5(b)). This picture indicates massive and uniform internalization of nanocrystals within the cells. Hence, the γFe₂O₃@di-HMBPs nanocrystals may act as a diagnostic and therapeutic system. A full biological study is in progress to understand the mechanism of such nanoparticles for osteoporosis treatment and diagnostic. The aim of this work is to test feasibility of such nanosystem, this system have been complexed to hydroxyapatite to demonstrate bone targeting and increasing bone mineral density to reduce the incidence of major osteoporotic fracture. Moreover, the superparamagnetic behavior of such nanoparticle allows them to be used as MRI contrast agent in order to improve the therapeutic diagnostic for osteoporosis.

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