Bisphosphonate Functionalized Gadolinium Oxide Nanoparticles Allow Long-Term MRI/CT Multimodal Imaging of Calcium Phosphate Bone Cement

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In the field of tissue engineering (TE) and regenerative medicine (RM), biological constructs have reached such a high similarity with native tissues that conventional imaging techniques tend to be inadequate for their detection.[1] However, it is of key importance to be able to monitor the performance of the material over the course of an in vivo trial. Therefore, there is a pressing need for the development of innovative noninvasive imaging approaches, for example, based on multimodal imaging strategies combining magnetic resonance imaging (MRI) and computed tomography (CT).[2] For example, calcium phosphate–derived cements (CPCs)—a class of advanced injectable and biodegradable bone substitutes—show such high structural similarity with the mineral phase of mammalian osseous tissue that their detection is hampered.[3] On conventional radiographs, CPC has similar radiopacity to cortical bone and a slightly more radiodense appearance than the surrounding trabecular bone, making the monitoring of the material performance over clinically relevant periods both problematic and inaccurate.[4]

Direct in vivo monitoring of bioconstructs using noninvasive imaging modalities such as magnetic resonance imaging (MRI) or computed tomography (CT) is not possible for many materials. Calcium phosphate–based composites (CPCs) that are applicable to bone regeneration are an example where the materials have poor MRI and CT contrast; hence, they are challenging to detect in vivo. In this study, a CPC construct is designed with gadolinium-oxide nanoparticles incorporated to act as an MRI/CT multimodal contrast agent. The gadolinium(III) oxide nanoparticles are synthesized via the polyol method and surface functionalized with a bisphosphonate (BP) derivative to give a construct (gadolinium-based contrast agents (GBCAs)-BP) with strong affinity toward calcium phosphate. The CPC-GBCAs-BP functional material is longitudinally monitored after in vivo implantation in a condyle defect rat model. The synthetic method developed produces nanoparticles that are stable in aqueous solution (hydrodynamic diameter 70 nm) with significant $T_1$ and $T_2$ relaxivity demonstrated in both clinical 3 T and preclinical 11.7 T MRI systems. The combination of GBCAs-BP nanoparticles with CPC gives an injectable material with handling properties that are suitable for clinical applications. The BP functionalization prolongs the residence of the contrast agent within the CPC to allow long-term follow-up imaging studies. The useful contrast agent properties combined with biological compatibility indicate further investigation of the novel bone substitute hybrid material toward clinical application.

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The demand for noninvasive imaging modalities makes MRI an ideal technique as it allows noninvasive anatomical imaging and functional 3D visualization of soft tissues with high spatial resolution. Imaging of CPCs using the magnetic resonance (MR) modality can be achieved by application of short or zero echo time acquisition sequences (i.e., ultrashort echo time (UTE) and zero echo time (ZTE), respectively), which acquire data quasi-simultaneously with the excitation pulse. Nevertheless, due to similar transverse relaxation values between cortical bone and CPC (i.e., $T_2 < 1$ ms), the contrast is not sufficient for the material characterization in vivo, thus incorporation of additional agent component is required.\[^5\]

Gadolinium(III) is a lanthanide element with seven unpaired electrons and a symmetric S-state, and it shortens $T_1$ relaxation times of the water in tissues in which it is taken up, leading to a signal enhancement in $T_1$-weighted MRI due to this paramagnetic properties.\[^6\] Commercially available gadolinium(III)-based contrast agent (GBCAs) have already been utilized to enhance the $T_1$ signal of CPC constructs in vivo.\[^7\] However, the strategy employed which utilized molecular agents that were not strongly linked to the material showed an insufficient performance level that did not meet the required contrast and longitudinal imaging properties. The CPC degradation profile in vivo and the highly porous nature of the material led to leaching of the contrast agent, limiting long-term monitoring properties. Therefore, in order to improve the contrast properties in both CT and MRI the use of gadolinium(III)-nanoparticles (NPs) versus molecular agents offers more efficient relaxivity, higher effective concentrations, and the potential to more firmly anchor the contrast agent into the material.

Bisphosphonates (BPs) are well known for their bone-targeting properties. Functionalization of pharmaceutics (e.g., osteoprotegerin) or nanoparticles (e.g., superparamagnetic iron oxide) with BP groups allows them to strongly interact with the hydroxyapatite mineral phase of the bone offering multiple bonding interactions with calcium ions ($\text{Ca}^{2+}$) from each BP component.\[^8\] These interactions can be used to specifically bind the GBCAs into the CPC matrix and prolong the residence of the imaging probe in situ, despite the on-going material degradation. To date, the CPC binding and targeting properties of BP remain unexplored.

Thus, in the presented study, we have developed a surface functionalized GBCAs that can be used for long-term noninvasive monitoring of a specific CPC composite (i.e., mix of alpha-tricalcium phosphate, $\alpha$-TCP, cryo-grinded poly(D,L-lactide-co-glycolide) microparticles, PLGA, and carboxymethylcellulose, CMC, see Experimental Section in the Supporting Information). To this end, gadolinium oxide ($\text{Gd}_2\text{O}_3$) nanoparticles ($< 5$ nm in diameter) for multimodal MR/CT imaging were synthesized via the polyol method. Surface functionalization of the nanoparticles to encapsulate them in a mesoporous silica shell by addition of 3-glycidyloxypropyl trimethoxysilane (GPTES) was used to stabilize the system in aqueous media and to facilitate further functionalization with BP derivatives. The presence of the BP groups in the final constructs enhanced the affinity toward the hydroxyapatite, the main component of the CPC composite, and created the CPC-specific contrast agent (Scheme 1). Elemental analysis and IR spectroscopy was used to characterize the BP functionalization of the GBCAs, while in vitro binding experiments confirmed

**Scheme 1.** Schematic illustration of the GBCA-BP synthesis and combination within the CPC. The multidentate bonding interaction between the phosphonate groups from the BP derivative and the calcium ions from the CPC increases the affinity of GBCA-BP to the mineral phase of CPC.
the high affinity of the BP functionalized GBCAs (GBCAs-BP) toward the solid state CPC. After in vitro toxicity tests and characterization of the handling and mechanochemical properties, the obtained CPC-GBCAs nanocomposite was implanted in vivo in a rat model and the behavior of the material was followed by CT and MRI. The dual-modality nanoparticle probe allowed the visualization of the implanted cement for the entire experimental time course of 8 weeks. Finally, histological assessments were performed to investigate the biological effect of the applied material on the surrounding bone tissues and showed no adverse reactions or inhibition of bone formation.

To form contrast agent, gadolinium oxide nanoparticles were coated with a biocompatible stable silica layer that gives high colloidal stability and contains epoxy rings (from GPTES) which were used to react with the bisphosphonate precursor and functionalize the surface of the GBCAs (Scheme 1). Elemental analyses (carbon, hydrogen, and nitrogen (CHN) combustion analysis and inductively coupled plasma optical emission spectroscopy, ICP-OES) were performed on the nanoparticles before and after functionalization, showing the chemical modification of the surface of the nanoparticles at each synthetic step (Figure 1). The results from before and after addition of GPTES offered an assessment of the amount of silica polymerized on the nanoparticles surface. Thus, allowing an estimation of the molar amount of epoxide groups available for covalent conjugation with BP derivative. As expected, analysis of the resulted nanoparticles showed a decrease in the percentage of gadolinium(III) and an increase

| Percentage (%) | C  | H  | N  | Gd | P  | Si |
|---------------|----|----|----|----|----|----|
| Gd₂O₃         | 7.67 | 2.06 | 0.00 | 52.11 | 0.00 | -  |
| GBCAs        | 14.28 | 2.53 | 0.00 | 39.48 | 0.00 | -  |
| GBCAs-BP     | 12.56 | 3.29 | 1.52 | 27.54 | 6.08 | 6.12 |

Figure 1. Chemical and morphological characterization of the synthesized nanoparticles. a) Table summarizing the elemental analysis results for the particles after the polyol synthesis, after GPTES stabilization and after BP functionalization, respectively. CHN analysis was performed by combustion using a CHN analyzer, while Gd and P were quantified by ICP-OES. Si content was determined by energy-dispersive X-ray spectroscopy using defined regions of interest from the TEM image. b) TGA analysis of the GBCAs after GPTES coating. c) TEM of GBCAs-BP. Yellow circles define a single particle. d–f) Fourier-transform infrared spectroscopy spectrum of the particles after the polyol synthesis, after GPTES coating, and after BP functionalization, respectively.
of the organic component (Figure 1a). The presence of carbon in the “uncoated” gadolinium oxide sample is due to the diethylene glycol, which is used as a solvent in the reaction as it adsorbs onto nanoparticles surface via interactions with the hydroxyl group stabilizing the NPs in solution.[10] The detection of nitrogen and phosphorous in the final product (i.e., GBCAs-BP) indicated the presence of the BP derivative on the surface of the nanoparticles. Thermogravimetric analysis (TGA, Figure 1b) was used to assess the overall mass of the organic layer, which was found to be around 30% of the total weight. Transmission electron microscopy (TEM) showed a slight size increase (≈ 1–2 nm) of the Gd$_2$O$_3$ nanoparticles core size after GPTES coating, while no differences were observed after BP functionalization (Figure S1, Supporting Information). GBCA-BP nanoparticles were shown to have homogeneous size and morphology with a final core diameter less than 5 nm (Figure 1c), while the measured hydrodynamic diameter was 70 nm. Successful BP functionalization was also confirmed by energy dispersive X-ray spectroscopy (Figure S1, Supporting Information) as well as by IR spectroscopy (Figure 1d–f). The IR spectrum of the precursor Gd$_2$O$_3$ nanoparticles contains a distinctive peak at 2871 cm$^{-1}$ corresponding to the stretching and bending of the methylene chain (CH$_2$), a sharp band at 1084 cm$^{-1}$ is assigned to the C$\equiv$O stretch, and the broad peak at 3100–3500 cm$^{-1}$ corresponds to the O$\equiv$H stretch (Figure 1d). After GPTES coating, the symmetric epoxy ring deformation gives an IR peak in the IR at 788 cm$^{-1}$, while the sharp band at 1248 cm$^{-1}$ is associated with ring stretching vibrations (Figure 1e), matching previous studies of epoxide derivatives.[9] In the BP functionalized derivative, the appearance of peaks at 1057 and 1521 cm$^{-1}$ corresponds to the phosphonate groups and to the N$\equiv$H amide bonds respectively, confirming that the BP functionalization had been achieved (Figure 1f). The characterization data for the GBCAs-BP are summarized in Table S1 in the Supporting Information.

An optimal GBCA for clinical applications needs to have high relaxivity showing significantly shortened T$_1$ relaxation values, which will allow the required signal enhancement to be achieved at a low enough concentration, and to be incorporated into the CPC without significantly disrupting the properties of the material. In vitro relaxivity studies were performed on the gadolinium nanoparticles with measurements at different magnetic field strengths that are typically found in clinical and preclinical settings (using 3T clinical scanner and 11.7T small bore system across a range of concentrations) (Figure 2 and Table S2, Supporting Information). Increasing the magnetic field strength is known to reduce T$_1$ relaxivity for gadolinium(III) agents and, in many cases, increase the T$_2$ relaxivity.[11]
To be an effective $T_1$ contrast agent, the nanoparticles should possess an ultrasmall core size and a hydrophilic coating surface, this ensures that a large surface area of Gd$_2$O$_3$ is available to directly interacts with the surrounding water molecules and that they can rapidly exchange. Aqueous suspensions of the GBCAs-BP with a concentration of gadolinium(III) varying between 0.2 and $2 \times 10^{-3}$ m were scanned using inversion recovery for $T_1$. The linear fit of the data acquired versus the concentration of gadolinium(III), gives an overall relaxation rate indicating the efficiency of the contrast agent under the experimental conditions. Specifically, GBCAs-BP showed an $r_1$ equal to $15.41 \times 10^{-3}$ m$^{-1}$ s$^{-1}$ at 3 T which is almost four times higher than the commercially available contrast agents (e.g., Magnevist or Omniscan). Furthermore, the nanoparticles showed only a slight decrease of $T_1$ relaxation at 11.7 T, with $r_1$ equal to $13.44 \times 10^{-3}$ m$^{-1}$ s$^{-1}$, while the final $r_2/r_1$ ratio remained similar at the two magnetic field strengths ($r_2/r_1 = 4.77$ at 3 T and $r_2/r_1 = 4.30$ at 11.7 T) (Figure 2a–d and Table S2, Supporting Information). The images of the GBCAs-BP phantoms at different concentrations (Figure 2e) show the dominant $T_2$ effect at higher contrast agent concentrations, indicating that the lower concentrations of GBCAs-BP (i.e., $1.5 \times 10^{-3}$ m) is effective for a $T_1$-weighted signal enhancement, which correlates with appropriate amounts to incorporate into CPC materials. In vitro binding tests demonstrated the high affinity of the GBCAs-BP (95%) toward the CPC material at up to 24 h incubation time (Figure 2f) with no dissociation observed.

In vitro cytotoxicity studies were performed on primary human osteoblast (HOb) showing a negative effect on cell viability at high concentrations of the GBCAs-BP (i.e., > $100 \times 10^{-6}$ m, Figure 2g). However, at low concentrations (0.1, 1, and $10 \times 10^{-6}$ m) of the GBCAs-BP showed a beneficial effect on the HOb viability when compared to the nontreated cells (i.e., the internal control) as the nanoparticles induced cell proliferation (Figure 2g). Such findings not only suggested a nontoxic and concentration-dependent effect of the GBCAs-BP nanoparticles, but also highlighted the beneficial potential of the BP functionalization on the cell behavior. This is as expected and in line with the known properties of bisphosphonate compounds, and the role of BP derivatives on the proliferation and differentiation of HOb cells has been investigated. With this study, we confirm the beneficial effect of BP-coated nanoparticles derivatives on HO and support further investigation of this strategy for bone regenerative applications.

Once the GBCAs-BP particles were synthesized, characterized, and found to have appropriate properties to enhance contrast in the application, it was important to determine the concentration of the nanoparticle contrast agent that could be added to the CPC composite without affecting its final handling and mechanical properties, and so preliminary studies were carried out (data not shown). Our findings corroborated the results in the available literature on CPC doping and suggested that adding GBCAs-BP into the CPC composite with a final concentration of 1 wt/wt% would be appropriate. Furthermore, this contrast agent concentration would allow direct comparison with the longitudinal in vivo imaging performances of the CPC-GBCAs-BP construct with the CPC composite doped with a commercial molecular gadolinium(III) contrast that has been described in previous studies. The handling and mechanical properties, as well as the imaging features of the prepared CPC-GBCAs-BP nanocomposite, were investigated in vitro (Figure 3). The setting time assessment showed an increase in the initial and final setting profiles for the CPC-GBCAs-BP nanocomposite when compared to the nonlabeled composite (Figure 3a). It is known that by increasing the alendronate concentration in the CPC matrix the setting time increases as a consequence of the coordination interaction between the phosphonate ions and the calcium salts present in the solution, preventing their rapid incorporation into the crystal lattice and hindering the crystal growth and agglomeration. The internal CPC control consisting of unfunctionalized nanoparticles (i.e., CPC-GBCAs), confirmed the role of the BP groups increasing the setting time of the CPC nanocomposite. However, the setting features observed for the CPC-GBCAs-BP nanocomposite, i.e., initial time of 11 min and final time of 23 min, are still acceptable for clinical use. The addition of the GBCAs-BP was shown to increase the compressive strength and the E-modulus of the final composite statistically when compared to the nonlabeled cement (Figure 3b,c). The nonfunctionalized control (i.e., CPC-GBCAs) showed no differences in properties when compared to the CPC-GBCAs-BP nanocomposite, suggesting that the mechanical properties were improved by the nanoparticles themselves rather than the BP functionalization on the surface. As measured by Brunauer–Emmett–Teller analysis, the CPC composite consisted of a nanoporous structure (pore width = 18.8 nm, surface area = 9.7 m$^2$ g$^{-1}$) with a reduction in pore size and surface area observed for the CPC-GBCAs-BP nanocomposite (pore width = 14.3 nm, surface area = 7.4 m$^2$ g$^{-1}$) possibly suggesting that the added GBCAs-BP were filling these pores to give rise to a more dense microstructure. The final mechanical properties of the CPC-GBCAs-BP were comparable with most of the studied calcium phosphate-based compositions; hence, it is suitable as cancellous bone filler. Finally, the GBCAs-BP labeled CPC composition showed excellent hydraulic properties as all the pastes could be extruded from the syringe through a 1.7 mm orifice in less than 30 sec by applying a minimal injection force (see Figure 3d).

Gadolinium has a high atomic number ($Z = 64$) and high X-ray attenuation per mass (3.11 cm$^2$ g$^{-1}$ at100 keV) and it has been used as CT contrast agent especially for angiography and aortography applications. Therefore, the capability of the GBCAs-BP to enhance the CT contrast of the nonlabeled CPC composite was also investigated. Gray value quantification, based on in vitro micro-CT acquisition, reported a shift of the values showing a darkening of the CPC-GBCAs-Bp nanocomposite versus the control (Figure 3e,f). The enhancement of the CT contrast of a calcium phosphate-based composite by using gadolinium-based nanoparticles could offer additional information, especially in cases of a multimodal imaging assessment of the CPC degradation (i.e., using both MRI and CT).

Finally, the MRI properties of the CPC-GBCAs-BP material were investigated after injection in pig bone blocks (Figure 3g) and compared to the nonlabeled CPC. ZTE-MRI acquisitions of the samples containing GBCAs-BP, performed at 11.7 T, showed a typical $T_1$-shringening effect which resulted in an imaging artifact that led to a sample size overestimation (Figure 3h). It is known that gadolinium(III) not only has a $T_1$-shringening effect, but also a $T_2$($T_2^*$) shringening effect,
depending on its concentration. Qualitative comparison of our results with previous studies where the CPC was combined with commercially available molecular gadolinium(III) agents (i.e., Gd-DTPA/MagneVist) the GBCA-BP nanoparticles showed enhanced contrast with a higher signal intensity, confirming the superior imaging performance of our nanoparticles. The contrast of CPC without nanoparticles supplementation can be qualitatively observed; however, it has been already proved to be insufficient for in vivo translation (Figure 3h).

To assess the longitudinal MRI and CT imaging behavior of the CPC-GBCAs-BP nanocomposite, an in vivo study was performed (Figure S2, Supporting Information). Respectively, labeled and nonlabeled CPCs were injected in a cylindrical defect prepared in a rat condyle, which is a well-established nonload bearing model commonly used for testing biomaterials. The contrast of CPC without nanoparticles supplementation can be qualitatively observed; however, it has already proved to be insufficient for in vivo translation (Figure 3h).

CT images showed enhanced signal intensity in the case of the CPC/GBCAs-BP nanocomposite when compared either to the nonlabeled CPC or to the natural bone phase. The CT signal enhancement persisted for all of the time points and allowed a facile morphological assessment of the implant shape.
and volume (Figure 4b). Interestingly, 4 weeks postsurgery, the CT acquisitions of the implanted CPC-GBCAs-BP showed heterogeneity in material density. Specifically, the central part of the implant appeared to be less dense compared to the outer area. Such findings were in line with the MRI acquisitions and confirmed that there is a lower GBCAs-BP concentration in the central part of the CPC composite. Moreover, the comparison of these findings with longitudinal studies performed in a similar animal model, but with nonfunctionalized contrast agents (i.e., molecular gadolinium(III) agents or superparamagnetic iron oxide particles), proved the feasibility of our strategy in prolonging the residence of the contrast agent in the CPC matrix up to at least 8 weeks postsurgery.[8,20] One potential issue is the observed implant size overestimation on MR.
images; however, this property could be considered an advantage for detection especially when small amounts of the cement need to be identified in the body. For instance, extravertebral CPC extrusion is a common problem after vertebroplasty that causes pain and neurological complications.[21] In these circumstances, the size-overestimation effect could serve to identify leakage of small amount of cement outside of the surgical site, hence supporting the surgeon in the postoperative neurological examination.

Histological assessment was performed 8 weeks after surgeries and showed a direct contact between bone and the cement, without sign of inflammation or fibrous encapsulation (see Figure 4c and Figure S3, Supporting Information). BP-loaded CPCs have been used to increase bone augmentation after in vivo implantation in femora and vertebra of osteoporotic rats. Specifically, release of BP derivatives from the CPC phase resulted in an increased bone density in the immediate proximity of the implant (i.e., in an area from 0.4 to 0.7 mm far the cement).[22] Our histological findings were in line with these previous studies indicating higher bone density may be present around the implant when compared to the CPC composition without BP components (Figure 4c). However, statistical t-testing did not show significant differences in new bone formation between the experimental groups of this size and so further studies are necessary to validate this observation (Figure 4c).

One area of further study that is ongoing is to look at the release profile in vivo and the biodistribution of the GBCA-BP on release from the CPC composite. Methods for radiolabeling are under investigation to determine a valid quantitative tracking. Previous studies have showed that GBCAs do not undergo to intracellular accumulation and are generally excreted by the hepatobiliary or renal systems.[23] The decrease in MRI and CT signal over time and the absence of background signals in the surrounding tissues indicate that any GBCAs-BP released from the materials did not accumulate and were eliminated from the body. Histological assessments using elemental analysis (e.g., inductively coupled plasma mass spectroscopy (ICP-MS) and inductively coupled plasma atomic emission spectroscopy (ICP-AES)) and TEM on tissue samples from different organs (e.g., kidney, liver, spleen, and brain) can provide useful and unequivocal information regarding the bioaccumulation of the nanoparticles once released from the implanted CPC. Additionally, hemolytic tests to investigate the lysis of erythrocytes in response to the release of the nanoparticles from the CPC in the bloodstream are suggested to further investigate their biocompatibility and potential use for medical applications.[24] The overall profile of the ultrasmall Gd$_2$O$_3$ nanoparticles that have been designed and produced in this work offers a significant advance over the current state-of-the-art for longitudinal imaging of calcium phosphate cements. The key image acquisition features are effective multiple modality imaging (combining contrast in both MR and CT from a single agent) and high relaxivity across appropriate MR field strengths. A feature of equally high importance for longitudinal studies is the high affinity for the cement material, which is due to the bisphosphonate coating added to the silica layer.

## Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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## Conflict of Interest
The authors declare no conflict of interest.

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bisphosphonate, calcium phosphate cements, computed tomography, gadolinium-based contrast agents, magnetic resonance imaging

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