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A theranostic agent to enhance osteogenic and magnetic resonance imaging properties of calcium phosphate cements

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ABSTRACT

With biomimetic biomaterials, like calcium phosphate cements (CPCs), non-invasive assessment of tissue regeneration is challenging. This study describes a theranostic agent (TA) to simultaneously enhance both imaging and osteogenic properties of such a bone substitute material. For this purpose, mesoporous silica beads were produced containing an iron oxide core to enhance bone magnetic resonance (MR) contrast. The same beads were functionalized with silane linkers to immobilize the osteoinductive protein BMP-2, and finally received a calcium phosphate coating, before being embedded in the CPC. Both *in vitro* and *in vivo* tests were performed. *In vitro* testing showed that the TA beads did not interfere with essential material properties like cement setting. Furthermore, bioactive BMP-2 could be efficiently released from the carrier-beads. *In vivo* testing in a femoral condyle defect rat model showed long-term MR contrast enhancement, as well as improved osteogenic capacity. Moreover, the TA was released during CPC degradation and was not incorporated into the newly formed bone. In conclusion, the described TA was shown to be suitable for longitudinal material degradation and bone healing studies.

1. Introduction

The growing need for bone treatment procedures keeps a high pressure on the development and optimization of artificial substitutes for bone regeneration. Calcium phosphate (CaP) based materials are amongst the most commonly used, as they closely resemble structure and composition of the natural bone. Especially, injectable CaP-based cements (CPCs) have the advantage of optimal bone defect filling capacity, and are applicable through minimally invasive surgery [1]. The osteoinductivity of such materials can be enhanced by incorporating biologically active molecules, like BMP-2, either via adsorption onto pre-set CPC scaffolds [2], or via loading before the CPC setting.

At the same time, assessment of tissue regeneration is essential to optimize the stages of bone healing. Most commonly, bone healing is assessed with X-ray or computed tomography (CT) assessments. Recently, also zero echo time (ZTE) magnetic resonance imaging (MRI) [3], has proved to be a powerful tool for bone tissue imaging. However, all commonly used techniques for bone imaging lack sensitivity to detect the presence of synthetic bone substitutes, without additional contrast enhancement. Advances in the fields of material science, imaging and formulation development lead to the development of theranostics, which aims at combining both therapeutic (e.g. the BMP-2) and diagnostic (e.g. the MR detection) functions within a single product. Theranostic medicine is currently explored to enhance the quality of cancer treatments, atherosclerosis, infections, and a variety of regenerative medicine applications [4]. In this study, we have developed a theranostic agent (TA), a silica-based nanocomposite MR contrast agent, which also functions as carrier for BMP-2 release. The specific formulation of such a composite, combined with the biocompatibility of silica, is meant to provide imaging contrast enhancement, while reducing unspecific reactivity and improving the osteogenic performance of CPCs. After

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in vitro characterization, the developed TA was tested *in vivo*, in a femoral condyle defect model in rats. Imaging properties were monitored by MRI, while osteogenic performance was measured by histomorphometry, up to 8 weeks post-implantation.

2. Materials and methods

2.1. CaP cement with PLGA microspheres

Calcium phosphate cement consisted of 85% of α-tricalcium phosphate, 10% dicalcium phosphate dihydrate and 5% hydroxyapatite [5]. The cement powder was sterilized using gamma radiation with 25 kGy (Isotron B.V., Ede, The Netherlands). As a porogen, acid-terminated PLGA microparticles were prepared using a double-emulsion solvent-extraction technique. Poly (DL-lactic-co-glycolic acid), (PURASORB, Purac, Gorinchem, The Netherlands) with a lactic to glycolic acid ratio of 50:50 and an average molecular weight of 4.55 ± 0.03 kDa, was used for microparticle preparation. The average size of the microparticles, as determined with image analysis, was 96 ± 16 μm [6]. PLGA microparticles were mixed with the CaP cement powder in a proportion of 20% wt/wt.

2.2. Theranostic agent

A customized theranostic agent (TA) was synthesized. The contrast agent particles were prepared by an inverse emulsion approach (patent WO2005/052581; Nano4Imaging GmbH, Aachen, Germany) using MRI responsive ultra small iron particles (USPIO) embedded within a mesoporous silica matrix. Two contrast agent formulations were tested: (i) MRI-1 with an iron oxide core size range of 200–300 nm at an end concentration of 40% wt/wt, and (ii) MRI-2 with an iron oxide core size range between 0.5 and 1.2 μm at an end concentration of 30% wt/wt.

BMP-2 (Pfizer, InductOs® former Wyeth Europe Ltd., Berkshire, UK) was immobilized onto the surface of the beads, functionalized with silane linkers carrying amino functions (3-aminopropyl-trimethoxysilane), as described by Elhert et al. [7]. A solution containing 10 μg of BMP-2 was mixed with a suspension of beads (12.5 μL at a concentration of 20 mg/mL) in phosphate-buffered saline (PBS) buffer (pH 6.8). PBS was then removed and the beads were resuspended in 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5) and placed at 4 °C, overnight.

Finally, the TA beads were given a calcium phosphate coating. For this, 500 μL of a 5 mg/mL contrast agent suspension, containing 10 μg of BMP-2, was mixed with 54 μL of 40 mM $CaCl_2$, 77 μL of 3.9 mM Na_2HPO_4 and 60 μL of 1 mM NH_3. The mixture was placed at 4 °C for 1 h. After pH had reached 7.9, 60 μL of 1 mM NH_3 was added into the solution, and again placed at 4 °C for 1 h. When pH was 8.5, again 80 μL 1 mM NH_3 was added and the solution remained at 4 °C for 1.5 h. The final pH was 9.5. The suspension was maintained under continuous rotation throughout the entire procedure. The resulting coated beads were magnetically separated and washed 4 times in PBS. The precipitate was then diluted to an end concentration of 20 mg/mL. Control samples were treated the same way, with exception that the beads did not contain BMP-2. After freeze-drying, the obtained solid phase was then mixed to reach a final end concentration of 0.1% (wt/wt) within the bone cement/PLGA composite.

2.3. Composite preparation

The cement was created by adding a filtered sterilized (0.2 μm filter) 2% aqueous solution of sodium phosphate (Na_2HPO_4) to the PLGA/CaP or PLGA/CaP/TA powder mixture using a 2-mL syringe (BD Plastipak, Becton Dickinson S.A., Madrid, Spain) with a closed tip. The components were shaken for 20 s using a mixing apparatus (Silamat Vivadent, Schaan, Liechtenstein).

2.4. Characterization of CPC and TA

2.4.1. Theranostic agent-beads

The morphology of the TA beads was determined by SEM and transmission electron microscopy (TEM). SEM images were obtained at 2000×, 5000× and 10,000× magnification, while TEM images were obtained at 30K and 50K.

Elemental analysis was performed using a scanning electron microscope (SEM, Philips XL30, Eindhoven, The Netherlands) coupled with an energy dispersive X-ray spectrometer (EDS, EDAX, AMETEK Materials Analysis Division, Mahwah, NJ). Samples were sputtered with a thin layer of gold using a common sputtering instrument (Cressington 108A, Watford, UK) to improve the surface conductivity. The EDS elemental maps were taken at accelerating voltage of 15 KeV and working distance of 10 mm.

2.4.2. CPC

Initial and final setting times for the different cement formulations were assessed using custom available Gillmore needles (ASTM C266). A plastic mould of 3 mm (diameter) × 6 mm (height) was used to prepare the scaffolds. Both initial and final setting times were determined and tests were performed at room temperature. Thereafter, for mechanical testing samples were placed on an 858 MiniBionixII® testing bench (MTS, Eden Prairie, MN) and the compressive strength in the longitudinal direction of the specimens was measured at 0.5 mm/min cross-head speed. Finally, to assess morphology all different cement formulations were assessed by scanning electron microscopy (SEM). For this purpose, destructed samples were obtained from the compression test. Images were obtained at 500×, 1000× and 2000× magnification.

Table 1
Experimental groups and implantation scheme. With the aim of testing both the MR contrast agent and BMP-2 performance, four experimental groups were selected: (i) Empty – untreated defect, (ii) CPC – defect filled with injectable calcium CPC, (iii) MRI – defect filled with injectable CPC incorporating the MR contrast agent and (iv) MRI/BMP2 – defect filled with injectable CPC incorporating the MR contrast agent and BMP-2.

Rat	Right defect	Left defect
1	Empty	CPC
2	CPC	MRI
3	MRI	MRI/BMP2
4	MRI/BMP2	Empty
5	Empty	CPC
6	CPC	MRI
7	MRI	MRI/BMP2
8	MRI/BMP2	Empty
9	Empty	CPC
10	MRI/BMP2	MRI

2.5. Ex vivo MRI

Sixteen explanted rat femoral condyles were collected, which were left-over material from unrelated experiments (RU-DEC 2012-317). In each condyle, a cylindrical defect was made of 3.0 mm depth and diameter; and filled bearing either the contrast agent MRI-1 or MRI-2 mixed into the CPC, at a concentration of both 0.1 and 0.5% wt/wt ($n = 4$ for each condition). After 30 min, condyles were immersed in 70% ethanol and MR imaging was performed on a 11.7T MR system (Biospec, Bruker, Germany) with a mouse brain surface coil. Zero echo time (ZTE) images were acquired for all samples at the same time and settings, to avoid confounding effects of individual calibration or thresholding, at 200 kHz bandwidth, TR = 4 ms, flip angle = 5°, FOV = 50 × 50 × 50 mm, matrix size 128 × 128 × 128, total acquisition time 3.27 min.

2.6. BMP-2 immobilization and release

Following BMP-2 immobilization, the amount of BMP-2 effectively bound to the surface of the beads was measured. Thereafter, BMP-2 release tests were performed and the protein concentration was measured by both ELISA, to quantify all released BMP-2, and BRE-Luc assay, to specifically quantify the fraction of bioactive BMP-2.

2.6.1. Quantification of effectively bound BMP-2

The fraction of BMP-2 bound to the silica beads was determined by enzyme-linked immunosorbent assay ELISA assays, with a Quantikine® BMP-2 immunoassay kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. The assay was performed for all process steps, i.e. (i) on the MES buffer used for the immobilization, and removed during the first washing step, (ii) on the PBS collected after the following washing steps, and finally (iii) on the resulting beads.

2.6.2. Quantification of released BMP-2

A 12.5 μL aliquot of the bead suspensions, loaded with BMP-2 was resuspended in 500 μL of minimum essential medium alpha (MEM-α). Both coated and non-coated samples remained in the medium for 2 h, 3 days and 7 days. The amount of BMP-2 released into the supernatants was determined using ELISA (R&D Systems) and BRE-Luc assay, described below.

2.6.3. BRE-Luc assay

M3T3-BRE-Luc cells were cultured in Dulbecco's modified eagle's medium (DMEM) containing 5% fetal bovine serum (FBS) and 800 μg/mL neomycine, at 37 °C in a humidified atmosphere of 5% CO_2. Cells were trypsinized and seeded at a concentration of 2×10^4 in 100 μL of 5% FCS-DMEM in a flatbottomed transparent plate for 24 h. After removing the culture medium, 100 μL of the samples medium, containing the released BMP-2, were added into the wells, together with 2% FBS. To estimate BMP-2 release, a standard curve obtained with soluble BMP-2 in dilutions from 0.4 until 50 ng/mL (100 μL/well) was performed in parallel. Following the incubation time of 16–20 h, the medium was removed and replaced by 25 μL of reporter lysis buffer (Promega, Madison, WI). The incubation was done at −80 °C for at least 1 h. After defrosting, 25 μL of Bright glow (Promega) were added to each well and the luciferase intensity was measured (LUMIstar Omega, BMG LABTECH GmbH, Ortenberg, Germany).