Pre-clinical Imaging Strategies for Bone and Dental Restorative Biomaterials

Simone Mastrogiacomo
Colophon

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Pre-clinical Imaging Strategies for Bone and Dental Restorative Biomaterials

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Paranymphs
Heinz Peter Janke
Daniela-Geta Petre
Alla mia famiglia
To my family
## CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chapter 1</strong></td>
<td>General Introduction</td>
<td>11</td>
</tr>
<tr>
<td><strong>Chapter 2</strong></td>
<td>MR Imaging of Hard Tissues and Hard Tissue Substitutes</td>
<td>19</td>
</tr>
<tr>
<td><strong>Chapter 3</strong></td>
<td>Visualization of Calcium Phosphate in Teeth by Zero Echo Time $^1$H MR Imaging at High Field</td>
<td>57</td>
</tr>
<tr>
<td><strong>Chapter 4</strong></td>
<td>A Theranostic Dental Pulp Capping Agent with Improved MRI and CT Contrast and Biological Properties</td>
<td>83</td>
</tr>
<tr>
<td><strong>Chapter 5</strong></td>
<td>Perfluorocarbon/Gold-Loading for Non-Invasive <em>in Vivo</em> Assessment of Bone Fillers Using $^{19}$F Magnetic Resonance Imaging and Computed Tomography</td>
<td>117</td>
</tr>
<tr>
<td><strong>Chapter 6</strong></td>
<td>Gadolinium Oxide Nanoparticles Functionalized with Biphosphonate for Multimodal Imaging of Calcium Phosphate Cement</td>
<td>149</td>
</tr>
<tr>
<td><strong>Chapter 7</strong></td>
<td><em>In Vivo</em> Evaluation and CT/MR Imaging of 3D Printed Gelatin Methacrylate (GelMA) Scaffolds For Bone Tissue Engineering</td>
<td>177</td>
</tr>
<tr>
<td><strong>Chapter 8</strong></td>
<td>Summary, Closing and Future Perspectives</td>
<td>203</td>
</tr>
<tr>
<td><strong>Chapter 9</strong></td>
<td>Samenvatting, Slotopmerkingen en Toekomstperspectieven</td>
<td>213</td>
</tr>
<tr>
<td><strong>Acknowledgments</strong></td>
<td></td>
<td>223</td>
</tr>
<tr>
<td><strong>List of Publications</strong></td>
<td></td>
<td>231</td>
</tr>
<tr>
<td><strong>Curriculum Vitae</strong></td>
<td></td>
<td>233</td>
</tr>
</tbody>
</table>
Chapter 1

General Introduction
Chapter 1

1. Bone Tissue Engineering

The loss and dysfunction of bone tissues lead to trauma, injuries, and diseases that result in a variety of socio-economic issues. More than two million patients worldwide undergo a bone transplantation every year, with an estimated total cost on the health system of 2.5 million dollars in the USA alone. To date, transplantation of autologous bone, i.e. autograft, is the gold standard. This procedure involves the use of bone from the same patient, which avoids immune and infection-related risks. However, recent studies have reported considerable shortcomings associated with autografts, such as the shortage of transplantable bone, the need for an additional surgical operation, and high donor site morbidity risks associated with bleeding, infection, and pain [1, 2].

Bone tissue engineering (BTE) has emerged over the last thirty years as promising alternative solution to autografts. Starting from the basic understanding of bone structure and biology, BTE aims to artificially recapitulate and enhance bone regeneration and bone repair mechanisms. Specifically, BTE relies on three key parameters: 1) a 3D biocompatible scaffold that mimics as close as possible the extracellular bone matrix, 2) stem cells that are able to express osteogenic potential, 3) specific growth factors that can trigger bone regeneration and healing [3]. The technically complex challenge of BTE has engaged a wide multidisciplinary scientific community consisting of engineers, biologists, surgeons, material scientist, etc., each of them focused on a specific aspect of the ultimate goal (e.g. develop artificial constructs that can be used for bone repair).

2. Biomaterials

One of the crucial aspects of BTE is the use of biologically compatible materials. Specifically, an ideal BTE material should be osteoinductive, i.e. able to promote the differentiation of stem cells in osteoblastic lineage, and osteoconductive, i.e. able to support bone growth. Furthermore, materials that can be degraded by biological systems, i.e. biodegradable, potentially can be tuned to ensure low toxic and immunological reactions from the host organism, while avoiding revision surgery [4]. The biomaterials used for BTE can be chosen from all material categories, i.e. ceramics, (bio)polymers, metals, and hybrid composites [5].

Ceramic materials have evoked special interest because of their high similarity to the calcified phase of mammalian bone, and likewise of dental tissues. For this reason, ceramics show remarkable biological, biodegradable and osteoconductive properties. Ceramic materials of clinical interest are usually based on calcium phosphate (CaP) salts, e.g. alpha-tricalcium phosphate (α-TCP), hydroxyapatite (HA), and bioglasses. New technical advances in ceramic
material manufacturing allow to obtain porous materials with injectable properties that can be implanted into the body through a minimally invasive surgical operation. Furthermore, the combination of ceramics with specific growth factors, gene activator complexes, antibiotics or therapeutics has been used to enhance the osteogenic and biological properties [6].

Polymers can have a natural origin, such as chitosan, silk, and alginate; or a synthetic origin, such as polylactic acid (PLA), polyglycolic acid (PGA), and poly-ε-caprolactone (PCL). Similar to ceramics, polymeric materials have become popular because of their biocompatible and biodegradable features. Main advantages of using polymers are that they can be manufactured in strictly controlled ways, through many processing techniques, such as fiber meshing, microsphere sintering, and 3D printing. Therefore, polymers can be designed with fine precision porosity and interconnectivity according to the specific bone application. Furthermore, especially synthetic polymers can be tailored to optimal mechanical strength, even suitable for load-bearing sites [7].

As usual, both ceramics and polymers are showing advantages and disadvantages. Therefore, hybrid composites have been designed using ceramics and different polymers, to combine the main material properties requested for BTE. A typical example is the combination of α-TCP and HA calcium phosphate-based cement with PLGA microparticles [8].

3. Imaging Challenges in BTE

For the translation of biomaterials in clinical applications, in vivo models for testing their biological properties are demanded [7]. To date, many preclinical BTE studies are based on destructive evaluation methods, such as histology and immunochemistry, which are limiting the possibility to perform longitudinal volumetric and functional assessments of the implanted scaffold. Therefore, there is a strong need of less-invasive imaging strategies with high resolution (i.e. ideally at subcellular level) that allow quantitative assessment of the tissue engineered constructs behavior once implanted into a living system [9, 10]. To date, the imaging characterization of typical BTE scaffolds is well established only at a pre-implantation stage (e.g. by using scanning and/or transmission electron microscopy), while it is more difficult to visualize the biomaterial degradation in vivo, as well as their interaction with cells and tissues [11].

Traditionally, bone imaging is based on X-ray radiography and X-ray tomography. These imaging techniques are widely used to assess bone graft substitutes and their tissue integration in vivo [12]. However, in recent studies, also other non-invasive imaging modalities have
been applied for BTE imaging. For instance: 1) optical coherence tomography (OCT) was used to study the distribution of cells labeled with magnetic beads within 3D polymeric scaffolds [13], 2) bioluminescence imaging was applied to follow ectopic bone formation within porous scaffolds by imaging mesenchymal stem cells (MSCs) expressing luciferase [14], 3) magnetic resonance microscopy (MRM) and X-ray microtomography (XMT) were used to assess newly formed bone and its mineral content within tissue-engineered phalange constructs [15], 4) regeneration and revascularization of large bone defects, as created in rabbits, were monitored over three months of time by single photon emission computed tomography (SPECT) [16], and 5) labeling bone morphogenetic protein-2 (BMP-2) with iodine-125 ($^{125}$I) allowed the observation of the BMP-2 release and its subsequent effect on the bone formation by SPECT imaging [17].

4. Objectives of the Thesis

In this context, this thesis focused on the development of bone-dedicated biomaterials with enhanced MRI and CT imaging properties. Therefore, various types of MRI and CT contrast agents (CAs) were incorporated in well-defined bone substituting materials and their subsequent behavior was tested in \textit{in vitro} and \textit{in vivo} models. Specifically, the following research questions were assessed:

1. What is the current state-of-the-art in MRI sequences for bone and bone-dedicated biomaterials?
2. Is zero echo time (ZTE)-based MR imaging suitable to monitor CPC degradation after \textit{in vivo} implantation in teeth?
3. Can the combination of CAs and growth factors be used to create a theranostic biomaterial for dental applications?
4. Are perfluorocarbons and gold nanoparticles suitable CAs for the enhancement of the MRI and CT signal of CPC?
5. Can functionalization of CAs with HA-targeting compounds be used to prolong the permanence of the CAs into the CPC matrix?
6. Can hydrogel based scaffolds be used for BTE, and if so, which imaging modality needs to be used for their \textit{in vivo} follow-up?
5. References


Chapter 2

MR Imaging of Hard Tissues and Hard Tissue Substitutes

Mastrogiacomo S., Dou W., Jansen J.A., Heerschap A., Walboomers X.F.
Manuscript in preparation
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACD</td>
<td>Analog-to-digital Converter</td>
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<tr>
<td>$B_0$</td>
<td>External Static Magnetic Field</td>
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<tr>
<td>$B_1$</td>
<td>Alternating magnetic field</td>
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<td>CNR</td>
<td>Contrast-to-noise</td>
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<td>CSI</td>
<td>Chemical Shift Imaging</td>
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<td>CW</td>
<td>Continuous Wave</td>
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<td>FA</td>
<td>Flip Angle</td>
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<tr>
<td>FOV</td>
<td>Field of View</td>
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<tr>
<td>FPGA</td>
<td>Field-Programmable Gate Array</td>
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<tr>
<td>FT</td>
<td>Fourier Transform</td>
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<td>fMRI</td>
<td>Functional Magnetic Resonance Imaging</td>
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<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<td>MRE</td>
<td>Magnetic Resonance Elastography</td>
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<td>MRS</td>
<td>Magnetic Resonance Spectroscopy</td>
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<td>MT</td>
<td>Magnetization transfer</td>
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<tr>
<td>NMI</td>
<td>Nuclear Resonance Imaging</td>
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<tr>
<td>RAID</td>
<td>Redundant Array of Independent Disks</td>
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<tr>
<td>RF</td>
<td>Radiofrequency Pulse</td>
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<tr>
<td>SAR</td>
<td>Specific Adsorption Rate</td>
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<td>SNR</td>
<td>Signal-to-noise</td>
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<td>SWIFT</td>
<td>Sweep Imaging With Fourier Transformation</td>
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<td>T</td>
<td>Tesla</td>
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<tr>
<td>TA</td>
<td>Acquisition Time</td>
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<td>TB</td>
<td>Terabyte</td>
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<tr>
<td>TI</td>
<td>Inversion Time</td>
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<td>TR</td>
<td>Repetition Time</td>
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<td>$T_1$</td>
<td>Longitudinal relaxation</td>
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<td>$T_2$</td>
<td>Transverse relaxation</td>
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<tr>
<td>$\mu$CT</td>
<td>Micro-Computed Tomography</td>
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<td>UTE</td>
<td>Ultrashort Echo Time</td>
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<td>ZTE</td>
<td>Zero Echo Time</td>
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1. Introduction

Magnetic resonance imaging (MRI) is a non-invasive screening technology that creates three-dimensional (3D) tomographic reconstructions of the body organs with high spatial resolution (< 1 mm). The primary advantage of MRI is to allow for obtaining cross-sectional anatomical information of the organs due to an excellent soft tissue contrast and no-depth penetration limits. At the same time, MRI offers functional information based on many tissues properties, such as proton density, temperature, biochemical content, oxygen level and pH [1, 2].

Since the introduction of MRI in clinical practice in the early 1980’s, the number of MR examinations has tremendously increased especially for pathologies related to brain, spine, abdomen, cardiovascular and muscular systems [3, 4]. To date, the state-of-art of MRI include many advanced technologies, such as: 1) MR spectroscopy (MRS) and chemical shift imaging (CSI), which permit the identification and classification of tumors through the assessment of the chemical metabolism of a specific area of the body, 2) functional MRI (fMRI), which is able to image the neuronal activity through the detection of the blood oxygenation level-dependent (BOLD) changes occurring in the brain, and 3) MR elastography (MRE), which can be used for the characterization of the biomechanical properties of soft tissues by the visualization of propagating shear waves [5-7].

Despite the wide and rapid use of MRI as a diagnostic tool for soft tissues, its application for hard tissues (i.e. bone and teeth) has been always challenging. The water content in such tissues is very low (< 20 % v/v) and mainly trapped in a solid phase leading to a very short relaxation profile. Therefore, the signal as created by conventional clinical MRI pulse sequences decays very rapidly, which results in a dark and undefined image [8]. Over the last decade, new ultrafast MRI sequences that are able to image tissues with very low water content have been developed. The most promising sequences are known as ultrashort echo time (UTE), zero echo time (ZTE) and sweep imaging with Fourier transformation (SWIFT). These ultrafast MRI sequences demand specific high-speed imaging hardware; therefore, their clinical translation has been limited thus far. Still, many small but significant steps have been made to allow the application of such sequences in the clinic. Thus, after a general introduction to the basic theory behind MRI, bone-specific MRI sequences, and the related strength and weakness, this review will focus on the basic description of the anatomical and chemical composition of the hard tissues, and all progresses that have been made on ultrashort MRI acquisition of such tissues in terms of sequences development and possible clinical
translations. Finally, an exotic but fast-growing application for such MRI sequences, i.e. acquisition and monitoring of biomaterials, is also reviewed.

2. Basic principles of MRI

MR imaging evolved from the basic physical principles of nuclear magnetic resonance (NMR). Typically, MRI is used to detect the nucleus of hydrogen (H\(^+\)), i.e. the single proton, in the water molecules as present in the body. Protons are positively charged subatomic particles that are constantly rotating along their axis. The motion of rotation of protons in an atom gives rise to a quantum-mechanic atomic property that is called spin. Only unpaired protons exhibit spin, which is the case of atoms with an odd number of protons and neutrons, whereas atoms with an equal number of protons and neutrons exhibit a null overall spin. The spinning motion of an electric charge generates a magnetic field, hence each proton can be considered as a small bar magnet and described as a vectorial force, named magnetic moment, with random orientation (Figure 1a). When an external magnetic field (B\(_0\)) is applied to a proton, its magnetic moment will align to the direction of B\(_0\) and have only two possible orientations, i.e. parallel to B\(_0\) (spin = 1/2), and antiparallel to B\(_0\) (i.e. spin = -1/2). The speed of the precession movement of the proton is described by the Larmor frequency (\(\omega\)) and depends on the strength of the applied B\(_0\) and on gyromagnetic ratio (\(\gamma\)) according to the following formula \(\omega = \gamma B_0\), where \(\gamma\) is a constant describing each atom species (e.g. for the hydrogen proton \(\gamma = 42.75 \text{ MHz/T}\)). If to the static B\(_0\) a second alternating magnetic field (B\(_1\)) is applied in the orthogonal direction, it is possible to disturb the magnetic moment of the proton from the B\(_0\) direction. The alternating magnetic field B\(_1\) is usually referred as radio frequency (RF) and must have the same Larmor frequency as the investigated nucleus. In this way, the applied RF is able to induce a transfer of energy to the protons and a shift in direction of 90° (Figure 1a). When the RF is switched off, the protons tend to return to their original position in a process known as relaxation.

During relaxation, protons lose energy according to two processes known as longitudinal relaxation (T\(_1\)) and transverse relaxation (T\(_2\)) (Figure 1b, c). The longitudinal relaxation describes the process whereby the adsorbed energy is released to the surrounding nuclei, inducing an increase of vibration within the lattice that results in an increase of thermal energy. Hence, longitudinal relaxation is also named spin-lattice relaxation, while T\(_1\) relaxation refers to the time needed by the protons to reach 63% of the original position (Figure 1b, d). Transverse relaxation describes the process whereby the adsorbed energy is released by spins as consequence of random mutual interference between each other, hence
also known as spin-spin relaxation. The $T_2$ relaxation refers to the time needed by the proton to have a reduction of the 37% of its original value (Figure 1c, e). Pure $T_2$ decay is happening theoretically only with a completely homogeneous $B_0$. However, this is never the case, as tissues with different susceptibility properties can affect the homogeneity of the magnetic field. These inhomogeneities are fixed in phase and time, while the random interactions between spins are time-depending. The sum of fixed and random effects is described by a $T_2^*$ constant (Figure 1e). As tissues have different relaxation properties, an image can be generated based on the differences in $T_1$ (i.e. $T_1$-weighted image, Figure 1d) and in $T_2$ (i.e. $T_2$-weighted image, Figure 1e) relaxation properties [1, 8, 9].

The position of the spins into the space can be defined based on the frequency analysis of the measured MRI signal, which is performed through a mathematical algorithm known as Fourier transform (FT). Specifically, FT is able to convert the frequency data (in Hz) in spatial information (in mm) that are collected in the so-called $k$-space. In a 2D acquisition, the $k$-space consists of a two-dimensional matrix with $K_x$ and $K_y$ coordinates corresponding to the x- and y-axis, respectively. Therefore, the location of the spins can be defined based on their spatial frequency in the $K_x$ and $K_y$ directions of the $k$-space [10, 11].

Tissues with a high free water content, such as blood, brain, skeletal muscle, and spinal cord, show long $T_2$ and $T_1$ relaxation, which can generate a very strong signal in MRI [12]. By contrast, in tissues with low water content where the protons are trapped in a rigid crystal phase, such as bone and teeth, the signal decays really quickly, resulting to a dark image with no discernible structured contrast (Table 1) [13, 14]. This review will focus on the tissues with short $T_2$ relaxation that are commonly classified as tissue with short (i.e. $T_2 = 1$-10 ms), ultrashort (i.e. $T_2 = 0.1$-1 ms), and supershort relaxation (i.e. $T_2 < 0.1$ ms) [15].
Figure 1. Schematic representation of the basic principles of MRI. In (a) protons are represented as red balls spinning around their own axis. When an external magnetic field $B_0$ (orange arrow) is applied, protons tend to align with the direction of $B_0$ and have only two possible orientation, spin-up and spin-down. The difference between the protons aligned parallel and antiparallel to $B_0$ (blue ball) represents the protons that are responsible for the MRI signal. The sum of these protons can be described by a magnetization vector ($M_0$, blue arrow). If a second magnetic field ($B_1$) orthogonal to $B_0$ is applied, it is possible to tilt $M_0$ of 90° along the $x$-$y$ direction ($M_{xy}$, green arrow). When $B_1$ is switched off $M_{xy}$ returns to the equilibrium through two processes: $T_1$ and $T_2$ relaxation. In (b) and (c) the schematic representation $T_1$ and $T_2$ relaxation are shown respectively. $T_1$ relaxation is defined as the time needed to achieve the 63% of the original longitudinal magnetization (d). Blue curve and the green curve represent tissues with short and long $T_1$ values, respectively. $T_2$ relaxation is defined as the time to dephase up to 37% of the original value (e). Blue curve and the green curve represent tissues with short and long $T_2$ values, respectively. Adapted from [8] and [9].
2.1. Basic principle of ultrashort echo time

UTE refers to MRI sequences that can be used to image tissues that have a $T_2$ shorter than 10 ms. The basic 2D-UTE basic sequence uses two short half RF pulses and acquires data as soon the excitation finishes. The first half RF pulse is collected by a negative slice selection gradient, while for the second half RF pulse the gradient is reversed (Figure 2a) [13, 14, 16]. The detected data are added together to provide one line into the k-space that starts from the center of the k-space and proceeds radially. This process, known as radial mapping of the k-space, is repeated through all 360°, typically in 128-512 steps (Figure 2b). The data are then interpolated onto a rectangular matrix, e.g. 512 x 512, and transformed into an image through a two-dimensional FT [17, 18].

![Figure 2. Schematic representation of a basic 2D UTE sequence. In (a) the pulse diagram is reported. Note that the half RF pulse is applied firstly with a negative and then with a positive slice selection (Gslice). When the RF is switched off the radial gradients $G_{xy}$ are applied and the acquisition starts. The acquired data give a line in the k-space (b). Each spoke represents the k-space trajectories due to the readout gradients. The small dots in the center are sampled during the gradient ramp; the big dots are sampled while the gradient reaches the plateau. For a typical acquisition are used 128-512 spokes and 256-512 point each spoke. Adapted from [13].](image)

As for the imaging of ultrashort $T_2$ components the signal has to be acquired as soon the signal excitation ends, fast transmit/receiving switching coils and dedicated hardware are demanded to minimize the signal decay. An optimal UTE acquisition does not necessarily imply to use a strong $B_0$. Still, high field strength for high signal-to-noise (SNR) is necessary when X-nuclei with lower signal sensitivity than $^1$H, such as phosphorous and sodium, have to be detected. Surely, the performance of the gradient, in terms of slew rate and amplitude, needs to be as high as possible, as it is necessary to ramp down the gradient really quickly.
after the RF. Also, a high RF receiver system is demanded to quickly recover the signal without losing any data [18].

Starting from conventional CSI sequences, a UTE-CSI sequence that is sensitive to ultrashort T₂ components and that can be used for the detection of sodium and phosphorous nuclei has been developed. The use of a gradient with high performance (i.e. peak 40 mT/m and slew rate of 200 T/m/s) and a short RF pulse (i.e. 200 µs) allowed the reduction of the echo time (i.e. the time between the application of the RF and the acquisition of the signal in the coil, typically referred as TE) to 170 µs, which is much lower compared to the TE used in the conventional CSI (i.e. 2.3 ms). Nevertheless, only low resolution images have been achieved thus far [19].

Safety concerns, mainly about tissue heating, are limiting the possibility to use stronger gradients or RF [20]. Recently, Grodzki et al. [21] proposed a new method of filling the k-space based on a combination of radial mapping and Cartesian single point acquisition, also known as pointwise encoding time reduction with a radial acquisition (PETRA). In this way, the gap in the middle of the k-space is filled with the exact value obtained from the single point acquisition. Therefore, every point in the k-space can be measured with the smallest encoding time. Afterwards, Lee et al. compared the conventional UTE sequence with the PETRA-based UTE method confirming the superiority of the latter approach. Currently, the only sequences clinically used on 3T MRI systems by UTE are based on PETRA acquisition [21, 22].

2.2. Basic principle of zero echo time

With the ZTE technique, the encoding gradient is switched on before the RF pulse excitation, hence resulting in an actual zero TE. This ZTE acquisition method uses a short hard-pulse excitation and small flip angle, while the gradients in the three directions are gradually reoriented (Figure 3a). Therefore, ZTE sequences have more restrictions in flip angles and readout bandwidths compared to the UTE. However, the fact that the gradient does not need to be switched off permits, at least virtually, an acquisition without any acoustic noise. The k-space is filled through a 3D radial center-out method and then transformed into an image by FT, as also is done for the UTE acquisitions. In reality, after the RF excitation, the acquisition starts with a delay (δ) that is determined by the time needed for the transmit/receive switching. This gap of information can be filled by acquisition oversampling, which is a linear algebra-based imaging reconstruction scheme (Figure 3b). However, large oversampling increases the amount of data that a computer has to process and the memory requirements [23,
The development of the RF switching techniques and the demand for strong hardware that can handle the enormous amount of generated data stands as one of the technical challenges for the application of ZTE sequences in humans. Recently, Weiger et al. [25] implemented the ZTE method for imaging in humans by using a custom console, including the spectrometer and the pulse generator that allowed the reduction of the excitation pulse to 3 µs and the transmit/receive switching to 471 µs. The custom-built console was based on a packaged analog-to-digital converter (ACD) and a field-programmable gate array (FPGA) combined with 1 terabyte (TB) redundant array of independent disks (RAID) for real-time data storage. However, a large excitation bandwidth with associated high specific absorption rate (SAR) and imaging artifacts were found to be the major concern [25]. A suitable solution to this problem was proposed by Li et al. [26] who initially combined the PETRA into the ZTE for the correction of imaging artifacts, allowing to operate with longer RF pulse and lower peak power. Furthermore, short amplitude-modulated and frequency-modulated pulses for high bandwidth RF excitation and long T₂ suppression methods were also optimized for ZTE leading to significantly improved sequences suitable for clinical use [27, 28].

Figure 3. Schematic representation of a basic ZTE sequence. In (a) the pulse diagram is reported. Note that the gradient in a given direction is ramped up and followed by and hard RF pulse. Signal acquisition starts immediately resulting in a TE = zero. In reality the acquisition starts after a delay (δ). The acquired data give a line in the k-space shown in (b). Big black dots and small empty dots represent the data points required for a complete projection. The small empty dots represent the points that are missed because of delay (δ). Gray dots indicate the additional point achieved through oversampling. Adapted from [23].

2.3. Basic principle of sweep imaging with Fourier transformation

The SWIFT method was firstly developed by Idiyatullin et al. [29, 30] SWIFT can be considered as a combination of all three basic NMR techniques, i.e. continuous wave (CW),
Chapter 2

pulsed, and stochastic. Specifically, SWIFT is based on a swept RF excitation, similar to CW NMR but with a faster rate, where the signal is acquired as the function of the time, as in the pulsed NMR, and extracted by using the correlation method as done in the stochastic NMR [31, 32]. This method leads to an MRI sequence that allows a nearly simultaneous excitation and acquisition scheme, hence suitable for the detection of ultrashort $T_2$ components. The scheme for SWIFT uses sequences of RF pulses with a specific ($T_p$) duration in the order of milliseconds. Each pulse is divided into $N$ segments, having the RF pulse on for a $\tau_p$ duration after a delay with the RF off. The acquisition is performed at $\tau_a$ after the pulse segment (Figure 4). In this way, the full set of frequency-encoding projections are firstly acquired and then reconstructed by using a 3D back-projection algorithm or gridding. The imaging process is then performed by using a cross-correlation method. However, this time-shared method, or gapped method, is limited by the time needed for the intermediate passage between each $N$ segment. Such restriction compromises the SNR and resolution of the images [30]. Several improvements of the SWIFT sequence have been already proposed by Idiyatullin et al., such as a continuous SWIFT acquisition mode (cSWIFT), a multiple excitation bands approach (MB-SWIFT), and a gradient-modulated SWIFT (GM-SWIFT) which are able to overcome the hardware limitations and to reduce the SAR and the acquisition time [33-36].

Figure 4. Schematic representation of a basic SWIFT sequence. Note that SWIFT uses sequences of RF pulses each of them having $T_p$ duration in the range of milliseconds. The pulse is divided into $N$ segments reported on the left of the figure. Data sampling is performed at $\tau_a$ time after each segment. Adapted from [31].

3. Anatomical considerations of the hard tissues

Hard tissues comprise all the tissues that show a mineralized component in their extracellular matrix, such as bone and teeth. The ratio of the mineral phase is not only different for each tissue but can vary with age, sex, gender, and site [37]. Such tissue heterogeneity results in
different $T_2$ properties according to the amount of available free water (Table 1). In the following paragraphs, a general overview of the specific chemical components of bone and teeth is provided.

**Table 1.** $T_1$ and $T_2$ of tissues and tissue components measured at 1.5 T

*Adapted from [13] and [14].*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$T_1$</th>
<th>$T_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>1441 ms</td>
<td>290 ms</td>
</tr>
<tr>
<td>Gray matter</td>
<td>1124 ms</td>
<td>95 ms</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>1008 ms</td>
<td>44 ms</td>
</tr>
<tr>
<td>Heart</td>
<td>1030 ms</td>
<td>40 ms</td>
</tr>
<tr>
<td>White matter</td>
<td>884 ms</td>
<td>72 ms</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>745 ms</td>
<td>74 ms</td>
</tr>
<tr>
<td>Kidney</td>
<td>690 ms</td>
<td>55 ms</td>
</tr>
<tr>
<td>Ligaments</td>
<td>-</td>
<td>4-10 ms</td>
</tr>
<tr>
<td>Knee menisci</td>
<td>-</td>
<td>5-8 ms</td>
</tr>
<tr>
<td>Periosteum</td>
<td>-</td>
<td>5-11 ms</td>
</tr>
<tr>
<td>Cortical bone</td>
<td>398 ms</td>
<td>0.4-0.5 ms</td>
</tr>
<tr>
<td>Dentin</td>
<td>-</td>
<td>0.15 ms</td>
</tr>
<tr>
<td>Dental enamel</td>
<td>-</td>
<td>70 $\mu$s</td>
</tr>
<tr>
<td>Protons in proteins</td>
<td>-</td>
<td>10 $\mu$s</td>
</tr>
</tbody>
</table>

3.1. Bone

Bone is a mineralized tissue that performs several functions in the body. Bone protects the vital organs, provides sites for muscle attachment to allow for motion and locomotion, produces the blood cells, and serves as a reservoir for several important ions (e.g. calcium, phosphate and others). The adult human skeleton consists of two main components: *compact bone* (~80% - also called *cortical bone*) and *trabecular bone* (~20% - also called *cancellous* or *spongy bone*). The ratio of cortical and trabecular bone is different depending on the different locations in the skeleton. As the name implies, compact bone shows a dense structure that is almost solid (i.e. 10% or less in porosity) and consists of parallel cylindrical units called *osteons* (or *Haversian systems*). Compact bone is present at the outer areas of long bone like femur and tibia, small bones like wrist and ankle, and flat bones like skull vault and other irregular bones. Trabecular bone is less dense than cortical bone, presents higher porosity usually between 50-90%, and is located near the ends of long and small bones and in between the surfaces of flat bones. The outer surface of the bone is covered by a connective
tissue, named the periosteum, which plays an important role in the skeletal development and bone healing (Figure 5) [38-40].

The mineral component of the bone consists of hydroxyapatite (HA). Depending on the site, the mineral component of most bones represents the 60% to 70% of the total dry weight, with the exception of the ossicles in the ear that show up to 98% mineral content. The remaining component of the bone consists of an organic phase (20-30%) and water (10-15 %). The main component of the organic phase is collagen type I (about 90%), which is stiffened with the mineral phase, while non-collagenous proteins, lipids, and water are present in minor amount, i.e. 5%, 3%, and 2% respectively [41-43].

Water content into the bone is found to be in three different forms. First, water can be associated with the mineral phase; second water can be associated with the organic collagen phase; and finally there is free bulk water located in the pores of the mineral phase [44, 45]. Naturally, the occurrence of both freely and tightly bound water results in two major relaxation components, which decay at different rate. Protons associated with the mineral phase and the organic phase are decaying quickly (i.e. $T_2 < 11.7 \, \mu s$, and $T_2 = 320 \, \mu s$, respectively), while bulk water decays slowly ($T_2 = 2.28 \, ms$) when measured on a 3 T MRI system [46-48].

**Figure 5.** Schematic representation of long bone morphology. From the outside towards the inside it is possible to distinguish periosteum, cortical bone (that consists of osteons) and the trabecular bone.

Note that each tissue has different $T_2$ values according to its composition. $T_2$ values reported in the figure are measured on a 1.5 T MRI system.
3.2. Teeth

Mammalian teeth consist of four main tissues: enamel, dentin, cementum, and pulp. Enamel, dentin, and cementum represent three differently mineralized tissues that are tightly attached to each other, while the pulp is the only soft tissue of the tooth (Figure 6). The enamel is a highly mineralized structure (up to 96%) forming the outer shell of the crown and is mechanically the hardest substance in the body. The remaining 4% consists of water and other organic proteins called amelogenin, ameloblastins, and enamelines. The dentin is formed by 70% mineral phase, 20% of organic phase, mainly collagen type I, and 10% water. The cementum is a thin layer present between dental root and the periodontium. The thickness of the cementum is ranging from 50 to 1500 µm depending on different location and tooth. Cementum consists of 45-50% of HA as inorganic phase, 50-55% organic phase (mainly collagen type I), and for the rest of water [49-54]. Because of the very low water content relaxation values for dentin and enamel are very low, i.e. T₂ < 1 ms and 70 µs, respectively (data measured on a 1.5 T system) [55].

![Figure 6. Schematic representation of human molar anatomy. From the outside to the inside it is possible to distinguish enamel, dentin, and dental pulp. T₂ values reported in the figure are measured on a 1.5 T MRI system. Adapted from www.charlesfamilydental.com](image)

4. Ultrashort echo time sequences for bone imaging

UTE-based MR sequences were firstly used to image human osseous tissues (e.g. periosteum, knee, lumbar spine, cortical bone in the tibia) by Bydder’s group [56, 57]. Aiming for a wider use of such sequences for clinical applications the authors tested a conventional UTE (CUTE) sequence as well as modified and optimized UTE sequences (see Table 2) [56]. Implementations of the CUTE sequence were based on two different methods: reduction of
Chapter 2

33

the signal from long-$T_2$ components, and on fat suppression. Reduction of long-$T_2$ components can be used to increase the conspicuity of the short-$T_2$ components in relation to normal tissues, while fat suppression can result in a reduction of the background signal. Based on these modifications of the UTE basic sequence, the authors developed a long-$T_2$ suppression UTE (LUTE), a fat suppressed UTE (FUTE), short inversion time (TI) with UTE (STUTE), and medium TI with UTE (MUTE) (Table 2). Among them, FUTE was found to be the most promising as this sequence was able to show the tibial periosteum and cortex in healthy patients. However, the signal from the cortical bone, which was associated to the collagen type I and the bound water, was decreasing with the increase of the distance from the coil [57].

Thereafter, through the subtraction of the images acquired by CUTE to the images acquired by FUTE it was possible to obtain in human volunteers high signal images of the periosteum and of the bone healthy condition at different locations (e.g. tibia, fibula, and spine). The thickness of the periosteum obtained from MRI was reported being consistent with the standard anatomical descriptions. Moreover, it was possible to show that the periosteum thickness is higher in case of patients with midtibial fracture when compared to healthy patients [58]. A similar approach based on the difference of images was also used to obtain a positive signal from the cortical bone with higher conspicuity. FUTE sequence was used to show callus formation in a fractured tibia of a 22-year-old male patient three weeks after the injury. Moreover, FUTE was able to show the difference in cortical bone density between healthy and osteoporotic patients. On the other hand, CUTE showed new bone formation occurring in 32-year-old female patients after four years from a tibia injury [59]. However, these modified UTE sequences were subject to susceptibility and gradient distortion artifacts.

Du et al. combined off-resonance saturation contrast to UTE acquisition (UTE-OSC) in order to suppress signals from long-$T_2$ and fat components and to create a high contrast for cortical bone (Table 2) [60]. Afterwards, the same authors developed an UTE spectroscopic imaging method (UTE) based on highly undersampled interleaved projection reconstructions with multiecho sequences (Table 2) [61, 62]. Through the UTESI sequence, it was possible to achieve high spatial resolution spectroscopic images of the cortical bone (Table 2). The success of this spectroscopic technique arises from three main factors: 1) a minimal TE of 8 $\mu$s that was achieved through the combination of a variable-rate selective excitation (VERSE), a radial ramp selection, and a fast transmit/receive switch; 2) an interleaved variable TE UTE acquisition that significantly improved the spatial resolution; 3) suppression of long $T_2$ components from muscle and fat. The feasibility to quantify mobile proton density by
pursuing the UTESI approach opened a new \textit{scenario} for MRI as a possible screening tool that could be used to detect bone disease related to bone pore size and water content changes, like in osteoporosis [62].

Preclinical studies were performed on an ovariectomized rat model that resulted in estrogen deficient and thus osteoporotic animals. After daily subcutaneous injection of alendronate, which was used as inhibitor of the bone resorption, bone mineral density was measured based on $^{31}$P and $^1$H UTE MRI and compared with the assessments performed by NMR and micro computed tomography (\textit{\mu}CT, i.e. \textit{gold standard} bone screening tool). The outcomes of this study proved the feasibility to use UTE-based MRI for the assessment of the water and mineral content in femora of ovariectomized rat, and to relate such findings to antiresorptive treatments. Phosphorous densities measured through $^{31}$P UTE MRI showed a positive correlation to the values obtained through $^{31}$P NMR, while water content measured through $^1$H UTE MRI showed a negative correlation with the \textit{\mu}CT-based bone density [63].

Once the potential for bone screening by the use of UTE sequences became consolidated in the scientific community, further improvements of such imaging methods followed. Firstly, a dual adiabatic inversion recovery UTE sequence (DIR-UTE) was released and tested for cortical bone (Table 2). DIR-UTE was able to suppress long-$T_2$ component and the signal from the surrounding fat and muscle, displaying the distal tibia of healthy volunteer with high contrast. However, DIR-UTE is a 2D technique, hence subject to partial-volume effect when tiny structures ($< 0.1$ mm) need to be acquired [64]. Finally, an adiabatic inversion recovery UTE sequence (IR-UTE) was developed. The IR-UTE provided excellent qualitative and quantitative depiction of the bone \textit{in vitro} and \textit{in vivo}, based on a robust long-$T_2$ species suppression achieved through the use of the inversion recovery and a short TR (Table 2). In comparison with standard UTE sequence, IR-UTE provided lower SNR and higher CNR [65].

However, despite the continuous improvements of the UTE sequences, the real translation towards clinical use was only possible after the introduction of the UTE-PETRA acquisition method, which combined radial and Cartesian filling of the k-space. Such a method was able to give 1 mm resolution images and high SNR with much shorter TA when compared to the conventional UTE, making ultrashort MRI sequences useful in orthopedic clinical applications (Table 2). Furthermore, UTE-PETRA was not sensitive to gradient imperfections and delay, and had low demand for fast gradient switching and ramping. Since its introduction, UTE-PETRA has been used for the imaging of knee, ankle, head and wrist in human patients [21, 22].
It is known that a deficit of bone mineral content is related to an increase in water content. Moreover, increase in free bulk porous water corresponds to increased bone porosity, while tightly-bound water is related to the mineral content. Gravimetric studies, $^1$H NMR, and three-point bending test performed on tibiae harvested from healthy and phosphorous-deficient rabbits, has proven that bound water is associated with bone strength and toughness, while free water is associated with the elasticity modulus of bone. Therefore, mineral features, and thus water content, can be associated to the bone biomechanical properties, and to the risk of bone fractures [66]. The importance of understanding the water content in bone lead to many studies where UTE-based approaches were used for the detection in vivo of the bound and free water. Chang et al. [67] combined the UTE sequence with magnetization transfer (MT) imaging, an indirect method that allows the performance of a quantitative and qualitative assessment of protons with extremely fast relaxation. They observed that UTE-MT provided accurate information about cortical bone tissue properties by using a whole-body clinical 3T MRI scanner in only two minutes acquisition time. Specifically, the off-resonance saturation ratio (OSR) measured through UTE-MT on human cadaveric femora and tibia correlates with the bone porosity measured via µCT and the mechanical properties, such as Young’s modulus and yield stress, measured through a four-point bending test. This study showed a moderate negative correlation between the OSR and the cortical porosity ($R^2 = 0.51$), and a positive correlation between OSR and both Young’s modulus ($R^2 = 0.12$) and yield stress ($R^2 = 0.30$). Although the correlation of the OSR and mechanical properties was low, UTE-MT appeared to be superior to the conventional UTE as it was able to detect the tightly-bound water components of the mineral bone phase on a clinical scanner [67]. Horch et al. [68] associated a preexisting UTE pulses with a double adiabatic full passage (DAFP) sequence and an adiabatic inversion recovery (AIR) for the detection of the pore and bound water, respectively. These sequences were used to acquire human cadaveric femora on a 4.7 T system. The estimated water content was correlated to the mechanical properties, such as Young’s modulus, yield stress, peak stress, and toughness to failure measured through three-point bending tests. Pearson’s correlation values between DAFP- and AIR-UTE and the various mechanical properties were ranging between 0.35 and 0.69, hence providing the proof-of-concept that this UTE based strategy can be used for the prediction of bone fractures [68]. Afterwards, the same authors translated in vivo the DAFP and AIR UTE protocols for the quantitative mapping of bound and porous water in radius and tibia of six healthy volunteers on a 3 T MRI clinical system. The outcomes of the study showed that the bound water concentration was approximately 28 and 35 mol $^1$H/liter of bone, while the porous
water was 7 and 6 mol $^1$H/liter of bone, for tibia and radius, respectively. These findings corroborated with the values reported in previous observations in femoral specimens [69]. Recently, few more UTE-based methods to detect water content in vivo with more accuracy and precision have been proposed, such as IR-UTE, 3D IR-UTE, UTE sequence associated with a numerical-based algorithm [70-72]. However, the correlation of these methodologies with the mechanical properties remains unexplored. Furthermore, water molecules bound to the collagen matrix or to the crystal phase are decaying really fast (< 10 µs) and their detection is still challenging even by using the most advanced UTE sequence.

The feasibility to use 3D UTE-MRI for the quantification of the mandibular condyle morphology, which is indicative of degenerative joint diseases, was investigated on human cadaveric specimens and compared with measurements performed via µCT. UTE-MRI was able to show the contour of the condyle cortical bone and the associated cartilage, which was not visible on µCT acquisitions. MRI resulted to give accurate information, with an isotropic voxel size of 100 µm, about joint surface curvature, incongruity, as well as on cartilage thickness [73]. In 2014, Serai et al. [74] proved the technical pediatric feasibility of musculoskeletal MRI using UTE sequences in a clinical 1.5 T system with less than 5 min TA, supporting its beneficial use for the evaluation of bone pathology, such as irregular ossification at the weight-bearing site of the femoral condyle.

As HA is the main component of bone, MRI imaging based on $^{31}$P detection gained attention to obtain information about the bone mineral density. However, $^{31}$P-MR imaging of cortical bone is challenged by the fact that $T_2^*$ of the cortical bone is very short (i.e. 179 µs at 1.5 T) and the $T_1$ is very long (i.e. 10.1 s at 1.5 T). The first UTE MRI of $^{31}$P was performed in 2004 by Robson et al. [75] on seven healthy patients. The authors reported a $^{31}$P-based image of the tibial cortical bone with pixel dimension of 0.3 mm that corresponded to 2.9 mm true resolution. $T_2^*$ and $T_1$ for the cortical bone were measured and resulted to be $207 \pm 12$ µs and $8.6 \pm 3.0$ s, respectively. Trabecular bone $^{31}$P-based visualization was achieved as well, although with a reduced SNR due to the lower $^{31}$P concentration per unit [75]. Afterwards, a $^{31}$P quadrature low-pass birdcage coil, which was able to provide 10 µs hard pulses for a 10° flip angle, was build and tested to image human wrist on a 3 T clinical system. The delay of transmit/receive switch was implemented through technical modifications of the circuit diode that lead to a switching speed in the order of nanoseconds. In this way, 3D $^{31}$P-based images of the human wrist with a resolution of 3.5 mm and with improved SNR were achieved in 37 min acquisition time [76].
5. **Zero echo time (ZTE) sequences for bone imaging**

Simultaneously with the development of UTE methods, ZTE sequences were also tested and improved for the MR imaging of bone. The first implementation of ZTE methods for humans was described in 2013 by Weiger et al. (Table 2 and paragraph 2.2) [25]. The authors reported the first 3D ZTE images of the human head, wrist, knee, and ankle on a 7 T human whole-body MRI system. The acquired images showed fine anatomical details with a well delineated bone-tissue interface, high SNR, and isotropic spatial resolution of 0.83 mm [25]. Also, these authors proved the feasibility of ZTE sequences to image trabecular microstructures in bovine bone samples, by comparing the µMRI images with the µCT acquisitions. By using imaging resolutions of 56 µm and 14.8 µm, respectively for the µMRI and µCT acquisitions, the trabecular micro-architecture was shown with excellent agreement on both modalities. Furthermore, the assessment of the bone volume fraction resulted in similar values, i.e. 0.34 and 0.36, for both µMRI and µCT [77]. However, because of the nonuniformity of the B$_1$, smooth intensity variations were observed in the head. This limitation was overcome by a combination of ZTE and a rotating ultrafast imaging sequence (RUFIS), which consisted of a nonselective hard pulse excitation followed by a 3D center-out radial sampling (Table 2). Image acquisition was started immediately at full speed leading to a nominal TE equal to zero. Furthermore, by using minimal gradient switching in between repetitions and short RF pulses, a robust and fast method with more efficient SNR was achieved. Such ZTE-based method provided high-resolution pictures of the cranium, facial skeleton, and cervical vertebrae of human volunteers. MRI acquisitions depicted anatomical details equivalent to the CT acquisitions obtained through a PET/CT scanner [78].

Similarly, to what was done for the UTE sequence, ZTE was associated to a PETRA acquisition method for the correction of the imaging artifacts within the current limits of the clinical scanner hardware (Table 2) [26]. Afterward, ZTE was associated with a MT method and tested for the assessment of the cortical bone composition in mice on a 4.7 T system (Table 2). Relaxation time properties of the femoral diaphysis were based on MT-ZTE and reported higher values when compared to previous studies. Specifically, $T_1$, $T_2$, and $T_2^*$ resulted to be $1107 \pm 203$ ms, $12.5 \pm 2.0$ µs, and $563 \pm 75$ µs, respectively. These differences were not only ascribed to a difference in magnetic field strength when compared to other studies, but also to the higher sensitivity of the MT-based approach [79].

Assessment of the mineral bone composition was also assessed through a $^{31}$P-ZTE-PETRA acquisition of human cadaveric tibia specimens. The mean of the bone mineral $^{31}$P content
was 6.7 ± 1.2 mol/l, and the values were positively correlated to the bone density measured by μCT ($R^2 = 0.46$). Furthermore, $^{31}$P $T_1$ relaxation time assessments showed a positive correlation with the bone density measured via μCT ($R^2 = 0.62$) and a negative correlation with the porosity ($R^2 = 0.45$) calculated from the water content. Based on these data, the authors proved that a decrease in $^{31}$P $T_1$ is associated with an increase in bone porosity. These findings can be explained by the fact that an increase in porosity results in a loss of minerals from the bone matrix. Therefore, the remaining $^{31}$P nucleus can interact with a greater number of protons that results in a reduction of the $^{31}$P $T_1$ value [80]. Recently, the same $^{31}$P-ZTE-PETRA method was used to acquire in vivo the tibia of healthy volunteers. Bone mineral content estimated through $^{31}$P-MRI showed a strong positive correlation with bone mineral content assessed through high-resolution peripheral quantitative CT ($R^2 = 0.96$) [81].

Larson et al. compared UTE and ZTE methods in vivo on a 7 T system [82]. After MRI acquisition of the bone in the brain, ankle, and knee of human volunteers the results dealing with the imaging quality, resolution capabilities, and off-resonance sensitivity were compared to each other. This study showed no differences in resolution capabilities and comparable image contrast and SNR for both UTE and ZTE sequence. Only subtle differences were found in the off-resonance response, due to the difference in k-space sampling for the two MRI techniques. ZTE acquisitions showed increased blurring around the skull, as well as signal dropout artifacts in proximity of the edge of the field of view, while UTE showed more flexibility in imaging volume selection resulting more adequate for clinical applications [82].

<table>
<thead>
<tr>
<th>Name of the sequence</th>
<th>MRI settings</th>
</tr>
</thead>
</table>
| CUTE (Conventional UTE) | FOV = 26 – 38 cm, section thickness = 4 – 8 mm  
Second TE = 2.87, 5.66, 11.08, 17.70 ms  
RF pulse = 0.4 – 6.0 ms, FA = 30° – 80°  
TA = 8.5 – 17 min. [56, 57] |
| FUTE (Fat-suppressed UTE) | FOV = 26 – 38 cm, section thickness = 4 – 8 mm  
Second TE = 2.87, 5.66, 11.08, 17.70 ms  
RF pulse = 10 ms, FA = 30° – 80°  
TA = 8.5 – 17 min. [56, 57] |
| STUTE (Short inversion time UTE) | FOV = 26 – 38 cm, section thickness = 4 – 8 mm  
Second TE = 2.87, 5.66, 11.08, 17.70 ms  
RF pulse = 0.4 – 0.6 ms, FA = 45° or 80°  
Inversion pulse = 4 ms, TI = 360 or 380 ms  
TA = 8.5 – 17 min. [56, 57] |
<table>
<thead>
<tr>
<th>Technique</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MUTE (Medium inversion UTE)</strong></td>
<td>FOV = 26 – 38 cm, section thickness = 4 – 8 mm, TR = 2500 ms, TE = 80 µs, Second TE = 2.87, 5.66, 11.08, 17.70 ms, RF pulse = 0.4 – 0.6 ms, FA = 45° or 80°, Inversion pulse = 4 ms, TI = 750 ms, TA = 8.5 – 17 min. [56, 57]</td>
</tr>
<tr>
<td><strong>UTE-OSC (Off-resonance saturation contrast UTE)</strong></td>
<td>FOV = 10 cm, section thickness = 2 – 3 mm, Gradient amplitude = 40 mT/m, Slew rate = 150 mT/m/ms, Bandwidth = 62.5 kHz, TR = 200 – 300 ms, TE = 8 µs, Second TE = 80 µs, FA = 60°, RF pulse = 90° x 8 ms, Spatial resolution = 0.78 x 0.78 mm², TA = 5 min. [60]</td>
</tr>
<tr>
<td><strong>UTESI (UTE spectroscopy imaging)</strong></td>
<td>FOV = 10 cm, section thickness = 8 mm, Gradient amplitude = 40 mT/m, Slew rate = 150 mT/m/ms, Bandwidth = 62.5 kHz, TR = 75 ms, TE = 8 µs, Second TE = 80 µs, FA = 60°, Spatial resolution = 0.78 x 0.78 mm², TA = 5 min. [62]</td>
</tr>
<tr>
<td><strong>DIR-UTE (UTE with dual inversion-recovery)</strong></td>
<td>FOV = 10 cm, section thickness = 2 mm, TR = 300 ms, TE = 8 µs, Number of projections = 51, bandwidth = 62.4 kHz, TA = ~ 5 min. [64]</td>
</tr>
<tr>
<td><strong>IR-UTE (inversion recovery UTE)</strong></td>
<td>FOV = 10 cm, section thickness = 6 mm, Gradient amplitude = 40 mT/m, Slew rate = 150 mT/m/ms, Bandwidth = 125 kHz, TR = 300 ms, TE = 8 µs, Second TE = 4400 µs, TI = 80, 100, 120, 140, 160 ms, FA = 60°, Spatial resolution = 0.2 x 0.2 x 6 mm³, TA = 9 min. [65]</td>
</tr>
<tr>
<td><strong>UTE-PETRA (UTE-pointwise encoding time reduction with radial acquisition)</strong></td>
<td>FOV = 200 mm, section thickness = 22 mm, Gradient amplitude = 8 – 15 mT/m, Slew rate = 100 mT/m/ms, Bandwidth = 86 kHz, TR = 2 – 5 ms, TE = 70 – 50 µs, Second TE = 4.6 ms, FA = 4° – 9°, Spatial resolution = 1.3 – 1.04 mm, TA = 5 – 10 min. [21]</td>
</tr>
<tr>
<td><strong>MT-UTE (magnetization transfer UTE)</strong></td>
<td>FOV = 4 mm, slice thickness = 3 mm, Gradient amplitude = 40 mT/m, Slew rate = 150 mT/m/s, Bandwidth = 62.5 kHz, TR = 100 ms, TE = 8 µs, FA = 60°, Spatial resolution = 1.35 x 1.35 x 1.35 mm³, MT = Fermi pulse 8 ms, Spectral bandwidth = 0.8 kHz, TA = 12 min. [67]</td>
</tr>
<tr>
<td><strong>ZTE</strong></td>
<td>FOV = 530 – 240 mm, slice thickness = 2 – 3 mm, Gradient amplitude = 22.2 – 24.5 mT/m, Slew rate = 200 mT/m/ms, Bandwidth = 500 – 250 kHz, TR = 471 – 793 ms, TE = 0, FA = 2.2°, RF hard pulse = 3 µs, Isotropic spatial resolution = 1.66 – 0.83 mm, TA = 2.31 – 3.12 min. [25]</td>
</tr>
</tbody>
</table>
### 6. Ultrashort MR sequences imaging of teeth

Dental tissues show the shortest relaxation profile of any tissue, and therefore their imaging represents the toughest challenge in MRI. Still, the use of MRI in dentistry is continuously increasing. The first UTE-MR image of teeth was shown by Boujraf et al. in 2009 [83]. Human volunteers were scanned using a whole-body 3 T system with a total scanning time of 10 min. The sagittal slice of the jaw acquired by using UTE sequences with TE = 50 µs provided a clear view of all the dental structures including enamel (Table 3). Subsequently, Bracher et al. [84, 85] imaged not only teeth by using 3D-UTE but also estimated and correlated T² values with the presence of dental caries. The mineral breakdown caused by caries was found to lead to a gradual increase in T² value depending on the extension of the lesion, therefore suggesting UTE based MRI as a feasible screening tool for the early detection of dental caries. Idiyatullin et al. [86] proposed a SWIFT sequence, which was able to give simultaneous acquisition in vivo of both soft and hard dental tissues with high resolution and short acquisition time (Table 3). SWIFT-MRI acquisitions were performed with a 4 T system and with a custom-made one-side shielded coil, which was located between the cheek and the teeth. The shielded coil minimized the signal from the surrounding soft tissue. To reduce artifacts associated with the patient motion, “rigid body” motions were also achieved by comparing 16 low resolution images. High-resolution ZTE images of extracted teeth (i.e. incisors, canine, molars, and wisdom teeth) was made ex vivo using an 11.7 T MRI system. Teeth showed various dental caries and dental fillers, including amalgam and ceramic materials. Three-dimensional ZTE images with 148x148x188 µm resolution were acquired in 6.55 min and compared with UTE-MRI and µCT acquisitions. ZTE-based MRI indicated
slight differences in dentin composition that were only barely or not recognizable at all by µCT. Furthermore, ZTE-MRI showed signal variation around the pulp, which was not visible in µCT. Dental fillings presented imaging artifacts in both modalities depending on their composition. In case of amalgam filling, which was used in a molar after endodontic treatment, a strong beam-hardening artifact was compromising the µCT image, while in ZTE images the artifact was reduced and confined to the filling location, which allowed better diagnosis. A strong blurring artifact was observed in the ZTE acquisition of a molar treated with an unknown filling. Despite the strong imaging artifact, local signal changes in the enamel and dentin were still detectable. However, µCT was superior to the ZTE-MRI and showed better signal from the enamel and the calculus as present on the tooth surface. UTE- and ZTE-based images were comparable, still ZTE was showing a better contrast of both dentin and enamel. These findings were confirmed by the quantification of the SNR for each imaging modalities [87, 88].

Information about the mineral density of the teeth and of their major component (i.e. HA) was obtained by quantification of the phosphorous content. Extracted human molars were scanned on a 9.4 T system with a home-build double resonance ($^{31}$P and $^1$H) probe through a $^{31}$P-SWIFT and $^{31}$P-ZTE sequence. These methods provided $^{31}$P-based images, which made it possible to distinguish the enamel from the dentin structures with a resolution of 0.5 mm. When comparing the two MRI methods, the $^{31}$P MRI images obtained through $^{31}$P-ZTR showed better SNR (>28 %) with respect to $^{31}$P-SWIFT [89].

However, the lack of coils and powerful gradient in a whole-body MRI system is hampering the translation of ZTE sequences in the clinic. Furthermore, ZTE sequences have to be optimized also for their use on 3 T and 7 T MRI systems [90]. An intraoral MRI coil that can be placed between the maxillary and mandibular teeth has recently been proposed by Idiyatullin et al. [91]. The coil consisted of a single loop of 10 mm width covered with sticky foam and suitable to fit in the adult maxillary arch. Such a coil was used for MRI acquisition of a human volunteer with a 4 T MRI scanner through a 3D radial SWIFT sequence. The intraoral coil showed high SNR and spatial resolution (0.3 mm$^3$) compared to an extraoral coil. However, the main limitation of the intraoral coil was the lower SNR in proximity of the cusps of the teeth [91]. Ludwig et al. [92] developed a wireless inductively-coupled intraoral coil consisting of two coaxial loops of 1.5 and 2 cm diameter covered with a dental resin and adapted to the human mouth anatomy by a home-made dental cast. The coil was used firstly ex vivo on a porcine mandible and then in vivo in a human volunteer using a 3 T MRI system. However, instead of an ultrashort TE sequence, a high-resolution 3D-FLASH was used for the
images acquisitions. The coil showed improved SNR when compared to other dental coils described in the literature, and displayed relevant anatomical details with an isotropic voxel size of 350 µm. The authors suggested the optimization and combination of the coil with ultrashort echo time sequences for further improvement of the image quality [92].

Table 3. Ultrashort MRI sequences dental tissues

<table>
<thead>
<tr>
<th>Name of the sequence</th>
<th>MRI settings</th>
</tr>
</thead>
</table>
| First UTE in teeth            | FOV = 80 mm³  
Gradient amplitude = 40 mT/m 
Slew rate = 200 T/m/ms, Bandwidth = 357 kHz 
TR = 9.4 ms, TE = 50 µs 
Isotropic spatial resolution = 250 µm³ 
FA = 10°, TA = 10 min. [83]    |
| SWITF                         | FOV = 110 mm, TR = 2.5 ms  
Isotropic spatial resolution = 430 µm³  
RF pulse = 2 ms, Bandwidth = 62 kHz  
FA = 8°, TA = 10 min. [86]      |
| High resolution ZTE           | FOV = 1.9 x 1.9 x 2.4 mm  
TR = 1.0 ms, TE = 0, FA = 3°  
Isotropic spatial resolution = 148 x 148 x 188 µm  
Bandwidth = 200 kHz  
RF pulse = 1 µs, T/R switching = 4.5 µs  
TA = 6.55 min. [87]             |

7. Ultrashort MR sequences for hard tissue substitutes and biomaterials

The development of MRI sequences that can detect really sort T₂ components, widens the use of MRI also to the imaging of artificial materials, as used in orthopedic and dental applications, like bone fillers and restorative materials [93]. Materials that are used for bone and dental application can be distinguished based on their degradation properties in degradable and non-degradable materials. Degradable materials, such as ceramics and certain polymers, have the property to be removed from the body through *in vivo* degradation, while non-degradable materials, such as metals and resins, are biologically compatible but mostly need to be removed from the body with an additional operation (e.g. metallic fracture stabilization plate) or left in situ (e.g. permanent dental fillers) [94, 95].

In 2011 Springer et al. [96] proposed a modified Ernst equation and variable flip-angle method combined with a 3D-UTE sequence to visualize polymeric materials with a 3 T whole-body MRI scanner. The variable flip-angle method allowed for the T₁ quantification of the polymeric material within a reduced acquisition time. Although the exact material composition was not described, the authors were able to estimate its relaxation properties, i.e.
T₁ = 223.1 ms and T₂* = 0.295 ms [96]. Dental restoration materials, such as amalgam and ceramic inlays, inserted in human molars could be imaged through high-resolution ZTE [87]. Grosse et al. [97] used a 3D-UTE sequence with specific optimized parameters to image several of the most common dental restoration materials, such as methacrylate resins (DErax, Ettingen, Germany), 2,2 bis [4-(2-hydroxy-3-methacryloyloxypropoxy)-phenyl] propane (VOCO, Hamburg, Germany), methacrylate-based cement (MAGNEDELTA, Büdingen, Germany), zinc phosphate cement (Harvard Dental International, Hoppegarten, Germany), etc. A great variety of dental restoration materials was visible using UTE-MRI, and for all these materials the relaxation profiles were quantified (Table 4). The reported T₁ and T₂ values of each dental restoration material now are available for further optimization of MRI sequences [97].

Table 4. T₁ and T₂* of most common dental fillers measured at 3 T. # means T₂* measure at 1.5 T, † measured on a 11.7T. Adapted from [13], [97-99]

<table>
<thead>
<tr>
<th>Material</th>
<th>T₁</th>
<th>T₂*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methacrylate resins</td>
<td>207 ms</td>
<td>235 µs</td>
</tr>
<tr>
<td>Triethylene glycol dimethacrylate</td>
<td>28 ms</td>
<td>180 µs</td>
</tr>
<tr>
<td>Methacrylate-based cement</td>
<td>214 ms</td>
<td>106 µs</td>
</tr>
<tr>
<td>Zinc phosphate cement</td>
<td>30 ms</td>
<td>40 µs</td>
</tr>
<tr>
<td>2,2 bis [4-(2-hydroxy-3-methacryloyloxypropoxy)-phenyl]</td>
<td>159 ms</td>
<td>96 µs</td>
</tr>
<tr>
<td>Urethandimethacrylat</td>
<td>227 ms</td>
<td>337 µs</td>
</tr>
<tr>
<td>Eugenol-free Polycarboxylate-based Cement</td>
<td>190 ms</td>
<td>113 µs</td>
</tr>
<tr>
<td>Hydroethyl dimethacrylate</td>
<td>295 ms</td>
<td>178 µs</td>
</tr>
<tr>
<td>Resin-reinforced, chemically curing glisopolyalkenoate cement</td>
<td>153 ms</td>
<td>600 µs</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>-</td>
<td>~ 1 µs²</td>
</tr>
<tr>
<td>CPC compositions</td>
<td>520-1000 ms †</td>
<td>270-450 µs †</td>
</tr>
</tbody>
</table>

Biodegradable calcium phosphate-based (CPC) compositions have also been imaged by MRI. Sun et al. [98] investigated the MRI visual properties of a specific injectable CPC composition consisting of a mix of 68% alpha-tricalcium phosphate (α-TCP), 8% dicalcium phosphate dehydrate, 4% HA, and 20% poly(lactic-co-glycolic acid) (PLGA). The CPC was first injected in vitro in a cylindrical defect created in bone blocks and subsequently implanted in vivo in cylindrical defects made in rat femora. The assessments were performed on an 11.7
MR IMAGING OF HARD TISSUES

T scanner with both UTE and ZTE sequence. Relaxation estimation for the CPC composition based on a 3D-UTE sequence showed a $T_2^*$ equal to 442 µs. This value was very close to the $T_2^*$ relaxation of the pig cortical bone (i.e. 597 µs), resulting in a similar MRI signal with both UTE and ZTE acquisition. This similarity in relaxation profiles hampered the identification of the CPC after implantation either in vitro or in vivo, which makes the use of MRI contrast agents the only solution for the enhancement of the CPC signal [98]. For example, Ventura et al. [99] combined already CPC with a dedicated dual contrast agent (DCA) that consisted of superparamagnetic iron oxide (SPIO) particles with a mean size of 200 nm, and gold nanoparticles (AuNPs) with a mean size of 4 nm as MRI and CT contrast agent, respectively. 

In vivo imaging by ZTE-MRI and CT of the CPC-DCA material after installation in rat femoral defects, revealed that ZTE-MRI allowed the identification of the implanted CPC until 8 weeks post-implantation, while the CT contrast of the CPC was lost 4 week after implantation. However, because of the strong susceptibility properties of the SPIO particles, a strong imaging artifact (i.e. blooming effect) was observed on the ZTE-MRI acquisitions. Although such a blooming effect leads to an easy identification and localization of the implanted CPC, it hampers proper analysis of the material [99]. The use of different type of MRI contrast agents is a possible solution to imaging artifact-related drawbacks. Therefore, CPC was combined with a fluorine-based MRI contrast agent (i.e. perfluoro-15-crown-5-ether) and gold nanoparticles, respectively for $^{19}$F-MRI and CT imaging [100]. Fluorine-based ZTE-MRI of the labeled CPC implanted in rat femora permitted the fine visualization of the CPC shape and made it possible to follow the material degradation longitudinally and to compare such findings with the CT images [100].

Another CPC composition, consisting of 59.1% $\alpha$-TCP, 1.5% carboxymethylcellulose (CMC), and 39.4% cryo-grinded PLGA, was used as dental pulp capping agent in extracted human molars. The specimens were scanned on an 11.7 T scanner through a 3D-UTE and ZTE sequence. This specific CPC reported a $T_2^*$ equal to 273 µs, which was lower than the $T_2^*$ from human dentin (i.e. $T_2^* = 476$ µs). Therefore, such a CPC can be distinguished from dentin on the ZTE acquisition (Figure 7). Also, the authors investigated the possibility to use MRI to follow CPC degradation after in vivo implantation in goat incisors. Relaxation studies were performed on the CPC before and after 7 weeks implantation. A lower $T_1$ value was found for the CPC composition after the implantation in vivo, i.e. 742 ms versus 1008 ms after and before the in vivo study, respectively. These results suggested the feasibility to use the relaxation properties of the CPC to follow its degradation in vivo [101].
8. Conclusions

Currently, MRI sequences for the imaging of hard tissue (e.g. bone and teeth) and of biomaterials used for restoration of such tissues are becoming more and more advanced. The huge potential of ultrashort TE MRI is evident, not only for the morphological high-resolution 3D reconstruction of bone and dental structures, but also for the early detection of bone diseases. By using ultrashort TE MRI it is possible to obtain information about the status of a pathological condition. Moreover, MRI is considered a very promising screening tool in dentistry not only because of the lack of radiation, but also because of the simultaneous multiplanar imaging of soft and hard tissue, the lack of artifacts generated by the presence of dental restoration materials fillers, as well as the ability to detect demineralization and dental caries [102].

However, many challenges still need to be solved before such MRI methods will be accessible to a wider patient population. While issues with hardware systems and fast transmit/receive switches can be solved in a relatively easy way, the need for high gradient performance remains still a challenge. Furthermore, the requirement of appropriate software, which allows for qualitative and quantitative analysis, also needs to be taken into account, as not all the MRI companies are updated with these recently developed sequences [16]. Finally, the extensive costs for MRI machines and their maintenance still hamper a global spreading of such imaging tools, especially in dentistry. The development of a relatively “cheap” MRI system is necessary. This review reported many studies that can be used as a proof-of-concept for the translation of MRI to dentistry. Therefore, the next step will be the refinement of the
available technologies to make them suitable for dental applications, while the use of short-bore systems and of low magnetic fields relying on the pre-polarization MRI concept can be suggested as a possible solution to reduce the manufacturing costs [103, 104].

In summary, ultrashort TE sequences represent an applicable MRI tool that allows quantitative and qualitative assessment of bone, teeth and solid-like biomaterials. Their clinical translation is an ongoing occurring process especially in the orthopedic field, while still many efforts have to be made in dental MR imaging. The use of biomaterials for bone and dental repair is well established in the clinic, which warrants the investigation of MRI sequences for their screening.

9. References
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Chapter 3

Visualization of Calcium Phosphate in Teeth by Zero Echo Time $^1$H MR Imaging at High Field

Dou W., Mastrogiacomo S., Veltien A., Alghamdi H.S., Walboomers X.F., Heerschap A.
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1. Introduction

For non-invasive imaging at high spatial resolution of hard tissues, such as bone and teeth, magnetic resonance imaging (MRI) is an attractive technique compared to X-ray or computer tomography (CT), as it does not make use of ionizing radiation, offers different options for contrast and simultaneously can provide information of soft tissues [1-3]. Due to the limited water content in these tissues and the very short $T_2^*$ of their protons [3-8], only sequences sensitive to ultra-short $T_2^*$ relaxation can be used to acquire images at high signal-to-noise ratio (SNR), such as ultra-short echo time (UTE), zero echo time (ZTE) or sweep imaging with Fourier Transform (SWIFT) [9-12]. These sequences have been applied in bone imaging for qualitative and quantitative analysis of cortical bone and direct depiction of bone microstructure [13,14]. Also, they have been applied in dental imaging to identify different tooth components, including the detection of caries lesions [3,15,16].

Calcium phosphate cement (CPC) is an injectable, biocompatible and biodegradable material with a high similarity to hard tissues and therefore commonly used for bone or tooth restoration [17-23]. To monitor CPC and its degradation in bone longitudinally, different imaging techniques like CT and MRI have been used, but none has been applied to CPC in teeth yet. Previously, we did not find differences in the MR relaxation properties between a CPC material and bone, necessitating the use of contrast agents for MR visualization of the CPC [24-26].

In this study, we aimed to examine the potential of ZTE MR imaging at high field to visualize a new CPC formulation for dental restorations that is designed for hard tissue formation with fast bio-degradation [27]. It is unknown if this CPC formulation has relaxation properties by which it can be distinguished by MRI from the surrounding dental components enamel and dentin after implantation in human teeth. Therefore, we first determined the $T_1$ and $T_2^*$ relaxation times of this new CPC material and of enamel and dentin, to optimize MR measurement parameters for the best image contrast of CPC in restored human teeth. As we encountered non-exponential $T_2^*$ decay, we applied the recently proposed Gaussian augmentation of mono-exponential (GAME) decay model for fitting $T_2^*$ [28]. This model assumes a Gaussian rather than a Lorentzian intra-voxel frequency distribution, and provides both $T_2^*$ and $T_2$ values. Subsequently, we tested if the relaxation optimized ZTE sequence could be used to assess the in vivo degradation of CPC in dental tissues. For this purpose CPC material was implanted into freshly extracted goat teeth, and into teeth of goats in vivo, which were extracted after seven weeks. Of the CPC implanted in vivo in the latter teeth, the MRI
properties, including $T_1$ and $T_2^*$ values, were estimated and compared with those of CPC implanted in the freshly extracted goat teeth.

2. Materials and Methods

2.1. CPC preparation

The calcium phosphate cement investigated in this study consisted of 59.1 weight percentage (wt%) alpha-tricalcium phosphate, 1.5 wt% carboxymethylcellulose (CAM bioceramics, Leiden, The Netherlands) and 39.4 wt% cryo-grinded poly-lactic-co-glycolic acid particles (PURASOB 5002A, Purac, Gorinchem, the Netherlands). The CPC powder was mixed in a 2 ml end-closed syringe and sterilized by 25 KGy of gamma irradiation (isotron B.V., Ede, The Netherlands).

All CPC materials were prepared by an experienced researcher who had received an extensive training in CPC preparation. To minimize the material variability all CPC materials applied in human and goat teeth were selected from the same batch.

2.2. Human teeth

Three extracted human teeth (molars) were applied for CPC implantation. They were selected as waste tissues and disconnected from any subject-indentifying data to allow their use exempt under the institutional review board. A vertical hole (diameter: 3 mm; length: 3 mm) was drilled in the occlusal surface of each tooth by using a dental drill, and filled with CPC paste ($\sim20 \text{ mm}^3$) which was obtained after mixing the powder with a sodium phosphate based setting solution (4% NaH$_2$PO$_4$•2H$_2$O). The teeth were then separately stored in three 15 ml falcon tubes, embedded in 5% gelatin type A (Sigma Aldrich, Saint Louis, USA). The applied gelatin solution prevented the teeth from drying out and also allowed for an easy localization of these samples on gradient-echo “localizer” images at the beginning of each MR experiment.

2.3. Goat teeth

Three healthy 1.5 years old Habsi male goats (weight: 40-50 kg) were included in this study. All in vivo experiments were performed after the animal protocol was approved (approval number: FR0263) by the animal ethical committee of King Saud University, College of Dentistry Research Center, Riyadh, Saudi Arabia. National guidelines for care and use of laboratory animals were obeyed.

All dental operations were performed under general anesthesia and sterile conditions. An intramuscular injection of ketamine hydrochloride (5 mg/kg) and diazepam (1 mg/kg) was
used to sedate the animals before the operations. To reduce the risk of perioperative infection, the goats received antibiotics pre-operatively (10 mg/kg intravenously; Amoxicillin®, Centrafarm, Etten-Leur, The Netherlands), and post-operatively at days 1 and 3 (50 mg/kg intramuscularly; Albipen®, Intervet BV, Boxmeer, The Netherlands). The analgesic Finadyne® (1 mg/kg, three times a day, Intervet BV, Boxmeer, The Netherlands) was administered for two days after the operation.

In total six teeth (lower incisors) were randomly selected from the goats for dental surgery involving CPC implantation. Three of these were subjected to this implantation after they were extracted from the animals. Because of the size difference between human molars and goat incisors, CPC material was implanted into smaller defects in goat teeth. A small cylindrical hole (diameter: 1 mm; length: 2 mm) was drilled on the labial surface of each tooth by a dental drill and immediately filled with the above mentioned CPC paste (~1.5 mm³), which are further denoted to be obtained at time point (TP) 1. The other three incisors were subjected to the same dental procedure but applied in vivo. At seven-week post-surgery, they were extracted from the goats for ex vivo MRI experiments (TP2). All goats were euthanized by an overdose of Nembuta® (Apharmo, Arnhem, The Netherlands). Each goat tooth was stored in a 15 ml falcon tube, embedded also in 5% gelatin type A (Sigma Aldrich, Saint Louis, USA).

2.4. MRI measurement

A 3D UTE sequence was used to investigate the MR relaxation properties and a ZTE sequence for imaging of the CPC restored teeth. While both sequences are able to detect tissues with very short transverse relaxation times at high SNR, their respective intrinsic features make them suitable for different purposes [13-16]. The selection of TE values in UTE enables to acquire images with T₂* contrast and also to suppress signals from materials with extremely fast decay like components of the RF coil. Moreover, UTE images at serial TEs can be used to evaluate the (very) short T₂* relaxation times of (semi-)solid tissues. For ZTE, the zero echo time results in images with higher SNR relative to the UTE images obtained at finite echo time. Additionally, the constant encoding gradients with maximal strength in ZTE, compared to the ones in UTE in which the maximum value is reached after the gradient ramp, allows faster k-space filling, resulting in images with higher resolution and less influence from local susceptibility gradients [15].

MRI measurements were performed on an 11.7T MR-system (BioSpec, Bruker, Germany) using a quadrature proton (¹H) volume coil (Bruker, Germany) with 40 mm inner diameter.
Each tooth sample was positioned parallel to the applied magnetic field. No signal contribution from the RF coil was seen in UTE and ZTE images likely because of the limited amount of protons in the coil materials and their ultra-short $T_2^*$ relaxation times.

A 3D UTE sequence (TE= 8 µs, repetition time (TR)= 8 ms, image resolution= 0.3 mm$^3$, 2 averages, gradient ramp duration=106 µs, bandwidth (BW)= 200 kHz, number of radial spokes= 51360 and $T_{RF}$ (the length of applied pulse)= 4 µs) was applied for $T_1$ estimation with 7 flip angles (FAs) from 3°-15° with 2° as an increment. Total scan time was about 1 hour 32 minutes. A similarly parameterized 3D UTE sequence was used for $T_2^*$ evaluation but with TR= 10 ms, FA= 7° and 16 TEs (18, 28, 38, 48, 58, 78, 98, 128, 158, 188, 228, 268, 308, 358, 408 and 608 µs). The corresponding total acquisition time was approximately 4 hours 52 minutes.

All human and goat teeth were imaged by a ZTE sequence with the scan parameters of TR= 2 ms, FA= 3°, image resolution= 0.16 mm$^3$, 4 averages, BW= 200 kHz and $T_{RF}$ = 1 µs. The resultant dead time is 6.6 µs, and each scan took about 27 minutes. The applied FA of 3° was calculated based on the estimated $T_1$ values to achieve near-optimal steady-state magnetization at TR of 2 ms.

### 2.5. Gravimetric analysis

Five blot-dried CPC cylinders (diameter: 4.5 mm; length: 9 mm) were dried in an incubator at 37 °C for 24 hours in order to remove pore water and subsequently oven-dried at 1300 °C for 6 hours to remove bound water, similar to the method applied in Chen et al. [29]. The cylinders were weighed before and after each drying step. The pore and the bound water content were calculated as follows:

$$V_{pore} = \frac{(W_{initial} - W_{incubator-dried})}{\rho_{water}} / V_{CPC} \quad (1)$$

and

$$V_{bound} = \frac{(W_{incubator-dried} - W_{oven-dried})}{\rho_{water}} / V_{CPC} \quad (2)$$

where $W_{initial}$, $W_{incubator-dried}$, $W_{oven-dried}$ represent the original weight, the weight of one CPC cylinder after incubator-drying and oven-drying respectively, and $V_{cpc}$ is the volume of one CPC cylinder and $\rho_{water}$ is the water density.
2.6. Data analysis

A custom-built program written in MATLAB R2014b (Math-Works, Natick, MA, USA) was used to calculate the T₁ and T₂* relaxation times of CPC as well as of human and goat teeth. From the multi-FA UTE data, T₁ was calculated from the slope of \( \exp \left( -\frac{TR}{T_1} \right) \) in the equation \([25, 30, 31]\)

\[
\frac{S(FA)}{\sin(FA)} = \exp \left( -\frac{TR}{T_1} \right) \frac{S(FA)}{\tan(FA)} + M_0 \exp \left( -\frac{TE}{T_2} \right) \left( 1 - \exp \left( -\frac{TR}{T_1} \right) \right)
\]  

(3)

where \( S(FA) \) represents the image intensity at a specific FA and \( M_0 \) is the net magnetization at thermal equilibrium. The slope was obtained by a linear regression between \( S(FA)/\sin(FA) \) and \( S(FA)/\tan(FA) \).

With the multi-TE UTE data, the T₂* values of fresh CPC, i.e., restored in human teeth and goat teeth at TP1 as well as the corresponding dental components (enamel and dentin) were estimated by using the mono-exponential (ME) model

\[
S(TE) = S_0 \exp \left( -\frac{TE}{T_2^*} \right)
\]  

(4)

and the GAME model \([28, 32, 33]\)

\[
S(TE) = S_0 \exp(-\frac{TE}{T_2}) \exp(-(TE\sigma)^2/2)
\]  

(5)

where \( S_0 \) is the pseudo-spin density and \(~2.35\sigma\) represents the full-width-at-half-maximum (FWHM) of the Gaussian frequency distribution responsible for the reversible transverse relaxation rate \([28]\).

As for a Lorentzian underlying frequency distribution with FWHM of 2R₂*, the T₂* was defined as \( (1/T_2 + R_2)^{-1} \), the corresponding T₂* under a Gaussian distribution with FWHM of \(~2.35\sigma\) was calculated using the following equation to keep the definition consistent:

\[
T_2^* = \left( \frac{1}{T_2} + 1.17\sigma \right)^{-1}
\]  

(6)

The T₂* values of CPC and dentin in goat teeth at TP2 were obtained with the bi-exponential (BE) model

\[
S(TE) = S_0 \left[ p \exp(-\frac{TE}{T_{2,1}^*}) + (1 - p) \exp(-\frac{TE}{T_{2,2}^*}) \right]
\]  

(7)

where \( S_0 \) is the pseudo-spin density, \( T_{2,1}^* \) and \( T_{2,2}^* \) are the effective transverse relaxation times of two different water components, and \( p \) is the fraction of the first water component.
The background noise \( S_{\text{noise}} \) for each dataset was estimated from a region of interest (ROI) selected in a homogeneous area completely outside the object without any visible artifact. A quadratic noise correction [31] was used to correct the noise effect on each dataset using the following equation:

\[
S_{\text{corr}} = \sqrt{(S_{\text{uncorr}})^2 - (S_{\text{noise}})^2}
\]  

(8)

in which \( S_{\text{uncorr}} \) and \( S_{\text{corr}} \) represent the signal intensities before and after noise correction.

The noise corrected signal intensities \( S_{\text{corr}} \) were used for \( T_1 \) and \( T_2^* \) estimation. The fits in ME, GAME and BE models were with unconstrained nonlinear optimization [34].

To evaluate the reproducibility of estimating the relaxation times for CPC as well as for enamel and dentin, coefficients of variation (CVs), defined as SDs/mean values across three tooth samples, were calculated using the correspondingly averaged e.g., \( T_1 \) and \( T_2^* \) values, across two or three consecutive slices in each tooth.

From the ZTE images, the SNR of ROI \( x \) and the contrast-to-noise-ratio (CNR) between ROIs \( x \) and \( y \) were calculated according to

\[
\text{SNR}_x = \left| S_x - S_{\text{background}} \right| / s
\]  

(9)

and

\[
\text{CNR}_{x-y} = \left| S_x - S_y \right| / s
\]  

(10)

where \( S_x, S_y \) and \( S_{\text{background}} \) represent the mean signal intensities of ROIs \( x, y \) and background respectively and \( s \) is the standard deviation (SD) of the noise.

To assess the signal homogeneity of the CPC regions in three goat teeth at TP1 and three at TP2 on ZTE images, the CVs of signal intensities in each CPC regions, defined by SDs/mean levels of signal intensities, were respectively calculated. The used signal intensities in CPC regions were corrected for noise effect by using Eq.8. A higher CV value for larger signal deviation represents a less homogeneous signal distribution. The corresponding mean CV levels were calculated across three consecutive slices of each goat teeth.

All ROIs were manually outlined on UTE and ZTE tooth images using MRIcro software [35]. The mean signal intensities for different ROIs were obtained.

3. Results

3.1. Human teeth

The relaxation times for CPC, dentin and enamel in all three human teeth were obtained for selected areas as indicated on the UTE MR images in Figures 1A and 1B. The signal
intensities recorded by the UTE sequence as a function of TE were fitted using the ME model to obtain $T_2^*$ relaxation times and were also fitted using the GAME model to obtain the irreversible relaxation times ($T_2$) and reversible relaxation times ($1/\sigma$) for CPC, dentin and enamel respectively (Figures 1C, D, E). The GAME model showed better fitting than the ME model as reflected in higher $R^2$ values for goodness of fit (mean: $0.994$ vs $0.955$). In the example shown in Figure 1, the GAME fits (red), compared to the ME fits (blue), performed significantly better in the CPC region (GAME: $T_2 = 1155 \mu$s, $1/\sigma = 367 \mu$s, $T_2^* = 247 \mu$s, $R^2 = 0.997$; ME: $T_2^* = 430 \mu$s, $R^2 = 0.884$), and slightly better in the enamel region (GAME: $T_2 = 1168 \mu$s, $1/\sigma = 752 \mu$s, $T_2^* = 415 \mu$s, $R^2 = 0.992$; ME: $T_2^* = 771 \mu$s, $R^2 = 0.962$) and in the dentin region (GAME: $T_2 = 651 \mu$s, $1/\sigma = 702 \mu$s, $T_2^* = 312 \mu$s, $R^2 = 0.990$; ME: $T_2^* = 508 \mu$s, $R^2 = 0.966$).

Using the GAME model for fitting, the mean $T_2$, $1/\sigma$ and $T_2^*$ values of similarly selected areas with the same size across three consecutive slices of each tooth were then calculated.
(Figures. 2A, B, C). The averaged 1/σ values for enamel in tooth 1 as well as for dentin in teeth 1 and 2 were not shown because of 1/σ >> 1000 µs, resulting in a comparable fitting performance by GAME and ME models.

The mean values across three teeth obtained for CPC were: \(T_2 = 1234\pm27\ \mu s\), \(1/\sigma = 412\pm38\ \mu s\) and \(T_2^* = 273\pm19\ \mu s\); for enamel: \(T_2 = 963\pm151\ \mu s\) and \(T_2^* = 562\pm221\ \mu s\); for dentin: \(T_2 = 577\pm41\ \mu s\) and \(T_2^* = 476\pm147\ \mu s\). The \(T_2\), \(1/\sigma\) and \(T_2^*\) values for CPC, enamel and dentin showed high inter-reproducibility across the three teeth with low CVs (\(T_2\): 2.2% for CPC, 15.6% for enamel and 7.0% for dentin; \(1/\sigma\): 9.2% for CPC; \(T_2^*\): 7.0% for CPC).

The \(T_1\) relaxation times of CPC and human tooth tissues were also estimated. Respective mean values were calculated across three consecutive slices for each tooth (Figure 2D). The mean \(T_1\) values were: \(1065\pm45\ \text{ms}\) for CPC; \(972\pm40\ \text{ms}\) for enamel and \(903\pm7\ \text{ms}\) for dentin. Reproducible \(T_1\) values were observed across the three teeth in each of CPC, enamel and dentin regions, indicated by low CVs (CPC: 4.3%; enamel: 4.2%; dentin: 0.8%).

**Figure 2.** Mean \(T_2\), \(1/\sigma\), \(T_2^*\) and \(T_1\) relaxation times of CPC and dental components in human teeth. A) mean \(T_2\) (µs); B) \(1/\sigma\) (µs); C) \(T_2^*\) (µs); D) \(T_1\) (ms) with error bars indicating ± 1 standard deviation (SD) of CPC, enamel and dentin across three consecutive slices for three human tooth samples. Mean 1/σ levels of dentin in teeth 1 and 2 as well as of enamel in tooth 1 are not shown because of 1/σmean >> 1000 µs.
**T**\textsubscript{1}, **T**\textsubscript{2} and **T**\textsubscript{2}\textsuperscript{*} values were also estimated for each voxel in the selected ROIs to construct histograms of their distributions for CPC, enamel and dentin. The median values were also shown and comparable to the obtained mean levels (Supplementary Material, Figures S1-S3). On ZTE images, acquired with optimized FA of 3° at TR of 2 ms based on these **T**\textsubscript{1} values, CPC has a much higher SNR compared to enamel and dentin (Figure 3). Across three consecutive slices over all three teeth, mean SNR values of 26.7±1.6, 10.9±0.8 and 19.3±1.3 were calculated for CPC, enamel and dentin respectively (Figure 4). Additionally, a strong image contrast for CPC is seen as reflected by high CNRs between CPC and surrounding enamel or dentin (mean: 15.7±1.2 or 7.4±0.4; Figure 4).

![Figure 3](image1.png)

**Figure 3.** MR images of human tooth with CPC obtained with ZTE. A) coronal view; B) transversal view. The corresponding signal-to-noise-ratios (SNRs) of CPC, enamel and dentin are 27.6, 11.1 and 20. The contrast-to-noise-ratios (CNRs) are thus 16.5 between CPC and enamel and 7.6 between CPC and dentin.

![Figure 4](image2.png)

**Figure 4.** Mean SNRs of CPC, enamel, and dentin and mean CNRs of CPC to enamel and to dentin in ZTE images across three consecutive slices over three human teeth. Error bars indicate ± 1 SD.

3.2. Goat teeth

The goat teeth were imaged *ex vivo* by ZTE at TP1 (with CPC implantation immediately after the extraction of teeth) and at TP2 (teeth extracted seven weeks after CPC implantation). Similar to the CPC in human teeth, the CPC in goat teeth also showed a hyperintense signal
and a strong image contrast compared to dentin at TPs 1 and 2 (Figures 5 and 6; mean: $\text{SNR}_{\text{cpc}} = 25.6 \pm 1.1$ and $\text{CNR}_{\text{cpc-dentin}} = 10.7 \pm 0.8$ at TP1; mean: $\text{SNR}_{\text{cpc}} = 26.4 \pm 1.2$ and $\text{CNR}_{\text{cpc-dentin}} = 11.3 \pm 1.3$ at TP2). Comparing the goat teeth at TP1 and TP2, the SNRs for dentin were not different (mean: 14.9±0.6 vs 15.1±0.7; Figure 6). However, on ZTE images the CPC in teeth extracted at TP2 was less homogeneous (Figure 5), reflected by a larger range of signal intensities than the CPC implanted after extracting these teeth at TP1 in all three slices over all teeth (mean CVs: 14.9±1% vs 10.5±2%). The SNRs of enamel and CNRs between CPC and enamel were not calculated since enamel in goat teeth is thin and therefore it is difficult to select a representative ROI for analysis (Figure 5).

The $T_1$ and $T_2$ values of fresh CPC in restored goat teeth at TP1 were also estimated to determine how comparable they are with those of CPC in human teeth. Robust $T_2$ fitting was obtained with the GAME model, indicated by high $R^2$ values (mean: 0.991±0.008). The mean levels of $T_2$ and also $T_1$ values were calculated across two consecutive slices over three goat teeth. The resultant values were comparable to those of fresh CPC in human teeth ($T_2$: 1271±335 µs vs 1234±27 µs; $T_1$: 1008±277 ms vs 1065±45 ms). As the estimated reversible relaxation times ($1/\sigma$) >> 1000 µs, the corresponding mean $T_2^*$ of fresh CPC in goat teeth at TP1 was also 1271±335 µs.

**Figure 5.** MR images of goat teeth obtained with ZTE. A) coronal view and B) transversal view at time point (TP) 1 with $\text{SNR}_{\text{cpc}}=24.8$, $\text{SNR}_{\text{dentin}}=14$, $\text{CNR}_{\text{cpc-dentin}}=10.8$ and CV= 12.6% for the signal intensities in CPC region. C) coronal view and D) transversal view at TP2 with $\text{SNR}_{\text{cpc}}=26.9$, $\text{SNR}_{\text{dentin}}=14.9$, $\text{CNR}_{\text{CPC-dentin}}=12$ and CV= 16.4% for the signal intensities in CPC region.
Figure 6. SNRs of CPC and dentin and CNRs of CPC to dentin in ZTE images of goat teeth. Across three consecutive slices over three goat teeth at TP1 and three at TP2, mean SNRs of CPC and dentin as well as mean CNRs of CPC to dentin. Error bars indicate ± 1 SD.

A BE model was used for $T_2^*$ estimations in the CPC and dentin regions for all three goat teeth at TP2. A robust fit was achieved with a high goodness of fit (mean $R^2$: 0.99; Figure 7). Two components with distinctly different $T_2^*$ and fraction values were found for CPC and dentin by using the BE model. The mean $T_2^*$ values for both components and the mean fraction values of the long $T_2^*$ components were calculated for CPC and dentin in each tooth across two (for CPC) and three (for dentin) consecutive slices (Figures 8A, B, C). The mean $T_2^*$ values across all three teeth for CPC were $1110±280$ µs and $94±38$ µs (with CVs 25.2% and 40.4%), and for dentin these were $2211±180$ µs and $91±2$ µs (with CVs 8.1% and 1.7%). The mean fraction percentage for the long $T_2^*$ component for CPC was $55.5±5.4%$ (CV: 9.8%), and for dentin this was $66.9±1.6%$ (CV: 2.5%).

The mean $T_1$ values in each goat tooth at TP2 were also estimated for CPC and dentin across two (for CPC) and three (for dentin) consecutive slices (Figure 8D). The average $T_1$ values and the corresponding CV values over all three teeth were: $742±103$ ms and 13.9% for CPC, and $930±57$ ms and 5.0% for dentin. Compared to freshly applied CPC in goat teeth at TP1, much lower $T_1$ values were found in the CPC region at TP2 ($742$ ms vs $1008$ ms).

3.3. Water content

Pore and bound water volume percentages of all five CPC cylinders were calculated. The mean value for pore water over all CPCs was $24.8±2.7\%$, and for bound water this was $10.4±0.8\%$.  


**VISUALIZATION OF CPC BY ZTE MRI**

**Figure 7.** MR images of goat teeth obtained with UTE at TE = 18 µs. A) coronal view at TP2. ROIs (pink) with identical size of 3 by 2 voxels were selected in CPC and dentin regions, respectively. A bi-exponential (BE) model fit the multi-TE UTE data (*) using a semi-log plot for the ROIs in CPC (B) and dentin (C) regions, which achieved high goodness of fit: $R^2 = 0.99$ (CPC: $T_{2.1^*} = 1310$ µs (fraction: 53.2%), $T_{2.2^*} = 114$ µs; dentin: $T_{2.1^*} = 2208$ µs (fraction: 68.5%), $T_{2.2^*} = 88$ µs).

**Figure 8.** Mean levels of the $T_{2^*}$ values in the long $T_{2^*}$ component (A) with its fraction (B), the $T_{2^*}$ values in the short $T_{2^*}$ component (C) and $T_1$ values (D) of the CPC and dentin across two (for CPC) or three (for dentin) consecutive slices for three goat tooth samples at TP2, indicated with error bars ± 1 SD.
4. Discussion

In this paper we present high field proton MR properties of a new CPC material as applied in the restoration of teeth. This includes $T_1$ and $T_2^*$ relaxation times of this material implanted in teeth and of the enamel and dentin tissues in these teeth. Because of the very short $T_2^*$ relaxation times of CPC and dental tissues, we used a ZTE sequence to obtain high SNR images of CPC restored teeth. In these images, CPC is hyperintense and has a strong image contrast with respect to dentin and enamel. In addition, we demonstrate that seven weeks after implantation in goat teeth CPC exhibits a less homogeneous signal distribution, a decreased $T_1$, and a change in $T_2^*$ values, implying a certain CPC degradation over time.

Previously, the $T_2^*$ value of a comparable CPC was reported, although the ME model used to analyze the multi-TE data in that study did not result in a satisfactory fit [36]. Because non-exponential behavior was also observed for the $T_2^*$ relaxation of the CPC investigated in this study, we applied the GAME model to fit our gradient-echo data [28]. This model assumes that the intra-voxel frequency distribution responsible for the reversible relaxation component is Gaussian—rather than Lorentzian—shaped, which is particularly relevant at higher field when this component becomes more prominent in $T_2^*$ relaxation. It has been successfully used for $T_2^*$ determinations from gradient-echo measurements of human brain and prostate and gynecologic cancers at 3T [28,32,33]. In this study it was applied for the first time to estimate the $T_2^*$ values of teeth and engineered tissue.

Unlike the ME model that gives a single $T_2^*$ value from fitting the signals of a multiple TE gradient-echo measurement, the GAME model provides both the irreversible relaxation time ($T_2$) and reversible relaxation time ($1/\sigma$) from such a measurement. As the reversible relaxation reflects local inhomogeneities, the relatively high CV (about 10%) that we obtained for the $1/\sigma$ times of CPC across three human tooth samples indicates differences in microstructures such as small (air) cavities in the material and at the interface of CPC with tooth tissues, which may cause complex susceptibility gradients. Fitting with the GAME model also appeared valuable for $T_2^*$ estimations of dentin and enamel. While $T_2$ relaxation dominates their $T_2^*$ relaxation times, the effect of $1/\sigma$ on $T_2^*$ of these tissues is not negligible in some teeth (Figures 1 and 2).

Previously, relaxation times for enamel and dentin have been investigated at 1T [7,37]. These studies determined for a semi-liquid component in enamel a $T_2^*$ of about 240 μs, a $T_2$ of 10 ms and a $T_1$ in the order of 300 ms. For dentin a $T_2^*$ of below 1000 μs and $T_2$ of 38 ms were reported. A study performed at 1.5T reported $T_2$ ($T_2^*$) values for dentin and enamel in adult teeth of 150 μs and 70 μs respectively [38]. At 3T human teeth were measured ex vivo.
and *in vivo* using a UTE sequence with multiple TEs [31,39]. Fitting by a standard ME model, the $T_1$ and $T_2^*$ relaxation times of dentin tissue *ex vivo* were 545 ms and 478 μs and the $T_2^*$ time of dentin *in vivo* was 324 μs, which are closer to the results we have obtained in the present study, than to those in any of the other previous studies. The discrepancies between the reported tooth relaxation properties of these studies might be because of the use of different field strengths, different MR sequences and different analysis and fitting methods, as well as of variations between the conditions of the tooth samples used in each examination.

Because of their very short $T_2^*$ relaxation times and limited water content, ZTE is a favorite sequence to record high SNR images of teeth [8,15]. As CPC, dentin and enamel appeared to have comparable $T_1$ values, a FA (3°) for near-optimum steady state magnetization at a TR of 2 ms could be applied in the ZTE sequence for tooth imaging, which essentially produces proton density weighted images. The observed hyperintense signal in CPC and strong image contrast to surrounding tissues can therefore only be explained by its higher water content relative to dentin and enamel. To confirm this assumption, a gravimetric analysis was performed to quantify water content in CPC. Two water components, namely, pore water and bound water, were found with a much higher water volume percentage (about 25 and 10% respectively) than that reported for enamel and dentin (4 and 10% respectively) [8]. As CPC thus has at least two water compartments, a bi-exponential $T_2^*$ decay may have been expected that would not have been possible to fit by the GAME model. A plausible reason that this is not observed is that the bound water probably has an ultra-short $T_2^*$. A $T_2^*$ value of 100 μs has been reported for collagen-bound water in cortical bone at 3 T [29]. Together with the substantial contribution from local field inhomogeneities, which follows from the GAME fit, the $T_2^*$ of bound water in CPC matrices is expected to be significantly shorter at 11.7 T. Also, considering the lower volume percentage of this bound water it is expected that in the UTE examinations for $T_2^*$ fitting, bound water contributed little to the recorded signal.

To investigate if CPC degradation in teeth can be monitored over time, we separately implanted CPC into three goat teeth *ex vivo* and three *in vivo*. While the fresh CPC implanted in teeth *ex vivo* was scanned immediately (TP1), the CPC injected in teeth *in vivo* was measured at seven weeks post-surgery (TP2). With respect to the CPC at TP1, we observed a less homogeneous signal distribution in ZTE images for the CPC component at TP2. We also compared their relaxation properties and found a decreased $T_1$ time and $T_2^*$ separated into two components (Figure. 9). Based on these findings, we infer that the CPC at TP2 had undergone compositional changes. The observed less homogeneous structure might indicate that a certain degree of CPC dissolution had occurred at TP2, giving rise to a different phase cement
The degradation of included PLGA particles in acidic monomers (i.e., lactic and glycolic acid) is expected to induce an initial dissolution of CPC with increased porosity. This porous structure is less rigid and could explain the decrease of $T_1$ in CPC at TP2. Moreover, the increased porosity could also allow more fluid flow through the implant and recruitment of odontoblast-like cells, which are responsible for reparative dentin deposition [40-41]. The newly emerging dentin component in CPC can explain why a bi-exponential model performed a robust fit for the CPC at TP2. This is also the reason that the short $T_2^*$ parts of CPC and dentin at TP2 were found comparable (mean: 94 μs vs 91 μs). Thus, these changes in relaxation properties may help to understand and monitor the interaction of CPC and dental tissues in vivo. Further histological analyses are required to assess the micro-structural alterations in the CPC area from TP 1 to 2.

![Figure 9](image-url)

_**Figure 9.**_ Comparison of $T_2^*$ and $T_1$ values between freshly applied CPC at TP1 and CPC at TP2. Mean levels of $T_2^*$ (A) and $T_1$ (B) relaxation times of CPC across two consecutive slices over three goat teeth at TP1 and three at TP2. Error bars indicate ± 1 SD.

It is striking that the human molars show a convex shape for the $T_2^*$ signal decay as a function of TE and the goat incisors a concave shape. This could be due to differences in chemical composition of these teeth. It is known that the chemical composition of human and animal teeth can vary widely [43]. In particular the calcium, phosphorous and magnesium content is not the same in teeth of different species, in different types of teeth of the same animal (molar and incisor) and in different tissues in the same tooth (enamel and dentin).

The images in our study were measured at high magnetic field strength and with rather lengthy acquisitions to obtain optimal SNR. However, it has been demonstrated that excellent MR images of teeth can be obtained at lower clinical field strengths with turbo spin echo (TSE), UTE or SWIFT sequences at relatively short acquisition times, including the detection
of bisphosphonate-induced necrosis, caries lesions, periodontitis and micro-cracks [8,16,39,44,45]. Therefore, we anticipate that our finding that the investigated CPC has $T_1$ and $T_2$ relaxation properties different from that of the host dental tissue also will provide useful image contrast of this material at lower field strength and shorter acquisition times. ZTE is now also available on clinical 3T MR systems and its applicability in dental imaging in vivo can be explored.

5. Conclusions
For the assessment of $T_2^*$ relaxation times in MRI of human teeth a Gaussian instead of a Lorentzian exponential model provides the best fit to the $T_2^*$ signal decays of CPC, dentin and enamel. Due to a high water content, excellent images of CPC in teeth can be obtained with ZTE at high field, showing high SNR and strong image contrast. Relaxation times and MR image properties of the regions filled with CPC in vivo were significantly altered after seven weeks, indicating that material decomposition, and possibly dentin formation, can be followed by MRI.

6. References


7. Supplementary Information

Figure S1. Histograms of $T_1$ (A), $T_2$ (B) and $T_2^*$ (C) distributions of CPC regions in three human teeth. The $T_1$, $T_2$, and $T_2^*$ estimations were performed voxel by voxel using Eqs. 3, 5 and 6 for 9 regions of interest with an identical size of 3 by 3 voxels selected in the CPC regions across three consecutive slices over three human teeth. The $T_1$ values in 81 voxels, and the $T_2$ and $T_2^*$ values in 75 out of 81 voxels were used for their distributions. Six voxels were excluded for $T_2$ and $T_2^*$ analysis because their $R^2$ values for goodness of fit were less than 0.90. Additionally, their median values are presented, which are comparable to the corresponding mean levels.

Figure S2. Similar to Figure S1, histograms for $T_1$ (A), $T_2$ (B) and $T_2^*$ (C) distributions of enamel regions in three human teeth are shown. In total 81 voxels were evaluated for $T_1$, $T_2$, and $T_2^*$ values. Their corresponding median values and mean levels are also presented and appear to be comparable.
**Figure S3.** Histograms, representing $T_1$ (A), $T_2$ (B) and $T_2^*$ (C) distributions of dentin regions in three human teeth, are shown. $T_1$, $T_2$, and $T_2^*$ values were estimated in total 81 voxels. In addition, the median and mean levels of $T_1$, $T_2$ and $T_2^*$ values are presented and appear to be comparable.
Chapter 4

A Theranostic Dental Pulp Capping Agent with Improved MRI and CT Contrast and Biological Properties


1. Introduction

The healthy state of dental pulp can be compromised after exposed to an extended caries or traumatic injury [1]. Different strategies for vital pulp treatment, such as direct and indirect pulp capping and partial pulpotomy, are performed to maintain the integrity and the vitality of the tooth. A capping material can serve to protect the exposed pulp and to promote dental pulp stem cells to differentiate into odontoblasts, which subsequently produce tertiary dentin (i.e. the “dentin bridge”) [2,3]. Based on the intensity of the external stimulus, tertiary dentin can be distinguished in reactionary dentin and reparative dentin [4]. The deposition of reactionary dentin occurs in the case of mild stimuli and is guided by post-natal odontoblasts that have survived to the initial damage. Reparative dentin is secreted in the case of severe external stimuli and is guided by newly formed odontoblast-like cells [5,6].

An ideal material for vital pulp therapy needs to be biocompatible, have optimal handling properties, show imaging contrast, and induce dentin deposition. Historically, calcium hydroxide has been widely used for this specific application especially because of its antimicrobial and reparative properties [7,8]. Therefore, calcium hydroxide is considered as gold standard. However, some limitations, such as lack of pulp sealing, high solubility and the formation of “tunnel defects”, reduce calcium hydroxide capability to fulfill optimal clinical requirements [9,10]. Among many others compositions that have been investigated over the last years, calcium phosphate cement (CPC) appears to be a valid candidate as it shows biocompatibility, biodegradability, injectable and self-setting properties [11-13]. Moreover, it has been proven that CPC can also induce dentin mineralization and inhibit caries [14,15]. The combination of CPCs with specific growth factors (e.g. transforming growth factor beta, TGF-β, bone morphogenetic protein 2, BMP-2) can further improve the biological properties of the final material resulting in enhancement of tissue formation [16-18].

Another important consideration in the development of a pulp capping biomaterial is the capacity for imaging, necessary to assess the quality of the filling proximal to the pulp [1]. Although X-ray based technologies are widely implemented, concerns about the use of ionizing radiations [19] have led to investigations of other imaging techniques, like magnetic resonance imaging (MRI), as a suitable alternative in dental screening. MRI is not only radiation free, but also allows for the simultaneous anatomical and functional tridimensional imaging of both soft and hard tissues [20,21]. Traditionally, the low presence of protons in bone and teeth results in a very short $T_2$ relaxation (i.e. 500 μs for cortical bone and 250 μs for enamel, respectively) hampering their visualization. However, the newly developed MRI
sequences with high sensitivity to ultra-short $T_2^*$ relaxation (i.e. ultrashort echo time, UTE, and zero echo time, ZTE), have opened new scenarios for MRI in dental imaging with the improved visibility of bone and dental tissues [22-26].

In this study, a defined CPC composition - previously tested favorably for bone application [27] - was used for direct pulp capping in an in vivo animal model (i.e. goat incisors). Specifically, we aimed to produce a theranostic biomaterial, which combined a dedicated dual contrast agent (DCA) and BMP-2. The DCA consisted of core-shell superparamagnetic iron oxide (SPIO)/colloidal gold nanoparticles respectively as MRI and CT contrast agent, while BMP-2 was used to trigger dentin regeneration. Imaging performances of the cement were monitored seven weeks’ post-surgery by µCT and MRI. Finally, the biological response, new dentin formation, and cement degradation were assessed.

2. Material and methods

2.1. CPC composition
Calcium phosphate cement (CPC) consisted of a weight/weight (wt/wt) mixture of 59.1% alpha-tricalcium phosphate (α-TCP; CAM Bioceramics BV, Leiden, the Netherlands), 1.5 % carboxymethylcellulose (CMC; CAM Bioceramics) and 39.4% cryo-grinded poly-(DL-Lactic-co-Glycolic acid) particles (<200 µm) with a 50:50 ratio of lactic to glycolic acid (PURASOB 5002A, Purac, Gorinchem, the Netherlands) [28].

2.2. Core-shell dual contrast agent (csDCA) preparation
Core-shell structured dual contrast agent (csDCA) particles were provided by Nano4Imaging GmbH (Aachen, Germany) and used without further purification. Briefly, superparamagnetic iron oxide (SPIO) nanoparticles (200 nm) were firstly synthesized according to the literature [29] and then coated with gold colloid. Through in situ reduction of suitable gold (III) salts a gold layer was deposited on SPIOS to form core-shell structures with a final SPIO/gold ratio equal to 3:1 (v/v). Afterward, an inverse emulsion approach was used in order to embed SPIO/gold nanoparticles within a silica matrix. Particles were treated with polyethylene glycol (PEG-400) and tetraethyl orthosilicate (TEOS) to introduce mesoporous silica matrix with a final concentration of 40% (wt/wt). Lastly, all particles were coated with an additional apatite-like layer in order to increase the affinity within the CPC phase. All csDCA particles had a heterogeneous size with a diameter ranging between 1 µm and 2 µm.
2.3. Theranostic dental pulp capping material (CPC/csDCA/BMP-2) preparation

CPC powders were combined with 5% (wt/wt) csDCA into an exit-closed 2 mL plastic syringe (Terumo Europe N.V., Leuven, Belgium). Syringes with pre-mixed components were sterilized by using 25kGy of gamma radiation (Synergy Health BV, Ede, the Netherlands). To improve biological properties, BMP-2 (R&D Systems, Tocris and Novus Biologicals, Abingdon, UK) was added to the powders through resuspension into the setting solution [30]. Briefly, BMP-2 was first resuspended in 4 mM HCl containing 0.1% bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO). Right before the in vivo interventions, 10 µg of BMP-2 were mixed with the setting solution and added to the syringe. For 100 mg of CPC powders, 60 µl of 4% NaH₂PO₄•2H₂O were used. Finally, all components were mixed vigorously for 30 seconds with a dental shaker machine (Silamet® Mixing apparatus, Vivadent, Schaan, Liechtenstein). As control CPC powders were prepared exactly in the same way, but without the addition of csDCA and BMP-2.

2.4. Morphological characterization of the material

Morphological assessment of the single CPC components, of the csDCA particles and of the final theranostic pulp capping agent was performed by scanning electron microscopy (SEM, JEOL 6310, Jeol, Tokyo, Japan). Energy dispersive x-ray spectrometry (EDS, Bruker, Kalkar, Germany) was used for the investigation of the csDCA particle distribution into the cement matrix. Morphology of the csDCA particles was further investigated by transmission electron microscopy (TEM, JEOL TEM1010). Briefly, particles were first embedded in a cold-setting embedding resin (Epofix™ Kit, Struers, Maassluis, the Netherlands). After polymerization, ultrathin sections (between 50 and 100 nm in thickness) were cut on a Leica Reichert Ultracut S microtome (Leica Microsystem B.V., Eindhoven, the Netherlands) equipped with a diamond knife (Drukker International B.V., Cuijk, the Netherlands) and collected on 100 mesh copper grids (Sigma-Aldrich).

2.5. Mechanical and handling properties characterization

To investigate the effect of csDCA particle incorporation into the CPC phase, mechanical and handling properties were assessed. Specifically, initial and final setting times were estimated using Gillmore needles (ASTM C266) [28]. Times were recorded after injection of CPC formulations in a brass mold (6 mm in diameter, 12 mm in height) immersed in a water bath kept at 37°C. Compressive strength and E-modulus were obtained from compression tests. Briefly, cylindrical blocks (4.5 mm in diameter, 9 mm in height) were prepared and placed
vertically in a testing bench machine (858 MiniBionixII, MTS, Eden Prairie, MN). A final loading force of 2.5 kN and constant speed of 0.5 mm/min was applied. Injectability properties were investigated by using the same testing bench machine (858 MiniBionixII) set in compression mode, with a custom made metallic cage fixture. Briefly, after mixing 500 mg of cement with 300 µl of setting liquid for 30 seconds, the syringe (orifice diameter 1.7 mm) was placed in the cage and then a compression force of 100 N (i.e. ~maximum force applicable by human operator) with a constant speed of 20 mm/min was applied until all material was extruded from the syringe (33 ± 2 s). The extrusion curve was calculated as applied force (N) by the time (sec) [31]. All cements extruded from the syringes were collected in 10 ml of PBS at 37°C and cohesive properties were qualitatively assessed, by counting the number of separate pieces.

2.6. Ex vivo direct pulp capping in human molars and goat incisors

To investigate the right type/concentration of DCA to be used for the enhancement of the CT and MRI contrast in respect of the natural dentin phase, *ex vivo* direct pulp capping was performed on both human molars and goat incisors. Human molars were provided by the Department Oral and Maxillofacial Surgery of RadboudUMC Nijmegen, The Netherlands. Goat incisors were collected at the College of Dentistry, King Saud University, Riyadh, Saudi Arabia from surplus cadavers from non-related prior experiments. Intact teeth were chosen and stored in ethanol 70% at room temperature until their use.

Briefly, on the occlusal surface of human molars and on the buccal surface of goat incisors a cylindrical defect (about 3 mm in diameter) was made through the enamel until the dentin was reached. Subsequently, a smaller cavity (about 1 mm in diameter) was made in the dentin to reach the pulp. Thereafter, a direct pulp capping procedure was performed using CPC with and without csDCA. Finally, the cavities were filled with light cured (LC) glass ionomer cement (GC Fuji II LC® CAPSULE, Cavex, Haarlem, the Netherlands). All samples were prepared in triplicate.

2.7. In vivo direct pulp capping in goat incisors

For the *in vivo* assay, animals involved in a non-related experiment were chosen (i.e. reduction of animal use). The work-protocol of the animal experiment was conducted using the facility and support of the College of Dentistry, King Saud University, Riyadh, Saudi Arabia, with the ethical reference number (PR0038). The animal study was not registered as a clinical trial. The *in vivo* experiment was requested to address three experimental hypothesis: 1) is csDCA able to give MRI and CT signal after 7 weeks implantation *in vivo*; 2) is
CPC/csDCA/BMP-2 able to induce more dentin formation when compared to the control groups; 3) is CPC/csDCA/BMP-2 degrading faster when compared to the control groups. The needed sample size was calculated through G*Power software (Erdfelder, Faul, & Buchner, 1996) [32] by using a significance level of 0.05 and 80% power. A standard deviation (SD) of 25% and effect size (d) of 40% were defined based on previous studies related to dentin regeneration [33, 34]. A split-mouth design was used [35]. This usually enhances the power of the study, but the extent of that increase could not be readily estimated. Therefore, the number of animals used was the one obtained in the conservative power calculation. The lower four incisors of each animal were distributed to the four experimental groups (i.e. empty defect, pulp capping with CPC, pulp capping with CPC/csDCA and pulp capping with CPC/csDCA/BMP-2) through a block randomization approach (n = 6 for all the experimental groups, see Table 1). Allocation of the animals to each split-mouth group was also performed through a simple randomization method (i.e. the first animal picked was named as Animal 1, etc.). The study population, used for in vivo direct pulp capping, consisted of 6 healthy, 18 months old, Habsi male goats. At this age the four lower goat incisors are all erupted and have a similar morphology, reducing the inter-group variability [36]. Pulp capping was performed by a single expert operator that was trained in the standardization of the surgical procedure according to previous work [33]. All procedures were performed under general anesthesia induced by intramuscular (IM) injection of ketamine hydrochloride (5 mg/kg) and diazepam (1 mg/kg). To reduce the risk of infection, animals were receiving antibiotics pre-operatively (10 mg/kg intravenously; Amoxicillin®), and post-operatively, at day 1 and day 3 (50 mg/kg intramuscularly; Albipen®). To alleviate pain analgesic Finadyne® (1mg/kg) was given three times a day. Animals were immobilized in a ventral position. The oral tissues were disinfected with 10% Povidone-iodine. Afterward, local anesthesia (Lidocaine 2% with 1:100,000 Epinephrine) was injected around the lower incisors. A cavity was drilled on the vestibular surface of the lower incisor by using a sterile round-shaped bur (1 mm in diameter) mounted on conventional dental handpiece with an appropriate speed and irrigation (Supplementary Information, Figure S1b). A cylindrical defect (about 1 mm in diameter and 2 mm in length) was prepared through the full enamel, dentin and pulp cavity until the dentin on the lingual side was reached (Figure S1c). In this way, pulp tissue along the direction of the defect was also ripped out from the pulp canal (i.e. partial pulpotomy). During the dental procedure, a sterile 0.9% saline solution was used to cool down the drill and to remove the debris. A cotton pellet was placed over the defect for 2-3 minutes to control the pulp hemorrhage until the bleeding was completely controlled.
Pulp capping was performed using one of the experimental cement pastes inserted in the prepared cavity with a passive application by means of a Dycal® placement instrument (Dentsply Sirona, York, PA). Light pressure was applied using a wet cotton pellet to ensure that the paste would reach the deepest part of the cavity. Also, one group was included leaving the cavities empty (n=6). Due to the chosen experimental groups, the main operator was only partially blinded. Although the cement pastes were prepared by another assistant operator, the difference in color between the pastes (i.e. white color for the CPC and brown color for the CPC/csDCA and the CPC/csDCA/BMP-2) hindered a completely blinded procedure. Still, the main operator was not able to recognize CPC/csDCA from CPC/csDCA/BMP-2 pastes. Two primary outcomes were evaluated during the surgical procedure, i.e. the ability to handle the prepared paste with a specific dental instrument (Dycal® placement instrument), and the ability of the paste to seal the defect. A defined score (i.e. very poor, poor, good, and very good) was attributed by the operator to each specimen. After material placement, all cavities were filled with light cured (LC) glass ionomer cement (GC Fuji II LC® CAPSULE, Cavex) (Figure S1e and S1f). Animals were housed together on a goat farm. Seven weeks post-surgery animals were euthanized by an overdose of Nembutal® (Apharmo, Arnhem, the Netherlands). Lower incisors were extracted and cleaned from the soft tissue. The apical part of the incisors was removed to allow fixative (i.e. 10% Formalin for one week) to flow through the tissues.

2.8. Ex vivo µCT

All specimens (i.e. human and goat teeth from ex vivo study and goat incisors from in vivo study) were wrapped in Parafilm® (SERVA Electrophoresis GmbH, Heidelberg, Germany) and scanned vertically along the direction of the X-ray beam by means of a micro-computer tomography imaging system (Skyscan 1072, Kontich, Belgium). The following parameters were used to get a two-dimensional (2D) reconstruction: X-ray source 100 kV/98 µA, exposure time 3.9 s and 1 mm Aluminium filter. Human molars were scanned using X15 magnification (pixel resolution = 18,88 µm). Goat incisors magnification was set at X25 (pixel resolution = 11,10 µm). Gray values of a defined volume of interest (VOI) were analyzed by CTAnalyser software (version 1.10.1.0, Skyscan) while two-dimensional reconstruction was obtained by DataViewer software (Skyscan 1.5.2.4).
Table 1. Randomization scheme of the implanted experimental groups and illustration of the goat lower incisors with individual tooth number.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Incisor 42</th>
<th>Incisor 41</th>
<th>Incisor 31</th>
<th>Incisor 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal 1</td>
<td>CPC/csDCA BMP-2</td>
<td>CPC/csDCA BMP-2</td>
<td>CPC</td>
<td>Empty defect</td>
</tr>
<tr>
<td>Animal 2</td>
<td>Empty defect</td>
<td>CPC/csDCA BMP-2</td>
<td>CPC/csDCA BMP-2</td>
<td>CPC</td>
</tr>
<tr>
<td>Animal 3</td>
<td>CPC</td>
<td>Empty defect</td>
<td>CPC/csDCA BMP-2</td>
<td>CPC/csDCA BMP-2</td>
</tr>
<tr>
<td>Animal 4</td>
<td>CPC/csDCA BMP-2</td>
<td>CPC</td>
<td>Empty defect</td>
<td>CPC/csDCA BMP-2</td>
</tr>
<tr>
<td>Animal 5</td>
<td>CPC/csDCA BMP-2</td>
<td>CPC/csDCA BMP-2</td>
<td>CPC</td>
<td>Empty defect</td>
</tr>
<tr>
<td>Animal 6</td>
<td>Empty defect</td>
<td>CPC/csDCA BMP-2</td>
<td>CPC/csDCA BMP-2</td>
<td>CPC</td>
</tr>
</tbody>
</table>

New dentin formation and cement degradation were both quantified based on µCT acquisitions by using CTAnalyser software (Skyscan). Discrimination between reactionary and reparative dentin was not possible from µCT acquisitions because of imaging resolution limits. Therefore, both kinds of dentin were considered together. Briefly, a cylinder of 1 mm in diameter and 2 mm in height was selected and located in the proximity of the formed dentin cap. Relative volume was independently quantified using a standardized gray value threshold and expressed in percentage. For the cement degradation, a cylinder of 2.5 mm in diameter and 1.5 mm in height was selected and placed in proximity of the performed defect. After applying a standardized threshold relative volume was quantified and expressed in percentage. To exclude bias from possible dentin debris left in the defect area after drilling values were normalized by the mean of the dentin-like components volume present in the empty defect group.

2.9. Ex vivo MRI

Prior to the MRI examination, all human and goat teeth specimens (i.e. from ex vivo and in vivo studies) were embedded in gelatin type A (Sigma-Aldrich) dissolved in MilliQ water with a final concentration of 5% (wt/v). Thereafter, samples were imaged on an 11.7 Tesla (T) MRI system (Biospec, Bruker) equipped with a proton quadrature volume coil. A zero echo time (ZTE) sequence was employed for the image acquisition. The corresponding scan parameters were repetition time (TR) = 2 ms, flip angle (FA) = 3°, averages = 3, acquisition
time (TA) = 27.34 minutes, matrix size = 256 x 256 x 256 and image resolution = 0.16 mm³. The acquired images were analyzed using OsiriX software (Pixmeo, Bernex, Switzerland). Qualitative evaluation of the MR images was performed to assess signal enhancement, pulp tissue integrity and new dentin formation.

2.10. Histological analysis

After fixation, goat incisors from the in vivo study were dehydrated in a graded series of ethanol (70-100%) and embedded in poly(methyl methacrylate) resin. Cross-sections of approximately 10 µm were prepared along the sagittal direction of the tooth. At least three sections per sample were obtained and stained with Methylene Blue and Basic Fuchsin. Images were acquired using a light microscope (Axio Imager Z1, Zeiss, Göttingen, Germany) equipped with a digital camera (AxioCam MRc5, Zeiss). Micrographs were obtained at X1, X10 and X20 magnification. Qualitative evaluation of the acquired micrographs was executed independently by three different operators. Tertiary dentin was classified as reactionary dentin or reparative dentin as described elsewhere [6]. Briefly, dentin showing tubular structures in continuity with the pre-existing secondary dentin was classified as reactionary dentin, while atubular calcified scar tissues were classified as reparative dentin.

2.11. Statistical analysis

Statistical analysis was performed by using IBM SPSS Statistic 22.0 software (IBM Corporation, Armonk, NY). Data were reported as mean ± standard deviation. Statistical significant differences in the mechanical properties were investigated by using a Student-t-test with Welch’s correction. Differences in setting times, in gray values, by one-way analysis of variance (ANOVA) with Tukey post hoc test. Differences in dentin formation and cement degradation were analyzed by using a Friedman’s analysis of variance by ranks. All differences were considered significant at p-values <0.05.

3. Results

3.1. Morphological characterization

Scanning electron micrographs of the CPC components (i.e. α-TCP, CMC and PLGA) are reported in the supplementary information (Figure S2). Core-shell DCA particles are shown in Figure 1a. On the external surface of the particles, it was possible to identify apatite-like structures as result of the coating step during the manufacturing process. TEM section images of the csDCA showed finest details on the complex structure of the particles. Specifically, a shell was distinguishable as a dark ring on the external side of the particles, which
corresponded to the apatite-like coated layer. The external shell was surrounding core-shell nanoparticles (i.e. SPIO/gold), which appeared homogeneously distributed in a bright phase (i.e. silica matrix) (Figure 1b). Visual inspection of the CPC/csDCA composition, after mixing with the setting solution, showed a uniform brown colored paste, indicating a homogeneous distribution of particles in the cement phase (Figure 2f). SEM micrographs and EDS analysis based on silicium detection confirmed that csDCA particles were evenly distributed over the CPC matrix (Figure 1c). Finally, connective apatite-like junctions between particles themselves and between particles and CPC phase were also identified (Figure 3d).

![Figure 1. Morphological characterization. In (a) Scanning electron micrograph of csDCA particles at X10000 magnification; in (b) Transmission electron micrograph of the cross-section of a csDCA particle. It is possible to identify the apatite-like layer (i.e. dark ring), the core-shell structures (i.e. dark spots) and the silica matrix (i.e. bright phase). Magnification X4000. In (c) overlapping between SEM image and EDS spatial resolution map based on Silicium (magnification X1000). In (d) SEM image of csDCA particles entrapped in the CPC phase (magnification X1000). Yellow arrows indicate junctions between particle/particle and particle/cement.]

3.2. Mechanical and handling properties

Assessment of the primary outcomes, related to the handling and sealing features of the cement pastes during the surgical procedure, showed no differences between CPC, CPC/csDCA, and CPC/csDCA/BMP-2 groups. All the pastes showed good consistency and
could be inserted in the defect in a relatively easy manner. The pulp bleeding could be stopped after pastes insertion suggesting good sealing properties for all the prepared CPC compositions.

Evaluation of the setting times showed a small (< 3 min) but statistically significant increase in the initial and final setting for the CPC/csDCA composite and for the theranostic CPC/csDCA/BMP-2 agent when compared to the CPC control (i.e. without csDCA and BMP-2; Figure 2a). Measurement from the compression tests reported no statistical differences in compressive strength and in E-modulus between the CPC with or without csDCA (Figure 2c and 2d). Extrusion curves from the injectability tests (Figure 2b) showed a similar behavior between CPC and CPC/csDCA, while qualitative evaluation of the cohesion reported a highest score for the CPC control when compared to the CPC/csDCA (Figure 2e, 2f, and S3). Finally, during the material preparation and implantation, no other differences were found in handling the CPC composition with and without csDCA.

3.3. Visual properties of CPC/csDCA implanted ex vivo in human molars and goat incisors

A ZTE sequence was used to image CPC restored human molars (Figure 3a) and goat incisors (Figure 3b) ex vivo. On the ZTE images teeth implanted only with CPC, showed a hyperintensity for this material compared to surrounding dental tissues (Figures 3c, 3d). However, for the teeth filled with CPC/csDCA, a stronger effect (i.e. “blooming effect”) on the MR signal is observed resulting in hypointensity for the CPC regions on the ZTE images (Figure 3e and 3f). Because of the blooming effect, which extends the contrast effect on the MR signal, the identification of the cement was easier for the CPC/csDCA composite when compared to the CPC alone. Glass ionomer filler was identified as a hypointense area (i.e. dark area; Figure 3).

Micro-CT reconstructions of the teeth showed a clear difference in contrast between the specimens treated with CPC/csDCA and the samples treated with only CPC (Figure 4a, 4b, 4d and 4e). Specifically, CPC/csDCA was clearly distinguished from the surrounding dental tissues in both kinds of specimens (i.e. human molar and goat incisors). Differently, the CPC control showed similar contrast to the dentin, hence the CPC was barely recognizable. Quantitative assessment of the mean level of the gray values distribution showed an increased frequency for CPC compositions with csDCA when compared to CPC alone (for both human and goat specimens). Moreover, the CPC/csDCA formulations showed a leftward shift of gray values curve. Beside such shift, no overlapping between gray values curve of the CPC/csDCA
and of the natural dentin occurred (Figure 4c and 4f). In all specimens, glass ionomer filler showed the highest CT attenuation features resulting in a completely dark area (Figure 4).

**Figure 2.** Mechano-physical properties. In (a) initial and final setting times from Gillmore test (n=3) for the CPC (in red), the CPC/csDCA (in blue) and the CPC/csDCA/BMP-2 (in green) composite. In (b) extrusion curves from injectability test (n=3), in (c) E-Modulus and in (d) compressive strength (n=8) for the CPC (in red) and the CPC/csDCA (in blue) are shown. In (e) and (f) representative pictures from CPC and CPC/csDCA from the cohesion tests (n=3) are reported.
3.4. Visual properties of CPC/csDCA implanted in vivo in goat incisors

After surgery, all animals appeared to be in good health condition throughout the entire experimental period. No visual damage or trauma was found in the treated incisors. Using ZTE MRI to acquire these goat incisors (Figure 5), differences were found between different groups. While a dark region was found on the images for the incisors with no filling (Figure 5a), teeth filled with CPC control (Figure 5b) showed a brighter signal when compared to the surrounding dentin tissue. A blooming artifact was found on the ZTE images for the teeth filled with either CPC/csDCA and CPC/csDCA/BMP-2 (Figures 5c and 5d). Interestingly, a visually stronger blooming effect was observed on the CPC/csDCA samples when compared to the ones treated with CPC/csDCA/BMP-2. ZTE MR images were also used to identify newly formed dentin (Figure S4).

Micro-CT 2D reconstructions showed that differences in contrast between samples with and without contrast agent vanished at 7 weeks post-surgery (Figure 5). These outcomes were confirmed by gray value quantification, which reported a reduced shift of the gray value curve for teeth treated with CPC/csDCA when compared to teeth treated with only CPC (Figure 6). Interestingly, samples treated with BMP-2 showed higher frequency intensity when compared to the other compositions, suggesting a higher radiodensity. Finally, the comparison between the CPCs implanted ex vivo (i.e. fresh paste) and in vivo (i.e. after 7 weeks degradation) was also performed (Figure S5). Our outcomes reported a higher CT contrast enhancement for the cements implanted ex vivo when compared to the same composition implanted in vivo resulting in a weaker material signal enhancement. These findings are in line with the obtained MRI data.

Similarly to the in vitro outcomes, glass ionomer filler was always identified as a hypointense area in the MR images and as a dark area in the µCT reconstructions respectively (Figure 5).
Figure 3. ZTE MR images of human molars (coronal view) and goat incisors (sagittal view) after \textit{ex vivo} pulp capping. In (a) and (b) an overview of a human molar and a goat incisor are shown respectively. In figures (c) and (d) teeth treated with CPC, while in (e) and (f) teeth treated with CPC/csDCA are shown respectively. Blue and yellow arrows indicate the applied ceramic material and the glass ionomer respectively. A strong blooming effect is noticed on the images only when csDCA was used.
Figure 4. µCT characterization of human molar (coronal view) and goat incisor (along with the sagittal view) after ex vivo pulp capping. Respectively in (a) human molars treated with CPC, in (b) human molar treated with CPC/csDCA, in (d) goat incisor treated with CPC and in (e) goat incisor treated with CPC/csDCA. Blue arrows indicate the installed ceramic material. Yellow arrows indicate the glass ionomer. In (c) and (f) are reported the graphs from gray value distribution assessment for human and goat teeth respectively (n=3). The curves of the gray values based on a defined VOI are shown respectively in red for CPC, in blue for CPC/csDCA and in green for dentin.

Figure 5. Goat incisors after the in vivo direct pulp capping. On the top MRI acquisitions, while on the bottom µCT reconstructions are reported respectively (sagittal view). Respectively, from the right to the left the same incisor from empty defect, CPC, CPC/csDCA and CPC/csDCA/BMP-2 group is reported. In the empty defect specimen the white region indicates the empty space. Blue arrows point out the cylindrical defect with or without implanted material, while yellow arrows indicate a thin layer corresponding to the glass ionomer filler.
3.5. Dentin and cement quantification

New dentin formation and cement degradation were quantified by µCT. Quantification of relative new dentin formation reported a statistically significant increase of the dentin deposition for the incisors treated with the theranostic CPC/csDCA/BMP-2 when compared to the empty defect group (Figure 7a). When looking at the same data clustered for each animal, the incisors treated with CPC/csDCA/BMP-2 were showing more dentin deposition when compared to the other experimental groups over all the studied population (Figure S6a). Quantification of the relative volume of the cement left in the defect after 7 weeks from the \textit{in vivo} installation reported statistically higher values for the groups treated with CPC and CPC/csDCA when compared to the theranostic CPC/csDCA/BMP-2 agent (Figure 7b). After plotting the same data clustered as function of the single animals all the incisors receiving CPC/csDCA/BMP-2 showed less cement volume percentage when compared to the other experimental groups over all the studied animals (Figure S6b).

\textbf{Figure 6.} Gray value distribution of cement compositions after installation \textit{in vivo} in goat incisors (n=6). Gray value curves from CPC composite doped with csDCA (i.e. blue curve) and csDCA/BMP-2 (i.e. green curve) showed a leftward shift compared with the CPC control (i.e. red curve) due to the presence of the contrast agent. CPC/csDCA/BMP-2 is also showing and increased radio-density. No overlapping with the gray value curve from the dentin phase (i.e. dark gray curve) was observed.

\textbf{Figure 7.} Quantitative evaluation of (a) the tertiary dentin formation and of (b) the cement degradation calculated based on µCT acquisitions (n=6). # means p<0.05.
3.6. Histology

Histological qualitative inspection showed that the defect was drilled through the full enamel, dentin and root canal until the dentin on the lingual side of the incisor was touched (Figure 8). Defects were homogeneous in size and shape and only sporadically were slightly downwards inclined (Figure 8a). All defects appeared still filled with the applied glass ionomer filler and with the cement (i.e. except for the empty defect group). All cement types showed a homogeneously distributed porosity and occasionally some micro cracks along the cement phase. These cracks were mainly located in the cement part, which was close to the root canal, while the cement far from the canal appeared to be always intact (Figure 8b, 8c, 8d). In all specimens, signs of inflammatory response were identified in the pulp canal in the proximity of the applied cement resulting in a violet stained area. Such area resulted visually wider in five out of six samples treated with BMP-2. In some cases, cement was found also in the pulp cavity in the proximity of the defect. In all samples, intact pulp tissue was found only in the lower part of the tooth (i.e. below the defect), while the upper part of the pulp cavity appeared completely or partially empty (i.e. partial pulpotomy). Dentin debris was always identified in the pulp cavity, right above and beneath the defect. Noticeably, all the samples were showing tertiary dentin formation. Newly formed dentin was clearly demarcated from the adjacent secondary dentin by a difference in color in the histological section from a dark to a brighter pink tint. The new dentin located into the lower part of the canal was forming a sealing cap between the defect and the underneath pulp. Such dentin showed an amorphous morphology as well as a lack of tubular structures, hence classified as reparative-like dentin. Reparative-like dentin was also visually identified around pre-existing debris of secondary dentin originated during the drilling. In such circumstances, new dentin was holding together all pre-existing pieces of dentin and forming a sealing layer that appeared as a dark circular region (i.e. corresponding to the debris) surrounded by bright pink tissue (Figure 8b, 8d). New dentin was observed also on the surface of the pulp canal, in the proximity of the sealing cap, where it was giving rise to a narrowing of the canal itself. This new type of dentin formed on the surface of the pulp canal walls showed irregular tubular structures, homogeneously distributed and in continuity with pre-existing tubules in the secondary dentin, hence classified as reactionary-like dentin (Figure 8 and S7).
Figure 8. Histological overview of different experimental groups after direct pulp capping performed in vivo in goat incisors. Respectively in (a) an example of empty defect incisor, in (b) a defect filled with CPC, in (c) a defect filled with CPC/csDCA and in (d) a defect filled with CPC/csDCA/BMP-2. In each panel, on the left an overview of the incisor (X1 magnification), on the top-right the cylindrical defect (X10 magnification), on the bottom-right the pulp canal with the newly formed dentin (X10 magnification). All sections were made along the sagittal direction and perpendicularly to the created incisor defect. In all specimens tertiary dentin formed a dentin bridge consisting of a combination of reactionary dentin and reparative dentin. Reparative dentin was identified in the central part of the pulp canal (yellow arrows). Reactionary dentin was observed on the surface of the pulp canal (green arrows). In all specimens the applied glass ionomer was still clearly visible (blue arrows). All cement composites showed a diffuse porosity, while the samples treated with BMP-2 showed more cement degradation.
4. Discussion

A variety of biomaterials, e.g. calcium hydroxide, mineral trioxide aggregate, calcium cement phosphate, have been used for pulp treatment [1-10]. Since calcium hydroxide, the “gold standard” material for pulp capping applications, has several shortcomings, this study considered CPC [11-13]. Besides the optimal biological and handling properties, calcium phosphate-based composites can induce dentin remineralization and inhibit caries [14,15]. Within this study, an alternative theranostic agent for pulp treatment was presented. The theranostic pulp capping agent combined a calcium phosphate-based composite with csDCA for the enhancement of the MRI and CT imaging properties. In addition, in a part of the samples, BMP-2 was incorporated to improve the biological properties. In general, it was found that contrast agents could be combined with the CPC without changing the final handling and mechanical properties. Enhancement of the MRI contrast allowed in vivo detection of the CPC up to 7 weeks after implantation, while BMP-2 promoted increased dentin deposition and faster cement degradation.

Several remarks can be made regarding the study set-up. First, pilot studies were performed to define the right DCA type to be added to the CPC with respect to a dental application. A previous study [27] had already confirmed that the combination of DCA particles with CPC at a final concentration of 5% wt/wt did not affect the handling and mechanical properties of the final composite. Therefore, in the current study, we decided to use the same ratio DCA to CPC. After testing different kind of DCA particles (Supplementary information, Table S1), csDCA was selected because of the best performance in terms of enhancement of the MRI and CT contrast (Figure S8 and S9).

A second technical remark should be made regarding the coating procedure. The morphological characterization of the csDCA revealed the external surface structure of the particles as well as their inner part. Although we did not specifically investigate, the external apatite-like coating layer appeared to be advantageous for the affinity of the csDCA to the CPC phase, due to the formation of connective apatite-based junctions between particles and CPC matrix.

Handling and mechanical properties of the hereby presented CPC/csDCA composite were investigated and compared to the CPC without csDCA. A slight increase in setting times was found, probably due to the physical incorporation of the csDCA into the CPC phase. Nevertheless, settings were occurring within the ranges that are acceptable for clinical use; i.e. initial time <10 min and final time <25 min [38]. Furthermore, the mechanical properties of
our cements were similar and in line with most of the ceramic compositions reported in the literature [11].

Incorporation of csDCA into the CPC, showed ex vivo a significant enhancement in MRI and μCT contrast. Consistently with previous studies, MRI acquisitions of the csDCA-doped CPC composite showed an overestimation of the shape and size of the CPC composite as a consequence of the strong magnetic susceptibility gradients caused by the iron oxide-based particles, which resulted in imaging artifacts (i.e. blooming effect) [26,27]. Such artifacts allow for an easy localization despite the relatively small size of the applied material (i.e. < 1 mm$^3$). Unequivocally, size overestimation results in a lack of anatomical information. New developed MR imaging techniques, such as slice encoding for metal artifact correction (SEMAC) and multi-acquisition variable-resonance imaging combination (MAVRIC) could be used to correct metal artifacts and improve image quality [39,40]. Furthermore, heteronuclear-based contrast agents, such as $^{19}$F-based compounds, could be used as a valid alternative to SPIO for the labeling of CPC composites [41].

After ex vivo characterization of the imaging and mechanical properties, an in vivo study was imminent to investigate the imaging properties and biological response to the theranostic agent. Our in vivo study was based on a direct pulp capping procedure, which is a relatively simple model, which is well established and commonly performed in clinics (Figure S1). The goat was used as animal model due to its big body size, which allows to perform multiple implants in the same entity and reduce the total number of animals needed. Furthermore, the number of experiments performed on goats, and sheep, is constantly growing as these animals are considered food producing animal, therefore more ethically accepted by either the scientific and the not scientific community when compared to other models (i.e. dogs) [37].

It is known that the natural condition of the exposed pulp influences the success rate of a pulp capping treatment. In the case of teeth with mechanically exposed pulp or with the pulp exposed after trauma, dentin has more chance to recover when compared to teeth with the pulp exposed as consequence of caries [5,6]. Moreover, partial pulpotomy (i.e. removal of a small portion of pulp tissue) can also increase the healing process [4]. For this reason, in the present study, during the drilling of the cylindrical defect, a small portion of pulp was mechanically removed. Afterward, by MRI, it was possible to assess the degree of pulpotomy achieved, i.e. how much tissue was removed, and correlate the pulp condition to the new dentin formation (Figure S4).

Within seven weeks from the implantation in vivo, a certain ratio of material degradation was expected [33]. Nevertheless, the CPC/csDCA/BMP-2 composite was still clearly
identifiable by MRI. Evidently, the blooming signals from cements implanted in vivo were visually reduced when compared to the ex vivo experiments. Such a decrease can be explained by the physical release of the entrapped csDCA particles from the CPC matrix [27]. Furthermore, the weaker signal from samples treated with CPC/csDCA/BMP-2 confirmed a faster occurring cement degradation. In other words, the different amount of cement present in the defect could be identified by different degree of blooming effect intensity. Still, differences in CT attenuation between CPC composites with and without csDCA almost vanished after 7 weeks of implantation in vivo. All these results supported the hypothesis that by using CPC/csDCA/BMP-2 as pulp capping agent, MRI evaluation of the signal can be used as non-invasive in vivo clinical screening tool for the monitoring of the material degradation.

Surely, radiation-free MRI has many potential advantages when compared to the conventional X-ray techniques [42]. Bracher et al. [23] showed that the performance of 3D MRI to identify caries lesions is similar to conventional X-ray, and even superior, in cases where X-ray is limited by a structural superimposition. MRI can be used for early detection of caries by assessing the increase in $T_2$ and $T_2^*$ values which occur as consequence of the local acidic environment due to the bacterial infection [23]. Furthermore, magnetic resonance spectroscopy (MRS) can be used in combination with MRI to quantitatively characterize dental structures and obtain more precise information about the microscopic mineral structures [43,44]. However, high costs and long acquisition times are limiting the use of MRI as screening tool in dental daily practice. To date, dental MRI is restricted to ex vivo and in vivo studies and few clinical validations. [21,23-24, 42-44] Therefore, before a real translation of MRI into dental clinics, dedicated low-cost systems, dental coils, short acquisition sequences and MRI compatible materials are strongly demanded. As the cost to purchase an MRI is estimated to be around 1 million $ per Tesla, a cost-effective solution could be the use very low magnetic fields (i.e. 20 – 50 mT), which can show high imaging performances relying on the pre-polarized MRI concept, as well as the use of short-bore systems [45]. Few dental-specific coils have been already developed, either cable-bound or wireless, and one is already commercially available (4-channel dental-array, Noras MRI Products GmbH, Höchberg, Germany) [46]. The hereby described MRI-dedicated material is pioneering in this context, despite the fact that its clinical applicability is not proven yet.

When considering the histology in none of the samples signs of unwanted dentin resorption were found. In our experimental model a severe damage was applied by drilling and partial pulpotomy, and therefore the formation of reparative dentin was expected. However, our
samples showed deposition of tertiary dentin consisting of a combination of reactionary and reparative dentin. New dentin deposition occurring on the surface of the pulp canal showed the typical structures of a reactionary dentin (i.e. tubular structures in continuity with the pre-existing secondary dentin). Differently, new dentin forming the sealing cap in the central part of the pulp canal was mainly atubular and amorphous. Evidently, the deposition of tertiary dentin represented a reparative process, most likely mediated by fibroblasts, which were responsible of the deposition of a permanent calcified scar tissue [6]. Since all samples showed deposition for similar tertiary dentin in the pulp canal, such biological response was interpreted as a normal dental reaction to the harmful stimulus. These outcomes are in line with results obtained in similar studies where calcium hydroxide was used instead of CPC [4,6,47].

Consistently to previous studies an early inflammatory response was expected in all groups as a resolutive mechanism in the dentin healing process [48]. Furthermore, it is known that BMP-2 can induce in vivo the activation of an inflammatory response as well as angiogenesis and increase of blood supply [47,49]. An inflammatory environment is characterized by a drop in local pH, which is considered one of the main causes of the faster cement erosion. Low levels of inflammation have been shown to be beneficial in stimulating bone metabolic activity and regeneration [48]. Therefore we speculated that the hereby developed theranostic CPC/csDCA/BMP-2 composite facilitated higher dentin deposition and cement degradation as a consequence of all the biological mechanisms triggered by the applied BMP-2.

There is an increasing concern about the validity of animal studies, which are supposed to be the last step before a human clinical trial. However, especially in dentin and pulp regeneration field, there is a lack of quantitative assessments of the new dentin deposition, as most of the studies are based on qualitative histological analysis [50]. Therefore, the translation of outcomes achieved in preclinical studies into clinical trials has been always difficult. In the current study qualitative assessment of dentin deposition performed via histology was supported by µCT quantification. However, neuronal and pulp regeneration were not investigated as well as the vitality of the teeth after the surgical procedure. Another limitation of this study is also related to the used small sample size, which could be used only for the detection of large size effect, possibly associated with an increasing risk of bias. Indeed, while a statistical difference between the group receiving CPC/csDCA/BMP-2 and the negative control (i.e. empty defect) was detectable, no differences could be found when the treated group was compared to the positive control (i.e. CPC group). Still, the promising results of this study could be useful to foster further studies with large sample size. For this
reason the report of our methodology was performed following as much as possible the ARRIVE guidelines for reporting \textit{in vivo} experiments [51].

5. Conclusion

By engineering biomaterials, it is possible to improve their specific properties according to the specific need and application. In this study, the visibility and biological features of a calcium phosphate based composite were enhanced by using csDCA and BMP-2, respectively. The resulted “theranostic” pulp capping agent presented herein was found to be suitable for dental applications bringing several advantages. Firstly, the contrast of the CPC composition is enhanced by allowing for a better visualization, identification and follow-up after implantation in an \textit{in vivo} clinical situation. Second, dentinogenic properties of the CPC were improved by using BMP-2, which resulted in an improved dentin deposition and a faster material degradation. In view of an upcoming wider use of dental MRI in the clinic, the presented theranostic pulp capping agent may help to improve MRI visibility of dental materials.

6. References


7. Supplementary information

**Figure S1.** Schematic representation of pulp capping procedure performed *in vivo* in goat incisors. The vestibular surface of goat incisors was drilled by means of a drill equipped with a dental bur (1 mm in diameter). A cylindrical defect was performed into the crown and into the underlying dentin until the pulp was reached (i.e. pulp punctuation). After partial pulpotomy, the pulp was covered with one of the experimental CPC composites by using an adequate dental tool. After pulp capping the defect was filled with standard glass ionomer and the external surface was shaped according to the anatomical morphology.
**Figure S2.** SEM pictures of α-TCP (a), CMC (b), PLGA (c) particles. Respectively, magnifications are X1000, X250 and X250.

**Figure S3.** Pictures from cohesion tests (n=3). Respectively are shown the images of the CPC and the CPC/csDCA pastes after 24h incubation in PBS at 37°C. A score based on the number of broken pieces obtained after extrusion from a syringe orifice (1.7 mm in diameter) was attributed to each sample. Specifically the following score were given: +++ = 1 single piece, ++ = > 2 pieces and <3 pieces, + = > 3 pieces. The table on the right summarizes the score for each group.
Figure S4. Representation of the same tooth acquired respectively by CT (a), MRI (b) and histology (c). In all figures, yellow arrows indicate the tertiary dentin. In figure (b) green arrow indicates empty pulp canal after the performed pulpotomy, while blue arrow indicates intact pulp tissue.

Figure S5. Comparison between gray values distribution of cement compositions implanted ex vivo (full line) and in vivo (dashed line). Respectively, is reported CPC in red and CPC/csDCA in blue.

Figure S6. Dentin volume (a) and cement volume (b) percentage, from each different experimental group, plotted as function of the animal. Note in (a) that the incisors receiving CPC/csDCA/BMP-2 (purple rectangles) showed more dentin formation when compared to the other incisors in each animal. In (b) the incisors receiving CPC/csDCA/BMP-2 showed less cement when compared to the other incisors in each animal.
**Figure S7.** Histology picture of tertiary dentin formed in the pulp canal (i.e. sagittal direction of one representative goat incisor). Tertiary dentin was forming a sealing *cap* in the pulp canal between the pulp and the applied defect. Tertiary dentin consisted of a combination of reactionary and reparative dentin. New dentin formed on the wall of the pulp canal was in continuity with the pre-existing secondary dentin and classified as reactionary dentin. Dentin formed in the deepest part of the pulp canal was classified as reparative dentin. Yellow arrows are pointing out newly formed tubular structures that are in continuity with the original dental tubules. In few cases tubular structures were found also in the dentin *cap* close to the healthy pulp tissue.

**Magnification X20.**

**Figure S8.** Gray value distribution of the CPC composites combined with different DCA (according to Table 1 from supplementary information). A cylindrical defect (2 x 3 mm) was performed on the buccal surface of human molars and filled with CPC combined to different DCA. csDCA means core-shell structured DCA. Mixed DCA means SPIO and gold independently included in the DCA particle. By brackets is reported the SPIO/gold ratio in volume/volume. All particles were combined with the CPC at a final concentration of 5% wt/wt. Notice that the cdDCA (3:1) gave the best CT enhancement compared to the CPC control (i.e. without contrast agents).
Figure S9. MRI acquisitions of human teeth along the sagittal direction. A cylindrical defect (2 x 3 mm) was performed on the buccal surface and filled with CPC combined to different DCA according to Table 1 from supplementary information. All particles were combined with the CPC at a final concentration of 5% wt/wt. Respectively, in (a) CPC control without DCA, in (b) CPC plus csDCA (SPIO/gold = 6/1), in (c) CPC plus csDCA (SPIO/gold = 3/1), in (d) CPC plus mixed_DCA (SPIO/Gold = 1/1), in (e) CPC plus mixed_DCA (SPIO/Gold = 2/1). SPIO/gold ratios are meant volume/volume. Notice that the CPC plus csDCA (SPIO/gold = 3/1) combination gave the strongest blooming artifact.

Table S1. List of the different DCA particles tested. In each column is reported the structure of the particles and the SPIO/Gold ratio, respectively. Core-shell_DCA indicates particles with a core of SPIO and a shell of gold. Mixed_DCA indicate that SPIO and gold are separate nanoparticles. All particles are embedded in a silica matrix and coated with an apatite-like layer. All particles were combined with the CPC at a final concentration of 5% wt/wt.

<table>
<thead>
<tr>
<th>Type of particle</th>
<th>SPIO/Gold ratio (v/v %)</th>
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<tbody>
<tr>
<td>Core-Shell_DCA</td>
<td>6:1</td>
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<td>3:1</td>
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<tr>
<td>Mixed_DCA</td>
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Chapter 5

Perfluorocarbon/Gold-Loading for Non-Invasive in Vivo Assessment of Bone Fillers Using $^{19}$F Magnetic Resonance Imaging and Computed Tomography

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†, ‡ equally contributed
1. Introduction

Bone grafting is the most common transplantation procedure, after blood, with more than two million patients worldwide receiving a bone transplant every year [1,2]. To date, autografting of autologous bone is still the gold standard procedure. However, drawbacks such as the need for a second surgery, high donor-site morbidity, and shortage of donor bone, are increasing the demand for artificial bone substitutes. Since 1920, calcium phosphate-based cement (CPC) has been extensively studied and used for orthopedic and dental applications, due to its high biocompatibility, biodegradability and osteoconductivity [3-5]. Moreover, the ability to be injected and to set at body temperature in vivo, makes CPC suitable for minimally invasive surgeries [6]. Two criteria are important in the application of CPC in bone repair: mechanical properties that are tailored to the specific application and an adequate imaging contrast that allows for monitoring cement after injection, particularly to monitor its degradation [6,7]. However, only a few strategies have been successful in the synthesis of CPC that meet both criteria, as labeling the cement typically affects its mechanical properties. X-ray and CT are the most common methods to monitor bone defects and the formation of new bone [8,9]. Non-labeled CPCs have a slightly higher radio-density than the natural bone, resulting in a brighter signal on conventional X-ray radiographs. However, the geometrical conformation of the cement is barely recognizable after surgery. Longitudinal monitoring becomes even more challenging when degradation of the material and the in-growth of the new bone begins [10-12]. Addition of radiopaque salts, such as barium sulfate or tantalum oxide, can enhance the radio-contrast of CPC without affecting its biological behavior [13-16]. However, the incorporation of such salts affects the setting and mechanical properties of the cement, making it less suitable for clinical application. To avoid changes in cement properties after labeling, different strategies, such as the encapsulation of probes into a silica carrier, have been described [17-18]. Also gold nanoparticles (AuNPs) are promising for CT because gold has a high atomic number (Z = 79) and high X-ray attenuation per mass (5.16 cm$^2$ g$^{-1}$ at 100 keV). Moreover, biocompatibility, low toxicity, and high affinity of gold nanoparticles to different functional groups, for example thiol or phosphine, make AuNPs suitable for labeling biomaterials [19-21]. However, no successful strategy for long term labeling of CPC with gold has been described thus far.

MR imaging of hard tissues (i.e. bone and teeth) requires acquisition sequences sensitive to their very short transversal relaxation such as ultra short echo time (UTE) and zero echo time (ZTE) sequences [22, 23]. However, in MR images with these sequences, bone transplants do
not provide enough contrast, making labeling essential [24]. Multimodal imaging with MRI and CT is advantageous, as it allows the acquisition of independent information using two different imaging techniques. The use of two independent labels with different physicochemical properties and different affinity to CPC, could reduce problems such as a decrease or complete loss of the signal due to, for example, diffusion of non-covalently attached label from the CPC matrix. The first CPC mixture that was labeled with multiple imaging agents contained superparamagnetic iron oxide particles (SPIO, 200 nm in diameter) and colloidal gold (4 nm in diameter), respectively as MRI and CT contrast agents [17, 18]. However, the strong susceptibility artifacts caused by SPIO particles resulted in a gross overestimation of the implant shape [25, 26]. Together with the fast decrease of the CT contrast over time, the use of this strategy became very limited. The labeling of CPC with heteronuclei, such as $^{19}$F, $^{31}$P or $^{23}$Na, natural isotopes with spin of \( \frac{1}{2} \) that can be directly detected with MRI, could be an excellent alternative to SPIO. Fluorine is especially attractive as it is almost completely absent from biological tissues, which results in a high MR contrast-to-noise ratio and specificity, and it has a similar intrinsic high sensitivity as that of protons [27-32]. Typical $^{19}$F MRI labels contain organofluorine compounds, in particular liquid perfluorocarbons (PFCs). Perfluorocarbons are biologically inert and exhibit low toxicity. Therefore, they have been used in the clinic as blood substitutes, and more recently as MR imaging agents [33-36].

In this study, we combined $^{19}$F MRI and CT for imaging the CPC in bone for the first time, using perfluoro-15-crown-5-ether (PFCE)-loaded poly(d,l-lactide-co-glycolide) (PLGA) nanoparticles and commercially-available gold nanoparticles (AuNPs). PFCE nanoparticles have previously only been applied to image labeled cells, i.e. soft tissues [35]. Here we use these nanoparticles for imaging of bone substitutes. We decided to use AuNPs because they are also effective for both promoting osteoblast differentiation and bone formation [37, 38]. This newly developed CPC/PFCE/gold composite was first characterized in vitro and then implanted in vivo in a rat femoral condyle model. We followed material degradation by in vivo $^1$H/$^{19}$F MRI and CT up to 8 weeks post-surgery, and correlated the images to the biological tissue response.

2. Materials and methods

2.1. CPC composite

Calcium phosphate cement (CPC) was prepared as a w/w mixture of 78.5% alpha-tricalcium phosphate ($\alpha$-TCP; CAM Bioceramics BV, Leiden, The Netherlands), 1.5%
carboxymethylcellulose (CMC; CAM Bioceramics) and 20% of cryo-grinded poly(d,l-lactide-
co-glycolide) microparticles (<200 µm) with a 50:50 ratio of lactic to glycolic acid
(PURASOB 5002A, Purac, Gorinchem, the Netherlands).

2.2. Contrast agents

PFCE-loaded PLGA nanoparticles were prepared as described elsewhere [35]. Briefly, PLGA
(100 mg) was dissolved in dichloromethane (3 mL) and mixed with PFCE (0.9 mL) and
Prohance® (1.78 mL). The resulting emulsion was added quickly under sonication to 25 mL
of 1.96% (w/w) solution of poly(vinyl alcohol) (9-10 kDa, 80% hydrolyzed) and sonicated for
3 min at 40% amplitude (Branson Digital Sonifier 250; Branson Sonic power, Danbury, CT).
After evaporation of the solvent over night the particles were washed four times with water at
16000 g, resuspended in water and freeze-dried, yielding of approximately 100-150 mg of
particles as white powder. PFCE was purchased from Exfluor, Rond Rock, TX; PLGA
Resomer RG 502H, lactide:glycolide molar ratio 48:52-52:48, from Evonic, Germany;
dichloromethane from Merck, Darmstadt, Germany; and PVA from Sigma-Aldrich, St Loius,
MO.

Gold nanoparticles (diameter 40 nm) stabilized by citrate in 0.1 mM phosphate buffered
saline solution (PBS) were purchased from Sigma and used without any additional
modification.

2.3. Material preparation

The CPC control (i.e. without contrast agents) was prepared by adding the mixed powders
(i.e. α-TCP, CMC and PLGA) into an exit-closed 2 mL syringe (Terumo Europe N.V.,
Leuven, Belgium). Afterwards 50 µL of sterile-filtered (0.2 µm filter) sodium dihydrogen
phosphate solution (4% NaH₂PO₄•2H₂O) were added into the syringe and shaken for 30 s by
means of a dental shaker machine (Silamat® Mixing apparatus, Vivadent, Schaan,
Liechtenstein).

In order to add the contrast agents to the starting CPC composition additional steps were
requested. Briefly, 100 mg initial powders of CPC were first mixed with 1 mL AuNPs
solution (corresponding to ~7.2 x 10⁹ particles), homogenized by vortex and freeze-dried over
night. Thereafter, 20 mg PLGA/PFCE nanoparticles were first resuspended in 1 mL of
MilliQ® water and then added to the CPC/gold powder. The solution was then vortexed and
freeze-dried again providing the final composition, which is indicated in this study as
CPC/PFCE/gold.
An additional CPC composite was prepared by performing the same freeze-dry steps as described before, but using MilliQ® water alone, without any contrast agents. This CPC/freeze-dry composite was used as an internal control for the characterization of the handling and mechanical properties. Samples were sterilized by using 25 kGy of gamma radiation (Synergy Health Ede B.V., Ede, the Netherlands).

2.4. Material characterization

Morphological assessment of the PFCE-loaded PLGA nanoparticles and of pre-set cylinders of CPC/PFCE/gold was performed by electron scanning microscopy (SEM, JEOL 6310, Jeol corporation, Tokyo, Japan). Images were acquired at 5 kV with X5000 magnification.

The size and the zeta potential of the PLGA/PFCE nanoparticles was measured by dynamic light scattering (DLS) using a Malvern Zetaziser Nano ZS (Malvern Instrument, Worcestershire, UK) at sample concentration of 0.1 mg/mL, using ultrapure water as a solvent for DLS and 5 mM sodium chloride solution for zeta potential measurements. The content of PFCE was determined by $^{19}$F NMR (Bruker Avance 400) using trifluoroacetic acid as an internal reference and deuterium oxide as solvent (both from Sigma-Aldrich).

2.5. Cement handling and mechanical properties

Setting times, elasticity, compression strength, injectability and cohesion properties of the CPC with and without contrast agents were investigated. As internal control the CPC/freeze-dried composite was also used.

For the setting times, a Gillmore apparatus according to ASTM C266 was used. The cement pastes were injected in a cylindrical bronze mould of 6 mm in diameter and 12 mm in height, and immersed in a water bath at 37 °C after which initial and final setting times were recorded. The elasticity and the compressive strength were calculated by using a testing bench machine (858 MiniBionixII, MTS, Eden Prairie, MN). Cylinders of 4.5 mm in diameter and 9 mm in height were prepared from the CPC mixtures, and compressed with a loading force of 2.5 kN at a constant speed of 0.5 mm min$^{-1}$. The injectability was assessed by using the same testing bench machine (858 MiniBionixII) set in a compression mode and adapted with a custom-made fixture metallic cage. A total weight of 500 mg for each cement composition was mixed into an exit-closed 2 mL syringe (Terumo Europe N.V.) with 250 µL of setting solution (4% NaH$_2$PO$_4$•2H$_2$O). After mixing the components for 30 s, the syringe was placed in the metallic cage, the exit was opened, and a compression force of 100 N (i.e. estimated maximum force applicable by human operator) was applied with a constant speed of 20 mm min$^{-1}$ until all the material was extruded from the syringe (33 ± 2 s). From the raw data, the
extrusion curve was obtained as reported in the literature as applied force (N) by the time (s) [39]. Finally all the cements extruded from the syringes were accumulated in 10 mL of PBS at 37°C and the cohesive properties were qualitatively assessed (i.e. by counting the number of fragments).

2.6. *In vitro* assay

A cylindrical hole (3 x 3 mm) was drilled into bone blocks (~1 cm³) obtained from pig cadaver jaw. The hole was filled either with CPC or CPC with contrast agents and cured overnight. All the samples were prepared in triplicate (n=3).

For MRI scan, the samples were first embedded in 5% gelatin type A (Sigma-Aldrich) in order to simulate a water environment. The gelatin was poured into a 15 mL plastic tube and solidified at + 4°C. For the micro-computed tomography (µCT) measurements the samples were wrapped in Parafilm® (SERVA Electrophoresis GmbH, Heidelberg, Germany) to avoid drying artifacts during the scan.

2.7. *In vitro* µCT

The bone blocks were scanned horizontally along the X-ray beam by using a µCT imaging system (Skyscan 1072, Kontich, Belgium). Samples were recorded using X15 Magnification (i.e. pixel resolution = 18.88 µm), X-ray source of 100 kV/ 98 µA, exposure time 3.9 s and 1 mm aluminium filter. The obtained projected files were reconstructed with NRecon software (Skyscan) and analyzed with CtAnalyser software (version 1.10.1.0; Skyscan). The volume of interest (VOI) was defined manually by selecting a total of 135 slices (in height; i.e. 2.55 mm) and a circle of 3 mm (in diameter). Two-dimensional (2D) reconstructions were finally obtained by DataViewer software (Version 1.5.2.4; Skyscan).

2.8. *In vitro* MRI

All the samples embedded in gelatin were scanned on a 11.7 Tesla (T) MRI system (Biospec, Bruker, Germany) equipped with a $^1$H/$^{19}$F volume coil. $^1$H/$^{19}$F MR images of the bone phantom were acquired by a zero echo time (ZTE) sequence with the following parameters: repetition time (TR) = 2ms/4ms, image resolution = 1.56 x 1.56 x 1.56 mm, 1 average/16 averages, flip angle (FA) = 2°/4° and acquisition time (TA) = 27sec/13.54 min. The obtained $^1$H and $^{19}$F MR images were processed with Matlab R2014b (MathWorks Inc, Natick, MA) and overlaid by using MRICro software (Smith Micro software, Aliso Viejo, CA) [40].
2.9. *In vivo* assay

The animal study was performed in agreement with the standards and the protocols of the Radboud University Medical Center, Nijmegen, the Netherlands. All the surgeries were performed after the approval of the Animal Ethics Committee (RU-DEC number 2015-0035) for the care and the use of laboratory animals. Sixteen healthy male Wistar rats (body weight: 250-300 g) were used as experimental animals. A rat femoral condyle defect model was used as described elsewhere [42]. In order to reduce the number of animals for this experiment both posterior legs of the animals were used. First the animal was anesthetized by inhalation of a mix of Isoflurane (Rhodia Organique Fine, Avonmouth, Bristol, UK) and oxygen, and then the legs were shaved and disinfected using povidone-iodine solution. The animal was located in the supine position on a heating mat in order to prevent hypothermia. A longitudinal incision was performed through the skin and the muscle on the medial surface of the knee (Supplementary Information, SI, Figure S1). After the exposition of the medial side of the distal femoral condyle, the patella was laterally dislocated in order to have a clear view of the knee joint. Three different dental burs with increasing diameter up to 2.5 mm were used in order to perform a cylindrical defect (2.5 mm in diameter and 3 mm in depth) along the same direction of the femur. A sterile 0.9% saline solution (Fresenius Kabi B.V., Emmercomposcum, the Netherlands) was used to cool down the dental bur and to clean the drilled cavity. In the meantime, the CPC powders were mixed with the setting solution as described before. The defect was filled with CPC with or without contrast agents, while it was left empty in the case of the positive control group. As negative control group, the leg was kept untreated (Table S1). After cement implantation, the patella was moved back to the original position and the muscle and the skin were closed with absorbable sutures (Vicryl ® 4.0 Ethicon, Somerville, NY). A subcutaneous injection of painkiller (5 mg/mL Rimadyl®, Pfizer animal health, NY) was performed post surgery in order to decrease postoperative discomfort. After the MRI and CT scans the skin was further fastened by using metallic wound-clips (Becton and Dickinson, Franklin Lakes, NJ). *In vivo* MRI and CT were performed right directly post-operative, and at 4 and 8 weeks post-surgery. After 8 weeks the animals were sacrificed by CO2/O2 inhalation and the femora were harvested for histological assessments.

2.10. *In vivo* CT

For the *in vivo* CT a small animal CT scanner was used (Inveon Micro-CT/PET, Siemens Medical Solution, Knoxville, TN). The animals were located in the supine position on a heating mat and always assessed under general anesthesia (Isoflurane/O2). Images were
recorded with an acquisition time of 6 min, spatial resolution of 30 µm, 80 kV tungsten anode source and exposure time of 1000 ms. Inveon Research Workplace (IRW, Siemens) software was used for 3D reconstruction of the projected files, and in order to define the VOI corresponding to the implanted material. As the shape of the implant was heterogeneous from leg to leg, the outline of the implant was carefully drawn. For each VOI the total volume in mm$^3$ and the mean attenuation intensity in Hounsfield units (HU) was calculated. For each implant, the level of attenuation intensity was adjusted by the corresponding volume. Finally the mean value of the signal intensity at each time point was computed. Signal decrease over time was also investigated. Based on a constant VOI (of 15 mm$^3$) the relative signal intensity at each time point was expressed as percentage and calculated as total signal intensity from the implants to the mean of the signal intensity from normal bone (i.e. no defect group) (Figure S2).

2.11. In vivo MRI

Animal MR experiments were performed with the same 11.7 T MRI system and $^1$H/$^{19}$F volume coil as for in vitro experiments. Animals were anesthetized by Isoflurane/O$_2$ and placed in the supine position. One by one each leg was immobilized inside the coil while body temperature and breathing were constantly monitored. For fluorine content quantification a 200 mL Eppendorf tube filled with 20 mg of PLGA/PFCE nanoparticles dispersed in MilliQ® water was used as reference. The Eppendorf tube was placed adjacent to the medial side of the leg. $^1$H/$^{19}$F images for each rat leg were acquired by ZTE sequence with TR=2ms, image resolution = 0.31mm$^3$/1.25mm$^3$, 1 averages/32 averages and FA = 2°/4°. The scan times were less than 7min and 14min for $^1$H and $^{19}$F respectively. The $^{19}$F signals in the rat legs as well as in the control sample were quantified in MRIcro software. Regions of interest (ROIs) were manually outlined based on the detected $^{19}$F signal on $^{19}$F ZTE images. $^{19}$F signal per ROI was calculated by multiplying the mean of the pixel intensity of the ROI by its volume (i.e. area per slice thickness). The total $^{19}$F amount was then summed over slices and expressed in arbitrary units (a.u.) [41]. Based on the quantified $^{19}$F signals of the control sample scanned together with each leg at day 0, 4 and 8 weeks, the normalization procedure for $^{19}$F signals has also been performed within each leg at three time points and between legs.

2.12. MRI, CT and implant volume linear correlation

Correlation analysis was performed by comparing $^{19}$F MRI signal (expressed in a.u.), CT signal (expressed in HU/mm$^3$) and volume of the implant (expressed in mm$^3$) at each time points. The volume of the implant at each time point was calculated by IRW (Siemens) based
on the \textit{in vivo} CT acquisitions. For each time point, two-tailed Pearson correlation coefficient ($\rho$) between MRI signal and volume of the implant, CT signal and volume of the implant and between CT signal and MRI signal, were calculated respectively. Afterwards, Pearson coefficients were converted to Fisher’s coefficient ($z$) and the interval of confidence at 95% was computed by back-converting $z$ to $\rho$. Pearson coefficients were ranked according to the rule of Thumb [43].

2.13. Histology

Samples were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for two weeks. The decalcified bones were then dehydrated in a gradual ethanol series (from 70% to 100%) and embedded in paraffin. Sections of 4 $\mu$m in thickness were cut along the axial direction of the femur by using a microtome (RM2165, Leica Microsystems, Rijswijk, the Netherlands). The sections were stained with Hematoxylin/Eosin (H/E) and with trichrome Elastic van Gieson (EVG). For each specimen, at least two images from three sections (at 100 $\mu$m of interdistance) were analyzed. Images were acquired by using a light microscope (Axio Imager Z1, Carl Zeiss AG Light Microscopy, Göttingen, Germany) equipped with a digital camera (AxioCam MRe5, Carl Zeiss AG Light Microscopy). Histomorphometrical analysis was performed based on the EVG-stained slices by using a computer image analysis technique based on ImageJ software (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). The amount of bone was calculated as area percentage inside a defined ROI (circle of 2.5 mm diameter, Figure S3).

2.14. Statistical Analysis

GraphPad Instat® (GraphPad Software, San Diego, CA) was used for all statistical measurements; data were reported as mean ± standard deviation. For comparison of data one-way analysis of variance (ANOVA) with a Tukey’s post hoc test was used. All differences were considered significant at p-values $<0.05$.

3. Results and Discussion

3.1. Simultaneous labeling of CPC with PFCE-loaded PLGA and gold nanoparticles

To label the cement for $^{19}$F MRI we used PFCE-loaded PLGA nanoparticles. These nanoparticles were developed at the Radboud University Medical Center (Nijmegen, the Netherlands) and are now being prepared for use in clinical trial for labeling dendritic cell-based vaccines (clinicaltrial.gov identifier NCT02574377) [35]. These particles typically have a hydrodynamic radius of 100 nm, as determined by DLS, and zeta-potential values in a range
of -1 mV to -5 mV, due to steric stabilization by poly(vinyl alcohol). Scanning electron microscope (SEM) image of the nanoparticles is shown in Figure 1A. The amount of PFCE in the particles was determined by nuclear magnetic resonance (NMR) using an internal reference and was around 20 wt%. As CT contrast agent, we used commercially available AuNPs that are stabilized with citric acid in PBS.

The largest initial challenge was the incorporation of both nanoparticle labels into the CPC matrix. Earlier combinations of CPCs and several contrast agents, were produced by mixing the dried powders, and therefore had only one easy production step [13-18]. However, our PFCE-loaded PLGA nanoparticles formed clusters in the freeze-dried state, making homogenous incorporation into the CPC difficult. Moreover, AuNPs were provided dispersed in PBS solution. Therefore, we developed an easy two-step method that allowed for the simultaneous incorporation of both nanoparticles into the CPC phase. All components were independently dispersed in water and mixed at high shear stress followed by fast freezing and freeze-drying the mixture to avoid the setting of CPC. In the second step, the obtained dual labeled-CPC powder could be mixed with the setting solution and handled further as the non-labeled CPC. This method was more efficient for incorporation of both labels than adding the imaging agents, mixing them in the setting solution, or mixing the cement directly with freeze-dried powder of nanoparticles. The color of the cement composite changed from white to pink after AuNPs were added (Figure 1C). The uniform distribution of color in CPC indicated a homogeneous distribution of AuNPs. Morphological analysis based on SEM (Figure 1B and Figure S4) proved that PFCE-loaded PLGA nanoparticles were incorporated into the CPC matrix. After incorporation and setting of the CPC, the initial PFCE-loaded PLGA nanoparticles formed clusters of approximately 1-5 µm in diameter. This cluster formation could be due to reduced colloidal stability of nanoparticles in the cement mixture and to the relatively high concentration of nanoparticles at 17% w/w [44]. However, MR imaging of labeled CPC revealed that these agglomerates were homogeneously distributed in the cement matrix (Figure 2C). Thus, the agglomeration did not influence the imaging properties of the final material. Likely, this agglomeration could even be an advantage for our purpose, due to possibly slower degradation of aggregated nanoparticles that allowed the PFCE signal to last longer in the CPC matrix. Unfortunately, the AuNPs were too small to be identified by SEM in the morphologically complex CPC matrix. However, the change of the color and the enhanced CT visibility indicated that the incorporation was successful (Figure 2E, Figure 2F and Figure 2G).
3.2. Imaging of cement injected \textit{in vitro} in pig jaw blocks

To find out the optimal ratio between the CPC powders and the contrast agents, we performed \(\mu\)CT and \(^{19}\)F MR imaging of the final material. Based on these results we determined that a concentration of 20 mg of freeze-dried PFCE-loaded PLGA nanoparticles per 100 mg of CPC powder provided sufficient labeling with PFCE. For CT contrast 1 ml of AuNPs suspension was necessary for significant enhancement (data not shown).

Next, we imaged CPC composites injected in pig bone blocks (Figure 2A) using \(^{1}\)H and \(^{19}\)F MRI. On the \(^{1}\)H MR image (Figure 2B), CPC could not be distinguished from pig bone. However, a strong fluorine signal was detected from the CPC/PFCE/gold composite in the \(^{19}\)F ZTE MR image (Figure 2C). Thus overlaying the \(^{19}\)F on the \(^{1}\)H image allowed identification of the labeled CPC (Figure 2D).

Two-dimensional gray scale images of the CPC injected in pig blocks were obtained by \(\mu\)CT acquisition. As shown in Figure 2, CPC with contrast agents (Figure 2F) appeared visually darker than non-labeled CPC (Figure 2E). A quantitative estimation of the gray value distribution confirmed this observation. CPC/PFCE/gold reported significantly higher pixel frequency compared to the CPC control as well as a rightward shift of the gray values curve (Figure 2G). Furthermore, the narrowing of the peak indicated an even distribution of the particles in the CPC phase.
3.3. Characterization of the handling and mechanical properties

The mechanical and handling properties of the final cement mixture are very important for the application as a bone filler. These mechanical properties should match those of the treated bone. Furthermore, the in situ setting should be fast, but also provide enough time for the surgeon to implant and model the paste. Further, hydraulic features should allow easy injection of the material, enabling minimally invasive operations [4-6]. To investigate whether the incorporation of the two types of nanoparticles affected the material properties of the CPC, we performed handling and mechanical testing. As a control we used CPC that was treated in the usual way (CPC-control), and another CPC that we prepared in the same way as the labeled CPC but without nanoparticles (CPC/Freeze-dried). The last control is important as possible changes in material properties can be caused either by incorporation of nanoparticles or by the extra steps involving resuspension in water and freeze-drying. Setting times of the CPC/PFCE/gold composite that we obtained from the Gillmore tests (Figure 3A and Figure 3B) showed an increase in the initial and final times by $1.7 \pm 0.3$ min and $3.2 \pm 0.3$ min respectively, compared to CPC-control. However, this small increase did not affect injection during surgery. Setting times of both non-labeled CPC controls -the freeze-dried and the non-freeze dried- were similar to each other. This behavior indicates that the incorporation of the particles did cause an increase in setting time. Ideally, the initial setting time should be

Figure 2. In vitro MRI and CT imaging of a bone block obtained from pig jaw. In (A) the morphological representation of the bone block; in (B) and (C) $^1$H and $^{19}$F ZTE MR images of CPC/PFCE/gold composite obtained at 11.7 T. Note that the $^{19}$F signal corresponds to the cylindrical defect in the $^1$H ZTE image as highlighted by the yellow lines. (D) The ZTE MR images of $^{19}$F overlaid on those of $^1$H ZTE. In (E) and (F) the gray scale 2D images from the µCT of the pig block filled with the CPC composite are shown without (E) and with (F) contrast agents. The yellow arrows indicate the implanted material. In (G) the graph from the gray values distribution calculated for a defined volume of interest (n=3). Note that the signal intensity is elevated and much more defined.
between 6-10 minutes, and the final hardening should be reached within 20 minutes [5,6]. Thus, the setting time of our labeled composite is within this range confirming that it can be used as a bone implant.

Comparing the compressive strength and the E-modulus of the CPC/PFCE/gold composite to the non-labeled CPC showed that labeling does not affect the mechanical properties of CPC (Figure 3C and Figure 3D). Particularly, both values are similar compared to the CPC that was only freeze-dried (i.e. CPC/freeze-dried), indicating that the freeze-drying rather than the incorporation of the nanoparticles affected the properties of the final material. Importantly, these differences were not significantly different when compared to standard CPC. Thus, compressive strength and E-modulus are comparable with the values of the most common α-TCP composites reported in the literature that are typically between 1 and 70 MPa and 0.5 and 9 GPa, respectively, depending on the exact composition of the material [5]. In particular, commercially available injectable bone cement, chronOSTM Inject, has a compressive strength of 3.0±0.6 MPa [44]. This value is very close to the results of this study (i.e. CPC/PFCE/gold = 4.6±2.1 MPa) meaning that our composite could be used for similar applications. Surely, CPC/PFCE/gold is indicated for non-load-bearing bone defects, specifically in cancellous bone, which shows similar compressive strength and elasticity (i.e. 4-12 MPa and 0.1-0.5 GPa, respectively) [46].

Finally, CPC/PFCE/gold composite showed excellent hydraulic and cohesive features. All samples could be completely extruded from the syringe through a 1.7 mm orifice, and thereafter set in an aqueous solution (Figure 3E, Figure 3F, Figure 3G and Figure S5). Even if, we used no needles in this study, our results indicate that CPC/PFCE/gold composite is suitable for use with needles up to 15 Gauge. Such needles are used, for example, in several back and face surgeries [6, 39].

3.4. Imaging of cement injected in vivo in rat bone model

Having promising in vitro results on both imaging and materials properties, we then investigated the performance of our labeled composite in vivo for longitudinal monitoring up to 8 weeks from the injection in a rat femoral condyle defect. This defect involves relatively simple surgery and is a well-established non-load bearing model to study bone biomaterials [42]. The time of 8 weeks was chosen, as we expected from our in vitro study (data not shown) and from previous works on CPC degradation in the same model that the CPC would stay in the bone for at least 4 weeks [17, 18].
Figure 3. Mechanical and handling properties of the CPC composite with and without contrast agents: respectively the initial (A) and the final (B) setting time from Gilmore tests (n=3); the E-Modulus (C) and the compressive strength (D) from the compression tests (n=5); the extrusion curves from the injectability tests (E) (n=3); CPC/PFCE/gold composite (F) and CPC (G) representative samples from cohesion tests (n=3). # and * mean p<0.05.

For in vivo testing, we used non freeze-dried CPC as a control as well as another group of animals with an empty defect. None of the animals showed any sign of discomfort, swelling or restriction in movement after surgery. To monitor the cement after injection, we performed MRI and CT image acquisitions immediately after surgery (i.e. day 0), and at 4 and 8 weeks post-surgery. High-resolution anatomical MR images of the rat leg were acquired at day 0, 4 weeks and 8 weeks after injection (Figure S6). As expected from in vitro imaging results, it was not possible to distinguish the non-labeled CPC composite in rat legs from the surrounding bone on 1H ZTE images. In contrast, after injection, the labeled composite could be imaged with both MRI and CT for the whole duration of 8 weeks.

The shape of the labeled composite was clearly recognizable on 19F ZTE MR images, shown in false color, providing precise geometrical information (Figure 4). Moreover, comparing the images at different time-points after injection showed that the 19F signal area was shrinking. Indeed, further quantitative analysis of 19F signal confirmed this observation, and showed a gradual decrease of the 19F-containing region over 8 weeks (Figure 5). Moreover, this reduction in signal area correlates to the decrease in the volume of the implant as a
consequence of cement degradation. This suggests that dissolution of the cement causes clearance of the PFCE-loaded PLGA nanoparticles, resulting in a signal decrease.

**Figure 4.** Representative *in vivo* ZTE MR images of $^{19}$F overlaid on those of $^1$H of a rat leg at day 0, 4 weeks and 8 weeks in axial and coronal directions. The presence of $^{19}$F is shown in false color. Yellow arrows indicate the implanted material, while the bright yellowish spot next to each leg corresponds to the reference (i.e. Eppendorf tube containing PFCE-loaded nanoparticles dispersed in water).

**Figure 5.** $^{19}$F MRI and CT signal quantification of the CPC/PFCE/gold composite at day 0, 4 weeks and 8 weeks. $^{19}$F signal (red) in $^{19}$F ZTE MR images and relative signal (blue) in CT images were quantified and expressed as arbitrary unit and in percentage of relative attenuation intensity, respectively ($n=8$). Lines indicate the trend of the data points.
Because of the high calcium salts content, CPCs generally show higher radiographic attenuation when compared to the natural bone phase [10]. However, there are many available calcium phosphate-based composites, which may consist of different mineral components (i.e. α-TCP, hydroxyapatite) often combined with different additives, polymers or fibers [4-6]. Depending on the specific mixture of components, CPCs may show different X-ray attenuation, with better or worse contrast when compared to the natural bone. Thus, a “natural” contrast between CPC and bone is not always a given, yet is dependent on parameters, such as the CPC composition, the type/density of the bone, and the biological phase of bone remodeling. We aimed to achieve a labeling methodology in which, irrespective of these circumstances, a reliable contrast can always be achieved. Composites with improved contrast are demanded especially when need to be injected in the vertebral spine. In such clinical circumstances, leakage of CPC from the vertebral body can occur leading to several symptomatic complications (i.e. neurological complication, collapse of the adjacent vertebrae) [11,12]. A clear view of the implanted CPC can be also hindered by the presence of extensive soft tissue around the implant that can obscure its precise shape identification [11]. Furthermore, CPC monitoring over time is changeling and less precise. It has been proven that only after disappearance of the 50% of the implanted material and subsequent replacement with new bone, it is possible to recognize a visible change in bone remodeling [10,11]. CPC/PFCE/gold composite showed not only visual enhancement of CT attenuation right after the surgery (i.e. Day 0), but the shape of the implant was clearly visible during the all duration of the experiment (Figure 6). By contrast, the non-labeled CPC used as control, shows similar attenuation as the natural bone phase. Although it is possible to identify the implanted non-labeled CPC after surgery, the border between the bone and the implanted cement was not always recognizable. The identification of the implanted control cement became more challenging at 4 weeks post surgery after the CPC degradation took place (Figure 6). Evaluation of the mean attenuation coefficients expressed in Hounsfield unit (HU) per mm$^3$ confirmed these results. At all the time points, the CPC/PFCE/gold composite showed higher values than both empty defect group and non-labeled CPC composite (Figure 6). However, this difference was statistically relevant only at 4 and 8 weeks. The higher signal intensity from the CPC/PFCE/gold composite allowed for a better definition of implant shape and volume, in comparison to the non-labeled CPC. A narrowing of the shape of the labeled implant was clearly visible for all the experimental times. For this reason, CT acquisitions enabled a more precise quantification of the volume of the labeled implant at each time point. Moreover, by comparing the relative signal intensities over time, it was possible to see a
gradual decrease of the signal intensity, indicating clearance of the label with cement degradation (Figure 5).

Figure 6. Qualitative and quantitative *in vivo* CT results. The CT images of the coronal direction of the rat femoral condyle are shown for defects left empty or/and filled with CPC alone or/and with CPC/PFCE/gold. From the top to the bottom, the pictures of the same specimen are shown directly after the surgery (Day 0) and at 4 and 8 weeks post-surgery. The bar graph presents the signal quantification estimated by IRW, based on a defined region of interest. Values are reported as mean of attenuation coefficients in HU per the total volume in mm$^3$ (n=8).
Only few studies on the long-term imaging of radiopaque bone substitutes are available. However, most of these studies concern in vitro tests or focus on in vivo biological response without imaging its degradation [13-16]. In a previous study, Ventura et al., used smaller AuNPs with 4 nm diameter that were embedded in silica matrix to enhance CT contrast [17]. However, these nanoparticles diffused from the silica particles 4 weeks after the implantation in vivo resulting in loss of CT contrast. In this study, larger nanoparticles with diameter of 40 nm provided long-term enhancement CT contrast, at least up to 8 weeks. We assume the bigger size of AuNPs hindered the diffusion of nanoparticles out of CPC resulting in prolonged enhancement of CT.

Finally, the combination of both imaging modalities enabled to monitor degradation of the cement. To compare quantitative MRI and CT signals, we investigated if there is a correlation between $^{19}$F MRI signal, CT attenuation coefficient and volume of the implant at each time point. The Pearson correlation coefficients and confidence intervals are summarized in Table 1. Pearson coefficients were ranked according to the rule of Thumb [43]. Very strong (i.e. $0.9<r<1$) and strong (i.e. $0.7<r<0.9$) statistically significant linear relationship (i.e. $p<0.05$) between the two variables, were found at day 0 and at 4 weeks respectively, for correlation of MRI signal to implant volume, CT signal to implant volume and MRI signal to CT signal. In contrast, at 8 weeks, a strong linear correlation was found between CT signal and volume of the implant, while the relationships between MRI signal and implant volume or MRI signal and CT signal were low and moderate respectively (i.e. moderate $= 0.5<r<0.7$, low $= 0.3<r<0.5$). This lower correlation of MRI signal could be due to limited MRI resolution for such a small implant volume (i.e. $< 4.41\pm1.96 \text{ mm}^3$), resulting in relatively few voxels over the implant and thus larger error due to the partial volume effect [29]. However, both MRI and CT demonstrated that the degradation of the cement mainly occurred as a gradual process of dissolution that starts from the edges of the implant. Thus, in total, it was possible to follow CPC degradation qualitatively and quantitatively up to 8 weeks post-surgery.

The versatility of a dual-labeling approach allows to produce patient-tuned composites that could include either both or only one of the two contrast agents depending on the specific bone applications. In imaging-guided vertebroplasty a CPC composite with initial enhanced contrast is preferred by the surgeons as it can help for the identification of any cement leakage [11]. By contrast, when the monitoring of the material degradation over time and the subsequent bone regeneration become the crucial aspect, e.g. in orthopedic and maxillofacial applications, a non-invasive imaging modality (i.e. MRI) is alternatively suggested [6].
Multimodal imaging approaches (i.e. PET/CT, PET/MRI) have gained clinical interest over the last years as two imaging modalities can provide synergistic benefits [23]. In this study a multimodal visible bone substitute (i.e. visible either by CT and MRI) was developed. Such dual-labeled composite could give to the radiologists the possibility to provide a patient-tailored imaging workflow according to each specific clinical circumstance (i.e. CT acquisition could be performed right after the surgery, while MRI applied for longitudinal monitoring). To date, a CT/MRI multimodal approach is aimed only in pre-clinical or clinical studies related to bone remodeling and biomaterials development. However, as MRI is superior to CT for many aspects (i.e. it is radiation-free, provides soft and hard tissues contrast, it gives 3D anatomical and functional information) an ultimate long-term focus for research should still be to make MRI as a preferential bone imaging screening tool [8, 22].

### 3.5. Bone-Growth Assessment by Histology and Histomorphometry

To investigate the tissue reaction on the CPC composites with and without imaging nanoparticles, we made histological sections at week 8. Due to the decalcification process during histological preparation, it was not possible to detect CPC remnants in the sections. However, the circular area of the original defect was still clearly recognizable and thus we used this area for qualitative and quantitative analysis (Figure 7). The empty defect group shows incomplete healing proving that the performed defect was a critical-size-defect for the 8-week period. Furthermore, both labeled and non-labeled CPC materials did not induce any immune response or fibrotic encapsulation. However, in several samples, a layer of fibrous tissue was present at the interface between bone and implant. This layer was not always

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<th>Table 1. Correlation analysis comparing mean intensities from $^{19}$F MRI signal, CT attenuation coefficient and implant volume.</th>
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continuous, and appeared to contain fibrous tissue and immature extracellular matrix fibers, yet often also showed red-stained calcified nodules, indicating the onset of mineralization. This effect was present in 1/8 samples of the CPC group and 4/8 samples of the CPC/PFCE/gold group. Chi-square testing demonstrated that such frequencies are not significantly different (p>0.05) between both groups. In all cases, there was direct contact between bone and CPC, and uniform bone growth in all the groups was observed. Such bone growth began at the edges and then proceeded throughout the entire contact area towards the inside of the implant.

Figure 7. Histological sections of the femora stained with EVG after 8 weeks of implantation. The original bone structure in (A), the empty defect in (B), the defect filled with CPC in (C) and the defect filled with CPC/PFCE/gold composite in (D) are shown. For each specimen, a picture of the general overview taken at 1X magnification (inset) and the respective zoomed view (magnification 10X) are shown. The markers correspond to 1 mm.
Histomorphometrical analysis of a defined ROI (i.e. circle of 2.5 mm in diameter) demonstrated the relative formation of the new bone, with approximate filling of defect of 50%, which is typical for this length of time [17]. No statistical differences in bone content were found between the empty defect group, the CPC group and the CPC/PFCE/gold group (Figure 8). This new bone formation is in the range of the decrease of both MRI and CT signals. Aiming at clinical application, further studies with longer experimental times and with materials of different degradation kinetics, could help to develop a reliable method for quantitative monitoring the degradation with MRI and CT contrasts in vivo.

Figure 8. The relative bone formation quantification based on histology performed after 8 weeks from the surgery (n=8). No statistical differences were found.

One limitation of presented experimental model was that we did not focus on the clearance of the applied labels from the body. However, many previous investigations studied body uptake of either perfluorocarbons (PFCs) and gold nanoparticles (AuNPs) as well as their biological compatibility [34, 36, 47-50]. Specifically, it has been proven that PFCs are firstly removed from the blood circulation by reticuloendothelial system filtration, and also can ultimately be exhaled through the respiration system [34, 36]. Differently, AuNPs uptake seems to be size-dependent. Small AuNPs (i.e. 5-15 nm) are excreted through kidney filtration, while large particles (>200 nm) are captured by the immune system and delivered into the liver or the spleen [47,48]. After 24 h from intravenous administration of 50 nm AuNPs in a rat model, particles showed accumulation only in the blood, liver and spleen [49]. Therefore, it could be speculated that the 40 nm AuNPs used in this study would undergo a similar fate and finally be eliminated from the body through the feces [50]. Another consideration is the concentration of contrast agents used for the preparation of the
CPC/PFCE/gold composite. Specifically, the estimated PFCE concentration is less than 5 mg per 100 mg of cement. Such concentration is considerably low when compared to the large concentration that is requested when PFCs are used in the blood circulation (i.e. about 10 g/Kg) [36]. Similarly, the used gold concentration (i.e. about 0.5 mg per 100 mg of cement) resulted to fall in the “therapeutic window” within which AuNPs can be used without any side effects, suggesting that an even higher concentration may be investigated for further improvement of the CT contrast [48].

4. Conclusions

Nowadays, one of the main challenges in tissue engineering is to develop detectable materials that can be followed longitudinally once implanted in the body. Here, we propose an innovative ceramic material with enhanced $^{19}$F MRI and CT contrast, for bone application. By labeling a CPC with PLGA/PFCE and AuNPs, we obtained a composite that can be qualitatively and quantitatively detected until 8 weeks post-surgery in an in vivo rat model of bone regeneration. Thus, our CPC/PFCE/gold provided more correct anatomical and functional information, with both MRI and CT, helping to overcome the imaging limitation of ceramics materials. Successful development of this CPC composite has substantial potential for clinical use.

The combination of the current contrast agents-based strategy is suggested for all kinds of ceramic materials as well as for polymeric scaffold or hydrogels. Certainly, for every different material or application, a certain degree of customization (e.g. fine-tuning the amount of nanoparticles) is required. For ceramics, PFCE could be incorporated in specific cement-dedicated carriers (e.g. microrods, fibers, bigger particles). Simultaneously, gold could be directly combined with the carrier containing PFCE by following many strategies, such as coating or incorporation in bigger beads. In this way, specific-cement-dedicated carriers can easily be developed and scaled-up for clinical applications. Furthermore, further improvement of the biological properties of the dual-labeled CPC composite could be pursued by adding specific growth factors (i.e. BMP-2) which can be used to trigger new bone formation.

5. References


6. Supplementary Information

![Figure S1](image1.png)

**Figure S1.** Femoral condyle defect, *in vivo* surgical procedure. A longitudinal incision was performed on the medial surface of the knee (a). After patella dislocation, the condyle is exposed (b). With a dental drill a cylindrical defect (2.5 mm in diameter) it is performed along the direction of the femur, (c) and (d). The CPC with or without contrast agents was used to fill the defect. In (e) the CPC/PFCE/gold composition is injected in the condyle defect. By using a spatula the shape of the cement was adapted to the anatomical shape (f), and thereafter the wound was closed in layers, using resorbable stitches.
Figure S2. CT representation of a rat condyle filled with CPC. The coronal direction in (a) and the sagittal direction in (b) are shown. By using IRW, a constant volume of interest (VOI) including the implanted material was drawn. Based on this VOI, the relative signal intensity in percentage was calculated.

Figure S3. Bone formation quantification based on the images from histology. In (a) the starting EVG stained picture of one section and the selected ROI (i.e. circle of 2.5 mm in diameter), in (b) the region outside of the circle is excluded, in (c) the selected area after threshold adjustment. By using ImageJ software, the total area (in $\mu m^2$) of the circle is calculated. The total bone formation is expressed as ratio between the bone area and the total selected area (in percentage).
**Figure S4.** Scanning electron micrographs of (A) an overview of the surface of CPC/PFCE/gold composite (magnification X2500). Yellow rectangles indicate (B, C) agglomerates of PLGA/PFCE, (D) agglomerates of PLGA/PFCE and cryo-grinded PLGA particle, (E) cryo-grinded PLGA particle. For figure B, C, D magnification is X15000; for Figure E magnification is X5000. White arrows indicate singular PLGA/PFCE nanoparticles attached to the overall agglomerate. Note that in (E) individual PLGA/PFCE nanoparticles resulted attached also to the cryo-grinded PLGA particles.

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**Figure S5.** Cohesion test. Pictures from the CPC and the CPC/PFCE/gold pastes are shown after 24 hours incubation in PBS at 37 °C. On the top three samples from the CPC composite, while on the bottom the same amount of samples from the CPC/PFCE/gold composite. In the table are reported the score from the cohesion test. Score was attributed as follows: +++ = the paste broke in 1 single piece, ++ = the paste broke in > 2 pieces and <3 pieces, + = the paste broke in > 3 pieces.
Figure S6. 1H ZTE images (coronal view) of an example rat condyle directly after surgery (A), at 4 weeks (B) and at 8 weeks (C).

Table S1. Randomization scheme of the experimental groups

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Chapter 6

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


Manuscript submitted

†, ‡ equally contributed
1. Introduction

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 detection [1,2]. Therefore, there is a pressing need for the development of innovative non-invasive imaging approaches, for example, based on multimodal imaging strategies combining magnetic resonance imaging (MRI) and computed tomography (CT) [3-5]. 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 [9-11].

The demand for non-invasive imaging modalities makes MRI an excellent option as it allows non-invasive anatomical imaging and functional 3D reconstruction of soft tissues with high spatial resolution. Imaging of CPCs using the MR modality can be achieved by application of short or zero echo time acquisition sequences (i.e. UTE and 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 [12-14].

Gadolinium(III) is a lanthanide element with seven unpaired electrons, a symmetric S-state, and it shortens $T_1$ relaxation times of the tissues leading to a positive signal enhancement in MRI due to these paramagnetic properties [15-17]. Commercially available gadolinium(III) based contrast agents (GBCAs) have already been utilised to enhance the $T_1$ signal of CPC constructs in vivo. However, the employed strategy utilised molecular agents that were not strongly linked to the material, showing 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 [18]. Therefore, the aim of this work was two-fold: to improve the contrast properties (both in CT and MRI) by the use of nanoparticles versus molecular agents, and to more firmly anchor the contrast agent into the material. Functionalization of the nanoparticles with biphosphonates (BPs) allows them to strongly interact with CPCs offering multiple bonding interactions with calcium ions ($\text{Ca}^{2+}$) from each BP component [19-21]. These interactions specifically bind the GBACs into the CPC matrix.
FUNCTIONALIZED GADOLINIUM OXIDE NANOPARTICLES

and prolong the residence of the imaging probe in situ, despite of the ongoing material degradation occurs.

Thus, in the presented study, we have developed a surface functionalized GBCAs that can be used for long-term non-invasive monitoring of a specific CPC composite. To this end, gadolinium oxide (Gd₂O₃) 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 trimethoxysilane (GTPES) 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 towards the hydroxyapatite, the main component of the CPC composite, and created the CPC-specific contrast agent (Scheme 1). Elemental analysis and infrared (IR) spectroscopy were used to characterize the BP functionalization of the GBCAs, while in vitro binding experiments confirmed the high affinity of the BP functionalized GBCAs (GBCAs-BP) towards 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 MR imaging. 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.

2. Materials and methods

2.1. Materials

Alpha-tricalcium phosphate (α-TCP) and carboxymethylcellulose (CMC) were provided by CAM Bioceramics BV, Leiden, the Netherlands. Cryo-grinded poly(D,L-lactide-co-glycolide) microparticles (<200 µm) with 50:50 ratio of lactic to glycolic acid (PLGA, PURASOB 5002A) was purchased from Purac, Gorinchem, the Netherlands. Sodium dihydrogen phosphate dehydrate (NaH₂PO₄•2H₂O) was purchased from Merck Chemicals BV, Amsterdam-Zuidoost, the Netherlands. Gelatin Type A, gadolinium chloride hexahydrate (GdCl₃), gadolinium acetate, diethylene glycol (DEG), and toluene were purchased from Alfa Aesar and Fisher, Karlsruhe, Germany. Sodium hydroxide (NaOH), polyacrylic acid (PAA, Mw = 2.1 kDa), 3-Glycidyloxypropyl trimethoxysilane (GPTES), pyridine (anhydrous) and O-(5-Norbornene-2,3-dicarboximido)-tetramethyluronium tetrafluoroborate (TNTU), were purchased from Sigma-Aldrich, St Louis, MO. All chemicals were used without further
purification. Bisphosphonate derivate (6-amino-1-hydroxyhexane-1, 1-diyl-bisphosphonic acid) was provided by Dr. Rafael Torres de Rosales (King’s College, London, UK).

![Scheme 1](image)

**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.

2.2. Synthesis of gadolinium based contrast agents (GBCAs)

GBCAs were synthesized according to a well-established synthetic method: the polyol method [22]. Briefly, GdCl₃ (1.86 g, 7 mmol) was dissolved in DEG (25 mL) at 70 °C and directly mixed with NaOH (0.6 g, 15 mmol) and dissolved in DEG (10 mL). The obtained white precipitate was heated up to 180 °C and the solution stirred for 24 h. The same steps of collection, purification, dialysis and freeze-dry were performed as done for the particles synthesized according to the traditional polyol-like method. These GBCAs were stabilized afterward by GPTES conjugation. Briefly, GBCAs (0.25 g) was suspended in toluene (50 mL), sonicated for 10-15 min and then stirred at 90 °C. While the solution was stirring, GPTES (2 mL) was added dropwise and then left stirring for 48 h at 90 °C. The obtained
silica capped GBCAs were collected by centrifugation (10 min, 13000 rpm at 4 °C), washed several times with toluene and ethanol and freeze-dried.

2.3. GBCAs functionalization with bisphosphonate (GBCAs-BP)

BP functionalization was based on a coupling rout method as reported elsewhere [22]. Silica capped GBCAs (0.15 g) and BP derivate (0.15 g) was suspended in pyridine anhydrous (10 mL) and sonicated for 5 min. The mixture was stirred at 65 °C for 48 h. The same steps of washing, dialysis and freeze-dry were performed as in the case of the PAA capped GBCAs.

2.4. GBCAs characterization

All synthesized GBCAs were chemically and morphologically characterized. CHN elemental analysis was used to measure the hydrogen, carbon and nitrogen content. An elemental analyzer 1108 CHN (CE Instruments ltd, Wigan, UK) was used for the measurements. The results were expressed in wt %.

Inductively coupled optical emission spectroscopy (ICP-OES) was performed on an Optima 5300 DV spectrometer system (Perkin Elmer, Waltham, MA). Phosphorous (P) and Gadolinium (Gd) content were determined for freeze dried powders and expressed as wt %.

Infrared (IR) spectroscopy was performed on a Fourier transform IR (FT-IR) spectrometer (Spectrum RX I, Perkin Elmer, Waltham, MA).

Dynamic Light Scattering (DLS) was performed in Zeta Nanosizer (Malvern Instruments, ZEN3600, Malvern UK) at 25 °C using disposable polystyrene cuvettes.

Transmission electron microscopy (TEM) analysis was performed on a JEOL 2010 TEM (Joel Ltd., Tokio, Japan) equipped with a Gatan US 4000 digital camera (Gatan, Oxford, UK) and 200 kV. EDS was performed on the central part of the acquired picture were the highest particle population was observed.

Relaxivity measurements on the synthesized GBCAs were performed either using a 3 Tesla (T) horizontal clinical MR scanner (Discovery MR750 general Electric scanner, GE Healthcare, Chicago, IL) or an 11.7 T vertical-bore preclinical MRI system (Bruker, Ettlingen, Germany). Longitudinal (T₁) and transverse (T₂) relaxation times were determined at range of gadolinium concentrations in water. A linear plot of concentrations versus 1/relaxation time gives r₁ and r₂ as the plot gradient.

Images were acquired at 3T using RARE (rapid acquisition with relaxation enhancement) inversion recovery sequence, field of view (FOV) = 4.0 cm, flip angle (FA) = 180°, repetition time (TR) = 3500 ms, echo time (TE) = 7.5 ms, inversion time (TI) = 1500 ms, bandwidth (BW) = 79365.1 Hz, acquisition time (TA) = 2.48 min.
2.5. MRI-visible bone filler (CPC-GBCAs-BP) preparation

The synthesized GBCAs-BP nanoparticles were combined with a defined CPC-PLGA-CMC composite. The CPC-PLGA-CMC composite labeled with GBCAs-BP is indicated in this study as CPC-GBCAs-BP nanocomposite. CPC was prepared as a weight/weight (wt/wt) mix of 78.5% α-TCP, 1.5% of CMC, and 20% of PLGA. To this starting CPC composition the selected GBCAs were added at different increasing wt/wt concentrations (i.e. 1%, 2.5%, 5% and 10%). Dried powders (final weight 100 mg) were added into an exit-closed 2 mL syringe (Termo Europe N.V., Leuven, Belgium) and mixed for 30s with a liquid phase (i.e. 4 wt% NaH$_2$PO$_4$•2H$_2$O, final volume 50 µL) through a dental shaker machine (Silmat® Mixing apparatus, Vivadent, Schaan, Liechtenstein). Two additional CPC-PLGA-CMC composites were prepared in the same way as internal controls. One control consisted of calcium phosphate-based, PLGA, and CMC without contrast agents (i.e. 78.5% α-TCP, 20% PLGA, and 1.5% CMC) and indicated in this study as CPC. The second control consisted of CPC-PLGA-CMC combined with GBCAs without bisphosphonate functionalization (i.e. 77.5% α-TCP, 1.5% CMC, 20% PLGA and 1% GBCAs) and is indicated in this study as CPC-GBCAs.

For the *in vivo* study, materials were sterilized by using gamma radiation (25 kGy; Synergy Health, Ede, the Netherlands), while the liquid solution was filtered by 0.2 µm filters (Whatman, GE Healthcare Life Sciences, Eindhoven, the Netherlands).

2.6. GBCA-BP towards CPC *in vitro* binding affinity study:

Briefly, cylindrical cylinders (4.5 in diameter, 9 mm in height and ~ 40 mg in weight) of CPC were prepared and incubated in Eppendorf tubes with different concentration of GBCAs-BP nanoparticles dispersed in water, i.e. 10, 100 and 1000 µM of Gadolinium (III) in 2 mL of water (n=3). The samples were gently mixed for 2, 6 and 24 h. Afterward, the supernatant was collected and analyzed by ICP-MS to detect any release of gadolinium (see above for ICP-MS). The binding efficacy was calculated according to the following equation:

\[
\text{Binding affinity} = \frac{(C_i - C_m)}{C_i}
\]

Where \( C_i \) is the initial concentration of used particles, while \( C_m \) is the concentration of gadolinium measured in the supernatant. Binding affinity was expressed as µg of gadolinium (III) per surface of CPC (m$^2$). The surface of the CPC composition was measured by Brunauer-Emmett-Teller (BET) analysis through a BET-Micromeritics system (Micromeritics Ltd., Dunstable, UK). Surface area was measured using nitrogen adsorption and desorption isotherms at -196.15 °C.
2.7. *In vitro* GBCAs-BP toxicity assay

Biocompatibility of the GBCAs-BP particles was investigated using primary human osteoblasts (HObs). Hob (CAI no: 406-05f) from the European Collection of Authenticated Cell Cultures (ECACC, PHE, London, UK) were seeded in 96 well-plates at a final concentration of 1000 cells/well and cultured with 200 µl of osteoblast growth medium (PromoCells GmbH, Heidelberg, Germany). After one day incubation at 37 °C and 5% CO₂ the medium was removed and the cells were treated with 100 µL of GBCA-BP water solution. GBCAs-BP were used at different concentration, i.e. 0.1, 1, 10, and 100 µM, and incubated for 48 h, 96 h, and 120 h (n=3). At each time point cell vitality was estimated by MTT calorimetric assay (Promega, Fitchburg, WI). Briefly, at each time point cells were incubated with 20 µL of MTT reagent (dimethylthiazol-2-yl-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) tetrazolium) for 4 h at 37 °C and 5% CO₂. Afterwards, adsorption rate was measured at 490 nm using a Synergy™HT microplate reader (Biotek, Winooski, VT). The obtained values were normalized by the control group (i.e. medium without particle) and expressed as percentage of cell viability.

2.8. *In vitro* MRI assessment of CPC-GBCAs-BP injected in pig bone blocks

CPC composite with and without GBCAs-BP was injected in cylindrical defect (3 mm in height and 3 mm in diameter) performed in bone blocks obtained from pig jaw (Figure 2g). Specimens were wrapped in Parafilm® (SERVA electrophoresis GmbH, Heidelberg, Germany) and embedded in 5 wt% gelatin type A. Then samples were scanned on a 11.7 T MRI system (Biospec, Brucker) equipped with a ¹H volume coil with inner diameter of 40 mm. Images were acquired by using a zero echo time (ZTE) sequence with the following scan parameters: TR = 2 ms, matrix size = 256 x 256 x 256, image resolution = 0.313 x 0.313 x 0.313 mm³, 1 average, FA = 3.7°, TA = 6.53 min. The obtained images were displayed by using MRICro software (Smith Micro software, Aliso Viejo, CA).

2.9. *In vitro* micro-computed tomography (µCT) assessment of CPC-GBCAs-BP injected in pig bone blocks

Cylindrical blocks (3 mm in height and 6 mm in diameter) made of CPC and CPC-GBCAs-BP were prepared as described before. Cylinders were scanned horizontally along the X-ray direction by using a µCT system (Skyscan 1275, Kontich, Belgium). The following settings were used for images acquisition: magnification X15 (i.e. pixel resolution 18.88 µm), source voltage 100 kV, source current 98 µA, rotation angle 180°, rotation step 0.9°, 1 mm
aluminum filter. Acquired files were reconstructed with NRecon software (Skyscan). Attenuation intensity for each sample was calculated by CtAnalyser software (version 1.10.1.0; Skyscan) based on a constant volume of interest (VOI) and expressed as frequency intensity of the gray values. Two-dimensional (2D) images reconstructions were obtained by Data Viewer software (Version 1.5.2.4; Skyscan).

2.10. Handling and mechanical properties characterization of CPC-GBCAs-BP nanocomposite

Only the CPC composition with 1% wt/wt of GBCAs was considered for further studies. Handling and mechanical properties of the chosen CPC-GBCAs-BP nanocomposite were investigated and compared either to the CPC control (i.e. non labeled CPC) and to the CPC-GBCAs composites (i.e. CPC combined with non functionalized GBCAs).

Setting time was investigated with a Gillmore apparatus (according to ASTM C266). Initial and final setting times were recorded after injection of cement pastes in a bronze mold of 6 mm in diameter and 12 mm in height immersed in a water bath at 37 °C.

Compressive strength and E-modulus were calculated from compression test performed through a testing bench machine (858 MiniBionixII, MTS, Eden Prairie, MN). Cement pastes were used to prepare cylinders of 4.5 mm in diameter and 9 mm in height. Cylinders were compressed along the vertical direction with a loading force of 2.5 kN and a constant speed of 0.5 mm min\(^{-1}\). Injectability features were investigated using the same testing bench machine (858 MiniBionixII) set in a compression mode and adapted with a custom-made metallic cage. Briefly, 500 mg of CPC mixtures were mixed with 250 µL of setting solution into an exit-closed 2 ml syringe (Terumo Europe N.V.). After mixing the components for 30 s the syringe was placed in the metallic cage, the exit was opened and a compression force of 100 N was applied with a constant speed of 20 mm min\(^{-1}\). When all the cement pastes were extruded from the syringe (after 33±2 s) compression force was stopped and the extrusion curve was calculated as applied force (N) by the time (s).

2.11. In vivo assay of CPC-GBCAs-BP injected in rat condyle model

To investigate the feasibility of long-term MRI imaging and to evaluate bone response to the CPC-GBCAs-BP nanocomposite an in vivo study was carried out. CPC composite with and without GBCAs-BP was injected in a rat condyle defect model. The animal study was performed after the approval of the Animal Ethics Committee for the care and use of laboratory animals (RU-DEC number 2015-0035) and in agreement with the standards of the Radboud University Medical Center, Nijmegen, The Netherlands. Six healthy male Wistar
rats (body weight: 250-300 g) were used as experimental animals. In order to reduce the number of animals for this experiment, both posterior legs of the animal were used. Furthermore, historical controls (i.e. leg left untreated and leg with defect left empty) were also included in this study.

For the surgery, the animals were anesthetized by inhalation of a mix of isoflurane (Pharmachemie, Haarlem, the Netherlands) and oxygen. Then, the animals were located in supine position on a heating mat in order to prevent hypothermia. Both legs were shaved and disinfected with povidone-iodine solution (Betadine, Kuinre, the Netherlands). A longitudinal incision was performed on the medial side of the knee through the skin and the underlying muscle (Supplementary Information, Figure S1). The patella was dislocated laterally in order to have a clear view of the articulating surface and patellar groove of the femoral condyle. A dental drill (Astra Tech Elcomed 100, Dentsply Implants, Zoetermeer, the Netherlands) equipped with three different dental burs with increasing diameter up to 2.5 mm was used to create a cylindrical defect (2.5 mm in diameter and 3 mm in dept) perpendicular on the patellar groove. During the drilling, a sterile saline solution (Fresenius Kabi, Emmer-Compascuum, the Netherlands) was used to cool down the dental bur and to clean the cavity. After defect preparation, pre-shaken cement paste was injected to fill the defect. Then the patella was moved back to the original position and the muscle and the skin were closed with absorbable sutures (Vicryl® 4.0 Ethicon, Somerville, NY). To decrease post-operative discomfort a subcutaneous injection of painkiller (5 mg/Kg Rimadyl®, Cattle, Capelle a/d Ijssel, the Netherlands) was performed post-surgery. Eight weeks after surgery, the animals were sacrificed by CO\textsubscript{2}/O\textsubscript{2} inhalation. Femora were harvested, placed in formalin 10 wt% for 48 h and then kept in ethanol 70% until histological assessment.

2.12. \textit{In vivo} MRI and CT imaging of the implanted CPC-GBCAs-BP nanocomposite

\textit{In vivo} MRI and CT acquisitions were performed post-surgery (i.e Day 0), and at 4 and 8 weeks after the surgery.

MRI acquisitions were performed on the same 11.7T MRI system (Biospec) as in the \textit{in vitro} study. Animals were anesthetized by inhalation of isoflurane/O\textsubscript{2} and located on the supine position. Body temperature and breathing were constantly monitored. One by one each leg was immobilized inside the coil and scanned by using same parameterized ZTE sequence applied for the \textit{in vitro} experiments. The obtained images were shown by using MRICro software (Smith Micro software).
CT acquisitions were performed on a small animal CT scanner (Inveon Micro-CT/PET, Siemens Medical solution, Knoxville, TN). Animals were kept under general anesthesia (isoflurane/O₂) and located in the supine position on a heating mat. Images were recorded with an acquisition time of 6 mins, spatial resolution of 30 µm, tungsten anode source (source voltage 80 kV) and exposure time of 1000 ms. Reconstructions of the acquired file were obtained by Inveon Research Workplace software (IRW, Siemens).

2.13. Histology and Histomorphometry

For histological analysis samples were first decalcified in 10 wt% ethylenediaminetetraacetic acid (pH 7.2) for two weeks, dehydrated in a gradual ethanol series (from 70% to 100%) and embedded in paraffin. At least three sections (4 µm in thickness at 100 µm of interdistance) were cut along the axial direction of the femur by using a microtome (RM2165, Leica Microsystems, Rijswijk, the Netherlands). Afterwards, sections were stained with Hematoxylin/Eosin (H/E) and with trichrome Elastic van Gieson (EVG). Images were acquired by using a light microscope (Axio Imager Z1, Carl Zeiss AG Light Microscopy, Göttingen, Germany) equipped with a digital camera (Axiocam MRc5, Carl Zeiss AG Light Microscopy). EVG-stained pictures were used for histomorphometrical analysis based on a computer image analysis technique through ImageJ software (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). Bone amount was calculated as area percentage inside a defined ROI corresponding to the original defect (i.e. circle of 2.5 mm in diameter)

2.14. Statistical analysis

Statistical analysis was performed by using GraphPad Instat® (GraphPad Software, San Diego, CA). Data were reported as mean ± standard deviation. Statistical significant differences were calculated by one-way analysis of variance (ANOVA) with Tukey post hoc test. Differences in new bone formation were calculated with Student’s T-test with Welch’s corrections. All differences were considered significant at p-values <0.05.
3. Results and Discussion

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) [22]. Elemental analyses (CHN combustion analysis and inductively coupled 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 of the organic component (Figure 1a). The presence of carbon in the “uncoated” gadolinium oxide sample is due to the diethylene glycol (DEG), which is used as a solvent in the reaction, as it adsorbs onto nanoparticles surface via interactions with the hydroxyl groups [23]. 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 Gd₂O₃ nanoparticles core size after GPTES coating, while no differences were observed after BP functionalization (Figure S2). 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 (EDX, Figure S2) as well as by infrared spectroscopy (IR) (Figure 1d, e, f). The IR spectrum of the precursor Gd₂O₃ nanoparticles contains a distinctive peak at 2871 cm⁻¹ corresponding to the stretching and bending of the methylene chain (CH₂), a sharp band at 1084 cm⁻¹ is assigned to the C-O stretch, and the broad peak at 3100 to 3500 cm⁻¹ corresponds to the O-H stretch (Figure 1d). After GPTES coating, the symmetric epoxy ring deformation gives a 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 [23]. In the BP functionalized derivative, the appearance of peaks at 1057 cm⁻¹ and 1521 cm⁻¹ corresponds to the phosphonate groups and to the N-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.

![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-OS.](image)

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![Figure 1. Chemical and morphological characterization of the synthesized nanoparticles.](image)

(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-OS. (b) TGA analysis of the GBCAs after GPTES coating. (c) TEM of GBCAs-BP. Yellow circles define a single particle. In (d), (e) and (f) FTIR spectrum of the particles after the polyol synthesis, after GPTES coating and after BP functionalization, respectively.
An ideal GBCA, 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 3 T clinical scanner and 11.7 T small bore system across a range of concentrations) (Figure 2 and Table S2). 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 [24,25].

To be an effective T$_1$ contrast agent, the nanoparticles should possess an ultra-small 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 mM were scanned using inversion recovery for T$_1$ [26-28]. 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 $r_1$ equal to 15.41 mM$^{-1}$s$^{-1}$ at 3 T, which is almost 4 times higher than the commercially available contrast agents (e.g. Magnevist® or Omniscan®) [24,25]. Furthermore, the nanoparticles showed only a slight decrease of T$_1$ relaxivity at 11.7 T with $r_1$ equal to 13.44 mM$^{-1}$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, b, c, d, Table S2). The images of the GBACs-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 mM) are 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 %) towards the CPC material at up to 24 hours 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 µM, Figure 2g). However, at low concentrations (0.1, 1, and 10 µM) of the GBCAs-BP showed a beneficial effect on the HOb viability when compared to the non-treated cells (i.e. the internal control) as the nanoparticles induced cell proliferation (Figure 2g). Such findings not only suggested a non-toxic 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 [29]. With this study, we confirm the beneficial effect of BP coated nanoparticles derivatives on HOb and support further investigation of this strategy for bone regenerative applications.

Figure 2. Characterization of the relaxation properties of the particles, their affinity towards the CPC mineral phase, and the in vitro cytotoxicity. (a) and (b) plot of $1/T_1$ and $1/T_2$ measured at 3 T, (c) and (d) plot of $1/T_1$ and $1/T_2$ measured at 11.7 T as a function of GBCAs-BP concentration respectively. $r_1$ and $r_2$ were calculated from the slope. (e) the $T_1$-weighted phantom of GBCAs-BP is shown at different concentrations (image recorded simultaneously). (f) the binding profile of GBCAs-BP after 2, 6 and 24 h incubation with CPC cylindrical blocks. (g) MTT assay performed on HOb.

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 nanoparticles 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 with the reports 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 of 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 [18]. 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 non-labeled 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 crystals growth and agglomeration [30-33]. The internal CPC control consisting of unfunctionalized nanoparticles (i.e. CPC-GBCAs), confirmed the role of the BP groups in 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 [34]. The addition of the GBCAs-BP was shown to statistically increase the compressive strength and the E-modulus of the final composite when compared to the non labeled cement (Figure 3b, c). The non-functionalized 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 BET analysis the CPC composite consisted of a nanoporous structure (pore width = 18.8 nm, surface area 9.7 m$^2$/g), thereby suggesting that the applied 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 [35]. 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$^{-1}$ g$^{-1}$ at100 keV) and it has been used as CT contrast agent especially for angiography and aortography applications [36-38]. Therefore, the capability of the GBCAs-BP to enhance the CT contrast of the non-labeled CPC composite was also investigated. Gray value quantification, based on in vitro micro-CT acquisition, reported an evident leftward shift of the values which resulted in 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 non-labeled CPC. ZTE-MRI acquisitions of the samples containing GBCAs-BP performed at 11.7 T showed a typical $T_2^*$ shortening 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 $T_1$-shorthening effect, but also a
T₂(T₂*) shortening effect, depending on its concentration [39-42]. Comparing our results with previous studies where the CPC was combined with commercially available molecular gadolinium(III) agents (i.e. Gd-DTPA from Magnevist®), the GBCA-BP nanoparticles showed excellent contrast with a higher signal intensity, confirming the superior imaging performance of our nanoparticles [18].

To assess the longitudinal MRI and CT imaging behavior of the CPC-GBCAs-BP nanocomposite, an in vivo study was performed (Figure S1). Respectively, labeled and non-labeled CPCs were injected in a cylindrical defect prepared in a rat condyle, which is a well established non-load bearing model commonly used for testing biomaterials [43]. MRI and CT acquisitions were performed post-surgery (i.e. Day 0), and at 4 and 8 weeks with the data shown in Figure 4. After the surgeries, high resolution ZTE images of the CPC-GBCAs-BP showed a T₂ weighted signal, which resulted in an implant size overestimation (Figure 4a). In contrast, the CPC composite without contrast agent appeared as a dark hypodense region on ZTE MR images. Such findings were in line with our in vitro assessments (Figure 3h). Longitudinal monitoring showed the superior imaging performances of the CPC-GBCAs-BP compared to the non-labeled CPC as in the former case it was possible to clearly identify the implanted material at every time point. Moreover, at 4 weeks post-surgery, the implant size of the CPC-GBCAs-BP nanocomposite was observed to have slightly decreased, while in the center of the implant a brighter area appeared. Interestingly, the relative intensity of this bright region increased at 8 weeks (Figure 4a). It is known that GBCAs can lead to a competitive behavior between T₁ and T₂ shortening effects resulting in a bright or dark signal respectively [44,45]. Such a competitive effect may explain the appearance of the brighter region in the central part of the implant, although variations in agent concentration could also be an important factor determining the change in signal intensity.

CT images showed an enhanced signal intensity in the case of the CPC-GBCAs-BP nanocomposite when compared either to the non-labeled 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 post-surgery, the CT acquisitions of the implanted CPC-GBCAs-BP showed a 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 non-functionalized 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 post-surgery [19-21,44,45]. 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.

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). 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) [46,47]. Our histological findings were in line with these previous studies and showed higher bone density 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 (Figure 4c).

One area of further study that is ongoing is to look at the release profile in vivo and the biodistribution of the GBCAs-BP when released from the CPC composite. Methods for radiolabeling are under investigation to determine a valid quantitative tracking. Previous studies have shown that GBCAs do not undergo to intracellular accumulation and are generally excreted by the hepatobiliary or renal systems [48,49]. 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. 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.
Figure 4. CPC composite with and without GBCAs-BP after injection in a rat condyle defect. (a) the ZTE MR images and (b) the CT acquisitions are shown respectively. From the top to the bottom the axial view of the same leg after surgery, at 4 and 8 weeks post-surgery are shown respectively. Note yellow dashed circles that indicate the difference in size between CPC with and without GBCAs-BP. Green arrows indicate the bright region that appears in the middle of the implant on the MRI acquisitions after 4 weeks from the surgery. The bright region corresponds on the CT images to a less dense material. Red arrow indicates the CPC that become indistinguishable from the surrounding bone after 8 weeks from implantation in vivo. In (c) histological sections after EVG staining (scale bar 500 µm). The inserts show the new bone formation quantification based on the histological sections. The red dashed lines indicate the bone volume observed in the non-treated samples. No significant differences between the groups were found (p<0.05).
4. References


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173
5. Supplementary Information

**Figure S1.** *In vivo* surgical procedure for a femoral condyle defect model. In (a) an incision was performed on the medial side of the knee. In (b) and (c) dental drill used to perform a cylindrical defect (2.5 mm in diameter) along the direction of the femur. In (d) pre-shaken paste of CPC with and without GBACs-BP was used to fill the defect. In (e) cement after injection in the condyle defect.

**Figure S2.** Core diameter and morphology of the nanoparticles was assessed by TEM. Energy dispersive spectroscopy (EDS) coupled to the TEM was used for elemental analysis. In (a) Gd$_2$O$_3$ nanoparticles synthesized via polyol-like method. The average size of such particles is below 5 nm (on the top), the main element detected was Gd (on the bottom). In (b) Gd$_2$O$_3$ after GPTES stabilization. The average size underwent a slight increase of ~1-2 nm compared to the Gd$_2$O$_3$ nanoparticles (on the top), while a typical silicon peak at 1.74 keV was shown by EDS analysis (on the bottom). In (c) the GBCAs-BP nanoparticles. The average size remained unchanged compared to the Gd$_2$O$_3$ nanoparticles after GPTES stabilization (on the top). A clear peak at 2 keV, corresponding to the P atoms belonging to BP molecules was observed by EDS analysis (on the bottom).
Figure S3. Histological sections of the femora after 8 weeks in vivo implantation. On the top a general overview of the femora (1X magnification) on the bottom the respective zoomed view (10 X magnification).

Table S1. Summary of the chemical properties of the GBCAs-BP

| GBCAs-BP |  
| --- | --- |
| **Synthesis Method** | Polyol method |
| **Hydrodynamic size from DLS** | 70 nm |
| **Elemental Composition (wt %)** | C = 12.28, H = 3.29, N = 1.52 |
| **ICP-MS (wt %)** | Gd = 27.54, P = 6.08 |
| **FT-IR (cm⁻¹)** | 3324, 2922, 1521, 1395, 1057, 967 |

Table S2. Relaxivity values of different synthesized GBCAs measured at 3T and 11.7T

<table>
<thead>
<tr>
<th>3 Tesla</th>
<th>11.7 Tesla</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r_1$ (mM⁻¹s⁻¹)</td>
<td>$r_2$ (mM⁻¹s⁻¹)</td>
</tr>
<tr>
<td>15.41</td>
<td>73.51</td>
</tr>
</tbody>
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Chapter 7

In vivo Evaluation and CT/MR Imaging of 3D Printed Gelatin Methacrylate (GelMA) Scaffolds for Bone Tissue Engineering

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Manuscript submitted
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1. Introduction

Gelatin methacrylate (GelMA) hydrogels are widely used for cartilage regeneration due to their optimal biological properties and their ability to induce extensive cartilage matrix formation [1,2]. Furthermore, GelMA is also used for the regeneration of 3D vascular structures [3,4]. Recent insights indicate that GelMA can also induce osteogenic differentiation and calcium deposition \textit{in vitro} as well as endochondral bone formation \textit{in vivo}, hence supporting its potential use also in bone tissue engineering (BTE) [5–7]. Additionally, the methacrylate moieties on GelMA’s backbone give rise to a stable crosslinked hydrogel upon exposure to UV light and in presence of a suitable photoinitiator. Thus, GelMA hydrogels are suitable materials for different manufacturing techniques such as photopatterning, micromolding and layer-by-layer 3D printing [8].

Layer-by-layer 3D printing is one of the most convenient techniques to create TE-dedicated scaffolds with well distributed and interconnected pores which ensure cell penetration, vascular in-growth and nutrient diffusion, as well as removal of waste products [9–12]. Furthermore, printing hydrogels with this technique allow creating patient-specific designed scaffolds with structural complexity, low-cost, and high-efficiency [13].

Once the appropriate material is selected and manufactured according to the tissue-specific anatomical requirements, non-invasive monitoring of the scaffold integration into an \textit{in vivo} environment, occurrence of the material degradation, and subsequent tissue regeneration are the important aspects for its final translation in BTE [14]. Up to date, computed tomography (CT) is considered the gold standard technique for pre-clinical imaging of bone tissues [15]. In CT imaging, the X-ray attenuation of a material is proportionally related to its atomic number (Z) and density (ρ), thereby the CT signal is high in mineralized tissues (e.g. bone and teeth), while it becomes weak in mainly-water containing tissues (i.e. soft tissues) [16]. For the same reasons, hydrogels show little or no signal on CT images as they consist mainly of water [17]. Enhancement of the CT signal can be achieved by combination with specific contrast agents (CA) [18,19]. To date, gold nanoparticles (AuNPs) has been suggested as promising CA, as they show high X-ray attenuation (5.16 cm$^2$ g$^{-1}$ at 100 keV), biocompatibility and low toxicity. Moreover, AuNPs showed ability to enhance CT contrast of ceramic-based bone substitutes as well as to induce osteoblast differentiation and new bone formation [20–22]. Alternatively to CT imaging, magnetic resonance imaging (MRI) is a valid screening option in BTE. The development of ultrashort echo time (UTE) MRI sequences allows the visualization of bone structures that are otherwise not visible by using conventional MRI sequences [23,24]. On the other hand, due to the high proton content,
hydrogels can be easily visualized and monitored in vivo through conventional T₁-weighted MRI [25].

Despite the huge potential in using GelMA in BTE, in vivo studies that prove its biocompatibility with respect to bone tissues remain unexplored. In this study for the first time, a 3D printed GelMA hydrogel for bone regeneration was designed and tested in a defined in vivo rat animal model (e.g. critical-size condyle defect). Furthermore, considering the advantages of non-invasive follow-up of BTE-dedicated scaffolds, two different imaging strategies were investigated as possible screening tools. The first strategy was based on the enhancement of the CT contrast of GelMA by means of labeling with AuNPs. The second strategy was based on the combination of T₁-weighted and UTE based MRI acquisitions for a dual imaging of GelMA and bone, respectively.

2. Materials and Methods

2.1. Preparation of gelatin methacrylate (GelMA) and GelMA pre-polymer solution

GelMA was synthesized as described elsewhere [26]. Briefly, type A porcine skin gelatin (Sigma-Aldrich, St. Luis, MO) was suspended at final concentration of 10 % (w/v) in phosphate buffer solution (PBS) at 60 °C and stirred until the gelatin was fully dissolved. Afterwards, 0.8 mL methacrylate (Sigma-Aldrich) per gram of gelatin was added under constant stirring. Once the reaction was completed, the mixture was dialyzed against distilled water using 12-14 kDa cut-off dialysis tubing (Spectra/Por 1 Dialysis Membranes, Spectrum Labs, Rancho Dominguez, CA) to remove exciting salts and methacrylic acid. Finally, the solution was lyophilized to generate a white porous foam and stored at -80 °C until further use.

The pre-polymer solution of GelMA was prepared as follows. The photoinitiator 2-Hydroxy-4’-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959, Sigma-Aldrich) was dissolved in 10 mM PBS at 37 °C at concentration of 0.05 % (w/v). After complete dispersion of Irgacure 2959, 5 % (w/v) GelMA was added to the solution to obtain GelMA pre-polymer solution.

2.2. Evaluation of mechanical properties, in vitro cytocompatibility and µCT visibility of GelMA and GelMA-AuNPs bulk hydrogels

To enhance CT contrast of GelMA-based scaffolds, AuNPs (Sigma-Aldrich) stabilized by citrate in 0.1 mM PBS, with different size and concentration were added to GelMA pre-polymer solution. Specifically, different concentrations of AuNPs were calculated as 0.08
mM, 0.16 mM and 0.40 mM. These same concentrations were used for two AuNPs size, i.e. 40 nm and 60 nm.

To assess the influence of AuNPs on the mechanical properties, *in vitro* cytocompatibility, and µCT visibility of GelMA, cylindrical discs of GelMA and GelMA-AuNPs (diameter = 10 mm, thickness = 1 mm) were prepared from pre-polymer solutions. To assess mechanical properties GelMA and GelMA-AuNPs hydrogels were detached from the molds and tested at a rate of 20% strain/min on a DMA Q800 apparatus (TA Instruments). The compressive modulus was determined as the slope of the linear region corresponding to 0-5% strain (n=5).

To evaluate the acute cytotoxic effect of AuNPs size and concentration, GelMA-AuNPs hydrogels (prepared in sterile conditions), were cultured with L929 fibroblasts (mouse C3H/An, ECACC, UK) in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Grand Island, MA), 10% FBS (EuroClone S.p.A., Pero, Italy), and 100 µg/mL Penicillin-Streptomycin (10,000 U/mL, Gibco) at 37°C and 5% CO₂ for 72 hours. Subsequently, CellTiter Cell Proliferation Assay (MTS, Promega, Fitchburg, WI) was performed to measure the metabolic activity colorimetrically (FLUOstar Omega UV/Vis spectrometer, BMG LabTech, Ortenberg, Germany) of the fibroblasts incubated with the GelMA control (i.e. non labeled) and the GelMA-AuNPs.

Radiopacity assessments of the GelMA-AuNPs hydrogels containing different size of AuNPs in different concentrations, were carried out using a Skyscan 1172 (Bruker, Kontich, Belgium) micro CT (µCT) system with the following settings: 40 kV, 250 µA, pixel size of 12.7 µm, exposure time 100 ms, and 1 mm aluminum filter. Acquired files were reconstructed through NRecon (Skyscan) reconstruction software, while assessment of the gray value of the hydrogels was performed using CTAnalyser software (version 1.10.1.0, Skyscan).

2.3. Rheological characterization of GelMA and GelMA-AuNPs pre-polymer solutions

Storage (G’) and loss modulus (G”) of the GelMA pre-polymer were studied with a stress-controlled rheometer (Discovery HR-1, TA Instruments, New Castle, DE). The rheometer was equipped with aluminum parallel plate geometry (40 mm diameter), while the gap between the plates was set to 250 µm. In order to verify the sol-gel transition point of the GelMA and GelMA-AuNPs pre-polymer solutions, rheological measurements were performed at controlled temperature gradient (i.e. from 3 to 40°C), with a heating rate of 2°C/min, a constant strain of 1% and a frequency of 1Hz.
2.4. Layer-by-layer 3D printing of GelMA and GelMA-AuNPs hydrogels

GelMA pre-polymer solution was prepared as previously described, decontaminated by filtering (PES membrane, 0.22 µm), and transferred into the plotting cartridge (Nordson EFD, Westlake, OH), which was kept under sterile conditions. The plotting cartridge was installed on the printing head of a bioplotter (Envisiontec, Gladbeck, Germany) and the GelMA solution was cooled down to 22 °C. GelMA solution was dispensed through a 22G needle, layer by layer with speed of 17 mm/s, forming 0° / 90° strand structures. A CAD file specifying the geometry of the scaffold was used as input to produce the physical model for the apparatus. Cuboids of 20 mm wide and 5 mm thick were fabricated during this process with 220 µm layer thickness and 800 µm strand distance. Later, they were exposed to 12.5 mW/cm² UV light (BlueWave® 75 UV Light Curing Spot Lamp, 365 nm, Torrington, CT) for 60 seconds. Subsequently, each scaffold was punched into discs of 3 mm in diameter (Figure 1). The same 3D printing parameters and steps were followed in the case of GelMA-AuNPs.

Figure 1. Schematic representation of the manufacturing steps followed for the preparation of 3D hydrogels for the in vivo assay. Firstly, cooled GelMA solution was 3D printed through layer-by-layer method. Secondly, the 3D-printed GelMA-based hydrogels were crosslinked by using UV light. Finally, the achieved 3D block was punched in the desired size. The same steps were followed for the production of 3D GelMA-AuNPs hydrogels. The insert is showing an overview of the morphology of 3D scaffolds used for the in vivo study. Notice that the GelMA scaffold appears slightly pink colored due to the presence of the AuNPs.
2.5. Pore and fiber size measurements by scanning electron microscopy (SEM) imaging

SEM imaging was used to determine the fiber size and pore size of 3D printed GelMA hydrogels. To this end, 3D printed GelMA hydrogels were rinsed with PBS and soaked for 5 minutes in Karnovsky fixative (2.5 % glutaraldehyde in 0.1 M sodium cacodylate) at 4 °C. The fixed samples were dehydrated through a series of ethanol solutions (i.e. 70 %, 80 %, 90 %, 100 %), and sputtered with a 7 nm layer of gold. Specimens were examined by using a SEM system (Phenom proX, PIK Instruments, Warsaw, Poland).

2.6. In vivo implantation in a rat defect model

The GelMA-AuNPs formulation which showed best performances in terms of mechanical properties, cytocompatibility and enhancement of the µCT, i.e. GelMA plus 0.16 mM AuNPs 60 nm, was printed as done for the non-labeled GelMA solution and used for further in vivo studies. The animal study was performed after the approval of the Animal Ethics Committee for the care and use of laboratory animals (RU-DEC number 2015-0035) and in agreement with the standards of the Radboud University Medical Center, Nijmegen, The Netherlands. The in vivo study is described according to the ARRIVE guidelines for reporting in vivo experiments [27]. The in vivo study was designed to answer three specific experimental hypothesis: 1) is 3D-printed GelMA scaffold suitable for BTE, hence allowing bone growth instead of adverse reaction (i.e. fibrotic encapsulation of the scaffold and hampering the bone formation); 2) are AuNPs able to enhance the CT contrast of GelMA and allow in vivo monitoring; 3) is MRI a suitable screening tool for the simultaneous monitoring of bone growth and GelMA-based hydrogel degradation.

For the in vivo assay sample size was calculated by using a significance level of 0.05 and power of 80%, while a standard deviation of 7% and effect size of 15% were defined based on values reported in the literature [22]. G*Power software was used for the power calculation [28]. Five samples (n=5) were assigned to two experimental groups (i.e. GelMA and GelMA-AuNPs 0.16 mM, 60 nm) and to two time points (i.e. 4 and 8 weeks), respectively. In order to reduce the number of animals for this experiment both posterior legs of the same animal were used. Therefore, the final study population consisted of 10 healthy male 6-7 weeks old Wistar rats (Rattus Norvegicus, bodyweight: 200-250 g, Charles River Laboratories, Leiden, The Netherlands). The animals were randomly allocated to each time point and to each experimental group. Surgical operations were performed by a single expert operator trained in the standardization of the surgical procedure. For the surgery the animal was anesthetized by inhalation of a mix of isoflurane (Rhodia Organique Fine, Avonmouth, Bristol, UK) and
oxygen and then located in supine position on a heating mat. Both legs were shaved and disinfected with povidone-iodine solution (Betadine, Kuinre, The Netherlands). A longitudinal incision was performed on the medial side of the knee through the skin and the underlying muscle until the femur was reached. Then, the patella was manually dislocated on the lateral side in order to have a clear view of the distal femoral condyle. A dental drill (Astra Tech Elcomed 100, Dentsply Implants, Zoetermeer, The Netherlands) equipped with three different dental burs with increasing diameter up to 3 mm was used to perform a cylindrical defect of 5 mm in depth (Figure SI a-b). During the drilling a sterile saline solution (Fresenius Kabi B.V., Emmer-Compuscum, the Netherlands) was used to cool down the dental bur and to clean the cavity. Sterile gauze was used to control the bleeding. After the defect preparation, pre-printed GelMA and GelMA-AuNPs hydrogels, prepared and kept in sterile condition, were taken through sterile tweezers and inserted into the defect. Due to the difference in color between the hydrogels from the two experimental groups (i.e. GelMA in white and GelMA-AuNPs in pink) the operator could not blindly operate. Once the 3D device was inserted, the patella was moved back to the original position; the muscle and the skin were closed with absorbable sutures (Vicryl® 4.0 Ethicon, Somerville, NY). To decrease post-operative discomfort a subcutaneous injection of painkiller (5 mg/Kg Rimadyl®, Cattle, Capelle a/d Ijssel, The Netherlands) was performed post-surgery. At 4 and 8 weeks from the surgery five animals were sacrificed by CO₂/O₂ inhalation and scanned again with both MRI and CT. Finally, femora were harvested, placed in formalin 10 wt% for 48 h and kept in ethanol 70% until histological assessments.

2.7. Ex vivo μCT

Ex vivo μCT examinations were used for the assessment of the hydrogel degradation and the bone healing. The scans were carried out using the SkyScan1172 (Skyscan) with the following settings: 100 kV, 100 μA, pixel size of 12.7 μm, exposure time 460 ms, and 1 mm aluminum filter. The new bone formation was quantified through CTAnalyser software. To this end, a defined volume of interest (VOI) of 3 mm in diameter and 2.5 mm in dept, and a standardized threshold selection were used. The new bone formation was normalized according to the initial bone volume in the same selected VOI.

2.8. In vivo CT

CT acquisitions were performed on a small animal CT scanner (Inveon Micro-CT/PET, Siemens Medical solution, Knoxville, TN). After the surgery (i.e. Day 0) the animals were kept under general anesthesia (isoflurane/O₂) and located on the supine position on a heating
Chapter 7

mat. At 4 and 8 weeks the animals were scanned right after the sacrifice. Images were recorded with an acquisition time of 6 min, spatial resolution of 30 µm, tungsten anode source (source voltage 80 kV) and exposure time of 1000 ms. Two-dimensional reconstructions of the acquired file were obtained by Inveon Research Workplace software (IRW, Siemens).

2.9. *In vivo* MRI

MRI acquisitions were performed on a 11.7 T MRI system (Biospec, Bruker, Germany) equipped with a $^1$H volume coil with inner diameter of 40 mm. Images were acquired by using T1-weighted rapid acquisition with relaxation enhancement (RARE) followed by and 2D-UTE sequence with the following scan parameters. For the T1-RARE: echo time (TE) = 9 ms, repetition time (TR) = 900 ms, RARE factor = 4, matrix size = 512 x 512 x 512, image resolution = 0.098 x 0.098 x 0.098 mm$^3$, averages = 4, acquisition time (TA) = 5.45 min. For the UTE: TE = 0.5 ms, TR = 5 ms, FA = 15°, matrix size = 256 x 256 x 256, slice thickness = 0.8 mm, image resolution = 0.234 x 0.234 mm$^2$, number of slice = 15, bandwidth = 200 kHz, averages = 1, acquisition time (TA) = 1.03 min. After the surgery (i.e. Day 0) the animals were kept under anesthesia by inhalation of isoflurane/O$_2$ and located on the supine position. Body temperature and breathing were constantly monitored. At 4 and 8 weeks the animals were scanned right after the sacrifice. One by one each leg was immobilized inside the coil and scanned. The obtained images were reconstructed by using MRIcro software (Smith Micro software, Aliso Viejo, CA). MRIcro was also used to define the size of the scaffold.

2.10. Histology and Histomorphometry

Specimens were decalcified in a solution of 10 wt% ethylenediaminetetraacetic acid (EDTA, pH 7.2) for two weeks under stirring, dehydrated in a gradual ethanol series (from 70% to 100%) and then embedded in paraffin. Three sections (4 µm in thickness at 100 µm of interdistance) were cut along the axial direction of the femur by using a microtome (RM2165, Leica Microsystems, Rijswijk, the Netherlands). Afterwards, sections were stained with Hematoxylin/Eosin (H/E) and with trichrome Elastic van Gieson (EVG). Images were acquired by using an automated light microscope (VisionTek, Sakura Finetek Europe B.V., Leiden, The Netherlands) with X10 magnification.

2.11. Statistical analysis

Samples were tested in quintet if otherwise indicated, and data are reported in mean value +/- standard deviation. Statistical analysis was performed using unpaired t-test for bone quantification based on µCT acquisitions, and two-way ANOVA followed by Sidak’s
multiple comparison test, for the metabolic activity and compressive modulus. Statistical differences were computed through Prism software (v5.00, GraphPad, San Diego, CA). Difference was considered statistically significant if \( p < 0.05 \) (*), \( p < 0.01 \) (**), \( p < 0.0003 \) (***) and \( p < 0.0001 \) (****).

3. Results

3.1. Characterization of GelMA-AuNPs bulk hydrogel

To enhance CT contrast of GelMA-based scaffolds AuNPs, with different size and concentration, were added to GelMA pre-polymer solution. No significant reduction in cellular metabolic activity for AuNPs concentrations below 0.16 mM, for both 40 nm and 60 nm sized nanoparticles, was observed (Figure 2a). Mechanical testing on GelMA-AuNPs in the concentration range they were biocompatible was performed subsequently (Figure 2b). Results indicated that the addition of the both 40 nm and 60 nm AuNPs did not significantly affect the compressive modulus of the hydrogels. Afterwards, both GelMA and GelMA-AuNPs hydrogels were scanned and compared regarding the changes in x-ray attenuation (Figure 2c). The histogram of the gray values distribution showed a shift in the gray values for the hydrogels compositions labelled with the AuNPs. Particularly, a distinctive shift in gray value and sharper curve with increased frequency was attained when 60 nm AuNPs were used with concentration of 0.16 mM. Therefore, the GelMA-AuNPs formulation containing 0.16 mM 60 nm AuNPs was selected as showing the best performance in terms of mechanical properties, cytompatibility and enhanced radiopacity. Such GelMA-AuNPs hydrogel was used to prepare 3D printed scaffold for the \textit{in vivo} assay.

3.2. 3D printing of GelMA and GelMA-AuNPs hydrogels

To understand the thermoresponsive properties of the GelMA hydrogels and the effect of AuNPs addition on thermoresponsivity, the rheological properties of the GelMA and GelMA-AuNPs pre-polymer solutions were investigated. A rapid decrease in \( G' \) around 18°C was observed, which also corresponded to the intersection point between \( G'' \) and \( G' \) for both GelMA and GelMA-Au pre-polymer solutions (Figure 3a). These findings indicate that pre-polymer solutions, at 5% GelMA (w/v) concentration, exhibited gel-like properties at temperatures below 18°C and liquid-like properties above 18°C. Therefore, printing temperature of both hydrogels was set to 22°C, which was considered the temperature that forms a weak gel and gives stable rheological properties just before the gelation occurs (Figure 3b, 3c, 3e, and 3f). In Figure 3d and Figure 3g, SEM images of high-resolution 3D printed dried GelMA and GelMA-AuNPs hydrogels were shown. Based on SEM observation
it was possible to observe that the scaffolds had regular pores, i.e. 285 ± 35 µm, and strands, i.e. 100 ± 15 µm.

Figure 2. Evaluation of in vitro cytocompatibility, mechanical properties, and µCT visibility of GelMA-AuNPs hydrogels. In (a) evaluation of the effect of AuNPs size and concentration on cell metabolic activity (*: p<0.01, ***: p<0.003). Note that at 0.08 mM and 0.16 mM, for both 40 nm and 60 nm size, no differences were observed when compared to the control. In (b) the assessment of the effect of AuNPs size and concentration on mechanical properties of the GelMA hydrogel in cytocompatible AuNPs concentration range; no differences between the experimental groups were observed. In (c) the effect of AuNPs size and concentration on radiopacity of GelMA-AuNPs hydrogels. In blue the composition that showed the best x-ray attenuation value.
Figure 3. Sol-gel transition of GelMA and morphology of 3D printed GelMA hydrogels. In (a) the storage modulus ($G'$) and the loss modulus ($G''$) were plotted as a function of temperature. The representative image of GelMA solution (b) and GelMA-AuNPs solution (e) at 37°C, i.e. the solution state of GelMA and GelMA-AuNPs, while in (c) and (f) the representative image of GelMA solution and GelMA-AuNPs at 22°C, respectively, i.e. the gel state of both hydrogels. In (d) and (g) SEM image of 3D printed GelMA hydrogels and GelMA-AuNPs hydrogels (magnification 250X). Both chemically dried hydrogels had regular pores of around 285 µm and strands with a thickness of 100 µm.

3.3. Longitudinal evaluation of 3D printed GelMA and GelMA-AuNPs implanted in rat model

To assess the longitudinal in vivo biological performance of the 3D GelMA and GelMA-AuNPs, pre-printed hydrogels were inserted into defects performed in rat leg. After the surgery, the animals were recovering quickly, were moving in an unrestricted manner, and no post-surgical complications were observed. Assessment of the primary outcomes showed good handling properties for both type of 3D hydrogels. Specifically, scaffolds could be easily handled and inserted in the defect by using a surgical tweezers. Furthermore, once the hydrogels were placed in the defect, by visual inspection it was possible to notice that the scaffolds were quickly perfused by bloodstream.

Estimation of bone regeneration over time was performed qualitatively and quantitatively via µCT (Figure 4). MicroCT images reconstructions showed that the size of the initial defect was gradually decreased at 4 weeks and 8 weeks followed by a subsequent new bone ingrowth (Figure 4a). Consistently, bone quantification showed a gradual increase in bone of 15% at 4 weeks and 28% at 8 weeks (Figure 4b).
Figure 4. Longitudinal evaluation of 3D printed GelMA. µCT tomograms of the rat condyle (a) and the implanted GelMA hydrogels at day 0, 4 weeks, and 8 weeks, GelMA-AuNPs at day 0, 4 weeks, and 8 weeks along the transverse plane. Yellow arrows indicate the defect area in the condyle. In (b) newly bone formation quantification in the defect area assessed from µCT scans. No differences between GelMA and GelMA-AuNPs were observed over time.

Histological assessment proved the optimal integration between the hydrogels and surrounding bone tissue (Figure 5). No fibrotic encapsulation of the hydrogel or extensive inflammation reactions was observed. The original 3D morphology of the scaffold persisted for the full experimental time and micro (< 10 µm) and macro (> 10 µm) porous structures could be still identified (Figure 5a). The new bone formation was mainly observed on the external side of the implanted scaffolds. However, bone deposition was also observed in the pores of the scaffold in correspondence of the big porous structures (Figure 5b). Such findings suggested the occurrence of enough nutrients efflux through all the scaffolds that promoted bone tissue ingrowth in the deepest area of the implant. In 6 out of 10 samples the bone
regeneration occurred in the central part of the implant, appeared to follow the square geometrical morphology of the 3D GelMA (Figure 5b).

**Figure 5.** Histological section of femoral condyle at 8 weeks post-surgery. In (a) overview of a histological section of the condyle filled with GelMA and GelMA-AuNPs, respectively. The sections were stained with EVG and acquired with x5 magnification. In (b) the enlarged view of the rectangles. The same section is reported after staining with EVG (on the top) and HE (on the bottom). Blue arrows indicate bone growing in contact with the scaffold, green arrows indicate expected onset of new bone following scaffold morphology. Magnification 20x.
3.4. In vivo monitoring by CT and MRI

The CT tomograms over 8 weeks monitoring for both GelMA and GelMA-AuNPs hydrogels, show that both scaffolds were only barely visible on CT acquisition performed after the surgery; still at this stage the porous structures of the scaffold could be recognized (Figure 6). However, the used AuNPs did not provide sufficient radiopacity enhancement of the CT contrast in vivo when compared to the GelMA control scaffolds, as no differences between the labeled and non-labeled hydrogels were observed. The morphology of the 3D printed scaffold became less distinguishable at 4 weeks post surgery.

![Figure 6](image)

*Figure 6. In vivo CT images of the GelMA/GelMA-AuNPs hydrogels implanted in rat condyle. Notice that hydrogels were visible only after surgery, red arrows indicate the porous structures of the 3D printed scaffolds.*

In Figure 7, the T₁-weighted and UTE MRI acquisitions of the implanted 3D GelMA hydrogels are shown at day zero, 4 weeks, and 8 weeks, respectively. On the T₁-weighted acquisition 3D GelMA scaffolds were clearly visible for the full experimental time. Furthermore, through T₁-weighted MR images it was possible to visualize the fine 3D morphology of the scaffold including the square-shaped porous structure. Over time, the size of the porous structure resulted to be smaller. MRI based on T₁-weighted acquisition could be also used for the quantitative assessment of the size of the scaffolds that resulted to be decreased over time (Figure 7b). As expected T₁-weighted acquisition could not provide
signal from the bone phase which resulted in a dark area (Figure 7a). Differently, MRI acquisitions based on UTE were able to give more information about the bone structures rather than the implanted GelMA hydrogel. Specifically, on UTE-MR images bone mineral phase was clearly recognizable over all the experimental time, hence allowing the visualization of the pre-existing and of the newly formed bone in the proximity of the implanted scaffold. Also, it could be observed that the fine morphology of the hydrogels was lost.

Figure 7. In (a) in vivo monitoring of 3D printed GelMA hydrogels via MRI based on T1-weighted (on the top) and UTE acquisition (on the bottom). Yellow arrows indicate the 3D printed GelMA hydrogels, red arrows indicate the pores in the 3D printed structure, blue arrows indicate the bone around the GelMA hydrogels. Note that only T1-weighted picture can give a clear view of the scaffold, while UTE can be used to visualize bone tissue. In (b) quantification of the scaffold size performed based on T1-weighted MRI. Note that over time the diameter was decreasing.
4. Discussion

The integration between BTE constructs and host tissue can be improved using hydrogels with distributed and interconnected pores [12,13]. However, a lack of proper porosity may cause insufficient vascularization into the defect site and lead to necrosis [29]. In this study, the possibility to use a 3D printing manufacturing process to obtain a porous hydrogel scaffold that could be used for bone regeneration was investigated in a rat condyle defect model, in addition to investigating its optimal imaging properties.

GelMA-based hydrogels can serve as 3D model systems for different tissue engineering applications as its chemical, biological and mechanical properties can easily be adjusted for the application of interest [30]. Our recent study showed the osteogenic differentiation of mesenchymal stem cells and the homogeneous calcification through the GelMA hydrogels [6]. After testing hydrogels containing different concentrations of GelMA, the composition with 5% (w/v) ratio showed higher MSC attachment. Similarly, more homogeneous and significantly higher calcium deposition was also observed when 5% GelMA hydrogels were used. These results supported our choice for GelMA as suitable material for BTE and also indicated the ideal concentration that needed to be used in further studies (i.e. 5% w/v).

Bone is a vascular tissue, thereby the integration of microchannels into GelMA hydrogels can further stimulate vascular network formation and increase the perfusion through the hydrogel [31]. Although microchannels can be created using 3D printing technology, 3D printing of GelMA is a challenging process. The current strategies for creating microchannels into GelMA hydrogels are blending with other biopolymers, pre-crosslinking, supporting with thermoplastic scaffolds, and thermoresponsive-based approach [9,32–34]. In previous studies, different polymers were added into GelMA hydrogels prior to the printing both to increase the viscosity of the pre-polymer solution or to cage the GelMA into a crosslinked polymer network [9,32]. For direct printing GelMA, without addition of different polymers or support constructs, different approaches were suggested by Bertassoni et al. [33] and Billiet et al. [34], respectively. Specifically, Bertassoni et al. suggested pre-polymerizing the GelMA hydrogel prior to the crosslinking. However, they indicated that this approach was not suitable for forming continuous well-structured fibers for the GelMA concentrations below 7% (w/v). On the other hand, Billiet et al. proposed to use the thermoresponsive characteristic of the GelMA hydrogel and adjusted the extrusion temperature to sol-gel temperature of the GelMA hydrogels to attain continuous fibers. However, they also implied that the printing potential was significantly limited, especially for hydrogels with 5% (w/v) concentration. The printing procedure suggested by Billiet et al., which utilizes the thermoresponsive characteristic of
GelMA, was an advantageous method for our study as this technique did not require further modification in printing system, and allowed us to print solely GelMA with high resolution. The GelMA 5% hydrogel solution used in this study, showed sol-gel temperature at significantly lower temperatures (i.e 18°C) with increased G’ and G” values which were sufficient to create continuous fibers and 3D constructs even when 5% GelMA concentrations were used. Besides, with further optimization on printing conditions suggested by Billiet et al., the 3D printing parameters for 5% GelMA hydrogels was achieved with 150 µm resolution, which certainly represents an improvement in resolution when compared to similar studies [35]. The achieved printing resolution obtained in this study warrants for further applications with definitive scaffold architecture for GelMA hydrogels.

Implantation in vivo of the 3D GelMA and GelMA-AuNPs demonstrated the occurrence of new bone formation over all the hydrogel surface suggesting optimal biological compatibility for both used materials. Fine integration of the newly formed bone tissue onto the surface of the scaffolds with and without AuNPs suggested potential osteoconductive characteristics [36]. The presence of viable tissues, also in the central part of the scaffold, suggested an appropriate nutrient supply through the scaffolds matrix. It can be expected that full ossification throughout the scaffold would even occur, at longer implantation times (>16 weeks), however, this was beyond the scope of the current study. In addition, the ossification process could further be enhanced by functionalizing the scaffold with added mineral, surface modification with attachment proteins, or simply by adding a BMP release system, to result in a “theranostic” medical device. Still, the current result was promising, showing the evident promotion of a defined bone ingrowth according to the microporous structure of the implanted scaffolds. The efflux of nutrients could have played an important role in this finding. Bulk hydrogels consist of an intrinsic micro-porosity nature [31]. However, such network may not be fully permeable to the nutrient efflux limiting the new bone formation mostly on bone-hydrogel junctions [21]. Differently, the high and interconnected porosity of 3D GelMA and GelMA-AuNPs hydrogels, promoted a more extend bone formation, hence supporting the use of 3D printing in manufacturing bone dedicated scaffolds. No differences in new bone formation between GelMA with and without AuNPs were observed in the current study. AuNps have been described to play a crucial role in vitro in the osteogenic differentiation of mesenchymal stem cells through the P38 mitogen-activated protein kinase pathway (MAPK) pathway [20]. Moreover, bulk GelMA combined with 14 µg of AuNPs, 27 nm in diameter, and implanted in critical-size defect performed in the skull of healthy rabbit has been shown to induce more bone formation (i.e. > 12%) when compared to GelMA without AuNPs [21].
These previous findings are in contrast with the outcomes of this study, hence questioning the ability of AuNPs to induce bone formation.

Besides manufacturing a suitable scaffold for BTE, non-invasive monitoring strategies that can allow the assessment of its integration and degradation in vivo was another important aspect of our study. Computed tomography is the most commonly used modality imaging for hard tissues as this technique provides high-resolution 3D anatomical information [37–39]. Therefore, CT was used in various studies to quantify and demonstrate bone regeneration in the performed defect area. Even though CT is the gold standard for hard tissue imaging, its translation to BTE applications is hampered by some challenges. Specifically, in case of hydrogel-based scaffolds, CT imaging results difficult due to their high water content. Thus, even though hydrogels have been used for BTE applications, CT was only used to image the newly formed bone [40-42] To overcome the challenge of imaging GelMA hydrogel via CT, it was hypothesized that the combination of GelMA with specific CA would improve the overall X-ray attenuation of the scaffold. Previously, different iodinated hydrogel formulations were already studied in embolization of blood vessels applications, nucleus pulposus replacements and as fiducial markers in gynecologic-cancer patients [43–45]. Hertig et al. [17], suggested iodixanol combined fibrin hydrogels for endodontic applications. Their ex vivo preliminary experiments in human teeth showed that the radiopacity of the fibrin hydrogels was only sufficient for a correct localization of the implant. Nonetheless, it was indicated that the swelling characteristics of hydrogels changed dramatically due to the addition of the iodixanol CA [17]. Among different CAs, AuNPs have been suggested as promising candidate as they show high X-ray attenuation depending on their size and concentration [46]. Our in vitro results showed that AuNPs could be used to enhance the CT signal of GelMA only to a certain extent, which resulted not enough for the in vivo monitoring. Most likely higher AuNPs concentrations (> 0.4 mM) could give better CT attenuation also in vivo, however this would have also dramatically affected the mechanical and biological properties of the GelMA. Therefore, CT imaging of GelMA hydrogel still remains a challenging issue.

MRI is the leading non-invasive screening technique as it is able to provide 3D anatomical and functional information especially in soft tissues [47]. To date, MR- imaging, spectroscopy (MRS), and elastography (MRE) have been used to provide respectively high-resolution images, assessment in changes of the molecular structures, and measurement of the mechanical properties of cartilage-dedicated TE constructs [48,49]. Furthermore,T1- and T2-weighted MRI was used for the non-invasive monitoring of hydrogel-based scaffolds
integration after implantation in trapezius muscle in rabbit model, and for the assessment of
the impact of injectable hydrogels on the ventricular remodeling after myocardial infarction
[25]. Recent developments of UTE MRI sequences allow the visualization also of hard tissues
(e.g. bone and teeth) that are otherwise showing little or no signal on conventional MRI
sequences [50]. Therefore, ultrashort acquisition-based MRI is expected to have a strong
impact also in the field of BTE. Specifically, we hypothesized its use for the non-invasive
longitudinal follow up of bone regeneration when dedicated 3D printed hydrogels are used.
The MRI workflow acquisition was based on a T1-weighted sequence, which was used to
obtain morphological reconstruction of the 3D GelMA, followed by an UTE sequence, which
was used to obtain morphological information of the pre-existing and the newly formed bone.
When comparing the imaging acquisitions performed by CT with the one obtained through
the MRI workflow proposed in this study, the latter method resulted to be superior in terms of
achievable information related to both scaffold degradation and new bone formation. Recent
advances on MRI based on chemical exchange saturation transfer (CEST) showed the
feasibility to specifically assess in vivo differences in hydrogels chemical composition [51].
On the other hand, high resolution UTE-based MRI of bone tissues has been already applied
in clinics, and is theoretically available also for BTE application [52]. Therefore, this study
can be used as proof-of-concept for further pre-clinical imaging investigations in BTE.

5. Conclusions
The longitudinal monitoring of TE constructs in vivo is of vital importance for translational
research. Thus, the design of new TE scaffolds should not only address the need of
biomaterials with optimal biological properties but also to focus on the imaging strategies
required to follow its fate once implanted in vivo. In this study, a 3D printed GelMA scaffold
with high porosity and interconnectivity was designed and tested in vivo. This GelMA showed
optimal biological properties and evident osteoconductive features. Furthermore, with these
scaffolds an MRI workflow consisting of T1-weighted MRI and UTE-MRI acquisition is
superior to CT imaging to visualize both scaffold and bone tissue.

6. References
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Chapter 7


Chapter 8

Summary, Closing and Future Perspectives
1. Summary

Every year more than two million of patients worldwide undergo bone transplantation due to bone diseases and trauma. The gold standard treatment is the transplantation of autologous bone. Nevertheless, a lack of transplantable bone and high morbidity risks associated with this procedure, increase the demand for suitable synthetic materials. The developments in this research field have resulted in several biocompatible materials that can be implanted in the body through minimally invasive surgery while promoting a faster bone healing and repair. The translation of such biomaterials to clinical use relies on pre-clinical in vivo animal models that are used to test their tissue integration properties.

One of the current challenges in the bone regeneration field is the development of less invasive pre-clinical imaging strategies that can be used to understand the biological behavior of biomaterials after installation into the body. An ideal imaging modality can give longitudinal quantitative and qualitative high-resolution information about the biomaterial integration and the subsequent new tissue replacement.

A general introduction to bone regeneration, biomaterials, and imaging challenges was provided in Chapter 1. Among all the pre-clinical imaging modalities, magnetic resonance imaging (MRI) is a technique that does not involve the use of ionizing radiations. Furthermore, MRI allows high spatial resolution, high soft tissue contrast and specificity, and unlimited penetration depth. However, MRI of hard tissues, such as bone and teeth, has been always challenging because of the low proton content in these tissues as well as a very short transverse relaxation ($T_2$). New MRI sequences, such as sweep imaging with Fourier transformation (SWIFT), ultra-short echo time (UTE), and zero echo time (ZTE), have been developed to image tissues with short $T_2$. Within Chapter 2, all MRI sequences used for the detection of hard tissues, such as bone and dental tissues, are reviewed. The main goal was to give a broad overview of the potential clinical and preclinical applications of these MRI sequences and on the future direction of such techniques. Furthermore, an insight in MRI of biomaterials for hard tissue application was also given. Evidently, Chapter 2 indicates that proton-based MRI by ultrashort echo time sequences is an excellent method to image bone, teeth, and biomaterials for bone and dental applications.

To date, different materials have been developed for bone and dental applications. Calcium phosphate cement (CPC) is a typical example as it shows optimal biological and handling properties. However, the high similarity between CPC and the natural osseous phase results in poor image contrast in most of the available in vivo imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI). To date, it remains still unclear if
CPC can be detected by MRI after insertion in vivo as a dental agent. Therefore, the aim of Chapter 3 was to optimize high field ZTE imaging to enable the visualization of a new CPC formulation implanted in teeth and to apply this in the assessment of its decomposition in vivo. To this end, CPC was implanted in three human and three goat teeth ex vivo and in three goat teeth in vivo. An ultra-short echo time sequence with multiple flip angles and echo times was applied at 11.7 T to measure $T_1$ and $T_2^*$ values of CPC, enamel, and dentin. Teeth with CPC were imaged by an optimized ZTE sequence. Goat teeth implanted with CPC in vivo were imaged after seven weeks ex vivo. $T_2^*$ relaxation of implanted CPC, dentin and enamel was better fitted by a model assuming a Gaussian rather than a Lorentzian distribution. For CPC and human enamel and dentin, the average $T_2^*$ was 273 ± 19 μs, 562 ± 221 μs and 476 ± 147 μs respectively; the average $T_2$ was 1234 ± 27 μs, 963 ± 151 μs and 577 ± 41 μs respectively and the average $T_1$ was 1065 ± 45 ms, 972 ± 40 ms and 903 ± 7 ms respectively. In ZTE images, CPC had a higher signal-to-noise-ratio than dentin and enamel due to a higher water content. Seven weeks after in vivo implantation the CPC filled lesions showed less homogeneous structures, a lower $T_1$ value and $T_2^*$ separated into two components. MRI by ZTE provided excellent contrast for CPC in teeth and allows to follow its degradation over time.

Still, the combination of CPC with specific contrast agents (CAs) results to be the better option for in vivo follow-up. Specifically, by using CAs it is possible to enhance the contrast of the CPC composition not only for MRI acquisition but also for other imaging modalities, such as CT. Furthermore, by combining diagnostic with therapeutic agents (i.e. theragnostic) it is possible to deliver materials with improved imaging and biological features. In Chapter 4, a theranostic agent for vital dental pulp treatment was designed. Specifically, a pulp capping agent that could show appropriate biological performance, excellent handling properties, and good imaging contrast was developed. Calcium phosphate-based composites are a potentially ideal candidate for pulp treatment, regardless their poor imaging contrast and poor dentino-inductive properties. Therefore, firstly the imaging properties of the CPC were improved by using a core-shell structured dual contrast agent (csDCA) consisting of superparamagnetic iron oxide (SPIO) and colloidal gold, as MRI and CT contrast agent respectively. Second, biological properties were implemented by using a dentinogenic factor (i.e. bone morphogenetic protein 2, BMP-2). The obtained CPC/csDCA/BMP-2 composite was tested in vivo, as direct pulp capping agent, in a male Habsi goat incisor model. Our outcomes showed no relevant alteration of the handling and mechanical properties (e.g.
setting time, injectability, and compressive strength) by the incorporation of csDCA particles. In vivo results proved MRI contrast enhancement up to 7 weeks post-surgery. Incisors treated with BMP-2 showed improved tertiary dentin deposition as well as faster cement degradation as measured by μCT assessment. In conclusion, the presented theranostic agent matches the imaging and regenerative requirements for pulp capping applications.

CPCs have commonly used also for bone repair. For accurate identification and localization during and after implantation in vivo, a composition with enhanced image contrast is needed for any potential bone applications. In Chapter 5, CPC was labeled with perfluoro-15-crown-5-ether-loaded (PFCE) poly(lactic-co-glycolic acid) nanoparticles (hydrodynamic radius 100 nm) and gold nanoparticles (diameter 40 nm), as $^{19}$F MRI and CT contrast agents respectively. The resulting CPC/PFCE/gold composite was implanted in a rat model for in vivo longitudinal imaging. Our findings showed that the incorporation of the two types of different nanoparticles did result in adequate handling properties of the cement. Qualitative and quantitative long-term assessment of CPC/PFCE/gold degradation was achieved in vivo and correlated to the new bone formation. Finally, no adverse biological effects on the bone tissue were observed via histology. In conclusion, an easy and efficient strategy for following CPC implantation and degradation in vivo was developed. As all materials used are biocompatible, this CPC/PFCE/gold composite is clinically applicable.

In Chapter 6, we designed a CPC construct with gadolinium-oxide nanoparticles incorporated to act as an MRI/CT multimodal contrast agent. The gadolinium(III) oxide nanoparticles were synthesized via the polyol method and surface functionalized with a bisphosphonate (BP) derivative to give a construct (GBCAs-BP) with a strong affinity towards calcium phosphate. The CPC-GBCAs-BP functional material was longitudinally monitored after in vivo implantation in a rat model (i.e. condyle defect). The synthetic method applied to produce nanoparticles that are stable in aqueous solution (hydrodynamic diameter 70 nm) with significant T₁ and T₂ relaxivity was demonstrated in both clinical 3 T and preclinical 11.7 T MR imaging systems. The combination of GBACs-BP nanoparticles with CPC gives an injectable material with handling properties that are suitable for clinical applications. The BP functionalization prolonged the residence of the contrast agent within the CPC that allowed long-term follow-up imaging studies. The useful contrast agent properties combined with appropriate biological response indicates further investigation of the novel bone substitute hybrid material towards clinical application.

Beside CPCs other materials have been proposed for BTE. Gelatin methacrylate (GelMA) is an inexpensive, photocrosslinkable, cell-responsive hydrogel. 3D printing GelMA was
previously demonstrated to promote cell penetration, vascular in-growth, and the diffusion of nutrients due to an evenly distributed and interconnected porosity. In Chapter 7, a printed GelMA hydrogel was designed and tested in vivo for BTE applications. Furthermore, two imaging strategies for the non-invasive monitoring of the scaffold degradation in vivo were investigated: i.e. 1) combination of GelMA with gold nanoparticles (AuNPs) for computed tomography (CT) imaging; 2) T1-weighted and ultrashort echo time (UTE) magnetic resonance imaging (MRI). To this end, 3D printed GelMA hydrogels were implanted in cylindrical defect created into the condyle of 6/7 weeks-old Wistar rats. GelMA showed to form a gel-like structure when it was cooled down to 22°C. The in vivo assay proved excellent biological properties for the 3D printed GelMA scaffolds and no signs of fibrotic encapsulation or inhibition of the bone formation as estimated via histology and micro-CT. Imaging assessment showed no feasible enhancement of the GelMA CT contrast by using AuNPs, while T1-weighted and UTE MRI can give more morphological information for both GelMA and bone tissue. In conclusion, the 3D printed GelMA designed in Chapter 7 was proven to be a good candidate scaffold material for BTE purposes, while MRI is suggested as optimal technique for its non-invasive longitudinal monitoring.

2. Closing remarks and future perspectives
This thesis aimed to investigate pre-clinical imaging strategies for the in vivo monitoring of specific biomaterials as used for the restoration of bone and teeth. Although CT remains the commonly used imaging modality for hard tissues, within this thesis MRI was considered as promising alternative candidate. For this purpose, the first main focus was the investigation of newly developed ultrashort echo time MRI sequences for the imaging of tissues and biomaterials characterized by very low water content. Nevertheless, with both CT and MRI modalities, the use of dedicated CAs was requested, especially for the in vivo monitoring of calcium phosphate-based composites. For this reason, CPC was combined with different types of CA (i.e. SPIO, Gadolinium, PFC) and tested in in vivo animal models for both dental and bone applications. Specifically, CAs were combined with bone-specific growth factors (i.e. BMP-2), incorporated in a CPC-dedicated carrier (i.e. silica carrier, PLGA nanoparticles) and functionalized with CPC-targeting compounds (i.e. bisphosphonate derivatives). Each contrast-enhanced CPC composition allowed long-term non-invasive in vivo follow-up. Therefore, with this thesis, a wide platform of visible materials for bone and dental applications was provided. Each composition presented specific strength and weaknesses, but still fulfilling all the biological requirements and thereby available for further investigations.
as demanded prior their clinical use. Furthermore, this thesis showed that the choice of the imaging modality and the need of CAs depends on the specific type of biomaterial. For instance, in case of hydrogel-based constructs, CAs were not essential, while MRI was found to be superior to CT.

Despite the successful achievements of this thesis, still many challenges exist. Although MRI of hard tissues and CPC through ultrashort echo time sequences, such as ZTE and UTE, is an established method, optimization of the acquisition protocols is still required. This optimization should focus on shortening the acquisition time, which is currently relatively long for the imaging of dental tissues. When CAs are used, easy and fast methods to quantify the achieved signal yet need to be developed. An in vivo highly-sensitive quantitative evaluation of the CA present in the implanted CPC will allow for a more precise understanding of CPC degradation over time. Furthermore, signal quantification is essential, especially when the combination of CPC with CAs results in typical MR imaging artifacts. For instance, such artifacts were observed when SPIO and Gadolinium were combined with CPC and acquired through ZTE sequences. Although it was always possible to visually recognize a decrease in CAs and the subsequent signal intensity, a quantitative evaluation was found to be very challenging. In view of this, quantitative susceptibility mapping based on MRI phase images can be a feasible strategy. It is known that CAs with different paramagnetic properties can affect the uniformity of the applied magnetic field (B₀). Phase images give information about local frequency distributions, which depend on the bulk magnetic susceptibility of the different tissues. Therefore, MRI phase image acquisitions can be used to quantify the imaging artifact as caused by the use of CA. Alternatively, T1 mapping based on UTE acquisition with variable flip angles (VFA) is also suggested for further investigation. The VFA-UTE method relies on MRI acquisition with multiple flip angles and can be used for the assessment of the relaxation properties of tissues with extremely fast signal decay.

To date, MRI seems the only technique that allows the non-invasive imaging of the tissue regeneration process. Theoretically, MRI can be used to image cell migration into the scaffold, the morphology and degradation of the scaffold, and the final biological response of the host tissue. However, the use of MRI in bone tissue engineering is still at an early stage and more investigations are required. Moreover, the use of MRI in dentistry is still considered a long-term scenario especially due to the high costs associated with this technique. This thesis proved the huge potential of using MRI as a dental screening tool, as MRI permits the visualization of soft and hard dental tissues simultaneously and to assess their chemical
composition. Consequently, the development of MRI-visible materials is needed and their further investigation for dental applications is warranted.

A screening approach based on different imaging modalities, i.e. CT/MRI, was also one of the topics in this thesis. A multimodal imaging approach is able to give complementary information as every limitation of one imaging modality can be potentially overcome by the other one. However, the lack of an integrated MRI/CT machine makes the imaging acquisition laborious and the interpretation of the data not always easy. Considering the technical challenge in building an MRI/CT scanner, a standardized and automated post-processing imaging approach is instead suggested for future studies.

Other considerations can still be made on the use of CAs. Different types of MRI-based CAs were successfully investigated in this thesis, yet still many other options remain unexplored. Metalloproteins (e.g. hemoglobin and ferritin), which have per se paramagnetic properties, are considered valid candidates for the development of a new generation of safe MRI probes for biomedical applications. In addition, it has to be noticed that the CA can have a significant effect on the properties of a biomaterial. For example, the incorporation of any new component in a calcium phosphate matrix, like CPC, will unavoidably interact with the crystallization process and change the handling properties of the final composition. On the other hand, although the optimal biological properties, most of the current forms of CPC are not showing osteoinductivity. Therefore, dedicated CAs have to be developed for a wide range of different biomaterials as used for dental restorations and bone regeneration, which have proven not to affect the final biomaterial properties.

The final consideration is dealing with the safety requirements of the used CAs. All the CAs presented in this thesis are already described as biocompatible and were used in relatively low concentrations, hence considered compatible for bone and dental tissues applications. However, it is known that nanoparticles can accumulate in specific organs (e.g. spleen, liver) instead of being excreted from the body. The assessment of the biodistribution of the CAs and the occurrence of any tissue accumulation was out of the scope of this thesis but is recommended in further studies. Such information will help in a better understanding of the fate of the CAs once they are released from the implanted material and solve any safety concerns about their use. Strong prove about the biological compatibility of the used materials will facilitate and speed up their clinical application.
Chapter 9

Samenvatting, Slotopmerkingen en Toekomstperspectieven
1. Samenvatting

Jaarlijks ondergaan er meer dan 2 miljoen patiënt een bottransplantatie als gevolg van ziekte of trauma. De behandeling die daarbij als *gouden standaard* geldt, is een transplantatie van autolog donorbot. Er bestaat echter een gebrek aan transplanteerbaar bots. Daarnaast brengt een dergelijke procedure risico op morbiditeit met zich mee. Hierdoor bestaat er een toenemende vraag naar geschikte synthetische materialen. Onderzoek heeft verschillende biocompatibele materialen opgeleverd, die geïmplanteerd kunnen worden door middel van minimaal invasieve operatie technieken en die resulteren in een snellere botgenezing. Het translateren van dergelijke biomaterialen van onderzoek naar klinisch bruikbare producten, maakt het noodzakelijk om pre-klinische *in vivo* modellen te gebruiken, om zo de weefselintegratie te kunnen bestuderen.

Een van de uitdagingen in het veld van botregeneratie, is de ontwikkeling van laag invasieve, pre-klinische beeldvorming, om daarmee het biologisch gedrag van de biomaterialen na plaatsing in het lichaam beter te kunnen begrijpen. Een optimale beeldvormende techniek kan longitudinaal en met hoge resolutie zowel kwantitatieve als kwalitatieve informatie opleveren over de integratie van het biomateriaal en vervolgens over het weefselherstel.

Een algemene inleiding met betrekking tot botregeneratie, biomaterialen en uitdagingen in de bijbehorende beeldvorming wordt gegeven in *Hoofdstuk 1*. Van alle manieren van pre-klinische beeldvorming, is magnetische resonantie (*magnetic resonance imaging; MRI*) een techniek die geen gebruik maakt van ioniserende straling. MRI kent een hoge resolutie, een goed zacht weefsel contrast, en een oneindige penetratie diepe. MRI van de harde weefsels, zoals botten en tanden, is echter nog steeds uitdagend vanwege het lage aantal protonen in deze weefsels en de zeer korte transverse relaxatie ($T_2$). Nieuwe MRI sequenties, zoals de zogenaamde *sweep imaging with Fourier transformation* (SWIFT), *ultra-short echo time* (UTE) en *zero echo time* (ZTE) sequentie, zijn specifiek ontwikkeld om weefsels met een dergelijke korte $T_2$ af te kunnen beelden. In *Hoofdstuk 2* wordt een overzicht gegeven van alle MRI-sequenties die gebruikt kunnen worden voor de harde weefsels. Het doel was om een zeer breed overzicht te geven van de potentiële klinische en pre-klinische toepassingen van deze MRI-sequenties, en daarnaast over de toekomstige ontwikkelingen van dergelijke technieken. Vervolgens wordt ook de toepassing van MRI bij het gebruik van biomaterialen beschreven. In hoofdstuk 2 wordt duidelijk dat bijvoorbeeld de UTE-sequentie een zeer geschikte methode is om botten, tanden, en biomaterialen weer te geven.
Op dit moment worden vele materialen ontwikkeld voor het gebruik in bot en tandweefsel. Een typisch voorbeeld is calcium fosfaat cement (calcium phosphate cement; CPC), een materiaal dat zeer geschikte biologische eigenschappen heeft en tegelijkertijd ook klinisch goed hanteerbaar is. De grote overeenkomst tussen CPC en natuurlijk botweefsel zorgt echter voor een slecht beeldcontrast bij het gebruik van conventionele in vivo beeldvorming, zoals computed tomography (CT) of MRI. Op dit moment is het ook onzeker of CPC herkend kan worden met MRI na plaatsing in een tand. Het doel van Hoofdstuk 3 was daarom de ZTE techniek te gebruiken om een CPC af te kunnen beelden, en de afbraak van het materiaal in vivo te kunnen volgen. Het CPC materiaal werd ex vivo geïmplanteerd in drie menselijke tanden en drie geitentanden, en eveneens in vivo in drie geitentanden. Een specifieke UTE-sequentie (UTE with multiple flip angles and echo times) werd toegepast bij een veld van 11.7T om de $T_1$ en $T_2^*$ waarden van CPC, glazuur, en dentine te bepalen. Tanden met CPC werden afgebeeld met een geoptimaliseerde ZTE-sequentie. Geitentanden die in vivo met CPC geïmplanteerd waren, werden na zeven weken ex vivo geanalyseerd. De $T_2^*$ relaxatie van CPC, humaan glazuur en dentine kon beter beschreven worden met een Gauss distributie dan met een Lorentz distributie. Voor CPC, humaan glazuur en dentine, was de gemiddelde $T_2^*$ waarde respectievelijk $273 \pm 19 \mu s$, $562 \pm 221 \mu s$ en $476 \pm 147 \mu s$; de gemiddelde $T_2$ was $1234 \pm 27 \mu s$, $963 \pm 151 \mu s$ en $577 \pm 41 \mu s$; en de gemiddelde $T_1$ was $1065 \pm 45 ms$, $972 \pm 40 ms$ en $903 \pm 7 ms$. In de ZTE beelden, had CPC een betere signaal-tot-ruis verhouding dan dentine of glazuur, vanwege de hoge hoeveelheid water. Zeven weken na implantatie werd het gebied dat gevuld was met CPC duidelijk minder homogeen van structuur, was er een lagere $T_1$ waarde, en bestond de $T_2^*$ uit twee losse componenten. De MRI ZTE vertoonde daarmee voldoende contrast en maakte het bovendien mogelijk de afbraak van het CPC materiaal in tanden over de tijd te volgen.

Desalniettemin, zou het samenvoegen van CPC met specifieke contrastmiddelen (contrast agents; CAs) een nog betere oplossing kunnen zijn voor het vervolgen van het materiaal in vivo. Bij het gebruik van CAs is het bovendien mogelijk niet alleen MRI acquisitie te gebruiken, maar tegelijk een combinatie te maken met andere beeldvormende technieken, zoals CT. Het zou zelfs mogelijk zijn om een samenstelling te bewerkstelligen waarin toevoegingen zitten die zowel de diagnostiek als de therapie ten bate zijn (de zogenaamde ‘theranostische’ materialen). Op deze manier is het mogelijk materialen te maken die tegelijkertijd verbeterde beeldvormende en biologische eigenschappen hebben. In Hoofdstuk 4 werd een theranostisch materiaal voor vitale pulpa behandeling ontwikkeld. Een materiaal
voor de overkapping van pulpaweefsel zou goede biologische eigenschappen moeten bezitten, goed hanteerbaar moeten zijn, en een bruikbaar contrast op moeten leveren. CPC composieten zouden een potentiële kandidaat vormen voor deze behandeling, maar beschikken over een slecht contrast en bevorderen de uitgroei van dentine niet. Daarom werd als eerste de beeldvorming van CPC verbeterd met een dubbel contrastmiddel (core-shell structured dual contrast agent; csDCA) wat bestond uit superparamagnetisch ijzeroxide (superparamagnetic iron oxide; SPIO) en colloïdaal goud, wat respectievelijk diende als MRI- en CT-contrast. Als tweede werden de biologische eigenschappen verbeterd, door toevoeging van een dentinogene groeifactor (*bone morphogenetic protein* 2; BMP-2). Het zodanig verkregen CPC/csDCA/BMP-2 composiet werd *in vivo* getest, als een materiaal voor directe pulp overkapping, in snijtanden van de Habsi geit. Het resultaat liet zien dat de toevoegingen geen relevante veranderingen opleverden in de hanteerbaarheid en mechanische eigenschappen, zoals uithardingsstijd, injecteerbaarheid, en compressiesterkte. De *in vivo* resultaten lieten een verbetering zien in het MRI-contrast tot aan 7 weken na de operatie. Snijtanden die behandeld waren met BMP-2 lieten meer afzetting van tertiair dentine zien en ook een snellere afbraak van het CPC in de µCT metingen. Er kan geconcludeerd worden dat het theranostische materiaal voldoet aan de vereisten voor beeldvorming en regeneratie bij een toepassing als pulp overkapping.

CPC materiaal wordt voornamelijk gebruikt voor toepassing in botrestauratie. Voor een accurate identificatie en lokalisatie, zowel gedurende en na afloop van de implantaatatie, is een CPC compositie met een aanmerkelijk verbeterd contrast noodzakelijk. In *Hoofdstuk 5*, werd het CPC materiaal voorzien van *perfluoro-15-crown-5-ether-loaded* (PFCE) poly(lactic-co-glycolic acid) nanopartikels (met een radius van 100 nm) en van goud nanopartikels (met een diameter van 40 nm), om respectievelijk te dienen als $^{19}$F MRI en CT contrastmiddel. Het resulterende CPC/PFCE/goud composiet werd geïmplanteerd in een rattenmodel voor *in vivo* longitudinale beeldvorming. De bevindingen lieten zien dat het incorporeren van de twee typen nanopartikels nog steeds resulteerde in goede hanteerbaarheid van het cement. Kwalitatieve en kwantitatieve metingen van de degradatie van CPC/PFCE/gold konden over langere tijd uitgevoerd worden en bleken gecorreleerd te zijn aan de vorming van nieuw botachtig weefsel. Tenslotte konden negatieve biologische effecten op het botweefsel uitgesloten worden door middel van histologie. Concluderend kan gezegd worden dater een eenvoudige en efficiënte strategie is ontwikkeld om het implanteren en degraderen van CPC *in vivo* te kunnen monitoren. Aangezien de materialen ook biocompatibel werden bevonden, zou dit CPC/PFCE/goud composiet op termijn klinisch toepasbaar zijn.
In Hoofdstuk 6, werd een CPC construct gemaakt met daarin gadolinium-oxide nanopartikels, wat diende als een multi-modaal MRI/CT contrastmiddel. De gadolinium(III) oxide nanopartikels werden gesynthetiseerd via de polyl methode en daarna aan het oppervlak gefunctionaliseerd met een bisfosfonaat (bisphosphonate; BP) derivaat om op die manier een sterke affiniteit te verkrijgen voor calciumfosfaat. Het verkregen CPC-GBCAs-BP materiaal werd vervolgens longitudinaal gevolgd na in vivo implantatie in een condylair defect in de rat. De synthetische methode die gebruikt werd om nanopartikels te maken – die stabil zijn in water (diameter 70 nm) en een significante T<sub>1</sub> en T<sub>2</sub> relaxatie vertoonden – werd effectief bewezen in zowel klinische 3 T als pre-klinische 11.7 T MR systemen. De combinatie van GBACs-BP nanopartikels met CPC leidt tot een injecteerbaar materiaal met een voldoende klinische hanteerbaarheid. Het functionaliseren met BP zorgde ervoor dat het contrastmiddel langer bij de CPC aanwezig bleef, wat beeldvormende studies over lange termijn mogelijk maakt. De bijdrage van het contrastmiddel, gecombineerd met de juiste biologische respons, maakt vervolgonderzoek van dit nieuwe hybride bot vervangende materiaal tot aan de fase van klinische toepassing aannemelijk.

Naast CPC zijn ook veel andere biomaterialen wellicht geschikt voor botweefselregeneratie. Gelatine methacrylaat (GelMA) is een goedkope, en lichtuithardende hydrogel die geschikt is voor celkweek. Er is eerder al aangetoond dat het 3D printen van GelMA een geschikte methode is om dragermaterialen te produceren die ingroei van cellen en vasculatuur bevorderen en die diffusie van nutriënten mogelijk maken dankzij de evenredig verdeelde open porositeit. In Hoofdstuk 7 werd een geprinte GelMA hydrogel ontworpen en getest voor botweefselregeneratie in vivo. Daarbij werden ook 2 niet-invasieve methoden onderzocht om de afbraak van het dragermateriaal te kunnen volgen, namelijk; 1) een combinatie van GelMA met goud nanopartikels (AuNPs) als contrastmiddel voor CT; 2) MRI scanning met de T<sub>1</sub>-weighted ultrashort echo time (UTE) methode. Voor deze doeleinden werd het 3D geprinte GelMA hydrogel materiaal geïmplanteerd in cilindrische defecten in de condyl van 6 a 7 weken oude Wistar ratten. Resultaten lieten zien dat de GelMA een gelachtige structuur vormde na afkoeling tot 22°C. Het in vivo experiment liet, zowel bij analyse met histologische technieken als met micro-CT, goede biologische eigenschappen zien. Er was geen sprake van de vorming van bindweefselkapsel of hindering van de botvorming. De beeldvorming liet geen duidelijke verandering van het beeld zien bij het GelMA CT signaal, bij het gebruik van de AuNps. In tegenstelling verschaft de MRI bruikbare morfologische informatie over de GelMA en het botweefsel. In conclusie kan gezegd worden dat het 3D
geprinte GelMA materiaal ontworpen in hoofdstuk 7 een goede kandidaat zou zijn als
dragermateriaal voor botweefselregeneratie, terwijl MRI aanbevolen kan worden als meest
optimale techniek voor langdurige niet-invasieve, longitudinale opvolging.

2. Afsluitende opmerkingen en toekomstperspectief

Dit proefschrift had als doel om pre-klinische beeldvormende technieken te onderzoeken die
bruikbaar zijn voor het opvolgen van specifieke biomaterialen, gebruikt voor het herstel van
botten en tanden. Alhoewel CT de meest gebruikte techniek voor het afbeelden van harde
weefsels blijft, werd er in dit proefschrift bijzondere aandacht geschonken aan MRI als
veelbelovend alternatief. Voor dergelijke doeleinden, lag de eerste focus bij het onderzoeken
van nieuwe UTE MRI-sequenties die ontwikkeld waren voor het afbeelden van weefsels en
materialen die gekarakteriseerd worden door een laag water gehalte. Desalniettemin was,
zowel voor CT als voor MRI, het toevoegen van een contrastmiddel vereist, zeker wanneer
het ging om de in vivo opvolging van calcium fosfaat composieten. Daarom werd dergelijk
CPC gecombineerd met verschillende typen CAs (zoals SPIO, Gadolinium, PFC) en
vervolgens getest in pre-klinische modellen voor zowel dentale- als bottoepassingen.
Specifiek werden de CAs verder gecombineerd met een bot stimulerende groeifactor (BMP-2),
geïncorporeerd in een dragermateriaal (silica, PLGA nanopartikels) en gefunctionaliseerd
met CPC-bindende stofjes (bisfosfonaat derivaat). Elke geteste CPC samenstelling met
contrastmiddel maakte het mogelijk om het materiaal over langere tijd niet-invasief, in vivo te
kunnen volgen. Ten gevolge kan zonder meer gesteld worden dat dit proefschrift voorziet in
een breed platform van visualiseerbare materialen voor toepassing in bot en tanden. Elke
samenstelling had daarbij specifieke sterke en zwakke eigenschappen, maar voldeed aan de
biologische vereisten die verdere studie naar klinische toepassing mogelijk maken. Tenslotte
liet dit proefschrift zien dat de keuze voor een bepaalde beeldvormende techniek en een
bepaald CA afhankelijk is van het type biomateriaal. Bij de hydrogel materialen was
bijvoorbeeld een CA niet essentieel, terwijl MRI beter geschikt was dan CT.

Ondanks de bereikte resultaten weergegeven in dit proefschrift, zijn er nog vele uitdagingen.
Alhoewel MRI van harde weefsels en CPC met sequenties zoals ZTE en UTE nu een goed
beschreven methode lijkt, is verdere optimalisering van de acquisitieprotocollen nog steeds
noodzakelijk. Een dergelijke optimalisering zou de nadruk moeten leggen op het verder
verkorten van de acquisitietijd, die nog steeds relatief lang is, voornamelijk voor het afbeelden
tandweefsels. Wanneer CAs gebruikt worden, is er verdere ontwikkeling nodig van
eenvoudige en snelle methoden om het verkregen signaal ook te kwantificeren. Een gevoelige
kwantitatieve evaluatiemethode voor de hoeveelheid CAs in een geïmplanteerd CPC zou het mogelijk kunnen maken om het proces van de degradatie van CPC gedurende de tijd zeer precies te kunnen gaan begrijpen. Een dergelijke signaalkwantificatie is zeker ook essentieel in gevallen waarbij de combinatie van CPC met CAs resulteert in typische MR artefacten. Zulke artefacten worden bijvoorbeeld gezien wanneer SPIO en Gadolinium gecombineerd worden met CPC en vervolgens bekeken worden met ZTE sequenties. Alhoewel het steeds mogelijk bleek om de afname van het gehalte Cas (en de daarbij behorende signaal intensiteit) visueel te herkennen, bleek een kwantitatieve evaluatie nog te uitdagend. Een mogelijke strategie zou kunnen zijn, om gebruik te maken van een techniek als *quantitative susceptibility mapping based on MRI phase images*. Het is bekend dat CAs met verschillende paramagnetische eigenschappen de uniformiteit van het toegepaste magnetische veld ($B_0$) kunnen beïnvloeden. Fase beelden geven informatie over de lokale verdeling van frequenties, die weer afhankelijk zijn van de bulk magnetische susceptibiliteit van de verschillende weefsels. En dus zou MRI fase beeldvorming gebruikt kunnen worden om beeldvormings-artefacten te kwantificeren die het gevolg zijn van het gebruik van CA. Als alternatief zou ook *T1 mapping based on UTE acquisition with variable flip angles (VFA)* voorgesteld kunnen worden voor vervolgonderzoek. De VFA-UTE method is gebaseerd op MRI acquisitie met verschillende flip angles en kan gebruikt worden om de relaxatie eigenschappen te beschrijven van weefsels met een extreem snelle afname van het signaal.

Op dit moment lijkt MRI de enige techniek die geschikt is voor niet-invasieve beeldvorming van alle processen betrokken bij weefselregeneratie. In theorie kan MRI gebruikt worden om te kijken naar celmigratie in een dragermateriaal, naar de morfologie en degradatie van dat materiaal en naar de uiteindelijke biologische reactie van de gastheer. Het gebruik van MRI in het veld van botweefselregeneratie is echter nog in een zeer vroege fase en er is aanvullend onderzoek nodig. Bovendien zijn de kosten voor MRI in de tandheelkunde aanzienlijk, en wordt een dergelijk scenario dus alleen voorzien op de langere termijn. Dit proefschrift liet zien dat er desondanks een groot potentieel in het tandheelkundig gebruik van MRI verscholen ligt, aangezien MRI toestaat zowel zachte en harde weefsels af te beelden, en zelfs gelijktijdig chemische eigenschappen te bestuderen. Het lijkt daarmee nog steeds aanlokkelijk om met MRI zichtbare materialen voor tandheelkundig gebruik verder te ontwikkelen.

Een volgend onderwerp van het proefschrift was een aanpak die gebaseerd is op het gelijktijdig gebruik van verschillende modaliteiten, zoals CT/MRI. Dergelijke multimodale beeldvorming kan complementaire informatie verschaffen, omdat de beperking van de ene
techniek gecompenseerd kan worden door de andere. Een gebrek aan geïntegreerde MRI/CT machines maakt beeldvorming op dit moment bewerkelijk en belemmert een heldere interpretatie van de verkregen data. Aangezien het technisch lastig is om een gecombineerde MRI/CT-scanner te produceren, zou voor verdere studies wellicht beter aan een gestandaardiseerde en volledig geautomatiseerde beeldverwerkingsmethode gewerkt kunnen worden.

Vervolgens kunnen nog diverse opmerkingen gemaakt worden over de CAs. Verschillende typen MRI-CAs werden met succes onderzocht in het proefschrift, desondanks zijn er vele mogelijkheden die nog verder uitgezocht dienen te worden. Metalloproteïnen (zoals hemoglobine en ferritine), die van zichzelf al paramagnetische eigenschappen vertonen, zouden beschouwd moeten worden als kandidaten voor de ontwikkeling van een geheel nieuwe generatie van veilige MRI signaalstoffen voor biomedische toepassing. Daarnaast moet opgemerkt worden dat CAs een significant effect kunnen hebben op de eigenschappen van een biomateriaal. Het insluiten van een extra ingrediënt in een calcium fosfaat matrix, zal bijvoorbeeld ontegenzegelijk de kristalvorming beïnvloeden, en dus ook zorgen voor een verandering in de klinische hanteerbaarheid van het eindproduct. Er zal waarschijnlijk voor elk biomateriaal in botregeneratie en in de tandheelkunde een specifiek geschikt eigen CA ontwikkeld moeten worden, dat geen invloed heeft op de uiteindelijk gewenste eigenschappen.

Een laatste overweging is de veiligheid van CAs. Alle CAs die in dit proefschrift zijn gebruikt staan bekend als biocompatibel, werden in relatief lage concentratie toegepast, en werden dus in het algemeen beschouwd al geschikt voor bot- en tandheelkundige toepassing. Het is echter bekend dat bepaalde typen nanopartikels zich kunnen ophopen in specifieke organen (zoals milt en lever) in plaats van uitgescheiden te worden door het lichaam. Het bekijken van de bio-distributie van CAs en het eventueel voorkomen van ophopingen in een bepaald weefsel werd niet bestudeerd, maar is zeker aan te bevelen voor vervolgstudies. Dergelijke informatie zal leiden tot een beter begrip van het uiteindelijke lot van CAs wanneer deze vrijkomen uit het geïmplanteerde materiaal en antwoorden kunnen geven op belangrijke vragen ten aanzien van veiligheid. Een sterk bewijs van biologische veiligheid van de gebruikte materialen zal het uiteindelijke klinische gebruik faciliteren en versnellen.
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List of publications

Related to the thesis


† and * authors equally contributed
LIST OF PUBLICATIONS

Other publications


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Curriculum Vitae

Simone Mastrogiacomo was born in 1985 in Benevento, Italy. He received the B.Sc. degree in 2009 in Biotechnology at the University of Parma, Italy. He performed his B.Sc. thesis at the Department of Hematology / Chemical Analysis at the Hospital Maggiore of Parma, Italy, focusing on the immunological and immunochemical methods for the detection of anti-neutrophil cytoplasmic auto-antibodies in systemic vasculitis. Mastrogiacomo achieved the M.Sc. degree in 2012 in Medical, Veterinary and Pharmaceutical Biotechnologies at the University of Parma, Italy. He conducted his M.Sc. thesis at the Department of Biomedical, Biotechnological and Translational Science at the University of Parma, Italy, focusing on the ex situ bioengineering of thyroid gland with specific emphasis on the morphological role of biocompatible materials. In 2013, Mastrogiacomo started his Ph.D. degree at the Department of Biomaterials at the Radboud University Medical Center, Nijmegen, The Netherlands. His Ph.D. project was part of a Marie Curie Initial Training Network (iTERM) funded by the European Union. His Ph.D. project focused on the preclinical imaging strategies for bone restorative materials. During his Ph.D. studies, he was a visiting researcher at the Department of Chemistry, Hull University, UK, and at the Nano4Imaging GmbH, Aachen, Germany.

In May 2018 Mastrogiacomo will start working as a postdoctoral fellow at the Laboratory of Functional Molecular Imaging (LFMI) of the Brain MRI Molecular Contrast Unit (BMMCU) at the National Institute of Neurological Disorders and Stroke (NINDS), National Health Institute (NIH), Bethesda, MD, USA.
You cannot hope to build a better world without improving the individuals. To that end, each of us must work for his own improvement, and at the same time share a general responsibility for all humanity, our particular duty being to aid those to whom we think we can be most useful

(Marie Skłodowska-Curie)