Label-free magnetic resonance imaging to locate live cells in three-dimensional porous scaffolds

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Porous scaffolds are widely tested materials used for various purposes in tissue engineering. A critical feature of a porous scaffold is its ability to allow cell migration and growth on its inner surface. Up to now, there has not been a method to locate live cells deep inside a material, or in an entire structure, using real-time imaging and a non-destructive technique. Herein, we seek to demonstrate the feasibility of the magnetic resonance imaging (MRI) technique as a method to detect and locate in vitro non-labelled live cells in an entire porous material.

Our results show that the use of optimized MRI parameters (4.7 T; repetition time = 3000 ms; echo time = 20 ms; resolution 39 × 39 μm) makes it possible to obtain images of the scaffold structure and to locate live non-labelled cells in the entire material, with a signal intensity higher than that obtained in the culture medium. In the current study, cells are visualized and located in different kinds of porous scaffolds. Moreover, further development of this MRI method might be useful in several three-dimensional biomaterial tests such as cell distribution studies, routine qualitative testing methods and in situ monitoring of cells inside scaffolds.

Keywords: magnetic resonance imaging; scaffolds; cell culture; in vitro

1. INTRODUCTION

Porous scaffolds have been extensively tested materials for various purposes in tissue engineering [1–9]. Irregular pore size ceramic scaffolds are used clinically for purposes of bone tissue formation, while solid free form (SFF) techniques—three-dimensional printing, stereo-lithography, fused deposition modelling, robocasting, phase-change jet printing and so on—constitute excellent methods for producing well-defined three-dimensional structures [10–14]. Within this SFF field, scaffolds produced from polycaprolactone (PCL) [15–21] and ceramics have attracted a lot of attention.

One of the most critical features of a porous scaffold is its biocompatibility, and hence the ability of seeded cells to migrate and grow on its inner surfaces. This colonization of the scaffold is especially relevant when cell-seeded materials have to be implanted in vivo. Cell migration to the inner structure is usually assessed in vitro, mainly by using microscopic techniques (scanning electron microscopy, confocal microscopy and so on). However, these techniques are dependent on light penetration, and material opacity limits the studies to superficial areas. Despite the fact that histology is the most commonly used and the most informative technique to locate cells inside the scaffolds, it requires sample fixation and processing. In addition, the slices obtained provide information about a part but not all of the material (figure 1). Therefore, locating live cells deep inside a material or in the entire structure, using a non-destructive real-time imaging technique, remains a challenge.

Magnetic resonance imaging (MRI) has already been reported as a useful technique in the tissue engineering field [22]. In vitro, MRI assays have been reported to define the structure of scaffolds [23,24]. In addition, the mineralization of the extracellular matrix could be
monitored by MRI. Accordingly, MRI has been used to assess bone and cartilage differentiation [25–33] and to follow up material properties after cell seeding [34].

In spite of the above, it is still difficult to follow up the cells combined with scaffolds by using MRI. First, cells should be labelled with a contrast agent, which usually requires the immobilization of the contrast agent on a nanoparticle [35]. Then, the nanoparticles should be internalized into the cells by means of transfection, electroporation, ultrasound pulses or other techniques [36]. Nevertheless, these cell manipulations have been reported to affect some major cell properties, such as viability, differentiation, migration and ability of colony formation [36].

In terms of image quality and interpretation, it is advantageous to have a positive signal (hyper-signal, ‘bright’). This can be provided by paramagnetic agents in T₁-weighted images (T₁WIs; i.e. gadolinium) as opposed to a negative signal (hypo-signal, ‘dark’), which is provided by super-paramagnetic agents in T₂-weighted images (T₂WIs; i.e. iron oxide). In this context, gadolinium- and manganese-based nanoparticle formulations have been increasingly used as T₁-based MRI contrast agents [35]. However, the use of these paramagnetic agents for cell-labelling purposes has some disadvantages. First, the fact that gadolinium requires direct contact with free water to alter image contrast can be troublesome in an intracellular and cellular compartmentalization environment [37]. Second, safety must be considered. Free gadolinium, highly toxic in its unchelated form, could be released as the cells are degraded [38]. Moreover, gadolinium-based contrast agents are currently associated with nephrogenic systemic fibrosis, particularly when being retained in cells without rapid clearance [39,40]. Finally, these methods are not very sensitive [35,41].

Thus, super-paramagnetic agents (i.e. iron nanoparticles and super paramagnetic iron oxide (SPIO) particles) are mainly used as contrast agents to label cells. They induce a highly sensitive signal loss in cells, improving the contrast with the surrounding tissue and allowing cell tracking [42–47]. These contrast methods have been used in tissue-engineered constructs to assess, in vitro, cell seeding in hydrated scaffolds [48–51].

To sum up, using MRI to locate labelled cells seeded in solid scaffolds is still a challenge. As solid materials look dark on MRI, contrast could not be obtained with more conventional SPIO cell-labelling methods. In addition, high-resolution images are required to observe the internal structure of porous materials.

Therefore, the aim of the current study is to locate non-labelled live cells in entire solid porous scaffolds with a non-destructive method (MRI). It would be a useful tool for several applications, such as cell distribution studies, routine qualitative testing methods, in situ monitoring of cells inside scaffolds and sample testing before an in vivo implantation. For this purpose, high-resolution MRI has been used and cells have been detected as hyper-signal objects. The assays were first focused on the optimization of MRI conditions to observe non-labelled cells as hyper-signal in vitro. Then, the usefulness of MRI for in situ evaluation of the cell seeding has been proven in solid three-dimensional materials, using SFF-designed PCL scaffolds as the first model and later extending these assays to other three-dimensional structures.

2. MATERIAL AND METHODS

2.1. Scaffolds

The preparation of the SFF-designed porous scaffolds was as previously reported [16,52,53]. Briefly, PCL (M_w 70 000–90 000; Sigma, Belgium) was processed into scaffolds with a Bioscaffold device (SysEng, Germany). The scaffolds were designed according to a 0/90° lay-down pattern and without shift. The final scaffold dimensions of the cylinder were 4.5 mm diameter and 3 mm high. The struts of the resulting structure were 250 μm in diameter and the pore size was 400 μm. The scaffolds were coated with a gelatin–fibronectin layer using a previously optimized multi-step coating protocol [53].

Ceramic scaffolds were made using a robocasting device (Robocasting Enterprises LLC, USA). Commercially available β-tricalcium phosphate powders, with an average particle size of 1.8 ± 0.8 μm (Fluka, Switzerland), were used to prepare inks for robocasting [52]. After making the scaffold using a layer-by-layer process and sample sintering, pieces of 3 × 3 × 3 mm were obtained with a structural pore size of 100 μm. Commercially available ceramic Bio-Oss blocks (Geistlich Pharma AG, Switzerland) were used as materials with non-regular internal pore structure.

2.2. Magnetic resonance imaging equipment

The MRI experiments were performed on a Bruker Biospec BMT 47/40 system (Bruker, Ettlingen, Germany) operating at 4.7 T and equipped with a 6 cm gradient...
system capable of a 450 G cm\(^{-1}\) gradient strength. A 3.5 cm Bruker-designed volume radiofrequency coil was used for both transmission and reception. The equipment was maintained at a constant temperature of 23°C.

2.3. Cell culture

A C2C12 mouse myoblastic cell line was used (CRL 1772; ATCC, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; 31966-021, Gibco, UK), containing 10 per cent fetal bovine serum (10500-064, Gibco) and antibiotics (100 U ml\(^{-1}\) penicillin and 100 μg ml\(^{-1}\) streptomycin sulphate; Gibco). Cell cultures were maintained at a temperature of 37°C under a humidified 5 per cent CO\(_2\) atmosphere. The cell passage was always performed at 80 per cent of confluence.

2.4. Cell seeding

Prior to the experiments, PCL scaffolds were sterilized using gamma radiation and ceramic samples were sterilized at 300°C. After cell seeding, the sample was centrifuged at 400g for 10 min to ensure the cell seeding process into each sterile scaffold (cell content and culture conditions used in each assay are specified in §3). The scaffolds were cultured at 37°C under a humidified 5 per cent CO\(_2\) atmosphere before testing.

2.5. Magnetic resonance imaging studies of cell pellets

Cell pellets were obtained during cell subculture by centrifugation of cell suspensions in 1.5 ml tubes (5 × 10\(^{9}\) cells, 10 min at 400g). Apparent diffusion coefficient (ADC), transverse relaxation time (T\(_2\)) and longitudinal relaxation time (T\(_1\)) image series were acquired at low resolution (LR) and at high resolution (HR). The geometry parameters for LR images were a field of view (FOV) of 2 × 2 cm and a matrix size of 128 × 128. The final resolution was 156 × 156 μm for a slice thickness of 1 mm. For HR images, the parameters were a FOV of 2 × 2 cm and a matrix size of 512 × 512. The final resolution was 39 × 39 μm for a slice thickness of 1.5 mm.

The imaging sequence analysis (ISA) utility of the ParaVision v. 3.0.1 package (Bruker) was used to perform the measurements of ADC, T\(_2\) and T\(_1\).

For ADC calculations, a series of four spin echo diffusion-weighted images (DWIs) were acquired. The parameters for these images were: (i) repetition time (TR) = 2500 ms; (ii) echo time (TE) = 55 ms; (iii) duration of diffusion gradients (Δ) = 10 ms; and (iv) time between diffusion gradients (D) = 25 ms. The b-factor for these series varied from 283 to 5034 s mm\(^{-2}\). The number of averages (NA) was 2 and the scanning was performed for 85 min for the LR image series and 170 min for the HR image series.

For T\(_2\) calculations, a multi-echo spin echo sequence was used. Forty-five echoes were acquired using as parameters a TR of 3000 ms and a TE ranging from 20 to 900 ms. The scanning times were 13 min for the LR image series and 25 min for the HR image series.

For T\(_1\) calculations, a series of seven saturation–recovery spin echo images were acquired using as parameters a TR varying from 250 to 6000 ms, a TE of 18 ms and an NA of 3. The scanning times were 166 min for the LR image series and 332 min for the HR images series.

2.6. Magnetic resonance imaging studies of scaffolds

Each scaffold was transferred into a 1.5 ml tube filled with fresh cell culture medium.

HR spin echo two-dimensional images were acquired using a TR of 3000 ms, a TE of 20 ms, an NA of 4, an FOV of 1 × 1 cm\(^2\) and a matrix size of 256 × 256 as parameters. The final resolution was 39 × 39 μm\(^2\) for a slice thickness of 1.5 mm. For the collection of these images, the scanning time lasted 51 min.

A fast spin echo sequence was used for HR three-dimensional proton-weighted images. The parameters used for these images were a TR of 3000 ms, an effective TE of 20 ms, an FOV of 1 × 1 × 1 cm\(^3\) and an acquisition matrix size of 256 × 192 × 192. When required, a pair of diffusion gradient pulses was added to achieve the diffusion weighting. The parameters for these diffusion gradients were: (i) δ = 5 ms; (ii) Δ = 30 ms; and (iii) diffusion gradient strength = 1.5 G cm\(^{-1}\). The acquired data were zero-filled to yield a reconstructed matrix of 256 × 256 × 256, resulting in a resolution of 39 × 39 × 39 μm\(^3\). These reconstructed data were imported to the ImageJ v. 1.42 program (NIH, Bethesda, MD, USA) for three-dimensional studies. The scanning time for these images varied from 7 to 30 h depending on the FOV used, the matrix size and the NA performed.

2.7. MTS cell viability assay

The protocol was performed following the manufacturer’s instructions (Aqueous MTS Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI, USA). Briefly, target scaffolds were transferred to new culture wells. Pre-warmed culture medium and reconstituted 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) were added (40 μl MTS and 400 μl medium). Samples were incubated at 37°C for 90 min. The medium was transferred to new wells to measure the absorbance (460 nm, Biotek FL-600). Blank readouts were subtracted.

2.8. alamarBlue cell viability assay

The protocol was performed following the manufacturer’s instructions (Biosource, Camarillo, CA, USA). Briefly, target scaffolds were transferred to new culture wells and pre-warmed culture medium (400 μl) and alamarBlue (AB) reagent (40 μl) were added. Samples were incubated at 37°C for 120 min. The medium was transferred to a new plate and fluorescence measurements were collected using a fluorescence excitation wavelength of 530 nm and a fluorescence emission wavelength of 590 nm (Biotek FL-600). Blank readouts were subtracted.

2.9. Live/dead viability/cytotoxicity assay

This assay is used to determine the intracellular esterase activity and plasma membrane integrity. Red fluorescent ethidium homodimer passes only through damaged cellular membranes and binds to nucleic
acids; it is not able to pass through the intact plasma membrane of live cells. By contrast, the green fluorescent polyanionic dye calcein allows for detection of live/viable cells. The protocol was performed following the manufacturer’s instructions (Molecular Probes, Eugene, OR, USA). Briefly, cell culture medium was removed and 100 μl of phosphate-buffered saline supplemented with 2 μM calcein AM and 2 μM ethidium homodimer was added. Plates were incubated for 1 h in the dark at 37°C. Then, materials were transferred onto microscope slides and fluorescence was detected using a fluorescence microscope (Olympus BX51; Olympus, Tokyo, Japan).

2.10. Histology

Cell-seeded and MRI-tested samples were fixed in formal 10 per cent for 24 h and processed for further paraffin embedding. Serial sections were collected from different scaffold locations for the histological study with haematoxylin and eosin staining. The histology of the sections mounted onto the slides was studied using an Olympus BX51 microscope.

3. RESULTS

3.1. Tuning cell contrast in magnetic resonance imaging

Two-dimensional MRI studies in cell pellets (10 × 10⁶ cells) were performed in order to assess whether or not the MRI is a useful technique to distinguish live non-labelled cells from the surrounding culture medium. These assays were performed at LR, which is commonly used in MRI. We also carried out the studies at a HR suitable to observe the porous structure of a solid scaffold in further assays. Figure 2 summarizes the results obtained using various image-weighting conditions (DWI, T2WI and proton density-weighted image (PDWI)) as well as using different voxel resolutions (HR and LR).

3.1.1. Diffusion studies

Figure 2 shows representative DWIs at LR (156 × 156 μm) and HR (39 × 39 μm). In both cases, the signal is higher in cells than in water, but the cell signal is weak in HR. Figure 2 also shows graphically the diffusion gradient (b-factor) effects on signal intensity. In these studies, the ADC (mobility of water protons) was also calculated at LR: ADCDMEM = 165 μm² s⁻¹ and ADCcells = 52 μm² s⁻¹. According to these data, we postulated that, in other image-weighting parameters, signal loss would occur owing to the fact that the voxel size at HR (39 × 39 μm) is lower than the ADC. At HR, signal loss should be higher in a medium likely to modify cell–medium contrast under other MRI conditions.

3.1.2. T2 studies

Figure 2 shows representative T2WIs at different resolutions: LR (156 × 156 μm) and HR (39 × 39 μm). A contrast between the cells and the medium can be observed, although, in this case, cells are observed as hypo-signal (dark when compared with the medium).
Graphical representation of multi-echo spin echo decay ($T_2$ calculation) at HR shows that the signal intensity corresponding to the cells and that corresponding to culture medium is similar under all tested TE conditions. This result indicates that changes in TE do not improve the contrast between cells and medium.

3.1.3. $T_1$ and proton density studies

As $T_2$ studies revealed that the lowest TE allows for the best amount of signal, a series of saturation–recovery spin echo images were obtained at a fixed TE (i.e. 20 ms), from T1WIs, (low TR) to PDWIs, (high TR). Figure 2 shows representative PDWIs at both resolutions, LR and HR. At LR, there is no contrast and the signal intensity obtained from cells and from the medium is comparable. However, in the HR image, not only a marked contrast is obtained but also a bright cell pellet could be observed. Graphical representation of saturation–recovery curves shows that, at HR, the signal intensity is higher in cells under PDWI conditions. We suggest that the contrast, obtained under HR PDWI conditions but not under LR PDWI conditions. A similar study performed in a three-dimensional case (voxel size $39 \times 39 \times 39$ $\mu$m), the signal of the medium tends to disappear with the diffusion gradient (see $b$-factor values and figure 3a,b), while the signal of the cells can still be observed in most of the applied diffusion gradients (figure 3b). This result indicates that the contrast is enhanced by a diffusion gradient. Figure 3c shows the results of a similar study performed in a three-dimensional case (voxel size $39 \times 39 \times 39$ $\mu$m). In this case, owing to the voxel size, the amount of cell signal is weak. Only the low diffusion gradient allows for contrast, while under higher diffusion gradients there is a complete signal loss.

Thus, our results indicate that the PDWI parameters used are useful for allowing the detection of cells in scaffolds. Moreover, diffusion gradients could be applied, improving the contrast even more, although it needs to be taken into account that full cell signal could be lost when applying very high diffusion gradients.

3.2. Magnetic resonance imaging in porous scaffolds

3.2.1. Improvement of magnetic resonance imaging conditions in scaffolds

Developed MRI parameters were used to explore the cells’ pattern behaviour once inside the scaffolds. With these parameters, different diffusion gradients were assayed in order to improve cell–material contrast. Figure 3 shows diffusion studies in control (figure 3a) and cell-seeded (figure 3b,c) scaffolds ($5 \times 10^6$ cells, 2 days in culture). In the two-dimensional HR studies (pixel size $39 \times 39 \mu$m), the signal of the medium tends to disappear with the diffusion gradient (see $b$-factor values and figure 3a,b), while the signal of the cells can still be observed in most of the applied diffusion gradients (figure 3b). This result indicates that the contrast is enhanced by a diffusion gradient. Figure 3c shows the results of a similar study performed in a three-dimensional case (voxel size $39 \times 39 \times 39$ $\mu$m). In this case, owing to the voxel size, the amount of cell signal is weak. Only the low diffusion gradient allows for contrast, while under higher diffusion gradients there is a complete signal loss.

Thus, our results indicate that the PDWI parameters used are useful for allowing the detection of cells in scaffolds. Moreover, diffusion gradients could be applied, improving the contrast even more, although it needs to be taken into account that full cell signal could be lost when applying very high diffusion gradients.

3.2.2. Three-dimensional studies: cell location in entire scaffolds

After determining the MRI of live-cell visualization parameters, scaffold samples were cell seeded ($5 \times 10^6$ cells, 2 days in culture) and cells were homogeneously located in all scaffold structures. Figure 4 shows slices obtained in three-dimensional studies. Figure 4a1–aR corresponds to a control scaffold and is provided for comparative purposes (see also the electronic supplementary material, figure S1). Figure 4b1–bR corresponds to a sample
mainly showing uniform distribution of cells in all scaffold structures. There were fewer cells in the top (figure 4b1) than in the centre of the scaffold (figure 4b3–4; see the reconstruction in the electronic supplementary material, figure S2) but a good cell distribution was achieved throughout the material. Figure 4c1–c5 corresponds to a sample that, although similarly cell seeded, shows most of the cells outside the structure. Thus, figure 4c1 shows some spaces filled with cells in the top; figure 4c3–4 shows a few cells in the centre of the structure; and it can be seen in figure 4c2–5 that most of the cells were outside the scaffold (see also the electronic supplementary material, figure S3). Samples with 1 × 10^6 cells were also assayed (figure 4d1–d5). Although a uniform cell distribution was observed (see also the electronic supplementary material, figure S4), the low number of cells allowed for cell detection only when a low diffusion gradient was applied.

3.2.3. Cell location in a narrow pore-size structure
In all previous assays, the scaffolds (SFF-designed PCL) had a 400 μm pore-size structure. We also aimed to study the suitability of the MRI technique for cell location in a narrower pore-size (100 μm) scaffolding material (an SFF-designed ceramic). To this end, scaffold samples with 100 μm pore size were cell seeded (5 × 10^6 cells, 7 days in culture) and visualized by MRI. Figure 5 shows that the reported MRI conditions are optimal to observe the cells inside the porous structure, with signal intensity higher than that from the culture medium. The three-dimensional study was also carried out showing a uniform cell distribution throughout the porous structure of the seeded scaffolds (see the reconstruction in the electronic supplementary material, figure S5).

3.2.4. Cell location in non-regular pore size scaffolds
The MRI conditions were also tested in Bio-Oss, which is a clinically available ceramic scaffold with non-regular pore structure. Owing to the non-regular pore characteristics of this structure, two-dimensional MRI is difficult to perform to visualize seeded cells. Thus, 1 × 10^6 cells were seeded in the Bio-Oss samples and cultured for 2 days before performing the three-dimensional MRI. Figure 6 shows four slices obtained from this study. It can be seen how cells (hyper-signal) adhere to the inner surfaces of the material.

3.3. Assessment of cell location inside scaffolds
Histological studies were carried out to confirm the results regarding cell distribution inside scaffolds that
we had obtained using the MRI technique. Figure 7 shows representative histological images of MRI-tested samples (PCL sample shown in figure 4b and ceramic sample shown in figure 5c). Slices from the inner part of the samples were obtained and stained. The images show that cells are located in the spaces between the rods of the scaffold while material rods can be seen as empty spaces owing to the histological processing.

3.4. Assessment of cell viability in magnetic resonance imaging conditions

As, during the MRI studies, samples are maintained at 23°C, we also aimed to test whether cells are able to maintain viability after the MRI testing. SFF-designed ceramic scaffolds were used for these studies and two cell-viability measurement methods (MTS and AB) were assayed. Figure 8 shows that, after overnight culturing, there are no differences in terms of cell viability between samples cultured under control conditions and samples cultured under MRI conditions in any of the assays. Also in figure 8, green fluorescence in images corresponds to the calcein cell-viability assay and again shows no differences in cell viability between the environmental conditions tested.
Herein, we present a novel MRI method to detect cellular distribution in a polymeric material or in vivo. In these studies, the contrast has been obtained for testing purposes is fairly innovative. Cell differentiation or by cell labelling. Since solid porous scaffolds provide a low signal in MRI, cells could not be located in these structures with the previously mentioned methods. Herein, we present a novel MRI method to detect non-labelled live cells in entire porous scaffolds that could be of potential interest for several in vitro or in vivo purposes.

Burg et al. [54] previously reported a method to observe cellular distribution in a polymeric material (TR = 600 ms, TE = 11 ms, resolution 23 μm). Similar to our method, they observed that the signal intensity was higher in cells than in the liquid phase and scaffold. However, it is important to note that the assays were performed in formalin-fixed specimens. Moreover, the method was presented by the authors themselves as a preliminary step addressing the feasibility of the technique. Herein, we show that the optimized HR MRI parameters used yield a most favourable contrast condition not only in fixed samples, as has been previously reported, but also in samples with live cells.

The MRI technique measures the proton’s response to magnetic fields in water molecules. However, water molecules have different mobility, known as diffusion, which depends on their location. Previous MRI studies established that, owing to the lower ADC in cells, a contrast between live cells and the liquid medium could be observed using a diffusion gradient [55]. We show (figure 2b) that, under our tested ADC values (cells, 52 μm² s⁻¹; culture medium, 165 μm² s⁻¹), we are able to obtain DWIs similar to those previously reported [55]. In addition, we prove that the diffusion image is hardly applicable under the conditions of HR and three-dimensional imaging required to observe the structure of porous scaffolds. This is owing to the low signal content in each pixel of these images and to the even higher loss of signal induced by the diffusion gradient.

In the cell pellet studies, we observed that, when working at HR, when the pixel size is lower than the ADC, the diffusion interferes with the contrast of PDWIs. Interestingly, we obtain a contrast in the PDWI at HR but not when voxels were larger (figure 2a). At HR, the contrast is obtained because the signal of the culture medium tends to be lost owing to diffusion while this effect is less noticeable in cells. Therefore, we report optimized MRI parameters that allow for visualization of bright live cells surrounded by medium. Moreover, the resolution is suitable enough to allow for observation of the porous structure of the scaffolds in further studies.

Subsequently, we tested these MRI parameters (HR and PDWIs) in solid porous scaffold samples. As mentioned, SFF–designed PCL scaffold was first selected because it has recently attracted significant attention [15–21], it is a US Food and Drug Administration-approved material, it can be applied in vivo and it has demonstrated great potential [56–58]. First, we tested with diffusion gradients. Our results demonstrated that, although it could be useful for cell visualization in two-dimensional images (Z = 1.5 mm), it is not applicable in thin slices (Z = 30 μm) with a very low signal from thin voxels (figure 3). Despite this caveat, the current developed PDWI parameters were effective to locate cells in the scaffolds, to assess differential cell content and to distinguish the well from the poorly seeded scaffolds (figure 4).

At this point in the study, we assessed whether or not this method would be useful when applied to other pore-size structures. We regarded this as a relevant question taking into account the variety of porous scaffolds with different pore-size architectures described in the literature. To address this issue, we used porous ceramic materials widely tested for tissue engineering purposes [4–14, 52, 59]. In SFF–designed ceramic scaffolds, with a pore size of 100 μm (figure 5), we were not only able to observe porous structure but also to locate cells as high signal intensity in the porous spaces, in both two-dimensional and three-dimensional assays. The MRI conditions described were also suitable to visualize cells in Bio-Oss ceramic block scaffolds. This is a type of scaffold widely used clinically and characterized by having non-regular pore size (figure 6). However, the study had to be restricted to three-dimensional assays owing to the characteristics of the material structure.

As final points, we confirmed the data obtained from the MRI studies regarding cell location (figure 7), by means of histological techniques commonly used to
visualize seeded cells in other three-dimensional SFF structures [60], and the cell viability under the environmental MRI conditions used by employing different methods (figure 8).

To summarize, we report here a novel MRI methodology suitable to visualize non-labelled live cells, to detect different content of cells and to assess the quality of cell colonization in a variety of solid scaffolds (different pore size and materials) that do not require previous sample preparation.

Nonetheless, some aspects and/or limitations need to be addressed. First, HR MRI requires high magnetic fields to increase the sensitivity and to allow for the detection of the weak signal obtained in such small voxels. The current results have been obtained at 4.7 T, which is higher than the usual clinical MRI (0.5–3 T) but lower than other research MRI equipment (7–11.7 T). We consider that the resolution of 39 μm is: (i) useful in most of the usual experimental equipment; (ii) adequate for most porous scaffolds; and (iii) comparable to resolution values routinely used in other non-destructive image methods (i.e. micro-computed tomography equipment).

Second, the practical aspects of the experimental procedure, including the time required for each assay, should be taken into account for this method to become a routine qualitative testing tool. Using the current selected parameters, the acquisition time depends mainly on matrix size. In addition, the average from multiple scans of the same sample (NA) increases the signal-to-noise ratio but also multiplies the scanning time. Thus, with the equipment used by us (4.7 T; single HR/PDWI/two-dimensional (similar to images in figure 5b,c) and HR/DWI/two-dimensional (similar to images in figure 3)), the scanning time would range from 15 to 60 min, which would be acceptable for a routine method. However, HR/PDWI/three-dimensional imaging (similar to images in figures 4 and 6) requires much longer scanning times, which can vary from 7 to 30 h.

Third, we show that cells are able to maintain their viability during the MRI experiments (figure 8). This is a crucial aspect when considering that samples would be later implanted. Nonetheless, specific equipment would be needed to perform other interesting specific studies such as monitoring and quantifying the cell development inside scaffolds by means of MRI in a time lapse. As mentioned above, it is noteworthy that a similar MRI method was reported by Burg et al. [54] to locate formalin-fixed cells. Thus, this method did not allow for identification of the cells that died during the culture process.

We conclude that the current MRI method is suitable for visualization and location of non-labelled live cells in various porous scaffolds. Moreover, further development of the MRI parameters described herein could be valuable to those interested in using non-destructive methods for purposes implying three-dimensional biomaterial testing; for instance, to assess cell distribution in an entire structure before an in vivo sample implantation, or to perform cell distribution studies, routine qualitative testing and in situ monitoring inside scaffolds.

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