High-Resolution Cellular MRI: Gadolinium and Iron Oxide Nanoparticles for in-Depth Dual-Cell Imaging of Engineered Tissue Constructs

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ABSTRACT Recent advances in cell therapy and tissue engineering opened new windows for regenerative medicine, but still necessitate innovative noninvasive imaging technologies. We demonstrate that high-resolution magnetic resonance imaging (MRI) allows combining cellular-scale resolution with the ability to detect two cell types simultaneously at any tissue depth. Two contrast agents, based on iron oxide and gadolinium oxide rigid nanoplates, were used to “tattoo” endothelial cells and stem cells, respectively, with no impact on cell functions, including their capacity for differentiation. The labeled cells’ contrast properties were optimized for simultaneous MRI detection: endothelial cells and stem cells seeded together in a polysaccharide-based scaffold material for tissue engineering appeared respectively in black and white and could be tracked, at the cellular level, both in vitro and in vivo. In addition, endothelial cells labeled with iron oxide nanoparticles could be remotely manipulated by applying a magnetic field, allowing the creation of vessel substitutes with in-depth detection of individual cellular components.

KEYWORDS: nanobiotechnology · magnetic resonance imaging · biomaterials · tissue engineering · stem cells

By combining biomaterials with living cells, tissue engineering is seeking to create biological grafts with specific features of native tissues, opening up new perspectives for tissue repair.1–3 Direct injection of therapeutic cells is another promising approach for tissue reconstruction.4 However, one obstacle to the development of such therapies is that current imaging techniques cannot visualize the fate of implanted cells deep within the body. Recent advances in nonlinear microscopy have led to new strategies for observation of living biological tissues,5,6 but such approaches are still restricted in depth. Advances in magnetic resonance imaging (MRI),7 scintigraphy,8 and fluorescence-based methods9 have taken in vivo imaging to a new level, by revealing the fate and functioning of cells. Optical methods for tracking cells tagged with fluorescent or luminescent labels are being developed for preclinical small animal studies,10 but these are also limited to a depth of a few millimeters, severely hindering their clinical application. Radiolabeled cells can be detected in vivo by means of positron emission tomography (PET), with excellent sensitivity and tissue penetration, but with insufficient spatial resolution or anatomic images, while the use of radioactive tracers raises safety issues. In contrast, MRI is a nonradiative and noninvasive method suitable for longitudinal studies, providing anatomic images at any depth with excellent spatial resolution. To detect cells by MRI, the cells of interest must first be tagged with a contrast agent. The current approach is to use superparamagnetic iron oxide nanoparticles (IONPs),11,12 which, by reducing the $T_2$ relaxation time, lead to a loss of signal; IONPs are therefore known as negative contrast agents. Many studies of IONP biocompatibility have been published in recent years, showing that
these nanomaterials have an extremely low impact on cell functions. With the advent of high-resolution MRI, individual cells can now be visualized in black on T2-weighted images (see ref 13 for a review). However, few studies have achieved cellular MRI imaging in biomaterials for the purposes of tissue engineering.14–17 IONP-labeled cells also have another property of particular interest to tissue engineering, namely, their capacity for remote magnetic manipulation, paving the way for the creation of organized cell assemblies.18,19 To our knowledge, the combination of a magnetically organized pattern with high-resolution MRI imaging has not previously been demonstrated.

Currently, the main limitation of cellular MRI is the lack of a multicellular imaging method, which is crucial to monitor multicellular grafts within their host tissue. Paramagnetic gadolinium (Gd) derivatives are another type of MRI contrast agent. These “positive” contrast agents reduce the T1 relaxation time, thereby enhancing the MRI signal. Although Gd chelates are already extensively used in the clinic to detect lesions or visualize vessels,20 only rare attempts have been made to exploit their properties for cellular imaging as gadolinium compounds21–25 or in combination with T2 agents for concurrent dual contrast.26 In this context, Gd-labeled cells have never been detected at a cell resolution by T1-weighted MRI. Among the candidates for a cellular paramagnetic label, gadolinium-based nanoparticles (GdNP) offer the advantages of efficient intracellular labeling and minimal toxicity.27

The method described here uses dual T1 (GdNP) and T2 (IONP) cell labels to image stem cells and endothelial cells simultaneously within a biomaterial used for tissue engineering, by means of high-resolution MRI. The use of endothelial cells and stem cells was motivated by the common need to vascularize reconstructed tissues, which is crucial for cell survival, nutrient transport, and waste removal.28 A promising approach is to seed scaffolds with two cell types: stem cells or specific cells of the targeted organ combined with endothelial cells or progenitors capable of facilitating in situ formation of blood vessels.29

After studying IONP and GdNP incorporation in endothelial cells and stem cells at various extracellular concentrations, we examined their impact on cell functions (especially differentiation capacity) and their contrast properties. Optimization of both cell seeding in biomaterials and imaging protocol provided high-resolution images of a multicellular construct with stem cells depicted in white and endothelial cells in black.

Still, it is important to emphasize that the MRI parameters used (specifically the gradient echo time and repetition time) are the key factors for detecting T1 and T2 signals exclusively. Indeed, IONP and GdNP shorten both the T1 and T2 relaxation times, differing only by the relative shortening of each parameter (the T1/T2 ratio is much higher for GdNP than for IONP). It is the optimization of the sequence parameters that allows simultaneous detection of the two agents (hypersignal versus hypovascular signal) on the same image.

Moreover, dual cell tracking could be performed, in vivo, for two weeks. Finally, a tubular template of the same biomaterial was used to create a cellular graft with a structure mimicking that of a blood vessel, thanks to a magnetic patterning of the endothelium. MRI images confirmed the dual imaging of both the muscle layer in white and the endothelium in black.

**RESULTS**

The aim of this study is to combine cellular-scale resolution with the ability to detect several cell types simultaneously at any tissue depth within a living body. The schematic of such cell-engineered tissue imaging is proposed in Figure 1.

**Nanotagging of Endothelial and Stem Cells.** The IONPs used here were maghemite nanoparticles with a diameter of 7 nm and a negatively charged surface (Figure 2a). They have already proved to be extremely effective agents for in vitro magnetic labeling of all cell types tested so far (cells from different species; primary cultures and cell lines; immune, cancer, muscle, endothelial, and stem cells).30

GdNP have been less extensively studied as cell labels. They are composed of a polysiloxane network bearing chelating species, e.g., DTPA (diethylenetriaminepentaacetic acid), at the surface. These particles display a size of about 1–3 nm (and an associated mass ranging from 3 to 10 kDa). They have already proven in vivo their efficiency as a multimodal theranostic contrast agent for MRI and radiosensitization.31 However, their possible functional impact on therapeutic cells (stem cell differentiation for example) remains to be determined.

To tune the cellular capture of these GdNPs, two types of nanoparticles were synthetized (GdNP1 and GdNP2, Figure 2a) according to different protocols. First, GdNP1 comprises a fluorescent FITC group. Second, the synthesis led to two different sizes (3–4 and 1–2 nm for GdNP1 and GdNP2, respectively) and, more importantly, different net charges at the surface: at physiological pH 7.4, GdNP1 had a slightly negative surface (zeta potential = –8 mV), whereas GdNP2 had a definitely positive charge (+17 mV) (supplementary Figure S1). Hydrodynamic diameter is found in the range 3–5 nm for both GdNPs, with a polydispersity index of 35% (supplementary Figure S1).

Having a diameter of less than 10 nm, both GdNPs and IONPs penetrate into the cell interior. Labeling of endothelial cells with IONPs and stem cells with GdNPs yielded internalized masses (Fe or Gd, respectively) of between 1 and 10 pg per cell (Figure 2b). Electron microscopy, optical microscope, and confocal microscope observations localized both nanoparticles within...
intracellular endosomal compartments (supplementary Figure S2). Thus, incubation for 1 h with IONP at [Fe] = 2.5 mM leads to the incorporation of 10 pg of Fe per endothelial cell, with no effect on cell functions (viability and proliferation; see supplementary Figure S3). Overnight incubation (12 h) is required for GdNP labeling, yielding 1 pg of Gd per stem cell after incubation with GdNP1 at [Gd] = 5 mM and 2 and 10 pg of Gd after incubation with GdNP2 at [Gd] = 1 mM and 5 mM, respectively. Note the more efficient incorporation of GdNP2 compared to GdNP1, attributed to the differences in their surface charges: the cellular negative membrane potentials likely promote the interaction between mesenchymal stem cells (MSCs) and GdNP2, in comparison with negatively charged GdNP1. Only the conditions corresponding to 10 pg of Gd per cell had a significant impact on cell viability (supplementary Figure S3). A comprehensive study of GdNP-labeled stem cell differentiation into bone (osteogenesis), fat (adipogenesis), and cartilage (chondrogenesis) was then conducted. For the three differentiation pathways and three intracellular amounts of Gd (1, 2, and 10 pg per MSC), no difference in the expression of differentiation-related genes (Figure 2c) or histological staining (Figure 2d) was found compared to control cells.

**MRI Contrast Properties of Gd- and Fe-Labeled Cells.** The longitudinal ($R_1$) and transverse ($R_2$) relaxivities of the two nanomaterials, both in solution and within cells, were then studied at different magnetic field strengths (Figure 3). In solution, IONPs exhibit the properties of $R_2$ agents ($r_2/r_1 > 10$, high $r_2$) and GdNPs the properties of $T_1$ agents ($r_1 \sim r_2$, high $r_1$). The NMRD profiles (changes in $R_1$ according to the applied magnetic field) always show a maximum at 0.1 T for IONPs and 1 T for GdNPs. At any field, the $R_1$ is superior for GdNP, in good agreement with the higher size of GdNP, At 4.7 T, the relaxation potential of both IONPs and GdNPs is thus favorable for negative and positive contrast, respectively: the $R_1$ of IONPs is greatly reduced, and the $r_2/r_1$ ratio therefore increases, while the $R_1$ of GdNPs is still high or increases.

Interestingly, the $R_1$ always decreased after the nanoparticles had been processed by the cells. With IONPs, the decrease following cell internalization was massive (100-fold or more), while $r_2$ fell only by a factor of 2.5. This resulted in a significant increase in the $r_2/r_1$ ratio, at 4.7 T, from 34 in solution to 330 intracellularly, thereby enhancing $T_2$-weighted cell detection. With GdNPs the $R_1$ fell far less, by a factor of 2.2 for GdNP1 (1 pg of Gd per cell) and 2.6 and 3.8 for GdNP2 (2 and 10 pg of Gd, respectively). Interestingly, $r_2$ did not decrease after cellular internalization; on the contrary, it rose by a factor of 1.1 in conditions of weak internalization (1 and 2 pg of Gd per cell) and 1.3-fold in conditions of stronger internalization (10 pg per cell). These changes led to a noteworthy increase in the $r_2/r_1$ ratio: at 4.7 T, $r_2/r_1$ rose from 2 in solution to respectively 4, 6, and 11 at 1, 2, and 10 pg of Gd per cell. Thus, intracellular confinement favored $T_2$-weighted detection of intracellular IONP but tended to undermine $T_1$-weighted detection of GdNP at high intracellular contents.

**$T_1$ Imaging of Gd-Labeled Stem Cells at Cellular Resolution.** To determine the $T_1$ imaging performance of Gd-labeled stem cells, they were seeded into porous polysaccharide biomaterials and were observed to distribute within the pores of the scaffold (see confocal microscopy images in Figure 4 and Figure S4 with zoomed observations of single cells, where stem cells exhibit green fluorescence, due to a membrane tag or to the FITC associated with GdNP). $T_1$-weighted and $T_2$-weighted images, described in detail in Materials.
Differentiations were obtained with nonlabeled and labeled cells. Incubation of endothelial cells with IONP for 1 h leads to 5 to 10 pg of iron accumulated per cell. The condition at 10 pg/cell was found to be detrimental to the resolution and prevent detailed imaging of cell pattern. Finally, we believe that in case of low Gd intracellular doses, the white cellular patterns observed are the first demonstration of the feasibility of Gd imaging at the cellular level.

Finally, at a higher intracellular Gd concentration (10 pg per cell), the cells appeared black on $T_1$-weighted sequences, and a strong black signal was detected on $T_2$-weighted sequences. At high levels of intracellular confinement, GdNPs therefore lose their ability to behave as a $T_1$ contrast agent, switching instead to $T_2$ contrast, in keeping with the increase in the $r_2/r_1$ ratio observed in these conditions. This highlights the role of intracellular confinement in the modulation of $T_1$ contrast properties. However, in the case of low Gd intracellular doses, the white cellular patterns observed are the first demonstration of the feasibility of Gd imaging at the cellular level.

The same imaging was performed with scaffolds seeded with endothelial cells labeled with IONPs ($T_2$ contrast agent). In this case the cells appeared black on both $T_1$ and $T_2$ sequences, with a total loss of signal on $T_2$ images for the 2 million cell condition shown in Figure 4, due to the excessive contrast of IONP-labeled cells. At lower cell numbers, IONP-labeled endothelial cells can be seen as individual black spots on $T_2$-weighted images (supplementary Figure S8), reaching a single-cell resolution, as previously reported in gels or in vivo. At this stage it must be noted that any improvement in $T_1$ relaxivity for the $T_1$ agents would be highly recommended to increase the sensitivity of cell detection. In contrast, given the large susceptibility artifact created by SPION-labeled cells, more efficient $T_2$ agents are not necessary since it could be detrimental to the resolution and prevent detailed imaging of cell pattern. Finally, we believe that in case both contrast agents are internalized in the same cell, only the $r_2$ effect dominates, preventing distinguishing double-labeled cells from single-IONP-labeled cells.

**Dual Black and White Imaging in Vitro and in Vivo.** Next, we examined the possibility of visualizing the two cell types simultaneously. Endothelial cells and stem cells were seeded in the same biomaterial, either on each side of the scaffold (symmetric seeding) or with and half million cells in supplementary Figure S5 and Figure S6, respectively. With 1 pg of Gd per cell (GdNP), the patterns of white spots observed on $T_1$-weighted images are remarkably similar to those obtained by confocal microscopy (Figure 4), whereas only the gel structure is visible on $T_2$-weighted images, resembling a control scaffold with no seeded cells (Figure S7). In this case the black spots on $T_2$-weighted images likely correspond to microbubbles that are not visible on $T_1$-weighted images. If the intracellular Gd content is increased to 2 pg of GdNP, the patterns of white spots resemble those obtained with 1 pg, but a black (cell) signal is now observed on $T_2$-weighted images, mostly co-localizing with the white pattern. As in these first two conditions, $r_2$ and $r_1$ are identical (see Figure 3); the difference is explained simply by the shortening of $T_2$ to half its value when the Gd content is increased by a factor 2.

Figure 2. Gadolinium and iron oxide nanoparticles for cell labeling. (a) Gadolinium-based nanoparticles made of a polysiloxane network surrounded by covalently bound gadolinium chelates presenting a size of about 3 nm for GdNP1 and 1.5 nm for GdNP2. The iron oxide nanoparticles, 7 nm in diameter, are stabilized by citrate coating. (b) Incubation of mesenchymal stem cells (MSC) overnight with GdNP1 and GdNP2 leads to a cellular capture of between 0.1 and 1 pg of Gd per cell for GdNP1 and GdNP2, respectively. Conditions at 1 pg Gd/cell (GdNP1); 2 and 10 pg Gd/cell (GdNP2) were further analyzed. Incubation of endothelial cells with IONP for 1 h leads to 5 to 10 pg of iron internalized per cell. The condition at 10 pg/cell was selected. (c) MSC differentiation into chondrocytes, adipocytes, and osteocytes. Expressions of chondrogenic cartilage matrix genes aggregan and collagen II (col2A); of adipogenic specific genes Glut4 and AdipoQ; and of osteogenic bone sialoprotein (BSP) gene were measured by real-time PCR, normalized to RPLP0 mRNA and expressed in arbitrary units (means of three separate experiments ± SEM), with the negative control values taken as 1. No statistical difference using the Student’s t test was observed between the different gadolinium labeling of the cells. (d) Adipogenic differentiation was also revealed for all conditions by accumulation of lipid vacuoles stained with oil red O. Osteogenesis was confirmed on histological sections by alkaline phosphatase activity (in blue) and calcium deposition (alizarin red). Similar adipogenic and osteogenic differentiations were obtained with nonlabeled and labeled cells, whatever the gadolinium concentration.

and Methods, were acquired on a 4.7 T MRI device equipped with a cryoprobe to image the biomaterials seeded with different numbers of cells (from 200 000 to 2 million). Images corresponding to a seeding with 2 million stem cells are shown in Figure 4, and with one
endothelial cells in the center and stem cells at the periphery (spot seeding). Both seeding patterns gave rise to images (Figure 5 and Figure 6) with bright white cells on one side and black cells on the other side in the symmetric conditions, and endothelial cells appearing as black spots in the center surrounded by a white cloud of stem cells in the spot-seeding conditions. The cell localization within the scaffold was confirmed with confocal microscopy. By comparing the two imaging techniques, we found that the MRI resolution of detection of the different cell populations is close to 200 \(\mu\)m (supplementary Figure S9).

In Figure 6, this new dual MRI technique was tested in vivo. Biomaterials seeded with both cell types were implanted subcutaneously in mice flanks. On whole-mouse views, the scaffolds were perfectly identifiable, yielding a black and white signal (shown at day 1 in Figure 6 for spot-seeding pattern; other mice and seeding patterns are shown in supplementary Figure S10). High-resolution MRI provided the same resolution as the one obtained in vitro, and the cellular patterns closely matched those obtained by confocal microscopy prior to implantation. Of importance, the time duration to get a high-resolution \(T_1\)-weighted image was less than 10 min, making this in vivo imaging easily manageable. Moreover, the majority of the cells embedded within the transplanted scaffold were still viable at different times, as shown by hematoxylin and eosin staining (supplementary Figure S11). Even if the cell distribution evolved over time, with a more widespread MSC distribution on day 15 (Figure 6c), MRI did not provide quantitative information on the proliferation of the implanted stem cells.

**Application to Vascular Tissue Engineering.** Endothelial and stem cells were then combined within the same porous biomaterial, this time with a tubular shape (internal diameter less than 2 mm), in order to create a cellular graft with a structure mimicking that of a small-diameter blood vessel. The main difficulty encountered with such small substitute vessels was attaching endothelial cells to the inner wall of the tube. Here we exploited IONPs not only as contrast tags but also as force mediators, by using an external magnet to force endothelial cells to form an endothelium (Figure 7). The tube was first seeded with a suspension of stem cells, which efficiently integrated the porous structure. Then, an endothelial cell suspension was introduced into the lumen, and the entire tube was placed inside a hollow cylindrical magnet. The endothelial cells were attracted efficiently to the inner walls (supplementary movie M2), leading to a rapid and uniform coverage. Confocal imaging unambiguously demonstrated the effectiveness of this approach by showing an endothelial layer lining the...
Fluorescence microscopy and confocal imaging confirmed the symmetric localization of the cells within the porous scaffolds (green, stem cells; red, endothelial cells).

DISCUSSION

MRI contrast agents, either paramagnetic (gadolinium chelates) or superparamagnetic (iron oxide nanoparticles), have been developed for human diagnostic purposes. By creating local magnetic fields that modify the relaxation times of surrounding protons, they increase target sensitivity and precision. The use of such contrast agents to monitor cells is more recent, with the first pioneering studies in 2000, and even more recently, the demonstration of MRI stem cell monitoring for therapy. Cell labeling with iron oxide nanoparticles has since been extensively studied in order to ensure biological safety and innocuousness for cell functions. In contrast, gadolinium has been used to label individual cells in only a few studies. The inherent toxicity of free gadolinium has led to the development of gadolinium chelates as molecular contrast agents or to label nano-objects.
therapy applications, it is crucial that the labeling process does not affect the capacity for stem cell differentiation. Only three such studies have been performed with gadolinium, using Gd nanotubes,\textsuperscript{49} mesoporous silica 100 nm particles,\textsuperscript{50} or citrate-coated gadolinium oxide nanoparticles.\textsuperscript{51} Here, we quantitatively investigated the dose-dependent uptake by mesenchymal stem cells of silica-coated gadolinium oxide nanoparticles with different surface charges and found no effect on cell viability for intracellular Gd load up to 10 pg. Moreover, the stem cell capacity to differentiate into three different lineages (adipogenesis, osteogenesis, and chondrogenesis) was preserved. This finding further confirms the potential of a Gd-based nanoplatform as a nontoxic cellular tattoo for MRI cell tracking.

Remarkably, the ability of Gd- and iron-based nanoplatforms to induce distinguishable positive and negative contrasts could be conserved once internalized by cells. It is well known that cellular compartmentalization into lysosomal structures drastically impacts longitudinal relaxivity, mainly due to the hampered accessibility of water protons to the confined $T_1$ or $T_2$ contrast agent.\textsuperscript{52,53} This effect favors $T_2$ (and $T_2^*$) detectability of labeled cells, by increasing the ratio $r_2/r_1$ in a dose-dependent manner. Here is the first observation of the effect of intracellular Gd confinement on the NMRD profile and first comparison of the effect of cellular processing on paramagnetic and superparamagnetic agents. While the NMRD profile of intracellular iron oxide is completely flattened, the profile of GdNPs is still peaked at high field following cell internalization. This suggests that the tumbling dynamics of GdNPs may not be affected by lysosome confinement, in contrast to iron oxide NPs, undergoing a drastic change in magnetic behavior upon cell internalization.\textsuperscript{54} Thus the relative quenching effect of GdNPs probably results mostly from the slow exchange of water molecules across the cell and endosomal membranes. In consequence, for moderate internalization of GdNP (<10 pg), labeled cells could still be detected in white on $T_1$-weighted images, whereas cells tagged with iron oxide were black in any case.

\textit{In vivo}, some transplanted labeled cells may die and release both Gd and Fe nanoparticles, potentially generating a confounding signal. Anyway, it is likely that these nanoparticles would remain entrapped in the scaffold pores and be recaptured by surrounding living cells, thus strongly limiting the risk of cellular signal loss. If, on the other hand, the released nanoparticles enter the bloodstream, they would be rapidly cleared by the reticuloendothelial system, ending up in the liver or spleen and again avoiding a false signal within the implant. However, if inflammatory cells are recruited.
at the site of the implantation, they could as well process the released nanoparticles and dead cells, then leading to a confounding signal.

Such multicontrast labeling provides novel opportunities for noninvasive multicellular tracking. Indeed, in order to mimic the intercellular interactions that maintain tissue homeostasis, cell therapy could greatly benefit from heterotypic cell graft. Recent tissue-engineered approaches highlight the advantages of coculture for promoting tissue repair. For example, myocardial engineered tissues cultured with a network of endothelial cells can be more easily connected to host vessels, contributing to improve cardiac function.59 Likewise, for cell therapy of a full-thickness burn, stem cells seeded in a biocompatible scaffold could be used to reconstruct the damaged area, while endothelial cells would be added to vascularize the graft and thus avoid necrosis.

As demonstrated here for the first time, a multilabeling technique would enable a personalized tracking of the different cell types involved in tissue regeneration. Regarding safety issues, the dose of the contrast agent (either iron oxide or GdNP) introduced in the body by the labeled cells is negligible in comparison to the intravenous dose administrated in current clinical practice. Nevertheless, the unique contrast properties of the iron- and Gd-based nanoplatform loaded in cells allow a differential monitoring of stem cell versus endothelial, together with anatomical details, in a single standard MRI acquisition. Nonconventional MRI contrast agents, such as chemical shift reagent (PARACEST agent)56 and multinuclear reagent (19F-containing label),57,58 were recently proposed to detect specific cell populations. However these frequency-encoding techniques suffer from relatively low spatial resolution in vivo and rely on the use of nonclinical 19F-MRI and MR spectroscopy. In addition, PARACEST agents are no more efficient when internalized in lysosomes and thus require electroporation for cell labeling.56 In contrast, the method presented here uses a very standard MRI protocol, naturally occurring endocytosis of platforms without a transfection agent, and take advantage of the excellent resolution (50 μm) provided by a 4.7 T MRI scanner and dedicated cryoprobe to reveal multicellular organization at the cellular level in a tissue-engineered construct. Moreover we demonstrate MRI monitoring of the multicellular construct after in vivo implantation in a mouse model. Remarkably the imaging signature of the stem cells and endothelial cells, their specific spatial organization, and beginning of the supporting scaffold could still be identified in the host tissue over two weeks after implantation. Such MRI cell imaging has a crucial advantage over 3D optical microscopy, which is limited by the depth of observation. While nuclear imaging methods could also potentially use different isotopes to label two cell types, their resolution is far poorer than that of MRI, and their anatomical correlation is low. Therefore high-resolution multiplex MRI, which has no depth limitation, will provide a unique tool for monitoring the fate of therapeutic cells used to repair tissue damage. It will then be possible to monitor the reconstruction and healing process in depth, as well as the degradation of the supporting scaffold, its ability to deliver the therapeutic cells, the ability of these cells to integrate, proliferate, and vascularize the implant, and the time course of all these processes. Last but not least, superparamagnetic labeling of therapeutic cells affords the unique capability of magnetic manipulation, which can be exploited to mimic cellular organization of native tissues in engineered tissue constructs. Such magnetic control of endothelial cell localization, together with multicellular imaging, was successfully used here to engineer vessels.

The main limitation of the proposed method in the clinical setting would be similar to those encountered with the use of MRI for cell tracking, i.e., nanoparticle dilution during cell division and leakage and intercellular transfer following cell death. Anyway, the first aim of the dual-cell tracking method is to monitor two cell
types within an engineered tissue, in which stem cells may differentiate and stop proliferating, while nanoparticles released from dead cells would likely be trapped within the scaffold and immediately transferred to adjacent living cells. The inherent limitation of this dual method is then that only two codes can be detected, but further developments with complex MRI sequences could be envisaged to modulate the signal as a function of the intracellular amount of each contrast agent.

CONCLUSIONS

Here we demonstrated that therapeutic cells (endothelial cells and stem cells) labeled with distinct gadolinium and iron oxide nanomaterials can be visualized by high-resolution MRI. The two cell types remain visible, one in black and the other in white, when embedded in a biomaterial intended for tissue engineering and cell therapy. This method provides cellular-level resolution, deep within the body, along with the corresponding anatomical image. In addition, the use of magnetic nanoparticles provides new possibilities for cellular manipulation prior to implantation, by simple application of an external magnetic field. Here, by way of an example, we described the creation of a potential substitute vessel. In conclusion, the dual-label, black and white, high-resolution cellular MRI represents a major step toward the goal of noninvasive, in vivo monitoring of cell therapy products.

MATERIALS AND METHODS

Chemicals. MSCGM medium was purchased from Lonza. Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), l-glutamine, penicillin—streptomycin, and phosphate-buffered saline (PBS) were obtained from Gibco. Parafomaldehyde, low-melting agarose, PKH26 red, and PKH67 green were purchased from Sigma-Aldrich. AlamarBlue was obtained from Life Technology. BD Matrigel Matrix was purchased from BD Bioscience.

Gadolinium Oxide and Iron Oxide Nanoparticles. The synthesis of the nanoparticles is a three-step process. The first step is the synthesis of a gadolinium oxide core followed by the growth of a polysiloxane shell in diethylene glycol (DEG). For GdNP1, the synthesis of a gadolinium oxide core followed by the growth of the nanoparticles is a three-step process. The first step is the precipitation of FeCl₂ (0.9 mol) and FeCl₃ (1.5 mol) salts, followed by the formation of a 4.0 mW He laser operating at 633 nm, an Avalanche photodiode detector, and an MPT-2 autotitrator. The zeta potential of GdNPs was determined by ranging the pH of the nanoparticle suspension from pH 12 to pH 4 (with steps of 0.5), by using 0.1 M solutions of NaOH and HCl. Each point corresponds to the average of 30 measurements.

Cell Labeling with Nanoparticles. Human mesenchymal stem cells were purchased from Lonza (HDF-D-00301). These adherent cells were cultured in MSCGM medium, in a humidified atmosphere at 37 °C and 5% CO₂. For gadolinium labeling, the stem cells were used at passages 4 to 8. Endothelial umbilical vein cells (HUVEC-C) were purchased from LGC Standards (CRL-1730). The cells were cultured in DMEM medium supplemented with 10% FBS, l-glutamine (0.002 M), and penicillin/streptomycin (100 IU/mL) at 37 °C in a humidified atmosphere at 5% CO₂. For iron oxide labeling, the cells were used at passages below 40.

Concerning gadolinium labeling, nanoparticle powders (GdNP₁ or GdNP₂) were resuspended in supplemented MSCGM medium and gently mixed for 30 min, in order to obtain a stable suspension at 0.5, 1, 2.5, or 5 mM of gadolinium. Then, the culture medium was replaced with nanoparticle suspension, and confluent stem cells were incubated for 12 h. Confluent HUVEC cells were labeled by incubating cells with an IONP suspension (at 2.5 mM iron) for 60 min, followed by a chase period of 12 h in supplemented culture medium. The surface of the magnetic nanoparticles was stabilized with a coating of negatively charged citrate molecules. The addition of free citrate in the culture medium prevents any aggregation of the nanoparticles, as previously established.

GdNP₂-MSC and IONPs-HUVEC were fluorescently labeled respectively with PKH67 (green) and PKH26 (red) dyes (Sigma Aldrich).

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Analysis. Determination of gadolinium or iron content in labeled cells was performed by ICP-MS analysis (Agilent 7500ce). The samples were digested in concentrated HCl/HNO₃ (3:1, v/v) solution and diluted with ultrapure water for the analysis.

Stem Cell Differentiation, Staining, and RT-PCR. Labeled (with GdNP₁ or GdNP₂) and unlabeled stem cells were subjected to adipogenic, osteogenic, and chondrogenic differentiation to evaluate whether gadolinium labeling has any effect on their differentiation potential. Adipogenic differentiation was performed in adipogenic medium consisting of DMEM high glucose containing 10% FBS, 100 μM penicillin, and 100 μg/mL streptomycin and supplemented with 100 μM insulin, 500 μM isobutylmethylxanthine, 1 μM dexamethasone, and 200 μM indomethacin. Cells maintained in medium without any supplementation served as negative control. After 21 days of incubation in adipogenic medium, cells were fixed in 10% formalin, washed with deionized water and then with 2-propanol 60%, and stained for 10 min at room temperature in 0.3% oil red O (Sigma-Aldrich), rinsed with tap water, and then counterstained with hematoxylin (Sigma-Aldrich).

For osteogenic differentiation, 3 × 10⁴ cells were seeded in six-well plates and grown for 21 days in DMEM low glucose containing 10% FBS, 100 μM penicillin, and 100 μg/mL streptomycin and supplemented with 0.1 μM dexamethasone, 50 μM L-ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate (Sigma-Aldrich). Alkaline phosphatase was then histochemically stained by using a commercial kit (85L2, Sigma-Aldrich). Calcium mineralization was also determined using alizarin red S staining. Cells were fixed in 10% formalin, washed...
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with deionized water, stained 5 min at room temperature with 2% alizarin red S (Sigma-Aldrich), rinsed with tap water, and then counterstained with 1% light green (Sigma-Aldrich).

For chondrogenic differentiation, 2.5 × 10^5 cells were centrifuged (260 g) to induce a high-density pellet, which was cultured in serum-free DMEM high glucose containing 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM L-ascorbic acid 2-phosphate, 0.1 μM dexamethasone, 1 mM sodium pyruvate, 0.35 mM L-proline (Sigma-Aldrich), 1% ITS-Premix (BD Biosciences), and finally 10 ng/mL TGF-β3 (Interchim). Negative control was a pellet cultured in the same medium without TGF-β3.

At the end of all three differentiation protocols, total RNAs were prepared using the total RNA isolation kit (Machery-Nagel). Complementary DNA was synthesized using SuperScript II Reverse Transcriptase kit (Life Technologies), from 1 μg of total RNA in a final volume of 100 μL. Real-time quantitative RT-PCR for five genes (Table 1) was performed in duplicate with the ABI PRISM 7900 sequence detection system and SYBRGreen dye (Applied Biosystems, Foster City, CA, USA). The fluorescence cycle threshold was calculated to quantify the relative amount of gene expression. The mRNA levels of genes of interest were expressed relative to levels of RPLP0. Student’s t-test for paired data was used to statistically analyze differences between the different labeling conditions.

Cell Seeding in Biomaterials. Polysaccharide-based scaffolds were prepared using a mixture of pullulan/dextran (75:25) with sodium trimetaphosphate at 11% (w/v) under alkaline conditions.59 Pores were created by a gas foaming technique using sodium carbonate in 20% acetic acid solution.60 Scaffolds were freeze-dried for 48 h for complete removal of water and stored at room temperature until use. Prior to cell seeding, the scaffolds were punched into discs having a cylindrical shape of 8 mm in diameter and 3 mm in thickness.

For the seeding in porous scaffolds, cells were trypsinized, counted, and resuspended at the desired concentration (0.5–2 × 10^5 cells) in 40 μL of culture medium. The suspension was slowly added to the dry scaffold, covering the entire surface. After hydration, scaffolds became transparent and maintained a regular internal lamellar pore structure, with porosity of about 200 nm.

For the dual-seeded scaffold, 2 × 10^5 IONPs-HUVEC-C were resuspended in 15 μL of culture medium and used to hydrate a half scaffold. After 2 min, the opposite scaffold side was hydrated with 20 μL of a suspension containing 2 × 10^6 Gd-NPs-MSCs. For the spot scaffold, 2 × 10^5 IONPs-HUVEC-C were resuspended in 2.5 μL of medium and seeded at the center of the scaffold. After 2 min, the entire scaffold was hydrated with 40 μL of Gd-NPs-MSC (2 × 10^6 cells). All the hydrated scaffolds were placed at 37 °C for 5 min in order to properly pack the cells in the pores; then culture medium was added to the culture dish in order to completely cover the seeded scaffold. Scaffolds were cultured for 36 h, washed twice with PBS (5 min per step), and fixed in 4% paraformaldehyde for 30 min at 4 °C. Fixed scaffolds were washed twice with PBS, analyzed by confocal microscopy, and then embedded in a 0.3% low-melting agarose gel for MRI characterization.

Toward the Fabrication of a Substitute Vessel. A tubular-shaped polysaccharide scaffold was used to simulate the vessel geometry. The nonhydrated tube had an external diameter of 6 mm, a lumen diameter of 2 mm, and a length of 8 mm. The scaffold was first seeded with a suspension of GdNPs-MSC (2 × 10^5 in 50 μL), by hydrating the tube’s outer surface. The tube was then inserted into a sterile hollow cylinder permanent magnet. The magnetic force experienced by the cells when inserted inside the lumen was calibrated by tracking cell migration (see supplementary movie M2) in concentric circles with an increment of 150 μm and balancing the measured viscous force (6π × viscosity × cell radius × cell velocity) by the magnetic one. The magnetic forces applied to the cells at the tube luminal surface were found to be 73 nm.

For the dual-seeded scaffold, 10^5 IONPs-HUVEC-C were resuspended in 2.5 L of culture medium. The suspension was mixed with 10^5 cells were cultured for 36 h before fixation in 4% paraformaldehyde.

A protein coating of the lumen was achieved by filling the cavity with cold BD Matrigel Matrix (BD Bioscience, #356234, 25 μL) for 30 s. After removing the excess of protein matrix, 20 μL of IONPs-HUVEC-C suspension (4 × 10^5 cells) was seeded in the lumen. After 4 h, the magnet was removed and the tube was cultured for 36 h before fixation in 4% paraformaldehyde.

Microscope Imaging of the Cellularized Constructs. Confocal microscope images were acquired by using an Andor Technology with Olympus BX81/BX61 device/Yokogawa CSU device spinning disk microscope (Andor Technology plc, Belfast, Northern Ireland), equipped with a 60 × Plan-Apo oil objective lens (60/1.42 oil, Olympus) or 4 × UPlan-Fl objective lens (4 × /0.13, Olympus). Samples were excited at 488 nm for detection of GdNP1-MSC and GdNP2-MSC (λem = 525 ± 15 nm) and at 561 nm for IONPs-HUVEC-C (λem = 608 ± 18 nm). Image acquisitions and 3D reconstruction were obtained by Andor iQ software and postprocessed with ImageJ opensource software.

Implantation in Mice. Animals were handled according to the European Community guidelines for the care and use of laboratory animals (European Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes and its amendment (2003/65/EC)). The Institutional Animal Care and Use Committee of Paris Cardiovascular Research Center (PARCC) approved animal protocols. Six-week-old NMRI nude mice (weighing 32 ± 1 g, provided by Janvier, France) were housed in polycarbonate cages and were provided with food and water ad libitum. For scaffold implantation, six mice were anesthetized with ketamine/xylazine intraperitoneally (10 and 50 mg/kg, respectively). Seeded scaffolds (diameter of 6 mm) were implanted subcutaneously on a flank, and the skin was closed with 4/0 sutures (Ethicon). At days 1, 4, 8, and 15, mice were anesthetized by administration of isoflurane in air (supplied at a flow rate of 1 L/min) and imaged by MRI (at least three animals were analyzed at each time point). The induction of anesthesia was achieved with a mixture of isoflurane at 3%, whereas a mixture at 0.5% was used for its maintenance. At the end of the experimental protocol, mice were sacrificed with a lethal injection of sodium pentobarbital.

MRI Imaging. Magnetic resonance imaging was performed on a BrukerBio-Spec 47/40 USR scanner or a 40 cm bore actively shielded 4.7 T scanner equipped with a cryogenic surface coil (CryoProbe) or a whole-volume radiofrequency (RF) coil, in the Small Animal Imaging Platform Paris-Descartes (PARCC-HEGP). The scanner was interfaced to ParaVision software for preclinical MRI research. All fixed scaffolds were imaged with the cryogenic probe by (i) 2D fast spin echo sequence (referred to as T1-weighted) with a TE of 12 ms and a TR of 190, 442, or 2000 ms, flip angle of 180°, in-plane resolution of 39 μm, 20 average, slice thickness of 200 μm for a total of seven slices; (ii) 3D gradient echo sequence (ran under fast imaging with steady-state precession

<table>
<thead>
<tr>
<th>Table 1: List of Primer Sequences for Gene Expression Analyses by Quantitative Real-Time PCR</th>
<th>gene</th>
<th>accession number</th>
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<th>antisense primer</th>
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<td>RPLP0</td>
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<td>5'-AAGGTGTATCTGTCCTCAGACAGTCA-3'</td>
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<tr>
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<td>5'-TCTCCTGACCTGTGTT-3'</td>
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</table>
protocol on free induction decay mode, referred to as 3D $T_1$-weighted, with a TE of 3 ms and a TR of 16 ms, flip angle of 180°, 30 averages, in-plane resolution of 150 μm obtained with the volume RF coil or 39 μm with the cryogenic probe, slice thickness of 500 μm with the volume RF coil or 200 nm with the cryogenic probe, for a total of seven slices for both probes; (ii) 3D gradient echo sequence with a TE of 3 ms and a TR of 16 ms, flip angle of 25°, resolution of 83 x 83 x 83 μm$^3$ (cryogenic probe), two averages. (See Table 2).

**Ex vivo Histological Imaging.** Scaffolds were retrieved from the mice after sacrifice. They were then fixed in 10% buffered formalin before being embedded in optimal cutting temperature (OCT) compound and frozen in a 2-propanol bath, cooled with liquid nitrogen. Cryosections (16 μm) were first observed by confocal microscopy to detect retained fluorescent signal from grafted cells.

**Conflict of Interest:** The authors declare no competing financial interest.

**Supporting Information Available:** Synthetic GdNP procedure and complementary details on cell labeling (Figures S1 to S3) and imaging in biomaterials in vitro and in vivo (Figures S4 to S13) are available free of charge via the Internet at http://pubs.acs.org.

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**REFERENCES AND NOTES**


