In Vivo Magnetic Resonance Imaging of Type I Collagen Scaffold in Rat: Improving Visualization of Bladder and Subcutaneous Implants

Yi Sun, MSc,¹ Paul Geutjes, PhD,² Egbert Oosterwijk, PhD,² and Arend Heerschap, PhD¹

Noninvasive monitoring of implanted scaffolds is important to understand their behavior and role in tissue engineering, in particular to follow their degradation and interaction with host tissue. Magnetic resonance imaging (MRI) is well suited for this goal, but its application is often hampered by the low contrast of scaffolds that are prepared from biomaterials such as type I collagen. The aim of this study was to test iron oxide particles incorporation in improving their MRI contrasts, and to follow their degradation and tissue interactions. Scaffolds with and without iron oxide particles were implanted either subcutaneously or on the bladder of rats. At predetermined time points, *in vivo* MRI were obtained and tissues were then harvested for histology analysis and transmission electron microscopy. The result showed that the incorporation of iron oxide particles improved MRI contrast of the implants, providing information on their location, shapes, and degradation. Second, the host tissue reaction to the type I collagen implants could be observed in both MRI and histology. Finally, MRI also revealed that the degradation and host tissue reaction of iron particles-loaded scaffolds differed between subcutaneous and bladder implantation, which was substantiated by histology.

Introduction

TISSUE ENGINEERING AIMS to repair or replace tissues and organs.¹ The major classes of materials to fabricate scaffolds in tissue engineering are synthetic polymers, and naturally derived substances (biomaterials).² Before clinical implementation can be achieved, their performance *in vivo* needs to be established. Currently, the quality and the degradation of scaffolds and tissue remodeling are mainly investigated in animals postmortem. Unfortunately, the remodeling and regeneration process remains somewhat obscure, as longitudinal follow-up of the same animal is not possible. Noninvasive monitoring of the materials can greatly assist in understanding the remodeling process and the effectiveness of the scaffold in regenerating and supporting tissues. For bone tissue engineering, imaging modalities like micro-computed tomography^{3–5} and magnetic resonance imaging (MRI)^{6–8} have been used. In contrast, proper tools are missing for imaging of materials used in soft tissue engineering: X-ray, ultrasound, and optical imaging are limited by radiation exposure, the lack of contrast, and penetration depth.⁹

Collagen has been used to fabricate scaffolds for functional genitourinary tissue¹⁰ and in skin tissue engineering.¹¹

Type I collagen-based scaffolds showed many advantages in soft tissue engineering, and its design and optimization has been investigated quite extensively.^{12–16} We also have used collagen scaffolds to engineer urethral and bladder tissue in animal models.^{17,18}

The human urinary bladder wall can be visualized in T₂-weighted MR images, because its signal intensity is low, while both the urine and the perivesical fat have high signal intensities.¹⁹ Most MRI of engineered bladder tissue has been restricted to *in vitro* studies.^{20–22} Monitoring the degradation of type I collagen scaffold as bladder implant is challenging: the high signal intensities regions, the irregular and individual shape of each bladder, the similarity of the material to adjacent normal tissue and the ambiguity to identify remodeled tissues make interpretation difficult. MRI of bladder implants in rodents is particularly challenging because their small size aggravates the former difficulties.

In this study, iron oxide particles were incorporated into type I collagen scaffolds to generate contrast for visualization by MRI. Iron oxide particle-loaded scaffolds were implanted either subcutaneously or on the bladder of rats, to monitor scaffold degradation. MR images were obtained at predefined time points and were compared with histological analysis.

Departments of ¹Radiology and ²Urology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

Materials and Methods

Scaffold preparation, contrast agent incorporation, and analyses

The collagen scaffolds (COL) were essentially prepared as described.¹³ In brief, collagen was isolated from bovine achilles tendon using extractions with diluted acetic acid, sodium chloride, urea, and acetone solutions. A 0.67 wt% collagen suspension was prepared by mixing insoluble type I collagen with 0.25M acetic acid. After 16 h at 4°C, the suspension was homogenized using a Potter-Elvehjem homogenizer, centrifuged to remove air and poured into a mould, frozen, and lyophilized, resulting in porous scaffolds. Cross-linking was achieved using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide in combination with N-hydroxysuccinimide, to strengthen the scaffolds. Ultra-small super paramagnetic iron oxide particles (USPIO; Thermo Scientific), 10 nm in diameter, were dissolved in acetic acid (1:4 in volume) before suspending the collagen, to fabricate the contrast agent-loaded scaffold (COL-USPIO). The rest of the process was as described above. Scaffolds were extensively washed using phosphate-buffered saline (PBS). Thereafter, all scaffolds were transferred to 70% ethanol and stored at -20°C until use.

The distribution of the iron particles and morphology of the scaffolds was analyzed using transmission electron microscopy (TEM). The samples were incubated on formvar-coated grids for 1 h at 21°C, washed with 0.1 M phosphate buffer (pH 7.4), then washed twice with MilliQ water, stained with 0.1% (w/v) uranyl acetate, and finally examined in a JEOL 1010 TEM.

Experimental design and surgical procedure

Animal experiments were performed according to local and national guidelines and after approval of the local IACUC (Institutional Animal Care and Use Committee). Adult male WAG/Rij rats were housed in temperature-controlled cages at 50–55% humidity with a 12 h light–dark cycle and free access to standard laboratory chow and tap water. Rats were divided into three groups (with six animals each), which were evaluated postsurgery at 2, 4, and 12 weeks. Surgery was performed under general anesthesia (induction: isoflurane (2–5%), followed by isoflurane (2%)/air (1 L/min) and sterile conditions. Preoperative Rimadyl (5.0 mg/kg) and morphine (1.0 mg/kg) was subcutaneously administered to reduce postoperative pain. Before implantation scaffolds were extensively washed with PBS. Scaffolds (1 cm in diameter) were subcutaneously implanted and on the bladder in the same animal, with one USPIO loaded and one unloaded scaffold. During the following 2 days, postoperative pain medication (temgesic 0.02 mg/kg) was administered subcutaneously. After evaluation by MRI at predetermined time points, the animals were sacrificed by CO₂ overdose and materials were harvested.

Magnetic resonance imaging

Magnetic resonance (MR) images were obtained on a 7T animal MR system (Bruker), equipped with a surface coil (3.5×3.5 cm). T₂-weighted images and T₂ relaxation time measurements were performed on control (COL) and iron oxide-loaded collagen scaffolds (COL-USPIO). The standard parameters for *in vitro* imaging by a turbo spin echo sequence

were as follows: flip angle=30°, FOV=40×40 mm, TR=2000 ms, TE=20 ms, slices thickness=1 mm, and turbo factors=4, matrix 128×128. To measure apparent T₂ values, several images were captured using the same parameters but with different echo time (TE=10, 20, 40, 60, 80, 100, 120, and 140 ms). Signal intensity versus echo time was fitted into a single exponential decay to calculate the apparent T₂ relaxation time.

In vivo MRI was performed using a similar 2D turbo spin-echo sequence (instead of a gradient-echo sequence, to minimize blooming and other artifacts). The surface coil was placed adjacent to the scaffold location, the FOV was 40×40 mm, TR=2000 ms, TE=14 ms, with 20 slices, slice thickness 0.5 mm, turbo factor=4, and matrix size 256×256; the total scanning time was 20 min. Images were acquired under general anesthesia postoperation, at 2, 4, and 12 weeks. A urinary catheter was inserted into the rat bladder before scanning, to facilitate filling of the bladder with PBS to maintain bladder volume.

The volumes of implanted scaffolds were assessed from the MR images by manually outlining their extension in each image slice using ImageJ. The entire volume was then calculated by multiplying the sum of these areas with the slice thickness (1 mm). Scaffold volumes were estimated for the subcutaneous implants immediately postimplantation, and at 2 and 4 weeks thereafter. For each group (COL and COL-USPIO), the average volumes with standard deviation (from three animals) were evaluated by Graphpad Prism (Graphpad Software). The rim thicknesses of subcutaneous implants were also measured at week 2 and 4 (ImageJ). The average of the maximum thickness of capsules from the central region of COL and COL-USPIO scaffolds were listed.

Immunohistological examination

Harvested scaffolds were macroscopically inspected, formalin fixed, and paraffin embedded. Sections were cut (5 µm), deparaffinized, and hydrated through a graded series of ethanol and stained with hematoxylin and eosin. To investigate the distribution of iron particles, sections were stained with Prussian blue.²³

Results

TEM and in vitro MR evaluation of scaffold

The collagen fibrils in the scaffolds showed a striated banded pattern (Fig. 1A, B). The USPIO particles were homogeneously distributed in the COL-USPIO scaffolds (Fig. 1B), even 4 weeks after implantation *in vivo* (Fig. 1C).

From the decay of signal intensity of scaffolds versus echo time, the following T₂ relaxation times were calculated: 22.8±3 ms for COL-USPIO and 80±10 ms for the plain (COL) scaffold. The decreased T₂ of the COL-USPIO scaffold resulted in enhanced contrast: the region of hypointensity in the MR image represented the scaffold (Fig. 2A), whereas the COL scaffold almost vanished in the background signals (Fig. 2B).

Histological evaluation of implanted scaffolds

Small cellular accumulations were observed at the periphery of the plain COL scaffolds, consisting of fibroblasts,

FIG. 1. Ultra-structural localization of iron particles in the collagen scaffold (images of transmission electron microscopy). (A) Plain collagen scaffold (COL), (B) ultra-small super paramagnetic iron oxide particles (USPIO)-loaded collagen scaffold (COL-USPIO), (C) USPIO loaded collagen scaffold after 4 weeks implantation. Small red arrows: collagen fibrils with striated banded pattern. White arrows indicate the small iron particles. Scale bar represents 200 nm. Color images available online at www.liebertpub.com/tec

macrophages, and some PMNs (polymorphonuclear leukocytes), lymphocytes, and giant cells (Fig. 3a). A cellular rim was present at the outer margins of the scaffold at week 2 in almost all scaffolds. At week 4, giant cells, macrophages, and some lymphocytes were still present. After 12 weeks, the number of infiltrating cells was similar but cells were more evenly distributed, and small capillaries with erythrocytes were present (Fig. 3e). Both at 4 and 12 weeks postsurgery, an increase of extracellular matrix formation between the COL scaffolds and the native tissue was observed (Fig. 3k). No major differences were observed between the subcutaneous skin and bladder COL implants.

In COL-USPIO bladder implants, more PMNs, macrophages, fibroblasts, and lymphocytes were observed at the outer margins compared with the subcutaneous implant, both after 2 and 4 weeks. Analysis of COL-USPIO scaffolds 12 weeks postbladder implantation demonstrated many macrophages and giant cells dispersed throughout the scaffold,

whereas this was much less apparent in skin-implanted COL-USPIO scaffolds (Fig. 3f, l). Small capillaries were observed at 2, 4, and 12 weeks in the COL-USPIO bladder group (Fig. 3b, f) comparable to COL-scaffolds 12 weeks postimplantation. Bladder-implanted COL-USPIO scaffolds always showed higher cellular infiltration density compared with the plain COL scaffold at all time points. In contrast, COL and COL-USPIO scaffolds showed comparable cellular infiltration density in the subcutaneous implants.

Prussian blue staining, used to visualize iron, demonstrated the presence of iron-containing cells in the COL-USPIO-loaded groups. Signs of positive Prussian blue could still be detected in material harvested 12 weeks post-implantation (Fig. 4).

Magnetic resonance imaging

Subcutaneous region. For the subcutaneously implanted COL scaffold, the T_2 weighted imaging clearly discriminated between the host tissue and the collagen implant (Fig. 5): the hyperintense region represented the shape and volume of the implant. Moreover, a capsule was present at the outer margins after 2 weeks and a rim was visible (Fig. 5, red arrow), most likely representing the cellular rim observed in the histological examination. At later time points the COL scaffold was still detectable, but with a reduced volume. At 4 weeks, a significant decrease in the volume was observed, while the rim of capsule present at the outer margins of the scaffold postsurgery was still visible. The scaffold became undetectable 12 weeks postimplantation.

The subcutaneously implanted COL-USPIO constructs rapidly degraded as judged by MRI: the central hypointense region disappeared 2 weeks after implantation and only some contrast remained visible in the periphery of the scaffold (Fig. 5). After 4 weeks, the hyperintense region in the scaffold became negligible (Fig. 5). From this time point onward, the MR image of the COL-USPIO scaffold is very similar to the subcutaneously implanted COL scaffold but much smaller. The average volume of the COL-USPIO implants decreased similar to the COL implants until week 2, but was lower at week 4 (Fig. 6).

Measurements of maximum thickness of the capsule in the central region of the scaffold clearly showed that the COL-USPIO implants had a thicker rim compared with the COL implants, at both week 2 and 4 (Table 1).

FIG. 2. T_2 -weighted imaging of plain scaffold and COL-USPIO in an Eppendorf tube filled with phosphate-buffered saline. (A) USPIO-loaded collagen scaffold (COL-USPIO), the region of hypointensity represents the scaffold. (B) Plain collagen scaffold, the scaffold is almost invisible due to the lack of contrast.

FIG. 3. Hematoxylin and eosin staining of sections from surgically removed skin and bladder implant. (a–f) From bladder implants, (g–l) are from skin implants. Scale bar represents 200 μ m. G, giant cells; n, newly formed extracellular matrix; c, new capillaries. Color images available online at www.liebertpub.com/tec

Bladder region. COL scaffolds implanted on the bladder could not be detected in MRI (Fig. 7) due to the hyperintensity of the fluid in the bladders. In animals in which COL-USPIO were implanted on the bladder, a hypointense region was observed, identifying the location and shape of

the implant (Fig. 6). Two weeks after implantation, the size of the hypointense region decreased, and was further reduced but still visible up to 4 weeks. At the last time point, 12 weeks postimplantation, no sign of the contrast agent was apparent on T₂-weighted imaging.

FIG. 4. Perls' Prussian blue staining of the bladder and subcutaneous implants (skin) after 12 weeks' implantation. Scale bar represents 200 μ m. Color images available online at www.liebertpub.com/tec

FIG. 5. T₂-weighted magnetic resonance imaging (MRI) of animals with subcutaneously implanted scaffolds, immediately after implantation and at 2, 4, and 12 week post-implantation. In the COL group, a hyperintensity region was seen. A clear rim could be observed at week 2, and it still existed at week 4. The size of the region decreases after implantation, and finally disappeared at week 12. The COL-USPIO presented as a region of hypointensity (yellow arrows), but the contrast quickly disappeared. After 2 weeks it was only present in the periphery of the implant. The decrease in size was faster for the COL-USPIO, the signal from the scaffold was mostly gone after 4 weeks, and not detectable at week 12. Yellow arrows point to the scaffolds, red arrows point to the rim. Color images available online at www.liebertpub.com/tec

Discussion

In this study we investigated the feasibility of *in vivo* MRI to monitor engineered soft tissue scaffolds, assisted by iron oxide particles incorporation. Regular and USPIO-loaded collagen scaffolds were subcutaneously implanted or on the bladder. We demonstrated that collagen scaffolds on the bladder could only be visualized in MRI with iron oxide doping. Additionally, we observed that the scaffold behavior such as degradation and the tissue reaction depended on the site of implantation.

In regenerative medicine the proper assessment of the degradation of engineered materials and of tissue remodeling requires longitudinal monitoring, ideally noninvasively and throughout the entire time span of these processes. MRI is particularly suited for this goal as it provides sub millimeter anatomical resolution without radiation and restriction of penetration depth, and offers contrast for both dense tissue and soft tissue. Moreover, it also can provide quantitative

FIG. 6. MRI measured volumes for COL and COL-USPIO, from week 0 till week 4.

information.^{24,25} Thus far, few MRI studies have addressed imaging of bladder tissue implants, and these mostly focused on measurements of vascularity, using a blood-pool contrast agent,²⁶ without showing long-term follow-up of scaffold remodeling. To the best of our knowledge, *in vivo* MRI monitoring implants on a small animal bladder has not been described.

In our study of bladder implantations, only the COL-USPIO scaffolds could be visualized and followed longitudinally. In these scaffolds, the homogeneously distributed USPIO perturb nuclear spin relaxation processes of surrounding protons from water molecules.²⁷ On T₂-weighted MR images, this resulted in hypointense regions that improved contrast and visibility of the scaffold implanted on the bladder. These hypointense regions were still visible up to 4 weeks, but not at 12 weeks postimplantation. This indicated that the level of USPIO particles present in the remaining scaffold was not sufficient for a complete follow-up, as the degradation of the scaffold was still in progress according to the histological analysis.

In the MRI of the subcutaneous implantation, the COL scaffold showed a hyperintense region immediately post-implantation. This most likely represented the stored bulk water present in the honeycomb structure and large interstitial spaces of the collagen scaffold.²⁸ After implantation, the PBS in the scaffold would be replaced by body fluids, maintaining the hyperintense appearance of the scaffold in MR images. In contrast, the COL-USPIO scaffold showed a region of hypointensity but this quickly disappeared within 2 weeks. From then on both the COL and COL-USPIO scaffold appeared as hyperintense regions up to week 4. After that the COL-USPIO scaffold appeared to degrade faster than the COL scaffold.

TABLE 1. THE MAXIMUM THICKNESS AT THE CENTRAL REGION OF COL AND COL-USPIO IMPLANTED SUBCUTANEOUSLY, AT WEEK 2 AND 4

Maximum thickness (mm)	Week 2	Week 4
COL-USPIO	0.45 ± 0.09	0.22 ± 0.03
COL	0.3 ± 0.04	0.15 ± 0.03

COL-USPIO, ultra-small super paramagnetic iron oxide particles-loaded collagen scaffold.

FIG. 7. T₂-weighted MRI of the bladder of the rat, immediately postimplantation and at 2, 4, and 12 weeks thereafter. The COL implant is not visible at all, no change could be observed from week 0 till week 12. In the COL-USPIO-implanted animals, the scaffold was observed as a hypointense region (red arrows). After 2 weeks, this hypointense region was substantially decreased. At week 4, a small region of hypointensity was still visible, whereas no sign of iron oxide-generated contrast was observed at 12 weeks. Color images available online at www.liebertpub.com/tec

In our study, USPIO incorporation caused no apparent changes in scaffold morphology. In contrast, doping with another commonly used MR contrast agent, gadolinium with diethylenetriaminepentaacetic acid, destroyed the 3D structure of the scaffold during fabrication (results not shown). Quantification of the iron oxide particle content in the COL-USPIO scaffolds is complex. First, the scaffolds were intensively washed, causing an unknown decrease of incorporated particles. Second, it is difficult to quantify the amount of particles based on their MR signal decrease. Thus, for quantitative assessments, new approaches in MR contrast are needed.

The implantation of the scaffold resulted in an inflammatory response, which was associated with the occurrence of polymorphonuclear cells and macrophages in the scaffold environment in agreement with other studies.²⁹ An invasion of a large number of macrophages was seen, in particular at the peripheral zone of the scaffold, which corresponded with MR observations. In MRI, this foreign body response was reflected by a hypointense capsule at the rim of the hyper-

intense region in the image of the subcutaneous COL implant. The thickness of the capsule could be used as an indicator of the biocompatibility of the material: biomaterials with poor biocompatibility are generally covered with thicker capsules, which restrict angiogenesis and integration.³⁰ We observed an enhanced foreign body reaction for the COL-USPIO scaffolds (a thicker capsule in MRI): the capsule from subcutaneous COL-USPIO implants was always thicker than the capsule from COL implants. This indicates that USPIO incorporation leads to a more severe inflammatory response, but there were no signs of iron oxide-related toxicity and it is very unlikely that the scaffolds contain amounts beyond their safety level (2.6 mg Fe/kg).³¹ Our assumption is this thicker capsule is due to more severe apparent inflammatory response by the introduction of iron particles³² and the hypointensity because of iron particles residuals. Higher resolution and/or functional MRI could obtain more detailed information of the capsule. However, this usually requires longer scanning times, which is not ideal for *in vivo* studies.

In this study, we also found that the remodeling of the COL-USPIO scaffold depended on the implantation site; for example, it was associated with more reactive cellular influx at the bladder site. It is likely that the different tissue microenvironment resulted in different degradation rates, cell response, and remodeling. Our results agree with other studies showing the importance of the microenvironment for the result of tissue engineering,³³ and also with observations in the interaction between tumor and organ-specific factors.³⁴ Thus, it is imperative to test the imaging capability and behavior of a specific scaffold at its target tissue or organ. This will be particularly important for “smart scaffolds” that contain tissue-specific cues such as growth factors.

Future studies could make use of the many imaging options of MR to improve evaluation of implants, such as quantitative MR measurements of *in vivo* T₁ and T₂ relaxation times or diffusion and perfusion processes. Apparent diffusion coefficient values would enable a nondirect measurement of the capsule density, since the dense capsule restricts the movement of free water.³⁵ MR cell labeling could be used to monitor transplanted cells and their behavior in the implant over time and under different physiological conditions. Moreover, gadolinium and iron oxide-based contrast agents can be injected, to track (neo) vasculature and measure vessel density. Noninvasive longitudinal experiments also can reduce the number of animals commonly used in the midterm experimental evaluation.

Conclusion

Our study indicates that next to regular histological analysis, longitudinal MRI of USPIO-loaded collagen scaffolds can reveal its location, size, and degradation, even at challenging anatomical locations. This demonstrated that degradation at different implantation sites may not be the same. Such information is valuable in guiding the further development and evaluation process of type I collagen scaffolds in soft tissue engineering.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Arend Heerschap, PhD

Department Radiology

Radboud University Nijmegen Medical Centre

Geert Grooteplein zuid 10

Nijmegen 6525GA

The Netherlands

E-mail: arend.heerschap@radboudumc.nl

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