Journal of
Tissue Science & Engineering
ISSN: 2157-7552

Kotecha et al. J Tissue Sci Eng 2013, S11
DOI: 10.4172/2157-7552.S11-007

Research Article Open Access

Monitoring Tissue Engineering and Regeneration by Magnetic Resonance Imaging and Spectroscopy

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Abstract

In this article, based on the invited talk at the "Tissue Science 2012" meeting in Chicago on October 1-3, 2012, we describe some examples of characterization of engineered cartilage and bone tissue using magnetic resonance spectroscopy and imaging. Two different models of engineered cartilage and engineered bone tissue constructs were used for these studies: 1) chondrocyte based cartilage tissue engineering constructs: human and bovine chondrocytes seeded in alginate beads (Hydrogel scaffold model) or bovine chondrocytes grown as pellets (scaffold free model); 2) mesenchymal stem cell (MSC) based cartilage and bone tissue engineering constructs: human mesenchymal stem cell (HMSCs) seeded in cartilage biomimetic scaffolds (collagen/chitosan scaffold integrated with extracellular matrix of cartilage) or HMSCs seeded in collagen/chitosan scaffolds. Magnetic resonance spectroscopy and imaging experiments using 9.4 T (400 MHz proton frequency), 11.7 T (500 MHz proton frequency) or 14.1 T (600 MHz proton frequency) MR spectrometers/Imager were performed on these constructs over two to four weeks of tissue culture time. Specifically, water suppressed proton NMR spectroscopy; proton and sodium multi-quantum coherence spectroscopy and proton T1, T2 and ADC parametric MRI were used to study the chondrogenesis and osteogenesis of these tissues. We found that the change in MR relaxation and diffusion coefficient parameters correlate well with the growth of engineered tissues. We found that the MR parameters and the change in these parameters in growing tissue are strongly influenced by the choice of scaffolds. We also found as expected that the tissue-engineered cartilage lacked order or preference in collagen orientation. Further work is underway to elucidate these findings. We anticipate that in future, MRI will augment histological and immunohistochemical techniques by providing a complimentary and real time quantitative assessment of engineered tissue growth at all growth stages: (i) cell seeding to pre implantation; (ii) preclinical validation studies post implantation in small and large animal models; (iii) clinical studies of performance of engineered tissues.

Keywords: Mesenchymal stem cells; Magnetic resonance imaging and spectroscopy; Tissue engineering

Introduction

The growth in tissue science during last decade is led by the belief that (i) it is possible to replace diseased or damaged tissue with engineered tissue which will function like a natural tissue [1], and (ii) it is possible to create a functional engineered tissue by using a combination of appropriate cells, scaffolds and growth conditions [2-4]. For example, engineered cartilage aims to provide a long-term relief to patients with osteoarthritis or to individuals with acute sports injuries [3], whereas engineered bone can provide a cure for large bone defects [4]. Cartilage tissue engineering uses chondrocytes or mesenchymal stem cells as the cell source, hydrogel or fibrin based scaffolds and biomimetic or growth factor delivery based growth conditions to produce a native like functional cartilage engineered tissue [5-8]. The growth conditions such as the cell density, the mechanical properties of scaffolds, the oxygen concentration and the growth factor strength are varied to test the effectiveness of engineered tissue for its target biochemical and mechanical properties [5,9,10]. Bone tissue engineering uses similar but bone-specific strategies, such as bone marrow derived mesenchymal stem cells (MSCs), suitable scaffolds and growth conditions to stimulate osteogenic differentiation of cells, to generate engineered bone with target biochemical and mechanical properties like a native bone [11-16]. The next step is to test the effectiveness of these strategies and to outline plans to move from bench to preclinical studies.

The growth of engineered tissues in the laboratory can be tested using a variety of analytical tools to characterize the tissue growth and to test the effectiveness of the engineering strategies at certain time points (every week or every few weeks). These tools include but are not limited to: histological assays, confocal microscopy, gene expression analyses, x-ray diffraction and micro CT (for engineered bone), biochemical analysis, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). This process is repeated until a successful strategy is found that can go beyond the bench to preclinical testing animal models. This flow is schematically shown in figure 1. In case of cartilage tissue engineering, the results are analyzed to quantify the production of cartilage extracellular matrix components, proteoglycans and collagen, and cartilage related protein expression as biomarkers for success. In case of bone tissue engineering, the extent of mineralization and vascularization are important predictors of success.

Many of the analytical techniques used for characterization of engineered tissues need fixing, sectioning, slicing of the tissue and can only provide a single time point characterization on a single tissue sample at a time. Since these processes invariably change the tissue's biochemical and mechanical properties, they do not give complete or accurate information about living tissue. Once the engineered tissues

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Received December 29, 2012; **Accepted** January 28, 2013; **Published** January 30, 2013

Citation: Kotecha M, Yin Z, Magin RL (2013) Monitoring Tissue Engineering and Regeneration by Magnetic Resonance Imaging and Spectroscopy. J Tissue Sci Eng S11: 007. doi:10.4172/2157-7552.S11-007

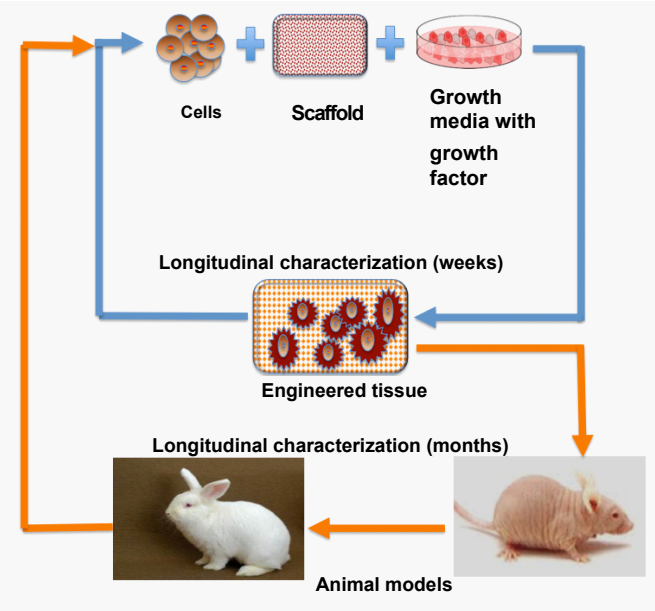

Figure 1: Schematic representation of strategies for testing engineered tissues.

pass the first level of scrutiny towards their effectiveness and moved to the next stage where they need to be tested on animal models, the engineered tissues are implanted in animals subcutaneously or at the injury sites and *in vivo* growth of engineered tissue is followed again for certain time points (typically few weeks to few months) [17]. At the designated time points, the animals are sacrificed and the same analytical techniques are used on *ex vivo* tissues to gauge the effectiveness of the tissue engineering techniques *in vivo*. Even though robust, these techniques for visualizing tissue growth are wasteful and do not give a complete *de facto* assessment of growing tissue; therefore, new analytical and imaging tools are needed to visualize the tissue growth noninvasively [18].

Magnetic Resonance Spectroscopy (MRS or NMR as it is known widely) and Magnetic Resonance Imaging (MRI) are increasingly used in tissue engineering laboratories as new non-invasive characterization tools for the engineered tissues [19-26]. MRS is an established technique to investigate the atomic level structure and molecular dynamics of biological samples, whereas MRI provides volumetric maps of tissue based on the spin density, relaxation times, or diffusion of water protons or other high abundance NMR visible nuclei (such as sodium in cartilage tissue and phosphorous in bone tissue) [27-30]. The changes in the structure and composition of the growing tissue is reflected in changes in MR parameters, such as chemical shift, line broadening, T1 and T2 relaxation times, diffusion coefficient and many other MR expressed parameters [19,20,31]. These MR-visible parameters reflect the physical and biological environment around the observed nuclei and therefore, can provide non-destructive information about changes in local biochemical and mechanical properties of engineered tissue during the growth phase (both *in vitro* and *in vivo*) quantitatively and qualitatively.

Some advantages of using NMR/MRI characterization for assessing the growth of engineered or regenerating tissues are: 1) The MR technique is noninvasive and does not need fixing, slicing, digestion or staining of the tissue; 2) It is possible to perform statistically significant longitudinal charaterization on the same set of tisssue samples (Figure 2(a)); 3) NMR spectra provide a charateristic signature associated with macromolecules (chemical shift) which can be further simplified by using the two-dimensional and double/triple-qunatum coherence spectrsoscopy [20,32]; 4) NMR provides direct information on molecular dynamics, which is not possible to obtain using other methods. The molecular dynamics is an indirect measure of tissue viscosity which is an important indicator of tissue functionality [33,34]; 5) Specific MRI technqiues that provide direct quantitative measure of tissue growth [35-37] include: sodium MRI and the $T1_{rho}$ MRI for quantifying the proteoglycan (GAG) molecules associated with proteoglycans, diffusion tensor imaging (DTI) for observing collagen fibril direction, and SWIFT MRI for visualizating soft tissue components.

These adavnatges makes MR technqiue an attarctive choice for biomolecular laboratory and clincal diagonstics are also useful while assessing longitudinal tissue growth. In the following sections, we give an overview of results obtained in our laboratory for the MRI and MRS charaterization of engineered cartilage and bone tissues.

Materials and Methods

Chondrocytes based cartilage tissue engineering constructs

Bovine chondrocytes were harvested from the metacarpophalangeal joints of 18-month-old bovines using the protocol developed by Petit et al. [38]. The incubation of articular chondrocytes suspended in alginate beads is a common tissue growth strategy in the field of cartilage tissue engineering [38-42]. Bovine chondrocytes (4 millions cells/ml) were

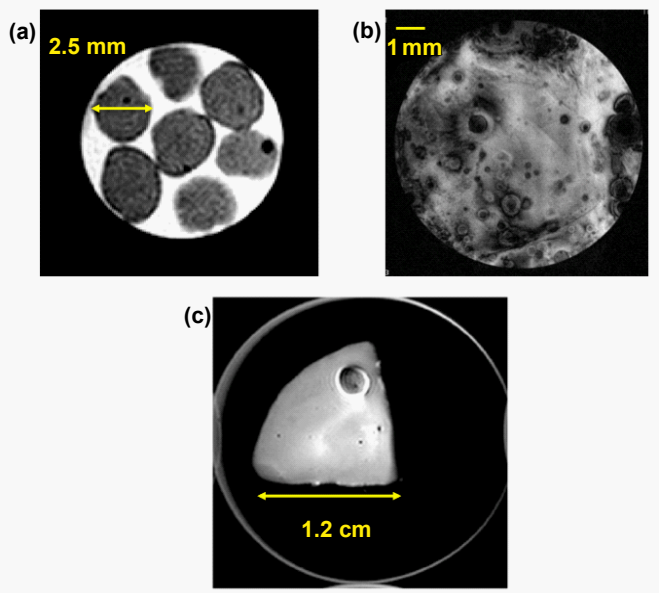

Figure 2: Examples of MRI images of cartilage and bone engineered tissue constructs. T_2 weighted axial slice of: (a) of alginate beads seeded with chondrocytes at 14.1 T. Seven beads are shown in the images indicating that statistically significant results can be obtained in one experiment; (b) Osteochondral tissue constructs (thanks to Prof. Wan-Ju Li at the University of Wisconsin-Madison) with very high resolution at 11.7 T showing dark spots presumably because of bone mineralization; (c) Cartilage monolayer culture purchased from "articular engineering (http://articular.com/)" at 14.1 T.

cultured using chondrogenic growth media in alginate beads according to the published protocol [39]. As an independent experiment, we also tested human chondrocytes (4 millions cells/ml, approx. 75 beads/ml of alginate) seeded in alginate beads purchased from Articular Engineering Inc. (Northbrook, IL).

Bovine chondrocyte pellets were formed by centrifuging 5×10^5 chondrocytes in tissue culture medium at 1000 g for 10 min [38]. The proteoglycan and collagen production were tested using biochemical analysis as described in published protocols [43,44].

Mesenchymal stem cell based cartilage and bone tissue engineering constructs

Human mesenchymal stem cells (HMSCs) were obtained from the NIH funded Tulane center (Tulane University, New Orleans, LA) for preparation and distribution of adult stem cells [43]. HMSCs (2×10^6 cells/ml) were embedded in 1:1 copolymer consisting of collagen, Type I and chitosan [25]. Cells were cultured for two weeks in chondrogenic and osteogenic growth media to generate the extracellular matrix of cartilage and bone respectively. These cartilage and bone ECM integrated scaffolds were used as a control scaffolds (biomimetic scaffolds) to induce chondrogenesis and osteogenesis of stem cells without the aid of growth factor and tested for their effectiveness as a engineered cartilage and bone tissues.

NMR and MRI data acquisition

NMR measurements were performed using a Bruker Avance 9.4 T spectrometer equipped with a broadband RF probe capable of multinuclear spectroscopy. The standard Bruker "zgesgp" pulse sequence was used for collecting the water suppressed proton spectra. The double quantum filtered spectra were collected using Jeener-Broekaert double quantum coherence pulse sequence [44]. Sodium triple quantum coherence spectroscopy was performed using standard four-pulse sequence to filter the triple quantum coherence filter signal [35].

MRI data acquisition

MRI experiments were performed at the Research Resource Center, UIC on an 11.7 T Bruker Avance spectrometer equipped with linear triple axis gradient system of maximum magnetic field gradient strength of 200 G/cm and micro 5 imaging probes equipped with 5 mm and 10 mm RF coils or using a 14.1 T Varian Spectrometer equipped with 30 mm RF coil probe at the Biomedical Imaging Center, Beckman Institute, UIUC. The T1 relaxation time measurements were performed using a RAREVTR (RARE with variable TR) or saturation recovery pulse sequence [45]. The T2 relaxation time was acquired using a CPMG pulse sequence modified by placing the bipolar read-refocusing gradient pair after the 180° pulse [46]. The apparent diffusion coefficient (ADC) was obtained using a diffusion weighted spin-echo sequence [47].

Representative Results

Figure 2 shows some examples of visualization of tissue growth for qualitative analysis using MRI T2 weighted images using human chondrocytes seeded in alginate beads (purchased from Articular Engineering Inc.), osteochondral constructs (obtained from Prof. Wan-Ju Li, university of Wisconsin-madison) and cartilage monolayer culture purchased from Articular Engineering Inc. The experimental parameters can be tailored to visualize the tissue growth at very high resolution (Figure 2b) or to visualize many tissues simultaneously (Figure 2a). The tissue engineers can benefit from such visual information available to them at different growth stage. Figure 3 gives an example of MRI assessment of human chondrocytes seeded in alginate beads over four weeks of culture time. The representative results of MR characterization of engineered tissues are presented below and are also summarized in table 1.

1. We monitored chondrogenic re-differentiation of bovine chondrocytes in alginate beads and in chondrocyte pellets using proton NMR spectroscopy [48]. Our ^{1}H water suppressed NMR data show much broader peaks for pellets as compared to the alginate beads suggesting a higher amount of macromolecule synthesis in the pellets. We also noticed that the peak intensities for all metabolites are higher in the case of pellets as compared to chondrocytes growing in alginate beads confirming higher amount of macromolecule synthesis in pellets. This is in line with a previously published study showing that the pellet culture produces higher amounts of proteoglycans and collagen as compared to the alginate bead system [49].

2. Using sodium triple quantum coherence spectroscopy in bovine chondrocytes seeded in alginate beads, bovine chondrocytes pellets, and HMSCs seeded in biomimetic scaffolds for four weeks, we found that the biexponential sodium relaxation can differentiate between chondrocytes seeded in alginate beads, chondrocytes grown as a pellets and HMSCs seeded in cartilage ECM integrated biomimetic scaffolds, based on the fast and slow relaxation times [20]. We also found that the average quadrupolar coupling was lower in engineered tissues as compared to the natural tissue, which indicates a lack of order in the engineered cartilage tissues investigated in our lab.

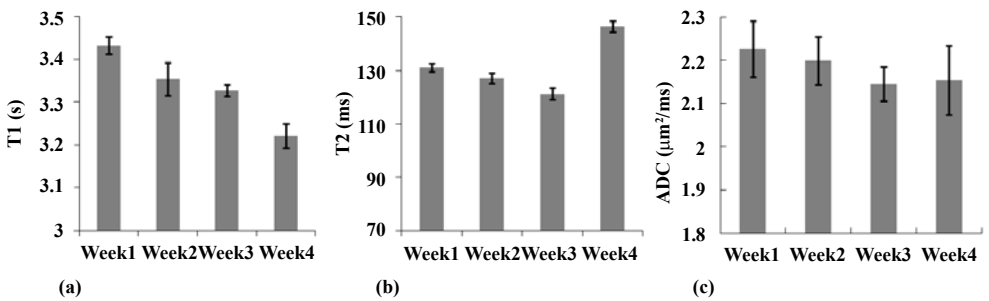

Figure 3: An example of MRI assessment of human chondrocytes seeded in alginate beads (a) T_1 (b) T_2 and (c) ADC values over the period of four weeks. T_1 decreased by 6%, T2 increased by 8% and ADC values decreased by 5%. In a separate experiment, it was confirmed that stiffness increased from 2.8 kPa to 3.8 kPa from week 2 to week 4 confirming the production of proteoglycans and collagen.

NMR/MRI experiment	Engineered tissue samples	Result	Ref.
Water suppressed proton NMR	1. Chondrocytes in Alginate beads for four weeks of culture time 2. Chondrocyte pellets for four weeks of culture time	1. NMR spectral lines associated with macromolecules are broader and stronger for pellets. 2. N-acetyl peak associated with GAG at 2ppm is found to be very week in engineered tissues. 3. Cell proliferation can be tracked using lipid peak at 1.3 ppm.	[21,49]
Sodium triple quantum coherence spectroscopy	1. Chondrocytes in Alginate beads for four weeks of culture time 2. Chondrocyte pellets for four weeks of culture time 3. Mesenchymal stem cells in biomimetic cartilage scaffold for four weeks of culture time	Average quadrupolar coupling are found to be lower in engineered cartilage tissues as compared to the native tissue indicating lack of order in engineered tissues.	[21]
Proton double quantum coherence spectroscopy	1. Chondrocytes in Alginate beads for four weeks of culture time 2. Chondrocyte pellets for four weeks of culture time	Low average dipolar coupling for both samples as compared to the native tissue. The average dipolar coupling was higher (~70 Hz) in alginate bead as compared to the chondrocyte pellets (~20 Hz).	[51]
T1, T2 and ADC MR parametric maps	Chondrocyte pellets for four weeks of culture time	T1 decrease by 18%, T2 decreased 42%, ADC decreased by 26% over the four-week period while tissue gained complexity and extra cellular matrix component that was visible in T2 weighted images.	[25]
T1, T2 and ADC MR parametric maps	Human Chondrocytes seeded in alginate beads and observed for four weeks of culture time	T1 decrease by 6%, T2 increased by 8%, ADC decreased by 5%.	
T1, T2 and ADC MR parametric maps	Human MSCs in collagen/chitosan scaffold for two weeks of culture time	T1 decrease by 66%, T2 decreased 49%, ADC remained unchanged.	[26]

Table 1: The representative results of MR characterization of engineered cartilage and bone tissues.

3. Using proton double quantum coherence spectroscopy for four weeks in bovine chondrocytes seeded in alginate beads and chondrocytes pellets [20,48], we found that the average residual proton dipolar coupling, which indicates the partial alignment of molecules leading to incomplete averaging, is also lower in chondrocyte pellets (~20 Hz) and chondrocytes seeded in alginate beads (~70 Hz) as compared to the native cartilage (~few kHz). This indicates that the collagen in engineered tissues does not have a preferred direction and exhibits a random isotropic spatial distribution.

4. Using T1, T2 and ADC MRI to follow the growth of bovine chondrocytes pellets for four weeks of culture time [24], we found that the T1 decreased by 14% (from 2.1 ± 0.01 sec to 1.8 ± 0.05 sec), T2 decreased by 41% (from 80 ± 0.8 ms to 47 ± 2.1 ms) and ADC value decreased by 29% (from 1.4 ± 0.01μm^2/ms to 1 ± 0.06 μm^2/ms). Biochemical analyses confirmed the production of chondrogenic extra cellular matrix components. Similar experiments with chondrocytes seeded in alginate beads did not show such marked reduction of parameters presumably because of low extracellular matrix production in alginate beads system.

5. Using T1, T2 and ADC MRI to follow the growth of human chondrocytes seeded in alginate beads for four weeks of culture time (see Figure 3), we found that the T1 decreased by 6 % (from 3.4 ± 0.02 sec to 3.2 ± 0.03 sec), T2 decreased for the first three weeks (from 131 ± 1.4 ms to 121 ± 2.1 ms), then increased in week 4 by 8% of initial value (142 ± 1.3 ms) and ADC value decreased by 5% (from 2.2 ± 0.06 μm^2/ms to 2.1 ± 0.08 μm^2/ms). These experiments in comparison with pellet experiments show that the scaffolds play an important role in MR assessment of tissue engineering constructs.

6. Using T1, T2 and ADC MRI to follow the growth human mesenchymal stem cells seeded in collagen/chitosan hydrogel scaffolds for two weeks of culture time [25], we found that the T1 decreased by 66% (from 3.75 sec to 2.46 sec), T2 decreased by 49% (from 65 ms to 32 ms) and the ADC value remain closed to water diffusion coefficient (a change from 2.3 μm^2/ms to 2.2 μm^2/ms) as shown in Figure 4. The production of osteogenic ECM component was confirmed by gene expression analysis. Further details of experiments can be found in the article by Ravindran et al. [25]. The reduction in T1 and T2 values with the growth of extracellular matrix component while keeping the diffusion coefficient close to the water diffusion coefficient indicates that the porous structure of these scaffolds allows the diffusion of nutrients throughout the engineering tissue.

Conclusions and Future Outlook

We have shown that magnetic resonance spectroscopy and imaging are sensitive characterization tools and can provide biochemical parameters related to changes from atomic scale to the tissue microstructure. We have also shown that the MR techniques are versatile and a variety of methods are available suitable for different needs, different cell and different tissue types. In most cases, a special toolbox needs to be created according to the expected results and tissue type for characterization. These techniques are noninvasive and do not need any special treatment of the tissue, hence are suitable for *in vitro*, *ex vivo* and *in vivo* characterization.

Although robust and useful, the MRS/MRI technqiues suffer from low sensitivity and cannot provide moelcular traces of extracellular components which optical techniques provide, hence limits thier use in tissue engineering laboratories. Another drawback is that in most cases, tissue engineers are not trained in magnetic resonance and need to pair up with MR specilist to take full advantage of the depth and the breadth which MR techniques can offer. NMR has been vastly popular for protein and organic molecule charaterization where the tools are more developed. In case of tissue charaterization, the efforts to develop tools are underway. MRI has been widely used for engineered tissue characterization until now. However, common MRI tools such as T1, T2 and ADC maps suffers from lack of specificity and more specific approaches such as sodium MRI, CEST MRI are needed for future development. These new tools can be extremely useful for quantification of extracellular matrix components as well as molecular dynamics which will be an added advantege for tissue engineering purpose. In order to standarize MR parameters for tissue engineering application, more MRS/MRI experiments with different combination

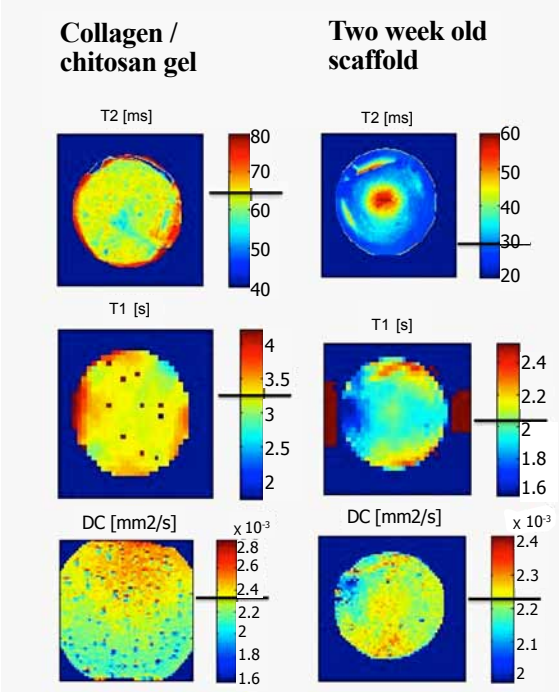

Figure 4: T_1 map, T_2 map and ADC map for collagen/ chitosan polymer gel (left panel) and two week old osteogenic scaffold with extra cellular matrix of bone (right panel). The changes in parameter values are evident from the figure. The experimental parameters were: pulse sequences used MSME (T_2), RAREVTR (T_1) and SE with PFG for diffusion measurements (ADC). Other parameters were: FOV = 1.2 cm×1.2 cm (except for lower left figure, where it was 0.8 cm×0.8 cm), matrix size = 128×128 (T_2), 32×32 (T_1), 64×64 (ADC).

of scaffolds and cells are needed. We welcome colloborations from tissue engineering community for further development of MR tools and for providing quantitative feedback to them regarding the engineered tissue designed for different application. As emphasized in recent NIH sponsered functional imaging for regenerative medicine workshop [18], the colloboration between imaging community and regenrative medicine community is crucial for successful translation of tissue engineering advancement to clinics.

Acknowledgment

This study was supported by NIH/NIBIB R01 grant EB007537. The authors wish to thank Dr. Thomas M. Schmid (Rush University, Biochemistry) for providing chondrocytes based tissue engineering constructs, Dr. Anne George (University of Illinois at Chicago, Oral Biology) and Dr. Sriram Ravindran (University of Illinois at Chicago, Oral Biology) for providing stem cells based tissue engineering constructs for MR studies.

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