Application of magnetic resonance microscopy to tissue engineering: A polylactide model

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Abstract: Absorbable polymers are unique materials that find application as temporary scaffolds in tissue engineering. They are often extremely sensitive to histological processing and, for this reason, studying fragile, tissue-engineered constructs before implantation can be quite difficult. This research investigates the use of noninvasive imaging using magnetic resonance microscopy (MRM) as a tool to enhance the assessment of these cellular constructs. A series of cellular, polylactide constructs was developed and analyzed using a battery of tests, including MRM. Distribution of rat aortic smooth muscle cells within the scaffolds was compared as one example of a tissue engineering MRM application. Cells were loaded in varying amounts using static and dynamic methods. It was found that the cellular component was readily identified and the polymer microstructure readily assessed. Specifically, the MRM results showed a heterogeneous distribution of cells due to static loading and a homogenous distribution associated with dynamic loading, results that were not visible through biochemical tests, scanning electron microscopy, or histological evaluation independently. MRM also allowed differentiation between different levels of cellular loading. The current state of MRM is such that it is extremely useful in the refinement of polymer processing and cell seeding methods. This method has the potential, with technological advances, to be of future use in the characterization of cell–polymer interactions. © 2002 Wiley Periodicals, Inc. J Biomed Mater Res 61: 380–390, 2002

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INTRODUCTION

Absorbable polymers have found recent application in the field of tissue engineering because they may be readily processed into a variety of porous constructs. 1–9 Their role is to provide a temporary scaffold for tissue ingrowth while gradually absorbing, therefore requiring no retrieval surgery after implantation. Because of their unique properties, they can be quite sensitive to conventional histologic processes. 10,11 This is particularly true for polymers with relatively low molecular weights and minimal tissue development, as can often be the case for a tissue-engineered construct before implantation. This limits the amount of information that may be obtained regarding the morphology of the material itself or the material/tissue interaction before implantation, both pertinent and critical pieces of information toward successful integration of the implant. Traditional microscopic techniques, such as scanning electron microscopy (SEM), are dependent on light penetration through the sample of interest. This limits the view to the surface and a few hundred micrometers below the surface. Confocal microscopy has been used in the assessment of porous constructs to observe cellular interaction; however, this technique is both thickness and opacity dependent. It is a noninvasive technique and allows real-time imaging. Unfortunately, it relies on laser penetration into a material, which is depth-limiting. The depth of possible view will be less for a more opaque, thicker sample; less porous also means less penetration. Porous beads on the order of several hundred micrometers have been successfully observed; superficial portions, on the order of several hundred micrometers, can be viewed of thicker specimens. 12,13

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This can provide very important information about the uppermost surfaces of a thick tissue-engineered device, but no information about the equally important central region.

Magnetic resonance microscopy (MRM) may be a critical tool to combine with standard analyses because it may allow noninvasive assessment of the complete volume in these specialized constructs. MRM relies on the same fundamental principles as magnetic resonance imaging (MRI) common in clinical practice. Unpaired nuclei possess a magnetic moment arising from the spin angular moment of the unpaired nucleon. The unpaired proton in $^1\text{H}$ is the most common source of signal biological imaging because of the abundance of protons in most tissues. Imaging relies on measurements of thermodynamic properties and therefore is not opacity or thickness dependent, but rather a function of the applied magnetic field strength. When placed in a strong magnetic field, $B_0$, the collection of protons in the sample tend to precess synchronously about the applied field at a very specific frequency $\omega$ (the Larmor frequency) according to the relationship $\omega = \gamma B_0$, where $\gamma$, the gyromagnetic, is a proportionality constant specific for a given nucleus. At $B_0$ of 7.1 T, the field used for this work, the Larmor frequency is 300 MHz. The synchronous precession of the collection of protons produces a net, macroscopically measurable magnetization, $M$, that can be manipulated by applying an additional magnetic field $B_1$ orthogonal to the main field. If the frequency of $B_1$ is at resonance, i.e., at the Larmor frequency, it will interact with the magnetization, causing it to nutate away from alignment with the main field. This in turn generates a radiofrequency (RF) signal that can be detected by a sensitive antenna placed around the specimen.

This phenomenon of nuclear magnetic resonance has been the basis of nuclear magnetic resonance spectroscopy, used by chemists for years. Spatial encoding of the local effects is reasonably recent with the first spectroscopy, used by chemists for years. Spatial encoding of the local effects is reasonably recent with the first. Unpaired protons and their environment, and extrinsic parameters chosen in a particular imaging strategy to highlight a specific intrinsic parameter. Contrast can be dependent, for example, on proton density, on $T_1$, the spin–lattice relaxation time, on $T_2$, the spin–spin relaxation time, or on the diffusion properties of the water. A shorter $T_1$ is a stronger interaction and represents the time for the perturbed system to return to equilibrium. Faster RF repetitions may not allow this equilibrium to be reached if $T_1$ is long in comparison; the intensity is then said to be "$T_1$ weighted." By weighting the image it is possible to obtain high resolution images (~4 µm) with biochemically dependent contrasts. Researchers have focused on instrumentation improvements, designing strong gradient fields and high sensitivity RF coils. These parameters can be manipulated to differentiate tissue types, for example distinguishing fat from smooth muscle.

MRM differs from MRI in three fundamental ways. MRM encodes at spatial resolution up to $10^6$ times smaller than is routine in MRI. The resulting signal is very weak so MRM is generally performed at very high magnetic fields to increase the sensitivity. Finally, MRM requires the use of much stronger magnetic field gradients. A complete discussion of MRM theory is beyond the scope of this article. Interested readers are directed to an excellent text and more recent discussions of applications specific to this work.

The major disadvantage to using conventional MRI in a tissue-engineered system is its low sensitivity. Tissue-engineering constructs are generally porous, a configuration that can easily entrap air and eliminate the signal. The RF coil configuration, the magnetic field magnitude, the voxel size, and the cellular presence will all influence the quality of detection. Select tissue-engineering studies have been undertaken to examine tissue-engineered constructs in bioreactor systems, specifically in gel-based systems. Alginate is a tissue-engineering carrier used frequently as a cell encapsulating agent or as a chondrocyte carrier in cartilage applications. MRI has been used to monitor cells growing within alginate in bioreactor systems. Using this method, it is possible to monitor cell metabolites, such as intracellular adenosine triphosphate, among others. MR has also been used to determine homogeneity of alginate gels, which may have large impact on its success as a cell carrier in the prevention or promotion of tissue development.

Contrast agents enhance the delineation of biologic and polymeric components in a tissue-engineering scaffold and hence are the subjects of many research foci. It is envisioned that before clinical use, a scaffold might be perfused in a sterile bioreactor with a contrast solution before scanning. Then, after imaging, the scaffold would be perfused with normal media to flush the construct of contrast agent. This allows the analysis to be performed in a sterile environment; thus, if it becomes evident that cellular growth and/or distribution is not adequate, adjustments in culture time and conditions may be made without sacrificing the tissue construct.

To consider MRM clinical use for tissue-engineering applications, it is first necessary to determine its efficacy in simplified in vitro tissue-engineered systems. The purpose of this research was to demonstrate the feasibility of MRM in assessing cellular distribution.
and homogeneity in tissue-engineered constructs, specifically by providing: 1. a three-dimensional (3D) view of the internal microstructure of a porous polymeric construct, and 2. a view of the cellular distribution throughout, thus providing a basis of comparison of two cellular seeding methods.

**MATERIALS AND METHODS**

**Cell isolation and culture**

Cells were isolated with the approval of and strict adherence to the guidelines of the Institutional Animal Care and Use Committee. Serial cultures of rat aortic smooth muscle cells (ASMC) were obtained from 160 to 200 g, 8- to 12-week female Lewis rats (Harlan Sprague-Dawley, Indianapolis, IN). The aorta was aseptically collected, stripped of adventitia, and washed three times in Hank’s balanced salt solution (Sigma, St. Louis, MO). The aorta was sliced into rings and placed in 1 mg/mL collagenase (Worthington Biochemical Corp., Lakewood, NJ), 0.125 mg/mL elastase (Sigma), and 1 µL/mL heat inactivated fetal bovine serum (Sigma) in 15 mL of Dulbecco’s modified Eagle’s medium (Fisher Scientific, Pittsburgh, PA) and incubated for 45 min at 37°C. The digested tissue was then triturated through a 15-gauge cannula and passed through a 70-µm sterile mesh. The cells were plated in smooth muscle growth medium (SMGM; Clonetics, San Diego, CA) supplemented with SMGM-2 in BioCoat™ flasks (Collaborative Laboratories, Bedford, MA). The cells were serially cultured to passage 8 in 150 cm² tissue culture flasks (Fisher Scientific) at which time the cells were washed with phosphate buffered saline (Sigma) and resuspended at 3 × 10⁷ cells/mL in supplemented SMGM.

**Porous constructs preparation**

Thirty-two porous polyactide (PLLA) (Resomer® L206, 0.8 dL/g inherent viscosity post processing; 125–200 micron pore size; Boehringer Ingelheim, Ingelheim, Germany) constructs, 9-mm diameter and 3-mm thickness, were obtained through a salt leaching technique. The constructs were soaked in 70% ethanol for 0.5 h before rinsing with modified smooth muscle growth media.

**Tissue-construct development**

Cells were added to the appropriate constructs using a “static” or a “dynamic” method. The static loading was achieved by placing the constructs in individual wells of a six-well tissue culture plate and pipeting a specific quantity of cells and 250 µL of media onto each porous construct, the amount of media corresponding to the estimated pore volume. After 24 h, the time considered adequate for cellular attachment, 5 mL of supplemented SMGM (denoted SMGMs) was added to each well. The dynamic seeding was attained by placing the polymers and cells into 250-mL stir flasks with 20-mL of media per polymer, four polymers per flask, and stirring at 24 rpm.

Eight matrices were seeded statically with 8.0 × 10⁶ ASMC/250 µL SMGMs/polymer; four matrices were incubated statically with 250 µL SMGMS; eight matrices were seeded dynamically with 2.0 × 10⁷ ASMC/polymer; eight matrices were seeded dynamically with 8.0 × 10⁶ ASMC/polymer, and four matrices were “seeded” dynamically with 250 µL SMGMs. This resulted in a high and low cellular level for dynamically seeded material and a low level of seeding for statically seeded material. The polymers were maintained in culture for 72 h before analysis.

**Tissue-construct assessment**

Presence/absence of cells and state of metabolic activity on the constructs were verified using a live/dead analysis (LIVE/DEAD Viability/Cytotoxicity Kit; Molecular Probes, Eugene, OR) and MTT (3-[4,5-dimethylthiazol-2]-2,5-diphenyltetrazolium bromide) (Acros Organics, Geel, Belgium) assay. Two cellular constructs and one acellular construct from each group were used for MTT analysis, whereas one construct from each cellular group was observed using a Live/Dead assay. One cellular construct and one acellular construct from each group were glutaraldehyde fixed and analyzed with SEM; two cellular constructs and one acellular construct from each group were embedded in glycol methacrylate, sectioned, and stained with hematoxylin and eosin. Two cellular constructs and one acellular construct from each group were formalin fixed and analyzed by MRM.

**Live/dead assay**

Cellular viability was monitored using fluorescence microscopy and a LIVE/DEAD Viability/Cytotoxicity Kit. The assay is based on the simultaneous detection of live and dead cells. Live cells are detected as bright green at a wavelength of 495 nm. This is caused by the enzymatic intracellular conversion of nonfluorescent calcein AM to fluorescent calcein. Dead cells are detected by the infusion of ethidium homodimer through damaged cell membranes and its binding to nucleic acids. This produces a bright red fluorescence at 530 nm. One polymer was assessed from each cellular case.

**Metabolic activity assay**

Metabolic activity was monitored by colorimetric assay of the uptake of MTT by the cells after a 72-h incubation time. The reaction product was extracted with isopropanol (Sigma) and read on a Dynatech MR 5000 microplate reader (Dynatech Laboratories Inc., Chantilly, VA) at 570 nm. Sig-
significant differences were determined based on a 95% confidence interval (Student’s t test). Two cellular polymers and one acellular polymer from each case were used for a total of eight polymers.

**Histology and SEM**

Samples were also retrieved for scanning electron microscopic (Philips CM-30, Eindhoven, The Netherlands) analysis as well as a histologic analysis. One cellular and one acellular sample were used from each group with a total of five samples. The six cellular and two acellular histology specimens were treated overnight in 10% formalin (Fisher Scientific) at 4°C. They were then embedded in glycol methacrylate and 5-micron sections were taken across (parallel to) the circular face of the specimen. The sections were mounted on slides and stained with hematoxylin and eosin. The histological sections were scored using a ranking of 0 to 5, where 0 represents no cellular presence and 5 represents complete cell coverage. Averages and standard deviations were determined for each case.

The five SEM samples, one from each group, were placed in 1% aqueous glutaraldehyde (Sigma) for 1 h and then treated overnight in 0.1% formaldehyde (Sigma). They were then dried with a series of increasing graded alcohols followed by critical point drier processing (EMS 850 critical point drier; Electron Microscopy Sciences, Fort Washington, PA) and gold-coated for SEM analysis with an EMS 550 sputter coater (Electron Microscopy Sciences).

**MRM**

A phantom experiment was performed using an acellular matrix from each group and formalin with Magnevist® [Formalin/gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA), in a volumetric ratio of 60:1]. There are no free protons in the polymer; therefore, it has no signal.27 The scan captures the microstructural features of the matrix by a “negative” effect; i.e., by measuring the proton-containing immersion fluid. Gd-DTPA reduces the T1 of formalin, providing a higher signal and thus highlighting and differentiating the matrix. To distinguish cellular presence, two cellular constructs from each group were placed in 10% formalin for MRM imaging. The resulting signal was displayed in gray scale, where black corresponded to no signal and white to a high signal. The diffusion coefficient of formalin is high and its T1 is long (resulting in a low signal); the opposite is true of tissue, resulting in a high signal (as denoted by bright white areas). Again, the polymer provides no signal (as denoted by black areas). The specifications were determined according to a pilot study,15 using a 7.1 Tesla scanner and a custom-designed Helmholtz pair coil. A 3D Fourier transform spin-echo pulse sequence with repetition time TR = 600 ms and echo time TE = 11 ms was used for the cellular samples. Spin-echo (or spin warp) imaging is the standard method for spatial encoding of the majority of MR imaging. This encoding method has been modified to provide diffusion weighting. The magnitude of the gradients required for this spatial resolution imply inherent diffusion weighting.28 Specifications of TR = 100 ms and TE = 10 ms were used for the acellular phantom samples. The slice thickness was 93.8 μm and the in-plane resolution was 23.4 μm and 46.9 μm for the cellular and acellular samples, respectively.

**RESULTS**

Figure 1(A) shows a 4-μm glycol methacrylate section of the PLLA porous control construct, after a 72-h dynamic treatment. The white areas are the PLLA; the light gray areas are the pores. It is impossible to quantify the precise location that this represents within the construct and it is also impossible to determine whether the structure is an accurate representation of the original specimen. Because of the thin section, an irregular pattern is evident—only where the section coincides with the pore plane can one truly envision the pore morphology created by the randomly distributed porogen. Figure 1(B) shows a 4-μm glycol methacrylate section of the PLLA porous cellular construct after a 72-h dynamic incubation with a high seeding level. The cells are evident within the structure (black areas); however, if polymer has been removed or dis-
torted during processing, then the true interfacial behavior between the cells and polymer will be impossible to discern.

Histological results showed that the highly loaded dynamic systems appeared to have a greater number of cellular aggregates distributed across the respective sections than single cells. There appeared to be less cellular presence at the edge of the sections than in the central regions. The dynamically loaded, low cellular concentration sections were inconsistent as to appearance histologically. One sample appeared to have an even distribution of cells across the face of the section, but both had spread cells and aggregates. The other section displayed a smaller number of spread cells, select aggregates, and some acellular areas. The statically loaded sections were consistent in appearance, both having an abundance of cells on one side unevenly distributed across that side of the section. The dynamic acellular samples resulted in very wrinkled sections. The static acellular samples also resulted in wrinkled sections but the sections appeared qualitatively to have a higher amount of polymer remaining. Both the dynamic case with low cell numbers and the acellular dynamic case appeared to have degraded in their mechanical environment, whereas the statically loaded polymers and the polymers loaded dynamically with a high cellular number had minimal disruption to their structure. Sections were ranked and scored by cellular presence and the results are shown in Table I.

The SEM analyses readily show the relatively intact state of static loading and a high cellular level with dynamic loading [Fig. 2(A–C)] versus the degraded nature of the dynamic seeding with low cell number and the acellular dynamic seeding cases [Fig. 2(D)]. The statically loaded constructs appear to have cellular growth across the surface, as compared with the dynamically loaded ones. The SEM shows a view of the base of the statically loaded constructs, agreeing with the MRM results (discussed below in more detail) that clearly show a cone-shaped distribution of cells throughout the scaffold, emanating from a continuous layer at the base. The dynamically seeded ones with more evenly distributed cells, as evidenced also by MRM, do not appear to have a similar high surface loading, as assessed by SEM. It is, however, difficult to distinguish cells on the polymeric scaffold, even at a high magnification. Unlike fibrous scaffolds in which the scaffold is a smooth circular structure, the solvent cast systems (particularly after the experimental time in media with serum) have a rough, irregular appearance that hides cellular components (Fig. 2). All cellular scaffolds exhibited measurable metabolic activity and all acellular scaffolds exhibited none. The dynamic culture cases with low cell number did not demonstrate significant differences in metabolic activity from those with high cell number (Fig. 3); however, both values were significantly greater than that of the statically loaded scaffolds.

MRM allows the opportunity to noninvasively observe the polymeric microstructure, as is demonstrated in Figure 4, showing a central section of 94-μm thickness, approximately at 1.5-mm depth of a 3-mm thickness, acellular specimen. Figure 4 shows the heterogeneous nature within a slice, where the edge has a “skin” of solid polymer and the interior region shows the mosaic caused by the leached salt crystals. The specimen provides no signal but is “revealed” by immersion in the solution containing Gd-DTPA, which provides a strong signal. The inverted images have a spatial resolution sufficient to readily appreciate the pore topography.

Figure 5 compares high and low levels of seeding in dynamic culture. The image shows the resolved cellular component for both top and side views. The images are rendered to show the volumes, i.e., the complete cellular content, including top and side views. The white areas are cells; the higher cellular density in the high level of cell seeding case is readily apparent. Figure 6 compares static and dynamic seeding for a low level of seeding. The static seeding with a low cellular number appeared to cause cellular sedimentation where there are more cells at the bottom than at the top of the polymer and, at the top, there are more cells evident in the middle than on the edges. Thus, static seeding causes a dome-shaped distribution of cells, as viewed from the side of the construct. Figure 6 shows both a top and side view of the statically loaded tissue construct, both rendered for cellular component into one plane. The dynamically loaded materials, in contrast, appear to have an even distribution of cells within each matrix.

**TABLE I**

<table>
<thead>
<tr>
<th>Method of Seeding, Cell Loading Level</th>
<th>Average Ranking</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamic, high loading</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Dynamic, low loading</td>
<td>3</td>
<td>1.4</td>
</tr>
<tr>
<td>Static, low loading</td>
<td>2.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Possible scores range from 0 to 5, where 0 represents no cellular presence and 5 represents complete cell coverage.

**DISCUSSION**

Pore size and structure are critical parameters influencing tissue ingrowth. The type of tissue introduced to a tissue-engineered system can be manipulated by modulating the scaffold design. For this reason, the ability to characterize the entire microstructure
and cellular distribution throughout such a scaffold is essential. Because most methods of processing porous constructs render the material heterogeneous in structure, it is important to a complete understanding and eventual optimization to thoroughly assess the scaffold–cell interaction.

Contrary to "traditional" implant materials which do not house cells on the interior and generally are solid materials, tissue-engineered implants usually are porous and contain cells within their volume as well.

**Figure 2.** (A) SEM of statically loaded, cellular PLLA scaffold with $8 \times 10^6$ ASMC. Porous cellular sample with cells. (B) SEM of statically loaded, control PLLA scaffold. The acellular samples maintained their integrity in the less mechanically demanding, static environment. (C) SEM of dynamically loaded, experimental PLLA scaffold with $2.0 \times 10^7$ ASMC. The cellular samples maintained their integrity in the mechanically demanding, dynamic environment. (D) SEM of dynamically loaded, acellular PLLA scaffold. The acellular samples were degraded in the dynamic environment, as can be seen by the fragmented appearance of this sample. Bars indicate the scale.

**Figure 3.** Cellular metabolic activity of cells, sample size of two, 95% confidence interval, error bars correspond to standard error.

**Figure 4.** (A) Top view, PLLA acellular scaffold in Magnevist, 1.5-mm depth of 3-mm thickness, 9-mm diameter specimen, 94-micron slice. Bar indicates the scale. (B) Closer view of microstructure, 1.5-mm depth of 3-mm thickness specimen, 94-micron slice. Pore structure is evident.
as on their surface. Because many are treated with cells before implantation, it is imperative to assess these very unique systems before implantation. Successful integration of a tissue-engineered implant relies on the controlled distribution of cells throughout a 3D volume. If cells adhere solely to the surface of the matrix and a sheet of cells forms, this results in an ineffective tissue-engineering matrix because it has effectively been reduced to a 2D cellular form, thus defeating the original purpose of providing a 3D carrier. Additionally, the coating may effectively be sloughed during or after implantation. If the desired arrangement of cells is an even distribution throughout the matrix, it is critical to verify that this has indeed occurred. Fibrotic tissue will infiltrate spaces that do not have other cell types present and this is generally undesirable in tissue reconstruction. Eventually it may be preferable to house multiple cell types in a heterogeneous manner (e.g., fat cells within the matrix and smooth muscle cells toward the surface) and it would be ideal to differentiate the cell/tissue types and verify this conformation within the 3D volume. The other key point regarding reasons for desiring a 3D analysis is the fractured view that histology provides. These are very different systems from the “traditional” systems from which standard histological techniques were derived.

Traditional methods of tissue-construct analysis include SEM, light microscopy, confocal microscopy, and biochemical assessments. SEM, although providing an excellent view of the surface of the material, does not lend information about the seemingly invisible central regions. This is often satisfactory for traditional biomaterials, particularly nonporous nonabsorbables, about which only surface information is desired. Tissue-engineering constructs, however, are generally both absorbable and porous. Not only is the interior structure crucial to enhancement of tissue development, but it is a dynamic structure, changing both with tissue development and with material degradation in vitro or absorption in vivo. SEM sample processing can also change the appearance of the sample because it involves several dehydration steps as well as a potentially artifact-inducing coating step.

The authors do not recommend the use of MRM as a standalone method, rather as a tool in an arsenal of methods, including histology. As such, it is critical to highlight the deficiencies of “traditional” histologic methods to appropriately design an optimized experiment (with maximum potential for data returns) where microscopy, histology, biochemistry, chemical characterization will be complementary. Light microscopy involves histological processing of specimens to acquire a thin section or slide. This is typically an excellent method of assessing both microstructure and cellular interaction with the biomaterial, particularly nonabsorbable materials. However, many absorbables, PLLAs, in particular, can be radically altered during this processing.10,11 The extent of degradation can depend on a number of factors, including molecular weight of the material, thermal transitional values, crystallinity, solubility, time of implantation or time degraded in vitro, and the amount of surrounding tissue retrieved. Obviously, the more shielded the material, the less problematic this phenomenon becomes. Therefore, the materials that have degraded in culture before processing will be more susceptible to histological processes than those that are relatively intact. As the material degrades or absorbs in vitro or in vivo, histological processing becomes increasingly difficult; it becomes impossible to positively differentiate between true absorption or hydrolytic degradation of the material and degradation due to histological pro-

Figure 5. Comparison of cell loading number in dynamic systems. Top row is side view (3-mm thickness), bottom row is top view. White indicates cellular presence resolved into one plane. Brighter white in high cell loading case (right) verifies the presence of a higher number of cells after seeding and incubation. Cells are evenly distributed in both cases. Bar indicates the scale.

Figure 6. Comparison of cell loading method for low cell number. Top row is side view (3-mm thickness), bottom row is top view. White indicates cellular presence resolved into one plane. Static method (left) results in a dome-shaped distribution of cells whereas the dynamic method results in a more even distribution. Bar indicates the scale.
cessing. A cellular, porous construct that has been cultured in vitro and has relatively less shielding tissue and high surface area is very susceptible to processing. Constructs with lower molecular weight, for example those used in soft tissue repair, are even more prone to degradation.

A second point making a histomorphometric analysis convoluted is that, as explained in a previous article, a higher amount of tissue mass does not necessarily indicate a better system. In fact, two systems can realistically have identical tissue mass but vastly different morphologies—e.g., a construct with cell aggregates only versus one with well-spread tissue development. It is very difficult to make direct comparisons based solely on histological results for these unique materials.

MRM analyses showed that both statically loaded constructs had a dome-shaped distribution of cells, whereas the dynamically loaded ones had an even distribution of cells. This was qualitatively obvious but was not quantitated—this was a feasibility study only; the next step is to develop enhanced methods of differentiation and a method of quantitation. There are two potential approaches to quantitation. Quantitation itself may be done through standard image analysis techniques (e.g., 3D image analysis packages are a standard tool in confocal microscopy); it is the enhancement of color contrast that is essential to minimizing error, thus the experimental set up or processing of the specimens is crucial. The diffusion coefficient of intracellular water is <10 times that of “unbound” water. By differentiating between the T₁s and/or diffusion coefficients of different proton sources, it may be possible to quantitate the cell density. The second method would be using alternate perfusion methods, perhaps a marker to preferentially label the intracellular fluid, thus enhancing the differentiation.

As contrast agent technology improves, so will the imaging potential of multicellular tissue-engineered devices. Conventional contrast agents, such as Gd-DTPA have a nonspecific extracellular distribution. Improvements may be made by using targeted agents that rely on selective uptake or clearance (e.g., lipophilic markers that target the liver) or that are directly injected to the site. An alternative is to use functional agents that are chemical respondents to tissue or organ function, thus shifting with changes in local physiological conditions. Combination agents may also be applied.

With the introduction of fluorescently labeled contrast agents, it is possible to integrate fluorescence microscopy with MRM, thus allowing features invisible to fluorescence microscopy to be highlighted in MRM and, conversely, to correlate cellular microstructure viewed via fluorescence microscopy with MRM. Other groups are investigating the use of magnetoimmuno agents that will immunohchemically bind to specific groups within tissue, using a receptor-ligand mechanism.

Gadolinium labeled monoclonal antibodies have been another avenue of interest, but have not had the success expected in MR imaging. Monoclonal antibodies are pricey, the amount necessary to deliver the requisite quantity of gadolinium depending on the strength and quality of the MR system; thus, this could be a very inefficient method given the current state of technology for the average MR system.

The apparent variability in this study between the dynamically seeded samples of low cell concentration, as judged solely by histology, may purely be a function of depth of section observed. It is impossible to know precisely what depth each histological section is taken, given that a sample may not be paraffin embedded in a perfectly horizontal manner and it may therefore require a different number of sections to face and to retrieve a complete section. A polymeric specimen embedded perfectly horizontally (denoted Case A) would obviously have less polymer removed to achieve a full section than that embedded even at a slight angle (denoted Case B). Additionally, the planes or sections assessed in Case A versus B are totally different; the one in B incorporates areas from the surface of the polymer to ones well into the central regions. It is also one view only, of an almost random section and gives no clue as to the three-dimensionality of the implant. The static cases were consistent in histological appearance; however, given the MRM view of a dome shaped, uneven distribution of cells, it is clearly a representative picture of one plane only. If one was to serially section the construct, it may be assumed that cellular distribution would vary enormously. It is apparent that statically loading the constructs allows the cells to congregate at the base of the polymer. The dynamic case allows all sides of the porous construct to be continually exposed to the media and cells, thus improving cellular distribution. PLLA is relatively hydrophobic, so constant exposure to media allows greater opportunity for wetting of the porous construct, throughout its entirety. Whereas these points are not critical to the traditional, “solid” implants, where the area of concern is the interface between the exterior of the material and surrounding tissue, it is of foremost importance to a porous tissue-engineered construct. It is in this scenario that all the interfaces are considered, including all those located centrally within the porous polymer.

All dynamically treated constructs, both low cellular level and acellular, showed higher mechanical degradation over the course of the study. The constant motion causes the constructs to collide with each other and the flask walls. It is hypothesized that the cell number and related protein content in the dynamic system played a hand in “bonding” the material to-
gether, resulting in less damage to the constructs with a high cellular level. It is probable that higher cell growth and/or less mechanically demanding flow conditions (in the static conditions) are responsible for the retention of mechanical integrity.

Standard biochemical assays are vital for assessing the metabolic state of the cellular component; however, they do not give information about the distribution of cells within a scaffold. Additionally, many of these assays are difficult to adapt from a 2D system such as a tissue culture plate, to a 3D scaffold. MTT analysis, for example, can be used in 2D systems to estimate cellular number; because, a standard curve may be derived from a 2D culture. In a 3D system, where the cellular growth and behavior can be quite different, it is difficult to accurately correlate these values using a standard curve derived from 2D culture. Therefore, for example, it may not be possible to differentiate between a high number of cells with low metabolic activity and a low number of cells with high metabolic activity. Figure 3 plots the cellular metabolic activity for each experimental case. This assay demonstrates that dynamic cellular seeding significantly enhances cellular metabolic activity (p < 0.05) as assessed using Student’s t test; however, this assay yields no information regarding spatial organization and, with this number of samples, one cannot differentiate between low and high dynamic seeding methods. Certainly, the number of samples could be increased; however, this becomes both cost and time intensive. The dynamically seeded polymers with low cellular number exhibited much higher variability (standard error of 0.10 for the dynamically seeded polymers with low cell number versus 0.01 for the dynamically seeded system with high cell numbers) which may be indicative of varying cellular content because of the mechanical degradation of the cell-carrying parts of the scaffold. DNA analyses, used to accurately assess cell number in 2D systems, can similarly be extremely inaccurate in 3D systems where repeated extractions are necessary and information is readily lost. Whereas it is not the intent to minimize the importance of biochemical assays, it is important to understand their limitations.

It should be noted that the MRM analyses used formalin-fixed samples, which is of interest and importance in research and nonclinical studies. Because observation of cellular distribution in 3D constructs is a previously unexplored area, this study was developed as feasibility only, to see if one could indeed differentiate cells from the matrix. A fixed specimen is the simplest case and only the preliminary step. Without fixative, one would expect to lose cellular viability before finding the optimal instrument settings. Once the optimal settings are determined and a standard operating procedure developed, live conditions (e.g., in an incubator, simulating a clinical specimen) may be studied without losing the true state of cellular activity. The short culture time in this study is regarded as an extreme case, testing the lower range of feasibility of cell detection with MRM. The results show that MRM can successfully demonstrate cellular distribution across a given section of polymer, information that is lost with histological analysis. The cells are denoted by the stronger (white) signal, the matrix by no (black) signal, and the formalin by the low (dark gray) signal. MRM operates on the same basic principles as MRI, but with a spatial resolution of roughly 6 orders of magnitude higher. The imaging is dependent on a number of physical parameters including the diffusion coefficient of a given free proton containing liquid and the proton density. Because the matrix is not a hydrogel, there are no free protons in the matrix. The scan in essence captures the microstructural features of the matrix by a “negative” effect, i.e., by immersing the matrix in a fluid and registering the proton containing immersion fluid. This is extended when a cellular matrix is analyzed; in this case, the difference between the diffusion of the protons in the relatively restricted environment of the cell and those unrestricted in the surrounding solution results in a stronger signal from the cell.

It is therefore possible to differentiate between seeding/proliferation methods and select an optimal method to enhance cellular distribution throughout the entire polymeric volume. The resolution of MRM is currently limited to approximately 10 to 20 μm, so it is not possible at the low culture time to observe the cellular/material interaction at the cellular level; however, with longer culture times and development of tissue, it can be assumed that this will be improved. MRM has the potential to be of great use in assessing the development of tubular and nontubular structures in tissue constructs, information that will be clinically relevant.

It is possible to differentiate between low and high cell seeding conditions using MRM. This capability is important because the two conditions are indistinguishable using other test methods, including assessment of metabolic activity as previously described. The comparison between seeding methods was only made for a low level of cells; however, it is interesting that these differences are evident at such a low level. The cell distribution influences the development of tissue throughout the polymer; certainly, the more even distribution of cells will allow enhanced infiltration of tissue. In the case in which large acellular areas are present within the polymer, it is possible that this material may simply absorb after implantation without development of the appropriate tissue. It has also been shown that a cellular component presence before implantation influences the appearance of vascular structures within the construct after implantation.35

MRM does by no means, at the present time, allow
assessment of the cell–polymer interaction at the cellular level; however, it allows a view of the cell distribution throughout a previously opaque volume. This may be extremely useful clinically because each patient may require a custom-fabricated tissue construct. Cellular growth and behavior depend on many variables, (e.g., patient age, body type, location in the body). It is therefore important, for autologous tissue-engineered implants, to monitor the development of tissue-engineered scaffolds accordingly and choose the appropriate time for implantation. A noninvasive approach is critical to minimize the donor tissue, polymer, and related supplies. Additionally, invasive monitoring techniques can lead to lost information. Because there is variability from implant to implant, an implant sacrificed for invasive assessment may actually yield slightly different information than the implanted one. This can be potentially influential on the successful development of 3D, viable tissue. Another important aspect to MRM is the advantage of real-time imaging; that is, no time is lost because of embedding and processing samples. A time lag between monitoring and implantation can result in minimally accurate information concerning the implanted tissue construct. Much research remains to be accomplished to make MRM truly efficient and cost effective as a tissue-engineering tool; however, the potential and utility are enormous for tissue-engineering clinical efficacy.

CONCLUSIONS

MRM may be successfully applied to a porous, absorbable tissue construct to optimize polymer microstructure design as well as optimize seeding/proliferation methods. In this particular study MRM showed that, for the cellular PLLA systems in question, static loading created an uneven distribution of cells throughout the scaffold. MRM was able to pinpoint and differentiate between high and low cellular number in dynamic loading. It was possible to visualize the polymeric microstructure at any depth within the 3D scaffold. It may be of clinical interest to use this approach before implantation.

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