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Monitoring tissue development in acellular matrix-based regeneration for bladder tissue engineering: multiexponential diffusion and T2* for improved specificity

Hai-Ling Margaret Cheng, PhD\textsuperscript{a,b,*}, Yasir Loai, BSc\textsuperscript{c}, Walid A. Farhat, MD\textsuperscript{c}

\textsuperscript{a} Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada
\textsuperscript{b} Physiology & Experimental Medicine, The Research Institute and Diagnostic Imaging, The Hospital for Sick Children, Toronto, ON, Canada
\textsuperscript{c} Developmental and Stem Cell Biology, The Research Institute and Division of Urology, The Hospital for Sick Children, Toronto, ON, Canada

Short title: MRI of acellular matrix-based bladder tissue engineering

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* Correspondence to:

Hai-Ling Margaret Cheng, PhD
Department of Diagnostic Imaging
The Hospital for Sick Children
555 University Avenue
Toronto, Ontario M5G 1X8, Canada
(tel) 416-813-5415 (fax) 416-813-7362
E-mail: hai-ling.cheng@sickkids.ca
ABSTRACT

Cell-seeded acellular matrices (ACMs) are a promising approach for tissue-engineering soft tissues and organs such as the urinary bladder. The ACM contains site-preferred structural and functional molecules, and degradation products derived from the ACM play important roles in tissue remodeling. Regeneration proceeds along concurrent trajectories of cell growth and matrix degradation, characterized by evolving biophysical and biochemical properties. Assessment of tissue development through a non-invasive imaging technique such as MRI must, therefore, be capable of distinguishing these concurrent biophysical and biochemical changes. However, although MRI provides exquisite sensitivity to tissue microstructure, composition, and function, specificity remains limited. In this study, multiexponential diffusion and effective transverse relaxation time T2* were investigated for their ability to assess cell growth and tissue composition, respectively. Bladder ACMs prepared with and without hyaluronic acid, and ACMs seeded with smooth muscle cells, were assessed on MRI. The slow diffusion fraction from multiexponential diffusion analysis demonstrated the best correlation with cellularity, with minimal influence from underlying matrix degradation. T2* measurements were sensitive to macromolecular content, specifically, the presence of hyaluronic acid, without confounding influence from tissue hydration. T2* also appeared sensitive to cell filling of matrix pore space. Compared to these metrics, commonly used MRI parameters such as T1, T2, and single diffusion coefficient were more limited in specificity. Use of T2 to measure tissue structure and composition is limited by its large dependence on water content, and single diffusion can only reflect the overall characteristics of the extra- and intracellular
environment. These findings are important for further development of more specific MRI methods for monitoring regeneration in tissue engineered systems.

**Keywords:** quantitative (MRI) magnetic resonance imaging; multiexponential diffusion; (T2*) effective transverse relaxation time; (ACM) acellular matrix; cellularity; (GAG) glycosaminoglycan; tissue hydration; bladder tissue engineering

**Abbreviations:**

2D, two-dimensional

3D, three-dimensional

ACM, acellular matrix

dH₂O, double distilled water

GAG, glycosaminoglycan

HA, hyaluronic acid

HBSS, Hank’s Balanced Salt Solution

H&E, hematoxylin and eosin

VEGF, vascular endothelial growth factor
**GRAPHICAL ABSTRACT**

Monitoring tissue development in acellular matrix-based regeneration for bladder tissue engineering: multiexponential diffusion and T2* for improved specificity

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Multiexponential diffusion MRI and T2* are investigated for monitoring acellular matrix-based bladder regeneration. The slow diffusion fraction was shown to correlate with cellularity in the presence of extracellular matrix changes, while T2* was sensitive to macromolecules without influence from tissue hydration. These metrics provide improved specificity over single T1, T2, and diffusion for assessing concurrent tissue engineering processes.
INTRODUCTION

The urinary bladder and bladder disorders

The primary function of the urinary bladder is to store urine at low pressure and allow voluntary voiding. These functions are regulated by nerves in the spine and around the bladder and depend on the unique composition and structure of the bladder wall. In a normal bladder, the wall is composed primarily of three layers: the urothelium, the lamina propria, and the detrusor muscle. The innermost urothelium consists of highly specialized cells that provide a barrier to urine penetration into underlying tissues and bloodstream, while the lamina propria is a connective tissue matrix that limits bladder wall distension and overall compliance. The detrusor layer consists of smooth muscle cell bundles that are coordinated by neural control to relax and contract during the filling-voiding cycle, thus maintaining low pressure and protecting the kidneys from damage.

Bladder dysfunction can result from a variety of congenital and acquired conditions and compromises quality of life for approximately 400 million people worldwide (1). In children, the causes are often congenital and include bladder extrophy (1 in 40,000), urethral defects (1 in 500) leading to obstructed urine flow, and neuropathic conditions such as myelomeningocele (1 in 800) that alter neuronal control of the bladder. Loss of proper bladder control, with subsequent changes in function, can arise from other disruptions to the neural pathways between the bladder and the micturition center in the brain. In adults, for example, neurogenic bladders are often induced from trauma to the spinal cord or from tumors. Obstructive uropathy can also present in adults for a variety of reasons, including stone formation, enlarged prostates, or tumors. Regardless of the cause of dysfunction, the
structure, thickness, and biomechanics of the bladder wall can be significantly altered. The result is a high-pressure, low-compliant bladder leading to incontinence and, in severe cases where pressure extends up the ureters, swelling and impaired function of the kidneys.

The current treatment strategy for bladder dysfunction uses gastrointestinal segments for replacing bladder tissue (2). This approach can be problematic because gastrointestinal tissue absorbs solutes that urinary tissue excretes. Due to this difference in tissue function, several complications can arise, such as infection, metabolic disturbances, urolithiasis, perforation, and malignancy (2). Alternative methods and materials have been investigated, most notably tissue expansion (3) and free grafts made from natural (e.g. small intestinal submucosa, bladder submucosa) or synthetic (e.g. gelatin sponge, polyvinyl sponge) materials (4,5). However, these attempts have met with limited success due to biocompatibility, mechanical, structural, and functional problems. Thus, the use of gastrointestinal segments has remained the gold standard despite its limitations.

**Tissue engineering for bladder replacement**

Alternative treatments for bladder dysfunction involving tissue engineering techniques have been pursued in an effort to overcome limitations associated with the conventional use of gastrointestines. Promising results have been reported in patients using autologous cells seeded on a collagen-polymer scaffold (6) and in dogs using cell-seeded allogeneic acellular bladder matrices (7). Prompt angiogenesis of thick tissues is critical to maintain viability and support continued growth and long-term survival (6,8). Cell-seeding also is required to ensure tissue regeneration beyond a distance of 0.5 cm (9) necessary for organ engineering. Compared to the unseeded scaffold, cell seeding reduces fibrosis and
MRI of acellular matrix-based bladder tissue engineering

Graft contracture and improves overall cellular organization (7). Another requirement is the use of a scaffold to guide 3D tissue formation. Both synthetic materials and biological matrices have been used. However, synthetic materials usually succumb to mechanical failure (6), and biological matrices are believed better able to reproduce the required biomechanics and also offer intrinsic biochemical characteristics that are difficult to achieve with synthetic materials. A promising biological scaffold material is the acellular matrix (ACM) derived from the extracellular matrix of the desired tissue type. The ACM has cellular components removed to reduce immunogenicity (10) and contains site-preferred structural and functional molecules for reconstructing tissue. Degradation of the ACM yields products that have the required angiogenic (11), chemotactic (11,12), and mitogenic (13) activities to facilitate tissue remodeling. Specifically, these products contain chemotactic and mitogenic factors that increase recruitment and proliferation of undifferentiated cells and inhibit the proliferation of differentiated cells. ACMS have been derived from different tissues, such as skin (14), liver (15), heart valves (16), and bladder (17), differing in their 3D structure but sharing very similar biochemical composition (13).

Despite early promise, much remains to be accomplished toward engineering a fully functional bladder. Obstacles include lack of prompt angiogenesis, adverse host-tissue reaction, inadequate mechanical properties, and lack of innervation. Angiogenesis must be established promptly for cell expansion and to avoid scarring, but it is currently very difficult to achieve functional vessels that are long-lasting (18). Another interference with regeneration is adverse host-tissue reaction, such as that arising from immunogenic response of cell components or nondegraded scaffold biomaterials. This reaction can be mitigated with the use of ACM and scaffold manipulation to prevent urine penetration and
its toxic effects (19). Achieving proper biomechanics presents even greater challenges. Evidence exists that in-vitro mechanical conditioning to simulate the in-vivo environment can improve tissue morphogenesis, proliferation, differentiation, and function (20,21,22). However, the type and degree of physical stimuli required and their role in the bladder remain to be determined. Finally, innervation is an important component to bladder function but is the least studied and understood. Strategies for nerve regeneration from other applications perhaps may be applied, such as a recent demonstration of innervating muscular tissue by seeding primary cultured neurons on a biological extracellular matrix (23).

**Imaging of the tissue engineered bladder**

Technology for non-invasive assessment such as MRI is recognized to enable accelerated progress in tissue engineering and eventual longitudinal monitoring of patients with tissue engineered organs. Current regeneration approaches need to look beyond standard histology for a more complete assessment of the function, structure, and composition of the developing tissue throughout its entire lifespan, from in-vitro cell seeding to long-term functioning in vivo, in order to identify more effective strategies. In the bladder, important parameters include cell growth, distribution, viability, and host-implant interaction in addition to ones more specific to large soft-tissue organs, particularly those with a mechanical function. These more specific parameters include biomechanics, functioning of an induced vascular supply, properties of new natural scaffolds for soft-tissue regeneration, and cell-scaffold interaction.

MRI studies of the tissue engineered bladder have been few but have focused on
several different aspects of regeneration. The earliest efforts addressed the importance of angiogenesis, investigating the potency of vascular endothelial growth factor (VEGF) and the ability of MRI to quantify neovessel formation (24,25,26). MRI measures of vascularity were shown to correlate with increased blood volume at higher VEGF doses (24), and quantification on an absolute scale was feasible using a blood-pool contrast agent (25). These studies also demonstrated that enhanced vascularization reduced fibrosis and improved overall cell repopulation in vivo (26).

The ACM scaffold has been the other main focus because of its role in regeneration, specifically, in providing an environment conducive to angiogenesis, tissue deposition, and restoration of structure and function specific to the grafted site (27). MRI characterization of the ACM has been lacking until recently but is important for establishing a baseline against which to gauge subsequent tissue development. In a couple of recent MRI studies of the bladder ACM (28,29), new imaging observations revealed unanticipated biological phenomena that may be relevant in other ACM-based systems. One important finding was matrix collagen degradation noted very early on after cell-seeding, possibly due to easier breakdown of biological tissue compared to synthetic materials (28). Degradation byproducts are known to modulate tissue remodeling (13), and matrix degradation concurrent with cell growth is expected in many regeneration paradigms. Distinction of these concurrent processes using more specific MRI techniques, including multicomponent T2 as demonstrated in Ref (28), will be critical to accurate assessment. Another valuable insight is that scaffold optimization from a single manipulation can produce concurrent biophysical and biochemical changes that have unpredictable effects on MRI parameters. Specifically, the incorporation of hyaluronic acid, a naturally occurring macromolecule that
can act as a carrier of bioactive molecules and growth factors and has shown potential in the development of engineered tissues (30,31), was shown to increase macromolecular content and ACM hydration (29). These concurrent changes exerted competing effects on MRI tissue relaxation and diffusion, and single MRI parameters failed to provide adequate specificity. These studies (28,29) highlight the ability of MRI to improve our understanding of uncharted development of engineered tissues by detecting changes for further investigation and validation. They also emphasize the need for more specific MRI methods to assess tissue structure, composition, and function.

In this study, we explore MRI methods for improved specificity in monitoring tissue development in a bladder ACM-based regeneration model. Multiexponential diffusion and effective transverse relaxation time, T2*, are investigated for their ability to discriminate cellularization and matrix changes without influence from concurrent or undesired sources.

**EXPERIMENTAL**

**Acellular matrix (ACM) preparation**

Acellular tissue matrices were prepared according to a published protocol (10). Fresh urinary bladders were harvested from pigs (20-50 kg) and immediately washed in sterile phosphate buffer saline (Sigma), longitudinally sectioned, and placed in a hypotonic solution [5 mM EDTA, 10 mM Tris HCl, pH 8.0, 1% Triton X-100, 0.1 mg/mL Pefabloc Plus™ (Alexis) and Penicillin/Streptomycin] (Sigma) for 72 hours, stirring at 4°C, to lyse all cell structures and inhibit proteases. Bladder tissues were then immersed in hypertonic solution [5 mM EDTA, 10 mM Tris HCl, pH 8.0, 1% Triton X-100 and 1.5 M KCl]
(Sigma), stirring for an additional 72 hours at 4°C to denature residual proteins. Subsequently, bladders were washed in Hank’s Balanced Salt Solution (HBBS), followed by overnight incubation at 37°C with Benzonase (2U/mL) diluted in HBSS to degrade DNA and RNA components. Final extraction was performed by transferring bladders to a 0.25% CHAPS (non-denaturing detergent) based solution [50 mM Tris HCl, pH 8.0, 1% Triton X-100 and Penicillin/Streptomycin]. The resulting ACM was washed with sterile double distilled water (dH$_2$O) and stored in 70% ethanol. Hematoxylin and eosin (H&E) sections were taken to confirm acellularity. All protocols were approved by the institutional Animal Care Committee and compliant with national policies on the humane use of laboratory animals (CCAC guidelines).

**Preparation of ACM and cell-seeded constructs**

ACMs of 3 mm thickness were cut into 2×2 cm$^2$ squares, dehydrated by gradual immersion in increasing concentrations of ethanol (50%, 70%, 80%, 90%, 100%) for 1 hour each at room temperature, and lyophilized for 24 hours (VirTis-temp, -70°C and vacuum, 120 millitorr). Hyaluronic acid (HA) was incorporated by rehydrating ACMs in increasing concentrations of HA (0.05, 0.1, 0.2 mg/100 mL) (Sigma) shaking for one hour, then in 0.5 mg/100 mL HA for 24 hours, all at 37°C. Excess HA was washed off with dH$_2$O. Alcian blue staining was performed to confirm HA uptake (Figure 1). ACMs with and without HA were immersed in medium (high glucose Dulbecco’s modified essential medium (Wisent Inc), 10% fetal bovine serum) at 4°C.

For cell-seeding, cell cultures were obtained from passage 2 smooth muscle cells isolated from porcine bladder (32) and expanded in medium. Cells were seeded on ACMs
in a 6 well plate at $1 \times 10^6$ cells/cm$^2$ in 100 µL of medium. The cells were allowed to attach on the ACM for 3 hrs (37°C in a humidified atmosphere of 5% CO$_2$) before incubating in 2 mL of medium. The medium was changed every 72 hours.

**Experimental design**

Cell-seeded ($N_s=20$) and unseeded ($N_u=15$) ACMs were prepared as described above. Six of the unseeded ACMs were further processed for HA incorporation. Imaging was performed on day 1, 3, and 7 and, for seeded ACMs, at an additional timepoint of 21 days after cell-seeding. Samples were prepared for MRI by transferring each ACM to a 5 mL, 12 mm diameter round Falcon tube containing medium, and placing the tubes in a water-filled container. Histology was also performed, and samples were taken prior to imaging to verify no changes occurred during MRI. Additional validation measurements involving immunofluorescence microscopy and biochemical assays from previous experiments are reported here to provide a benchmark.

**Histology**

All tissue samples were fixed in 10% formalin for 24 hours, embedded in paraffin, and sectioned into 5 µm thick slices. Sections were stained with H&E and Masson’s Trichrome and examined under a light microscope (Nikon Eclipse E 400). Cell density was assessed qualitatively at all time-points on H&E sections. Four different classes of cellularity were defined: I (very few cells), II (few cells), III (patches of cells), and IV (good cellularity). A blinded histology assessment was performed by a pathologist.
Quantitative MRI

MRI was performed on a 1.5 Tesla clinical scanner (Signa EXCITE TwinSpeed, GE Healthcare, Milwaukee, WI) using a single 3-inch surface coil positioned under a water-filled container in which ACM-containing tubes were placed. Pilot scans were taken to determine placement of imaging slices perpendicular to the tubes and encompassing the upper and lower ends of the ACMs.

Quantitative T1, T2, T2*, and diffusion imaging was performed. The longitudinal relaxation time T1 was measured using a rapid 3D technique (33) based on a fast spoiled gradient echo sequence with radio-frequency field correction [flip angle = 2, 3, 10, 20°, number of averages (N_{AVG}) = 4]. The transverse relaxation time T2 was measured using a 2D spin echo sequence [9 echoes with echo times (TE) = 9 - 300 ms, repetition time (TR) = 3000 ms, N_{AVG} = 1]. The effective transverse relaxation time T2* was measured using a 2D multi-echo gradient-echo sequence [16 equally spaced TEs = [2.6 – 34.4] ms, TR = 118 ms, FA = 20°, N_{AVG} = 4]. Diffusion imaging was performed using a spin echo diffusion-weighted imaging sequence up to high b-values [b = 0 – 3000 s/mm² in steps of 200 s/mm², TR = 4000 ms, N_{AVG} = 16]. In addition to high b-value acquisition for multiexponential diffusion analysis, single diffusion coefficients were also obtained by imaging at a b-value of 1000 s/mm². Slice thickness was 3 mm for all sequences; in-plane resolutions were 0.4 mm (T1, T2, T2*) and 0.8 mm for diffusion.

All image post-processing of quantitative T1, T2, and T2* data was performed on a pixel-wise basis using in-house scripts developed in Matlab (V.7.0, Mathworks Inc., Natick, MA, USA). T1 parameter maps were generated as previously described, with
analytical-based flip angle correction using B1 field maps acquired separately (33). Both T2 and T2* parameter maps were computed by fitting a mono-exponential signal decay function to signal intensities obtained at various echo times (34). The mean T1, T2, or T2* value for each ACM was then obtained by averaging within the ACM and through all imaging slices through which the ACM traversed. For diffusion data, a region encompassing the ACM was drawn and the average signal intensity at each b-value was taken for analysis. Non-linear least squares analysis, which requires no a priori knowledge of the number of the exponentials, was used to estimate diffusion coefficients and their fractions. All data are expressed as mean value ± standard deviation.

**Statistical analysis**

Differences between groups were assessed using a two-tailed Student’s t-test. Significance was declared at $P < 0.05$.

**RESULTS**

**Histology**

Cellularity varied over the time interval of 1 to 21 days post-cell seeding, with cells aggregated mainly at the surface of the ACM. Representative H&E sections showing the degree of cellularity associated with each of the four classifications are given in Figure 2. No difference in cellularity was observed in samples taken before and after MRI from the same ACM, indicating that the time spent for MRI did not affect cell viability.

Masson’s Trichome sections (Figure 3) illustrate the main constituents of the ACM: smooth muscle bundles within a collagen extracellular matrix. Histologically, the ACM
appears consistent amongst the different preparations: cell-seeding and HA incorporation. However, it is known from previous immunohistochemistry and biochemistry experiments that cell-seeding results in matrix collagen degradation from cell-released collagenase (28), and HA incorporation increases both macromolecular content and tissue hydration (29). Table 1 summarizes these biochemical changes. These concurrent changes present conflicting influences on single MRI parameters such as T1 and T2 and thereby limit their specificity. For example, increased T1 and, to a much greater extent, increased T2 from hydration can confound our assessment of cell growth or matrix glycosaminoglycan (GAG) content. The T2 spectrum was previously shown to distinguish these concurrent changes (Figure 4), but its assessment of biophysical boundaries (such as cells) would be limited due to its sensitivity to biochemical composition.

**Quantitative MRI**

Examples MR images of the construct are shown in Figure 5. From high b-value diffusion imaging, sensitivity to presence of cells in the ACM is shown. Figure 6 shows that cell-seeded and unseeded ACMs exhibit biexponential and monoexponential diffusion decay, respectively. This behaviour is consistent with a fast diffusion component associated with the extracellular compartment and a slow diffusion component associated with the intracellular space. Figure 7 shows that the slow diffusion fraction has the highest correlation with cellularity \( r = 0.954, P < 10^{-5} \) compared to single diffusion coefficient \( r = -0.153, P = 0.654 \), T1 \( r = -0.614, P < 0.05 \), and T2 \( r = -0.309, P = 0.355 \).

New observations on T2*, which is predominantly used in tissue iron and oxygen measurements, suggest its potential role as a more specific parameter for assessment of
tissue composition. Figure 8 shows that cell-seeding produces the greatest relative change on T2* ($P < 10^{-4}$) compared to T2 ($P < 0.05$) or T1 ($P = 0.11$), although the correlation between T2* and cellularity was not significant ($r = 0.152$, $P = 0.676$). Figure 9 shows that the presence of the macromolecule HA in the unseeded ACM produces significant changes on T2*, T2, and T1 ($P < 0.05$), but the largest relative change is seen on T2* and appears to be due to reduced sensitivity to tissue hydration. Note that while both T1 and T2 changed in the same direction with cell-seeding or incorporation of HA, only T2* changed in opposing directions with these two manipulations.

**DISCUSSION**

Improved specificity in MRI measurements was investigated in this study for monitoring tissue development in a bladder ACM-based paradigm for soft-tissue regeneration. The ACM as a biological scaffold is a relatively new approach for guiding 3D tissue formation and is especially promising for whole organ tissue engineering, especially those with a mechanical function. Aside from reducing an immunogenic response, the ACM possesses the required structural and mechanical properties and its degradation releases by-products that facilitate tissue remodeling. This approach may provide truly off-the-shelf replacement tissue if animal-derived ACMs can be used in humans. For instance, given the similar composition and biomechanical properties between porcine and human ACMs (35), some investigators have taken the next step and seeded human bladder cells on porcine ACM (36). Nonetheless, experience with biological matrices is in early stages, and there are few related imaging studies. The first MRI studies of ACM-based soft-tissue regeneration reported early matrix degradation (28), which is not seen or probably arises
much later in conventional scaffold materials. This concurrency of matrix degradation and cell growth can confound assessment of either process if common MRI parameters such as T1, T2, and single diffusion coefficient are used. Also, tissue hydration can vary substantially with matrix manipulation (29), likely much more so than in synthetic polymer scaffolds. Because of these properties, the ACM may require more specific MRI techniques to distinguish relevant processes amidst less important concurrent changes, such as those related to tissue water content.

For the assessment for cell growth, this study shows that multiexponential diffusion analysis may provide the best specificity by minimizing sensitivity to other changes, such as matrix degradation or tissue hydration. These other changes exert an appreciable effect on more commonly used parameters in tissue engineered systems, such as T1, T2, and single diffusion coefficient (37), and underlie the weak correlation between these parameters and cellularity as observed in this study. Amongst these three parameters, only T1 exhibits a trend (decrease) consistent with cell growth. The other two parameters, T2 and diffusion, are more unpredictable probably due to their greater sensitivity to water content and changes in the extracellular matrix structure. In contrast, the slow diffusion component in multiexponential diffusion decay is sensitive to the intracellular compartment and quite insensitive to changes in the extracellular environment. It also provides a more specific technique to measure cellularity without confounders from tissue biochemistry.

New properties on the effective transverse relaxation time $T2^*$ were observed in this study and suggest improved specificity over T2 measurements. One consistent observation was that unlike T2, $T2^*$ was relatively insensitive to water content. This is best appreciated by comparing ACMs with and without HA (Figure 9). In principle, macromolecules
including proteins induce a lowering in T2 and, therefore, T2* compared to free water. This is due to more restricted water in the hydration layer on the surface of the macromolecules. However, being hydrophilic in nature, HA also elevates tissue hydration and, thus, free water content, therefore increasing T2. The competing T2 effects from a higher GAG content and increased hydration resulted in a net increase, whereas T2* remained low. The only plausible explanation is that T2* is much less sensitive to water content, likely because an increase in free water does not introduce local centers of magnetic non-uniformities; thus, T2* dephasing is not affected. This reduced sensitivity of T2* to hydration was speculated in a recent study of chondrocyte transplantation in patients (38), but histological validation was lacking. In this study, biochemistry validation confirms that T2* is much more specific to macromolecular content than T2 or T1. A second observation in this study was a substantially larger T2* in cell-seeded relative to unseeded ACMs (Figure 8). This result is more difficult to interpret. A possible explanation is that cell-seeding, which is known to fill pores of the ACM, reduces T2* dephasing associated with pore interfaces. This result is particularly relevant in tissue engineering applications, because scaffolds are designed to be porous for the purpose of embedding cells. The ability to identify when cells have occupied these pores is very valuable for determining how firmly entrenched cells are.

While this study has suggested new potential in tissue engineering applications for multiexponential diffusion and T2* measurements, our results are consistent with MRI studies in other applications that attempt to elucidate underlying biological mechanisms. For example, diffusion studies of perfused breast cancer cells in vitro showed that a second slow diffusion component associated with restricted intracellular diffusion arose when cells
were added to a gel medium (39). Other studies have shown a correlation between the slow diffusion fraction and intracellular space in cell suspensions (40,41). However, caution must be taken in interpreting the slow diffusion fraction in terms of absolute cellular volume. The biexponential diffusion model is valid only when there is no exchange between intra- and extracellular water compartments and no restriction of intracellular water by cell membranes. When these conditions are unmet, diffusion decay deviates from a pure biexponential, and the diffusion fractions do not correspond to the actual sizes of the two pools (42,43,44). In order to achieve the ideal situation, water exchange must be very slow, such as when there are short diffusion times, slower diffusion rates, large cells, or low membrane permeability. Nonetheless, the slow diffusion fraction provides a useful metric to estimate cellularity even if not on an absolute scale.

Our T2* findings are more challenging to interpret, as there are relatively few literature comparisons. Nonetheless, our hypothesis that increased T2* is due to cell filling of matrix pores, decreased T2* to higher macromolecular content, and relative insensitivity of T2* to hydration are plausible given the known behaviour of T2* decay. The association with pores, however, requires more explanation. Materials with pores larger than 15Å exhibit increased transverse relaxation due to the ability of large pores to take up water (45). Scaffolds contain much larger pores, in the microns range, that are expected to contain free water and give rise to susceptibility differences at pore interfaces. This difference leads to T2* dephasing, the magnitude of which is likely lessened when pores are replaced with cells.

There are a number of different avenues for future investigation, and new challenges will present themselves as the regeneration strategy becomes increasingly
complex. For instance, matrix- and cell-related changes, which can exert opposing or constructive influences, may need to be distinguished. Also, other essential layers of the bladder such as the urothelium and lamina propria need to be formed, and it remains to be determined if cells for these layers can be recruited in vivo and not require direct seeding as does the smooth muscle layer. To identify these multiple signatures will be a challenge, likely to require finding matrix- or cell-type-specific molecular signatures that can be detected on MRI. Improvement can also be made in assessing cell repopulation of the construct. First, the multiexponential diffusion method for measuring cellularity would benefit from imaging at higher field strengths, which allows shorter diffusion times and approach to the slow exchange limit. Measuring the number of viable cells would be even more informative, and this may be achieved through 1H spectroscopy, where a linear correlation between cell number and the choline peak has been previously observed (46,47). Quantitative validation of cell number counts (e.g. DNA quantification) will be undertaken in future studies. Further investigation is also needed to understand the T2* changes observed in this study. Our hypothesis that T2* may identify cell filling of pore space should be evaluated in polymer scaffolds where pore size and number can be systematically controlled. Another finding related to T2*, namely, its insensitivity to tissue hydration that was also observed in chondrocyte transplantation (38), is potentially valuable in a wide variety of applications. For example, inflammation, which often results from tissue construct-host interaction, should be investigated in the future to determine if T2* is sensitive to infiltration of inflammatory bodies but not to accompanying edema.

In conclusion, this study has demonstrated the potential role of multiexponential diffusion and T2* for more specific assessment of cell growth and tissue development. The
results of this study are relevant for regeneration paradigms based on biological scaffolds such as the acellular matrix for engineering soft tissue and large organs. They also extend to other applications where concurrent biological changes exist, for example, where cellularity needs to determined independent of biochemical changes or where tissue hydration is a confounder and needs to be removed.
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### Table 1

Biophysical and biochemical changes in ACM from different manipulations

<table>
<thead>
<tr>
<th>Manipulation</th>
<th>Tissue Changes</th>
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<tbody>
<tr>
<td>Incorporation of hyaluronic acid (HA)</td>
<td>Total GAG concentration increases, with a 2:1 ratio of non-sulphated (i.e. HA) versus sulphated GAG. Water content nearly doubles.</td>
</tr>
<tr>
<td>Seeding with smooth muscle cells</td>
<td>Matrix collagen I degrades</td>
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FIGURE LEGENDS

**Figure 1.** Alcian blue staining confirms the uptake of hyaluronic acid (HA) in HA-ACM constructs, which have a blue hue compared with ACM constructs without HA. (magnification, 10×).

**Figure 2.** Representative H&E stained sections for the different classifications of cellularity: I (very few cells), II (few cells), III (patches of cells), and IV (good cellularity). (magnification, 10×).

**Figure 3.** Masson’s Trichome staining of the ACM shows a collagen extracellular matrix and smooth muscle bundles (upper left corner). (magnification, 10×).

**Figure 4.** Changes in the T2 spectrum (solid to dotted curves) can distinguish a) increased tissue water content accompanying incorporation of hyaluronic acid and b) degradation of the extracellular matrix with cell-seeding. In a) the higher water content is evident in a shift of the long T2 component towards higher values. In b) progressive loss of matrix collagen is evident in a reduced fraction of the short T2 component.

**Figure 5.** a) Diffusion-weighted and b) T2*-weighted MR images of an ACM in a 12-mm diameter tube. Diffusion-weighted images are illustrated for b-values of 200 s/mm² (left) and 1600 s/mm² (right). T2*-weighted images are illustrated for echo times of 2.6 ms (left) and 34.4 ms (right).

**Figure 6.** Diffusion decay characteristics are different between unseeded ACMs (filled circles) and seeded ACMs (open circles). Unseeded ACMs exhibit monoexponential decay, whereas biexponential behaviour emerges in seeded ACMs due to the presence of both an extra- and intracellular compartment.
Figure 7. Quantitative MRI parameters for discriminating various levels of cellularity. The slow diffusion fraction, which represents the proportion of intracellular space, is significantly correlated with increasing cellularity (I, II, III, IV). The other parameters (single diffusion coefficient D, T1, and T2) show much lower correlation or none at all.

Figure 8. Effect on MRI parameters from seeding cells on ACM. T2* exhibits a significant and greater relative change compared to either T2 or T1. The T2* increase is postulated to arise from cell-filling of matrix pores. Mean values and standard deviation are shown in units of ms for unseeded (shaded) and seeded (non-shaded) ACMs. ** P < 0.01, * P < 0.05.

Figure 9. Effect on MRI parameters from incorporating hyaluronic acid in ACM. T2* exhibits a significant and greater relative change compared to either T2 or T1. The T2* decrease is consistent with higher macromolecular content in the ACM and suggests that unlike T2, T2* is relatively insensitive to tissue hydration. Mean values and standard deviations are shown in units of ms for ACM (shaded) and HA-ACM (non-shaded). * P < 0.05.
FIG. 1
FIG. 3
FIG. 5
FIG. 5
FIG. 8
FIG. 9