Designation: F3224 – 17

Standard Test Method for Evaluating Growth of Engineered Cartilage Tissue using Magnetic Resonance Imaging[1]

This standard is issued under the fixed designation F3224; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This standard is intended as a standard test method for engineered cartilage tissue growth evaluation using MRI.

1.2 This standard is intended for use in the development of tissue engineering regenerative medical products for cartilage damages, such as in knee, hip, or shoulder joints.

1.3 This standard has been prepared for evaluation of engineered cartilage tissue growth at the preclinical stage and summarizes results from tissue growth evaluation of tissue-engineered cartilage in a few notable cases using water spin-spin relaxation time, T_2, *in vitro* and *in vivo* in small animal models.

1.4 This standard uses the change in mean T_2 values as a function of growth time to evaluate the tissue growth of engineered cartilage.

1.5 This standard provides a method to remove the scaffold contribution to the tissue growth evaluation.

1.6 Information in this standard is intended to be applicable to most porous natural and synthetic polymers used as a scaffold in engineered cartilage, such as alginate, agarose, collagen, chitosan, and poly-lactic-co-glycolic acid (PLGA). However, some materials (both synthetic and natural) may require unique or varied methods of MRI evaluation that are not covered in this test method.

1.7 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.8 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document applies.

2.2 *ASTM Standards:*[2]

F2312 Terminology Relating to Tissue Engineered Medical Products

F2529 Guide for *in vivo* Evaluation of Osteoinductive Potential for Materials Containing Demineralized Bone (DBM)

F2603 Guide for Interpreting Images of Polymeric Tissue Scaffolds

F2664 Guide for Assessing the Attachment of Cells to Biomaterial Surfaces by Physical Methods

F2978 Guide to Optimize Scan Sequences for Clinical Diagnostic Evaluation of Metal-on-Metal Hip Arthroplasty Devices using Magnetic Resonance Imaging

2.3 *ISO Standard:*[3]

ISO/TR 16379-2014 Tissue-engineered medical products — Evaluation of anisotropic structure of articular cartilage using DT (Diffusion Tensor)-MR Imaging

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *biomaterial, n*—any substance (other than a drug), synthetic or natural, that can be used as a system or part of a system that treats, augments, or replaces any tissue, organ, or function of the body. **F2664**

3.1.2 *chondrocyte, n*—a cell that has secreted the matrix of cartilage and becomes embedded in it.

3.1.3 *chondrogenic differentiation, n*—the biological process of stem cells changing their lineage into chondrocytes. If the starting cells are chondrocytes, this term refers to differentiation of cells into the same phenotype.

[1] This test method is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.44 on Assessment for TEMPs.

Current edition approved Nov. 1, 2017. Published February 2018. DOI: 10.1520/F3224-17.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

3.1.4 *chondrogenic extracellular matirx (chondrogenic ECM), n*—an extracellular matrix containing cartilaginous matrix proteins such as proteoglycan, collagen type II, collagen type X and other matrix proteins found in cartilage.

3.1.5 *echo time (TE), n*—time after 90° pulse in an MRI pulse sequence until an echo signal is formed.

3.1.6 *fast low angle shot (FLASH) MRI, n*—a gradient echo MRI acquisition technique with low flip angle radiofrequency pulse excitation and short repletion time for fast image acquisition.

3.1.7 *field of view (FOV), n*—MR image acquisition parameter that defines the dimensions of the imaging plane (expressed in cm × cm or mm × mm).

3.1.8 *histological assessment of engineered cartilage tissue growth, n*—histological assessment is used to assess the presence of cartilage extracellular matrix proteins in the engineered cartilage to evaluate the tissue growth (e.g. Safranin O staining for proteoglycan assessment).

3.1.9 *hydrogel, n*—a water-based open network of polymer chains that are cross-linked either chemically or through crystalline junctions or by specific ionic interactions. **F2603**

3.1.10 *in-plane resolution, n*—the spatial resolution of an image (typically expressed in mm × mm or µm × µm). It is given by = FOV/acquired matrix size.

3.1.11 *magnetic resonance imaging (MRI), n*—an imaging technique that uses static and time-varying magnetic fields to provide tomographic images of tissue by the magnetic resonance of nuclei. **F2978**

3.1.12 *matrix size, n*—the number of pixels in each image dimension of FOV.

3.1.13 *mesenchymal stem cell (MSC), n*—a multipotent cell derived from mesenchyme that is capable of proliferating and differentiating in chondrogenic lineage and can produce a cartilage extracellular matrix.

3.1.14 *multi slice multi echo (MSME) MRI, n*—an MRI pulse sequence for the measurement of T_2 where a series of 180° RF pulses (number of echoes) is followed by a 90° RF pulse in a multi-slice MRI pulse sequence. This pulse sequence is the MRI extension of similar nuclear magnetic resonance (NMR) spectroscopy sequence named Carr-Purcell-Meiboom-Gill (CPMG) echo train pulse sequence for T_2 measurement.

3.1.15 *number of averages (NA), n*—the number of times an identical MRI experiment is repeated to improve the SNR.

3.1.16 *pulse sequence, n*—programmed train of RF and gradient pulses. In MRI, it is a time protocol for obtaining images.

3.1.17 *quantitative real-time polymerase chain reaction (qRPCR), n*—a laboratory technique for the detection, selection, and amplification of specific gene transcripts based on their genetic sequence. Commonly, it is used to assess the presence of chondrogenic markers such as Sox9, RUNX2, ECM proteins, etc. in a tissue-engineered cartilage.

3.1.18 *radiofrequency pulse (RF pulse), n*—a short duration radiofrequency electromagnetic pulse used for changing the direction of magnetization vector.

3.1.19 *rapid acquisition with refocused echoes (RARE) MRI, n*—an MRI pulse sequence for fast image acquisition. This MRI pulse sequence is characterized by a series of 180° RF rephasing pulses followed by a 90° RF pulse, with each echo is individually phase-encoded for fast image acquisition.

3.1.20 *region of interest (ROI), n*—a user-defined area of an image in which parameter of interest is calculated.

3.1.21 *relaxation rate (R_2), n*—inverse of spin-spin relaxation time ($R_2 = 1/T_2$).

3.1.22 *repetition time (TR), n*—time interval between consecutive 90° RF pulses or the time interval when the basic unit of MRI pulse sequence is repeated. **ISO/TR 16379-2014**

3.1.23 *scaffold, n*—three-dimensional natural or synthetic biomaterial typically made out of one or more polymers (natural or synthetic) and used as a skeleton for cell seeding. **F2603**

3.1.24 *signal to noise ratio (SNR), n*—the ratio of the amplitude of any signal of interest to the amplitude of the average background noise which includes both coherent and non-coherent types of noise.

3.1.25 *slice thickness, n*—the thickness of the 2D imaging plane in an MRI image. **ISO/TR 16379-2014**

3.1.26 *spin echo (SE) MRI, n*—a method for acquiring MR images based on the spin-echo pulse sequence.

3.1.27 *spin-spin relaxation time (T_2), n*—T_2 refers to the characteristic exponential time constant of the transverse magnetization. This is typically the time taken for the transverse magnetization to decrease to 37% of the initial value It is typically depicted in milliseconds (ms).

3.1.28 *stem cell, n*—an undifferentiated cell that is capable of developing into many different cell types.

3.1.29 *voxel, n*—the minimum unit volume of a three-dimensional MRI image. **ISO/TR 16379-2014**

4. Significance and Use

4.1 Tissue-engineered cartilage is prepared by seeding stem cells or chondrocytes in a three-dimensional biodegradable scaffold under controlled growth conditions. It is expected that the cells will differentiate towards chondrogenic lineage and produce an ample amount of cartilage extracellular matrix proteins, proteoglycans, and collagen type-II. Longitudinal assessment is needed weekly for the first few weeks *in vitro* and monthly at a later stage *in vivo* to determine the growth rate of tissue-engineered cartilage. Traditional testing methods such as histological staining, mechanical testing, and qPCR are invasive, destructive, and cannot be performed *in vivo* after the transplantation of engineered tissue as a regenerative treatment. In the regenerative medicine of cartilage, it is important to evaluate whether the implanted tissue regenerates as an articular cartilage over time. MRI is the only available non-invasive imaging modality that is utilized for post-operative monitoring and assessment of cartilage regeneration in clinics. Therefore, it is important to evaluate tissue-engineered cartilage using MRI at the preclinical stage as well.

4.2 Preclinical *in vivo* assessment of tissue-engineered cartilage is performed in small animal models such as mice, rats

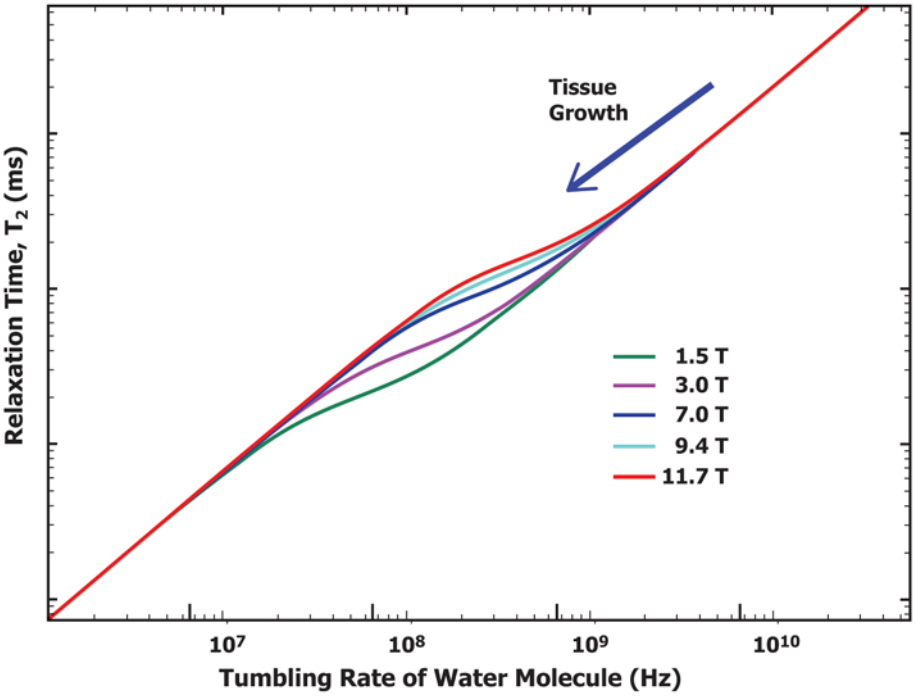

FIG. 1 The change in the water relaxation time T_2 as a function of the magnetic field and the tumbling rate of the water molecule using BPP theory of relaxation (1). Note that the tumbling rate of the water molecule decreases with increasing tissue growth. The blue arrow shows the direction of change of the relaxation time, T_2, as a function of the tissue growth.

or rabbits, and in large animal models such as goats, pigs, and horses. It is possible to evaluate engineered cartilage tissue growth at each stage of development non-invasively using MRI. This may reduce the number of animals needed for the assessment and will provide a good estimate of cartilage regeneration.

4.3 Parametric MRI technique allows non-invasive quantitative assessment of tissue growth *in vitro* and *in vivo*. When the amount of extracellular matrix increases over time, the interaction of the water molecule with its surroundings changes, and this creates a change in T_2. The amount of change in T_2 is directly correlated with the amount of matrix generated with high sensitivity and specificity. The T_2 MRI is thus used to observe tissue growth for use commonly in longitudinal diagnosis following cell seeding in a scaffold *in vitro* or following tissue implantation *in vivo*.

4.4 The T_2 MRI for preclinical evaluation of engineered cartilage takes into account the presence of a scaffold in the developing tissue-engineered cartilage. These data are published in refereed journals and book chapters, and included here as a guide for preclinical quantitative evaluation for engineered cartilage tissue growth (**2-12**).[4] Additional data utilizing T_2 MRI for tissue growth evaluation of engineered cartilage can be found in the references (**13-15**).

4.5 Magnetic resonance parameters of water protons in tissue are sensitive to the tissue microstructure. In cartilage tissue engineering, cells produce primarily cartilage extracellular matrix proteins, proteoglycans, and collagen, type-II. As tissue matures with the production of ECM, the matrix changes the environment around water molecules. The water nuclear spins find several new pathways for relaxation and the T_2 generally is lower from the original value. Fig. 1 shows the effect of water tumbling rate and magnetic field strength on T_2. As shown by the blue arrow, when the engineered cartilage tissue matures, the tumbling rate of the water molecule is lower and as a result, the T_2 is lower. The reduction of T_2 as a function of tissue growth is the basis of engineered cartilage assessment using MRI. Fig. 1 also shows that this principle holds true from low to high magnetic field strengths (1.5 T – 11.7 T) that are commonly used in MRI assessment.

4.6 As shown in Fig. 1, the change in T_2 is dependent on the magnetic field strength and initial tumbling rate of the water molecule that signifies its surrounding.

4.7 The principle of reduced T_2 with increased tissue growth generally holds true for scaffold-free cartilage tissue engineering. However, in scaffold-based cartilage tissue engineering, the following relationship should be used to assess the tissue growth (**3, 6**):

$$R_2(ECM) = R_2(TEC) - R_2(Control) \quad (1)$$

[4] The boldface numbers in parentheses refer to the list of references at the end of this standard.

where:

R_2 = $1/T_2$,
$R_2(ECM)$ = the calculated relaxation rate arising from cartilage extracellular matrix,
$R_2(TEC)$ = the measured experimental relaxation rate of the tissue-engineered cartilage graft, and
$R_2(Control)$ = the relaxation rate of the scaffold without cells.

4.7.1 The change in calculated relaxation rate, R_2(ECM), using Eq 1 have been found to be positively correlated with tissue growth (**3**, **6**).

5. MRI Assessment of Engineering Cartilage Tissue Growth

5.1 *Sample Preparation:*

5.1.1 *In Vitro MRI Assessment*—Typically, MRI tissue assessment of early stage *in vitro* samples is performed using vertical bore high field MRI scanner and a small diameter radiofrequency probe (~ 5-10 mm) that is equipped with gradients in the x-, y-, and z-directions and relevant acquisition software for pulse sequence generation and data acquisition. The samples that are small (~ 3-5 mm in diameter) can be packed using the technique shown in Fig. 2. As shown in the figure, samples are placed in an MRI-compatible tube on top of a susceptibility matched plug (or agar gel) to keep them in the center of the RF coil. The top of the tube is typically filled with a culture medium or Fluorinert oil. The use of Fluorinert oil allows better image visualization because it does not contain any protons thus removing the background signal completely. The use of growth culture medium is recommended for the assessment in the natural tissue-growth environment. The culture medium T_2 can be used for normalization of all measurements. The *in vitro* MRI assessment can also be performed using a dedicated small animal MRI scanner typically equipped with a bigger RF coil (~ 30-60 mm) where multiple such sample tubes can be arranged for simultaneous MRI assessment. If possible, it will be beneficial to measure both TEC sample and acellular control samples at the same time to reduce systematic errors.

5.1.2 *In Vivo MRI Assessment—In vivo* assessment of engineered cartilage can be performed using a dedicated small animal MRI system or using a clinical MRI scanner. It is expected that different MRI hardware will be used according to the need. Volume coils are more suitable for mouse or rat models of tissue assessment whereas receive-only surface coil may be used for rabbit or other large animal models that will allow the better visualization of small test samples implanted in the animal. The sample outlines can be drawn using an MRI image as shown in Fig. 3.

5.2 *MRI Measurement Process:*

5.2.1 The process flowchart shown in Fig. 4 is used for acquisition and calculation of data for each time point of tissue assessment. Typically for *in vitro* assessment, the time points may be every week whereas for *in vivo* assessment, it could be every other week or every month. This standard envisions that different MRI hardware and pulse sequences will be used at different locations.

5.3 *Notes on MRI Set Up:*

5.3.1 *Radiofrequency Coil*—Preclinical assessment of tissue-engineered cartilage uses a wide range of small test samples *in vitro* or implanted in an animal. Therefore, different RF coils may be used, depending upon the availability of hardware and available equipment time for MRI data collection. The data in Annex were selected to show a different coil setup for different image acquisition scenarios (**3**, **6**, **7**).

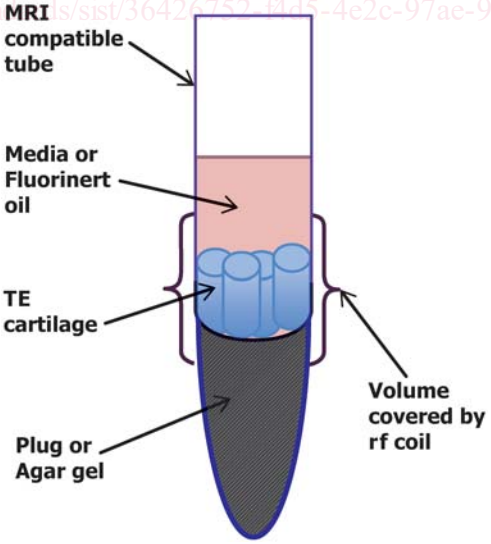

FIG. 2 Schematic of sample preparation for *in vitro* assessment in a vertical bore system. The bottom of the tube is filled with a susceptibility matched plug or agar gel to keep the samples at required location. Only the volume covered by RF coil is imaged. The samples are covered with the culture medium or Fluorinert oil.

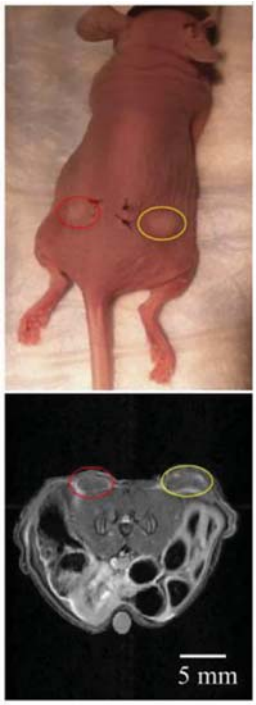

FIG. 3 Schematic of sample preparation for *in vivo* engineered cartilage tissue grafts assessment using mouse subcutaneous model. The bottom panel shows the T_2-weighted MRI image slice for locating the grafts and for drawing ROI for T_2 calculations. The red outline shows the tissue engineered cartilage graft whereas the yellow outline shows the location of the acellular control graft.

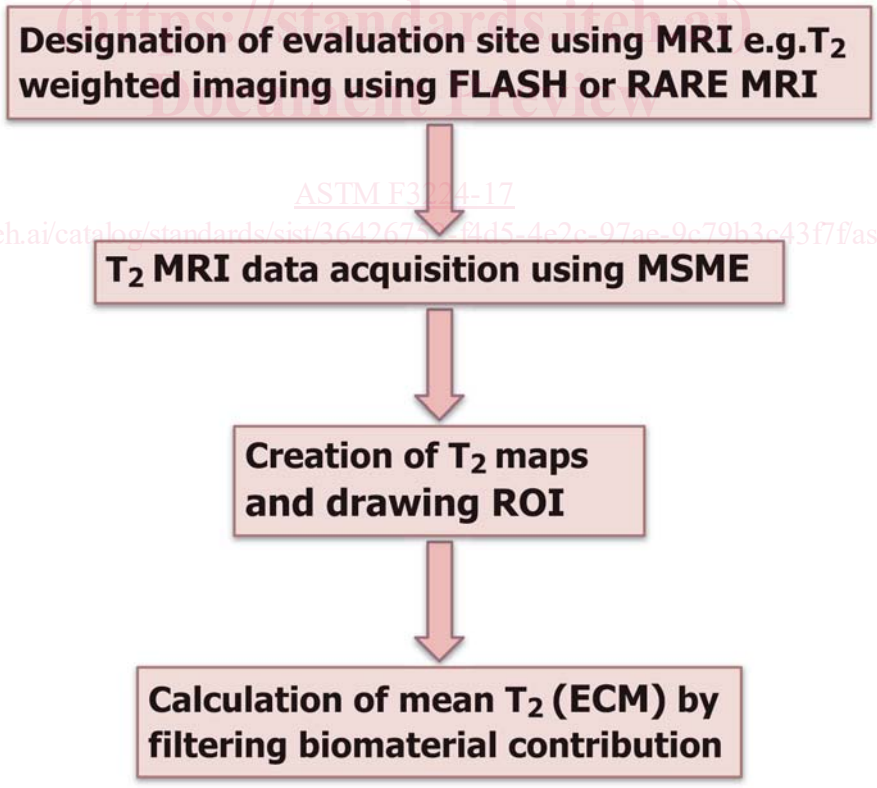

FIG. 4 Flowchart for T_2 map creation at each time point

5.3.2 *Image Resolution*—Typically, MRI at the preclinical stage is performed at a higher magnetic field strength that allows signal acquisition with a higher SNR. *In vitro* samples are especially suitable for very high-resolution image acquisition (~ 50 μm). Since engineered cartilage has an intrinsic structure based on the biomaterial chosen as a scaffold, the

matrix and slice thickness parameters should be chosen to allow acquisition of images with clear visualization and removal of scaffold contribution of tissue-engineered cartilage (4, 6).

5.3.3 *Choice of the Pulse Sequences*—Because of the nature of preclinical samples, it is possible to choose spin echo-based pulse sequences that take longer but produce fewer artifacts and produce higher quality images compared to fast spin echo pulse sequences. It is important to optimize the pulse sequence parameters using a standard sample such as water, agar or agarose before applying them to tissue engineered cartilage (16, 17).

5.3.4 *Repetition Time*—Longer repetition time (~ $5T_1$) improves image quality but lengthens the imaging time.

5.3.5 *Number of Echoes in T_2 MRI*—The end goal of data acquisition in T_2 MRI is to calculate the T_2 values by fitting the signal intensity into a single exponential model. Higher numbers of echoes will allow better T_2 estimation.

5.3.6 *Acceptable SNR*—Better SNR in T_2 images will result in higher confidence in result interpretation.

5.3.7 *T_2 Map Creation*—T_2 maps are extracted using voxel-by-voxel single exponential fit of imaging data. The average value of the parameter and the standard deviation are calculated from the region of interest (ROI) within the tissue. For each tissue-engineered cartilage samples, corresponding data acquisition in the control sample is needed. ROIs for calculation of mean parameter value shall be selected to cover the entire sample.

6. Calculation or Interpretation of Results

6.1 *Calculation of $T_2(ECM)$*—The change in the values of T_2(ECM) with time provides an estimate of tissue growth during the period of observation. T_2(ECM) should be calculated as follows:

$$T_2(ECM) = 1/R_2(ECM) \quad (2)$$

6.2 *Interpretation of Result*—The mean T_2(ECM) is plotted against the time point of tissue growth. The reduction in the mean T_2(ECM) value reflects the extent of extracellular matrix production, both proteoglycans and collagen, in the engineered cartilage being investigated. It should be noted that the extent of change in T_2 reflects the growth, not the absolute T_2 values.

7. Keywords

7.1 biomaterials; cartilage tissue engineering; MRI; MRI assessment of engineered cartilage tissue growth; MRI in tissue engineering; stem cell and regenerative medicine

ANNEX

(Mandatory Information)

A1. INFORMATIVE

NOTE A1.1—This standard envisions different MRI hardware and acquisition parameters used at different locations. Thus observational data are presented for *in vitro* and *in vivo* engineered cartilage tissue assessment using a vertical bore and a horizontal bore MRI apparatus, respectively. Table A1.1 presents the MRI apparatus and imaging parameters used in the observation that are presented in this Annex. This table is presented here as a guide and as an example only. Fig. A1.1 presents the 11.7 T Bruker microimaging instrument along with gradient systems and RF coils available at the University of Illinois at Chicago facility.

A1.1 *In Vitro* MRI Assessment of Chondrocyte Pellets (2, 9, 10, 18)

A1.1.1 *Sample Preparation*—The bovine chondrocyte pellets were created using 5×10^5 chondrocytes and grown for three weeks in a chondrogenic tissue culture medium. At days 3, 7, 14, and 21, MRI assessments were performed. At each time point, the pellets (n = 3) were stacked on top of 1% agar

TABLE A1.1 MRI apparatus, RF coils, and Imaging Parameters used for tissue growth evaluation data presented in this document

NOTE 1—MSME is Bruker pulse sequence that is the MRI version of classical CPMG sequence for T_2 data acquisition.

Imaging Facility	University of Illinois at Chicago (Research Resource Center)			The University of Chicago (Lynn S. Florsheim MRIS lab)
Apparatus (Manufacturer model)	11.7 T (Bruker Avance DRX)			9.4 T (Bruker Biospec)
rf coil	5 mm saddle coil			72 mm Bruker quadrature coil
Sample	Chondrocyte pellets	PLGA – Puramatrix™ scaffolds + HMSCs	Collagen/Chitosan gel + HMSCs	Biomimetic ECM + HMSCs
Sequence	MSME	MSME	MSME	MSME
FOV (mm)	8.4 × 4.2	10 × 10	10 × 10	30 × 30
Matrix	128 × 64	128 × 128	128 × 128	256 × 256
In-plane resolution (μm)	67 × 67	78 × 78	78 × 78	117 × 117
TE (ms)	5.8 ms	7.2 ms	7.2	9.167
TR (ms)	5000	4000	4000	5000
Slice thickness (mm)	0.5	0.5	0.5	1.0
Number of averages	1	1	1	1
Number of echoes in T_2 measurement	64	32	32	16
Imaging plane	Sagittal	Sagittal	Sagittal	Axial
Parameter calculation model	Single exponential	Single exponential	Single exponential	Single exponential
Time points	3, 7, 14, and 21 days	0, 7, 14, and 28 days	0 and 28 days	7, 14, 21, and 28 days