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The Application of Sodium Triple-Quantum Coherence NMR Spectroscopy for the Study of Growth Dynamics in Cartilage Tissue Engineering

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Abstract

We studied the tissue growth dynamics for tissue-engineered cartilage at the early growth stage after cell seeding for four weeks using sodium triple-quantum coherence NMR spectroscopy. The following tissue-engineering constructs were studied: 1. Bovine chondrocytes cultured in alginate beads, 2. Bovine chondrocytes cultured as pellets (scaffold-free chondrocyte pellets), 3. Human Marrow Stromal Cells (HMSC) seeded in Collagen/Chitosan based scaffolds of chondrogenic extracellular matrix environment expecting chondrogenic differentiation, named as biomimetic scaffolds. We found that the sodium triple quantum coherence spectroscopy can differentiate between different tissue-engineering constructs and the native tissues based on the fast and the slow relaxation rates as well as based on the average quadrupolar coupling. Both the fast (T_f) and the slow (T_s) relaxation times were found to be longer in the chondrocyte pellets and the biomimetic scaffolds as compared to the chondrocytes suspended in alginate beads and the human articular cartilage tissues. In all cases, it was found that the relaxation rates and the motion of sodium ions, as measured from correlation time, were dependent on the amount of macromolecules, high cell density and the anisotropy of the cartilage tissue engineering constructs. Average quadrupolar couplings were found to be lower in the engineered tissue as compared to the native tissues, presumably due to the lack of order in collagen accumulated in the engineered tissue. These results indicate the use of sodium triple quantum coherence spectroscopy as a tool to investigate anisotropy and growth dynamics of cartilage tissue engineered constructs in a simple and reliable way.

Keywords

Cartilage; Cartilage Tissue engineering; Sodium NMR; Sodium MRI; Triple-quantum coherence spectroscopy; Biomimetic scaffolds; Chondrocyte pellets; alginate beads; collagen; proteoglycans

Introduction

Osteoarthritis (OA) is a degenerative joint disease and a major public health issue with the aging adult population in developed countries (1–3). Osteoarthritis is characterized by focal and progressive loss of articular cartilage of joints, which inherently has a poor self-healing

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capacity in adults (4, 5). Over the years, many types of treatments have evolved to treat damaged or diseased cartilage. These include marrow stimulation, autologous chondrocyte implantation, and osteochondral autograft transfer system (6, 7). However, none of the available treatments have the potential to regenerate the biological composition and biomechanical properties of native cartilage (8, 9). Tissue engineering has the potential to provide a long-term relief to patients with OA by using bio-engineered constructs to replace damaged or diseased cartilage.

Articular cartilage, schematically illustrated in Figure 1, consists of chondrocytes cells and extracellular matrix (ECM), which in turn is made up of tissue fluid and structural macromolecules: collagens, mainly type II, proteoglycans and non-collagenous proteins and glycoproteins (10). Proteoglycans are made of a protein core and one or more glycosaminoglycan chains (GAG) that contain repeating disaccharides. Each unit of these disaccharides has at least one negatively charged carboxylate and sulfate group. These long chains of negatively charged glycosaminoglycans attract cations such as sodium. The high concentration of sodium ions (240 – 300 mM) inside the tissue and low concentration of sodium ions (145mM) in surrounding tissue fluid creates a concentration gradient governed by the Donnan osmotic effect (11). This high concentration of cations is responsible for 50% of the cartilage tissue stiffness and is one of the indicators of cartilage health (12). Collagen is a positively charged protein at neutral pH. It forms a cross-banded fibril network in the extracellular matrix of tissues. The orientation and composition of these fibrils change along the depth of the tissue and provides the tensile stiffness and strength of articular cartilage. This cross-banded collagen network mechanically and biochemically traps proteoglycans and thus, both macromolecule units are interwoven throughout the tissue depth (10, 13).

The goal of cartilage tissue engineering is to regenerate native cartilage in its biochemical composition and mechanical strength. In order to mimic native cartilage, tissue engineers focus on the production of the main ECM components, proteoglycan and collagen, using variety of approaches. The main differences between these approaches are absence (scaffold-free) or presence of biocompatible scaffold and the cell sources used for chondrogenic differentiation (14). In the case of scaffold based approach, the alginate bead or collagen in combination with other biocompatible materials are used as scaffolds for scaffold based cartilage tissue engineering constructs (15–18). Chondrocytes isolated from the native tissue or Human Marrow Stromal cells (HMSC) differentiating towards chondrocytes can be used as a cell source (15, 19, 20). Each of these methods has its own advantages and their effectiveness depend on their ability to produce primary chondrogenic extracellular matrix components proteoglycans and collagen, and their mechanical properties matched with the native one. To ensure high quality engineered cartilage, this ECM production needs to be monitored at various time points using various biochemical, mechanical and histological monitoring techniques, such as expression analysis using qRT-PCR and immunohistochemical analysis to be matched with the properties of the native tissue. Although quantitative in terms of ECM amount, these techniques give no information about anisotropy and dynamics involved in cartilage tissue engineering. We present a first such study using sodium triple-quantum coherence spectroscopy, which provides a window into inherent dynamics in the cartilage tissue growth.

Numerous studies have shown the advantages of using nuclear magnetic resonance (NMR) spectroscopy and MR parametric studies for the tissue engineering problems (21–23). Weber *et al* and Shulz *et al* have shown the advantages of using ^{31}P and ^{13}C solid state NMR for bone tissue engineering and regeneration (24, 25). Jeffries *et al* and Constantinidis *et al* have used the high-resolution ^1H , ^{13}C , ^{19}F and ^{31}P NMR to study the growth, metabolism and vitality of artificial liver and pancreatic constructs respectively. Cheng *et al* have applied T_2^* and multi-exponential diffusion to study the growth dynamics of bladder

tissue regeneration (26). Li *et al* have applied magnetization transfer to study the growth of cartilage tissue engineering (27). Miyata *et al* have used proton T_1 maps to calculate fixed charged density in tissue-engineered cartilage, and Irrechukwu *et al* and Reiter *et al* have used T_2 relaxation analysis for tissue-engineered cartilage matrix characterization (28–30). However, to the best of our knowledge, sodium (^{23}Na) NMR and MRI have not been used to study the development of tissue engineering cartilage.

Sodium (^{23}Na) is the second most abundant NMR active nuclei after water proton found in biological tissues. Sodium concentration in tissues varies independently of proton distribution depending upon the physiological and pathological conditions; therefore, sodium spectroscopy and imaging have the potential to provide complementary information to proton spectroscopy and imaging. The sodium spin-lattice relaxation (T_1) and spin-spin relaxation (T_2) relaxation are dominated by quadrupolar interaction between the electric quadrupolar moment of the nucleus and the electric field gradient (EFG) at the location of the nucleus. The fluctuation in EFG, which is influenced by its environment, is the main mechanism of relaxation in case of sodium nuclei. Thus, sodium ion concentration and relaxation are sensitive to the physiological conditions of the tissue. As stated earlier, sodium concentration in cartilage is higher because of attractive binding between sodium ions and proteoglycan. Therefore, sodium NMR is an attractive tool to investigate the structure and dynamics of native and engineered cartilage. Sodium NMR and MRI have been widely applied to study the cartilage dynamics and degeneration in its native state and Sodium double and triple quantum coherence spectroscopy has been applied to the study of anisotropy, order and degeneration of cartilage tissue by a number of researchers (12, 31–40). It has been shown that in the early stages of osteoarthritis, the loss of proteoglycan causes change in sodium ion concentration and relaxation rates that can be measured quantitatively using sodium triple-quantum coherence spectroscopy and imaging (12, 41, 42). Recent studies using sodium MRI on cartilage has been focused on GAG assessment from sodium signal intensity of cartilage repair tissues after surgical treatment in patients (43–46). Keinan-Adamsky *et al* have applied sodium triple-quantum coherence spectroscopy to study the pig cartilage maturation from newborn to 39 months in pigs and found that the short relaxation time T_{2f} decreases and the average quadrupolar coupling, ω_Q , increases with age (47). We present the novel application of sodium triple-quantum coherence spectroscopy for the study of tissue-engineered cartilage.

In the present study, we have investigated the application of sodium triple quantum (TQ) coherence NMR spectroscopy for monitoring tissue growth dynamics for tissue-engineered cartilage at the early growth stage after cell seeding. For comparison, we have also investigated human cartilage tissue. Sodium triple-quantum coherence filter is insensitive to the sodium ions undergoing fast isotropic motion and therefore, it filters out signal from free sodium ions (12, 48). This allows us to observe the interaction of sodium ions with the tissue macromolecules and to gauge the anisotropy and dynamics in the engineered tissue. The triple-quantum coherence signal in this case is characterized by biexponential relaxation decay with a fast (T_f) and a slow relaxation (T_s) time constant and an average residual quadrupolar coupling, ω_Q (12, 48). These rates are indicative of the tissue physiologic state. Keinan-Adamsky *et al*, found that the fast relaxation time constant and average quadrupolar coupling measured from sodium triple-quantum coherence spectroscopy are diagnostic for patients with different stages of osteoarthritis (31). In addition, Insko et al, have shown an increase in the slow relaxation time constant (T_s) and a decrease in the fast relaxation time constant (T_f) with decreasing proteoglycan content in an enzymatically treated bovine cartilage tissue (38). To the best of our knowledge, the potential of sodium triple-quantum coherence spectroscopy has not been used to study growth and development of tissue-engineered cartilage, post-cell seeding.

We calculated the fast and the slow relaxation time constants and the average quadrupolar coupling for three common engineered cartilage tissue constructs: 1. Bovine chondrocytes cultured in alginate beads, 2. Bovine chondrocytes cultured as pellets (scaffold-free pellets), and 3. Human Marrow Stromal Cells (HMSC) cultured in scaffold embedded with chondrogenic extracellular matrix environment, called biomimetic scaffolds. These constructs were chosen to represent three commonly used approaches in the cartilage tissue engineering. At the early stages of tissue growth, cells generate collagen and proteoglycan, primarily. The deposition of these macromolecules change the immediate environment around the sodium ions and therefore, their relaxation behavior post-cell seeding is representative of the accumulation of these macromolecules. Although sodium ions bind primarily to negatively charged proteoglycan, our triple-quantum coherence data indicate, that the sodium quadrupolar properties may be affected by both proteoglycan and collagen. The effect of the order of collagen fibers and the amount on the anisotropy in the native tissue has also been discussed before (31, 37). We found that the sodium triple quantum coherence spectroscopy can differentiate between different tissue-engineering constructs and the native tissues based on the fast and the slow relaxation rates as well as based on the average quadrupolar coupling. We found that both the fast and the slow relaxation rates are sensitive to the tissue growth in all the tissue-engineered cartilage constructs, and vary according to the engineered cartilage and the growth stage. The residual average quadrupolar coupling in the case of tissue-engineering constructs was found to be lower than the native tissue, which indicates the significantly low order of structure in the case of tissue-engineered cartilage constructs. Finally, we propose a schematic model of tissue-engineered constructs.

Theory

Quadrupolar spin probes, such as sodium (^{23}Na) with spin-quantum number $3/2$, are important in investigating dynamics in biological tissues. These nuclei possess a electric quadrupole moment, Q that interacts with the electric field gradient (EFG) at the location of the nucleus. The electrical environment around the nucleus produces the EFG, thus sodium NMR is very sensitive to the local environment surrounding the nucleus. In isotropic liquids, this interaction is averaged to zero. However, in a system, where sodium nuclei are involved in a slow molecular motion or reside in an ordered environment, multiple-quantum coherence can be evolved. In such cases, the sodium nuclei experiences a non-zero EFG, and a residual quadrupolar coupling can be observed through multiple quantum coherence (48). Because of the non-zero quadrupolar interaction, the transverse relaxation rate for sodium nuclei is biexponential with one short (T_f) and one long (T_s) decaying component owing to the satellite transitions ($\pm 3/2 \leftrightarrow \pm 1/2$) and the central transition ($-1/2 \leftrightarrow +1/2$). The triple-quantum transition signal is detected by the pulse sequence and phase cycling designed to filter the triple-quantum signal (49).

The motion of sodium ions in engineered or native cartilage tissue is anisotropic because of the interaction of sodium ions with extracellular matrix components, proteoglycan and collagen fibers. The strong triple-quantum signal is observed in this case with a biexponential decay and average residual quadrupolar coupling (12, 31, 50). The triple quantum signal intensity in this case can be written as (12);

$$S(\tau, t) \sim M_0 \frac{9}{80} \left[\left\{ e^{-(R_1 - i\omega_Q)\tau} - 2e^{-R_2\tau} + e^{-(R_1 + i\omega_Q)\tau} \right\} \cdot \left\{ e^{-(R_1 - i\omega_Q)t} - 2e^{-R_2t} + e^{-(R_1 + i\omega_Q)t} \right\} \right], \quad (1)$$

where $R_1 (=1/T_f)$ and $R_2 (=1/T_s)$ are the fast and the slow decaying components of biexponential transverse relaxation times and ω_Q is the average residual quadrupolar coupling. Figure 2 shows an example of triple quantum signal intensity as a function of

preparation time τ and its best-fit using equation 1. Using the fast and slow relaxation times, one can calculate the motional averaging parameter, $\omega_0\tau_c$, using the equation below (50);

$$\omega_0\tau_c = \left\{ \frac{1}{8} \left[5 \frac{R_1}{R_2} - 9 + \left(\left(5 \frac{R_1}{R_2} \right)^2 - 58 \frac{R_1}{R_2} + 49 \right)^{1/2} \right] \right\}^{1/2} \quad (2)$$

The motional averaging parameter, $\omega_0\tau_c$ where τ_c is rotational correlation time), represents how fast (or slow) sodium ions can tumble depending upon the anisotropy in its immediate environment (12). In the fast motion limit ($\omega_0\tau_c \ll 1$, typically in liquids), average quadrupolar coupling is zero. In the slow motion regime, such as the motion of sodium ions in biological tissues, the motional averaging parameter is equal or greater than 1 and there is a residual quadrupolar coupling. The combination of motional averaging parameter with average quadrupolar coupling gives accurate information about the dynamics and the order in the tissue. The large residual average quadrupolar coupling, such as the one found in the native cartilage tissue represents the order in the tissue (31).

Materials and methods

Cartilage tissue engineering construct preparation

Alginate Beads—The culture of articular chondrocytes suspended in alginate beads has the advantage of maintaining cell phenotypes over prolonged periods and is frequently used in the field of cartilage tissue engineering (17, 19, 51). Bovine chondrocytes (4 millions cells/ml) were cultured using chondrogenic growth media in alginate beads using established protocol (19). Typical size of the beads was 2–3 mm in diameter at the start of the experiments (~0.5 millions cells/bead) and increased up to 3–4 mm diameter during the course of the study. It has been shown previously that the chondrocytes generate cartilage extra-cellular matrix macromolecules, proteoglycan and collagen with increasing cell density in alginate bead culture (19, 51, 52). Each week, 4 beads were removed from the incubator and placed in a 5 mm NMR tube for sodium NMR studies.

Scaffold-free Pellets—Scaffold-free chondrocytes pellets are also used in cartilage tissue engineering as a preferred method to treat osteochondral defect (20). Each chondrocyte pellet was formed by centrifuging 5×10^5 bovine chondrocytes harvested according to standard protocols in tissue culture medium at $1000 \times g$ for 10 min (51). The increasing amount of proteoglycan and collagen were confirmed by biochemical analysis using published protocols (53, 54). The NMR measurements on these pellets were performed from week 1 after the cell seeding in order for pellets to gain some mechanical strength and cartilage macromolecules. Each week, three pellets were stacked in layers and placed into a 5 mm NMR tube with media for sodium NMR studies.

Biomimetic scaffolds—Biomimetic scaffolds are found to be effective for bone regeneration and similar strategies are under active consideration for cartilage tissue engineering (55). Biomimetic scaffolds were prepared using a technique similar to our published protocol (55). Briefly, human marrow stromal cells (HMSCs) were subjected to chondrogenic differentiation in a collagen/chitosan scaffold for a period of 2 weeks. The scaffolds were then decellularized as published previously leaving behind the cell-secreted extracellular matrix (ECM). This procedure resulted in scaffold that contained within, the ECM of HMSCs undergoing chondrogenic differentiation. We used these scaffolds to induce chondrogenic differentiation of undifferentiated HMSCs (1 millions cells/ml) without the use of differentiating agents. The chondrogenic differentiation of HMSCs at the end of four week period was confirmed by up-regulation of chondrogenic marker genes using gene expression analysis. Each week, three scaffolds (about 1–2 mm in diameter) were taken out

of the incubator and placed into a 5mm NMR tube with growth media for sodium NMR study.

Human and Bovine articular cartilage explants—The human and bovine articular cartilage explants were acquired from Articular Engineering (<http://articular.com/>) and cultured in the incubator at 37 °C with 5 % CO₂ with growth media. The explants were about 3mm in diameter and approximately 1mm thick. Therefore, they do not represent the complete articular cartilage with all the three zones intact. Three samples of each type were taken out of the incubator and triple-quantum coherence spectroscopy experiments were performed using the experimental parameters described above.

NMR experiments—Sodium NMR measurements were performed at room temperature on a 9.4T (¹H frequency = 400.132 MHz and ²³Na frequency, 105.83 MHz) Bruker Avance spectrometer equipped with a broadband probe capable for multinuclear NMR measurements. A capillary filled with D₂O was immersed in NMR tube for locking the deuterium signal. Samples were placed on top of doty aurum plug (14mm) to keep the samples at the center of rf coil. The 90° pulse width was 9.25μs for standard 150mM NaCl solution and was recalibrated for cartilage tissue samples. The relaxation delay in all experiments was set to 0.5 s. The T₁ relaxation measurement was done using inversion recovery pulse sequence 180-tau-90 with tau set to 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 0.75 and 1s and were found to be between 35 –50 ms. The T₂ measurements were done using standard cpmg pulse sequence with following parameters: echo time: 1ms, number of steps: 2, 4, 8, 10, 12, 16, 32, 48, 64, 128, 176, and 256 and resulting T₂ values were between 28 – 52 ms. Sodium triple quantum coherence signal were acquired using standard four pulse triple-quantum coherence filter sequence, 90° Φ_1 - τ /2-180° Φ_2 - τ /2-90° Φ_3 - δ -90° Φ_4 , with $\Phi_1 = \Phi_2 = \Phi$; $\Phi_3 = \Phi + \pi/2$, $\Phi_4 = 0$, with Φ was cycled through 30°, 90°, 150°, 210°, 270° and 330° to select the triple-quantum coherence (12). The other experimental parameters were, no. of scans = 432, no. of points in FID = 1024, sw = 4 kHz. The preparation delay, τ , was varied as 0.1, 0.2, 0.4, 1, 2, 4, 10, 20, 40, 60, 100 and 140 ms. In case when signal didn't decay for the lowest or highest values of the preparation delay τ , further experiments with τ lower than 100 μs or higher than 140 ms were performed. The delay δ was set to 5 μs. No line broadening was used for processing these data. The standard error of mean (SEM) values were calculated by taking the mean of signal intensity of all τ values and dividing it by the square root of the number of samples, n. The fast (T_f) and the slow (T_s) relaxation times and the average residual quadrupolar coupling were measured by fitting triple-quantum signal intensity to equation 1 as a function of preparation delay, τ , using a custom written MATLAB program. The standard errors associated with the residual quadrupolar coupling were found to be high because of the small number of data points and therefore discussed only qualitatively in this article. The motional parameter, $\omega_0\tau_c$, was calculated using the obtained relaxation rates in equation 2. Proton double quantum coherence spectra was collected using the Jeener-Broekaert double quantum coherence pulse sequence, 90° Φ_1 - τ /2-180° Φ_2 - τ /2-45° Φ_3 - δ -45° Φ_4 with phase cycle $\Phi_1 = 0^\circ$; $\Phi_2 = 0^\circ$ 90° 180° 270°; $\Phi_3 = 16(90^\circ)$ 16(270°); $\Phi_4 = 4(0^\circ)$ 4(90°) 4(180°) 4(270°) and Rx = 2(0° 180°) 2(90° 270°) 2(180° 0°) 2(270° 90°) 2(180° 0°) 2(270° 90°) 2(0° 180°) 2(90° 270°) to select the double quantum coherence (31). The experimental parameters for this experiment were: $\delta = 5 \mu s$, $\tau = 100 \mu s$, proton 90° pulse width = 11 μs, relaxation delay = 1 s, number of points in FID = 4096, number of scans = 256, sw = 12kHz.

After cell seeding, the tissue engineering construct samples were taken out from the incubator for four consecutive weeks and the NMR experiments were preformed immediately in the growth media to preserve their natural environment.

Results and discussion

Each tissue-engineering construct had a significantly different pattern for the change in triple-quantum coherence signal intensity as a function of preparation delay, τ , from week to week. Figure 3 shows the rise and the decay triple quantum coherence curve as a function of preparation delay, τ , for chondrocytes in alginate beads, HMSCs in biomimetic scaffolds and chondrocyte pellets for 2 weeks in culture. It is clear that in the alginate beads, the build up and decay of the triple-quantum coherence signal is faster than the biomimetic scaffolds and the chondrocyte pellets indicating smaller fast and slow relaxation times and an average higher values of quadrupolar coupling. Biomimetic scaffolds had a fast rise indicating shorter T_f relaxation times, but slow decay indicating longer T_s relaxation times. Chondrocyte pellets had a slow rise and a slow decay for the observed triple-quantum signal intensity curve.

Table 1 gives the calculated fast and slow relaxation time constants along with the residual quadrupolar coupling using the best fit to equation 1 for all three cartilage tissue-engineering constructs. For comparison, similar parameters are also given for human cartilage explants. The relaxation times and average quadrupolar coupling in native tissue is in the close range as reported by Keinan-Adamsky *et al* (31). However, in this study, the native tissues were approximately 3mm in diameter and 1 mm thick disk-like shaped. Therefore, they did not possess a complete zonal layered cartilage tissue structure. The calculated values of quadrupolar coupling in the native tissues, as well as the values reported in the literature (31), are much higher as compared to the calculated average quadrupolar parameter in the engineered tissues. The high values of average quadrupolar coupling in the human cartilage tissue, is possibly due to the order of collagen network found in the native state. In comparison, average quadrupolar coupling was also high in the chondrocytes suspended in alginate beads. We suspect that this is also because of the polymeric chain in the beads. The fast and the slow relaxation times are also lower in the native tissues as compared to the biomimetic scaffolds and chondrocyte pellets, which might be because of the higher amount of macromolecules providing relaxation pathway for the sodium ions. In the following section, we will focus on the understanding of relaxation and dynamics behavior of sodium ions in the engineered tissues.

It is clear from the table 1 and the figure 3 that the chondrocytes cultured in alginate beads had a smaller values of both relaxation time constants T_f and T_s as compared to the chondrocyte pellets and the biomimetic scaffolds. Masuda *et al* have shown that the bovine chondrocytes cultured in alginate beads maintain their phenotype and continue to produce proteoglycans and collagen with decreasing PG/collagen ratio while in cell culture (52). Alginate bead forms a gel using calcium ion network with L-guluronic acid polymeric residues (G-blocks) (56). This Ca^{+} ion network with L-guluronic acid residues provides a charged ordered environment and creates an osmotic pressure in the beads. The strong triple-quantum coherence build-up and fast relaxation times in alginate beads might be due to this bead specific environment that is not present in the other tissue-engineering constructs. Because of the divalent network of calcium ions and polymeric chain of guluronic acid, sodium ions reside in a charged ionic network environment, which might be contributing to the fast relaxation of sodium ions in the alginate beads. This environment gets modified when the chondrocyte cells produce extracellular matrix components proteoglycans and collagen. The signal intensity rose from week 0 to week 4 except at week 2, where there was a sudden drop. If we look into the relaxation times from week to week, both fast T_f and slow T_s relaxation time constants, decrease for the first three weeks, possibly due to the production of extracellular matrix component proteoglycans, which might bind with sodium and calcium ions in the gel, thus providing additional relaxation pathway. However, at week three, relaxation time constants increase and then slightly

increase again at week 4. This is an interesting trend. In the other two engineering constructs, the fast and the slow relaxation time constants change in different directions with the production of extra-cellular matrix components, which was also the case in the published study by Insko *et al* for enzymatically degraded bovine cartilage (38). This suggests a possible role of gel like structure and calcium ion network in the sodium relaxation pathway. The average sodium motional parameter, $\omega_0\tau_c$, for the alginate beads was 6.54 ± 0.5 ($\tau_c = (6.18 \pm 0.47) \times 10^{-8} \text{ s}^{-1}$).

In case of biomimetic scaffolds, the slow relaxation time T_s increases for first week, then decreases until week 3 before slightly increasing again at week 4. The fast relaxation rate, T_f , had a reverse trend; it decreased for the first two weeks, then increases again at week 3 and week 4. Insko *et al* have shown that the T_s values decrease and the T_f value increase with increasing proteoglycan content (38). In order to explain the relaxation behavior of these constructs, we need to understand the physical and biochemical properties of these samples. These scaffolds contained cartilage extra-cellular matrix components proteoglycans and collagen, as part of the hybrid 'scaffolds plus matrix' growth strategy right from the beginning. The base scaffold was 1:1 combination of collagen and chitosan porous material. These scaffolds are porous with pore size ranging from $1 \mu\text{m} - 10 \mu\text{m}$, similar to our published results for osteoblasts, and this affects the environment around sodium ions and their relaxation (57). The growth strategy was such that when the mesenchymal stem cells perceive a cartilage extracellular matrix in their environment, they will undergo chondrogenic differentiation. The chondrogenic differentiation of HMSC cells and the production of cartilage extracellular matrix components, proteoglycans and collagen, in these biomimetic scaffolds at the end of 4 week culture time were confirmed by qRT-PCR and immunohistochemical analysis and will be published elsewhere. This is the first such study of chondrogenic differentiation of HMSCs without the aid of growth factors.

There might be two factors affecting the relaxation behavior in the biomimetic scaffolds. First, the starting cells in these scaffolds are HMSCs, and as they undergo chondrogenic differentiation, it is possible that there is a reduction in the amount of original proteoglycan for the first week while the HMSCs are starting to go through the chondrogenic differentiation. At the beginning of second week, when these newly formed chondrocytes start generating cartilage-like extra-cellular matrix components, the relaxation pattern changes as expected. The slow relaxation time (T_s) decreases while the fast relaxation time (T_f) increases as expected. The motional parameter, $\omega_0\tau_c$, increases during the first two weeks, which means the motion is getting slower with the production of extra-cellular matrix components. It drops suddenly at week 3 and week 4. Thus the sodium ions move faster at week 4 as compared to week 2 as shown in Figure 4. Further studies are underway to understand this peculiar behavior. Our assumption is that the random order collagens are responsible for reduced anisotropy in the system. The average sodium motional parameter, $\omega_0\tau_c$, for the biomimetic scaffolds was 5.45 ± 1.1 ($\tau_c = (5.15 \pm 1.04) \times 10^{-8} \text{ s}^{-1}$).

The most interesting case for the sodium triple-quantum coherence study was for the chondrocyte pellets. The triple-quantum coherence signal had much slower rise and decay time constants, as compared to other two tissue-engineering constructs, and was observed for the first two weeks of cell culture. The triple-quantum coherence signal intensity dropped to almost zero with no distinct rise or fall for pellets at week 3 and only recovered slightly at week 4 as shown in figure 5. No such sharp change in the triple-quantum coherence intensity was observed in the other two tissue engineered constructs. This was surprising as proteoglycan and collagen continue to grow with reduced proteoglycan to collagen ratio in the pellets as confirmed by the biochemical analysis in our lab. The pellet experiments were repeated again with fresh samples and similar trend in the triple-quantum

filtered signal intensity was observed. As can be seen from Table 1, both the slow and the fast relaxation times are large, with the slow rise and fall of triple quantum coherence spectroscopy for week one and week two of the culture period. The decrease in the value of T_s and the increase in the value of T_f with the increasing amount of proteoglycan are expected for week 1 and week 2. The calculated average motional parameter was 1.84 ± 0.49 ($\tau_c = (1.174 \pm 0.46) \times 10^{-8} \text{ s}^{-1}$), which signifies faster sodium ion motion as compared to the other two tissue-engineering constructs and the native cartilage tissues. The production of random order collagen fibrils is represented by a small value of average quadrupolar coupling in this case. The drop in the triple-quantum coherence signal intensity at week 3 and 4 is again probably due to the isotropic environment created by this random order collagen. The isotropic environment created with the production of extra-cellular matrix component was also confirmed by the small average dipolar coupling in proton double-quantum coherence spectra as shown in figure 6. This splitting is much smaller than the reported values for bovine Achilles tendon which show the basic difference between the native and the engineered tissues (58).

Based on the sodium triple-quantum coherence data and based on the known immunohistochemical data on these tissue-engineered constructs, we propose a schematic model for tissue engineering constructs. This model is more apt for chondrocyte pellets, but this might be suitable for other cartilage tissue-engineered constructs as well. We propose that there are three major differences between the tissue-engineered constructs and the native cartilage tissue. 1. The lack of order: the collagen type II produced in the engineered tissues is of random nature and possibly with a small fibril size, which contributes to the reduced anisotropy in the engineered tissues. 2. The ratio of proteoglycans to collagen: The native tissue has a higher amount of collagen as compared to the proteoglycan, whereas in the engineered tissue, as found in our lab as well as in published studies by other groups, the amount of proteoglycans accumulation is higher as compared to the collagen production (52). This ratio (PG/collagen) reduces as tissue matures, but it does not reach to the level of native tissue in the time frame studied. 3. The cell density: In the mature native cartilage tissue, the number of chondrocytes is only 1% of wet weight, whereas in the engineered tissues it is significantly higher in order to achieve the high production of extra-cellular matrix component. The effect of cell proliferation and differentiation on the sodium relaxation and dynamics is not accounted for in the present study, however we expect that it does have a non-negligible contribution. The proposed model based on these three main features of engineered tissues is presented in figure 7.

Conclusions and future outlook

In the present study, we have investigated the application of sodium triple-quantum coherence spectroscopy for the study of tissue-growth dynamics in three different cartilage tissue engineering constructs. These experiments were performed in the normal growth media in order to preserve the natural environment of the engineered tissues. Human HMSCs seeded in biomimetic scaffolds, bovine chondrocyte seeded in alginate beads and bovine chondrocyte pellets were studied. The average quadrupolar couplings were found to be smaller in the engineered tissues as compared to the native tissue and it was attributed to the lack of order in the engineered cartilage. The fast (T_f) and the slow (T_s) relaxation time were found to be lower in alginate beads as compared to the biomimetic scaffolds and the chondrocyte pellets. In case of biomimetic scaffolds, the motional parameter increased during the first two weeks of cell-culture and then dropped at week 3. This sudden drop in motional parameter is attributed to the production of random order collagen fibrils. The slow relaxation time constants and the smaller value of motional parameter, $\omega_0\tau_c$, in case of chondrocyte pellets are attributed to the increased isotropy due to random order collagen fibrils in these samples. This study shows that the sodium triple-quantum coherence

spectroscopy can be a useful tool to study the dynamics and anisotropy of proteoglycans and collagen in the engineered cartilage tissue. We plan to further utilize these findings in sodium triple-quantum *in vivo* MRI experiments using the engineered cartilage tissues implanted in a mouse model. It would be interesting to see if the tissue gains anisotropy after implantation. The effect of cell proliferation and differentiation on the sodium relaxation and average quadrupolar coupling parameters was not accounted for in this study. We plan to address this parameter by varying the initial cell density and accounting for cell numbers during the growth stage of engineered cartilage tissues in our future studies. These studies will shed much needed light on the growth dynamics of cartilage tissue engineering, which can be used to enhance the performance of the cartilage tissue engineering.

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Abbreviations

NMR	Nuclear Magnetic Resonance
MRI	Magnetic Resonance Imaging
ECM	extra cellular matrix
OA	Osteoarthritis
T₁	Spin-lattice relaxation time
T₂	spin-spin relaxation time
HMSCs	Human mesenchymal stem cells

References

1. Tchetina EV. Developmental Mechanisms in Articular Cartilage Degradation in Osteoarthritis. Arthritis. 2011; 2011 doi: 10.1155/2011/683970.
2. Prevalence and most common causes of disability among adults--United States 2005. MMWR Morb Mortal Wkly Rep. 2009; 58(16):421–426. Epub 2009/05/02. doi: mm5816a2 [pii]. PubMed PMID: 19407734. [PubMed: 19407734]
3. Centers for Disease Control and Prevention, Arthritis Foundation. A National Public Health Agenda for Osteoarthritis 2010. Available from: [<http://www.cdc.gov/arthritis/docs/OAagenda.pdf%5D>].
4. VanItallie TB. Gout: epitome of painful arthritis. Metabolism. 2010; 59(Supplement 1):S32–S36. doi: DOI: 10.1016/j.metabol.2010.07.009. [PubMed: 20837191]
5. Buckwalter JA, Mankin HJ. Articular cartilage .2. Degeneration and osteoarthrosis, repair, regeneration, and transplantation. J Bone Joint Surg. Am. 1997; 79A(4):612–632. PubMed PMID: ISI:A1997WU51500022.
6. Clair BL, Johnson AR, Howard T. Cartilage Repair. Foot & Ankle Specialist. 2009; 2(4):179–188. doi: 10.1177/1938640009342272. [PubMed: 19825772]
7. Ahmed TAE, Hincke MT. Strategies for Articular Cartilage Lesion Repair and Functional Restoration. Tissue Engineering Part B: Reviews. 2010; 16(3):305–329. doi: 10.1089/ten.teb.2009.0590. [PubMed: 20025455]
8. Mithoefer K, Hambly K, Villa SD, Silvers H, Mandelbaum BR. Return to Sports Participation After Articular Cartilage Repair in the Knee. The American Journal of Sports Medicine. 2009; 37(1 suppl):167S–176S. doi: 10.1177/0363546509351650. [PubMed: 19861696]

9. Duchow J, Hess T, Kohn D. Primary Stability of Press-Fit-Implanted Osteochondral Grafts. *The American Journal of Sports Medicine*. 2000; 28(1):24–27. [PubMed: 10653539]
10. Buckwalter JA, Mankin HJ. Articular cartilage .1. Tissue design and chondrocyte-matrix interactions. *J Bone Joint Surg Am*. 1997; 79A(4):600–611. PubMed PMID: ISI:A1997WU51500021.
11. Lesperance LM, Gray ML, Burstein D. Determination of Fixed Charge-Density in Cartilage Using Nuclear-Magnetic-Resonance. *J Orthopaed Res*. 1992; 10(1):1–13. PubMed PMID: ISI:A1992GX48200001.
12. Borthakur A, Mellon E, Niyogi S, Witschey W, Kneeland JB, Reddy R. Sodium and T-1 rho MRI for molecular and diagnostic imaging of articular cartilage. *Nmr Biomed*. 2006; 19(7):781–821. doi: Doi 10.1002/Nbm.1102. PubMed PMID: ISI:000242646700005. [PubMed: 17075961]
13. Roemhildt ML, Coughlin KM, Peura GD, Fleming BC, Beynnon BD. Material properties of articular cartilage in the rabbit tibial plateau. *Journal of biomechanics*. 2006; 39(12):2331–2337. Epub 2005/09/20. doi: S0021-9290(05)00343-X [pii] 10.1016/j.jbiomech.2005.07.017. PubMed PMID: 16168420; PubMed Central PMCID: PMC2933407. [PubMed: 16168420]
14. Klein TJ, Malda J, Sah RL, Huttmacher DW. Tissue Engineering of Articular Cartilage with Biomimetic Zones. *Tissue Eng Part B-Re*. 2009; 15(2):143–157. doi: DOI 10.1089/ten.teb.2008.0563. PubMed PMID: ISI:000266574000004.
15. Harley BA, Lynn AK, Wissner-Gross Z, Bonfield W, Yannas IV, Gibson LJ. Design of a multiphase osteochondral scaffold III: Fabrication of layered scaffolds with continuous interfaces. *Journal of Biomedical Materials Research Part A*. 2010; 92A(3):1078–1093. doi: 10.1002/jbm.a.32387. [PubMed: 19301263]
16. Raimondi MT, Falcone L, Colombo M, Remuzzi A, Marinoni E, Marazzi M, et al. A comparative evaluation of chondrocyte/scaffold constructs for cartilage tissue engineering. *J Appl Biomater Biomech*. 2004; 2(1):55–64. Epub 2004/01/01. PubMed PMID: 20803451. [PubMed: 20803451]
17. Chubinskaya S, Huch K, Schulze M, Otten L, Aydelotte MB, Cole AA. Gene expression by human articular chondrocytes cultured in alginate beads. *J Histochem Cytochem*. 2001; 49(10):1211–1219. PubMed PMID: ISI:000171138900003. [PubMed: 11561005]
18. Kotecha, M.; Ravindran, S.; Vaidyanathan, A.; George, A.; Magin, RL. Characterization of ECM embedded biomimetic scaffolds for cartilage tissue engineering using sodium triple quantum coherence spectroscopy. *Proceedings of the International Society for Magnetic Resonance in Medicine (ISMRM) 20th Annual Meeting and Exhibition; May 5–11; Melbourne, Australia*. 2012.
19. Ceuninck F, Lesur C, Pastoureau P, Caliez A, Sabatini M. Culture of Chondrocytes in Alginate Beads. 2004:15–22.
20. Cheuk Y-C, Wong MW-N, Lee K-M, Fu S-C. Use of allogeneic scaffold-free chondrocyte pellet in repair of osteochondral defect in a rabbit model. *J Orthopaed Res*. 2011; 29(9):1343–1350. doi: 10.1002/jor.21339.
21. Othman SF, Li J, Abdullah O, Moinnes JJ, Magin RL, Muehleman C. High-resolution/high-contrast MRI of human articular cartilage lesions. *Acta Orthop*. 2007; 78(4):536–546. doi: Doi 10.1080/17453670710014194. PubMed PMID: ISI:000250516600014. [PubMed: 17966009]
22. Othman SF, Curtis ET, Plautz SA, Pannier AK, Butler SD, Xu HH. MR elastography monitoring of tissue-engineered constructs. *Nmr Biomed*. 2012; 25(3):452–463. doi: Doi 10.1002/Nbm.1663. PubMed PMID: ISI:000300670600008. [PubMed: 21387443]
23. Xu HH, Othman SF, Magin RL. Monitoring Tissue Engineering Using Magnetic Resonance Imaging. *J Biosci Bioeng*. 2008; 106(6):515–527. doi: Doi 10.1263/Jbb.106.515. PubMed PMID: ISI:000263490500001. [PubMed: 19134545]
24. Weber F, Böhme J, Scheidt HA, Gründer W, Rammelt S, Hacker M, et al. 31P and 13C solid-state NMR spectroscopy to study collagen synthesis and biomineralization in polymer-based bone implants. *NMR in Biomedicine*. 2012; 25(3):464–475. doi: 10.1002/nbm.1649. [PubMed: 22351643]
25. Schulz J, Pretzsch M, Khalaf I, Deiwick A, Scheidt HA, Salis-Soglio G, et al. Quantitative monitoring of extracellular matrix production in bone implants by C-13 and P-31 solid-state nuclear magnetic resonance spectroscopy. *Calcified Tissue Int*. 2007; 80(4):275–285. doi: DOI 10.1007/s00223-007-9007-3. PubMed PMID: ISI:000246103100008.

26. Cheng H-LM, Loai Y, Farhat WA. Monitoring tissue development in acellular matrix-based regeneration for bladder tissue engineering: Multiexponential diffusion and T2* for improved specificity. *NMR in Biomedicine*. 2012; 25(3):418–426. doi: 10.1002/nbm.1617. [PubMed: 22351641]
27. Li W, Hong L, Hu L, Magin RL. Magnetization Transfer Imaging Provides a Quantitative Measure of Chondrogenic Differentiation and Tissue Development. *Tissue Engineering Part C: Methods*. 2010 online Ahead of print:1-9. doi: 10.1089/ten.tec.2009.0777.
28. Miyata S, Numano T, Homma K, Tateishi T, Ushida T. Feasibility of noninvasive evaluation of biophysical properties of tissue-engineered cartilage by using quantitative MRI. *Journal of biomechanics*. 2007; 40(13):2990–2998. doi: 10.1016/j.jbiomech.2007.02.002. [PubMed: 17442320]
29. Irrechukwu ON, Reiter DA, Lin PC, Roque RA, Fishbein KW, Spencer RG. Characterization of Engineered Cartilage Constructs Using Multiexponential T-2 Relaxation Analysis and Support Vector Regression. *Tissue Eng Part C-Me*. 2012; 18(6):433–443. doi: DOI 10.1089/ten.tec.2011.0509. PubMed PMID: ISI:000304797500004.
30. Reiter DA, Irrechukwu O, Lin P-C, Moghadam S, Thaer SV, Pleshko N, et al. Improved MR-based characterization of engineered cartilage using multiexponential T2 relaxation and multivariate analysis. *Nmr Biomed*. 2012; 25(3):476–488. doi: 10.1002/nbm.1804. [PubMed: 22287335]
31. Keinan-Adamsky K, Shinar H, Shabat S, Brin YS, Nyska M, Navon G. Na-23 and H-2 Magnetic Resonance Studies of Osteoarthritic and Osteoporotic Articular Cartilage. *Magn Reson Med*. 2010; 64(3):653–661. doi: Doi 10.1002/Mrm.22479. PubMed PMID: ISI:000281346300005. [PubMed: 20806373]
32. Blumich B, Munnemann K, Boni T, Colacicco G, Ruhli F. Noninvasive H-1 and Na-23 nuclear magnetic resonance imaging of ancient Egyptian human mummified tissue. *Magn Reson Imaging*. 2007; 25(9):1341–1345. PubMed PMID: ISI:000250824900011. [PubMed: 17490843]
33. Borthakur A, Shapiro EM, Akella SVS, Gougoutas A, Kneeland JB, Reddy R. Quantifying sodium in the human wrist in vivo by using MR imaging. *Radiology*. 2002; 224(2):598–602. PubMed PMID: ISI:000177025400043. [PubMed: 12147862]
34. Reddy R, Insko EK, Noyszewski EA, Dandora R, Kneeland JB, Leigh JS. Sodium MRI of human articular cartilage in vivo. *Magn Reson Med*. 1998; 39(5):697–701. PubMed PMID: ISI: 000073234200004. [PubMed: 9581599]
35. Shapiro EM, Borthakur A, Gougoutas A, Reddy R. Na-23 MRI accurately measures fixed charge density in articular cartilage. *Magn Reson Med*. 2002; 47(2):284–291. PubMed PMID: ISI: 000173578700009. [PubMed: 11810671]
36. Vega AJ, Ooms KJ, Cannella M, Marcolongo M, Polenova T. Na-23 TQF NMR imaging for the study of spinal disc tissue. *J Magn Reson*. 2008; 195(1):112–115. PubMed PMID: ISI: 000260398000014. [PubMed: 18774321]
37. Navon G, Shinar H, Eliav U, Seo Y. Multiquantum filters and order in tissues. *Nmr Biomed*. 2001; 14(2):112–132. PubMed PMID: ISI:000168413000006. [PubMed: 11320537]
38. Insko EK, Kaufman TH, Leigh JS, Reddy R. Sodium NMR evaluation of articular cartilage degradation. *Magn Reson Med*. 1999; 41(1):30–34. PubMed PMID: ISI:000078336800006. [PubMed: 10025608]
39. Ooms KJ, Cannella M, Vega AJ, Marcolongo M, Polenova T. Na-23 TQF NMR imaging for the study of spinal disc tissue. *J Magn Reson*. 2008; 195(1):112–115. doi: DOI 10.1016/j.jmr.2008.07.024. PubMed PMID: ISI:000260398000014. [PubMed: 18774321]
40. Ooms KJ, Cannella M, Vega AJ, Marcolongo M, Polenova T. The application of Na-23 double-quantum-filter (DQF) NMR spectroscopy for the study of spinal disc degeneration. *Magn Reson Med*. 2008; 60(2):246–252. doi: Doi 10.1002/Mrm.21637. PubMed PMID: ISI:000258105800001. [PubMed: 18666105]
41. Borthakur A, Shapiro EM, Beers J, Kudchodkar S, Kneeland JB, Reddy R. Sensitivity of MRI to proteoglycan depletion in cartilage: comparison of sodium and proton MRI. *Osteoarthr Cartilage*. 2000; 8(4):288–293. PubMed PMID: ISI:000088511800007.
42. Wheaton AJ, Borthakur A, Shapiro EM, Regatte RR, Akella SVS, Kneeland JB, et al. Proteoglycan loss in human knee cartilage: Quantitation with sodium MR imaging - Feasibility

- study. *Radiology*. 2004; 231(3):900–905. doi: DOI 10.1148/radiol.2313030521. PubMed PMID: ISI:000221585200043. [PubMed: 15163825]
43. Trattnig S, Welsch GH, Juras V, Szomolanyi P, Mayerhoefer ME, Stelzeneder D, et al. 23Na MR Imaging at 7 T after Knee Matrix–associated Autologous Chondrocyte Transplantation: Preliminary Results 1. *Radiology*. 2010 doi: 10.1148/radiol.10100279.
  44. Zbý Š, Stelzeneder D, Welsch GH, Negrin LL, Juras V, Mayerhoefer ME, et al. Evaluation of native hyaline cartilage and repair tissue after two cartilage repair surgery techniques with 23Na MR imaging at 7 T: initial experience. *Osteoarthritis Cartilage*. 2012; 20(8):837–845. doi: 10.1016/j.joca.2012.04.020.
  45. Krusche-Mandl I, Schmitt B, Zak L, Apprich S, Aldrian S, Juras V, et al. Long-term results 8 years after autologous osteochondral transplantation 7 T gagCEST and sodium magnetic resonance imaging with morphological and clinical correlation. *Osteoarthritis Cartilage*. 2012; 20(5):357–363. doi: 10.1016/j.joca.2012.01.020.
  46. Schmitt B, Zbý Š, Stelzeneder D, Jellus V, Paul D, Lauer L, et al. Cartilage Quality Assessment by Using Glycosaminoglycan Chemical Exchange Saturation Transfer and 23Na MR Imaging at 7 T. *Radiology*. 2011; 260(1):257–264. doi: 10.1148/radiol.11101841. [PubMed: 21460030]
  47. Keinan-Adamsky K, Shinar H, Navon G. Multinuclear NMR and MRI studies of the maturation of pig articular cartilage. *Magn Reson Med*. 2006; 55(3):532–540. doi: Doi 10.1002/Mrm.20775. PubMed PMID: ISI:000235858400010. [PubMed: 16450338]
  48. van der Maarel JRC. Thermal relaxation and coherence dynamics of spin 3/2. II. Strong radio-frequency field. *Concept Magn Reson A*. 2003; 19A(2):117–133. doi: Doi 10.1002/Cmr.A.10088. PubMed PMID: ISI:000186653800006.
  49. Kemp-Harper R, Brown SP, Hughes CE, Styles P, Wimperis S. Na-23 NMR methods for selective observation of sodium ions in ordered environments (vol 30, pg 157, 1997). *Prog Nucl Mag Res Sp*. 1997; 31:287. PubMed PMID: ISI:000071715400006.
  50. Jaccard G, Wimperis S, Bodenhausen G. Multiple-Quantum NMR-Spectroscopy of S=3/2 Spins in Isotropic-Phase - a New Probe for Multiexponential Relaxation. *J Chem Phys*. 1986; 85(11):6282–6293. PubMed PMID: ISI:A1986F086500005.
  51. Petit B, Masuda K, DSouza AL, Otten L, Pietryla D, Hartmann DJ, et al. Characterization of crosslinked collagens synthesized by mature articular chondrocytes cultured in alginate beads: Comparison of two distinct matrix compartments. *Exp Cell Res*. 1996; 225(1):151–161. PubMed PMID: ISI:A1996UM49100016. [PubMed: 8635508]
  52. Masuda K, Sah RL, Hejna MJ, Thonar EJMA. A novel two-step method for the formation of tissue-engineered cartilage by mature bovine chondrocytes: The alginate-recovered-chondrocyte (ARC) method. *J Orthopaed Res*. 2003; 21(1):139–148. doi: 10.1016/s0736-0266(02)00109-2.
  53. Chandrasekhar S, Esterman MA, Hoffman HA. Microdetermination of Proteoglycans and Glycosaminoglycans in the Presence of Guanidine-Hydrochloride. *Anal Biochem*. 1987; 161(1):103–108. PubMed PMID: ISI:A1987G131900016. [PubMed: 3578776]
  54. Creemers LB, Jansen DC, vanVeenReurings A, vandenBos T, Everts V. Microassay for the assessment of low levels of hydroxyproline. *Biotechniques*. 1997; 22(4):656–658. PubMed PMID: ISI:A1997WT81500019. [PubMed: 9105617]
  55. Ravindran S, Gao Q, Kotecha M, Magin RL, Karol S, Bedran-Russo A, et al. Biomimetic ECM Incorporated Scaffold Induces Osteogenic Gene Expression in Human Marrow Stromal Cells. *Tissue Engineering Part A*. 2012; 18(3–4):295–309. doi: 10.1089/ten.tea.2011.0136. [PubMed: 21867449]
  56. Martinsen A, Skjakbraek G, Smidsrod O. Alginate as Immobilization Material .1. Correlation between Chemical and Physical-Properties of Alginate Gel Beads. *Biotechnol Bioeng*. 1989; 33(1):79–89. PubMed PMID: ISI:A1989R362300010. [PubMed: 18587846]
  57. Ravindran S, Gao Q, Kotecha M, Magin RL, Karol S, Bedran-Russo A, et al. Biomimetic Extracellular Matrix-Incorporated Scaffold Induces Osteogenic Gene Expression in Human Marrow Stromal Cells. *Tissue Engineering Part A*. 2012; 18(3–4):295–309. doi: DOI 10.1089/ten.tea.2011.0136. PubMed PMID: ISI:000300003300008. [PubMed: 21867449]

58. Eliav U, Navon G. Multiple quantum filtered NMR studies of the interaction between collagen and water in the tendon. *J Am Chem Soc.* 2002; 124(12):3125–3132. doi: Doi 10.1021/Ja011791n. PubMed PMID: ISI:000174520500043. [PubMed: 11902901]

Figure 1. Schematic diagram of the native articular cartilage tissue with collagen type II fibers, proteoglycans and chondrocytes.

Figure 2. Representative example of TQ signal intensity as a function of creation time and its best fit with eq. 1 for chondrocytes seeded in alginate beads at week 0. Inset shows the TQ pulse sequence used in the study.

Figure 3.

Sodium triple-quantum coherence signal intensity at week 2 for chondrocytes seeded in alginate beads (SEM = $\pm 16\%$), HMSC seeded in biomimetic scaffold (SEM = $\pm 3\%$) and chondrocyte pellets (SEM = 6.4%) as a function of preparation delay, τ . The markers are the experimental data points and the line is for guide to eyes only. The signal to noise ratio (SNR) for these experiments was ~ 29 for the optimum τ value.

Figure 4.
Triple-quantum spectra for the HMSCs seeded in biomimetic scaffolds at week 2 and week 4. Week 4 spectra show a narrow line width that indicates a faster motion.

Figure 5.
Sodium triple-quantum coherence spectra of chondrocyte pellets as a function of creation delay τ for 4 weeks of culture time.

Figure 6. Proton double-quantum (DQ) coherence spectra for the chondrocyte pellets at four weeks of culture time showing a very small splitting $2\nu_D$ (~ 20 Hz). This indicates a small average dipolar coupling. In native tissue, this splitting is found to be in kHz range (31).

Figure 7.
Proposed model of engineered cartilage and its macromolecule composition with short collagen fibrils and higher amount of proteoglycans to collagen.

Table 1

The fast (T_f) and the slow (T_s) relaxation times, residual quadrupolar coupling, ω_Q , and motional parameter, $\omega_0\tau_c$, for sodium ion in alginate beads embedded with chondrocytes, scaffold free chondrocyte pellets, and ECM embedded scaffolds seeded with HMSC cells as a function of growth time at 400 MHz (9.4T). The values are given with parameter standard error. For comparison, similar parameters from the human articular cartilage (1 mm thick, 3 mm in diameter) explants are included in the table.

Time points	Biomimetic scaffolds (Human HMSC) (n=3)				Alginate Beads (Bovine Chondrocytes) (n=4)				Scaffold free chondrocyte pellets (Bovine Chondrocytes) (n=3)			
	T_s (ms)	T_f (ms)	ω_Q (Hz)	$\omega_0\tau_c^{\#}$	T_s (ms)	T_f (ms)	ω_Q (Hz)	$\omega_0\tau_c$	T_s (ms)	T_f (ms)	ω_Q (Hz)	$\omega_0\tau_c$
Week 0	44.5 ± 5.6	2.09 ± 0.32	211 ± 190	4.98 ± 0.26	16.3 ± 1.0	0.51 ± 0.04	829 ± 459	6.15 ± 0.16	-	-	-	-
Week 1	55.0 ± 14.8	1.65 ± 0.55	*	6.30 ± 0.71	15.2 ± 0.7	0.60 ± 0.04	*	5.45 ± 0.12	73 ± 35.6	12.0 ± 4.6	*	2.42 ± 0.49
Week 2	51.1 ± 11.1	1.4 ± 0.37	*	6.69 ± 0.58	14.8 ± 1.2	0.29 ± 0.05	2601 ± 1357	7.84 ± 0.39	49 ± 1.33	15.5 ± 0.33	9.6 ± 13.2	1.26 ± 0.03
Week 3	36.0 ± 10.6	2.2 ± 0.68	*	4.63 ± 0.47	16.4 ± 1.2	0.44 ± 0.04	*	6.75 ± 0.20	-	-	-	-
Week 4	41.0 ± 7.0	2.37 ± 0.43	*	4.65 ± 0.29	17.8 ± 1.0	0.50 ± 0.03	628 ± 458	6.5 ± 0.14	-	-	-	-
Human Cartilage	18.66 ± 1.08	0.32 ± 0.03	2463 ± 798	8.42 ± 0.24								

* Values not included in the table because the parameter standard error exceeded many fold than the calculated parameter values.

Quadratic term in equation 2 was not included in the calculation of error.