Extended Monitoring of Osteogenesis Using MRI-compatible Tissue Culture System

BY

AISHWARYA VAIDYANATHAN

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THESIS

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Defense committee:

Dr. Richard Magin, Chair and Advisor
Dr. Anne George, Advisor, Department of Oral Biology
Dr. Sriram Ravindran, Department of Oral Biology
This thesis is dedicated to appa and amma without whom it would have never been accomplished.
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ADC</td>
<td>Apparent Diffusion Coefficient</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenic Protein</td>
</tr>
<tr>
<td>DMP</td>
<td>Dentin Matrix Protein</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
</tr>
<tr>
<td>EthD</td>
<td>Ethidium Homodimer</td>
</tr>
<tr>
<td>FID</td>
<td>Free Induction Decay</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of View</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infra Red Spectroscopy</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HMSC</td>
<td>Human Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>HFBR</td>
<td>Hollow Fiber Bio Reactor</td>
</tr>
<tr>
<td>MRE</td>
<td>Magnetic Resonance Elastography</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MRM</td>
<td>Magnetic Resonance Microscopy</td>
</tr>
<tr>
<td>MRS</td>
<td>Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>MSME</td>
<td>Multi Spin Multi Echo</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NTP</td>
<td>Nucleotide Tri Phosphate</td>
</tr>
<tr>
<td>PA</td>
<td>Peptide Amphiphiles</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>RARE</td>
<td>Rapid Acquisition with Relaxation Enhancement</td>
</tr>
<tr>
<td>RAREVTR</td>
<td>RARE with Variable Repetition Time</td>
</tr>
<tr>
<td>RF</td>
<td>Radio Frequency</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribo Nucleic Acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal to Noise Ratio</td>
</tr>
<tr>
<td>SPECT</td>
<td>Stimulated Positron Emission Computed Tomography</td>
</tr>
<tr>
<td>SPIO</td>
<td>Super Paramagnetic Iron Oxide</td>
</tr>
<tr>
<td>TE</td>
<td>Echo Time</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition Time</td>
</tr>
<tr>
<td>VTU</td>
<td>Variable Temperature control Unit</td>
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1. **INTRODUCTION**

1.1 **Understanding Tissue engineering**

Tissue engineering originated as an extension of biocompatibility. Earlier, materials that remained inert and provided structural strength after implantation were considered biocompatible. But as the demands of the medical devices and tissue substitutes changed, so did the definition of biocompatibility. A biocompatible material is now viewed as a device that can interact with the surrounding environment to direct and control tissue response in a desirable way. [1] Langer, Lanza and Vacanti define clinical tissue engineering as “the use of a synthetic or natural biodegradable material, which has been seeded with living cells when necessary, to regenerate the form and/or function of a damaged or diseased tissue or organ in a human patient.” [2] It can be noted that the basic requirements for tissue regeneration are: cells and an appropriate biomaterial that can act as a scaffold. Another latent factor in the process of tissue engineering is the dynamic interaction of the seeded cells with the biomaterial/surroundings, which is termed as the **cell microenvironment or the cell niche**. The basic principle and requirements of tissue engineering is illustrated in Figure 1.

Most efforts in the field of tissue engineering and regenerative medicine are directed towards understanding this complex and dynamic interaction amongst the cells and between the cells and the microenvironment *in vivo*. This is given so much importance because the best
way to control and direct the behavior/response/interaction of cells in vitro is by understanding the in vivo dynamics and emulating it within tissue engineered constructs. [3]

![Image](image.jpg)

**Figure 1: Illustration showing basic requirements of tissue engineering [4]**

### 1.1.1 Cells

In order to regenerate tissue, it is necessary to seed the scaffolding biomaterial with either the desired cell type or stem cells that possess the potential to differentiate into the necessary cell type based on the biochemical cues supplied to them. Stem cells are of 2 types: embryonic stem cells and adult stem cells. Embryonic stem cells are pleuripotent and can differentiate into all cell types. They are derived from the blastocyst of the embryo. Mesenchymal stem cells are multipotent ie they can differentiate into many cell types. Bone marrow is the most popular source of adult stem cells, although other sources are being discovered.
1.1.2 Biomaterials/scaffold

The complex interactions that take place between the cells and its microenvironment are the major players in directing cell lineage. [5, 6] In order to emulate this complex environment found in vivo, it is necessary to choose a suitable scaffolding material and cell type. The microarchitecture found in vivo can be emulated in vitro by making a smart choice of scaffolding materials and cell type. A carefully chosen scaffold will interact with the cells and the cell niche to direct differentiation and dynamically control body response to the tissue engineered construct when implanted.

Different types of scaffolds have been used in the past, both natural and synthetic. Table I lists the commonly used natural and synthetic polymers in tissue engineering.

<table>
<thead>
<tr>
<th>NATURAL</th>
<th>SYNTHETIC</th>
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<tr>
<td>Type I Collagen</td>
<td>Poly-(α hydroxyl acids)</td>
</tr>
<tr>
<td>Glycosaminoglycans</td>
<td>Poly-(lactic acid), poly-(glycolic acid) and their copolymers</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Polydioxanone</td>
</tr>
<tr>
<td>Polyhydroxy alkanoates</td>
<td>Poly-(ε caprolactone)</td>
</tr>
<tr>
<td></td>
<td>Poly orthoesters</td>
</tr>
<tr>
<td></td>
<td>Polyurethanes</td>
</tr>
<tr>
<td></td>
<td>Polyanhydrides</td>
</tr>
<tr>
<td></td>
<td>Polyphosphazenes</td>
</tr>
<tr>
<td></td>
<td>Poly-(amino acids) and pseudo poly-(amino acids)</td>
</tr>
</tbody>
</table>

TABLE I: POLYMERS USED IN TISSUE ENGINEERING APPLICATIONS [2]
1.1.3 Cell niche

The cell niche is constituted by the cell surroundings formed by the supporting cells and the signals emanating from them. [7, 8] The key parameters that contribute to the formation of a niche are discussed below:

- ECM composition of the embedded stem cells: The ECM is largely composed of different types of collagen, laminins, proteoglycans etc that interact with receptors on the cells and direct cell proliferation, differentiation, adhesion, migration and matrix remodeling. [5, 9]

- Biophysical properties of ECM: Biophysical properties of ECM like the elasticity, stiffness, cell shape also affect the cell fate. Many studies have been published that prove the existence of the dependence of cell fate on stiffness and elasticity of the microenvironment. [10, 11]

- 3D structure and microarchitecture of ECM: Cells within scaffolds made from the same biomaterial but with different porosity or volume ratio exhibit different behavior. [12]

- Soluble factors: Soluble factors serve as an effective means of communication within the cell niche. They can act locally or diffuse to other regions and direct cell behavior. Cytokines, growth factors and chemicals are the common soluble factors. [13,14]

- Environmental factors like Oxygen tension also have an effect on the cell behavior. [15]

It can be seen that major changes occurring during the tissue engineering process are
dictated by the ECM. Hence, monitoring the dynamic ECM provides valuable information for successful tissue regeneration [3, 5].

1.2 Bone tissue engineering: Mechanisms and prevailing methods

Bone tissue engineering has been viewed as a potential method to replace autografts. As explained in the previous section, in order to successfully regenerate bone, it is important to emulate and mimic the conditions of bone formation and mineralization occurring in vivo. Culture of different cell types has been performed to study the cell behavior and the response of cells to different microenvironments.

The cells that form bone and control mineralization are osteoblasts, which can be obtained through differentiation of osteoprogenitor cells and Human Marrow Stromal Cells (HMSCs). The osteoblasts proliferate and migrate through the 3-dimensional environment of the substrate, forming and reforming focal adhesions mediated through transmembrane proteins like integrins. As the osteoblasts proliferate, they synthesize Alkaline Phosphatase, an enzyme that can convert organic phosphate to inorganic phosphate, causing formation of calcium phosphate crystals. The deposition of these crystals on the newly synthesized ECM provides further cues for mineralization. [16]

Stages of bone formation and mineralization have been studied by several analytical techniques:

a) Histology and staining to examine crystal deposition using light/confocal microscopy:

Alizarin Red and Von Kossa are popular staining methods to visualize crystal
deposition in an osteogenic culture. Alizarin Red dye binds to free Calcium and Calcium compounds, imparting a red stain. Von Kossa staining protocol involves addition of Silver Nitrate to the osteogenic culture, which reacts with Calcium Phosphate deposits to undergo a precipitation reaction to form Silver salts, which is further reduced to metallic Silver after addition of a reducing agent. The black Silver deposits represent Calcium Phosphate deposits in the culture.

b) Gene expression analysis to study expression of different genes at different stages during bone formation: Different genes are expressed during different stages of mineralization. For example, alkaline phosphatase is an early marker of osteogenesis. Other genes that are analyzed to confirm osteogenic differentiation are Osteocalcin, Collagen Type I, Osteopontin, Bone Sialo Protein, Osteonectin etc. Gene expression studies require invasive treatment of cells with chemicals to extract RNA.

c) X-ray diffraction studies: X-ray diffraction method enables one to analyze the size and orientation of minerals deposited in the culture, but this requires grinding and lyophilization of samples, making the procedure invasive. [17]

d) Electron microscopy: SEM and TEM also enable evaluation of individual crystals deposited within a culture. The method has been used in certain cases to prove that the mineralization has occurred along the axis of collagen deposition. [18] This method is also invasive as it involves sample preparation which may result in dissolution of scaffolds and loss of mineral deposits in the organic solutions that are added during the process. [19]
e) Atomic Force Microscopy (AFM): AFM enables visualization of cells, associated proteins as well as crystals formed by “tapping” the surface with a cantilever, where forces between the tip of the cantilever and sample produces a deflection which is measured using a laser spot on the tip of the cantilever that is reflected onto an array of photodiodes. AFM has been applied in tissue engineering to study adherence of osteoblasts to different biomaterials. [20]

f) Vibrational spectroscopy: Infrared spectroscopic methods provide information about the local environment of ions through asymmetric vibrations. Several studies have applied FTIR to study mineralization as a ratio of phosphate peak. [21] FTIR requires very thin sections of samples and requires high degree of dehydration. Raman spectroscopy can be performed on thicker slices and without dehydration. Studies have been performed to study mineralization using Raman spectroscopy. [22]

g) Micro Computed Tomography (Micro CT): Micro CT has been used to study the porosity as well as mineral deposition. It enables 3-dimensional visualization of mineral deposition as a result of osteogenesis. This method has been used by [23] to study mineralization in osteogenic culture.

The above described methods, even though routinely used, have their own shortcomings. For instance, histological analysis gives the most conclusive results, but is invasive, precluding any further characterization. Gene expression studies, also invasive, provide a quantitative measure of the levels of proteins expressed, giving us a clear picture of cell fate, but failing to give a description about the distribution and 3-dimensional information
of cells. Modern microscopy techniques like confocal microscopy do not possess sufficient depth penetration. Also, live cell imaging may be difficult to perform without modification of the genetic material of the cells by transfecting fluorescent protein-synthesizing genes. Other optical techniques have limited depth resolution and local area coverage and are dependent on external fluorophores. [24, 25] Other options like FTIR, electron microscopy also require the samples to be destructively sectioned. SPECT, PET, despite their high depth penetration, are not commonly used as they depend on radioactive labeling of cells.

MRI, being a non-destructive technique, capable of providing a resolution in the order of tens of microns has been viewed as a potential analytical technique to monitor bone tissue engineering.

1.3 Magnetic Resonance Imaging (MRI)

MRI has been used as a tool to investigate the magnetic relaxation response of water in the ECM by measuring the changes in spin-lattice (T1) and spin-spin (T2) relaxation times noninvasively with sub-millimeter resolution [26]. Relaxation is the process by which nuclear spins within the sample that are excited by a Radio Frequency (RF) pulse return back to their ground energy state. This process consists of 2 independent events: longitudinal relaxation in which the spins release excess energy into the surrounding lattice, the time constant of which is T1 and transverse relaxation in which spins interact with each other to go back to their ground states, the time constant of which is T2.
MRI is a non destructive technique with resolution of tens of microns and an ability to generate tomographic images without actually slicing or dicing the sample. [27] MRI is an emission tomography technique in the sense that the nuclear spins (dipole moments) in a sample are excited by Radio Frequency pulse (RF pulse) and they dissipate this energy to the surroundings as they relax back to their ground state. It is this energy that constitutes the NMR signal and is recorded over time. A notable feature is that the excitation is by RF pulses, which is low energy non ionizing radiation. [28] Spatial information is lent to this NMR signal by the application of known spatially varying gradients of magnetic field, to produce the MR image using the NMR signals. Like other imaging techniques, MRI also generates a multi dimensional array representing some physical quantity. A very specific attribute is that, it can produce 2-dimensional and 3-dimensional images without any sectioning or slicing or making any adjustments to the hardware and machinery. [27]

1.3.1 Hardware components

The MRI scanner consists of the following components, which are illustrated in Figure 2.

1. A large main magnet that produces a static magnetic field $B_0$, in which the sample (patient) to be imaged is placed. The nuclear spins (dipole moments) in the sample align with respect to this external magnetic field and generate a net magnetization $M$ along the $Z$ axis. The direction of this main magnetic field is along the $Z$ axis by convention. This main magnetic field has 2 main attributes: strength and uniformity. These 2 factors
play an important role in the quality of the signals generated. The main magnetic field $B_0$ is usually produced by a superconducting magnet, immersed in liquid nitrogen and surrounded by a Helium jacket to maintain the temperature. It follows that the maintenance of a low temperature is crucial.

![Figure 2: MRI system and components [29]](image)

2. The RF system consists of a transmitter coil that can transmit RF energy to the aligned nuclear spins in the sample to excite them to a higher energy level. This is done in the
form of an oscillating $B_i$ field. The RF coil also contains a receiver coil that converts NMR signals obtained from precessing spins to electrical signal.

3. The gradient system consists of 3 orthogonal gradient coils that produce known spatially varying gradients in the main magnetic field. These gradients play an important role in the spatial encoding and localization of the received NMR signals.

### 1.3.2 Principles of MRI

The process of acquiring an image using MRI can be succinctly described by the following flow diagram. Each step in the flow diagram below is explained briefly in the following sections to understand the imaging process.

$$
\vec{\mu} \rightarrow \vec{M} \rightarrow \vec{M}_{xy} \rightarrow S(t) \rightarrow S(\vec{k}) \rightarrow I(\vec{x})
$$

(1.1)

All atoms have a specific atomic and mass number. The atoms that contain an odd number of protons, neutrons or both possess an intrinsic angular momentum $J$, also called spin. An ensemble of nuclei possessing the same type of atoms is referred to as a spin system. When such a sample is placed in an external magnetic field, the isotopes possessing nuclear spins realign to give rise to nuclear magnetization. The origin of this magnetization is often described by both classical and quantum mechanical models.
**Magnetic moment $\mu$**

A nucleus possessing an odd mass number carries a charge. Since it has an odd number of protons and/or neutrons, it also carries a spin. This system is nothing but a charged particle spinning about its axis, creating a magnetic field around it. This magnetic field is described by the quantity $\mu$, magnetic moment which is related to the angular momentum by the following relationship

$$\vec{\mu} = \gamma \vec{J} \quad (1.2)$$

where $\gamma$ is the gyromagnetic ratio of the specific isotope.

The magnitude of $\mu$ is given by

$$\mu = \gamma h \sqrt{I(I+1)} \quad (1.3)$$

where $h$ is Plank’s constant, $I$ is the spin quantum number, which takes half integer values for nuclei with odd mass number, integral values for nuclei with odd atomic number and zero for nuclei with even mass and atomic number. $^1\text{H}$, $^{13}\text{C}$ are spin-half systems. Isotopes that have zero spins are not NMR-visible.

The direction of $\mu$ is completely random under thermal equilibrium. When the sample is placed in the presence of a main external magnetic field $B_0$, the spins realign according to a few of its intrinsic properties. (Figure 3)

According to the quantum mechanical model, a magnetic moment can align itself in a discrete set of directions. The $z$-component of the magnetic moment vector is given by

$$\mu_z = \gamma m_1 h \quad (1.4)$$
where \( mI \) is the magnetic quantum number of the spin system, which can take the values \(-mI\) to \(+mI\). Hence, a spin system with magnetic quantum number \( mI \), can take \( 2mI + 1 \) discrete directions when placed in an external magnetic field.

For \(^1\text{H} \) which is a spin-half system, there exist a total of 2 directions. The angle \( \theta \) made by the magnetic moment vector with respect to the main magnetic field \( B_0 \) is given by

\[
\cos \theta = \frac{\mu_z}{\mu} = \frac{mI}{\sqrt{1(I + 1)}}
\]  
(1.5)

For proton, \( \theta \) is 54.4°. ie the proton spins align either parallel or anti-parallel to the main magnetic field \( B_0 \), making an angle of 54.4° with the main magnetic field \( B_0 \).

When a spin system is placed in an external magnetic field, the magnetic moments experience a torque, which is the rate of change of angular momentum, given by equation 1.6.

\[
\frac{d\vec{J}}{dt} = \vec{\mu} \times B_0 \vec{k}
\]  
(1.6)

Hence, (1.7) represents the equation of motion of isolated spins.

\[
\frac{d\vec{\mu}}{dt} = \gamma \vec{\mu} \times B_0 \vec{k}
\]  
(1.7)
Figure 3: Magnetic moment vectors (µ) a) pointing in random directions under thermal equilibrium b) aligned in the presence of external magnetic field [27]

The solution to the above equation is

\[
\begin{align*}
\mu_{x}(t) &= \mu_{x}(0) e^{-i \gamma B_{0} z} \\
\mu_{z}(t) &= \mu_{z}(0)
\end{align*}
\]

which describes the free nuclear precession of the magnetic moment vectors. The angular frequency at which the precession occurs is known as the Larmor frequency, given by

\[
\omega_{0} = \gamma B_{0}
\]  

(1.9)

The direction of precession is clockwise when observed against the direction of the main magnetic field $B_0$.

\[ \text{Macroscopic Magnetization } M \]
M is the macroscopic magnetization which is the vector sum of the magnetic moments of all the microscopic spins within the sample.

\[ \vec{M} = \sum_{n=1}^{N_s} \vec{\mu}_n \]  \hspace{1cm} (1.10)

In a spin-half system, the spins align themselves in 2 orientations, as explained earlier, making an angle of ±54.4°. The energy associated with each orientation is different and is given by

\[ E = -\vec{\mu} \cdot \vec{B}_0 = -\mu_z B_0 = -\gamma h m_1 B_0 \]  \hspace{1cm} (1.11)

The energy is lower for spins pointing upwards (\( m_I = 1/2 \)) than for the spins pointing downwards, giving rise to an energy difference between the two spin states.

\[ \Delta E = E_\downarrow - E_\uparrow = \gamma h B_0 \]  \hspace{1cm} (1.12)

Due to the existence of a non-zero energy difference between the two spin states, more spins exist in the lower energy state than in the higher energy state (against the main magnetic field \( B_0 \)), giving rise to an observable macroscopic magnetization vector \( M \).

\[ M_s^0 = |\vec{M}| = \frac{\gamma^2 h^2 B_0 N_s}{4 k T_s} \]  \hspace{1cm} (1.13)

where \( g \) is the gyromagnetic ratio, \( h \) is the planck’s constant/2\( \pi \), \( B_0 \) is the main magnetic field, \( N_s \) is the total number of spins in the object, \( T_s \) is the temperature at which the sample is kept, \( K \) is the Boltzmann constant. It can be seen from this relation that the magnetization is proportional to the main magnetic field, from which stems the practice of imaging at high magnetic fields.
As described earlier, the magnetic spins in an object precess freely with an angular frequency given by the Larmor relationship. [27] This is probably the most important piece of information as far as understanding MRI is concerned.

\[
\omega_0 = \gamma B_0 \tag{1.14}
\]

As evident, the frequency with which the spins precess is proportional to the gyromagnetic ratio, which enables us to selectively disturb/excite the nuclei of interest. For example, 1H spins precess at 42.58 MHz while \(^{31}\)P spins precess at 11.26 MHz at 1T magnetic field. A group of spins with the same Larmor frequency is known as an isochromat. In reality, a sample may contain a range of Larmor frequencies, giving rise to the existence of more than 1 isochromat, due to 2 reasons a) inhomogeneities in the main magnetic field b) chemical shift effect. Even the best magnets in the world suffer from inhomogeneities, consequently creating a range of Larmor frequencies. The chemical shift effect is caused by the differences in the chemical structures of the compounds within the sample. For instance, a water molecule contains 2 protons, which are present in a much different environment than the protons in a fat molecule. Different molecules contain different number of orbiting electrons, which produce their own magnetic field that “shield” the protons/nuclei of interest from the main magnetic field \(B_0\). This structural difference among compounds is quantified by shielding constant \(\delta\). This property is exploited in NMR spectroscopy in elucidating the structure of molecules, based on the difference in the resonance frequencies.
Transverse magnetization $M_{xy}$

Under static conditions, we have randomly precessing nuclear spins, which get aligned in discrete orientations when placed in an external magnetic field, with an observable bulk magnetization $M$ pointing in the Z direction and a net zero magnetization in the transverse (XY) plane. Hence it is necessary to achieve some kind of phase coherence among these spins. In order to do that, external energy in form of a Radio Frequency pulse (RF pulse) is given to the system to excite the spins from lower energy state to a higher energy state. The energy of the RF pulse has to be equal to the energy difference between the lower and the higher energy states of the spins. It follows that

$$\hbar \omega_{rf} = \Delta E = \gamma \hbar B_0 \quad (1.15)$$

The RF pulse is applied to the sample in the form of an oscillating magnetic field $B_1$, which is usually of much smaller magnitude than the main magnetic field $B_0$. The result of applying an oscillating RF pulse of magnitude $B_1$ for time $t$ is that the nuclear spins are excited. The RF pulses are applied in the form of circularly polarized pulses through the RF transmitter coil.

The effect of applying the RF excitation using $B_1$ field is the deflection and precession of the main magnetization initially in the Z direction to the transverse plane, creating a measurable transverse magnetization. The smaller angle between $M$ and the Z axis is referred to as the flip angle ($\alpha$). The flip angle can be controlled by the used by adjusting the $B1$ strength and the duration of the pulse. The flip angle for a rectangular pulse is given by
\[ \alpha = \omega_1 \tau_p = \gamma B_1 \tau_p \] (1.16)

---

**Relaxation**

Once the RF pulse is applied for a particular duration to cause a 90° flip, the magnetization is flipped from Z axis to the transverse plane, flipping all the available magnetization to the transverse plane, leaving none in the Z axis. Following thermodynamic laws, the spins tend to go back to their low energy state after the excitation pulse has been turned off. This process is known as *relaxation*. The relaxation comprises of two independent processes: recovery of magnetization to the longitudinal plane (Z axis) and decay of magnetization from the transverse plane (XY plane) as illustrated in Figure 4.

The recovery of magnetization to the Z axis occurs by the transfer of the energy gained from the RF pulse to the surrounding lattice of the sample; hence T1 relaxation time is also known as spin-lattice relaxation time. T1 is the time constant of the recovery curve; it takes T1 units of time for the Z magnetization to recover to 63% of the magnetization at thermal equilibrium. T2 relaxation time or the spin-spin relaxation time is the time constant of the *decay* of the magnetization from the transverse plane (XY plane) where it takes T2 units of time for transverse magnetization to be reduced to 37% of the thermal equilibrium value. The recovery of magnetization in the Z axis usually occurs slower than the decay of magnetization from the transverse plane, in biological samples. Consequently the T1 is usually higher than T2 for biological samples. [30]

\[ M_z(t) = M_0 (1 - e^{-\frac{t}{T1}}) \] (1.17)
The decay of magnetization from the transverse plane can be interpreted as the loss of signal coherence and spin dephasing in the transverse plane, which is facilitated by 2 simultaneous processes.

**a) Spin-spin interaction:** Spinning of one proton can create a magnetic field, which in turn affects the net magnetic field experienced by a neighboring spin, thereby altering the Larmor frequency at which it precesses. This leads to different spins precessing at
slightly different Larmor frequencies, causing loss of coherence and hence the decay of magnetization in the transverse plane.

b) **Magnetic field inhomogeneities:** Even the best magnets have variations in the magnetic field they produce. This means that not all spins in the sample are experiencing the same magnetic field, causing them to precess at different Larmor frequencies. This in turn leads to loss of phase coherence and decay of transverse magnetization.

➢ **Signals in MRI S(t)**

a) **Free induction Decay (FID):** FID is the basic transient signal in MRI, occurring as a result of pulse excitation of the spin system. As the name suggests, it is a decaying signal, that decays with a time constant $T_2^*$. $T_2^*$ is lower than $T_2$ and is determined by the spectral distribution of spins within the sample and the magnetic field inhomogeneity.

$$\frac{1}{T_2^*} = \frac{1}{T_2} + \gamma \Delta B_0$$

(1.18)

The above equation indicates that the FID decays faster in the presence of a highly non-homogenous field.

b) **RF echo:** An RF echo occurs when dephased spins reach new phase coherence as a result of application of refocusing RF pulse. The RF echoes are 2-sided, with the echo itself decaying with time constant $T_2^*$ while the amplitude of the spin echoes obtained after repeated pulses is $T_2$- weighted. The time after which the echo is obtained after applying
the excitation RF pulse is known as the Echo time (TE). The time period between 2 successive excitation RF pulses is known as Repetition time (TR). It is in fact the time over which the user observes the recovery of longitudinal magnetization before the next excitation pulse is applied.

c) **Gradient echo:** A gradient echo also occurs as a result of refocusing of a large number of spins and attainment of phase coherence. The difference between an RF echo and a gradient echo is that while RF echo occurs as a result of refocusing RF pulse, a gradient echo occurs as a result of a gradient of equal magnitude applied in the opposite direction for the same period of time. The amplitude of gradient echoes is T2*-weighted with repeated gradient switching.

The process of acquiring an MR image begins with choosing an appropriate pulse sequence. Selection of pulse sequence offers a method by which the user can select the Field of View (FOV), geometry of the image, pulse characteristics like the shape, duration, strength of the RF pulses and other parameters that contribute to the SNR and resolution. By varying TE and TR, one can acquire a T1, T2 or a proton density-weighted image

- **Encoding S(k)**

  Encoding comprises of 2 components, slice selection and spatial encoding within the slice. Slice selection is done by the choosing a slice-selective RF excitation pulse that excites the spins in the chosen slice. Spatial encoding is done by the application frequency and phase encoding gradients that lend spatial meaning to the received signals. By applying fixed frequency encoding gradient, the spatial information is encoded along the gradient direction.
Similarly a phase encoding gradient relates the phase angle of spins to their spatial location. Phase encoding is done on pre-frequency encoded spins. This concept is better understood using k-space perspective which establishes a relationship between the spatial frequency and the gradients applied using Fourier transform.

The ultimate goal of the imaging methods and sequences is to collect enough signals and sample the k-space effectively to reconstruct the image. The k-space is sampled in different ways by different sequences. For example, the k-space is filled sequentially and symmetrically by spin echoes. Spin echoes can be modified to perform radial or rectilinear sampling of the k-space as elucidated below. The number of points filled in the k-space is dictated by the number of phase encoding and frequency encoding steps indicated in the pulse sequence. The resolution, FOV and imaging time depend on the number of phase and frequency encoding steps in the sequence.

- **Contrast in MRI**

**T1 and T2 as contrast agents:** The relaxation characteristics of a sample often provide us with information about the distribution of spins. In the case of {1}H MRI, information about water distribution is obtained. By setting the echo time (TE) and repetition time (TR) appropriately, it is possible to obtain images that are T1 or T2 weighted. Contrast can be obtained from other parameters like T1rho, magnetization transfer, apparent diffusion coefficient etc. Here, we focus on just T1 and T2 as contrast agents.
a) **T1**: T1 relaxation occurs by the transfer of energy gained from the RF pulses to the surrounding lattice. The T1 relaxation is fast if the energy transfer from spins to lattice is efficient. Efficient energy transfer occurs when the natural resonant frequency of the proton sample is more or less equal to the Larmor frequency (frequency of the RF pulse to initiate the excitation). For water, the natural frequency of protons is higher than the Larmor frequency of protons. Similarly, in solids, the natural frequency of protons in the compact structure is lower than the Larmor frequency of protons. In the above 2 cases, the energy transfer from the spinning protons to lattice is not efficient, hence the T1 is long. On the contrary, the natural frequency of protons in fat usually is similar to the Larmor frequency of protons, increasing the efficiency of energy transfer and lowering T1.

T1-weighted images can be obtained from saturation recovery or inversion recovery sequences. The equation describing the saturation recovery sequence is given below:

\[
M_z^{(n)}(0_-) = M_z^0 \left( 1 - e^{-T_w/T_1} \right) + M_z^{(n-1)}(0_+) e^{-T_w/T_1} \quad n > 1
\]

Where \(M_z^{(n)}(0_-)\) is the longitudinal magnetization before the application of the nth pulse, \(M_z^{(n-1)}(0_+)\) is the longitudinal magnetization after the application of the (n-1)th pulse. This sequence is known as saturation recovery because it is assumed that the decay of transverse magnetization is complete before the next pulse is applied. This condition is known as saturation condition and is reached when the TR set by the user is
much longer than T2. The above equation reduces to a single term as the post-pulse longitudinal magnetization is zero.

\[
M_x^{(n)}(0-) = M_x^0 \left(1 - e^{-T_n/T_1}\right) \quad n \geq 2
\]  \hspace{1cm} (1.20)

It is this magnetization that contributes to the FID signal. It is evident that the FID signal bears a relation to the T1 of the sample being imaged. By setting a short T1 in the saturation recovery sequence, one can obtain images with T1-weighted contrast. In practice, saturation recovery spin echo sequence is used in order to obtain echoes and to symmetrically fill k-space.

b) **T2:** Faster the dephasing of spins, faster is the decay of magnetization from the transverse plane and shorter is the T2. In water, the protons are situated wide apart from each other, in comparison to solids, which have a compact, tightly packed structure. Hence the interaction among the protons is minimal and the dephasing is lower in water than in solids, resulting in a longer T2 for water than solids. Proteins and fat have T2 that lies intermediate to water and solids.

In order to symmetrically fill k-space, the saturation recovery sequence described above is modified to incorporate spin echoes, where the amplitude of the spin echo signal is given by

\[
A_E = M_x^0 \left(1 - e^{-T_R/T_1}\right) e^{-T_E/T_2}
\]  \hspace{1cm} (1.21)
T1, T2 or spin density weighting can be obtained by selecting TE and TR appropriately.

Table II summarizes the TE and TR selections to obtain images with different contrast.

Figure 5 represents the pulse sequence diagram of a spin echo imaging sequence.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>$T_E$</th>
<th>$T_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_1$-weighting</td>
<td>short</td>
<td>appropriate</td>
</tr>
<tr>
<td>$T_2$-weighting</td>
<td>appropriate</td>
<td>long</td>
</tr>
<tr>
<td>$\rho$-weighting</td>
<td>short</td>
<td>long</td>
</tr>
</tbody>
</table>

Table II: CHOICE OF TE AND TR TO OBTAIN WEIGHTED IMAGES [27]

1.4 Progress of MRI in tissue engineering- Literature review

A number of studies have been done in the past that prove the ability of MRI and MRM to detect changes in tissue engineered constructs over time and correlation of the MRI
parameters with composition of extra cellular matrix has been obtained which has been reviewed in [31] The rationale behind the use of MRI for monitoring tissue engineering is not intuitive. As cell-biomaterial interaction progresses within the tissue engineered constructs, changes in the extra cellular matrix composition, tissue hydration or water/lipid ratios occur, which in turn are reflected in MRI parameters or as contrast in weighted images. [31]

1.4.1 Cell tracking in tissue engineered constructs using contrast enhanced MRI

Super Paramagnetic Iron Oxide (SPIO) particles have been used to label cells, enabling cell tracking using MRI. SPIO-aided MRI tracking has proven to be useful in studying the remodeling of the implants in vivo, non-invasively. It also paves the way to address questions regarding the migration of native cells in the body into the tissue engineered construct post implantation, migration of immune cells or migration of seeded cells. [32] Several groups have tested the efficiency of SPIO-contrast enhanced cell and molecular tracking using MRI. Ko et al. used pre-SPIO (feridex)-labeled HMSCs to seed gelatin based scaffolds and cultured the constructs. The constructs were later implanted subcutaneously in nude mice to non-invasively monitor cellular motility around the implant using MRI and correlated the findings to histology.[33] Another study by Ramaswamy and co workers demonstrated the use of SPIO-labeling of cells (vascular smooth muscle cells and vascular endothelial cells) in tissue engineered pulmonary valves made from 4% agar gel. The authors were able to successfully visualize labeled cells as hypo intense regions in T2 weighted images. [32]
Apart from labeling cells with contrast agents, few groups have ventured other ways of imparting contrast. Shen et. al. performed a series of studies to determine if ferrofluid-enhanced MRI can be used to study microcapsules containing recombinant cells, which when introduced inside the body, play a therapeutic role by the virtue of their genetically engineered DNA. Their studies have proven that the ferrofluid-incorporated capsules can be used both in vitro and in vivo to track the progress of gene therapy and that the ferrofluid capsules were well tolerated in the animal model. [34] Bull et al. used MRI to study the nanofiber network (scaffolds) derived from self assembly of Peptide Amphiphiles conjugated with Gd(III) contrast agents. These nanofiber networks have proven to act as a scaffold to direct differentiation of neural progenitor cells and to mimic bone ECM in vitro. Using MRI, they optimized the appropriate crosslinking structure of PA that would provide uniform signal intensity within the gel, which in turn would enable easy tracking of scaffolds in vivo. [35]

1.4.2 In vitro monitoring using MRI

a) Bone tissue engineering

Washburn et. al used MRM and X ray tomography to monitor the changes occurring on Poly (ethyl methacrylate) scaffolds seeded with chick osteoblasts. They observed a decrease and loss of MR signal over time as formation of bone occurred. Cortical bone does not appear brightly on MR images due to low proton content. But the cancellous bone produces a relatively bright signal due to the intervening bone marrow, which enables visualization of the microscopic architecture [36]. A similar loss of MR signal was reported by Peptan et. al. This
group studied osteogenic differentiation of Human Mesenchymal Stem Cells (HMSCs) in a
gelatin based scaffold. They observed a decrease in the relaxation times over 4 weeks, due to
mineralization, which induces magnetic susceptibility changes within the scaffold. They
established a correlation curve between the trend in T2 and biomarkers for osteogenic
differentiation (ALP and osteocalcin activity), indicating that osteogenesis imparts
microarchitectural details that lower the relaxation times. [37]

b) Cartilage tissue engineering

Miyata and co workers correlated MR parameters, biophysical and biochemical
properties of tissue engineered cartilage made from chondrocyte-seeded agarose discs. They
observed a decreasing trend in T1 and D and a slight increase in T2. They found good
correlation of T1 and D, but not T2 to glycosaminoglycans and biophysical properties. They
speculate that the increase in T2 may be due to the insufficient reorganization of collagen in
agarose model. [38]

Li and co workers correlated Magnetization transfer properties to histochemical and
biochemical markers in tissue engineered cartilage. Magnetization transfer probes the
subpopulation of water protons in the tissue that are bound to macromolecules. They
stimulated differentiation of stem cells into chondrocytes in a gelatin-based scaffold.
Quantitative Magnetization transfer parameters (rate of magnetization exchange and ratio of
bound water proton to free proton) were found to correlate well with the GAG and collagen
content in the scaffolds. This proved the differentiation of stem cells to chondrocytes to
synthesize elevated amounts of such molecules. [39] Neeves et al carried out a study to optimize bioreactor conditions to culture bio artificial meniscal cartilage with properties that resemble most to the native cartilage. [40]

In tissue engineering of cartilage, parameters like flow rate, perfusion, mass transfer, geometry of bioreactor play an important role in the quality of the construct. Hence it is crucial to optimize such parameters. Neeves et al have demonstrated the use of MR imaging and $^{31}$P spectroscopy to study and compare tissue engineered constructs grown in 2 different flow rates within their perfusion bioreactor and correlated their results to histological analysis. Diffusion and $^{31}$P measurements gave an estimate of the water content and Nucleotide Tri Phosphate (NTP) content which is a measure of cell content. [40] In another study, Neeves and co workers have used the same perfusion bioreactor system to cultivate meniscal cartilage constructs of different geometries and compared those using MRI and $^{31}$P MRS analyses. [41]

Reiter et al moved a step forward in the application of MRI for cartilage tissue engineering by proposing to use multi parametric data analysis and the use of multiexponential T2 to increase sensitivity. Their goal was to discriminate between different groups of cartilage in pathological conditions. [42]

Cheng et al published a study in 2010 that explored the use of quantitative MRI to understand the progress of seeding smooth muscle cells on urinary bladder-derived-acellular matrix. They observed an interesting T1 and T2 behavior of the seeded matrices in comparison with the control matrices. As opposed to most studies that have reported a shortening of T1 and T2 in cell seeded matrices in comparison to unseeded controls, this study reported T1 and T2 of
seeded matrix to be higher than that of the control. They attributed this behavior to the
degradation of collagen due to the secretion of collagenase by the smooth muscle cells in the
matrix, which is validated by histological analyses. [43]

c) Need for MRI-compatible culture system

MRI, being a non invasive technique, it is possible to make serial measurements
repeatedly on the same sample. The above discussed studies do not take advantage of this
possibility as they have no means of maintaining the tissue engineered constructs viable while
performing MRI analyses which could take several hours. The long imaging period involved in
analyzing the tissue engineered constructs is detrimental to the cells within them as they are
removed from the optimum conditions maintained in the incubator and placed within the bore
of a magnet. This largely affects the viability of the cells within the constructs. Hence the
prospect of using the MRI-analyzed constructs as an implant within an animal or the possibility
of further characterization is lost.

To circumvent this limitation, few groups have devised MRI-compatible culture systems
and bioreactors to culture as well as monitor tissue engineered constructs. Peterson et. al.
constructed an MRI compatible Hollow Fiber Bioreactor (HFBR) to facilitate serial monitoring of
the process of native neocartilage formation. Figure 6 illustrates the schematic of the HFBR.
They performed $^{31}$P NMR spectroscopy, MRI, histology as well as biochemical assays to find
glucose utilization and lactose production in the bioreactor to analyze and compare the
characteristics of neocartilage formed to typical cartilage. [44]
The next year, Potter et. al. used the same system to culture neocartilage and used MRI, biochemical and histological analyses to monitor neocartilage formation over a 4 week period. They observed a correlation of magnetization transfer and collagen content, along with correlation between collagen and T1. They concluded that the stages of neocartilage formation in the HFBR closely resembled the stages in the in vivo cartilage formation. [45] Another study using the same in vitro HFBR model was performed by Chesnick et al with primary osteoblasts adhering to the fibers within the bioreactor. The T2 relaxation times increased over week 4 and week 6 due to loss of proteoglycan as a result of mineral deposition, which was supported by the decrease in T2 of the surrounding media. [46]

Planchamp and group developed a HFBR to monitor the kinetics of entry of hepatobilary contrast agents (Gd-BOPTA and Gd-DTPA) into hepatocytes. Their system consisted of oxygen regulation unit as well as a pump to alter the perfusion rates of the contrast agents into the hepatocytes that were cultured in the extracapillary space of the HFBR. The authors were able to observe the contrast agents perfusion within the system and have obtained results similar to the results obtained from clinical studies. [47]

**d) A simpler solution**

The above studies have contributed to real time imaging of live cell cultures and maintaining them in highly regulated conditions for extended period of time successfully. But for various reasons, none of the above mentioned systems can be a universal solution to real time live imaging using MRI. A few reasons are: a) they all involve making serious large scale
alterations to the existing scanner; b) the MRI scanner has to be dedicated to just one study, preventing other users/groups to perform normal imaging.

Figure 6: Schematic of MRI-compatible Hollow Fiber Bioreactor (HFBR). The system is maintained at 37 C and is perfused continuously while within the magnet [44]

Hence we have developed an MRI compatible culture system to support culture of tissue engineered constructs as well as to enable live real time imaging of the constructs using
MRI, without making large scale alterations to the MRI scanner or the culture system. We hypothesize that this culture system would permit cell viability when the constructs are being analyzed within the magnet without compromising on the behavior of the cells or the integrity of the MR imager. This altered system would enable the MRI-analyzed scaffolds to be characterized further in *in vivo* experiments or other live cell-based analyses.

The MRI-compatible culture system is purported to maintain temperature, pH and supply nutrients to the cells within the tissue engineered constructs. These 3 basic requirements were met by incorporating a Variable Temperature control unit, a CO₂-independent culture media and supplying enough nutrition media to the constructs respectively. Experiments were performed with the aim of drawing a comparison between the parameters measured in the constructs that were grown in the conventional culture system to the constructs grown in the MRI-compatible housing to evaluate the efficiency and suitability of the MRI-compatible culture system.
2. MATERIALS AND METHODS

2.1. Cell culture

HMSCs were used throughout this study. HMSCs isolated from the iliac crest of normal adult donors were obtained from NIH-funded center for research resources, Tulane Center for preparation and distribution of adult stem cells. [48] Cells from passage 4 were used for the experiments. Cells were cultured in minimum essential medium–alpha (Gibco) containing 20% fetal bovine serum (Gibco), 1% L-glutamine (Gibco), and 1% antibiotic–antimycotic cocktail (Gibco).

Once the cells reached confluence, they were trypsinised to form a cell suspension. HMSCs at the concentration of 2×10⁶ cells/mL were embedded in a 1:1 copolymer matrix consisting of 1 mg/mL type I collagen (BD Biosciences, San Jose, CA) and 1 mg/mL chitosan (Sigma, St. Louis, MD). This blend of monomers was chosen based on an earlier study that indicated that this ratio was ideal for mesenchymal cell culture. This ratio provided the perfect mix of hydrogel stability combined with proliferation rate similar to that of type I collagen hydrogel. [3] The collagen and chitosan were both monomers in 0.1 M acetic acid.

Along with the monomers, 1× Hank's balanced salt solution and 5% (w/v) NaHCO₃ were added in order to maintain the osmotic pressure of cells within the hydrogel. The polymerization reaction mixture was kept in ice throughout the process to prevent localized polymerization reactions. The cell suspension was added to the reaction mixture, followed by 1
M NaOH solution. NaOH was added to initiate polymerization by raising the pH of the reaction mixture. The volume of NaOH to be added was estimated by titration for this particular blend of collagen and chitosan. 300 µL of the reaction cocktail was added to each well of a chambered cover glass plate (Lab tek II, Rochester, NY) and was placed in an incubator at 37 °C and 5% CO₂ atmosphere for 45 minutes. Once the hydrogel had solidified, 500 µL of growth media was added to each well. [49]

The constructs were cultured in growth media for 24 hours to enable proper cell attachment. The constructs were then split into 4 groups, to be cultured in 4 different culture conditions:

a) 3 constructs were transferred to an MRI-compatible housing and were fed with growth media
b) 3 constructs were transferred to another MRI-compatible housing and were fed with osteogenic media
c) 12 constructs were cultured in conventional tissue culture dish (chambered) and were fed with growth media
d) 12 constructs were cultured in conventional tissue culture dish (chambered) and were fed with osteogenic media.

The osteogenic media consisted of ascorbic acid at the concentration of 100 µg/mL, β-glycerophosphate at the concentration of 10 mM, and dexamethasone at 10 mM in the normal growth media. All the constructs were cultured for 28 days. The constructs maintained in the MRI-compatible housing (conditions a) and b)) were monitored bi-weekly using MRI for 4
weeks. 3 constructs cultured in conventional tissue culture system (conditions c) and d)) were removed from culture and fixed in Neutral Buffered Formalin after every week and were later imaged and analyzed using MRI. Figure 7 represents the different experimental groups in consideration.

Figure 7: Representation of the different experimental conditions in which the tissue engineered constructs were cultured

2.2. MRI compatible tissue culture system

The new MRI-compatible culture system (Figure 8) consisted of a susceptibility-matched Ultem plug (Doty Scientific, SC, USA) placed within a 10 mm MRI tube on which three constructs were placed. The MRI tube also housed a capillary containing 10 mM Copper Sulphate to act as a standard for MRI measurements. The capillary was filled with Copper Sulphate while making sure that there were no air bubbles that could cause susceptibility
artifacts in the MR images. Both ends of the capillary were then sealed with flame to ensure that there are no leak holes. Care was taken to maintain all the components of this system sterile and contamination-free. Gas exchange to the scaffolds within the tube was made possible by covering the tube with sterile cotton plugs. This setup was maintained at 37°C and 5% CO₂ by placing in an incubator.

![Figure 8: MRI-compatible culture system with an inset representative MSME image of the tissue engineered constructs](image)

### 2.3 Pre-MRI

Prior to performing MRI on the constructs cultured in the MRI-compatible housing (conditions a and b), the media was removed and was substituted by a CO₂-independent media prepared by adding HEPES buffer at a concentration of 2.4 g/L to the normal HMSC growth media. This is to maintain the pH of the media independent of CO₂. Usually, commercial media contains a particular concentration of bicarbonate ions that equilibrate with the 5% CO₂ inside
the incubator to maintain the pH of the culture at 7.2. [50] When the constructs are placed within the MRI scanner, the CO\textsubscript{2} level is not at 5%. Hence drastic changes in pH occur in the absence of a controlled CO\textsubscript{2} environment. Addition of HEPES ensures maintenance of pH independent of the CO\textsubscript{2} atmosphere. [51] The cotton plugs are replaced by sterile MRI tube-caps to prevent air-borne contamination during the MR analysis.

2.4 MRI Analyses

The 11.7 T Bruker microimager uses a 56 mm vertical bore magnet (Oxford instruments, UK), a Bruker DRX-500 MHz Avance Spectrometer (Bruker Instruments) controlled by a Silicon Graphics SG12 and a Bruker imaging software Paravision 4.0. The system consists of a Bruker triple axis gradient system with a maximum magnetic field gradient of 200 G/cm and micro 5 imaging probes. All experiments were performed using commercial Bruker 10 mm RF saddle coils in an imaging probe equipped with a variable temperature control unit in which the MRI-compatible culture system was maintained at a constant 37\textdegree C throughout all the imaging experiments. Experiments were performed in the presence of standard 10 mM Copper sulphate solution, following tuning and matching.

Standard MSME pulse sequence was used to measure T2 relaxation time of the tissue engineered constructs, with 32 linearly spaced echoes, TE 6 ms, TR 5000 ms, measured on axial slices of thickness 1 mm on an FOV 1 cm x1 cm, with frequency and phase encoding matrix size of 128x128, amounting to a spatial resolution of 78 \( \mu \text{m} \times 78 \mu \text{m} \). [52] In MSME, multiple
echoes are generated by repeated application of combination of slice-selective RF pulses. Standard RAREVTR pulse sequence was used to measure T1 relaxation time of the tissue engineered constructs, with 8 variable repetition times ranging from 104.6 ms to 15000 ms and with TE 11.45 ms, measured on axial slices of thickness 1 mm on a FOV 1 cm x1 cm, with frequency and phase encoding matrix size of 128x128, amounting to a spatial resolution of 78 µm x 78 µm. [53] The raw MRI data were processed using custom-written Matlab code, which uses non linear least squares method and the fitting function \[ S = S_0 \times \exp(-\text{TE}/T2) + A_0 \] to calculate T2 and \[ S = S_0 \times (1 - \exp(\text{IR}/(-T1))) \] to calculate T1.

The MRI analysis was performed with media containing HEPES as the surrounding solution in the case of the live constructs being cultured in the MRI-compatible housing. The MRI analysis was performed with formalin as the surrounding solution in the case of constructs cultured in the conventional tissue culture system that were already fixed with neutral buffered formalin.

The calculated T2 values of the tissue engineered constructs for each time point were normalized with respect to a mean T2 value for CuSO\(_4\) obtained from the distribution of CuSO\(_4\) values. The normalization was done after checking for normality of the distribution of the T2 values of CuSO\(_4\). The normalization was performed by assigning Z-scores to every set of measurements. [54]

The Z score for a particular CuSO\(_4\) measurement was calculated by using the following expression
\[ Z = \frac{(Y - M)}{S} \] (2.1)

Where \( Y \) is the measured T2 of CuSO4 to be normalized, \( M \) is the mean of the T2 distribution of CuSO4 and \( S \) is the standard deviation of the T2 distribution of CuSO4. The calculated Z score indicates the deviation of the particular T2 measurement of CuSO4 from the mean T2 of CuSO4. A Z score of -1 indicates that the measurement has dropped from the mean by a standard deviation of 1.

The T2 values of the constructs were corrected using the expression

\[ T2^c = T2 - (Z \times S) \] (2.2)

Where \( T2^c \) is the corrected (normalized T2) of the tissue engineered constructs, \( Z \) is the calculated Z score for the particular measurement and \( S \) is the standard deviation of the distribution of T2 of CuSO4.[55]

This was done to eliminate variations caused by difference in signal intensity or artifacts, if any. The same normalization and correction was done for T1 values also.

### 2.5 Live-dead assay

Fluorescence based live-dead assay (Molecular Probes, Life technologies, NY, USA) was performed on live constructs from all 4 groups that were cultured for 28 days. The live-dead assay involves addition of 2 reagents: Calcein AM and Ethidium homodimer. Conversion of cell-permeable non fluorescent calcein to fluorescent calcein indicates esterase activity in live cells, making the live cells fluoresce in green. Ethidium homodimer (EthD III) enters cells with
damaged cell membranes as present in dead cells, imparting red fluorescence to the dead cells. This was done to compare the number of live and dead cells in the constructs grown in the conventional culture to the constructs grown in the housing. [56]

Multiple images were obtained from different regions of each construct. The number of dead cells was counted by using Cell Counter Macro available in ImageJ from all images. [57] The images were all obtained using the same magnification, making the field of view of the images constant. The dead cells within this constant field of view (320 x 430 µm) were counted in the stained constructs from each group.

2.6 Confocal microscopy

The live dead assay only indicates the number of live and dead cells, without providing any information about the morphology and orientation of the cells within the 3-dimensional environment. Actin staining of the formalin-fixed constructs using fluorescent phalloidin conjugated with Oregon Green (for green fluorescence) at the end of 4 weeks was done to study and compare the morphology of the cells within the constructs cultured in the conventional tissue culture dish and the housing for 4 weeks. Phalloidin specifically binds to polymerized actin filaments within the cell. The constructs were imaged using Zeiss LSM 510 and 710 confocal microscopes. Z stack of the constructs was performed in which a set of images of planes varying at a constant interval of 1 µm from the surface of the sample was obtained and was reconstructed using Zeiss imaging software.
2.7 Histology

The constructs that were fixed with neutral buffered formalin after 4 weeks in culture from all 4 groups were embedded in OTC compound and cryosectioned. All sections were 8 µm thick. Alizarin red and Von Kossa staining were done to visualize mineral deposits as a result of osteogenic differentiation and compare them. [58] Safranin O staining was done to visualize proteoglycan deposits as a result of osteogenic differentiation. All the staining procedures involved rigorous washing of the sections with distilled water as the first step.

The Alizarin red stain was then added to the washed sections and the slides were incubated for 30 minutes. The sections were washed with distilled water after incubation to remove excess dye. The Alizarin Red dye binds to Calcium in the sections and imparts a red color to the stained regions.

The washed sections were stained with 1% w/v solution of Silver Nitrate and were incubated in the dark for 30 minutes. Counter stain was added and the slides were washed with tap water right after black coloring develops in the sections. Silver Nitrate reacts with the phosphates in the sections (mostly Calcium Phosphate) to precipitate Silver Phosphate which is reduced to metallic Silver by the counter stain.

Safranin O staining was carried out by immersing rehydrated sections in 0.5% solution of Safranin O in 0.1 M Sodium Acetate buffer at pH 4.6.
The stained sections were then dehydrated by immersing them in an increasing gradient of alcohol solutions and xylene and were mounted using a coverslip. The mounted sections were imaged using a Carl Zeiss light microscope and associated Axio Vision software.
3. RESULTS

The main objective of the MRI-compatible tissue culture system is to maintain the tissue-engineered constructs in incubator-like conditions within the MRI scanner when the analysis is being performed. We have carried out experiments to compare the conventional tissue culture system to the MRI-compatible housing to assess the suitability of the developed MRI-compatible housing. The following section describes the results obtained from conventional tissue culture system, from the MRI-compatible housing and a comparative analysis of the results.

3.1 Assessment of cell viability and cell organization within constructs

After 4 weeks of maintaining collagen-chitosan constructs in the conventional culture and the MRI-compatible housing, fluorescence-based live-dead assay was performed on live constructs. Figure 9 shows representative images of green live cells and red dead cells within the constructs. The fraction of dead cells within the constructs grown in conventional culture and MRI-compatible housing are similar. The top 2 panels display live-dead stained constructs cultured in the conventional tissue culture system for 4 weeks and fed with normal (left) and osteogenic (right) media.
Figure 9: Representative images of live-dead assay showing green live cells and red dead cells - Top: Constructs cultured in normal media (left) and osteogenic media (right) in conventional culture system, Bottom: Constructs cultured in normal media (left) and osteogenic media (right) in the MRI-compatible culture system. Note the difference in the lengths of the live cells between the top and the bottom rows.

The bottom 2 panels display stained constructs that were cultured in MRI-compatible housing for 4 weeks, fed with normal (left) and osteogenic (right) media. The green-live cells in the conventional tissue culture system (top panels) appear more branched and longer than the green live cells in the MRI-compatible housing (bottom panels). The red dead cells were quantified for each image using the cell counter macro in ImageJ software [57]. Since the images were all acquired with the same magnification, the field of view is constant for all.
images. The area of the region in which the dead cells were counted was 320x430 µm². Multiple images from different regions of the same construct were obtained and the red dead cells were counted from each image. This was repeated for constructs grown in all the 4 conditions. A comparison chart of the red dead cells in the conventional tissue culture system and the MRI-compatible housing is shown in Figure 10. The Y axis represents the number of dead cells per 320x430 µm².

![Figure 10: Comparison of number of dead cells, as counted from live-dead assay images using ImageJ.](image)

The number of dead cells in the constructs cultured in the conventional tissue culture system and the housing were not significantly different. The number of dead cells in constructs fed with osteogenic media is higher than the number of dead cells in constructs fed with normal growth media. This is expected as the constructs grown in osteogenic media are subjected to a
greater stress as a result of the chemical cues added to the media. [59] It is evident that the number of dead cells in the constructs cultured in conventional tissue culture system and in the housing is similar in both normal as well as osteogenic media.

To get a better insight into the morphology of cells grown in the conventional and MRI-compatible housing, confocal microscopy images were obtained after staining the constructs that were cultured for 4 weeks with fluorescent analog of Phalloidin. Phalloidin binds and localizes actin filaments within the cells making visualization of cell structure and morphology possible using confocal microscope.

The top panels of Figure 11 represent the images of the constructs that were cultured for 4 weeks in conventional tissue culture system and fed with normal (left) and osteogenic (right) media. The bottom panels of Figure 11 represent the images of the constructs that were cultured for 4 weeks in the MRI-compatible culture system and fed with normal (left) and osteogenic (right) media. The representative images have all been depth coded using Zeiss software. The confocal microscopy images reveal that the branched and extended nature of the cells [60] is consistent in all 4 groups.
Figure 11: Representative confocal microscopy images that have been depth coded. Top: Constructs cultured in normal media (left, depth coded from 0 to 70 µm) and osteogenic media (right, depth coded from 0 to 45 µm) in conventional culture system. Bottom: Constructs cultured in normal media (left, depth coded from 0 to 45 µm) and osteogenic media (right, depth coded from 0 to 50 µm) in the MRI-compatible culture system. Note that the cells are morphologically similar.

3.2 Assessment of cell behavior by studying mineralization in constructs

The groups of constructs that were fed with osteogenic media and maintained in the conventional and MRI-compatible housing for 4 weeks were subjected to histological analysis to
test for mineral deposits as a result of osteogenic differentiation after fixing in Neutral Buffered Formalin. Figure 12 shows representative images of Alizarin red stained cryosections from constructs fed with osteogenic media cultured in the conventional tissue culture system (left) and the MRI-compatible system (right).

Figure 12: Alizarin red staining on cryosections obtained from constructs fed with osteogenic media and cultured in conventional tissue culture system (left) and MRI-compatible housing (right)

Alizarin red staining is a standard method to evaluate mineralization contributed by calcium deposition. The constructs fed with osteogenic media grown in the conventional and MRI-compatible housing both revealed many clusters of Calcium deposits. The Calcium clusters appeared more concentrated and dense in the sections from constructs cultured in the conventional system (Figure 12, left) than the sections from constructs cultured in the MRI-compatible housing. The sections of constructs grown in the MRI-compatible housing looked more diffuse and well spread out. (Figure 12, right)
Figure 13 displays representative images of Von Kossa stained cryosections made from constructs fed with osteogenic media grown in the conventional system (leftmost) and in the MRI-compatible housing (centre). The rightmost panel of Figure 13 displays a Von Kossa stained section from a construct fed with normal growth media and cultured in conventional tissue culture system, which served as the negative control.

The black silver deposits seen in Figure 13 indicate aggregation of phosphate. The phosphate deposits (mostly in the form of Calcium Phosphate) get precipitated as Silver Phosphate after addition of Silver Nitrate. Addition of a counter stain reduces the Silver Phosphate deposits to metallic silver deposits which appear black. Such metallic silver deposits were absent in the cryosections from constructs fed with normal media in conventional culture system. (Figure 13, rightmost) The phosphates were seen as black dense concentrated clusters in the cryosections of constructs fed with osteogenic media and cultured in the conventional
system. (Figure 13, leftmost) The phosphate deposits appeared less intense, less concentrated and more diffuse in the sections of constructs fed with osteogenic media cultured in the MRI-compatible housing. (Figure 13, centre)

Safranin O staining was performed to reveal proteoglycan deposition as a result of osteogenesis in constructs cultured in conventional tissue culture system and MRI compatible housing for 4 weeks. The representative images are shown in Figure 14.

Figure 14: Safranin O staining on cryosections obtained from constructs fed with osteogenic media and cultured in conventional tissue culture system (left) and in MRI-compatible housing (right)

The proteoglycan deposits are much higher in constructs cultured in conventional tissue culture system than the constructs cultured in the MRI-compatible housing. This is evident from the vast difference in the stain intensity imparted to the sections by Safranin O dye.
3.3 Monitoring osteogenic differentiation using MRI-compatible housing and comparison of the behavior with conventional tissue culture system

The T2 trends of the tissue engineered constructs fed with normal and osteogenic media cultured in conventional tissue culture system are shown in Figure 15. 3 constructs were removed from the culture each week and fixed in Neutral Buffered Formalin. MRI was performed with the constructs in formalin along with a capillary filled with 10 mM CuSO$_4$ acting as a standard. As evident, the T2 of the constructs grown in both normal and osteogenic media exhibit a decreasing trend over a period of 4 weeks.

![Figure 15: T2 trend of constructs fed with normal and osteogenic media cultured in conventional tissue culture system](image)

The T2 of constructs grown in normal media dropped from 31 ms on Day 01 to 12 ms on Day 28, while the T2 of the constructs grown in osteogenic media dropped from 31 ms on Day
01 to 12 ms on Day 21 and raised again to 19 ms on Day 28. The T2 of the constructs fed with osteogenic media at each point was significantly lower than the corresponding T2 of constructs fed with normal media until Day 21.

The T1 trends of tissue engineered constructs fed with normal and osteogenic media cultured in conventional tissue culture system are shown in Figure 16. There is absence of any specific trend in T1. The T1 values range from 1.7 s to 2.7 s over the period of 4 weeks.

Figure 16: T1 trend of constructs fed with normal and osteogenic media cultured in conventional tissue culture system

The MRI monitoring of tissue engineered constructs grown in the MRI-compatible housing was performed bi-weekly. The T2 trends of the constructs maintained in normal and osteogenic media and cultured in the housing are shown in Figure 17. As opposed to the
decreasing T2 trend exhibited by constructs grown conventionally, the constructs grown in MRI-compatible housing do not exhibit any specific trend. It is notable that the T2 of constructs maintained in osteogenic media is lower than the T2 of constructs maintained in normal growth media at all time points. The T2 values of the constructs range from 33 ms to 53 ms over the period of 4 weeks.

![Figure 17: T2 trend of constructs fed with normal and osteogenic media cultured in MRI-compatible housing](image)

The T1 of the constructs grown in the MRI-compatible culture system (Figure 18) also did not exhibit any specific trend. Also, it is to be noted that the T1 values of the constructs grown in the MRI-compatible housing range from 3.5 s to 4.5 s which is much higher than the range of T1 observed in constructs cultured in the conventional tissue culture system.
A difference in the range of T2 and T1 values between the conventional tissue culture system and the MRI-compatible tissue culture system exists because the MRI measurements were performed on formalin-fixed constructs in the former case and live constructs in the latter case. As expected, formalin fixation has introduced a downward shift in the ranges of both T1 and T2 measurements. Also, the MRI measurements were performed with formalin as the surrounding fluid in the former case and growth media as the surrounding fluid in the latter case. Experiments were performed to verify if the difference in the surrounding fluid introduced any variations in the T1 and T2 values. It was found that the MR values were not affected by these two choices of surrounding fluid.
4. DISCUSSION

The first step in the application of MRI to monitor the progress of tissue engineering is to conduct optimization and proof-of-principle studies to elucidate correlation between MRI parameters (T1, T2, T1rho, ADC) and tissue specific markers (gene expression, differentiation markers etc.) Several groups have examined this aspect in different tissue engineering models, both in vitro and in vivo. [26, 61, 43, 45, 66]

The next step in the application of MRI in tissue engineering has been perceived as the development of an MRI-compatible culture system that would permit real time continuous imaging of the tissue engineered constructs. Even though the technique of NMR and MRI is non-invasive, performing extended MRI analyses on tissue engineered constructs is derogatory to the cells within the constructs. Hence the non-invasive nature of MRI is lost when MRI is performed on cell-based samples. Potter [45], Peterson [44], Chesnick [46] etc have designed extensive MRI-compatible bioreactor systems to study the effect of perfusion or to study bioreactor-cultivated tissue using MRI. Such reactors demand a dedicated MRI scanner solely for the purpose of the experiment at hand. Also it would involve making large scale changes to the existing system that would potentially skew the calibrations performed by other users.

We have developed a simple but elegant way to maintain cell viability within constructs while performing MRI analyses by satisfying 3 basic requirements for cell viability: temperature regulation, pH regulation and nutrition supply. The present study was performed to assess the
suitability of the MRI-compatible housing to culture tissue engineered constructs. All experiments were performed with the aim of drawing a comparison between the results shown by constructs grown in conventional tissue culture system and the constructs grown in the MRI-compatible housing.

The results from live-dead assay and actin-stained confocal microscopy images reveal that the MRI-compatible housing has been successful in maintaining the viability and morphology of the cells within the constructs. This also means that the addition of HEPES buffer to substitute for carbonate-CO$_2$ equilibrium-based-pH maintenance did not pose any derogatory effects on the cells. This is in accordance with previous studies that have confirmed pH maintenance and cell survival in the absence of CO$_2$ regulation by addition of HEPES. [50, 51] The health of mesenchymal stem cells is evaluated by their branched morphology and their thin extended nature. [60] This feature is conserved in constructs grown in the MRI-compatible housing.

The presence of dead cells within the constructs that have been cultured for about 4 weeks is inevitable as a result of apoptosis and handling. [62] Hence the occurrence of red-fluorescent dead cells was not unexpected. The number of dead cells in the osteogenic culture is greater than the number of dead cells fed with normal growth media. This was expected too, as inducing osteogenesis by addition of chemical cues increases cell death in osteogenic culture. [59] The number of dead cells in the constructs maintained in conventional culture and the MRI-compatible housing were not significantly different, indicating that the MRI-compatible housing has been successful in maintaining the viability of cells.
Even though the cells’ health and viability were maintained satisfactorily by the MRI-compatible housing, the cell behavior of the constructs maintained in the MRI-compatible housing was not similar to that of the constructs maintained in the conventional tissue culture system. The osteogenic induction was carried out with the aim of comparing the differentiation and hence the behavior of cells within the constructs grown in conventional tissue culture and MRI-compatible tissue culture systems. The constructs fed with osteogenic media grown in the conventional culture system exhibited ECM deposition and mineralization as indicated by a decrease in T2 and histological studies respectively. On the other hand, the constructs fed with osteogenic media grown in the MRI-compatible culture system did not exhibit a decreasing trend in T2 and showed much lower extent of mineralization in the histological sections.

To explain the absence of ECM deposition and mineralization in the osteogenic constructs grown in the MRI-compatible system, a little insight into the mechanism and precursors for ECM deposition and mineralization is provided in the following paragraphs.

**Mechanism of mineralization**

It has been proven that the osteoprogenitor cells that exist in the bone (also referred to as the Marrow Stromal Cells) that have been used in our studies, have the potential to differentiate into bone and undergo mineralization.[16] The culture conditions and requirements for initiating the differentiation of HMSCs into osteogenic lineage and inducing mineralization are the following:
3-dimensional environment: The encapsulation of HMSCs in hydrogel/scaffold has been proven advantageous as it mimics the in vivo 3-dimensional environment and also prevents the dedifferentiation of differentiated cells into fibroblasts.

Chemical cues: The nutrition media to enable osteogenic differentiation usually consists of dexamethazole, ascorbic acid and β glycerophosphate which act as chemical cues to induce osteogenesis and mineralization. Dexamethazone is added to act as a promoter for certain osteogenic lineage-specific genes [16]

Apart from choosing a suitable cell type and an appropriate media with the necessary factors and chemicals to support differentiation into osteogenic lineage, the cells MUST also be synthesizing ECM, over which mineralization could occur.

The process of osteogenic differentiation can be divided into 3 phases. [63] The phases are illustrated in Figure 19.

a) **Cell proliferation:** The MSCs migrate within the substrate by undergoing several cycles of forming weak adhesions, tractions and detachments. They begin to differentiate into osteoblasts at the end of their migratory phase by forming strong focal adhesions mediated by transmembrane proteins like integrins.

b) **Maturation:** The cells secrete and accumulate ECM proteins like Collagen I post proliferation. In the initial phase of maturation, the cells express Alkaline Phosphatase.

c) **Matrix mineralization:** Deposition of ECM and expression of Alkaline Phosphatase are precursors to mineralization. Alkaline Phosphatase is one of the early indicators of osteogenic lineage, among others like Osteocalcin, Osteopontin, Collagen I etc. This
enzyme is responsible for the hydrolysis reaction that converts organic phosphate into inorganic phosphate, which is in turn deposited on the ECM. The precipitation and attachment of phosphate is crucial to initiate more complex mineralization. It has been shown that mineralization depends on the expression of specific ECM proteins like BMP-7, DMP-1 etc, which are synthesized along with ECM deposition. [64] Other glycoproteins like Osteocalcin, Osteopontin and Bone Sialo Protein are expressed during matrix mineralization, which also influence further mineralization. [63]

Figure 19: Reciprocal and functionally coupled relationship between cell growth and differentiation-related gene expression [65]

It follows that the extent of mineralization is influenced by the previous steps of ECM formation, alkaline phosphatase upregulation and Calcium Phosphate crystallization. [63]
MRI has been used as a non invasive tool to monitor progress of differentiation in tissue engineered constructs. [39, 43, 66, 67] The present study has once again proved the ability of MRI to detect osteogenic differentiation in constructs grown in conventional tissue culture system. The T2 and T1 of the constructs fed with both normal and osteogenic media exhibit a decreasing trend, exemplifying cellular proliferation in normal growth culture and accumulation of ECM components and mineralization in osteogenic culture. This is in accordance with the observations made by [37, 39, 66].

It is well accepted that increase in cellularization creates focal adhesions with the substrate that leads to contraction of the constructs and restricted water diffusion in the constructs. This is the reason behind the decrease in T2 observed in the constructs fed with normal growth media. On the other hand, in osteogenic constructs, an increase in macromolecular deposition and mineralization is the reason behind the observed decrease in T2. When there is an increased deposition of macromolecules like proteins, proteoglycans, a hydration layer is formed along the surface of the macromolecules, hence constricting the free movement of protons, causing a reduction in T2. [30, 37, 39, 66] The decrease in T2 observed in the osteogenic constructs, interpreted as deposition of ECM and mineralization has been validated by histological studies that clearly exhibit mineralized deposits and proteoglycan deposits (Figures 12, 13, 14)

Even though a decreasing T2 trend is observed in both normal and osteogenic constructs grown in the conventional culture, the T2 of the constructs grown in normal media is higher than the T2 of constructs grown in osteogenic media at every time point until Day 21. There is a slight increase in the T2 of the constructs grown in the osteogenic media on Day 28, above the
T2 of constructs grown in normal media. (Figure 15) We hypothesized that the slight increase in T2 may be due to loss of proteoglycan occurring as a result of increased mineralization. This assumption was based on the findings documented by previous groups that had observed a decreased proteoglycan content as a result of mineralization. [68] We tested this theory by performing Safranin O staining on constructs fixed at Day 21 and Day 28, to find no apparent loss of proteoglycan after Day 21. We speculate that the increase in T2 may be due to break down of certain ECM components as a part of matrix remodeling. Further studies need to be performed to explain the slight increase in T2 after Day 21.

The absence of any trend or mineralization in the constructs grown in the MRI-compatible housing fed with osteogenic media can be explained by the constant perturbations in the orientation of the constructs in the MRI-compatible housing. The influence of orientation of cells in formation of ECM has been studied by Yoshida et al. [69, 70] According to [70], the orientation of the cytoskeleton inside the cell can dictate the orientation of the ECM produced. This, in fact has been one of the motivations for micropatterning and guided cell culture strategies. [69, 71] In the conventional culture, the constructs are not disturbed by any external motion; hence the cells remain in a particular orientation, enabling them to synthesize ECM properly and promote subsequent mineralization. In the case of MRI-compatible culture system, the constructs rest on the Ultem plug placed inside the MRI tube. The constructs are constantly subjected to motion and disturbances in an attempt to remove bubbles or during transport to the MRI scanner. This affects the direction and orientation of ECM synthesis, consequently affecting the dense mineralization. This explains the diffuse appearance of calcium and
phosphate deposits in the alizarin red and Von Kossa-stained cryosections from constructs maintained in the MRI-compatible housing, while the cryosections from constructs maintained in the conventional culture system exhibited deposits of minerals in clusters.

Another contributor to the diffuse less-intense appearance of mineral deposits in the constructs cultured in the MRI-compatible housing is the temperature variations occurring in the live culture during transport of constructs from the incubator to the MR facility. Variation in temperature is an additional source of stress to the constructs being cultured in the MRI-compatible tissue culture system. This is understandable as several proteins like ATP are temperature-dependent. [72]
5. CONCLUSIONS

MRI has once again proved to be useful in monitoring the progression of osteogenic differentiation in collagen-chitosan based tissue engineered constructs. The collagen-chitosan based tissue engineered constructs used in this study have shown to support osteogenic differentiation and subsequent mineralization, which can be detected by MRI as shortening of T2 over 4 weeks.

The developed MRI-compatible tissue culture system has been successful in maintaining the cell viability and morphology but is not successful in supporting osteogenic differentiation. This is believed to be due to absence of control over the orientation of the constructs in the MRI-compatible housing, hence perturbing ECM deposition and mineralization.

We have identified orientation as an important parameter influencing the cell behavior in the developed MRI-compatible housing. A possible development would be to design the MRI-compatible housing with grooves or cassettes to hold the tissue engineered constructs in place, thereby enabling the cells within to synthesize ECM along their cytoskeletal direction and pave way for subsequent mineralization.
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## 7. APPENDIX

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8. VITA

AISHWARYA VAIDYANATHAN

Education

- Master of Science in Bioengineering
  University of Illinois at Chicago with CGPA 4.0/4.0
  Aug 2010-Aug 2012

- Bachelor of Technology in Industrial Biotechnology
  SASTRA University, India with CGPA: 9.01/10
  Aug 2006-May 2010

Professional membership

- Society for Women Engineers
- Biomedical Engineering Society

Research Experience

- Department of Bioengineering, University of Illinois at Chicago. Jan 2011-Aug 2012
  Fashioned an MRI-compatible housing for the tissue engineered constructs that can maintain the constructs in incubator-like conditions while inside the MRI scanner

- Department of Cell Biology and Anatomy, University of Illinois at Chicago. Aug 2010-Jan 2011
  Investigated the effect of "enriched environment" on the neurogenesis in brain, as a possible therapy for Alzheimer's disease, findings indicate enhanced expression of certain markers.

- Biochemistry lab, Vision Research Foundation, India Dec 2009-Mar 2010
  Conducted research to explore a possible surgical treatment for diabetic retinopathy, a severe ailment in the eye, occurring as a result of diabetes; this served as preliminary basis for using Retinoic acid in eye surgery. This involved isolating Retinal Pigment Epithelium (RPE) from eyes and culturing them.

- Chemical engineering pilot plant, Central Leather Research Institute, India May-Aug 2009
  Created a pilot effluent-treatment system, working on the principle of bioremediation using blue green algae (Blue green algae culture derived nutrition from effluents), which has now been scaled up for regular use to reduce the toxicity (BOD) of effluent chrome liquor from the leather industry.

Conference Publications

- M. Kotecha, S. Ravindran, A. Vaidyanathan, R. Magin, A. Geroge; “Sodium Triple Quantum Characterization of ECM Embedded Biomimetic Scaffolds for Cartilage Tissue Engineering” at 20th annual meeting and exhibition-ISMRM 2012 at Melbourne, Australia

- A. Vaidyanathan, S. Ravindran, A. George, R. Magin; “Extended Monitoring of 3-Dimensional Tissue Engineered Scaffolds using MRI-Compatible Culture System” at Biomedical Engineering Society (BMES) 2012, Atlanta, USA