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Three-Dimensional Culture of Cells and Matrix Biomolecules for Engineered Tissue Development and Biokinetics Model Validation

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Abstract

There has been considerable progress in cellular and molecular engineering due to recent advances in multiscale technology. Such technologies allow controlled manipulation of physiochemical interactions among cells in tissue culture. In particular, a novel chemomechanical bioreactor has recently been designed for the study of bone and cartilage tissue development, with particular focus on extracellular matrix formation. The bioreactor is equally significant as a tool for validation of mathematical models that explore biokinetic regulatory thresholds (Saha, A. K., and Kohles, S. S., 2010, “A Distinct Catabolic to Anabolic Threshold Due to Single-Cell Nanomechanical Stimulation in a Cartilage Biokinetics Model,” *J. Nanotechnol. Eng. Med.*, **1**(3), p. 031005; 2010, “Periodic Nanomechanical Stimulation in a Biokinetics Model Identifying Anabolic and Catabolic Pathways Associated With Cartilage Matrix Homeostasis,” *J. Nanotechnol. Eng. Med.*, **1**(4), p. 041001). In the current study, three-dimensional culture protocols are described for maintaining the cellular and biomolecular constituents within defined parameters. Preliminary validation of the bioreactor’s form and function, expected bioassays of the resulting matrix components, and application to biokinetic models are described. This approach provides a framework for future detailed explorations combining multiscale experimental and mathematical analyses, at nanoscale sensitivity, to describe cell and biomolecule dynamics in different environmental regimes.

Keywords

biomechanical signaling; nanomedicine; bioreactor; extracellular matrix

1 Introduction

Cartilage and bone defects are a leading cause of disability, an economic burden that exceeds 28 billion dollars in orthopedic repair in the United States alone [1]. More than

250,000 knee and hip replacements are performed each year [2], and the number continues to increase with an aging population suffering from defects or deterioration of articular cartilage caused by osteoarthritis and trauma. Because cartilage is avascular, and has a limited capability for self-repair, it has been the focus of many bioengineering investigations in nanomedicine. Tissue engineering is an evolving field that has the potential to provide functional cartilage tissue to help the millions of people who suffer from degenerated, diseased, or damaged articular cartilage. There have been rapid advancements in cell-based therapies, which includes the in vitro engineering of functional tissue substitutes for subsequent in vitro transplantation [2]. To date, however, the properties and structure of native cartilage tissue have not been entirely mimicked by engineered tissue (Fig. 1). Limitations of the current solutions such as autologous chondrocyte transplantation (ACT) include difficulty in obtaining a required amount of cells for a construct and cellular redifferentiation during expansion, as well as degeneration at the donor site. Although recorded success rates of ACT are between 60% and 90% depending on the localization of the injury [3], long-term success rates are considerably lower.

Tissue engineering is the process of creating functional three-dimensional (3D) tissues using cells combined with scaffolds, signaling factors, and devices that facilitate growth, organization, and differentiation. The development of mathematical models based on the interaction between cells and biomaterials can help understand the basic mechanisms of cartilage physiology [4]. In order to create an environment for tissue development, the mathematical models need to characterize the organization, mechanical properties, and physiologic response at multiple scales, from cellular to molecular to tissue levels. By harnessing nanobiotechnology, this approach can help design new drug development and delivery systems, regenerative medicine, and tissue remodeling procedures [5–7].

In healthy connective tissue, structural protein fibers such as collagen and elastin fibers have diameters ranging from several tens to several hundreds of nanometers. The nanoscale protein fibers entangle with each other to form a nonwoven mesh that provides tensile strength and elasticity for the connective tissue [8]. Articular cartilage is composed of immobilized chondrocytes, which secrete the cartilage specific biomolecules that make up the tissue's extracellular matrix (ECM) [9,10]. Physiological regulation, synthesis, and maintenance of ECM are maintained by the chondrocytes (although only 10% of the volume) through biokinetic reactions [5–7]. The structural integrity of cartilage is derived from the fibrous network formed by the cartilage specific protein, collagen type II, which is immersed in a gelatin formed by proteoglycans rich in sulfated glycosaminoglycans (GAG), and water, which binds with such high affinity that it makes up ~70% of the total weight [11]. It is this unique ECM that allows low friction movement of joint surfaces. In return, joint loading from body movement induces fluid velocities in the tissue that maintain waste and nutrient flow, as well as ion movement. Recreating the unique mechanical properties that give cartilage tissue the capability of bearing compressive loads during daily activities is a major challenge in tissue engineering.

1.1 Biomaterial Scaffolds and Three-Dimensional Culture

Recent work has shown that cells often exhibit unnatural behavior when they are excised from their native 3D environment and confined to a two-dimensional (2D) monolayer. The acute disparities in cellular function between 2D and 3D cultures warrant further biological examination [12]. When articular chondrocytes are cultured in a static 2D monolayer, they lose their overall hyaline structure and functional elasticity. In vitro expansion of such cultures is associated with cellular redifferentiation and reduced or total loss of their ability to redifferentiate [13,14]. A 2D environment allows chondrocytes to attach and gradually spread, which causes them to lose their spherical morphology and acquire an elongated fibroblastlike phenotype. In our current work, we observed morphological changes that

occur within the first few days of cells cultured in a static monolayer, as the freshly isolated cells begin to adhere and spread along the culture vessel.

Morphological alterations acquired in monolayer are accompanied by critical biochemical changes. Loss of the hyaline morphology is associated with the expression or total loss of aggrecan and collagen type II synthesis, and an increase in the expression of fibrocartilage associated ECM molecules collagen type I and versican [13,14].

Hydrated biomaterials may recreate the native 3D environment and mimic the physical structure, biological function, and biochemical nature of ECM [15]. Native ECM provides physical support for cells, as well as a substrate with specific ligands for cell adhesion and migration, and regulates cellular proliferation and function by providing growth factors [8]. During healthy balanced tissue regulation, growth factors stimulate the anabolic synthesis of essential ECM macromolecules, a process that is complemented by the catabolic degradation of ECM by proteinases during tissue remodeling and repair [5–7]. In order for this process to take place in vitro the biomaterial scaffold needs to degrade at a rate that compliments the synthesis of neotissue and be responsive to the local biological environment [16].

For the experimental design described herein, hydrated polymers (hydrogel) offer a biomaterial scaffold with customizable and biomimetic properties. This material is manipulated by photopolymerization for quick formation of the liquid mixture into gel when exposed to ultraviolet (UV) rays. Photopolymerization has been examined as a method to create scaffolds with specific nanoscale topography to promote control of cell migration and function [17].

1.2 Mechanical Stimulation and Biological Response

Redifferentiated chondrocytes have the capacity to redifferentiate if they are transferred into a 3D environment that supports a spherical morphology; however, their original phenotype is not fully re-acquired [13]. When 3D cellular constructs are grown in static culture, cells on the outer surface of the constructs are typically viable and proliferate, while cells within the construct may be less active or necrotic [18]. 3D culture techniques that encompass biomaterials and so allow chondrocytes to remain round yield cells that continue to express cartilage specific genes, but are limited relative to proliferation capacity and lack the zonal distribution of cells and ECM molecules found in native tissue [19]. Mechanical stimulation (fluid, solid, thermal, etc.) of 3D constructs consisting of cells, biomaterials, and growth stimulant inhibitors have shown promising results in the production of viable load-bearing tissues such as cartilage and bone [20,21]. The transmission of biochemical and biomechanical signals from the cell-cell interaction determines the formation of healthy morphology. Thus, preserving these interactions is essential in recreating the native tissue. In one cartilage engineering approach, it has been suggested that isolating cells from distinct histological zones and seeding the cells layerwise within a scaffold may help to preserve the native zonal distribution that contributes to the function of absorbing compressive and tensile forces. Utilizing a multiscale bioreactor as described in this design innovation paper, which can recreate native biomechanical and biochemical stimuli, will facilitate tissue development investigations and validate ongoing biokinetic modeling.

2 Engineering and Experimental Design Methods

2.1 Bioreactor Design and Application

A chemomechanical bioreactor was recently designed [22,23] with the primary objectives of providing mechanical stimulus (solid and fluid), mechanical characterization, optical inspection, and ultrasonic assessment of engineered tissue development (Fig. 2). In humans,

articular cartilage is a thin layer of 0.5–5 mm thick hydrated soft tissue [24], as such the 3D culture environment was designed to support constructs 5 mm in height to mimic native cartilage geometry.

A prototype bioreactor has now been fabricated for stimulating and monitoring cell, ECM, and scaffold development of 3D engineered cartilage constructs (Fig. 3). The device combines a linear actuator (ElectroForce, Bose, Eden Prairie, MN) connected to an array of five load transducers (1000 g, ± 0.15 –25%, model 31 Mid, Sensotec/Honeywell, Golden Valley, MN). The actuator can be controlled via displacement (± 2.5 mm) or force (20 N) to apply a variety of waveforms (WinTest, Bose) for dynamic stimulation of five concurrent cube-shaped test samples (Fig. 4). Access for a digital video microscope and ultrasound transducers facilitate minimally invasive construct assessment. The entire bioreactor is sized to fit inside an incubator (Galaxy R Series Incubator, New Brunswick, Edison, NJ) with sensor control of the internal environment chemistry, carbon dioxide (CO₂), oxygen (O₂), and nitrogen (N₂).

Elastic moduli of 3D hydrogel-cell constructs are typically measured in compression after 2, 4, and 6 weeks of in vitro culture time. Samples are initially unloaded, and then subjected to compressive strain at a rate of 0.5 mm/min [25]. The compression modulus will be determined by analyzing the linear regions of the stress versus strain curve on samples at low deformation (<15%).

Initial mechanical stimulation will relate dynamic constituent masses with elastic properties applying a basic rule of mixtures. More complex spherical-composite models will eventually be applied [20,21]. Bioassay quantification will be described in Secs. 2.5 and 2.6. The modeled (and measured) scaffold and cell-ECM masses (m_i) can be converted to constituent volumes (V_i) using density values (ρ_i):

$$\text{Cell-ECM: } V_{\text{CE}} = \frac{m_{\text{CE}}}{\rho_{\text{CE}}}$$

(totaled for multiple ECM constituents)

$$\text{Scaffold: } V_s = \frac{m_s}{\rho_s} \quad (1)$$

The total porosity (P) of the construct can be calculated from constituent volumes estimated from the biomarkers and the current gross volume of the construct composite (V_o) using the relationship

$$P = \frac{V_o - (V_s + V_{\text{CE}})}{V_o} = 1 - \frac{(V_s + V_{\text{CE}})}{V_o} \quad (2)$$

For constructs with distinct porosities, the elastic modulus of the cell-ECM-scaffold composite structure ($E_{\text{structure}}$) may be characterized by the phenomenological relationship [26]:

$$E_{\text{structure}} = E_{\text{solid}}(1 - P)^k \quad (3)$$

where k is a curve-fit parameter and E_{solid} can be defined by the cell-ECM (E_c) and polymeric scaffold (E_s) moduli using the two-phase, Voigt model [27]:

$$E_{\text{solid}} = v_{\text{CE}} E_{\text{CE}} + v_s E_s \quad (4)$$

Here, the solid volume of the engineered construct is defined by the volume fractions of the cell-ECM (v_{CE}) and polymeric scaffold (v_s), respectively:

$$\begin{aligned} \text{Cell-ECM: } v_{\text{CE}} &= \frac{V_{\text{CE}}}{V_s + V_{\text{CE}}} \\ \text{Scaffold: } v_s &= \frac{V_s}{V_s + V_{\text{CE}}} \text{ where } v_{\text{CE}} + v_s = 1 \end{aligned} \quad (5)$$

This preliminary micromechanical arrangement assumes that the scaffold and neo-ECM experience equivalent deformation in response to an applied load [28]. These metrics will provide a quantitative means of comparing the developing engineered tissues with native tissues as an approach for assessing the designed 3D culture environment.

2.2 Cartilage Dissection and Disaggregation

In the initial preparations of cell-scaffold constructs, which will be developed into cell-ECM-scaffold constructs using the bioreactor, fresh tissue is excised from the condylar surface cartilage of 6 month old bovine metacarpophalangeal joints contributed by a local abattoir within 1–8 h of death (Mark's Meat, Canby, OR). To decrease the chances of contamination, the joints are dissected in cold, sterile dissection solution containing a high concentration of antibiotic/antimycotic (AB-AM) solution (100×) stabilized (Sigma) in Dulbecco's phosphate buffered saline (PBS, Sigma Aldrich Co., St. Louis, MO) to give 250 µg/ml or 0.625 µg/ml amphotericin B. The soft muscle and connective tissue is removed utilizing a sterile scalpel (sizes 11 and 23), and cartilage is carved from the bone utilizing a sterile scalpel (size 15) from the bone and separated from any remaining soft tissue. The excised tissue is rinsed several times with cold PBS and 2.5% AB-AM solution through a 70µ mesh filter (Cell Mirco Sieves TM, BioDesign Inc.), weighed, and measured for ultrasonic propagation as described in Sec. 2.7. The resulting tissue fragments are mechanically disaggregated and finely minced with surgical scissors and opposing scalpels into ~1 mm³ pieces. The cells released from supporting matrix during disaggregation are centrifuged and resuspended in 5 ml of media containing Dulbecco's modified eagle medium mixture and Ham's F12 nutrient mixture (DMEM/F12 (1:1), Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, HyClone), 5% L-Glutamine (L-Glu, Lonza-BioWhittaker), 1% AB-AM, and stored at 4°C until digestion is complete. To allow timely detection of microbial contamination, it has been suggested to incubate the fragments for 24–48 h before digestion [29].

2.3 Enzymatic Digestion

The explanted tissue fragments are consecutively digested with 0.25% trypsin (2.5% trypsin at 1:250) (BioWhittaker, Lonza, Basel, Switzerland) and 0.5% collagenase (Liberase Blendzyme III, Roche Diagnostics, Madison, WI) to release the chondrocytes from the ECM with a high cell count of ~5×10⁶/ml per calf hoof. Warm trypsinization is carried out at 37°C on a hot-plate/magnetic stirrer for 1 h in Hank's buffered salt solution (HBSS) (Lonza) supplemented with 1% AB/AM solution. Culture media containing fetal bovine serum (FBS) (Thermo Scientific HyClone, Logan, UT) is added to inhibit trypsin activity and the cell suspension obtained is filtered through a 70µ sterile nylon filter to remove any tissue debris. Cells are collected by centrifugation at 1500 rpm for 10 min and rinsed several times

with culture media, which consisted of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12: Sigma), and resuspended in 5 ml chondrocyte culture media containing DMEM/F12 supplemented with 10% FBS, 5% L-Glutamine (Lonza), and 1% AB-AM and stored at 4°C until further digestion with collagenase is complete. The undigested tissue debris collected after trypsinization, which appeared more gelatinous than solid, is digested overnight in 0.5% collagenase at 37°C for 16–24 h. Sterile disposable 15 ml transfer pipets (VWR Int., Radnor, PA) are used to aspirate the fragments several times in order to release loosened cells from the gelatinous tissue debris. Digestion is continued in the samples that appeared to have tissue fragments still intact or a low cell count, at a higher concentration (0.75%) for an additional 1 hour. Isolated viable chondrocytes from each collection set (i.e., cells from disaggregation, trypsin, and collagenase digestion) were counted using a hemocytometer and Trypan-blue exclusion dye (0.4% in PBS). The sample collections having a cellular health viability of >75% as indicated by a lack of Trypan dye incorporation are resuspended in chondrocyte media at a concentration of $\sim 5 \times 10^6/\text{ml}$.

2.4 Cell-Seeding and Biomaterial Polymerization

The hydrogel scaffold mixture, consisting of poly(ethylene oxide) (PEO), poly(ethylene glycol) dimethacrylate (PEGDM), and the photoinitiator, 1-hydroxycyclohexyl phenyl ketone (Sigma Aldrich), is dissolved in sterile saline, penicillin G, and streptomycin to form a 20% (w/v) solution [30]. The hydrogel constituents are combined in a 3:2 ratio (PEO:PEGDM). The UV photoinitiator creates a final concentration of 0.05% [17]. Individual 125 μl aliquots of chondrocyte suspension (5×10^6 cells/ml in growth media as applied previously [11]) are added and polymerized layerwise in cubic molds of 125 mm^3 under 365 nm UV light for 3 min, a wavelength not harmful to the living cells [31]. The resulting hydrogels (five per batch) are removed from the 125 mm^3 molds using a sterile spatula, weighed, and measured for ultrasonic propagation (as input for Eqs. (1)–(5)) before being placed into each of the five culture wells fabricated for the bioreactor (Figs. 3 and 4).

2.5 Biomolecular Assays

Constituents representing the greatest total volume fraction and primary influence on cartilage biology and biomechanics will be monitored during cartilage development and biokinetic interactions. These are the chondrocytes, proteoglycan, and collagen content. Cell density can be measured through fluorometric assay as an indicator of proliferation or death overtime [25]. Being the primary components of the ECM are easily measured by colorimetric assay after construct digestion, and their distribution is assessed through histological staining of the neotissue sections. Specific proteoglycans and collagen isoforms, namely, aggrecan and collagen type II, can be quantified through immunoassays or visualized by antibody staining [25]. Total GAG content is quantified utilizing dimethylmethylene blue assay; total collagen content is quantified by a hydroxyproline assay. DNA content can be measured by Hoeschst-33258 and related to cell number by dividing DNA content by 7.7 pg per chondrocyte [25]. While these techniques provide useful information about the tissue grown in vitro, they require digestion and destroy the samples at specific end points. Ongoing design efforts are directed toward development of a bioreactor in which the quality of the neo-ECM and scaffold may be measured in real-time, utilizing digital imaging and ultrasonic sensors.

2.6 Histochemical Staining

At typical selected time points of 2 weeks, 4 weeks, and 6 weeks, constructs are removed from the chemomechanical culture environment, fixed in 10% formalin for 8–24 h, dehydrated, paraffin-embedded, and partially sectioned (10 μm thick) using a microtome. Sections are then stained with fast green and safranin-O (GAGs stains red-orange), and Masson's trichrome method (collagen stains blue) (Fig. 5). Colorimetry assays will

determine planar and volumetric densities of the assayed constituents as harvested time points. Remaining section geometries will be weighed and measured for ultrasonic propagation (as input for Eqs. (1)–(5)). The biomolecular assays characterize the state of the ECM as assessment of the tissue development as well as input for validation of the biokinetic models (Table 1). The primary assays will characterize antibodies associated with immunohistochemistry and histochemical staining to include the following:

1. *Type II Collagen* as the predominant collagen in healthy articular cartilage. Type II collagen forms a three-dimensional fibrillar network essential for the tensile stiffness and strength of cartilage [32].
2. *Aggrecan* as the third major component of articular cartilage, constituting the majority of the proteoglycanhyaluronic acid polymers. These large macromolecules are embedded within the fibrous network of collagen type II, providing the compressibility and elasticity of cartilage. During tissue turnover aggrecan and its fragments originating from the core protein part of aggrecan are released into the supernatant of cartilage explant cultures. The total aggrecan can be identified via an enzyme-immunoassay for the quantitative determination of aggrecan and its fragments containing the G1 and/or G2 domains that are released into the supernatant from articular cartilage explants [32].
3. *Type I Collagen* as an indicator of redifferentiation of articular chondrocytes during expansion of cells grown in a monolayer and as an indicator for fibrocartilage. An assay kit is designed to quantify the amount of type I collagen from bovine cell and/or tissue cultures (Chondrex, Inc.).
4. *Type X Collagen (COL10)* as an indicator of hypertrophic cartilage.

2.7 Ultrasound Analysis

Ultrasound transmission velocities are measured in vitro with the objective of defining acute and post facto elasticity metrics for characterizing developing engineered cartilage constituents. Ultrasound analysis is a nondestructive technique that can assess macroscopic construct properties and constituent quality. Ultrasonic longitudinal (v_{ij}) and transverse (v_{ij}) wave propagation velocities are measured in three orthogonal orientations (planar and thickness directions) for six measurements per sample. The test setup includes a pulser-receiver (model 5058PR, Panametrics, Olympus Corp., Waltham, MA), a multi-channel oscilloscope (model TDS460A, Tektronix, Beaverton, OR), and an array of sending-receiving transducers: 5 MHz (model V156), 10 MHz (model V112), 50 kHz (model X1021), and 100 kHz (models X1020 and V1548) transducers (Panametrics) facilitating a total of at most 1050 measurements. Aggregate cell-ECM-scaffold densities (ρ) of the samples are determined and system time delays are accounted for during each transducer arrangement. Bulk ($K=\rho_f v_{ii}^2$) and aggregate ($H_a=\rho_s v_{ii}^2$) moduli can be measured from separated fluid (f) and solid (s) ultrasound propagations, respectively [33], and used as input for Eqs. (3) and (4). The measurement of intrinsic ultrasonic properties may indicate the structure and composition of the tissue under investigation, as well as its mechanical and physical properties [25]. Initial cartilage pieces are excised as described earlier under aseptic conditions, sliced into 800–1000 μm sections with a microtome. Preliminary native cartilage testing confirmed density ($\rho = 1330 \text{ kg/m}^3$) and propagation velocities similar to that reported in the literature (1500–1720 m/s) [34].

2.8 Mathematical Model Input

Previous mathematical frameworks characterized nanoscale biokinetic mechanisms as stimulated within the micromechanical environment of a single cell [5–7]. The model results described the influence of mechanical stress on the anabolic and catabolic pathways

including the interactions with cytokines (C) and growth factors (G), fundamental regulatory processes that lead to matrix homeostasis. The engineering and experimental designs presented here monitor the catabolic (negative influence) and anabolic (positive influence) effects of chemomechanical stimuli (T), C, and G on a multicellular level using the mathematical framework exploring these differential interactions through time (dt):

$$\begin{aligned} \frac{d}{dt} \begin{bmatrix} C \\ G \end{bmatrix} &= [f_i(C, G, \alpha_i, \beta_i, \Omega_i)] + [\xi_i(T)] \\ \frac{d}{dt}(\text{collagen} + \text{GAG}) &= f(C, G, \text{collagen} + \text{GAG}, \lambda, \delta) \end{aligned} \quad (6)$$

This computational framework defines the secretion rates of C and G as functions (f) of their current levels and associated rate constants derived from the tissue engineering literature (α_i , β_i , and Ω_i) and the bioreactor controls (ξ_i), for $i=C$ or G , as previously described [5–7]. The dynamic state of the C and G is then applied as a positive or negative influence on the accumulation rate of ECM, defined as the total amount of collagen plus GAG. Boundary conditions for these levels will be determined from the protocols described in Secs. 2.5 and 2.6 (Table 1). The dynamic state of the ECM is defined as a function of the matrix molecules themselves and rate constants (λ and δ) modified to optimize description of the model with the collected data. This is an iterative modeling process, which will allow us to initiate an assumed biokinetic state and “back calculate” the levels of C and G from the measured ECM abundance through multiple iterations. Combinations of positive and negative feedback/influences can be explored by varying the chemomechanical bioreactor control system. Iterative analyses of the biokinetic models (Eq. (6)) are run through 5×10^3 to 10×10^5 normalized time steps using commercial software (MATLAB, Mathworks, Inc., Natick, MA) at each sample harvest/collection period (Fig. 6). Recreating the tissue in vitro by utilizing the bioreactor’s capability provides an approach to validate biokinetic models as a descriptor of dynamic inter- and intra-cellular interactions, necessary to promote tissue synthesis, regulation, and maintenance of the resulting ECM. Statistical validation and assessment of influencing variables will be conducted with multiple analysis of covariance (MANCOVA) techniques (JMP v9, SAS Concepts, Inc., Cary, NC).

3 Discussion

The successful large-scale production of engineered tissues requires an adequate source of healthy expandable cells, the optimization of biomaterial scaffolds, and the application of bioreactors, which mimic the in vivo environment of the body and are amenable to scale-up [35]. The in vitro environment provided by the chemomechanical bioreactor described here provides an experimental platform to explore cell-cell and cell-matrix molecular communication associated with ECM synthesis and degradation as a nanomedicine application. The importance of static or dynamic chemical and/or mechanical stimulation as characterized in biokinetic models for cartilage has indicated mechanisms, which increase proteoglycan and collagen expression, thus suggesting an active role in maintaining healthy chondrocytic phenotype [4].

The engineering and experimental design innovation described here recreates the in vivo environment of load-bearing articular cartilage on micro- to milliscales (cells and scaffold architecture) by measuring/modeling nanoscale biomolecular interactions. As an approach to exploring highly resolved, intrasample heterogeneity, distinct zonal distributions of ECM macromolecules and chondrocyte morphology in articular cartilage may be controlled through layerwise construct fabrication. Articular cartilage is stratified in a depth-dependent manner with distinct layers (superficial, middle, and deep). Each distinct zone is well characterized by cellular shape, ECM ultrastructure, and material properties (Fig. 1). The

superficial region has a dense network of collagen fibers primarily aligned parallel to the joint surface, whereas collagen fiber orientation is more random in the middle and perpendicular in the deepest zone [36]. Being avascular in nature, it is not a surprise that articular cartilage has reduced oxygen tension, and each zone has varying oxygen levels that decrease from superficial (~6% O₂) to deep (~1% O₂) zones [37]. Oxygen tension can alter matrix synthesis, as well as the material properties of articular cartilage in vitro, which can be a testable explanation for the zonal distribution along the depth of the tissue. The bioreactor described here has the potential to test the influence of many such parameters including oxygen tension and offers a novel arrangement in comparison to current bioreactor designs [17,38].

Ongoing efforts are exploring the chemomechanical environments in support of dynamic cell and tissue culture. These efforts include optimizing the scaffold's material and structural properties (local stiffness, porosity, etc.) in a manner that supports cell, nutrient, waste, and other biomolecular transport concomitant with controllable mechanical properties. Future capabilities will monitor and evaluate the developing neotissue in a nondestructive manner, accommodating real-time measurement. Utilizing a bioreactor that encompasses the ability to culture tissue, as well as monitor matrix growth and maintenance, may have a much broader scope than facilitating tissue development; it has the potential of replacing animal models in drug response studies, cellular and molecular engineering, and regenerative medicine. It may be possible to better understand the roles of specific mammalian genes involved with essential metabolic pathways without manipulating an animal model [39].

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Fig. 1. Three-dimensional reconstruction via microcomputed tomography of a through-thickness section of human articular cartilage (after [40]) The total transverse slice dimension is 1500 μm representing the articular surface (top), three histologic zonal layers, and the bony osteochondral transition (bottom).

Fig. 2.

A solid model of the assembled chemomechanical bioreactor for three-dimensional culture (after [22]) The image includes the upper electromechanical actuator applying high-resolution load-deformation control to the “hat,” which distributes the mechanical stimulus to the five load arms and, as such, to the five culture wells below. The wells can be rotated into the view of single ultrasonic or digital imaging sensors.

Fig. 3.

The now-fabricated, novel chemomechanical bioreactor with (a) five culture wells and (b) a load-train applying load-displacement for measuring exact loads. The high-resolution, copper-colored load-cells measure the dynamic loads transferred via the load-rods through the square load-platens into the culture wells and onto the eventual cell-biomaterial constructs.

Fig. 4.

Open-view (model) of a bioreactor test chamber for engineered tissue chemomechanical stimulation indicating the (a) cell-biomaterial construct (125 mm^3 pink cube) and (b) three current well designs for static and dynamic three-dimensional culture. The middle culture well, which provides perfused flow and dynamic culture, will be described in a future article [21].

Fig. 5. Expected cell-matrix preparations (after [25]) During matrix synthesis, cells will be distributed throughout the (a) collagen (to be stained with Masson's Trichrome) and (b) aggrecan (to be stained with safranin-O/fast) constituents as are typical for this cell-scaffold arrangement. The solid dark circles each represent a single cell at 20 μm in diameter.

Fig. 6.

Representative biokinetic modeling results (applying Eq. (6)) from expected biomolecular accumulation data as gathered from the chemomechanical bioreactor (as boundary conditions and validation) Here the model is initially allowed to run as the ECM concentration (% , GAG and collagen) peaks in the response to local resources and then drops to a steady state level. A chemomechanical stimulus is then applied at 5×10^5 relative time units, driving the accumulation upward in an anabolic response.

Table 1

Focal biomarkers and the assay products applied to evaluate the state of extracellular matrix biokinetics. The biomarkers and the listed typical secretion standards will be measured as boundary conditions characterizing the experimental progress of the engineered tissue development. These data will also provide input for model initiation and validation during statistical comparison.

Biomarkers	Assay Product	Cell Secretion Rate (in vitro values)
Type II collagen	Rhuemera® native type II collagen capture ^a	~1.5–3.0 µg per 10 ⁶ chondrocytes (basal media) [32]
Glycosaminoglycan (GAG)	Rhuemera® assay kit for proteoglycan ^a	~55–105 µg per 10 ⁶ chondrocytes (basal media)
Type I collagen	Rhuemera® type I collagen kit ^a	~10–15 ng per 10 ⁶ fibroblasts
Type X collagen	Kit for collagen type X ^b	~1–10 µg per 10 ⁶ chondrocytes
Total collagen	Soluble collagen content ^c	~5–15 µg per 10 ⁶ chondrocytes

^aEnzyme-linked immunosorbent assay (ELISA), Rhuemera and Immunodiagnostics System. (Astarte Biologics, LLC, Redmond, WA).

^bELISA (Antibodies-online, Atlanta, GA).

^cSircol (Biocolor Life Science Assays, Carrickfergus, UK).