New Approaches to Solute Transport Measurement in Mechanically Loaded Articular Cartilage*

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Diffusive and convective transport of bioactive solutes within the extracellular matrix of articular cartilage plays an important role in the regulation of tissue physiology and the cell biological response to mechanical compression. With a view toward identifying means to employ mechanical control of solute transport for the optimization of in vitro methods of cartilage tissue engineering, we have developed techniques for real-time observation and quantification of solute transport within statically compressed cartilage explants in the presence of interstitial fluid flows and matrix inhomogeneities, and also within dynamically compressed cartilage explants. Initial results confirm the potential usefulness of the new methods, and suggest roles for transport phenomena in regulation of the cartilage biological response to static and dynamic compression. Further characterization of solute transport using these methods may therefore guide the optimization of in vitro mechanical loading protocols used in cartilage tissue engineering.

Key Words: Cartilage, Solute Transport, Diffusion, Convection, Anisotropy

1. Introduction

Adult articular cartilage may be up to a few millimeters thick, but contains no blood vessels, nerves, or lymphatic system. Cartilage cells occupy only about 5% of the tissue volume; the remaining 95% is occupied by the mechanically functional extracellular matrix⁽¹⁾. Cartilage cells therefore depend heavily upon transport of solutes through the extracellular matrix for many of their biological activities including acquisition of nutrients, rejection of wastes, synthesis and deposition of matrix molecules, remodelling of the matrix with secreted enzymes, and cell-to-cell signalling via cytokines and growth factors. Solute transport within cartilage matrix is therefore a central aspect of cartilage physi-

ology.

Articular cartilage functions as a load-bearing and lubricating surface in synovial joints. In response to tissue loading, cartilage cells alter their biological activities in various ways which are closely related to the nature of mechanical loads applied. Static compression of cartilage is associated with decreases in rates of cell metabolism⁽²⁾, while low-amplitude dynamic compression and associated fluid flows within the poroelastic matrix have been associated with increases in cell metabolic rates⁽³⁾. The biological response to compression has significant potential for the optimization of methods for cartilage tissue engineering⁽⁴⁾. For this reason, the mechanisms by which tissue loading is transduced into changes in cartilage cell activity is of significant practical interest. It is reasonable to hypothesize that one important mechanism by which this transduction occurs is via changes in transport rates of bioactive solutes in the matrix⁽⁵⁾. Therefore, mechanical control of transport within cartilage-like tissues in vitro may represent a possible means for the promotion of the development of tissue-

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engineered constructs for clinical applications^{(6)–(8)}.

The spectrum of bioactive solutes in cartilage covers very wide ranges of molecular weight, chemical composition, and physicochemical properties⁽⁹⁾. In addition, the cartilage matrix is itself inhomogeneous in composition and structure at cell⁽¹⁰⁾ and tissue⁽¹¹⁾ length scales. Variations in tissue composition and structure may underlie variations in transport characteristics between cartilage zones^{(12)–(14)}. Furthermore, recent theoretical work⁽¹⁵⁾ has suggested that tissue compression may give rise to anisotropic (direction-dependent) transport phenomena. The study of inhomogeneity and anisotropy of solute transport in compressed cartilage may contribute to the understanding of tissue structure-function relationships and highlight the particular roles of different matrix functional zones in regulating the cell microenvironment and response to compression. Therefore, the notion of “solute transport within cartilage-like matrix” refers to a very large class of physical situations, which may be addressed by a broad range of experimental techniques. Many methods have been developed for solute transport measurements in cartilage-like tissues^{(16),(17)}, including tools for investigation of effects of static and dynamic mechanical compression on solute transport^{(18)–(20)}. Methods which permit the direct observation of solute concentration distributions^{(13),(21)} present particular advantages since tissue-scale average transport rates may be determined in combination with relationships between cell-scale variations in matrix composition, structure, mechanics, and solute localization. In comparison to radioactivity or magnetic resonance-based techniques, fluorescence methods are technically simple. Our goals have therefore included the development of fluorescence-based methods for real-time observation and quantification of solute transport within statically compressed cartilage explants in the presence of interstitial fluid flows and matrix inhomogeneities, and also within dynamically compressed cartilage explants.

2. Methodological Background

2.1 Cartilage explants

Adult (18-month old) bovine articular cartilage explants were acquired as previously described⁽²²⁾. Except where stated otherwise, the first $\sim 100\ \mu\text{m}$ of superficial cartilage was removed, then a tissue sheet $650\ \mu\text{m}$ thick was cut, consisting primarily of intermediate and deep zone cartilage. Rectangular explant blocks were acquired from this sheet with a custom cutting tool, and cylindrical explant disks were acquired using a biopsy punch (Stiefel Laboratories, Sligo, Ireland). Explants were stored frozen in phosphate buffered saline (PBS) with 0.01% sodium azide

(Sigma, Buchs, Switzerland) and protease inhibitors (Complete tablets, Boehringer Mannheim, Rotkreuz, Switzerland).

2.2 Solute preparation and characterization

Solutes used in experiments included tetramethylrhodamine alone (TMR; MW 430 Da), and dextrans of mean molecular weights 3, 10, and 40 kDa conjugated to TMR (Molecular Probes Europe, Leiden, The Netherlands). Dextrans were resuspended at 10 mg/mL in PBS with 0.01% sodium azide; 0.5 mL samples were loaded onto Sephadex G-25 PD-10 columns (Amersham Pharmacia Biotech, Dübendorf, Switzerland) and the eluant between elution volumes 2 mL and 4 mL (containing macromolecules) used in experiments. This preparative step reduced small molecular weight contaminants (possibly trace amounts of free TMR) to below our ability to detect. Further control studies were performed to verify that fluorescent dextrans which had been desorbed from cartilage had not been degraded or changed their apparent molecular weight during experiments⁽²²⁾.

2.3 Quantitative fluorescence measurement

All experiments involved desorption of solute from explants into adjacent baths, with real-time measurements of fluorescence intensities in order to determine relative solute concentrations either inside explants or in the baths. This approach was simplified by having a linear relationship between image fluorescence intensities and solute concentrations. Pilot studies were therefore conducted wherein several explants were equilibrated at a range of solute concentrations, and simulated desorption baths were prepared over a range of concentrations. Fluorescence measurements were then performed under standardized conditions, and those ranges of solute concentration in which intensity varied linearly with concentration were used in experiments.

3. Diffusion-Convection under Static Compression

As previously described⁽²²⁾, articular cartilage explant blocks ($10\ \text{mm} \times 3\ \text{mm} \times 650\ \mu\text{m}$) were compressed between a microscope slide and a custom-built plexiglass block (Fig. 1) such that they formed the only path for fluid and solute transport between two adjacent, well-mixed PBS baths of equal volume ($\sim 8\ \text{mL}$). Solute was allowed to desorb from the explant into each bath as the experiment progressed. A hydrostatic pressure gradient was applied between the baths by water (PBS) columns communicating with each bath via 1 mm diameter $\times$ 10 mm long channels. Control studies indicated that the columns contributed an effective dilution rate of only a few percent per hour to bath solute, which was typically negligible in experiments. Consideration of cartilage

Fig. 1 Schematic diagram of apparatus used for measurement of diffusion-convection in cartilage under static compression. Rectangular block explants (cut to $10\text{ mm} \times 3\text{ mm} \times 650\text{ }\mu\text{m}$) were compressed statically (along the direction of the nominally $650\text{ }\mu\text{m}$ dimension) while solute desorbed out of the cartilage and into adjacent baths. Water columns provided a hydrostatic pressure gradient between baths. Fluid flow and solute flux were directed along the nominally 3 mm dimension of explants.

as a poroelastic material using typical values for tissue material properties⁽²³⁾ indicates that a pressure gradient equivalent to a 1 m difference in water column heights would be associated with a deformation gradient of a few percent across the tissue. Therefore, this 1 m difference was not exceeded in experiments, so that uniformity of tissue density and material properties could reasonably be assumed.

Solute flux Γ was modelled by contributions from the effective diffusivity D within cartilage and a convection term involving the fluid velocity (volumetric flux) U and a solute-specific convection coefficient ω :

$$\Gamma = -D\nabla c + \omega U c \quad (1)$$

Solute concentration c is defined relative to tissue fluid volume, whereas fluxes (Γ and U) are defined relative to the total tissue area including both fluid and solid. Conservation of solute requires

$$\frac{\partial c}{\partial t} = -\frac{1}{\phi} \nabla \cdot \Gamma \quad (2)$$

where ϕ is tissue fluid volume fraction. At relatively early times (t), these experiments may be modelled as desorption from a semi-infinite space with initially uniform internal concentration and a zero concentration boundary condition⁽²²⁾ (Fig. 2). The solution to this problem may be obtained by analogy with previous results⁽²⁴⁾:

$$c(x, t) = \frac{c_0}{2} \left[\left(1 + \operatorname{erf} \left[\frac{x - U_1 t}{\sqrt{4D_1 t}} \right] \right) - \exp \left(\frac{U_1 x}{D_1} \right) \left(1 - \operatorname{erf} \left[\frac{x + U_1 t}{\sqrt{4D_1 t}} \right] \right) \right] \quad (3)$$

where $U_1 = \omega U / \phi$ and $D_1 = D / \phi$. Insertion of this result into Eq. (1) and integration to determine bath solute concentration $c_B(t)$ provides

Fig. 2 Governing equation with boundary and initial conditions the evolution of solute concentration within statically compressed cartilage explants during solute desorption via diffusion (D_1 -term) and convection (U_1 -term). At early times, desorption into each adjacent bath may be modelled as occurring from a semi-infinite space. The distance into the explant from the bath interface is represented by x .

Fig. 3 Accumulation of 3 kDa dextran in upstream and downstream baths during desorption from a cartilage explant in a diffusion-convection experiment. A hydrostatic pressure difference was applied across a $\sim 3\text{ mm}$ thick explant by a water column height difference of 1 m . Therefore, solute was expected to accumulate more rapidly in the downstream bath than upstream due to solute convection in addition to diffusion.

$$c_B(t) = c_B(0) + \frac{A_c \phi}{V_B} c_0 \left[\frac{D_1}{U_1} \left(\frac{2\alpha}{\sqrt{\pi}} \exp[-\alpha^2] + \operatorname{erf}[\alpha] \right) - \frac{U_1 t}{2} (1 - \operatorname{erf}[\alpha]) \right] \quad (4)$$

where A_c is the surface area of cartilage touching the bath, V_B is bath volume, and $\alpha = \sqrt{\frac{U_1^2 t}{4D_1}}$. Symmetry implies that on the side of the explant at relatively high hydrostatic pressure (upstream) U_1 is positive (Fig. 2), while on the downstream side it is of equal magnitude but negative. Therefore, solute accumulation in the downstream bath is expected to be faster, with the difference from the upstream bath reflecting the magnitude of U_1 . Initial experiments with 3 kDa dextran-TMR appear promising (Fig. 3); however, results of theoretical fits to experimental data are inconsistent. Possible reasons include inhomogeneities in explant material properties (inducing

asymmetry), the small fluid velocities accessible with this technique (~ 10 nm/s for a 1 m water column⁽²³⁾), and the potentially low convection coefficient (ω) of 3 kDa dextran-TMR (an open-coil polymer which may bind slightly to the cartilage matrix⁽¹⁹⁾). In addition, convection is more difficult to observe when combined with diffusion over relatively short distances and times, as these methods use. Future work will emphasize longer experiments in order to maximize the convection “signal” within the “background noise” of diffusion. Therefore, this new method shows promise for the quantification of D and ω for solutes in cartilage-like materials within a single experimental protocol.

4. Inhomogeneous and Anisotropic Diffusion under Static Compression

We are also developing two-directional diffusion measurements that are an extension of existing techniques for measuring unidirectional diffusion with a confocal microscope⁽¹⁹⁾. A cartilage explant block, $3\text{ mm} \times 2\text{ mm} \times \text{full thickness}$ (including the superficial zone; typically $\sim 1\text{ mm}$) is compressed statically in the direction of tissue thickness. Compression is applied by custom-made plexiglass blocks containing rigid porous inserts (Fig. 4(a)) made of unsintered aluminum oxide with a characteristic pore size of $2\text{ }\mu\text{m}$ (Kerafol, Eschenbach, Germany). One uncompressed face of the explant is held against a glass microscope slide, and mounted in the viewing field of an inverted confocal microscope (Zeiss LSM 410). The

three remaining uncompressed explant faces are unconfined and communicate directly with a well-stirred saline bath introduced at time $t=0$ to initiate solute desorption perpendicular to the direction of compression. In addition, saline is circulated through the porous inserts to allow desorption parallel to the compression direction (Fig. 4(a)).

At early times of desorption, the distributions of solute concentration within each of the four imaging windows (Fig. 4(b)) are expected to correspond to that of diffusion from a semi-infinite space with uniform initial concentration and a zero concentration boundary condition (Eq. (3) with $U_1=0$):

$$c(x, t) = c_0 \operatorname{erf} \left(\frac{x}{\sqrt{4D_1t}} \right) \quad (5)$$

where x represents the distance inward (into the cartilage) from the interface with each adjacent saline bath. This local simplification will allow for the use of existing methods of analysis to determine solute diffusivities⁽¹⁹⁾. Therefore, this apparatus is capable of measuring anisotropy (e.g. by comparing desorption from the superficial zone to that from the unconfined edge) and inhomogeneity (e.g. superficial versus deep zones) in solute diffusion processes occurring within a single statically compressed cartilage explant.

Optimizing the size of the porous insert to ensure that the evolution of solute concentrations within the image windows could be considered as locally one-directional diffusion processes for suitably long times was an important design issue. Finite difference

Fig. 4 (a) Schematic diagram of a rectangular, full-thickness cartilage explant within the two-directional diffusion apparatus, as viewed from the microscope objective. Static compression, which is variable and can be measured by direct observation, is applied by rigid surfaces contacting the tissue superficial and deep zones. A saline bath is circulated next to explant unconfined edges and within the rigid porous inserts during solute desorption from explants. (b) Confocal image of cartilage explant containing TMR and mounted within a prototype of the two-directional diffusion apparatus.

Fig. 5 (a) Contour plots of two-directional diffusion simulations ($D_1=50 \mu\text{m}^2/\text{s}$ in both directions) from a cartilage explant with dimensions $1 \text{ mm} \times 3 \text{ mm}$ and a porous insert width of 1 mm . Results are shown for 300 and 600 s of desorption from an explant with initially uniform solute concentration. (b) Simulations of solute concentrations within cartilage explants after 300 and 600 s of desorption, for experimental image windows (Fig. 4(b)) $200 \mu\text{m}$ wide at the explant articular surface (■) and centered on a free edge (●). Results follow the solution for unidirectional diffusion from a semi-infinite space (Eq. (5)) for at least 300 s at the articular surface and the free edge.

simulations of the two-directional experiment were therefore performed using Mathematica with the Crank-Nicholson differencing scheme⁽²⁵⁾ to solve the partial differential equation of diffusion (obtained by combining Eqs. (1) and (2) with $|U|=0$: $\frac{\partial c}{\partial t} = D_1 \nabla^2 c$, Fig. 5(a)). Each iteration was solved by singular value decomposition. The spatial grid employed had $33.3 \mu\text{m}$ spacing, the time step was 0.5 s , and D_1 was $50 \mu\text{m}^2/\text{s}$ in both directions^{(19),(22)} (Fig. 5(a)).

Comparisons of Eq. (5) with the simulated evolution of concentration for a 1 mm wide porous insert and a $200 \mu\text{m}$ wide image window⁽¹⁹⁾ (Fig. 5(b)) indicated that each of the imaging locations are expected to obey locally one-dimensional diffusion kinetics for satisfactorily long times (up to 300 s at least).

5. Diffusion-Convection under Dynamic Compression

Articular cartilage explant disks (diameter 2 mm , thickness $650 \mu\text{m}$) are obtained from adult bovine proximal humeri. Disks are mounted in a novel compression apparatus that enables real-time confocal imaging of explant deformation and solute desorption during dynamic compression (Fig. 6). The

Fig. 6 Schematic diagram of apparatus for measurement of solute diffusion and convection in dynamically compressed cartilage explants.

apparatus frame rests on an inverted confocal microscope stage and supports an ultra-precision motorized actuator (Newport, Irvine, USA) in series with a precision miniature load cell (Sensotec, Columbus, USA) and stainless steel compression rod. Explant disks are compressed axially without radial confinement between the rod and the flat bottom of a plexiglass bath container just above the microscope objective. A pump provides for introduction of an

external bath at $t=0$ and mixing of the bath during the desorption period (which lasted 1 100 s in initial experiments). Three modes of compression (10% static strain, 50% static strain, or dynamic ramp compression between 10% and 50% strain; Fig. 7) were applied during desorption. Analysis of fluorescence images recorded every 100 s was performed using NIH Image (NIH, Bethesda, USA). Average

Fig. 7 (a) Mean concentration (within fixed-diameter regions of interest) normalized to initial values for TMR (◆) and 10 kDa dextran (●) desorption from cartilage explant disks under (a) 10% static compression, (b) 50% static compression, and (c) quasi-static ramp compression from 10% to 50% and back to 10% compression over 1 000 s.

fluorescent intensity was measured within a circular region of interest centered on the disk and held constant for each sample throughout compression. For comparisons between explants, intensities were normalized to the initial ($t=0$) values.

In an initial series of experiments, concentration decreased with desorption time for all solutes and compression modes (Fig. 7). The concentration decrease at 10% and 50% static compression was quasi-exponential for each solute (Fig. 7(a), (b)), consistent with desorption by diffusion only⁽²⁰⁾. Concentration in dynamically compressed samples appeared to additionally follow changes in compressive strain (Fig. 7(c)). The fraction of the initial concentration remaining at $t=1\,100$ s was greatest under 50% static strain for both solutes studied (Fig. 8), consistent with decreased diffusivity under increased static compression. The greatest efflux of TMR occurred under dynamic loading, though no significant differences were detected.

In initial experiments, our compression and real-time imaging apparatus therefore illustrated an inhibitory effect of static loading on transport, and suggested a stimulatory effect of dynamic loading. These results support the hypothesis that mechanical loading may influence chondrocyte metabolism via alterations to solute transport in the cartilage extracellular matrix. Concentration increases seen during dynamic compression (Fig. 7(c)) may suggest attractive interactions between these solutes and the matrix that limit solute convection. Diffusivities (D) and convection coefficients (ω), and fluid velocities associated with dynamic compression remain to be quantified in future work.

Fig. 8 Mean concentration at 1 100 s normalized to initial ($t=0$) values for solute desorption from cartilage explant disks under 50% static compression (white bars), 10% static compression (grey bars), and quasi-static ramp compression (black bars). $K=p<0.01$ on two-tailed Student's t -test.

6. Conclusions

Solute transport over tissue length-scales is of fundamental biological significance within avascular (adult) articular cartilage and tissue substitute materials. With a view toward control of transport processes by mechanical compression for the promotion of in vitro development of tissue-engineered cartilage, present methods provide several tools for the characterization of transport within cartilage-like materials. These could therefore be valuable for basic studies of transport and effects of compression in cartilage-like tissues, and for the identification of compression protocols which optimize solute transport conditions and cell biological activities for tissue engineering applications.

Initial results using methods described above are consistent with the hypothesis that the cartilage biological response to compression may be mediated by changes in transport of bioactive solutes through the extracellular matrix. In general, tissue static compression is associated with matrix compaction, reduced rates of solute transport, and decreased rates of matrix synthesis. Fluid flow through cartilage, as occurs during tissue dynamic compression, appears to be associated with augmented rates of solute transport and may thereby contribute to the increased rates of matrix synthesis associated with low amplitude dynamic loading⁽³⁾. However, dynamic compression of cartilage necessarily combines matrix compaction together with interstitial fluid flows, and it is unclear under which mechanical conditions solute transport will be augmented or reduced. Furthermore, effects of loading on transport may vary between tissue functional zones. Therefore, exploration of a wide range of mechanical loading "waveforms" will be necessary in the future in order to characterize the range of their effects on solute transport.

In addition, the wide range of physical interactions which may occur between bioactive solutes and the cartilage matrix imply that a single mode of mechanical loading may have different effects on transport rates of different solutes. Steric interactions related to solute size and shape⁽⁹⁾, electrostatic interactions⁽¹⁾, and biochemical binding⁽¹⁷⁾ may all play important roles in determining solute transport rates within the cartilage extracellular matrix⁽²⁶⁾. In addition, consumption or production by cartilage cells introduces additional complexities to the transport characteristics and physiological roles of some matrix solutes⁽²⁷⁾. Therefore, it is possible that different modes of tissue compression may be optimal for transport of different solutes, such that identification of an "optimal" loading regime for transport is a solute-

specific challenge. Furthermore, since cell biological activities may depend upon the local concentrations of several different solutes, a loading regime which is optimized with respect to cell activity may also depend upon the tissue loading history. We therefore believe that characterization of solute transport within mechanically compressed cartilage, by methods such as those described here, represents a rich class of biophysical phenomena with significant potential for practical application in tissue engineering.

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