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Modeling the Dynamic Composition of Engineered Cartilage

Christopher G. Wilson
Worcester Polytechnic Institute

MODELING THE DYNAMIC COMPOSITION OF ENGINEERED CARTILAGE

by

Christopher Garrison Wilson

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APPROVED:

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Dr. Sean S. Kohles, Research Advisor

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Dr. Lawrence J. Bonassar, Research Advisor

_____

Dr. Jill Rulfs, Committee Member

_____

Dr. Christopher H. Sotak, Committee Member

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ABSTRACT

Experimental studies indicate that culturing chondrocytes on biodegradable polymeric scaffolds may yield “engineered” cartilage for the replacement of tissue lost to injury or diseases such as osteoarthritis. A method of estimating the outcome of cell-polymer cultures would aid in the design and evaluation of engineered tissue for therapeutic use. The goals of this project were to develop, validate, and apply first-generation mathematical models that describe the kinetics of extracellular matrix (ECM) deposition and scaffold degradation in cell-polymer constructs cultured *in vitro*. The ECM deposition model is based on a product-inhibition mechanism and predicts an asymptotic, exponential increase in the concentration of ECM molecules found in cartilage, including collagen and glycosaminoglycans (GAG). The scaffold degradation model uses first-order kinetics to describe the hydrolysis of biodegradable polyesters in systems not limited by diffusion. Each model was fit to published data describing the accumulation of GAG and collagen, as well as the degradation of poly glycolic acid (PGA) and poly lactic acid (PLA), respectively. As experimental validation, cell-polymer constructs (n = 24) and unseeded scaffolds (n = 24) were cultured *in vitro*, and biochemical assays for GAG and collagen content, as well as scaffold mass measurements, were performed at 1, 2, 4, 6, 8, or 10 weeks of culture (n = 8 per time point). The mathematical models demonstrate a moderate to strong goodness of fit with the previously published data and our experimental results (R^2=0.75-0.99). These models were also combined to predict the temporal evolution of total construct mass with reasonable accuracy (30% RMS deviation). In ongoing work, estimates of biochemical composition derived from these models are being proposed to predict the mechanical properties and functionality of the constructs. This modeling scheme may be useful in elucidating more specific mechanisms governing ECM accumulation. Given their potential predictive power, these models may also reduce the cost of performing long-term culture experiments.

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SPECIFIC AIMS

The objective of this project was to develop methods of estimating the dynamic composition of engineered articular cartilage in culture, for the purposes of understanding the biological mechanisms governing neotissue deposition and, in the future, predicting the mechanical properties of the constructs as a function of culture time. Mathematical models capable of describing the biochemical composition and mechanics of these constructs may lend insight into the effects of various culture conditions on *in vitro* tissue formation. This thesis was directed by three specific aims:

- Develop first-generation mathematical models of extracellular matrix deposition, polymeric scaffold degradation, and total construct mass from hypothesized mechanisms of action.
- Validate the matrix accumulation and scaffold degradation models by fitting to published data sets on engineered cartilage constructs and experimental data from this study.
- Apply the matrix accumulation, scaffold degradation, and total mass models to data generated in an *in vitro* culture system.

BACKGROUND

ARTICULAR CARTILAGE ANATOMY & PHYSIOLOGY

Articular cartilage is a dense, highly hydrated (60-85%) tissue found on the ends of bones in the knee, hip, shoulder, and other joints (Fig. 1); it is classified as a hyaline cartilage and is distinguished by its smooth, bluish-white, glistening appearance (Mow and Ratcliffe 1997). It provides a low friction surface for joint motion, and along with tendons and ligaments, aids in the transfer of load between bones. Articular cartilage also acts as a cushion, absorbing some of the compressive stress associated with physiologic movement. The functionality of articular cartilage is closely related to the tissue's stratified extracellular matrix (ECM) and the properties of the ECM molecules (Fig. 2). Within the tissue is a relatively sparse, nonuniform distribution of chondrocytes (Fig. 3, nuclei stained purple) that produce and maintain an ECM rich in structural proteins (Fig. 3, acidophilic collagen fibers stained pink) and proteoglycans.

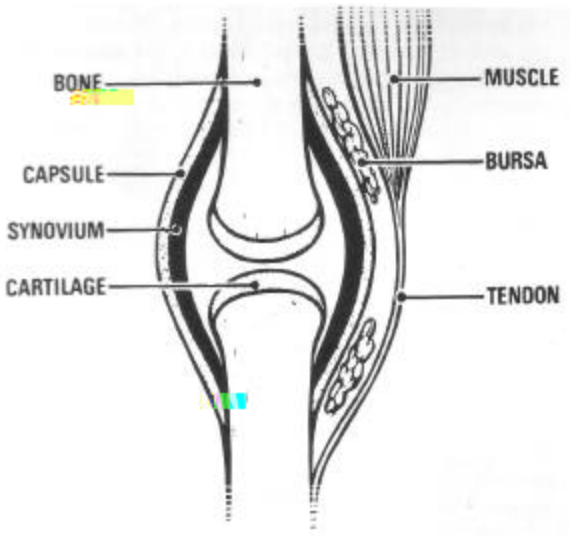

Figure 1. Schematic of a simple synovial joint (Dowson and Wright 1981).

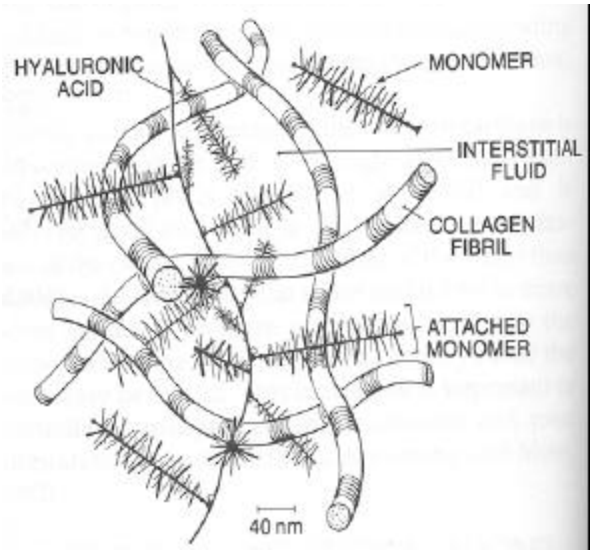

Figure 2. Extracellular matrix molecules of articular cartilage (Mow and Ratcliffe 1997).

The cells in the layer closest to the tissue surface (i.e., tangential layer) are elongated along the surface (Fig. 4) and continuously remodel an ECM consisting mostly of

collagen. The collagen fibrils in the superficial zone are parallel to the tissue surface, and this arrangement provides the tensile strength necessary to maintain the tissue's resistance to shear stresses. Chondrocytes along the bone-cartilage interface (i.e. radial layer) are stacked, with a cuboidal morphology. In this deep layer, collagen fibrils are arranged perpendicular to the tissue surface and penetrate the tide mark between cartilage and bone, anchoring the tissue to the underlying bone. A transitional layer corresponding to 40-60% of the tissue thickness consists of randomly oriented collagen fibers and a relatively high concentration of proteoglycans with spherical chondrocytes. The properties of articular cartilage are due, in part, to the layers of varying matrix molecule composition and organization and cell morphology.

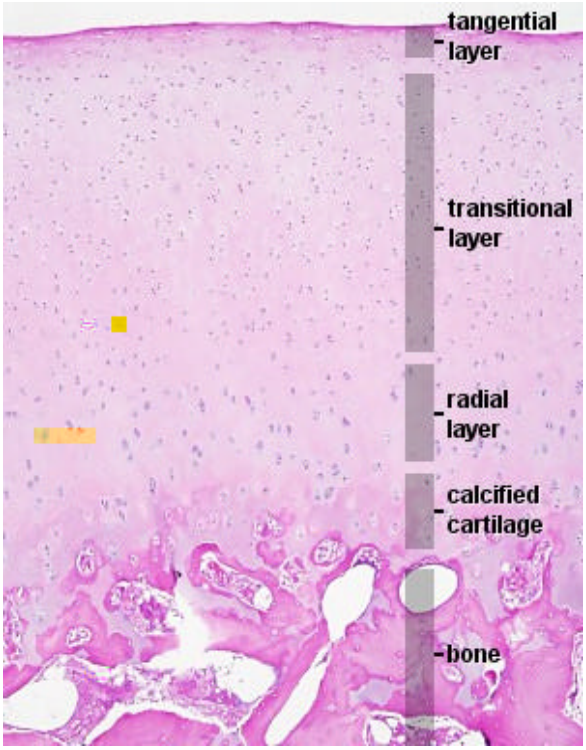

Figure 3. Tissue section of articular cartilage, hematoxylin and eosin stained (Slomianka

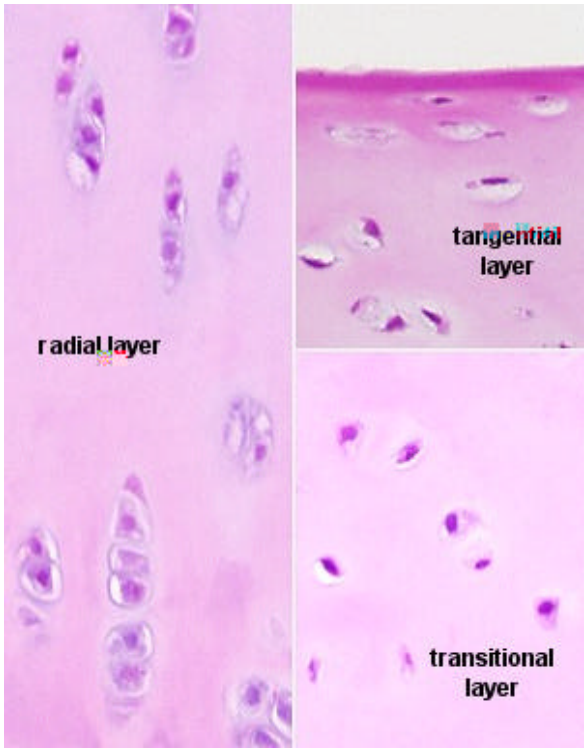

Figure 4. Cell morphology in cartilage layers, hematoxylin and eosin stained (Slomianka

The functionality of articular cartilage is also due, in part, to the properties of the matrix molecules. The most common proteoglycans, including aggrecan, decorin, and

versican, are assembled with glycosaminoglycans (GAGs), like chondroitin sulfate and keratan sulfate, in a bottlebrush configuration (Fig. 2). It is important to note that these GAGs contain electronegative sulphate groups, and are bound to aggrecan (the predominant proteoglycan in articular cartilage) in high densities. High molecular weight hyaluronic acid chains provide a backbone for anchoring aggrecan-GAG units and localize the high GAG density within the tissue. Due to Donnan osmotic pressure – the pressure required to maintain equilibrium between concentration and electrochemical gradients – water and cations are drawn from the synovial fluid into the extracellular space. It is this "swelling pressure" that allows articular cartilage to absorb and dissipate compressive stresses.

The collagen fibers play an important role in determining the properties of cartilage as well. First, the collagen network, composed primarily of collagen types II, IX, and XI, provides steric hindrance to the extrusion of large proteoglycans. Collagen molecules are arranged in fibers, and are organized as a network that interferes with the movement of large molecules. Without this constraint, the tissue would behave like a sponge, releasing interstitial fluid and proteoglycans as compressive stress is applied. In addition, collagen fibers provide the tensile strength needed to endure shear stresses imposed under physiologic loading conditions. (Mow and Ratcliffe 1997)

The extracellular matrix of articular cartilage may be further separated into three zones, by distance from the chondrocytes. A pericellular matrix, approximately 200nm thick, consists of a dense shell of proteoglycans, non-collagenous proteins, and glycoproteins in direct contact with the cell membrane. The territorial matrix is marked by a network of

unoriented collagen fibers and a lower proteoglycan content than the pericellular matrix. Cells interact with the territorial zone via processes that traverse the pericellular matrix. The interterritorial matrix, which comprises the bulk of the matrix mass in articular cartilage, is characterized by oriented, large diameter collagen fibrils. (Buschmann 1992) The heterogeneous distribution and arrangement of ECM molecules with distance from the cells may lend insight to the assembly of matrix constituents and the regulation of their synthesis.

Articular chondrocytes can adapt to changing biomechanical conditions by changing the rates and ways in which ECM is remodeled. It has been shown that cyclic compression of cartilage explants and engineered cartilage constructs can differentially stimulate or suppress GAG or protein synthesis, depending on the frequency and magnitude of applied stress (Gray et al. 1989; Davisson et al. 2001). Static compression, in contrast, generally suppresses synthesis of proteoglycans and protein (Gray et al. 1988; Gray et al. 1989; Ragan et al. 2000). In addition, chondrocytes exhibit a similar rapid biosynthetic response to changes in pH and osmolarity of the interstitial fluid; it is thought that these changes in pH and osmolarity are related to the tissue's fixed charge density (FCD) and water content, which may be altered during loading of cartilage explants or constructs (Gray et al. 1988; Urban et al. 1993). As a compressive load is applied to the tissue, ECM molecules are compacted and interstitial fluid is driven to regions of lower pressure. This compaction of the ECM brings charged groups on the proteoglycans and collagen fibrils closer together, resulting in a local increase in the fixed charge density. Since pH and osmolarity are sensitive to fluctuations in electro- and chemical gradients, the local increase in fixed charge density influences local pH

and osmolarity, which in turn, may trigger a biosynthetic response through cell-surface ion channels, changes in cell volume, or an unknown mechanism. The relationships between fixed charge density, physicochemical parameters, and chondrocytic biosynthetic behavior are unclear, and require further investigation.

CARTILAGE PATHOLOGY

Cartilage is damaged through trauma, resulting in focal defects or disease, in which osteo- or rheumatoid arthritis leads to global joint degeneration (Mow and Ratcliffe 1997). Focal defects may be manifested as tears or punctures in the surface of the tissue, which result in disruptions of the otherwise continuous and smooth cartilage-cartilage joint interface. Such injury can lead to the onset of arthritic diseases as well. Osteoarthritis, most often diagnosed in older patients, similarly interferes with normal joint motion via increased joint surface roughness. The quantity of proteoglycans (and thus water), retained within cartilage has been shown to decrease with age, and the resultant increasing friction between joint surfaces leads to mechanical degradation of the cartilage; ultimately, arthritis is an adverse response to interactions between changing mechanical and degrading biochemical conditions within the joint. In addition, the normal wound repair responses – closure, scarring, remodeling – common to many tissues are highly attenuated in articular cartilage because the tissue lacks sufficient blood supply and cell population density for rapid matrix biosynthesis and motile activity. The biological limitations of the tissue permit perpetual joint degeneration from traumatic injury and diseases such as osteoarthritis.

Both injury and disease lead to some loss of joint function and are typically accompanied by pain; the most common clinical interventions include pharmaceuticals (analgesics), physical therapy, and corrective surgery. Analgesics relieve pain at the injury or disease site, and can restore some joint function (Pavelka 2000). Physical therapy has been shown to relieve pain and restore some function of osteoarthritis patients (Fransen et al. 1997). When a patient's joint has degenerated to the point of low- or immobility, a total joint replacement may be performed. In the case of a total knee replacement, the femoral chondyle and tibial head are shaved from the ends of the bones, exposing the medullary cavity of each bone. A prosthetic joint, made of titanium and ultra-high molecular weight polyethylene, is pressure-fit into the cavities of the bones and may be further fixed with bone cement. Contemporary joint replacements provide the patient with several years of use and approximately natural range of motion and functionality. The limitations of prosthetic joints, however, are a relatively short service life (~10 years), adverse redistribution of stress on the bones (which can lead to bone resorption), and lack of an adaptive response to changing biomechanical stimuli.

Newer approaches to repairing damaged cartilage include treating focal defects with autologous tissue or cells. Fragments of cartilage are harvested from the perimeter of the joint and surgically fixed in the defect; alternatively, chondrocytes may be isolated from the autologous tissue and implanted in the wound site (Hunziker 1999). These techniques are intended to restore the structure of the joint surface and underlying tissue by filling the defect space with viable tissue or a population of cells capable of producing new matrix. The reported short- and long-term results of these approaches are mixed, and donor site availability and morbidity may be limiting factors. The primary

benefit of biologic devices for cartilage repair is the potential for retaining the native tissue's adaptability to changing biomechanical conditions. The preservation of tissue remodeling mechanisms may yield a permanent repair of damaged tissue.

There is a clear need for tissue replacement therapies capable of restoring the structure and function of articulating joint surfaces while retaining the native tissue's adaptation mechanisms. The methods of clinically treating cartilage damage have historically focused on pain management, physical rehabilitation, or implantation of synthetic prosthetics. Biologic devices – including autologous tissue or cells – have emerged within the last five years as viable alternatives to traditional joint replacement techniques, and may offer the benefits of a longer service life and the ability to respond to changing mechanical forces.

ENGINEERED CARTILAGE

The field of research known as "tissue engineering" is a broad interdisciplinary approach to the design, construction, and evaluation of biological tissue replacement therapies (Langer and Vacanti 1993). It combines the study of cell biology, mechanics, and transport phenomena with the development of devices that generate new tissue through inductive or conductive mechanisms (Lanza et al. 1997; Glowacki 2001). Devices for cartilage repair, for example, may be entirely cell-based, in which a population of differentiated cartilage cells – chondrocytes – are grown *ex vivo* and implanted at the defect site; presumably, these cells will produce and remodel new cartilaginous tissue and eventually restore joint function. Another approach is entirely biomaterial-based, in which a biodegradable material is used as a temporary prosthetic and scaffold for

infiltration by cells in the surrounding healthy tissue (Solchaga et al. 2000; Gastel et al. 2001). Alternatively, cells may be used in conjunction with a biomaterial to increase the rate of new tissue formation; in this case the biomaterial acts as a scaffold for new tissue formation from the implanted cells and from native cells at the periphery of the defect site. Growth factors, like bone morphogenetic protein (BMP), transforming growth factor beta (TGF-β), basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF) have been shown to influence extracellular matrix production in chondrocytes (Sah et al. 1994; Sah et al. 1996; Arevalo-Silva et al. 2001; van den Berg et al. 2001). These and other growth factors may be delivered to the damaged tissue directly, within a degradable carrier, or from cells genetically engineered to express the growth factors, and may stimulate healthy cells to repair a joint defect (Hidaka et al. 2001). The roles of cells, scaffolds, and growth factors, as well as the interactions among them, in the formation of functional tissue are investigated in tissue engineering research.

The relatively simple structure and avascular, anueral physiology of articular cartilage make this tissue a good candidate for development as an “engineered” tissue. Some documented methods of engineering cartilage include seeding hydrogels, like alginate and agarose, or other polymeric scaffolds made of poly-lactic acid (PLA), poly-glycolic acid (PGA), or collagen with chondrocytes (Buschmann et al. 1992; Freed et al. 1993; Mooney et al. 1996; Ragan et al. 2000; Lee et al. 2001). Construct culture conditions can be static, in which media has no bulk flow, or dynamic, in which media flows through the constructs as in roller bottles, spinner flasks, or mixed plate type bioreactors (Freed et al. 1993; Freed et al. 1994; Freed et al. 1994; Freed et al. 1998). The scaffold

materials are biocompatible and degradable, and provide temporary mechanical support and favorable substrate chemistry as cells manufacture and deposit neotissue (Freed et al. 1994). In addition, these scaffolds may be shaped to guide the formation of anatomically correct tissue (Cao et al. 1997; Kim and Mooney 1998). Some challenges in finding a suitable scaffold include optimizing the degradation kinetics and ensuring that the cells of interest retain their phenotype upon adhesion to the scaffold surface. In addition, matching the material properties and shape of the scaffold with those of the native tissue to be replaced is an important clinical consideration.

Given that one goal of tissue engineers is to develop biological devices capable of restoring native tissue structure and function, it is clear that methods of predicting and evaluating the performance and behavior of these devices would be useful (Langer and Vacanti 1993). Though much empirical data has been reported for devices cultured *in vivo* and *in vitro*, a mechanistic approach to interpreting and modeling such data is necessary for its proper use in the rational design of future tissue engineered devices. Galban and Locke have proposed several models to describe cell growth in engineered scaffolds, and these models are based on fundamental equations of mass transfer (Galban and Locke 1999; Galban and Locke 1999; Galban and Locke 2000). With respect to the problem of modeling matrix synthesis in a cartilage repair device, one phenomenological model has been proposed (Gray et al. 1989), and one mechanistic model based on mass transport of oxygen to chondrocytes has been proposed (Obradovic et al. 2000). In reporting the latter model, which is most relevant to this work, Obradovic et al. hypothesized that regulation of GAG synthesis is governed by oxygen delivery to cells and can be described by equations of mass transfer. The

model predicts that as new tissue is formed, permeability of the construct drops and oxygen becomes less available to the cells. The model accounts for spatial variations in oxygen concentration within a cell-scaffold construct (due to local differences in permeability and diffusivity) and can estimate the temporal changes in quantity and location of GAGs within a construct. There are, to my knowledge, no published mechanistic models of collagen deposition or total construct mass for developing engineered cartilage.

The aim of this study is to develop mathematical models describing accumulation of the two major extracellular matrix constituents of articular cartilage – GAG and collagen – and the degradation of a polymeric scaffold in an engineered tissue construct. The models are based on general, hypothesized mechanisms governing these processes, which for this study are assumed to be independent, and the models are intended to guide future studies of the biophysics of cell metabolism and cell-matrix interactions. Elucidating the mechanisms underlying matrix biosynthesis and reliably estimating scaffold degradation kinetics through the use of these models may contribute to the design and optimization of engineered cartilage and other tissues. In addition, a model based on these accumulation and degradation models is proposed for predicting the total construct mass as a function of time. This modeling regime may, in the future, may be applied with known structure-property relationships to estimate the mechanical properties of maturing engineered cartilage constructs.

BIOLOGY AND MATHEMATICAL MODELING

Mathematical models are increasingly used to investigate the behavior and mechanisms of chemical, mechanical, and electrical phenomena observed in biological systems. For example, blood circulation, control of cell volume, muscle mechanics, nueral systems, population dynamics, and genetics are now rigorously studied through the development and application of mathematical models (Marsden et al. 2002).

Models are used to describe patterns in the observed data, and may be empirically derived (a.k.a. phenomenological modeling) or based on known mechanisms and derived from fundamental equations. In general, empirical models are designed to describe and predict the observed data well, and may consist of complex interactions between dependent and independent variables with little or no physical significance. In contrast, mechanistic models are intended to describe the processes governing observed phenomena with known and definable physical relationships between parameters. In engineering and other applications where "what" happens is more important than "how" it happens, empirical models are often adequate; for example, heat and mass transfer properties of non-biological systems are routinely calculated from empirical relationships (Incropera and Dewitt 1996). When studying and characterizing biological systems, however, a mechanistic description of events is often more useful than empirical descriptors.

As described above, there are few published models, empirical or mechanistic, for describing the macroscopic structural and functional properties of tissue constructs. Mathematical models may be beneficial to future investigations by providing information

about the long-term fate of the constructs and the biological mechanisms regulating construct development. This work focuses on the development of first-generation mathematical models, with mechanistic underpinnings, capable of describing construct composition as a function of *in vitro* culture time. In future work, these models may be adapted to incorporate the influences of more parameters and combined with empirical and mechanistic structure-property relationships to estimate construct mechanical properties.

METHODS

MODEL FORMULATION – MATRIX ACCUMULATION

A typical data set generated in a study of engineered cartilage (Buschmann et al. 1992) shows that as the construct GAG concentration increases with time, the GAG synthesis rate drops (Fig. 5). This response is also observed in chondrocytes in monolayer fed exogenous proteoglycans (Fig. 6) (Handley and Lowther 1977).

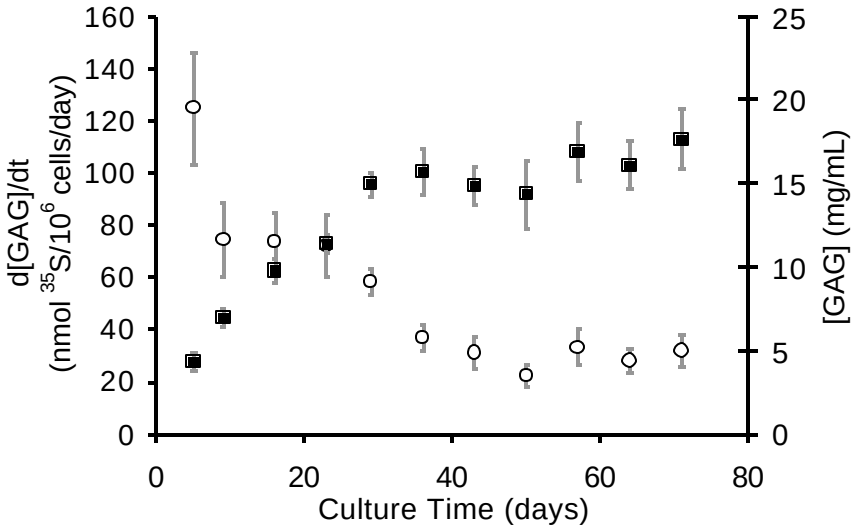

Figure 5. GAG concentration (■, right axis) and synthesis rate (○, left axis) as a function of culture time (Buschmann et al. 1992). Data reported as mean ± standard deviation (s.d.) (n=10—12).

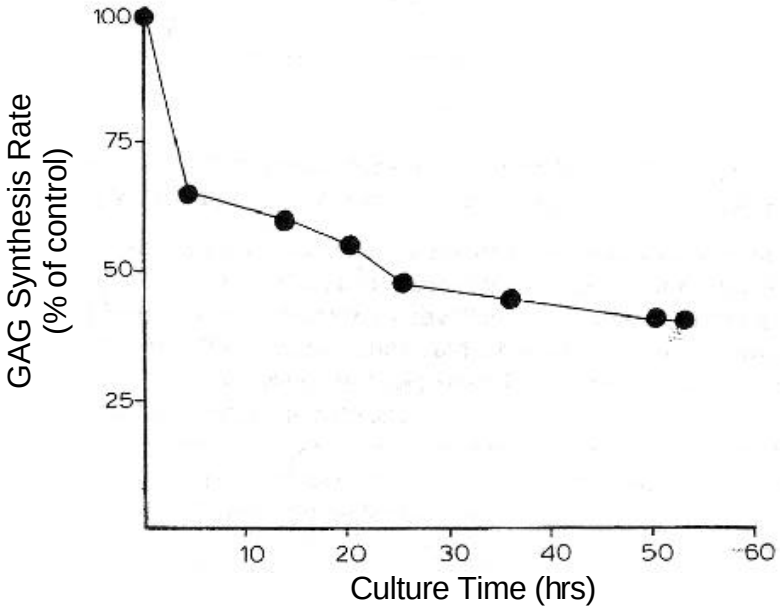

Figure 6. Rate of GAG synthesis (relative to controls) with time in culture with 10mg/mL exogenous proteoglycan (Handley and Lowther 1977).

The GAG synthesis rate data were determined by measuring the incorporation of radiolabeled sulfate or acetate. Using Buschmann's data, a strong negative correlation (r^2 = 0.82) was found between concentration and synthesis rate (Fig. 7). Chondrocyte monolayers have also been shown to respond to exogenous proteoglycans in an analogous, dose-dependent manner (Fig. 8) (Handley and Lowther 1977).

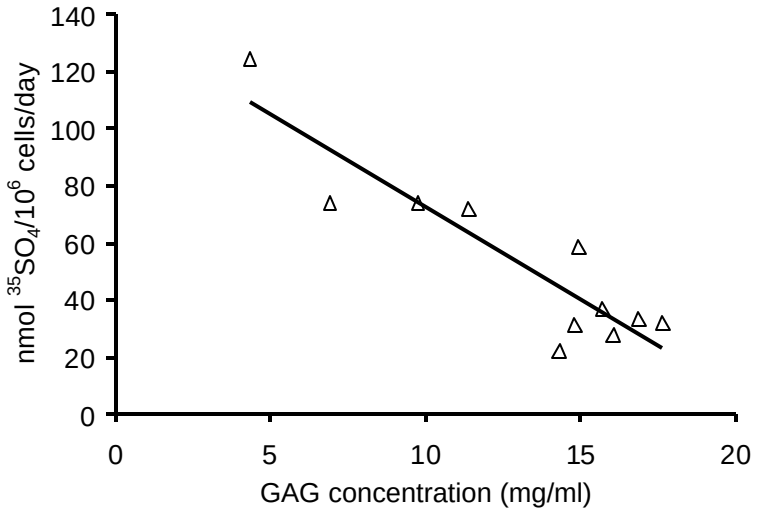

Figure 7. GAG synthesis rate vs. concentration.

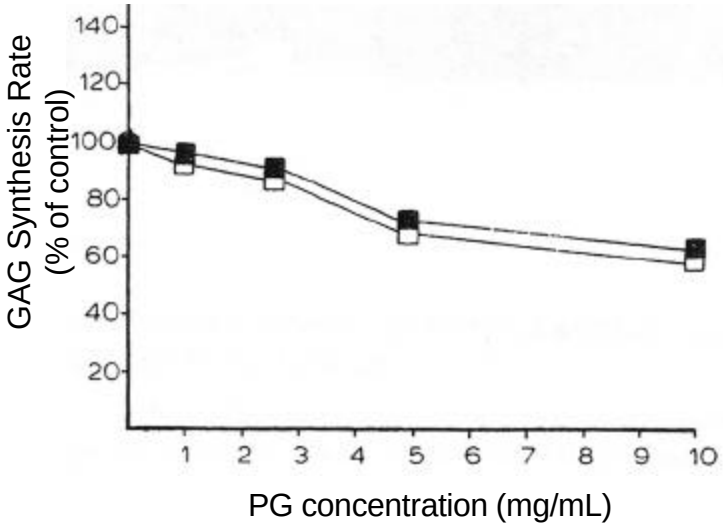

Figure 8. GAG synthesis rate (relative to control) vs. concentration of exogenous proteoglycans (Handley and Lowther 1977).

The negative correlation between synthesis rate and concentration of proteoglycan suggests that the accumulation of GAG is governed by a negative feedback, or product inhibition, loop. In other words, there is a "target" or steady-state concentration of each

matrix molecule that chondrocytes seek to maintain; as the concentration of each matrix component approaches the target concentration, the cells stop accumulating matrix by reducing synthesis and/or increasing turnover. Once the target concentration is reached, the cells balance synthesis and turnover, continuously remodeling the ECM. The idea of chondrocyte GAG synthesis regulation via GAG concentration in the matrix is not new; the mechanisms and biochemical mediators governing chondrocytes metabolism have been investigated since the 1970's (Handley and Lowther 1977; Sandy et al. 1980; Hascall et al. 1983; Boyle et al. 1995; Knudson et al. 2000).

In vivo, chondrocytes deposit matrix molecules in the pericellular space and rely on diffusion and fluid convection via the physiologic pump for transport of matrix molecules into the interterritorial space, where aggregation and polymerization occurs. During static *in vitro* culture of engineered constructs, diffusion may be limited by the scaffold material and convective forces may be absent altogether. As a result, the matrix molecules accumulate in the pericellular spaces and interterritiorial regions where diffusion is greatest, including peripheral regions of the construct (Fig. 9).

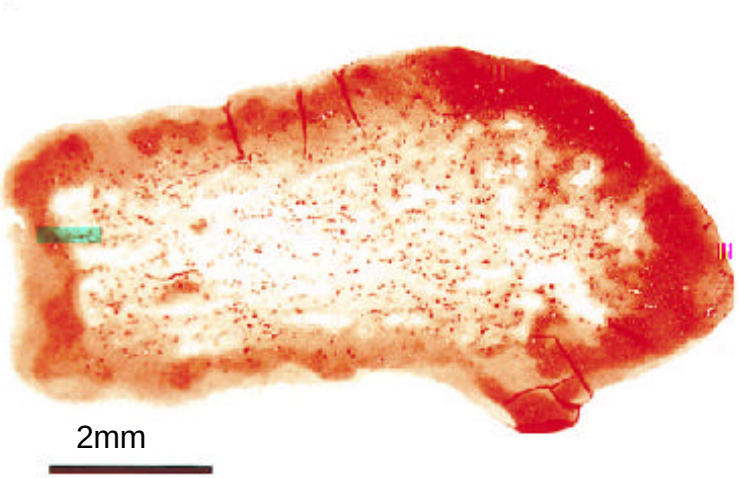

Figure 9. Chondrocyte-PGA construct after 12 days of *in vitro* culture in a bioreactor (Freed et al. 1998). Safranin-O (red) stains sulfated GAGs.

GAG and collagen molecules, when densely packed around a cell, provide a mechanically competent native tissue-like housing capable of changing pericellular osmolarity, permeability, and pH (Wilkins et al. 2000). These local physicochemical changes, which gradually occur with matrix molecule accumulation, may control cell metabolism by interfering with nutrient uptake and waste withdrawal, adjusting cell volume, or changing ion channel activity (Gray et al. 1988; Wilkins et al. 2000). Chondrocytes have also been shown to alter their biosynthetic activity in response to static and dynamic compression and hydrostatic pressure, in both engineered constructs and explants (Kim et al. 1994; Buschmann et al. 1996; Smith et al. 1996; Quinn et al. 1998). The mechanisms of this apparent mechanotransductive response are unclear, and may involve deformation of the cell membrane or nucleus, generation of streaming potentials, or compaction of the deposited matrix. Another potential negative feedback mechanism is based on a model of cell adhesion. Chondrocytes express adhesion proteins, known as integrins, on their cell surface and can form focal adhesions with extracellular matrix proteins like collagen. Integrins not only serve as anchors, however, since many have been associated with intracellular signaling pathways that govern cell phenotype and function (Qi and Scully 2000). Chondrocytes also express a hyaluronate-binding surface protein, CD44, which anchors the cells to proteoglycans in the pericellular spaces (Knudson and Knudson 1991). Such surface receptors and integrins, when binding with locally accumulated matrix molecules, may be a way for chondrocytes to “sense” the location and quantity of GAG and collagen. Through surface protein binding and subsequent signaling cascades, production of

matrix molecules may be down regulated in response to accumulation in the pericellular space.

Given the complexity of the system to be modeled, the first-generation matrix accumulation model is based on product-inhibition. The definition of product-inhibition, as adapted from The Compendium of Chemical Terminology, is the following:

> *A metabolic control mechanism in which the end product of a biochemical sequence is able to inhibit the rate of a process early in the sequence, thereby controlling the metabolic flux through this pathway* (McNaught and Wilkinson 1997).

This hypothesized relationship is stated mathematically:

(1) $$\frac{d[ECM]}{dt} \propto [ECM]_{SS} - [ECM]$$

The rate of net synthesis of a matrix molecule, $\frac{d[ECM]}{dt}$, is proportional to the difference between a steady state ("*SS*") concentration of that molecule ($[ECM]_{SS}$) and its current concentration. A rate constant, *k*, is substituted to form an equation,

(2) $$\frac{d[ECM]}{dt} = k\{[ECM]_{SS} - [ECM]\}$$

which upon rearrangement and integration,

(3) $$\int \frac{d[ECM]}{[ECM]_{SS} - [ECM]} = k \int dt$$

yields the following first-order model equation for matrix molecule accumulation:

(4) $$[ECM](t) = [ECM]_{SS}(1 - e^{-kt})$$

Once *k* is substituted with one over a characteristic time constant, τ, the final model equation is:

(5) $$[ECM](t) = [ECM]_{SS}(1 - e^{-\frac{t}{t}})$$

where $[ECM]_{SS}$ and τ are adjustable parameters and may be dependent on system factors including cell type and seeding density, the presence of growth factors, scaffold geometry and chemistry, and culture conditions (static vs. dynamic). In fitting this model to data on the biochemical composition of cell-scaffold constructs, least squares estimates of $[ECM]_{SS}$ and τ were found by non-linear regression. Each matrix component is assumed to accumulate independent of the other; this is an important assumption that needs to be validated in the future.

The form of the accumulation model describes an asymptotic exponential increase in matrix molecule concentration with time.

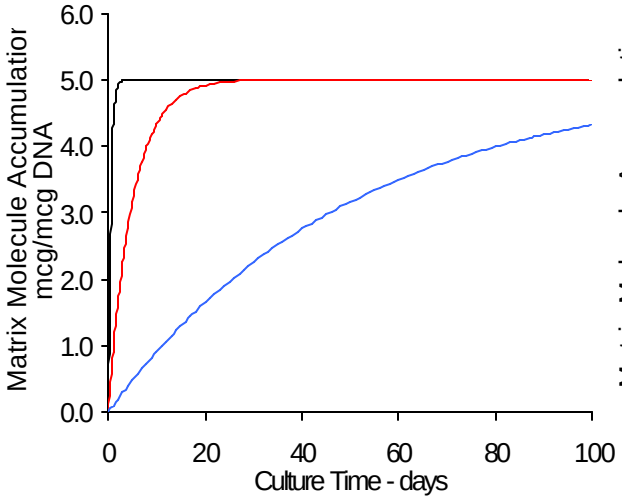

Figure 10. Effect of changing the time constant.
τ_1 (—) < τ_2 (—) < τ_3 (—)

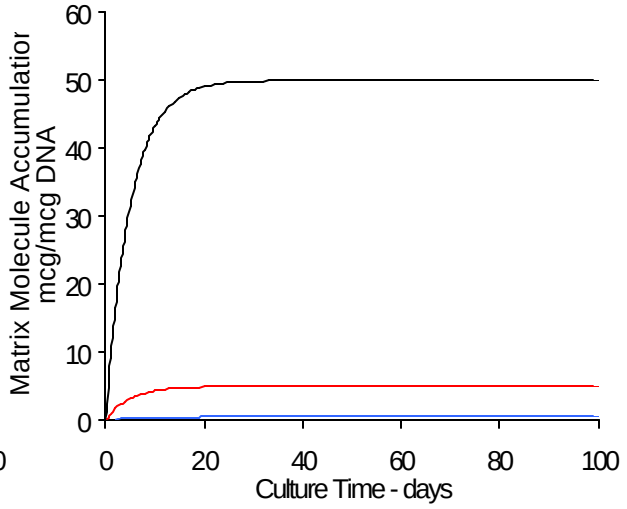

Figure 11. Effect of changing the steady-state matrix molecule concentrations.
$[ECM]_{SS1}$ (—) < $[ECM]_{SS2}$ (—) < $[ECM]_{SS3}$ (—)

In Fig. 10, $[ECM]_{SS}$ is held constant for changing τ, and Fig. 11 illustrates constant τ with changing $[ECM]_{SS}$:. At t = τ, approximately 63% of the steady state matrix molecule concentration has accumulated in the system. The time constant is large in

systems that produce ECM slowly, and the steady state matrix molecule concentration is largest for culture systems that yield the most matrix.

MODEL FORMULATION – SCAFFOLD DEGRADATION

There is a variety of scaffold materials being investigated for use in engineered tissues (Grande et al. 1997; Ishaug-Riley et al. 1998; Hutmacher 2000). Hydrogels and poly(α-hydroxy esters) are some of the most rigorously researched polymers since they exhibit long-term biocompatibility, are non-toxic, and have degradation kinetics that can be tailored to compliment neotissue growth kinetics (Hakkarainen et al. 1996).

For the purposes of this study, the scaffold degradation model was designed to describe the behavior of the poly(α-hydroxy esters). Poly-glycolic acid, poly-lactic acid, and their copolymers have been shown to support chondrocyte attachment and neotissue formation *in vitro* and in *in vivo* implantation experiments (Laitinen et al. 1993; Freed et al. 1994; Puelacher et al. 1994; Puelacher et al. 1994; Grande et al. 1997). These polymers undergo hydrolysis in which chain scission occurs at the ester bond, releasing monomers of glycolic or lactic acid (Hakkarainen et al. 1996). Under conditions in which diffusion is not greatly limited by scaffold geometry, porosity, or culture conditions, the rate of monomer release (and thus mass loss) is proportional to the amount of scaffold material remaining (Fig. 12) (Rodriguez 1996).

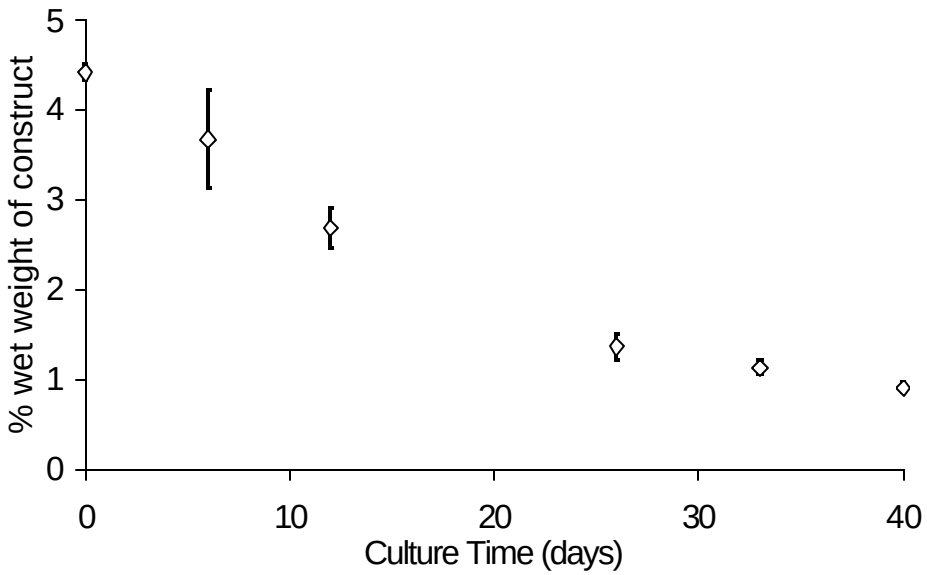

Figure 12. Degradation profile for a highly porous PGA scaffold (Freed et al. 1994). Data reported as mean + s.d..

In this way, the scaffold degradation obeys first-order decay kinetics:

(6) $$\frac{d[Scaffold]}{dt} \propto [Scaffold]$$

Again, a rate constant, *k*, converts the relationship to an equation:

(7) $$\frac{d[Scaffold]}{dt} = k[Scaffold]$$

which upon rearrangement and integration,

(8) $$\int \frac{d[Scaffold]}{[Scaffold]} = k\int dt$$

yields the following first-order model equation:

(9) $$[Scaffold](t) = [Scaffold]_0 e^{-kt}$$

Again, *k* is replaced by $1/t$ for the final model equation:

(10) $$[Scaffold](t) = [Scaffold]_0 e^{-\frac{t}{t}}$$

The initial scaffold mass, $[Scaffold]_0$, is known, leaving τ as the only adjustable parameter in this model. The effects of adjusting τ are illustrated in Fig. 13; a larger time constant indicates a longer degradation time.

Figure 13. Effect of changing the degradation time constant. τ_1 (—) < τ_2 (—) < τ_3 (—)

The model estimates that at t = τ, 63% of the initial scaffold mass has been lost through degradation. An important assumption is that degradation is independent of neotissue formation and cell activity. It is also significant that this model is dej -243uce

its place. In order to assess the maturity of an engineered construct, it may be useful to model the total mass as a function of culture time.

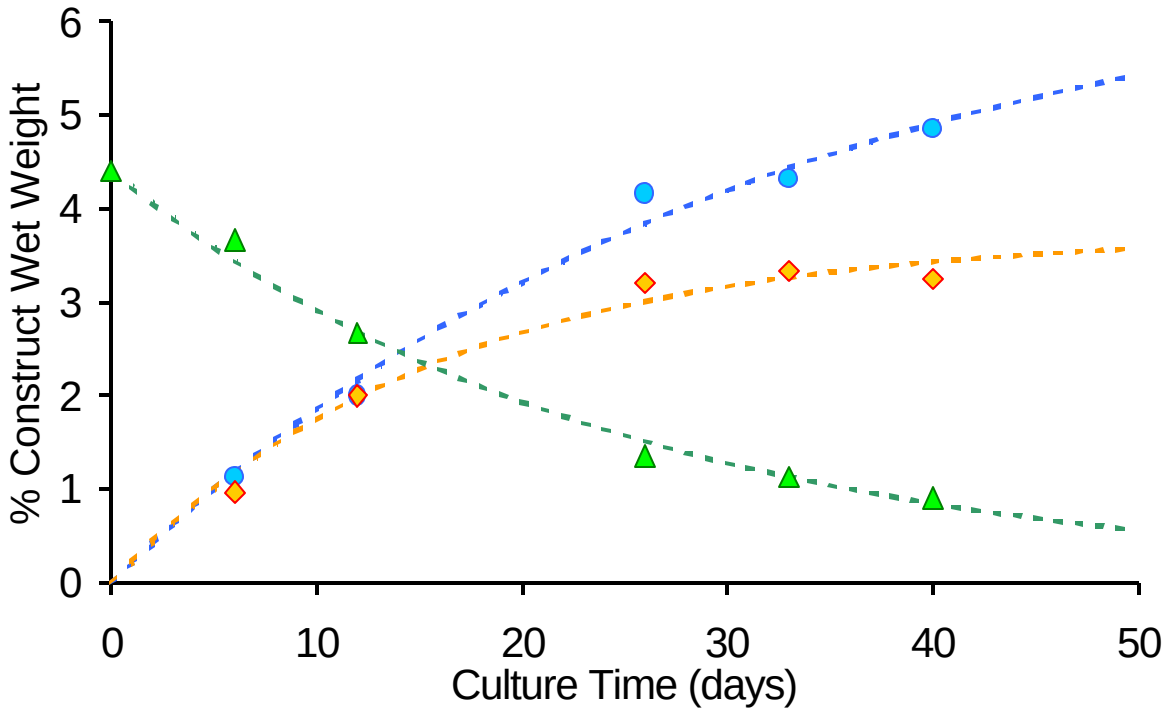

Figure 14. Competing processes of scaffold degradation and ECM accumulation (Freed et al. 1998): mean scaffold mass (▲); mean type II collagen (◈) content; mean GAG (⊙) content.

Given the previously described assumption of independence between degradation and accumulation, a preliminary approach to modeling the total construct mass is the simple addition of the scaffold degradation and matrix accumulation models. This approach is thus hypothesized to be capable of describing the total construct mass as a function of time, where construct mass, M(t) is given as:

(11)

$$\begin{aligned} M(t) = \ & \text{cell mass} + [GAG]_{SS}\left(1 - e^{-t/t_{GAG}}\right) \\ & + [Collagen]_{SS}\left(1 - e^{-t/t_{Collagen}}\right) \\ & + [Scaffold]_0 e^{-t/t_{Scaffold}} \end{aligned}$$

Important assumptions for this model include: 1) cell mass is constant, and 2) matrix accumulation and scaffold degradation are mutually independent processes.

Mathematically, the behavior of the total construct mass is governed by the competing degradation and accumulation models. For the purposes of engineering tissue replacements, it may be optimal for the scaffold degradation and matrix accumulation kinetics to be similar, leading to a near-constant scaffold mass with time. The behavior for different combinations of degradation and accumulation time constants can lead to rapid accumulation, rapid loss, or approximately steady-state mass conditions (Fig. 15). In both cases where the time constants are not equal, however, the model predicts an asymptotic approach to the initial construct mass at long times.

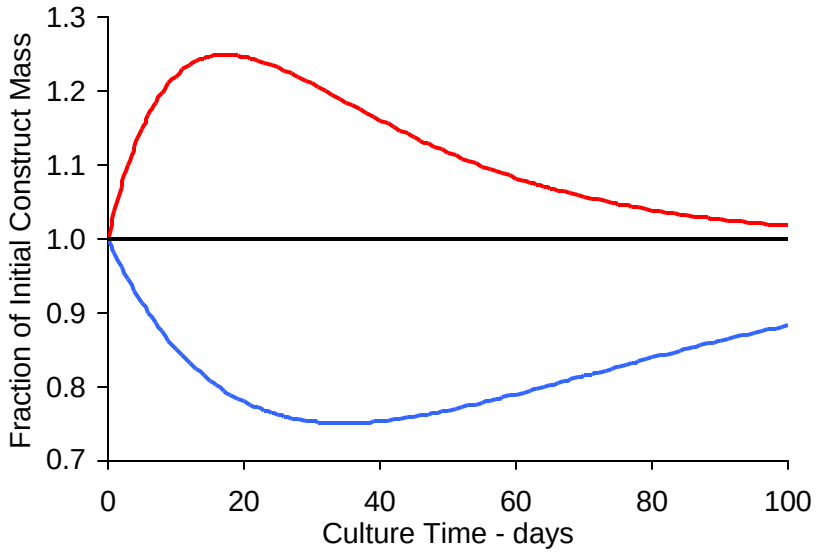

Figure 15. Effect of differing degradation and accumulation time constants on estimated construct mass: $\tau_{scaffold} = 2 \cdot \tau_{matrix}$ (——); $\tau_{scaffold} = \tau_{matrix}$ (——); $\tau_{scaffold} = 0.5 \cdot \tau_{matrix}$ (——).

FITTING THE MODELS

Data on matrix accumulation and scaffold degradation were gathered from previously published reports on engineered cartilage and typical scaffold materials. In most cases, image analysis software (Adobe Photoshop 5) was required to take average and standard deviation values from charts; in some cases, the data were obtained from tables. The accumulation and degradation models were fit to average data for each culture time by finding least squares estimates of the time constants ($\tau_{Collagen}$, τ_{GAG}, $\tau_{Scaffold}$) and steady-state matrix molecule concentrations ($[Collagen]_{SS}$, $[GAG]_{SS}$) via an unweighted quasi-Newton convergence method with Microsoft Excel spreadsheet software (initial conditions: $\tau = 1$, $[ECM]_{SS} = 1$). More specifically, the "Solver" analysis tool in Excel allowed the root mean square (RMS) deviation to be minimized by adjusting the relevant adjustable parameters (τ and $[ECM]_{SS}$ for accumulation, τ for degradation) as described above. The quasi-Newton minimization algorithm iteratively calculates RMS deviation as it changes the values of the adjustable parameters. Depending on the magnitude and sign of the difference in RMS deviation between iterations, the algorithm increases or decreases each of the adjustable parameters (two adjustable parameters for matrix accumulation, one for scaffold degradation until the RMS deviation is minimized. The quasi-Newton algorithm is considered a fast and reliable method of regressing up to as many as 100 parameters (Seber and Wild 1989). Strength of model fit was assessed via the coefficient of determination (R^2) and percent root mean square deviation (RMS deviation divided by the average value of the data). Fits were considered strong with $R^2 \geq 0.80$, and RMS deviation <50% were considered

reasonably accurate. For post-hoc tests of multiple comparisons, $p < 0.05$ were considered statistically significant.

EXPERIMENTAL METHODS

A two-week pilot study was performed to evaluate the expected variance in GAG and collagen mass measurements and reveal any difficulties with the experimental protocols. A total of 15 scaffolds (n = 5 at 4, 7, and 16 days of culture) were seeded with ~2.5 million chondrocytes and cultured in 6-well polystyrene culture plates with standard complete media. Increases in GAG and collagen content with culture time were observed and confirmed that our culture system behaves similarly to previously published culture systems. The pilot work revealed some difficulties in seeding the constructs, and it was determined that prewetting the scaffolds and seeding under dynamic conditions (on a horizontal shaker), rather than statically, would improve cell attachment and seeding efficiency. Initial cell counts and data from a DNA assay indicate that seeding efficiency ~85% was achieved with this method.

Variance data on the GAG and collagen contents from the pilot work were used to perform a sample size calculation for the main experiment. The SAS 8.0 package with the "UnifyPow" macro (O'Brien 1998) was used to find a minimum sample size that would provide enough data to find a significant difference between 6 culture time groups with 95% confidence. The results of this calculation indicated that for an experiment with 6 time points, twenty-four samples (four at each time point) would provide enough data to detect significance differences at that power. The main study was designed to include culturing twenty-four seeded constructs, with four constructs harvested after 1,

2, 4, 6, 8, and 10 weeks of *in vitro* culture. In parallel with this experiment, twenty-four unseeded (i.e., without cells), but otherwise similar, PGA/PLA constructs were cultured in standard complete media to gather data on scaffold degradation kinetics unique to this system. Again, four scaffolds at each culture duration were used. In all cases, scaffolds were randomly assigned to each group.

Bovine articular chondrocytes were harvested from the femoropatellar and glenohumoral grooves of a 2-3 month old calf (A. Arena Brothers, Hopkinton, MA), within six hours of slaughter. On a benchtop sterile field, chips of cartilage were carefully excised and placed in a room temperature solution of phosphate buffered saline (PBS) treated with antibiotics (penicillin and streptomyocin) and antimycotics (amphotercin). After washing with the PBS solution, the cartilage chips were treated with 0.3% collagenase in standard complete media (Ham's F-12, 10% Fetal Bovine Serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and .25μg/mL amphotericin B) overnight at 37°C on a horizontal shaker. The digest solution was then filtered through a 180μm nylon filter (Millipore) and the cell suspension was washed three times in the PBS solution. Cell counts were performed using a hemocytometer and viability assessed via trypan blue exclusion; the cell suspension was then stored in a 50mL polyethylene conical tube in a 37°C incubator until seeding (<48hrs). Cell viability was found to be approximately 95%.

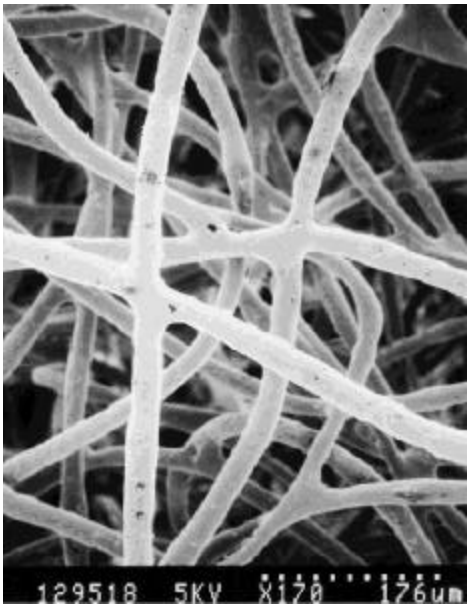

Figure 16. Scanning electron micrograph (SEM) of PGA fiber bonded with a 5% solution of PLA (Kim and Mooney 1998).

Construct scaffolds were made from non-woven poly-glycolic acid (PGA) fleece (Albany International Research, Mansfield, MA). A ½" diameter machinist's punch was used to cut forty-eight circular patches approximately 1mm thick from the fleece (Pazzano et al. 2000). The patches were immersed in a 1% w/v poly-l-lactic acid (PLLA) solution in methylene chloride for 10s seconds and allowed to dry in a fume hood for at least 10 minutes. PLLA is used to bond the PGA fibers, and enhances the stiffness of the scaffold (Fig. 16). The scaffolds were weighed, sterilized with UV light and ethanol for 30 minutes, and allowed to dry in a dessicator for at least 24 hours prior to seeding.

For 6 hours prior to seeding with cells, the sterilized scaffolds were prewet with complete media and incubated at 37°C in 95% humidity and 5% CO_2. The scaffolds to be seeded were then transferred to 15mL conical tubes with a 1mL solution of complete media with approximately 2.5 million cells for 12 hours at 37°C on a horizontal shaker. Once seeded, the cell-polymer constructs were transferred to 12-well plates for culture

under standard incubation conditions in 3mL media, with media changes every 2-3 days (Fig. 17). Unseeded scaffolds were cultured under identical conditions.

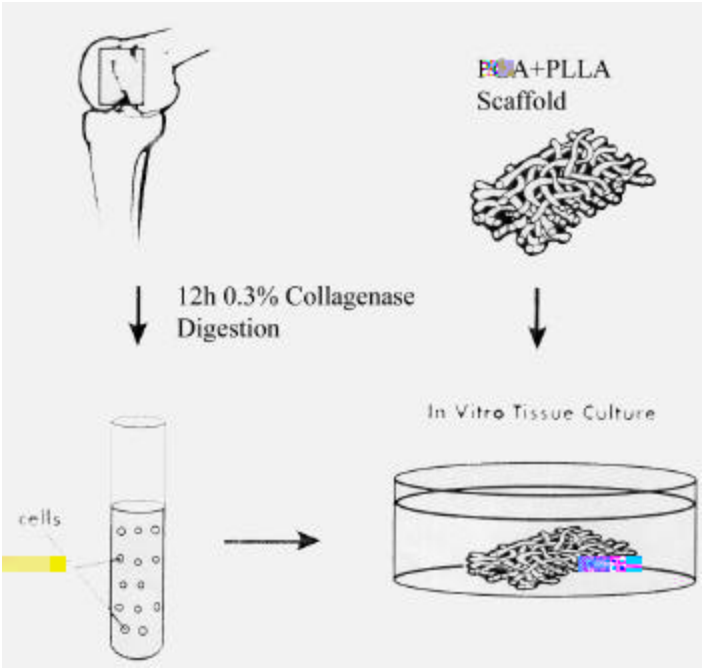

Figure 17. Schematic summary of cell isolation and construct culture methods. Adapted from (Freed et al. 1993).

At the end of the prescribed culture periods, constructs were removed from the plates, and laid on three Kimwipes prior to determining the wet weight. Next, the constructs were placed in 15mL conical tubes or 1.5mL cryovials, frozen, and lyophilized. After lyophilization, all constructs were weighed again to find the dry weight, and the cell-polymer constructs were treated with a 1mL papain (0.125mg/mL) digest solution overnight at 60°C. This digestion liberates extracellular matrix molecules and cells from the scaffold and allows for quantitative analysis of the construct biochemistry. All digests were stored at -20°C until use.

To determine the composition of the constructs, assays for GAG (Farndale et al. 1986), hydroxyproline (Woessner 1961), and DNA (Kim et al. 1988) content were performed. To measure the sulphated GAG content, the dimethylmethylene blue

spectrophotometric (525nm excitation, 592nm absorbance) method was used. Collagen content is correlated to the amount hydroxyproline, an amino acid found with known frequency within collagen molecules, in a construct. The assay includes acid hydrolysis of the construct digest, followed by reaction with chloramine-T and para-dimethylaminobenzaldehyde (pDMAB). A spectrophotometer (560nm absorbance) is used to assess the amount of hydroxyproline is present in the sample, and a 1:10 w/w ratio of hydroxyroline to collagen is used to calculate collagen content. The assays for GAG and collagen content were performed in triplicate. DNA content, an indicator of cell number, was measured by Hoechst 33258 dye binding using a fluorometer (358nm excitation/458nm absorbance); the DNA assay was performed in duplicate for each sample.

Additional Statistical Analysis

The GAG, collagen, scaffold, and DNA contents were normalized to construct dry weight, and Tukey's post-hoc test for multiple comparisons was used to determine statistical differences ($p < 0.05$ significant) between culture time groups.

RESULTS

The objectives of this work were to 1) develop a first-generation modeling regime for describing the composition of engineered cartilage constructs with culture time, 2) validate the models by fitting to previously published data and data generated in our culture system, and 3) apply the models to our culture system. Model development was described above, and the following shows that the second and third objectives were also met. The matrix accumulation model was validated by fitting to nine published data sets on GAG and collagen, as well as data generated in an experiment with our *in vitro* culture system; by both statistical metrics (R

construct dry mass) showed statistically significant differences between most groups with culture time, with increasing trends.

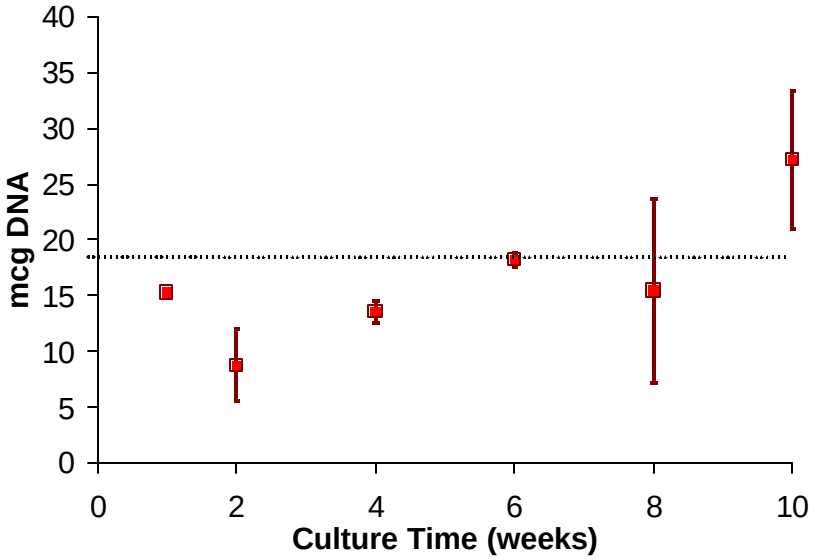

Figure 18. DNA content with time in culture. Dashed line indicates target DNA content from initial cell seeding density. Data reported as mean ± s.d. (n=3-4).

ACCUMULATION MODEL VALIDATION

The matrix accumulation model was fit to nine published data sets from a variety of scaffold systems and culture conditions. These nine data sets represent, to my knowledge, all publicly available reports of engineered cartilage with data on the temporal accumulation of GAG and/or collagen. Grande et al., studied the effects of different scaffold materials and culture conditions (static vs. dynamic) on matrix accumulation, but the reported data was limited to three time points, and consequently found inadequate for model fitting (Grande et al. 1997). In general, the model described the data for accumulation of two major matrix constituents – GAG and collagen – well with coefficients of determination (R^2) ranging from 0.77-0.99 and generally low root mean square deviations (4.6-37%) (Table 1, Figs. 19 & 20). The data are reported in several different units, though each matrix molecule mass measurement is normalized

to construct volume, mass, or cell count; it is also important to note that quantities of GAG and collagen in healthy native tissue are at least an order of magnitude higher than those observed in these studies. The strength of model fits and reasonably high accuracies found with these published data sets serve as a preliminary validation of the form of the model.

Table 1. Results of matrix accumulation model fits

SCAFFOLD	CELL TYPE	SOURCE	MATRIX COMPONENT	$[ECM]_{SS}$	τ_{ECM} (DAYS)	R^2	% RMS DEV.
Agarose	BAC	(Buschmann et al. 1992)	GAG	17.2 mg/mL	17.1	0.96	6.6
	Immortalized Mouse Chondrocytes	(Mallein-Gerin et al. 1995)	GAG	2.0 mg/mL	24.7	0.77	21.7
PGA	BAC	(Vunjak-Novakovic et al. 1996)	GAG	11.7 %dw	18.6	0.97	5.4
			Collagen	50.4 %dw	131.5	0.98	6.9
	BAC	(Freed et al. 1998)	GAG	6.8 %ww	31.3	0.99	5.9
			Collagen II	3.7 %ww	15.9	0.98	6.8
	BAC	(Vunjak-Novakovic et al. 1998)	GAG	1.6 %ww	22.2	0.99	4.6
			Collagen	2.4 %ww	16.7	0.98	9
	BAC	(Stading and Langer 1999)	Collagen	3.5 %w w	22.3	0.92	8.1
Alginate	BAC	(Beekman et al. 1997)	GAG	118.3 µg/µg DNA	86.4	0.89	29.0
			Collagen	27.5 µg/µg DNA	17.9	0.77	37.0
	BAC	(Ragan et al. 2000)	Collagen	15.7 µg/µg DNA	22.4	0.89	24.4
Self-Assembling Peptide	BAC	(Kisiday et al. 2001)	GAG	5.9 mg/mL	7.4	0.96	6.1
PGA/PLA	**BAC**	**This study**	**GAG**	**6.1% dw**	**187**	**0.95**	**13.5**
			Collagen	**6.5% dw**	**18.9**	**0.80**	**19.8**

The matrix accumulation behavior observed in this study (Fig. 21) is qualitatively similar to that reported by other investigators. In general, there was less GAG and collagen in the constructs than expected, though the synthesized matrix consisted of these components in nearly physiologic proportions (~3:1 collagen:GAG). All constructs were free of infection during culture, though one sample from the 8 week seeded group became mechanically unstable and disintegrated prior to harvest. The accumulation model was fit to the data for GAG and collagen accumulation with strong goodness of fit (R^2 = 0.95 and 0.80, respectively) and reasonable accuracy (RMS deviation = 13.5 and 19.8%, respectively).

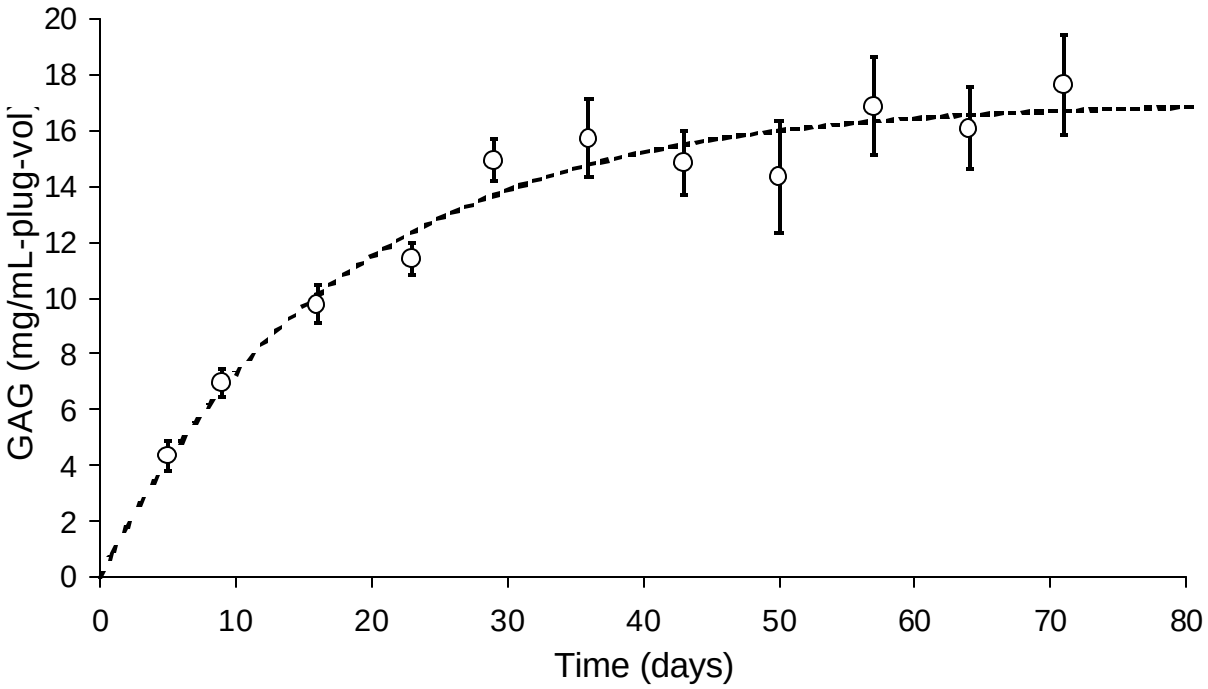

Figure 19. Best-fit curve for GAG accumulation in an agarose culture system (Buschmann et al. 1992). Mean ± s.d..

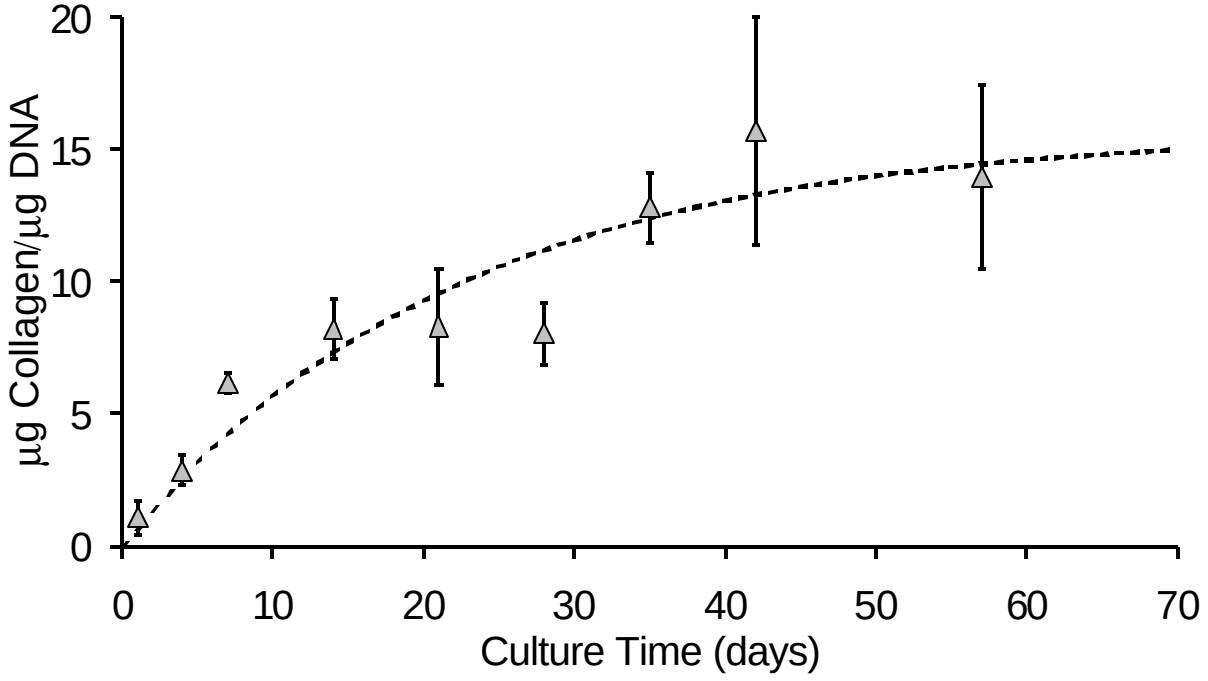

Figure 18. Best-fit curve for collagen accumulation in an alginate culture system (Ragan et al. 2000). Mean ± s.d.

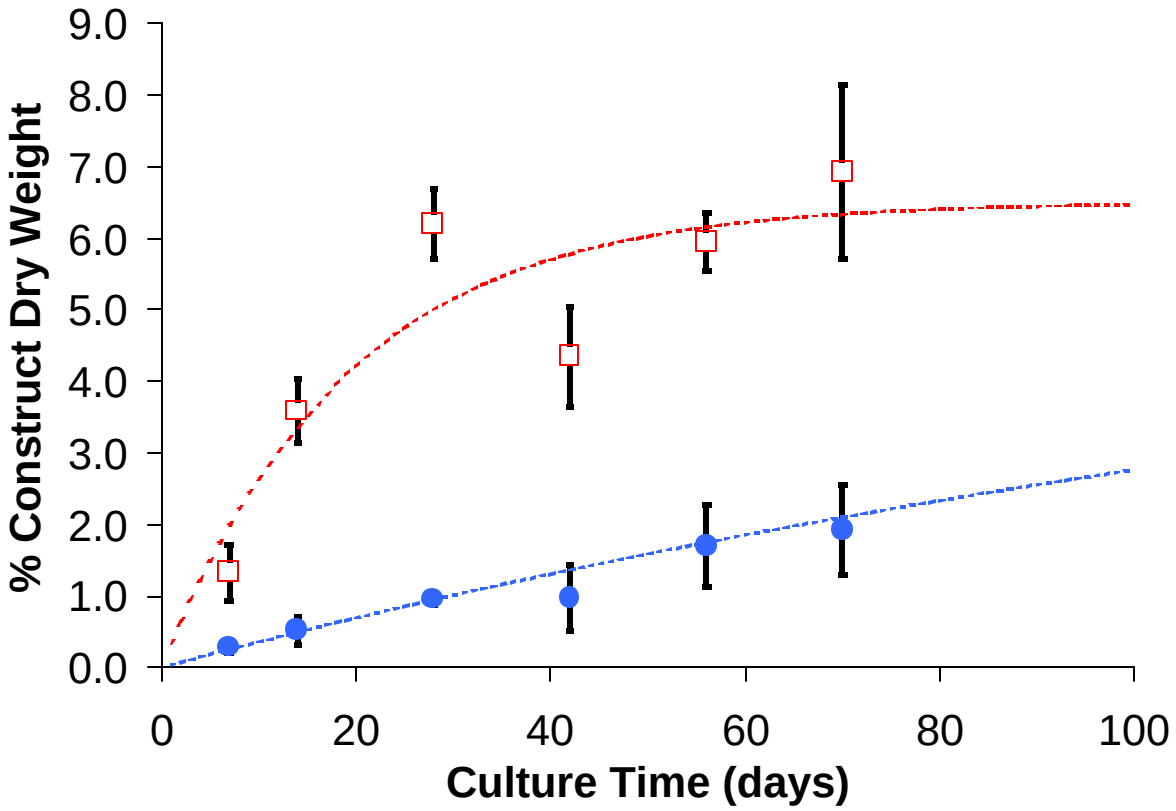

Figure 21. GAG (•) and collagen ( ) accumulation from this study, with best-fit curves. Data reported as mean ± s.d., n=3-4

DEGRADATION MODEL VALIDATION

The degradation model was fit to five published data sets for degradation of scaffolds with a variety of porosities, and each is based on PGA or PGA/PLA chemistry. In general, the degradation model described the data well with coefficients of determination between 0.75 and 0.99, and generally low root mean square deviations (4.8-27.5%); the best fits were to data on high porosity scaffolds (Table 2, Fig. 22). For scaffolds of relatively low porosity, the model fits were characterized by lower coefficients of determination and higher deviations. The model also described the degradation behavior observed in this study well (R^2 = 0.95), though with moderate accuracy (25% RMS dev.) (Fig. 23).

Table 2. Results of scaffold degradation model fits

Scaffold Material	Scaffold Porosity	Source	$\tau_{Scaffold}$ (days)	R^2	% RMS Dev
PGA	97%	(Freed et al. 1994)	56.1	0.94	13.8
		(Freed et al. 1998)	24.3	0.99	4.8
PGA/PLA	97%	**This Study**	**28.1**	**0.90**	**27.3**
50:50 PLGA	92%	(Agrawal et al. 2000)	70.0	0.89	10.1
	73%	(Lu et al. 2000)	175.1	0.87	18.8
75:25 PLGA	0%	(Li et al. 1990)	114.6	0.75	27.5

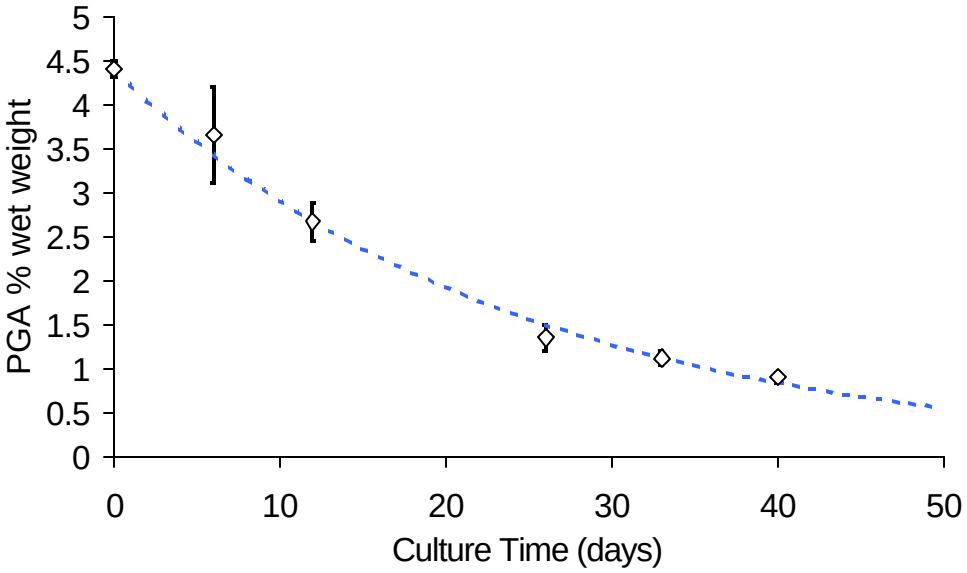

Figure 22. Best-fit curve for a highly porous PGA scaffold (Freed et al. 1994). Mean ± s.d.

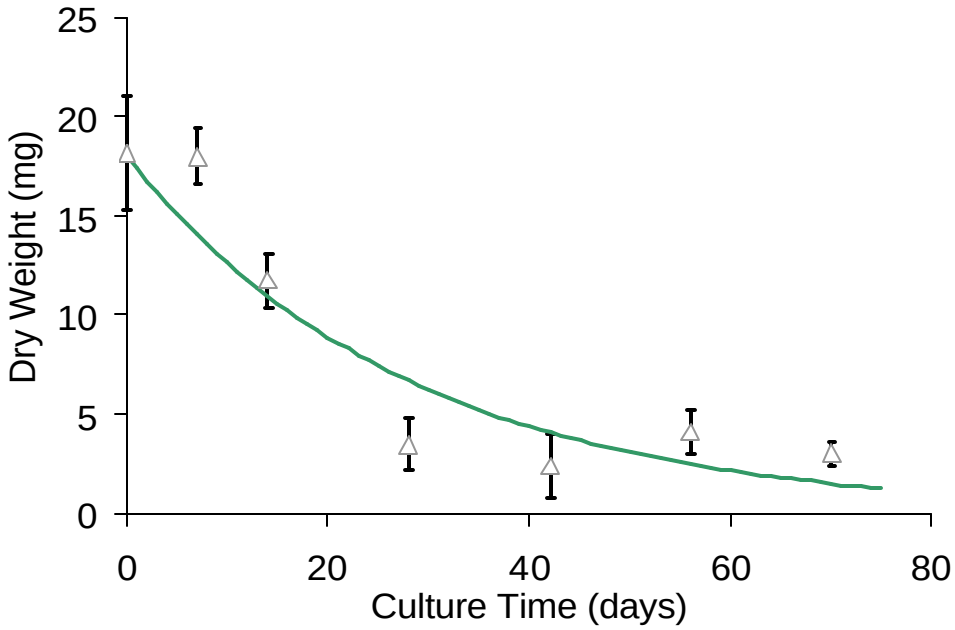

Figure 23. Best-fit curve for scaffold degradation in this study. Mean ± s.d., n = 4

ESTIMATING TOTAL CONSTRUCT MASS: APPLICATION OF THE MODELS

The predictive power of the matrix accumulation and scaffold degradation models was evaluated by using them to calculate total construct mass as a function of culture time. Construct dry mass measurements in this study are independent of the GAG, collagen, and scaffold degradation data, so a comparison of predicted and measured construct masses provides some insight to the predictive power of our models. The total mass calculation is an additive combination of the matrix accumulation model (fit to data on mass of GAG and collagen), the scaffold degradation model (fit to data on mass of scaffold), and a constant cell mass term (determined to be 0.213mg, using 10^{-11}g dry mass/chondrocyte). The calculated values at 7, 14, 28, 42, 56, and 70 days of culture were compared with measured values; again R^2 and RMS deviation were used as metrics for goodness of fit. In general, the total mass model describes the data well, with a coefficient of determination of 0.81 (Fig. 24). A root mean square deviation of 31% indicates that the total mass of an engineered tissue construct can be predicted with reasonable accuracy using data on GAG, collagen, and scaffold contents.

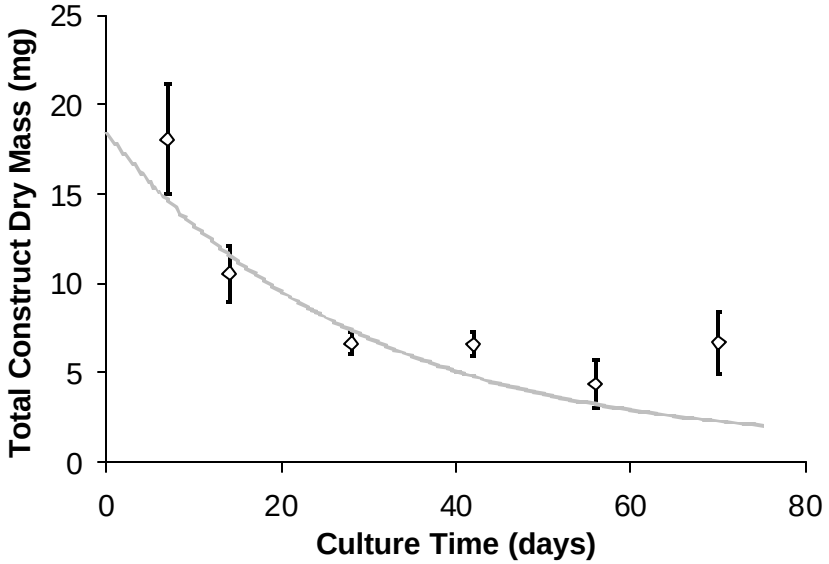

Figure 24. Predicted total construct mass (- - -) and measured construct mass over culture time. Mean ± s.d., n = 3-4.

parameters in our models may serve as metrics of construct performance, allowing for ready comparison between engineered tissue systems.

The scaffold degradation model, based on simple hydrolysis, was shown to describe the degradation of high porosity (> 90%) scaffolds well. The degradation of the polyesters PGA and PLA in aqueous media has been shown to occur by hydrolytic chain scission of the ester bonds. During conditions of essentially unlimited diffusion, the rate law for this reaction is simply first-order, and is characterized by an exponential decay of the mass of the polymer. This kinetics model was found to be suitable for some PGA and PGA/PLA scaffolds, and there appears to be a relationship between model fit and scaffold porosity. Given similar degradation conditions, scaffolds with lower porosity have lower coefficients of determination (R^2) and higher root mean square deviations than those with high porosity (>90%). The lower porosity scaffolds may interfere with the diffusion of water to, and of degradation products away from, some reaction sites; this would reduce the rate of scaffold mass loss, especially at early times. In addition, limiting diffusion may alter the degradation profile further by facilitating autocatalysis, which occurs when acidic monomers accumulate near reaction sites and catalyze the hydrolysis reaction (Li and McCarthy 1999; Lu et al. 2000). The presented degradation model cannot describe the effects of these factors well, and may only be suitable for scaffolds with some minimum porosity.

Applying our models to the problem of predicting total construct mass as a function of culture time yielded estimates in fair agreement with the measured values. The total mass model diverged from measured values most significantly at long culture times, but

described the general behavior well. Since the total mass estimates are dominated by the scaffold mass, it is difficult to draw any conclusions about the predictive power of the accumulation model.

RELEVANCE TO PUBLISHED MODELS

The model proposed by Obradovic et al. was reported to describe the temporal and spatial deposition of GAGs engineered cartilage with high accuracy (Obradovic et al. 2000). The mechanistic basis for their model lies in the hypothesis that the rate of GAG deposition depends on local oxygen concentration, as it was previously shown that GAG synthesis and tissue formation are sensitive to oxygen tension (Obradovic et al. 1997; Obradovic et al. 1999). The model equations are based fundamentally on conservation of mass, and describe the temporal changes in local oxygen concentration ($[O_2]$) and GAG concentration ([GAG]):

(12) $$\frac{\partial [O_2]}{\partial t} = D_{O_2}\left(\frac{\partial^2 [O_2]}{\partial r^2} + \frac{1}{r}\frac{\partial [O_2]}{\partial r} + \frac{\partial^2 [O_2]}{\partial z^2}\right) - r \cdot \frac{Q_m [O_2]}{[O_2]_m + [O_2]}$$

(13) $$\frac{\partial [GAG]}{\partial t} = D_{GAG}\left(\frac{\partial^2 [GAG]}{\partial r^2} + \frac{1}{r}\frac{\partial [GAG]}{\partial r} + \frac{\partial^2 [GAG]}{\partial z^2}\right) + r \cdot k \cdot \left(1 - \frac{[GAG]}{[GAG]_{SS}}\right)[O_2]$$

The first term on the right side of each equation describes the diffusion of each species through the construct. The second term in Eq. 12 describes oxygen consumption by the cells, and is assumed to follow Michaelis-Menten kinetics where ρ is the cell density, Q_m is the maximal oxygen consumption rate, and $[O_2]_m$ is the oxygen concentration at half-maximal oxygen consumption. The second term in Eq. 13 describes GAG synthesis kinetics, and shows a first-order dependence on $[O_2]$, where $[GAG]_{SS}$ is the maximum

GAG concentration and k is the rate constant. The authors note that the diffusion term in Eq. 13 is negligible throughout the construct (except at the surface) since D_{GAG} is low. Considering this simplification, the GAG accumulation model appears similar to the model presented in this work, with an added term for oxygen-dependence; in the model from this work, the cell density and oxygen concentration parameters are assumed to be lumped into the time constant, τ. Notably, the authors' sensitivity analysis indicate that adjustments of $[GAG]_{SS}$ and k generate the greatest deviation in predicted values from observed values of [GAG]. This suggests that despite the inclusion of several other adjustable parameters, including Q_m, D_{O_2}, D_{GAG}, and $[O_2]_m$, their model is largely consistent with the accumulation model proposed in this work. In addition, it indicates that the rate constant k (or time constant, τ, from this work) describing GAG synthesis kinetics is influenced by factors besides O_2 concentration. Overall, the model proposed by Obradovic et al. is more powerful than the matrix accumulation model from this work, since it is capable of describing both the temporal and the spatial distributions of GAG in developing engineered cartilage. When the first-order dependence on O_2 concentration is eliminated through spatial-averaging, however, the GAG accumulation kinetics equation is consistent with the model presented in this work.

LIMITATIONS OF THE PROPOSED MODELS

In general, the accumulation model can describe the rates and extent to which extracellular matrix components accumulate in engineered constructs with low to moderate deviation. The behavior of collagen and GAG accumulation in published reports and the data generated in this study is an exponential asymptotic increase with time. It is difficult, however, to compare the least-squares estimates of the time

constant and the steady state matrix molecule contents between data sets. First, the data are reported in several different units, and conversion to a common set of units requires data on construct volume, construct mass, or cell number that is not available in all reports. In addition, variation in the best-fit parameters between data sets is often the effect of multiple, confounded differences in experimental materials and methods. For example, the best-fit time constant that describes collagen accumulation data by Ragan et al. is 25% higher than the time constant for collagen data by Beekman et al., and the best-fit steady state collagen concentration is 43% lower in Ragan et al.'s study. These apparently dramatic differences in matrix accumulation are the result of numerous potential factors, including cell seeding density, concentration of alginate, scaffold geometry, age of the cell source, and cell isolation procedure. Both studies involve the *in vitro* culture of bovine articular chondrocytes seeded in an alginate scaffold, and the time-dependent biosynthesis pattern of the constructs is similar. But, it is important to consider that the observed differences in rate and extent of collagen accumulation may be attributed to numerous factors, and the effects of these factors are lumped within the adjustable parameters of the matrix accumulation model.

Another, less critical, limitation of the presented models is that they are not readily applicable to *in vivo* development of engineered tissue constructs. This is important because one premise of this work is that models can be used to estimate the properties – and by implication, therapeutic potential – of implantable engineered tissues. Rather than cultivate the constructs *ex vivo* under controlled conditions, some investigators have studied the development of the new tissue through subcutaneous implantation in immuno-deficient ("nude") animals (Laitinen et al. 1992; Puelacher et al. 1994;

Puelacher et al. 1994; Cao et al. 1997; Britt and Park 1998; Arevalo-Silva et al. 2001). A potential benefit of this approach is that the construct can be supplied with physiologic quantities of nutrients, growth factors, and other regulatory molecules by nearby blood vessels. In addition, observations can be made about the physiologic response to the constructs. Implanting these constructs into the body introduces several new factors that may influence scaffold degradation and matrix accumulation. For example, the immune-competent body's foreign body response (leading to fibrotic encapsulation) may expose the construct to a continuously changing cellular and biochemical environment. In addition, *in vivo* culture conditions may impose nonuniform stresses and strains from surrounding organs or structures on the scaffold and cells, which may influence the rate of growth or the organization of new tissue. The presented models are not capable of predicting the effects of these factors, and the differences in cell-scaffold behavior between *in vivo* and *in vitro* culture are unclear. So, it is important to note that our models may be used to estimate the composition of what goes into the body, but may not be useful in predicting the compositional fate of constructs *in vivo.*

FUTURE WORK

The modeling work presented here is a preliminary step in the development of more complex models capable of describing and revealing the nuances of matrix accumulation and scaffold degradation in engineered constructs. Investigation of the simplifying assumptions made in this study is a necessary next step.

The independence of proteoglycan and collagen accumulation is a critical assumption in the ECM accumulation model. The assumption is supported by experimental data

suggesting that chondrocytes turnover proteoglycans more rapidly than collagen; the half-life of proteoglycans *in vitro* is from days to weeks, whereas collagen is more stable and has a half life ranging from 300 days to years (Hay 1991; Boyle et al. 1995). In addition, the synthetic pathways for proteoglycans and collagen are markedly different, and this is expected given their distinct molecular structures. Some characteristics of the extracellular matrix structure and assembly process, however, indicate that the mechanisms regulating proteoglycan and collagen deposition may be coupled. The two biopolymers form an interpenetrating network in which steric and electrochemical interactions between the individual GAG and collagen molecules provide the bulk properties necessary for transmitting and absorbing physiologic loads *in vivo*. There is, presumably, some optimal ECM composition that balances the contributions of energy absorbing GAGs (and associated water) and reinforcing collagen. In addition, the extracellular formation of collagen fibrils is thought to rely on the presence of proteoglycans, and aggregation of proteoglycans has been shown to occur in interterritorial spaces of the extracellular matrix (Mow and Ratcliffe 1997), indicating another potential level of interaction between ECM components. These points of functional dependence between matrix components suggest that collagen accumulation may depend directly or indirectly on GAG content and vice versa.

It has been shown that treating cartilage explants with collagenase stimulates proteoglycan synthesis (Lee et al. 1994). It is unclear, however, whether collagen synthesis is influenced by depletion of matrix GAGs and whether engineered tissue would exhibit similar responses. One way of evaluating these questions is to perform an experiment in which engineered cartilage is cultured in the presence of an enzyme

that digests either the newly formed proteoglycans or collagen, and the relative collagen or GAG synthesis rates are measured. Conversely, constructs may be cultured in media supplemented with free collagen or GAGs. A control for these experiments is engineered cartilage cultured without the digestive enzymes or supplemented media. These experiments would provide data about how the *quantity* of each matrix component influences the synthesis and turnover of the other matrix component. An important aspect of the hypothesized dependence is the potential role of matrix molecule *arrangement* because chondrocytes may be sensitive not only to the composition of the surrounding matrix but also to its structure.

Another assumption made in this preliminary study is that scaffold degradation and matrix accumulation are independent. As discussed above, chondrocyte adhesion to substrates and neotissue in the pericellular spaces via surface proteins may modulate biosynthesis behavior, and it has been shown that surface chemistry (primarily hydrophilicity and polypeptide activation) influences cell adhesion to different polymeric scaffold materials (Ishaug-Riley et al. 1999; Pakalns et al. 1999). However, the effects of time dependent drops in scaffold mass, molecular weight, and surface area on chondrocyte biosynthesis have not been investigated. An experiment to assess the interaction between matrix deposition and scaffold degradation might involve seeding chondrocytes on scaffolds that have undergone various degrees of degradation and measuring the biosynthetic response, perhaps via radiolabeled sulfate and proline incorporation rates. This experiment may be performed with a variety of scaffold geometries and cell seeding densities in order to obtain information on these factors as well. Conversely, it is important to note that cell metabolism or neotissue formation may

contribute to scaffold degradation that is faster or slower than the degradation of an unseeded scaffold.

Second-Generation Models

The biosynthetic behavior of chondrocytes in an engineered tissue construct may be better modeled at the cell scale. A second-generation model for extracellular matrix accumulation may be developed to predict the response of individual cells. This modeling approach would be more useful in probing the regulatory mechanisms of ECM synthesis and turnover since the presented model inherently averages the responses of millions of cells. Measuring and modeling the temporal changes in pericellular environmental parameters like pH, osmolarity, and fixed charge density may clarify the relative contributions of each to the behavior of single cells.

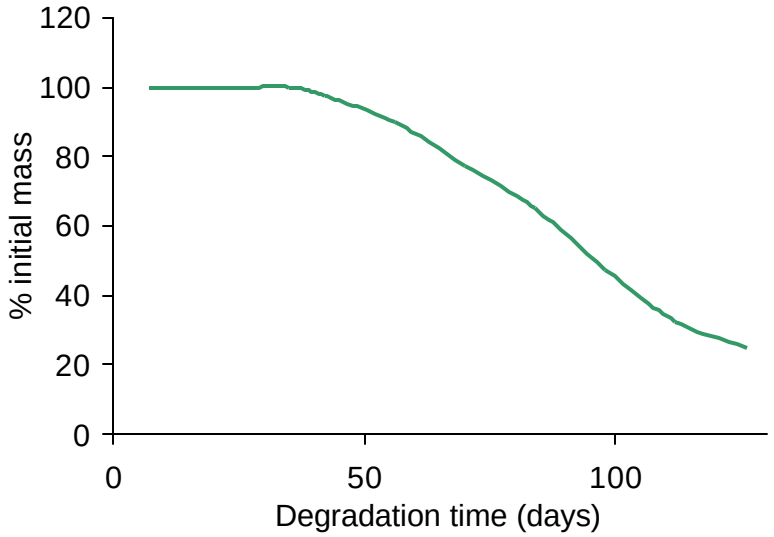

Figure 25. Degradation kinetics for a lower porosity (73%) PLGA scaffold (Lu et al. 2000).

The degradation of some polymeric scaffolds, including those with lower porosity and more hydrophobic surface chemistry, are not as well-described by the presented model. Deviation consistently arises at early degradation times when the model predicts an immediate drop in scaffold mass and the experimental data indicate a delay of days to

weeks before this drop occurs. This behavior is marked by a sigmoidal mass decay pattern, as illustrated in Fig. 25.

The plateau at early degradation times may be due to limited diffusion of water into the scaffold and degradation products from the scaffold. Erosion requires uptake of water by the scaffold and mass loss requires diffusion of degradation products from the scaffold into the aqueous environment. Low porosity, hydrophobic chemistry, and large geometries inhibit the transfer of water into, and diffusion of degradation products out of the scaffold. Scaffolds with these characteristics exhibit an initial "induction" period in which water is slowly absorbed, hydrolysis begins and molecular weight drops, but degradation products are still too big to move out of the scaffold. At the end of the induction period, the molecular weight of the degradation products has decreased sufficiently to allow for rapid diffusion into the media and scaffold mass loss is observed.

It has been suggested that Prout-Tompkins (PT) kinetics may be used to describe the degradation behavior of poly lactide-co-glycolide (PLGA) beads (Dunne et al. 2000). The PT equation (14) uses adjustable parameters t_{max} and τ, indicating the time to maximum rate of mass loss and the time constant, respectively:

(14)
$$[Scaffold](t) = \frac{[Scaffold]_0}{1 + e^{[(t - t_{\max})/t]}}$$

The PT equation has applications in drug delivery and other pharmacuetical applications (e.g., describing the hydrolysis of aspirin), though it was initially used to describe the thermal degradation of crystalline salts and was based on a nucleus branching mechanism (Prout and Tompkins 1944; Brown 1997; Brown and Glass

1999). The equation yields a sigmoidal decay curve, but a mechanistic understanding of its use in the context of this study is required.

Finally, a second-generation modeling scheme will include methods of predicting the mechanical properties of engineered constructs. It has been shown previously that the shear modulus of chondrocyte-PGA constructs depends on biochemical composition and microstructure (Stading and Langer 1999). Rarely, however, is a quantitative connection between construct composition and mechanical properties developed. A preliminary approach to developing this correlation will involve applying a rule of mixtures to determine the elastic modulus. Volumes of the scaffold and new tissue components will be found using the accumulation and degradation models ([Scaffold](t), [Collagen](t), and [GAG](t)) and the density of each constituent ($\rho_{Scaffold}$, $\rho_{Collagen}$, and ρ_{GAG}):

(15) Tissue: $$V_{Tissue} = \frac{[Collagen](t)}{r_{Collagen}} + \frac{[GAG](t)}{r_{GAG}}$$

Scaffold: $$V_{Scaffold} = \frac{[Scaffold](t)}{r_{Scaffold}}$$

The porosity of the construct may be estimated from these volumes and the gross volume of the construct (V_0) using the relationship:

(16) $$P = \frac{V_0 - (V_{Tissue} + V_{Scaffold})}{V_0} = 1 - \frac{(V_{Tissue} + V_{Scaffold})}{V_0}$$

For a structure with macroscopic porosity, like a cell-polymer tissue construct, the elastic modulus may be determined using the phenomenological relationship (Martin et al. 1998):

(17) $$E_{Structure} = E_{Solid}(1-P)^k$$

where *k* is an adjustable parameter. The elastic modulus of the solid part of the structure, E_{Solid}, is calculated by a volume-fraction-weighted (v_{Tissue} and $v_{Scaffold}$) addition of the moduli of the new tissue and the scaffold according to the two-phase Voigt model (Hashin and Shtrikman 1963):

(18) $$E_{Solid} = v_{Tissue}E_{Tissue} + v_{Scaffold}E_{Scaffold}$$

The volume fractions are defined in the following way:

(19) Tissue: $$v_{Tissue} = \frac{V_{Tissue}}{V_{Tissue} + V_{Scaffold}}$$

Scaffold: $$v_{Scaffold} = \frac{V_{Scaffold}}{V_{Tissue} + V_{Scafffold}}$$, and $v_{Tissue} + v_{Scaffold} = 1$

One simplifying assumption in this modeling scheme is that under applied stress, the scaffold and tissue components of the construct undergo equivalent deformation (Jones 1999). This may not be a reasonable assumption because the tissue and the polymeric scaffold are expected to have highly variable, spatially dependent viscoelastic properties. To address this issue, phenomenological relationships describing the elastic properties of a composite as a function of the constituents will be investigated (Kohles and Martinez 2000). Another significant simplification in this approach is the omission

of a fluid phase that is thought to contribute appreciably to the mechanical properties of native cartilage (Mow et al. 1980).

CONCLUSIONS

The three specific aims of this project were to 1) develop mathematical models for the description of construct matrix accumulation and scaffold degradation kinetics in engineered cartilage systems, 2) validate the models with previously published and experimental data, and 3) apply the models to experimental data. The models were derived from hypothesized mechanisms and are relatively simple: two adjustable parameters in an increasing, asymptotic exponential function for the accumulation model, and one adjustable parameter in a decreasing exponential function for the degradation model. A rigorous literature search revealed several studies of engineered cartilage with data on GAG and collagen accumulation, as well as several studies of polyester scaffold degradation; these data sets were used to validate the form of our models. In addition, a long-term cell-scaffold *in vitro* culture experiment yielded data for model validation. The fit of the matrix accumulation model to both GAG and collagen data from the literature and our experiment was moderate to strong as judged by the coefficient of determination (R^2), and the model described the behavior with reasonable accuracy as judged by the RMS deviation. Finally, the models were applied in the estimation of total construct mass as a function of culture time. This total mass model, an additive combination of the matrix accumulation and scaffold degradation models, predicted the observed evolution of construct mass well as judged by R^2 and with reasonable accuracy as judged by RMS deviation.

This work represents one of the first published attempts to characterize the patterns of temporal changes in biochemical composition of developing, engineered cartilage. It may be useful in the elucidation of mechanisms and factors contributing to the regulation of chondrocyte biosynthesis and scaffold degradation in *in vitro* culture systems. These models may be useful in designing cell-scaffold constructs and the culture conditions necessary for the generation of tissue with specified properties. In addition, these models may reduce the need for long-term culture experiments by reliably predicting long-term effects from data gathered in short-term studies.

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