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# Regeneration of Hyaline Cartilage Using a Mechanically-Tuned Chondrocyte-Seeded Biomimetic Tissue-Engineered 3D Scaffold: A Theoretical Approach

**Addie Hicks**

School of Biological and Health Systems Engineering, Arizona State University, Tempe, USA
Email: arhicks@asu.edu

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## Abstract

**The limited ability of cartilage tissue to repair itself poses a functionally impairing health problem. While many treatment methods are available, full restoration of the tissue to its original state is rare. Often, complete joint replacement surgery is required to obtain long-term relief. Tissue engineering approaches, however, provide new opportunities for cartilage replacement. They seek to provide mechanisms to repair or replace lost tissue or function. A theoretical method is presented here for regenerating hyaline cartilage *in vitro* using a chondrocyte-seeded three-dimensional biomimetic engineered scaffold with mechanical properties similar to those occurring naturally. The scaffold composition, type II collagen, aggrecan, hyaluronan, hyaluronan binding protein (for link protein), and BMP-7, were chosen to encourage synthesis of hyaline cartilage by providing a more native environment and signaling cue for the seeded chondrocytes. The scaffold components mimic the macrofibrillar collagen network found in articular cartilage. Type II collagen provides tensile strength, and aggrecan, the predominant proteoglycan, provides compressive strength.**

## Keywords

**Hyaline/Articular Cartilage, Type II Collagen, Aggrecan, BMP-7, Scaffold**

## 1. Introduction

The structural arrangement of hyaline cartilage allows for its unique function. It has an extracellular matrix

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(ECM) that consists predominantly of type II collagen, the proteoglycan aggrecan, and 80% by weight of tissue fluid. In addition, it contains a collection of other proteins (e.g. link protein, other collagens) and about 1% of its volume in chondrocytes [1] [2]. The type II collagen provides tensile strength to the tissue while aggrecan molecules and tissue fluid provide compressive strength [3]. The collagen fibrils combine with other proteins and macromolecules to form a macrofibrillar collagen network [1]. This type of connective tissue is avascular, and generally lacks the ability to repair itself. Damage to it can result in bone-to-bone contact and wear in joints and result in pain and disability. Injuries can persist for years and lead to further degeneration [2] [4]. In normal healthy adult tissue, chondrocytes don't proliferate, but maintain the extracellular matrix. When injury occurs and chondrocytes are damaged and die, there is only limited proliferation. The remaining lower density of the cells can dedifferentiate into a fibroblast-like phenotype and produce fibrocartilage instead of hyaline [5]. Chondrocytes have a rounded morphology and synthesize type II collagen as the dominant fibril while fibroblasts have a more flattened appearance and predominantly synthesize type I collagen (prevalent in fibrocartilage) [1] [5] [6].

Phenotype expression is influenced by signaling proteins such as TGF-$\beta$ and bone morphogenetic proteins (BMP's), which regulate growth, differentiation, and matrix synthesis [5] [7] [8]. BMP-7 plays a strong role in bone and cartilage formation and serves as a signaling cue during development [9] [10]. It is also present in adult cartilage. In a study conducted by Christian Kaps *et al*. [7], BMP-7 was shown to decrease production of type I collagen and simultaneously maintain synthesis of type II collagen in genetically modified bovine chondrocytes. The hyaline-specific proteoglycan aggrecan was also synthesized by these cells. R. T. Louwerse *et al*. [11] reported a linear increase in aggrecan production with increases in BMP-7 concentration. These data suggest that BMP-7 may be a good candidate growth factor to enhance regeneration of hyaline cartilage *in vitro*. Use of this growth factor could encourage maintenance of the chondrocytic phenotype and promote cell proliferation and production of hyaline extracellular matrix.

Tissue engineered scaffolds have emerged as a useful mechanism to regenerate tissue both *in vivo* and *in vitro* [12] [13]. They provide a tailorable substrate for cell attachment and growth and can be fabricated in two- and three-dimensional configurations. Three-dimensional scaffolds better simulate the *in vivo* environment and allow cells to move in any direction and behave more naturally [4] [14]-[16]. Scaffolds are fabricated from a variety of materials and with varying properties such as biocompatibility, porosity, mechanical strength, and biodegradability [17]. Natural materials (e.g. collagen, chitosan) as well as synthetic ones (e.g. poly (lactic-co-glycolic acid)(PLGA) or poly(L-lactic acid)(PLLA) have been the subject of many investigations. Roberts *et al*. reported that PEG hydrogels encapsulating hyaluronan and/or Link-N (a link protein peptide) aided in retaining cell-secreted glycosaminoglycans under loading [18]. Incorporation of chondroitan sulfate and hyaluronic acid into collagen II sponges was reported to increase chondrocyte phenotype maintenance and ECM synthesis [19]. Additionally, interest in the use of decellularized ECM for cartilage repair has grown and shows promise [20]-[22].

The approach proposed here is that for *in vitro* regeneration of hyaline cartilage, a tissue-engineered scaffold consisting of type II collagen, aggrecan, hyaluronan, link protein, and the growth factor BMP-7 can be synthesized to form a hyaline-like macrofibrillar collagen network scaffold (**Figure 1**) that is mechanically tuned. Since chondrocytes sense and respond to their environment [23] [24], when seeded and cultured on the extracellular matrix mimicking scaffold, the ECM produced by them may tend towards hyaline. Chondrocytes can be harvested from adult bovine articular cartilage, or differentiated from mesenchymal stem cells or adult stem cells [25]. Type II collagen was chosen because it composes approximately 90% of the fibrils present in hyaline cartilage and gives the tissue its tensile strength. Aggrecan was chosen because it is the most prominent proteoglycan in this tissue type and provides compressive strength [1] [4]. Hyaluronan binds to aggrecan, link protein, and chondrocytes and, therefore, will facilitate cross-linking of the network [26]. Link protein also binds directly to aggrecan [27]. BMP-7 was chosen because of its known effects on cartilage generation both during development and in experimental studies [28] [29]. Mimicking the naturally occurring macrofibrillar collagen network may provide an environment for chondrocytes that encourages them to synthesize hyaline. Unlike decellularized ECM, donor tissue would not be required. Also, seeding an adequate density of chondrocytes in the matrix and adding BMP-7 may create a more "developmental state" and induce production of this type of cartilage.

## 2. Theoretical Methods

Several combinations of type II collagen and aggrecan concentrations may be used to formulate the scaffolds

and determine the most effective mixture. Sample formulations are shown in **Table 1**. Comparison of the ECM synthesized by chondrocytes seeded on the different scaffold compositions will determine how the formulations affect ECM production and which one results in the most hyaline-like cartilage. Proteins and products for the scaffold are commercially available (Sigma). Protocols similar to those established by J. S. Pieper and colleagues [15] [30] may be used for scaffold synthesis and determination of collagen and aggrecan content.

## 2.1. Scaffold Synthesis and Cross-Linking

For the formulations in **Table 1**, porous collagen matrices can be prepared by incubation of 0.8 g (or 0.4 g) of insoluble type II collagen in 50 ml 0.5 M acetic acid (pH 2.5) at 4˚C for 16 hr, adding 50 ml of ice-cold DI water, and homogenizing the mixture in a blender. The collagen is then filtered (using 90 μm nylon gauze filters), deaerated under vacuum, and poured into polystyrene culture flasks. Next, it is frozen at −80˚C and lyophilized to produce the porous collagen matrices. A pore size of 100 μm may be a good target based on studies performed by J. K. Sherwood [16]. Cross-linking of the matrices is initiated by immersing them in an appropriate mixture containing 2 mg/ml (or 1 mg/ml) aggrecan, 0.3 mg/ml hyaluronan, 0.3 mg/ml hyaluronic acid binding protein (used for link protein), and 400 μg/ml BMP-7.

To establish collagen content, it is removed from the matrix with collagenase and the residual matrix weighed. Collagen is then expressed as a percentage of the original weight. Aggrecan (chondroitin sulfate) content can be determined by hexosamine analysis using p-dimethylaminobenzaldehyde after acid hydrolysis. Measurement of collagen and aggrecan quantities in the scaffold samples before and after ECM synthesis by the chondrocytes will allow determination of whether their content increased. Scanning electron microscopy (SEM) images of the scaffolds will enable examination of their structure and the incorporated molecules.

## 2.2. Characterization of Scaffold Mechanical Properties

To mimic the physical characteristics of articular cartilage, the mechanical properties of the scaffolds must be measured and optimized. The compressive modulus of bovine articular cartilage has been reported to be about

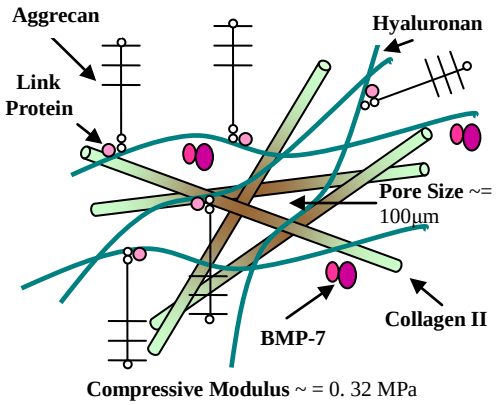


**Figure 1.** Depiction of biomimetic tissue-engineered 3D scaffold for generating hyaline cartilage.

**Table 1.** Representative scaffold formulations.

| Formulation | Concentration | |
|---|---|---|
| | Type II Collagen | Aggrecan |
| 1 | 8 mg/ml | 1 mg/ml |
| 2 | 8 mg/ml | 2 mg/ml |
| 3 | 16 mg/ml | 1 mg/ml |
| 4 | 16 mg/ml | 2 mg/ml |

0.32 MPa [31]. This value may be useful as a target. Tests to measure the scaffold stress, strain, yield and tensile strengths will facilitate determination of the compressive modulus. Protocols similar to J. K. Sherwood and colleagues [16] may be used for testing. Briefly, an Instron Testing machine (model 4201, Canton, MA) is used to test both tensile and compressive properties. For tensile testing, the scaffolds are placed in pneumatic grips with an external pressure of 30 psi. A strain rate of 0.1 mm/min is applied and the load (stress) recorded. The amount of displacement is measured with extensometers. For compression tests, the samples are placed between a compression plate on the top and an anvil on the bottom. Compression is carried out to between 7% and 20% strains (deformations) at a rate of 0.5 mm/min. Uniform deformation is assumed.

### 2.3. Chondrocytes

For *in vitro* studies, potential sources of chondrocytes are through differentiation of mesenchymal stem cells or adult stem cells into chondrocytes, or by isolation from the articular cartilage of adult bovine metaphalangeal joints. For isolation, the methods of J. S. Pieper *et al.* [15] can be utilized. Briefly, chondrocytes are harvested from the joints using RPMI 1640 medium containing collagenase, 0.1% penicillin, and 0.1% streptomycin. The cells are retrieved by centrifugation and resuspended in RPMI 1640, 10% fetal calf serum, 1% pyruvate, 0.1% penicillin, and 0.1% streptomycin. Use of the trypan blue staining/hemacytometer method will determine cell viability. Toxicity tests should also be performed to confirm scaffold biocompatibility. Chondrocytes can then be seeded onto the fabricated scaffolds of varying collagen and aggrecan concentrations (**Table 1**) at a determined cell density. One scaffold would be placed per substrate for each of the experimental conditions and replicates. Maintaining cultures for approximately 15 days will allow observation and characterization of cell behavior by examining proliferation, morphology, and ECM synthesis.

### 2.4. Proliferation

Measurement of DNA content using the Hoechst 33258 fluorescent dye assay will reveal cell proliferation activity. Cell-seeded scaffolds are incubated in 250 µl 0.2 M NaCl, 0.1 M NaAc, 0.01 M L-cysteine-HCl, and 0.05 M EDTA-$Na_2$ (pH 6.0) containing 16U papain for 16 hr at 65˚C. One hundred microliters of the mixture is combined with 100 µl 0.02 M Tris-HCl (pH 8.0) containing 0.1% sodium dodecyl sulphate. The solution is then incubated for 30 min at 60˚C. A 100 µl aliquot is removed and added to 1 ml of the Hoechst solution and analyzed for DNA. Fluorescence is measured at 365 nm for excitation and 458 nm for emission.

### 2.5. ECM Synthesis and Cell Morphology

Determination of the amount of ECM synthesized by chondrocytes on the scaffolds through measurement of the collagen and aggrecan content is described above. Further analysis of ECM may be made by comparing SEM images taken at various intervals (e.g. Days 1, 4, 7, 10, and 14) and determining if increases have occurred. Immunohistochemistry (IHC) staining will verify the presence of type II collagen and aggrecan. Cell morphology can also be observed at these intervals. Rounded somas are suggestive of the chondrocyte phenotype. A flattened somal appearance suggests that dedifferentiation has occurred and cells have reverted into a fibroblastic phenotype. IHC staining for the cellular marker Cathepsin B would also identify chondrocyte dedifferentiation.

## 3. Results

### 3.1. Scaffold

Results from the stress, strain, yield, and tensile strength measurements will provide information on the mechanical properties of the scaffold at the different formulations examined. Calculation and tuning (by adjustments to the formulations) of the compressive modulus in response to chondrocyte behavior will facilitate optimization of the scaffold. Analyzing the SEM images will identify the structural arrangement of the fibers, pore size, and macromolecule attachments.

### 3.2. Hyaline Regeneration

Analysis of the cell morphology, IHC staining, proliferation, and ECM synthesis results will inform as to phenotype maintenance (e.g. chondrocytes vs. fibroblasts) and whether or not the cells have proliferated or pro-

duced new matrix material. Increased quantities of ECM and immunohistochemistry identification of type II collagen and aggrecan would suggest that the biomimetic scaffold induced chondrocyte production of hyaline cartilage.

## 4. Conclusions of Theoretical Approach

Establishment of a mechanism to effectively regenerate hyaline cartilage *in vitro* with mechanical properties (e.g. compressive modulus about 0.32 MPa) similar to native tissue may have potential applications in cartilage repair and replacement. The tissue-engineered approach proposed here may minimize or eliminate dedifferentiation of chondrocytes and the subsequent synthesis of non-hyaline cartilage. Further studies *in vivo* using the chondrocyte-scaffold construct may show efficacy for cartilage replacement therapies.

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