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# Extraction techniques for the decellularization of tissue engineered articular cartilage constructs

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ABSTRACT

Several prior studies have been performed to determine the feasibility of tissue decellularization to create a non-immunogenic xenogenic tissue replacement for bladder, vasculature, heart valves, knee meniscus, temporomandibular joint disc, ligament, and tendon. However, limited work has been performed with articular cartilage, and no studies have examined the decellularization of tissue engineered constructs. The objective of this study was to assess the effects of different decellularization treatments on articular cartilage constructs, engineered using a scaffoldless approach, after 4 wks of culture, using a two-phased approach. In the first phase, five different treatments were examined: 1) 1% SDS, 2) 2% SDS, 3) 2% Tributyl Phosphate, 4) 2% Triton X-100, and 5) Hypotonic followed by hypertonic solution. These treatments were applied for either 1 h or 8 h, followed by a 2 h wash in PBS. Following this wash, the constructs were assessed histologically, biochemically for cellularity, GAG, and collagen content, and biomechanically for compressive and tensile properties. In phase II, the best treatment from phase I was applied for 1, 2, 4, 6, or 8 h in order to optimize the application time. Treatment with 2% SDS for 1 h or 2 h significantly reduced the DNA content of the tissue, while maintaining the biochemical and biomechanical properties. On the other hand, 2% SDS for 6 h or 8 h resulted in complete histological decellularization, with complete elimination of cell nuclei on histological staining, although GAG content and compressive properties were significantly decreased. Overall, 2% SDS, for 1 or 2 h, appeared to be the most effective agent for cartilage decellularization, as it resulted in decellularization while maintaining the functional properties. The results of this study are exciting as they indicate the feasibility of creating engineered cartilage that may be non-immunogenic as a replacement tissue.



## 1. Introduction

Injuries to articular cartilage, whether traumatic or from degeneration, generally result in the formation of mechanically inferior fibrocartilage, due to the tissue's poor intrinsic healing response [1]. As such, tissue engineering strategies have focused on developing replacement tissue *in vitro* for eventual *in vivo* implantation. One such strategy employs a "self-assembly process" [2] in which chondrocytes can be used to form robust tissue engineered constructs without the use of a scaffold.

Although engineered articular cartilage tissue has recently been created with biochemical and biomechanical properties in the range of native tissue values [3], there are currently two significant limitations to cartilage tissue engineering. First, human cells are scarce in number and difficult to procure, and passage of these cells leads to dedifferentiation [4]. These issues make the use of autologous cells for cartilage repair difficult. Additionally, the majority of cartilage tissue engineering approaches have employed bovine or other animal cells, and tissues grown from these cells are xenogenic. Thus, their use may result in a severe immune response following implantation, though this has not been fully elucidated.

It is believed that a decellularized xenogenic tissue may be a viable option as a replacement tissue, as the antigenic cellular material will be removed while preserving the relatively non-immunogenic extracellular matrix (ECM), as described in an earlier review [5]. Ideally, this will also preserve the biomechanical properties of the tissue. For instance, an acellular dermal matrix [6] has seen successful use clinically as the FDA approved Alloderm product. Additionally, acellular xenogenic tissues have been created for many musculoskeletal applications, including replacements for the knee meniscus [7], temporomandibular joint disc [8], tendon [9], and ACL [10], as well as in other tissues including heart valves [11–17], bladder [18], artery [19], and small intestinal submucosa [20,21].

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However, studies demonstrating the effects of tissue decellularization on cartilage are limited [22], and there are no studies demonstrating the effects of decellularization on musculoskeletal tissue engineered constructs. In the only other study examining cartilage decellularization [22], a photo-oxidation approach was employed to decellularize bovine xenografts, after which they were implanted *in vivo* into a sheep model. It was determined that the photo-oxidation approach, which resulted in nonviable chondrocytes, resulted in a reduced monocyte and plasma cell infiltration in the implant after 6 months. In this study, the primary benefit of using an approach involving tissue engineered cartilage constructs, rather than explants, is the potential to modulate the geometry and functional properties of the construct to match those of a specific patient or joint surface. Additionally, xenograft approaches are appealing, as animal tissue provides a relatively limitless and cost-effective cell source. However, decellularization of the xenograft tissue may be required to eliminate the immunogenicity of the tissue. While the use of decellularized xenograft explants can also be considered, both approaches must be explored before determining which approach should be pursued. As such, we are also pursuing an explant-based approach [23].

Therefore, the objective of this study was to determine the effects of multiple decellularization treatments on tissue engineered construct cellularity, biochemical, and biomechanical properties. A two-phased approach was used in which an appropriate agent for decellularization was selected in phase I, and an appropriate treatment time was selected in phase II. It was hypothesized that cells could be removed from self-assembled constructs while preserving the biochemical and biomechanical properties. To test this hypothesis, self-assembled articular cartilage constructs were cultured for 4 wks, and then treated with 1% sodium dodecyl sulfate (SDS), 2% SDS, 2% Tributyl Phosphate (TnBP), 2% Triton X-100, or a hypotonic/hypertonic solution, for either 1 or 8 h. These treatments were selected from prior literature [7–17,20,21], as well as from pilot experiments in our lab. Next, the treatment selected in phase I was applied for 1, 2, 4, 6, or 8 h in phase II. The effects of the decellularization treatments were assessed on construct cellularity and functional properties.

## 2. Materials and methods

### 2.1. Chondrocyte isolation and seeding

Cartilage was harvested from the distal femur of wk-old male calves [24–26] (Research 87, Boston, MA) shortly after slaughter, and chondrocytes were isolated following digestion with collagenase type 2 (Worthington, Lakewood, NJ). To normalize variability among animals, each leg came from a different animal, and cells from all legs were combined together to create a mixture of chondrocytes; a mixture of cells from five legs was used in the study. Cell number was determined on a hemocytometer, and a trypan blue exclusion test indicated that viability remained >90%. Chondrocytes were frozen in culture medium supplemented with 20% FBS (Biowhittaker, Walkersville, MD) and 10% DMSO at −80 °C for 1 day prior to use. After thawing, viability was greater than 90%. To construct each agarose well, sterile, molten 2% agarose was added to wells of a 48-well plate, and a stainless steel mold consisting of 5 mm dia. × 10 mm long cylindrical prongs was placed into a row of the plate to construct the cylindrical molds. The agarose solidified at room temperature for 60 min, after which the mold was removed from the agarose. Two changes of culture medium were used to completely saturate the agarose well by the time of cell seeding. The medium was DMEM with 4.5 g/L-glucose and L-glutamine (Biowhittaker), 100 nM dexamethasone (Sigma, St. Louis, MO), 1% Penicillin/Streptomycin/Fungizone (P/S/F) (Biowhittaker), 1% ITS+ (BD Scientific, Franklin Lakes, NJ), 50 μg/mL ascorbate-2-phosphate, 40 μg/mL L-proline, and 100 μg/mL sodium pyruvate (Fisher Scientific, Pittsburgh, PA). To seed each construct, $5.5 \times 10^6$ cells were added in 100 μL of culture medium. Constructs formed within 24 h in the agarose wells and were cultured in the same well until $t = 10$ days, after which they were unconfined for the remainder of the study, as described previously [27]; $t = 0$ was defined as 24 h after seeding. Throughout the studies, constructs were cultured in an incubator at 37 °C and 10% $CO_2$.

### 2.2. Decellularization phase I

At $t = 4$ wks, self-assembled constructs ($n = 6$/group) were removed from culture and exposed to one of five decellularization treatments, for either 1 h or 8 h. The decellularization treatments included:

1) 1% SDS
2) 2% SDS
3) 2% TnBP
4) 2% Triton X-100
5) Hypotonic/hypertonic Solution (half-time of each)
   a. Hypotonic: 10 mM Tris HCl, 5 mM EDTA, 1 μM PMSF
   b. Hypertonic: 50 mM Tris HCl, 1 M NaCl, 10 mM EDTA, 1 μM PMSF

All treatments included 0.5 mg/mL DNase Type I, 50 μg/mL RNase, 0.02% EDTA, and 1% P/S/F, in PBS. Both 1 h control and 8 h control groups were exposed to this same solution without detergent treatments. These treatments were applied at 37 °C with agitation. Following the 1 h or 8 h treatment, the constructs were washed for 2 h in PBS at 37 °C with agitation. Additionally, an untreated control was assessed immediately following construct removal from culture, without the treatment or wash steps.

### 2.3. Decellularization phase II

At $t = 4$ wks, self-assembled constructs ($n = 6$/group) were removed from culture and exposed to 2% SDS for 1, 2, 4, 6, or 8 h. As in phase I, all treatments included 0.5 mg/mL DNase Type I, 50 μg/mL RNase, 0.02% EDTA, and 1% P/S/F, in PBS. These treatments were applied at 37 °C with agitation. Following the SDS treatment, the constructs were washed for 2 h in PBS at 37 °C with agitation. Additionally, an untreated control was assessed immediately following construct removal from culture, without the treatment or wash steps.

### 2.4. Histology

After freezing, samples were sectioned at 14 μm. To determine construct cellularity, a hematoxylin & eosin (H&E) stain was used. A Safranin-O/fast green stain was used to examine GAG distribution [28,29], and picrosirius-red was employed for collagen content.

### 2.5. Quantitative biochemistry

Samples were frozen overnight and lyophilized for 48 h, followed by resuspension in 0.8 mL of 0.05 M acetic acid with 0.5 M NaCl and 0.1 mL of a 10 mg/mL pepsin solution (Sigma) at 4 °C for 72 h. Next, 0.1 mL of 10× TBS was added along with 0.1 mL pancreatic elastase and mixed at 4 °C overnight. A Picogreen® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) was used to assess total DNA content. GAG content was quantified using the Blyscan Glycosaminoglycan Assay kit (Biocolor), based on 1,9-dimethylmethylene blue binding [30,31]. After hydrolysis with 2 N NaOH for 20 min at 110 °C, total collagen content was determined using a chloramine-T hydroxyproline assay [32].

### 2.6. Indentation testing

Samples were assessed with an indentation apparatus, as described previously [33]. A 0.7 g (0.007 N) mass was applied with a 1 mm flat-ended, porous indenter tip, and specimens crept until equilibrium, as described elsewhere [2]. For the constructs treated for 1 h with the hypotonic/hypertonic solution and 8 h with 1% SDS, 2% TnBP, or 2% Triton X-100, a 0.27 g (0.0027 N) mass was applied instead such that all constructs would experience a similar strain during indentation testing. Examined over the entire study, strains from 3 to 9% were recorded. The thickness was measured using digital calipers with accuracy to 0.01 mm. Preliminary estimations of the aggregate modulus of the samples were obtained using the analytical solution for the axisymmetric Boussinesq problem with Papkovich potential functions [34,35]. The sample's biomechanical properties, including aggregate modulus, Poisson's ratio, and permeability were then calculated using the linear biphasic theory [36].

### 2.7. Tensile testing

A uniaxial materials testing system (Instron Model 5565, Canton, MA) was employed to determine tensile properties with a 50 N load cell, as described previously [37]. Briefly, samples were cut into a dog-bone shape with a 1-mm-long gauge length. Samples were glued to paper tabs with cyanoacrylate glue outside of the gauge length. The 1-mm-long sections were pulled at a 1% constant strain rate. All samples broke within the gauge length. The gauge length, thickness, and initial cross-sectional area were measured using digital calipers. For each construct, a stress–strain curve was created from the load–displacement curve and Young's