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# Time-dependent functional maturation of scaffold-free cartilage tissue analogs

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ABSTRACT

One of the most critical parameters in cartilage tissue engineering which influences the clinical success of a repair therapy is the ability to match the load-bearing capacity of the tissue as it functions in vivo. While mechanical forces are known to positively influence the development of cartilage matrix architecture, these same forces can induce long-term implant failure due to poor integration or structural deficiencies. As such, in the design of optimal repair strategies, it is critical to understand the timeline of construct maturation and how the elaboration of matrix correlates with the development of mechanical properties. We have previously characterized a scaffold-free method to engineer cartilage utilizing primary chondrocytes cultured at high density in hydrogel-coated culture vessels to promote the formation of a self-aggregating cell suspension that condenses to form a cartilage-like biomass, or cartilage tissue analog (CTA). Chondrocytes in these CTAs maintain their cellular phenotype and deposit extracellular matrix to form a construct that has characteristics similar to native cartilage; however, the mechanical integrity of CTAs had not yet been evaluated. In this study, we found that chondrocytes within CTAs produced a robust matrix of proteoglycans and collagen that correlated with increasing mechanical properties and decreasing cell-matrix ratios, leading to properties that approached that of native cartilage. These results demonstrate a unique approach to generating a cartilage-like tissue without the complicating factor of scaffold, while showing increased compressive properties and matrix characteristics consistent with other approaches, including scaffold-based constructs. To further improve the mechanics of CTAs, studies are currently underway to explore the effect of hydrodynamic loading and whether these changes would be reflective of in vivo maturation in animal models. The functional maturation of cartilage tissue analogs as described here support this engineered cartilage model for use in clinical and experimental applications for repair and regeneration in joint-related pathologies.

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## 1. Introduction

Articular cartilage is a dense tissue lining the joint surface that is defined by the unique zonal architecture of extracellular matrix constituents and chondrocytes, as well as by its load bearing capacity during daily physiological activities. As a consequence of the avascular and aneural nature of this specialized tissue, cartilage has limited healing capacity following injury. Surgical methods to repair chondral defects include abrasion arthroplasty (Johnson, 1986), subchondral drilling (Insall, 1974), and osteochondral autografts (Hangody et al., 2004). Currently, there exists only one FDA approved cell-based regenerative approach, termed, autologous chondrocyte implantation (Brittberg et al., 1994). This approach utilizes patient cartilage biopsies that are digested for chondrocyte harvest and expansion, with subsequent re-implantation of the cells at the site of cartilage damage to induce regeneration. All of these techniques encounter limited success due to issues which include fibrocartilage formation, chondrocyte de-differentiation, and lack of tissue integration and mechanical support (Furukawa, 1980; Hunziker, 2002). To address these limitations, cartilage tissue engineering aims to repair cartilage by recapitulating the matrix architecture, cellular composition, and mechanical properties through the use of various design platforms, including scaffolds, cell sources, and environmental signaling cues to guide tissue regeneration and achieve native cartilage properties.

Research in this field is active, with numerous approaches to cartilage tissue engineering, and these methods can be generally divided into two groups: scaffold-based vs. scaffold-free constructs. Scaffold-based methods include the use of natural and

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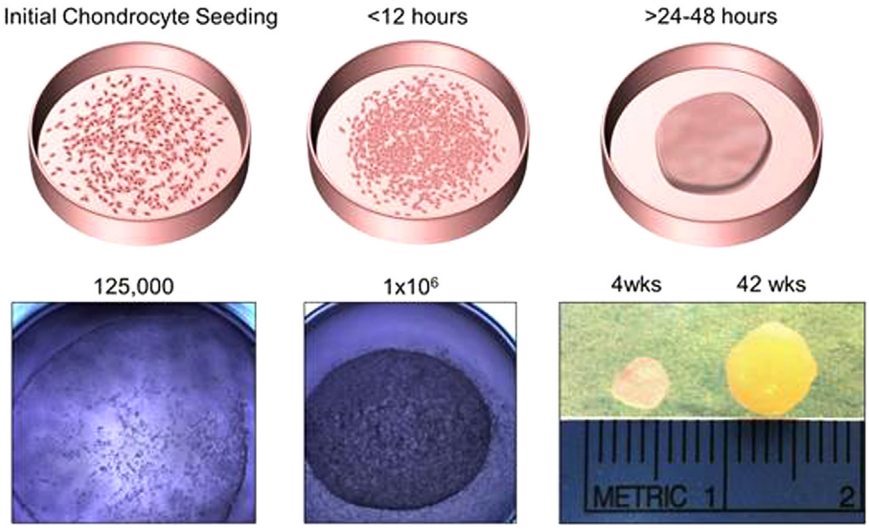


**Fig. 1.** Schematic of CTA fabrication: chondrocytes seeded in 96 well plates begin to coalesce within 12 h and form stable tissue analogs 24–48 h post-seeding. CTAs 4 h post-seeding show chondrocytes beginning to coalesce, form a mass and with increasing time, contract (125,000 chondrocytes/CTA), while higher seeding densities (1 million chondrocytes/CTA) result in more complete contraction and formation of a uniform construct. CTAs 4 and 42 weeks post seeding are cylindrical in shape and relatively uniform in size.

synthetic hydrogels (Mauck et al., 2002; Erickson et al., 2009; McCall et al., 2012; Ingavle et al., 2013; Rahman et al., 2013) and fibrous meshes (Moutos et al., 2007; Kim et al., 2011) with or without cells (e.g. chondrocytes and stem cells) and other cues (e.g. growth factors) to guide and stimulate cartilage formation. Advantages of scaffold-based methods include the ability to temporarily re-differentiate passaged chondrocytes (Capito and Spector, 2006), near native mechanical properties (Moutos et al., 2007; Erickson et al., 2009; Erickson et al., 2012), sufficient stiffness for bioreactor mechanical stimulation (Hu and Athanasiou, 2006), and ability to entrap cells in a locally controlled environment. However, some significant disadvantages of material-based approaches include phenotype instability, toxicity of degradation products and crosslinking methods, cell adhesion, and inappropriate biomaterial remodeling; each depends and varies based on the materials and methods utilized (Hu and Athanasiou, 2006).

Alternatively, scaffold-free methods utilize high cell density cultures in combination with low adhesion surfaces (Novotny et al., 2006; Ofek et al., 2008; Revell et al., 2008; Kim et al., 2011), bioreactors (Furukawa et al., 2003; Kelm and Fussenegger, 2004), or centrifugation methods (Muraglia et al., 2003) to form aggregates (Anderer and Libera, 2002; Furukawa et al., 2003; Revell et al., 2008; Huey and Athanasiou, 2011), pellets (Zhang et al., 2004; Bernstein et al., 2009) and micro-tissues (Kelm and Fussenegger, 2004). These techniques avoid scaffold-based issues, can retain chondrocyte phenotype, promote cell–cell interactions, elaborate a natural ECM (Anderer and Libera, 2002; Kelm and Fussenegger, 2004; Bernstein et al., 2009), and are additionally compatible with high throughput assay systems (Bhadriraju and Chen, 2002; Huang et al., 2008) which utilize small volumes and cell numbers, primarily due to uniformity of characteristics. Additional enhancement of ECM characteristics (and thus improved cartilage-like characteristics) has been achieved with the addition of real-time mechanical loading applied to these scaffold-free constructs (Elder and Athanasiou, 2009; Kraft et al., 2011).

We have previously developed a scaffold-free approach to cartilage tissue engineering that uses primary chondrocytes situated in hydrogel coated culture vessels that prevent adhesion and thus promote the formation of a self-aggregating suspension of cells, which subsequently forms into a cartilage-like biomass. In this model, chondrocytes are cultured at high density in tissue culture vessels coated with poly 2-hydroxyethyl methacrylate (polyHEMA); this hydrogel coating prevents cell attachment to the plastic substrate. Within 24 h, chondrocytes coalesce to form a stable construct that remains in suspension and progressively increases in mass with time. We refer to these constructs as cartilage tissue analogs (CTA). Chondrocytes in CTAs possess phenotypic characteristics and deposit ECM that is similar to native cartilage (Estrada et al., 2001; Kim et al., 2011) and can be produced from several species, including neonatal porcine, bovine, equine, and human chondrocytes (Dodge et al., 1998; Richardson and Dodge, 2000; Estrada et al., 2001). We have shown that the CTAs in culture continue to produce collagen type II and do not produce collagen type I, which would be indicative of their dedifferentiation to a fibroblastic phenotype (Novotny et al., 2006). However until this study, the mechanical properties of CTAs have not yet been evaluated over an extended time course. One of the primary limiting factors in the clinical application of cartilage tissue engineering is the insufficient load-bearing capacity of the repair tissue (Hunziker, 2002; Khoshgoftar et al., 2013). As such, this study evaluated the temporal development of CTA mechanical and biochemical properties and the relationship between mechanics and cell–matrix content in order to determine patterns of growth and maturation in this scaffold-free engineered cartilage.

## 2. Methods

### 2.1. CTA fabrication

Articular cartilage was harvested from juvenile bovine knees ($N=5$, age 2–6 months), minced, washed with PBS with 2X PSF (2% penicillin, streptomysin, fungizone) and digested in type II Collagenase (298 U/mg) in Basal Medium for up to 36 h (1 mg/mL in Dulbecco's Modified Eagles Medium with 10% Fetal Bovine Serum and 1% PSF). Digested cartilage was filtered through 70 μm strainers to separate cells from undigested matrix, diluted with 2 × PBS-PSF, and centrifuged at 1750 rpm for 20 min at 12°C. The wash and centrifugation process was repeated 3 × to pellet and isolate juvenile bovine chondrocytes.

Chondrocytes were seeded at $1 \times 10^6$ cells/well (200 μL/well) in poly 2-hydroxyethyl methacrylate (polyHEMA) hydrogel coated 96 well plates (Ultra-Low Adhesion 96 well plates, Corning) to form small cartilage tissue analogs (CTA). As shown in Fig. 1, chondrocytes initially form clusters at the bottom of the well (Fig. 1, left) that eventually condense into a uniform mass (Fig. 1, right). The CTA constructs coalesce within 24–48 h and continue to mature with time in culture;

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