Mimicking Cartilage Tissue Zonal Organization by Engineering Hydrogels with Dual Gradient of Biochemical and Mechanical Cues

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Introduction: Cartilage tissue is characterized with zonal organizations, with mechanical and biochemical cues transition in a gradient manner. However, most scaffolds developed for cartilage repair so far are homogeneous in nature and fail to recapitulate the zonal organization in native cartilage. The goal of this investigation is to mimic cartilage zonal organization by engineering hydrogels with dual gradients of biochemical and mechanical cues. Specifically, we seek to design a facile method that allows fabrication of tissue scale (cm) hydrogels with mechanical and biochemical gradient cues that support chondrocyte encapsulation, proliferation and extracellular matrix (ECM) deposition in 3D over time.

Methods: To control hydrogel stiffness, eight-arm poly(ethylene glycol) - norbornene (8-arm PEG-NB, MW 10K) was chosen due to its bio-inert nature, which can be crosslinked by linear PEG dithiol (MW 1.5K) (Fig.1A). To mimic biochemical content of cartilage, methacrylated chondroitin sulfate (CS-MA) was incorporated (Fig.1A). Primary (passage 0) bovine neonatal chondrocytes were mixed with hydrogel precursor solution before loaded to a gradient generator (Fig.1B). Cell-containing hydrogel precursor solutions were loaded to two chambers in the gradient generator, with one containing 20% PEG and 8% CS, and the other containing 2% PEG and 3% CS (Fig.1B). The mixed precursor solution was continuously pumped into a mixed chamber (3cm*1cm*3mm) to produce hydrogel solution with dual gradients of PEG and CS (Fig.1B). Chondrocytes were also encapsulated in single gradient hydrogels (CS gradient only or PEG gradient only) as controls. Gelation was induced by exposure to light (365 nm, 5min). As-formed gradient hydrogels were divided into 5 zones for characterization of biochemical and mechanical cues. Hydrogel diffusion was measured using fluorescence recovery after photobleaching (FRAP)-based assay. To examine the effects of gradient cues on cartilage tissue formation, chondrocytes were encapsulated in gradient hydrogels and cultured for up to 21 days in chondrocyte growth medium (high-glucose DMEM containing 50μg/mL ascorbate-2-phosphate, 40μg/mL proline, fetal bovine serum, and 100 U/mL penicillin and 0.1 mg/mL streptomycin).

Outcomes were analyzed using biochemical assays (DNA, sGAG, and hydroxyproline), gene expression, and histology. To further understand the differential cellular response observed in dual-gradient hydrogels and interactive effects of combined biochemical and mechanical cues, chondroitin-sulfate only gradient and mechanical-only hydrogels were made using the same method described above and analyzed using similar methods to better interpret the cellular behaviors within dual-gradient hydrogels.

Results: Our method allows rapid formation of tissue scale (3cm * 1cm) gradient hydrogels in less than 1 min, which allows homogeneous encapsulation of chondrocytes in every zone over 90% viability (Fig.1E). Compressive mechanical testing confirmed the stiffness of hydrogels increase linearly from zone 1 (5kPa) to zone 5 (120kPa) (Fig.1C). Similarly, quantitative sGAG assay confirmed biochemical gradient across different zones (Fig.1D). When encapsulated in 3D gradient hydrogels, chondrocytes exhibited
zonal specific responses in gene expression, cell proliferation as well as cartilage ECM deposition (sGAG and type II collagen). Increasing hydrogel stiffness and CS content led to enhanced cartilage gene expression (aggrecan and type II collagen), increased cell proliferation (DNA), as well as markedly enhanced collagen deposition (as measured by hydroxyproline per wet weight) (Fig.2D).

Consistent with gene expression and biochemical results, immunostaining showed more extensive type II collagen deposition in stiffer/high CS region (i.e. zone 4/5) vs. softer/low CS zones (i.e. zone 1/2). Cells residing in zone 5 proliferated and formed large cell clusters with extensive type II collagen staining (Fig.3). In contrast, cells in softer gels (i.e. zone 1) remained single with only marginal new collagen II deposition (Fig.3). Increasing CS concentration in gradient hydrogels led to larger nodules of neocartilage and more ECM matrix deposition, which may be achieved by facilitating cellular remodeling of matrix through degradation. Immunostaining of cytoskeleton actin also showed similar trend, with stiffer hydrogels leading to more extensive cell spreading (Fig.3).

**Discussion:** Here we report a facile method for rapid fabrication of tissue scale dual gradient hydrogels that support homogeneous cell encapsulation in 3D. Chondrocytes encapsulated in gradient hydrogels responded to zonal-specific biochemical and mechanical cues. The trend of resulting ECM deposition and cell morphology mimics the superficial to deep zones of native articular cartilage.

**Significance:** Mimicking zonal organization in cartilage tissue is important for restoring tissue function and integration, which remains a great challenge. The platform reported here offers a valuable tool to overcome this challenge and may be broadly useful for mimicking zonal organizations in other tissue types of tissue interfaces such as soft tissue -bone interfaces.

![Figure 1](image-url)  
**Figure 1:** Gradient Hydrogel Fabrication and Characterization. A) Gradient hydrogel is composed of 8-arm-poly(ethylene glycol) (PEG)-norbornene (MW 10,000 g/mol), PEG-dithiol (MW 1500 g/mol), and 25% methacrylated chondroitin sulfate (CSMA); B) Gradient hydrogel is bulk polymerized after the prepolymer solution is mixed with bovine primary chondrocytes. C) Measurement of CSMA concentrations and compressive modulus from zone 1 to zone 5 in dual-gradient hydrogel; D) Cell viability within selected zones of the gradient hydrogel on day 1 (scale bar = 50 μm).
**Figure 2:** Cellular responses within 3D dual gradient hydrogel. A) Cartilage markers (Sox9, Coll II, Apg) gene expressions within each zone of the gradient hydrogel; B) Chondrocyte DNA content, hydroxyproline, and sGAG per wet weight measured within each zone of the gradient hydrogel. C) Immunostaining of cartilage marker (Collagen Type II) within gradient hydrogel on Day 21. Scale bar = 50 μm.

**Figure 3:** Immunostaining of cellular morphology within gradient hydrogel. Collagen II and actin within each zone of gradient hydrogel are shown. (Scale bar = 50 μm)