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# Effect of stiffness of chitosan-hyaluronic acid dialdehyde hydrogels on the viability and growth of encapsulated chondrocytes

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ABSTRACT

Substrate elasticity or stiffness can influence the phenotypic and functional characteristics of chondrocytes. This work aimed to study the effect of varying stiffness compositions of a two-component injectable hydrogel based on chitosan (CH) and oxidized hyaluronic acid (HDA) on the growth and functionality of encapsulated chondrocytes. Three different ratios of the gel were prepared (10:1,10:3 and 10:5 CH-HDA) and characterized. The stiffness of the gels was evaluated from the force displacement curves using force spectroscopy AFM analysis. Rabbit articular chondrocytes were harvested and the cells from Passage 2 to 4 were used for the encapsulation study. The viability and ECM production of encapsulated chondrocytes were assessed at 7 day, 14 day and 28 day post culture. The results of the study show that as the ratio of hyaluronic acid dialdehyde component was increased, the stiffness of the gels increased from $130.78 \pm 19.83$ kPa to $181.47 \pm 19.77$ kPa which was also evidenced from the decrease in gelling time. Although there was an increase in the percentage of viable encapsulated cells which also maintained the spherical phenotype in the less stiff gels, decreased expression of ECM markers- Collagen type II and Glycosaminoglycans was observed compared to the stiffer gels. These findings indicate that gel stiffness strongly impacts the chondrocyte microenvironment both in maintenance of phenotypic integrity and ECM production.



## 1. Introduction

Damage to the articular cartilage due to trauma and injury is mainly due to mechanical disruption of the articular surface limited to the articular cartilage or disruption of both articular cartilage and subchondral bone. The therapeutic options available pose a real challenge as regeneration capability of the cartilage is limited since it is an avascular, aneural and alymphatic tissue. Articular cartilage in its native form is a hyaline tissue where chondrocytes are within a hydrated framework of extracellular matrix (70–80%) which is composed mainly of collagen type II and proteoglycans mainly aggrecan and hyaluronic acid. The rheological properties of cartilage tissue provides a viscoelastic environment acting both as a lubricant and a shock absorber for the underlying subchondral bone. Apart from the limited regeneration capability, the repair process is also impaired due to the poor migration capacity of chondrocytes that prevents the infiltration of repair tissue into the host environment. The most promising treatment options in recent years includes the use of autologous chondrocytes and the marrow stimulating technique. However the use of autologous chondrocytes requires the chondrocytes to be expanded in vitro before implantation and this leads the cell population to lose their phenotype. The secretion of extracellular matrix is reversed from collagen II production to collagen type I and III typical of fibrocartilage formation which ultimately leads to matrix formation with poor biomechanical properties. In the marrow stimulation technique using microfracture technique, the mesenchymal progenitor cells flowing into the affected or injured site is seen to have variable results usually with fibrocartilage tissue formation rather than the hyaline tissue. [1–3] This is where hydrogel systems have found advantage as an encapsulating system for chondrocytes where they can not only encapsulate cells but also maintain both cell viability and phenotype and support neocartilage formation.

Injectable chondrocyte based hydrogel systems for cartilage tissue regeneration is an attractive therapeutic option for cartilage repair which is finding a lot of potential in recent years [4,5]. The physical cues provided by the hydrogel substrate should ideally mimic the extracellular matrix environment especially the stiffness of the gel system to regulate the cell biology. *In vitro* long term culture of chondrocytes in monolayers have shown the chondro-

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cytes to dedifferentiate to a fibroblast like morphology and cease to secrete the cartilage specific collagen type II and proteoglycans [6]. Several studies have shown the culture of chondrocytes in 3D natural and synthetic polymer hydrogel matrices like alginate, agarose, fibrin, collagen, chitosan, hyaluronic acid, polyacrylamides etc. both in their native and modified forms [7–10]. Some of the major challenges in the development of an injectable hydrogel system has been the ease of preparation for non-invasive delivery intended for immediate use in surgical interventions and the maintenance of viability, stable phenotype and functionality of the chondrocytes that are encapsulated within the hydrogel environment. These challenges can be met by altering the physical structure of these hydrogels mainly the crosslinking density which regulates all the functions including the stiffness or elasticity of these gels thereby impacting chondrocyte behavior.

In this study we have attempted to study the effect of stiffness of hydrogels prepared from low molecular weight chitosan and oxidized hyaluronic acid in varying ratios on the viability and functionality of encapsulated chondrocytes. The gelation is attributed to the formation of Schiff's base and hence avoids the use of external crosslinking agents. Chitosan is a linear polysaccharide that is biocompatible and biodegradable and has found widespread application as a scaffold for tissue engineering. [11–13]. Hyaluronic acid, a major component of Glycosaminoglycan (GAG) is a linear disaccharide with repeat units of *N*-acetyl-D-glucosamine and D-glucuronic acid. They contribute 1–10% of the total GAG content in cartilage tissue and is responsible for tissue hydration, resistance to compressive load, intracellular signaling, protein up-take, and cell migration. Previous reports have already shown the efficacy of these hydrogels in chondrocyte cultures [14–16]. In one study, the mixture of crosslinked hydrogels with varying concentrations of hyaluronic acid methacrylate (HA-MA) showed distinct differences in the mechanical properties of the construct after 8 weeks of culture. Injectable chitosan hydrogel mixture has already reached phase I/IIa trials together with a radioactive agent for the treatment of knee synovitis in patients with rheumatoid arthritis (RA) [17]. The design of injectable hydrogels with varying stiffness and their influence on the maintenance of the chondrocyte phenotype and functionality has been the major focus in this study. We hypothesize that changing the stiffness of the Chitosan-HDA hydrogel prepared with varying the concentration of oxidized hyaluronic acid could impact the behavior of encapsulated chondrocytes. The study was done using Rabbit articular chondrocytes and cellular characteristics were studied using live dead staining, immunostaining and histological staining of ECM components and its biochemical estimation after 7.14 and 28 days culture period.

## 2. Materials and methods

Chitosan ~92.7% deacetylated, (100–150 kDa based on viscosity, Koyo Chitosan FM-80), hyaluronic acid sodium salt (Sigma, USA) and sodium periodate (Sigma, USA) was used for this study. Dialysis tubing (Spectra/Por®, M.W.C.O. 12000) is from Spectrum Laboratories Inc., CA, USA. Phosphate buffer saline (PBS), Dulbecco's Minimum Essential Medium (DMEM), antibiotic/antimycotic solution, sodium pyruvate, glutamine, nonessential amino acid (NEAA), and 0.25% Trypsin–EDTA were obtained from Gibco Invitrogen Corporation. All the other reagents were of analytical or equivalent grade.

### 2.1. Preparation of Chitosan–Hyaluronic acid dialdehyde (CH-HDA) hydrogels

For preparing the Chitosan–Hyaluronic acid dialdehyde hydrogels, hyaluronic acid was oxidized to form its dialdehyde. Briefly, 1 g HA was dissolved in 100 ml distilled water and the required amount of periodate was added to obtain 50% oxidized hyaluronic dialdehyde (HDA) as described earlier [18]. The reaction was allowed to proceed in the dark for 6 h at 25 °C with constant stirring. The solution was then dialyzed against distilled water for 3–4 days with three changes of water everyday till the dialysate was periodate-free. The absence of periodate was checked by adding a 0.25 ml aliquot of the dialysate to 0.25 ml of a 1% (w/v) solution of silver nitrate and ensuring the absence of any precipitate. The dialysate was then freeze-dried and stored in dark. For preparing the hydrogel, 1.2% Chitosan was prepared in 1% acetic acid solution and then neutralized with buffer solution containing $NaHCO_3$ (0.3 M), HEPES (0.2 M) and NaOH (0.05 M) to bring the final chitosan concentration to 1%. Similarly, 5% HDA was prepared in PBS and neutralized with the same buffer solution. Three gel compositions were prepared by mixing the two component solutions in the ratio as shown in Table 1.

**Table 1**
Composition chart of the three gels

| Sample | Composition | Chitosan (μl) | HDA (μl) |
|---|---|---|---|
| A | 10:1 CH-HDA | 50 | 5 |
| B | 10:3 CH-HDA | 50 | 15 |
| C | 10:5 CH-HDA | 50 | 25 |

### 2.2. Characterization of chitosan hyaluronic acid dialdehyde hydrogel

#### 2.2.1. FTIR spectroscopy

FTIR was used to characterize the chemical structure of the three gels prepared. FTIR with Attenuated Total Reflection (ATR) was the sampling tool used for analysis the spectra were recorded at room temperature in the range of 4000–600 $cm^{-1}$ region using a NICOLET 5700 FTIR spectrophotometer with (Thermo Corporation, USA) Diamond ATR (Attenuated Total reflectance) accessory. Fifty scans were recorded per collection and the spectrum was analyzed using OMNIC software.

#### 2.2.2. % degree of crosslinking

The degree of crosslinking was determined based on the free amine groups present in the gel and comparing with un crosslinked chitosan using ninhydrin assay. Briefly 10 mg of sample was boiled with freshly prepared ninhydrin reagent at 100 °C for 15 min. After boiling the solution was cooled and 1 ml of ethanol was added to stabilize the color formed. The optical absorbance of the solution was measured at 590 nm with a spectrophotometer (Cary Win UV). Glycine at various known concentration was used as standards. The amount of free amino groups is proportional to the optical absorbance of the solution. Triplicate samples were used for determining the% degree of crosslinking.

The degree of crosslinking of the solution is calculated using the equation

$$\%Degree\ of\ crosslinking = \frac{Ci - Cf}{Ci} \times 100$$

Where Ci is the optical absorbance of bare chitosan solution and Cf is the optical absorbance of the crosslinked gels.

#### 2.2.3. Gelling time determination of chitosan–hyaluronic acid dialdehyde gel

Gelling time was determined by measuring determining the viscosity change during gelation reaction when the two components chitosan and oxidized hyaluronic acid is mixed using a programmable viscometer (Brookfield, Model DV-11+) at 25 °C with spindle SLV-64 and small sample adaptor (5 ml) at 150 rev/min.

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