Injectable, Redox-Polymerized Carboxymethylcellulose Hydrogels for Stem Cell-Based Nucleus Pulposus Tissue Engineering

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Disclosures:

Introduction: Intervertebral disc (IVD) degeneration is associated with dehydration of the nucleus pulposus (NP) and results in the loss of proteoglycans and collagen important for disc function [1]. Given the limitations of current surgical treatments, tissue engineering is a promising approach that may potentially restore biological and functional properties of damaged NP. Photocrosslinked carboxymethylcellulose (CMC) hydrogels seeded with human mesenchymal stem cells (hMSCs) were recently shown to support cell viability and functional extracellular matrix production in the presence of transforming growth factor-β3 (TGF-β3) [2]. However, an injectable hydrogel system that does not rely upon the application of UV light for polymerization may be more readily translated to the clinic. This may be achieved by employing a water-soluble reduction/oxidation (redox) initiation system. Herein, a redox system comprised of ammonium persulfate (APS) and tetramethylethyldiamine (TEMED) was investigated for the first time in CMC hydrogels to evaluate its potential as an injectable vehicle for stem cell-based NP tissue formation. Specifically, the effects of cell seeding density on the viability and NP-like differentiation capability of hMSCs was evaluated in redox-polymerized CMC hydrogels. It was hypothesized that a higher seeding density would result in superior constructs with respect to biochemical composition and mechanical functionality.

Methods: Macromer Synthesis/Hydrogel Preparation: Methacrylation of 250 kDa CMC (Sigma) (7.5%) was based on previous protocols [3]. Passage 4 hMSCs were encapsulated in CMC hydrogels at 20×10⁶ cells/mL (20M) and 40×10⁶ cells/mL (40M). Briefly, cells were suspended in the polymer solution along with APS (10mM) and TEMED (10mM) in separate barrels of a dual-barrel syringe. The solutions were mixed and injected into a casting device to obtain hydrogels at a final macromer concentration of 2% (w/v). Cell Culture: All hydrogels were maintained in serum-free, chemically defined medium [5] supplemented with 10ng/mL rhTGF-β3 (R&D Systems). Swelling Properties: The equilibrium weight swelling ratio (Qₑ) of the gels was measured at days 7, 21 and 35 (n=4-5) [2]. Biochemistry: Sulfated glycosaminoglycans (GAGs) and Collagen II (Col II) were quantified at day 35 using the DMMB assay and indirect ELISA, respectively (n=4-5) [3]. Histology/Immunohistochemistry: ECM localization was assessed using antibodies to Col II with a peroxidase-based system and Alcian Blue (AB) staining [2, 3].

Mechanical Testing: Unconfined compression testing was conducted to measure the equilibrium Young's modulus (Eₑ) (n=5) [3, 4]. Rheology: Rheological analysis was performed using an AR2000ex Rheometer (TA Instruments) equipped with a cone and plate geometry (2°, 40-mm) at 25°C to determine gelation onset and completion (n=3). Cytotoxicity: Cell proliferation was determined via the PicoGreen assay (Invitrogen) (n=4-5) and cell viability was quantified via an MTT assay and visualized using Live/Dead staining (Invitrogen) (n=1-3). Statistical Analysis: A 2-way ANOVA with a Tukey's post-hoc test was used to determine the effects of cell seeding density and time on the material properties and biochemical content. Significance was set at p<0.05. Data represent mean ± s.d.

Results: The Qₑ of both 20M and 40M groups decreased with time and had significantly lower values at day 35 (21.54±0.80 and 18.18±0.47, respectively) compared to the earlier time points. The GAG content significantly increased with time in both 20M and 40M groups and the 40M group produced significantly higher GAG than the 20M group on days 21 and 35 (Table 1). Col II content increased significantly with time in both groups. However, the 20M group produced significantly more Col II than the 40M group on days 21 and 35 (Table 1). The DNA content in the 40M group was significantly higher than the 20M group at all time points and no significant differences were observed between Days 7 and 35 in both groups. Histological staining confirmed the biochemical results, with more extensive interterritorial AB staining and less Col II staining in the 40M gels. In particular, Col II staining in the 40M gels was less intense in the center, while the 20M gels had a more homogeneous Col II distribution. (Fig. 1) At days 21 and 35, the 40M group exhibited a significantly higher Eₑ, with respect to the 20M group (Fig. 2). Cell viability via the MTT assay showed relatively constant levels in both groups over 35 days which was consistent with the Live/Dead staining images (Fig. 3), which indicated minimal cell death. Rheological analysis measured a gelation onset time of 2.91±0.31 min and a gelation completion time of 10.93±0.43 min.

Discussion: This is the first study to report an injectable redox-polymerized, CMC hydrogel system for stem cell-based nucleus pulposus tissue engineering. Overall, the constructs demonstrated high potential for NP-like differentiation of MSCs in terms of biochemical content and material properties. The Qₑ decreased with time, and by day 35, reached values comparable to that seen in native bovine NP tissue in both 20M and 40M groups [3]. Although the 40M group exhibited significantly higher GAG content, the Col II content was significantly higher in the 20M group. This discrepancy in matrix deposition in the 40M group might be indicative of a negative feedback mechanism affecting collagen matrix accumulation over time, which has been
previously reported in other hydrogel systems. [6, 7] Additionally, staining revealed lower GAG and Col II deposition in the gel center, suggesting limited nutrient diffusion into the gel interior in the 40M group. However, the greater GAG deposition in the 40M group may have contributed to its superior compressive properties. The E, of both 20M and 40M groups by day 35 (~18 kPa and ~28 kPa, respectively) exceeded reported values for native human NP (~5 kPa) [8], but may be beneficial in the harsh in vivo environment associated with IVD degeneration [9]. Also, the GAG:Col II ratio in both 20M and 40M groups (~5:1 and ~16:1, respectively) fell within the range reported for native human NP (27:1) [10]. Overall, the 40M group displayed the highest mechanical properties, consistent with our hypothesis, but the 20M constructs retained more Col II and may be sufficient to produce functional NP-like tissue in redox-polymerized hydrogels. Moreover, the findings suggest that an optimal density is required to maintain functionality and that higher densities above the threshold might disrupt the matrix distribution and differentiation capacity [7]. The lack of cytotoxicity in both groups further supports the utility of these gels as an injectable material for NP replacement, given that prior studies have indicated cytotoxic effects of other redox-initiated hydrogel formulations [11]. In terms of ease of delivery, a gelation completion time of ~11 min provides sufficient time for surgical manipulation and is on the order specified by ISO standard 5833/1-1999 E for injectable materials. Future work will test the efficacy of this hydrogel system in organ culture and in vivo models.

**Significance:** These redox-initiated CMC hydrogels may lead to injectable, tissue-engineered NP replacements to treat IVD degeneration.

**Acknowledgments:** NSF for funding.


<table>
<thead>
<tr>
<th>Cell density (M/mL)</th>
<th>Time (Days)</th>
<th>Collagen II (ng/mg)</th>
<th>GAG (ng/mg)</th>
<th>DNA (ug/disk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>7</td>
<td>5.040 ± 0.592</td>
<td>684.107 ± 60.699</td>
<td>5.734 ± 0.810</td>
</tr>
<tr>
<td>20</td>
<td>21</td>
<td>319.044 ±91.858</td>
<td>1740.726 ±485.136*</td>
<td>2.626 ± 0.840*</td>
</tr>
<tr>
<td>20</td>
<td>35</td>
<td>1204.238 ±940.65*</td>
<td>6173.517 ±441.970* #</td>
<td>5.231 ± 0.565</td>
</tr>
<tr>
<td>40</td>
<td>7</td>
<td>2.168 ± 0.809</td>
<td>425.892 ± 128.950</td>
<td>9.900 ± 0.100 #</td>
</tr>
<tr>
<td>40</td>
<td>21</td>
<td>250.176 ±50.592</td>
<td>4832.450 ±64.995*</td>
<td>6.590 ± 0.568 # +</td>
</tr>
<tr>
<td>40</td>
<td>35</td>
<td>626.724 ±72.610</td>
<td>10161.344 ±382.072* #</td>
<td>9.035 ± 1.216 #</td>
</tr>
</tbody>
</table>

**Table 1:** Biochemical content of CMC hydrogels. * - Sig. diff. from all other groups. # - Sig. diff. from other group within time point. + - Sig. diff from all other time points within group. + - Sig diff from earlier time points.

**Figure 1.** Histological and immunohistochemical staining of D35 CMC constructs. Scale bar = 50 μm.
Equilibrium Modulus

![Equilibrium Modulus Graph](image)

**Figure 2.** Equilibrium modulus of CMC constructs. * - Sig diff from all other groups.

![20M and 40M Images](image)

**Figure 3.** Live/Dead staining of D35 CMC constructs. Scale bar = 50 μm

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