Effect of Cell Environment on ECM Production and Gene Expression in Poly(ethylene glycol) / Chondroitin Sulfate Hydrogels

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Introduction

A common approach to engineer cartilaginous tissue is to encapsulate chondrocytes in hydrogels that provide a three-dimensional environment to regulate cell function and guide extra-cellular matrix production. Cell environment can be controlled through gel chemistries and exposure to soluble factors. Our group has been particularly interested in poly(ethylene glycol) (PEG) based hydrogels; however, we recently synthesized chondroitin sulfate (ChSA) based macromers that can be systematically copolymerized with PEG macromers to form copolymer networks. The rationale behind incorporating ChSA into the networks is multifold. First, ChSA is the principle proteoglycan in cartilage. The presence of ChSA in gels provides a more natural environment for the chondrocytes and might stimulate cartilage production. Second, chondrocytes should recognize ChSA and be able to degrade and reorganize it as needed. Thirdly, the negative charge on ChSA imparts a high degree of swelling while simultaneously improving their mechanical properties. The goal of this work was to investigate the effect on gel mechanics, extracellular matrix production, and gene expression of incorporating ChSA into PEG based hydrogels.

Experimental

Macromers

Poly(ethylene glycol) dimethacrylate (PEGDM, 3400 MW) was purchased from Shearwater Polymer, Inc. and used without further purification. The primary alcohol on chondroitin sulfate-A (ChSA, 14,000-60,000 MW, Sigma) was modified with methacrylate groups to form multivinyl ChSA (ChSA-MA) macromers. Briefly, ChSA was dissolved in water and reacted with an excess of methacrylic anhydride at 60°C overnight at pH ~10. The ChSA-MA macromer was precipitated in methanol and dialyzed in dH_2O. The ChSA-MA employed in this study was modified with <1 mol% methacrylate groups per molecule. The vinyl peaks were not detectable by ^1H NMR, but their presence was verified by gel formation. Unmodified ChSA did not form a crosslinked gel. A simple Baeyer test, in which potassium permanganate reacted with the vinyl groups in less than a minute as seen by a shift in the solution color, provided further evidence of the presence of methacrylate groups[1].

Hydrogel Synthesis

Hydrogels were formulated using a 10-20% (w/w) macromer solution. Cytocompatible photoinitiating conditions were employed[2]. The photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (I2959, Ciba-Geigy), was dissolved into the macromer solution to a final concentration of 0.05% (w/w). The solution was filter-sterilized using a 0.2 μm syringe filter. The macromer solution was photopolymerized using a longwave ultraviolet lamp (UVP, model XX-20) at an intensity of ~4 mW/cm^2 for 10 minutes. PEG gels were formed with macromer concentrations ranging from 10-20% (w/w). Gels with tailored chemistries were formed by copolymerizing 20% (w/w) macromer solutions of ChSA-MA and PEGDM in the following ratios: (i) 100:0, (ii) 40:60, (iii) 25:75, and (iv) 10:90 ChSA-MA:PEGDM and used in the cell encapsulation studies described below.

Hydrogel Characterization

Hydrogel discs (5mm in diameter and ~2mm in thickness) were swollen to equilibrium in phosphate buffered saline (PBS, pH 7.4) at 37°C for 24 hours. The swollen discs (n=3) were weighed and lyophilized to determine the swollen and dry polymer mass, respectively. The equilibrium mass swelling ratio, q, was determined by ratioing the equilibrium swollen mass to the dry polymer mass. The compressive modulus of elasticity was measured on the swollen discs (n=5) using a dynamic mechanical analyzer (DMA-7, Perkin Elmer) in unconfined compression at a rate of 40 mN min^{-1} at room temperature.

Chondrocyte Isolation and Encapsulation

Articular cartilage was obtained under aseptic conditions from the femoral-patellar groove of a young calf (Research 87, Marlboro, MA) as described elsewhere[3]. The isolated chondrocytes were resuspended in chondrocyte medium (DMEM, without phenol red (Gibco), supplemented with 10 mM Hepes (Gibco), 0.4 mM L-Proline (Sigma), 50 mg l^{-1} l-ascorbic acid (Sigma), 0.1 M MEM non-essential amino acids (Gibco), 1% penicillin-streptomycin (Gibco), 0.5 μg ml^{-1} fungizone (Gibco), and 10% fetal bovine serum (Gibco)). Chondrocyte viability was determined using trypan blue exclusion and a hemacytometer. Isolated chondrocytes were then combined with the sterile macromer/initiator solution at a concentration of 75 x 10^6 cells ml^{-1} and encapsulated under the cytocompatible photoinitiation conditions described above. The cell-hydrogel constructs (5 mm in diameter and ~2 mm in thickness) were placed in 12 well plates and incubated at 37°C on an orbital shaker in a humid environment with 5% CO_2. The medium was replaced biweekly. The constructs were cultured 4 weeks

mRNA Isolation and RT-PCR

Immediately after encapsulation and at 3, 7, and 14 days, constructs were removed from culture and their total RNA isolated in a manner similar to that described by Chomczynski *et al*[4]. Briefly, constructs were homogenized in 1 mL of TRIzol™ Reagent (Invitrogen), centrifuged (12,000g, 4°C, 15 min.) to remove excess scaffold components and incubated for 5 min. at room temperature (RT) to ensure complete dissociation of nucleoprotein complexes. Chloroform (Sigma, 200 μl) was added to the samples, which were subsequently vortexed and incubated for 10 min. at RT. Aqueous and organic phases were separated by centrifugation (12,000g, 4°C, 15 min.) followed by removal of the RNA containing aqueous phase. Precipitation of RNA was induced by adding 0.75 ml 75% ethanol in DEPC treated water followed by vortexing and a 15 min. incubation at RT. Centrifugation (12,000g, 4°C, 15 min.) created an RNA pellet which was washed with 75% ethanol in DEPC treated water, air dried, and resuspended in DEPC treated water.

Isolated RNA (7 ng) was transcribed to DNA using a commercially available reverse transcription kit (Invitrogen, SuperScript™ First Strand Synthesis System for RT-PCR) following kit instructions and a thermal cycler (Eppendorf Mastercycler Personal). Prior to reverse transcription, any contaminating DNA was removed from the isolated RNA through amplification grade DNase I digestion following kit instructions. 500 picograms of the resulting single stranded DNA was amplified using Platinum *taq* DNA Polymerase (Invitrogen) and gene specific primers (Gibco) following kit instructions. Glyceraldehyde-phosphate-dehydrogenase (GAPDH), an enzyme used in the metabolic pathway and uniformally expressed across all cell types, was used as the housekeeping gene. Gene expression was determined for collagen type I, collagen type II, and aggrecan relative to GAPDH expression. Amplified DNA was mixed with 10x BlueJuice™ Loading Buffer (Invitrogen), run on a 1.5% agarose gel, and subsequently stained with 0.5 μg ml^{-1} ethidium bromide in 1x Tris–Acetate-EDTA (Fisher)[5]. NIH Image 1.62 was used to compare the total amount of DNA in each band.

Results and Discussion

By formulating copolymer gels with these chemistries, the macroscopic gel properties were manipulated to obtain high compressive moduli coupled with a high degree of swelling. With the incorporation of ChSA, the gel compressive modulus increased from 34 to 140 kPa for similar degrees of swelling (table 1). Interestingly, when chondrocytes were encapsulated in pure ChSA gels and cultured *in vitro*, collagen and glycosaminoglycan synthesis was inhibited. However, in the copolymer gels, synthesis of both extracellular matrix components was stimulated (data not shown). Furthermore, current research is aimed at characterizing enzymatic hydrogel degradation and its effect on extra-cellular matrix production and distribution.

TABLE 1. Properties of PEG/ChSA hydrogels.

% (w/w) total macromer	% (w/w) ChSA*	Mass Equilibrium Swelling Ratio (q)	Compressive Modulus (kPa)
10	0	9.3 ± 1.0	34 ± 3
20	40	9.5 ± 0.6	140 ± 10

Data reported as mean±standard deviation. * Macromer concentrations are given as percent of total macromer in solution.

The effect of scaffold chemistry on the gene expression of chondrocytes was also investigated. Chondrocytes were encapsulated in copolymers of PEG and ChSA of different ratios and cultured *in vitro* for up to four weeks (Figure 1). Gene expression for collagen type II was enhanced by the incorporation of PEG into *in vitro* cultured gels at day 14, illustrating an important influence of gel chemistry on chondrocyte function; however, aggrecan gene expression was unaffected. This study demonstrates that the macroscopic properties of chondrocyte gel carriers can be controlled through the incorporation of charge into networks by ChSA, but the neutral, non-interactive base PEG chemistry facilitates extracellular matrix deposition.

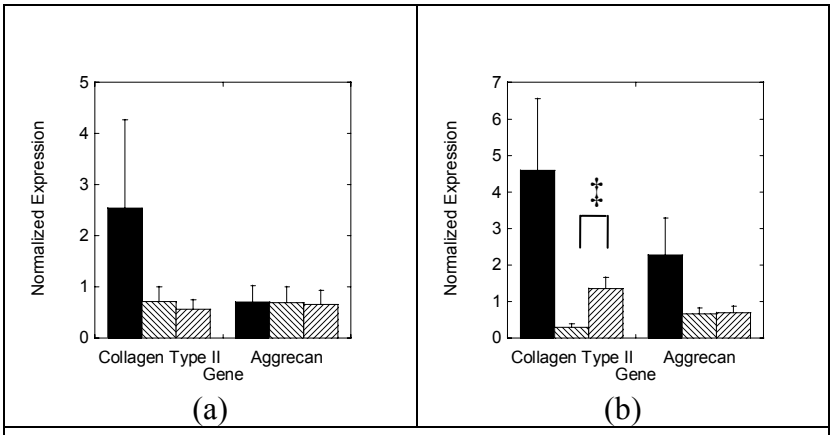

Figure 1. Expression of collagen type II and aggrecan normalized by GAPDH immediately after encapsulation (■), after 7 days (▨), and 14 days (▨) *in vitro* for chondrocytes photoencapsulated in hydrogels composed of a) pure ChSA and b) 25%ChSA/75%PEG. ‡ $p<0.05$

References

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