

Incorporation of tissue-specific molecules alters chondrocyte metabolism and gene expression in photocrosslinked hydrogels

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Abstract

Hydrogels are highly swollen, insoluble networks which can entrap chondrocytes and provide a 3-D environment necessary for the re-growth of cartilaginous tissue. In this study, hydrogels were formulated with a synthetic poly(ethylene glycol) (PEG) component to provide control over the macroscopic gel properties and from a cartilage specific compound, chondroitin sulfate (ChSA), to capture features of the chondrocytes' native environment. PEG was chosen as the base hydrogel chemistry, because it forms a 3-D environment that maintains chondrocyte function. ChSA, a highly negatively charged main component of proteoglycans, was then selectively incorporated into the PEG gel. Macroscopic gel properties were manipulated to obtain high compressive moduli coupled with a high degree of swelling by formulating copolymer gels with these chemistries. The gel compressive modulus of cell-free PEG gels increased from 34 to 140 kPa with the incorporation of ChSA for similar degrees of swelling. When chondrocytes were encapsulated in pure ChSA gels, synthesis of collagen and glycosaminoglycans was inhibited. However, when PEG was introduced into the copolymer gels, both extracellular matrix components were stimulated. Total collagen content increased from non-detectable in the pure ChSA gels to 0.48 ± 0.05 mg/g wet weight in the copolymer gels (40/60 ChSA/PEG). Gene expression for collagen type II was also enhanced by the incorporation of PEG into the gel, 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.

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1. Introduction

Numerous chemistries derived from both natural and synthetic polymers have been used to develop cell-carriers for cartilage tissue engineering. Prefabricated scaf-

folds prepared from poly(glycolic acid) (PGA), poly(L-lactic acid) (PLLA), and their copolymers (PLGA) have been extensively investigated as promising scaffolds for regenerating cartilaginous tissue [1–3]. Alternatively, approaches to create 3-D environments that emulate the natural environment of cartilage offer biomimetic cell matrices. For example, chondrocytes have been seeded onto porous scaffolds formulated from type I collagen–glycosaminoglycan (GAG) copolymers [4], type II collagen [4], and hyaluronic acid, a molecule that makes up the backbone structure of proteoglycans [5,6]. The

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scaffold chemistry is an important component in the scaffold design and can influence chondrocyte metabolism; for example, canine chondrocytes seeded onto type II collagen scaffolds had higher biosynthetic activity than cells seeded onto type I collagen-GAG scaffolds [4].

An alternative scaffold approach uses in situ forming hydrogels that simultaneously serve as an injectable carrier while providing a 3-D niche to support cell function. A variety of chemistries have been used to form hydrogels in the presence of chondrocytes and successfully generate cartilaginous tissue, e.g. natural polymers derived from alginate [7] and fibrin glue [8,9]. However, to develop cell-type specific carriers, an important design feature may be the incorporation of components that mimic the native extracellular matrix (ECM) of cartilage. For example, hyaluronic acid has been modified by esterification to form hydrogels for chondrocyte carriers [10,11]. Chondrocytes cultured in monolayer on chondroitin sulfate modified chitosan membranes maintained their phenotype, as seen by the production of proteoglycans and type II collagen [12].

Although natural based hydrogels provide an environment that facilitates ECM formation, the gel properties are often limited and typically inferior to that of native articular cartilage [13,14]. In contrast, synthetic hydrogels provide the unique advantage of greater control over the macroscopic gel properties. We are particularly interested in multifunctional macromers that can be copolymerized using a photoinitiation process to form hydrogels. Photoinitiated polymerization of hydrogels utilizes light to convert liquid macromer solutions to solid gels at physiological temperature and pH with spatial and temporal control over the initiation reaction. Furthermore, photocrosslinkable macromers can be easily synthesized from a variety of chemistries, and gels with tailored chemistries can be fabricated by simple changes in the initial macromer compositions in solution and subsequent copolymerization.

In this study, hydrogels were formulated from macromers containing a synthetic poly(ethylene glycol) (PEG) component to control the hydrogel properties and a natural chondroitin sulfate (ChSA) component to emulate the native cartilage environment. PEG was chosen for the base hydrogel chemistry, because it can easily be modified with photopolymerizable and crosslinkable groups to form a 3-D matrix which maintains chondrocyte viability and promotes the deposition of ECM rich in proteoglycans and type II collagen [15,16]. Several groups have modified ChSA with photocrosslinkable groups [17,18] which allows it to be selectively introduced into the PEG gel by copolymerization. ChSA has several attractive features that make it desirable for tissue engineering scaffolds including: (i) it is one of the main components of proteoglycans, (ii) it is highly negatively charged, and (iii) it can be enzymatically degraded by cellular secretion of chondroitinase.

Crosslinked hydrogels were formulated by copolymerizing the PEG and ChSA macromers. The resulting hydrogels were characterized with respect to their swelling and compressive moduli. To assess the ability of these scaffolds to provide a suitable environment for cartilage tissue engineering, chondrocytes were photoencapsulated in gels prepared from pure ChSA and from copolymer gels with varying PEG:ChSA ratios and cultured in vitro. The neotissue formed was analyzed biochemically to quantify GAG and collagen production and histologically to examine the spatial distribution of the ECM components. Finally, gene expression was analyzed by measuring the mRNA levels of collagen type I, collagen type II, and aggrecan using RT-PCR.

2. Materials and methods

2.1. Macromers

Poly(ethylene glycol) dimethacrylate (PEGDM, 3400 MW) was purchased from Shearwater Polymer, Inc. and used without further purification. Pendant alcohol groups on chondroitin sulfate-A (ChSA, 14,000–60,000 MW, Sigma) were modified with methacrylate groups to form multivinyl ChSA (ChSA-MA) macromers [17]. 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 dH₂O. 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 [19].

2.2. Hydrogel synthesis

Hydrogels were formulated using a 10–20% (w/w) macromer solution. Cytocompatible photoinitiating conditions were employed [20]. 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 min. PEG gels were formed with macromer concentrations ranging from 10% to 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.

2.3. Hydrogel characterization

Hydrogel discs (5 mm in diameter and ~2 mm in thickness) were swollen to equilibrium in phosphate buffered saline (PBS, pH 7.4) at 37 °C for 24 h. 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 in the elastic region of swollen discs ($n = 5$) using a dynamic mechanical analyzer (DMA-7, Perkin–Elmer) in unconfined compression at a constant stress rate of 40 mN min⁻¹ up to 20% strain at room temperature.

2.4. 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 yielding 30–40 g of cartilage per joint [21]. The isolated chondrocytes were resuspended in chondrocyte medium (DMEM, without phenol red (Gibco), supplemented with 10 mM Hepes (Gibco), 0.04 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 75×10^6 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% CO₂. The medium was replaced biweekly. The constructs were cultured for either 8 weeks (pure ChSA gels) or for 4 weeks (copolymer gels). Data resulting from biochemical and RT-PCR experiments were obtained from independent chondrocyte isolations and one to two joints were used per experiment. Isolated chondrocytes from different joints were thoroughly mixed prior to encapsulation.

2.5. Neotissue analysis

At prescribed time points, the constructs ($n = 3$) were weighed to determine their wet weight, lyophilized for 24 h to determine their dry weight then digested in a papain solution (125 µg ml⁻¹ papain type III (Worthing-

ton), 10 mM L-cysteine (Aldrich), 100 mM phosphate (Fisher Scientific), and 10 mM EDTA (Fisher Scientific) at pH 6.3) at 60 °C for 15 h. Total glycosaminoglycan content was determined using dimethylmethylene blue and a UV–Vis spectrophotometer (Perkin–Elmer, Lambda 40) [22]. Total collagen content was measured by the hydroxyproline content [23] in which collagen is comprised of 10% hydroxyproline [24]. One cell–hydrogel construct from each condition was also fixed overnight in 10% formalin, embedded in paraffin and microtomed in 8 µm sections following standard histological techniques. The sections were stained with Safranin-O and fast green for negatively charged proteoglycans (glycosaminoglycans), which stain red and with Masson's trichrome for collagen, which stains blue. Hematoxylin was used to visualize the cell nuclei, which stain black.

2.6. mRNA isolation and RT-PCR

Immediately after encapsulation and at 3, 7, and 14 days, constructs were removed from culture ($n = 3$) and their total RNA isolated in a manner similar to that described by Chomczynski et al. [25]. 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. Quantification of RNA content was determined with a UV–Vis spectrophotometer at 260 nm.

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 uniformly expressed across all cell types, was used

Table 1
Primer sequence and PCR reaction conditions

Gene	Strand	Primer (5'-3')	Cycle conditions	Number of cycles
Collagen type I ^a	Sence	TGCTGGCCAACCATGCCTCT	95 °C 30 s, 55 °C	40
	Anti-sence	TTGCACAATGCTCTGATC	60 s, 72 °C 75 s	
Collagen type II ^b	Sence	CTGGATGCCATGAAGGTTT	93 °C 60 s, 58 °C	36
	Anti-sence	TAGTCTTGCCCCACTTACCG	120 s, 72 °C 60 s	
Aggrecan ^a	Sence	CACTGTTACCGCCACTTCCC	93 °C 60 s, 58 °C	37
	Anti-sence	GACATCGTCCACTCGCCCT	120 s, 72 °C 60 s	
GAPDH ^c	Sence	AACACCCTCAAGATTGTGACGA	93 °C 60 s, 58 °C	37
	Anti-sence	TCCACCACCTGTTGCTGTA	120 s, 72 °C 60 s	

^a Primers adapted from Saldanha et al. [48].

^b Primers from Allemann et al. [49].

^c Primers adapted from van Sussante et al. [42].

as the housekeeping gene. Gene expression was determined for collagen type I, collagen type II, and aggrecan relative to GAPDH expression. Primers and amplification conditions are listed in Table 1. Amplified DNA was mixed with 10 × BlueJuice™ Loading Buffer (Invitrogen), run on a 1.5% agarose gel, and subsequently stained with 0.5 µg ml⁻¹ ethidium bromide in 1 × Tris–Acetate–EDTA (Fisher) [26]. NIH Image 1.62 was used to compare the total amount of DNA in each band.

2.7. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with a confidence level of 0.05. All values are reported as the mean ± standard error of the mean unless otherwise stated.

3. Results and discussion

Hydrogels formulated by copolymerizing poly(ethylene glycol) and chondroitin sulfate macromers form unique networks that contain both a synthetic and natural component. A schematic of the network formed through the copolymerization of these macromers is illustrated in Fig. 1. Poly(ethylene glycol) was end-capped with photocrosslinkable methacrylate groups to form a divinyl macromer while chondroitin sulfate was modified to form a multivinyl macromer. Upon copolymerization of these macromers, a final network structure results with polymethacrylate kinetic chains connected via PEG and chondroitin sulfate crosslinks. Since chondroitin sulfate has negatively charged pendant sulfate groups within each repeat unit, the network charge is readily controlled by compositional changes in the copolymer solution prior to polymerization. Furthermore, changes in the functionality and concentration of the macromers in solution enable facile control of the network structure and crosslinking density. As a result, the overall macroscopic properties of the final hydrogel can be widely varied.

3.1. Hydrogel characterization

Crosslinking density influences many of the macroscopic properties of hydrogels. Two properties that are particularly important for cartilage tissue engineering are the equilibrium water content (equilibrium mass swelling ratio, q) and the compressive modulus. In general, an increase in the crosslinking density results in a decrease in the water content and an increase in the compressive modulus. By varying the macromer molecular weight, functionality, and the percent macromer in solution prior to polymerization, networks with a range of crosslinking densities can be obtained [27–29]. The macroscopic gel properties of hydrogels synthesized from PEGDM macromers are summarized in Table 2. By varying the macromer concentration from 10% to 20% (w/w), q decreased 56% from 9.3 ± 1.0 to 5.2 ± 0.1 ($p < 0.001$), but the compressive modulus increased an order of magnitude from 34 ± 3 to 360 ± 14 kPa ($p < 0.001$) in these uncharged gels.

In designing an in situ forming cell-scaffold for engineering cartilage at the articulating surface of a joint, the compressive modulus of the scaffold is an important parameter. Native articular cartilage has an equilibrium modulus of elasticity of 500–1000 kPa [30]. Therefore, the mechanical properties obtained from gels formulated with a 20% PEGDM macromer concentration approach that of the native tissue. However, we have shown previously that an equilibrium mass swelling ratio somewhere between 5.7 and 7.9 is required in these PEG hydrogels to promote proteoglycan diffusion into the extracellular regions of the scaffold [31]. Therefore, a trade-off exists between networks with a high degree of swelling to facilitate diffusion of ECM molecules and networks with a high compressive modulus to restore function. With the incorporation of degradable units into the PEG macromer, it is possible to obtain networks with an initially high modulus, and as the crosslinks degrade with time, the equilibrium mass swelling ratio increases to facilitate proteoglycan diffusion [31].

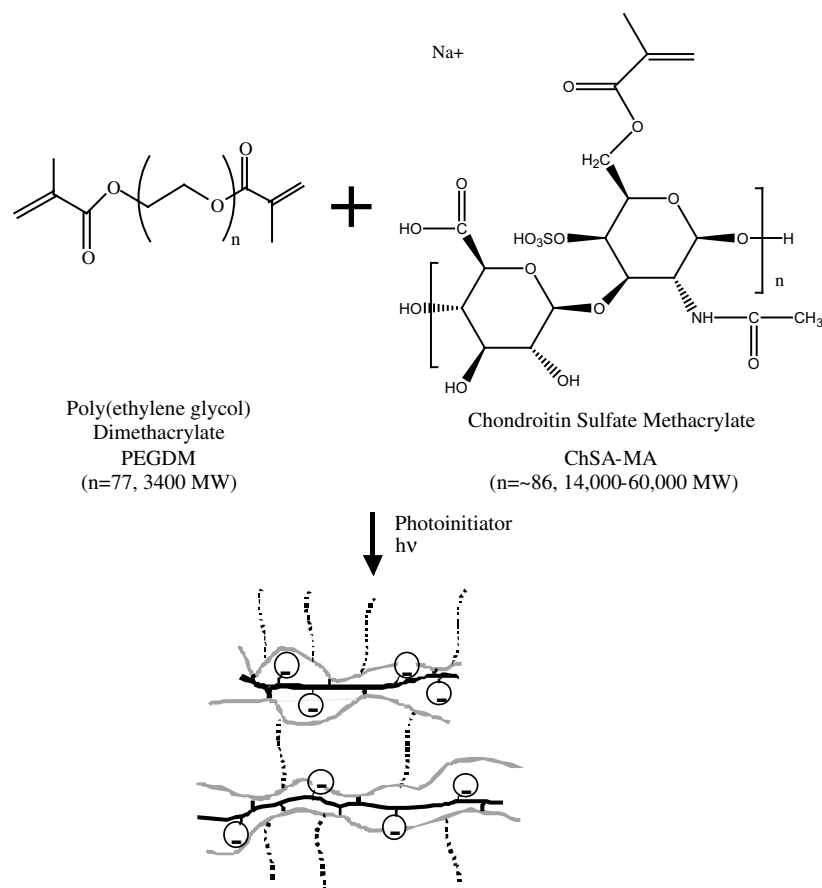


Fig. 1. An idealized hydrogel network formulated by copolymerizing PEGDM with ChSA–MA. The network consists of polymethacrylate kinetic chains (—) connected via PEG (⋯) and ChSA (—) crosslinks.

Table 2
Properties of PEG hydrogels

% (w/w) PEGDM	Mass equilibrium swelling ratio (q)	Compressive modulus (kPa)
10	9.3 ± 1.0	34 ± 3
20	5.2 ± 0.1	360 ± 14

Data reported as mean \pm standard deviation. $p < 0.001$ for comparisons between gel composition for q and compressive modulus.

In this contribution, we were interested in developing networks that possess a high modulus and a high equilibrium swelling ratio. To accomplish this goal, negative charges were incorporated into the base PEG hydrogel through the chondroitin sulfate chemistry. When bound to the network, negative charges repel each other creating a higher swelling pressure within the gel. The resulting macroscopic properties of networks formed by copolymerizing PEGDM macromers with chondroitin sulfate macromers are summarized in Table 3. An increase in the ChSA–MA macromer concentration resulted in a 61% increase in the gel water content from 5.9 ± 0.1 to 9.5 ± 0.6 ($p < 0.001$). A pure ChSA gel gave the highest degree of swelling with a q of 20.9 due to its negative charges and low double bond concentration,

Table 3
Properties of PEG/ChSA hydrogels

% (w/w) PEGDM ^a	% (w/w) ChSA ^a	Mass equilibrium swelling ratio (q)	Compressive modulus (kPa)
90	10	5.9 ± 0.1	280 ± 20
75	25	7.5 ± 0.2	190 ± 20
60	40	9.5 ± 0.6	140 ± 10
0	100	20.9 ± 0.6	20 ± 6

Data reported as mean \pm standard deviation.

^a Macromer concentrations are given as per cent of total macromer in solution (20% w/w). $p < 0.001$ for comparisons between gel composition for q and compressive modulus.

leading to a more loosely crosslinked gel. With the incorporation of 40% ChSA–MA into the copolymer network, the compressive modulus increased fourfold compared to pure PEG gels with statistically similar q values (9.3 vs. 9.5). In addition, gels with a q of 7.5, sufficient for proteoglycan diffusion in pure PEG gels, were obtained by incorporating 25% ChSA–MA into the network. In these gels, the compressive modulus was 190 ± 20 kPa. By incorporating charge into copolymer gels, the macroscopic hydrogel properties can be manipulated to obtain networks with properties that are important for creating permissive 3-D cell environments

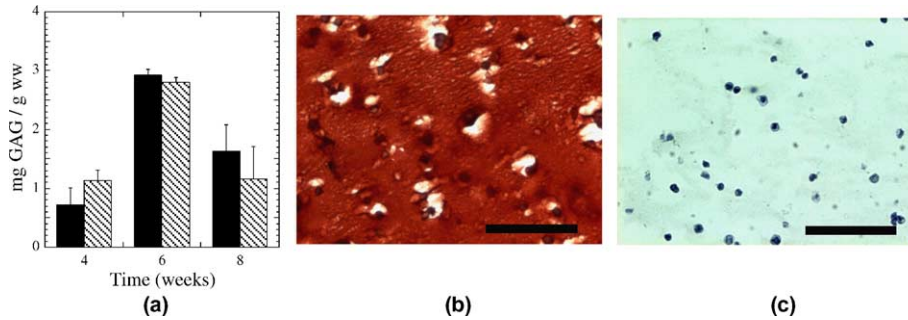


Fig. 2. (a) Glycosaminoglycan content of ChSA hydrogels in the absence of chondrocytes (■) and in the presence of chondrocytes (▨) as a function of culture time in vitro. Panels (b) and (c) are histological micrographs of chondrocytes photoencapsulated in ChSA gels after 6 weeks in vitro. Safranin-O was used to stain the glycosaminoglycans red which also stains the ChSA within the gel red (b). Masson's trichrome stain was used to stain collagen blue (c). Scale bar is 100 μ m for (b) and (c).

that facilitate tissue formation and restore function immediately.

3.2. Cell encapsulation studies

Chondrocytes were photoencapsulated into pure ChSA hydrogels to assess their ability to form cartilaginous tissue in vitro in these novel hydrogel matrices. Interestingly, when the GAG content was quantified and compared to control ChSA gels in the absence of cells (Fig. 2a), the GAG content was statistically similar, suggesting that the embedded chondrocytes did not synthesize any proteoglycan molecules within these constructs. Furthermore, collagen was not detected during the 8 week culture period as measured by the hydroxyproline content. When examined histologically, the micrographs in Fig. 2b show viable rounded chondrocytes after 6 weeks. However, it is difficult to assess the distribution of newly synthesized, if any, GAG molecules in Fig. 2b since Safranin-O used to stain for negatively charged proteoglycans also stains ChSA within the hydrogel. The histological micrograph for collagen (Fig. 2c) illustrates the absence of collagen, which is in agreement with the biochemical analysis. Similar histological results were observed at 4 and 8 weeks (data not shown). These findings demonstrate that cells are present and viable (further verified by the gene expression discussed below) within these novel ChSA gels, but the ChSA chemistry appears to inhibit synthesis of extracellular matrix components.

This inhibition of ECM synthesis was further examined through gene expression for type I collagen, type II collagen, aggrecan, a proteoglycan composed of GAGs, and GAPDH, the housekeeping gene. The results are shown in Fig. 3. The agarose gel demonstrated the presence of cartilage ECM mRNA (type II collagen and aggrecan) and the absence of type I collagen mRNA, an indicator of chondrocyte de-differentiation, at day 7. When normalized by GAPDH, the mean collagen type II expression decreased, although not statisti-

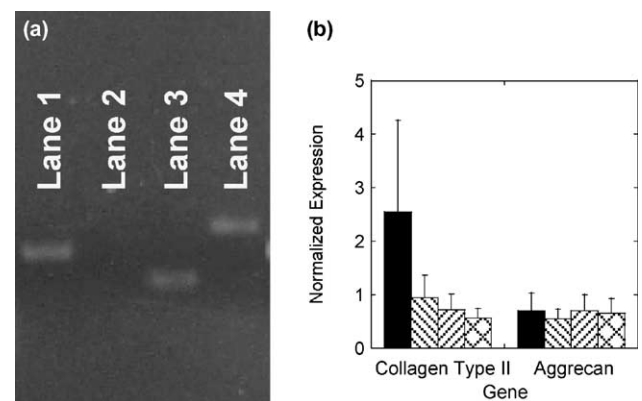


Fig. 3. (a) An agarose gel for gene expression of collagen type II (lane 1), collagen type I (lane 2), aggrecan (lane 3), and GAPDH (lane 4) at 7 days. (b) Expression of collagen type II and aggrecan normalized by GAPDH immediately after encapsulation (■), after 3 days (▨), 7 days (▧), and 14 days (▩) in vitro for chondrocytes photoencapsulated in hydrogels composed of pure ChSA.

cally significantly, with culture time suggesting a down-regulation of type II collagen mRNA. Aggrecan expression levels, however, remained constant throughout the 2 week study. Collagen type I was not expressed in the ChSA gels over the course of the experiment, but was detected in controls of chondrocytes cultured on tissue culture polystyrene for 10 days (data not shown). The absence of type I collagen indicates that the chondrocytes maintained their differentiated phenotype while encapsulated in these gels. These results support the evidence from the biochemical and histological data that pure ChSA hydrogels inhibit collagen synthesis, and provide a striking example of the influence of synthetic ECM analogs on cell expression and function.

In previous studies, our group and others have shown that PEG, as a crosslinked hydrogel [15,31–33] and as a highly viscous linear polymer [34], maintains chondrocyte viability and promotes the formation of cartilaginous tissue. When chondrocytes were encapsulated in ChSA/PEG copolymer gels, GAG and collagen synthe-

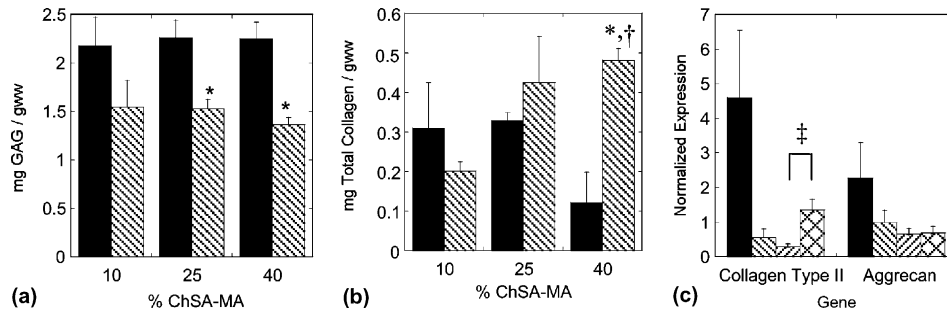


Fig. 4. (a) GAG and (b) total collagen contents after 2 (■) and 4 weeks (▨) in vitro for chondrocytes photoencapsulated in PEG/ChSA copolymer hydrogels as a function of ChSA composition. (c) Expression of collagen type II and aggrecan immediately after encapsulation (■), 3 days (▨), 7 days (▩), and 14 days (▧) in vitro for chondrocytes photoencapsulated in hydrogels composed 25%ChSA/75%PEG. (*) $p < 0.05$ when compared to 2 week. (†) $p < 0.01$ when compared to 4 week, 10% ChSA-MA and (‡) $p < 0.05$.

sis were stimulated as indicated by the biochemical data in Fig. 4a and b. GAG stimulation was verified by comparing the GAG content at 4 weeks in the copolymer gels to the pure ChSA gels, which had at least 60% more ChSA present in the gel (Fig. 2a). The mean GAG content was higher in all of the copolymer gels compared to the pure ChSA gels, but the difference was not statistically significant. The GAG content was similar in all three gels at 2 and 4 weeks, although the ChSA content differed from 10% to 40% among the copolymer gels. In all cases, the mean GAG content decreased from 2 to 4 weeks, and this decrease was statistically significant in the 25% and 40% ChSA-MA gels. In native cartilage, chondrocytes remodel their surrounding cartilage by secreting enzymes that degrade proteoglycans [35]. Therefore, it is possible that the chondrocytes sense the presence of chondroitin sulfate (the main component found in proteoglycans) within the hydrogel and secrete enzymes to degrade the chondroitin sulfate. However, additional experiments are necessary to elucidate the role, if any, of enzymatic degradation. Total collagen content was significantly lower than the GAG content, a phenomenon which has been observed in non-degrading gels in vitro [31]. A significant increase in collagen content was observed in the 40% ChSA-MA gels from 2 to 4 weeks.

Gene expression of chondrocytes encapsulated in copolymer hydrogels formulated with 25% ChSA-MA was also characterized and the results are shown in Fig. 4c. As with the pure ChSA gels, collagen type II expression was down-regulated in the days following encapsulation. However, by day 14, collagen type II expression increased significantly compared to day 7 ($p = 0.01$). Furthermore, collagen type II expression in copolymer gels was significantly higher than expression in pure ChSA hydrogels at day 14 ($p = 0.035$), suggesting that the presence of PEG up-regulates collagen type II mRNA. Expression of aggrecan also appeared to drop immediately after encapsulation, though not significantly, but quickly leveled off to approximately the same expression levels seen in pure ChSA hydrogels.

Histological evidence (Fig. 5) demonstrates viable rounded chondrocytes in the copolymer gels. Again, it is difficult to assess the accumulated GAGs due to the presence of ChSA. The degree of staining appears considerably lighter in the copolymer gels compared to the pure ChSA gels (Fig. 2b) due to the larger amount of ChSA in the pure gels. On the other hand, collagen was present in the copolymer gels as indicated by the blue staining (panels b and c). Interestingly, collagen was present in the extracellular regions, a phenomenon not observed before in non-degrading PEG hydrogels

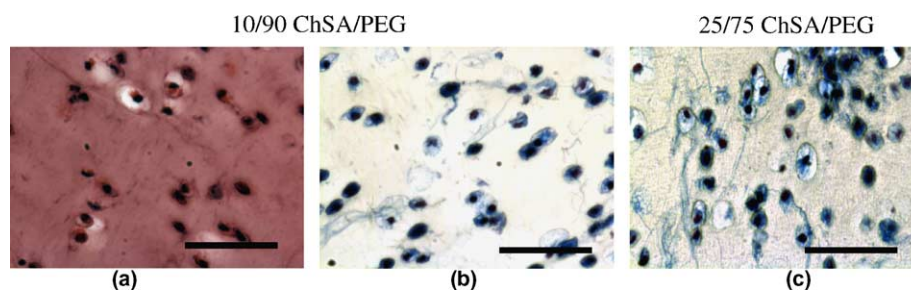


Fig. 5. Histological micrographs of chondrocytes photoencapsulated in PEG/ChSA hydrogels after 2 weeks of in vitro culture: (a) is a representative micrograph of Safranin-O which stains proteoglycans red, and subsequently chondroitin sulfate within the gel red (micrograph from the 10%ChSA/90%PEG gel); and (b) and (c) are histological sections from 10%ChSA/90%PEG and 25%ChSA/75%PEG gels, respectively, stained with Masson's trichrome which stains collagen blue. Scale bar is 50 μm.

[31,32]. This observation suggests that there may be proteolytic degradation in these copolymer gels, and additional experiments are ongoing to confirm this hypothesis. Furthermore, the collagen has an unusual fiber-like morphology not seen before in degrading or non-degrading PEG gels and this change in collagen morphology may be due to the presence of ChSA. By four weeks, the distribution of collagen appeared similar to the two week gels for all systems including the 40% ChSA copolymer gel (data not shown).

The results presented here demonstrate that hydrogels consisting of pure chondroitin sulfate inhibit collagen and GAG synthesis and down-regulate type II collagen expression under in vitro culture conditions. Chondroitin sulfate adds additional components to the gel system in terms of its potential biological activity as the primary component of proteoglycans and its negative charges, which may influence chondrocyte metabolism differently compared to the neutral, stealth-like chemistry of PEG.

There have been several studies investigating the effects of glycosaminoglycans on chondrocytes. Handley et al. [36,37] found that the presence of exogenous proteoglycans in suspended cultures of aggregated chondrocytes inhibited collagen production due to a decrease in the synthesis of the collagen polypeptide chain, a precursor of procollagen [38]. It is possible that the presence of exogenous chondroitin sulfate in the hydrogel may inhibit endogenous secretion of proteoglycans and collagen. Numerous groups are also investigating novel scaffolds with chondroitin sulfate [39] and other polysaccharides [40], but few publications to date report on chondrocytes seeded in these scaffolds. One study demonstrated that chondroitin sulfate modified surfaces of chitosan did not alter GAG production compared to polystyrene controls, but did increase type II collagen synthesis by chondrocytes cultured in monolayer [41]. When chondrocytes were seeded onto type I collagen scaffolds, cell proliferation and total proteoglycan production were enhanced in scaffolds with covalently linked ChSA, but collagen synthesis was not addressed [42]. In these studies, chondroitin sulfate was incorporated into the scaffold in conjunction with other chemistries. Here, we demonstrate hydrogel scaffolds comprised of solely ChSA inhibit chondrocyte biosynthetic activity.

The biological role of glycosaminoglycans was explored in an effort to elucidate this inhibition. GAGs are known to interact with a plethora of proteins that are important in both extracellular and intracellular events [43]. Interestingly, the dry weights of the ChSA controls increased significantly from 3.3 ± 0.6 mg at 4 weeks to 5.5 ± 0.4 mg at 8 weeks ($p < 0.01$), a 67% increase. A similar increase, although not significant, was observed in the ChSA gels with cells. It is possible, that proteins (e.g., growth factors and other signaling molecules) present in the serum interact with the immo-

bilized ChSA causing an increase in the dry mass; and as a result the proteins are hindered from diffusing to and interacting with the encapsulated chondrocytes. In addition, the high concentration of fixed negative charge in the ChSA gels may attract free cations from the medium, resulting in an increase in osmolality within the hydrogel. Chondrocytes are known to be highly osmotic sensitive cells [44]. For example, when the osmolality was altered by adding sodium ions to the culture medium of suspended isolated chondrocytes, proteoglycan and collagen synthesis was affected in a concentration dependent manner [45]. Specifically, maximum synthesis was observed near physiological osmolality while above and below the physiological range synthesis decreased. The osmolality in the ChSA/PEG copolymer gels may be closer to physiological levels of osmolality compared to that of the pure ChSA gels. The degree of osmolality will depend on the amount of free ions in the medium solution. Therefore exploring different levels of culture medium osmolality may provide insight into osmolality effects on chondrocyte biosynthesis within these charged ChSA gels.

With the incorporation of PEG, a neutral, non-interactive molecule, both GAG synthesis and collagen synthesis were stimulated in chondrocytes. How the ChSA, particularly within the pure ChSA gels, interacts with the cells to inhibit synthesis of ECM components remains unclear, but is likely a combination of events. Further studies are necessary to understand this complex interaction between ChSA and the embedded chondrocytes. It is important to note that the in vitro environment is not always a good predictor for what happens in vivo. For instance, chondrocytes have been encapsulated in type I collagen gels, and under in vitro conditions, the chondrocytes dedifferentiated into fibroblast-like cells [46]. However, when placed in vivo into full-thickness articular cartilage defects, hyaline cartilage formed in 4 weeks [47]. Our current studies indicate that the combination of the in vivo environment and the incorporation of degradation into these copolymer gels significantly enhances chondrocyte biosynthetic activity (unpublished data).

PEG gels create a desirable 3-D niche for cell encapsulation and this 'permissive' environment enables chondrocytes to secrete and distribute ECM components when the degradation is properly tuned [16]. The goal of incorporating ChSA is to control further the gel macroscopic properties (the degree of methacrylation can be tightly controlled [17] in order to minimize the ChSA concentration while maximizing the effects of charge), but also to create a cell 'promoting' environment (e.g., cell responsive degradation and biological effects of ChSA). Interestingly, our results demonstrate that pure ChSA gels are not 'promoting' for chondrocytes and this may have important implications for tissue engineering. The gel composition and ChSA levels must be appropri-

ately tuned to create a developing or healing environment, rather than a mature extracellular matrix that down-regulates cell expression of these proteins and ECM molecules.

4. Conclusions

Hydrogel properties were manipulated by copolymerizing a negatively charged chondroitin sulfate macromer into a base PEG hydrogel cell carrier. For a given equilibrium mass swelling ratio of ~ 9 , the gel compressive modulus increased from 34 to 140 kPa with the incorporation of ChSA into the gel. When chondrocytes were photoencapsulated in pure ChSA gels, neither GAG nor collagen was present after 8 weeks in vitro. However, when PEG was introduced into the network, GAG and collagen synthesis was stimulated in the copolymer gels. Gene expression studies confirmed these results, showing significantly higher collagen type II expression in PEG/ChSA copolymer gels compared to pure ChSA gels after 2 weeks. This study demonstrates that ChSA enhances gel macroscopic properties, but inhibits chondrocyte biosynthetic activity while the PEG base chemistry enhances tissue formation that is comprised of collagen and glycosaminoglycans.

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References

- [1] Freed LE, Marquis JC, Nohria A, Emmanuel J, Mikos AG, Langer R. Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers. *J Biomed Mater Res* 1993;27(1):11–23.
- [2] Schreiber RE, Dunkelman NS, Naughton G, Ratcliffe A. A method for tissue engineering of cartilage by cell seeding on bioresorbable scaffolds. *Ann NY Acad Sci* 1999;875(Bioartificial Organs II):398–404.
- [3] Rotter N et al. Cartilage reconstruction in head and neck surgery: comparison of resorbable polymer scaffolds for tissue engineering of human septal cartilage. *J Biomed Mater Res* 1998;42(3):347–56.
- [4] Nehrer S et al. Canine chondrocytes seeded in type I and type II collagen implants investigated in vitro. *J Biomed Mater Res* 1997;38(2):95–104.
- [5] Brun P et al. Chondrocyte aggregation and reorganization into three-dimensional scaffolds. *J Biomed Mater Res* 1999;46(3):337–46.
- [6] Aigner J et al. Cartilage tissue engineering with novel nonwoven structured biomaterial based on hyaluronic acid benzyl ester. *J Biomed Mater Res* 1998;42(2):172–81.
- [7] Paige KT, Cima LG, Yaremchuk MJ, Schloo BL, Vacanti JP, Vacanti CA. De novo cartilage generation using calcium alginate–chondrocyte constructs. *Plast Reconstr Surg* 1996;97(1):168–78.
- [8] Sims CD et al. Tissue engineered neocartilage using plasma derived polymer substrates and chondrocytes. *Plast Reconstr Surg* 1998;101(6):1580–5.
- [9] Silverman RP, Passaretti D, Huang W, Randolph MA, Yaremchuk MJ. Injectable tissue-engineered cartilage using a fibrin glue polymer. *Plast Reconstr Surg* 1999;103(7):1809–18.
- [10] Butnariu-Ephrat M, Robinson D, Mendes DG, Halperin N, Nevo Z. Resurfacing of goat articular cartilage by chondrocytes derived from bone marrow. *Clin Orthop Relat R* 1996(330):234–43.
- [11] Robinson D, Halperin N, Nevo Z. Regenerating hyaline cartilage in articular defects of old chickens using implants of embryonal chick chondrocytes embedded in a new natural delivery substance. *Calcif Tissue Int* 1990;46(4):246–53.
- [12] Suh JK, Matthew HW. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. *Biomaterials* 2000;21(24):2589–98.
- [13] Lee KY, Rowley JA, Eisele P, Moy EM, Bouhadir KH, Mooney DJ. Controlling mechanical and swelling properties of alginate hydrogels independently by cross-linker type and cross-linking density. *Macromolecules* 2000;33(11):4291–4.
- [14] Bryant SJ, Nuttelman CR, Anseth KS. The effects of crosslinking density on cartilage formation in photocrosslinkable hydrogels. *Biomed Sci Instrum* 1999;35:309–14.
- [15] Elisseff J et al. Transdermal photopolymerization of poly(ethylene oxide)-based injectable hydrogels for tissue-engineered cartilage. *Plast Reconstr Surg* 1999;104(4):1014–22.
- [16] Bryant SJ, Anseth KS. Controlling the spatial distribution of ECM components in degradable PEG hydrogels for tissue engineering cartilage. *J Biomed Mater Res* 2003;64A(1):70–9.
- [17] Bryant SJ, Davis-Arehart KA, Luo N, Shoemaker RK, Arthur JA, Anseth KS. Synthesis and characterization of photopolymerized multifunctional hydrogels: water-soluble poly(vinyl alcohol) and chondroitin sulfate macromers for chondrocyte encapsulation. *Macromolecules* 2004;37(18):6726–33.
- [18] Li Q, Wang DA, Elisseff JH. Heterogeneous-phase reaction of glycidyl methacrylate and chondroitin sulfate: Mechanism of ring-opening-transesterification competition. *Macromolecules* 2003;36(7):2556–62.
- [19] Pavia DL, Lampman GM, Kriz GS. Introduction to organic laboratory techniques: a contemporary approach. Fort Worth: Saunders College Publishing; 1988.
- [20] Bryant SJ, Nuttelman CR, Anseth KS. Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 fibroblasts in vitro. *J Biomater Sci-Polym E* 2000;11(5):439–57.
- [21] Freed L, Vunjak-Novakovic G. Tissue engineering of cartilage. In: Bronzind J, editor. *The biomedical engineering handbook*. Boca Raton, FL: CRC; 1995. p. 1788–806.
- [22] Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* 1986;883(2):173–7.
- [23] Woessner Jr JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* 1961;93:440–7.
- [24] Hollander AP et al. Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. *J Clin Invest* 1994;93(4):1722–32.
- [25] Chomczynski P, Sacchi N. single-step method of Rna isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal Biochem* 1987;162(1):156–9.
- [26] Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. NY: Cold Spring Harbor Laboratory Press; 1989.
- [27] Sawhney AS, Pathak CP, Hubbell JA. Bioerodible hydrogels based on photopolymerized poly(ethylene glycol)-co-poly

- (α -hydroxy acid) diacrylate macromers. *Macromolecules* 1993; 26(4):581–7.
- [28] Metters AT, Anseth KS, Bowman CN. Fundamental studies of a novel, biodegradable PEG-*b*-PLA hydrogel. *Polymer* 2000;41(11):3993–4004.
- [29] Martens P, Metters AT, Anseth KS, Bowman CN. A generalized bulk-degradation model for hydrogel networks formed from multivinyl cross-linking molecules. *J Phys Chem B* 2001; 105(22):5131–8.
- [30] Armstrong CG, Mow VC. Variations in the intrinsic mechanical properties of human articular cartilage with age, degeneration, and water content. *J Bone Jt Surg Am* 1982;64(1):88–94.
- [31] Bryant SJ, Anseth KS. Hydrogel properties influence ECM production by chondrocytes photoencapsulated in poly(ethylene glycol) hydrogels. *J Biomed Mater Res* 2002;59(1):63–72.
- [32] Bryant SJ, Anseth KS. The effects of scaffold thickness on tissue engineered cartilage in photocrosslinked poly(ethylene oxide) hydrogels. *Biomaterials* 2001;22(6):619–26.
- [33] Elisseff J, McIntosh W, Anseth K, Riley S, Ragan P, Langer R. Photoencapsulation of chondrocytes in poly(ethylene oxide)-based semi-interpenetrating networks. *J Biomed Mater Res* 2000; 51(2):164–71.
- [34] Sims CD et al. Injectable cartilage using polyethylene oxide polymer substrates. *Plast Reconstr Surg* 1996;98(5):843–50.
- [35] Hall BK. *Cartilage*. New York: Academic Press; 1983.
- [36] Handley CJ, Brooks PR, Lowther DA. Extracellular matrix metabolism by chondrocytes. VI. Concomitant depression by exogenous levels of proteoglycan of collagen and proteoglycan synthesis by chondrocytes. *Biochim Biophys Acta* 1978;544(2):441–4.
- [37] Handley CJ, Lowther DA. Extracellular matrix metabolism by chondrocytes. 3. modulation of proteoglycan synthesis by extracellular levels of proteoglycan in cartilage cells in culture. *Biochim Biophys Acta* 1977;500(1):132–9.
- [38] Handley CJ, Brooks P, Lowther DA. Suppression of collagen synthesis by chondrocytes by exogenous concentrations of proteoglycan subunit. *Biochem Int* 1980;1(3):270–6.
- [39] Pieper JS, Hafmans T, Veerkamp JH, Van Kuppevelt TH. Development of tailor-made collagen–glycosaminoglycan matrixes: EDC/NHS crosslinking, and ultrastructural aspects. *Biomaterials* 2000;21(6):581–93.
- [40] Smeds KA, Grinstaff MW. Photocrosslinkable polysaccharides for in situ hydrogel formation. *J Biomed Mater Res* 2000;54(1): 115–121.
- [41] Sechrist VF et al. GAG-augmented polysaccharide hydrogel: a novel biocompatible and biodegradable material to support chondrogenesis. *J Biomed Mater Res* 1999;49(4):534–41.
- [42] van Susante JLC et al. Linkage of chondroitin–sulfate to type I collagen scaffolds stimulates the bioactivity of seeded chondrocytes in vitro. *Biomaterials* 2001;22(17):2359–69.
- [43] Jackson RL, Busch SJ, Cardin AD. Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiol Rev* 1991;71(2):481–539.
- [44] Hall AC. Differential effects of hydrostatic pressure on cation transport pathways of isolated articular chondrocytes. *J Cell Physiol* 1999;178(2):197–204.
- [45] Urban JPG, Hall AC, Gohl KA. Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes. *J Cell Physiol* 1993;154(2):262–70.
- [46] van Susante JL et al. Culture of chondrocytes in alginate and collagen carrier gels. *Acta Orthop Scand* 1995;66(6):549–556.
- [47] Wakitani S, Goto T, Young RG, Mansour JM, Goldberg VM, Caplan AI. Repair of large full-thickness articular cartilage defects with allograft articular chondrocytes embedded in a collagen gel. *Tissue Eng* 1998;4(4):429–44.
- [48] Saldanha V, Grande DA. Extracellular matrix protein gene expression of bovine chondrocytes cultured on resorbable scaffolds. *Biomaterials* 2000;21(23):2427–31.
- [49] Allemann F, Mizuno S, Eid K, Yates KE, Zaleske D, Glowacki J. Effects of hyaluronan on engineered articular cartilage extracellular matrix gene expression in 3-dimensional collagen scaffolds. *J Biomed Mater Res* 2000;55(1):13–9.