
Hydrogel properties influence ECM production by chondrocytes photoencapsulated in poly(ethylene glycol) hydrogels

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Abstract: When using hydrogel scaffolds for cartilage tissue engineering, two gel properties are particularly important: the equilibrium water content (q , equilibrium swelling ratio) and the compressive modulus, K . In this work, chondrocytes were photoencapsulated in degrading and nondegrading poly(ethylene glycol)-based hydrogels to assess extracellular matrix (ECM) formation as a function of these gel properties. In nondegrading gels, the glycosaminoglycan (GAG) content was not significantly different in gels when q was varied from 4.2 to 9.3 after 2 and 4 weeks *in vitro*. However, gels with a q of 9.3 allowed GAGs to diffuse throughout the gels homogeneously, but a $q \leq 5.2$ resulted in localization of GAGs pericellularly. Interestingly, in the moder-

ately crosslinked gels with a K of 360 kPa, an increase in type II collagen synthesis was observed compared with gels with a higher (960 kPa) and lower (30 kPa) K after 4 weeks. With the incorporation of degradable linkages into the network, gel properties with an initially high K (350 kPa) and final high q (7.9) were obtained, which allowed for increased type II collagen synthesis coupled with a homogenous distribution of GAGs. Thus, a critical balance exists between gel swelling, mechanics, and degradation in forming a functional ECM. © 2001 John Wiley & Sons, Inc. *J Biomed Mater Res* 59: 63–72, 2002

Key words: tissue engineering; cartilage; hydrogels; photopolymerization; poly(ethylene glycol)

INTRODUCTION

Hydrogels are attractive materials for tissue engineering because of their high water content and tissue-like elastic properties. In recent years, hydrogels have been investigated as suitable materials for tissue engineering cartilage,^{1–5} bone,^{6–8} tendons,⁹ and nerves.^{10–12} Hydrogels provide a highly swollen three-dimensional environment with a high water content, resembling to some degree the environment of the native tissue, which helps to promote cell proliferation and cell function. The high equilibrium swelling allows increased diffusion of nutrients into the gel and cellular waste out of the gel. Hydrogels are attractive materials for tissue engineering, particularly cartilage tissue engineering, because they provide an environment for

the chondrocytes to retain their native form, surrounded by a lacunae.

Another advantage of hydrogels for tissue engineering applications is the relatively mild gelation conditions for numerous naturally occurring polymers that enable encapsulation of cells under physiological temperature and pH. Examples of these materials include alginates and fibrin glue, which have been used to encapsulate chondrocytes and successfully generate cartilaginous tissue. Specifically, autologous chondrocytes were mixed with a solution of alginate and calcium ions and injected subcutaneously into immunocompetent porcine animal models. After 6 weeks *in vivo*, there was histological evidence of cartilaginous tissue within the alginate gel.¹ Ting et al.² encapsulated chondrocytes in a fibrin glue gel, and the glycosaminoglycan content was similar to that of native cartilage tissue after 6 and 12 weeks *in vivo*. Although these naturally based hydrogel systems provide many advantages for tissue engineering, limitations exist with respect to gel properties and gelation process. For example, gelation begins upon mixing of two components, which limits temporal control of the process; the stability of the crosslinks in the gels is limited

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and difficult to control *in vivo*, and the mechanics of these gels are inferior to that of native cartilage.

In designing a suitable hydrogel for tissue engineering cartilage, the scaffold should be able to withstand the normal loads and stresses of native cartilage, especially in the initial stages, before the cells begin producing their own functional extracellular matrix (ECM). For example, a fibrin glue gel polymerized in the absence of cells and swollen in PBS at 37°C for 24 h has a compressive modulus (K) of ~30 kPa,¹³ which is considerably lower than that of native cartilage (where K ranges from 500 to 1000 kPa).¹⁴ Lee et al.¹⁵ incorporated covalent crosslinks into alginate gels in an effort to increase the mechanics of these natural-based systems. By the addition of adipic dihydrazide as a crosslinking agent, the shear modulus was increased from 24.4 ± 0.6 kPa (when the crosslinker was calcium ions) to 36.9 ± 3.7 kPa, which was still significantly lower than the shear modulus reported for articular cartilage in canine models (380 to 650 kPa).¹⁶

As an alternative to these natural hydrogels, we are interested in synthetic and photocrosslinkable hydrogels based on poly(ethylene glycol) (PEG). Although alginates and fibrin glue have certain advantages based on their natural structure and composition, synthetic materials provide greater control over the final macroscopic hydrogel properties. In addition, photopolymerizable hydrogels allow temporal and spatial control of the polymerization and gelation process, which increases the ease of handling for the surgeon as well as the ability to form complex architectures *in vivo*. For a given macromer chemistry, a range of gel properties can be obtained by controlling the network crosslinking density (e.g., by varying the molecular weight of the macromer or varying the percent macromer in solution before polymerization). As the crosslinking density increases, the equilibrium water content decreases, but the compressive modulus increases.¹⁷ These two properties are particularly important from a tissue-engineering perspective. Hydrogel swelling can impact transport and overall cell viability, whereas the gel mechanics can limit the practical application of the materials and influence cell behavior.¹⁸

Hence, this study attempts to elucidate the role of hydrogel crosslinking density on the chondrocytes' ability to produce cartilaginous tissue when photoencapsulated in PEG hydrogels. From a practical perspective, we aim to maximize gel mechanics while maintaining a critical water content for cell viability and cell function. From a fundamental perspective, we aim to elucidate the role of crosslinking density on ECM formation. Chondrocytes were photoencapsulated in PEG gels of similar composition but varying crosslinking density, and the cell-hydrogel constructs

were examined biochemically and histologically to assess the cartilaginous tissue formed in the constructs. Initially, gels that did not degrade on the time scale of the experiment were investigated to eliminate temporal changes in the gel properties during the study. Subsequent to these studies, a degradable PEG macromer¹⁹ was incorporated into the network to further understand the role of crosslinking density and the changes in the gel properties with degradation, on ECM formation.

MATERIALS AND METHODS

Chondrocyte isolation

Chondrocytes were isolated from the femoral-patellar groove of a young calf (Research 87, Marlboro, MA). Briefly, the articular cartilage was excised under aseptic conditions and digested using a solution of 0.2% Collagenase Type II (Gibco) and 5% fetal bovine serum (Gibco) in Dulbecco's modified eagle's medium (Gibco) without additives for a maximum of 17 h at 37°C on an orbital shaker. The solution was filtered through a 70- μ m nylon cell strainer and centrifuged at 1000 rpm for 10 min; the supernatant was then aspirated off, and the pellet resuspended in PBS (Gibco) supplemented with 1% penicillin-streptomycin (Gibco) and 0.02% ethylenediaminetetraacetic acid (EDTA, Aldrich). The solution was centrifuged an additional two times and resuspended in PBS. The chondrocyte viability was determined using trypan blue exclusion and a hemacytometer.

Hydrogel preparation

Poly(ethylene glycol) dimethacrylate (PEGDM; MW, 3400) was purchased from Shearwater Polymers, and a triblock copolymer, poly(lactic acid)-*b*-poly(ethylene glycol)-*b*-poly(lactic acid) with acrylate end groups (PEG-LA-DA; MW, 4600) was synthesized as described elsewhere.¹⁹ The poly(lactic acid) (PLA) chemistry was chosen because of its degradation pathway via hydrolysis as well as PLAs wide use in preparing scaffolds for tissue engineering.²⁰⁻²² On average, five lactide repeat units were added to each side of the core PEG molecule. The chemical structures of the macromers are shown in Figure 1. The solid macromers were sterilized for 20 min under UV irradiation. The macromers were then dissolved in sterile PBS (Gibco) or water at a concentration of 10%, 20%, and 30% (w/w). The UV photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methylpropanone (D2959, Ciba-Geigy), was used under previously determined cytocompatible initiating conditions.²³ The photoinitiator was dissolved in water in concentrated amounts and filter-sterilized, and a small amount was added to the macromer solution to a final concentration of 0.05% (w/w).

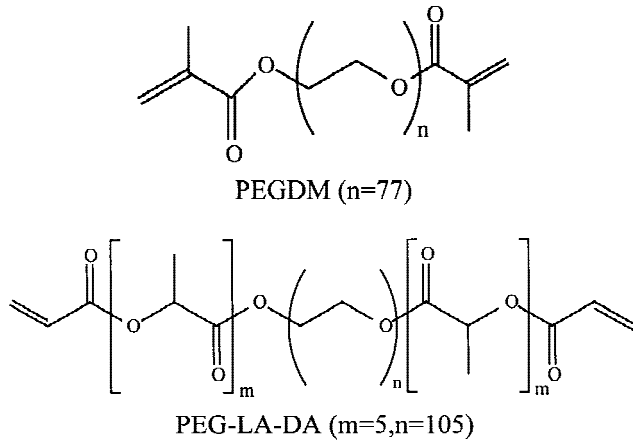


Figure 1. Chemical structures of the PEG-based macromers.

Cell encapsulation

Chondrocytes were seeded at a cell density of 75 million cells per milliliter of the macromer solution. A volume of 40 μL of cell-macromer solution was photopolymerized using a long-wave UV lamp (Model B100AP, Blak-Ray) at an intensity of $\sim 10 \text{ mW}/\text{cm}^2$ for 10 min. The resulting cell-hydrogel constructs were incubated at 37°C under static conditions in a humid environment with 5% CO_2 in 12-well plates. The constructs were cultured for 4 weeks using Dulbecco's modified Eagle's medium (Gibco), without phenol red, supplemented with 10 mM HEPES, 0.04 mM L-proline, 50 mg/L L-ascorbic acid, 0.1M MEM nonessential amino acids (Gibco), 1% penicillin-streptomycin (Gibco), 0.5 $\mu\text{g}/\text{mL}$ fungizone (Gibco), and 10% fetal bovine serum (Gibco). The medium was replaced twice a week.

Biochemical assays

The constructs were freeze-dried for 24 h and then digested in a papain solution [125 $\mu\text{g}/\text{mL}$ papain type III (Worthington), 10 mM L-cysteine (Aldrich), 100 mM phosphate, and 10 mM EDTA at pH 6.3] for 15 h at 60°C . Total glycosaminoglycan content was determined using the dimethylmethylene blue dye method.²⁴ Total collagen content was determined using the hydroxyproline assay²⁵ in which hydroxyproline makes up $\sim 10\%$ of collagen.²⁶ DNA content was measured using Hoechst 33258 (Polysciences), and the chondrocyte number was determined by using the conversion factor 7.7 pg of DNA per chondrocyte.²⁷ The GAG and total collagen contents were normalized by the number of chondrocytes. This normalization is particularly important in hydrogels, which do not degrade over the course of the experiment. The difference in macromer concentration within the gels leads to differences in overall water content and polymer mass. Therefore, to minimize the influence of variations from gel to gel with respect to wet weight and dry weight, the biochemical content was normalized by cell number. A sample size of three was used.

Histological analysis

After 4 weeks *in vitro*, two cell-hydrogel constructs of each system were fixated overnight with 10% formalin, embedded in paraffin, and microtomed in 8- μm sections. Following standard histological staining procedures, the sections were stained with Safranin O and fast green for negatively charged proteoglycans (GAG), which stain red, and stained for collagen using the Masson's trichrome stain, which stains collagen blue.

Immunohistochemistry

The paraffin sections were stained with a labeled streptavidin-biotin immunoenzymatic antigen detection system (UltraVision Detection System, NeoMarkers, Inc.). The paraffin sections were enzymatically digested with pepsin (1 mg/mL in 0.01N HCl) for 30 min at 37°C . Collagen type II mouse monoclonal antibody was obtained from NeoMarkers Inc. Collagen type II mouse monoclonal antibody was obtained from Chemicon Inc. Healthy bovine articular cartilage and bovine achilles tendon were used as positive controls for collagen type II and type I, respectively.

Swelling studies

Hydrogel discs prepared from the PEGDM macromer were weighed immediately after polymerization and then allowed to swell in PBS at 37°C for 48 h to reach equilibrium. After equilibration, the discs were then weighed again to determine the equilibrium swollen mass. The dry (or polymer) mass was obtained for each disk by drying the discs for at least 24 h in a vacuum oven and subsequently weighing. The equilibrium mass swelling ratio, q , was calculated by ratioing the equilibrium swollen mass by the polymer dry mass. Partially degradable hydrogel discs were prepared by copolymerizing PEGDM with PEG-LA-DA. The discs were weighed immediately after polymerization and degraded in PBS at 37°C . During degradation, the discs were weighed daily until hydrolysis of the degradable crosslinks was complete and a stable equilibrium swelling ratio was reached. A sample size of three was used.

Compression tests

The compressive modulus of elasticity was measured using a dynamic mechanical analyzer (DMA-7, Perkin Elmer) in unconfined compression at a rate of 40 mN/min at room temperature. Hydrogels prepared from PEGDM were allowed to swell to equilibrium in PBS at 37°C for 48 h. Discs of 5 mm in diameter and approximately 2 mm thick were used in the tests. The hydrogels prepared from copolymerizing PEGDM with PEG-LA-DA were degraded in PBS at 37°C . The compressive modulus was measured daily for the partially degrading hydrogels until a stable modulus was reached. A sample size of five was used.

Hydrogel characterization

The average mesh size, ξ , in the hydrogels was determined as described by Canal and Peppas.²⁸

$$\xi = v_{2,s}^{-1/3} l C_n^{1/2} n^{1/2} \quad (1)$$

where $v_{2,s}$ is the equilibrium polymer volume fraction in the gel, l is the bond length, C_n is the characteristic ratio of the polymer; and n is the number of bonds between the crosslinks. The equation was modified slightly because, in these hydrogels, the crosslinker is the PEG macromer and not a point source. The modified equation provides a good approximation for determining the mesh size in these PEG hydrogels.²⁹ To determine n , the molecular weight between crosslinks, \overline{M}_c , was estimated using a modified Flory-Rehner equation neglecting chain ends in which the polymer-solvent interaction parameter, χ_{12} , was assumed constant over the macromer concentration used in this study.³⁰ Merrill et al.³¹ found χ_{12} to be 0.426 and constant over a range of $v_{2,s}$ values from 0.04 to 0.2 for PEG in water and PBS.

Statistical analysis

Statistical analysis was performed using a Student's t test with a confidence level of 0.05. All values in this article are reported as the mean with a standard deviation.

RESULTS AND DISCUSSION

Understanding the role of gel properties on ECM formation is important for numerous tissue-engineering applications. In this contribution, chondrocytes were encapsulated in both degrading and nondegrading gels, and the biochemical composition and distribution of the ECM was examined as a function of the gel properties, particularly swelling and mechanics. In the first part of this study, chondrocytes were suspended in a solution of the PEGDM macromer and photopolymerized to produce hydrogels where the network structure was fixed and would not

degrade during the course of the experiment. In the second part of the study, the PEGDM macromer was copolymerized with a degradable PEG macromer so a certain percentage of the network crosslinks degraded over the course of the experiments. By introducing degradation, we aimed to further understand how temporal changes in the gel properties influence ECM formation.

Nondegradable hydrogels

PEG hydrogels were synthesized by photopolymerizing the PEGDM macromer at different concentrations to yield gels with the same chemistry, but different crosslinking densities. These gels did not degrade under the *in vitro* conditions used and on the time-scale studied; however, the ester linkage has been shown to degrade slowly *in vivo*.³² The photocrosslinked PEG hydrogels had varying degrees of crosslinking and spanned a range of properties (Table I). Bovine articular cartilage is comprised of ~80% water, which corresponds to a q of ~5, and has a compressive modulus of 500–1000 kPa.¹⁴ With these synthetic PEG gels, we can obtain swelling and static compressive modulus properties that closely approach that of native cartilage, which is difficult to achieve in the natural hydrogel systems. By varying the concentration of macromer in the PEG gels, we obtained equilibrium swelling ratios that varied from 9.3 to 4.2 and compressive moduli that spanned nearly two orders of magnitude from 34 to 1370 kPa. The mesh size of the gels was shown to decrease with increasing macromer concentration from 140 to 40 Å. The dependence of the final network crosslinking density on the initial macromer concentration is due, in part, to cyclization, which occurs with an increase in solvent concentration. Cyclization decreases the crosslinking efficiency and leads to a more loosely crosslinked gel.³³

The gel properties were also measured in the presence of chondrocytes as a function of macromer con-

TABLE I
Properties of Nondegradable Hydrogels^a

% PEGDM ^b	q^c	Compressive Modulus (kPa)	Mesh Size (Å)	q (w/cells) ^c	Compressive Modulus (kPa) (w/cells)
10	9.3 ± 1.0	34 ± 3	140	12.6 ± 0.2	30 ± 1
20	5.2 ± 0.1	360 ± 14	60	6.4 ± 0.05	260 ± 30
30	4.5 ± 0.1	940 ± 60	50	5.2 ± 0.2	400 ± 100
40	4.2 ± 0.1	1370 ± 20	40	—	—

^aHydrogels do not degrade on the time scale and conditions of the experiment.

^bWeight percent macromer before polymerization.

^cMass swelling ratio (equilibrium swollen mass/dry polymer mass).

^d—, not measured.

centration (Table I). The incorporation of cells into the network adds complexity to understanding the gel properties and quantifying parameters such as mesh size. The water content significantly increased and the compressive modulus decreased in all systems when chondrocytes were present in the gels. The increase in swelling was attributed, in part, to the cells diluting the macromer concentration and, in part, to the high water content of the cells. With respect to the reduction in the compressive modulus, the cells may absorb some of the load applied to the construct and subsequently deform, which may influence the measurements of the compressive modulus (i.e., if the cells are more compressible than the gel alone). Lee et al.³⁴ showed that chondrocytes encapsulated in alginate gels deform under a compressive load. The properties of the gel with 40% macromer were not measured in the presence of cells. In our preliminary studies, the viability of the chondrocytes was significantly compromised during the polymerization of the gel with 40% macromer, and therefore, further experiments with this gel were not performed in the presence of cells.

For chondrocytes encapsulated in 10, 20, and 30% PEGDM hydrogels, the biochemical composition of the evolving neocartilaginous tissue was analyzed at 2 and 4 weeks and examined as a function of the gel properties. The results are shown in Figure 2. The GAG content was significantly higher than the total collagen content in all three systems, which is typical of *in vitro* culture. In a previous study by Elisseff et al.,⁴ cartilaginous tissue formed in PEG semi-interpenetrating networks was comprised of 4.5% GAG per wet weight (ww) and 0.4% total collagen content per ww for constructs cultured *in vitro*, whereas the same system *in vivo* was comprised of 1.5% GAG per ww and 2.5% total collagen per ww⁵ after 2 weeks.

Both the GAG and total collagen content increased from 2 to 4 weeks of culture. The GAG content was

statistically similar in all three systems at each time point, suggesting that the gel properties do not influence the amount of GAG present in the cartilaginous tissue. The mean total collagen content was higher in the gels with a q of 9.3 compared with the other gels with a lower q after 2 and 4 weeks of culture. In evaluating the total collagen content, the most loosely crosslinked system shows increased total collagen synthesis over the more tightly crosslinked systems. However, overall, the PEGDM gels provide an environment that maintains cell viability and promotes cell function (i.e., the secretion of GAGs and collagen) in gels with a q of at least 4.5, which corresponds to a gel that is composed of 78% water.

In addition to monitoring the total biochemical composition, the distribution of the newly synthesized matrix molecules is extremely important when engineering a tissue equivalent. The primary components of articular cartilage, GAG and collagen, were evaluated histologically to gain a better understanding of the role of crosslinking density on the spatial distribution of the ECM components. Gels with increasing crosslinking density were stained for negatively charged GAGs (Fig. 3). Although, the GAG content was similar in all three hydrogels when measured biochemically, the distribution of the GAG molecules is clearly affected by the gel properties. In the most loosely crosslinked system with a q of 9.3, the GAG molecules are distributed throughout the gel. However, in the gels that are more densely crosslinked, the GAG molecules are retained in the pericellular regions. The mesh size in these gels was calculated to be ~ 140 Å in the most loosely crosslinked system and ~ 50 Å in the most densely crosslinked system (Table I). Interestingly, the size of a proteoglycan aggregate is approximately 90 Å in width as estimated by electron micrography.³⁵ Here, the data clearly illustrate the impact of hydrogel structure on the diffusion and distribution of proteoglycans in the cell-hydrogel construct.

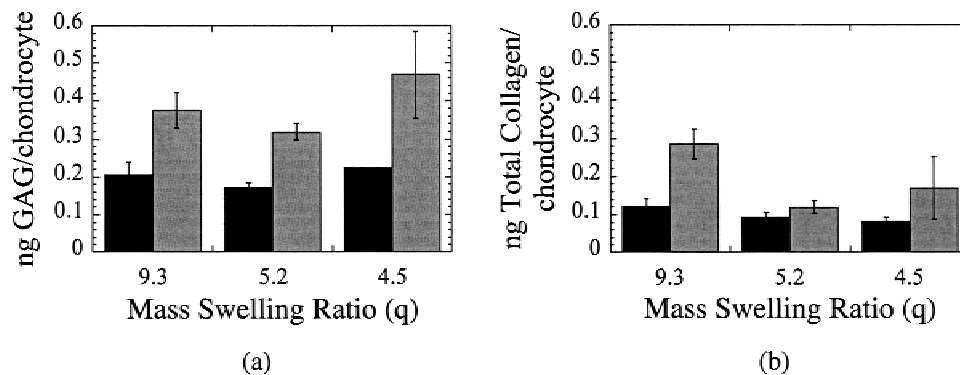


Figure 2. Biochemical composition of cartilaginous tissue as a function of gel properties in gels that do not degrade on the time scale of the experiment. (a) GAG content and (b) total collagen content. ■ and ▒, cell-hydrogel constructs cultured for 2 and 4 weeks *in vitro*, respectively. q is the equilibrium mass swelling ratio defined as the mass of the swollen gel divided by the mass of the dry polymer.

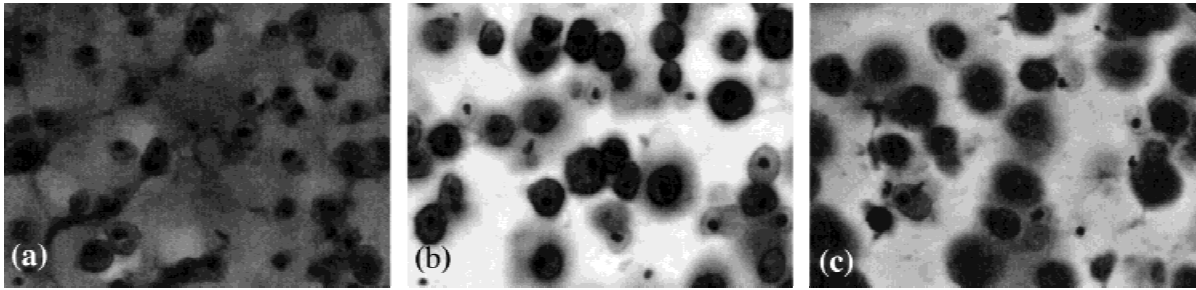


Figure 3. Histological analysis of glycosaminoglycans as a function of gel properties after 4 weeks *in vitro*. Safranin O stains proteoglycans red (shown in grayscale). (a) $q = 9.3$ and $\xi = 140 \text{ \AA}$, (b) $q = 5.2$, $\xi = 60 \text{ \AA}$, and (c) $q = 4.5$, $\xi = 50 \text{ \AA}$. The gels do not degrade on the time scale of the experiment. Original magnification $\times 400$.

The distribution of collagen in the cell-hydrogel constructs was also examined. As anticipated from the GAG results, collagen was present only in the lacunae in all three systems, and a representative histology micrograph is shown in Figure 4. This data suggest that the collagen distribution is inhomogeneous even in the most loosely crosslinked, nondegrading gels. Mature collagen is highly organized into microfibrils, which can range from 40,000 to 300,000 \AA in diameter, depending on the species.³⁶ Although beyond the scope of this article, we have shown elsewhere that a homogenous distribution of collagen is attainable by controlling the hydrogel degradation during cartilaginous tissue formation.³⁷

Furthermore, in assessing the extracellular matrix formed from chondrocytes encapsulated in hydrogels, it is important to assess the type of collagen synthesized in addition to the total collagen and collagen distribution. The collagen found in articular cartilage is comprised of primarily type II collagen. In each of the gels, immunohistochemistry was performed to examine the distribution of type II collagen (Fig. 5). These results suggest that not only the amount of collagen produced, but also the type of collagen produced, is dependent on the gel properties. Interest-

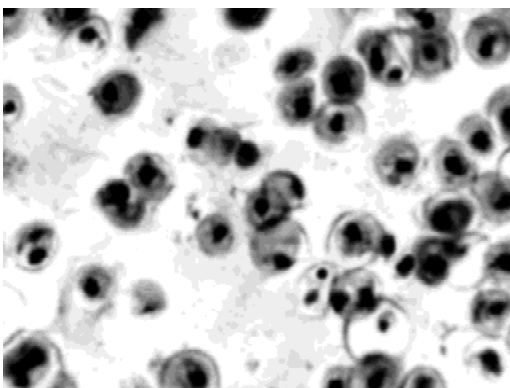


Figure 4. A representative histological analysis of collagen in a PEG hydrogel where $q = 5.2$ and $K = 360 \text{ kPa}$ after 4 weeks *in vitro*. Collagen stains blue (shown in grayscale). Original magnification $\times 400$.

ingly, in the most loosely crosslinked system with a compressive modulus of 30 kPa only a few lacunae stained positive for type II collagen. In the system with a compressive modulus of 360 kPa, a maximum of type II collagen production was observed after 4 weeks *in vitro*. The most densely crosslinked gel examined with a K of 940 kPa had only a few lacunae staining positive for type II collagen and was similar to the most loosely crosslinked gel. We hypothesize that the crosslinked gel imparts some strain on the cells, is indicated by the shape of the lacunae in the histological pictures. The lacunae appear slightly deformed and less rounded compared with native articular cartilage. This external force may influence the chondrocytes production of collagen. As seen in previous studies, which investigated dynamic stimulation on isolated chondrocytes, an upregulation of type II collagen mRNA synthesis was observed.^{38,39} However, further studies are required to better understand the mechanisms that influence the increased production of type II collagen in the moderately crosslinked PEG gels examined here. In an effort to elucidate the type of collagen found in these gels as seen by the Masson's trichrome stain, the gels were also stained for type I collagen. Interestingly, minimal staining was observed (data not shown). Articular cartilage also contains minor collagens of type IX and type XI,⁴⁰ which may account for the type of collagen seen in these gels. Further investigations are ongoing.

Partially degradable hydrogels

An important criteria in designing a suitable scaffold for tissue engineering cartilage is degradation, including the time scale for complete degradation and the overall mass loss or erosion profile. In the previous section, we examined the role of gel properties, in the absence of degradation, on the biochemical content, and histological appearance of ECM production. In these studies, degradable linkages were incorporated into the PEG hydrogels in a systematic and controlled

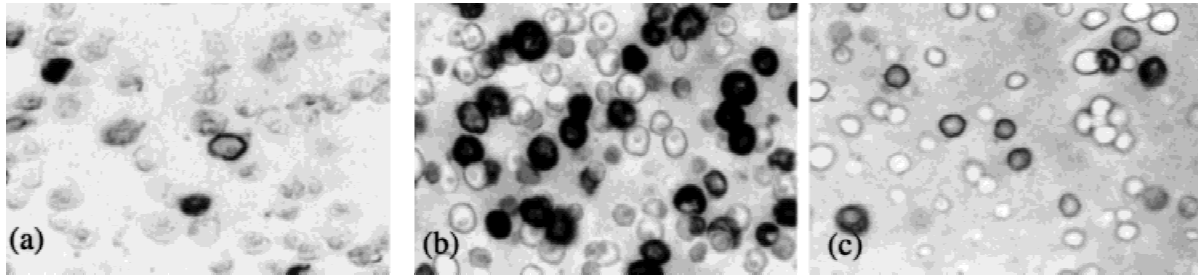


Figure 5. Immunohistochemical staining for type II collagen as a function of gel properties after 4 weeks *in vitro*. (a) $K = 34$ kPa, (b) $K = 360$ kPa, (c) $K = 940$ kPa. The gels do not degrade on the time scale of the experiment. Original magnification $\times 400$.

manner to examine the role of dynamic changes in the crosslinking density during ECM production. Specifically, two gels were prepared with a total macromer concentration of 10% and 20% (w/w). In each system, 57.5 mol% of the total macromer concentration was PEGDM (nondegradable macromer) and 42.5 mol% was PEG-LA-DA (degradable macromer).

The gel properties were measured as a function of degradation time and compared with initial gel properties. After ~ 7 days, both systems had reached their new equilibrium in which the degradable crosslinks were completely hydrolyzed. In the gel synthesized from 10% macromer, the equilibrium swelling was initially 9.8 ± 0.1 and increased to 16.6 ± 0.3 after the degradable linkages were completely hydrolyzed. The initial compressive modulus was 36 ± 2 kPa and decreased to 11 ± 1 kPa once degradation was complete. In the gel initially polymerized from a 20% macromer solution, the equilibrium swelling increased from 5.7 ± 0.6 to 7.9 ± 0.2 , and the compressive modulus decreased from 350 ± 30 kPa to 140 ± 10 kPa upon partial degradation.

To ensure that all of the degradable linkages were hydrolyzed, a previously determined scaling law was used to estimate how the crosslinking density, ρ_x , varies with measured properties such as swelling. For example, in these degrading PEG systems, it has been shown that the equilibrium volume swelling ratio is proportional to $\rho_x^{-3/5}$.⁴¹ If all the degradable linkages were hydrolyzed, then 42.5% of the crosslinks would

be broken, based on the initial molar concentration of degradable macromer incorporated into the network. Thus, the volume swelling ratio should increase by a factor of 1.4. Here, we observe that the model agrees very closely with the 20% macromer gel ($q_{\text{final}}/q_{\text{initial}} = 1.4$), but under-predicts for the 10% macromer gel ($q_{\text{final}}/q_{\text{initial}} = 1.6$).

In these partially degrading PEG gels, chondrocytes were encapsulated, and the biochemical composition was assessed at 2 and 4 weeks and compared with nondegrading gels. A slight increase was observed in both the GAG and total collagen contents in the partially degrading gels from 2 to 4 weeks *in vitro* as shown in Figure 6. The GAG and total collagen contents were statistically similar to their nondegrading equivalents, suggesting that the dynamic changes in gel properties do not influence the total biochemical content in gels. Therefore, degradation can be incorporated into the hydrogels without sacrificing the ECM formation, as measured biochemically.

In addition, the distribution of GAGs was examined histologically in the partially degradable systems (Fig. 7). In the gel with an initial q of 9.8, the GAGs are evenly distributed throughout the gel at 2 and 4 weeks, which is what we would expect based on the results from the nondegradable gels with a similar q . However, the system with an initial q of 5.7, the GAGs are located in the pericellular regions at 2 weeks, similar to its nondegrading equivalent at 4 weeks, but by 4 weeks the GAGs are evenly distributed throughout

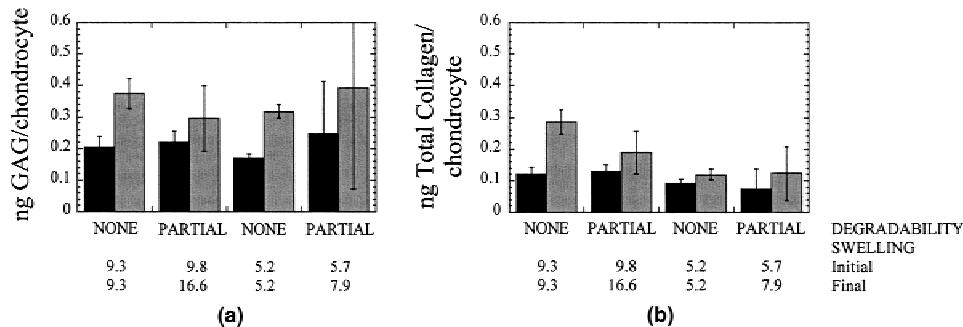


Figure 6. Biochemical composition of cartilaginous tissue as a function of gel properties and degradation. (a) GAG content and (b) total collagen content. ■ and ▣, cell-hydrogel constructs cultured for 2 and 4 weeks *in vitro*, respectively. q is the equilibrium mass swelling ratio defined as the mass of the swollen gel divided by the mass of the dry polymer.

the gel. After partial degradation, the q increases from 5.7 to 7.9. The mesh size of this system increases from ~ 75 to ~ 115 Å, based on the measured q . These results further support the results from the nondegradable gels that a minimum mesh size exists ($> \sim 90$ Å) and is important in facilitating GAG diffusion through the gels.

The distribution of collagen was also assessed in the gels, and after 4 weeks, the collagen was localized in the lacunae, similar to the nondegrading systems (as shown in Fig. 4). Even in the system with the highest final q of 16.6, the mesh size is ~ 250 Å, which is lower than that reported for the diameter of collagen fibers.³⁶ Therefore, we would expect that the newly synthesized collagen is restricted to the pericellular regions within the gel. To achieve uniform distribution of collagen in these gels requires the incorporation of at least 75% degradable crosslinks in the gel.³⁷

When the collagen was typed through immunohistochemistry (Fig. 8), again we see a dependence of K on the number of lacunae staining positive for type II collagen. In the partially degrading system, which has an initial compressive modulus similar to the 20% PEGDM, nondegradable system, we see an increase in the number of lacunae staining positive for type II collagen when compared with the more loosely crosslinked system. This data also supports the finding in the nondegradable systems that the compressive modulus may span from 140 to 360 kPa for increased type II collagen synthesis. It is not clear, if the increased type II collagen synthesis is a function of the initial compressive modulus or the final compressive modulus. Again, minimal staining for type I collagen

was observed. In these PEG hydrogels, a critical balance exists between q (swelling) to maintain cell viability and function, mechanics to maximize type II collagen synthesis, and degradation to allow for distribution of ECM molecules throughout the gel.

CONCLUSIONS

The aim of this study was to determine an optimum crosslinking density in PEG hydrogels such that the gel mechanics are maximized (an important criteria in tissue engineering cartilage), while a critical water content for cell viability and a critical mesh size for ECM distribution is maintained. We demonstrated that chondrocytes photoencapsulated in PEG gels with a range of equilibrium swelling and compressive moduli produce a similar biochemical content of newly synthesized cartilaginous tissue. However, to obtain a uniform distribution of proteoglycans throughout the gel, the mesh size must be greater than ~ 90 Å. For the PEGDM-based hydrogels, this mesh size criteria correspond to an equilibrium swelling ratio of ~ 7.9 or higher. However, a maximum type II collagen production was observed in moderately crosslinked gels with a compressive modulus of 360 kPa, corresponding to a q less than that required for homogenous distribution of proteoglycans. In an effort to circumvent this issue, we systematically incorporated a fraction of degradable linkages into the network. We demonstrated that the biochemical composition was not compromised in these systems and that we can initially obtain a high compressive modulus

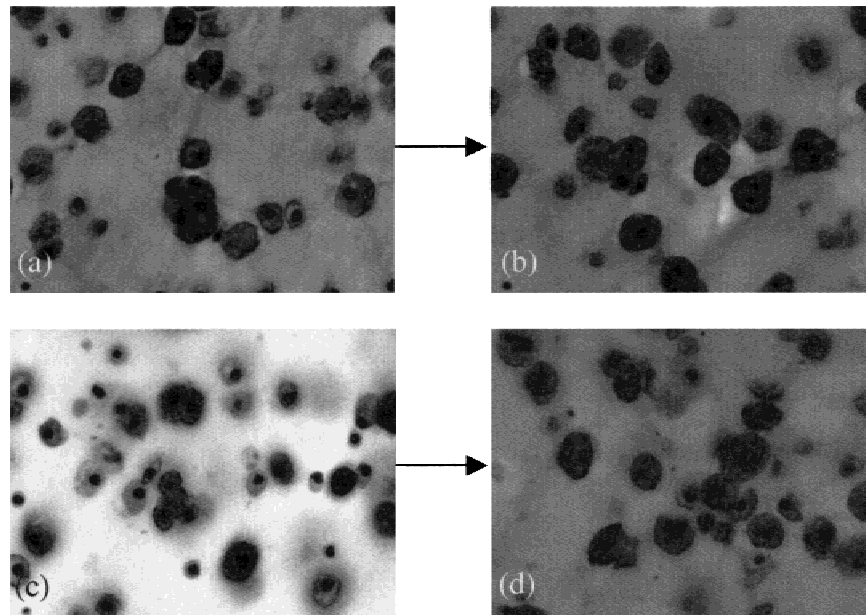


Figure 7. Histological analysis of glycosaminoglycans as a function of degradation and gel properties in partially degrading gels. Safranin O stains proteoglycans red (shown in grayscale). (a) and (c) After 2 weeks *in vitro*; (b) and (d) after 4 weeks *in vitro*. (a) and (b) Gel properties vary from $q = 9.8$ to 16.6 and $\xi = 140$ to 250 Å. (c) and (d) Gel properties vary from $q = 5.7$ to 7.9 and $\xi = 75$ to 115 Å. Original magnification $\times 400$.

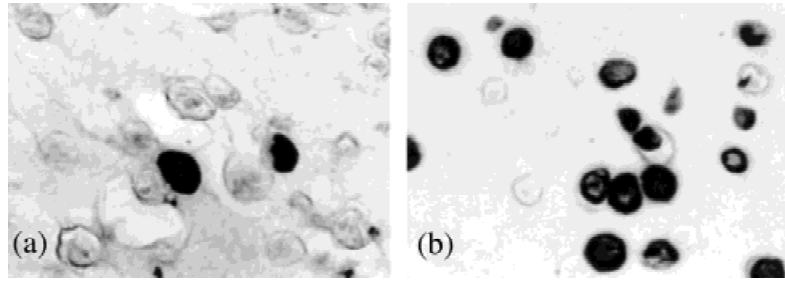


Figure 8. Immunohistochemical staining for type II collagen as a function of gel properties in partially degrading hydrogels after 4 weeks *in vitro*. (a) $K = 36$ to 11 kPa; (b) $K = 350$ to 140 kPa. Original magnification $\times 400$.

(of 350 kPa) to restore function and increase type II collagen synthesis. As the gel degrades and the mesh size increases, the proteoglycans are then able to diffuse throughout the gel. By proper design of partially degrading hydrogels, one can maximize the initial gel mechanics without compromising the final distribution of proteoglycans.

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