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Tailoring the Degradation of Hydrogels Formed from Multivinyl Poly(ethylene glycol) and Poly(vinyl alcohol) Macromers for Cartilage Tissue Engineering

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Tuning the degradation profiles of polymer cell carriers to match cell and tissue growth is an important design parameter for (cartilage) tissue engineering. In this study, degradable hydrogels were fabricated from divinyl, tetrafunctional poly(ethylene glycol) (PEG) and multivinyl, multifunctional poly(vinyl alcohol) (PVA) macromers to form homopolymer and copolymer gels. These gels were characterized by their volumetric swelling ratio and mass loss profiles as a function of degradation time. By variation of the macromer chemistry and functionality, the degradation time changed from less than 1 day for homopolymer PVA gels to 34 days for pure PEG gels. Furthermore, the degrading medium influenced mass loss, and a marked decrease in degradation time, from 34 to 12 days, was observed with the PEG gels when a chondrocyte-specific medium containing fetal bovine serum was employed. Interestingly, when copolymer gels of PEG and PVA were formed, PVA was released throughout the degradation (as determined by gel permeation chromatography) suggesting that covalent cross-linking of the PVA in the network was facilitated by copolymerizing with the PEG macromer. To assess these novel gels for cartilage tissue engineering applications, chondrocytes were photoencapsulated in the copolymer networks and cultured in vitro for up to 6 weeks. DNA, glycosaminoglycan (GAG), and total collagen contents increased with culture time, and the resulting neocartilaginous tissue at 6 weeks was homogeneously distributed as seen histologically. Biochemical analysis revealed that the constructs were comprised of 0.66 ± 0.04 μg of DNA/mg wet weight (ww), 1.0 ± 0.05% GAG/ww, and 0.29 ± 0.07% total collagen/ww at 6 weeks. Furthermore, the compressive modulus increased during culture from 7 to 97 kPa as the neocartilaginous tissue evolved and the gel degraded. In summary, fabricating hydrogels through the copolymerization of PEG and PVA macromers is an effective tool for encapsulating chondrocytes, controlling gel degradation profiles, and generating cartilaginous tissue.

Introduction

Hydrogels are crosslinked networks that provide many advantages when used as cell carriers to engineer a variety of tissues.[1−6] The highly swollen 3-D environment maintains a high water content, resembling to some degree the environment of biological tissues, 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. For cartilage tissue engineering, hydrogels provide a particularly ideal environment for chondrocytes to retain their native form, surrounded by a lacuna.

To develop a suitable cell carrier with hydrogels that will promote tissue growth, the degradation rate and mechanical properties of the scaffold must be fine-tuned. In general, these properties can be controlled through variations in the gel crosslinking density, and crosslinking in hydrogels can occur through physical, ionic, or covalent crosslinks. However, in many of the natural hydrogels (e.g., fibrin glue and alginates) that have been used to tissue engineer cartilage,[2,7−11] the physical and ionic crosslinking mechanisms have been difficult to control and, therefore, limit the final network structure and properties. In contrast, covalently crosslinked hydrogels formed from a chain polymerization of multivinyl macromers offer many advantages. Particularly, the ability to control the crosslinking density provides the flexibility and tailorability to design a wide range of polymeric networks for cell encapsulation and tissue growth.

We are particularly interested in designing degradable, photoreactive, and crosslinkable macromers based on poly(ethylene glycol) (PEG) and poly(vinyl alcohol) (PVA). The hydroxy groups on the ends of the PEG polymer can be easily functionalized with degradable units and endcapped with vinyl groups to form tetrafunctional, divinyl macromers.[12] PVA, on the other hand, can be modified into multifunctional, multivinyl macromers through the plethora of pendant hydroxy groups, which can be derivatized with a variety of substituents and to varying degrees, adding greater flexibility in the macromer design.[13−15] Changing the number of degradable units, macromer molecular weight, functionality (particularly, in the case of PVA), and concentration in

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solution prior to polymerization influences many of the macroscopic properties and degradation profiles in PEG and PVA hydrogels.[12,15,16] Furthermore, copolymerization of the PEG and PVA macromers enables additional control of functionality and properties that are especially important from a tissue engineering perspective.

These multi(meth)acrylate macromers readily react to form crosslinked hydrogels via photoinitiated chain polymerizations. Photopolymerization is a process that enables *in situ* formation of crosslinked networks at physiological pH and temperature. The mild gelation conditions allow for cells to be encapsulated in these photocrosslinked hydrogels and remain viable.[17,18] The unique advantage of chain polymerization is the ease with which a variety of chemistries can be incorporated into the hydrogel by simply mixing derivatized macromers of choice and subsequently copolymerizing. For example, we functionalized chondroitin sulfate (found in native cartilage) with methacrylate groups and systematically incorporated it into PVA gels through photopolymerization to create gels that mimic critical biochemical aspects of cartilage.[19]

In this study, the degradation behavior and subsequent network structural changes were evaluated in gels fabricated by homopolymerizing either acrylated PVA or methacrylated PEG and by copolymerizing the PEG and PVA macromers. The fractional mass loss and equilibrium volumetric swelling ratio were measured as a function of degradation time. In the copolymer gels, the release of degradation products was evaluated using gel permeation chromatography to gain a better understanding of the molecular-level structural features in the copolymer networks. Finally, to assess these novel degradable copolymer gels for cartilage tissue engineering applications, chondrocytes were photoencapsulated in the gels and cultured for up to 6 weeks. The resulting cartilaginous tissue was analyzed biochemically to quantify the extracellular matrix (ECM) components, histologically to examine the spatial distribution of the ECM, and mechanically to measure the compressive modulus as a function of tissue formation.

Experimental Methods

Materials. Triblock copolymers of lactic acid and poly(ethylene glycol) (PEG) endcapped with methacrylate functionalities (PEG-LA-DM) were synthesized by commonly used procedures.[12] Briefly, PEG-LA was produced by the ring opening polymerization of DL-lactide using stannous octoate in the presence of PEG (Fluka, 3000 g/mol), and lactides were added to the ends of the PEG molecules through a polycondensation reaction. Then, PEG-LA was endcapped with methacrylates by the addition of a 3-fold molar excess of methacryloyl chloride and triethylamine. The PEG-LA-DM macromer was precipitated three times in a 1:100 excess of cold ethyl ether to recover and purify the macromer. The resulting macromers contained an average of seven lactic acid repeat units flanking each side of a core PEG molecule, with ~100% methacrylation efficiency, as determined by ^{1}H NMR. The efficiency was calculated by comparing the area under the integral for the vinyl resonances ($\delta = 6.1$ ppm, s, $\delta = 5.7$ ppm, s), as well as for the lactide resonances ($\delta = 5.1$ ppm, $\delta = 1.3$ ppm), to the resonance for the PEG backbone (methylene protons, $\delta = 4.2$ ppm). The spectra were collected in $CDCl_3$.

Poly(vinyl alcohol) (PVA, Clairant, 14 000 g/mol) was modified with degradable, photocrosslinkable pendant groups, as described elsewhere.[14,15] Briefly, mono-2-(acryloyloxy)-ethyl succinate (AOES) was extended with succinic anhydride to produce a 5-ester acrylate molecule, which was then attached to the PVA via the pendant hydroxyl groups. The presence of resonances for the vinyl protons ($\delta = 6.4$ ppm, d, $\delta = 6.1$ ppm, q, $\delta = 5.8$ ppm, d) was seen in ^{1}H NMR, and the spectra were collected in D_2O. However, to quantify the average number of pendant ester-acrylate groups on the PVA, the polymerization of the Acr-Est-PVA was monitored using differential scanning calorimetry (DSC) (Perkin-Elmer DSC-7). Because of the low concentration of vinyl groups, DSC was more accurate in quantifying the degree of acrylation compared to ^{1}H NMR.[20] Briefly, the polymerization of the Acr-Est-PVA macromer solution is an exothermic reaction, and the heat released by the sample is proportional to the rate of polymerization. The integrated rate curve is directly related to the conversion of the acrylate functional groups in the system. Therefore, if one independently measures the conversion of the acrylate groups, and if the heat of reaction for the acrylate functional groups (ΔH_{theor}) is known, then the concentration of acrylate groups in the sample can be calculated. The ΔH_{theor} was determined by polymerizing a small sample of AOES in the DSC to 100% conversion, as verified using FT-IR. From these experiments, ΔH_{theor} for these acrylates was ~16.5−17 kcal/mol.[20] On the basis of this analysis, three 5-ester acrylate molecules were coupled to the PVA (Acr-Est-PVA).

The photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy)phenol]-2-methyl-1-propanone (Irgacure 2959, I2959, Ciba-Geigy), was used as supplied and dissolved in deionized (DI) H_2O or phosphate buffer saline solution (PBS, pH 7.4) to a final concentration of ≤0.05 wt % in all formulations. For the cell encapsulation experiments, the macromer/initiator solutions were filter-sterilized using a 0.2 μm filter.

Macromer Polymerization. The Acr-Est-PVA macromers were dissolved in DI-H_2O or PBS at 80 °C to a final concentration of 10 wt %. Similarly, the PEG-LA-DM macromers were dissolved in DI-H_2O or PBS at 25 °C to a final concentration of 10 wt %. DI-H_2O was used for the gel characterization studies, while PBS was used for the cell encapsulation studies. Copolymers were formulated by mixing the above solutions at room temperature in a 25:75 ratio (on a weight basis) for the Acr-Est-PVA and the PEG-LA-DM solutions, respectively. The macromer/initiator solution was photopolymerized under cytocompatible conditions using an ultraviolet light source (Novacure, EFOS, Inc. (for the hydrogel characterization studies) and UVP, model XX-20 (for the cell encapsulation studies)) at an intensity of 5−20 mW/cm^2 for ≤10 min.[17]

Mechanical Analysis. The compressive modulus of elasticity was measured using a dynamic mechanical analyzer (DMA-7, Perkin-Elmer) in unconfined compression at a rate of 10−40 mN/min at room temperature. Hydrogel disks of

5 mm in diameter and 2 mm in thickness were used. A sample size of 5 was used for the initial time points. A sample size of 3 was used for the cell-hydrogel constructs at 6 weeks.

Volumetric Swelling and Mass Loss Characterization. Volumetric swelling and mass loss were measured throughout the degradation for the homopolymers of both the PEG-LA-DM and Acr-Est-PVA macromers, as well as the copolymer gel. Degradation of the polymerized hydrogels was carried out in PBS at 37 °C. Disks (10 mm in diameter and 1 mm thick) were polymerized in molds, weighed immediately following polymerization, placed in a permeable, plastic tissue cassette, and degraded in buffer solution under sink conditions. At various time points, a tissue cassette was removed from the buffer solution, patted dry, and weighed to obtain the swollen wet weight of the disk (m_s). The disk was then thoroughly dried by lyophilization, and a final dry weight was obtained (m_{fd}). The initial dry polymer mass (m_{id}) was calculated by multiplying the initial wet weight of the disk by the weight fraction of macromer in solution. The percent mass loss of each sample was determined using the following equation:

$$\% \text{ mass loss} = \frac{(m_{id} - m_{fd})}{m_{id}} \times 100\% \quad (1)$$

PEG hydrogels were also degraded in chondrocyte medium (described in the following sections) using sterile techniques. The gels were placed under similar conditions as the cell encapsulation experiments (37 °C with 5% CO_2 under humid conditions).

The volumetric swelling ratio (Q) was calculated via the mass swelling ratio ($q = (m_s/m_{fd})$) according to the following equation:

$$Q = 1 + \frac{\rho_{polymer}}{\rho_{solvent}}(q - 1) \quad (2)$$

Here, $\rho_{polymer}$ is the macromer density and was approximated by the density of PEG ($\rho_{PEG} = 1.07$ g/mL), PVA ($\rho_{PVA} = 1.2619$ g/mL), or an appropriate ratio of the two densities. $\rho_{solvent}$ is the density of the buffer solution and was approximated as 1.0 g/mL. In all swelling and mass loss plots presented in this paper, the y-axis error bars were calculated from at least three samples taken at the same time point, and x-axis error bars are a result of variation in either the time the sample was taken or the variation in the total time required for degradation (i.e., the final data point).

Gel Permeation Chromatography (GPC). GPC was used to confirm the functionalization of the PEG and PVA macromers and to characterize the soluble and released gel components that were collected throughout the degradation process. The GPC was equipped with a Waters 515 pump, a Waters U6K manual injector, Polymer Standards service supreme columns (linear, 30 Å, 100 Å, 1000 Å), and a Waters 2410 refractive index detector. Samples were run in an aqueous environment (0.1 M $NaNO_3$, 1 mL/min) at 35 °C and compared against linear PEG standards using ethylene glycol as an internal standard. In most cases, the GPC traces were normalized so that the highest peak represents 100% detector response.

Chondrocyte Isolation and Cell Encapsulation. Chondrocytes were isolated from the femoral-patellar groove of a young calf (Research 87, Marlboro, MA), as described elsewhere.[21] The isolated chondrocytes were mixed with the macromer/initiator solution described above and polymerized into a thin disk (∼2 mm in thickness and ∼5 mm in diameter) at a concentration of 75×10^6 cells/cm^3. The cell-hydrogel constructs were cultured in nontreated tissue culture plates (12 well) on an orbital shaker using chondrocyte medium (Dulbecco's Modified Eagle Medium (Gibco) supplemented with 1% penicillin-streptomycin (Gibco), 0.5 μg/mL fungizone (Gibco), 0.01 M MEM nonessential amino acids (Gibco), 10 mM Hepes, 0.04 mM l-proline, and 10% fetal bovine serum). The constructs were incubated at 37 °C in a humid environment with 5% CO_2. Medium was replaced every 2−3 days.

Biochemical Assay. Three cell-hydrogel constructs were analyzed at 2, 4, and 6 weeks. The constructs were lyophilized for 24 h and then digested with a papain solution (125 μg/mL papain type III (Worthington) and 10 mM L-cysteine (Aldrich) in PBE buffer (10 mM phosphate, 10 mM EDTA, pH 6.5)) for 16 h at 60 °C on an orbital shaker. Total glycosaminoglycan (GAG) content was determined using dimethylmethylene blue dye.[22] The total collagen content was determined by measuring the hydroxyproline content[23] in which the hydroxyproline content is 10% of the total collagen content.[24] DNA content was measured using Hoechst 33258.[25] A sample size of 3 was used for all assays.

Histology. Two cell-hydrogel constructs were fixed in formalin, embedded in paraffin, and analyzed histologically at 2 and 6 weeks using standard histological procedure. Cross sections (8 μm thick) were stained with Safranin-O, which stains proteoglycans red, and Masson's trichrome stain, which stains collagen blue.

Statistical Analysis. Statistical analysis was performed on the biochemical and compressive modulus data using a Student's t-test. A confidence level of 0.05 was considered significant. All values are reported as the mean ± standard deviation.

Results and Discussion

Degradable hydrogel networks that span a wide a range of erosion profiles during degradation were synthesized from multivinyl macromers via photoinitiated, radical chain polymerizations. Specifically, the hydrogel networks were fabricated from divinyl PEG macromers, multivinyl PVA macromers, and comacromer solutions of PEG and PVA. These polymer chemistries have many advantages for use in tissue engineering applications, because they are highly hydrophilic, biocompatible, and easily cleared from the body.[26−28] Furthermore, photocrosslinked PEG hydrogels provide a 3-D environment that promotes the formation of cartilaginous tissue rich in both proteoglycans and type II collagen, the main components found in native cartilage.[29,30] By copolymerizing macromers with varying chemistry and functionality, our aim was to gain greater control

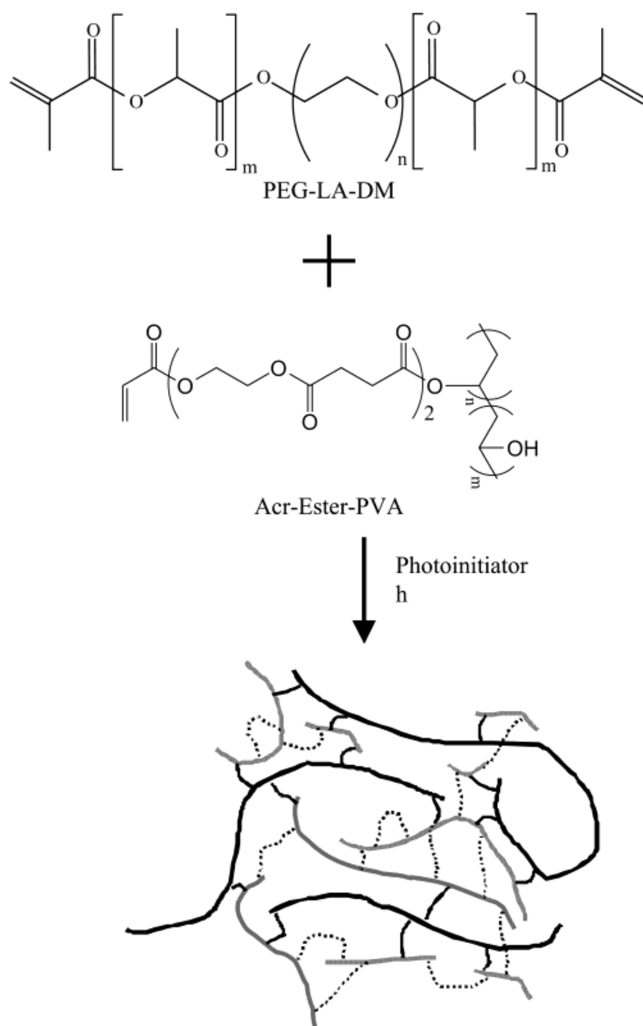

Figure 1. An idealized schematic of the structure of hydrogels formulated by copolymerizing PEG-LA-DM with Acr-Ester-PVA. The network consists of kinetic chains (light lines) connected via PEG crosslinks (dotted lines) and the multifunctional PVA chains (bold lines).

over the degradation rates, mass loss profiles, and macroscopic gel properties (e.g., mechanics and swelling) to develop suitable hydrogels for chondrocytes and cartilage growth.

During polymerization, the radicals propagate through pendant (meth)acrylate groups to form poly(meth)acrylate kinetic chains. Through the copolymerization of PEG-LA-DM and Acr-Est-PVA macromers, a complex 3-D network structure is formed that will be highly entangled and interconnected with hydrolyzable blocks between the kinetic chains and the PEG or PVA chains (Figure 1). Furthermore, coreacting acrylates with methacrylates, which are known to have different reactivities, generates heterogeneous networks on the molecular level and provides another means to tailor the network structure. In addition, using different ester chemistries within the degradable blocks in the PVA and PEG enables one to manipulate further the network hydrolysis and erosion profile.

Due to their hydrophilicity, these networks swell in the presence of water, and the ester bonds within the crosslinks are cleaved homogeneously at a rate dictated by the pseudo-first-order hydrolysis kinetic constant and the number of degradable linkages. For a PEG chain to erode from the network, an ester bond on each side of the PEG crosslinker must be cleaved. However, since the PVA chains can be connected into the network by more crosslinks, a higher fraction of the degradable blocks must be cleaved along the PVA chain before it is released from the gel. During degradation, PEG, PVA, and polymer kinetic chains, as well as low molecular weight species from the degradable blocks, are eroded from the network resulting in an overall mass loss.

Typical swelling and mass loss profiles for a PEG-based hydrogel are shown in Figure 2. In this case, the gels were degraded in a standard PBS buffer solution at physiological pH and temperature. The volumetric swelling ratio (Figure 2A) was greater than 10 throughout degradation, illustrating that these are highly swollen gels containing at least 90% water. From the volumetric swelling ratio, the network crosslinking density (ρ_{xl}) and degradation rates can easily be estimated. For example, for highly swollen gels ($Q > 10$), the Flory–Rehner equation[31] relates the ρ_{xl} to the volumetric swelling ratio by

$$Q \sim {\rho_{xl}}^{-3/5} \tag{3}$$

Thus, as the crosslinks are hydrolytically cleaved, the network crosslinking density decreases corresponding to an increase in the swelling ratio.

Furthermore, the degradation kinetics are also related to the volumetric swelling ratio. Following psuedo-first-order hydrolysis kinetics, the concentration of crosslinks in the network decays exponentially as a function of degradation time,[15,16] and therefore, the volumetric swelling ratio will increase exponentially and is directly related to the degradation kinetic constant by the equation

$$Q \sim e^{3/5k't} \tag{4}$$

where k' is the degradation kinetic constant and t is the degradation time.

To determine if the degradation kinetics are influenced by the surrounding medium, PEG gels were also placed in a more relevant medium (chondrocyte medium), which is rich in cell nutrients, growth hormones, and growth factors necessary for cell growth and tissue synthesis. Interestingly, the degradation behavior of the PEG gels, as illustrated by the volumetric swelling data, was markedly different in the two mediums (Figure 2A). The kinetic constant (k') for the gels degraded in chondrocyte medium was $\sim$0.18 day^{-1}, which was significantly greater than k' for PBS buffer ($\sim$0.047 day^{-1}). Although these values are different, they are within the range of previously reported hydrolysis rate constants for poly(lactic acid) (2.45×10^{-3} day^{-1} to 3.17 day^{-1}).[32,33]

The mass loss profiles in Figure 2B also illustrate a great dependence on the degrading medium. The gels in the presence of chondrocyte medium were completely hydrolyzed in $\sim$12 days; however, the gels in PBS had lost no appreciable mass over this same time period and were not completely solubilized until $\sim$34 days. Gels degraded in medium without serum showed no difference in degradation

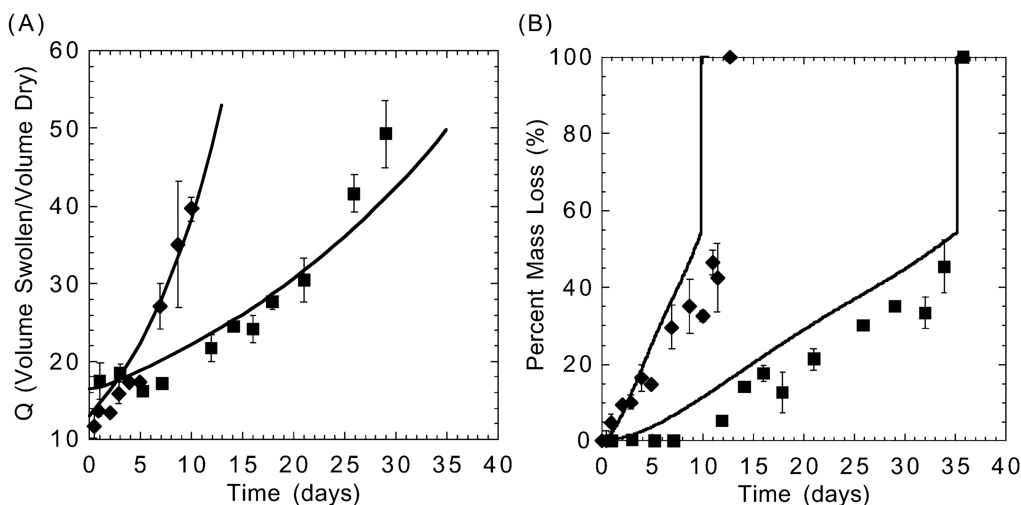

Figure 2. (A) Volumetric swelling ratio and (B) percent mass loss of degradable PEG-LA-DM hydrogels as a function of degradation time. All gels were made from a 10 wt % solution of the PEG-LA-DM and were degraded in either PBS (■) or chondrocyte medium (♦) at 37 °C. The lines in Figure 2A are exponential fits of the data, and the lines in Figure 2B were mass loss predictions from a previously developed theoretical model.[34]

times when compared to PBS (unpublished data). Previous studies have shown that serum proteins, such as bovine serum albumin (BSA), increase the rate of hydrolytic de-esterification in PLA films[35] due to BSA's esterase-like activity.[36] Furthermore, poly(glycolic acid) and poly(glycolic-*co*-lactic acid) can be enzymatically degraded by esterases.[37] Thus, the complex mixture of proteins present in the serum has a significant impact on the degradation rates of these hydrolytically labile hydrogels.

The general degradation behavior of homopolymer hydrogel networks formed from the chain polymerization of divinyl PEG macromers[12,16,38] and multifunctional PVA macromers[34] have been previously studied. However, we believe that by copolymerizing these two macromers, copolymer networks can be formed that exhibit many of the benefits of each macromer type and will provide an additional tool to tailor the mass loss profile.

Changing the macromer chemistry and functionality in the macromer solution has many implications on the resulting hydrogel and its cross-linking density. For example, the Flory polymer-solvent interaction parameter, χ, influences the hydrogel's water content and, consequently, the compressive modulus. Homopolymer gels were fabricated from PEG-LA-DM and Acr-Est-PVA, and copolymer gels were comprised of 75% PEG-LA-DM and 25% Acr-Est-PVA from a 10 wt % macromer solution. Interestingly, the initial equilibrium volumetric swelling ratios were 17.5 after 24 h for both the copolymer and pure PEG gels (PVA gels were completely eroded by 24 h). Since PEG gels tend to swell more than PVA gels at equal crosslinking density, due to a lower χ value,[39,40] the similar Q values suggest that the copolymer gels are less crosslinked. This observation is further supported by the fact that the initial double bond concentration is higher in the pure PEG system (Table 1), which should lead to a more crosslinked network.

During polymerization, these macromers lead to different rate profiles and network structures. The PVA macromer contains multiple functional groups along the backbone, and therefore as the first acrylate group on a PVA chain is reacted into the evolving network, the local radical environment is one with a high concentration of pendant vinyl groups. This phenomenon typically leads to an increase in cyclization and a lower crosslinking density, especially when a high solvent concentration is present (e.g., 90%). This decrease in ρ_{xl} is further evident by the low initial compressive modulus of the pure PVA gels (Table 1). In addition, the soluble fraction in these systems is ≥50%, which is indicative of a decrease in crosslinking efficiency. The PEG macromers, on the other hand, contain two photoreactive groups per chain (i.e., one on each end of the macromer), and for the given macromer molecular weight, a much higher concentration of double bonds is present in solution initially compared to the PVA during polymerization (Table 1). The initial compressive modulus in the PEG gels was 2 orders of magnitude higher compared to the PVA gels. When copolymer gels were formed, the initial double bond concentration, as well as the initial compressive modulus of the resulting hydrogel, more closely resembled the pure PEG gels. It is likely that as the PVA chains are incorporated into the network, the unbound PEG macromers in solution can more easily diffuse to the pendant acrylate groups on the PVA resulting in a more crosslinked network.

Table 1. Properties of PEG and PVA Hydrogels

hydrogel composition	[DB],[a] mM	compressive modulus (kPa)
PVA	19.9	0.5 ± 0.03
PVA/PEG (25:75)	40.9	12 ± 2
PEG	47.8	48 ± 3

[a] Concentration of double bonds in the system.

To gain a better understanding of these network structures and how they change during degradation, the volumetric swelling ratios and mass loss profiles are shown in Figure 3 as a function of degradation time for these three gel compositions. Initially, the volume swelling ratios for the PEG homopolymer and the PEG/PVA copolymer were similar. Both systems reached an equilibrium Q value of >10 in less than 24 h (data for the PVA homopolymer was not obtained due to the fast erosion of the network). As expected, deviations in the swelling behavior of the homopolymer and

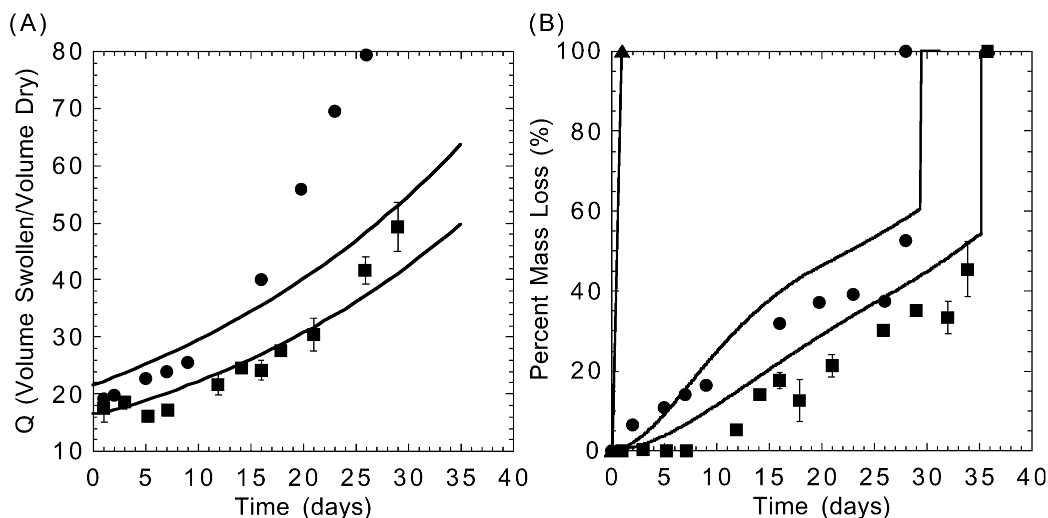

Figure 3. (A) Volumetric swelling ratio and (B) percent mass loss of degradable PEG-LA-DM homopolymers (■), Acr-Est-PVA homopolymers (▲), and PEG-LA-DM/Acr-Est-PVA copolymerized hydrogels (●) as a function of degradation time. All gels were made from a 10 wt % solution of macromer, and the copolymer was composed of 75% PEG-LA-DM and 25% Acr-Est-PVA. The lines in Figure 3A are exponential fits of the data, and the lines in Figure 3B are mass loss predictions from a previously developed theoretical model.[34]

copolymer gels were evident with time, and the copolymer gel degraded more quickly than the PEG gel. The degradation kinetic constant for the PEG homopolymer was 0.047 day^{-1}, as determined by a pure exponential fit of the data. However, the PEG and PVA crosslinks in the copolymer gel will likely have different hydrolysis rate constants resulting from variations in the degradable ester group chemistries. To account for this variation, the swelling data was fit to a function containing two exponential time constants with a pre-exponential weighting factor based on the relative fraction of PVA versus PEG crosslinks. This function captured the basic trends seen in the swelling data, and the hydrolysis of the PVA crosslinker was estimated to be 0.12 day^{-1}. During the late stages of degradation, the predicted exponential swelling curve deviates from the observed experimental data, possibly due to the changes that occur in the gel chemistry with degradation. For example, as the crosslinks are cleaved and eroded from the network, the gel becomes more ionic in nature from the noneroded poly((meth)acrylic acid) chains and the increasing ionic nature.[41]

As illustrated in Figure 3B, the variations in the hydrolysis kinetics also influence the mass loss profiles for the three different polymer networks. The PVA hydrogels were completely degraded in less than a day, even though the initial macromer solution contained a significant concentration of crosslinkable double bonds. Previous studies by the authors have shown that similar PVA gels fabricated from a 20 wt % solution resulted in a high soluble fraction (e.g., 50%) attributed to a high degree of cyclization.[15] With the lower macromer concentration used in this study (i.e., 10 wt %), an equally high, or higher, soluble fraction is expected, leading to a loosely crosslinked gel with a short time for complete degradation. Since these gels degraded so quickly, it was not possible to determine accurately the difference between the soluble fraction and the eroded mass.

The PEG hydrogels exhibited significantly longer degradation times of ~34 days, and the PEG/PVA copolymers were not completely degraded until ~28 days. Interestingly, no soluble fraction was measured in either the PEG or copolymer gels. This result suggests that copolymerization with the PEG macromers facilitates the incorporation of the PVA macromer into the hydrogel.

To gain a better understanding of how and when the PEG and PVA chains are released from the networks, the eroded products were collected during the degradation cycle for the copolymer gels and analyzed using GPC. To identify accurately the degradation products, the elution volume of the modified PEG and PVA macromers, as well as their unmodified precursors, was measured. The unmodified PEG corresponded well to the PEG standards of similar molecular weight as expected, but the molecular weights of the PVA and the modified macromers were strongly underestimated by the PEG standards. Since in this study we were most interested in the types of chains released, and not necessarily the molecular weights, elution volumes were used as references. The results obtained from the GPC for the unmodified PEG and PVA precursors are illustrated in Figure 4A (curves C and A, respectively). The two polymers exhibit distinct curves. The broadness of the PVA peak suggests a highly polydisperse system, whereas, the narrower PEG peak suggests a more uniform distribution. When these hydrophilic polymers were functionalized with hydrophobic, degradable grafts, the shapes of the resulting macromer peaks were similar but were shifted to longer elution times (Figure 4A). This shift is likely due to an enhanced interaction between the macromers and the GPC columns, rather than to a decrease in the molecular weight of the macromer.

The degradation products of the copolymer gels were collected at 1, 9, and 16 days, as well as after complete degradation. GPC analysis did not detect any polymer chains in solution after 1 day, which agrees with the mass loss results and suggests that there was little to no mass erosion from the network at this early time point. In addition, the absence of a soluble fraction, as observed in the homopolymerization of the PVA macromers, indicated that all the macromer was incorporated into the network during the copolymerization. By 9 and 16 days, an appreciable amount

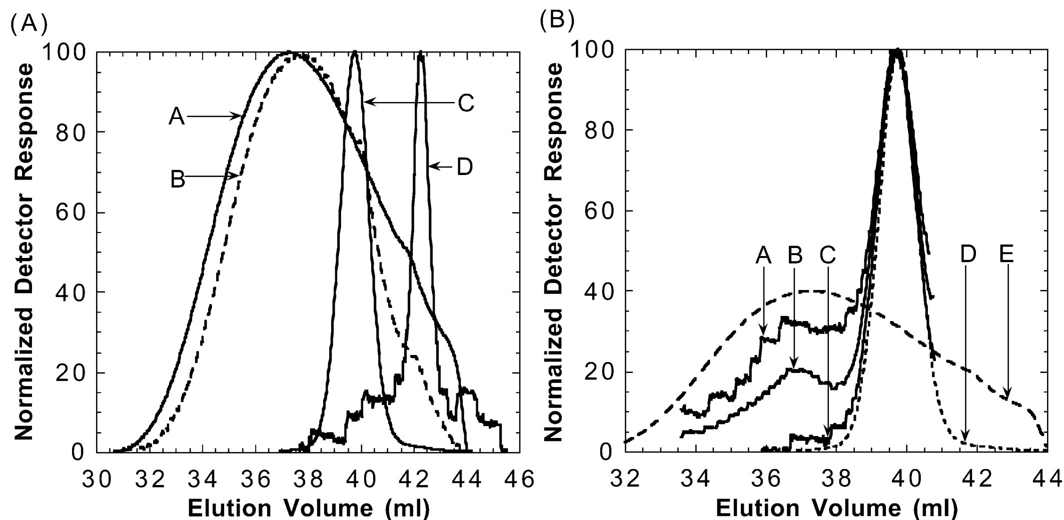

Figure 4. Normalized GPC traces as a function of elution volume. Panel A shows the unmodified 14 000 g/mol PVA (A), PVA modified with an average of three ester acrylate molecules (B), unmodified 3000 g/mol PEG (C), and PEG modified with seven lactides per side and endcapped with methacrylates (D). Panel B shows the unmodified 3000 g/mol PEG (D), unmodified 14 000 g/mol PVA (E), and the degradation products from the 25:75 PVA/PEG hydrogels were collected at 9 days, 16 days, and complete degradation (curves A, B, and C, respectively).

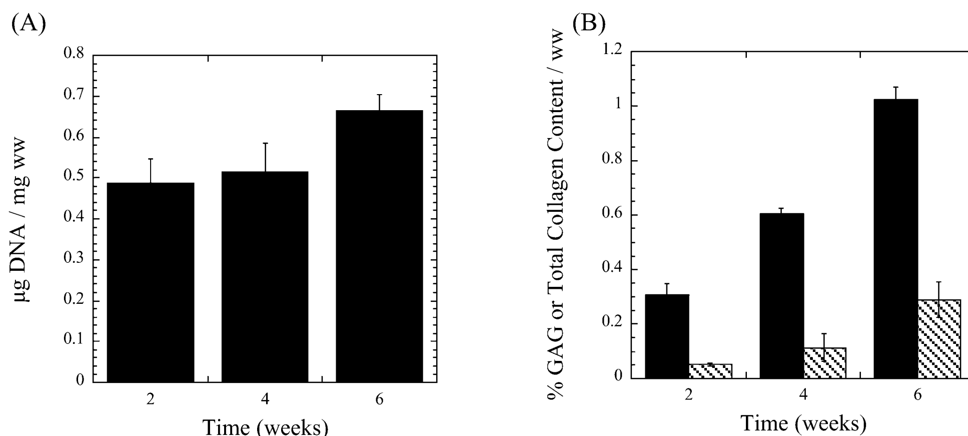

Figure 5. DNA content (A) and biochemical composition (B) of neocartilaginous tissue produced by chondrocytes encapsulated in PEG/PVA copolymer hydrogels and cultured *in vitro*. Solid bars and line hatched bars are the GAG and total collagen contents, respectively.

of polymer chains were released, and the GPC traces are shown in Figure 4B (curves A and B, respectively). The elution volume of the degradation products agreed well with those for the unmodified PEG and PVA polymers (curves D and E, respectively). Interestingly, both the PEG and PVA chains were present in solution after 9 and 16 days. The release time for the PVA chains in this system was significantly longer than that for the pure PVA gels, which were completely degraded within 1 day. Therefore, in the copolymer gels, the PEG macromer facilitated an increased incorporation of the PVA macromer into the hydrogel, resulting in longer retention of the PVA within the network, in this case, for at least 16 days. After complete degradation, only PEG was observed in solution, although a small amount of the PVA may have been present (Figure 4B, curve C). It is also important to note that the eroded PEG and PVA chains were similar to their *unmodified* precursors, suggesting that most or all of the degradable linkages were hydrolyzed from the core polymers. Unfortunately, the kinetic chains make up $<3\%$ of the total network and their elution volumes may overlap with the PEG and PVA peaks,[42] and therefore, characterization of the kinetic chains released was not feasible.

Clearly, understanding the degradation behavior of these gels is essential in the design of cell carriers, which are to be used as temporary initial supports until the cells begin producing their own functional tissue. There are many factors that influence the hydrolytic degradation of these scaffolds, for example, the aqueous medium (Figure 2) and the presence of cells and growing ECM. Chondrocytes were encapsulated in the PEG/PVA copolymer gels to evaluate the potential of these novel hydrogels to serve as appropriate scaffolds for cartilage tissue engineering.

The DNA, glycosaminoglycan (GAG), and total collagen contents were measured as a function of culture time and are shown in Figure 5. A statistical increase in DNA content was observed from 4 to 6 weeks, suggesting that the chondrocytes are proliferative in these degrading copolymer gels. Both GAG and total collagen contents increased over the 6-week culture time. Interestingly, the GAG content was significantly higher compared to the total collagen content in these gels over the course of the experiment. At 6 weeks, the GAG content was 3.5-fold greater than the collagen content. If the ratio of total collagen to GAG is evaluated with culture time from 2 to 6 weeks, an increase in the ratio is observed from 0.17 ± 0.02 to 0.28 ± 0.07, respectively.

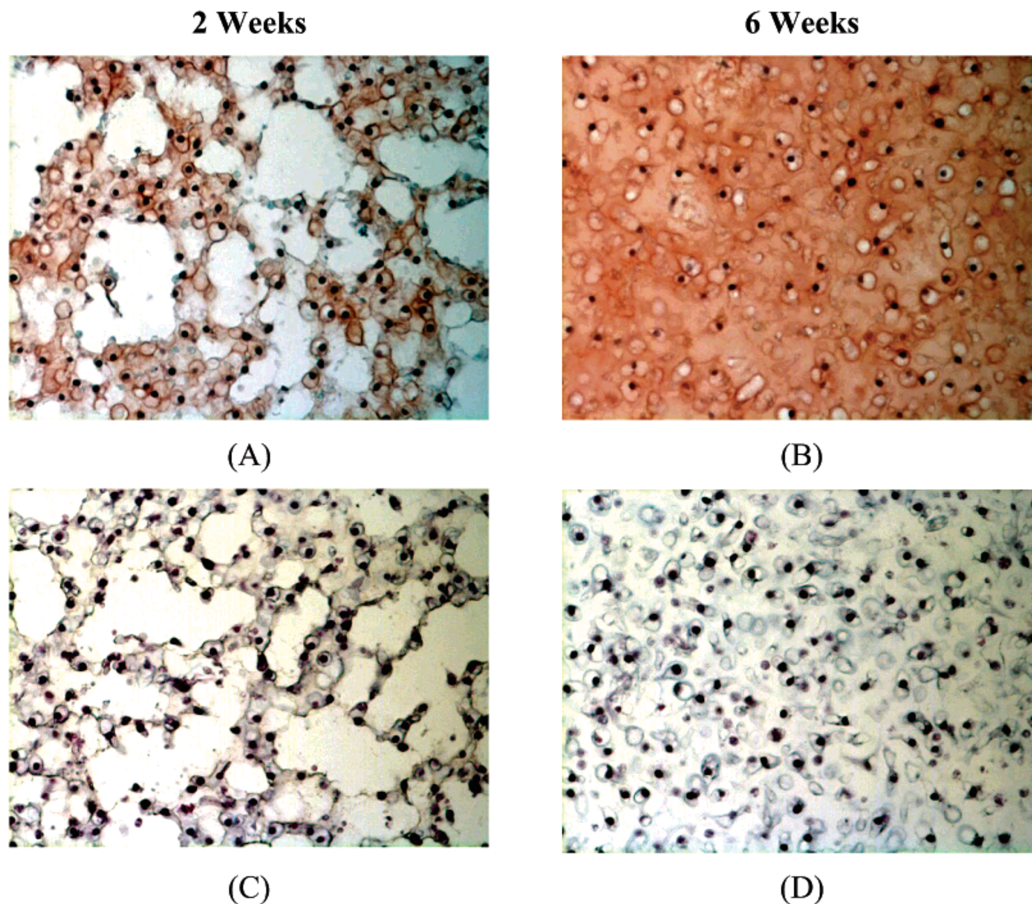

Figure 6. Histological micrographs of neocartilaginous tissue produced by chondrocytes encapsulated in PEG/PVA copolymer hydrogels after 2 weeks (A, C) and 6 weeks (B, D) of *in vitro* culture. Safranin-O was used to stain proteoglycans red (A, B) and Masson's trichrome strain was used to stain collagen blue (C, D). Original magnification is 200×.

This increase can be attributed to a greater percent increase in collagen synthesis by the cells compared to GAG synthesis with culture time. Interestingly, previous studies by the authors have shown that neocartilaginous tissue produced in pure degrading PEG gels was comprised of 2.6-fold greater collagen content than GAG content[43] after 6 weeks. Therefore, chemistry may play a significant role in the time evolution of ECM components in these hydrogels.

To gain a better understanding of the way that the neocartilaginous tissue is formed in these degrading copolymer gels, the spatial distribution of the extracellular matrix (ECM) components was assessed. The histological micrographs are shown in Figure 6. By 2 weeks, the chondrocytes are healthy and exhibit a round morphology in these copolymer gels. This healthy appearance suggests that the degradation products do not adversely affect the cells' viability and function. Furthermore, the chondrocytes are located in their characteristic lacunae resembling that of native cartilage. Significant degradation of the gel was observed by 2 weeks as illustrated by the void spaces (i.e., acellular regions with no tissue present) in panels A and C of Figure 6. The Masson's trichrome stain used to stain collagen blue also stains the PEG polymer brown (unpublished observations). Based on the characterization of the degradation products described above, the PEG polymer will be present throughout the degradation. Some staining is present near the cells, but no staining was observed in the void spaces suggesting that these areas degraded quickly (in less than 2 weeks) before the cells could lay down significant tissue. However, the tissue formed appears connected throughout the construct despite the localized regions of increased degradation.

By 6 weeks, GAG and collagen ECM molecules were distributed throughout the construct. The GAG staining was significantly darker compared to collagen, which is in agreement with the biochemical data in Figure 5. Interestingly, no void spaces were visible by 6 weeks throughout the construct (Figure 6B,D). Previous studies by the authors have shown that in degrading PEG hydrogels (i.e., gels which degraded on a faster time scale than the copolymer gels described in this study), when larger void spaces were present in the constructs, they were not filled by the chondrocytes or ECM with increased culture time.[30] In contrast, the smaller void spaces seen here with the copolymer gels at 2 weeks were filled completely with neotissue by 6 weeks. This observation suggests that there may be a critical distance in which the chondrocytes are able to proliferate into and lay down new tissue. In addition, gel chemistry as well as the initial gel mechanics may influence cell proliferation and ECM deposition. Thus, further studies are necessary to elucidate the mechanism(s) by which the cells are able to fill in void space with ECM.

In engineering a tissue that functions to absorb shock and protect the underlying bone during normal activities, it is important to assess the mechanical properties of the neotissue. Cartilage is predominantly subjected to compressive strains,

Table 2. Compressive Modulus of the Cell-Laden Copolymer Gels as a Function of Culture Time

	compressive modulus (kPa)
week 0	7 ± 1
week 6	97 ± 3
native articular cartilage[44]	500−1000

and the compressive modulus of articular cartilage has been reported to be 500−1000 kPa.[44] In these copolymer constructs, the initial compressive modulus is a function of both the gel and embedded chondrocytes. Interestingly, the presence of cells lowered the initial modulus from 12 ± 2 (Table 1) to 7 ± 1 kPa (Table 2). By 6 weeks with the evolution of neocartilaginous tissue and with the gel no longer present, the modulus increased an order of magnitude to 97 ± 3 kPa, which is comparable to other reported values for tissue-engineered cartilage.[29,45] However, the compressive modulus was 5-fold lower than that of the native tissue, which is indicative of the lower biochemical content, particularly GAGs, found in the tissue-engineered cartilage. The proteoglycan molecules (rich in GAGs) are responsible for giving cartilage its compressive properties. The GAG content in native cartilage from which the chondrocytes were harvested was found to be 4.7%/ww, which was 5-fold higher than the GAG content in the copolymer gels by 6 weeks. Interestingly, the equilibrium modulus of articular cartilage has been shown to increase with increasing GAG and decreasing water content.[46]

Conclusions

In this study, we have shown that degradation media and the network chemistry and functionality influence the swelling and mass loss profiles for hydrolytically labile, bulk degrading hydrogel networks. Specifically, the degradation rates of the hydrogels significantly increased when the gels were degraded in medium containing fetal bovine serum compared to PBS. With a change of the network chemistry (PEG vs PVA), the initial compressive moduli ranged 2 orders of magnitude, and the degradation time varied from 1 to 34 days. Furthermore, copolymerizing macromers with different chemistries resulted in gels with crosslinks that were eroded from the network at different rates. From a tissue engineering perspective, these copolymer gels provided an environment that promotes cell proliferation and extracellular matrix deposition. During tissue formation, the compressive modulus increased by an order of magnitude, and by 6 weeks, a homogeneous neocartilaginous tissue rich in proteoglycans and collagen was formed throughout. Fabricating gels through the copolymerization of these PEG and PVA macromers can be an effective tool for controlling and tuning the degradation of hydrogels to promote cartilaginous tissue evolution.

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