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# Controlling the spatial distribution of ECM components in degradable PEG hydrogels for tissue engineering cartilage

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**Abstract:** In developing a scaffold to support new tissue growth, the degradation rate and mass loss profiles of the scaffold are important design parameters. In this study, hydrogels were prepared by copolymerizing a degradable macromer, poly(lactic acid)-*b*-poly(ethylene glycol)-*b*-poly(lactic acid) endcapped with acrylate groups (PEG-LA-DA) with a nondegradable macromer, poly(ethylene glycol) dimethacrylate (PEGDM). The resulting hydrogels exhibited a range of degradation behavior and mass loss profiles. Chondrocytes were photoencapsulated in gels formulated with 50:50, 25:75, and 15:85 (mol % PEGDM: mol % PEG-LA-DA) and cultured for 6 weeks *in vitro*. The neocartilaginous tissue formed was examined biochemically and histologically. After 6 weeks, the DNA content in gels with 75 and 85% degradable crosslinks was nearly twice that of the DNA con-

tent in the 50% gels. The total collagen content was significantly higher in the 85% gel [ $2.4 \pm 0.8\%$  wet weight (ww)] compared to the 50% gel ( $0.22 \pm 0.29\%$  ww). In examining the neocartilaginous tissue with immunohistochemistry, type II collagen was localized in the pericellular region in the 50% gel; however, when increased degradation was incorporated into the gel, type II collagen was found throughout the neotissue. In summary, the important role of hydrogel degradation in controlling and influencing the deposition and distribution of extracellular matrix molecules was demonstrated and quantified. © 2002 Wiley Periodicals, Inc., *J Biomed Mater Res* 64A: 70–79, 2003

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

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## INTRODUCTION

In designing a cell scaffold to provide temporary support for new tissue growth, the scaffold degradation rate and mechanism are important parameters. As the scaffold degrades, extracellular matrix (ECM) fills the void space and, ultimately, the final product is a new living tissue equivalent. Several approaches have been explored to design a suitable, biocompatible, and *degradable* scaffold for tissue engineering applications. For example, cells have been seeded onto prefabricated porous meshes prepared from poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymers or encapsulated into natural and synthetic hydrogels. These three-dimensional scaffolds provide an

environment that promotes cell proliferation and ECM deposition.

In tissue engineering cartilage, a considerable amount of research has been focused on using prefabricated meshes. Degradation of these scaffolds occurs through hydrolysis of the ester groups in the polymer backbone and can be controlled by variation in the polymer chemistry (e.g., PGA versus PLA),<sup>1</sup> the polymer molecular weight and its distribution, crystallinity, and the scaffold porosity.<sup>2,3</sup>

In studies characterizing scaffold degradation, PGA mesh scaffolds were found to degrade completely in 4 weeks<sup>4,5</sup> whereas poly(L-lactic acid) (PLLA) scaffolds did not degrade significantly over 6 months<sup>4,6</sup> due to its increased hydrophobicity and crystallinity. Freed et al.<sup>4</sup> seeded chondrocytes onto PGA and PLLA scaffolds, and after 2 months *in vitro*, little proteoglycan synthesis was observed throughout the PLLA scaffolds while mostly cells and ECM were present in the PGA scaffolds. The difference in the ECM formation in this study was, in part, attributed to the slow degradation of the PLLA scaffolds. In another study, chondrocytes were seeded onto PLA scaffolds, and the proteoglycan distribution was inhomogeneous and the

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polymer scaffold still was present after 9 weeks of *in vitro* culture.<sup>7</sup>

An alternative design approach for cartilage tissue engineering scaffolds involves encapsulating cells into *in situ*-forming degradable hydrogels derived from natural components, such as alginates and fibrin glue. *In situ*-forming gels provide several advantages over the prefabricated scaffolds described above. For example, hydrogels are highly hydrophilic polymer networks that swell, rather than dissolve, in the presence of water because of their crosslinks. In addition, *in situ*-forming gels provide a minimally invasive vehicle for transplantation.

However, the stability and degradation of these natural-based gels are not easily controlled. For example, in alginate gels, a “de-crosslinking” mechanism occurs in which the divalent cations are exchanged with monovalent cations from the surrounding media, a mechanism that is difficult to control.<sup>8</sup> In addition, little degradation was observed in alginate gels cultured for 3 months *in vitro* when cells were either present or absent in the gels.<sup>9</sup> In a study by Cao et al.,<sup>10</sup> chondrocytes were encapsulated in calcium alginate gels, and after 6 months of implantation, alginate still was present and nodules of cartilaginous tissue had formed in the gel construct.

In contrast, fibrin glue degrades more quickly than alginates. Fibrin glue degrades via an enzymatic degradation pathway, and the degradation can be controlled to some degree by varying the fibrinogen concentration. When a fibrinogen concentration of 80 mg/cc was used under *in vivo* conditions, the fibrin glue gel lost ~50% of its initial mass in 2 weeks and ~80% of its mass by 5–6 weeks.<sup>11</sup> In a study by Sims et al.,<sup>12</sup> chondrocytes encapsulated in fibrin glue gels for 12 weeks produced a cartilaginous tissue throughout the construct, comprised of both glycosaminoglycans and collagen, as seen histologically. Furthermore, the GAG content was similar to that of native cartilage controls.

However, in numerous cartilage tissue engineering applications, both scaffold degradation and mechanics are important. The scaffold must not only degrade at a rate similar to new tissue formation, but it also must provide temporary support to withstand the normal loads and stresses of the native tissue. A shortcoming of many of the natural hydrogels is that, in addition to the degradation behavior, the gel mechanics are limited and difficult to control.

As an alternative to these natural hydrogels, we are particularly interested in degradable, photocrosslinkable hydrogels based on poly(ethylene glycol) (PEG). Photopolymerization enables *in situ* scaffold formation in which a liquid macromer solution combined with cells is polymerized under mild cytocompatible conditions, and complex architectures easily are

formed *in vivo* with temporal and spatial control over the gelation.

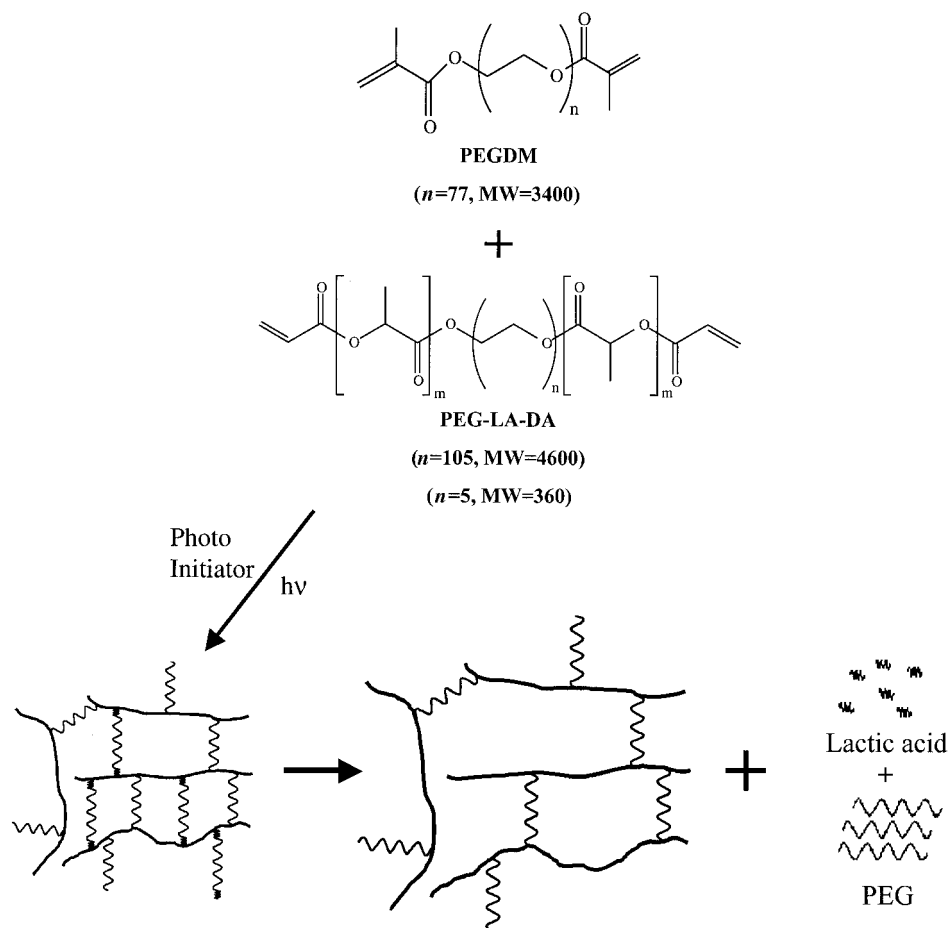
In addition, synthetic gels afford greater control over the macroscopic mechanical properties and degradation profiles. For example, Sawhney et al.<sup>13</sup> described the synthesis of a triblock copolymer, poly(lactic acid)-*b*-poly(ethylene glycol)-*b*-poly(lactic acid) endcapped with photocrosslinkable acrylate groups (PEG-LA-DA). The degradation of these PEG gels is readily controlled through the macromer chemistry, molecular weight, and the percent macromer in solution prior to polymerization.<sup>13,14</sup> Gels prepared from these degradable PEG macromers degrade via hydrolysis within the PLA block present in the network crosslinks, and by increasing the molecular weight of the PLA block, the degradation rate can be increased without significantly changing the gel chemistry.<sup>13</sup>

In this study, chondrocytes were photoencapsulated into PEG hydrogels with varying degradation profiles. Specifically, PEG gels were formed by copolymerizing poly(ethylene glycol) dimethacrylate, a macromer that does not degrade on the time scale of the experiments, and a degradable triblock copolymer, PEG-LA-DA. By varying the ratio of these two macromers, hydrogels with distinct mass erosion profiles were formed. The aim of this study was to examine the influence of the scaffold degradation behavior and mass loss profile on extracellular matrix deposition and spatial distribution by chondrocytes photoencapsulated in these PEG gels. Chondrocytes were cultured for 6 weeks *in vitro*, and the neocartilaginous tissue formed was examined biochemically and histologically.

## MATERIALS AND METHODS

### Macromer

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, PEG MW = 4600) was synthesized as described elsewhere.<sup>13</sup> The chemical structures are shown in Figure 1. On average, five repeating lactic acid units were added to each side of the core PEG molecule, as determined by <sup>1</sup>H NMR. The macromers were dissolved at varying concentrations with molar ratios of 50:50, 25:75, and 15:85 (PEGDM:PEG-LA-DA) in sterile phosphate-buffered saline (PBS) to a final macromer solution concentration of 10% (w/w). The UV photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (I2959, Ciba Geigy), used in this study is cytocompatible under the given concentrations and initiating conditions, as previously determined.<sup>15</sup> A concentrated solution of I2959 in PBS was prepared and filter sterilized. A small volume was added to the macromer solution to obtain a final initiator concentration of 0.05% (w/w). The mac-



**Figure 1.** An ideal schematic of the formation and degradation of PEG hydrogels formed by copolymerizing macromers that do not degrade on the time scale of the experiment (PEGDM) with macromers that degrade completely in 2 weeks (PEG-LA-DA).

romer/initiator solution was irradiated with 365-nm light at an intensity of  $\sim 10$  mW/cm<sup>2</sup> for 10 min in the presence of chondrocytes.

### Modeling degradation

A previously developed, theoretic model that accounts for both structural and kinetic parameters was used to describe the degradation behavior of the PEG hydrogels.<sup>16,17</sup> The model has been used to predict accurately erosion (i.e., mass loss), swelling, compressive modulus, and drug release from gels prepared from the PEG-LA-DA macromer.<sup>16,18,19</sup> In this study, gels formulated from 10% (w/w) PEG-LA-DA were degraded in phosphate-buffered saline (pH 7.4, 37°C), and the volumetric swelling ratio,  $Q$  (equilibrium swollen volume/dry polymer volume), was measured as a function of degradation time. The volumetric swelling ratio is proportional to the crosslinking density, which decays exponentially with degradation.

Because these are highly swollen gels, the rate of hydrolysis follows pseudo first-order rate kinetics. By combining these relationships, we can obtain the following relationship,  $Q \propto e^{-kt}$ , in which the kinetic time constant,  $k$ , is a model

parameter.<sup>19</sup> Using the experimentally determined  $k$ , mass loss is readily predicted from the model. The eroded molecules will include oligomeric lactic acid, poly(ethylene glycol), and poly(meth)acrylate chains. The size of the eroded chains is relatively small (compared to essential growth factors that diffuse in and maintain cell function), and therefore their diffusion out of the scaffold should not be impeded by the presence of cells or tissue. The model was modified to predict the degradation behavior of copolymerized gels containing degradable ( $k = 0.00009$  min<sup>-1</sup>) and nondegradable crosslinks ( $k \approx 0$ ).

### Chondrocyte encapsulation

Chondrocytes were isolated from the femoral-patellar groove of a young calf (Research 87, Marlboro, MA), as described elsewhere.<sup>20</sup> The isolated chondrocytes were combined with the macromer/initiator solution described above and polymerized into a thin disk ( $\sim 2$  mm in thickness and  $\sim 5$  mm in diameter) at a concentration of  $75 \times 10^6$  cells/cc. Using this technique, chondrocytes are seeded uniformly throughout the gel, as previously seen.<sup>15</sup> The constructs were cultured in nontreated tissue culture plates (12-well)

on an orbital shaker using Dulbecco's modified eagle medium (Gibco) supplemented with 1% penicillin-streptomycin (Gibco), 0.5  $\mu\text{g}/\text{mL}$  of fungizone (Gibco), 0.01M of MEM nonessential amino acids (Gibco), 10 mM of Hepes, 0.04 mM of 1-proline, and 10% fetal bovine serum. The constructs were incubated at 37°C in a humid environment with 5% CO<sub>2</sub>. The medium was replaced every 2–3 days.

### Biochemical assay

Three cell-hydrogel constructs were analyzed at 2, 4, and 6 weeks. The constructs were freeze dried for at least 48 h and then digested with a papain solution (125  $\mu\text{g}/\text{mL}$  of papain type III (Worthington) and 10 mM of l-cysteine (Aldrich) in PBE buffer (10 mM of phosphate, 10 mM of EDTA, pH 6.5)). The constructs were crushed and then digested for 16 h at 60°C on an orbital shaker. Total glycosaminoglycan (GAG) content was determined using dimethylmethylene blue dye.<sup>21</sup> The total collagen content was determined by measuring the hydroxyproline content<sup>22</sup> in which the hydroxyproline content was 10% of the total collagen content.<sup>23</sup> DNA content was measured using Hoechst 33258.<sup>24</sup> A sample size of three was used for the 2- and 4-week samples for all three hydrogel compositions and for the 6-week samples prepared from 15:85 (PEGDM:PEG-LA-DA). A sample size of two was used for the 6-week samples prepared from 50:50 and 25:75 (PEGDM:PEG-LA-DA).

### Histology

The cell-hydrogel constructs were fixed in formalin, embedded in paraffin, and analyzed histologically at 4 weeks using standard histologic procedures. Cross sections (8  $\mu\text{m}$  thick) were obtained by slicing, as a function of depth, through the disk. A representative section of each construct was stained with Safranin-O, which stains proteoglycans red. Immunohistochemical staining was performed on the paraffin sections with a labeled streptavidin-biotin immunoenzymatic antigen detection system (UltraVision detection system, NeoMarkers, Inc.). The paraffin sections were digested enzymatically with pepsin (1 mg/mL in 0.01N of HCl) for 30 min at 37°C. Collagen type II mouse monoclonal antibody was obtained from NeoMarkers Inc., and healthy bovine articular cartilage was used as the positive control. Collagen type I mouse monoclonal antibody was obtained from Chemicon Inc., and healthy bovine achilles tendon was used as the positive control.

### Statistical analysis

Statistical analysis was performed on the biochemical data using a Student's *t* test with a confidence level of 0.05 unless

otherwise specified. All values reported in the paper are reported as the mean  $\pm$  standard deviation.

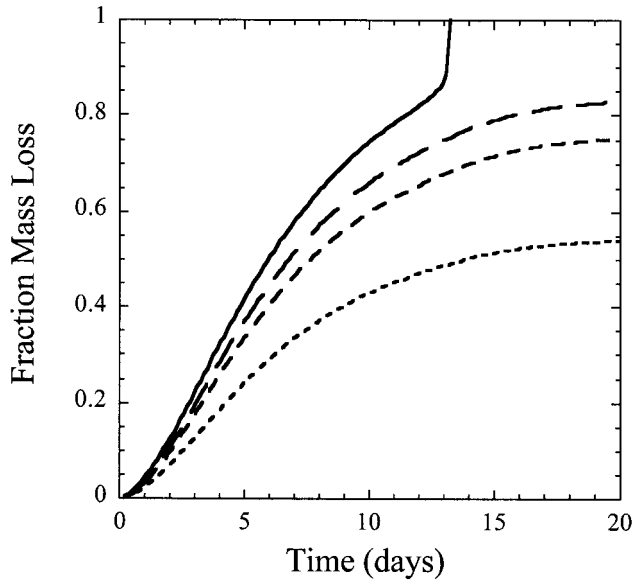
## RESULTS AND DISCUSSION

Understanding how degradation and mass loss influence ECM deposition in degradable scaffolds is important when designing a scaffold for new tissue growth. By fabricating hydrogels through the copolymerization of macromers with varying degradation kinetics, networks can be formed that span a wide range of behavior during degradation. In this study, chondrocytes were photoencapsulated in hydrogels that were synthesized by copolymerizing PEGDM with PEG-LA-DA in varying molar ratios of 50:50, 25:75, and 15:85 (PEGDM:PEG-LA-DA). By comparing ECM formation in these gels, we can begin to understand the influence of gel degradation on ECM deposition within the PEG hydrogels.

PEGDM does not degrade on the time scale of the experiments, but the ester linkage will cleave over time *in vivo*.<sup>25</sup> On the other hand, PEG-LA-DA degrades completely in 2 weeks *in vitro* following pseudo-first-order hydrolysis kinetics of the crosslinks.<sup>14</sup> As the crosslinks are hydrolyzed, the concentration of the crosslinks in the network decays exponentially as a function of degradation time. Figure 1 shows an ideal schematic of the degrading network structure when a portion of the crosslinks is degradable. For example, in a gel prepared with 50 mol % PEGDM and 50 mol % PEG-LA-DA, half of the crosslinks will be hydrolyzed during degradation.

To understand the mechanisms that occur during gel degradation, it is helpful to understand how these networks are formed. During polymerization, (meth)acrylate endgroups on the macromer backbone react to form kinetic poly(meth)acrylate chains. These kinetic chains are connected through PLA-*b*-PEG-*b*-PLA crosslinks to form a three-dimensional network. The crosslinks degrade via hydrolysis of labile ester bonds, which reside within the PLA blocks of the crosslinker. For the scaffold to lose mass, an ester bond within the PLA block must be cleaved on each end of the crosslink. Once the crosslinks are cleaved from the kinetic chain, the unbound PEG chains, oligomeric lactides (bound to the PEG chains), and monomeric lactic acid can diffuse out of the network. The kinetic chains are attached to the network by numerous crosslinks and therefore are not released until late in the degradation. In this work, the incorporation of 15–50 mol % of nondegradable crosslinks prevents erosion of the kinetic chains from the network.

Representative mass-loss curves are shown in Figure 2 as a function of degradation time and composition for the PEG gels. The percent mass loss was pre-



**Figure 2.** Percent mass loss as a function of degradation time and gel composition as predicted from a theoretic model<sup>21,22</sup> for gels prepared from 100% PEG-LA-DA (—) and from varying molar ratios of 85:15 (PEG-LA-DA:PEGDM) (— —), 75:25 (— · —), and 50:50 (· · · · ·).

dicted using a previously developed theoretic model in which the hydrolysis kinetics (a model parameter) were based on experimental swelling data measured as a function of degradation time for a gel prepared from 10% (w/w) PEG-LA-DA.<sup>16,17</sup> The model was modified to predict the degradation behavior of copolymerized gels containing degradable and nondegradable crosslinks.

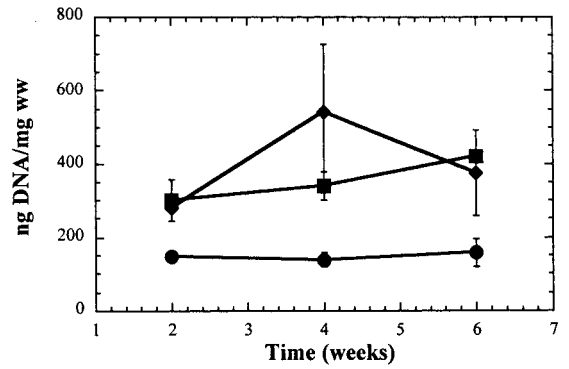
In the case when 100% of the crosslinks are degradable, the mass loss increases as crosslinks are hydrolyzed and removed from the network. As more and more crosslinks are broken, kinetic chains no longer attached to the 3-D network diffuse out of the gel. When fewer than two crosslinks remain per kinetic chain, the system transitions from a 3-D insoluble network to a highly branched, soluble polymer. At this point, a sharp increase in mass loss is observed, and this phenomenon is referred to as reverse gelation.

Degradation behavior of the PEG gels can be controlled to some degree by incorporating nondegradable crosslinks into the network. When a portion of the crosslinks do not degrade and on average more than two nondegradable crosslinks are connected to each kinetic chain, the network will not dissolve. Figure 2 shows the predicted mass loss of gels with 15, 25, and 50% nondegradable crosslinks as a function of degradation time. When a higher percentage of the crosslinks is nondegradable, the rate of mass loss decreases. For example, to reach 40% mass loss, a gel with 100% degradable crosslinks takes only 5 days whereas a gel with 50% degradable crosslinks takes 9 days.

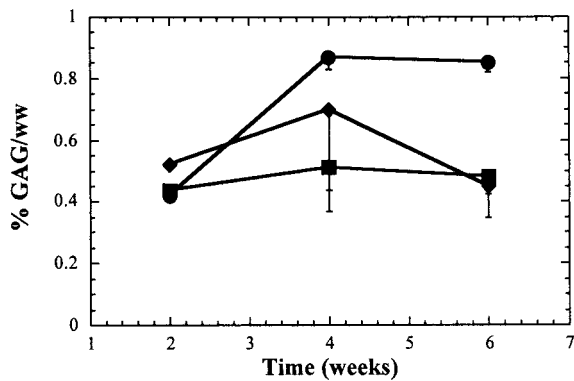
By varying the number of nondegradable crosslinks in the network, one has control over the degradation profile as well as over the evolving network properties. From a practical perspective, the degrading network mass loss can be controlled to match ECM formation. As the crosslinks are broken, the network mesh size increases, and the diffusion of secreted ECM molecules increases to facilitate macroscopic tissue formation. However, as the gel degrades, the mechanical properties of the gel also decrease. In cartilage tissue engineering, it is important to design a material that maintains mechanical integrity during degradation until the newly formed ECM can support the normal loads and stresses that exist in native cartilage. Therefore, by copolymerizing macromers, with varying mass erosion profiles, the degradation behavior of the gel can be tailored to control ECM formation and the network properties. For example, crosslinks that degrade quickly will allow for initial ECM diffusion while crosslinks that degrade much more slowly will give mechanical integrity to the cell-hydrogel construct until the newly formed ECM organizes into a functional tissue.

The neocartilaginous tissue formed in degrading gels of varying composition was examined to understand the influence of network degradation and changing network properties on ECM formation. The biochemical composition of the evolving cartilaginous tissue was analyzed after 2, 4, and 6 weeks *in vitro*. The DNA, GAG, and total collagen contents are shown in Figure 3 as a function of gel composition (i.e., varying mass loss profiles in the gel). The DNA content was statistically lower in the gel with 50% degradable crosslinks compared to gels with increased degradation ( $\geq 75\%$  degradable crosslinks) for each time point with the exception of the 85% gel at 6 weeks, which was significantly different with a  $p < 0.10$ . No statistical differences in DNA content were found between the gels with 75 and 85% degradable crosslinks over the course of the experiment. The three degrading systems were seeded with the same initial cell-seeding density (75 million cells/cc), which suggests that cell proliferation may occur in gels with increased degradation during the first 2 weeks. However, the DNA content did not increase significantly from 2 to 6 weeks in all three systems.

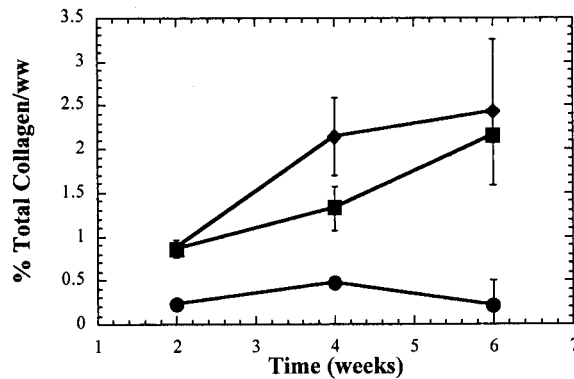
The GAG content was statistically similar in the gels with 50 and 75% degradable crosslinks and statistically higher in the 85% gel at 2 weeks. After 6 weeks of culture, the GAG content in the gel with 50% degradable linkages was  $0.85 \pm 0.03\%$  wet weight (ww), nearly twice that of the gels with increased degradation ( $0.45 \pm 0.02\%$  ww in the 85% gel). In contrast, over the course of the experiment, the total collagen content was statistically higher in the gels with 75 and 85% degradable crosslinks than it was in the gel with 50%



(a)



(b)



(c)

**Figure 3.** Biochemical content of the neocartilaginous tissue formed by chondrocytes photoencapsulated in PEG hydrogels with varying degrees of degradability with (●) 50%, (■) 75%, and (◆) 85% degradable crosslinks. The cell-hydrogel constructs were cultured for 6 weeks *in vitro*.

degradable crosslinks. After 6 weeks, the neocartilaginous tissue was comprised of  $2.4 \pm 0.8\%$  total collagen/ww in the 85% gel, which was nearly an order of magnitude higher than in the 50% gel ( $0.22 \pm 0.29\%$  total collagen/ww).

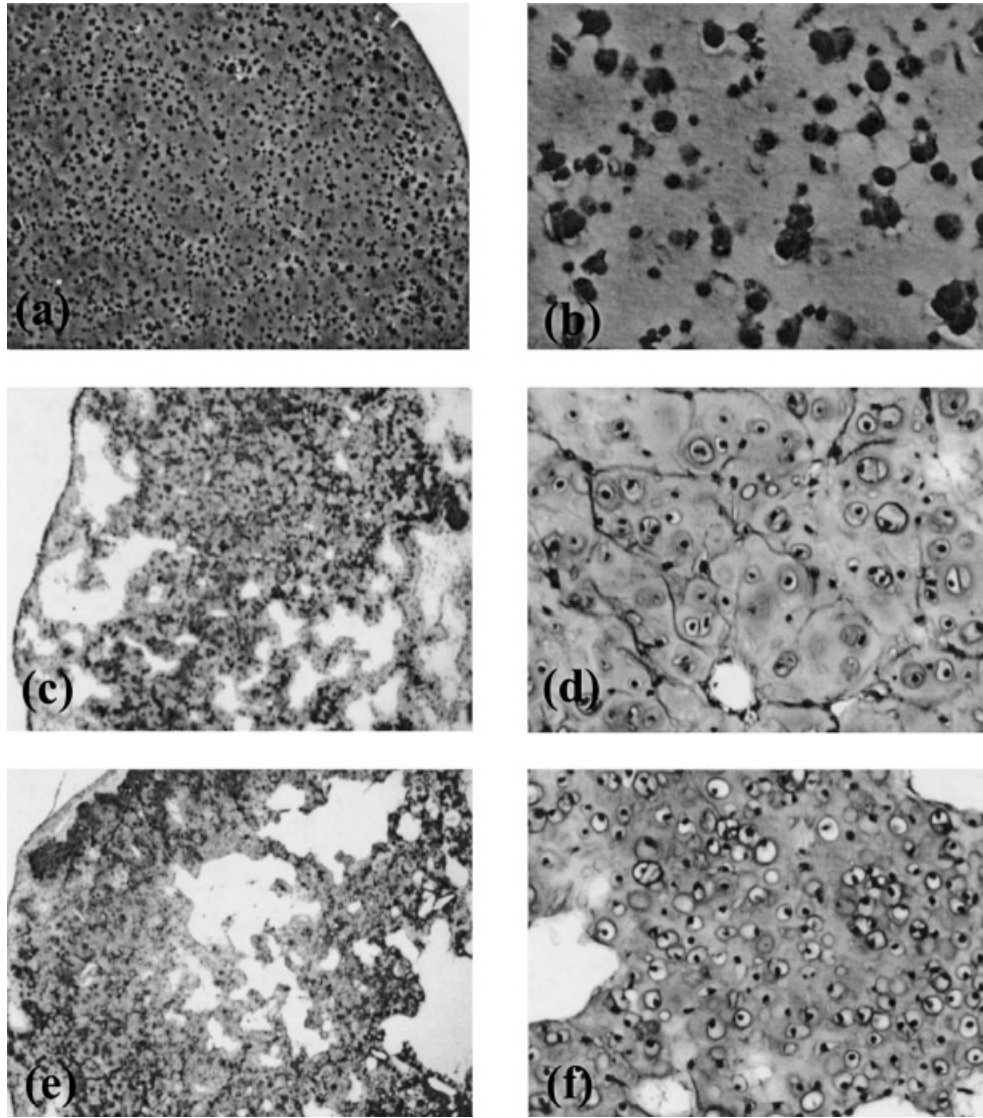
The bovine articular cartilage that was used for our primary cell isolation consisted of 8.8% total collagen/ww and 4.7% GAG/ww, as determined in our lab following the same protocol for the biochemical assays described in the Materials and Methods section. In the gels with increased degradation ( $\geq 75\%$  degradable crosslinks), the total collagen content was higher than the GAG content. However, in the system with 50% degradable crosslinks, the neocartilaginous tissue formed consisted of more GAGs than collagen. Thus, with increased gel degradation, the formed neotissue more closely approaches the composition of native articular cartilage.

In addition to evaluating the biochemical content of the gels, it is equally important to examine the spatial distribution of the ECM molecules. As the gel degrades and crosslinks are hydrolyzed (as drawn in Fig. 1), network properties, such as the water content, gel mechanics, and mesh size change.

In a previous study, we demonstrated that network properties in nondegrading gels influence ECM formation both spatially and biochemically.<sup>26</sup> Therefore, to elucidate the role of scaffold degradation on the formation and distribution of new tissue, understanding how network properties evolve during scaffold degradation can be helpful. For example, the mesh size of the network structure controls specie diffusion through highly swollen gels; however, as the gel degrades and crosslinks are broken, the average mesh size increases. The initial mesh size of the 50% degradable gel was previously determined to be  $\sim 140 \text{ \AA}$ ,<sup>26</sup> and since all the gels in this contribution were formulated under the same conditions (i.e., macromer concentration and photoinitiating conditions), the initial mesh size of the gels with different degradation kinetics should be similar. This assumption was further validated experimentally by insignificant variations in the initial equilibrium swelling of the gels. However, significant variations in the mesh size will evolve during degradation, and these variations depend on the gel composition.

Finally, the size of the ECM components found in native cartilage is important when understanding the diffusion and distribution of these components during scaffold degradation. For instance, the two main components of cartilage are proteoglycans and collagen. A moderately sized proteoglycan was determined to be  $\sim 90 \text{ \AA}$  in width,<sup>27</sup> and a typical collagen fiber diameter can range from 40 to 300 nm depending on the specie.<sup>28</sup>

Safranin-O was used to stain negatively charged proteoglycans red, and the histologic micrographs are shown in Figure 4. In all three systems, the chondrocytes appeared healthy in their characteristic lacunae. In addition, the initial mesh size of all three degrading systems ( $\sim 140 \text{ \AA}$ ) is larger than a moderately sized proteoglycan ( $\sim 90 \text{ \AA}$ ). In the 50% gel, the proteogly-



**Figure 4.** Histologic analysis of chondrocytes photoencapsulated in PEG hydrogels with varying degrees of degradability with (a,b) 50%, (c,d) 75%, (e,f) 85% degradable crosslinks after 4 weeks of *in vitro* culture. Sections were stained using Safranin-O, which stains proteoglycans red (shown in grey scale). Original magnification is  $\times 40$  for (a), (c), and (e) and  $\times 200$  for (b), (d), and (f).

cans were homogeneously distributed throughout the construct after 4 weeks *in vitro*. In the gels with 75 and 85% degradable crosslinks, GAGs also were present throughout the neotissue; however, in these gels void spaces were visible in the cell-hydrogel construct, likely the result of decreased mechanical integrity due to the high degree of degradation.

Furthermore, in examining the GAGs within the pericellular region, heavy staining was observed in the gel with 50% degradable crosslinks while little or no staining was observed pericellularly in the gels with increased degradable crosslinks. In the gel with 50% degradable crosslinks, the mesh size increased to  $\sim 250$  Å once all the degradable crosslinks were broken.<sup>26</sup> It is likely that larger proteoglycans diffuse much more slowly through the network, and therefore an increase

in GAG staining in the lacunae was observed in the gels with only 50% degradable crosslinks compared to gels with increased degradation. In addition, the shape of the lacunae is rounded and more typical of native tissue in gels with increased degradation ( $\geq 75\%$  degradable crosslinks). In contrast, the lacunae in the gels with only 50% degradable crosslinks exhibit a slightly strained appearance, which may be attributed to the relatively higher compressive modulus (initially 30 kPa) of the partially degraded gel.<sup>26</sup>

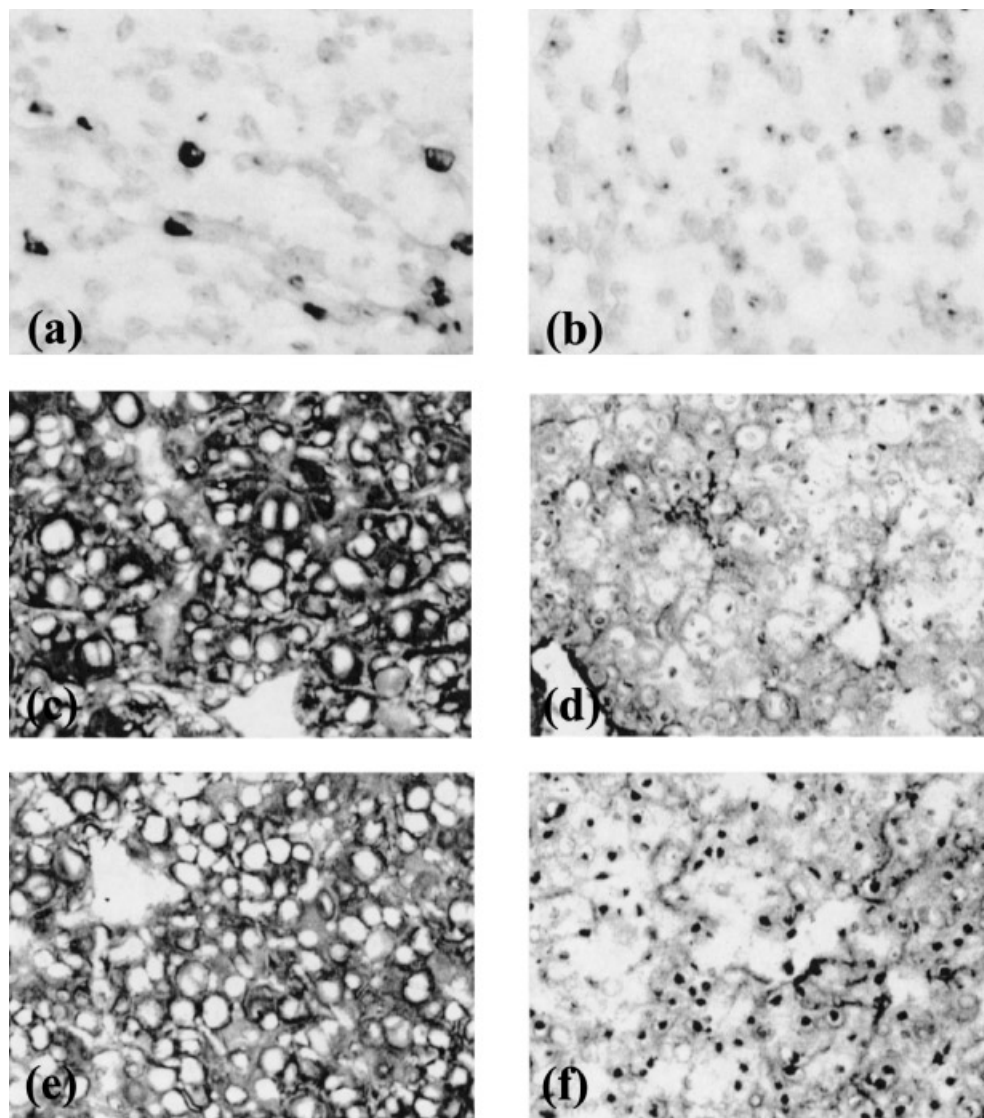
In engineering a functional tissue equivalent, in addition to the total collagen content and distribution of collagen, the *type* of collagen produced is important. In articular cartilage, the most abundant type of collagen is type II, which comprises nearly 90% of the total collagen. However, chondrocytes are known to un-

dergo a phenotypic change and develop into fibroblast-like cells, which primarily produce type I collagen. Therefore, immunohistochemistry was performed on the gels that exhibited a range of degradation behaviors to examine the distribution of both type I and type II collagen in the gels. The resulting histologic micrographs are shown in Figure 5.

Interestingly, in examining after 4 weeks the gels with 50% degradable crosslinks, only a few cells were producing type II collagen, and the collagen was localized in the pericellular region. Again, the chondrocytes appeared somewhat strained in those gels, which may have influenced the type of ECM formed. When increased degradation was introduced into the networks, type II collagen was observed throughout the neocartilaginous tissue, as illustrated by the dark,

relatively uniform staining. In addition, the size of a collagen fiber (40–300 nm in diameter) is much larger than the final mesh size of the 50% gel (~250 Å); therefore collagen was not able to diffuse into the extracellular regions and was localized in the pericellular region. However, with increased degradation, a critical mesh size was reached, which allowed collagen to diffuse into the extracellular region.

With respect to type I collagen, little collagen synthesis was observed in the gel with 50% degradable crosslinks whereas some staining was observed in the gels with 75 and 85% degradable crosslinks. But in all cases, a greater amount of type II than type I collagen was present in the neocartilaginous tissue, as seen histologically. Previous *in vivo* studies have shown similar results in which chondrocytes seeded onto PGA/



**Figure 5.** Immunohistochemical analysis of chondrocytes photoencapsulated in PEG hydrogels with varying degrees of degradability with (a,b) 50%, (c,d) 75%, and (e,f) 85% degradable linkages after 4 weeks of *in vitro* culture. Panels (a), (c), and (e) were stained for type II collagen (shown in grey scale). Panels (b), (d), and (f) were stained for type I collagen (shown in grey scale). Original magnification  $\times 200$ .

PLLA and pure PLLA scaffolds and encapsulated in agarose gels produced primarily type II collagen, but small amounts of type I collagen also were present.<sup>29</sup>

## CONCLUSIONS

The mass-loss profile of PEG hydrogels easily can be tailored to control deposition and distribution of the ECM molecules that make up the neocartilaginous tissue. For example, we have shown through a theoretic model that the incorporation of nondegradable crosslinks into the network slows the rate of mass loss and eliminates reverse gelation. Chondrocytes encapsulated in hydrogels with at least 75% degradable crosslinks produced a cartilaginous tissue that had nearly twice the DNA content and nearly an order of magnitude higher total collagen content than gels formed with 50% degradable crosslinks.

In addition, gels with increased degradation ( $\geq 75\%$  degradable crosslinks) supported an increase in type II collagen synthesis that was distributed homogeneously throughout the neocartilaginous tissue. During scaffold degradation, network properties (e.g., mesh size) change and influence the formation of new tissue. In this study, we demonstrated that a critical mesh size exists ( $\geq 75\%$  degradable crosslinks) that promotes diffusion of both collagen and proteoglycan molecules through the scaffold. Therefore, by controlling the degradation behavior of the PEG hydrogels, one has control over the formation of neocartilaginous tissue.

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