

# The effects of scaffold thickness on tissue engineered cartilage in photocrosslinked poly(ethylene oxide) hydrogels

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## Abstract

The thickness of human articular cartilage has been reported to vary from less than 0.5 up to 7 mm. Hence, tissue engineered cartilage scaffolds should be able to span the thickness of native cartilage to fill defects of all shapes and sizes. In this study, we demonstrate the potential for using photopolymerization technology to encapsulate chondrocytes in poly(ethylene oxide) hydrogels, which vary in thickness from 2 to 8 mm. Chondrocytes, encapsulated in an 8 mm thick, photocrosslinked hydrogel and cultured in vitro for 6 weeks, remained viable and produced cartilaginous tissue throughout the construct comparable to a 2 mm thick gel as seen both histologically and biochemically. In addition, the total collagen and glycosaminoglycan contents per wet weight of the 8 mm thick cell-polymer construct were  $0.13 \pm 0.01$  and  $0.25 \pm 0.03\%$ , respectively, and did not vary significantly as a function of spatial position in the construct. The histological evidence and the biochemical content were similar in all constructs of varying thickness. The results suggest that photocrosslinked hydrogels are promising scaffolds for tissue engineering cartilage as cell viability is readily maintained; uniform cell seeding is easy to achieve; and the biochemical content of the extracellular matrix is not compromised as the scaffold thickness is increased from 2 to 8 mm. © 2001 Elsevier Science Ltd. All rights reserved.

*Keywords:* Tissue engineering; Cartilage; Photopolymerization; Hydrogels; Poly(ethylene oxide)

## 1. Introduction

Damaged cartilage has a limited ability to repair itself due to the absence of vascularization and nerve endings in the tissue [1]. Traditional therapies to repair damaged cartilage include alloplastic and allogenic implants and more recently autologous chondrocyte transplantation. The former therapies are limited by donor tissue availability and donor site morbidity, while the latter therapy requires surgical removal of healthy cartilage and is limited by the size of the defect [2]. As an alternative to these current therapies, efforts in tissue engineering of cartilage have led to the development of biocompatible, biodegradable scaffolds onto which cells are seeded.

In developing a successful scaffold for tissue engineering, several questions arise such as: how to tailor the architecture of the scaffold to provide adequate transport, mechanics, and degradation and how to obtain

a uniform cell density as the scaffold thickness is increased. The second question is especially important in cartilage tissue engineering. Ateshian et al. [3] measured the thickness of human articular cartilage (average age of 34 years) and found that in the tibial plateau the thickness varied from 0.35 to 6.25 mm and in the patella from 0.89 to 5.94 mm. Kladny et al. [4] also measured the thickness of human articular cartilage (average age of 37.5 years) and found the thickness varied from 0.5 to 7.1 mm. Therefore, cartilage tissue engineering scaffolds should be able to span the thickness of native cartilage, i.e., over the range of potential defects, from partial to full thickness defects.

Many of the early successes in tissue engineering cartilage in vivo and in vitro used  $\sim 2$  mm thick (and smaller) poly(glycolic acid) (PGA) meshes [5–12]. To the best of our knowledge, Freed et al. [13,14] are the only investigators who have published results related to the effects of scaffold thickness in these meshes. The authors reported that cell density and glycosaminoglycan content decreased with increasing thickness (from 0.88 to 3.84 mm) after 23 days in vitro. Although these scaffolds are promising in promoting new cartilage tissue growth,

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limitations exist. For example, PGA meshes must be prefabricated and seeded with chondrocytes; hence, as the scaffold thickness increases, a uniform cell density becomes difficult to obtain.

An alternative to seeding cells on prefabricated scaffolds is to encapsulate cells in hydrogel matrices. Hydrogels are beneficial because of their high water content, which facilitates transport of nutrients, and tissue-like elastic properties. In situ forming hydrogels such as alginates [11,15], fibrin glue [16–18], and modified poly(ethylene oxide) (PEO) [19] have been successful in encapsulating chondrocytes. Alginates and fibrin glue are derived from natural products; although attractive for in vivo applications, limitations exist with respect to controlling the mechanics and uniformity of the gels. Hence, we are particularly interested in photocrosslinkable hydrogels based on PEO chemistry. Elisseff et al. [19] provided the first successful investigation of transdermally photopolymerizing chondrocytes into athymic mice for tissue engineering cartilage. The advantages of photopolymerization include the ability to convert rapidly a liquid monomer or macromer to a gel at physiological temperatures with temporal and spatial control during polymerization to form complex three-dimensional architectures in vivo with controlled mechanics.

In this contribution, we investigate the formation of cartilaginous tissue as a function of scaffold thickness using photocrosslinked hydrogels. In particular, we wanted to assess the feasibility of photopolymerization technology and photocrosslinked hydrogels to fill cartilage defects of all sizes. In this regard, this paper aims to answer several fundamental questions including: (1) Will the polymerization conditions permit curing of thick constructs on a clinically acceptable time scale? (2) Will the chondrocytes remain viable and uniformly distributed as the scaffold thickness is increased? and (3) Will the chondrocytes produce cartilaginous tissue throughout the construct? To answer these questions chondrocytes were photoencapsulated in PEO hydrogels of varying thickness from 2 to 8 mm and cultured in vitro for 6 weeks. Cell viability was determined qualitatively using a membrane integrity assay, and the neocartilage was characterized both quantitatively, by measuring the biochemical content, and qualitatively, through histological sections.

## 2. Materials and methods

### 2.1. Chondrocyte isolation

Chondrocytes were isolated from the femoral-patellar groove of a young calf (Research 87, Marlboro, MA). Two isolations were performed. Briefly, the articular cartilage was excised under aseptic conditions and digested at 37°C on an orbital shaker using a solution of 0.2%

Collagenase Type II (Gibco) and 5% fetal bovine serum (Gibco) in Dulbecco's modified Eagle medium (Gibco) without additives for a maximum of 17 h. The suspension was filtered through a 70 µm nylon cell strainer, centrifuged at 1000 rpm for 10 min. The supernatant was 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 twice and resuspended in media. The chondrocyte viability was determined using trypan blue exclusion and a hemacytometer.

### 2.2. Hydrogel preparation and characterization

The macromer, poly(ethylene oxide) dimethacrylate (PEODM, Shearwater Polymer, Inc., MW 3400), was sterilized under UV light overnight. The macromer was dissolved in sterile phosphate-buffered saline (PBS) to a final solution concentration of 10% (w/w). The UV photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (D2959, Ciba Geigy), used in this study is cytocompatible under the given concentrations and initiating conditions as previously determined [20]. A concentrated solution of D2959 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 polymerization behavior of six samples was monitored using differential scanning calorimetry (DSC, Perkin Elmer DCS-7) in which the heat flow is proportional to the rate of polymerization. The DSC head was modified with a quartz window to allow for transmission of initiating light. A small amount of the macromer solution was placed in a DSC pan and photopolymerized at 25°C in the presence of air with 365 nm light at 9 mW/cm<sup>2</sup>. Quartz lids were used to minimize the evaporation of water.

### 2.3. Chondrocyte encapsulation

Isolated chondrocytes were suspended in the macromer/initiator solution at a concentration of  $50 \times 10^6$  and  $100 \times 10^6$  cells per ml and polymerized using a long-wave ultraviolet lamp (Model B100AP, Blak-Ray) at an intensity of  $\sim 10$  mW/cm<sup>2</sup> for 10 min. The cell-macromer solution was polymerized in a cylindrical shape with a diameter of  $\sim 9$  mm at a thicknesses of 2, 4, 6, or 8 mm. The constructs were incubated at 37°C in a humid environment with 5% CO<sub>2</sub> in untreated tissue culture 12 well plates under static conditions. The cell-polymer constructs were cultured using Dulbecco's modified Eagle medium (Gibco) without phenol red supplemented with 10 mM HEPES, 0.04 mM L-Proline, 0.1 M MEM non-essential amino acids (Gibco), 1% penicillin–streptomycin (Gibco), 0.5 µg/ml fungizone (Gibco), and 10% fetal bovine serum (Gibco), and the media was replaced every 2–3 days.

#### 2.4. Cell viability

Cell viability was determined after 2 weeks of in vitro culture on one of the 4 and 8 mm thick constructs (initially encapsulated at  $100 \times 10^6$  cells/ml). Each construct was divided into  $\sim 2$  mm sections. Cell viability was determined on each section using a membrane integrity assay, the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, L-3224) which contains calcein AM and ethidium homodimer. Briefly, calcein AM diffuses through the membrane of living cells and reacts with intracellular esterase to produce a green fluorescence, while ethidium homodimer, which cannot diffuse across the healthy cell membrane but can diffuse across the damaged cell membrane, binds to nucleic acids to produce a red fluorescence. A Nikon Eclipse TE300 with epi-fluorescence capabilities was used to visualize the cell viability.

#### 2.5. Biochemical analysis

A cell-polymer construct for each thickness (initially encapsulated at  $50 \times 10^6$  cells/ml) was analyzed to quantify the biochemical content of the neocartilage. The constructs were divided into  $\sim 2$  mm sections, as illustrated in Fig. 1, to examine quantitatively cartilaginous tissue formation as a function of depth. The sections were freeze-dried for at least 48 h, crushed and then digested in a papain solution (125  $\mu$ g/ml papain type III (Worthington), 10 mM l-cysteine (Aldrich), 100 mM phosphate, and 10 mM EDTA at pH 6.3) for 16 h at 60°C. The glycosaminoglycan (GAG) content was determined using the dimethylmethylene blue dye method [21]. Total collagen content was determined by quantifying the hydroxyproline content [22], which makes up 10% of collagen [23]. The GAG and total collagen contents are reported as a percentage of the wet weight, where the wet weight includes the neocartilage, polymer and water. The constructs were comprised of  $\sim 90\%$  water.

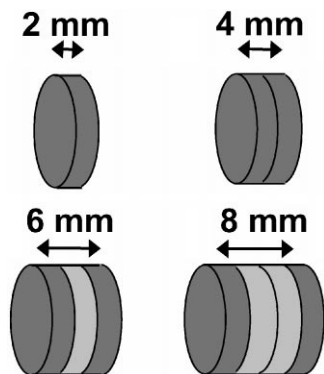


Fig. 1. Four cell-polymer constructs were polymerized with varying thickness. The constructs were analyzed by sectioning each construct into approximately 2 mm thick disks in which  $\blacksquare$  represent the edge sections while  $\square$  represent the middle sections.

#### 2.6. Histological analysis

For each thickness, a cell-polymer construct (initially encapsulated at  $50 \times 10^6$  cells/ml) was divided into  $\sim 2$  mm thick sections and analyzed histologically. Each section was fixed in formalin overnight, embedded in paraffin, and sectioned into 10  $\mu$ m sections using standard histological procedures. The sections were stained for positively charged GAGs with Safranin O and counterstained with Fast Green, in which the nuclei stain black and GAGs stain orange/red, and stained for collagen using the Masson trichrome technique, in which nuclei stain blue-black and collagen stains blue.

### 3. Results and discussion

Chondrocytes encapsulated in photocrosslinked hydrogels of varying thickness were examined to assess the potential for using photopolymerization technology to tissue engineer cartilaginous tissue in defects of different sizes. To photopolymerize the thick cell-polymer constructs, several factors are important including: choosing a clinically suitable photoinitiating system with respect to cytocompatibility (and viability), the degree of light attenuation in the sample to insure complete conversions as a function of depth, and the polymerization time scale.

In previous work [20], we identified one such cytocompatible photoinitiating system based on the photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (D2959), at a concentration of 0.05% (w/w) and exposed to 365 nm light at an intensity of  $\sim 8$  mW/cm<sup>2</sup> for up to 10 min. In this contribution, we employ this initiating system along with a PEO-based photocrosslinkable macromer. PEO was chosen because of its long history in biomedical applications, and PEODM, the modified photocrosslinkable macromer, has been recently used in vivo as an adhesive for gluing severed blood vessels [24] and as a protective barrier around islets cells [25,26]. Finally, by varying the molecular weight of the macromer, a wide range of macroscopic properties (e.g., equilibrium water content and compressive modulus) can be achieved in the final network.

Typically, when photopolymerizing thin polymer films with a low initiator concentration, light attenuation is negligible; however, as the sample thickness increases light attenuation can become appreciable. The cytocompatible photoinitiator chosen in this study (D2959) has a low molar absorptivity at 365 nm (6.71/mol cm) [20], and the amount of light absorption is readily calculated from the Lambert–Beer law. For the thickest sample polymerized in this study (8 mm), 2.7% of the initiating light is absorbed; therefore, the overall polymerization behavior can be approximated from thin film studies and assumed to be fairly uniform as a function of depth.

To determine if the polymerization occurs on a clinically acceptable time scale using cytocompatible initiating conditions, differential scanning calorimetry (DSC) was used to monitor the rate of polymerization as a function of exposure time. Due to the high water content (90%) in our polymerizing samples, extreme care was taken to minimize evaporation, but the heat of vaporization of water is so large that even trace amounts of evaporation can lead to errors in the absolute rate values. Thus, further investigation is necessary to determine the absolute polymerization rate; however, the polymerization time scale is readily obtained. From the results shown in Fig. 2, the polymerization is complete in  $\sim 3$ –4 min, and therefore, suitable for many *in vivo* applications. In addition, the shape of the polymerization rate curve with respect to exposure time is characteristic of crosslinking polymerizations exhibiting many of the typical features of autoacceleration and autodeceleration [27].

With this understanding of the polymerization behavior, which occurs on a clinically acceptable time scale under cytocompatible photoinitiating conditions, chon-

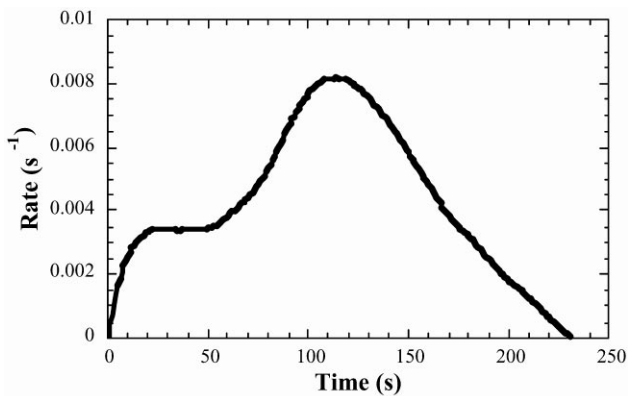


Fig. 2. A typical polymerization rate curve as a function of time for a 10% (w/w) PEO DM solution polymerized with 0.05% (w/w) D2959 and  $9 \text{ mW/cm}^2$  of 365 nm light.

drocytes were then encapsulated in photocrosslinked hydrogels 9 mm in diameter and varying in thickness from 2 to 8 mm. The constructs were analyzed by sectioning the gels into  $\sim 2$  mm thick disks, as pictured in Fig. 1, and the cell viability, cell uniformity, and neocartilage formation was examined as a function of depth.

After 2 weeks *in vitro*, 4 and 8 mm hydrogel constructs were assessed qualitatively for cell viability and cell uniformity as a function of thickness. The constructs were sectioned (as shown in Fig. 1), and a small sample located in the core of each section was removed and analyzed using a fluorescent cell viability assay. The results for the 8 mm construct are shown in Fig. 3. Nearly all the cells remained viable throughout the construct suggesting that the hydrogel provides adequate transport of nutrients at increased thickness to maintain cell viability; however, further studies are required to determine how matrix deposition is affected by transport issues with respect to oxygen and other signaling molecules. In addition, the cells are uniformly distributed both radially and longitudinally. Results from the 4 mm hydrogel (not shown) were similar to the 8 mm construct. These data suggest that an increase in hydrogel thickness from 2 to 8 mm does not compromise cell viability and cell uniformity as seen qualitatively over a period of 2 weeks in PEO hydrogels.

To further understand the effects of scaffold thickness on chondrocyte functionality, the biochemical content of the cartilaginous tissue formed in these networks was quantified. The glycosaminoglycan (GAG) and the total collagen contents per wet weight (ww) of the construct were measured as a function of depth after 6 weeks *in vitro*, and the results are shown in Fig. 4. Since the constructs are symmetric, the biochemical content is presented as a function of depth by averaging the edge sections and the middle section(s) (in the case of the 6 and 8 mm constructs) for the 4, 6 mm, and the 8 mm constructs. The GAG and total collagen contents in each section were similar as a function of depth and scaffold

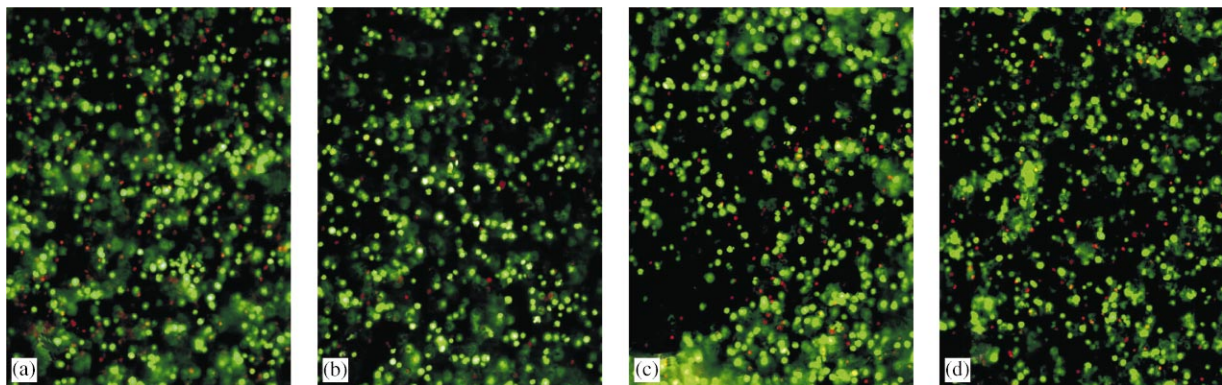


Fig. 3. Chondrocytes encapsulated in an 8 mm thick PEO hydrogel after 2 weeks *in vitro* and stained with a LIVE/DEAD cell assay in which the live cells fluoresce green and the dead cells fluoresce red. (A) and (D) are an inner view of the edge sections and (B) and (C) are an inner view of the middle sections.

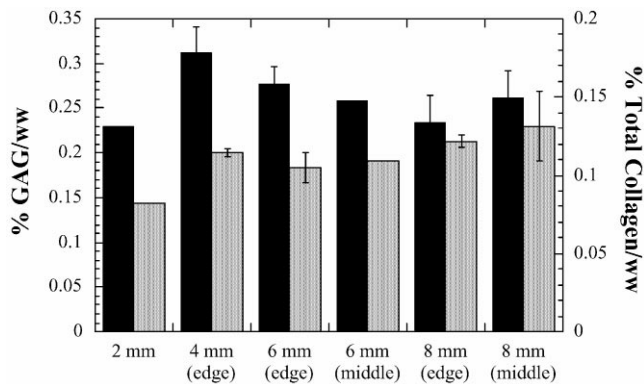


Fig. 4. Biochemical content of cell-polymer constructs with varying thickness in which (■) is the % glycosaminoglycan content/ww of the cell-polymer construct and (▨) is the % total collagen content/ww of the cell-polymer construct. ( $N = 2$  for the 4, 6, and 8 mm edge sections and the 8 mm middle section;  $N = 1$  for the 2 and 6 mm middle section).

thickness. For example, in the 8 mm construct, the GAG content in the edge and middle sections was  $0.23 \pm 0.03\%$  GAG/ww and  $0.26 \pm 0.03\%$  GAG/ww, respectively, which is comparable to the 2 mm construct comprised of  $0.23\%$  GAG/ww. The total collagen content of the 8 mm construct in the edge and middle sections was  $0.12 \pm 0.004\%$  total collagen/ww and  $0.13 \pm 0.02\%$  total collagen/ww, respectively, which is slightly higher than the 2 mm thick construct with  $0.08\%$  total collagen/ww. In the 6 mm construct, the GAG and total collagen contents were similar in the edge and the middle sections and comparable to the 8 mm construct. The data suggest that chondrocytes encapsulated in these hydrogels, which span the thickness of human articular cartilage, are capable of producing cartilaginous tissue; and the biochemical content is not compromised as the construct thickness is increased from 2 to 8 mm.

In previous work, Elisseff et al. [19], transdermally polymerized a semi-interpenetrating network based on PEO, and after 6 weeks in vivo the biochemical content of the neocartilage was  $\sim 1.8\%$  GAG/ww and  $\sim 6\%$  total collagen/ww. In this in vivo study, the total collagen content was higher than the GAG content, as seen in native cartilage [28]. This difference in the biochemical make up of the neocartilage produced in vivo versus in vitro may be attributed, in part, to the complex nature of the in vivo environment containing growth factors, hormones, and other signaling factors, which may influence and regulate the synthesis of collagen. To further illustrate this difference, Freed et al. [5] seeded chondrocytes on poly-L-lactic acid meshes, which were cultured in vitro and transplanted subcutaneously in athymic mice, and the former resulted in minimal neocartilage growth while the latter resulted in significant cartilage formation.

A great deal of the research in cartilage tissue engineering has focused on seeding chondrocytes on PGA and PLA meshes [5–14,29]. For example, Schreiber et al.

[12] investigated ovine articular chondrocytes seeded onto PGA scaffolds (2 mm thick) cultured in vitro under static conditions, similar conditions to our study. The cartilaginous tissue formed in these meshes comprised  $2\%$  GAG/ww and  $0.9\%$  total collagen/ww when cultured between 4 and 6 weeks; the ratio of GAG to collagen is comparable with our in vitro data. However, when Freed et al. [13,14] investigated the effects of scaffold thickness in PGA meshes, both GAG content and cell density decreased with increasing thickness. PGA and PLA meshes must be prefabricated and seeded with cells, and therefore obtaining a uniform seeding density is difficult especially as thickness is increased. Gugala et al. [29] seeded chondrocytes on 2 mm thick PLA scaffolds cultured in vitro under static conditions, and after 9 weeks, histological evaluation showed that more proteoglycans were present at the seeding side of the scaffold. The data presented in this paper suggest that photopolymerized hydrogels may have advantages over PGA meshes of appreciable thickness with respect to cell seeding and maintaining cell viability throughout the construct.

In addition to quantifying the biochemical content of the neocartilage, the constructs were examined histologically to evaluate qualitatively the distribution of the GAGs and collagen in the constructs both radially and longitudinally. Safranin O and Masson trichrome, which stain for positively charged proteoglycans and collagen, respectively, were used to evaluate the constructs, and histological sections are presented in Figs. 5 and 6. A negative control of the hydrogel without cells was used to insure that the polymer matrix did not contribute to the histological evaluation.

The histological evidence shows that the GAGs are distributed throughout the hydrogel both radially in each section of the constructs and longitudinally as thickness increases. The GAGs produced in the 2 mm construct are similar to the middle sections of the 6 and 8 mm constructs as shown in Fig. 5. At a higher magnification (as seen in Fig. 6), chondrocytes are rounded and located within the lacunae resembling healthy native cartilage and, as well, remain distributed throughout the gel after 6 weeks in vitro. The constructs stained positive for collagen within the lacunae as seen in Fig. 6, which agrees with previous studies using chondrocytes seeded on PGA meshes (1.5 mm thick) and cultured in vitro for 7 weeks [5]. However, Rotter et al. [30] demonstrated that when chondrocytes are seeded on meshes of PGA and PLA (2 mm thick) and transplanted subcutaneously in athymic mice for 24 weeks, collagen was produced throughout the extracellular matrix. The neocartilage formed in vitro in these 8 mm thick photocrosslinked hydrogels is comparable to the in vitro results with 1.5 mm thick PGA meshes which further suggests that photocrosslinkable hydrogels provide a promising scaffold for tissue engineering cartilage especially as scaffold thickness increases.

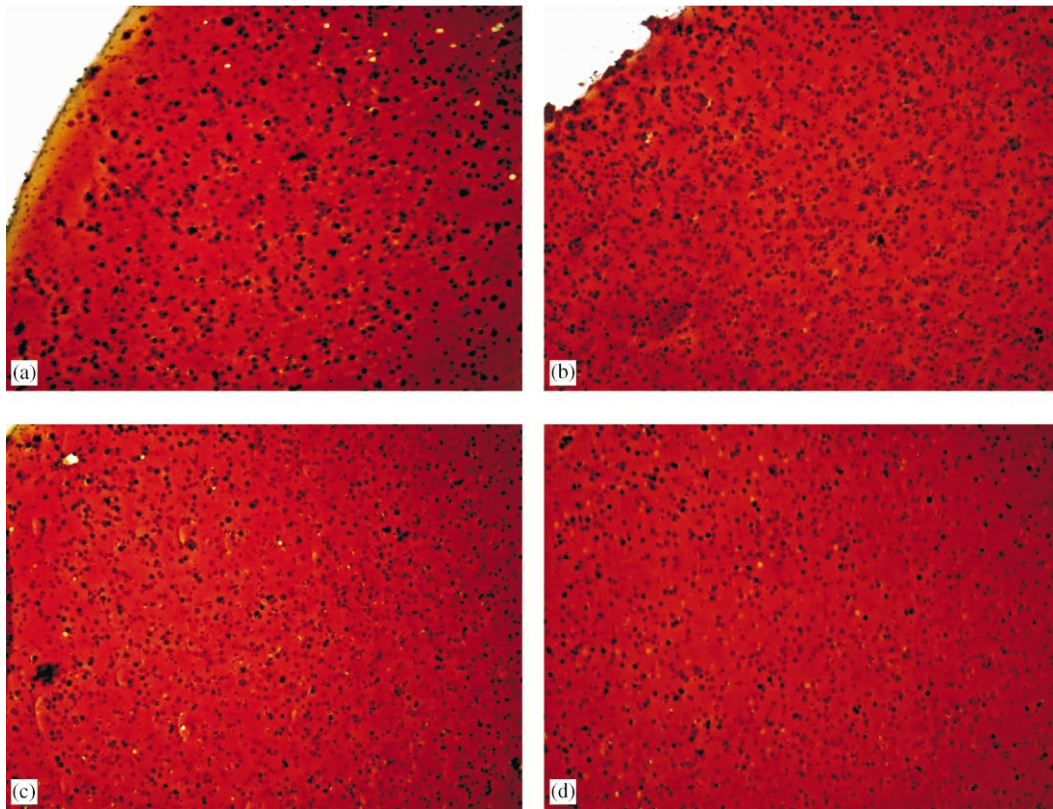
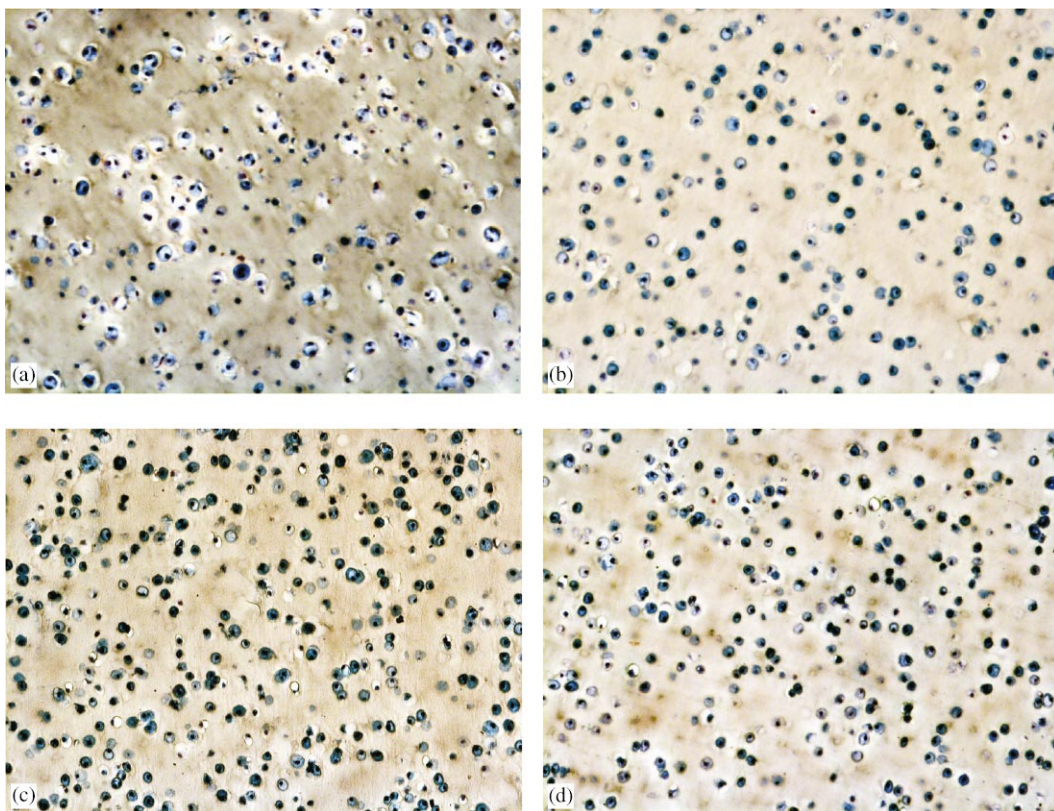


Fig. 5. Histological analysis of glycosaminoglycans as a function of scaffold thickness in PEO hydrogels after 6 weeks in vitro. Safranin O stains proteoglycans red, nuclei black, and cytoplasm green. (a) and (b) are edge sections from 2 and 4 mm thick gels, respectively. (c) and (d) are middle sections from 6 and 8 mm thick gels, respectively. Original magnification  $40\times$ .



#### 4. Conclusions

This study demonstrates the potential for using photopolymerized cell-hydrogel constructs in regenerating cartilaginous tissue for partial and full thickness defects by examining the biochemical content and histology of cartilaginous tissue formed as a function of thickness in PEO-based hydrogels. Under in vitro conditions, photocrosslinked hydrogels can be used to encapsulate chondrocytes and regenerate cartilaginous tissue in scaffolds up to 8 mm in thickness and produce, throughout the construct, a similar tissue to that found in 1.5 mm thick PGA meshes. The advantages of photoencapsulating cells in a hydrogel matrix are the ease with which uniform seeding can be achieved and the high transport of nutrients that these gels allow to maintain cell viability even at increased thickness. We have shown that chondrocytes encapsulated in an 8 mm thick photocrosslinked hydrogel and cultured in vitro for 6 weeks produced cartilaginous tissue throughout the construct as seen histologically and comprised  $0.25 \pm 0.03\%$  GAG/ww and  $0.13 \pm 0.01\%$  total collagen/ww.

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Fig. 6. Histological analysis of collagen content as a function of scaffold thickness in PEO hydrogels after 6 weeks in vitro. Masson trichrome stains collagen blue and nuclei black. (A) and (B) are edge sections from 2 and 4 mm thick gels, respectively. (C) and (D) are middle sections from 6 and 8 mm thick gels, respectively. Original magnification 100 $\times$ . The slight variation in the color between A and B–D is a processing artifact.

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