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# Photoencapsulation of chondrocytes in poly(ethylene oxide)-based semi-interpenetrating networks

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Received 29 July 1999; revised 12 November 1999; accepted 3 December 1999

**Abstract:** A photopolymerizing hydrogel system provides an efficient method to encapsulate cells. The present work describes the *in vitro* analysis of bovine and ovine chondrocytes encapsulated in a poly(ethylene oxide)-dimethacrylate and poly(ethylene glycol) semi-interpenetrating network using a photopolymerization process. One day after encapsulation, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide) (MTT) and light microscopy showed chondrocyte survival and a dispersed cell population composed of ovoid and elongated cells. Biochemical analysis demonstrated proteoglycan and collagen contents that in-

creased over 2 weeks of static incubation. Cell content of the gels initially decreased and stabilized. Biomechanical analysis demonstrated the presence of a functional extracellular matrix with equilibrium moduli, dynamic stiffness, and streaming potentials that increased with time. These findings suggest the feasibility of photoencapsulation for tissue engineering and drug delivery purposes. © 2000 John Wiley & Sons, Inc. *J Biomed Mater Res*, 51, 164–171, 2000.

**Key words:** cartilage; tissue engineering; hydrogel; photopolymerization

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## INTRODUCTION

Tissue engineering employs cells seeded on three-dimensional natural or synthetic scaffolds. Both solid and gel scaffolds are used as matrices. Cells may be directly encapsulated in gels, allowing increased efficiency and uniformity in cell seeding on the scaffold compared with solid scaffolds. Gels are often injectable, making implantation easier than systems that require surgical implantation. To design a system to encapsulate cells directly, polymer scaffolds must be identified or developed that (a) are water soluble to produce cell-polymer suspensions, and (b) undergo a physical or chemical change causing gelation. Control of the gelation process and cell compatibility of the reaction are desirable. Current gelling systems under investigation include alginate, Pluronics, and fibrin

glue. Alginate, a polysaccharide, forms an ionic network. Many groups have investigated the activity and biological properties of chondrocytes entrapped in alginate *in vitro* (i.e., Ref. 1). Alginate has been examined for use in craniofacial cartilage replacement and as cartilage plugs to prevent vesicoureteral reflux.<sup>1,2</sup> Similarly, chondrocytes have been encapsulated in agarose gels and the biochemical and mechanical properties of the resulting tissue have been studied.<sup>3</sup> Fibrin glue is another biological gel that has been used to encapsulate chondrocytes, but the resulting gel degrades too quickly to maintain structural integrity before tissue is formed.<sup>4</sup> Although the above systems are injectable, there is little control over the gelation process after injection.

Recent attention has focused on materials that may be implanted in a minimally invasive manner for use in plastic and orthoscopic surgery.<sup>5</sup> Injectable materials allow implantation through a large-bone needle or arthroscopic instrument.<sup>6</sup> Our laboratory has developed a technique—transdermal photopolymerization—which uses light that is transmitted through tissue to photopolymerize an injected macromer solution.<sup>7</sup> Photopolymerization provides direct control over the gelation process while transdermal or trans-

No benefit of any kind will be received either directly or indirectly by the authors

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Contract grant sponsor: NIH; Contract grant number: DE13023

tissue exposure to the light source that causes polymerization decreases the invasiveness of the gel placement.

Photopolymerization provides a unique way to form a gel in a fast, controllable manner. Gels can be formed *in situ*, providing easy *in vivo* placement of gels for tissue engineering or drug delivery purposes. Photopolymerization is also being examined as a method to create scaffolds with specific nanoscale topography to promote control of cell migration and function.<sup>8</sup> Direct photoencapsulation of cells in a gel would benefit the application of both of the above techniques to tissue engineering.

The purpose of this present work was to photoencapsulate chondrocytes in a gel scaffold. Substituted poly(ethylene oxide) and a photopolymerization process were used to transform a liquid polymer solution to a gel while directly encapsulating the chondrocytes. The biochemical and mechanical properties of the extracellular matrix produced by the chondrocytes were studied.

## MATERIALS AND METHODS

### Cell isolation

Saddle sections from 1- to 2-week-old calves were obtained from a local abattoir (A. Arena, Hopkington, MA). The intact femoropatellar groove was removed and chondrocytes were immediately isolated using type II collagenase (0.2% in complete media with overnight incubation; Worthington, Freehold, NJ), washed three times with phosphate-buffered saline (PBS), and passed through a Nytex filter. Ovine chondrocytes were obtained from ovine saddle sections in a similar manner. Cell number was determined by hemocytometer counting. Cells were centrifuged (1000 rpm, 10 min) to form a pellet before addition of polymer.

### Polymer preparation

Poly(ethylene oxide) (PEO) (100,000; Shearwater Polymers, Knoxville, TN) and poly(ethylene oxide)-dimethacrylate (PEODM) (3400; Shearwater Polymers) were combined in a 3:2 ratio and dissolved in PBS with 100 U/mL penicillin G and 100 µg/mL streptomycin to form a 20% (w/v) solution. The photoinitiator 1-cyclohexyl phenyl ketone (HPK; Polysciences) was added to the polymer solution (3-µL/mL polymer solution from a stock of 120 mg/mL in 70% ethanol). After thorough mixing to dissolve the polymer, the solution was added to a pellet of bovine chondrocytes to make a final concentration of  $50 \times 10^6$  cells/cc. Ovine chondrocyte concentration was  $40 \times 10^6$  cells/cc. The chondrocytes were suspended in the viscous polymer solution by thorough mixing.

### Photopolymerization

One hundred fifty microliters of the chondrocyte/polymer suspension was placed in either sterile Eppendorfs or tissue culture inserts (diameter 6 mm) and placed under a UVA lamp for 3 min (Fig. 1). The lamp was at a height so that the polymer/chondrocyte solutions received a light intensity of approximately 2–3 mW/cm<sup>2</sup> as determined by radiometer measurements. Resulting hydrogels were removed from the Eppendorf or tissue culture insert using a sterile spatula and placed in 12-well tissue culture plates with media [high-glucose Dulbeccos modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 10 µg/mL vitamin C, 12.5 mM HEPES, 0.1 mM nonessential amino acids and 0.4 mM proline]. Media were refreshed biweekly. Hydrogels were incubated statically at 37°C, 5% CO<sub>2</sub>. Control hydrogels were synthesized and incubated as described above, but contained polymer without chondrocytes.

### Biochemical characterization

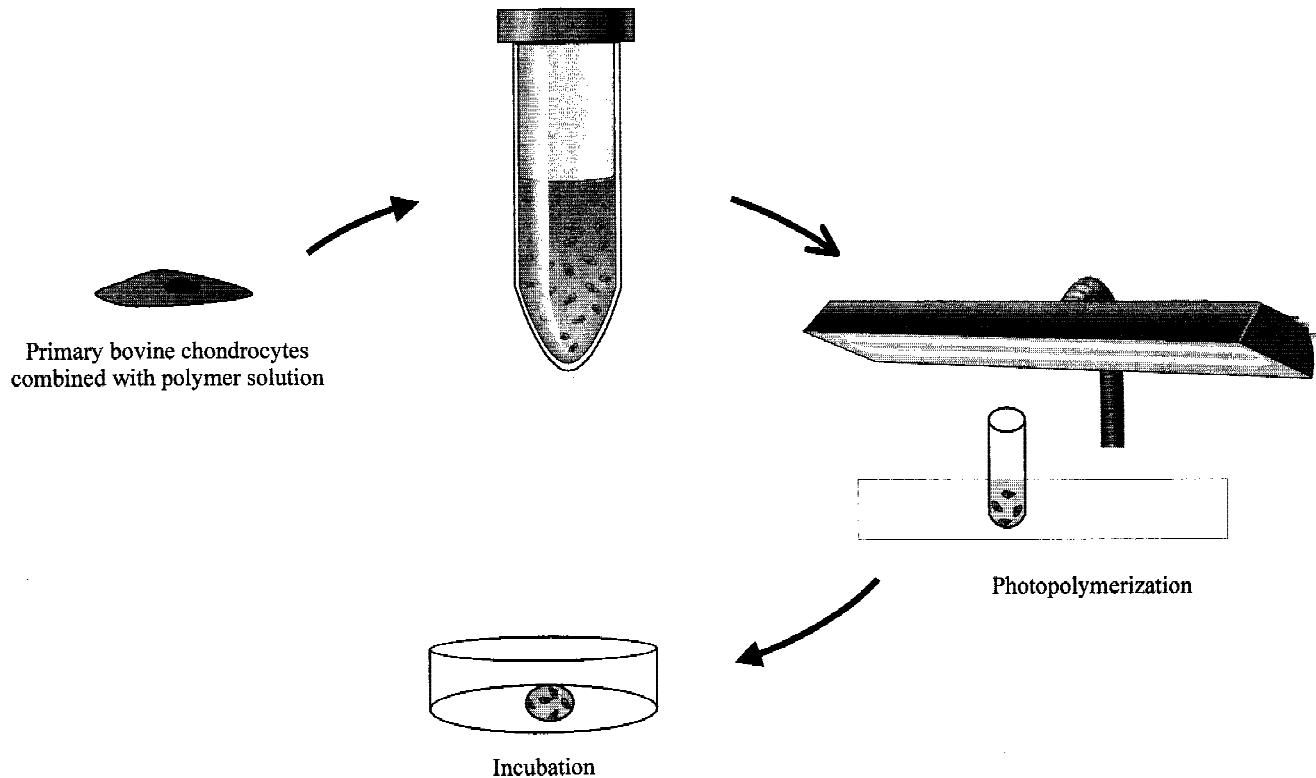
Hydrogels were analyzed by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide) (MTT) and light microscopy 1 day after chondrocyte encapsulation. Three milliliters of MTT (0.5 mg/mL in DMEM with 2% FBS) was added to hydrogels in 12-well plates. The hydrogels and MTT were incubated for 2 h. The MTT solution was removed and the hydrogels were rinsed twice with PBS and micrographs (×4.5 magnification) were obtained.

Biochemical analysis was performed on hydrogel constructs formed in Eppendorf tubes with bovine chondrocytes after 3, 7, 10, and 14 days of incubation. Hydrogels without cells were used as controls. Constructs were removed from the tissue culture plates and blotted dry, and wet weights (ww) were obtained. Gels were subsequently lyophilized for at least 48 h after which dry weights (dw) were determined. The hydrogels were digested in 1 mL papainase overnight at 60°C. GAG content was estimated by chondroitin sulfate using dimethylene blue dye and a UV-VIS spectrophotometer.<sup>9</sup> Total collagen content was determined by the hydroxyproline determined after acid hydrolysis and reaction with *p*-dimethylaminobenzaldehyde and chloramine-T using 0.1 as the ratio of hydroxyproline to collagen.<sup>10</sup> The cell content of the hydrogels was determined using Hoechst 33258, spectrofluorometry, and a conversion factor of 7.7 pg DNA/chondrocyte.<sup>11</sup> One-way analysis of variance (ANOVA) was performed using Minitab software. Results are presented as mean and standard deviation.

Histology was performed on hydrogels incubated for 2 weeks. The hydrogels were preserved in 10% formalin and prepared according to standard histological technique. Sections were stained with hematoxylin and eosin (H&E) and Safranin-O.

### Biomechanical characterization

Hydrogels were photopolymerized in tissue culture inserts with ovine chondrocytes for biomechanical analysis to



**Figure 1.** Experimental protocol. Cells isolated from bovine or ovine articular cartilage were mixed with poly(ethylene oxide)-dimethacrylate and poly(ethylene glycol) to a final concentration of  $50 \times 10^6$  cells/cc. Aliquots of the cell/polymer suspension were then placed under  $2 \text{ mW/cm}^2$  UVA light for 3 min and the resulting gels were incubated under static conditions.

form constructs in a disk shape. We prepared 40% PEODM and 100% PEODM control gels without cells the day before testing and allowed them to swell overnight in PBS. Hydrogel constructs with cells were incubated at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  and analyzed after 3 and 6 weeks of incubation. Six-millimeter-diameter disks were cored from the hydrogels that averaged approximately 7–8 mm in diameter. Thickness was measured by a current-sensing micrometer and was generally on the order of 1.5 mm.

Mechanical and electromechanical properties were measured in uniaxial confined compression in an apparatus described previously.<sup>3</sup> The equilibrium stress-strain behavior was assessed by stepwise compressions of 10%, 20%, and 30%, and load was recorded for 15 min. After stress relaxation reached equilibrium at the 30% static offset, a dynamic compression was superimposed at a frequency of 0.01–1 Hz. The resulting oscillatory load and streaming potential were detected.

## RESULTS

### Cell encapsulation

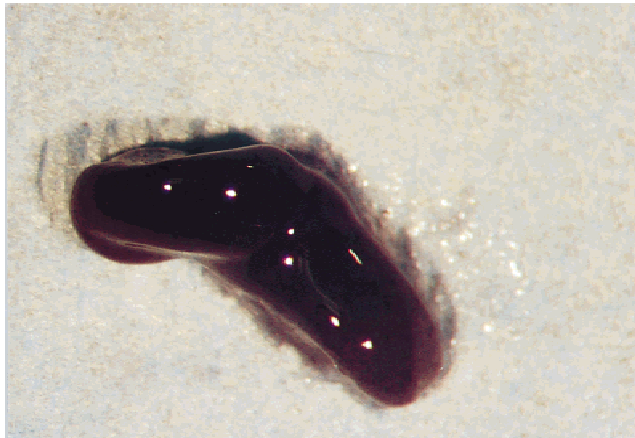
One day after photopolymerization, hydrogels grossly demonstrated a uniform distribution of viable cells as determined by MTT ( $n = 3$ ) [Fig. 2(a)]. Upon

further examination by light microscopy, a dispersion of cells within the hydrogel was observed [Fig. 2(b)]. Clusters of two to three cells were observed, although most cells were individually dispersed. Heterogeneous chondrocyte morphology was observed with both ovoid and elongated cells present.

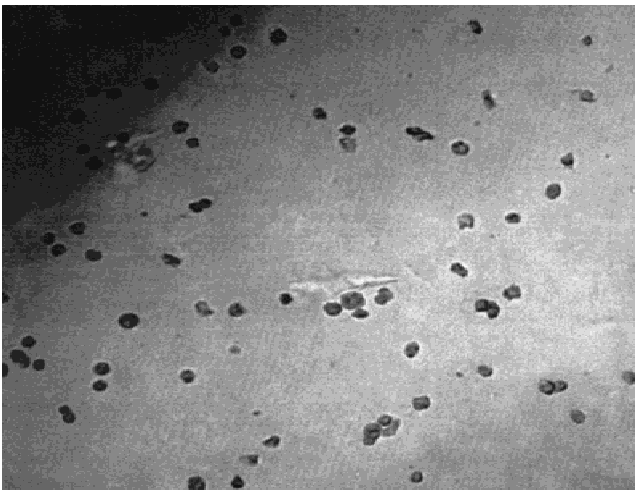
### Construct composition and structure

Bovine constructs exhibited increases in total collagen and GAG contents (% ww) after 3, 7, 10, and 14 days of static incubation ( $n = 4$ ) (Fig. 3). A statistically significant increase in collagen occurred on days 3–10 ( $p = .034$ ), with a maximum value of 0.39% after 14 days of incubation. GAG increases were statistically significant at all time points with a maximum value of 3.9% (ww) ( $p < 10^{-4}$ ) (Fig. 3). The ovine constructs showed lower matrix production than the bovine constructs. GAG and total collagen were  $0.87 \pm 0.36\%$ , and  $0.28 \pm 0.012\%$  ww, respectively at 2 weeks and  $1.32 \pm 0.29\%$  and  $0.44 \pm 0.086\%$  ww after 4 weeks' incubation.

Cell content of the bovine constructs decreased significantly from  $41,000 \pm 4000$  cells/mg ww at day 3 to  $22,000 \pm 8000$  cells/mg ww on day 14 ( $p = .043$ ) (Table 1). Ovine constructs, which had lower initial cell load-



(A)

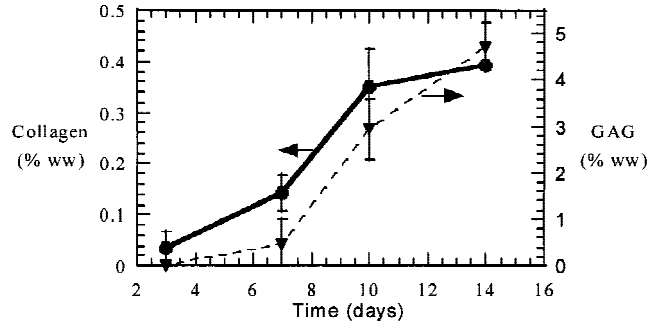


(B)

**Figure 2.** Encapsulation. One day after photoencapsulation: (A) MTT staining of gels demonstrated viable ovine chondrocytes (original magnification  $\times 4.5$ ), and (B) light microscopy of bovine cells dispersed in the gel with both ovoid and elongated morphology (original magnification  $\times 100$ ).

ing, showed substantial increases in cell number over 4 weeks. Ovine cell number increased from  $12,200 \pm 6700$  to  $63,700 \pm 6200$  cells/mg ww after 4 weeks of static incubation ( $p < 10^{-3}$ ) (Table I).

Histological analysis demonstrated a structure that resembled cartilage-like tissue [Fig. 4(a-d)]. Gels incubated for 2 weeks and stained with H&E demonstrate ovoid and elongated cells surrounded by a basophilic extracellular matrix [Fig. 4(a)]. Safranin-O-stained sections of 2-week specimens showed the distribution of negatively charged proteoglycans [Fig. 4(b-d)]. Grossly, cells were present in varying densities in the gel. Figure 4(b,c) shows regions of increased cell density surrounded by a matrix staining strongly for proteoglycans. Figure 4(d) demonstrates a region of the gel with both small clusters and individual cells. The



**Figure 3.** Biochemical analysis. Evolution of GAG and total collagen contents (% wet weight) over 14 days of photoencapsulated bovine chondrocytes.

pericellular region of individual cells and small clusters of cells stained for proteoglycans.

**Biomechanical analysis**

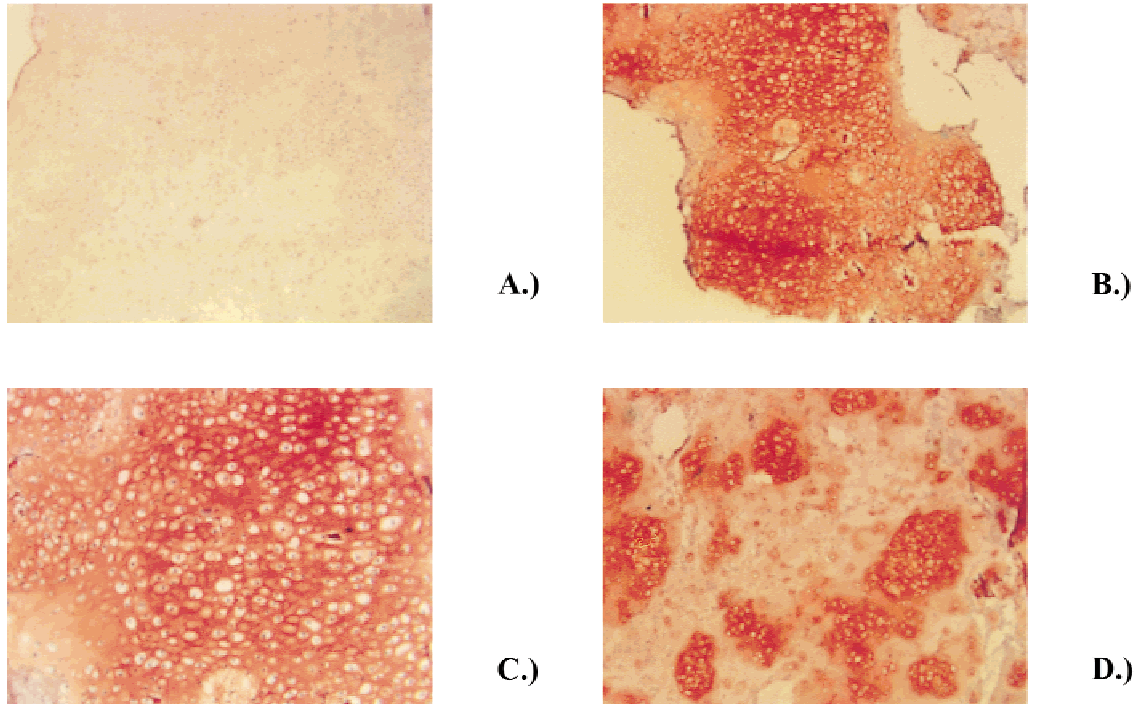
Construct mechanical and electromechanical properties increased with time. Ovine chondrocytes encapsulated in gels had significantly lower mechanical and electromechanical properties than natural cartilage, generally on the order of one magnitude below native cartilage ( $n = 4$ ) (Fig. 5). The equilibrium modulus increased steadily from an initial (40% PEODM control) value of  $<1$  kPa to over 70 kPa after 6 weeks of static incubation [Fig. 5(a)]. Control hydrogels synthesized using 100% PEODM exhibited a higher static equilibrium modulus than the 40% PEODM semi-IPNs. The 100% and 40% PEODM gels showed similar properties in dynamic stiffness. The dynamic stiffness of the gels increased from 0.001 MPa in control gels to 0.1 and 1 MPa at 3 and 6 weeks, respectively [Fig. 5(b)]. The streaming potentials of the gels were 0.08, 0.01, and 0.03 mV/% after 0, 3, and 6 weeks of incubation [Fig. 5(c)].

**DISCUSSION**

Photopolymerization provides a fast, efficient method to convert a liquid to a gel *in situ* under ambient conditions.<sup>12</sup> Photopolymerization is used extensively in the dental community to form sealants on teeth and in caries and is being studied for use in minimally invasive surgical procedures including prevention of postsurgical tissue adhesions and resteno-

**TABLE I**  
Cell Content of Hydrogel/Chondrocyte Constructs Incubated Statically for 2 Weeks

Time (d)	Cell Content (cells/mg wet wt)
3	$41,000 \pm 4000$
7	$35,000 \pm 6000$
10	$32,000 \pm 6000$
14	$22,000 \pm 8000$



**Figure 4.** Tissue morphology. Histological cross sections of bovine hydrogel constructs after 2 weeks' incubation stained with (a) hematoxylin and eosin (original magnification  $\times 100$ ), (b) Safranin-O/Fast green demonstrating proteoglycan distribution in a region of cartilage-like tissue (original magnification  $\times 100$ ), (c) (original magnification  $\times 200$ ) and (d) a region of sparsely distributed cells (original magnification  $\times 200$ ).

sis after angioplasty.<sup>13,14</sup> Superior temporal and spatial control of gelation may be achieved with photopolymerization. The reaction or gelation process proceeds only when and where light is exposed to the photopolymerizing polymer solution. Increased control in spatial resolution with photopolymerization has led to research aiming toward materials or processing techniques that allow design of scaffold architecture on the micrometer or even nanoscale level.<sup>8</sup> This may allow formation of scaffolds that mimic more closely the natural *in vivo* environment of a cell, or promote specific behaviors to enhance tissue engineering.<sup>15</sup> The ability to encapsulate cells using a photopolymerization process would provide a method to efficiently place cells in a hydrogel system for tissue engineering or even drug delivery purposes.

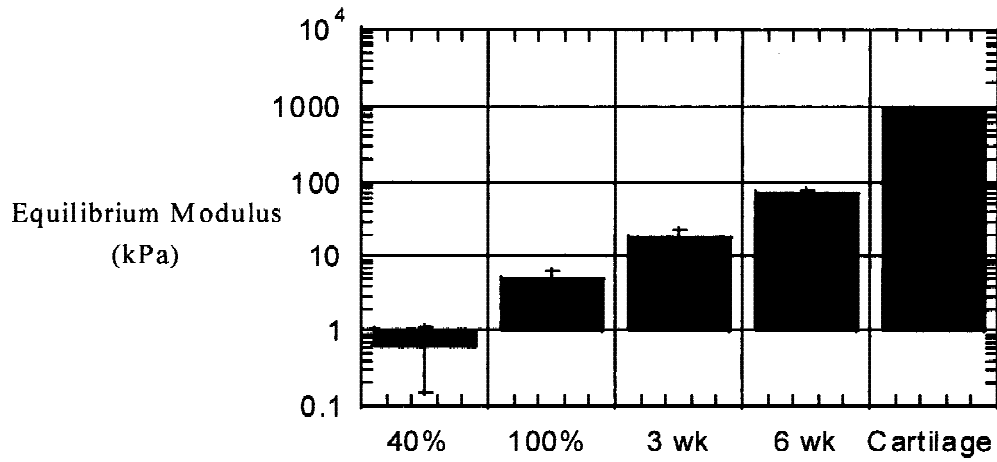
Successful photoencapsulation of cells requires a uniform cell suspension and mild photopolymerizing conditions. Low concentrations of photoinitiator and low-intensity UVA light were used for the encapsulation. These conditions were determined previously to be cell compatible yet still allow photopolymerization to proceed efficiently.<sup>6,7</sup>

Semi-interpenetrating networks, gels that contain both crosslinked and noncrosslinked polymer, were used for photoencapsulation. Gels made from 40:60 PEODM:PEO were used for photoencapsulation of chondrocytes. Through the addition of PEODM to the PEO system, a covalently crosslinking network may

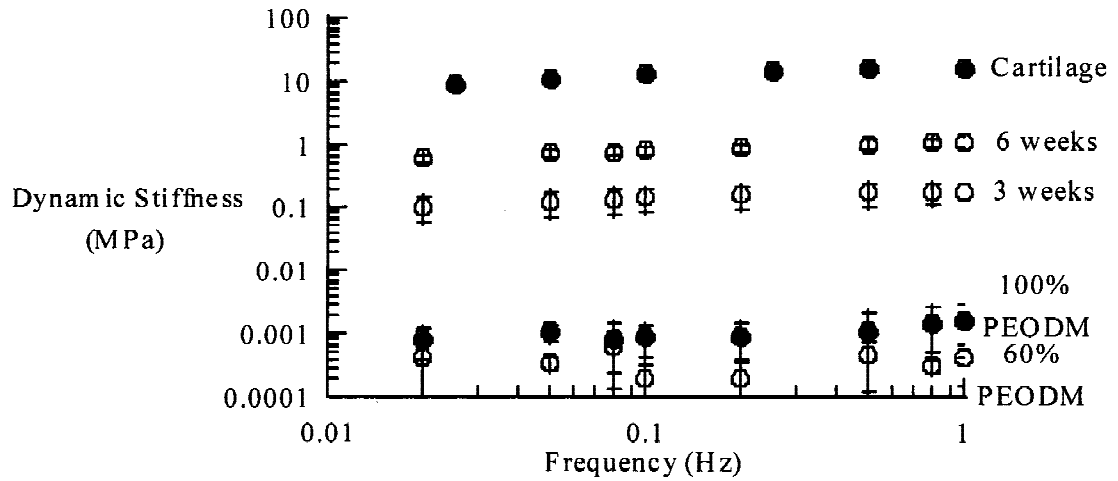
be formed. Increasing the PEODM content of the semi-IPN increases the crosslinking density or mechanical strength of the gel. The increase in crosslinking density may be observed in both the decreased swelling of the hydrogels previously determined and the enhanced biomechanical properties found in this study (Fig. 5).<sup>6</sup> PEODM:PEO semi-IPNs provide some mechanical strength with PEODM that is covalently crosslinked, yet the uncrosslinked PEO entrapped within the network allows nutrient and waste transport and space for tissue development. This polymer combination was used as a model system for studying photoencapsulation of chondrocytes because of the biocompatibility of PEO with chondrocytes.<sup>16</sup> Other photoreactive polymers or polymer/initiator systems designed to influence cell movement or tissue formation, or with tailored degradative properties could potentially be used.

Cartilage is composed of proteoglycans and collagen secreted by chondrocytes to form a functional, organized extracellular matrix. Chondrocytes react to both the biochemical and biomechanical signals provided *in vivo* to stimulate either matrix synthesis or degradation. Whereas proteoglycan and collagen concentration and organization is heterogenous in native cartilage, measuring these matrix components provides information regarding the proclivity of the chondrocytes to form cartilage-like tissue within a particular scaffold or encapsulation system. The concentra-

A.)



B.)



C.)

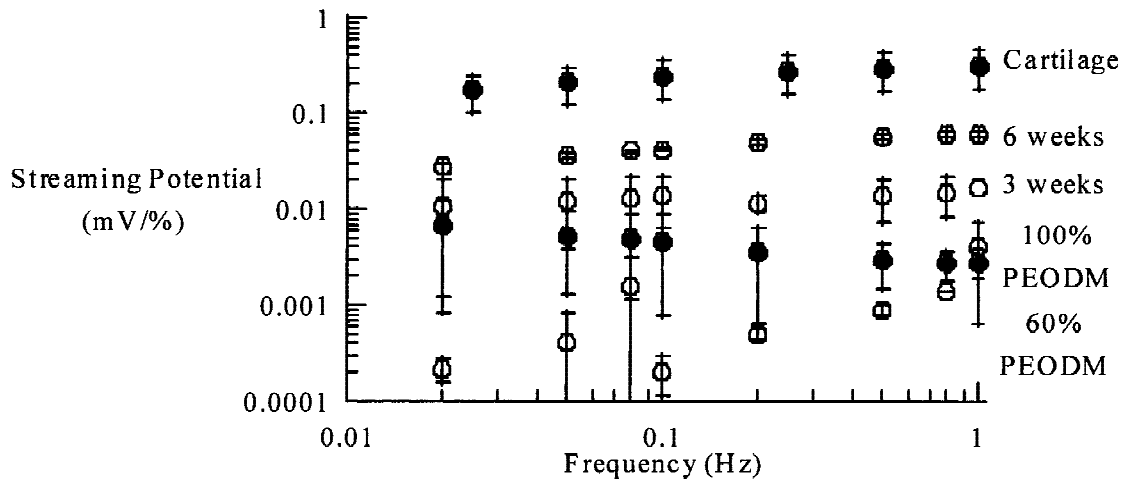


Figure 5. Biomechanics. (a) Equilibrium confined compression moduli, (b) dynamic stiffness, and (c) streaming potential of gels without cells and gels with ovine chondrocytes after 3 and 6 weeks of incubation.

tion of both proteoglycans and collagen increased with incubation time. Although the concentration of GAG in the gels approached physiological levels, the concentration of collagen was below other tissue-engineered cartilage.<sup>17</sup> Injection of the photopolymerizing gel system *in vivo* may provide the biochemical and mechanical signals necessary for matrix production and further enhance matrix production and organization. Modification of the photoencapsulating polymer may also help increase collagen concentrations.

Biochemical composition of the gels was studied at 3, 7, 10, and 14 days to learn the early matrix production capabilities of the gel/chondrocyte constructs. Initial matrix deposition is crucial to the survival and functionality of the constructs, both *in vitro* where scaffolds may be degrading and providing less support, and *in vivo* where harsh mechanical forces are present. It may be that with longer incubation times further matrix will be produced and secreted. PGA scaffolds incubated *in vitro* showed continual increased matrix production and organization for over 6 months of *in vitro* incubation.<sup>17</sup>

The histology sections of the 2 week hydrogels constructs also demonstrated extracellular matrix production along with interesting morphological features (Fig. 4). The safranin-O/Fast green staining showed clearly large regions of cartilage-like tissue, which appeared to be hypercellular and secreting large amounts of proteoglycans. On the other hand, there were also some regions with small clusters and individual cells staining for proteoglycans. The sparsely distributed cells appeared viable and secreted matrix. Eventually, these clusters and individual cells may continue to grow and coalesce to form a uniform matrix with further incubation depending on factors including the cell type, state of differentiation, and initial cell loading. The high viscosity of the polymer solution before gelation or the gelation process itself may be responsible for the variations in cell density within the gel constructs.

Comparison of the ovine and bovine constructs demonstrates the importance of cell source and cell biology. Both constructs have similar collagen contents, but the bovine constructs have significantly more GAG after shorter incubation. On the other hand, the ovine constructs show large increases in cell content with a fivefold increase from day 1 to 4 weeks. The ovine chondrocytes are proliferating yet not producing large amounts of extracellular matrix. These results suggest that the ovine cells are dedifferentiating. The bovine chondrocytes may be more differentiated with their lack of proliferation and higher GAG production. Further studies may focus on tailoring the gel environment to better control cell proliferation and matrix production.

The mechanical properties of tissue engineered con-

structs provides information on the functional and matrix properties of the tissue. It has been demonstrated that the concentrations of GAG, collagen, and water in cartilage are related to the physical properties of the tissue. For example, it has been reported that the equilibrium modulus increases with increasing GAG and decreasing water content.<sup>18</sup> The dynamic stiffness and streaming potential provide information regarding the total matrix deposition and quality.<sup>18</sup> The increase in dynamic stiffness and streaming potential with frequency is caused by increasing fluid velocity in the constructs as higher compression rates are applied.<sup>3</sup> In the case of dynamic stiffness, the observed increase in dynamic stiffness of the gels with time is due to the increased frictional interaction between the interstitial fluid and matrix. The streaming potential is caused by the flow of fluid past immobilized fixed charge. The majority of fixed charged (at physiological pH) is found on GAG. The increasing streaming potential implies that GAG is being functionally immobilized into a matrix in the gels.<sup>3</sup> The increase in the equilibrium modulus, dynamic stiffness, and streaming potential with time suggests that the encapsulated chondrocytes synthesized functional extracellular matrix (Fig. 5). Residual polymer present may also contribute to the mechanical properties observed. The photoencapsulated chondrocytes demonstrated mechanical properties approximately one order of magnitude below native bovine cartilage (Fig. 5). However, the equilibrium modulus, dynamic stiffness, and streaming potential of the photopolymerized gel constructs were comparable to tissue-engineered cartilage using PGA incubated under static conditions.<sup>19</sup>

The authors thank Dr. Gordana Vunjak-Novakovic for critical review of the manuscript, Dr. Ivan Martin for helpful discussions, and Dr. Alan Grodzinsky for use of biomechanical equipment.

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