

Crosslinking Density Influences Chondrocyte Metabolism in Dynamically Loaded Photocrosslinked Poly(ethylene glycol) Hydrogels

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(Received 3 July 2003; accepted 25 November 2003)

Abstract—In approaches to tissue engineer articular cartilage, an important consideration for *in situ* forming cell carriers is the impact of mechanical loading on the cell composite structure and function. Photopolymerized hydrogel scaffolds based on poly(ethylene glycol) (PEG) may be synthesized with a range of crosslinking densities and corresponding macroscopic properties. This study tests the hypothesis that changes in the hydrogel crosslinking density influences the metabolic response of encapsulated chondrocytes to an applied load. PEG hydrogels were formulated with two crosslinking densities that resulted in gel compressive moduli ranging from 60 to 670 kPa. When chondrocytes were encapsulated in these PEG gels, an increase in crosslinking density resulted in an inhibition in cell proliferation and proteoglycan synthesis. Moreover, when the gels were dynamically loaded for 48 h in unconfined compression with compressive strains oscillating from 0 to 15% at a frequency of 1 Hz, cell proliferation and proteoglycan synthesis were affected in a crosslinking-density-dependent manner. Cell proliferation was inhibited in both crosslinked gels, but was greater in the highly crosslinked gel. In contrast, dynamic loading did not influence proteoglycan synthesis in the loosely crosslinked gel, but a marked decrease in proteoglycan production was observed in the highly crosslinked gel. In summary, changes in PEG hydrogel properties greatly affect how chondrocytes respond to an applied dynamic load.

Keywords—Cartilage, Tissue engineering, Photopolymerization, Dynamic loading, Hydrogel properties, Crosslinking density.

INTRODUCTION

Cartilage, unlike other tissues, has a limited ability to self-repair upon injury. This issue has led to the development of a plethora of cell carriers for regenerating cartilage tissue. *In situ* forming carriers are especially advantageous, because cells can be entrapped uniformly throughout the scaffold⁴; the cell solution contours to the irregularly shaped defect prior to gelling¹⁸; and the surgical procedure is minimally invasive.¹³ However, cell scaffolds that

are formed *in situ* in a load-bearing joint will immediately experience mechanical loading, and it is important to understand how cells embedded in these scaffolds will sense and respond to physiologic loads.

During daily activities, the synovial joint is routinely subjected to static and dynamic loads. It is well known that joint immobilization and joint overloading result in degradation of cartilage to a mechanically inferior tissue.^{26,44,48} However, moderate activity is essential to maintain a functional tissue. These phenomena may be recapitulated *ex vivo*. For example, the application of a static unconfined compressive strain to cartilage explants *in vitro* induced an inhibition in proteoglycan and total protein synthesis.⁹ However, an oscillatory compressive strain influenced proteoglycan synthesis in a frequency-dependent manner. Low frequencies (≤ 0.001 Hz) inhibited proteoglycan synthesis, while higher frequencies (0.01–1 Hz) stimulated proteoglycan synthesis.⁴⁹

Chondrocytes sense mechanical loading through a variety of complex changes that occur in the tissue environment. These changes include cell deformation, streaming potentials, fluid flows and changes in hydrostatic pressure, pH, and osmolality.^{3,21,23} To isolate the complex changes that occur in native cartilage during mechanical loading, several investigators have embedded isolated chondrocytes in agarose, a neutral hydrogel. This model system provides a method in which to examine cell deformation in the absence of the complex processes that occur *in vivo*.^{19,31,32} For example, chondrocytes embedded in agarose hydrogels were found to deform to a similar degree as the global applied strain on the cell-agarose constructs.³² When the constructs were subjected to mechanical loads, static compressive strains inhibited chondrocyte proteoglycan synthesis, while dynamic compressive strains at a frequency of 1 Hz resulted in an upregulation of proteoglycan synthesis.³⁴ Nitric oxide, an inter- and intracellular messenger molecule, has been implicated as a constituent in the mechanotransduction pathways that influence chondrocyte proliferation in agarose gels.³⁶ The equilibrium aggregate modulus of the

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cell-agarose constructs increased sixfold when an intermittent oscillating strain was applied over 28 days.⁴⁰ Although agarose maintains the chondrocyte phenotype and chondrocyte metabolism,^{2,10,35} limitations exist with respect to the gel's ability to function as a suitable cell scaffold for cartilage tissue engineering. The shortcomings of agarose include the limited control over the gelation process and final gel mechanics, as well as the minimal degradation that occurs *in vitro* and *in vivo*.^{10,47}

To serve as an *in situ* forming chondrocyte carrier, we are particularly interested in synthetic hydrogels that are photopolymerizable. Synthetic gels provide the unique advantage of greater control over the macroscopic gel properties and scaffold degradation, while photopolymerization allows *in situ* gelation with greater control, both temporally and spatially, over the polymerization reaction. Photopolymerization is a process that utilizes light to convert a liquid macromer (or prepolymer) solution to a solid gel under physiological temperature and pH. By careful selection of photoinitiating conditions, this process can occur under mild and cytocompatible conditions.⁷ The macroscopic gel properties can be tailored by varying the macromer molecular weight, functionality, and concentration in solution prior to polymerization.^{39,42} Furthermore, the degradation can be controlled by incorporating a variety of degradable chemistries into the macromer backbone.⁵⁰

Previous work^{6,14} demonstrated that photocross-linkable hydrogels formulated from poly(ethylene glycol) (PEG) macromers provide a synthetic matrix which maintains chondrocyte viability and promotes deposition of extracellular matrix rich in proteoglycans and type II collagen. The present study tests the hypothesis that changes in the hydrogel crosslinking density influence the metabolic response of encapsulated chondrocytes to an applied load. Accordingly, chondrocytes were photoencapsulated in PEG hydrogels with two different crosslinking densities, in which the static and dynamic properties were fully characterized. Dynamic compressive strain was then applied to the cell-hydrogel constructs, and the response of the chondrocytes as a function of their local environment and loading was assessed by measuring nitrite production, cell proliferation, and proteoglycan synthesis after 48 h.

MATERIALS AND METHODS

Chondrocyte Isolation

Full-depth articular cartilage was excised from the proximal surface of the metacarpal-phalangeal joint in 18-month-old steers (four animals were used for a total of 8 feet) and washed in Earle's balanced salt solution (EBSS, Gibco, Paisley, Scotland). The cartilage slices were diced finely and incubated at 37°C for 1 h in Dulbecco's minimal essential medium supplemented with 20% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 20 mM HEPES, 100 unit ml⁻¹

penicillin, 100 μg ml⁻¹ streptomycin, and 150 μg ml⁻¹ L-ascorbic acid (DMEM + 20% FCS, all Gibco, Paisley, UK) + 700 unit ml⁻¹ pronase (BDH Ltd., Poole, UK) and for 16 h at 37°C in DMEM + 20% FCS + 100 unit ml⁻¹ collagenase type IX (Sigma, Poole, UK). The supernatant, containing released chondrocytes, was passed through a 70-μm pore size sieve (Falcon, Oxford, UK), washed twice in DMEM + 20% FCS, and finally resuspended in DMEM + 20% FCS. Cell viability was determined using the trypan-blue exclusion test. Isolated chondrocytes from different animals were thoroughly mixed prior to encapsulation.

Construct Preparation

Poly(ethylene glycol) dimethacrylate (PEGDM) was synthesized from PEG (3000 MW, Fluka, USA). PEG was dissolved in methylene chloride and reacted with an excess of methacryloyl chloride and triethylamine under argon atmosphere for 12 h at 4°C and an additional 24 h at room temperature. The resulting PEGDM was precipitated in ethyl ether three times and excess ether removed under vacuum. ¹H NMR (Varian VYR-500S) was used to assess purity and to determine that the degree of methacrylation of the PEG endgroups approached 100%. Specifically, the area under the integrals for the vinyl resonances ($\delta = 5.7$ ppm, $\delta = 6.1$ ppm) was compared to that for the PEG backbone (methylene protons, $\delta = 4.4$ ppm). To prepare gels, the PEGDM macromer was first dissolved in sterile phosphate buffered saline (Gibco, Paisley, UK) or deionized water to a final concentration of 10 and 20% (w/w), respectively. These solvents were found to maintain the highest level of cell viability in their respective gel systems postencapsulation (unpublished observations). Cytocompatible photoinitiating conditions were employed using Irgacure 2959 (2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone, Ciba-Geigy) to a final concentration of 0.05% (w/w) and 0.0125% (w/w) for the 10 and 20% (w/w) macromer solution, respectively.⁷ Where stated, chondrocytes were combined with the macromer/initiator solution at a concentration of 50×10^6 cells ml⁻¹ and irradiated with 365-nm light (UVP, model XX-20) at an intensity of ~ 2 mW cm⁻² for 10 min.

Hydrogel Characterization

Cylindrical hydrogel constructs (4–5 mm in diameter and 5 mm in height) were fabricated from the PEGDM macromers for both swelling and mechanical characterization studies. The constructs were swollen to equilibrium in PBS at 37°C for at least 48 h, weighed to determine the equilibrium swollen mass, M_s , and lyophilized overnight to obtain the dry polymer mass, M_d , for each sample. The equilibrium volumetric swelling ratio (Q) was calculated

by the following relationship:

$$Q = 1 + \frac{\rho_{\text{pegdm}}}{\rho_{\text{solv}}} \left(\frac{M_s}{M_d} - 1 \right) \quad (1)$$

where ρ_{pegdm} and ρ_{solv} are the density of the PEGDM macromer and the solvent, respectively. The gel cross-linking density, ρ_x , was estimated from the equilibrium swelling data using a modified version of the Flory–Rehner equation neglecting chain ends¹⁷ and is given by the following equation:

$$\rho_x \frac{-1}{v} \left(\frac{\ln(1 - v_p) + v_p + \chi v_p^2}{\bar{v}_p^{1/3} - \frac{v_p}{2}} \right) \quad (2)$$

where \bar{v} is the specific volume of the solvent; v_p is the equilibrium polymer volume fraction (Q^{-1}); and χ is the solvent–polymer interaction parameter. The value of χ was previously determined to be 0.426 and found constant over a range of v_p values from 0.04 to 0.2 for PEG in water and PBS.⁴¹ The average mesh size, ξ , in the hydrogels was determined as described by Canal and Peppas¹²:

$$\xi = v_p^{1/3} l C_n^{1/2} n^{1/2} \quad (3)$$

where l is the bond length; C_n is the characteristic ratio of the polymer; and n is the number of bonds between the crosslinks calculated from the cross-linking density.

The tangent modulus of each hydrogel construct formulation was determined using a dynamic mechanical analyzer (DMA-7, Perkin Elmer) in unconfined compression with nonporous platens at a rate of 40–120 mN min⁻¹ at room temperature.⁵ Stress-relaxation tests and cyclic unconfined compression tests were performed using a material tester (MTS, BioNIX 100) also with nonporous platens. The hydrogel constructs were compressed at a constant strain rate (0.2 mm s⁻¹) up to 15% strain and subsequently maintained for a period of 1000 s, and the resulting force was measured as a function of time. Cyclical unconfined compression was applied to the PEG constructs. Each sample was subjected to a tare load of 0.0196 N to simulate the force of the loading pins described below in the cell-straining apparatus. An axial displacement of 15% with a sine waveform at a frequency of 1 Hz was applied, and the resulting force was measured as a function of time.

Cell Morphology

Chondrocytes were embedded in 3D rectangular PEG constructs (4 × 4 × 3 mm) and allowed to equilibrate for 24 h in DMEM + 20% FCS. The constructs were incubated for 1 h in DMEM + 20% FCS + 5 μM calcein AM, a cell-permeant esterase substrate, (Molecular Probes, Cambridge, UK). The constructs were mounted in a specially designed apparatus and placed on the stage of an inverted microscope (Nikon TE300, Kingston upon Thames, England) associated with a confocal laser scanning unit

(UltraView, Perkin Elmer, Cambridge, UK).³² This system was previously calibrated with fluorescent polymer spheres, with a nominal diameter of 10 μm, which had been seeded in agarose constructs.³⁰ A population of 50 cells per construct was selected randomly, and a horizontal scan was obtained through the center of each individual cell at 0 and 20% gross strain. A total of 200 cells from 4 separate constructs was examined. Cell diameters [full-width half-maximum] were measured parallel (x) and perpendicular (y) to the direction of the applied strain for each cell. The cell deformation was quantified by the following equation:^{19,32,35}

$$\text{Diameter ratio} = \frac{x}{y} \quad (4)$$

Dynamic Loading

A well-characterized cell-strain system³³ (Zwick-Roell, Leominster, UK), incorporated into a standard tissue culture incubator (Hereaus, Brentwood, UK), was employed to apply dynamic compression to the PEG hydrogel constructs, as previously described. All cylindrical constructs (4 mm in diameter and 5 mm in height) containing 3 million cells per construct were allowed to equilibrate for 24 h in free swelling conditions prior to the application of strain. Twenty-four PEG constructs were transferred into a 24 well plate (Costar, High Wycombe, UK) and mounted within the apparatus. Impermeable loading pins (2 g in weight, 4 mm in diameter) rested on the constructs. This force resulted in a static strain of 2.8 and 1.3% for the 10 and 20% PEGDM gel systems, respectively. Five constructs serving as free swelling controls were placed in a separate 24 well plate. One milliliter of DMEM + 20% FCS supplemented with 1 μCi ml⁻¹ [³H]thymidine and 10 μCi ml⁻¹ ³⁵SO₄ (both Amersham Biosciences, Amersham, UK) was introduced into each well. Twelve of the twenty-four constructs in the cell-straining apparatus were subjected to dynamic compressive strain from 0 to 15% strain at 1 Hz, using a sinusoidal waveform. The remaining 12 constructs were cultured under identical conditions as the specimen placed in the loading apparatus (i.e., loading pins rested on the construct), but were not subjected to dynamic compression and served as controls for the dynamically loaded constructs. All constructs were cultured for an additional 48 h at 37°C and 5% CO₂. Two separate experiments were performed, providing a total of 24 dynamically compressed constructs, 24 controls within the cell-straining apparatus, and 10 free swelling constructs.

Biochemical Analysis

At the end of the 72 hour (24 + 48 hour) experiment, culture media were stored for subsequent biochemical analysis. Absolute concentrations of nitrite (μM), a stable end-product of nitric oxide metabolism, were measured in the media of cultured cells using a spectrophotometric method based on the Griess assay, as described previously.^{22,36}

Absorbance was measured at 550 nm, and nitrite (μM) was determined by comparison with standard solutions of sodium nitrite. The constructs were digested in phosphate buffered saline supplemented with 0.1 units ml^{-1} papain (Sigma, Poole, UK), 10 mM l-cysteine (Aldrich, Poole, UK) and 10 mM EDTA at pH 6.3 for 15 h at 60°C . [^3H]-Thymidine incorporation was measured in the papain digests by 10% (w/v) trichloroacetic acid precipitation onto filters using the Millipore Multiscreen system (Millipore, Watford, UK), as described previously.³⁴ $^{35}\text{SO}_4$ incorporation was determined in both medium and papain digests using the Alcian blue precipitation method as described by the authors.³⁶ The total glycosaminoglycan content in the constructs was measured using the dimethylmethylene blue dye method.¹⁶ Total DNA, determined using the Hoescht 33258 method,²⁹ was used to normalize nitrite production, $^{35}\text{SO}_4$ incorporation, [^3H]-thymidine incorporation, and glycosaminoglycan content.

Statistical Analysis

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

RESULTS

The PEGDM macromer concentration was varied from 10 to 20% (w/w) to create gels with different cross-linking densities that exhibit a range of macroscopic properties. A schematic of the polymerization process is shown in Fig. 1, and selected macroscopic properties are given in Table 1. A twofold increase in macromer concentration led to a threefold increase in the cross-linking density from 0.116 to 0.376 mol l^{-1} . Crosslinking density influences many of the gel macroscopic properties such as the volumetric equilib-

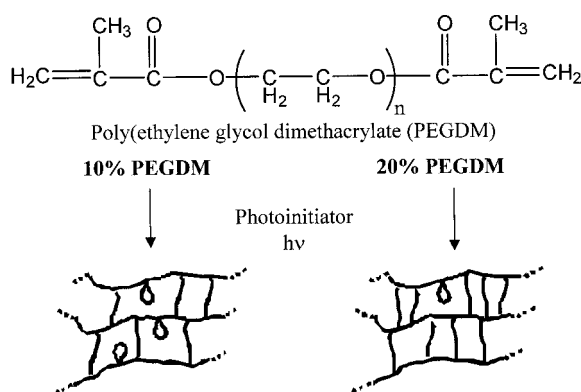


FIGURE 1. The formation of PEG hydrogels, formulated from 10 and 20% PEGDM macromer concentrations. The 20% PEGDM gel forms a more tightly crosslinked gel while the 10% PEGDM gel forms a more loosely crosslinked network because of non-idealities in the network formation.

TABLE 1. PEG hydrogel properties.^a

	ρ_x (mol l^{-1}) ^b	Q ^c	K ^d (kPa)	ξ ^e (\AA)
10% PEGDM	0.116 ± 0.001	11.3 ± 0.1	60 ± 3	160 ± 1
20% PEGDM	0.376 ± 0.033	6.5 ± 0.3	670 ± 120	80 ± 4

^aData are reported as mean \pm standard deviation.

^bCross-linking density.

^cEquilibrium volumetric swelling ratio.

^dTangential compressive modulus.

^eMesh size.

rium swelling ratio (Q), which is a measure of the amount of water the gel imbibes, the compressive modulus, and the mesh size (ξ), which is a measure of the average length between crosslinks and controls solute diffusion through the gel. This increase in crosslinking density resulted in a 42% decrease in Q , a 50% decrease in ξ , but a 1000% increase in compressive modulus.

To understand the behavior of these PEG hydrogels during mechanical loading, the stress-relaxation response of the two crosslinked hydrogels was measured in the absence of cells, and representative curves are shown in Fig. 2. A small degree of stress relaxation was observed in the crosslinked gels over a period of 1000 s. In the 10% gel, the stress decreased from 11.9 to 10.6 kPa, and in the 20% gel, the stress decreased from 136 to 122 kPa [Figs. 2(a) and 2(b)]. However, the overall crosslinked nature of the gels gives the network a dominating elastic component. Dynamic unconfined compression was applied to the hydrogels in the absence of cells, and maximum stress values of 8.4 and 120 kPa were recorded for the 10 and 20% gel formulations, respectively [Figs. 2(c) and 2(d)]. In both gels, the stress profile was symmetrical with no indication of lift-off between the indenter and the top surface of the construct [Figs. 2(c) and 2(d)].

Cell deformation was examined in unstrained and strained PEG gel constructs using confocal laser scanning microscopy.³² Representative micrographs, presented in Fig. 3, illustrate the deformation of a group of chondrocytes, embedded in the 10% gel at 0 and 20% strain. Upon the application of a static strain, the cells deformed to a more discoid shape. The mean diameter ratios of a population of cells embedded in unstrained and strained gels made up of the two cross-linking densities are presented in Table 2. In unstrained gels, the mean diameter ratios obtained from both the 10 and 20% gel constructs were indistinguishable and approached unity, suggesting a spherical morphology. Upon application of a 20% static compressive strain, the diameter ratios decreased significantly in both gel formulations, but the reduction was most marked in the 20% gel constructs (Table 2).

Total DNA content presented in Table 3 was 40–50% greater in the 10% PEG gels compared to the 20% PEG gels, but was unaffected by the experimental conditions. Nitric oxide production was assessed by measuring nitrite

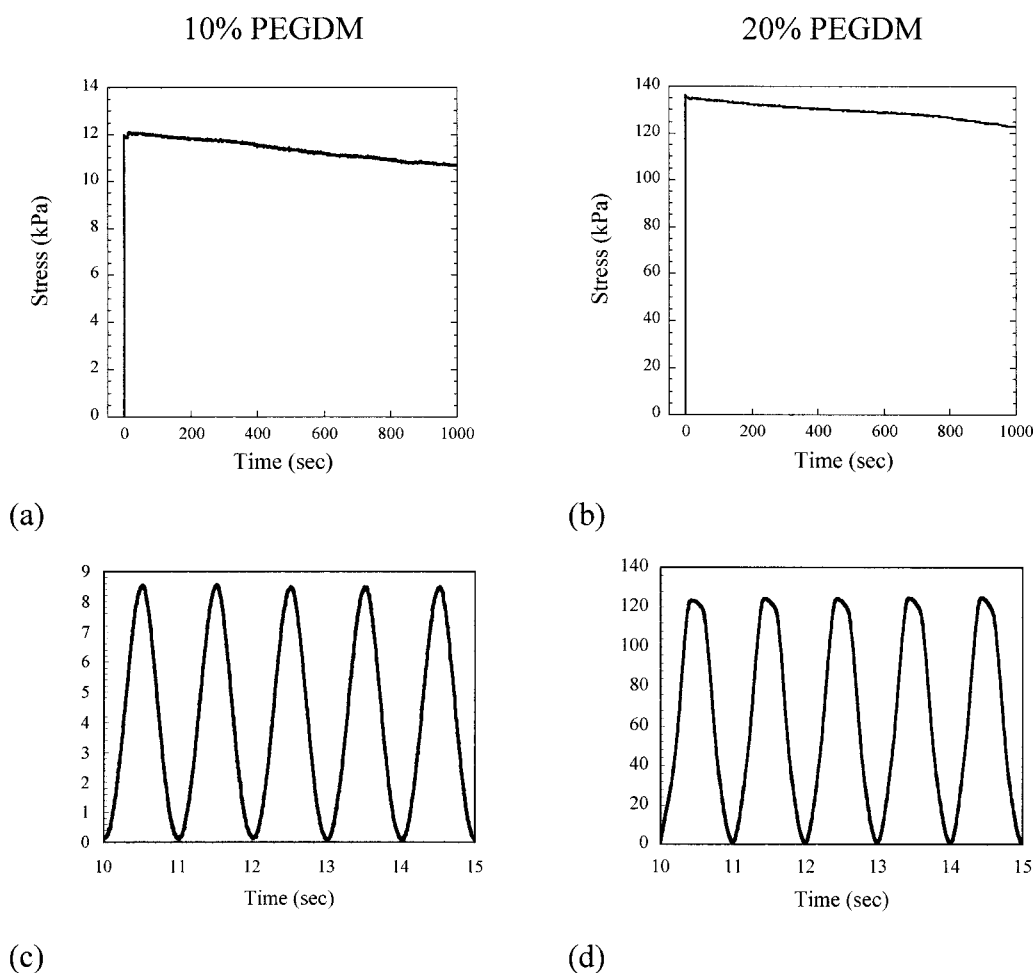


FIGURE 2. The stress-relaxation behavior (a) and (b) and the stress versus time response to a cyclical (1 Hz) axial compression at 15% compressive strains in a sinusoidal waveform (c) and (d) for PEG hydrogels formulated with 10 (a) and (c) and 20% (b) and (d) PEGDM.

release into the medium surrounding the constructs. The mean values for nitrite release, for each experimental group, are presented in Fig. 4. The values were similar for both the 10 and 20% gel constructs in the free swelling controls. However, when the gels were placed in the cell-straining apparatus, nitrite production decreased significantly in the 20% gel constructs compared to free swelling controls, but was unaffected in the 10% constructs [Fig. 4(a)]. Accordingly, nitrite release was significantly reduced in the 20% gels compared to 10% gels ($p < 0.001$). However, upon application of a dynamic strain, nitrite release was enhanced, although not significantly, within constructs prepared from both gel formulations [Fig. 4(b)]. [^3H]-Thymidine incorporation into newly synthesized DNA was used as a measure of cell proliferation. Figure 5 indicates that [^3H]-thymidine incorporation was significantly greater in the 10% gel constructs for all experimental conditions ($p < 0.05$). Upon the application of a dynamic strain, [^3H]-thymidine incorporation decreased by $26 \pm 5\%$ and $47 \pm 6\%$ in the 10 and 20% gel constructs, respectively, differences which were

significant at the 5% level [Fig. 5(b)]. Furthermore, [^3H]-thymidine incorporation was significantly inhibited in the dynamically loaded gels compared to the free swelling gels in both gel systems.

[^{35}S]-sulfate incorporation into newly synthesized sulfated glycosaminoglycans was used as a measure of proteoglycan synthesis. As illustrated in Fig. 6, [^{35}S]-sulfate incorporation was significantly greater in the 10% gel compared to the 20% gel for all experimental conditions. In the 20% gel constructs, [^{35}S]-sulfate incorporation was significantly reduced by the presence of the platens in the cell-strain system (in the absence of a dynamic load) when compared to the free swelling controls [$p < 0.05$, Fig. 6(a)]. Proteoglycan synthesis during dynamic strain was unaffected in the 10% gel constructs, but was significantly inhibited in the 20% gel constructs [Fig. 6(b)]. When compared to free swelling gels, proteoglycan synthesis was similarly unaffected in the 10% gels, but significantly reduced in the 20% gels under dynamically loading. Total glycosaminoglycan (GAG) content (Table 4) measured for the entire

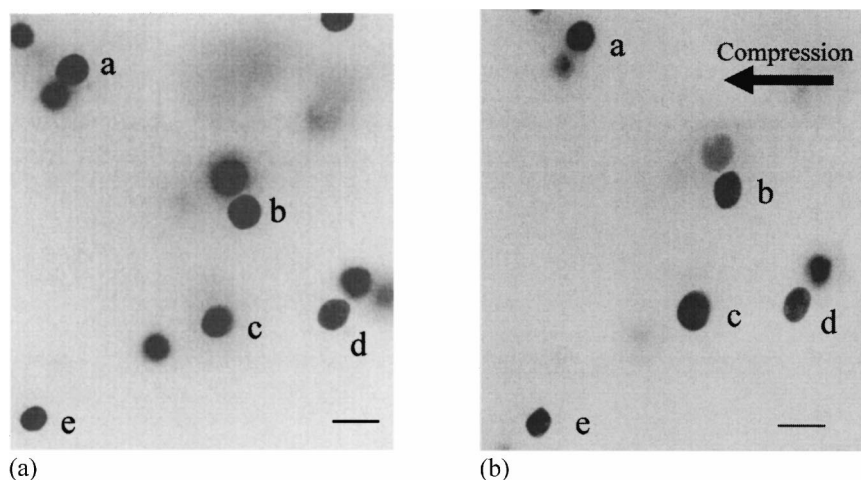


FIGURE 3. A representative group of chondrocytes embedded in a 10% PEGDM gel were followed as the gel was statically compressed from 0% gel strain (a) to 20% gel strain. Scale bar represents 17 μm . The letters correspond to the same cell at 0 and 20% gel strain.

72 h culture period demonstrated similar findings as the [^{35}S]-sulfate incorporation results. However for the 10% gels, the application of a dynamic load resulted in a significant 5% decrease in GAG content. In addition, [^{35}S]-sulfate was measured in the medium to determine the percent of newly synthesized proteoglycans released from the construct and found to be between 3.7 and 6.7% of the total [^{35}S]-sulfate incorporation (i.e., in the construct and released to the medium). The mean values were higher in the 20% PEG gel in all experimental groups [although only values for the control gels within the cell-strain apparatus were significant ($p < 0.001$)].

DISCUSSION

Hydrogels based on PEG are promising scaffolds for cartilage tissue engineering because the PEG chemistry is biocompatible,⁴⁶ is easily cleared from the body,⁴⁵

and maintains the chondrocyte phenotype.¹³ In addition, PEG can be readily modified with photoreactive and crosslinkable endgroups, such as methacrylates, to form 3D crosslinked networks. These networks are formed through a radical initiated chain polymerization in which polymethacrylate chains are connected via PEG crosslinks (Fig. 1). By fabricating hydrogels from macromer solutions of varying concentrations, networks with the same-gel chemistry, but with different structures, can be formed that span a wide range of crosslinking densities. The dependence of the final network crosslinking density on the initial macromer concentration is due, in part, to cyclization, which occurs with higher solvent concentrations. Cyclization lowers the crosslinking efficiency and leads to a more loosely crosslinked gel.¹⁵

For cartilage tissue engineering, the gel properties that are particularly important are the compressive modulus, equilibrium water content (equilibrium swelling ratio), and the mesh size. The tangent compressive modulus was varied by an order of magnitude by simply increasing the macromer concentration from 10 to 20% (w/w). When cells were present within the gel, the modulus was unaffected after 24 h of culture (as seen with the 20% gel) suggesting that the overall crosslinked nature of these elastic gels

TABLE 2. Diameter ratio of chondrocytes photoencapsulated in PEG hydrogels subjected to a 0 and 20% applied strain.^a

	Diameter ratio ^b	
	0% gel strain	20% gel strain
10% PEGDM	0.99 \pm 0.08	0.80 \pm 0.10 ^c
20% PEGDM	0.99 \pm 0.08	0.72 \pm 0.11 ^{c,d}

^aData are reported as mean \pm standard deviation.

^bDiameter ratio = X/Y where X and Y are the diameters in parallel and perpendicular to the applied strain, respectively.

^c $p < 0.001$ compared to 0% gel strain for the respective gel system ($n = 200$).

^d $p < 0.001$ compared to 10% PEGDM gels at 20% gel strain ($n = 200$).

TABLE 3. Total DNA content ($\mu\text{g/ml}$).^a

	Free swelling	Dynamically loaded	
		Control	1 Hz
10% PEGDM	13.8 \pm 0.7	14.2 \pm 0.5	14.9 \pm 0.3
20% PEGDM	10.0 \pm 0.2 ^b	9.6 \pm 0.4 ^b	10.0 \pm 0.2 ^b

^aData are reported as mean \pm standard error.

^b $p < 0.001$ compared to the respective 10% PEGDM gels.

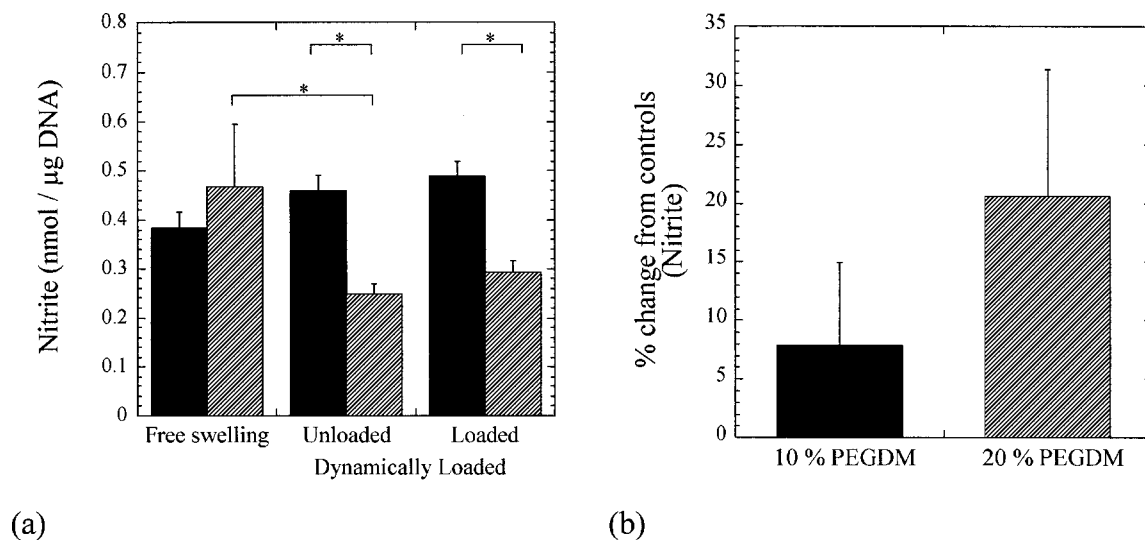


FIGURE 4. Nitric oxide production (as measured by nitrite) by chondrocytes embedded in PEG hydrogels. (a) Nitrite production for 10% (■) and 20% (▨) (w/w) PEGDM gels cultured in free swelling, control gels within the cell-strain apparatus and dynamically loaded (15% amplitude strain at 1 Hz) gels. The control gels experience a small static strain of 2.8 and 1.3% for the 10 and 20% gels, respectively, exerted by the loading pins. (b) The percent change of the dynamically loaded constructs from the control gels within the cell-strain apparatus. Data are given as the mean \pm standard error ($N = 24$). * $p < 0.05$.

dominate the gel modulus. The equilibrium swelling ratio and the mesh size decreased by a factor of 2 with an increase in crosslinking density. In engineering gel scaffolds that will be formed *in situ*, specifically in a load-bearing joint, a balance must be sought between networks that imbibe a minimum amount of water to facilitate nutrient diffusion and maintain cell viability with networks that possess reasonably good mechanical integrity to withstand physiological strains. Interestingly, the gel formulated with 20% (w/w) PEGDM macromer consisted of 84% water with a compressive modulus of 670 ± 120 kPa. These macroscopic hydrogel properties are within the range of

those reported for native articular cartilage (50–90% tissue fluid⁸ with an equilibrium compressive modulus of 500–1000 kPa).¹

In both crosslinked systems in the absence of cells, minimal stress relaxation was observed following the application of a 20% static strain, suggesting that these gels have a greater elastic, than viscous, component (Fig. 2). In the 10% gel, the stress decreased by 11% and similarly to 10% for the 20% gel over 1000 s. In contrast, native cartilage is highly viscoelastic with stress relaxations of 75% of the peak stress occurring in 1–5 s.⁴³ As a result of the elastic nature of these PEG hydrogels, lift-off between the platen

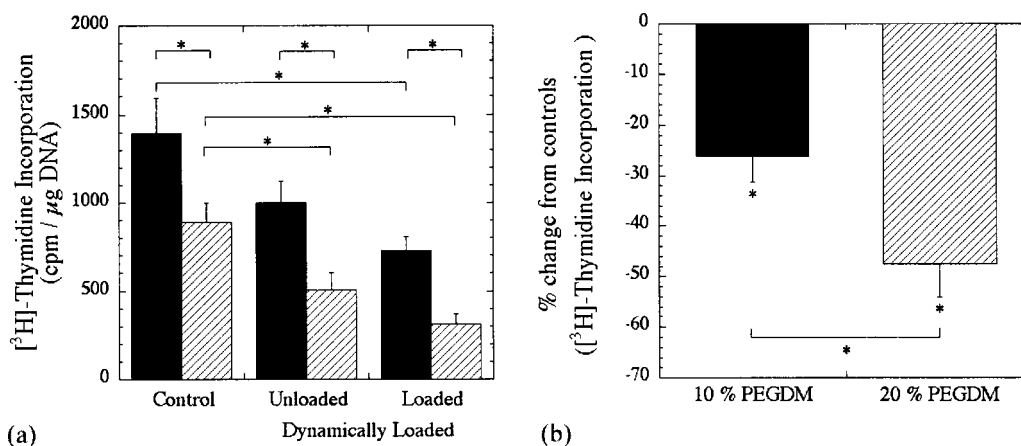


FIGURE 5. [³H]-Thymidine incorporation by chondrocytes embedded in PEG hydrogels. (a) [³H]-Thymidine incorporation for 10% (■) and 20% (▨) (w/w) PEGDM gels cultured in free swelling, control gels within the cell-strain apparatus and dynamically loaded (15% amplitude strain at 1 Hz) gels. The control gels experience a small static of 2.8 and 1.3% for the 10 and 20% gels, respectively, exerted by the loading pins. (b) The percent change of the dynamically loaded constructs from the control gels within the cell-strain apparatus. Data are given as the mean \pm standard error ($N = 24$). * $p < 0.05$, where the symbols above the bars in (b) illustrate statistically significant changes from control gels.

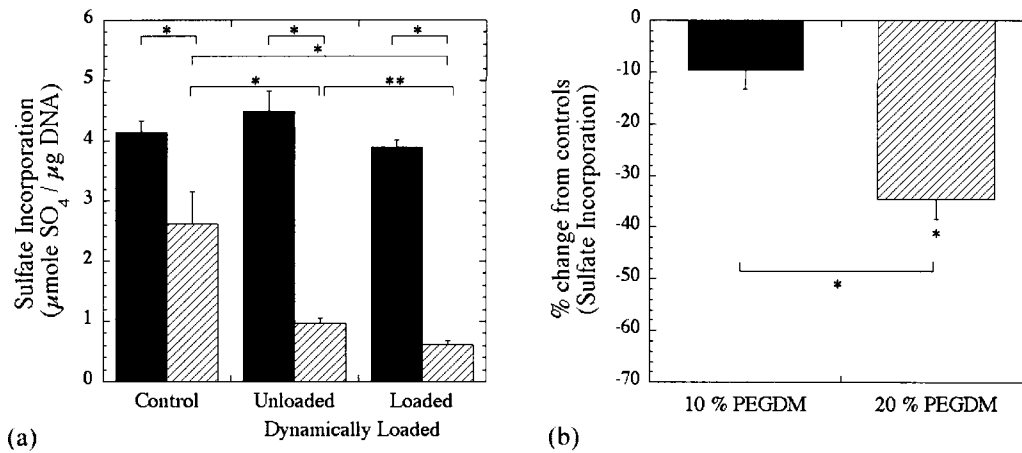


FIGURE 6. $^{35}\text{SO}_4$ incorporation by chondrocytes embedded in PEG hydrogels. (a) $^{35}\text{SO}_4$ incorporation for 10% (■) and 20% (▨) (w/w) PEGDM gels cultured in free swelling, control gels within the cell-straining apparatus, and dynamically loaded (15% amplitude strain at 1 Hz) gels. The control gels experience a small static strain of 2.8 and 1.3% for the 10 and 20% gels, respectively, exerted by the loading pins. (b) The percent change of the dynamically loaded constructs from the control gels within the cell-strain apparatus. Data are given as the mean \pm standard error ($N = 24$). * $p < 0.05$ and ** $p < 0.01$, where the symbols above the bars in (b) illustrate statistically significant changes from control gels.

and the hydrogel was not observed in either crosslinked system during dynamic loading. Lift-off occurs during cyclic loading when viscoelastic materials are unable to reach their original height, as dictated by the creep behavior, before the platen lowers again. Lift-off has been observed in agarose gels during unconfined, compressive strain amplitudes between 15 and 20%.^{38,40} Although these materials exhibit a different response compared to native cartilage or agarose, the highly elastic nature of the PEG gels may enable more efficient transfer of the applied strain to the cells during each cycle.

Since cell deformation is one process that is thought to be important in the mechanotransduction pathway, a static compressive strain of 20% was applied to the PEG constructs, and the morphology of the chondrocytes was assessed. The diameter ratios decreased significantly as the embedded chondrocytes adapted an oblate ellipsoid morphology (Fig. 3). The morphology of the deformed cells [Fig. 3(b)] appears to differ somewhat across the cells shown in the micrograph; this observation is discussed in detail

elsewhere (Bryant, Anseth, Lee and Bader, under review). This reduction in the diameter ratio was greater in the highly crosslinked gels (20% PEGDM) compared to the loosely crosslinked gels (10% PEGDM), and the mean diameter ratio was similar, in the 20% PEGDM gels, to that reported in agarose constructs.³² This finding suggests that the average local strain surrounding the cells is greater in the more tightly crosslinked gels because of the associated higher modulus.

In the free swelling state, an increase in cross-linking density resulted in a reduction in total DNA content, cell proliferation, and proteoglycan synthesis. This phenomenon may be due to changes in the environment surrounding the cells, potentially related to variations in mesh size and hydraulic permeability. A smaller mesh size may result in slower diffusion of growth factors through the gel that promote cell proliferation, but may also physically hinder cell expansion preventing the cells from entering into their proliferative phase. Chondrocytes within gels of higher cross-linking density exhibit decreased cell proliferation,

TABLE 4. Total glycosaminoglycan (GAG) content ($\mu\text{g GAG}/\mu\text{g DNA}$).^a

	Free swelling	Dynamically loaded		
		Control	1 Hz	% change
10% PEGDM	3.9 \pm 0.2	3.6 \pm 0.2	3.4 \pm 0.1	-5.0 \pm 1.6*
20% PEGDM	2.5 \pm 0.1 ^b	1.7 \pm 0.1 ^{b,c}	1.5 \pm 0.1 ^{b,c,d}	-13.1 \pm 3.0 ^{e**}

^aData are reported as mean \pm standard error.

^b $p < 0.001$ compared to the respective 10% PEGDM gels

^c $p < 0.001$ compared to free swelling gels for the 20% gels.

^d $p < 0.05$ compared to dynamically loaded controls.

^e $p < 0.05$ compared to the respective 10% PEGDM gels.

* $p < 0.05$; ** $p < 0.01$.

which is in agreement with the lower total DNA content. Accordingly an increase in crosslinking density may reduce the mass transfer of nutrients, metabolites and peptide mitogenic/morphogenetic factors in a manner similar to that reported for high concentration agarose and alginate gels. However, previous studies by the authors have shown that the glycosaminoglycan content was similar in 10 and 20% PEG gels after 2 and 4 weeks of culture.⁵ Therefore, it is possible that during the initial culture periods, a higher cross-linking density, and subsequently a smaller mesh size, delays the production of proteoglycans. Interestingly, the mean values of [³⁵S]-sulfate measured in the medium was greater in the 20% gels, suggesting that diffusion of newly synthesized proteoglycans may not be inhibited by the smaller mesh size. Larger proteins, such as growth factors, however may still be affected.

When compared to free swelling gels, the application of a small static load (<3%) to the constructs (in the control gels within the cell-straining apparatus) reduced cell proliferation in the 20% gels ($p < 0.05$) and in the 10%, although not significantly, and proteoglycan synthesis was inhibited in the 20% gels, but not in the 10% gels. It is possible that the impermeable platens impede nutrient diffusion into the gels. However, in a separate experiment where nutrient diffusion was restricted from the ends of the constructs, there were no gross differences in glycosaminoglycan accumulation in the radial direction as seen histologically. Previous studies examining nutrient diffusion of small solutes in compressed cartilage have shown that increasing the medium concentrations of proline and sulfate did not alter the inhibition of extracellular matrix synthesis during static loading.^{20,28} However, diffusion of larger macromolecules such as growth factors is likely to be impaired in the 20% gel because of the smaller mesh size and may affect the overall proteoglycan production and cell proliferation when diffusion is restricted.

Upon the application of a cyclic strain, chondrocytes embedded in the PEG hydrogels with two crosslinking densities responded in a distinct manner with regard to intracellular signaling events, proliferation, and ECM production. For example, levels of nitric oxide production, measured as nitrite release, were similar in both crosslinked gels in the absence of any applied strains, but on the application of a small static strain (as a result of the platens) and a dynamic strain, nitrite levels were greater in the more loosely crosslinked gels compared to the more tightly crosslinked gels. It has been suggested that nitric oxide plays an important role in the mechanotransduction pathway that influences cell proliferation.³⁶ In particular, chondrocytes seeded in agarose constructs responded to dynamic strains by a decrease in nitric oxide production, which correlated to a stimulation in cell proliferation. In this study, a similar, but opposite, trend was observed. Dynamic loading of the PEG constructs resulted in an increase in nitrite production, although not significantly, but an inhibition of cell prolif-

eration, which was statistically significant. Interestingly, an increase in crosslinking density resulted in a greater percent increase in the nitrite production [Fig. 4(b)], although not significant, and a greater percent decrease in cell proliferation in response to a cyclic load [Fig. 5(b)].

The application of a dynamic load did not alter the total DNA content, suggesting that the chondrocytes within these crosslinked PEG gels were not adversely affected. Cyclic loading did not influence proteoglycan synthesis in the low crosslinked gels, but did affect the total GAG content. An increase in crosslinking density resulted in a greater inhibition of proteoglycan synthesis (and similarly of total GAG content). In general, dynamic loading imparts increased fluid flow along the edge of the construct that is dependent on the frequency and the dynamic stiffness of the scaffold.²⁷ The two crosslinked gels examined in this study exhibit a range of stiffness, which may alter the fluid flow during compression and, subsequently, influence extracellular matrix formation.

These findings are contrary to those found in chondrocyte seeded agarose hydrogels that were cultured under identical loading conditions with similar adult bovine chondrocytes. In agarose gels, dynamic loading resulted in a downregulation of nitric oxide production, an upregulation in cell proliferation, and a frequency-dependent proteoglycan synthesis.³⁷ However, when juvenile bovine chondrocytes were encapsulated in fibrin glue gels that were subjected to an oscillatory compression for 20 days, a downregulation in cell proliferation, sulfated glycosaminoglycan synthesis, and collagen synthesis was observed.²⁵ In this study, a single dynamic amplitude and frequency were examined on the basis of the optimal loading conditions for chondrocytes in agarose gels; however, the chondrocytes' environment is very different in these PEG gels (i.e., the crosslinking mechanism, gel chemistry, and gel macroscopic properties are all different). The optimal loading conditions (i.e., amplitude and/or frequency) may depend on the gel type, and furthermore, in the case of the PEG gels, on the crosslinking density. There are numerous factors that can influence the cells' response to external signals, such as scaffold structure (i.e., porosity) and/or chemistry, cell seeding density, the time in culture at which the gel is subjected to mechanical loading, and the total loading time. Furthermore, during mechanical loading, a series of complex events occur that have been implicated in altering cell metabolism such as cell deformation, fluid flow, streaming potentials, and hydrostatic pressures.^{11,24,28,33} These complex processes will differ greatly with gel chemistry, crosslinking density, and the presence of elaborated tissue. For example, in this study we have shown that an increase in crosslinking density results in greater cell deformation during early culture times. Clearly, additional experiments are necessary to determine optimal loading conditions (amplitude, frequency, and duration) for chondrocytes embedded in PEG hydrogels as a function of crosslinking density.

Furthermore, we are interested in incorporating degradable crosslinks into the PEG gels and examining the influence of cyclic loading on the development of a macroscopic tissue as the gel resorbs.

In summary, this study demonstrates that chondrocytes respond to mechanical loads when encapsulated in PEG hydrogels through cell deformation and changes in cell function. This response is further mediated by changes in crosslinking density and suggests that determining optimum loading conditions may be scaffold (and scaffold structure) specific.

ACKNOWLEDGMENTS

The authors thank NIH for their support through a research Grant (R01 DE12998), and NSF and GAANN for fellowships to SJB. Support was also provided by the Engineering and Physical Sciences Research Council of Great Britain. The authors also thank the anonymous reviewers for their comments.

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