

Crosslinking density influences the morphology of chondrocytes photoencapsulated in PEG hydrogels during the application of compressive strain

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Abstract

Chondrocyte deformation, which occurs during mechanical loading, is thought to play an important role in the mechano-transduction pathway. In designing a scaffold that can be gelled in situ for cartilage tissue engineering, an important consideration is the influence of mechanical loading. This study tested the hypothesis that changes in the crosslinking density of a hydrogel scaffold influence the morphology of encapsulated chondrocytes in response to an applied load. Chondrocytes were entrapped in photo-crosslinkable hydrogel scaffolds based on poly(ethylene glycol) (PEG) with two crosslinking densities, 0.119 and 0.376 mol/l, with the higher density having a 11-fold higher compressive modulus. The cell-embedded hydrogels were subjected to static compressive strains between 0% and 20% after 1 and 6 days of culture. Using confocal laser scanning microscopy, chondrocytes in the highly crosslinked gel at day 1 deformed more than gels in the more loosely crosslinked gel. By day 6, this finding was reversed. When single cells within a region were followed, heterogeneities in cell deformation were observed on both a macroscopic and microscopic scale. These heterogeneities were greater in the highly crosslinked gel. These findings demonstrate that different levels of cell deformation and heterogeneity may be obtained by varying the crosslinking density in PEG hydrogels.

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Introduction

During normal activity, articular cartilage is subjected to mechanical loads that exert stresses of up to 20 MPa [23] and strains of 2–9% [14] within the tissue. Cartilage is mainly comprised of type II collagen fibrils intertwined with highly negatively charged proteoglycans in gel form, and this unique composition provides the tissue with its ability to withstand and distribute the applied loads. Mechanical loading is important in maintaining the functional composition of cartilage via the chondrocytes, which are responsible for the turnover

of newly synthesized functional matrix molecules. Specifically, the chondrocytes detect and respond to loading by metabolic alterations that are mediated through specific mechanotransduction pathways.

Mechanical loading produces complex changes within cartilage tissue through processes such as cell deformation, streaming potentials, and fluid flow with changes in pH, osmolality, and hydrostatic pressure. To understand the importance of mechanical loading in vivo, several investigators have examined the influence of loading on cartilage explants [4,22,24,34,37]. For example, chondrocytes in cartilage explants visualized by confocal laser scanning microscopy all deformed to a similar degree, parallel to an applied strain of 15% [20]. However, cell deformation varied with depth from the articular surface in line with the local tissue strain [22].

To isolate the related processes that occur in native cartilage during mechanical loading, various studies

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have utilized a model system comprising chondrocytes embedded in a neutral hydrogel prepared from agarose [19,26,29]. Cell deformation can be examined without the complex processes associated with deformation of a charged matrix that occur in vivo. Isolated chondrocytes embedded in an agarose hydrogel deform to a similar degree as the strain applied to the cell-agarose constructs [26]. In addition, during early stages of culture when matrix production is limited, chondrocytes respond to dynamic compressive strain, applied at a specific frequency of 1 Hz, by an upregulation of proteoglycan synthesis [30]. These data suggest that chondrocyte deformation plays an important role in mechanotransduction pathways.

Although agarose provides a 3D matrix, which maintains both chondrocyte phenotype and metabolism with time in culture [2,12,28], it has a limited ability to function as a suitable cell-scaffold for tissue engineering. For example, the gelation mechanism of agarose occurs via a temperature dependent conformational change in the polysaccharide chain and is difficult to control. The mechanical properties of agarose hydrogels are controlled to some degree by varying the agarose concentration, but are limited to values, which are less than 10% of those of native cartilage. In addition, in vitro degradation is reported to be minimal [12], although enzymatic degradation was observed in vivo [33].

Crosslinked poly(ethylene glycol) (PEG) hydrogels have been used as chondrocyte-carriers to generate successfully cartilaginous tissue in vitro and in vivo for tissue engineering [7–9,15–17]. Chondrocytes encapsulated in PEG hydrogels exhibit a range of properties (approaching that of native articular cartilage), without compromising cell viability and cell function [9]. However, the influence of mechanical loading on these tissue engineering cell-scaffolds has not been addressed. Since cell deformation is thought to play an important role in the mechanotransduction pathway, the objective of this study was to examine cell deformation in PEG hydrogels during the first 6 days in culture. In particular, this study tests the hypothesis that changes in hydrogel properties, in the form of the crosslinking density, influence the morphology of encapsulated chondrocytes in response to an applied load.

Materials and methods

The front feet of freshly slaughtered 18 month old steers were obtained from a local abattoir. Full depth articular cartilage was removed from the proximal joint surface of the metacarpal-phalangeal joint under aseptic conditions. The cartilage slices were washed in Earle's balanced salt solution (EBSS, Gibco, Paisley, UK), diced finely, and incubated at 37 °C for 1 h in Dulbecco's minimal essential medium supplemented with 20% (v/v) fetal calf serum, 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 number and viability were determined using a haemocytometer and trypan blue staining.

Poly(ethylene glycol) dimethacrylate (PEGDM, 3000 MW) was synthesized as previously described [35]. The PEGDM macromer was dissolved in methylene chloride and precipitated three times in ethyl ether to remove any contaminants. ¹H NMR was used to determine the methacrylation efficiency of ~100% and to confirm that no contaminants were present in the PEG macromer. The PEGDM macromer was dissolved in either sterile phosphate buffered saline (PBS, Gibco, Paisley, UK) or sterile deionized water to a final concentration of 10% and 20% (w/w), respectively. Previously determined cytocompatible photoinitiating conditions were employed using Irgacure 2959 (2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone, Ciba-Geigy) at a final concentration of 0.05% (w/w) and 0.0125% (w/w) for the 10% and 20% macromer solution, respectively [10]. Where applicable, chondrocytes were combined with the macromer/initiator solution to give a final concentration of 50 × 10⁶ cells ml⁻¹ and encapsulated using 365 nm light (UVP, model XX-20) at an intensity of ~2 mW cm⁻².

Cell free hydrogel constructs, 4–5 mm in diameter and 5 mm in height, formulated from the PEGDM macromer were allowed to swell in PBS at 37 °C for at least 48 h to reach equilibrium. After equilibration, the discs were weighed to determine the equilibrium swollen mass (M_s), then lyophilized to determine the dry (or polymer) mass (M_d). The equilibrium volumetric swelling ratio, Q , was calculated from the equilibrium mass swelling ratio (M_s/M_d) [31]. The crosslinking density, ρ_x , was estimated from Q using a modification of the Flory–Rehner equation neglecting chain ends [18].

The compressive moduli of the hydrogel constructs was measured using a dynamic mechanical analyzer (DMA-7, Perkin–Elmer, Shelton, CT, USA) in unconfined compression at a rate of 40–120 mN min⁻¹ at room temperature. Stress relaxation tests of the two hydrogel systems were performed using a materials tester (MTS, Bionix 100, Cirencester, UK). The hydrogel constructs were compressed at a constant rate of 0.2 mm s⁻¹ up to a strain of 20%, which was subsequently maintained for a period of one hour. The resulting force was measured as a function of time.

For each of the characterization procedures, a sample population of five constructs was used. Values are reported as the mean and standard deviation.

Cell-hydrogel constructs of size 4 × 4 × 3 mm³ were prepared as described above and cultured in DMEM + 20% FCS at 37 °C and 5% CO₂ in a humid environment for up to 6 days. Medium was replaced every 2 days. At days 1 and 6, constructs were removed and incubated in a 5 µM solution of calcein AM (Molecular Probes, Cambridge, UK) in DMEM for 1 h. Calcein AM is a cell-permeable fluorogenic esterase substrate that stains live cells. The specimens were mounted in a specially designed test rig [26] placed on the stage of an inverted microscope (Nikon TE300, Kingston upon Thames, UK) associated with a confocal laser scanning system (Perkin–Elmer, UltraView, Cambridge, UK). This system had been previously calibrated using fluorescent polymer spheres, with a nominal diameter of 10 µm, which had been seeded in agarose constructs [25]. Horizontal scans through the center of individual cells were obtained for a population of cells. The full-width, half-maximum (FWHM) diameter of each cell was measured parallel (x) and perpendicular (y) to the direction of the applied strain. The FWHM approach is an intensity adjusted procedure [27,28]. The deformation of each cell in the sample population was quantified in terms of a diameter ratio given by x/y [19,26,28]. A population of 50 cells per construct was randomly selected. A total of four constructs was used to determine the diameters and diameter ratios of chondrocytes in PEG gels at 0% and 20% strain.

In a separate experiment, a single cell approach was adopted [3,25,26] for day 1 constructs, in which approximately 10 cells were randomly selected from a region of ~0.6 mm² per construct. These cells were followed during compression from 0% to 20% strain in 10% strain increments. A total of six constructs were analyzed for a total of ~60 single cells. The cell strain, ϵ_x , was determined by $(X_u - X_s)/X_s$, where X_u is the x diameter of the unstrained cell and X_s is the x diameter of the strained cell.

On day 6, three 20% PEGDM cell constructs were removed from culture and incubated with a 2 ml solution of testicular hyaluronidase (Sigma, Poole, UK) at a concentration of 100 unit ml⁻¹ in DMEM + 20% FCS for 1 h at 37 °C. The hyaluronidase solutions were removed and each specimen was washed twice with 2 ml of EBSS. The specimens were subjected to a 20% static compressive strain to evaluate cell deformation. Approximately 40 cells per construct were analyzed for a total of 126 cells.

After 6 days in culture, two cell-hydrogel constructs from each system were fixed overnight with 4% (w/v) formaldehyde and subsequently processed to paraffin. Sections were prepared, dewaxed, and rehydrated. The sections were stained with hematoxylin and Safranin-O/fast green, which stain nuclei black and negatively charged glycosaminoglycans (GAG) red.

Statistical analysis

Statistical analysis was performed using an ANOVA. A two-factor ANOVA with a completely randomized design was used for the *x* and *y* diameters where the two factors were time and gel composition. A three-factor ANOVA with a completely randomized design was used for the diameter ratios where the three factors were time, gel composition, and gel strain. For the single cell approach, an ANOVA with repeated measures was used. All values are reported as the mean and standard error. A confidence level of 0.05 was considered statistically significant.

Results

By varying the concentration of PEGDM macromer in solution prior to polymerization, gels with different degrees of crosslinking density were obtained. A schematic of the crosslinking mechanism for gels prepared from 10 and 20 wt% PEGDM macromer concentrations and their corresponding gel properties is shown in Fig. 1. A 2-fold increase in the concentration of the multifunctional macromer leads to a statistically significant 3-fold increase in crosslinking density. The increased crosslinking density influences many of the hydrogel's macroscopic properties, including a 42% decrease in equilibrium swelling ratio, but an 11-fold increase in compressive modulus.

Stress values decreased from 214 to 203 kPa for the 20% gel constructs and from 13 to 9 kPa for the 10% gel constructs during the application of a 20% static strain for 1 h. These small degrees of stress relaxation reflect movement of polymer chains or fluid movement within the network. However, the overall crosslinked nature of the gels gives the network a dominating elastic component. To ensure no changes in cell morphology occurred during the course of the experiment, the cell diameters and diameter ratios were plotted as a function of time.

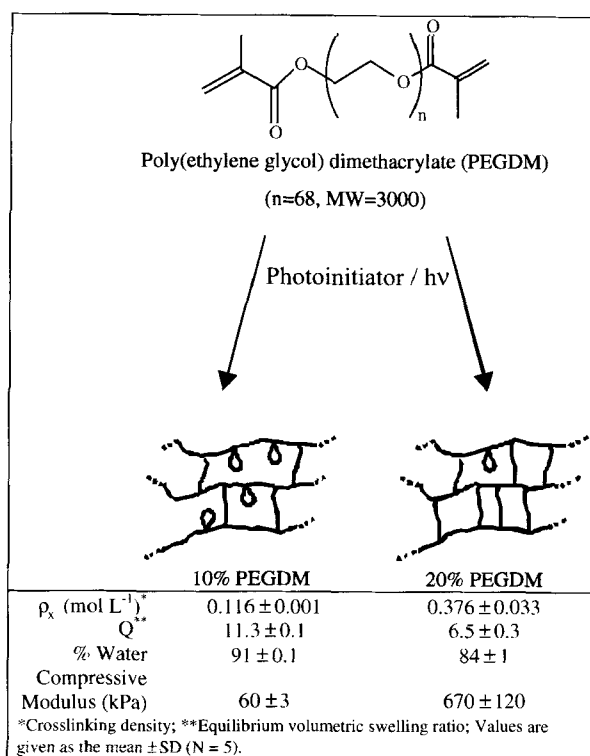


Fig. 1. A schematic of the formation of PEG hydrogels fabricated from 10% and 20% PEGDM macromer concentrations and their corresponding properties. The 20% PEGDM gel forms a more tightly crosslinked gel while the 10% PEGDM gel forms a more loosely crosslinked network.

For four specimens, the correlation coefficient, r^2 , varied from 0.005 to 0.16 ($p > 0.05$) for the 10% gel and from 0.02 to 0.14 ($p > 0.05$) for the 20% gel with no observed trends in the data. Therefore, the cell diameters and diameter ratios were not influenced by the small degree of stress relaxation observed in the PEG gels.

Cell diameters within both systems increased significantly with time in culture at a rate that varied between the two hydrogels (Table 1). When examining cell morphology as a function of the diameter ratio, the unstrained diameter ratio varied from 0.97 to 1.01 over the 6 days of culture, suggesting the cells retained a rounded morphology when embedded in both the 10% and 20% gel constructs. No statistically significant differences were observed between the unstrained diameter ratios in either of the PEGDM gels during the 6 day culture period. On application of a static 20% compressive

Table 1
Diameters and diameter ratios of chondrocytes encapsulated in PEG gels and subjected to 0% and 20% gel strain

% PEGDM	Day 1				Day 6			
	0% gel strain		20% gel strain		0% gel strain		20% gel strain	
	<i>x</i>	<i>y</i>	<i>x/y</i>	<i>x/y</i>	<i>x</i>	<i>y</i>	<i>x/y</i>	<i>x/y</i>
10	11.5 ± 0.09	11.6 ± 0.09	0.99 ± 0.005	0.80 ± 0.007	13.1 ± 0.14	13.0 ± 0.16	1.01 ± 0.006	0.85 ± 0.007
20	12.1 ± 0.11	12.2 ± 0.10	0.99 ± 0.006	0.72 ± 0.008	12.5 ± 0.12	12.9 ± 0.13	0.97 ± 0.005	0.89 ± 0.008

Values are given as the mean ± SE; N = 200 for each subpopulation at each time point.

strain, chondrocytes in both gel constructs deformed significantly, as reflected by the decrease in the diameter ratio. Comparisons between the two systems revealed that at day 1 chondrocytes in the 20% gel constructs deformed greater than cells in the 10% gel constructs, but by day 6 these differences were reversed.

The chondrocytes embedded in the PEG hydrogels were also examined histologically (Fig. 2), revealing that chondrocytes had synthesized a proteoglycanaceous pericellular matrix surrounding individual cells. A similar distribution was observed in both the 10% and 20% PEGDM gel constructs.

Using the single cell approach, 10 chondrocytes, located within a small region (chosen at random, but centrally located) of an individual construct, were followed during the application of compressive strain to the construct. A selection of micrographs of single cells (Fig. 3), revealed that cell deformation is heterogeneous,

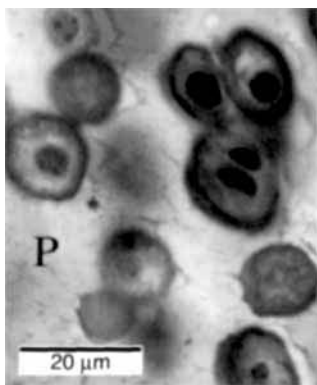


Fig. 2. A representative micrograph of a histological section of chondrocytes photoencapsulated in PEG hydrogels. Sections were stained using hematoxylin and Safranin-O, which stains nuclei black and proteoglycans red (shown in greyscale). This section is from a 10% PEGDM sample after 6 days of culture. The PEG polymer stains lightly and is indicated by P while the proteoglycans are dark grey. Original magnification was 600x.

with cell strain values ranging from 0% to 32% at an applied strain of 20%. When the single cell data were analyzed as a function of specimen or region, clear differences were revealed (Fig. 4). In the strained state, differences existed between the regions for both the 10% gels ($p = 0.04$) and the 20% gels ($p < 10^{-5}$). A stronger interaction between region and strain was found for the 20% gels ($p < 10^{-6}$) compared to the 10% gels ($p < 0.01$). Therefore, the differences, which existed between the regions, were more pronounced in the 20% gels.

Discussion

The poly(ethylene glycol) chemistry has a well established history in biomedical applications and is easily modified with photoreactive and crosslinkable endgroups, such as methacrylates or acrylates. Chondrocytes photoencapsulated in hydrogels formulated from PEG macromers maintain their viability and phenotype both in vitro and in vivo [7–9,15–17], and this environment promotes the formation of an extracellular matrix composed of type II collagen and proteoglycans. The crosslinking mechanism of the photopolymerized PEG gels occurs through a radical initiated chain propagation mechanism that produces kinetic chains with covalent PEG crosslinks, which entraps the chondrocytes uniformly through the gel [10]. By fabricating hydrogels from macromer solutions of varying concentrations, networks with the same gel chemistry can be formed that exhibit a wide range of crosslinking densities, ρ_x . Indeed, ρ_x influences macroscopic gel properties; for example, an increase in ρ_x results in a decrease in water content, but a substantial increase in compressive modulus (Fig. 1). However, for tissue engineering cartilage, a balance must be sought between maintaining a high water content for cell viability and exhibiting adequate mechanical integrity to restore function. By tailoring the crosslinking density of the gels, properties can

Cell No.	10% PEGDM				20% PEGDM			
	1	2	3	4	5	6	7	8
0% gel strain	●	●	●	●	●	●	●	●
10% gel strain	●	●	●	●	●	●	●	●
20% gel strain	●	●	●	●	●	●	●	●
% cell strain for 20% gel strain	19	20	0	11	20	32	1	4

Fig. 3. A selection of micrographs of chondrocytes photoencapsulated in 10% and 20% PEGDM gels subjected to 0%, 10%, and 20% gel strain. Each column represents the same single cell followed through the deformation of the gel.

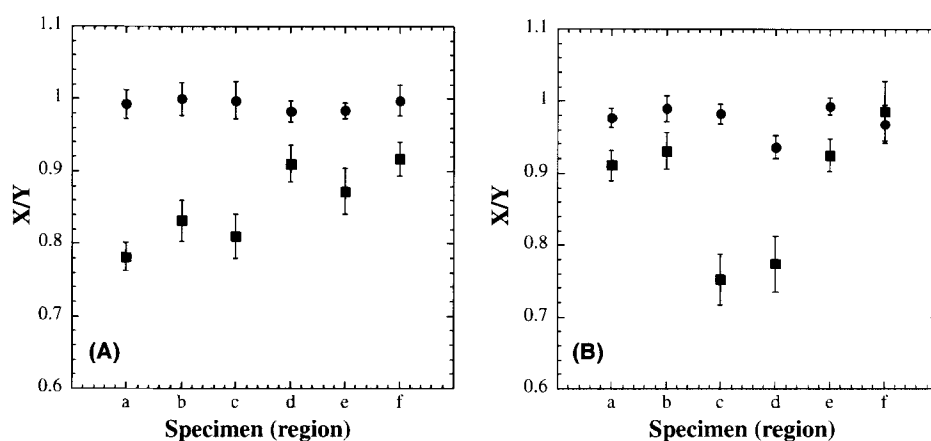


Fig. 4. Diameter ratios of chondrocytes photoencapsulated in PEG hydrogels formulated from (A) 10% and (B) 20% PEGDM macromer concentrations. Ten cells were analyzed per specimen to represent a *region* within the gel. (●) and (■) are the diameter ratios at 0% and 20% gel strain, respectively. Error bars represent SE.

be obtained that closely resemble native cartilage. The gel formulated with 20% PEGDM with a crosslinking density of $0.376 \pm 0.033 \text{ mol L}^{-1}$, contained 84% water and exhibited a compressive modulus of $670 \pm 120 \text{ kPa}$, similar to the $\sim 80\%$ water and modulus of 500–1000 kPa of articular cartilage [1,11].

In the present study, the x and y diameters of the isolated chondrocytes prior to embedding in the PEG hydrogels were 12.5 ± 0.2 and $12.5 \pm 0.2 \text{ } \mu\text{m}$ ($n = 11$), respectively. When subsequently embedded and cultured for 24 h, the diameters were similar for cells in the 20% PEGDM gels, but significantly smaller in the 10% PEGDM gels. This decrease suggests that the encapsulation process and/or the 24 h of subsequent culture caused a cell volume decrease in the more loosely crosslinked gel. Cell volume can vary due to changes in osmolality [13,36]. Certainly, changes in the macromer concentration prior to polymerization can influence the solution osmolality, which could induce relative changes in volume for cells seeded within the 10% and 20% gels. Further studies are necessary to confirm these hypotheses.

During 6 days of culture, the hydrogel cell constructs maintained their 3D shape and were firm to the touch. Previous studies have reported no statistically significant changes in the tangent modulus of cell laden 10% PEGDM gels during 1 week of culture [6]. The cell diameters increased significantly in both gel systems (Table 1), which may be a direct result of the culture-induced upregulation of cell metabolism, as evident from the presence of proteoglycans in the histological micrograph (Fig. 2). The increases in cell diameters were approximately 13% and 4.5% in the 10% and 20% PEGDM gels, respectively. These differences could be due to the fact that in the more highly crosslinked stiffer gel, the chondrocytes were less metabolically active and/or sterically impeded from increasing in cell diameter.

In all cases, the mean diameter ratios were approximately unity suggesting a rounded morphology for chondrocytes in the unstrained state. The ratios became significantly smaller as the chondrocytes adopted an oblate ellipsoid morphology at 20% strain. At day 1, this reduction was particularly marked in the highly cross-linked system, where the mean diameter ratio of 0.72 was similar to that reported in agarose constructs [26]. By day 6, cell deformation was significantly reduced in both gel systems, associated with an increased production of proteoglycans. To test this hypothesis, gels with the higher crosslinking density were subject to the enzyme hyaluronidase, which cleaves hyaluronan and chondroitin sulfate, thereby disrupting accumulated aggrecan. The diameter ratio decreased significantly from 0.89 to 0.81 ± 0.009 , confirming that by day 6 a stiff pericellular matrix had formed that inhibited cell deformation. However, cell deformation was not completely restored by hyaluronidase treatment suggesting that additional extracellular matrix components were present that enhanced the pericellular matrix. Similar behavior was reported in agarose constructs of constant concentration [21,27].

The difference in the mean diameter ratios for the two gel systems may be due to the different crosslinking densities (ρ_x) and/or ρ_x -dependent matrix synthesis. The authors have found that after 3 days of culture, proteoglycan synthesis was slower in the 20% gels compared to the 10% gels (Bryant, Chowdhury, Lee, Bader, and Anseth, unpublished results), which could explain why the cells deformed less in the 10% gels at day 1. However, by day 6 cell deformation was significantly reduced in the 20% gels. The authors previously showed that the spatial distribution of proteoglycans after 4 weeks in vitro was dependent on the degree of crosslinking, but not the overall content [9]. In particular, the proteoglycans were distributed throughout the gel in the 10%

PEGDM gel and localized in the pericellular regions in the 20% PEGDM gel. Although the spatial distribution of proteoglycans (Fig. 2) appears similar in both cross-linked systems, the smaller proteoglycans might be able to diffuse further away from the pericellular regions in the lower crosslinked gels, resulting in slightly more expansive, but less stiff pericellular matrix. Future studies will examine the thickness of the pericellular matrix in gels with different crosslinking densities.

In both PEGDM systems, considerable variation existed in the cell deformations within a population (Fig. 3). Indeed, some cells in the highly crosslinked, 20% gels exhibited an equivalent local strain of 32% (e.g., cell 6), which was significantly greater than the applied strain. Close examination of the horizontal section of this cell revealed the presence of pointed apices at the equators and an indication of membrane buckling. This phenomenon has been reported with myoblasts seeded in agarose constructs, which were subjected to applied strains of 40%, and was attributed to high local strains associated with the cell membrane [3]. Interestingly, buckling was only apparent with the 20% PEGDM gels, which also contained cells where local strains were less than 5% (cells 7 and 8). These findings suggest considerable heterogeneity in the crosslinking density, particularly with the 20% gel, which was further illustrated by the single cell approach. Considerable variation in diameter ratios for both gel systems was noted when examining a group of cells within a region (Fig. 4). Cells encapsulated in the higher crosslinked gel appear to experience greater heterogeneity. For example, the cells in *region f* did not deform compared to the unstrained gel, whereas the cells in *regions c* and *d* deformed considerably. Moreover, differences existed in the local strain surrounding the cells within a given region. For example, the local cell strain within *region d* for the 20% gel varied from 0% to 25%, which was considerably higher than the applied strain. Although, the overall macroscopic properties of the PEG gels are highly reproducible, heterogeneities in the crosslinking density may be formed during gelation due to the high solvent concentration and/or the presence of cells. Future studies are necessary to determine if cell strain depends on position within the strained constructs as reported in cartilage explants [32] and to elucidate the source of inhomogeneity.

Strain transfer to the gel is influenced by the heterogeneity of the crosslinking density. The strain might be expected to follow the path of least resistance and be highest in areas of low crosslinking density and associated low compressive modulus. The cell morphology in the unstrained constructs could also influence the subsequent level of cell deformation. Thus, cells that are less spherical in unstrained constructs were less likely to deform (e.g., cell 8 in Fig. 3). A few cells also deformed in the y direction, i.e. perpendicular to the applied strain,

suggesting that the applied load is transferred through the gel anisotropically.

Close examination of the cell shapes in the unstrained constructs (Fig. 4) revealed that the cells appeared slightly deformed even though the mean diameter ratios were unity. If the diameter ratios are recalculated based on the ratio of the minimum to maximum diameters, then the ratio is significantly lower than the previously described diameter ratio (x/y) of the population approach ($p < 10^{-5}$). The modified min/max diameter ratio is 0.95 ± 0.003 and 0.94 ± 0.003 for the 10% and 20% PEGDM gels, respectively, at day 1. The isolated chondrocytes were analyzed similarly, and the mean min/max ratio was 0.97 ± 0.08 , which was not significantly different from the originally defined diameter ratio (1.00 ± 0.01). These data suggest that an internal strain may be present in the crosslinked gel and applied to the chondrocytes prior to the application of a gross construct strain. Previous studies have shown that crosslinking density influences the production of type II collagen, which may be related to this internal strain applied to the chondrocytes [9]. In addition, no trends were observed in the morphology of the unstrained cells suggesting that the internal strain is anisotropic.

The data suggest that in the formation of PEG hydrogels in the presence of cells, heterogeneity exists at multiple levels. Measuring the diameter ratios of a population of cells is important to obtain the mean values for cells in unstrained and strained constructs. The diameter ratios of unstrained and strained cells using the population approach were statistically similar between gels with the same ρ_x at a given culture period. However, the population approach experiments masked the heterogeneity evident in the crosslinked gels. This was observed using a single cell approach in which discrete regions of the gel were analyzed. On a macroscopic level, differences in crosslinking density of the gels existed as a function of region that spanned many cells, as well as within a region that influences the deformation characteristics of neighboring cells. These differences can also exist on a microscopic level, which will influence the surface of individual cells. Such an argument was recently formulated in a new multilevel finite element approach to examine tissue engineered constructs [5]. Interestingly, the heterogeneities seen in the PEG gels have not been observed in the agarose gels, and this difference may be attributed to the mechanism of crosslinking and gelation. In this study, we showed that chondrocytes deform in photocrosslinked PEG hydrogels, a process that may be crucial to the development of a functional cartilage matrix in vivo. In addition, the crosslinking density of the PEG gels appears to influence cell morphology in a heterogeneous manner at different hierarchical levels. With an increase in macromer concentration, the heterogeneity is more pronounced. Close observations of the unstrained cells suggest that the

crosslinked network applies an internal strain to the surface of the cells. Thus, further understanding of how to control the heterogeneities within the PEG gels may allow simulation of the heterogeneities of native cartilage tissue.

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