Photocrosslinkable laminin-functionalized polyethylene glycol hydrogel for intervertebral disc regeneration

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1. Introduction

Intervertebral disc (IVD) disorders and age-related degeneration are believed to contribute to lower back pain. There is significant interest in cell-based strategies for regenerating the nucleus pulposus (NP) region of the disc; however, few scaffolds have been evaluated for their ability to promote or maintain an immature NP cell phenotype. Previous studies have shown that NP cell–laminin interactions promote cell adhesion and biosynthesis, which suggests a laminin-functionalized biomaterial may be useful for promoting or maintaining the NP cell phenotype. Here, a photocrosslinkable poly(ethylene glycol)–laminin 111 (PEG-LM111) hydrogel was developed. The mechanical properties of PEG-LM111 hydrogel could be tuned within the range of dynamic shear moduli values previously reported for human NP. When primary immature porcine NP cells were seeded onto PEG-LM111 hydrogels of varying stiffnesses, LM111-presenting hydrogels were found to promote cell clustering and increased levels of sGAG production as compared to stiffer LM111-presenting and PEG-only gels. When cells were encapsulated in 3-D gels, hydrogel formulation was found to influence NP cell metabolism and expression of proposed NP phenotypic markers, with higher expression of N-cadherin and cytokeratin 8 observed for cells cultured in softer (<1 kPa) PEG-LM111 hydrogels. Overall, these findings suggest that soft, LM111-functionalized hydrogels may promote or maintain the expression of specific markers characteristic of an immature NP cell phenotype.

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subunits [16,21,22]. Additional studies have shown that immature primary NP cells cluster and produce more sulfated glycosaminoglycan (sGAG) when cultured on soft, laminin-rich substrates [23]. These findings suggest that a soft, laminin-functionalized biomaterial may maintain or promote the immature NP cell phenotype and biosynthesis.

A variety of synthetic, natural and hybrid materials have been investigated as scaffolds for NP regeneration [24], mainly in the form of hydrogels, which mimic the highly hydrated nature of the native NP. Natural components of the ECM such as hyaluronan [25–27], collagen [28,29] and fibrin [28], and naturally derived polysaccharides such as alginate [28,30,31], chitosan [32–34] and agarose [28], have all been studied as potential scaffolds for NP tissue engineering. One limitation of using natural polymers for tissue engineering is that their mechanical properties cannot be easily controlled. To overcome this, hybrid biomaterials of both natural and synthetic materials [27,34–36], and natural polymers modified to contain functional groups that allow for photocross-linking [37,38] have been explored as potential scaffolds for NP tissue engineering. Photopolymerizable poly(ethylene glycol) (PEG) hydrogels have been extensively investigated as scaffolds for numerous tissue engineering applications due to their hydrophilicity, biocompatibility and tunable mechanical properties [39]. Since PEG can be easily modified with biofunctional moieties [40,41], ECM-derived peptides and full-length ECM proteins such as collagen [42,43], fibrinogen [42,44] and laminin [45] have been incorporated into PEG hydrogels as a means to control cell–material interactions in three dimensions. We have previously developed a PEG–laminin-111 (PEG-LM111) conjugate capable of supporting NP cell adhesion, which can be crosslinked via the addition of PEG-dithiol and PEG-octoacrylate for use as a biomaterial carrier for cells delivered to the disc [46]. Few studies have attempted to evaluate a scaffold for its ability to maintain or promote the immature or notochordal-like NP cell phenotype [47]. This is likely due to the lack of specific markers that distinguish notochordal-like NP cells from smaller, more chondrocyte-like NP cells, anulus fibrosus cells and articular chondrocytes. A number of recent studies have focused on defining the NP cell phenotype by evaluating biomarker expression in immature or non-degenerate NP cells as compared to that in degenerate NP cells, anulus fibrosus cells or articular chondrocytes [48–51]. Laminin-binding integrin subunits α3, α6 and β4 have been shown to be uniquely expressed in cells of the immature NP [17,52]. N-Cadherin has been shown to be more highly expressed in NP cells as compared to articular chondrocytes [50] and anulus fibrosus cells [53]. Finally, cytokeratin 8, an intermediate filament protein, is known to be expressed in notochordal disc cells [54] and more recently has been shown to be differentially expressed in NP cells [48,50]. These findings provide useful biomarkers for choosing those scaffold biochemical and physical properties that can promote or maintain an immature NP cell phenotype.

The objective of this work was to develop a laminin-functionalized PEG hydrogel based on well-characterized photocrosslinkable chemistry, and to investigate the effect of laminin ligand presentation and matrix stiffness in maintaining or promoting the immature NP cell phenotype. Here, PEG-LM111 conjugates were crosslinked to form hydrogels upon the addition of PEG-diacylate and exposure to UV light. We evaluated immature NP cell organization and proteoglycan synthesis when cells were cultured on top of PEG-LM111 hydrogels. Additionally, we describe here the effects of hydrogel stiffness and LM111 concentration on immature NP cell metabolism and NP phenotypic marker expression, including integrin subunits α3 and α6, as well as cytokeratin 8 and N-cadherin, when cells were cultured within 3-D PEG-LM111 hydrogels.

2. Materials and methods

2.1. LM111 PEGylation and PEG-LM111 hydrogel preparation

PEG-LM111 hydrogels were prepared via a two-step process in which LM111 was PEGylated to introduce functional acrylate groups and crosslinked by photopolymerization with additional PEG-diacylate (PEG-DA) (Fig. 1). LM111 (Trevigen®, Gaithersburg, MD) was PEGylated with acrylate–PEG–N-hydroxysuccinimide (Ac–PEG–NH2, MW = 10 kDa, Creative PEGworks, Winston Salem, NC) as described previously [46]. Briefly, Ac–PEG–NH2 was added to a LM111 solution at varying molar excess Ac–PEG–NH2 (10:1, 25:1, 100:1 or 500:1) to synthesize PEG-LM111 conjugates with varying degrees of modification. PEG-LM111 conjugate solution was dialyzed to remove unreacted Ac–PEG–NH2. The LM111 concentration in each PEG-LM111 conjugate precursor solution was determined by measuring the absorbance at 280 nm, and conjugates were stored at −80 °C until further use. PEG-DA (10 kDa, Creative PEGworks) was weighed, sterilized by exposure to UV light (265 nm) for 30 min, and transferred to sterile Eppendorf tubes. To form PEG-LM111 hydrogels, varying amounts of PEG-LM111 conjugate (0–1000 μg ml−1) and 10 kDa PEG-DA (2–10% (w/v), Creative PEGworks) were mixed, injected into custom molds [55] using a 22 gauge needle and 1 ml syringe and polymerized upon exposure to UV light (3–4 mW cm−2) in the presence of 0.1% (w/v) photoinitiator (Irgacure 2959®, Ciba Specialty Chemicals, Tarrytown, NY). A number of experiments were performed to evaluate LM111 distribution within PEG-LM111 hydrogels, and cell interactions with PEG-LM111 hydrogels in both two and three dimensions. A summary of PEG-LM111 hydrogel formulations tested and experimental output measures obtained for each formulation is presented in Table 1.

2.2. LM111 distribution in PEG-LM111 hydrogels

Immunostaining of PEG-LM111 hydrogels was performed to evaluate the effects of the Ac–PEG–NH2 to LM111 ratio used in conjugate synthesis on the amount of protein incorporated into PEG-LM111 hydrogels. PEG-LM111 hydrogels were crosslinked as described above to obtain four different hydrogel formulations containing 5% (w/v) PEG-DA and 200 μg ml−1 PEG-LM111 conjugate synthesized at either 10:1, 25:1, 100:1 or 500:1 M ratio of

![Image](https://i.imgur.com/5J5J5J5.png)

**Fig. 1.** Schematic of photocrosslinkable PEG-laminin (PEG-LM111) hydrogel preparation. Acta Biomater (2013), http://dx.doi.org/10.1016/j.actbio.2013.11.013
Ac-PEG-NHS to LM111. Blank 5% (w/v) PEG-DA hydrogels and 5% (w/v) PEG-DA gels mixed with 200 µg ml⁻¹ LM111 prior to cross-linking were photocrosslinked in the same manner as compared to other groups. Samples were cored using a 6 mm diameter biopsy punch and frozen in Tissue-Tek® OCT compound (Sakura Finetek USA, Torrance, CA) and cryosectioned to obtain 20 µm thick sections. For each gel formulation, 6–8 sections were stained with a primary antibody specific to the γ chain of LM111 (L9393, Sigma–Aldrich, St Louis, MO), followed by a goat anti-rabbit secondary antibody (Alexa Fluor 488®), Invitrogen, Carlsbad, CA). Samples were imaged by confocal microscopy to obtain three image fields per section (Zeiss LSM 510, 10× objective; Zeiss, Jena, Germany). Quantitative image analysis was performed using a custom-written MATLAB script. Briefly, images were converted to grayscale, thresholded by subtracting out a blank PEG-DA image, and mean fluorescence intensity per image field was obtained as a measure of the amount of LM111 incorporated into each hydrogel. Mean intensity values were normalized to that of hydrogels containing entrapped LM111. A one-way ANOVA was performed to analyze mean fluorescence intensity per image field, using Tukey’s post hoc test to detect differences between PEG-LM111 conjugates synthesized with different Ac-PEG-NHS to LM111 ratios (P < 0.05, n = 6 or 8 separate sections per hydrogel formulation, 3 images per section).

2.3. Mechanical properties of PEG-LM111 hydrogels

PEG-DA was dissolved in PEG-LM111 conjugate solution (synthesized at 25-fold molar excess Ac-PEG-NHS) and 0.1% Irgacure 2959® solution to obtain six different hydrogel formulations (5 or 10% PEG-DA and 0, 100 or 500 µg ml⁻¹ PEG-LM111 conjugate). Hydrogel precursor solutions were injected into a custom injection mold as described above. After crosslinking, cylindrical samples 5 mm in diameter and 2 mm thick were cored and allowed to equilibrate in PBS before testing. All samples were tested in oscillatory shear in a 37 °C temperature-controlled phosphate-buffered saline bath using a stress-controlled rheometer (AR-G2, TA Instruments, New Castle, DE). Immediately prior to testing, each gel was digitally photographed to obtain the sample diameter (average diameter = 4.7 ± 0.6 mm). Samples were placed in the center of a preheated sintered stainless lower platen (8 mm diameter) and subjected to a compressive tare load (1–2 g) with a sintered steel upper platen (8 mm diameter). After equilibrating, samples were subjected to a 10% compressive strain followed by 20 min relaxation. Samples were subjected to an oscillatory torsional strain (1–20 rad s⁻²) with a maximum amplitude of shear strain (γ, 0.01). The complex shear moduli (|G|) were reported at a frequency of 10 rad s⁻¹. Differences in shear moduli amongst hydrogels with varying concentrations of PEG-DA and PEG-LM111 conjugate were analyzed via two-way ANOVA with Tukey’s post hoc test (P < 0.05, n = 5 per hydrogel formulation).

2.4. Cell isolation and culture

Porcine NP cells were used for study as the majority of cells isolated from the immature porcine NP are known to resemble notochordally derived NP cells [52], and are therefore useful for identifying scaffold formulations that maintain the notochordal-like NP cell phenotype. Lumbar spines were obtained from pigs shortly after the animals were killed (L1–L5, 4–7 months, Nahunta Pork Outlet, Raleigh NC). Cells were isolated from the NP regions of IVDs by enzymatic digestion [56] and cultured in monolayer for 1–7 days in culture media (F-12 media supplemented with 5% fetal bovine serum (FBS), 10 mM HEPES, 100 U ml⁻¹ penicillin, and 100 U ml⁻¹ streptomycin) prior to experiments.

2.5. Primary NP cell behavior on PEG-LM111 hydrogels in two dimensions

2.5.1. sGAG production

Primary porcine NP cells (88,000 cells cm⁻²) were seeded onto PEG-LM111 hydrogels of varying concentrations of PEG (2 or 10% PEG-DA) and LM111 (0 or 1 mg ml⁻¹ LM111) for a total of four hydrogel substrate formulations (n = 6 per substrate). PEG concentrations were chosen to form “soft” and “stiff” hydrogels, with “soft” 2% PEG gels having similar stiffness to laminin-presenting gels previously shown to induce NP cell clustering and sGAG production [23]. Primary NP cells (88,000 cells cm⁻²) were also seeded onto a laminin-rich, ECM-derived basement membrane extract (BME) product ( Trevigen®, Gaithersburg, MD; growth-factor reduced, 13.8 mg ml⁻¹) as a positive control. BME is a solubilized basement membrane preparation extracted from the Engelbreth–Holm–Swarm (EHS) mouse sarcoma tumor, which contains high concentrations of several ECM proteins: LM111 (~60%), type IV collagen (~30%), entactin (~8%) and heparin sulfate [57]. When polymerized into a “thick” gel, BME provides a laminin-rich environment of stiffness close to that of the native NP tissue (0.2 kPa) [23,27,58]. Cells were cultured on top of each hydrogel formulation for 4 days in culture media, after which sGAG production by NP cells was analyzed with the dimethylmethylen blue (DMMB) spectrophotometric method as previously described [23]. All media overlay from culture samples was collected, while cells and gel proteins remaining in the corresponding wells after removal of media were digested in papain solution (125 µg ml⁻¹ in PBS with 5 mM EDTA and 5 mM cysteine, 2 h, 65 °C). Samples from control wells that only contained gels (no cells) were collected and processed similarly to control for background sGAG content in substrates; two acellular samples were used per substrate as prior work has shown values to be highly repeatable and very low [23]. sGAG content was measured by mixing samples with DMMB dye, and absorbance (553 nm) was measured on a plate reader (Perkin-Elmer Enspire Multimode Reader, Waltham, MA). sGAG concentrations were calculated from a standard curve prepared from chondroitin-4-sulfate (Sigma). Total concentration of sGAG (media overlay plus cell digest) was normalized to total DNA content (Quant-iT PicoGreen dsDNA Kit, Invitrogen) for each sample. Differences in sGAG production (sGAG/DNA) across hydrogel substrate formulations were analyzed via one-way ANOVA with Tukey’s post hoc analysis (P < 0.05, n = 6 per substrate formulation).

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2.5.2. Cell morphology

Immature porcine NP cells (88,000 cells cm$^{-2}$) were seeded onto PEG-only hydrogels (2 or 10% PEG-DA), and PEG-LM111 hydrogels (2 or 10% PEG-DA, 1 mg ml$^{-1}$ LM111) as described above (n = 6 per substrate formulation). The same number of NP cells were seeded onto BME gels for comparison. After 4 days of culture upon gels, NP cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA; diluted in DPBS) for 20 min at room temperature. NP cells were then washed with DPBS and labeled for actin (Alexafluor-488 phallolidin, Invitrogen, 200× dilution in DPBS for 30 min at room temperature) followed by a cell nuclei stain (propidium iodide, Sigma, 0.33 mg ml$^{-1}$ for 20 min at room temperature). Immediately after staining, NP cells were imaged via confocal microscopy (Zeiss LSM510, 10× magnification).

2.6. Cell viability in PEG-LM111 hydrogels in three dimensions

Preliminary cell encapsulation experiments were performed to evaluate PEG-LM111 conjugate bioactivity and to assess the ability of LM111 to promote NP cell survival in 3-D culture under low-cell-density, serum-free conditions. Primary porcine NP cells were encapsulated in blank PEG-DA hydrogels (6% PEG-DA), PEG-DA hydrogels with unmodified LM111 that had been mixed in prior to crosslinking (6% PEG-DA, 200 μg ml$^{-1}$ entrapped LM111) or PEG-LM111 hydrogels (6% PEG-DA, 200 μg ml$^{-1}$ PEGylated LM111). Briefly, porcine NP cells were pelleted and resuspended in hydrogel precursor solutions (2 million cells ml$^{-1}$), injected into custom injection molds and polymerized upon exposure to UV light in the presence of 0.1% (w/v) photoinitiator (Irgacure 2959) as described above. After polymerization, cylindrical constructs were cored using a 6 mm biopsy punch and cultured in 24 well plates in serum-free media (F-12 supplemented with 10 mM HEPES, 100 U ml$^{-1}$ penicillin and 100 U ml$^{-1}$ streptomycin) with gentle agitation. At each time point, cell–gel constructs (n = 5 per formulation) were submerged in live/dead staining solution (Invitrogen) and incubated at 37°C for 30 min. Cell viability and distribution were visualized by confocal microscopy on days 0 and 7. Images (four per cell–gel construct) were analysed using ImageJ (NIH) to determine the percentage of live cells present at each time point. Differences in cell viability amongst hydrogel formulations were analyzed via two-way ANOVA (time point, hydrogel formulation) with Tukey’s post hoc test (P < 0.05, n = 5 per hydrogel formulation).

2.7. Primary NP cell behavior in PEG-LM111 hydrogels in three dimensions

Primary porcine NP cells (20 million ml$^{-1}$) were mixed with PEG-DA (5 or 10%), PEG-LM111 conjugate (0, 100 or 500 μg ml$^{-1}$) and 0.1% Irgacure 2959$^a$ for a total of six material formulations. Precursor solutions were injected into custom molds and crosslinked by UV exposure (5 min, 3–4 mW cm$^{-2}$). Samples were cored using a 3 mm biopsy punch (n = 9 per formulation) and cultured in vitro (F-12 media supplemented with 10% FBS, 10 mM HEPES, 100 U ml$^{-1}$ penicillin, and 100 U ml$^{-1}$ streptomycin) with gentle agitation. 50% volume media changes were performed every 3–4 days for 28 days.

2.7.1. Measurement of media metabolites

Early metabolite levels have been shown to predict long-term matrix accumulation for chondrocytes in elastin-like polypeptide scaffolds [59], and were therefore measured to evaluate hydrogel formulation effects on cell metabolism. Media aliquots from NP cell–PEG samples, and media from wells containing no PEG or cells were obtained on day 4 and stored at −80°C until further use. On the day of analysis, media aliquots were thawed and filtered through a 10 kDa MW cut-off centrifugal filter device (Nanosep, Pall Life Sciences, East Hills, NY). Samples (15 μl) were then transferred to plastic vials, briefly spun and analyzed for glucose, lactate and pyruvate concentrations on a CMA 600 microdialysis analyzer (CMA Microdialysis, North Chelmsford, MA). The difference between each metabolite concentration in media collected from wells containing cell-laden PEG hydrogel samples and day 0 media that had been cultured for the same period was calculated for each sample. For each metabolite, differences amongst hydrogel formulations were analyzed via two-factor ANOVA (% PEG-DA, PEG-LM111 conjugate concentration) with Tukey’s post hoc test (P < 0.05, n = 9 per hydrogel formulation).

2.7.2. Phenotypic marker immunostaining

Cell-laden hydrogel samples were immunostained for N-cadherin, cytokeratin 8, integrin α3 and integrin α6 to evaluate the effects of PEG-LM111 hydrogel stiffness and LM111 concentration on NP cell phenotype. On day 28, samples (n = 3 per formulation) were embedded in Tissue Tek® OCT embedding compound and flash frozen in liquid nitrogen. Samples were stored at −80°C until further use. Frozen cell-laden PEG-LM111 hydrogel sections (8 μm thick) were fixed in 4% paraformaldehyde (10 min at room temperature) for labeling with antibodies detecting N-cadherin and cytokeratin 8. For labeling integrin subunits α3 and α6, sections were fixed in acetone (10 min at −20°C). Following fixation, all sections were incubated with blocking solution (3.75% BSA/5% goat serum) for 45 min at room temperature, and then incubated for 2 h at room temperature with one of the following primary antibodies: N-cadherin (ab12221, Abcam, Cambridge, MA), cytokeratin 8 (SM3079P, Acris Antibodies, San Diego, CA), integrin α3 (AB1920, EMD Millipore, Billerica, MA) and integrin α6 (555734, BD Biosciences, San Jose, CA). Sections were washed twice with PBS and incubated with secondary antibody (AlexaFluor 488, Molecular Probes, Eugene, OR) for 30 min in blocking solution. Control sections were incubated with appropriate IgG controls or secondary antibody alone as a negative control for polyclonal antibodies. All sections were counterstained with propidium iodide (Sigma) at room temperature for 30 min to label cell nuclei. Sections were imaged via confocal microscopy (Zeiss, 20× NA 0.5 objective). Digital images acquired for each cell-laden hydrogel (n = 9 images per hydrogel formulation) were evaluated by two blinded graders coming to consensus for fluorescence staining uniformity with the following ordinal scale: 0 (no stain), 1 (<10% cells positively stained), 2 (10–50% cells positively stained), 3 (>50% of cells positively stained) for each image. Grades of immunohistochemical staining were analyzed for differences amongst hydrogel formulations using Kruskal–Wallis one-way ANOVA (P < 0.05). When significance was observed, post hoc Wilcoxon tests with Bonferroni correction for multiple comparisons were performed to detect differences amongst hydrogel formulations.

3. Results

3.1. LM111 distribution in PEG-LM111 hydrogels

Immunostaining of PEG hydrogels was performed to determine the effect of the Ac-PEG-NHS to LM111 ratio used in the PEGylation reaction on the amount of LM111 incorporated into each hydrogel formulation. Immunostaining results demonstrate that the ratio of PEG to LM111 used in PEG-LM111 conjugate synthesis affects the amount of LM111 incorporated into the hydrogel (Fig. 2). Fluorescence images show more intense staining for LM111 in hydrogels containing PEG-LM111 conjugate synthesized with a high Ac-PEG-NHS PEG to LM111 ratio (500:1) as compared to those containing PEG-LM111 conjugates synthesized with a low ratio.
of Ac-PEG-NHS to LM111 (25:1) or physically entrapped LM111 (Fig. 2B). Mean fluorescence intensity per image field significantly increased with increasing molar excess of Ac-PEG-NHS:LM111 ratio in the PEG-LM111 conjugate synthesis reaction (mean ± SEM, n = 6 sections, 3 images per section, conditions labeled with different letters significantly different, P < 0.03). (B) Representative images of LM111 immunostaining in PEG hydrogels. Scale bars = 100 μm.

3.2. PEG-LM111 hydrogel mechanical properties

PEG-LM111 hydrogels were tested in oscillatory torsional shear to investigate the effects of PEG-LM111 conjugate concentration and PEG-DA concentration on hydrogel mechanical properties. Photocrosslinked PEG-LM111 hydrogel stiffness (|G'|) significantly increased with increasing PEG-LM111 conjugate concentration for gels formed with either 5% or 10% PEG-DA (two-way ANOVA, P < 0.03) (Fig. 3). PEG-LM111 hydrogel stiffnesses (|G'|) were significantly higher in gels containing 10% PEG-DA as compared to 5% PEG-DA (two-way ANOVA, P < 0.001).

3.3. Primary NP cell behavior on PEG-LM111 hydrogels in two dimensions

To determine if immature NP cells form multicell clusters and synthesize ECM on PEG-LM111 hydrogel substrates, primary NP cells were seeded on PEG-LM111 hydrogels containing 1 mg ml⁻¹ LM111 and varying concentrations of PEG (2 or 10%). NP cells seeded on soft, PEG-LM111 hydrogels containing low amounts of PEG synthesized similar levels of sGAG as compared to cells plated on laminin-containing, polymerized BME substrates (one-way ANOVA, Tukey’s post hoc test, P > 0.05) (Fig. 4A). Primary NP cells seeded onto PEG-LM111 hydrogel substrates containing high amounts of PEG synthesized significantly less sGAG (Tukey’s post hoc test, P < 0.0001), with total sGAG production similar to that of cells seeded onto PEG hydrogels containing no protein. Over 4 days in culture, primary NP cells were found to form large, multicell clusters when seeded on PEG-LM111 hydrogels containing low concentrations of PEG (2%) or polymerized BME substrates (Fig. 4B). In contrast, NP cells seeded on PEG-LM111 substrates containing high concentrations of PEG (10%) or PEG-only substrates (no LM111) remained attached as single cells with uniform distribution.

3.4. Cell viability in 3-D PEG-LM111 hydrogels

To verify the bioactivity of crosslinked PEG-LM111 conjugate, and to assess the ability of LM111 to promote NP cell survival in 3-D PEG hydrogels under low-cell-density, serum-free conditions, primary porcine NP cells were entrapped in PEG-DA hydrogels containing the same concentration of PEG-LM111 conjugate or entrapped unmodified LM111 and cultured for 7 days. Cells remained
viable through photocrosslinking, with >82% cell viability for all hydrogel formulations on day 0. Cell viability was significantly lower after 7 days in culture for all hydrogel formulations (P < 0.001); however, NP cell survival on day 7 was significantly higher in PEG hydrogels containing entrapped LM111 or PEG-LM111 conjugate as compared to blank PEG-DA gels (Tukey’s post hoc test, P < 0.02) (Fig. 5).

3.5. Primary NP cell behavior in 3-D PEG-LM111 hydrogels

Media aliquots from NP cell-laden PEG-LM111 hydrogels were analyzed for glucose, lactate and pyruvate to evaluate the effects of hydrogel stiffness and LM111 concentration on NP cell metabolism. Lactate and pyruvate levels were significantly affected by hydrogel stiffness and LM111 concentration (two-way ANOVA, P < 0.004) as compared to cells cultured in gels containing 0 or 500 μg ml⁻¹ PEG-LM111 conjugate (Fig. 6).

Cytokeratin 8, N-cadherin, integrin α3 and integrin α6 expression by NP cells cultured in PEG-LM111 hydrogels was evaluated by immunohistochemical analysis (Table 2, Fig. 7). The percentage of cells staining positively for cytokeratin 8 was significantly higher in softer PEG-LM111 gels containing 5% PEG and 100 or 500 μg ml⁻¹ LM111 (one-way ANOVA, Bonferroni correction, P < 0.004) as compared to stiffer gels containing 10% PEG and equivalent amounts of LM111, and PEG-only (no LM111) gels. N-cadherin expression was significantly higher for NP cells cultured in PEG-LM111 hydrogels containing 5% PEG and high concentrations of LM111 (500 μg ml⁻¹) (one-way ANOVA, Bonferroni correction, P < 0.004) as compared to cells cultured in all other hydrogel formulations. Integrin α3 expression varied significantly with hydrogel formulation (P < 0.0001) with the highest percentage of cells staining positively for cells cultured in PEG-LM111 gels containing 5% or 10% PEG and 100 μg ml⁻¹ LM111, or 5% PEG and 500 μg ml⁻¹ LM111. Very low or no staining of integrin α6 was observed for all hydrogel formulations.

4. Discussion

The goal of this work was to develop a laminin-functionalized hydrogel with tunable mechanical properties and to evaluate the…
effects of matrix stiffness and LM111 ligand concentration on immature NP cell phenotype. Previous work has shown that BME-polymerized gels or soft BME upon polyacrylamide promote formation of multicell clusters for cultured primary disc cells [23,60]. While polyacrylamide substrates systems are useful for 2-D studies, they are not suitable for cell entrapment and 3-D cell culture; therefore, we engineered a biomaterial by attaching LM111 to PEG-based hydrogels, as PEG is non-cytotoxic, non-fouling, and has a long history of clinical use as a drug delivery agent for therapeutic proteins [61]. In addition, the mechanical properties of PEG hydrogels formed by UV photopolymerization of PEG-DA can be easily manipulated [40]. Results reveal that specific formulations of the PEG-LM111 hydrogel appear to preserve features of the immature NP cell phenotype.

Increasing the degree of LM111 PEGylation (ratio of Ac-PEG-NHS to LM111 in the PEGylation reaction) was shown to significantly increase the amount of LM111 incorporated into PEG-LM111 hydrogels. While increased ligand density within PEG-LM111 hydrogels is desirable, our previous work has shown that a high degree of LM111 modification by Ac-PEG-NHS significantly reduces its bioactivity and inhibits immature NP cell adhesion to the modified protein [46]. Therefore, PEG-LM111 conjugates synthesized at low molar ratios of Ac-PEG-NHS to LM111 (25:1) were utilized in all subsequent experiments. Characterization of the bulk mechanical properties of PEG-LM111 hydrogels showed that PEG concentration (% w/v) was a dominant variable in determining the stiffness of PEG-LM111 hydrogels, with dynamic stiffness values for gels containing 10% PEG an order of magnitude higher than those of gels formed with 5% PEG. This was expected since increasing the polymer concentration results in an increased number of polymer chains in the hydrogel, thereby increasing the number of entanglements in the gel. PEG-LM111 hydrogel stiffness, however, could not be completely decoupled from ligand concentration, as increasing the amount of PEG-LM111 conjugate also increased PEG-LM111 hydrogel stiffness for gels containing both 5% and 10% PEG. This finding differs from that of a recent study, in which increasing the amount of LM111 conjugate in PEG-DA hydrogels was reported to significantly decrease hydrogel stiffness [45]. Our finding may be related to the PEG-LM111 conjugate having an increased degree of modification by the addition of PEG onto LM111, thereby increasing the number of acrylate groups on LM111 that can participate in the photocrosslinking reaction. This feature may reduce the likelihood that a single PEGylated LM111 molecule can result in chain termination. The inability to independently tune the dynamic stiffness and LM111 concentration of PEG-LM111 hydrogels demonstrates a limitation of the PEG photocrosslinking system for evaluating the effects of matrix stiffness and ligand concentration on cell behaviors. However, since PEG-LM111 hydrogel stiffness is largely controlled by the total PEG concentration of the gel, this is a useful system for furthering our understanding of ECM stiffness and composition on NP cell phenotype.

Dynamic shear moduli of photocrosslinked PEG-LM111 hydrogels were found to be approximately 10× higher than that of injectable PEG-LM111 hydrogels formed by a Michael-type addition reaction [46], despite having the same total PEG and LM111 concentrations. This is likely due to differences in the molecular weight of functionalized PEG molecules used to form the two different hydrogels, and intrinsic differences between the different crosslinking systems. PEG-LM111 hydrogel dynamic shear moduli can be tuned within the range of values previously reported for non-degenerate human NP (7–21 kPa) [62] by altering both the total PEG concentration and PEG-LM111 conjugate concentration, with hydrogel stiffness being dominated by the former. Human NP dynamic shear moduli have been shown to increase significantly with age and grade of degeneration, up to 60 kPa [63], values which could be achieved in the PEG-LM111 hydrogel system by increasing the total PEG concentration. While PEG-LM111 hydrogel dynamic shear values closely match that of the human NP, phase angle (δ) values were more characteristic of an elastic material (δ = 0.9–8.2°) as compared to the human NP (δ = 23–31°) [58]. Overall, these findings suggest that PEG-LM111 hydrogels can be formulated to match certain NP tissue mechanical properties; however, they may have reduced capacity to dissipate energy in comparison to the native NP.

While 2-D studies afford very good control of the substrate stiffness and ECM chemistry, culturing cells within a 3-D matrix is much more representative of the native environment. As expected, the majority of primary NP cells encapsulated in PEG-based hydrogels at a very low cell density and cultured under serum-free conditions died over 7 days in culture; however, significantly more cells survived when encapsulated in hydrogels containing LM111 as compared to PEG-only gels. It is well known that LM111 promotes cell survival for a number of cell types in vitro [19]; therefore, this finding suggests that LM111 may be a survival ligand for primary NP cells. Viability was similar for cells cultured in PEG-LM111 hydrogels and PEG hydrogels containing an equal concentration of entrapped, unmodified LM111. This finding suggests that PEGylated LM111 retains the bioactivity of the native protein in three dimensions, and that survival is mediated by cell–LM111 interactions irrespective of ligand presentation. Overall, cell viability was likely affected by the small mesh size of PEG hydrogels formed by photopolymerizing PEG-DA, which limits nutrient and waste diffusion in cultured hydrogels [64], and may inhibit cell–cell interactions when cells are encapsulated at very low densities. For primary NP cells cultured within 3-D PEG-LM111 hydrogels of varying stiffnesses and LM111 ligand concentration, media metabolite concentrations suggest that LM111 ligand density, but not stiffness of the material, has a significant effect on NP cell metabolism, particularly lactate production. NP cells rely on diffusion of...
LM111 may improve matrix production in PEG scaffolds. Notch–cell interactions at early time points has been shown to be a strong predictor of glycosaminoglycan and hyaluronan accumulation in chondrocytes [59], suggesting that low levels of LM111 may improve matrix production in PEG scaffolds.

Although it has been hypothesized that promoting or maintaining a notochordal-like immature NP cell phenotype may be important for tissue engineering strategies aimed at NP regeneration, there has been limited assessment of the effects of scaffold design on cell phenotype. This is in part due to the lack of specific markers that distinguish immature, notochordal NP cells from small, more chondrocyte-like NP cells, as well as from articular chondrocytes and anulus fibrosus cells. Recently, numerous studies have focused on markers uniquely expressed in the immature or non-degenerate NP, and suggest integrin α3 [17], integrin α6 [17,52], N-cadherin [50,53] and cytokeratin 8 [48,50] as potential NP phenotypic markers. Here, N-cadherin was found to be highly expressed when immature NP cells were cultured in soft, PEG-LM111 hydrogels containing 5% PEG and high concentrations of LM111 (500 mg ml⁻¹), characteristic of a soft hydrogel with high LM111 ligand density. Cytokeratin 8 is known to be highly expressed in the human notochord [54] and immature porcine NP [48], and was higher for NP cells cultured in PEG-LM111 gels containing 5% PEG and either low (100 mg ml⁻¹) or high (500 mg ml⁻¹) amounts of LM111. This is in contrast to the findings for PEG-LM111 hydrogels formed from 10% PEG or PEG-only (no LM111). The findings for higher N-cadherin and cytokeratin 8 expression in LM111 containing PEG gels of 5% PEG suggests that soft (<1 kPa), LM111-functionalized gels may lead to maintenance of this key feature of immature NP cell phenotype. NP cells were found to express varying levels of integrin α3 across hydrogel formulations; however, very little immunostaining for integrin α6 was observed for all cell-laden constructs. This was unexpected since porcine NP cells have previously been shown to attach to LM111 via integrin α6 [16], while adult human NP cells make use of α3, α5 and β1 subunits to attach to LM111 [21]. Integrin α3 has been shown to be expressed in the immature human and porcine NP, but not in the adjacent anulus fibrosus [17], and to play a functional role in human NP cell adhesion to laminins [21]. In this study, integrin α3 expression was highest for primary NP cells cultured in PEG-LM111 hydrogels that contained 5% PEG and either 100 or 500 mg ml⁻¹ LM111; however, moderate expression was observed for cells cultured in the stiffer gels containing similar amounts of LM111. This finding may reflect that NP cell–LM111 interactions are mediated at least in part by the integrin α3 subunit when attaching to the modified PEG-LM111 conjugate. Taken together, immunostaining of NP cell-laden PEG-LM111 hydrogels suggest that soft, LM111 functionalized hydrogels may promote both cell–cell and cell–matrix interactions characteristic of an immature NP cell phenotype.

In addition to expression of specific phenotypic markers, immature NP behavior on softer, PEG-LM111 substrates was found to correlate closely to that of NP cells seeded onto BME gels, with cells seeded onto stiffer, PEG-LM111 hydrogels with low levels of PEG and high concentrations of LM111 found to cluster and to synthesize higher levels of sGAG, as compared to cells seeded onto blank PEG-only or stiffer, PEG-LM111 hydrogels. Hydrogel formulations tested in 2-D studies differed from those evaluated in 3-D studies, which inhibits our ability to draw clear relationships between these experiments; however the goal of this work was not to compare 2-D and 3-D situations, as those have long been known to induce very different phenotypes in cells [68]. Overall, this finding suggests that specific formulations of the PEG-LM111 hydrogel are able to mimic the physical and biochemical properties of polymerized BME previously shown to promote an immature NP cell phenotype.

5. Conclusions

We report here on the development and characterization of a photocrosslinkable LM111-functionalized hydrogel with tunable mechanical properties for evaluating the effects of matrix stiffness and LM111 concentration on NP cell behaviors in both two and three dimensions. In two dimensions, the softer, LM111-presenting hydrogels were found to promote primary NP cell clustering and increased levels of sGAG production as compared to stiffer LM111 presenting and PEG-only gels. In three dimensions, hydrogel formulation was found to influence NP cell metabolism and expression of proposed phenotypic markers, with higher expression of N-cadherin and cytokeratin 8 observed for cells cultured in softer (<1 kPa), LM111-functionalized PEG-LM111. Overall, these findings suggest that softer, LM111-functionalized scaffolds may promote or maintain an immature NP cell phenotype, and that incorporation of the LM111 ligand into scaffolds might be useful for studies aimed at NP regeneration. In future work, soft PEG-LM111 hydrogels (total PEG concentration ≤5%) containing high concentrations of LM111 (≥500 μg ml⁻¹) will be evaluated as tissue engineered scaffolds for NP replacement.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 2,4,5,7, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2013.11.013.

References


