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Injectable laminin-functionalized hydrogel for nucleus pulposus regeneration

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ABSTRACT

Cell delivery to the pathological intervertebral disc (IVD) has significant therapeutic potential for enhancing IVD regeneration. The development of injectable biomaterials that retain delivered cells, promote cell survival, and maintain or promote an NP cell phenotype *in vivo* remains a significant challenge. Previous studies have demonstrated NP cell – laminin interactions in the nucleus pulposus (NP) region of the IVD that promote cell attachment and biosynthesis. These findings suggest that incorporating laminin ligands into carriers for cell delivery may be beneficial for promoting NP cell survival and phenotype. Here, an injectable, laminin-111 functionalized poly(ethylene glycol) (PEG-LM111) hydrogel was developed as a biomaterial carrier for cell delivery to the IVD. We evaluated the mechanical properties of the PEG-LM111 hydrogel, and its ability to retain delivered cells in the IVD space. Gelation occurred in approximately 20 min without an initiator, with dynamic shear moduli in the range of 0.9–1.4 kPa. Primary NP cell retention in cultured IVD explants was significantly higher over 14 days when cells were delivered within a PEG-LM111 carrier, as compared to cells in liquid suspension. Together, these results suggest this injectable laminin-functionalized biomaterial may be an easy to use carrier for delivering cells to the IVD.

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1. Introduction

Intervertebral disc (IVD) degeneration contributes to disability and symptomatic pain in affected individuals, and affects more than half the US population at some point in their lifetime [1]. A number of biological and anatomical changes are associated with IVD degeneration, initially occurring in the nucleus pulposus (NP), where degeneration is characterized by decreased cellularity, decreased water content and loss of proteoglycan deposition in the extracellular matrix (ECM) [2]. These changes in matrix composition coincide with the loss of a distinct cell population derived from the embryonic notochord [3,4]; therefore, it has been hypothesized that notochordal cell disappearance may be an initiating or contributing factor to degenerative disc disease [5–7]. Since current surgical treatments of disease resulting from disc degeneration often do not restore IVD function [8] and can lead to increased mechanical load and adjacent segment disease [9], there exists significant interest in tissue engineering strategies for regenerating the IVD.

Cell transplantation to the IVD has been performed clinically [10], and aims to repopulate the disc with cells capable of synthesizing new ECM to restore function to the diseased IVD. In animal models of IVD regeneration, reimplantation of autologous disc cells or stem cells has been shown to delay degeneration, as measured by histological and radiographic changes [8,11–16]. Although the need for a biomaterial carrier to improve cell retention immediately after delivery has been demonstrated [17], few studies have evaluated the role of the carrier in long-term cell survival and retention [16]. Injectable materials for cell delivery to the IVD have received

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considerable attention since they provide a minimally invasive tissue engineering approach for enhancing IVD regeneration. Numerous hydrogels derived from natural components of the ECM, including fibrin [17–19], hyaluronan [11,20,21], and collagen [12,22,23], and natural biopolymers such as chitosan [24–26] have been investigated as carriers for cell delivery to the disc. Although these naturally derived materials mimic many features of the native ECM, few peptide and protein functionalized scaffolds have been developed that can direct biological responses of cells for NP regeneration. The development of injectable biomaterials that support cell retention, cell survival and maintain or promote an NP cell phenotype *in vivo* remains a significant challenge.

Laminins are heterotrimeric ECM proteins consisting of α, β, and γ polypeptide chains that mediate a number of cellular functions including adhesion, survival, migration and differentiation [27,28]. Previous studies in our laboratory have demonstrated NP cell – laminin interactions that are unique to the immature disc, suggesting that laminins may be important contributors to NP-specific cell biology. Immunohistochemistry and flow cytometry results demonstrated higher expression levels of the laminin α5 and γ1 chains, laminin receptors (integrin α3, α6, β4 subunits, CD239), and related binding proteins in NP cells as compared to cells from the adjacent anulus fibrosus [29–31]. Additional studies have shown that soft, laminin containing ECM substrates promote immature NP cell morphology, cell–cell interactions, and proteoglycan synthesis for cells of the NP [32]. Finally, immature porcine NP cells adhere to laminins in higher numbers as compared to cells from the adjacent anulus fibrosus [33]. These findings provide support for known interactions between immature NP cells and multiple laminin isoforms that regulate NP cell biology, and suggest that a soft, laminin-functionalized hydrogel may be desirable for promoting primary NP cell phenotype and biosynthesis.

Poly(ethylene glycol) (PEG) hydrogels have been widely used in tissue engineering applications since they are hydrophilic polymers that readily allow for incorporation of biological signals derived from the native ECM [34]. The non-fouling nature of PEG, combined with its tunable mechanical properties, allows for control over biological signal presentation and hydrogel stiffness. Therefore, full length ECM-derived proteins, including collagen, fibrinogen and laminin, have been covalently coupled to PEG hydrogels for a variety of tissue engineering applications and shown to influence cell behavior in three dimensions [35–38]. The majority of these studies rely on photocrosslinking of acrylate functional groups on PEGylated proteins and PEG-multiacrylates to form three-dimensional hydrogels; however, conjugate addition between free thiols and PEG-acrylate or PEG-vinylsulfone allows gelation to occur *in situ* under physiological conditions [39,40] without the need for UV light. This chemistry enables a utility of the PEG-crosslinking hydrogel that is more suitable for the delivery of cells *in vivo*.

The objective of this work was to develop an injectable laminin-functionalized PEG hydrogel with tunable mechanical properties, and to assess its potential use as a carrier for cell-based IVD regeneration. We describe here the synthesis and characterization of a PEG-laminin (PEG-LM111) conjugate with functional acrylates for crosslinking, and subsequent hydrogel formation via the addition of PEG-dithiol and PEG-octoacrylate. Additionally, we describe the generation of luciferase expressing primary NP cells, and the delivery of these cells to the disc space within our injectable biomaterial carrier.

2. Materials & methods

2.1. PEG-laminin (PEG-LM111) conjugate synthesis and characterization

Laminin-111 (LM111, Trevigen®, Gaithersburg, MD) was PEGylated with acrylate-PEG-N-hydroxysuccinimide (Ac-PEG-NHS, MW = 10 kDa, Creative PEGworks, Winston Salem, NC) to introduce functional acrylate groups for crosslinking. LM111 was dialyzed into a 0.1 M sodium bicarbonate buffer, pH 8.5, and diluted to a concentration of 2 mg/ml. Ac-PEG-NHS was solubilized in ice cold 0.1 M sodium bicarbonate buffer and added to LM111 solution at 10:1, 25:1, 100:1 or 500:1 M ratio of Ac-PEG-NHS to LM111. Reactions were carried out for 2 h at room temperature. Precursor PEG-LM111 conjugate solutions were dialyzed against 1x PBS to remove any unreacted Ac-PEG-NHS. 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.

A TNBS (2,4,6-trinitrobenzene sulfonic acid) assay [41] was modified to measure the free amino groups in each PEG-LM111 conjugate compared to unmodified protein, and to estimate the degree of modification due to Ac-PEG-NHS substitution. Each PEG-LM111 conjugate and unmodified LM111 were diluted to 500 μg/ml in PBS, which was determined to be within the linear range for LM111 using this assay. TNBS was diluted to 0.01% in 0.1 M sodium bicarbonate buffer, pH 8.5. Samples (PEG-LM111 conjugates or LM111) were mixed with 0.01% TNBS at a 1:1 ratio by volume, and incubated for 30 min at 60 °C. Absorbance was measured at 340 nm using a microplate reader (Enspire, PerkinElmer, Waltham, MA) and used to calculate degree of modification (%): 100 x {1−((A_{340} PEG-LM111)/(A_{340} LM111))}. An ANOVA was performed to analyze degree of modification, using Tukey's post hoc test ($p < 0.01$, $n = 3$) to detect differences between PEG-LM111 conjugates synthesized with different Ac-PEG-NHS to LM111 ratios.

2.2. Cell isolation and culture

Cells isolated from the porcine NP have a unique notochordal phenotype, and were therefore used to evaluate both PEG-LM111 conjugate bioactivity and for *in vitro* and *in vivo* cell delivery experiments. Lumbar spines were obtained from pigs shortly after sacrifice (L1–L5, 4–7 months, Nahunta Pork Outlet, Raleigh NC). Cells were isolated from the NP regions of IVDs by enzymatic digestion [42] and cultured in monolayer for 3–7 days in culture media (Ham's F-12 media supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 100 U/ml penicillin, and 100 U/ml streptomycin) prior to experiments. For control studies, cells from a lung epithelial cell line (WI26VA4, ATCC No. CCL-95-1) were cultured in monolayer (37 °C, 5% CO_2, 20% O_2) with media changes every 3–4 days (Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 10 mM HEPES) prior to experiments.

2.3. PEG-LM111 conjugate bioactivity

To evaluate cell attachment to PEG-LM111 conjugates, wells of 96-well plates were coated with PEG-LM111 conjugates synthesized with various ratios of Ac-PEG-NHS to LM111 at 5, 10, and 25 μg/ml LM111 by overnight incubation at 4 °C. Coated wells were blocked with 3.75% bovine serum albumin (BSA) for 3 h at 37 °C to prevent non-specific adhesion. LM111 coated wells and BSA only coated wells were used as positive and negative controls, respectively. Porcine NP cells in monolayer were detached using trypsin/EDTA, washed with trypsin neutralizing solution and resuspended in serum free media. Cells (4000 cells/well) were allowed to adhere to the LM111 and PEG-LM111 conjugate coated surfaces for 2 h at 37 °C. Wells were rinsed with serum free media to remove non-adherent cells, and the number of adherent cells per well was determined using CellTiterGlo® (Promega Corporation, Madison, WI) luminescent cell viability reagent. Differences in attachment numbers to PEG-LM111 conjugates at 25 μg/ml LM111 were detected via ANOVA with Tukey's post hoc test ($p < 0.05$, $n = 3$ separate cell isolations).

To determine if PEG-LM111 conjugate maintains the ability to induce ERK activation upon cell adhesion, wells of 6-well tissue culture plates were coated by overnight incubation at 4 °C with 20 μg/ml LM111 or PEG-LM111 that had been PEGylated at the highest ratio of Ac-PEG-NHS shown to promote cell attachment at levels similar to native protein. All wells were blocked with 3.75% BSA to prevent non-specific adhesion. Cells from a lung epithelial cell line WI26VA4 (ATCC No. CCL-95-1) that had been cultured to confluence, then serum deprived for 24 h, were seeded onto LM111 or PEG-LM111 conjugate coated surfaces (500,000 cells/well). WI26VA4 cells cultured in suspension served as negative controls. After 30 and 60 min, cells were lysed with ice cold cell lysis buffer containing protease and phosphatase inhibitors (RIPA, Cell Signaling Technologies, Danvers, MA), spun down at 4 °C, and cell lysates were stored at −80 °C. Total protein concentration in each of the cell lysates was determined using the BCA Protein Assay (Thermo Scientific, Waltham, MA). All lysates were diluted in cell lysis buffer to equal concentrations of total protein and a phospho-ERK ELISA (Cell Signaling Technologies) was used to detect relative levels of phosphorylated ERK.

2.4. PEG-LM111 hydrogel synthesis and mechanical properties

PEG-octoacrylate (20 kDa, Creative PEGworks) and PEG-dithiol (3.4 kDa, Creative PEGworks) were dissolved separately in PEG-LM111 conjugate (25:1 Ac-PEG-NHS to LM111) solutions and PBS to final concentrations of 10% (w/v) PEG and 0, 100, or 500 μg/mL PEG-LM111 conjugate (25:1 Ac-PEG-NHS to LM111). All samples were tested in oscillatory shear under physiological conditions (37 °C, pH 7.4) in a humidified atmosphere. Briefly, appropriate volumes of PEG-octoacrylate and PEG-dithiol solutions that had been dissolved in PEG-LM111 conjugate solution were

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