Acta Biomaterialia 10 (2014) 1102–1111

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Photocrosslinkable laminin-functionalized polyethylene glycol hydrogel for intervertebral disc regeneration

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ARTICLE INFO

Article history:
Received 17 August 2013
Received in revised form 15 October 2013
Accepted 17 November 2013
Available online 25 November 2013

Keywords:
Nucleus pulposus
Intervertebral disc
Polyethylene glycol
Photocrosslinking
Laminin

ABSTRACT

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.

1. Introduction

Intervertebral disc (IVD) disorders, including herniation, stenosis, spondylolysis, and degeneration, resulted in more than 663,000 inpatients stays for back surgery or other back disorder treatments in the US in 2008 alone, costing more than $9.5 billion and making back problems the ninth most expensive condition treated in US hospitals [1]. IVD degeneration is associated with a loss of disc height and hydration, diminished blood supply in the endplates, and anulus fibrosus tears [2–4]. Current therapies for treating disc degeneration include conservative non-surgical approaches and surgical intervention such as discectomy, spinal fusion and total disc replacement; however, these therapies do not restore the structure and function of the native IVD.

Disc degeneration is believed to originate in the nucleus pulposus (NP) region of the disc [3]; therefore, there is significant interest in tissue engineering strategies to regenerate the NP. The native NP is composed primarily of water, proteoglycans and collagen type II. Age-related degeneration of the NP is characterized by decreased water content, decreased cellularity, loss of proteoglycans in the extracellular matrix (ECM) and increased matrix stiffness [5,6]. Aging itself is associated with an early loss of the juvenile NP cell population that is originally derived from the embryonic notochord [7–9]. These large, highly vacuolated notochordal-like NP cells organize in cell clusters [7,10,11], synthesize a proteoglycan-rich ECM and secrete soluble mediators that regulate proteoglycan synthesis by other cell types [12–15]. Therefore, the aging-associated loss of this notochordal NP cell population has been hypothesized to be a contributing factor to IVD degeneration, and suggests that promoting or maintaining an immature, notochordal-like NP cell phenotype may be useful for NP tissue regeneration.

It is widely known that ECM protein composition and matrix elasticity play important roles in regulating cellular function. Previous studies have shown region-specific expression of the laminin family of ECM proteins and their receptors in immature IVD tissues [16–18]. Laminins are heterotrimeric ECM proteins that mediate numerous cellular processes such as adhesion, differentiation, migration and survival [19,20]. NP cells have been shown to attach to laminins in higher numbers as compared to cells from the adjacent anulus fibrosus, with cell attachment to the laminin-111 (LM111) isoform mediated by the integrin α3, α5, α6 and β1

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http://dx.doi.org/10.1016/j.actbio.2013.11.013

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 photocrosslinking [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-diacrylate 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-diacrylate (PEG-DA) (Fig. 1). LM111 (Trevigen®, Gaithersburg, MD) was PEGylated with acrylate–PEG–N-hydroxysuccinimide (Ac-PEG-NHS, MW = 10 kDa, Creative PEGworks, Winston Salem, NC) as described previously [46]. Briefly, Ac-PEG-NHS was added to a LM111 solution at varying molar excess Ac-PEG-NHS (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-NHS. 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 Speciality 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-NHS 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

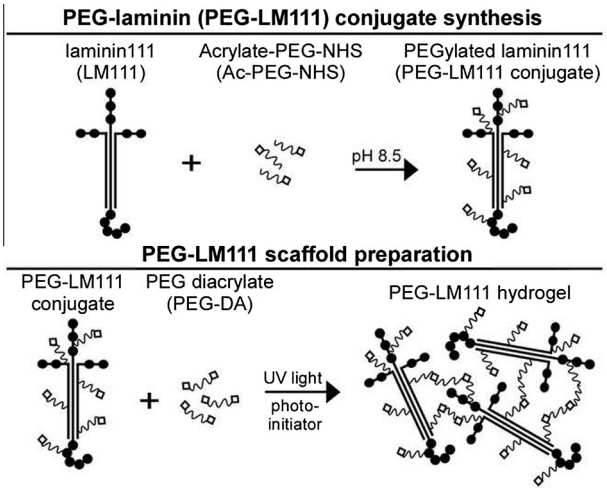

Fig. 1. Schematic of photocrosslinkable PEG-laminin (PEG-LM111) hydrogel preparation. PEG-LM111 conjugates were synthesized by the addition of Ac-PEG-NHS to introduce functional acrylate groups for crosslinking (top). Conjugates were mixed with additional PEG-DA and crosslinked via exposure to UV light to form PEG-LM111 hydrogels of varying stiffness and LM111 concentration.