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Evaluation of cell-laden polyelectrolyte hydrogels incorporating poly(L-Lysine) for applications in cartilage tissue engineering

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ABSTRACT

To address the lack of reliable long-term solutions for cartilage injuries, strategies in tissue engineering are beginning to leverage developmental processes to spur tissue regeneration. This study focuses on the use of poly(L-lysine) (PLL), previously shown to up-regulate mesenchymal condensation during developmental skeletogenesis *in vitro*, as an early chondrogenic stimulant of mesenchymal stem cells (MSCs). We characterized the effect of PLL incorporation on the swelling and degradation of oligo(poly(ethylene) glycol) fumarate) (OPF)-based hydrogels as functions of PLL molecular weight and dosage. Furthermore, we investigated the effect of PLL incorporation on the chondrogenic gene expression of hydrogel-encapsulated MSCs. The incorporation of PLL resulted in early enhancements of type II collagen and aggrecan gene expression and type II/type I collagen expression ratios when compared to blank controls. The presentation of PLL to MSCs encapsulated in OPF hydrogels also enhanced N-cadherin gene expression under certain culture conditions, suggesting that PLL may induce the expression of condensation markers in synthetic hydrogel systems. In summary, PLL can function as an inductive factor that primes the cellular microenvironment for early chondrogenic gene expression but may require additional biochemical factors for the generation of fully functional chondrocytes.

1. Introduction

Articular cartilage is the specialized connective tissue that enables frictionless bone articulation within synovial joints in the body. Its elegantly organized extracellular matrix comprises specialized collagens and proteoglycans that together contribute to the favorable viscoelastic and swelling properties of the tissue. Unlike bone, articular cartilage is devoid of any vascularity, lymphatics, and nerves [1]. Due to this avascularity and the low mitotic activity of its resident chondrocytes, articular cartilage inherently exhibits a limited endogenous capacity for regeneration. In the absence of long-lasting clinical solutions, such cartilage-related injuries continue to impose a significant economic burden on society [2] and remain a leading cause of global disability [3,4]. To address these shortcomings in the management of cartilage defects, current efforts are focused on the use of tissue engineering principles to develop better solutions.

By definition, hydrogels are three-dimensional (3D) polymer networks that consist of various hydrophilic components cross-linked to form water-insoluble matrices and have been investigated for applications in cartilage repair due to their highly favorable material properties [5]. These water-absorbent constructs can be fabricated from a wide variety of synthetic [6–9] or natural [10–16] materials for the encapsulation of mesenchymal stem cells (MSCs) and to provide *in vivo*-like conditions reminiscent of native articular cartilage microenvironments. While strategies aimed at maximizing their reparative capacity through the optimization of *in vitro* chondrogenic induction protocols have proven relatively successful [17–20], the ability to induce complete healing of damaged cartilage reliably [21] has not yet been achieved.

Recent strategies in cartilage tissue engineering are beginning to mimic specific stages in skeletogenesis with the goal of generating functional articular cartilage tissues [22–24]. Pre-cartilaginous condensation, a developmental process characterized by dense cellular aggregation and significantly increased cell–cell contacts, is believed to be critical in the governance of natural cartilage

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formation *in embryo* [25]. During condensation, the rapid onset of cell–cell interactions is largely mediated by a small number of key cell adhesion molecules, of which N-cadherin plays a special role [26–29]. Interestingly, previous research has demonstrated that the cationic polypeptide poly(L-Lysine) (PLL) possesses chondrostimulatory properties and can stimulate the chondrogenesis of mesenchymal micromass cells in developing chick limbs [30,31]. Several mechanisms for the physiological effects of PLL on chondrogenic differentiation have been proposed, including alteration of glycosaminoglycan biosynthesis and distribution, crosslinking of proteoglycans and cells, changing of cell morphology, and interaction with other biochemical cues [27]. Despite these promising reports, the use of PLL to induce embryonic signaling processes during chondrogenesis in cartilage tissue engineering remains heavily unexplored, especially within the context of 3D culture systems.

In this study, we hypothesized that the physical incorporation of PLL within hydrogels can stimulate the chondrogenic gene expression of encapsulated MSCs. To test this hypothesis, we utilized oligo(poly(ethylene glycol) fumarate) (OPF), a PEG-based macromer that can be crosslinked to yield hydrolytically degradable hydrogels of highly tunable mechanical and swelling properties [32,33], as a synthetic platform to isolate the effects of PLL. Considering the cationic nature of PLL and the presence of negative charges from the OPF macromers comprising the hydrogel backbone, it is anticipated that both moieties will form strong electrostatic interactions, leading to the bulk retention of PLL. Further, it is expected that the incorporation of PLL will affect the swelling and degradation of these hydrogel constructs. In order to evaluate these hypotheses, a number of specific objectives were established as follows: (i) to determine the amount of PLL retained or released from OPF hydrogels when incorporated at various concentrations; (ii) to characterize the swelling and degradation of PLL-laden OPF hydrogels as functions of PLL molecular weight (MW) and PLL loading amount; (iii) to assess the effects of PLL incorporation on encapsulated MSC chondrogenic gene expression as functions of PLL MW and PLL dosage; and (iv) to evaluate potential additive or synergistic effects between PLL presentation and OPF MW on MSC chondrogenic gene expression and condensation.

2. Materials and methods

2.1. Experimental design

The specific objectives of this overall investigation were completed via a series of individual studies, which were designed as follows. In order to determine the amount of PLL retained or released from OPF hydrogels, two experiments were designed as outlined in Table 1. For a third experiment, fluorescein isothiocyanate (FITC) conjugated PLL (PLL-FITC, 50 kDa) was loaded into OPF hydrogels at increasing concentrations (500 ng/construct, 5 μg/construct, or 20 μg/construct) according to Table 2.

Table 2
Experimental design for the determination of PLL-FITC release.

Group (n = 3)	OPF MWb (g/mol)	PLL MW (kDa)	PLL dosage (per hydrogel)
OPF	10K, 35K	50	–
OPF-500ng	10K, 35K	50	500 ng
OPF-5μg	10K, 35K	50	5 μg
OPF-20μg	10K, 35K	50	20 μg

A full factorial study was designed to characterize the effects of PLL MW and PLL loading on the swelling and degradation of synthetic OPF hydrogels. Specifically, the factors of (A) PLL MW, (B) PLL loading per construct, and (C) OPF MW were investigated as outlined in Table 3 in order to identify main and combinatory effects on OPF swelling and degradation. The PLL MWs of 50 and 225 kDa were determined based on previously reported values used in the chondrogenic stimulation of chick limb mesenchymal cells in micromass culture *in vitro* [30]. The PLL loading concentrations were chosen to yield a sufficiently wide range in order to elucidate the effects of PLL on bulk hydrogel swelling, where the low concentration levels reflect those used for the chondrogenic stimulation of cells in monolayer [34].

The effects of PLL presentation on cells encapsulated in such composite hydrogels were also investigated. As shown in Table 4, the effects of PLL MW and PLL dosage on MSCs encapsulated in OPF hydrogel composites were first investigated using a factorial design, where the MW factor comprised two levels (50 or 225 kDa) and the dosage factor consisted of two levels (250 or 500 ng/construct). Cell-laden hydrogels without any PLL incorporation were used as negative controls. The PLL MW (225 kDa) and amount (500 ng/construct) yielding the highest chondrogenic gene expression in encapsulated MSCs were chosen in the follow-up experiment, which investigated any potential additive or synergistic effects of PLL presentation in combination with OPF MW on MSC chondrogenic gene expression as shown in Table 5. These OPF MWs were chosen based on a previous study from our laboratory, which demonstrated that an increase in swelling ratio (due to an increase in nominal OPF MW from 10K to 35K) resulted in enhanced type II collagen gene expression [35].

2.2. OPF synthesis and characterization

OPF was synthesized using poly(ethylene glycol) (PEG) of nominal MWs of either 10,000 g/mol or 35,000 g/mol, according to previously described methods [32]. In brief, PEG was first dried via azeotropic distillation in toluene and dissolved in anhydrous dichloromethane. Triethylamine and fumaryl chloride were then added drop-wise to initiate the synthesis reaction, which was allowed to proceed for 2 days. The resultant product was purified by removing dichloromethane using rotoevaporation, separating salt precipitates with ethyl acetate, washing with ethyl ether, and

Table 1
Experimental design for the determination of PLL retention.

Group (n = 3)	Incubation condition	PLL loading method	PLL dosage (μg/hydrogel)
i.			
OPF	pH 7.4, 13	–	–
OPF-Pre	pH 7.4, 13	During Fabrication	20
OPF-Post	pH 7.4, 13	After Fabrication	20
ii.			
OPF	pH 7.4	–	–
OPF-Pre	pH 7.4	During Fabrication	20

i.) Samples were incubated at both pH conditions in parallel, and supernatants were collected and analyzed at 2 and 24 h post-fabrication. ii.) Supernatants were collected at 2 h, 1, 7, 14, and 21 days for analysis. Samples were then transferred to pH 13 incubation conditions for 24 h before collection and analysis.