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Promoting chondrocyte cell clustering through tuning of a poly(ethylene glycol)-poly(peptide) thermosensitive hydrogel with distinctive microarchitecture

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ABSTRACT

Hydrogels are considered to be attractive cell-matrix for chondrocytes due to their similarity in properties to the natural cartilage. However, the formation of chondrocyte cell clusters in hydrogels has been mostly limited to naturally-derived or relatively fast degrading materials. In this study, a series of diblock copolymer poly(ethylene glycol)-poly(alanine) (mPEG-PA) was synthesized and investigated as injectable biomimic hydrogels for the culturing of chondrocytes. Depending on the poly(alanine) chain length, afforded hydrogels exhibited variable mechanical property and microarchitecture due to difference in secondary structure arrangement. After 21 days of culture, cell clusters were observed in all hydrogels with longer PA chains and these hydrogels supported more homogenous and established clustering as well as significantly higher glycosaminoglycan and collagen deposition. Interestingly, scanning electron microscopy revealed a distinct micron range fibrillar-like microarchitecture that may be responsible for maintaining chondrocyte phenotype and matrix production. In addition, micrographs revealed the presence of collagen fibrils and an extensive extracellular matrix network. Therefore, it is reasonable to conclude that mPEG-PA hydrogels possess the desirable properties for chondrocyte cluster formation and serve as potential candidate in cartilage tissue engineering.

1. Introduction

The design and development of biomaterials suitable for cartilage tissue engineering is imperious due to the cartilage's inability to undergo self-repair. In addition, Rapid aging of the population is equated to drastic changes in medical demands, of which include treatment for age-related cartilage degeneration. Hydrogels are attractive candidates for cartilage generation due to their highly elastic and hydrated three dimensional networks that mimics the native cartilage tissue. Furthermore, thermosensitive hydrogels possess the additional clinical advantage of application via a minimally invasive procedure. Cells can be homogenously incorporated with the aqueous copolymer solution and injected into the desired site, where immediate gelation occurs due to physical crosslinking of the copolymer at the physiological temperature [1].

Classical thermoreversible hydrogels such as poly(ethylene oxide-b-propylene oxide-b-ethylene oxide) (PEO-PPO-PEO) [2,3], N-isopropylacrylamide-based copolymers [4,5], and PEO-poly(ester)-based systems [6,7] have been studied for their compatibility as cell carrier. The native extracellular matrix (ECM) has been described as a frame work of fiberous proteins embedded in a gelatinous polysaccharides matrix [8]. An important aspect of hydrogel design is the physical mimicking of the natural ECM. Scaffold microarchitecture and surface chemistry directly affect cell phenotype, attachment, and nutrient availability. In addition, the scaffold should be able to localize ECM proteins and biological cues to create a cell-friendly niche [9,10]. However, most hydrogels to date fall short of mimicking the delicate architecture of the natural ECM. Electrospinning is a technique that has been used to provide nano and micro-scale fibers valuable for cartilage engineering [11,12]. Poly(caprolactone) nanofibers has been shown increase the chondrogenic differentiation of mesenchymal stem cells [13,14].

Self-assembling polypeptide hydrogels, such as the marketed HydroMatrix™ or PuraMatrix™, exhibit striking similarity to the natural ECM and thus have been exploited for a variety of cell-carrying applications [15–18]. These poly(peptides) arrange into nanofibers that interweave to form stable β-sheet hydrogels [19]. Kisiday et al. has shown

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that chondrocytes seeded in peptide hydrogels retained their morphology after four weeks of culture [20]. Recently, Jeong et al. has reported a series of poly(ethylene)-poly(peptide)-based thermosensitive hydrogels that are capable of gelation at low concentrations and supporting 3D cell culture [21–23]. Therefore, theoretically, the formation of fiber-like thermosensitive hydrogels is possible through tuning of the copolymer.

In this study, we prepared and evaluated a hybrid hydrogel consisting of a hydrophilic polyethylene glycol (PEG) segment and a hydrophobic poly(peptide) chain, which can arrange into stable secondary structures that mimics the arrangement of poly(amino acids) inside the body. The poly(alanine) chain length was adjusted to provide hydrogels with different architecture arising from dissimilarity in secondary structure arrangement. Gelation and structural properties were investigated, and their applicability in cartilage generation was evaluated using chondrocytes. To the best of our knowledge, this is the first observation of significant growth in chondrocyte cell clusters as observed via fluorescent staining and scanning electron microscope with extended culture in a synthetic fiber-like thermosensitive hydrogel.

2. Experimental section

2.1. Material and methods

2.1.1. Materials

Methoxypolyethylene glycol Mw2000, L-alanine, triphosgene, triethylamine (TEA), mesyl chloride, and dimethylformamide (DMF) were purchased from Sigma (St. Louis, MO). Dichlormethane (DCM), tetrahydrofuran (THF), chlroform, and ammonia water (31–33%) were obtained from J.T. Baker (Phillipsburg, NJ). Dimethyl sulfoxide (DMSO), hexane, and ethyl ether were purchased from Echo Chemicals (Taiwan). Basal mediums and additives used for the preparation of cell culture medium were obtained from Gibco (Carlsbad, CA) and prepared according to instructions. All other materials were purchased and used as received and according to manufacturers' instructions.

2.1.1.1. Synthesis and characterization of mPEG-poly(alanine). Methoxypolyethylene glycol-poly(alanine) (mPEG-PA) was obtained by ring-opening polymerization of N-carboxyanhydride form of L-alanine using amine-terminated mPEG. Preparation of L-alanine-NCA was carried out by the addition of triphosgene (63.3 g, 213.3 mmol) dissolved in THF to finely grounded L-alanine (10 g, 110 mmol). The reaction was carried out for 8 h and the resulting product was filtered, concentrated, and precipitated to obtain L-alanine-NCA. Ring opening polymerization of L-alanine-NCA with amine terminated mPEG was carried out in the solvent DMF/CHCl3 = 1/3 for 3 days. The amount of L-alanine-NCA in the feed was adjusted according to the final poly(alanine) chain length desired. Briefly, 5 g(43.5 mmol) of L-alanine-NCA 5 g(2.5 mmol) of mPEG were mixed to provide mPEG-poly(alanine) of theoretical weight 2000–1500. Final products were precipitated, dissolved in DMSO for dialysis against deionized water (MW cut off 3000), and lyophilized.

Nuclear magnetic resonance (^{1}H NMR) spectra were recorded using a Varian Unity Inova 500 instrument with deuterated trifluoroacetic acid as solvent for synthesized mPEG-poly(alanine). TMS was used as the internal standard. Number average molecular weight and polydispersity (PDI) were determined by gel permeation chromatography (GPC) using an OHpack SB-803HQ column with water as the eluent at a flow rate of 1.0 ml/min and PEG (MW 400–5000) as the standard. GPC samples were prepared on ice and filtered through a 0.22 μM filter prior to sampling. An attenuated total reflectance (ATR) module was used on a Perkin Elmer BX FT-IR system for verification of chemical structure.

2.1.1.2. Preparation and gelation properties of copolymer solutions. Characterization of secondary structures in diluted copolymer solutions (0.02% *w*/*v* in deionized water and filtered) and as a function of temperature was recorded using an Aviv 202 circular dichroism spectrometer. Results are the representation of the average of ten scans per sample. Secondary structure predictions were made with a CDNN deconvolution software [24].

Sol–gel transition profile of copolymers in PBS was determined using the inverted test tube method from 4 °C to 80 °C at 2 °C increments. Briefly, copolymer solutions were prepared at various concentrations and placed on a rotary mixer overnight to allow complete dissolution. The solution to gel transition point was taken as the temperature where the gel become non-flowing when the tube was inverted and lightly agitated.

Rheological measurements were conducted using a TA instrument AR-2000Ex rheometer. Approximately 600 μL of the copolymer dissolved in PBS was loaded onto the pre-chilled system equipped with a 40 mm parallel plate geometry. The temperature was varied from 10 °C to 45 °C at a heating rate of 1 °C/min with a controlled stress of 10 Pa and frequency of 1 Hz. The gelation temperature was defined as the point where storage modulus (G′) > elastic modulus (G″).

ATR-FTIR was conducted on D_2O solubilized copolymers (5% *w*/*v*) to determine the presence and change in secondary structure as a function of poly(alanine) chain length.

2.1.1.3. Incorporation of chondrocytes. Chondrocytes were isolated from ears of New Zealand white rabbits. The cartilage tissue was minced with scissors, cut into 1 mm^3 fragments, and washed thoroughly with PBS. Each fragment was digested for 5 h at 37 °C with 1 mL of collagenase solution (4 mg/mL). Digested tissue was passed through a 100 μm filter to obtain chondrocytes. The cells were washed with PBS, counted, and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% anti-anti with 5% CO^2. At 80% confluency, chondrocytes were harvested by trypsin, suspended in DMEM, centrifuged, and diluted to a concentrated cell suspension. Copolymer solutions and cells were mixed to obtain the final copolymer concentration of 5% (*w*/*v*) and cell density of 1×10^6 cell/mL. The copolymer/cell mixture (30 μL) was injected into 1 mL of prewarmed chondrogenic defined medium (CDM) (DMEM, 10^{-7} M dexamethasone, 50 mg/mL ascorbic acid, 50 mg/mL L-proline, 1% anti-anti, and 1% ITS(insulin, transferrin, selenium)). Medium was changed every 3 days by removing 500 μL and replenishing 500 μL.

2.1.1.4. Biochemical analysis. DNA, glycosaminoglycan (GAGs), and total collagen quantification were conducted by first digesting the cell-laden scaffolds with papain for 24 h in a 60 °C oven. DNA quantification was carried out using the Invitrogen Quant-iT DNA Assay Kit, high sensitivity. Then, GAGs content was analyzed using the 1,9-dimethylmethylene blue dye-binding (DMMB) assay with known concentrations of chondroitin-6-sulfate as the standard. Samples were read with a p Biotek Synergy HT plate reader at 540 nm. Total collagen content was determined according to a previously described method, with hydroxyproline as the standard. Hydroxyproline:collagen ratio is approximately 1:10.

2.1.1.5. Gene expression of encapsulated chondrocytes. For quantitative real-time polymerase chain reaction (PCR), RNA was isolated from the hydrogels at predetermined time points using the TRIsure® reagent according to protocol. Then, a MMLV HP RT kit was used for reverse transcription of total RNA to cDNA, which was stored at −20 °C prior to use. Three different genes were analyzed including type I collagen (COLI), type II collagen (COLII), and aggrecan (AGG) using Applied Biosystems SYBR Green PCR master mix on an ABI Prism 7300 system. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was selected as the housekeeping gene. Table 1 lists the primer sequences used.