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Nondestructive evaluation of a new hydrolytically degradable and photo-clickable PEG hydrogel for cartilage tissue engineering

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ABSTRACT

Photopolymerizable and hydrolytically labile poly(ethylene glycol) (PEG) hydrogels formed from photo-clickable reactions were investigated as cell delivery platforms for cartilage tissue engineering (TE). PEG hydrogels were formed from thiol-norbornene PEG macromers whereby the crosslinks contained caprolactone segments with hydrolytically labile ester linkages. Juvenile bovine chondrocytes encapsulated in the hydrogels were cultured for up to four weeks and assessed biochemically and histologically, using standard destructive assays, and for mechanical and ultrasound properties, as nondestructive assays. Bulk degradation of acellular hydrogels was confirmed by a decrease in compressive modulus and an increase in mass swelling ratio over time. Chondrocytes deposited increasing amounts of sulfated glycosaminoglycans and collagens in the hydrogels with time. Spatially, collagen type II and aggrecan were present in the neotissue with formation of a territorial matrix beginning at day 21. Nondestructive measurements revealed an 8-fold increase in compressive modulus from days 7 to 28, which correlated with total collagen content. Ultrasound measurements revealed changes in the constructs over time, which differed from the mechanical properties, and appeared to correlate with ECM structure and organization shown by immunohistochemical analysis. Overall, non-destructive and destructive measurements show that this new hydrolytically degradable PEG hydrogel is promising for cartilage TE.

Statement of Significance

Designing synthetic hydrogels whose degradation matches tissue growth is critical to maintaining mechanical integrity as the hydrogel degrades and new tissue forms, but is challenging due to the nature of the hydrogel crosslinks that inhibit diffusion of tissue matrix molecules. This study details a promising, new, photo-clickable and synthetic hydrogel whose degradation supports cartilaginous tissue matrix growth leading to the formation of a territorial matrix, concomitant with an increase in mechanical properties. Nondestructive assays based on mechanical and ultrasonic properties were also investigated using a novel instrument and found to correlate with matrix deposition and evolution. In sum, this study presents a new hydrogel platform combined with nondestructive assessments, which together have potential for *in vitro* cartilage tissue engineering.

1. Introduction

Hyaline, articular cartilage is the tissue that covers the osseous ends in diarthrodial joints and is responsible for absorbing forces and allowing for a smooth, almost frictionless motion between the articulating surfaces [1]. Once damaged, its intrinsic capacity for repair is low. Cartilage defects that are left untreated can lead to the onset of secondary osteoarthritis [2], which has a high socio-economic burden [3]. Clinically available treatment options for defects in articular cartilage, such as microfracture [4], mosaicplasty [5] and autologous chondrocyte implantation [6], still fail to demonstrate reproducible success. The poor outcome is associated with the formation of a fibrous, mechanically inferior tissue [7]. Matrix assisted autologous chondrocyte implantation, whereby

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cells are delivered within a three-dimensional (3D) matrix, has the potential to overcome this shortcoming. However, the ideal matrix has yet to be identified.

Poly(ethylene glycol) (PEG) hydrogels have shown promise as a cell encapsulation platform for cartilage tissue engineering (TE) [8–11]. However, the tight mesh of the hydrogel crosslinks, which maintains cells within the 3D hydrogel, impedes diffusion of the cell-secreted extracellular matrix (ECM) molecules and thus inhibits macroscopic tissue growth [12,13]. The introduction of labile bonds within the crosslinks is therefore necessary to promote tissue growth. However, the challenge is matching the rate of degradation with the rate of neo-tissue synthesis, which is necessary to preserve the mechanical integrity of the material while at the same time, providing adequate space for the deposition of newly synthesized ECM. Degradable hydrogels formed from purely synthetic based chemistries remain a promising approach given the high fidelity and reproducibility of synthetic materials. Previous studies have used PEG hydrogels formed from PEG dimethacrylate monomers containing poly(lactic acid) (PLA) segments to introduce hydrolytically labile esters within each crosslink. These studies have demonstrated that if degradation of the hydrogel occurs too fast, the overall construct mechanical properties drop dramatically with time despite the presence of elaborated ECM molecules [14].

In an effort to identify an improved purely synthetic and degradable PEG hydrogel for cartilage TE, the present study investigated degradable PEG hydrogels synthesized from a thiol-ene photoclickable hydrogel platform [15], but with the introduction of caprolactones. Previous work from our group demonstrated that free-radical polymerization using thiol-norbornene macromers led to a mild encapsulation environment when compared to acrylate macromers due to the nature of the types of radicals formed during encapsulation [8]. This resulted in a deposition of neo-tissue that more closely resembled hyaline cartilage. In addition, the introduction of ester bonds within the crosslinks offers a strategy to introduce hydrolytic degradation. The rate of hydrogel degradation can be, to some extent, controlled by changing the crosslink density of the material, the chemistry of the ester linkage (e.g., caprolactone) and by changing the number of ester bonds within the crosslink [16–18].

Standard assays, used for assessing cartilaginous tissue production or quantifying the cell number within hydrogel constructs, are typically destructive [19–21]. A method that allows for evaluating the progression of cartilage matrix production *in situ*, without the need to sacrifice the sample would harbor many advantages, such as reducing sample numbers, enabling real time adjustments to the culture conditions, and offering an on-line assessment for clinically, *in vitro* grown engineered cartilage. In the current study, an instrument with combined capabilities of mechanical assessment and ultrasound was investigated as a means for nondestructive evaluation of the hydrogel constructs [22]. Ultrasound measurements of hydrogels, specifically agarose, have been correlated with mechanical properties of the material [23]. In addition, ultrasound measurements of cartilage have been correlated with mechanical properties of the tissue as well as its collagen content [24,25]. Ultrasound has also been previously demonstrated to show promise when investigating neo-cartilage tissue deposited in synthetic hydrogels [22,26].

The overall goal of the present study was to assess whether non-destructive measurements could be used to track the progress of neo-tissue development in a modern degradable scaffold system and perhaps then be useful as a quality control variable in the *ex-vivo* manufacture of engineered tissue. A new, photo-clickable and hydrolytically degradable PEG hydrogel formed from thiol-norbornene macromers containing caprolactone segments for cartilage TE was used for the assessment. Hydrogel degradation was first confirmed in acellular hydrogels. Juvenile bovine chondrocytes were encapsulated into the hydrogel and neo-tissue assessed for up to 4 weeks, and analyzed weekly. Standard, destructive assays were conducted to quantify the amount of DNA, sulfated glycosaminoglycans (sGAGs), and total collagen, as well as the spatial distribution of ECM within the constructs. Nondestructive assays were conducted based on mechanical properties and ultrasound measurements. The non-destructive assays were compared to the standard destructive assays to further evaluate their potential in assessing the evolution and quality of the neo-tissue in synthetic degrading hydrogels.

2. Materials and methods

2.1. Materials

Penicillin-Streptomycin (P/S), Fungizone and GlutaGRO™ were from Corning® Cellgro (Manassas, VA)[1]. Hoechst 33258 was from Polysciences, Inc. (Warrington, PA). Irgacure 2959 (I2959) was from BASF (Tarrytown, NY). Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). Retrievagen A antigen retrieval solution was from BD Biosciences (San Jose, CA). Keratanase I was from MP Biomedical (Solon, OH). Collagenase type II and papain were from Worthington Biochemical (Lakewood, NJ). Ethyl ether, ethylene diamine tetra acetic acid (EDTA) and Triton X-100 were from Fischer Scientific (Fair Lawn, NJ). The LIVE/DEAD® assay, phosphate-buffered saline (PBS), gentamycin, HEPES buffer, minimal essential medium non-essential amino acids (MEM-NEAA), trypan blue, DAPI, AlexaFluor 488-conjugated goat anti-rabbit IgG and AlexaFluor 546-conjugated goat anti-mouse IgG were from Invitrogen (Carlsbad, CA). PEG (M_W 20,000) was from JenKem Technology USA (Plano, TX). L-proline, L-ascorbic acid, bovine serum albumin (BSA), dimethyl methylene blue (DMMB), ε-Caprolactone, stannous octoate, 5-norbornene-2-carboxylic acid, N,N′-Diisopropylcarbodiimde (DIC), pyridine, 4-(Dimethylamino)pyridine (DMAP), chondroitinase ABC and hyaluronidase were from Sigma-Aldrich (St Louis, MO). Mouse anti-aggrecan antibody (A1059-53E) and rabbit anti-collagen II antibody (C5710-20F) were from US Biologicals (Swampscott, MA). Fluoromount-G® was from SouthernBiotech (Birmingham, AL).

2.2. Macromer synthesis

Macromers of PEG-caprolactone (PEG-CAP) endcapped with norbornene (PEG-CAP-NOR) were synthesized by first reacting 8-arm PEG-hexaglycerol (20 kDa) with ε-caprolactone and stannous octoate as the ring-opening catalyst following protocols adapted from [27]. All reactions were conducted in argon purged reaction vessels unless mentioned otherwise. Briefly, the 8-arm PEG-hexaglycerol was melted at 90 °C and then reacted with 1.5-fold molar excess of ε-caprolactone (per arm of the PEG molecule) in the presence of stannous octoate under vacuum at 140 °C for 6 h. The PEG-CAP product was purified by precipitation in ice-cold ethyl ether. PEG-CAP was subsequently reacted with 5-norbornene-2-carboxylic acid (10 M excess), DIC (10 M excess), DMAP (1 M excess), pyridine (10 M excess) in dichloromethane under argon and overnight at room temperature (RT). The final PEG-CAP-NOR product was purified by filtration over active carbon and precipitation in ice-cold ethyl ether. The product was then dried, re-dissolved in a minimum amount of chloroform and further purified by two washes in glycine buffer (0.05 M sodium chloride, 0.05 M sodium hydroxide, 0.05 M glycine) and one wash in

[1] Certain commercial equipment, instruments, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.