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Comparison of photopolymerizable thiol-ene PEG and acrylate-based PEG hydrogels for cartilage development

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ABSTRACT

When designing hydrogels for tissue regeneration, differences in polymerization mechanism and network structure have the potential to impact cellular behavior. Poly(ethylene glycol) hydrogels were formed by free-radical photopolymerization of acrylates (chain-growth) or thiol-norbornenes (step-growth) to investigate the impact of hydrogel system (polymerization mechanism and network structure) on the development of engineered tissue. Bovine chondrocytes were encapsulated in hydrogels and cultured under free swelling or dynamic compressive loading. In the acrylate system immediately after encapsulation chondrocytes exhibited high levels of intracellular ROS concomitant with a reduction in hydrogel compressive modulus and higher variability in cell deformation upon compressive strain; findings that were not observed in the thiol-norbornene system. Long-term the quantity of sulfated glycosaminoglycans and total collagen was greater in the acrylate system, but the quality resembled that of hypertrophic cartilage with positive staining for aggrecan, collagens I, II, and X and collagen catabolism. The thiol-norbornene system led to hyaline-like cartilage production especially under mechanical loading with positive staining for aggrecan and collagen II and minimal staining for collagens I and X and collagen catabolism. Findings from this study confirm that the polymerization mechanism and network structure have long-term effects on the quality of engineered cartilage, especially under mechanical loading.

1. Introduction

Free-radical polymerizations have emerged as a promising strategy to encapsulate cells in hydrogels owing to their fast gelation times occurring at physiological temperature and pH [1–3]. Photopolymerization offers additional spatial and temporal control over the reaction allowing for *in situ* polymerization of materials with well-defined structures [4,5]. Numerous natural [6–9] and synthetic [10–14] polymers have been functionalized with vinyl groups to form hydrophilic macromolecular monomers, or macromers, that can be polymerized via free-radical polymerization to form hydrogels. To this end, a wide range of macromer chemistries and hydrogel properties can be achieved and subsequently tailored to a particular cell type. Furthermore, reaction conditions for free-radical photopolymerization have been identified which enable encapsulation of cells while maintaining high viability [11,15,16], making photopolymerization promising for tissue engineering and *in vivo* cell delivery. However, the choice of polymerization mechanism (e.g., the type of polymerizable moiety) and resulting network structure could have a significant effect on cell function and long-term tissue development.

Depending on the polymerizable moiety chosen for free-radical mediated polymerization, multi-functional macromers can undergo chain or step-growth polymerization [11,17,18]. These polymerization mechanisms can be initiated under similar conditions (e.g., photoinitiation involving the same photoinitiator, wavelength, and light intensity). However distinct differences arise during polymerization especially when oxygen is present. The primary differences include the type of propagating radical and its reactivity with oxygen. Molecular oxygen acts as a radical scavenger resulting in the formation of reactive oxygen species (ROS) such as peroxy radicals [19,20]. Free-radical polymerization of chain-growth (meth)acrylates is notorious for being inhibited by oxygen leading to an accumulation of ROS. On the other hand, free-radical step-growth polymerization between a thiol and vinyl group (referred to as 'ene') can be propagated by ROS and thus consume ROS [21].

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Extracellular ROS is known to generate oxidative stress in cells [22,23] and has been linked to inhibition of tissue synthesis and upregulation of tissue degrading enzymes [24,25]. The differences in reactivity with oxygen between the thiol-ene and acrylate systems may therefore differentially affect cells during encapsulation.

Another distinct difference that arises, depending on the polymerization mechanism, is polymer network structure. When radical mediated chain-growth polymerizations of water soluble (meth)acrylate macromers are performed in aqueous solutions, polydisperse and hydrophobic poly(meth)acrylate kinetic chains are formed. These kinetic chains lead to heterogeneities in crosslink density and therefore network structure [17,26]. In contrast, thiol-ene polymerizations proceed by an orthogonal, step-growth mechanism where one thiol reacts with one ene leading to a more homogenous distribution in crosslinks [11,17]. For applications in tissue engineering where mechanical forces are prevalent, differences in network structure may impact how stresses and strains are translated through the hydrogel [27] into local mechanical cues perceived by the cells.

This study therefore aimed to determine whether differences arising between free-radical polymerized hydrogels formed by two distinct mechanisms, acrylates through chain-growth and thiol-enes through step-growth, have an effect on encapsulated cells for cartilage tissue engineering. Specifically, this study posed three research questions. 1) Does photopolymerization by chain-growth and step-growth differentially affect cells during encapsulation in hydrogels? 2) Does the network structure formed by chain-growth and step-growth polymerizations differentially translate mechanical cues to encapsulated cells in the form of cell deformation? 3) Does the combination of polymerization mechanism and network structure have long-term effects on tissue production and composition when cells are encapsulated in hydrogels and subjected to mechanical loading? To address these questions, synthetic macromers based on poly(ethylene glycol) (PEG) were employed, which were functionalized to polymerize by radical mediated chain-growth (e.g., PEG diacrylate [28]) or step-growth (e.g., PEG tetranorbornene and PEG dithiol [11]) mechanisms and which form stable or degrading hydrogels [11,12,17]. Cartilage cells (i.e., chondrocytes) were investigated because chondrocytes maintain their phenotype when encapsulated in synthetic hydrogels [29] and mechanical forces are critical to their function [30,31].

2. Materials and methods

2.1. Macromer synthesis

Poly(ethylene glycol) diacrylate (PEGDA, 4600 Da) and hydrolytically degradable oligo(lactic acid)-*b*-PEG-*b*-oligo(lactic acid) diacrylate (PEG-LA-DA, 4600 Da, 1.3 lactic acid per side) were synthesized as described previously [13]. PEG-tetranorbornene (PEGTNB) was synthesized by reacting 4arm-PEG-NH_2 (5000 Da, JenKemUSA) with 4 M excess 5-norbornene-2-carboxylic acid (Sigma) in dimethylformamide in the presence of 2 M excess 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU, AKSci) and 2 M excess N,N-diisopropylethylamine (DIEA, Sigma). PEGTNB was precipitated in ice-cold diethyl ether, dialyzed against diH_2O, sterile filtered and the final product was collected after lyophilization. Hydrolytically degradable PEG-tetranorbornene (degPEGTNB) was synthesized as described previously [11]. PEG-dithiol (PEGDSH) was purchased (3400 Da, LaysanBio). Functionalization of PEG with acrylate or norbornene was determined using ^{1}H nuclear magnetic resonance (NMR) imaging. Percent substitution was determined by comparing the area under the carbon–carbon double bond peak associated with the acrylate (5.8, 6.2, 6.4ppm) or the area under the alkene peak associated with the norbornene (~6 ppm) to the area under the peaks of the methyl groups in PEG (~3.6 ppm). Acrylate substitution (two per PEG molecule) was confirmed to be 70–100%. Norbornene substitution (four per PEG molecule) was confirmed to be 95–100%.

2.2. Cell isolation, encapsulation and viability

Full-depth articular cartilage was harvested from the patellar-femoral groove of up to two 1–3 week-old calves (Research 87) within 24 h of slaughter. The tissue was digested in 500 units/mL collagenase type II (Worthington Biochemical) following previously published protocols [32]. Freshly isolated cells were >85% viable determined by trypan blue exclusion. PEGDA, PEG-LA-DA, PEGTNB:PEGDSH (1 ene:1 thiol), degPEGTNB:PEGDSH (1 ene:1 thiol) were dissolved in PBS to final concentrations of 6–11% (w/w) with 0.05% (w/w) photoinitiator I2959 (1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one) (Ciba-Geigy). Macromer concentration was varied such that the tangent compressive modulus of the hydrogels in their swollen state was similar for the two different hydrogel systems. Freshly isolated chondrocytes were suspended in sterile macromer/photoinitiator solutions at a final concentration of 50×10^6 chondrocytes/mL. A 50 μL volume of cell suspension was polymerized using 352 nm light at 6 mW/cm^2 for 10 min into cylindrical constructs (~5 mm in diameter and ~2.5 mm in height). Cell-hydrogel constructs were incubated at 37°C and 5% CO_2 in chondrocyte medium (DMEM supplemented with 10% FBS (v/v), 0.04 mM L-proline, 50 mg/L L-ascorbic acid, 10 mmol HEPES per L, 0.1 mol Minimum Essential Medium with nonessential amino acids/L, 1% penicillin/streptomycin, 0.5 μg/mL fungizone, and 20 μg/mL gentamicin). Cell viability of the encapsulated cells was determined by a membrane integrity assay (Live/Dead® Cell Viability Assay (Invitrogen)), which stains live cells green and dead cells red, and imaged on a Zeiss LSM 5 Pa confocal microscope.

2.3. Cell culture and mechanical loading

Hydrogels were cultured under free swelling conditions or in custom bioreactors [32] at 37°C and 5% CO_2 for up to 46 days. Culture medium was replaced every 2–3 days. After 24 h of free swelling culture (referred to as day 0), hydrogels were placed into the bioreactor between permeable platens made of Porex® HDPE (40–70 μm) and subjected to unconfined compressive loading applied intermittently from 0 to 10% strain in a sinusoidal waveform at a frequency of 1 Hz (8 cycles/day of 0.5 h on, 1.5 h off, followed by 8 h off).

2.4. Hydrogel degradation

Acellular PEG-LA-DA and degPEGTNB:PEGDSH were photopolymerized using identical sterile conditions as described above. Each hydrogel was weighed immediately after polymerization to determine initial polymer mass (m_{pi}), then placed in chondrocyte medium, which was replaced every 2–3 days, and in an incubator at 37°C and 5% CO_2. At specified time points, hydrogels were lyophilized and their dry polymer mass (m_p) determined. Percent mass loss was determined by

$$\text{Mass loss} = \left(\frac{m_{pi} - m_{pi}}{m_{pi}}\right) \times 100$$

2.5. Intracellular reactive oxygen species (ROS)

Intracellular ROS was qualitatively assessed immediately after polymerization in PEGDA and PEGTNB:PEGDSH constructs. In brief, chondrocytes in suspension were incubated with carboxy-H_2DFFDA (carboxy-2,7-difluorodihydrofluorescein diacetate, Invitrogen) for 20 min, rinsed in PBS via centrifugation, and encapsulated in PEG hydrogels as described above. Immediately post-encapsulation, hydrogels were imaged on a Zeiss LSM 5 Pa confocal microscope. A sample size of 3 was used.

2.6. Mechanical testing

Shear: *In situ* dynamic photorheology [33] was used to measure evolution of shear storage (G′) and loss (G″) moduli during photopolymerization of PEGDA and PEGTNB:PEGDSH in the absence or presence chondrocytes at 50 million cells/mL ($n = 4$ per condition). A rheometer (Ares 4400, TA Instruments) was modified to enable simultaneous irradiation (320–390 nm; 6 mW/cm^2) of the sample and moduli measurements during a dynamic time sweep with a frequency of 10 Hz and strain of 10% [15]. Plate diameter was 20 mm and plate separation was 50 μm.

Compression: Tangent compressive modulus was determined for chondrocyte-laden constructs at specified time points during culture under unconfined compression (MTS Synergie 100) applied at a constant strain rate (0.5 mm/min).

2.7. Cell deformation

Chondrocyte-laden PEGDA and PEGTNB:PEGDSH constructs with similar tangent moduli were cultured under free swelling conditions in chondrocyte medium for 18 h. Cell-laden hydrogels were treated with 2 μM calcein-AM to stain the cytosol green and then placed in a custom-built straining device and imaged on an inverted confocal microscope [31]. A static compressive strain was applied to the hydrogel at 0% strain and then at 20% strain. Fifty cells per hydrogel ($n = 4$) per strain condition were imaged. The diameter at full-width half-maximum height of each cell was measured along the axis parallel (x) and perpendicular (y) to the applied strain using NIH ImageJ software. Cell deformation was assessed by a diameter ratio (x/y).