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PEG hydrogels formed by thiol-ene photo-click chemistry and their effect on the formation and recovery of insulin-secreting cell spheroids

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ABSTRACT

Hydrogels provide three-dimensional frameworks with tissue-like elasticity and high permeability for culturing therapeutically relevant cells or tissues. While recent research efforts have created diverse macromer chemistry to form hydrogels, the mechanisms of hydrogel polymerization for in situ cell encapsulation remain limited. Hydrogels prepared from chain-growth photopolymerization of poly(ethylene glycol) diacrylate (PEGDA) are commonly used to encapsulate cells. However, free radical associated cell damage poses significant limitation for this gel platform. More recently, PEG hydrogels formed by thiol-ene photo-click chemistry have been developed for cell encapsulation. While both chain-growth and step-growth photopolymerizations offer spatial-temporal control over polymerization kinetics, step-growth thiol-ene hydrogels offer more diverse and preferential properties. Here, we report the superior properties of step-growth thiol-ene click hydrogels, including cytocompatibility of the reactions, improved hydrogel physical properties, and the ability for 3D culture of pancreatic β-cells. Cells encapsulated in thiol-ene hydrogels formed spherical clusters naturally and were retrieved via rapid chymotrypsin-mediated gel erosion. The recovered cell spheroids released insulin in response to glucose treatment, demonstrating the cytocompatibility of thiol-ene hydrogels and the enzymatic mechanism of cell spheroids recovery. Thiol-ene click reactions provide an attractive means to fabricate PEG hydrogels with superior gel properties for in situ cell encapsulation, as well as to generate and recover 3D cellular structures for regenerative medicine applications.

1. Introduction

Hydrogels are a class of hydrophilic, crosslinked polymers that serve as ideal matrices for cell encapsulation and delivery [1,2], as well as for controlled release of biomacromolecules for tissue regeneration [3,4]. Many polymers, synthetic or natural, have been utilized to create hydrogels for biomedical applications. For example, derivatives of poly(ethylene glycol) (PEG) macromers have been widely used due to their tissue-like elasticity, well-defined chemistry, and tunable biochemical, biophysical, and biomechanical properties. Coupling with photopolymerizations as a gelation mechanism, PEG hydrogels can be synthesized with spatial-temporally defined features and properties to control cellular activities, such as spreading, migration, and differentiation [5–7].

PEG diacrylate (PEGDA) hydrogels crosslinked from radical-mediated chain-growth photopolymerizations have been used in numerous drug delivery and cell encapsulation studies [8–16]. The crosslinking density of PEGDA hydrogels can be easily controlled to yield gels with different levels of elasticity and water content, which affect biomolecular transport and cell survival. To obtain high hydrogel mesh size (ξ) for facile biomolecular transport, and thus enhancing cell survival in PEGDA hydrogels, PEG macromers with higher number average molecular weights ($\overline{M_n} > 10$ kDa) are usually preferred [11,17,18]. The use of higher molecular weight PEGDA, however, often leads to decreased radical propagation rate since high $\overline{M_n}$ polymers have lower molar concentrations of functional groups (e.g., acrylates) per unit mass. This also results in decreased polymerization efficiency and higher sol fraction at lower polymer contents. Furthermore, free radicals initially generated from the photoinitiators have long half-life in chain-growth polymerizations because radicals can propagate through vinyl groups on PEGDA, causing high cellular damage during in situ cell encapsulation.

Recently, PEG-peptide hydrogels based on step-growth thiol-ene photopolymerizations have been developed to overcome the disadvantages of chain-growth polymerizations while retaining the advantages of photopolymerizations [19]. Multi-arm PEG-norbornene macromers (e.g., 4-arm PEGNB or PEG4NB) are crosslinked by

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matrix metalloproteinase (MMP) cleavable peptides flanked with bis-cysteines via step-growth photopolymerizations [19]. The resulting thiol-ene networks are more homogeneous and have higher functional group conversion when comparing to chain-growth polymerized gels with similar crosslinking density. Thiol-ene photopolymerization is considered a 'click' reaction due to the rapid and orthogonal reaction between the ene and thiol functionalities. Furthermore, all advantages offered by photopolymerizations (e.g., spatial-temporal control over reaction kinetics) are retained in thiol-ene hydrogels [19].

Thiol-ene hydrogels have emerged as an attractive class of hydrogels for studying 3D cell biology [20,21], for controlled release of therapeutically relevant proteins [22], for directing stem cell differentiation [23], and for promoting tissue regeneration [24]. A variety of cell types have been successfully encapsulated in PEG-norbornene hydrogels, including fibroblasts [19,20], valvular interstitial cells (VICs) [21], mesenchymal stem cells (MSCs) [23], and fibrosarcoma cells (HT-1080) [20]. In addition, enzyme-sensitive, surface-eroding thiol-ene hydrogels have also been developed for enzyme-responsive protein delivery [22].

One emerging application of photopolymerized PEG hydrogels is the fabrication of bioactive and immuno-isolating barriers for encapsulation of cells, including insulin-secreting pancreatic β-cells [11,13–15,25]. Photopolymerizations offer an attractive means for rapid and convenient encapsulation of β-cells, while PEG hydrogels provide a framework from which to conjugate diverse functionalities for promoting or suppressing specific cell functions. Despite tremendous efforts toward creating permissive and promoting microenvironments for β-cells, challenges remain and the field of β-cell delivery may benefit from a highly cytocompatible gel system that causes minimum, if any, cellular damage during in situ cell encapsulation. The major hurdle to the success of photopolymerized PEG hydrogels in β-cells encapsulation is the necessary use of photoinitiator, which, upon light exposure, generates free radicals that may cause stresses and cellular damage during the encapsulation processes [11].

In this contribution, we report the superior cytocompatibility of step-growth thiol-ene click reactions and hydrogels for pancreatic β-cells (MIN6). Using chain-growth photopolymerized PEGDA hydrogels as controls, we studied the cytocompatiblity of the thiol-ene reactions, as well as the physical properties of the resulting hydrogels. We further developed a thiol-ene hydrogel system composed of a PEG4NB macromer and a simple bis-cysteine-terminated and chymotrypsin-sensitive peptide sequence (CGGY↓C, arrow indicates enzyme cleavage site) for the encapsulation of MIN6 β-cells. The survival, proliferation, and formation of β-cells spheroids in this thiol-ene hydrogel system were systematically studied. Finally, we characterized the erosion of this unique chymotrypsin-sensitive gel system and utilized it for the rapid recovery of viable and functional 3D β-cell spheroids formed naturally in these thiol-ene hydrogels.

2. Materials and methods

2.1. Materials

4-arm PEG (20 kDa) and PEG monoacrylate (PEGMA, 4 kDa) were obtained from JenKem Technology USA and Monomer-Polymer Dajac & Labs, respectively. Fmoc-amino acids were purchased from Anaspec. CellTiter Glo® and Alamarblue® reagents were obtained from Promega and AbD Serotec, respectively. Trypsin-free α-chymotrypsin was obtained from Worthington Biochemical Corp. Live/Dead cell viability kit for mammalian cells was purchased from Invitrogen. All other chemicals were obtained from Sigma–Aldrich unless noted otherwise.

2.2. PEG4NB, PEGDA, and photoinitiator lithium arylphosphanate (LAP) synthesis

4-arm PEG-norbornene (PEG4NB) was synthesized according to an established protocol [19] with slight modification. Briefly, *N,N′*-dicyclohexylcarbodiimide (DCC, 2.5X) was reacted with norbornene acid (10X) in dichloromethane (DCM) to form an intermediate product - norbornene carboxylic acid O-acyl-urea, followed by the formation of norbornene anhydride and by-product dicyclohexylurea. Norbornene anhydride was filtered through a fritted funnel and added into a second flask containing pre-dissolved 4-arm PEG-OH, 4-(dimethylamino)pyridine (DMAP, 0.5X), and pyridine (5X) in DCM. All reactions were performed under nitrogen. The flask was placed in an ice bath and the reaction was allowed to proceed overnight. The product was filtered and redissolved in DCM and then precipitated in cold ethyl ether. The precipitated product was extracted with ether in an Allihn condenser extractor system at 50 °C for 48 h, followed by drying in a desiccator. The degree of functionalization (>90%) was characterized by proton NMR.

The synthesis of PEGDA [12,26] and photoinitiator lithium arylphosphanate (LAP) [27] was performed according to published protocols.

2.3. Peptide synthesis

All peptides were synthesized using standard solid phase peptide synthesis in a microwave peptide synthesizer (CEM Discover SPS). Briefly, Fmoc-Rink-amide-MBHA resin was swell in dimethylformamide (DMF) for 15 min. The deprotection procedures (in 20% piperidine/DMF with 0.1M HOBt) were performed in the peptide synthesizer for 3 min at 75 °C with microwave power set at 20 W. Fmoc-protected amino acids (5-fold molar excess) were dissolved in an activator solution (0.28M DIEA in DMF) containing HBTU (5-fold molar excess). The activated Fmoc-amino acid solution was added to the deprotected resin and the coupling reactions were performed in the synthesizer for 5 min at 75 °C and 20 W. The coupling of Fmoc-Cys(Trt)-OH was performed at 50 °C to decrease the racimification reaction. Ninhydrin test was conducted after each coupling and deprotection step to ensure completion of each step. In rare cases, amino acid coupling reactions were repeated until a negative Ninhydrin test result was obtained. The synthesized peptides were cleaved in 5 mL cleavage cocktail (95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIPS), 2.5% distilled water, and 250 mg of phenol) in the synthesizer for 30 min at 38 °C and 20 W. Cleaved peptides were precipitated in cold ether, dried in vacuo, lyophilized, and stored in −20 °C. The concentrations of the sulfhydryl group on the cysteine-containing peptides were quantified using Ellman's reagent (PIERCE).

2.4. Non-gelling photopolymerizations and cell viability assay

MIN6 β-cells at desired densities were suspended in Hank's Balanced Salt Solution (HBSS) containing required macromolecular components. For non-gelling chain-growth photopolymerizations (Scheme 1a), PEGMA (4 kDa) at 16 mM was used. For non-gelling step-growth thiol-ene photopolymerizations (Scheme 1b), 8 mM PEG4NB (20 kDa) and 8 mM mono-cysteine peptide CGGGG were combined to yield a total functionality of 16 mM. Photoinitiator LAP was added at 1 mM (0.028wt %) when needed. Half of the pre-polymer solutions containing cells were exposed to UV (365 nm, 5 mW/cm^2) for 3 min (identical to that used in cell encapsulation). Following photopolymerizations, 5 μL of cell solutions (with or without UV exposure) were combined with 50 μL of HBSS and 50 μL of Celltiter Glo® reagent for quantification of intracellular ATP concentrations using a microplate reader (BioTek Instruments). Standard curves using known concentrations of ATP monohydrate were generated for interpolation of unknown ATP concentrations.

2.5. Dynamic viscometry

Viscosity of the macromer solutions with or without UV exposure was measured on a Bohlin CVO 100 digital rheometer (Viscometry mode, 4° cone/plate geometry, gap = 150 μm). Dynamic viscosity measurements were conducted at 25 °C and in controlled shear rate (100–400 s^{-1}).

2.6. Hydrogel fabrication and characterization

Chain-growth PEG hydrogels were photopolymerized from desired concentrations of PEGDA (10 kDa) and in the presence of 1 mM LAP (3 min UV at 365 nm, 5 mW/cm^2). Step-growth thiol-ene hydrogels were formed from PEG4NB (20 kDa) and a chymotrypsin-sensitive peptide crosslinker (CGGYC). Gels were formed in 1 mL syringes with open tips for gel removal. To characterize gel fraction, all components were dissolved in double distilled water (ddH_2O), and gels (60 μL) were dried in vacuo immediately following photopolymerization. The dried weights obtained gravimetrically contain both crosslinked (gel fraction) and uncrosslinked (sol fraction) polymers. The gels were then incubated in ddH_2O on an orbital shaker for 24 h for removing sol fraction. Gels were dried again to obtain crosslinked polymer weight, from which to calculate the gel fractions (ratios between the two dried weights). For equilibrium gel swelling, polymerized hydrogels were placed in ddH_2O for 24 h to allow diffusion of unreacted macromers, followed by drying in vacuo to obtain dried gel weight (W_{dried}). The dried gels were then placed in PBS for 48 h. The swollen weights of the gels ($W_{swollen}$) were obtained gravimetrically for determining swelling ratios based on the following equation: $Q = W_{swollen}/W_{dried}$.

The obtained swelling ratios were used to calculate hydrogel mesh sizes as described elsewhere [3,26,28].