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Enzymatically degradable poly(ethylene glycol) hydrogels for the 3D culture and release of human embryonic stem cell derived pancreatic precursor cell aggregates

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ABSTRACT

This study aimed to develop a three dimensional culture platform for aggregates of human embryonic stem cell (hESC)-derived pancreatic progenitors that enables long-term culture, maintains aggregate size and morphology, does not adversely affect differentiation and provides a means for aggregate recovery. A platform was developed with poly(ethylene glycol) hydrogels containing collagen type I, for cell–matrix interactions, and peptide crosslinkers, for facile recovery of aggregates. The platform was first demonstrated with RIN-m5F cells, showing encapsulation and subsequent release of single cells and aggregates without adversely affecting viability. Aggregates of hESC-derived pancreatic progenitors with an effective diameter of 82 (15) μm were either encapsulated in hydrogels or cultured in suspension for 28 days. At day 14, aggregate viability was maintained in the hydrogels, but significantly reduced (88%) in suspension culture. However by day 28, viability was reduced under both culture conditions. Aggregate size was maintained in the hydrogels, but in suspension was significantly higher (~2-fold) by day 28. The ability to release aggregates followed by a second enzyme treatment to achieve single cells enabled assessment by flow cytometry. Prior to encapsulation, there were 39% $Pdx1^+/Nkx6.1^+$ cells, key endocrine markers required for β-cell maturation. The fraction of doubly positive cells was not affected in hydrogels but was slightly and significantly lower in suspension culture by 28 days. In conclusion, we demonstrate that a MMP-sensitive PEG hydrogel containing collagen type I is a promising platform for hESC-derived pancreatic progenitors that maintains viable aggregates, aggregate size, and progenitor state and offers facile recovery of aggregates.

1. Introduction

The dimensionality of culture platforms is increasingly being recognized as an important factor for the *in vitro* culture of cells [1–3]. In three dimensional (3D) culture, cells are often embedded within a material where they can migrate and experience cell–matrix interactions and cell–cell contacts in all directions. On the contrary, two dimensional (2D) cultures polarize these interactions, restricting movement and cell interactions to a single plane. The difference between 2D and 3D cultures is striking, leading to very different cell morphologies [4], gene expression profiles [5], and tissue production [6]. These differences have been highlighted in 3D cancer cell models [7], the maintenance of pluripotency for embryonic stem cells [8,9], and the differentiation of stem cells [10]. For these reasons, 3D culture platforms represent a promising and important approach to *in vitro* cell culture that better captures the native tissue environment.

In their native environment, β-cells are located within aggregates known as the islets of Langerhans, which are comprised of endocrine cells and extracellular matrix (ECM) molecules and are found embedded in pancreatic tissue. Within this 3D environment, β-cells experience cell–matrix and cell–cell interactions. In 3D cultures within a biomaterial, extracellular matrix (ECM) molecules can readily be incorporated to facilitate cell–matrix interactions. For β-cells isolated from islets and other insulin producing cells (e.g., pancreatic precursor cells and β-cell lines), the inclusion of

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ECM proteins has been shown to be important for their survival and insulin secretion [11]. For example, Mason et al. [12] showed that the incorporation of collagen into a poly(ethylene glycol) hydrogel supported the differentiation of encapsulated rat pancreatic precursors into glucose responsive β-like cells. Culturing cells within aggregates in 3D culture platforms enables cell–cell contacts, which has also been shown to be important for survival and insulin secretion of β-cells and β-like cells [13], but depends on aggregate size [14]. There have been a few studies investigating 3D culture platforms for human embryonic stem cell (hESC) derived pancreatic precursors with the most common being suspension cultures (e.g., spinner flasks). While suspension cultures support cellular aggregates, they do not afford tight control over aggregate size and are not able to easily provide ECM cues. Alternative strategies such as microwells have been used to control aggregate size [15], but long-term culture and incorporating ECM molecules are more challenging. Given the clinical potential of hESC derived pancreatic precursors [16] and the recent evidence demonstrating for the first time the ability to achieve hESC derived insulin producing cells [17,18], there is a need to establish improved 3D culture platforms for hESC derived pancreatic precursors.

The goal of this study was therefore to develop a biomaterial-based 3D culture platform for aggregates of hESC-derived pancreatic progenitor cells, which meet the following criteria: (a) enable their long-term culture, (b) maintain aggregate size and morphology, (c) not adversely affect differentiation and (d) provide a means for aggregate recovery. The ability to recover aggregates enables this system to serve as a temporary 3D culture platform for *in vitro* differentiation from which aggregates could be recovered and subsequently analyzed or implanted following established clinical protocols. Previous works have demonstrated that hydrogels formed with protease sensitive crosslinks are promising for the culture and release of murine cardioprogenitor clusters [19] and MIN6 β-cell spheroids [20]. This study, therefore, builds off of this prior work, but for the clinically relevant cell population – hESC-derived pancreatic progenitor cells. A platform was chosen based on a poly(ethylene glycol) (PEG) hydrogel containing entrapped collagen type 1 [12] to support cell–matrix interactions and matrix metalloproteinase (MMP)-sensitive crosslinks to enable facile recovery of the encapsulated aggregates using an enzyme suitable for islet isolation. The utility of this hydrogel platform to recover encapsulated single cells and aggregates upon exposure to a collagenase enzyme blend was first demonstrated using the RIN-m5F cell line. Aggregates of hESC-derived pancreatic progenitor cells were then assessed for viability, aggregate size, and aggregate recovery and compared to aggregates grown in suspension culture. The recovered aggregates were subjected to a second enzyme treatment to obtain single cells, which were then used to evaluate the progenitor state by expression of key transcription factors associated with endocrine differentiation by flow cytometry. This study demonstrates that encapsulation in a MMP-sensitive PEG hydrogel is a promising platform for hESC derived pancreatic precursors and an improvement over suspension culture. Overall, the hydrogel maintained pancreatic progenitor markers similar to those in suspension culture, but led to improved long-term viability up to several weeks and maintained aggregate size, while allowing for the facile recovery of encapsulated aggregates at prescribed times through a cytocompatible enzymatic degradation of the hydrogel.

2. Materials and methods

2.1. Macromolecular monomer (macromer) synthesis

Poly(ethylene glycol) (PEG) tetranorbornene was synthesized following previously established protocols [21,22]. Briefly, 4-arm PEG-NH_2 (5000 Da, JenKemUSA) was dissolved in a sparing amount of dimethylformamide (Sigma) and combined with a 6 M excess of 5-Norbornene-2-carboxylic acid (Sigma) in the presence of 3 M excess of 2-(1H-7-Azabenzotriazol-1-yl) -1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (AKSci) and N,N-Diisopropylethylamine (Sigma). The product, PEG tetranorbornene, was recovered by precipitation in ice-cold diethyl ether, dialyzed against de-ionized H_2O, sterile filtered, and lyophilized. Functionalization of PEG with norbornene was determined using ^{1}H NMR (Fig. S1) and confirmed to be ~100% (i.e., each arm of a PEG molecule was functionalized with a norbornene) by comparing the area under the alkene peaks for norbornene (6, 6.2 ppm) to the area under the peaks of the methyl groups in PEG (3.6 ppm).

2.2. Hydrogel formation and characterization

A precursor solution was prepared with 10% (g/g) PEG tetranorbornene and a bis-cysteine crosslinker (CVPLSLYSGC) (GenScript) at a 1:1 thiol:ene ratio, along with 0.05% (g/g) photoinitiator (Irgacure 2959), and 0.25 mg/ml rat tail collagen type 1 (Life Technologies) in phosphate buffered saline (PBS, at pH 7.4). Hydrogels were formed by polymerizing this solution with long-wave UV light (352 nm) at ~6 mW/cm^2 for 6 min into cylinders that were ~2 mm in height and 4.5 mm in diameter. The tangent compressive modulus was determined for acellular hydrogels after a 24 h free swelling period by subjecting samples to unconfined compression at a rate of 0.5 mm/min up to 15% strain (Synergie 100, 10 N; MTS). The mass swelling ratio was determined by measuring the mass of hydrated gels and dividing by the mass of the same sample after freeze-drying. Hydrogels were characterized by their degradation in a solution of 1.3 Wünsch units of Liberase TL (Roche) per mL PBS. Hydrogel wet weights were measured at 10 min intervals until reverse gelation (i.e., solubilization of the gel). Distribution of collagen within the hydrogels was analyzed after embedding in freezing medium and sectioning without further processing. Sections (~40 μm) were incubated with a rabbit anti-rat collagen I (Abcam, 1:100) followed by incubation in goat anti-rabbit Alexa Fluor® 488 (1:300, Life Technologies). Images were acquired by confocal microscopy (Zeiss LSM 5 Pascal).

2.3. RIN-m5F cell culture, encapsulation and recovery

A rat insulinoma cell line RIN-m5F (ATCC® CRL-11605™) was cultured in RPMI 1640 media (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologics), l-glutamine (1X) (Life Technologies), and penicillin streptomycin (1%) (Corning). RIN-m5f cells were cultured to ~90% confluency, aggregated on an orbital shaker (60 rpm) overnight in non-tissue culture treated plates, encapsulated in PEG hydrogels as described in Section 2.2, cultured for 24 h, and then released with Liberase TL at a concentration of 1.3 Wünsch units/mL until reverse gelation, which occurred in 45–60 min. Encapsulated and recovered cells were stained with the Live/Dead® Cell Viability Assay (Life Technologies) and imaged on a confocal microscope.

2.4. Differentiation of human embryonic stem cells (hESCs) into pancreatic precursor cells

Human embryonic stem cell (hESC)-derived pancreatic progenitors were generated in a monolayer format directly from hESCs grown on mouse embryonic fibroblasts (MEFs) (Nostro et al., submitted). The hESC line used was MEL1 PDX1:GFP (gift from Dr. Elefanty). To generate definitive endoderm, hESCs on MEFs were induced with 100 ng/ml ActivinA (R&D Systems) and 1 μM CHIR 99021 for 1 day in RPMI supplemented with 2 mM glutamine (Gibco-BRL) and 4.5×10^{-4} M MTG (Sigma), followed by 100 ng/ml

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