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Microfluidic fabrication of bioactive microgels for rapid formation and enhanced differentiation of stem cell spheroids ☆

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ABSTRACT

A major challenge in tissue engineering is to develop robust protocols for differentiating ES and iPS cells to functional adult tissues at a clinically relevant scale. The goal of this study is to develop a high throughput platform for generating bioactive, stem cell-laden microgels to direct differentiation in a well-defined microenvironment. We describe a droplet microfluidics system for fabricating microgels composed of polyethylene glycol and heparin, with tunable geometric, mechanical, and chemical properties, at kHz rates. Heparin-containing hydrogel particles sequestered growth factors Nodal and FGF-2, which are implicated in specifying pluripotent cells to definitive endoderm. Mouse ESCs were encapsulated into heparin microgels with a single dose of Nodal and FGF-2, and expressed high levels of endoderm markers Sox17 and FoxA2 after 5 days. These results highlight the use of microencapsulation for tailoring the stem cell microenvironment to promote directed differentiation, and may provide a straightforward path to large scale bioprocessing in the future.

Statement of Significance

Multicellular spheroids and microtissues are valuable for tissue engineering, but fabrication approaches typically sacrifice either precision or throughput. Microfluidic encapsulation in polymeric biomaterials is a promising technique for rapidly generating cell aggregates with excellent control of microenvironmental parameters. Here we describe the microfluidic fabrication of bioactive, heparin-based microgels, and demonstrate the adsorption of heparin-binding growth factors for enhancing directed differentiation of embryonic stem cells toward endoderm. This approach also facilitated a ~90-fold decrease in consumption of exogenous growth factors compared to conventional differentiation protocols.

1. Introduction

The study of developmental biology has led to the design of numerous embryonic stem cell (ESC) directed differentiation protocols. For example, the identification of growth factor gradients that drive regional specification during gastrulation has shed light on the biochemical microenvironment needed for *in vitro* production of ESC-derived definitive endoderm (DE), the germ layer of origin for adult tissues including the liver, pancreas, gut, and lung [1]. Directed differentiation protocols for engineering endoderm-derived tissues aim to initially mimic gastrulation-related signaling pathways, involving Nodal, FGF, Wnt, and BMP, to generate DE cells capable of responding to subsequent differentiation signals [1,2]. For instance, mouse ESCs can be differentiated *in vitro* to hepatocyte-like cells by sequentially mimicking certain developmental stages: formation of embryoid bodies (EBs), specification to

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DE *via* supplementation with Activin A and FGF-2, and finally induction to hepatic differentiation *via* exposure to HGF and other biomolecules produced by non-parenchymal cells of the liver [3]. However, controlling these tightly regulated microenvironmental factors represents a significant challenge when biomedical applications require large quantities of stem cell-derived tissue constructs, such as for cell replacement therapy or high throughput screening. The purpose of this study is to identify a scalable strategy for controlling the stem cell microenvironment using biomaterials. More specifically, we demonstrate the microencapsulation of ES cells in growth factor-binding heparin hydrogels for rapid preparation of EBs and subsequent growth factor mediated directed differentiation.

Microencapsulation is a large-scale cell bioprocessing technique that facilitates rapid nutrient/waste transport, while limiting hydrodynamic stresses associated with stirred suspension culture [4]. ES and iPS cells have been microencapsulated in alginate and other biomaterials to generate 3D aggregates for propagation and differentiation (e.g. EB formation) [5–7]. Of particular interest for directed differentiation applications, droplet microfluidic technologies enable rapid generation of emulsions with excellent monodispersity, a highly desirable feature for EB-based protocols where aggregate size influences cell fate [8–14]. In addition to bio-inert alginate [8,15–18], there are a few examples of biofunctional matrices used for cell microencapsulation with droplet microfluidics. For example, RGD-functionalized polyethylene glycol (PEG) microgels have been shown to support hMSC spheroids in isolation from allogeneic IgG molecules [19]. However, we are unaware of reports describing growth factor-binding microgels for ES cell encapsulation and differentiation.

Herein, we describe a bioactive copolymer microgel system composed of heparin and PEG. Heparins are highly sulfated glycosaminoglycans that regulate cell signaling through reversible sequestration of numerous growth factors (GFs) expressing heparin-binding domains [20,21]. Accordingly, heparin-functionalized synthetic hydrogels have been developed for controlled release of GFs, and as matrices for cell cultivation and differentiation [21–25]. In this work we demonstrate microfluidic fabrication of hydrogel microcapsules containing heparin and explore the possibility of incorporating GFs into the microgels to direct endodermal differentiation of encapsulated mESCs.

2. Materials and methods

2.1. Heparin-methacrylate synthesis

Methacrylate-modified heparin (heparin-MA) was prepared as previously reported [26]. Briefly, 2 g/L heparin sodium salt (16–18 kDa MW) (Sigma) was dissolved in DI water with methacrylic anhydride (5-fold molar equivalent). The reaction solution was adjusted to pH 8.5 with 5 N NaOH, stirred for 2 days at RT, then dialyzed and lyophilized.

2.2. Microfluidic device fabrication

Microfluidic flow focusing devices were fabricated by standard soft lithographic techniques: PDMS elastomer (Sylgard) was poured over SU-8 master molds and cured for 1 h at 70 °C. The PDMS chips were O_2 plasma bonded to glass, then treated with Aquapel™ solution for ~30 s and perfused with air to render channel surfaces hydrophobic. For droplet characterization experiments, flow focusing channel dimensions (H × W) were 45 × 45 μm or 100 × 100 μm.

2.3. Microgel fabrication

Acrylate/methacrylate- and thiol-modified polymer solutions were prepared separately, then mixed on-chip immediately prior to microfluidic flow focusing to generate crosslinked hydrogel droplets (Fig. 1). These solutions consisted of (i) 2× concentration of PEGDA (6 kDa) (Creative PEGworks) and Heparin-MA (16–18 kDa) dissolved in PBS or cell culture medium with 15 mM sodium carbonate (pH 7.4) and (ii) 2× concentration of 8-arm PEG-thiol (10 kDa) (Creative PEGworks) dissolved in PBS or culture medium. Microgels (4%, 7%, and 10% w/v) were prepared by mixing precursor solutions at PEGDA:Heparin-MA:8-arm PEG-thiol ratio of 3.5:2:1.5 to maintain stoichiometric equivalence of reactive groups (i.e. thiol and acrylate groups). Upon mixing, prepolymer solutions were flow focused with an immiscible carrier phase composed of HFE-7500 oil (3 M) with 2% v/v PEGylated fluorosurfactant (Ran Biotech). For droplet characterization experiments, oil and aqueous phase flow rates were varied from 5 to 50 μL min^{-1} and 0.5 to 10 μL min^{-1}, respectively. Microdroplets were collected and incubated off-chip at 37 °C for 10–20 min for gelation. Finally, emulsions were destabilized by replacing carrier oil with a 20% v/v solution of perfluorooctanol (Sigma Aldrich) in HFE-7500, and crosslinked microgels were partitioned into aqueous media.

2.4. Rheometry

Shear moduli were measured with a Discovery HR2 hybrid rheometer (TA Instruments) with parallel-plate geometry in time sweep and strain sweep modes. For gelation kinetics experiments, heparin-MA, PEGDA, and 8-arm PEGSH were mixed and immediately pipetted between instrument plates. Time sweeps were performed by holding constant strain and frequency at 10^{-3} rad and 10 rad s^{-1}, respectively. For steady state rheometry experiments, shear moduli were measured in 8 mm prefabricated hydrogel disks (n = 3) under strain sweep test mode (0–4% strain).

2.5. Growth factor-heparin binding experiments

2.5.1. Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed using a four-channel BIAcore T3000 instrument. For surface immobilization on bare gold SPR chips, heparin oligomers were modified with thiol moieties (~40% conversion of COOH groups) as previously described [27]. Thiolated heparin was dissolved in PBS to 1 μM concentration and injected into the SPR instrument at a flow rate of 5 μL min^{-1} for 5 min. Heparin immobilization was followed with surface passivation by flowing 3 mM mercaptohexanol (MCH) in PBS at 20 μL min^{-1} for 30 s. Recombinant Activin A and Nodal (100 ng/mL in PBS) (R&D) were then sequentially introduced into the instrument at 5 μL min^{-1} for 5 min.

2.5.2. GF sequestration in heparin microgels

Growth factor sequestration in heparin microgels was assayed by determining the relative depletion of GFs added to microgel suspensions. In brief, recombinant FGF-2, Activin A, and Nodal (1 ng/mL) were added to DMEM containing a 1:6 volume fraction of microgel droplets (120 μm, 7% w/v) and incubated at RT for 16 h. Supernatant was then collected and analyzed by ELISA (R&D [Activin A and FGF]); MyBioSource [Nodal]) (n = 3), wherein remaining GF concentration was measured and normalized to control samples prepared in DMEM.

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