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One-dimensional self-assembly of mouse embryonic stem cells using an array of hydrogel microstrands

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ABSTRACT

The ability of embryonic stem (ES) cells to self-renew indefinitely and to differentiate into multiple cell lineages holds promise for advances in modeling disease progression, screening drugs and treating diseases. To realize these potentials, it is imperative to study self-assembly in an embryonic microenvironment, as this may increase our understanding of ES cell maintenance and differentiation. In this study, we synthesized an array of one-dimensional alginate gel microstrands and aqueous microstrands through an SU-8 filter device by means of capillary action. Furthermore, we investigated self-assembly behaviors and differentiation potentials of mouse ES cells cultured in microstrands of varying diameters. We found that microstrands with an aqueous interior facilitated high density cell culture and formed compact microtissue structures, while microstrands with gelled interiors promote smaller cell aggregate structures. In particular, we noticed that ES cells collected from one-dimensional aqueous microstrands favored the differentiation towards cell lineages of endoderm and mesoderm, whereas those from gelled microstrands preferred to differentiate into ectoderm and mesoderm lineages. In addition to providing a "liquid-like" tubular microenvironment to understand one-dimensional self-assembly process of ES cells, this alginate hydrogel microstrand system also offers an alternative way to manipulate the stem cell fate-decision using bioengineered microenvironments.

1. Introduction

Embryonic stem (ES) cells hold promise in combating various diseases and have the potential to be exploited in drug discovery and diagnostics due to their capacity to self-renew indefinitely and their ability to differentiate into multiple cell types [1–3]. Living cells organize into functional units through self-assembly [4–8], and understanding cellular self-assembly is of paramount importance for engineering various tissue constructs and understanding life [4,5]. Although self-assembly at the molecular level has been intensively investigated, the study of self-assembly at the cellular level is somewhat lacking, partly due to its complexity [9]. Better understanding of ES cell self-assembly in an embryonic microenvironment will provide insights in tissue morphogenesis/organogenesis and offer strategies for effective expansion and differentiation of ES cells, leading to advanced cell therapy, tissue regeneration, and disease modeling.

According to the concept of semi-solid "soft matter" (the living system possessing complexity and flexibility) [6,10], and "tissue fluidity" (monodispersed cells reconstructing tissues through morphogenetic movement, differential adhesiveness and cell aggregation) [11–16], embryonic tissues can be considered as liquids [7]. Therefore, it is highly desirable to develop a soft and "liquid-like" system platform to mimic the embryonic microenvironment to study the self-assembly behavior of ES cells.

One commonly used approach to assemble mouse ES cells into embryoid bodies under gravity-driven self-assembly is the "hanging drop" technique, which has been used for self-assembly of neuronal microtissue and other structures as well [17,18]. However, this technique is tedious, and not suitable for high-throughput and long-term culture. Microfabrication techniques in combination with hydrogels have been used to assemble cells into a variety of structures [19–23], such as spheroids [24], toroids [11,25], rods [26], and honeycombs [27], with the purpose to investigate dynamic cellular self-assembly [28]. It has been shown that microtissue self-assembly is driven by cell–cell contact and intercellular adhesion, which involves connexins [8], cadherins [29], and actin cytoskeletal tensions [30]. Considering the requirement of "tissue fluidity", alginate hydrogel has great potential to mimic the embryonic microenvironment due to its gentle gelling behavior [31], reversible cross-linking [32], tunable flexibility [33], and non-adhesive property [34]. Alginate hydrogel can be fabricated into macrobeads (with a diameter in several mm) [35], microbeads (with a diameter <1 mm and with a hydrogel core) [36–39], microcapsules (with a diameter <1 mm and a liquid core

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enclosed within a spherical hydrogel membrane) [40,41], microfibers (solid hydrogels) [42,43] and microtubes (liquid core with hydrogel shell structures) [44–46]. It has been shown that alginate microbeads or microcapsules support the maintenance and/or differentiation of mouse or human ES cells [47–56]. Alginate microcapsules or microtubes with a liquid core can provide a "liquid-like" microenvironment for self-assembly of cells. Small diameter structures (≤200 μm) are desirable and create a microenvironment with better mass transfer of oxygen, nutrients, and metabolic wastes. In this regard, alginate hydrogels shaped into micro-tubular structures with diameters ≤200 μm are more readily fabricated, compared to spherical alginate microcapsules with similar diameter.

Here we present an approach to fabricate an array of alginate hydrogel microstrands (long microfibers and microtubes) through a microfabricated SU-8 filter device by means of capillary action. We name long microfibers with a homogenous gel core "alginate gel microstrands", while long microtubes with a liquid core and an alginate/polylysine (ALG/PLL) shell are referred to as "ALG/PLL aqueous microstrands". The diameter of these microstrands can be controlled from 30 to 300 μm, while the length is 3 cm or longer. Since the aspect ratio (length/diameter) of the microstrand is larger than 100, we consider them to be one-dimensional microstructures. When the diameter of alginate microstrands are around 30 μm, which is close to the size of ES cells, cells can line-up in the microstrand in a pearl-on-a-string fashion. We investigate the self-assembly behavior of mouse ES cells in one-dimensional alginate microstrands with various diameters having either a gel or liquid core. We further examine the potential of these encapsulated ES cells to differentiate into ectodermal, mesodermal, and endodermal cell types.

2. Materials and methods

2.1. Mouse ES cell culture

Mouse CCE ES cells were obtained from StemCell Technologies (Vancouver, Canada) [57,58]. Mouse ES cells were cultured in gelatin coated flasks in a standard cell culture incubator at 37 °C, 5% CO_2 and maintained in an undifferentiated state in growth medium consisting of Dulbecco's Modified Eagle's Medium (DMEM with 4.5 g/l D-glucose) supplemented with 15% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids, 10 ng/ml murine recombinant leukemia inhibitory factor (LIF; StemCell Technologies, Vancouver, Canada), 0.1 mM monothioglycerol, 2 mM L-glutamine and 1 mM sodium pyruvate (Sigma Aldrich, St. Louis, MO).

2.2. Fabrication of SU-8 filter

The SU-8 filter was fabricated using standard photolithography techniques (Fig. 1). First, a silicon substrate was cleaned using Piranha (3:1 H_2SO_4:H_2O_2), rinsed with DI water and then dried with nitrogen. H_2SO_4 was purchased from Transene Company (Danvers, MA.) and H_2O_2 from Puritian Products (Bethlehem, PA). Then a release layer of omnicoat (Microchem, Boston, MA) was spun on the wafer at 3000 rpm using a spin coater (Brewer Science, Rolla, MO) and baked on a hot plate at 200 °C for 1 min. SU-8 50 (Microchem) was spin-coated to a final thickness of approximately 50 μm and baked at 65 °C for 5 min, 95 °C for 20 min, and was then cooled to room temperature. The SU-8 was then exposed to 365 nm light using an EVG 640 Contact Aligner (EV Group, Albany, NY). Two separate UV exposures were used to create the support structure and the open vertical channels. The support structure surrounding the "hole area" was exposed with a dose of 500 mJ/cm^2, while the area with the through-holes was exposed with a dose of 200 mJ/cm^2. Lastly, the substrate was baked at 65 °C for 1 min, 95 °C for 10 min, cooled to room temperature and placed in the PGMEA developer (Microchem) overnight. The SU-8 filter was developed and released from the substrate by immersion in PGMEA. SU-8 filters were removed from the silicon substrate and then sterilized by soaking it in 70% ethanol for 20 min and then washing 3 times with sterile 1X phosphate buffered saline (PBS).

2.3. Fabrication of alginate hydrogel microfibers and alginate/PLL aqueous microstrands

Mouse ES cells were added to 1.5% alginate of 250 cps at 25 °C (Sigma Aldrich, St. Louis, MO) to make the ES cell-alginate mixture at 1 × 10^6 cells/ml. The SU-8 microfilter was placed floating on 100 mM of $CaCl_2$ solution. 2.5 μl of ES cell-alginate mixture was pipetted on top of the open microchannels/holes in the SU-8 filter and flowed immediately through the microfluidic channels by capillary action. Once the alginate and ES cell blend made contact with the $CaCl_2$ solution, carboxylate and hydroxyl groups in alginate coordinated to calcium ions and formed the alginate gel microstrands. To produce an aqueous environment, microstrands were soaked in 0.01% poly-L-lysine (PLL) (Molecular weight = 30,000–70,000 kDa; Sigma) for 10 min, removed and replaced by 1.6% sodium citrate for 5 min, which acts as

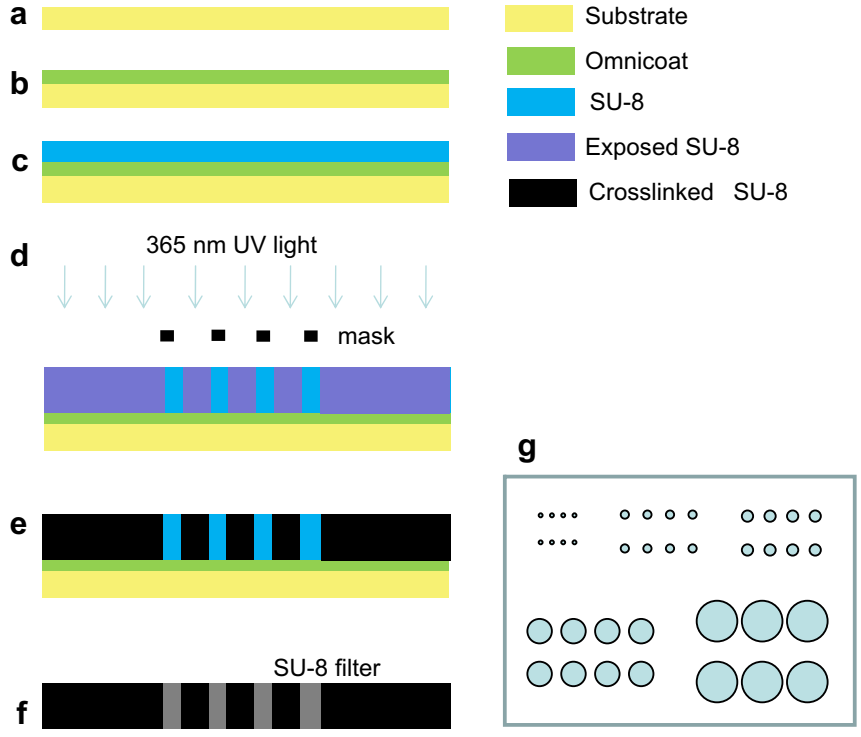

Fig. 1. Schematics of the fabrication of the SU-8 microfluidic filter device using photolithography. (a) Piranha clean of the silica wafer. (b) Omnicoat treatment for the release layer. (c) SU-8 50 coating. (d) Exposure. (e) Post-exposure bake. (f) Develop. (g) Schematics of the microchannel array of the SU-8 filter device.