Available online at www.sciencedirect.com
ScienceDirect
Biomaterials 29 (2008) 752–763
Biomaterials
www.elsevier.com/locate/biomaterials

A microwell array system for stem cell culture

Hannes-Christian Moeller[a,b], Matthew K. Mian[c], Shamit Shrivastava[a,d], Bong Geun Chung[a,c], Ali Khademhosseini[a,c,*]

[a]*Department of Medicine, Center for Biomedical Engineering, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA*
[b]*École Supérieure de Biotechnologie de Strasbourg, F-67412 Illkirch-Cedex, France*
[c]*Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA*
[d]*Department of Mechanical Engineering, Center for Nanotechnology, Indian Institute of Technology, Guwahati, India*

Received 1 August 2007; accepted 17 October 2007
Available online 14 November 2007

Abstract

Directed embryonic stem (ES) cell differentiation is a potentially powerful approach for generating a renewable source of cells for regenerative medicine. Typical *in vitro* ES cell differentiation protocols involve the formation of ES cell aggregate intermediates called embryoid bodies (EBs). Recently, we demonstrated the use of poly(ethylene glycol) (PEG) microwells as templates for directing the formation of these aggregates, offering control over parameters such as size, shape, and homogeneity. Despite these promising results, the previously developed technology was limited as it was difficult to reproducibly obtain cultures of homogeneous EBs with high efficiency and retrievability. In this study, we improve the platform by optimizing a number of features: material composition of the microwells, cell seeding procedures, and aggregate retrieval methods. Adopting these modifications, we demonstrate an improved degree of homogeneity of the resulting aggregate populations and establish a robust protocol for eliciting high EB formation efficiencies. The optimized microwell array system is a potentially versatile tool for ES cell differentiation studies and high-throughput stem cell experimentation.

Keywords: Microwells; Poly(ethylene glycol) (PEG); Embryoid bodies (EBs)

1. Introduction

Embryonic stem (ES) cells hold therapeutic potential as renewable source of cells in tissue engineering and regenerative medicine [1,2]. ES cells are characterized by the capacity to differentiate into specific tissue lineages in response to temporally and spatially regulated extrinsic and intrinsic signals [1,3,4]. Recent work has interrogated protocols for directing ES cell fate *in vitro* [5–12]. Typical ES cell differentiation protocols involve the formation of embryoid bodies (EBs)—structures which recapitulate features of early embryonic development and give rise to a wide spectrum of cell types [13–16]. EBs are usually formed using the hanging drop method [16,17] or in suspension culture [15]. The hanging drop method permits some control over EB size, but these cultures are cumbersome and not suitable for scale-up. Although suspension culture has advantages in that it is easily scalable and requires little expertise, the resulting EBs are heterogeneous in size and shape [18,19].

It is known that ES cell differentiation is affected by microenvironmental stimuli that directly or indirectly depend on EB size [4,18,20]. Such environmental stimuli influences cell–cell, cell–extracellular matrix (ECM), and cell–soluble factor interactions as well as other physicochemical factors including temperature, pH, and oxygen availability. Since these parameters can be functions of EB size, cell populations obtained from suspension culture EBs can vary dramatically—even when they were cultured under identical conditions [21]. To uniformly direct EB

*Corresponding author. Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, 65 Landsdowne Street, Room 252, Cambridge, MA 02139, USA. Tel.: +1 617 768 8395; fax: +1 617 768 8477.
E-mail address: alik@mit.edu (A. Khademhosseini).

0142-9612/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2007.10.030

differentiation, microenvironmental stimuli must be precisely controlled by homogenizing EB parameters such as size and shape.

To overcome the challenges associated with traditional EB culture techniques, a variety of approaches have been developed. For example, stirred vessel bioreactors have been used to improve EB homogeneity [12,19,22]. Also, encapsulation of EBs in agarose capsules and the use of E-cadherin-blocking antibodies have been employed to reduce agglomeration of EBs in stirred cultures [23]. Rotary shakers have also been used to provide constant circular motion to suspension cultures, resulting in improved EB homogeneity [24,25]. However, ES cell proliferation, viability, and aggregation are sensitive to hydrodynamic forces and shear stresses [26].

An ideal system for directing ES cell differentiation would provide uniform microenvironments to EBs while also being amenable to large-scale culture. Such a system should allow for *in situ* analysis, but EBs should also be accessible for further experimentation. Ideally, such a platform would also be simple, inexpensive, and applicable in standard biological laboratories.

We have previously developed a microfabricated platform of poly(ethylene glycol) (PEG) microwell arrays that showed advantages over suspension culture in controlling size, shape and homogeneity of EB populations [27]. This system can be integrated into microfluidic platforms to enable high-throughput experimentation [28]. However, our initial approach had drawbacks in that cells often adhered to the microwells and cell seeding and EB retrieval yields were suboptimal.

In this study, we develop an optimized microwell platform. We enhance the cell-repellent properties of the microwell substrate and establish robust seeding procedures and aggregate retrieval methods. We also use computational simulations to guide selection of microwell geometry. Using our array system, we are able to grow large populations of cell aggregates that are both homogenous and easily retrievable.

2. Materials and methods

2.1. Master fabrication

Photomasks were designed using the layout editor software CleWin Version 2.8 (WieWeb Software, Hengelo, Netherlands) and printed on Mylar™ clear films at Fineline Imaging, Inc. (Colorado Springs, CO) with a high plot resolution of 20,230 dpi. Patterns of microwells with 50, 75, 100, 150 and 175 μm diameters were created on silicon wafers. The wafers were cleaned and spin coated with hexamethyldisilizane (Arch Chemical Industries, Norwalk, CT) adhesion promotor before the permanent epoxy negative photoresist SU-8 2025 (MicroChem Corp., Newton, MA) was deposited. Spin coating was performed at 4000 rpm, yielding the desired film thickness of 20 μm. Wafer were softbaked at 65 °C for 3 min, followed by a second softbaking at 95 °C for 6 min. For crosslinking of the photoresist, the coated wafers were exposed to UV light of 350–400 nm for 90 s through a photomask. Subsequently, wafers were post-exposure baked at 65 °C for 1 min and then at 97 °C for 6 min. The photoresist-patterned silicon master was developed using SU-8 developer, rinsed with isopropyl alcohol for 10 s, and air dried with pressurized nitrogen. The pattern and depth of the microwells was analyzed using a Dektak surface profiler (Veeco Instruments, Santa Barbara, CA).

2.2. PDMS-stamp fabrication

Poly(dimethylsiloxane) (PDMS) molds were fabricated by curing a 10:1 mixture of silicone elastomer base solution and curing agent Sylgard 184 (Dow Corning Corporation, Midland, MI) on a silicon master patterned with SU-8 photoresist. The PDMS elastomer solution was degassed for 15 min in a vacuum chamber and cured at 70 °C for 2 h before the PDMS molds were peeled from the silicon masters. The generated PDMS replicas had patterns corresponding to the silicon master with protruding columns and were subsequently used for molding of PEG microwells.

2.3. Microwell fabrication

Non-adhesive microwells were fabricated using micromolding on UV-photocrosslinkable polyethylene glycol diacrylate and methacrylate (PEG-DA and PEG-MA) (Sigma-Aldrich Co., St. Louis, MO and Monomer-Polymer & Dajac Labs, Inc., Feasterville, PA) of different average molecular weights (MWs) (258, 330, 575 and 1000 Da) mixed in a 1% (w/w) ratio of the photoinitiator 2-hydroxy-2-methyl propiophenone (Sigma-Aldrich Co., St. Louis, MO). Glass substrates were treated with 3-(trimethoxysilyl) propylmethacrylate (TMSPMA) (Sigma-Aldrich Co., St. Louis, MO) for 5 min and baked at 70 °C for 1 h. A patterned PDMS stamp was placed on an evenly distributed film of PEG monomer solution on a glass support and then photocrosslinked by exposure to light of 350–500 nm wavelength for 16 s at an intensity of 100 mW/cm^2 using the OmniCure® Series 2000 curing station (EXFO, Mississauga, Canada). After polymerization, the PDMS stamp was peeled from the substrate. The stability of microwells micromolded on TMSPMA-treated and untreated glass slides was assessed by incubating microarrays in Dulbecco's phosphate-buffered saline (PBS) and analyzing the integrity of the arrays over time. In all cases, 1% photoinitiator was added and dilutions were made in PBS. Experiments performed to assess array stability were conducted in triplicates.

2.4. Murine ES cell culture

Pluripotent murine ES cells (R1 strain) [29] were manipulated under tissue culture hoods and maintained in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. All tissue culture components were purchased from Gibco-Invitrogen Corporation (Carslbad, CA) unless otherwise indicated. Culture medium for maintenance of ES cells consisted of knockout Dulbecco's modified Eagles medium (DMEM) supplemented with 15% (v/v) ES qualified fetal bovine serum (FBS), 1% (v/v) non-essential amino acid solution MEM NEAA, 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol and 10^3 U/ml mouse leukemia inhibitory factor (LIF), ESGRO® (Chemikon Int. Inc., Eugene, OR). Cells were kept undifferentiated by changing media daily and passaging every 2 days with a subculture ratio of 1:4. Tissue culture plates (T75) were treated with 0.1% gelatin in distilled water and incubated for 24 h. For EB formation, LIF was omitted from the medium and ES cells were allowed to differentiate either in suspension culture using non-tissue culture-treated dishes without gelatin coating or by seeding cells onto non-adherent PEG microwells arrays.

2.5. Protein adsorption

Fluorescein isothiocyanate (FITC) conjugated bovine serum albumin (BSA) was dissolved in PBS at 100 μg/ml. To test protein adsorption to PEG hydrogels made from macromers with different average MW (PEG 258, PEG 330, PEG 575 and PEG 1000), 50 μL of the protein solution was evenly distributed on the surfaces and incubated for 20 min at room temperature in the dark. After incubation, samples were washed twice in