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Microfluidic environment for high density hepatocyte culture

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Abstract We present a microfluidic bioreactor for culturing high-density arrays of hepatocytes in a tissue-like micro-architecture. The microfluidic environment mimicked physiological liver mass transport, enabling sustained culture of high density cells (>2,000 cells/mm^2) without nutrient limitation for over 1 week. The key feature of this design was a microporous microfluidic barrier that formed a sieved-pocket to concentrate cells during loading. Nutrient depletion within the cell mass was avoided by maintaining a continuous flow of medium (10 μl/day) that diffused across the porous barrier. Human hepatoma cells (HepG2/C3A) remained viable and functional as demonstrated by fluorescent viability assays and secretion of albumin for the one-week culture period.

Keywords Microfluidics · Cell culture · Hepatocytes · Bioreactor

1 Introduction

Upon isolation, rapid loss of liver-specific function in traditional monolayer culture of hepatocytes limits their application in drug screening and as a dependable cell-source for bioartificial liver support (Allen and Bhatia 2002; Park and Lee 2005; Guguen-Guillouzo et al. 1986). In native liver tissue, hepatocytes rely on a complex three-dimensional network of blood capillaries to receive adequate oxygen and soluble factors transport. A typical liver micro-unit, the sinusoid, consists of a cord of hepatocytes with extensive cell–cell contact located within tens of micrometers from a nutrient flow. Thus, a bioreactor that is able to provide a more physiologic microenvironment is well-suited to maintaining a differentiated hepatocyte phenotype after isolation (Strain and Neuberger 2002).

The most commonly applied methods for improving *in vitro* hepatocyte culture are in spheroidal aggregates and on extracellular matrix (ECM) treated surfaces. Hepatocyte aggregates have characteristics similar to native liver function, and have been found to be more effective in maintaining long-term hepatocellular functions *in vitro* than their monolayer counter-parts (Elkayam et al 2006; Fukuda et al. 2005; Nakazawa et al. 2006; Fukuda et al. 2003; Fukuda and Nakazawa 2005; Nyberg et al. 2005; Torok et al. 2001).

The ability to control hepatocyte behavior requires precise combinatorial studies with the ability to optimize a variety of important culture parameters such as flow rate, cell density, media composition, and geometric configuration. In recent years, microfluidic technologies have been pioneered to offer the advantages of high-density, high-throughput arrays, to address the cellular length scale with nano- to micro-liter reagent volumes, to effectively control fluid flows, and to improve cell culture microenvironments. It is generally acknowledged that microfluidic cell culture devices are advantageous due to their small length scale and flow control (Sia and Whitesides 2003). Microfabricated devices have previously been demonstrated on a variety of silicon and poly-dimethylsiloxane (PDMS) substrates for the purpose of hepatocyte culture, such as membrane-based bioreactors (Ostrovidov et al. 2004; De Bartolo et al. 2000),

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multi-layer polymer structures (Leclerc et al. 2004), and devices that include integrated oxygen and nutrient delivery systems with online monitoring (Lee et al. 2006a; Powers et al. 2002).

Our current work addresses the development of a microfluidic environment specifically for improved hepatocyte culture *in vitro*. The core concept is to controllably maintain cells in a "tissue-like" state by using a microfluidic mass transport design that mimics the tissue microvasculature. We hypothesize that this approach will maintain the biological function of liver cells *in vitro* as well as provide a cost effective screening platform for pharmaceutical development.

2 Microfabrication

The microfluidic cell culture array was fabricated by using a two-layer SU-8 negative photoresist process in soft-lithography and replicate molding with PDMS as previously described (Lee et al. 2006b). The overall device consisted of three ports: one for medium inlet, one for medium outlet, and one for cell loading. The ports were interfaced to plastic wells glued on top of the PDMS such that liquid could be directly filled into the well without the use of syringes. The microfluidic culture unit consisted of a cell loading area, a microfluidic barrier, and a medium flow channel (Fig. 1). The microfluidic cell barrier was fabricated with a height of 2 μm and consisted of a grid of 5 μm wide channels. This mesh was designed to localize and concentrate cells into the cell culture area while enabling diffusion of nutrients to/from the flow cannel (Lee et al. 2007). The cell culture area was 150 μm wide by 440 μm long by 30 μm tall. The flow channel was patterned directly opposite of the perfusion barrier, and was 50 μm wide by 30 μm tall.

3 Finite element simulation

The microfluidic cell culture unit was modeled using Femlab Multiphysics software (Comsol). The key consideration was to simulate the effect of continuous perfusion on glucose concentration within the solid cell mass. The glucose consumption rate of the HepG2 cells was set at 10^{-11} g/cell/hr as previously described (Enozawa et al. 2001). A homogenous cell mass was assumed to reside within the microfluidic culture area. Fluidic resistance through the 3D channels were approximated in 2D and verified against theoretical values for laminar flow. Simulations of glucose concentration (with an initial value of 25 mM) were analyzed at 0.1, 0.5, 1.0, 10, and 100 μl/day flow through the microfluidic bioreactor.

4 Cell culture

Human hepatoma HepG2/C3A cells (#CRL-10741) purchased from the American Type Culture Collection (ATCC) were cultured in Dulbecco's minimum Essential medium

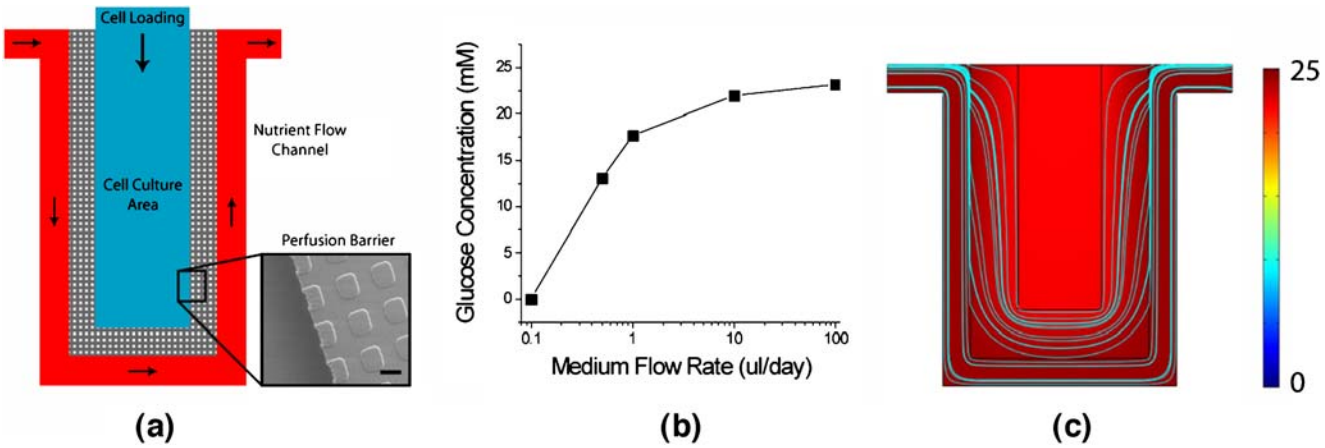

Fig. 1 (**a**) Microfluidic culture unit design. The microfluidic unit consisted of three parts: a 150 μm wide by 440 μm long cell culture area (*blue*), a microfluidic perfusion barrier (*gray*), and a medium flow channel (*red*). Cells were introduced from the top port, and localized into the cell culture area. The perfusion barrier consisted of a grid of channels 5 μm wide and 2 μm tall, serving to prevent cells from passing through, while enabling nutrient exchange from the flow channel on the opposite side. *Inset* shows SEM micrograph of the perfusion channels. Scale bar represents 5 μm. (**b**) Glucose concentration at the center of cell culture mass was determined using finite element analysis software and plotted against medium flow rate. (**c**) Surface plot of glucose concentration throughout one culture unit with an inlet glucose concentration of 25 μmM. At a flow rate of 10 μl/day, glucose concentration at the center of the cell mass after consumption was theoretically determined to be 22 mM

(DMEM) supplemented with 0.01125% (*w*/*v*) BSA Fraction V (Gibco, Grand Island, NY), 1% (*w*/*v*) non-essential amino acids, 1mM sodium pyruvate, 4 mM L-Glutamine, and 1% (*w*/*v*) penicillin/streptomycin and incubated in a humidified 37°C incubator with 5% CO_2. HepG2/C3A cell viability and proliferation was determined daily using Trypan Blue and the Live/Dead fluorescence assay (Molecular Probes).

For control cultures, cells were plated in standard tissue-culture (TC) 12-well plates at a density of 10^5 cells/ml in each well. Media samples were collected and refreshed with 1 ml fresh media every 24 h. For device cultures, 75 μl of media was added to each media inlet well, and a total volume of 40 μl of 10^6 cells/ml cell suspension was added to the cell inlet. Cells were loaded into the device at 3 psi and media was loaded at 5 psi using an air-pressure regulator through a custom made manifold. The device was then incubated in a humidified 37°C incubator with 5% CO_2 on a 45° sloped rack for continuous gravity driven flow. Flow rates measured by collecting the outlet medium every 24 h for 37 culture units was 10.3±1.6 μl/day/well. Media was refilled to a final volume of 75 μl/well each day in order to preserve constant head pressure throughout the culture period.

Figure 2 shows cell growth inside the microfluidic cell culture array in one sinusoid chamber from days 1 to 8. Cells were seeded at approximately 10^6 cells/ml and loaded until each chamber contained approximately 100 cells. Cell viability in device cultures was approximately 80% after one week incubation. On a standard 12-well tissue culture plate, the cells appeared strongly attached and more spread, with distinct cell membranes. By day 8 in the microfluidic culture device, the cells exhibited a "tissue-like" morphology with features such as dense packing, cuboidal geometry, and indistinguishable fused membranes (Fig. 3). The microfluidic format enabled a threefold increased cell density to be maintained compared with monolayer culture.

Fig. 2 HepG2/C3A human hepatoma cell growth inside the microfluidic cell culture array. Scale bar represents 100 μm

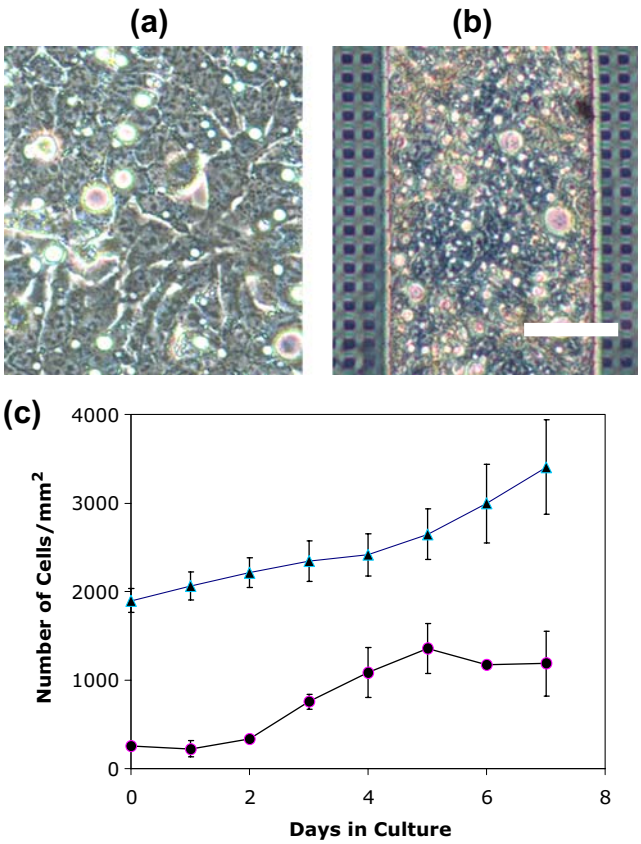

Fig. 3 High density cell culture. Phase contrast micrographs of confluent cell morphology on a standard 12-well tissue culture plate (**a**) and inside one sinusoid of the microfluidic cell culture array (**b**) after 8 days of culture. Images are at equal magnification, with scale bar representing 50 μm. (**c**) Cells were seeded at 10^5 cells/well for controls (*filled circles*) and about 500 cells/well for devices (*filled triangles*) and measured for cell density over 1 week

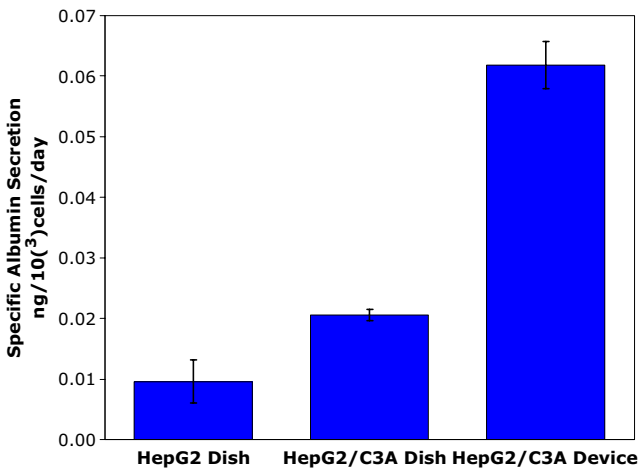

Fig. 4 Albumin secretion was characterized in TC dish and the microfluidic device after 4 days of culture. Albumin secretion from cells cultured in the microfluidic device increased threefold compared with on a dish

Liver-specific synthetic function of the protein albumin was assessed via a quantitative dot-blot assay as previously described (Heinicke et al 1992). Briefly, 100 μl samples were homogenized and taken from each well in control cultures and 10 μl from each outlet well in the microfluidic devices. All samples were stored at −20°C for subsequent analysis of albumin. Human albumin standards, undiluted samples, and negative controls (media) were blotted in triplicate at 2 μl each on a nitrocellulose membrane. Before immunostaining, membranes were incubated with 5% ECL block (RPN2106, Amersham Biosciences, Buckinghamshire, England) in TBS-T overnight at 4°C to prevent non-specific binding and washed three times with TBS-T. Membranes were then incubated with goat derived anti-human albumin antibodies (Fitzgerald, Concord, MA, diluted 1:10,000 in TBS-T) for 90 min at room temperature and incubated with horseradish peroxidase (HRP)-conjugated donkey derived anti-goat IgG antibodies (1:10,000) for 45 min at room temperature followed by staining with ECL western blotting detection reagents. The membrane was then exposed to Kodak Biomax Light Film with densitometric processing of the developed images. After 4 days of culture in the microfluidic format, the HepG2/C3A cells were producing three times the albumin on a per-cell basis compared with dish cultures (Fig. 4). The HepG2 cell line was used as a baseline to demonstrate the improved albumin production of the HepG2/C3A variant.

5 Discussion

This work demonstrates that engineering of a microfluidic environment can affect the behavior of cells cultured *in vitro*. The current design enables a high cell density to be maintained without nutrient depletion. The continuous flow "tissue bioreactor" format was modeled using finite element software, and validated for maintaining the human liver derived cell line HepG2/C3A in culture with a flow rate of 10 μl/day. This configuration enhanced the specific production of albumin in the cells by threefold when compared to a 12-well plate. We chose to use the model HepG2/C3A cell line since this HepG2-derived cell line has a protein synthesis profile that most closely resembles native human liver cells and is able to synthesize seventeen of the twenty normal major human plasma proteins (Knowles et al. 1980). As a proof-of-concept, we examined albumin secretion, as it is one of the primary plasma proteins secreted by hepatocytes. Continued optimization of this approach can lead to improved bioreactors for tissue-based studies.

The microfluidic culture format enabled cells to be assembled in close proximity to each other without nutrient limitation. This mimics the natural liver tissue configuration, where a high density of hepatocytes is in close contact with the microcirculation. This bio-mimetic culture format may enhance cell–cell contacts such as tight junctions and desmosomes normally found in native liver tissue. It is also hypothesized that the flow dynamics of media may also provide necessary cues to maintain hepatocyte differentiation and function. The use of microscale bioreactors offers a promising avenue to investigate these physical effects on cell behavior.

There is growing attention on the effect of the cellular microenvironment on tissue specific behavior (Albrecht et al. 2006). The use of microfabrication technology to address this question may yield powerful advances for the future of cell biology. In this work, we have developed a method for fabricating microfluidic devices with physiologically relevant mass transport conditions and showed how this can affect maintenance of differentiated phenotype and biological function. In the future, this approach will be extended for the optimization of primary hepatocyte culture to be applied to drug metabolism and fundamental physiology studies.

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