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Monolayer microbubbles fabricated by microfluidic device for keratocytes observation

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ABSTRACT

In order to construct 3D culture system for tracking cell migration, microfluidic techniques were applied to generate micro bubbles composed of gelatin, and then the bubbles were fabricated into monolayer in the micro-channel which is cell-traceable under phase microscope. The keratocytes were harvested through primary culture from *Hpsophrys nicaraguensis* (a fish). Live cell imaging was used to record the migration trajectory and the morphology changes. Moreover, galvanotaxis, the tendency of cells directed by an electrical field was studied. Without the electrical field, random pattern was observed in the 2D micro-channel. When the electrical field was applied, the keratocytes in 3D gelatin bubbles migrated along the rim of bubbles and went across the interconnecting pore between the adjacent bubbles, causing the tortuous trajectory of migration patterns. However, in the 2D micro-channel, the keratocytes' migration paths are closer to straight lines. The speed and velocity of keratocytes for both 2D and 3D environment were also compared under the condition with electric field. We believe that the understanding of the keratocytes' migration behavior in 2D and 3D condition under electric field will provide valuable information in succeeding the model system of wound repairs and electrophysiological environment *in vivo*.

1. Introduction

Cell migration has received many attentions in physiological process and bio-medical field since it is highly related to wound repair, vaccine development, embryonic development [1], blood vessel formation, anti-tumor therapy, immune surveillance, and inflammation. One of the mechanisms for cell migration is galvanotaxis, which is a tendency that cells are directed by an electric current, galvanotaxis has been researched in human keratinocytes and mammalian corneal epithelial cells in wound-repairing field [2]. Recently, some studies observing cell migration in two-dimensional substrate indicating that migration is affected by chemical gradient or electric field without any architectural barriers. However, studying cell migration, there are significant differences between two-dimensional microenvironment and three-dimensional microenvironment due to the following reasons: First, 3D culture add the third dimension to cellular environment, which causes significantly different cellular characteristics and behaviors compared with traditional 2D culture. Second, 3D culture mimics the *in vivo* microenvironment of the cells under *in vitro* condition; in other words, 3D culture is closer to nature than 2D culture. Furthermore, cells migrate through 3D environment is needed for overcoming the physical barriers, and cells would have some interactions with the scaffold they attached which may give some factors to direct cells migration [3,4].

To study galvanotaxis, epidermal keratocytes isolated from fish or amphibian skin as a model cell are often used [5,6], Those cells crawl with little change in shape or speed as a result of tight coupling between protrusion at the front and retraction at the rear. And, keratocytes are able to maintain nearly constant speed and direction during movement over many cell lengths. Moreover, the size of a keratocyte is about 0.1 mm thickness which is suitable for observing under microscopy [7,8]. To establish 3D cell culture scaffolds, encapsulation technique [9] and lyophilization [10] are often used. Nevertheless, these scaffolds have random pore sizes and variable mechanical properties which may cause difficulties for constantly repeatable tracking of cell migration. Therefore, to seek for not only biocompatible but also homogeneous 3D scaffolds for as a template for examination of cell movement is an important issue. Lin and coworkers apply microfluidic techniques to generate micro bubbles, and then fabricated the bubbles into porous scaffolds for 3D cell culture [11,12]. Furthermore, using microfluidic techniques for 3D cell culture scaffold production is a current trend since the cells are micro-sizes [13,14]. Thus, we followed their strategy to build up structures for observing the movement of keratocytes in a microbubble system.

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In our studies, in order to construct a physiologically-relevant microenvironment, we fabricated (extracellular matrix)-based micro bubbles for mimicking 3D cell culture environment using microfluidics principles. Additionally, we fabricated a monolayer which is composed of gelatin bubbles for cell culture, and tracked the cell movement under phase microscopy. Gelatin, which is one of the extracellular matrix molecules [15], was selected as the scaffolding material for cell seeding. Gelatin is derived from the collagen inside animals' skin and bones, also is biodegradable and biocompatible. Regarding cells, we harvested the keratocytes through primary culture from *Hpsophrys nicaraguensis*, which is a kind of fish. After keratocytes seeding into the monolayer, we used live cell imaging to record the migration trajectory and the morphology change of the cells. 2D micro-channel without gelatin bubbles was used as the control group.

Furthermore, the mechanism of cell migration "galvanotaxis", which is the tendency of cells directed by an electrical field, was also examined in this study. The electric field was imposed to exhibit the galvanotaxis phenomenon of keratocytes. Galvanotaxis between microbubbles and control group was compared. Finally, the speed and velocity were calculated and analyzed in both 2D and 3D environments under the electric field. According to our results, we believe that understanding of the keratocytes' migration behavior in both 2D and 3D microenvironment under electric field may increase the probability of succeeding in anti-tumor therapy, and even vaccine development or wound repairing.

2. Material and methods

2.1. Generating bubbles by microfluidic device

A planar flow-focusing microfluidic device made of polydimethylsiloxane [12] was used, and this device contains two inputs and one output (Fig. 1a). One of the inputs is the liquid flow, and the flow rate was controlled by PhD 2000 syringe pump (Harvard Apparatus). The other one is gas flow, which is adjusted through a Heise PM pressure gauge (Fig. 1c). Generally, the liquid flow rate ranged from 20 to 80 ml min^{-1} and the air pressure was 5–35 psi, and adjustment of these two parameters would result in different bubble sizes. Using microfluidic channels, bubbles were formed when liquid and gas encountered each other at the orifice at the middle of the channel (Fig. 1b). The bubbles were collected into the other type of micro-channel which was for cell culture through PE 20 tube from the output of the microfluidic device.

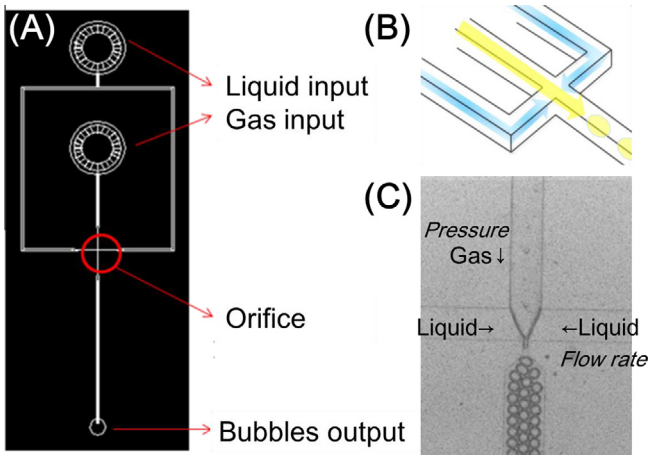

Fig. 1. Bubble formation through microfluidic channel. (a) Overall scheme of the microfluidic device, (b) 3D image of the orifice, including liquid section (yellow), gas section (blue), and bubbles section (yellow ball), and (c) a picture of bubble formation under microscope, gas could be tuned by pressure and liquid could be adjusted by flow rate with a syringe pump. (For interpretation of color in figure, the reader is referred to the web version of this article.)

2.2. Monolayer gelatin bubbles in the micro-channel

For collecting bubbles, a micro-channel formed between PDMS and glass slide was used. The SU-8 photoresist structure was used directly as a master for replica molding [16] (Fig. 2a). After removing the cured PDMS replica from the master, the PDMS mold was bonded to a glass cover by O_2 plasma machine (Fig. 2a). After these steps, micro-bubbles from microfluidic device were able to be collected in micro-channels. The height of the channel should be determined upon the sizes of bubbles to make sure that there was monolayer in the channel. Monolayer bubbles allowed easy observation of locomotion of cells compared with multiple ones.

For bubbles, gelatin, a type of extracellular matrix (ECM), was chosen. When making gelatin bubbles, the liquid was composed of 7% gelatin (from porcine skin, SIGMA, Lot# 070M0081V) and 1% Pluronic® F127 surfactant (SIGMA, Lot# BCBH4538V). It has been shown that cells proliferate and maintain high viability at this concentration of Pluronic® F127 [11], and this surfactant can reduce the surface tension required to stabilize bubbles. N_2 and perfluorohexane (Synquest Laboratories, CAS# 355-42-0) were used for gas (sometimes C_2F_6 would be substituted for N_2). Perfluorohexane and C_2F_6 prevent a coarsening phenomenon of bubbles at longer time. The liquid bubbles were collected into the micro-channel whose length was about 1–2 cm and the width was 200 μm. The height of the channel should be determined on the sizes of bubbles to make sure that there was monolayer bubbles in the channel and 40–75 μm of bubble sizes would be ideal in this case with the height of the micro-channel ranges 45–80 μm (Fig. 2a). Furthermore, the sizes of keratocytes are about 20–30 μm, so 40–75 μm of bubble sizes would be proper for the experiment. During the process of bubbles formation, the temperature was maintained above 45 °C to keep the solution in liquid phase. The forming bubbles were imaged in a Leica Z16 APO stereomicroscope with an ultrafast camera Miro3 (Vision Research) at an exposure time of 40 ms and 22,000 frames per second (ESI, Supplementary Movie S1†). After the micro-channel was filled with micro-bubbles, it was placed in a 4 °C refrigerator for solidification. Gelatin was crosslinked by soaked in 2% glutaraldehyde (SIGMA–ALDRICH, Lot# MKBD9857) in the 4 °C refrigerator overnight. Notice that the temperature for storing the liquid foam was not allowed to be lower than the freezing temperature, to avoid the formation of tiny ice crystals inside the gelatin and the creation of microscopic pores that weaken the mechanical properties of the final scaffolds. According to these procedures, the liquid foam was turned into solid foam.

Next day, the remaining glutaraldehyde was washed with 1 M glycine and DI water with a syringe pump. Finally, an ordered three dimensional, monolayer gelatin scaffold with tunable pore size, solid fraction, and material stiffness was fabricated (Fig. 2b).

2.3. Monolayer fluorescence labeling

The monolayer composed of gelatin bubbles was labeled with the fluorescent molecule Cy5 [17]. Briefly, unlabeled gelatin bubbles in the micro-channel were injected with Cy5 solution. The solution is composed of original Cy5 reagent and buffer: 0.25 M sodium bicarbonate containing 0.4 M sodium chloride, and the pH was adjusted to 10 through the addition of 1 M sodium hydroxide. To avoid exposure to light, the samples were placed in a dark room, and reacted overnight at room temperature. Next day, the micro-channels were washed by PBS solution.