

3D Scaffolds Fabricated by Microfluidic Device for Rat Cardiomyocytes Observation

Chih-Wei Chao, Jiashing Yu

Abstract—To mimic the natural circumstances of cell growth in an organism, we present three-dimensional (3D) scaffolds fabricated by microfluidics for cultivation. This work investigates the cellular behaviors of rat cardiomyocytes in gelatin 3D scaffolds compared to those on 2D control, such as proliferation, viability and morphology. We found that the scaffolds may induce skeletal differentiation of H9c2 cells.

Keywords—Microfluidic device, H9c2, tissue engineering, 3D scaffolds.

I. INTRODUCTION

NOWADAYS, in most studies, cells are cultured on a dish, and expand in two-dimensional circumstances. However, in an organism, cells proliferate under three-dimensional conditions. The 2D cultured cells lack essential interactions present in 3D conditions or *in vivo*. [1]

In the tissue engineering field, in order to mimic both the form and functionality of the native extracellular matrix (ECM), the design of three-dimensional (3D) scaffolds for *in vitro* cell cultures and *in vivo* tissue regeneration is continuously developing. [2] Hydrogels are appealing as scaffolds because of their similarity with ECM, and to make them more biomimetic, several approaches were conducted to produce porous 3D hydrogel scaffolds, such as freeze-drying [3], salt leaching [4], cryogel [5], gas foaming [6] and so on. The scaffolds must simultaneously promote desirable cellular functions like proliferation and differentiation relating to the specific tissue and cell type. [7] Specifically, fusion of myoblasts into multinucleated myotubes is critical in muscle tissue engineering.

H9c2, which is a cell line derived from embryonic BD1X rat heart tissue, is unique for its ability to differentiate into either skeletal or cardiac myocytes, depending primarily on whether they are treated with retinoic acid. [8] But also, the differentiation level is influenced significantly by cell confluency and the environment. [9], [10]

In this work, we took advantage of microfluidic techniques to construct gelatin microbubble-based scaffolds. [11] In addition to the observation of cellular morphology, we further conducted assays to test the cytotoxicity and proliferation rate. From LDH assays and myosin heavy chain labeling, we have found that the myoblasts are able to differentiate into myotubes

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in the microenvironment provided by the gelatin microbubble-based scaffold.

II. MATERIALS AND METHODS

A. Fabrication of 3D Scaffolds by Microfluidic Device

The microfluidic device contains one gas input, one liquid input and an output for bubbles to flow out.

Different flow rate and air pressure result in different bubble sizes. Within this device, bubbles appeared at the cross-section where gas and liquid flow encountered.

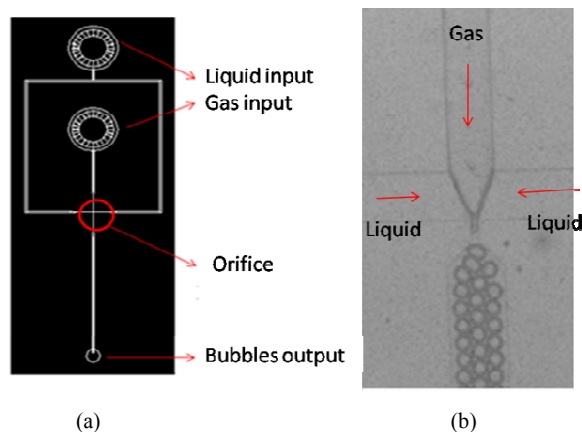


Fig. 1 Image of (a) microfluidic device on computer (b) bubbles formation under high-speed camera

B. Cell Culture

Rat H9c2 cardiac myoblasts were purchased from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, l-glutamine (2 mM), and penicillin (100 IU/mL). All cells were incubated in humidified air at 37°C and 5% CO₂ for the entire duration of the study.

C. Live/Dead Assay

In order to assess the cell viability in scaffolds, the seeded H9c2 cells were stained by live / dead assay on day7. After rinsing in PBS, We stained cells by immersing samples in the solution with 2 μ M calcein AM and 4 μ M EthD-1 in PBS.

D. Cell Nuclei and F-Actin Labeling

Cell morphology was further observed with the aid of immunofluorescence after 1, 3 and 7 days of initial culture. At each time point, medium was first removed. Next, the cells were then fixed with 3.7% formaldehyde for 30 min at room

temperature and followed by incubated in 0.2% Triton-X 100 for 10 min to permeabilize the cells. Samples were then immersed in phalloidin stain at a concentration of 500nM for 30 minutes. After 25 minutes of phalloidin staining, DAPI at a concentration of 300 mM was added.

E. Myosin Heavy Chain Labeling

The differentiation of cardiomyoblasts into myotubes was confirmed by myosin heavy chain labeling. After 14days of culture in growth medium, cells were first permeabilized and fixed as described earlier. In order to prevent non-specific binding, cells were then immersed in 10% fetal bovine serum solution in PBST. After rinsing in PBST buffer, we incubated the samples with mf-20 primary antibody overnight at -4°C . Following primary antibody incubation, the samples were immersed in secondary antibody solution for two hours in the dark followed by rinsing for three times. Finally, samples were further stained by DAPI.

F. LDH Assay

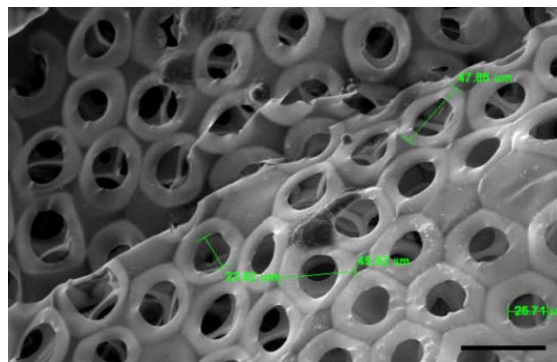
Before the conduction of LDH assay, 2×10^4 of H9c2 cells were seeded on 2D control and 3D scaffolds. To precisely quantify the cells with cell activity, to precisely quantify the cells with the cell activity, cell proliferation was determined by conducting LDH-release assay after 1 day, 7 days and 14 days from cell seeding.

LDH (Lactic dehydrogenase) is a cytosolic enzyme present in a wide variety of organisms including plants and animals. LDH-release assay quantitatively measures LDH from cells whose plasma membranes are damaged by surfactants Triton-X 100. The released LDH is measured with a coupled enzymatic reaction that causes Iodonitrotetrazolium chloride (INT) reacting into a red color formazan. Therefore, the level of formazan formation is directly proportional to the amount of released LDH. Before conducting LDH-release assay, the LDH working solution was prepared by mixing the LDH stocking solution at the final concentration of 12 mg/mL sodium L-lactate, 1 mg/mL β -Nicotinamide adenine dinucleotide hydrate (NAD^+), 0.45 mg/mL diaphorase, 0.1 mg/mL bovine serum albumin, 4 mg/mL sucrose, and 0.67 mg/mL iodonitrotetrazolium violet. Cells were then rinsed twice by PBS carefully followed by incubation of 0.2 % Triton-X 100 to lyse the cells for 30 min. And 50 μL of the lysates were added into 96-well microplates. The enzymatic reaction was initiated after mixing 100 μL LDH working solution. Absorbance at 492 nm was measured using an ELISA spectrophotometer after the mixtures were incubated in 37°C for 15 minutes.

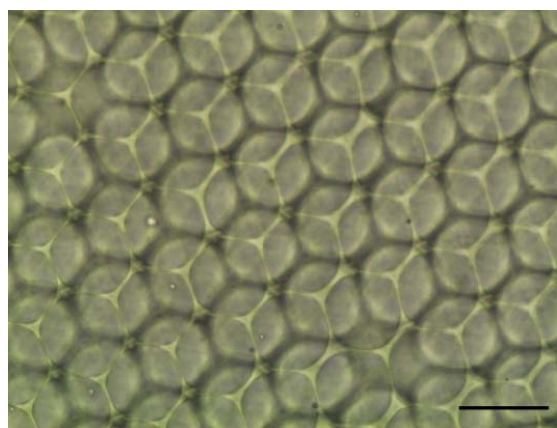
III. RESULTS

A. Scaffold Fabrication

The dimensions of the fabricated scaffolds structure are determined from the SEM and microscope images. Fig. 2a shows that each scaffold is composed of many microbubbles with approximately $50\mu\text{m}$ in diameter and $25\mu\text{m}$ for the diameter of the connecting channels between bubbles. The image taken by a reversal microscope (Fig. 2 (b)) further confirms the dimensions of scaffolds.



(a)

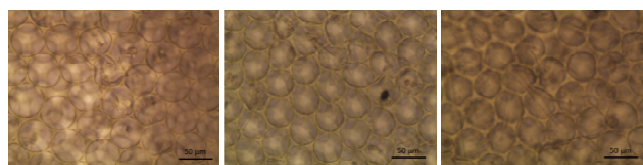


(b)

Fig. 2 Characterization of microbubble-based scaffolds: (a) a SEM image (b) an image taken by reversal microscope. Scale bar = $50\mu\text{m}$

B. Cell Morphology on 3D Scaffolds

H9c2 cardiomyocytes were seeded in 3D gelatin scaffolds, and we took images of the scaffolds at the 1st, 3rd and 7th days after seeding (Fig. 3). From the outline of cells, we know that most cells begin attaching after 24 hours, and stretched well after 72 hours. But unlike 2D culturing on a dish, not much proliferation was observed.



(a)

(b)

(c)

Fig. 3 Images taken by a reversal microscope after seeding for (a)1, (b) 3 and (c) 7 days

C. Live/Dead Assay

The results of a qualitative viability test are reported in the following, based on the live/dead assay after culturing for 7 days. The images show intense green fluorescence and nearly no visible red spot. The ratio of the live cells to total cells is 94.9%. (n=15) This suggests a good viability.



Fig. 4 Fluorescence microscopy images displaying the live/dead assay of H9c2 cells in 3D scaffolds after culturing for 7 days (green cells are live, dead cells are red). Scale bar = 200µm

D. Cell Nuclei and F-Actin Labeling

DAPI/Phalloidin dye was used to stain cell nuclei and F-actin. According to these images, we can observe that some nuclei were in the same cytoskeleton. This shows that: besides cardiomyocytes, many myotubes were formed in the scaffolds without the stimulation of differentiation medium.

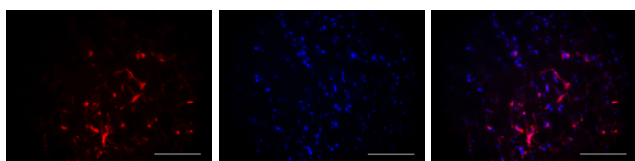


Fig. 5 Nuclei and F-actin staining of H9c2 cells in 3D scaffolds after culturing for 7 days

E. Myosin Heavy Chain Labeling

We assumed that the microbubble-based scaffolds are capable of inducing myoblasts into myotubes. In order to validate this hypothesis, cells are seeded in scaffolds treated with growth medium only. After 14 days of culture, without a stimulation of chemical cue, many myoblasts had differentiated into myotubes. This suggests that the microenvironment supplied by scaffolds might stimulate myoblasts into fusion.

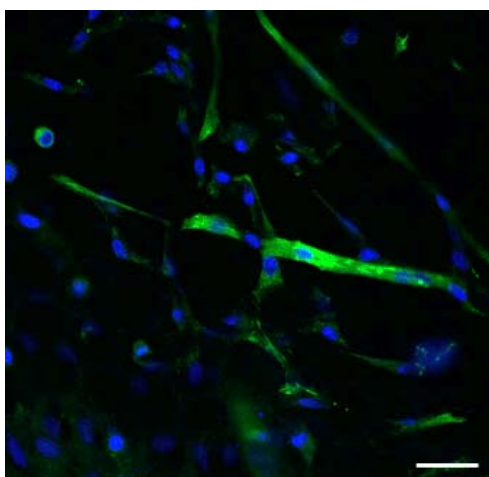


Fig. 6 Confocal immunofluorescence pictures of differentiating H9c2 cells in scaffolds on day 14. The nuclei are stained with DAPI (blue), and myotubes are labeled with myosin heavy chain antibody (mf-20). Scale bar = 50µm

F. Cell Proliferation Rate

The proliferation rate was quantitatively confirmed by LDH assay. To compare the results of cells in 3D circumstances with

2D, we further seeded H9c2 on gelatin coated surface as control. The result is shown on Fig. 5. H9c2 cells on gelatin film grow 4.6 folds from day1 to day3, and 2.1 folds from day3 to day7. For cells in scaffolds, cells grew 1.7 folds and 1.4 folds from day1 to day3 and day3 to day7, separately. Significantly lower proliferation rate was observed for the scaffolds set. In fact, one crucial factor of the differentiation of myoblasts into myotubes is the cessation of proliferation. This result may thus be related to the differentiation capability in scaffolds.

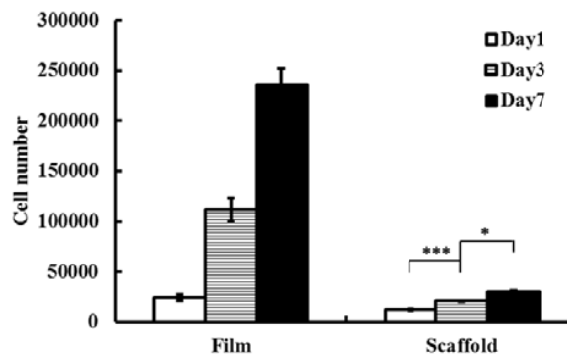


Fig. 7 Quantification of cell proliferation after 1day, 3days, and 7days of culture on gelatin film and in scaffold

IV. CONCLUSIONS

Two different assays were conducted to qualitatively assess cell viability, proliferation and the structure of actin. With the images of the nuclei and F-actin staining, we observed formation of myotubes without the stimulation of differentiation medium.

The results of live/dead assay suggest that the rat cardiomyoblasts seeded in scaffolds are viable. Also, the total amount of cells does not seem to increase, and this is consistent with the result of the 1st assay: the differentiation of cells caused the low proliferation rate.

We can come into a conclusion: our 3D gelatin scaffolds caused the cells to differentiate into myotubes rather than proliferate

Our future work might focus on getting more proves about the promotion effect of differentiation which is cause by these micro-bubbles scaffolds.

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