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LETTER

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Feasibility study of a biocompatible pneumatic dispensing system using mouse 3T3-J2 fibroblasts

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Abstract

This paper presents results for dispensing living cells using a pneumatic dispensing system to verify the feasibility of using this system to fabricate biomaterials. Living cells (i.e., mouse 3T3-J2 fibroblast) were dispensed with different dispensing pressures in order to evaluate the effect of dispensing process on cell viability and proliferation. Based on the results of a live-dead assay, more than 80% of cell viability has been confirmed which was reasonably similar to that in the control group. Furthermore, measurement of cell metabolic activity after dispensing confirmed that the dispensed cell proliferated at a rate comparable to that of the control group. These results demonstrate that the pneumatic dispensing system is a promising tool for fabrication of biomaterials.

Keywords: Pneumatic dispensing, Living cell, Viability, Proliferation

Background

Technologies to manipulate living cells are widely employed in biological and tissue engineering applications. For example, depositing living cells at a specific location is useful to help understand how cell behavior such as proliferation, differentiation, and migration is related to local environment (e.g., extracellular matrix and neighboring cells) [1–4]. Also, in tissue engineering, which seeks to repair injured or ill organs, it is necessary to seed living cells at a precise position in three-dimensional (3D) scaffolds to achieve successful production of artificial tissues or organs [5–7].

To construct 3D cellular structures, many cell patterning technologies have been developed, including micro-contact printing using soft-lithography [8], and microfluidic technology [9]. These technologies can produce a high resolution pattern, but they are inconvenient to form 3D structures and expensive to change patterns [10].

Recently, dispensing (e.g., inkjet printing) technology has attracted much attention for use as a tool to fabricate structures consisting of various cell types [11–15]. Because the dispensing technology can make a precise cell pattern directly by dispensing small droplets of biological materials containing living cells, it has advantages of simplicity and flexibility for fabrication of complex cellular structures. The dispensing technology is flexible to pattern various designs by simply controlling the position to dispense living cells in droplets from a nozzle. Furthermore, because the dispensing technology can be easily integrated with computer-assisted manufacturing systems, it can use solid free-form fabrication to precisely form complex 3D cellular structures [16, 17]. Also, it can form cellular structures that consist of different cell types by using and controlling multiple nozzles, each with a corresponding reservoir [5].

For tissue engineering applications, considerable research has been devoted to extending the capability of dispensing technology to create artificial tissues or organs. Because living cells are easily damaged by heat and mechanical stress [5], the biocompatibility of the dispensing system must be assessed by evaluating the

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effect of the dispensing process on cell viability and proliferation. Living cells have been dispensed using various printing systems such as thermal inkjets [11], piezoelectric inkjets [12–15], electrostatics [5], lasers [18], and electrohydrodynamic jets [19].

This paper presents results for dispensing living cells using a pneumatic dispensing system to verify the feasibility of using it as a biocompatible fabrication tool. The pneumatic dispensing system uses a simple yet effective mechanism with a backflow stopper and a flexible membrane, so it is easy to control droplet volumes and to eject highly viscous liquid [20, 21]. To assess the biocompatibility of the dispensing system, experiments were conducted using mouse 3T3-J2 fibroblast cells at various operation conditions, then cell viability and proliferation were evaluated using a live-dead assay and a cell counting kit assay, respectively.

Materials and methods

Pneumatic cell dispensing system

To dispense the liquid droplet containing living cells, an experimental setup using a pneumatic dispensing system was developed as shown in Fig. 1a. A cell suspension from a reservoir is delivered to the dispenser by applying a steady air pressure (i.e., inlet pressure) to the reservoir. Using a magnetic stirrer at the bottom of the reservoir, cells were prevented from sedimentation by gravity and uniform dispersion of cells was maintained.

The dispenser has the top (glass) and bottom (silicon) substrates separated by a flexible membrane made of a polydimethylsiloxane (PDMS) film as shown in Fig. 1b. A large through-hole connected to controlled pressures (positive and negative) was machined through the top substrate. The flexible membrane (thickness ~ 70 μm) is deformed by the applied pressure. The bottom substrate including a liquid chamber, an inlet, and a sideway outlet (Table 1) was fabricated using conventional micromachining technology as described in our previous paper [20]. The reservoir and the dispenser were washed with 70% ethanol and then with phosphate-buffered saline (PBS) prior to use.

The flexible membrane is deflected by the applied pressure, and thus draws in or dispenses liquid. The membrane is either pulled (negative pressure) or pushed (positive pressure) depending on the programmed electric signal. The applied pressure is normally negative; a pulsed signal switches a solenoid valve to provide positive pressure (during duration time) to dispense the liquid. The liquid is drawn into the chamber when the membrane is pulled (during delay time) and dispensed when it is pushed.

Preparation of cell suspension

A cell suspension was prepared using mouse fibroblasts (3T3-J2 cells) obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD) supplemented with 10% bovine calf serum (Gibco), 1% mixture of penicillin and streptomycin (Gibco). Cells were cultured at 37 °C in a humidified incubator in an atmosphere containing 10% CO_2 [22]. To apply living cells to the dispensing system, cells in culture flasks were trypsinized, then cell pellets were collected, and resuspended in a phosphate-buffered saline (PBS, Gibco) solution. The suspension contained 500,000 cells mL^{-1}, as quantified using a hemocytometer.

Cell viability assay

The viability of dispensed cells as compared with a pipetted control was quantified using a LIVE-DEAD® assay Kit (Lonza, Walkersville, MD). Live cells fluoresce green unless the cell membrane is damaged in which case they fluoresce red. Before dispensing cells on a substrate (96-well plate), a collagen gel layer was formed on the substrate surface (Fig. 2a). Collagen is a well-known universal cell binder and is widely applied in cell culture [23]. A dilute (1.25 mg mL^{-1}) aqueous solution of type-I rat tail collagen (BD Biosciences, Bedford, MA) was prepared using sterilized DI water, then diluted 9:1 (volume ratio) with 10 × DMEMS (Gibco). A 40 μL of the mixture was pipetted into each well on the plate, then incubated at 37 °C for 30 min to form the collagen gel layer. After collagen gelation process, 30 μL of a mixture of 2 μM calcein AM and 4 μM ethidium homodimer-1 solution from the live-dead kit was added to each well (Fig. 2b). Then a single liquid droplet (volume ~ 250 nL) containing cells was dispensed directly onto each well of the plate (Fig. 2c). At the same time, 1 μL of the four times diluted cell suspension (i.e., 125,000 cells mL^{-1}) was pipetted on the plate, as a control. Dispensed cells were incubated for 30 min at room temperature, then a fluorescence microscope (IX71 microscope, Olympus) was used to obtain images of stained cells.

Cell proliferation assay

To confirm the proliferation of dispensed cells, their metabolic activity was measured using a cell counting kit (CCK-8, Dojindo, Kumamoto, Japan). Twenty droplets of cell suspension (total volume ~ 5 μL) were dispensed into each well of a 96-well plate supplemented with 100 μL of fresh culture medium (Fig. 3a). After dispensing the cells, 10 μL of CCK-8 solution was added to each well and the plates were incubated for 2 h at 37 °C in an atmosphere containing 10% CO_2 (Fig. 3b). The emitted fluorescence of samples at 450 nm was measured using a micro-plate

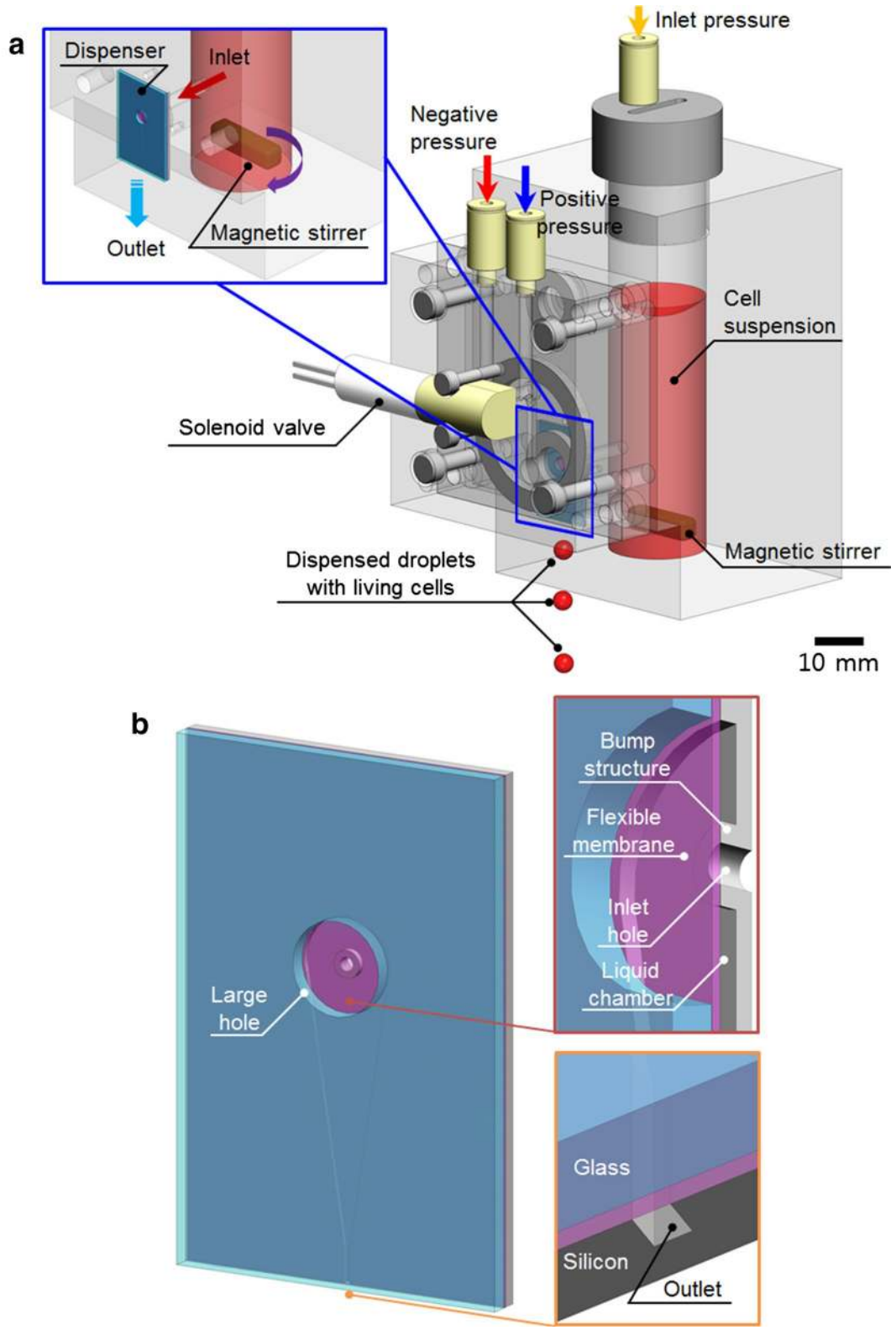

Fig. 1 Schematic views of **a** cell dispensing system and **b** dispenser design with a width of 10 mm, a height of 20 mm and a thickness of about 0.8 mm

Table 1 Summary of design parameters

Design parameters	Size (μm)
Chamber	
Diameter	2000
Height	100
Bump	
Diameter	400
Height	80
Inlet hole	
Diameter	200
Outlet nozzle	
Width	100
Height	100

reader (UVM 340, Biochrom, Cambridge, UK). Samples were measured repeatedly for continuous monitoring of the same sample at each time point (4, 24, 48, and 72 h) after dispensing the cells. After the fluorescent was measured, wells including cells were washed with PBS solution and incubated in fresh culture media (Fig. 3c). The culture media were changed and the cells returned to the incubator. At the same time and well plate, 5 μL of the same cell suspension was pipetted as a control.

Results and discussion

To test the biocompatibility of our dispensing system, cell viability and proliferation were analyzed qualitatively using live-dead and CCK-8 assays. A statistical significance was determined using analysis of variance (ANOVA) on MINITAB version 14.2 (Minitab Inc., State College, PA, USA). A P value less than 0.05 was considered statistically significant.

Cell viability

Damages to cell membranes by physical forces (i.e., shear stress) during dispensing process were observed in optical images using a live-dead assay. Cell suspension was dispensed at positive pressures from 25 to 100 kPa

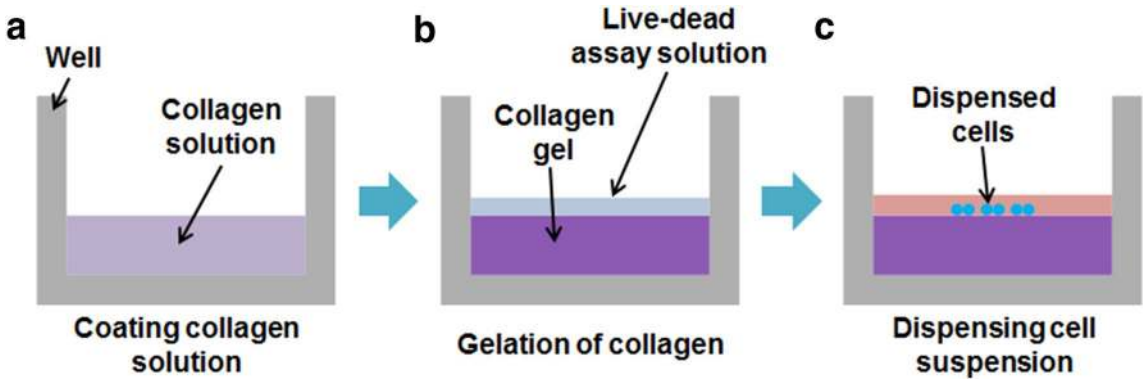

Fig. 2 Schematic views of the cell viability test procedure: **a** injection of the collagen solution into the well, **b** thermal gelation of the collagen solution, and **c** dispensing of a cell-containing droplet

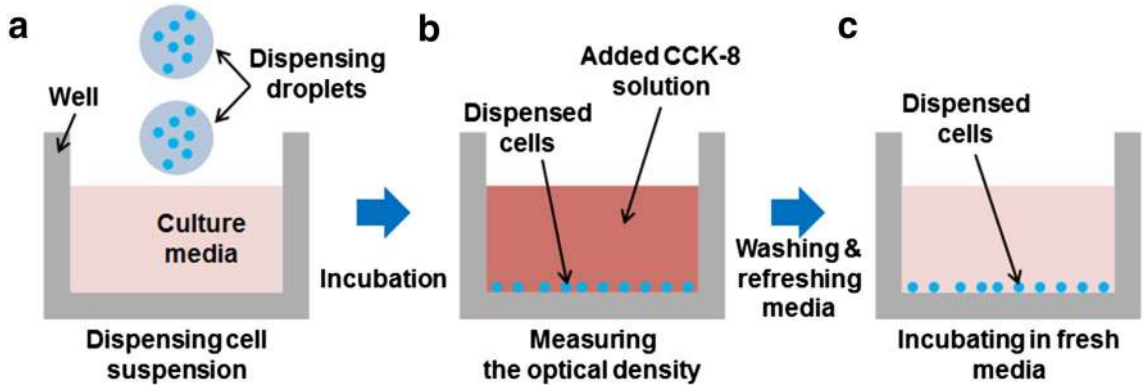

Fig. 3 Schematic views of the cell proliferation test procedure: **a** dispensing of cell droplets into the well, **b** addition of the CCK-8 solution into the well and measurement of light absorbance after incubation, and **c** replacement with fresh cell media

while the droplet volume was varied from 182 to 274 nL (CV < 1.2%). Images of stained cells which were almost all of cells containing a single droplet (90–130 cells per droplet) were shown in Fig. 4. The cell viability was calculated by counting live or dead cells for each image at each dispensing pressure (n = 8). The mean cell viability and standard deviation (SD) were plotted in Fig. 5. The viability of dispensed cells ranged from 81 to 83% for applied pressure values (25–100 kPa), and was not affected significantly by applied pressure ($P > 0.05$). The average viability of dispensed cells was slightly less than that of the control group (~ 94%). This difference in viability may have occurred because the higher droplet velocity subjected dispensed cells to a larger shear stress than those experienced by cells in the pipetted control. For the current design of the dispenser, the measured droplet velocities were in range from 1.6 to 5.4 m/s while applied pressures were changed [24], which are higher than typical inkjet printing system such as piezoelectric or thermal bubble jet [25].

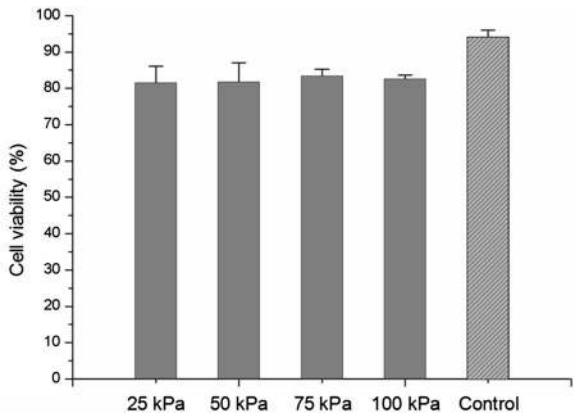

Fig. 5 Cell viability result for various positive pressures. The graph shows a slight loss of cell viability after cell dispensing

Cell proliferation

Even if a cell membrane is not damaged, its function may be impaired by the stress experienced during the dispensing process [11]. So cell metabolic activity after dispensing was measured using a CCK-8 assay. Measurements were made starting at 4 h after dispensing, which is needed for the cells to settle and adhere to the substrate, then repeated daily for up to 3 days (72 h). Under the same operating conditions, 20 droplets were dispensed repeatedly into each well with the control (n = 5). All samples were dispensed in one sequence over no more than 10 min using a single batch of cells in suspension. Figure 6 shows the mean value of optical density (OD) at 450 nm and SD for each time interval. The measured OD values of all samples show a progressive increase over time ($P < 0.05$). These results suggest that the cells were proliferating at a rate comparable to that of the control.

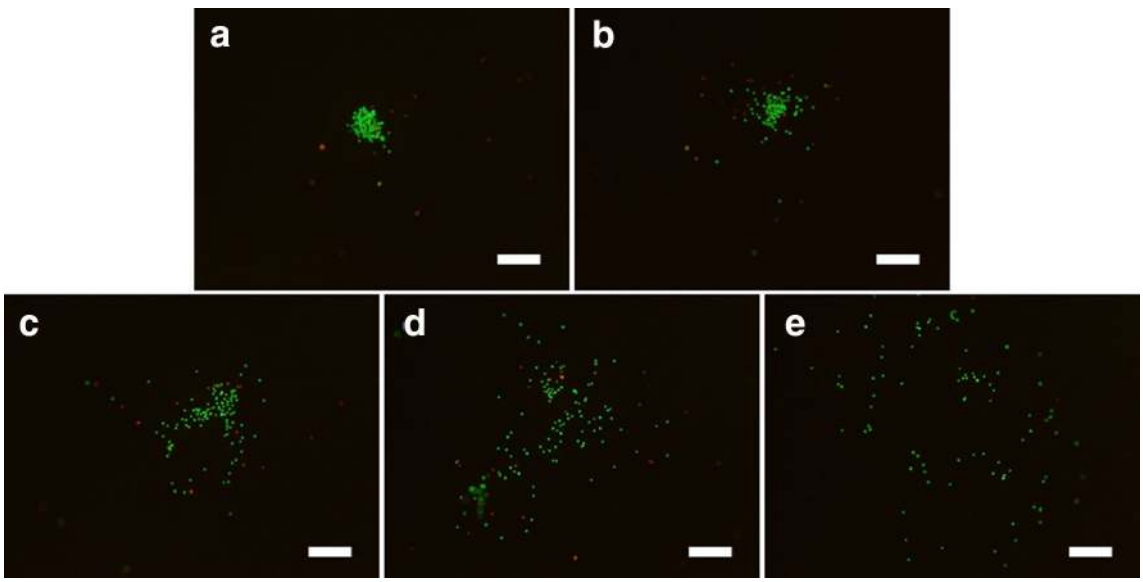

Fig. 4 Live-dead assay of fibroblast cells dispensed onto a collagen gel at positive pressures of **a** 25 kPa, **b** 50 kPa, **c** 75 kPa, **d** 100 kPa, and **e** the control (pipetting). All scale bars are 500 μm

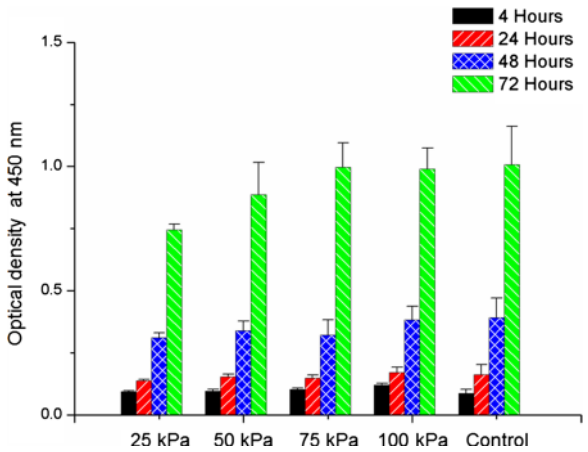

Fig. 6 Cell proliferation result as a function of time in culture after dispensing at different positive pressures. The graph demonstrates that the cell proliferation rate is similar for various positive pressures ($P < 0.05$)

Conclusion

The effect of our dispensing system on cell viability and proliferation was assessed at various applied pressures. Based on the results of a live-dead assay, more than 80% of cell viability has been confirmed which is reasonably compatible to the control group. Cell metabolic activity measurements confirmed that the dispensed cells were proliferating at a rate comparable to that of the control. These results confirm the feasibility of using our pneumatic dispensing system to dispense living cells for fabrication of biomaterials.

Authors' contributions

LS and KH performed the experiments, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Ethics approval and consent to participate

Not applicable.

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