Development of an effective three dimensional fabrication technique using inkjet technology for tissue model samples

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Abstract
Engineering of a functional tissue model is considered integral to the development of alternative methods to animal testing. Tissues and organs have three dimensional (3-D) structures and are composed of variety of components, multiple types of cells and extracellular matrices proteins. For engineering those structures in vitro, effective 3-D fabrication technique is required. We have attempted to fabricate 3-D structures using inkjet printing technology to arrange cells and various biomaterials freely in 3-D space. In order to fabricate 3-D structures, hydrogel was used, and we have developed an original inkjet system specialized for fabrication of 3-D hydrogel structures. In this study, we performed spatially controlled hydrogel structures including cells in 3-dimensions using our original inkjet system. Cells were suspended in gel precursor solution, and hydrogels including the cells were formed by printing into reactant solution for sol-gel reaction. Using our inkjet system, we successfully fabricated several 3-D hydrogel structures such as sheet and tube with cells. We demonstrated an inkjet-based 3-D fabrication technique using hydrogel/cells. Those results suggested that 3-D tissue model samples can be designed and fabricated by using inkjet-based 3-D fabrication technique. Such 3-D bio-manufacturing technology will contribute to the development of alternative methods to animal experiments.

Keywords: tissue model, 3-D fabrication, inkjet technology

1. Introduction
Animal experiments have been contributing to the developments in medicine and biology. Toxicity tests, dose tests and biocompatibility tests using experimental animals have also been contributing to safeguard human beings against various chemicals and pharmaceutical agents. However, there are some important considerations involving scientific and ethical concerns, such as the ethical concerns involving use of laboratory animals, the differences of species between human and experimental animal and the intrinsic variability of the animal test. Therefore, the need for more sophisticated validation method has been promoting the development of in vitro systems using human cells for alternatives to animal experiments. Recently, artificially constructed human tissue models of skin and cornea have been utilized for the alternative methods to skin or ocular irritation (Tornier et al., 2006; Doucet et al., 2006; Vinardell & Mitjans, 2008). In addition to the simple cell culture systems, the functional tissue model in vitro is considered integral to further development of alternative methods to animal experiments. However, native tissues and organs have three dimensional (3-D) structures and are composed of a variety of components such as multiple types of cell and extracellular matrices proteins. In addition, in tissues, there are special microstructures, such as tubular, sheet and solid structures, which bring structure-specific biological functions. To obtain the functional tissue model in vitro, some effective 3-D fabrication techniques with biological materials, which enable to manufacture such biological microstructures, are required. Then, we focused on an inkjet technology. It is known that inkjet printers print pictures with small droplets, with multicolor inks, and at high resolution on the printing media. These characteristics are considered advantageous for arrangement of cells and biological materials (Fig. 1). Then, we have attempted to develop an inkjet-based 3-D fabrication technique.
Previously, we and other research groups have already reported that inkjet can be used in ejecting living cells (Nakamura et al., 2005; Xu et al., 2006; Saunders et al., 2008). Furthermore, we recently made a prototype of original inkjet 3-D printing system and developed an effective 3-D fabrication technique specialized for high resolution cell positioning. In this work, we shall report the inkjet-based 3-D fabrication technique using the gelation technique of inkjet droplets.

Materials and methods

Preparation of cells

A human cervical carcinoma cell line, HeLa cells (JCRB 9004), was provided by the Health Science Research Resources Bank (Osaka, Japan). HeLa cells was maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin, Sigma, St. Louis, MO) in 5% CO₂ at 37°C. Human umbilical vein endothelial cells (HUVECs) and endothelial cell growth medium (Humedia EG2) were obtained from Kurabo Industries, Ltd. (Osaka, Japan). HUVECs were used between passages 2 and 5.

Development of an original inkjet system (3-D bio-printer) for 3-D fabrication

We have developed an original inkjet 3-D printing system specialized for fabrication with living cells and biological materials (Fig. 2). The whole printer unit was set up inside a clean bench for the cell culture, which required a clean environment.

For this study, we used a piezoelectric inkjet head, whose structure have been described previously (Nishiyama et al., 2007), and the inkjet head can be freely moved in 3-D space. Both the hardware and software used in the printing system were designed and developed originally in our laboratory.

The gelation technique of inkjet droplets

In order to arrange cells in 3-D space, the gelation technique of inkjet droplets was used. Cells were suspended in the gel precursor solution, and ejected into reactant solution for sol-gel reaction. On gelling of inkjet droplets, cells were embedded into hydrogel. In this study, we used sodium alginate and CaCl₂ solution, and fibrinogen and thrombin solutions, as the pairs of gel precursors and gel reactant solutions, respectively. Micro gel fibers containing cells could be fabricated, when straight lines were printed, ejecting at a suitable frequency and moving inkjet head at a suitable speed.
A 3-D fabrication technique

i) 3-D tubular structures

By using the gelation technique, 3-D tubular structures were fabricated as followings. 0.8% sodium alginate solution containing HeLa cells (6×10^6 cells/ml) was ejected continuously into a CaCl₂ solution with inkjet head moving continuously in a circle pattern of 1 mm in diameter for a suitable period of time, where the ink was ejected at 800 Hz and the inkjet head was moving at a speed of 20 mm/second, respectively.

ii) Fabrication of sheet structure using fibrin gel

For fabrication of sheet structure, fibrin hydrogel was used. HUVECs were suspended with 30 mg/ml fibrinogen solution and then ejected into 500 U/ml thrombin solution. To form a sheet structure, the inkjet nozzle head was moved sequentially with line pattern for painting the area of programmed square. The inkjet head were moved at a speed 20 mm/second while ejecting the inkjet droplets at a frequency of 800 Hz.

Results

Gelation technique of inkjet droplets for cell patterning

Although we could print living cells by inkjet technology in our previous study, we found two serious problems with inkjet-based cell printing for 3-D fabrication. The first was the problem that printed cells were dried immediately, because the inkjet droplets were so small to be dried immediately. The second problem was that positioning of printed cells was very difficult in the wet substrate. When the droplets were printed onto wet substrates to avoid from drying, the printed cells were spread and distribute randomly by blotting. Therefore, we have developed gelation technique of inkjet droplets to overcome these problems. When inkjet droplets containing gel precursor were ejected into reaction solution for sol-gel reaction, the droplet changed form to hydrogel (Fig. 3A). By using the gelation technique, cells were embedded into hydrogel such as alginate gel and fibrin gel to protect from drying, and were able to be arranged into the micro-gel fiber without blotting, printed in even a watery substrate (Fig. 3B and 3C).

Fabrication of 3-D tubular structure by 3-D bio-printer

Tubular structures are often observed morphologically in various organs, such as blood vessels, bile ducts, and bronchus. Therefore, we tried to fabricate tubular structure by z-directional-laminated printing of circle pattern. Figure 4 shows a tubular structure of 1mm in diameter and more than 1cm in length, which contained HeLa cells in the alginate hydrogel.

Fabrication of sheet structure by 3-D bio-printer

Sheet structures are also observed frequently in
various tissues and organs. Furthermore, the sheet structure is a basic component in laminated structures. For these reasons, we tried to fabricate a sheet structure using 3-D bio-printer by printing linear patterns tightly. Figure 5 shows a sheet structure of $7 \times 5$ mm containing HUVECs, which was fabricated with fibrin hydrogel.

Discussion
Classically, most cell culture methods have been performed under adherent 2-D conditions. However, some limitations in 2-D culture were reported on cellular morphology and function (e.g. Smalley et al., 2006; Abbot, 2003). Therefore, 3-D cell culture methods are focused in biological research area. Cells in tissues and organs live within extracellular matrices and other types of cells. Therefore, 3-D cellular microenvironment is critical for cells to express several functions, including proliferation, migration, differentiation and drug responses. To engineer such a suitable microenvironment, it is necessary to arrange not only cells but also other biomaterials such as extracellular matrices and growth factors.

In this study, we were able to fabricate 3-D tubular structures and sheet structure with cells / hydrogel using original 3-D bio-printer. By developing gelation technique, cells could be protected from drying, and printed without blotting even in the watery medium. It was demonstrated that inkjet-based 3-D fabrication technique is an effective cell positioning method for creation of more sophisticated 3-D tissue model samples.

Another research group reported that inkjet technology had been used to print a gradient pattern of growth factors on 2-D fibrin film, and that cell proliferation was enhanced within the growth factor patterned region (Campbell et al., 2005; Miller et al., 2006). This indicates that inkjet technology has a potential to control cellular functions and tissue growth by controlling the concentration of gradients of growth factors in the fabricated structures.

In the future, we believe that inkjet-based 3-D fabrication technique will contribute to fabricating and developing more functional tissue models for alternatives to animal experiments.

Conclusion
In this study, we demonstrated the development of our inkjet-based 3-D fabrication technique using hydrogel and cells. Using the gelation technique and the 3-D bio-printer, spatially controlled hydrogel structures could be fabricated including living cells in three dimensions. Those results suggested that the more sophisticated tissue models can be designed and fabricated by engineering method using inkjet-based 3-D bio-fabrication technique. Such 3-D bio-manufacturing technology will contribute to the development of alternative methods to animal experiments.

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References