Application of inkjet in tissue engineering and regenerative medicine: Development of inkjet 3D biofabrication technology

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Abstract

Tissue engineering and regenerative medicine are hoped as the most promising advanced medicine of 21st century. Both are the most promising and reasonable approaches to save patients with organ failure, instead of transplantation. To date, simple and thin tissues have been successfully engineered such as skin and cartilage, however, a number of challenges are needed in engineering other thicker, larger and more complicated tissues and finally available organs. Biological tissues are composed of several types of cells and biomaterials, and have 3D architectures with micro-scaled resolution and macro-scaled mass. To engineer such tissues, printing technologies are promising, because the printer must print pictures on macro-scaled papers simultaneously with micro-scaled resolution. Then, we have developed 2D to 3D biofabrication using inkjet and hydrogel. 3D bioprinter has been developed using inkjet by our selves and several structures with hydrogel and living cells were fabricated. In this presentation, we introduce our progress of the research and development using inkjet technology. Digital fabrication including inkjet will provide promising and innovative approaches for sophisticated tissue engineering.

Introduction

Organ and tissue replacement therapy is indeed the ultimate final therapeutic method for irreversible organ failure which can not be recovered with any other therapies. Tissue engineering and regenerative medicine, which are the researches aiming finally to make alternative tissues and organs for transplantation therapies, are the most reasonable and promising approaches to solve many serious problems in transplantation therapy, such as donor organ shortage and several socio-ethical problems.

To date, tissue engineering has successfully achieved in engineering of some tissues such as skin, cornea and cartilage, which are thin and simple, and composed of mono-type of cells. On the other hands, the tissues that should be engineered in next decades are thick and large 3D tissues, composed of multiple different kinds of cells, with unique microstructures, and with sufficient vasculature. Major approaches to obtain donor tissues and organs addressed by biologists and medical researchers are embryological approaches using ES cells and stem cells, or molecular and genetically engineering technologies. Those are indeed important researches, however, it needs many years to make alternative organs for transplantation. Some effective engineering methods are needed to obtain donor tissues and organs by just like off-the-shelf manner. Then, we have challenged another approach, in which tissues and organs are directly built up using cells and biomaterials, applying manufacturing and fabrication technologies. Considering the characteristics of the next targeted complicated tissues, it is speculated that the techniques to construct 3D structures with multiple different cells and biological materials with micro-scaled resolution are required.

Based on such speculations, we have applied inkjet technology and have developed innovative approach to fabricate biological tissues, which we call inkjet bioprinting or inkjet 3D biofabrication. As we and some other researchers have ever reported, inkjet technology has many advantages and meets many of such requirements for building up biological tissues [1-6]. Besides, we previously reported the feasibility of an inkjet technique not only for ejecting living cells, but also for fabrication of 3D structures using gelation technique of ejected droplets [3, 4]. Then, we have developed an original 3D bioprinter to fabricate 3D hydrogel structures containing living cells [3, 4]. Using the 3D bioprinter, several 2D and 3D hydrogel structures and cell contained structures have been successfully constructed [3, 4].

Purpose

As mentioned above, we have addressed to develop inkjet 3D bioprinting technique and biofabrication technology. This technique of inkjet 3D biofabrication is one of the rapid prototyping technologies, or one of the digital fabrication technologies. In our technique, alginate hydrogel was used mainly. The ejected droplets form micro gel beads, which fuse to form fibers and sheets, and finally 3D hydrogel structures can be built up by laminating. Therefore, a micro gel bead, which is produced by one droplet of inkjet ejection, is the minimum elemental unit for inkjet 3D biofabrication, or a dot of inkjet digital fabrication, just as the pictures printed by inkjet are printed with micro dots of several color inks (Figure 1). In addition, downsizing of inkjet droplets have developed the quality of inkjet printing. Then, in this study, we investigated the micro gel beads first and next tried downsizing to develop inkjet 3D biofabrication technology further more.

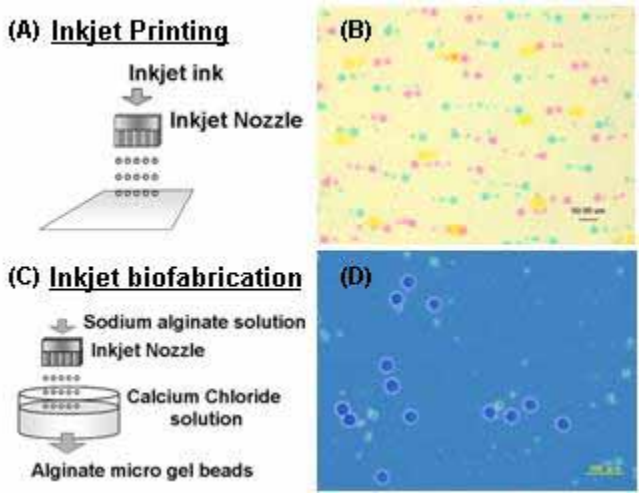


***Figure 1**, Investigation of the minimum dots of inkjet printing (A, B) and of inkjet biofabrication (C, D).*
Microscopic photograph of the inkjet dots printed by commercial inkjet printer (PIXUS860i, Canon Inc., Tokyo, Japan) (B), Phase contrasted microscopic photographs of alginate micro gel beads produced by inkjet system used in our experiments (D)

Materials and Methods

1) Inkjet 3D bioprinter

We have developed 3D bioprinter [3, 4]. For the purpose of dealing with living cells, the size was designed for use in the clean bench and all the control systems were set outside of the bench. Inkjet head can move XYZ axis, and 3D structures are fabricated in the calcium chloride solution.

As an inkjet system, a static electricity actuated inkjet system (SEAJetTM inkjet head, Seiko Epson Corp. Suwa, Japan) was used in this study [2, 7]. This inkjet system is one of the mechanical type of inkjet systems. The pusher plate of each nozzle was actuated by static electricity power. This head has 12 nozzles and all of the nozzles can be controlled independently. The shapes of the nozzle orifices are triangle with 60µm in bottom length and 40µm in height. This system doesn't generate heat and is composed of glass and silicon and stable against many chemicals. We have recognized it as one of the biocompatible inkjet systems for cell printing [1,2].

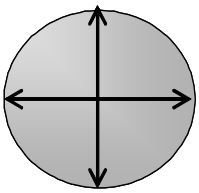
We used another inkjet head, when we tried to make smaller gel beads. It was an experimental SEAJet head with small sized nozzle orifices and the pusher plates.

2) Producing alginate micro gel beads

Basic procedure of making alginate micro gel beads was followings. 0.8 to 1.0% sodium alginate solution was prepared and ejected into 2.0% calcium chloride solution by inkjet. As alginate hydrogel itself is colorless and transparent, the produced micro gel beads could not be seen easily without using phase contrast microscopy. For visualization, cyan colored printer ink (ICC32 Cyan, Seiko Epson, Suwa, Japan) was added into sodium alginate solution. We usually stirred calcium chloride solution with hands or using a magnetic stirrer.

Based on this procedure, we obtained alginate micro gel beads and then evaluated them.

3) Morphological analysis of micro gel beads



$$\text{Diameter of micro gel beads} = \frac{L_{Long} + L_{short}}{2}$$

L_{Long} ; Length of long axis

L_{Short} ; Length of short axis

***Figure 2**, Quantitative analysis of the size of micro gel beads by image analysis*

In general, micro gel beads obtained seemed sphere shape. Then, the sizes of the micro gel beads were measured and analyzed using image analysis. We used a particle-analyzing program developed with IMAQ Vision Builder™ (National Instruments Corp., Austin, USA). In this program, the image of every gel bead was estimated as ellipsoidal shape. Fitting to ellipsoidal shape was done based on the particle area and the length of the outer contour of the particle. And next, the lengths of the long and the short axis were measured and their average was estimated as a diameter of a micro gel bead. Then the average and standard deviation were calculated for total of all the micro gel beads counted, and the variability of them was evaluated with histogram.

Results

1) Production of alginate micro gel beads and size analysis

According to the basic procedure mentioned above, alginate micro gel beads were obtained (Figure 3). In general observation, almost all of the micro gel beads showed circular shapes with homogeneous size of approximately 40□m in diameter. As almost all the floating micro gel beads also showed round shapes, the shapes of micro gel beads were thought almost sphere.

Then, the further quantitative analysis was performed with image analysis. The sizes of all micro gel beads were measured according to the analyzing procedure mentioned in materials and methods. 30 different microscopic photographs of micro gel beads were taken and those images were analyzed. As a result, in total, 506 beads were measured. The histogram of the diameter is shown in figure 4. The average and the standard deviation of the diameters were 38.50µm and 3.58µm, respectively. And 94% of micro gel beads were within 35 to 45µm.

In close observation, the smaller micro gel beads with the diameter of 10 to 30µm were also found (Figure 3B, Arrowed). Those were thought to be generated by the satellite droplets of inkjet ejection. We could recognize again that the control of satellite is an important issue in inkjet biofabrication, too, as has ever and still been a major issue for inkjet printing technology.

2) Small sized micro gel beads

We speculated the size of the micro gel beads produced was dependent on the inkjet droplet size, and then we tried to make smaller size of micro gel beads using another experimental inkjet head with smaller nozzle orifice. As a result, alginate micro gel beads with smaller diameter were successfully produced. They were compared with same-scaled micro gel beads produced with usual inkjet head and those images were shown in Figure 5. The remarkable difference was seen. The diameters of the small micro gel beads were almost 10µm, while those of usual micro gel beads were 40µm. Thus, it was demonstrated that the sizes of the micro

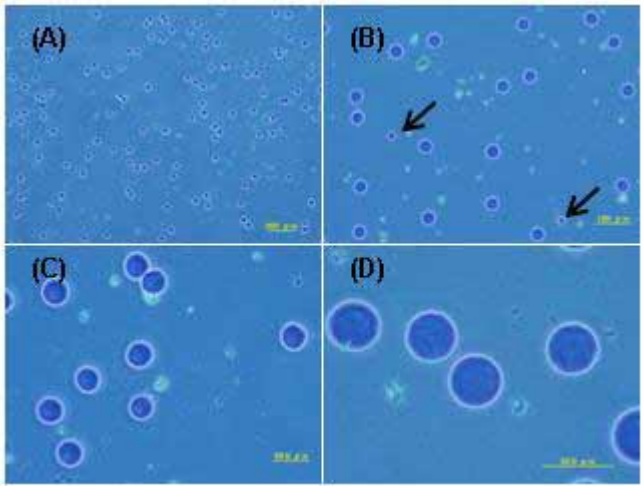


***Figure 3**, Produced alginate micro gel beads. Smaller micro gel beads are indicated by the arrow (B).*

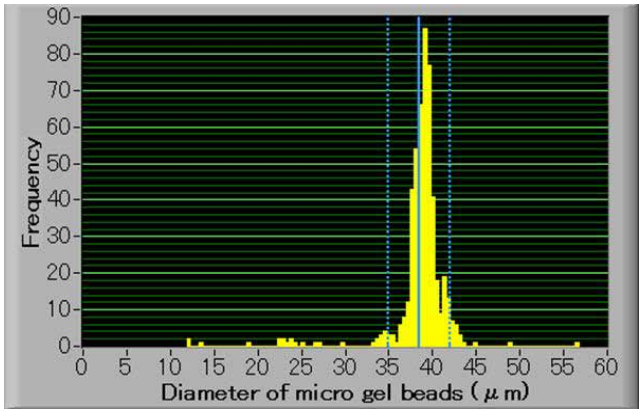


***Figure 4**, Histogram of the diameter of micro gel beads produced by inkjet. Average diameter: 38.50µm, Standard Deviation: 3.58µm, n=506*

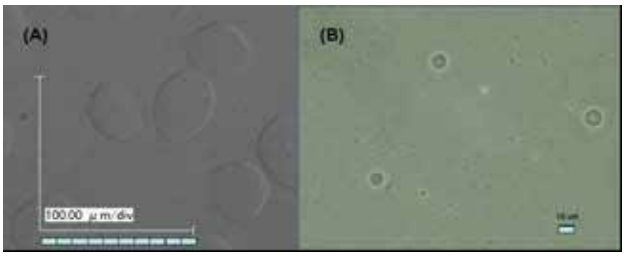


***Figure 5**, Alginate micro gel beads by usual SEAJet inkjet head (A), and smaller gel beads by the experimental inkjet head with smaller orifice (B). Magnification of image (A) and (B) was adjusted to same scale.*

gel beads could be controlled by changing the inkjet droplet size.

Because the actual ejecting volume is dependent on the several conditions of ink used and circumstances of experiments very much, precise volume of both usual inkjet head and new prototype one. However, remarkable downsizing of minimum dots could be achieved.

Discussions

To overcome several obstacles and limitations in the researches in tissue engineering and regenerative medicine, we have explored to develop some effective methods and finally developed inkjet 3D biofabrication technique, using inkjet printing technique and gelation technique of inkjet droplets. Our basic strategy is based on application of CAD/CAM (computer aided designing/manufacturing) techniques to tissue engineering field. Similar approach for tissue engineering was demonstrated as computer aided tissue engineering [8]. We have developed this inkjet 3D biofabrication technique as one of the specified CAM methods for manufacturing of biological tissues with biological materials including living cells.

From the views on manufacturing technology, our technique itself can be classified as one of the rapid prototyping techniques, or one of the particle deposition methods. Recently, inkjet 3D printers and several other types of rapid prototype machines have been applied to tissue engineering [9-12]. Those industrial methods have been shown to have high abilities to perform 2D and 3D structures. However, they are not suitable for dealing with living cells and biological materials. Those are useful only in fabrication of the scaffolds. How to control the distribution of cells, how to control the location of different types of cells, how to control the growth factor concentration, those essential issues are still big problems in the scaffold based tissue engineering. In this point, the inkjet 3D biofabrication that we have developed makes it possible to handle living cells and build up 3D hydrogel structures in which living cells are embedded, protected from drying and positioned directly in 3D space. This approach has a good potential to overcome those problems mentioned above.

From the point of views of digital fabrication technology, the minimum elemental unit for fabrication is micro gel beads in our technique, and the investigation of them is important. As a result, we could recognize that the micro gel beads with homogeneous size could be produced effectively by inkjet. It is just owing to the highly qualified inkjet head and control technology of ink droplets. Such homogeneous micro gel beads can not be obtained without sorting process in other techniques, such as splaying and emulsion method. Besides, the capacity of inkjet to produce these high qualified micro gel beads also represents the potential of high qualified fabrication by inkjet 3D biofabrication, too, because the micro gel beads are the minimum elemental unit of inkjet 3D biofabrication technique. And the success in downsizing of micro gel beads also indicates the improvements of the precision of this fabrication technique. Therefore, the potential of inkjet 3D biofabrication technique was supported. However, usual living cells, which diameters are usually more than 20μm, cannot be embedded in the small gel beads. Therefore, multiple sized inkjet heads are necessary for biofabrication.

In clinical medicine, CT scan, MRI scan, and ultrasonic echo scan have been used to obtain 2D images and recently 3D images of the inner organs of the patients for diagnosis. Such images, especially 3D images are very useful to understand the positional relationships and the morphology of the organs. Those images are all generated using computer technologies based on the digital 3D data. In addition, histological information of several tissues and organs has also been obtained and understood. The technology to make CAD data for tissue engineering has almost been established. If some digital fabrication technique or bio-CAM technologies will be established and available in future, such 3D data must be useful not only for diagnosis but also for fabricating patient's tissue and organs. As those 3D CAD data are based on the patient's own organs, the most suitable organs for the patients can be effectively designed and fabricated with 3D fabrication technology. The true tailor-made tissue engineering medicine will come true.

We demonstrated here some of the excellent data we obtained, however, we also recognized that the inkjet ejection was easily influenced by ink condition and surrounding conditions. Further developments are needed until this technique will be established and be an effective building up technology for tissue engineering and regenerative medicine.

Conclusion

In this study, we introduced our recent progress in the development of inkjet 3D biofabrication technique. Digital fabrication by homogeneous inkjet droplets and by much smaller inkjet droplets will provide more sophisticated fabrication. In conclusion, inkjet 3D biofabrication will contribute to the development of an innovative approach of direct building up of biological tissues.

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Author Biography

Makoto Nakamura graduated from the School of Medicine, Kobe University (1986), and worked as a clinical pediatrician for 10 years. After receiving an M.D., Ph.D. (1996), He concentrated his activities on research and development of artificial organs, and worked in the National Cardiovascular Center (1996-1999), then at Tokyo Medical and Dental University as an associate professor (1999-). Currently, his primary focus is on tissue engineering and he has promoted the "Bioprinting Project" (2005-), with the support of Kanagawa Academy of Science and Technology.

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