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Ink Jet Three-Dimensional Digital Fabrication for Biological Tissue Manufacturing: Analysis of Alginate Microgel Beads Produced by Ink Jet Droplets for Three Dimensional Tissue Fabrication

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Abstract. *Ink jet technology has advantages such as high-resolution and a multicolor printing capability and has a good potential for computer-based three-dimensional (3D) fabrication. The authors have developed an ink jet 3D bioprinter to manufacture biologically viable 3D structures using living cells. They have developed an effective method for the fabrication of 3D hydrogel structures by using ink jet technology with a liquid aqueous gelating medium, which is essential in fabricating 3D structures with living cells. In the present study, they evaluated the feasibility of the ink jet approach for digital 3D biofabrication, analyzing the microgel beads produced by the ink jet droplets. The ink jet droplets of sodium alginate solution, which were ejected into* $CaCl_2$ *solution, gelled to form microgel beads. The resulting beads were analyzed by means of image-based particle analysis to show that homogeneously sized microgel beads were effectively produced. Ink jet 3D biofabrication has a high potential for effective digital fabrication with such homogeneous microgel beads and will provide promising approaches for sophisticated computer assisted tissue engineering. © 2008 Society for Imaging Science and Technology.*
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INTRODUCTION

Tissue engineering and regenerative medicine are expected to merge into one of the most promising technologies in advanced medicine in the 21st century. The final goal of both research areas is to save organ failure patients by replacing or supporting damaged organ functions with engineered tissues and organs, as an alternative to organ transplantation, which has a number of serious problems. Several recent research developments have brought about significant achievements in engineering simple tissues, and further developments are expected to lead to the capability of handling other types of more complicated organs. In addition, developments in tissue engineering have also had significant impact on the life science field.[1,2] The technology for engineering biological tissues promises to be a useful tool to provide tissue samples in vitro for investigating the mechanisms of diseases and developing innovative drugs. For these reasons, tissue engineering is one of the important key technologies to develop in the biomedical field in the 21st century.

One of the major issues in tissue engineering is the development of the ability to fabricate large, thick three-dimensional (3D) composite tissues, which are composed of multiple types of cells, with unique microstructures, and with sufficient vasculature (blood vessels) in order to profuse all of the cells in the engineered tissue. Scaffold-based tissue engineering, in which acellular 3D fabricated scaffolds are used as a foundation, has been a major approach to engineering large 3D tissues. Several intrinsic problems still remain to be solved, however, such as the engineering of composite tissues with multiple cell types and the control of cell distributions inside the 3D structures.[3,4]

Therefore, considering the characteristics of the targeted composite tissues and the problems referred to above, we have developed an innovative tissue engineering technique,

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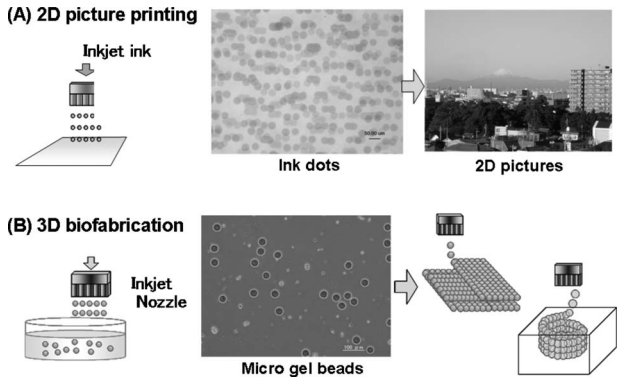

Figure 1. Investigation of the minimum dots of ink jet 2D picture printing (a) and those of ink jet 3D biofabrication (b). Microscopic photograph of the ink jet dots printed by commercial ink jet printer (PM950C, Seiko EPSON Corp., Japan) (a), Phase contrasted microscopic photograph of alginate micro gel beads produced by ink jet technique (b).

which we have referred to as either ink jet 3D bioprinting or ink jet 3D biofabrication with living cells.[5–7] With this technique, viable 3D tissue and organ structures are directly built up with living cells and other biomaterials by means of ink jet technology. As we and other researchers have previously reported, ink jet technology has many advantageous characteristics, such as "on demand" controllability, high-resolution, and color printing capability with various multicolor inks.[5–10] If we can apply such characteristics in direct 3D tissue fabrication, this approach may be able to solve some of the intrinsic problems of the conventional acellular scaffold approach. Thus, we have examined viable cell printing by ink jet techniques and have reported its feasibility for cell printing.[5,7]

In addition, we have now developed an effective method for the fabrication of 3D hydrogel structures containing living cells by ink jet techniques. Hydrogels (i.e., hydrated polymers) are essential both for mechanical construction of 3D structures and to ensure cell viability by preventing drying and other harmful effects during and after fabrication. In our technique, a gelation reaction is used, in which ink jet droplets consisting of liquid aqueous gel precursor react with a gelating medium. In addition, we have designed a custom ink jet 3D bioprinter specialized for biofabrication.[7,11,12] Based on this background, a new manufacturing technique, which is essentially a type of digital 3D fabrication, has been made possible using biological materials that include viable cells.

OBJECTIVE

As mentioned above, the ink jet 3D biofabrication technology we have developed belongs to the group of digital 3D fabrication techniques. In our technique, sodium alginate solution was mainly used as a gel precursor material because it gels at once on treatment with calcium ions to form an alginate hydrogel, which is a well known biocompatible hydrogel, and which has often been used in the tissue engineering field. The ejected droplets containing sodium alginate form alginate micro-gel beads by contact with Ca^{2+} ions and fuse to form fibers and sheets; finally, 3D hydrogel structures can be built up by lamination printing. Such a gelling property is thought to be useful for digital 3D biofabrication. Therefore, a single microgel bead, which is produced by one droplet of ejected ink, is the minimum elemental unit for ink jet 3D biofabrication, i.e., one dot of ink jet digital fabrication, just as one dot of ink is used in printing pictures by conventional ink jet printers (Figure 1). Therefore, in the present study, we analyzed microgel beads produced by this technique and discuss the potential of the ink jet 3D bioprinting approach.

In addition, we speculated that the size of the microgel bead should limit the resolution of 3D fabrication, and that this size should depend on the ink jet droplet size. Thus, we sought to fabricate smaller microgel beads with a smaller nozzle orifice, and here we report our achievements.

MATERIALS AND METHODS

Ink Jet Head and Ink Jet Biofabrication System

As the ink jet system, a static electricity-actuated ink jet system (SEAJet™ ink jet head, Seiko Epson Corp., Suwa, Japan) was used in this study. Details of this head were described previously.[5,13] This ink jet system is one of the mechanical ejection type ink jet systems, and the pusher plate of each nozzle is actuated by static electricity. This system does not generate heat; it is composed of glass and silicon and is therefore stable against many different types of chemicals. We have recognized it as one of the biocompatible ink jet systems for cell printing and have developed an ink jet 3D bioprinter in which this SEAJet™ system is applied.[7,11,12] This 3D bioprinter was designed and developed especially for the purpose of fabricating 3D biological tissues including living cells; the ink jet head can be moved along *X*, *Y*, and *Z* axes, and, by use of a gelling medium as an ink; 3D hydrogel structures can be fabricated in the aqueous medium of the gel reactor by laminar printing.

Production of Alginate Microgel Beads

Alginate hydrogel was used as a gel-forming medium in this study. The basic procedure of making alginate microgel beads was as follows: 0.8% sodium alginate solution was prepared and ejected into 2.0% calcium chloride solution by means of the ink jet nozzle. The ejected droplets containing sodium alginate form alginate microgel beads. As the alginate hydrogel itself is colorless and transparent, the microgel beads produced could not be seen easily without using phase contrast microscopy. For visualization, a cyan colored printer ink (ICC32 Cyan, Seiko Epson, Suwa, Japan), or a fluorescent marker ink (Pilot Ink Co., Tokyo, Japan) was added into the sodium alginate solution at concentrations of 5.0, and 10.0 volume percent, respectively. We typically stirred the calcium chloride solution by hand agitation or by using a magnetic stirrer during ink jet ejection. Based on this procedure, we obtained alginate microgel beads.

Morphological Analysis of Microgel Beads

The size of the microgel beads was measured and analyzed by use of image analysis with a particle analysis program developed with IMAQ™ Vision Builder (National Instru-

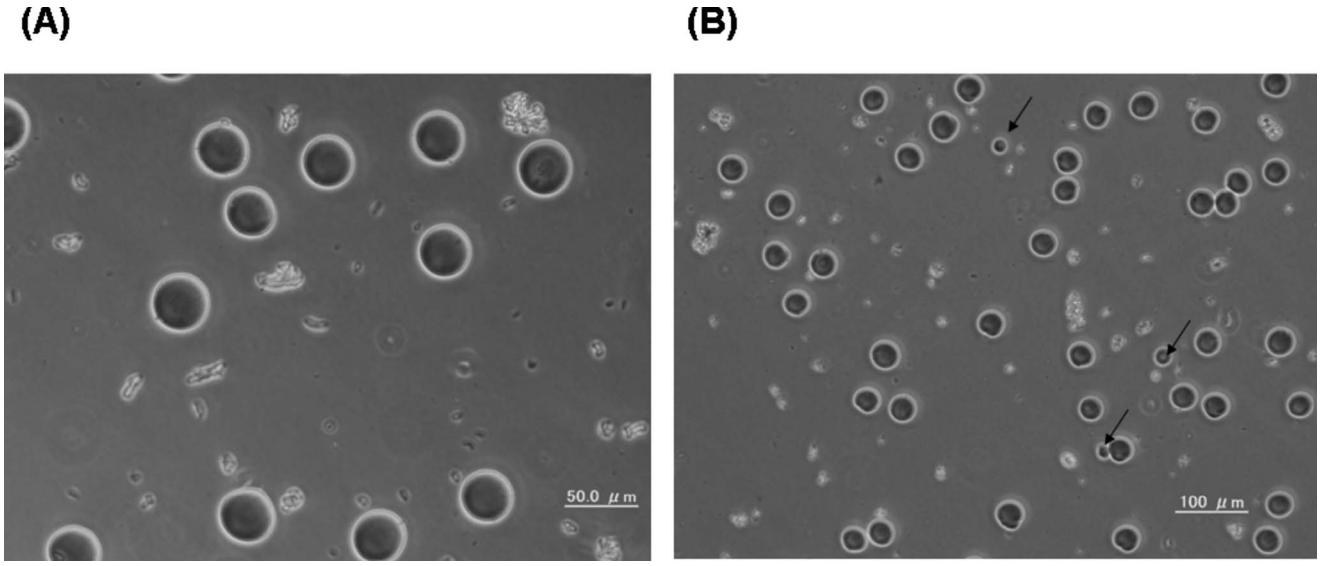

Figure 2. Phase contrasted microscopic photograph of alginate microgel beads produced by ink jet technique at the different magnified views (a), (b). Arrows indicates the smaller gel-beads compared to surrounding microgel beads.

ments Japan, Tokyo, Japan). In this program, the shapes of every gel bead in the image were fitted as ellipsoids. This fitting was carried out based on the particle area and the length of the outer contour of the particle. Next, the lengths of the long and short axes were measured, and the diameter of the microgel bead was estimated as their average. Thirty images were analyzed, and then the average and standard deviation were calculated for all of the microgel beads counted, and the variability was evaluated with a histogram.

Downsizing of Microgel Beads

We speculated that the size of the microgel beads should be dependent on the ink jet droplet size, and therefore we attempted to fabricate smaller microgel beads using another experimental SEAJet™ ink jet head with a smaller nozzle orifice. This head was made only for experimental purposes, and its details have not been disclosed yet. The microgel beads produced with this experimental head were also analyzed in the same way as described above.

RESULTS

Production of Alginate Microgel Beads

According to the basic procedure already described, alginate microgel beads were obtained (Figure 2(a)). In general, almost all of the microgel beads showed circular shapes, with a homogeneous diameter of approximately 40 μm. The shapes of the microgel beads are nearly spherical, because all of the floating beads exhibited spherical shapes. In detailed observation, smaller microgel beads with diameters of 10–30 μm were sometimes found (Fig. 2(b), see arrows). These were thought to be generated from satellite droplets of the ink jet ejection.

Downsizing of Microgel Beads

Using the experimental ink jet head with the smaller nozzle orifice, alginate microgel beads with smaller diameter were successfully produced and are compared to the microgel beads produced by the conventional ink jet head as shown in Figures 3(a) and 3(b). For the small microgel beads, fluorescent ink was added to the sodium alginate solution for visualization. In general, the size of these microgel beads was approximately 25 μm.

Quantitative analysis of microgel beads

As the next step, further quantitative analysis was performed by image analysis for the microgel beads that had been produced. The sizes of all of the microgel beads were measured according to image analysis procedures described in the Materials and Methods section. Thirty different microscopic images of microgel beads were analyzed, and 506 beads in total were measured. The average (±standard deviation) of the diameters was (38.50+3.58) μm. Next, a histogram of the bead diameters was plotted and is shown in Figure 4. This histogram shows that the size distribution of the microgel beads obtained was within a narrow range, and 94% of the beads were within 30–45 μm; thus, it was confirmed quantitatively that homogeneous microgel beads could be obtained. The presence of much smaller microgel beads, with diameters of 10–30 μm, which were thought to be generated by the satellite droplets of the ink jet ejection, was also reconfirmed.

In the same way, a quantitative study by image analysis was performed for the smaller microgel beads. Again, 30 images were analyzed, and 938 microgel beads were measured. The average (±standard deviation) of the diameters was (26.33+3.25) μm. A histogram was again made and is shown in Fig. 4. The size was distributed within a 10 μm range, from 20 to 30 μm; the distribution was clearly shifted to smaller diameters, although the distribution was somewhat broader, compared to that of the larger microgel beads.

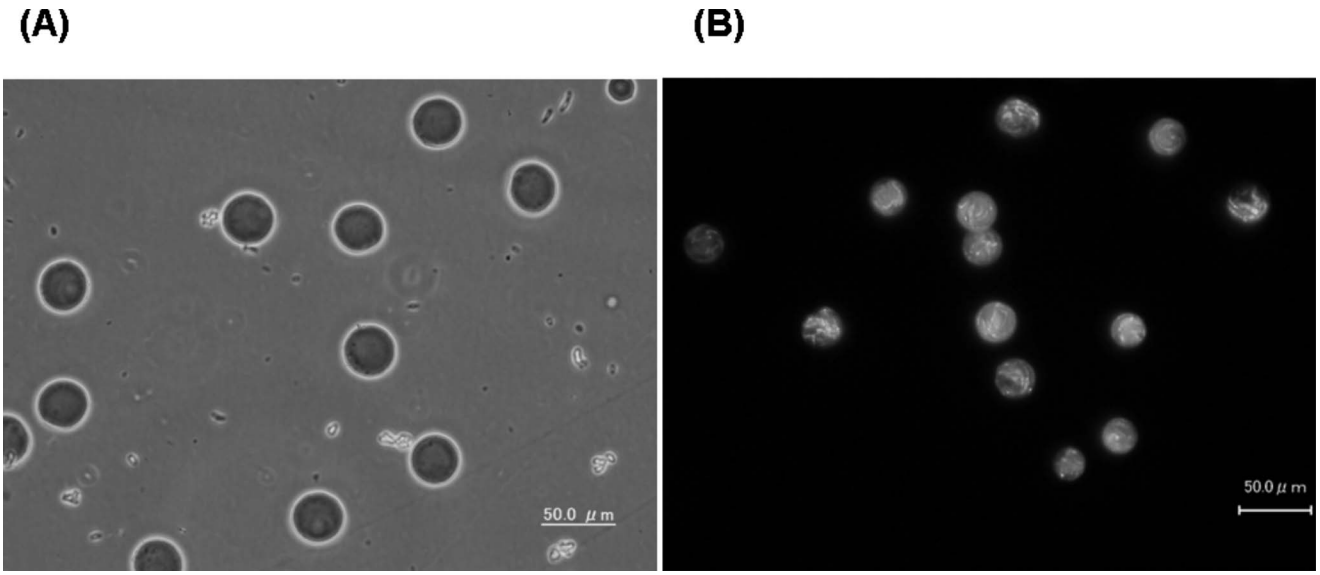

Figure 3. Alginate microgel beads produced with the conventional ink jet head (a) and the down-sized alginate microgel beads by new ink jet head with smaller nozzle orifices (b). For the latter, fluorescence ink was added to the sodium alginate solution and the produced microgel beads were observed with a fluorescent microscope. (a) and (b) are the photographs at the same magnification.

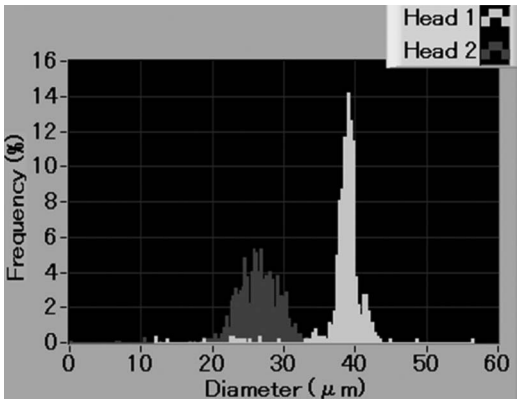

Figure 4. A histogram of the diameters of the microgel beads by conventional ink jet head (head 1: green), and that by the new ink jet head with smaller nozzle orifices (head 2: pink).

3D Fabrication With Smaller Microgel Beads

We fabricated 3D tubular structures using the 3D bioprinter, in which the new, experimental ink jet head with the smaller nozzle orifice was mounted. The ink jet head was moved in a circular pattern, thus a tubular structure was fabricated by laminar printing of into the $CaCl_2$ solution in the well. Figure 5 shows the diagram of the fabrication of the tubular structure by ink jet 3D bioprinting technique and a fabricated alginate gel tubes. It can be seen that the fabricated tubular structure is composed of many homogeneous sized microgel beads regularly arrayed. By use of the new ink jet head the diameter of the fabricated tube was also scaled down from the previously demonstrated 1 mm to 200 μm.

DISCUSSION

Ink jet technology has been developed over a period of many years, and ink jet printing performance has improved greatly, so that extremely high-quality, high-resolution images can now be printed digitally. In the development of ink jet printing, one of the essential concerns was the control of the ink jet droplets, including satellite droplets. We investigated the microgel beads at first for this reason; however, we now speculate that the ink jet droplet size will be more important for 3D ink jet biofabrication than for two-dimensional (2D) printing. The fabricated structures are constructed in all three dimensions on a very small scale via the deposition of the microgel beads, and thus, the quality of the beads will directly affect the 3D architecture. In the present investigation, we have demonstrated that homogeneous microgel beads can be produced effectively by the ink jet technique. The results have indicated the stability of the ink jet ejection, which is thought to be due to the highly developed ink jet head and the excellent control technology for ejection of the ink jet droplets. The capacity of ink jet technology to produce high-quality beads should also lead to high-quality 3D biofabrication. However, we also recognized that the actual ejection volume is easily influenced by several factors, including both the type of ink used and the exact experimental conditions. Therefore, the control of the ink jet droplet size is still an important issue in ink jet biofabrication.

As for the size reduction of the microgel beads, we used a special prototype of the SEAJet™ head, in which the nozzle orifices and pusher plates were smaller than those of the conventional head. As a result, the downsizing of the minimum dots could be achieved. The size reduction of the nozzle orifice appeared to be effective in the downsizing of the microgel beads via downsizing of the ink jet droplets. The size reduction of the microgel beads will be effective in the precise fabrication of high-resolution microscale structures. As we demonstrated, a high-quality 200 μm hydrogel

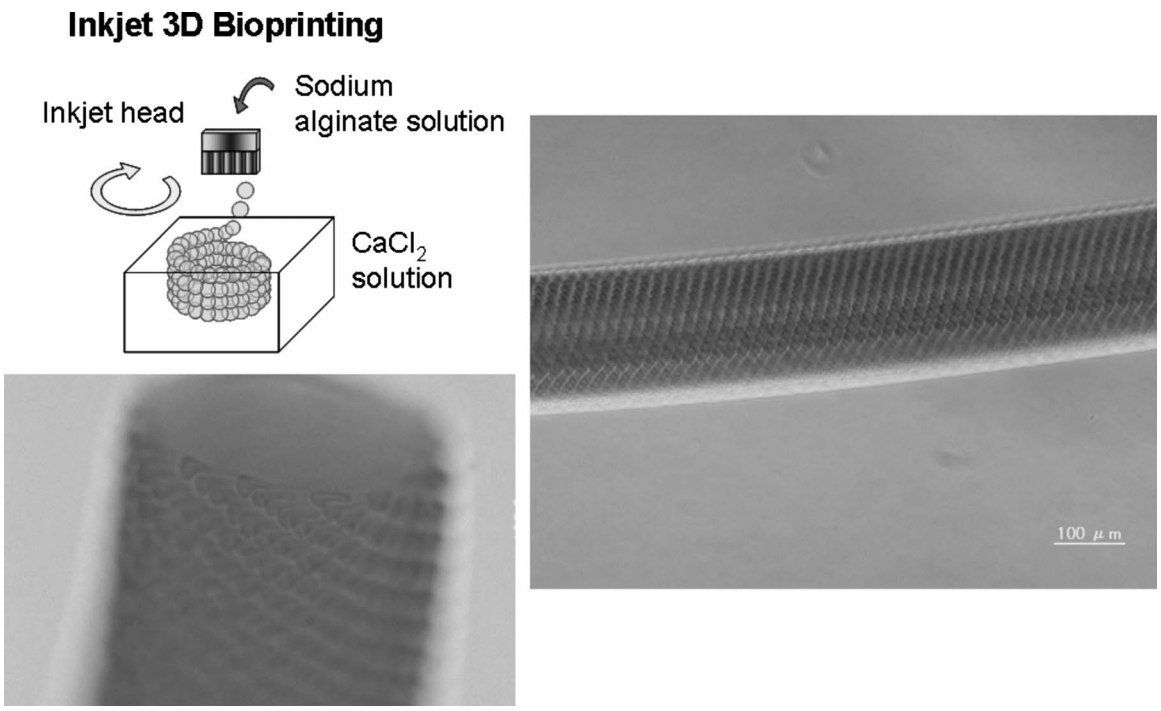

Figure 5. Ink jet 3D biofabrication of tubular structures by laminating print using the new ink jet head with smaller nozzle orifices. The diameter of the tubular structure was 200 μm, and this structure was composed of alginate hydrogel.

tube was fabricated with the smaller orifice ink jet head; it will be possible to fabricate even finer hydrogel structures using much finer ink jet droplets, although the size reduction will be limited by the actual cell size, in the case of ejection of living cells.

Several 2D and 3D printing and rapid prototyping techniques have been applied in various bioprinting strategies, including dip pens, dispensers, ink jet printers, photolithography, laser ablation, laser fabrication, solid free forming, and 3D printing.[1,14–19] However, many of these have been targeted towards the fabrication of acellular scaffolds for conventional scaffold-based tissue engineering.

To overcome several of the intrinsic limitations of scaffold-based tissue engineering, effective methods to manufacture cell-containing 3D structures should be developed. Direct 3D cell positioning and the use of hydrogels are thought to be essential, and the fabrication of fine structures is also needed. To address these points, we consider the approach of ink jet 3D biofabrication most promising. In this study, we have referred to the downsizing of ink jet microgel beads for the fabrication of finer hydrogel structures. This is indeed one of the superior aspects of ink jet technology. In addition, if a multicolor printing system is used, the simultaneous printing of multiple types of cells will also be realized. We believe that these are the most promising forthcoming extensions of the application of ink jet technology to biofabrication.

CONCLUSIONS

We have developed a specially designed ink jet 3D bioprinter based on our previous studies of ink jet cell printing and ink jet gel formation techniques. In this study, we were able to confirm the feasibility of the ink jet technique in fabricating stable 200 μm structures through the confirmation of the capability of producing homogeneously sized microgel beads by the ink jet technique and the downsizing of the microgel beads. Just as ink jet technology had required a development period of 20 years prior to the achievement of photoquality printing, further developments of the bioprinting technology are also necessary and are expected. However, we have now taken some initial steps, in an innovative approach to ink jet 3D biofabrication, toward the manufacturing of viable 3D tissues and organs, which are designed for medical use, as an alternative to organ transplantation, in saving the lives of diseased patients.

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