Development of a Three-Dimensional Bioprinter: Construction of Cell Supporting Structures Using Hydrogel and State-Of-The-Art Inkjet Technology

Yuichi Nishiyama
Kanagawa Academy of Science and Technology,
Think-E Building, 1-23, Minamiwatarida,
Kawasaki-ku, Kawasaki 210-0855, Japan

Makoto Nakamura[1]
Graduate School of Science and Engineering for Research (Engineering),
University of Toyama,
3190, Gofuku, Toyama 930-8555, Japan;
Kanagawa Academy of Science and Technology,
Kanagawa, Japan
e-mail: maknaka@eng.u-toyama.ac.jp

Chizuka Henmi

Kumiko Yamaguchi

Shuichi Mochizuki

Hidemoto Nakagawa

Koki Takiura

Kanagawa Academy of Science and Technology,
Think-E Building, 1-23, Minamiwatarida,
Kawasaki-ku, Kawasaki 210-0855, Japan

We have developed a new technology for producing three-dimensional (3D) biological structures composed of living cells and hydrogel in vitro, via the direct and accurate printing of cells with an inkjet printing system. Various hydrogel structures were constructed with our custom-made inkjet printer, which we termed 3D bioprinter. In the present study, we used an alginate hydrogel that was obtained through the reaction of a sodium alginate solution with a calcium chloride solution. For the construction of the gel structure, sodium alginate solution was ejected from the inkjet nozzle (SEA-Jet™, Seiko Epson Corp., Suwa, Japan) and was mixed with a substrate composed of a calcium chloride solution. In our 3D bioprinter, the nozzle head can be moved in three dimensions. Owing to the development of the 3D bioprinter, an innovative fabrication method that enables the gentle and precise fixation of 3D gel structures was established using living cells as a material. To date, several 3D structures that include living cells have been fabricated, including lines, planes, laminated structures, and tubes, and now, experiments to construct various hydrogel structures are being carried out in our laboratory.
[DOI: 10.1115/1.3002759]

1 Introduction

We have developed a new technology for producing living tissues in vitro, utilizing the direct and accurate printing of cells with an inkjet printing system. Organ transplantation is one of the ultimate medical treatments, however, the shortage of donor organs is an urgent and serious problem because waiting periods for donor organs have become longer and longer, and a number of million patients have died during the waiting period every year. Tissue engineering is one of the promising approaches to solve such problems, and many researchers have addressed the development of available tissues and organs by engineering approaches. To date, engineered epithelium, cornea, and cartilage have been developed and applied clinically and successfully; however, any effective methods to produce complicated tissues and organs have not been established, especially in composite tissues composed of multitype cells. Then, we have speculated and addressed the development a new technology to manipulate individual cells and to construct and regenerate composite biological tissues.

The main technique used in our approach is the direct and accurate printing of living cells using the inkjet technique. We previously confirmed that the cells ejected from an inkjet nozzle were alive and able to proliferate [1]. In addition, we were able to eject the growth factor and other chemicals along with the cells by the inkjet technique. These results indicate that the cells precisely placed using the inkjet technique have a potential to attach to each other, and the regenerative tissues can be engineered through the culturing process. However, with this method using the inkjet technique, the cells must be fixed onto the printed position in order to prevent blotting and migration, and we aimed to construct 3D structures using liquid materials such as cell suspended media. Thus, we employed some liquid materials that have an ability to solidify after ejection, such as sodium alginate solution and fibrinogen solution, which gelate after mixing with calcium chloride solution and thrombin solution, respectively. With these materials, we were able to control the position of the ejected cells and precisely form 3D structures. These gels are biocompatible and can be formed in a liquid substrate to prevent the cells from drying. Similar techniques have been reported in other works [1–6].

With conventional rapid-prototyping techniques, it is possible to fabricate 3D structures using resin, plaster, and molten-metal using inkjet technology. However, these methods are not capable of fabricating structures using living cells and biological materials. In contrast, our method was designed to fabricate some living tissue structures, and to also be able to utilize cells and biomaterials simultaneously. Furthermore, hydrogel 3D structures have been successfully constructed using our novel technique described in this paper.

Herein, we present our technique for fabricating 3D cell supporting structures using the inkjet technique and hydrogels. First, we describe our custom-made 3D bioprinter, which is able to eject cells and other liquid materials with precise positioning. Next, our new method of fabrication of 3D gel structures is presented, in which soft fixation with improved viscosity is used to construct gel structures precisely. We mainly used alginate gel in the present study, as it is inexpensive and easy to handle. Nevertheless, we intend to discuss details of gel structures formed from other materials that have better biocompatibility at the next stage of our research. We have investigated a number of different gel materials; however, only the combination of fibrinogen and hyaluronan is described in this study.

2 Experiments and Results

2.1 Materials. Three kinds of materials, sodium alginate, calcium chloride, and polyvinyl alcohol (PVA), were used in this study. The sodium alginate was the precursor of the gel and calcium chloride was used as the substrate. Alginate gel was obtained by mixing them. PVA was used as a viscosity enhancer for the

[1]Corresponding author.

Contributed by the Bioengineering Division of ASME for publication in the JOURNAL OF BIOMECHANICAL ENGINEERING. Manuscript received March 6, 2007; final manuscript received May 16, 2008; published online December 31, 2008. Review conducted by Cheng Zhu.

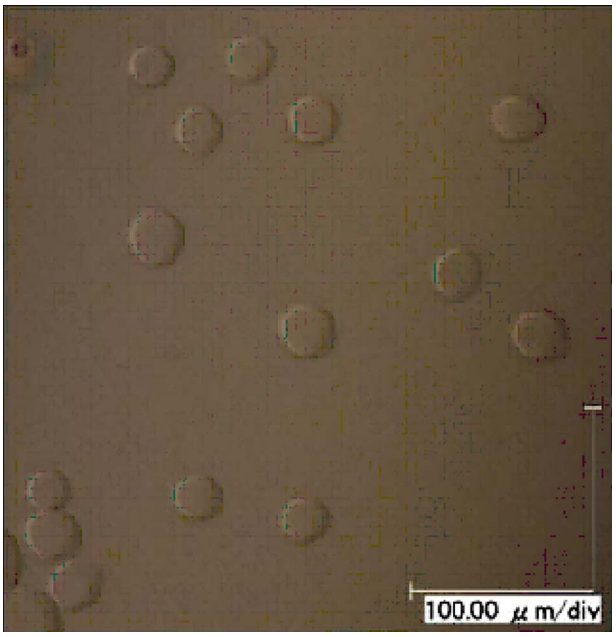


Fig. 1 Alginate gel beads

substrate. When the sodium alginate solution was ejected from the inkjet nozzle into a substrate of calcium chloride, alginate gel beads with the same size as the droplets supplied by the inkjet were obtained, as shown in Fig. 1. The bead was the minimum unit of gel structure used in this study. Since the beads suspended in a low viscosity substrate moved freely, a gel structure could not be formed with precise positioning. Therefore, we mixed the substrate with PVA to increase its viscosity. Both the alginate gel and the PVA are known to have good biocompatibility. For example, the alginate gel was used for the matrix material of cell transplantation and the PVA was applied for the soft contact lens material [7–9]. Figure 2 shows living HeLa cells in alginate gel beads. The viability of ejected cells was confirmed by time lapse monitoring, which showed that cells were moving in the alginate gel beads.

For improved biocompatibility, fibrinogen and hyaluronan could also be used for the gel precursor and substrate viscosity enhancer, respectively. The fibrinogen is contained in the blood. If necessary, it can be harvested from each patient. However, alginate gel and PVA were mainly used in order to study their feasibility with our 3D bioprinter, as they are inexpensive and adequate materials.

2.2 Inkjet Nozzle. In general, inkjet nozzles have originally been developed for the printer heads. The very high resolution print can be outputted by the recent inkjet printers. The size of the

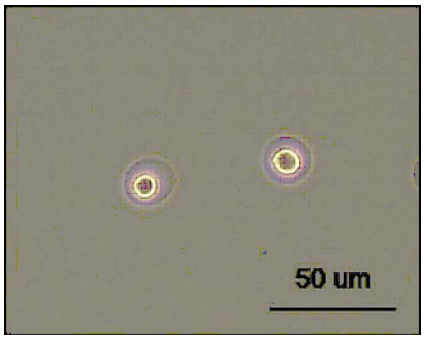


Fig. 2 Gel beads including HeLa cells

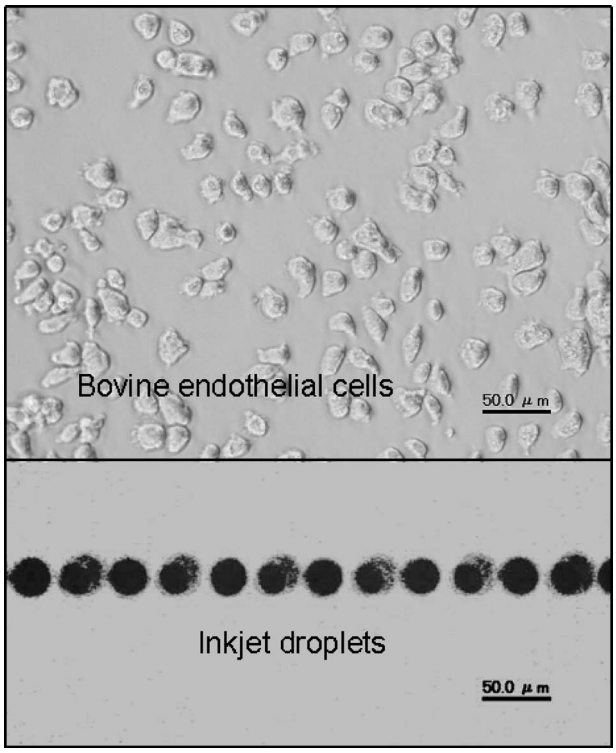


Fig. 3 Comparison of inkjet droplets and bovine endothelial cells

droplets ejected from the inkjet nozzles ranges from 1 pl to 100 pl. Figure 3 shows a comparison of the inkjet droplets by a commercial inkjet printer and the suspended cells. The size of the droplets was nearly equal to that of the cells [1]. Therefore, the inkjet printer has a potential to form 2D biological tissues at a similar resolution with the cells.

We used an EPSON SEA-Jet™ inkjet nozzle head (static electricity actuated inkJet, SEA-Jet™, Seiko Epson Corp., Suwa, Japan), as shown in Fig. 4 [10]. It is driven by electrostatic force using different electrical potentials and does not use any heat generation. Thus, it was able to eject cells through the inside capillary without heating the droplets, which were sized at 8–160 pl, at a frequency of 2 kHz or less. Although the inkjet nozzle head has 12 channels, each nozzle head is able to eject only a single kind of fluid because the inlets of the channels connect to the same tank of materials.

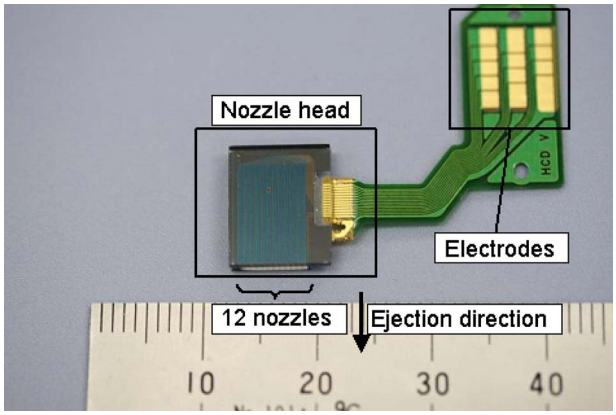
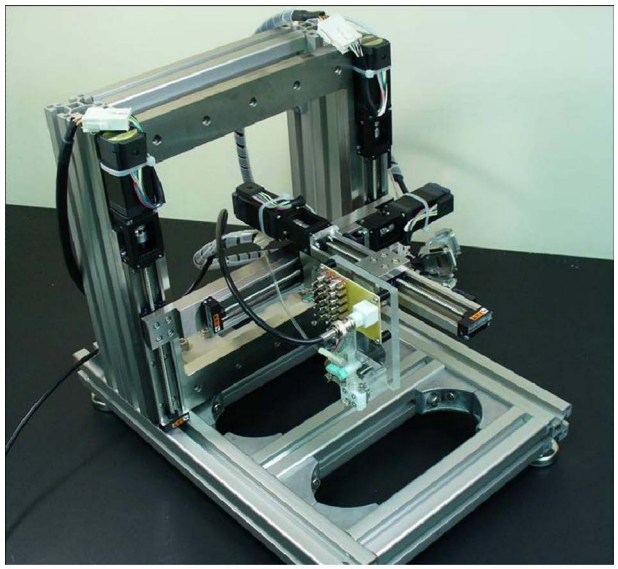
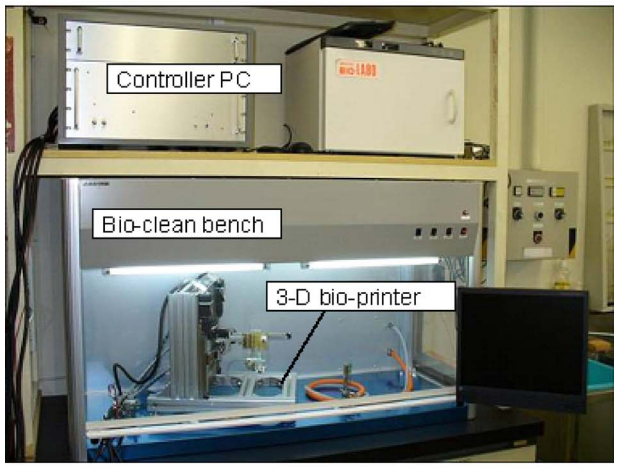


Fig. 4 EPSON SEA-Jet™ inkjet nozzle head



(*a*)



(*b*)

Fig. 5 Newly developed bioprinter: (*a*) 3D bioprinter developed in our laboratory and (*b*) 3D bioprinter in a bioclean bench environment

2.3 Our 3D Bioprinter. Our 3D bioprinter was manufactured to be capable of operating the inkjet nozzle head and fabricating 3D structures (Fig. 5(*a*)). We found that the printer had a positioning accuracy of less than a few micrometers and could operate in a bioclean bench setting (Fig. 5(*b*)). The actuators of the printer are equipped with a feed mechanism of ball screws with stepping motors (ASC36AK, Oriental Motor Co., Ltd., Tokyo, Japan), which are controlled by a closed-loop control method. The printing process is controlled by a Windows PC.

We also developed power and signal sources for the nozzle using a signal generator (H8/3052 microprocessor, Renesas Technology Corp., Tokyo, Japan) and amplifier (PA92 Operational Amplifier, Apex Microtechnology Corp, Tucson, AZ), respectively. With this system, a signal of 0–2 V is amplified up to 20 times of the original voltage. We drove the EPSON SEA-Jet™ nozzles by a rectangular waveform using this custom-made controller. Table 1 shows the fundamental specification of the 3D bioprinter.

2.4 Fabrication of Gel Structures. The minimum gel structure printed by our bioprinter is a bead, as shown in Fig. 1, thus the resolution of the gel structure was as small as the size of the bead. Although the bead sizes varied depending on the nozzles and the fluid type, they were approximately 10–60 μm in diameter in our examination. In this study, several gel structures were constructed by precisely distributing beads.

Table 1 Specification of the 3D bioprinter

Nozzle	Epson SEA-Jet™
Maximum ejecting frequency	2 kHz
Dimensions ($W \times D \times H$)	$400 \times 300 \times 380$ mm^3
Positioning resolution	0.2 μm
Positioning repeatability	± 4 μm
Maximum feed speed	130 mm/s
Effective feed stroke (all axis)	100 mm

Figure 6 describes the method used to fabricate gel structures. Droplets ejected from the inkjet nozzle are gelated by the contact with the substrate surface. Therefore, 2D gel structures with arbitrary patterns were able to be formed on the surface of the substrate. 3D gel structures were obtained by stacking 2D gel structures and allowing them to sink into the substrate.

2.4.1 Fabrication of a Gel Line. Figure 7(*a*) shows a gel line made by the continuous operation of the EPSON SEA-Jet™. The substrate was a 2% calcium chloride solution on a slide glass, and the droplets ejected from the nozzle were a 0.8% solution of sodium alginate. The nozzle was easily able to eject up to 1% solution of sodium alginate. In this case, the line had no gap and had a constant diameter, while lines with other configurations, such as a broken line, were also fabricated by the same nozzle head, as shown in Fig. 7(*b*). The line shapes varied depending on the ejection frequency and the nozzle speed, and the results are shown in Table 2. Although solid lines were formed under the condition of high ejection frequency and low nozzle speed, a more refined line was formed at lower ejection frequency and higher nozzle speed limits.

2.4.2 Fabrication of a Gel Sheet. A gel sheet that formed a plain gel structure was made by the parallel arrangement of gel lines, as shown in Fig. 8. In this case, a 2% calcium chloride solution was used as the substrate, and the ejection frequency and nozzle speed were set to 800 Hz and 20 mm/s, respectively.

2.4.3 Fabrication of 3D Gel Structures. 3D gel structures were constructed by the lamination of some gel sheets. However, the initial trials ultimately failed because the 3D gel structures were not fixed firmly and easily broken down. Therefore, several other materials were examined to construct firmer 3D gel structures. After several exploring tests, we found that the use of calcium chloride mixed with some viscosity improver was effective. Then, we tried various viscosity enhancers, such as dextran, xanthan gum, guar, carrageenan, tragacanth gum, and pectin, and

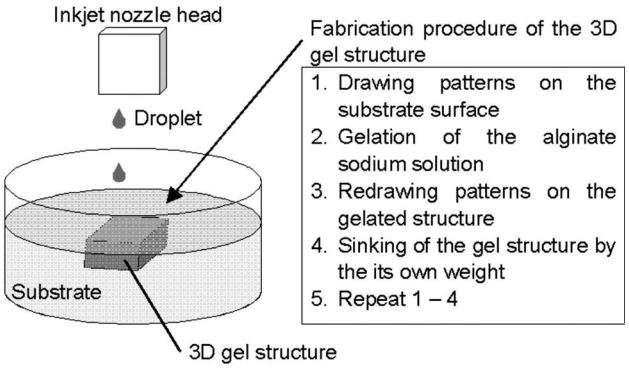
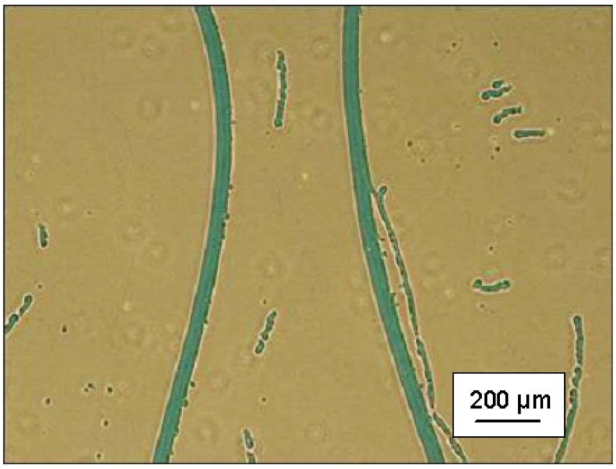
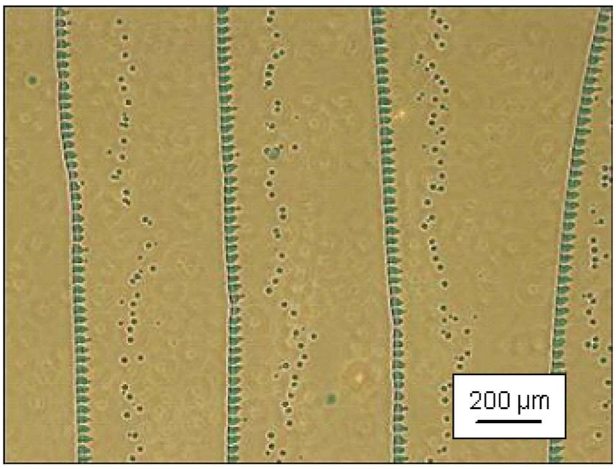


Fig. 6 Illustration of the gel structure fabrication method



(*a*)



(*b*)

Fig. 7 Gel lines formed using the 3D bioprinter (nozzle head speed: 35 mm/s, ejecting frequency: 500 Hz): (*a*) alginate gel solid line (nozzle head speed: 40 mm/s, ejecting frequency: 1 kHz) and (*b*) alginate gel broken line

found that PVA and hyaluronan are the best candidates with acceptable properties, such as biocompatibility, homogeneous solubility, and stability. Finally, 3D gel structures by the lamination of gel sheets were successfully formed using a substrate, whose viscosity was enhanced by PVA, as shown in Fig. 9. In addition, gel rings were formed by drawing circles and 3D gel tubes were successfully made by continuously drawing a circle at the same position, as the gel rings were stacked to form a 3D tube, which sank into the substrate (Fig. 10). We found that the substrate mixed with greater than 15% PVA showed good supporting properties for

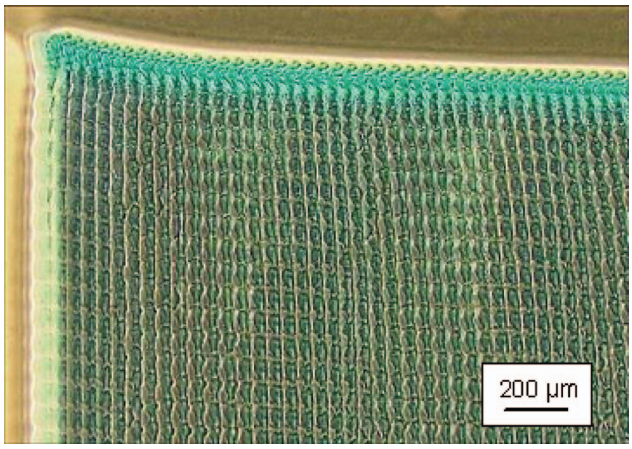


Fig. 8 Gel sheet (nozzle head speed: 25 mm/s, ejecting frequency: 800 Hz)

the 3D gel structures, while 10% calcium chloride in the substrate was the most suitable for the firm gelation of the sodium alginate.

2.4.4 Fabrication of Gel Tubes Containing HeLa Cells. By means of the method described above, the 3D gel structures containing living cells can be fabricated. Figure 11 shows a representative 3D gel tube containing HeLa cells, which was fabricated by the use of sodium alginate solution containing HeLa cells (6×10^6/ml) as ink, and 10% calcium chloride solution with 15% PVA as a substrate, respectively. In these 3D structures, hydrogel functioned well as a morphological cell supporting structure.

2.5 Viscosity. The viscosity of the liquid materials, such as the sodium alginate solution, is an important factor for our fabrication methods. The inkjet nozzles are not capable of ejecting a high viscosity solution, while the substrate could not fix the gel structure effectively with a low viscosity solution. Then, the viscosities of the liquid materials were measured using a rotational viscometer (LVDV-II+PRO CP, Brookfield Engineering Laboratories, Inc., Middleboro, MA), and the results are shown in Figs. 12(*a*) and 12(*b*). The viscosities of the fibrinogen and the hyaluronan solutions were compared to those of the sodium alginate and PVA solutions, with the results shown in Figs. 13(*a*) and 13(*b*).

3 Discussion

3.1 Gel Structure Formation Using 3D Bioprinter. The main objective of this study was to demonstrate the fabrication of 3D gel structures as cell supporting structures, which have the potential to grow and develop to regenerative tissues with cultured living cells. Although the gel structures produced in our laboratory were not as complex as actual tissues, basic 3D forms, such as fibers, sheets, laminated sheets, and tubes, were able to be fabricated using our 3D bioprinter and our method of fixing the gel

Table 2 Conditions necessary for forming gel lines. (○) Gel line could formed. (△) A part of the gel line could not be formed continuously. (×) Gel line could not be formed continuously.

		Nozzle head speed [mm/s]												
		5	10	15	20	25	30	35	40	45	50	75	100	125
Shooting frequency [Hz]	100	△	×	×	×	×	×	×	×	×	×	×	×	×
	500	○	○	○	○	△	△	×	×	×	×	×	×	×
	1000	○	○	○	○	○	○	○	○	○	△	×	×	×
	1500	○	○	○	○	○	○	○	○	○	○	△	×	×
	2000	○	○	○	○	○	○	○	○	○	○	△	△	△

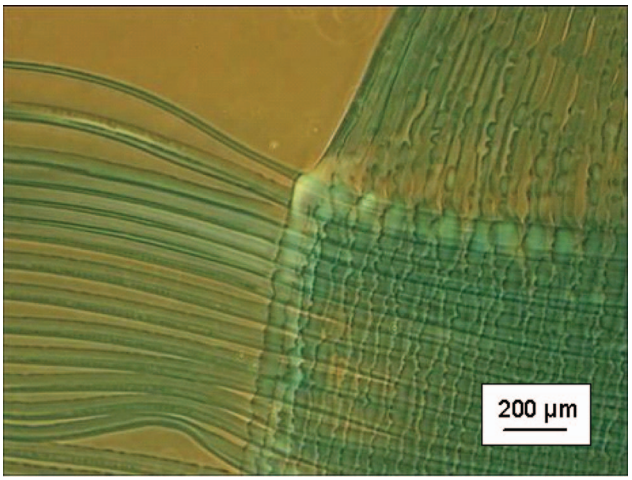


Fig. 9 Gel lamination (nozzle head speed: 25 mm/s, ejecting frequency: 800 Hz)

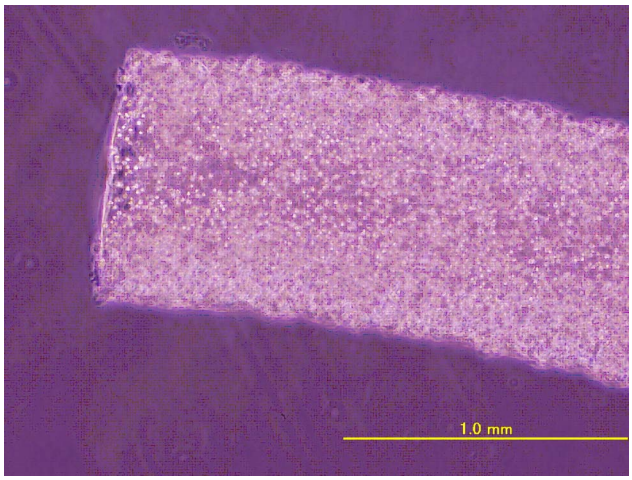


Fig. 11 Gel tube including HeLa cells (nozzle head speed: 25 mm/s, ejecting frequency: 800 Hz)

structure (Figs. 8–10). They were directly fabricated in the liquid substrate. Then, if the gel structure includes the cells, the cells inside of the gel are not desiccated and are protected from the impact shock due to the shock absorption characteristics of the liquid. Furthermore, since the gel structures were comprised of the gel bead 10–60 μm in diameter, they were formed in minimum resolution of the bead size, whose size was the same as the usual cell size, using the 3D bioprinter. Therefore, our custom-made 3D bioprinter showed sufficient potential to fabricate cell supporting structures, such as 3D gel structures and precise cell positioning, which were able to provide protection for the cells by the use of hydrogel materials. In addition, we demonstrated that a tubular gel structure was able to be fabricated as a cell supporting structure by our method, as shown in Fig. 11, and confirmed that a number of cells embedded in the gel tube were alive. We speculated that if a gel tube containing vascular endothelial cells, vascular smooth muscle cells, and fibroblast cells was fabricated by the present method, and the cells inside the gel attached to each other and self-assembled, a tubular tissue with a similar construction as a blood vessel could be obtained.

3.2 Effects of Viscosity. The nozzles were able to eject easily up to a 1% solution of sodium alginate. Hence, a solution with components sized up to 10 mPa could also be ejected by the nozzles, as shown in Fig. 12(*a*). Although we have yet to evaluate only in terms of viscosity, viscosity is an important property to consider from the aspect of the ejecting ability and ejection stability of the nozzles. A substrate with more than 15% PVA showed a good ability to support the gel structures and had a viscosity of about 400 mPa (Fig. 12(*b*)).

Considering the effects of viscosity, we propose here the fibrinogen solution and hyaluronan as other candidates of available biocompatible materials for the gel precursor and substrate, re-

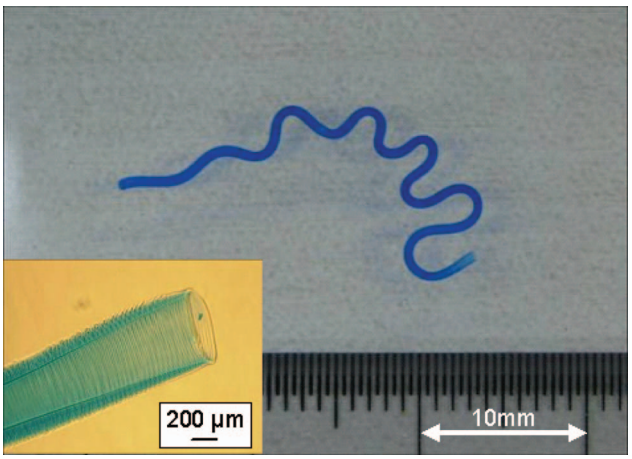


Fig. 10 Gel tube with enlarged view of the opening at the tip of the tube (nozzle head speed: 25 mm/s, ejecting frequency: 800 Hz)

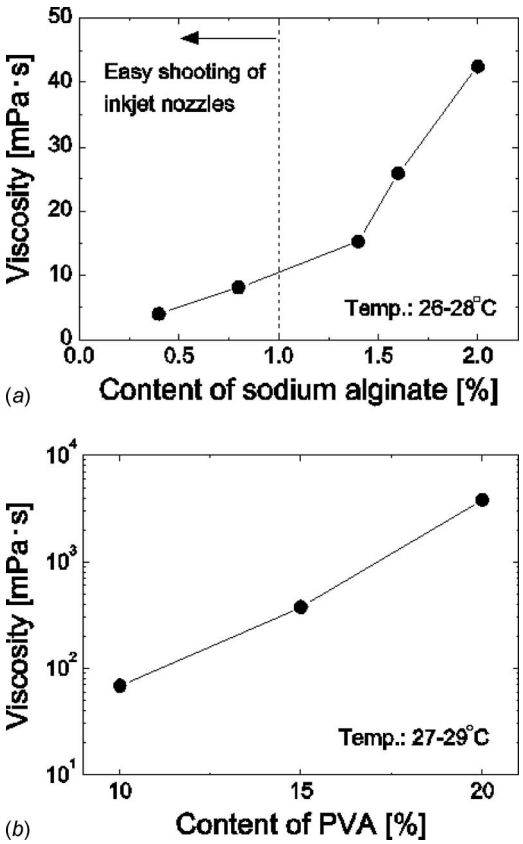


Fig. 12 Viscosity of the sodium alginate and PVA solutions determined using a viscometer: (*a*) sodium alginate solution and (*b*) PVA solution

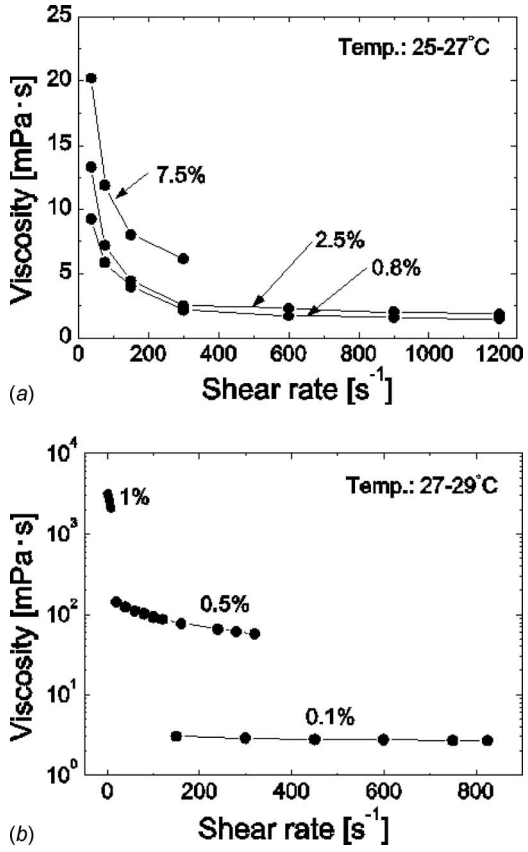


Fig. 13 Viscosity of the fibrinogen and hyaluronan solutions determined using a viscometer: (*a*) fibrinogen solution and (*b*) hyaluronan solution

spectively. According to preliminary experiments on viscosity, a fibrinogen solution of up to 0.8% can be ejected by the inkjet nozzles (Fig. 13(*a*)), while a greater than a 0.5% solution of hyaluronan can be used as the substrate, as well as 15% of PVA (Fig. 13(*b*)).

3.3 Biocompatibility of the Materials. The biocompatibility of the materials was not evaluated completely in the present study. Although alginate hydrogel itself is known to be biocompatible and we have also confirmed that the cells in alginate gel beads were living by time lapse monitoring, a 10% calcium chloride solution was used in some experiments in this study to make the hydrogel stiffer. In this case, we examined the viability of the cells by trypan blue staining after dissolving of the alginate gel, and confirmed that 70% of the cells were alive. However, such high osmotic solutions must have some adverse effects on the cells. Therefore, further additional exploration of more suitable materials and further development of gelation techniques are needed. This is one of the important issues at the next stage of development of this technology. According to our experiments on the effects of viscosity, as discussed above, we found that fibrin gel and hyaluronan solution are thought to be promising candidates for gel and viscosity regulator, respectively. Applying such gels and substrate materials, we may avoid such harmful conditions.

Nevertheless, it is considered that viable 3D tissues can be engineered and developed, if living cells in the 3D biocompatible gel structures adhere to each other and proliferate in a 3D environment after fabrication. Such potential was demonstrated in the present study.

4 Conclusion

We have developed a 3D bioprinter using state-of-the-art inkjet technology and its feasibility was shown in this study. Using our bioprinter, several simple 3D cell supporting structures, such as fibers, 2D sheets, multilayered sheets, and 3D tubes, were constructed in a liquid substrate. Our results also show that viscosity control had a critical effect on stabilizing the 3D cell supporting structures in the fluid. Our bioprinter also showed a potential to simultaneously eject multiple materials, including hydrogel precursor, living cells, growth factors, and nutrients. Presently, we are developing a second generation bioprinter that can organize 3D gel structures by embedding different types of living cells inside or outside. We are aiming at the establishment of an effective bioprinter that can fabricate 3D structures comprised of living cells, toward the final goal of engineering living tissues and organs in the future.

Acknowledgment

We thank Seiko Epson Corp. for their kind support in regard to the inkjet head and Dr. K. Suyama for the expert technical assistance. This work was supported by Kanagawa Academy of Science and Technology and grants-in-aid for Scientific Research (Grant Nos. 17300146, 18760518, and 18880042) from Japan Society for the Promotion of Science.

References

[1] Nakamura, M., Kobayashi, A., Takagi, F., Watanabe, A., Hiruma, Y., Ohuchi, K., Iwasaki, Y., Horie, M., Morita, I., and Takatani, S., 2005, "Biocompatible Inkjet Printing Technique for Designed Seeding of Individual Living Cells," Tissue Eng., **11**, pp. 1658–1666.

[2] Goldmann, T., and Gonzalez, J. S., 2000, "DNA-Printing: Utilization of a Standard Inkjet Printer for the Transfer of Nucleic Acids to Solid Supports," J. Biochem. Biophys. Methods, **42**, pp. 105–110.

[3] Campbell, P. G., Miller, E. D., Fisher, G. W., Walker, L. M., and Weiss, L. E., 2005, "Engineered Spatial Patterns of FGF-2 Immobilized on Fibrin Direct Cell Organization," Biomaterials, **26**, pp. 6762–6770.

[4] Miller, E. D., Fisher, G. W., Weiss, L. E., Walker, L. M., and Campbell, P. G., 2006, "Dose-Dependent Cell Growth in Response to Concentration Modulated Patterns of FGF-2 Printed on Fibrin," Biomaterials, **27**, pp. 2213–2221.

[5] Roth, E. A., Xu, T., Das, M., Gregory, C., Hickman, J. J., and Boland, T., 2004, "Inkjet Printing for High-Throughput Cell Patterning," Biomaterials, **25**, pp. 3707–3715.

[6] Xu, T., Jin, J., Gregory, C., Hickman, J. J., and Boland, T., 2005, "Inkjet Printing of Viable Mammalian Cells," Biomaterials, **26**, pp. 93–99.

[7] Lim, F., and Sun, A. M., 1980, "Microencapsulated Islets as Bio Artificial Endocrine Pancreas," Science, **210**, pp. 908–910.

[8] Chrisine, W., Erich, K. O., Jorg, K., Zofia, F., Tim, W., and Dorothee, F., 2004, "Poly(Vinyl Alchol) Membranes for Adhesion Prevention," J. Biomed. Mater. Res., Part B: Appl. Biomater., **70B**, pp. 191–202.

[9] Hyon, S. H., Cha, W. I., Ikada, Y., Kita, M., Ogura, Y., and Honda, Y., 1994, "Poly(Vinyl Alcohol) Hydrogels as Soft Contact Lens Material," J. Biomater. Sci., Polym. Ed., **5**, pp. 397–406.

[10] Kamisuki, S., Hagata, T., Tezuka, C., Fjii, M., and Atobe, M., 1988, "A Low Power, Small, Electrostatically-Driven Commercial Inkjet Head," *Proceedings of MEMS 98*, pp. 63–68.