2013 IEEE/RSJ International Conference on
Intelligent Robots and Systems (IROS)
November 3-7, 2013. Tokyo, Japan

Microstructuring Thermoresponsive Gel using Hysteresis towards 3D Cell Assembly

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***Abstract*— In this paper, we conducted an assembly of microstructures made of a thermoresponsive gel using hysteresis character of the thermoresponsive polymer solution. This method can be used for 3 dimensional cell assembly by embedding cells in the thermoresponsive gel structures. Gel sheets can be fabricated by micrheaters on a substrate and maintained in the gel condition by the hysteresis character. The microstructures can be formed by assembling the gel sheets using a probe device. The generation of a thermoresponsive gel was conducted using the microheaters made of ITO on a substrate and the generated gel sheets were manipulated by a micromanipulator. The fabrication of a gel sheets was achieved using the hysteresis character and the fabricated gel sheets were picked and placed by the probe. The positioning of the gel blocks can be precisely controlled by the micromanipulator. The results indicate the method we propose has a great possibility to achieve 3D cell assembly without large stress to cells during the assembly and cell culture.**

I. Introduction

Recently, tissue engineering takes much more attentions with the development of pluripotent stem cells such as embryonic stem (ES) cells [1] and induced pluripotent stem (iPS) cells [2]. The market for tissue engineering is expected to grow more and more in near future [3]. 3 dimensional assemblies of cells in vitro is one of the useful ways to get important information for tissue engineering. Environments around cells can be controlled easily and precisely in vitro. Hence, in vitro cell systems have a great potential to reveal control method of cell growth, differentiation, histogenesis efficiently and precisely.

To achieve the 3D cell assembly, we propose a new method using a hysteresis character of thermoresponsive polymer solution. In this method, cells are embedded in the thermoresponsive gel blocks and the gel blocks are assembled by a probe device. Some thermoresponsive polymer solutions show hysteresis character during so-gel phase transition [4, 5]. The temperature to change the phase from sol to gel (gelation temperature) is higher than the temperature from gel to sol (solation temperature) by the hysteresis. Therefore, once the gel is generated, the gel can maintain the gel condition by the hysteresis when the thermoresponsive polymer solution is in between the gelation and solation temperature. Hence, a microheater is required only for the generation of a thermoresponsive gel block and the generated gel block is kept in the gel condition even if the microheater is turned off. 3D microstructures are formed by assembling the gel blocks. If cells are dispersed in the thermoresponsive polymer solution before gelation, cells are embedded in the gel blocks and the 3D cell structures can be formed in the gel blocks. Cells can be assembled in the similar condition of cell culture by the thermoresponsive gel.

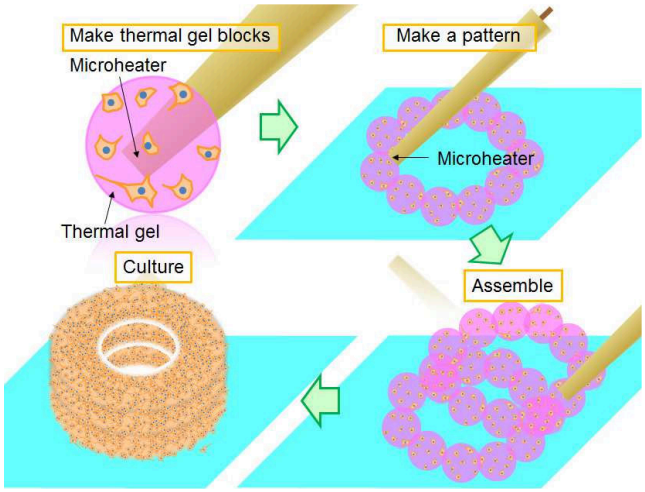

Fig. 1 Concept of the microstructure assembly using the hysteresis character of thermoresponsive solution

In this paper, the concept of 3D cell assembly using the hysteresis character is explained at first. The fabrication of a gel sheet is conducted using microheaters on a substrate. The handling of fabricated gels was conducted by a micromanipulator. The size change of gel sheets during the fabrication was evaluated from the experimental results. The cell viability was checked when cells were cultured inside the thermoresponsive gel. Finally, the gel sheets were assembled in 3D using the probe device.

Manuscript received March 15, 2013. This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grans-in-Aid for BioAssembler (23106006)).

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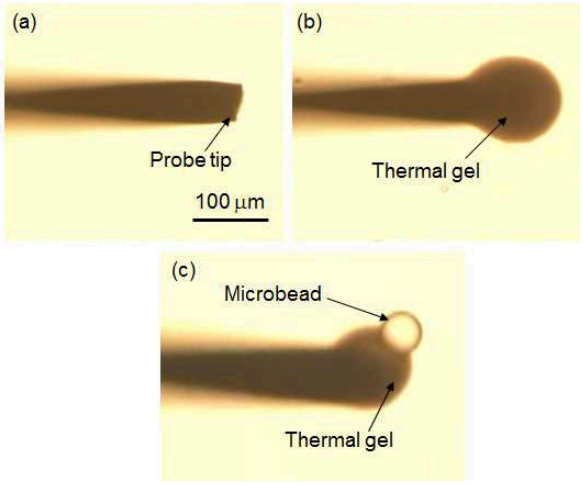

Fig. 2 Thermal gelation at the tip of GeT probe (a) Before applying a voltage (b) When 1.8 V was applied to the probe (c) When 50 μm microbead was handled by the probe

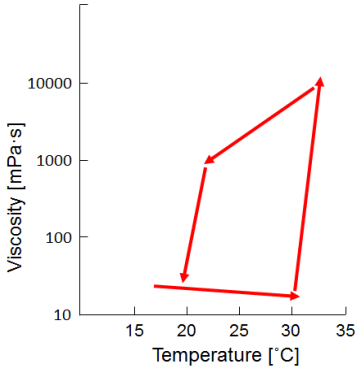

Fig. 3 The hysteresis character of 10 wt% PNIPAAm solution [5]

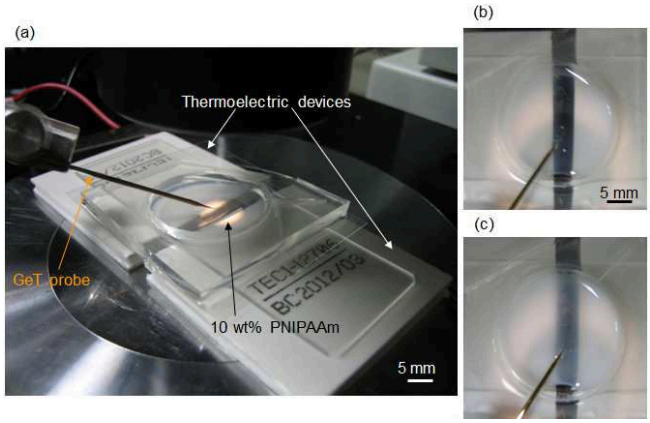

Fig. 4 Experimental setup for fabrication of thermoresponsive gel structures (b) Temperature control for the thermoresponsive solution by the large thermoelectric devices (b), (c) When the temperature of PNIPAAm solution was 30 ˚C and 31.5 ˚C respectively.

II. THERMORESPONSIVE GEL SHEETS

A. Concept

Lots of researches are conducted research works to achieve 3 dimensional cell assemblies in recent years. For example, a photocrosslinkable polymer was used to embed cells in the hydrogel structures. The cells were dispersed in the photocrosslinkable polymer solution and UV light was locally exposed to solidify the solution to fabricate hydrogel structures [6-8]. Bioprinting technology and magnetic manipulation are also used for 3D cell assembly. In the bioprinting method, cells are ejected from inkjet nozzles and embedded in the alginate gel [9, 10]. The magnetic manipulation uses magnetic nanoparticle to manipulate cell structures [11]. Some methods does not require scaffold to make 3D structures. For example, spheroids were formed using flow or gravity to assemble cells in 3D [12, 13].

However, those methods have some disadvantages. In the photocrosslinkable hydrogel, cell growth is restricted by the hydrogel. Because the hydrogel is not a biodegradable material and the long term cell culture is difficult. In the case of the bioprinting method, cell growth will be limited by the alginate gel because the alginate gel is also not a biodegradable material. Cells may have mechanical stresses during the ejection from the inkjet nozzles. The methods using magnetic, flow or gravity forces to assemble cells are not suitable for precise positioning of cells, especially in 3D assembly. Hence, conventional methods are not enough to achieve assembly of 3D cell structures precisely without large stress to cells during the assembly and culture process. The development of a new method which can realize the precise assembly of cells without applying large stress has great possibility to achieve 3D cell systems in vitro.

In this paper, we conducted an assembly of thermoresponsive gel structures using the hysteresis character of thermoresponsive solution. Figure 1 shows the concept of this method. At first, cells are dispersed in the thermoresponsive solution and the solution temperature keeps in between the solution and gelation temperature. Then, thermoresponsive gel is generated in the polymer solution using a microheater. The generated gel can keep the gel condition even if the microheater is turned off because of the hysteresis. Consequently, a gel block or sheet with cells can be made. Repeating the fabrication process of the gel structures and placing them in 3D using micromanipulator, 3D cell structure is assembled. After flushing the cells around the structure, the 3D cell structure is cultured and finally 3D cell system can be made in vitro.

A “thermoresponsive gel (GeT) probe” [14] was used to handle fabricated gel structures in this paper. The GeT probe we proposed has a microheater at the probe tip and the thermoresponsive gel can be generated at the probe tip in the thermoresponsive polymer solution as shown in Fig. 2 (b). The probe is used to handle microobjects by the generated gel (Fig. 2 (c)). The probe can handle soft materials like liposome [14] without large deformation. It means that the GeT probe is suitable to handle thermoresponsive gel structure which is soft and easily deformed.

The cell assembly can be conducted without toxicity to cells if the biocompatible polymer is used. The gel structure can be returned to sol condition by controlling the

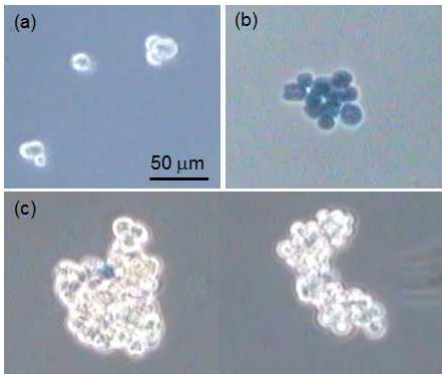

Fig. 5 Experimental results of cell staining by 0.2 % trypan blue (a) In culture condition, (b) On 55 ˚C hotplate for 20 minutes (c) After 1 hour in culture medium+10 wt% PNIPAAm solution on 29 ˚C

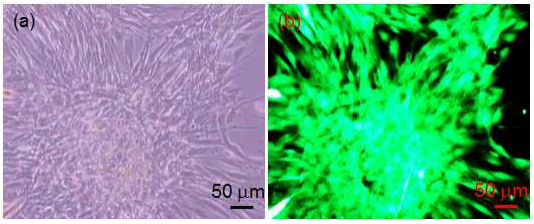

Fig. 6 Cell viability check using Calcein-AM (a) After culturing C2C12 cells in culture medium + 10 wt% PNIPAAm solution for 1 week inside the CO_2 incubator (b) After staining by Calcein-AM

temperature after cells grow enough. The positioning of the gel sheets is precisely controlled when the sheets are manipulated by the probe device. Hence, the method can achieve the fabrication of 3D cell structures precisely without large stress to cells during the assembly.

B. Fabrication of gel sheets using hysteresis

So far, we conducted the fabrication of gel blocks [19] using the GeT probe. However, this method has the limitation in size of fabricated gel structures. Fabricating large gel structure (more than 100 μm square) at once is difficult because of the probe tip size. Therefore, we use another microheater to fabricate large gel structures, i.e. gel sheets at once.

The fabrication of gel sheets was conducted using the hysteresis character of the thermoresponsive solution. In the experiment, 10 wt% poly(*N*-isopropylacrylamide) (PNIPAAm) was used as a thermoresponsive polymer solution. The 10 wt% PNIPAAm solution becomes clouded and gelled over 32 ˚C [15]. It is expected that less energy and high response speed for gelation can be achieved by the PNIPAAm because the gelation temperature is close to the room temperature. The PNIPAAm has a biocompatibility and used for biological applications like the scaffold of cells and the seat on the petri dishes to detach cells from the dishes after culture [16, 17]. Cells can be cultured even if the cells are embedded in the PNIPAAm gel because the culture medium can spread into the gel [18]. Hence, the 10 wt% PNIPAAm solution has advantages to use cell assembly.

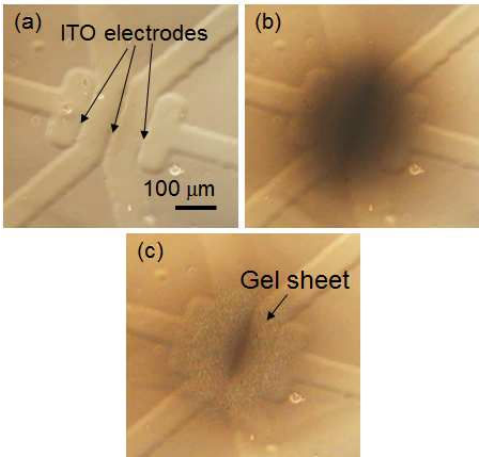

Fig. 7 Experimental results about fabrication of thermoresponsive gel sheet using the ITO electrode (a) Before applying a voltage (b) During the heating (c) After heating

When the temperature is decreased after gelation, the viscosity of PNIPAAm solution dropped rapidly under 22 ˚C and the gel is returned to the sol condition as shown in Fig. 3 [5]. In the experiment, two large thermoelectric devices (40 mm square size) were used as heaters to keep the temperature of the PNIPAAm solution around 30 ˚C as shown in Fig. 4 (a). 5 mm interval was made in between the two large thermoelectric devices to observe the gelation around the microheater under the vertical optical microscope. Figure 4 (b) and (c) shows the PNIPAAm solution when the temperature was 30 ˚C and 31.5 ˚C respectively. The PNIPAAm solution became clouded when the temperature was closed to the gelation temperature 32 ˚C. To achieve the uniform temperature distribution of the PNIPAAm solution in the bath, a slide glass (0.8 mm to 1 mm thickness) was placed on the two thermoelectric devices because the glass has higher thermal conductivity than the air. The bottom substrate of the bath was too thin (0.12 mm to 0.17 mm thickness) to keep the temperature uniformly. The bath was put on the slide glass. The GeT probe was fixed on the micromanipulator (WR-6, Narishige) and placed into the bath.

Cell viability in the PNIPAAm solution was checked using the rat liver cells (RLC-18) and the mouse myoblast cells (C2C12). The RLC-18 cells were dispersed into the culture medium (Dulbecco's Modified Eagle Medium (DMEM) and 5 % Fetal Bovine Serum (FBS)). 200 ml culture medium with cells and 350 ml DMEM+5 % FBS+10 wt% PNIPAAm solution were mixed and put into the bath. The bath was heated around 29 ˚C by the thermoelectric heaters.

Figure 5 shows the results. The cells in culture condition showed the white color which means the live condition (Fig. 5 (a)), while the cells on the hotplate showed the blue color which means the dead condition (Fig. 5 (b)) by using 0.2 % trypan blue. After 1 hour in our experimental setup, most of cells showed white color as shown in Fig. 5 (c). The results validate that the experimental setup can maintain cells in live condition and can be used for 3D cell assembly method we

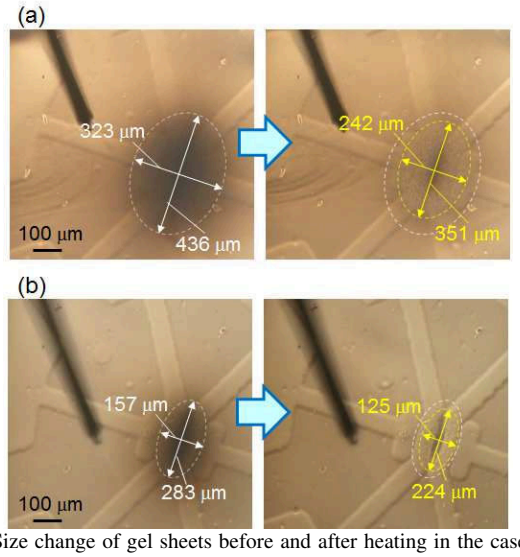

Fig. 8 Size change of gel sheets before and after heating in the case of (a) large gel and (b) small gel

proposed.

The C2C12 cells were dispersed into the culture medium (DMEM + 10 % FBS) with 10 wt% PNIPAAm solution and cultured in a CO_2 incubator for 1 week. The cells were stained by Calcein-AM to check their survivability. Calcein-AM solution was mixed into the culture medium. The cells were cultured for 90 minutes with 4 μM Calcein-AM to stain living cells in green fluorescence. Figure 6 shows the results. Hence, cells showed the green fluorescence. The result indicates that the cells can be cultured inside the thermoresponsive gel.

To fabricate thermoresponsive gel structures, indium tin oxide (ITO) electrodes were fabricated on the substrate as microheaters as shown in Fig. 7 (a) by the photolithography techniques and etching of ITO. The thermoresponsive gel can be generated on the heater by applying a voltage to the electrodes. Figure 6 shows the fabrication results of a gel sheet using the ITO electrode. In the experiment, the ITO electrodes were placed in the 10 wt% PNIPAAm solution and the solution was kept around 30 ˚C by the thermoelectric heaters. When the ITO heater was turned on, a thermoresponsive gel was generated on the heater (Fig. 7 (b)), and it was kept in gel condition even if the heater was turned off again (Fig. 7 (c)) because of the hysteresis character. Hence, the results show that the thermoresponsive gel structures can be fabricated using the hysteresis character.

During the fabrication of gel sheets, the size of the sheet was changed during turning off the ITO heater. To evaluate the size change, two gel sheets which have different sizes were fabricated. As shown in Fig. 8, the length of ellipsoidal gel axis reduced around 80 % after turning off the ITO heater and gel size became around 60 % after turning off the heater in both case large and small gel sheet. The results indicate that the gel size after turning off the heater can be estimated from the size before turning off the heater and the size of the gel sheets can be controlled by changing the applied voltage to the ITO electrodes.

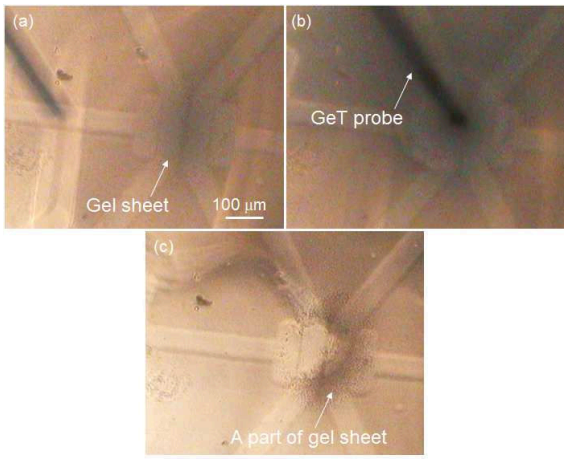

Fig. 9 Handling of a gel sheet when the bottom surface was hydrophobic condition (a) Before handling, (b) When the GeT probe was attached to the gel sheet, (c) After remove the GeT probe

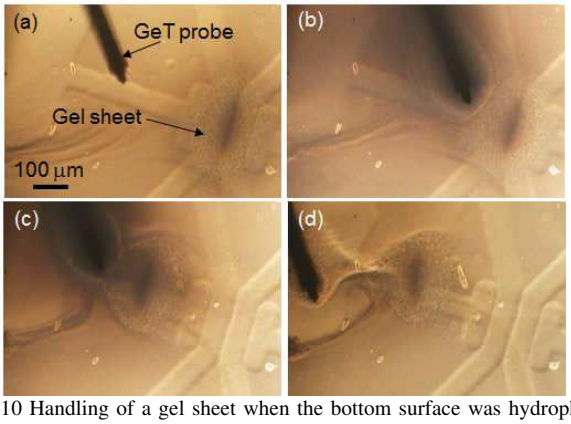

Fig. 10 Handling of a gel sheet when the bottom surface was hydrophilic condition (a) Before handling, (b) When the gel sheet was handled by the GeT probe, (c) Move the handled gel sheet to the left side, (d) After release the handled gel sheet

C. *Gel sheet handling*

After fabricating the gel blocks and gel sheets, they have to be handled for 3D assembly. Therefore, the handling of fabricated gel block and gel sheet was conducted by the GeT probe. Figure 9 shows the results of handling a gel sheet when the bottom substrate was not treated by the oxygen plasma. The bottom surface was coated by PDMS as an insulator as written in chapter 2.3.1, the surface shows the hydrophobic condition. The thermoresponsive gel also shows the hydrophobic condition, and consequently the gel sheet was attached on the substrate strongly. As shown in Fig. 9. (c), only the area the probe tip gel was attached was removed from the substrate and a part of the gel sheet was remained on the bottom surface.

To prevent the attachment of the gel sheet on the bottom substrate, the substrate was treated by the oxygen plasma (PIB-10, Vacuum Device) for 3 minutes in hard mode and the surface was changed to the hydrophilic condition. Then, the handling of the gel sheet was conducted again. Figure 10

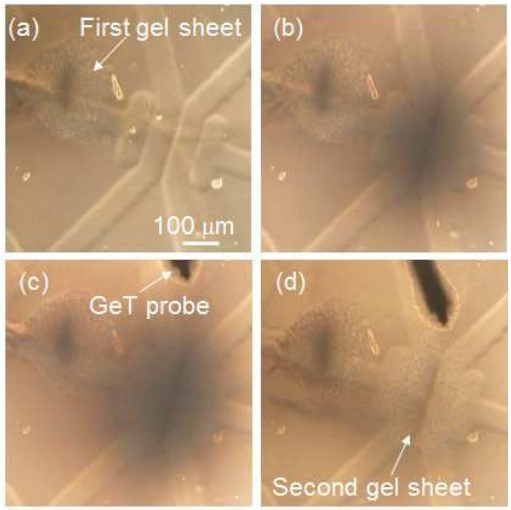

Fig. 11 Fabrication of 2nd gel sheet on the substrate (a) Before fabrication, (b), (c) When the ITO heater was turned on (d) After fabrication

shows the results when the surface was hydrophilic condition. In this condition, the gel sheet was not attached on the substrate strongly, and the gel sheet was moved to the other place by the GeT probe. The results validate that the fabricated gel sheets or gel blocks can be handled by the GeT probe, and assembled to the arbitrary shape in 3D.

D. 3D gel assembly

After making several gel sheets, they are assembled in 3D as shown in the conceptual schematic (Fig. 1). Therefore, assembly of two gel sheets was demonstrated. In the experiment, second gel sheet was fabricated using the ITO electrode after handling the first gel sheet (Fig. 10). The experimental results are in Fig. 11. The second gel sheet was fabricated without affecting the position of first gel sheet. Then, the second gel sheet was handled by the GeT probe to put on the first gel sheet.

Figure 12 shows the results when the second gel sheet was handled and moved up by the GeT probe. In the experiment, the edge of the gel sheet was handled to observe entire gel sheet clearly. While the gel sheet was ellipsoidal shape before handling as shown in Fig. 12 (a), the gel was deformed and changed its shape during lift up process as shown in Fig. 12 (b)-(d).

After handling the second gel sheet, the GeT probe was moved to the first gel sheet. The second gel sheet was released on the first gel sheet to assemble the gel sheets in 3D by turning off the GeT probe. Figure 13 shows the results. The second gel sheet was moved on the first gel sheet (Fig. 13 (b)) and released from the GeT probe by turning off the microheater at the probe tip (Fig. 13 (c)). The second gel sheet was not attached on the GeT probe and released on the first gel sheet (Fig. 13 (d)). Figure 14 shows the magnified images of assembled two gel sheets. Because the second gel sheet was deformed during the lift up process, the second gel sheet showed deformed shape while the first gel sheet was

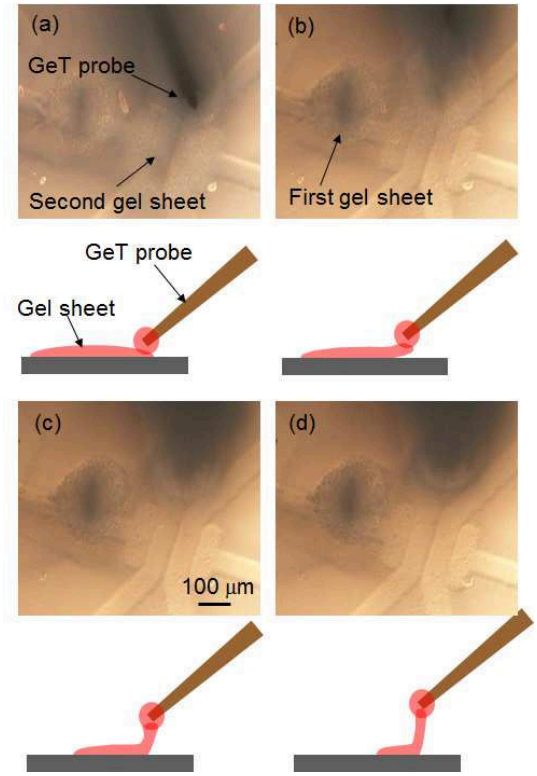

Fig. 12 Handling and lift up of gel sheet for 3D assembly (a) When the edge of the gel sheet was attached on the GeT probe tip, (b)-(d) When the GeT probe was moved up (Schematics in the bottom of each microscopic images show the side view condition of gel in each steps)

ellipsoidal shape. Although the gel shape of the second sheet was deformed, two layered gel structure was assembled by the thermoresponsive gel.

The results validate that the gel sheets or gel blocks can be handled, moved and released by the GeT probe. The gel structures can be also assembled using the GeT probe in 3D. However, the gel sheet was too soft to maintain its shape during the lift up process. Therefore, the handled position of the gel sheet has to be moved to close to center of gravity to prevent large deformation.

III. Conclusion

In this paper, we conducted 3D assembly of microstructures made of thermoresponsive gel using the hysteresis character of the thermoresponsive polymer solution. The gel sheets were fabricated using the ITO heaters on the substrate and the gel condition was kept after turning off the heater by the hysteresis character. The cell viability can be kept in the experimental condition and the experimental results validate the method can be used to keep the gel in the polymer solution and cells can be caught in the gel if cells are dispersed into the thermoresponsive solution before experiments.

The fabricated gel sheets were handled by the GeT probe and they were assembled in 3D. The method will be extended

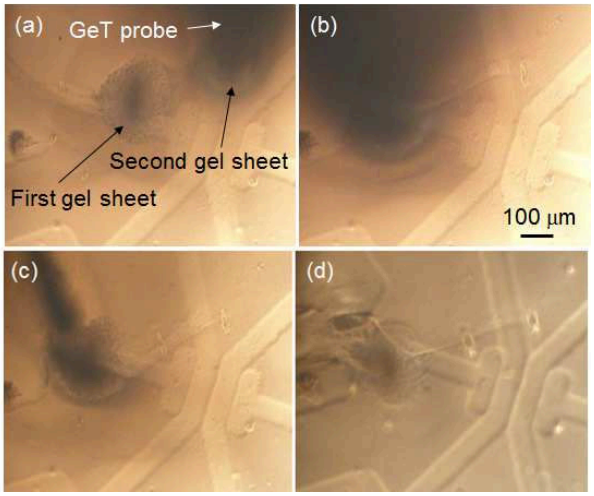

Fig. 13 Experimental results to fabricate 3D gel structure using two gel sheets (a) After lifting up the second gel sheet, (b) When the GeT probe was moved on the first gel sheet, (c) When the GeT probe was turned off to release the second gel sheet, (d) After releasing the second gel sheet on the first gel sheet

to 3D cell assembly and in vitro cell system to apply for the tissue engineering.

ACKNOWLEDGEMENT

This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grans-in-Aid for BioAssembler (23106006)).

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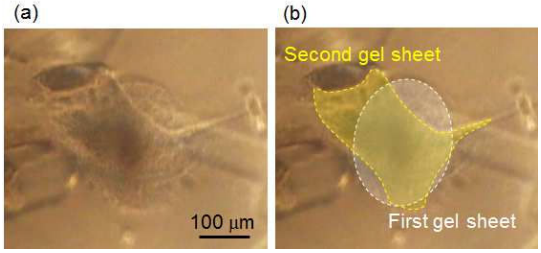

Fig. 14 Magnified images after assembling two gel sheets (a) Microscopic image of two gel sheets after assembly, (b) Indicate the edge of each gel sheets

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