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Two-dimensional microarray of HepG2 spheroids using collagen/polyethylene glycol micropatterned chip

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Abstract A new cell chip technology in the form of a two-dimensional microarray of HepG2 spheroids was developed by using the microcontact printing technique. The chip consisted of several collagen spots in a triangular arrangement within a 100-mm^2 area at the center of a glass plate (24 × 24 mm), which served as the cell adhesion area; the region excluding the collagen spots that was modified with polyethylene glycol (PEG) served as the non-adhesion area. HepG2 cells inoculated onto the chip gradually formed spheroids with smooth surfaces and high circularity on each collagen spot due to cell proliferation; the spheroid diameters remained constant after 10 days of culture. Such a two-dimensional microarray configuration of HepG2 spheroids could be maintained for at least 2 weeks. The spheroid diameter was directly proportional to the pitch between the collagen spots on the chip. This indicates that we can factitiously control the spheroid diameter. In addition, albumin secretion activity of HepG2 spheroids increased with the increase of spheroid diameter. This chip technology may be applicable as a cellular platform for developing two-dimensional spheroid microarrays.

1 Introduction

Recently, a cell chip or a cell array, in which living mammalian cells are patterned on a plate, has advocated owing to its various applications [1–8] such as those in drug screening, cell transfection, cell differentiation studies, and fundamental cell biological studies. This is because it can facilitate the observation of cell responses and the use of various techniques including high-throughput screening. Among the various cell chips, a cell chip using liver-derived cells, including primary hepatocytes, or cell lines, has attracted attention as an *in vitro* model for studying liver functions, including drug metabolism and hepatotoxicity [9–18], because the liver plays many essential roles in maintaining normal physiology.

Generally, the liver-derived cells are cultured with a monolayer configuration. However, the differentiated functions of cells in such a monolayer decrease or are lost. In contrast, the spheroid (spherical multicellular aggregate) configuration, which is formed following the rearrangement and compaction of single cell aggregates, has several advantages over a monolayer configuration. These are as follows: (i) it possesses a tissue-like structure that includes cuboidal cells and abundant cytoplasmic organelles, and expresses intercellular adhesion molecules that are required for cell communications and the reconstruction of cellular polarity; and (ii) it exhibits functions of higher levels for extended periods of time compared to a monolayer configuration [19–21]. Therefore, a cell chip technique using homogeneous spheroids may lead to the development of a superior cell chip. Some chips using spheroid cultures have been reported, for example, Kataoka et al. have developed a two-dimensional multiarray of hepatocyte spheroids on a microfabricated polyethylene glycol (PEG)-brush surface [13], while Griffith et al. have developed a microarray bioreactor of hepatocyte spheroids by using microfabricated silicon [14]. Matsue et al. have developed a spheroid array of HepG2 cells by microfabrication of silicon [15]. We have also succeeded in developing a microarray of

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hepatocyte spheroids by microfabrication and microcontact printing [16–18]. Although we believe that these are an outstanding technique for the cell chip, it is difficult to control the spheroid diameter. In particular, the spheroid diameter of liver-derived cell lines, such as HepG2, easily varies by cell proliferation [9–12]. Therefore, a technique that can achieve the control of spheroid diameter will have advantages over the previous spheroid chip.

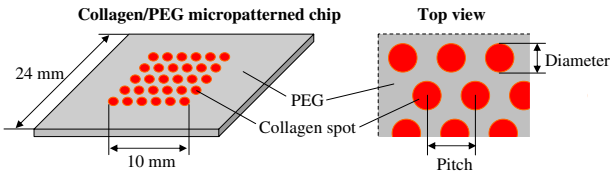
Microcontact printing, which is based on the pattern transfer of materials from a stamp onto a substrate surface, is widely used as a simple method for surface modification and can be applied to a variety of purposes including cell immobilization at a defined location [22, 23]. In this study, we developed a new cell chip with a simple pattern configuration by employing microcontact printing and by using collagen and PEG to create a microarray of HepG2 spheroids. Furthermore, we established a method to control the spheroid diameter by evaluating the effect of the chip structure on the spheroid microarray formation.

2 Materials and methods

2.1 Collagen/PEG micropatterned chip

Figure 1 shows a schematic diagram of the designed chip. The chip consisted of several collagen spots in a triangular arrangement within a 100-mm^2 region in the center of a glass plate (24 × 24 mm) that served as the cell adhesion area, and the region excluding the collagen spots was modified with PEG to form the non-adhesion area.

The chip was fabricated using microcontact printing (Fig. 2). First, the surface of the glass plate was coated with a



Chip condition	Diameter	Pitch	Spot number
Chip 1	100 μm	600 μm	288
Chip 2	200 μm		288
Chip 3	300 μm		288
Chip 4	100 μm	200 μm	2744
Chip 5		300 μm	1216
Chip 6		400 μm	672
Chip 7		500 μm	418
Chip 8		600 μm	288

Fig. 1 Schematic diagram of the collagen/PEG micropatterned chip and the chip designs used. The chip had several collagen spots in a triangular arrangement within a 100-mm^2 area in the center of the chip (24 × 24 mm), which served as the cell adhesion area, and the regions excluding the collagen spots that were modified with PEG served as the non-adhesion area

10-nm-thick layer of platinum in an ion sputter unit (Hitachi High-Tech Science Systems Corp., Ibaraki, Japan) to serve as a substrate on the chip. A stamp was molded by casting the liquid prepolymer of poly(dimethylsiloxane) (PDMS; Sylgard 184, Dow Corning Co., Midland, MI, USA) over the master stamp, which had small convex wells constructed in a poly-methylmethacrylate plate. The PDMS stamp was briefly oxidized by air plasma (Harrick Scientific Co., Ossining, NY, USA) and inked with a 0.15% collagen solution (Cellmatrix, Nitta Gelatin Co., Osaka, Japan). The inked stamp was microscopically contacted with the center of the chip to create the cell adhesion area. The stamp was carefully peeled off from the substrate of the chip. Next, the chip was immersed in 5 mM PEG possessing a thiol group (PEG-SH; molecular weight, 30,000) (NOF Co., Tokyo, Japan) in ethanol solution to obtain a cell non-adhesion area around the collagen spots. With this procedure, the thiol group allowed the covalent attachment of PEG onto a platinum-coated surface. The collagen/PEG micropatterned chip was thoroughly rinsed with distilled, deionized water, followed by rinsing in 70% ethanol for sterilization and the removal of unattached PEG-SH. The chip was then immersed in the culture medium until use.

To understand the effect of the chip structure on the formation of the HepG2 spheroid microarray, eight different chip designs were fabricated (Fig. 1). The effect of the diameter of the collagen spots on spheroid formation was evaluated by using three types of chip designs where the pitch between the collagen spots was kept the same but the diameter of the spots was varied (chips 1, 2, and 3 in Fig. 1). The effect of the pitch between the collagen spots on spheroid formation was evaluated by using five different chip designs where the diameters of the spots were the same, but the pitch between the spots were different (chips 4, 5, 6, 7, and 8 in Fig. 1).

2.2 Preparation of HepG2

HepG2 cell lines (RCB1648, Cell Bank, RIKEN BioResource Center, Ibaraki, Japan) were cultured as a continuous monolayer in a 55-cm^2 tissue culture dish (Corning Inc., NY, USA) containing 13 mL Williams' medium E (Sigma-Aldrich Co., MO, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.3 HepG2 culture on the collagen/PEG micropatterned chip

A HepG2 suspension was obtained from a confluent monolayer on the tissue culture dish by treating with 0.25%

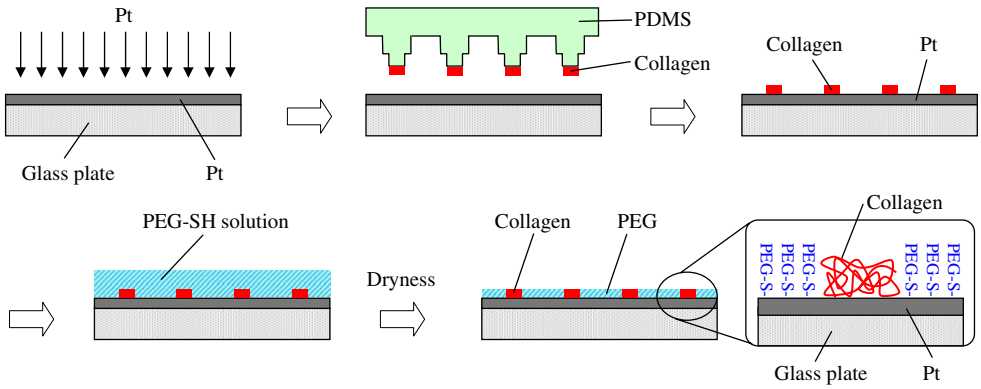


Fig. 2 Fabrication process of the collagen/PEG micropatterned chip

trypsin treatment. The suspension with a cell density of 2.5×10^5 cells/mL was inoculated onto the chip in a polystyrene dish (diameter, 35 mm) containing 2 mL of the culture medium. After 2 h of culture, the cell-inoculated chip was transferred to another polystyrene dish containing 2 mL of fresh culture medium in order to remove the cells that did not adhere to the chip. The culture medium was changed at one-day intervals. All cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

2.4 Evaluation of spheroid conformation

The three-dimensional conformation of HepG2 spheroids on the chip was observed using a wet scanning electron microscope (wet-SEM; Hitachi Ltd., Tokyo, Japan).

To evaluate the time course of changes in the spheroid diameter, images of 100 spheroids that were formed on the chip were taken using a phase-contrast microscope at 3, 5, 7, 10, and 14 days of culture. The diameters were measured using a Windows personal computer installed with a two-dimensional image analysis program (Win ROOF, Mitani Corp., Fukui, Japan). The diameter was calculated using the equivalent circle diameter (ECD) method whereby the spheroid area was converted into an ECD.

The conditions for the survival of the cells inside the spheroids were evaluated by hematoxylin and eosin (HE) staining; spheroids at 14 days of culture were fixed in 3.7% buffered formalin, embedded in paraffin, sectioned, and stained with HE.

2.5 Evaluation of liver functions

The albumin secretion activity of the HepG2 cells was evaluated as a typical liver function. The concentration of albumin that was secreted in the culture medium during 24 h was detected by an enzyme-linked immunosorbent assay (ELISA) conducted using a commercially available kit (Albuwell II; Exocell Inc., PA, USA). The albumin secretion activity was evaluated at 3, 7, and 14 days of culture, and their values were normalized with the cell number during that culture time.

The cell number was determined by a modified DNA-DAPI (4′,6-diamidino-2-phenylindole) fluorescence method [18]. Briefly, the DNA from the HepG2 spheroids was extracted by a DNA extraction solution that consisted of TEN buffer (50 mM Tris–HCl, 5 mM EDTA, 100 mM NaCl; pH, 7.5) containing 100 μg/mL proteinase K (Wako Pure Chemical Industries Ltd., Osaka, Japan). A DAPI solution, which consisted of TEN buffer containing 1 μg/mL DAPI (Wako Pure Chemical Industries Ltd., Osaka, Japan), was prepared for the measurement of DNA-DAPI fluorescence. The extracted DNA solution was mixed with the DAPI solution in a 1:1 ratio, and the fluorescence was measured using a fluorescence plate reader (excitation wavelength, 355 nm and emission wavelength, 460 nm) (Fluoroskan Ascent; Thermo Electron Corp., Vantaa, Finland). A standard curve of cell-DNA was constructed using cell suspension values. The constructed standard curve was used to convert the DNA-DAPI fluorescence values obtained from the plate reader to the cell number.

2.6 Statistical analysis

Data obtained from experiments of spheroid diameter and liver function represented the mean ± standard deviation of four chips from two independent cell preparations and four points from one independent cell preparation, respectively. Statistical analysis of the numerical variables was performed by using a repeated-measures ANOVA test. A value of $p < 0.05$ was considered to be significant.

3 Results

3.1 Spheroid formation on the collagen/PEG micropatterned chip

The HepG2 cells inoculated onto the chip attached to the collagen spot within 2 h and formed a monolayer. The

monolayer configuration on the collagen spot remained until approximately 3 days of culture (Fig. 3a, d, g). Thereafter, the HepG2 cells gradually formed spheroids on each collagen spot by cell proliferation (Fig. 3b, e, h); with further proliferation the spheroid configuration was maintained. After approximately 10 days of culture, the resulting spheroids attained smooth surfaces and high circularity (Fig. 3c, f, i). Furthermore, an SEM image revealed that they had a domed structure on the collagen spot (Fig. 4). There were no hepatocytes attached to the PEG area on the chip during the culture. This two-dimensional microarray configuration of HepG2 spheroids was maintained for at least 2 weeks of culture.

Figure 5 shows the changes in the spheroid diameters in different chip designs where the diameters of the collagen spots were varied. In chips with collagen spots of diameters 100 and 200 μm (chips 1 and 2, respectively), the spheroid diameter increased drastically from the third till the 10th day of culture, following which it remained constant. In contrast, in the chip with the collagen spots of diameter 300 μm (chip 3), the spheroid diameter was not greater than the diameter of the collagen spots during the culture period. As a result, the spheroid diameters in these three chip designs were observed to remain in the range of 250–300 μm.

3.2 Effect of the pitch between collagen spots

Figure 6 shows the spheroid morphologies at 14 days of culture in chips with different pitches between the collagen spots. The HepG2 cells in chips of all designs formed spheroids with smooth surfaces and high circularity. Although each spheroid existed independently on chips with pitches longer than 300 μm (chips 5, 6, 7, and 8), some spheroids were bridged together in the chip with a 200-μm pitch (chip 4).

The spheroid diameter in each chip design drastically increased during the initial 10 days of the culture; however, the diameter remained constant thereafter (Fig. 7a). Furthermore, the spheroid diameter increased with an increase in the pitch between the collagen spots on the chip. For example, the spheroid diameters of chip 4 (200-μm pitch), chip 5 (300-μm pitch), chip 6 (400-μm pitch), chip 7 (500-

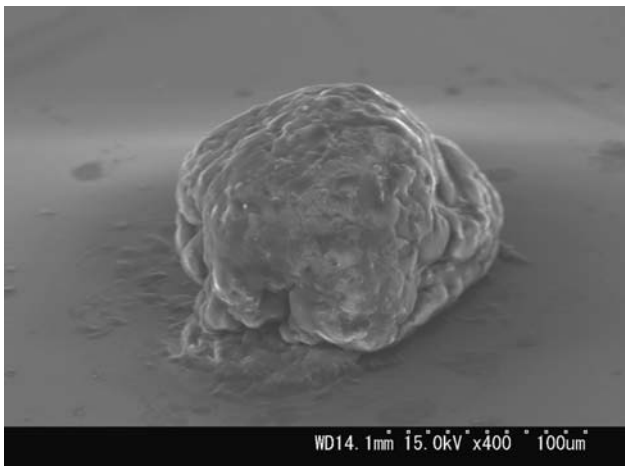


Fig. 4 Wet-SEM micrograph of a representative HepG2 spheroid formed on the collagen/PEG micropatterned chip at 14 days of culture

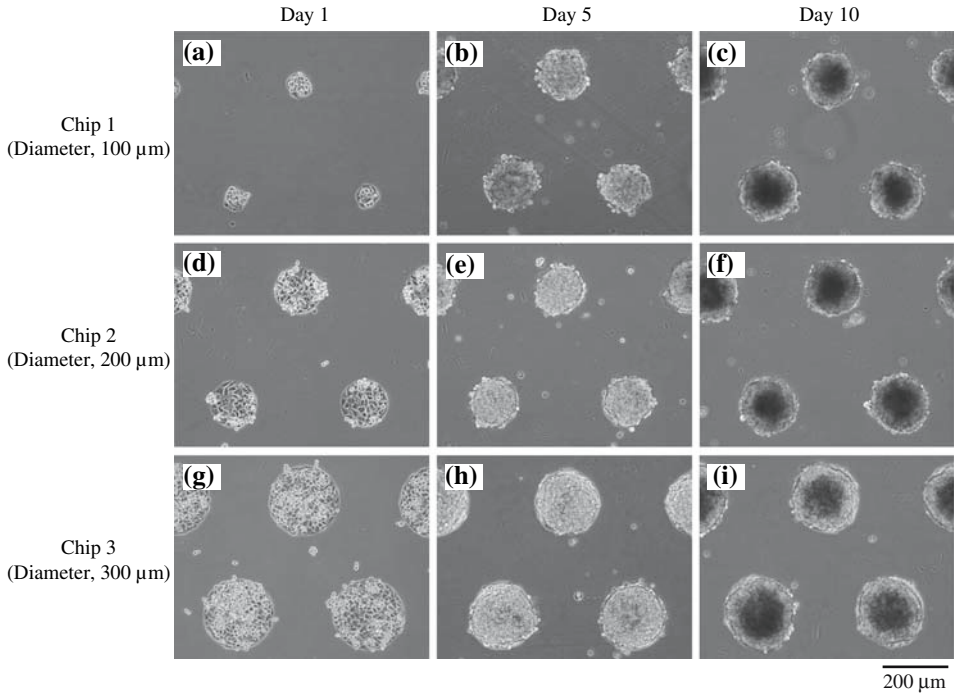


Fig. 3 Phase-contrast micrographs of cell configurations on chip 1 (diameter of collagen spot, 100 μm) (**a**–**c**); chip 2 (diameter of collagen spot, 200 μm) (**d**–**f**); and chip 3 (diameter of collagen spot, 300 μm) (**g**–**i**). (**a**, **d**, **g**), 1 day of culture; (**b**, **e**, **h**), 5 days of culture; and (**c**, **f**, **i**), 10 days of culture

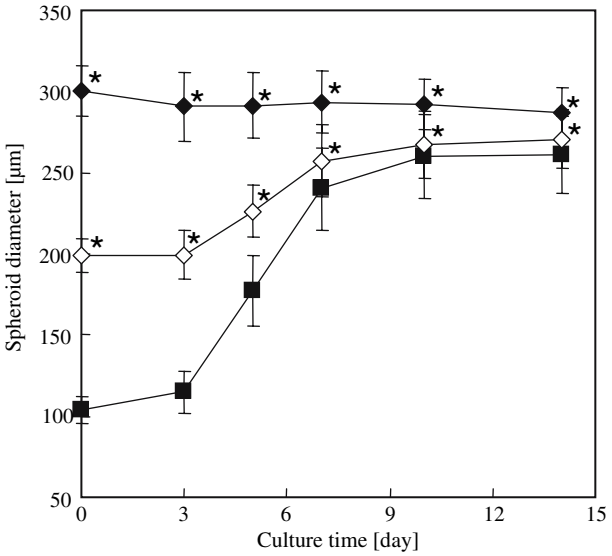


Fig. 5 Change in the spheroid diameters on chip 1 (diameter of collagen spot, 100 μm) (■), chip 2 (diameter of collagen spot, 200 μm) (◇), and chip 3 (diameter of collagen spot, 300 μm) (◆). Error bars represent SD. $*p < 0.05$ compared with the value of chip 1

μm pitch), and chip 8 (600-μm pitch) at 14 days of culture were 133 ± 11, 174 ± 15, 219 ± 17, 247 ± 18, and 267 ± 22, respectively. These results revealed that the pitch is positively correlated with the spheroid diameter (Fig. 7b).

3.3 Internal conditions of the spheroids

Conditions for the survival of cells in the spheroids of different diameters were observed by HE staining (Fig. 8). The spheroids that were approximately less than 180 μm in diameter consisted of viable nucleated cells that were observed even in the core of the spheroids; however, spheroids with diameters more than 180 μm revealed cell necrosis within their core.

3.4 Liver functions

The albumin secretion activity in the HepG2 spheroids in different chip designs was compared at 3, 7, and 14 days of culture. Although the level of albumin secretion activity per cell was almost the same in all the chip designs at 3 days of culture, the level increased with the spheroid formation. Furthermore, the level of albumin secretion activity per cell increased with the increase of spheroid diameter at 7 and 14 days of culture (Fig. 9).

4 Discussion

Spheroid culture has been strongly advocated as a highly useful *in vitro* model that should be used in place of the traditional monolayer culture. Therefore, many researchers have begun using spheroid culture of various cells for a variety of applications.

Among the cell lines used in these cultures, HepG2 cells have highly differentiated functions, and it has been reported that these functions are further upregulated by spheroid formation [24–26]. Therefore, HepG2 spheroid culture is a useful technique for an *in vitro* model for studying hepatotoxicity and tumorigenesis, and as a cell source of bioartificial liver. In this study, we succeeded in developing a two-dimensional microarray of HepG2 spheroids. In the chips used, the location of spheroid formation was controlled by micropatterned collagen spots.

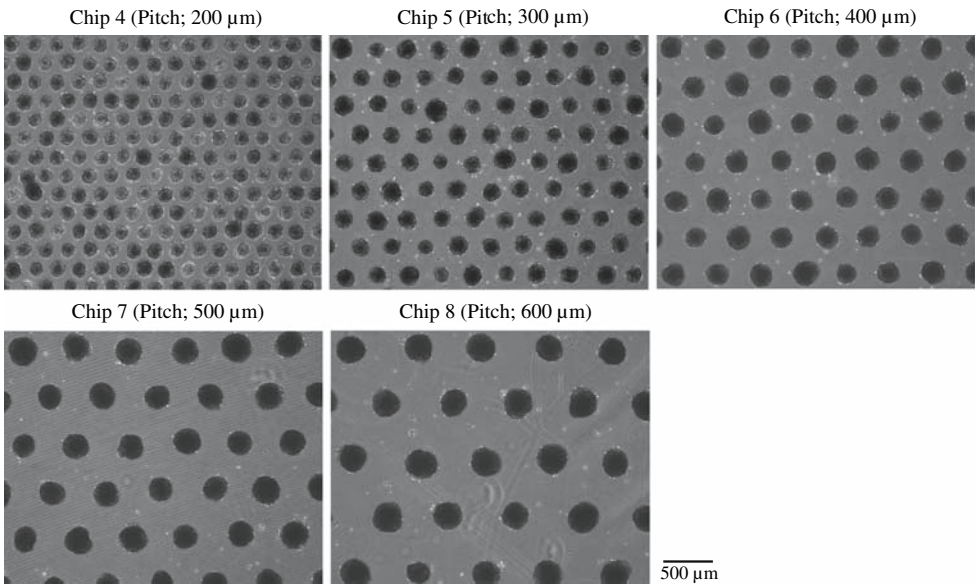


Fig. 6 Phase-contrast micrographs of hepatocyte configurations on chip 4 (pitch between collagen spots, 200 μm), chip 5 (pitch between collagen spots, 300 μm), chip 6 (pitch between collagen spots, 400 μm), chip 7 (pitch between collagen spots, 500 μm), and chip 8 (pitch between collagen spots, 600 μm) at 14 days of culture

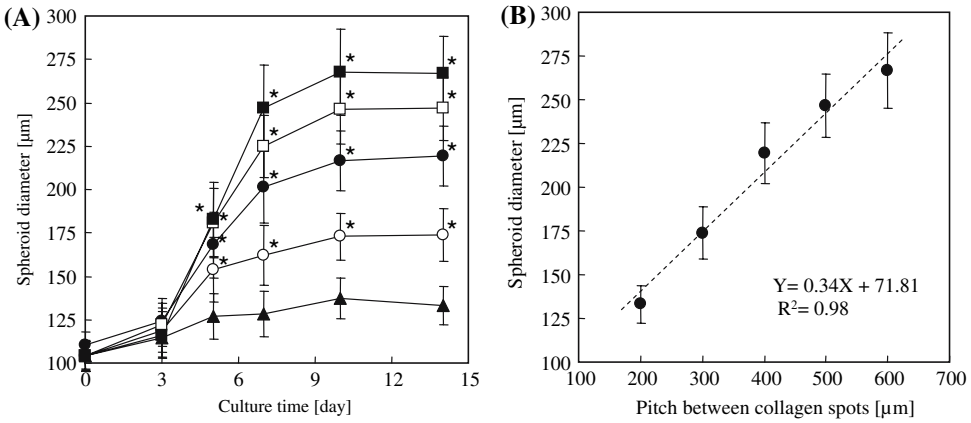


Fig. 7 (**a**) Change in the spheroid diameters on chip 4 (pitch between collagen spots, 200 μm) (▲), chip 5 (pitch between collagen spots, 300 μm) (○), chip 6 (pitch between collagen spots, 400 μm) (●), chip 7 (pitch between collagen spots, 500 μm) (□), and chip 8 (pitch between collagen spots, 600 μm) (■). (**b**) Relation between the spheroid diameter and the pitch between collagen spots at 14 days of culture. Error bars represent SD. $*p < 0.05$ compared with the value of chip 4

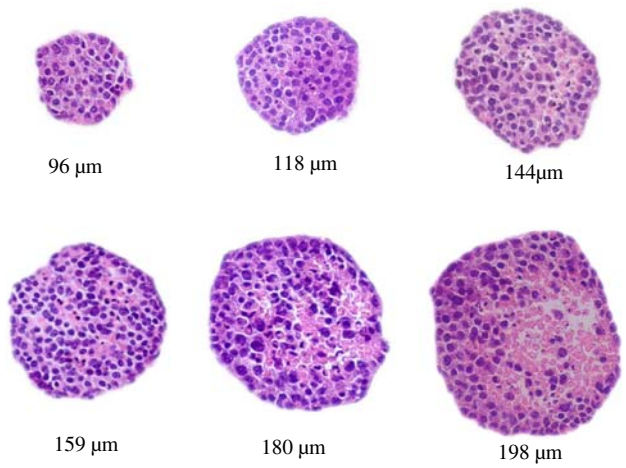


Fig. 8 HE staining photographs of representative HepG2 spheroids formed on the collagen/PEG micropatterned chip at 14 days of culture

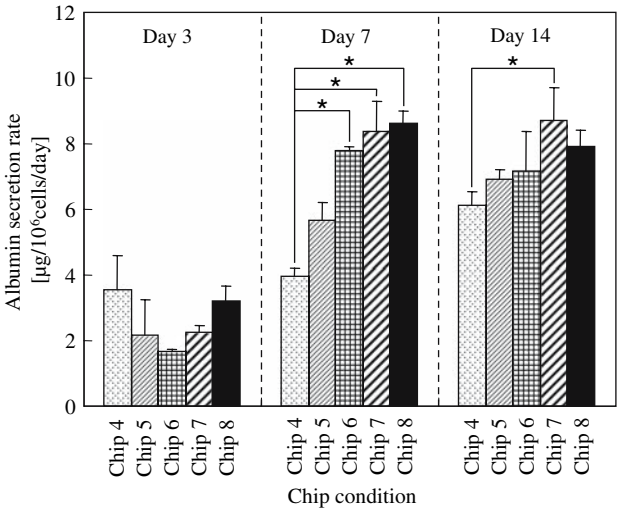


Fig. 9 Albumin secretion activities of HepG2 spheroids on chip 4 (pitch between collagen spots, 200 μm), chip 5 (pitch between collagen spots, 300 μm), chip 6 (pitch between collagen spots, 400 μm), chip 7 (pitch between collagen spots, 500 μm), and chip 8 (pitch between collagen spots, 600 μm) at 3, 7, and 14 days of culture. Error bars represent SD. $*p < 0.05$ compared with the value of chip 4

Thus, the HepG2 spheroids were immobilized on the chips at regular predetermined intervals. Furthermore, the spheroid diameter was strongly positively correlated with the pitch between the collagen spots on the chip. This important finding indicates that we can factitiously control the spheroid diameter by using chips with different pitches between cell adhesion spots. Such a technique for the control of spheroid diameter has not been reported in other studies. Therefore, this may be a more useful technique for an *in vitro* model than previous techniques using spheroid formation.

An interesting observation from the study was that the diameter of the HepG2 spheroids in all chip designs increased from the third till the 10th day of culture and subsequently remained constant for at least 2 weeks. The mechanism responsible for the constancy of the spheroid diameters is still unclear. However, it may be hypothesized that the spheroid size and cell growth are regulated by the concentrations of oxygen and the nutrients around spheroids and/or by the secretions from each spheroid. We believe that an analysis of the culture medium surrounding the spheroids may lend support to our hypothesis. Another observation with regard to the HepG2 spheroid microarray was that each spheroid existed independently on chips that possessed a pitch of more than 300 μm between the collagen spots; however, some spheroids were bridged together on chips with a 200-μm pitch. In case of chips with small pitches, some HepG2 cells that were inoculated on the chips adhered to each other to form links between

adjacent collagen spots, thereby giving rise to some spheroid bridges. This phenomenon is similar to the one reported by Kataoka et al. where bovine aortic endothelial cells could not be patterned in cases where the distance between the cell adhesion regions was less than 100 μm [13]. Consequently, a chip with a distance of more than 100 μm between adjacent collagen spots (as observed in this study, a chip with a pitch greater than 300 μm and the diameter of collagen spots, 100 μm) may be a more suitable design for precise spheroid microarray.

The level of albumin secretion activity per cell increased with the increase of spheroid diameter. Increased cell density and extensive cell–cell contacts in the large spheroids may explain the enhanced differentiated function of HepG2 cells. An evaluation of the detailed relationship between spheroid diameter and many liver functions including mRNA expressions will be the focus of our next study. On the other hand, the HepG2 spheroids of more than 180 μm in diameter revealed dead cells in their core. This result is similar to that of a previous report [26]. The spheroids are avascular structures; hence, oxygen and nutrients that are necessary for cell survival are supplied by diffusion through the spheroid surface to the core. Thus, cell death occurs within the core of large spheroids. Generally, the presence of a necrosed cell layer within the spheroids is not desirable for studying drug metabolism, hepatotoxicity, and bioartificial liver. Therefore, the HepG2 spheroids that were less than 180 μm in diameter will be better for these applications. On the other hand, the large spheroids having necrotic or hypoxic core may be a useful cancer model.

The chip designed in this study is simple in structure and easy to handle. Although we used collagen to form cell adhesion areas in this study, a chip micropatterned with other extracellular matrixes and artificial materials can also be designed by using the microcontact printing technique. Therefore, we believe that the chip developed by us may be applicable as a generic two-dimensional spheroid microarray of various cells.

5 Conclusion

A simple cell chip micropatterned with collagen and PEG was designed using the microcontact printing technique. HepG2 cells formed spheroids with smooth surfaces and high circularity on each collagen spot patterned onto the chip following cell proliferation, thereby establishing a two-dimensional microarray of HepG2 spheroids. In addition, we found that the spheroid diameter could be factitiously controlled by changing the pitch between the collagen spots on the chip. This technique may be applicable as a cellular platform for the development of two-dimensional spheroid microarrays of various cells.

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