Two-Dimensional Multiarray Formation of Hepatocyte Spheroids on a Microfabricated PEG-Brush Surface

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A two-dimensional microarray of ten thousand (100×100) hepatocyte heterospheroids, underlaid with endothelial cells, was successfully constructed with 100 μm spacing in an active area of 20×20 mm on microfabricated glass substrates that were coated with poly(ethylene glycol) brushes. Cocultivation of hepatocytes with endothelial cells was essential to stabilize hepatocyte viability and liver-specific functions, allowing us to obtain hepatocyte spheroids with a diameter of 100 μm, functioning as a miniaturized liver to secret albumin for at least one month. The most important feature of this study is that these substrates are defined to provide an unprecedented control of substrate properties for modulating cell behavior, employing both surface engineering and synthetic polymer chemistry. The spheroid array constructed here is highly useful as a platform of tissue and cell-based biosensors and detects a wide variety of clinically, pharmacologically, and toxicologically active compounds through a cellular physiological response.

Introduction

Recently, microarray-based high-throughput technologies, dealing with DNA and proteins, have been developed for genome and proteome screening. These microarrays are a useful tool for characterizing gene[1–3] and protein[4–6] expression patterns in human disease processes in order to identify novel molecular targets for therapy. However, this represents only the first step in understanding the biological and medical significance of these molecules.[1–6] Intensive investigation of the proteins and chemical networks that comprise the cells and tissues of an organism, and the specific roles of proteins in these networks, will be a necessary next step to understanding cellular functions in healthy and diseased states. New high-throughput techniques based on tissue and cell microarrays will facilitate clinical and pharmaceutical analysis of molecular targets, because living cells can monitor the targets through the physiological changes that are induced in them by exposure to drugs and environmental perturbations, such as toxicants, pathogens or other agents.[7–11] In this regard, primary hepatocytes are the most useful candidates for constructing tissue- and cell-based biosensors (TBB and CBB). Novel applications of this approach include an alternative to animal experiments in a variety of situations in which the reliance on animals would prove objectionable or impractical, because TBB and CBB do not involve the use of live animals as detectors. Here, we report for the first time a simple and effective way to form two-dimensional microarrays of hepatocyte heterospheroids, underlaid with endothelial cells. Cocultivation of hepatocytes and endothelial cells was used to stabilize hepatocyte viability and liver-specific functions, forming a microarray of ten thousand (100×100) hepatocyte spheroids and functioning as a miniaturized liver—as seen in Figure 1.

Figure 1. *Microarray of ten thousand (100×100) hepatocyte heterospheroids prepared on 100 μm diameter circular glass domains with l = 100 μm spacing on 20×20 mm glass substrate coated with α-lactosyl-PEG/PLA.*

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Since isolated hepatocytes are known to readily lose many liver-specific functions during culture, the most crucial issues in hepatocyte-based biosensors are long-term viability and retention of liver-specific functions of cultured hepatocytes.[11–21] In this regard, the formation of multicellular spheroids is of particular interest, since spheroids show not only morphological but also functional similarities to tissues and organs; unlike conventional monolayer cell cultures.[16–18,21] Most of the spheroids developed so far by liquid-overlay culture[22] have been homospheroids, composed of a single population of cells. Nevertheless, tissues and organs in vivo are structures made from cells of different types that are systematically and functionally combined and exhibit dynamically interrelated functions in the mature organism.[23,24] A novel approach is essential for the formation of heterospheroids of hepatocytes that are layered on feeder cells such as endothelial cells. It is necessary to control the size, shape, and spacing to construct proper functioning spheroid-based biosensor systems.

Results and Discussion

Micropatterned poly(ethylene glycol) (PEG) treated substrates with two-dimensional arrays of plasma-etched circular domains (ø = 100 μm) were prepared by sequential spin-coating of polylactide (PLA) and α-lactosyl-PEG/PLA block copolymer on silanized glass slide dishes, followed by plasma-etching through a metal mask pattern with circular holes (Figure 2a). The PEG-treated region on the patterned substrate works to repel proteins and, consequently, inhibits cell adhesion.[25,26] Proteins are expected to adsorb from the serum-containing medium onto the plasma-etched circular domains, exposing the base glass surface. Indeed, the circular glass domains, separated by PEG-treated regions, substantially adsorb fibronectin (FN), as detected by immunofluorescence microscopy after treating with a rhodamine-conjugated anti-FN antibody (Figure 3). The presence of a *p*-aminophenyl lactosyl group on the distal end of the PEG brush was also confirmed by fluorescence through a conjugation of fluorescein-5-isothiocyanate (FITC) to a secondary amino group of the *p*-aminophenyl lactose moiety. Note that the fluorescence image was well correlated with the data generated by surface elemental analysis; conducted by scanning electron microscopy with energy-dispersed analysis of X-rays (SEM/EDX) and X-ray photoelectron spectroscopy (XPS) experiments.[27]

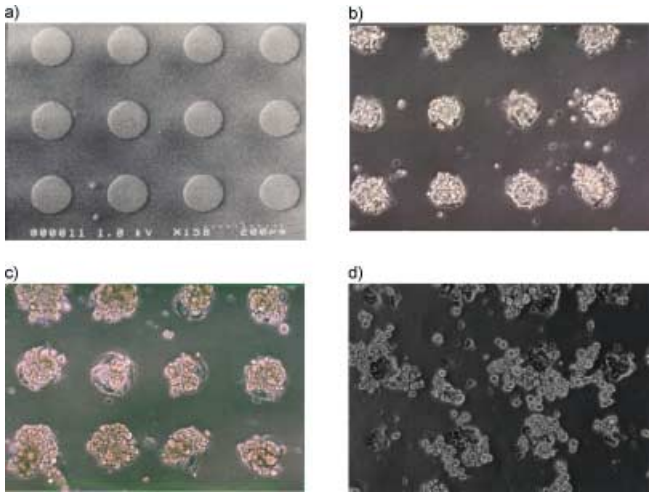

Figure 2. *Patterned 3D coculture of hepatocyte spheroids and endothelial cells (BAECs). a) Micropatterned α-lactosyl-PEG/PLA coated dish with ø = 100 μm circular domains spaced at l = 100 μm intervals. b) Patterned culture of BAECs on substrate a for 24 h at 37 °C. c) Organized pattern of hepatocytes spheroids underlaid with BAECs. d) Hepatocytes directly seeded on substrate a without preadhered BAECs.*

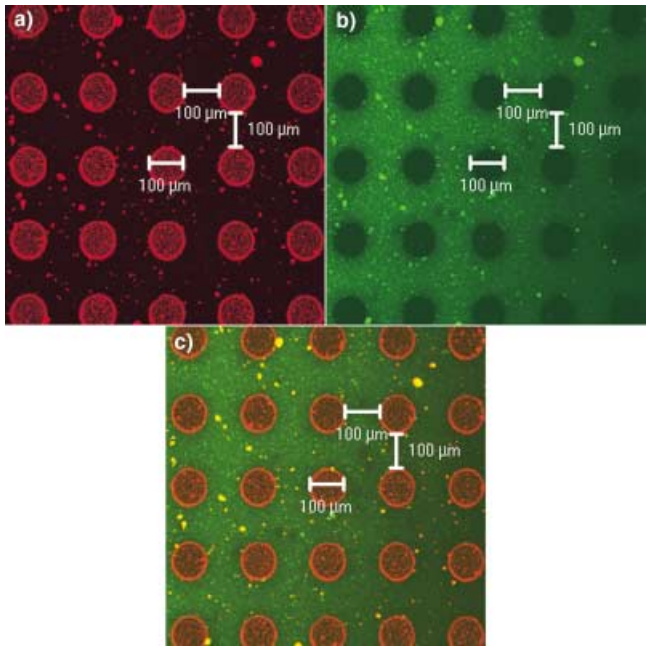

Figure 3. *Patterned substrate surfaces visualized by fluorescence microscopy: a) the circular glass domain was confirmed by the adsorption of FN from an aqueous solution, immunostained in situ with rabbit antibovine FN polyclonal antibody, followed by the rhodamine-conjugated goat antibody against rabbit IgG; b) the α-lactosyl groups were confirmed by staining with FITC, treated with secondary amine of* p*-aminophenyl-β-*D*-lactopyranoside, tethered at the PEG-chain end. c) Superimposition of a and b. The three images were obtained from the same view field.*

Bovine aortic endothelial cells (BAECs) at passage 13 were then seeded onto the patterned surfaces with ø = 100 μm circular domains that were edge-to-edge spaced at *l* = 100 μm intervals (Figure 2a), and cultured at 37 °C for 24 h in a 10% fetal bovine serum medium. Obviously, BAECs adhered only onto the circular domains, exposing a glass substrate (Figure 2b). Preferentially adsorbed extracellular-matrix (ECM) proteins, including fibronectin, vitronectin, and laminin on the glass circular domains, may promote the adhesion of anchorage-dependent BAECs. This is supported by the fact that the BAEC pattern is consistent with FN adsorption results, as seen in Figures 2b and 3.

Rat primary hepatocytes, suspended in a culture medium, were then applied to the patterned dishes with cultured endothelial cells selectively located in the circular domains. Interest-

ingly, rat primary hepatocytes formed spheroids within 24 h only on the circular regions of existing endothelial cells, generating a two-dimensionally arrayed structure of the hepatocyte spheroids (Figure 2c). In contrast, on the same patterned α-lactosyl-PEG/PLA surface without preadhered BAECs, hepatocytes attached to and spread on both the PEG layer and the glass regions, without spheroid formation (Figure 2d). These results demonstrate the significant role of BAEC as a feeder layer for the formation of hepatocyte spheroids.

Furthermore, the spacing between circular domains was found to play a substantial role in the patterned alignment of BAECs, and subsequently in hepatocyte-spheroids formation. On a micropatterned surface coated with α-lactosyl-PEG/PLA (ø = 100 μm) with edge-to-edge spacing of $l = 100$ μm (Figure 4a), BAECs attached only in the circular domain and continued to form active ruffling extensions at the PEG boundary. At first, the extensions protruded several micrometers into the ECM-nonadhesive PEG regions, but rapidly retreated within minutes of the protrusion. Consequently, they were unable to spread deeply into the nonadhesive PEG-treated regions (Figure 4b). However, when the spacing was reduced to 80 μm (Figure 4c), the BAECs started to bridge across multiple islands (Figure 4d). This bridging was more pronounced to form the complete cell sheet (Figure 4f), when the spacing was further reduced to 50 μm (Figure 4e). The threshold spacing for BAEC bridging seems to be in the range between 50 μm and 100 μm. Note that no spheroid formation of hepatocytes occurred on the monolayered sheet of BAEC, as seen in Figure 4f.

The functionality of the PEG chain end is another crucial factor for the spatial alignment of BAEC. In contrast to PEG with lactose end groups (Figure 4b), a patterned surface with methoxy-ended PEG as matrix region resulted in the spreading sheet formation of BAEC (Figure 4g), despite the same patterning of ø = 100 μm and $l = 100$ μm. The difference in PEG functionality seems to alter the structure of the PEG brush and result in ECM-protein adsorption, and consequently cell adhesion. Most importantly, the surface engineering and chemical approach described here provide unprecedented control in tailoring the substrate for modulating cell behavior.

The cell viability of the obtained spheroid was assessed with a LIVE/DEAD viability/cytotoxicity assay kit. Living cells are distinguished by the presence of ubiquitous intracellular esterase activity and are determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent and cell-impermeable calcein. The polyanionic calcein is well retained within living cells and produces an intense, uniformly green fluorescence (excitation/emission; ~494 nm/~517 nm). Indeed, the intense green fluorescence of calcein was observed intracellularly in the cytoplasm of every spheroid hepatocyte, even after three weeks of culture (Figure 5a). It should be noted that no such green fluorescence was observed for isolated hepatocytes without any underlaid

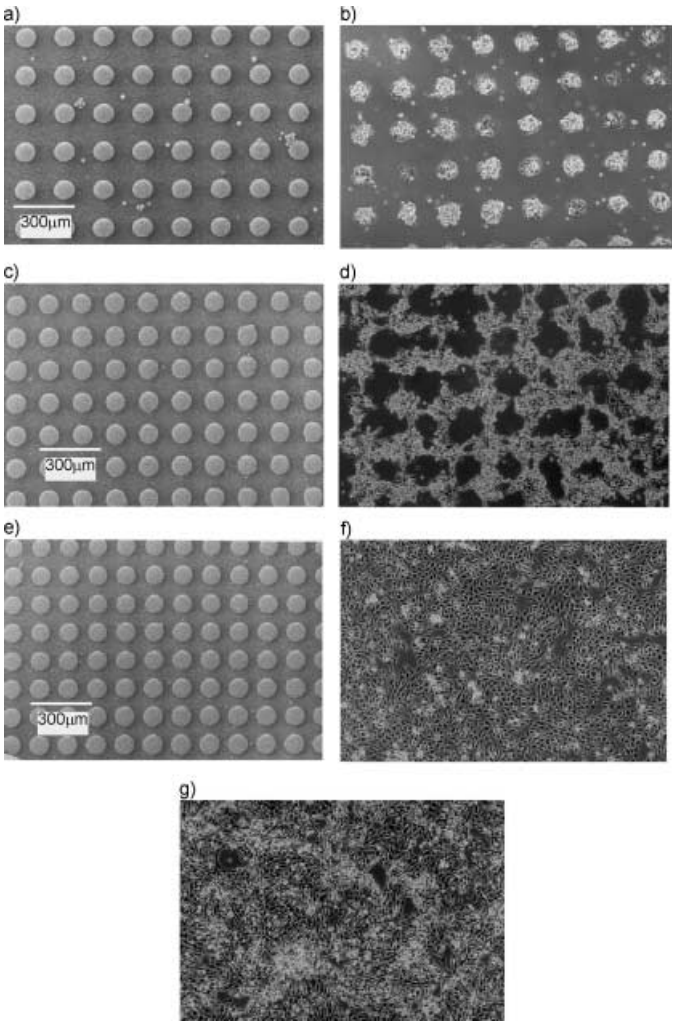

Figure 4. *a), c), e) SEM images of micropatterned an α-lactosyl-PEG/PLA surface with different spacings: a) ø = 100 μm, l = 100 μm, c) ø = 100 μm, l = 80 μm, and e) ø = 100, l = 50 μm. b), d), f), g) Phase contrast micrographs of BAECs: b) cultured on substrate a, d) cultured on substrate c, f) cultured on substrate e, and g) cultured on a micropatterned α-methoxy-PEG/PLA surface with ø = 100 μm and l = 100 μm.*

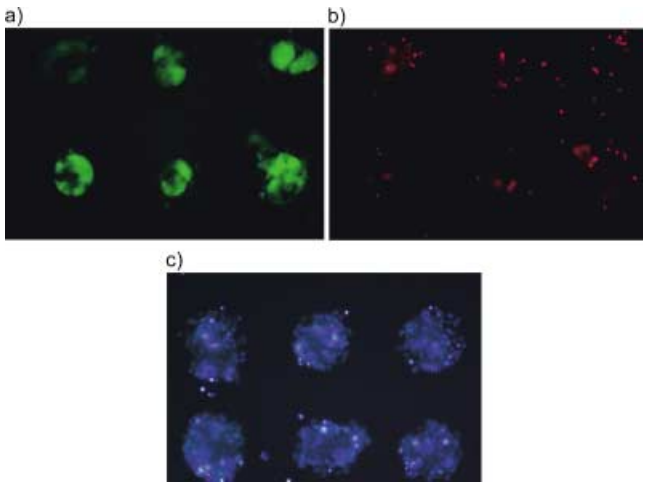

Figure 5. *Viability assay by LIVE/DEAD viability/cytotoxicity assay kit of three-dimensionally cocultured spheroids on α-lactosyl-PEG/PLA-pattern-coated dishes for three weeks at 37°C. a) Live-cell image stained with calcein. b) Dead-cell image stained with EthD-1. c) Distinct nuclei stained with a DNA-binding dye (Hoechst 33342).*

BAECs. On the other hand, ethidium homodimer-1 (EthD-1) enters only into cells with damaged plasma membranes and binds to nucleic acids, thereby producing a bright red fluorescence (excitation/emission; ~528 nm/~617 nm) in dead cells. No such red fluorescence was observed for spheroids underlaid with BAECs, in line with the result from calcein AM. In contrast, a bright red fluorescence was observed for isolated hepatocytes without underlaid BAECs (Figure 5b). Staining with the fluorescent dye (Hoechst 33342) for nuclei further demonstrated nuclear morphology (Figure 5c). These results suggest that cell viability was well retained in the spheroid structure, interacting with the underlaid BAEC layer.

Hepatocyte spheroids in contact with BAECs were characterized by an immunohistochemical double-staining method. In situ fluorescent staining was done with an anti-rat albumin antibody for cellular albumin synthesis, a characteristic phenotype of hepatocytes. Rhodamine-conjugated phalloidin was used for F-actin (Figure 6). Figure 7 further demonstrates a 3D view of a multicellular spheroid of hepatocytes underlaid with BAECs as a feeder cell. This was reconstructed from a stack of 2D image volumes. The procedure permits the precise 3D determination of a variety of functional, geometric, and densitometric parameters, for example, volume and surface.[28] It should be noted that spheroids significantly express a stable level of liver-specific functions (albumin secretion) even after three weeks, showing intense green fluorescence, compared to the usual cell monolayers. In multicellular organization intimately coupled to the dynamics of the actin cytoskeleton, most of the actin was localized in the cell cortex, as opposed to the stress fiber, which is linked to the cell-substratum contact via a focal adhesion complex.[29] The obtained spheroids have ultrastructural similarities to native liver tissue, such as junctional complexes; this leads to a high level of retained liver-specific functions. Indeed, Tzanakakis et al.[29] reported that an intact F-actin network is required for both efficient spheroid self-assembly and liver-specific functions, including albumin secretion and cytochrome P450 activity, by using cytochalasin D, an inhibitor of actin polymerization.[30] Note that these albumin secretions, cytoskeleton as well as cell–cell junction, are maintained intact in the spheroids, presumably due to the heterotypic cell interaction through the hepatocyte–BAEC contact.[19,23,24] To further investigate the cellular function in the hepatocyte heterospheroids, hepatic albumin secretion was evaluated as a function of time by using a sandwich enzyme-linked immunosorbent assay (ELIZA). The results demonstrate that continuous albumin secretion in hepatocytes cocultured with BAECs was observed for over 31 days of culture (Figure 8). Note that continuous secretion of albumin for 31 days has rarely been accomplished in other culture methods reported so far, and this is a direct demonstration that the surviving hepatocytes have functions comparable to the ones seen in the liver.

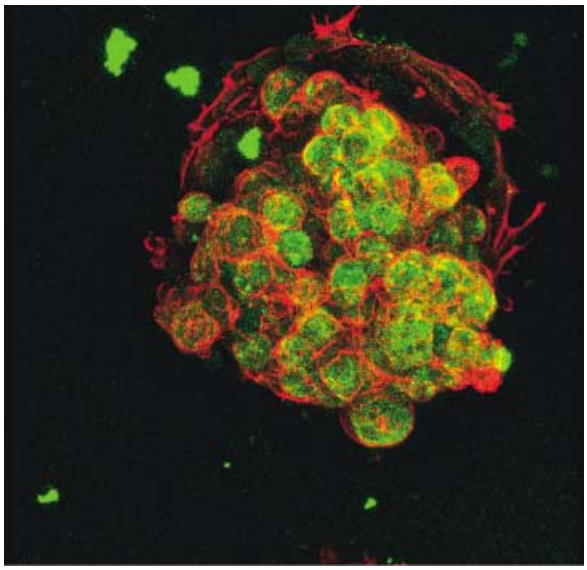

Figure 7. *3D view of spheroids shown in Figure 6, underlaid with endothelial cells as a feeder layer.*

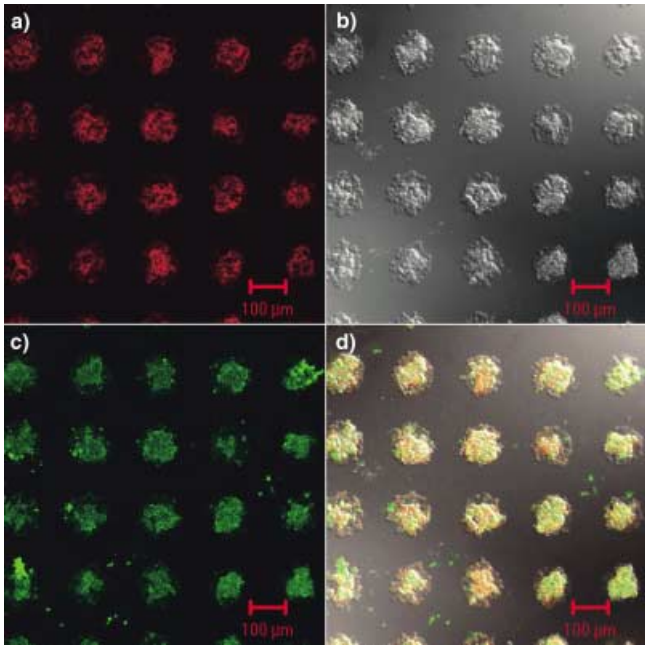

Figure 6. *Confocal-laser scanning microscopy of patterned three-dimensional spheroids after the double staining of F-actin and albumin, cocultured for three weeks at 37°C. Spheroids were fixed and double stained with a) rhodamine-conjugated phalloidin for F-actin and b) anti-rat albumin antibody and FITC-conjugated second antibody for albumin synthesis activity. c) Interference reflection microscopy. d) Superimposition of a, b, and c. The four images were obtained from the same view field.*

Conclusion

In conclusion, a two-dimensional heterospheroid array composed of bovine aortic endothelial cells and rat primary hepatocytes was prepared on micropatterned substrates with circular glass domains 100 µm in diameter, surrounded and separated by 100 µm spacing of α-lactosyl-PEG/PLA. Eventually, ten

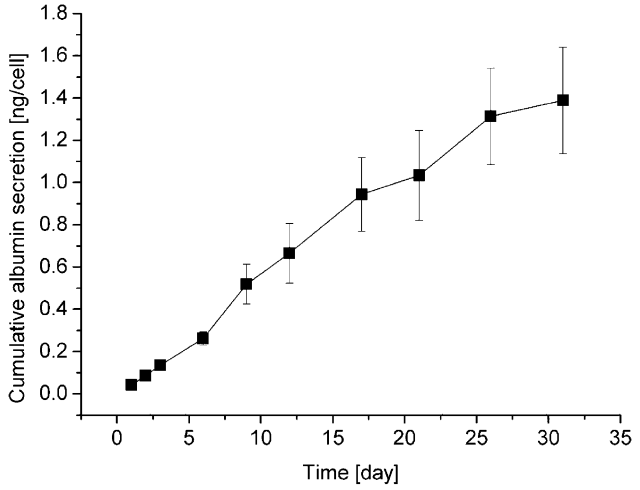

Figure 8. *The change of albumin secretion from the hepatocyte heterospheroids underlaid with BAECs as a function of time.*

thousand (100×100) spheroids on a circular glass domain 100 μm in diameter, were arrayed densely in an area of 20× 20 mm (Figure 1). The most important feature of this study is that these substrates are defined to provide an unprecedented control of substrate properties for modulating cell behavior, employing both surface engineering and synthetic polymer chemistry. These hepatocyte heterospheroids exhibited stereotypical polygonal morphology with distinct nuclei and well-demarcated cell–cell borders for at least one month, and expressed liver-specific proteins such as albumin.

TBB and CBB, based on the spheroid formation presented here, offer the promise of responding to environmental perturbations such as toxicants, pathogens or other agents in a physiologically relevant manner. In contrast to identification assays, such as those based on antibodies or nucleic acids that rely on structural determinants, cells respond only to biologically active threats. This miniaturized artificial liver array has the ability to rapidly evaluate drugs and environmental perturbations for potential risk to health, and to make predictions for effects of exposure. Furthermore, the patterned array of cell-organized structures such as spheroids on surfaces may have a particular importance for constructing tissue-engineered liver by seeding spheroids into three-dimensional scaffolds. This is also a useful tool for obtaining insights into the mechanism of cell–cell interactions, a central research topic in cell biology.

Experimental Section

Materials: Commercial tetrahydrofuran, 3,3-diethoxypropan-1-ol (Aldrich), and L-lactide (LA; Aldrich) were purified by conventional methods. Ethylene oxide (EO; Saisan) was dried over calcium hydride and distilled under an argon atmosphere. Potassium naphthalene was used as a solution in THF, the concentration was determined by titration.[31] The following reagents were used as received: bovine plasma fibronectin (FN) from Nitta Gelatin, Japan; rabbit anti-bovine FN polyclonal antibody from Biogenesis, UK; rabbit anti-rat albumin antibody, peroxidase-conjugated sheep anti-rat albumin, and rhodamine- or FITC-conjugated goat anti-body against rabbit immunoglobulin G (IgG) from Cappel, Aurora, OH; Dulbecco's modified Eagle's medium (DMEM) from IWAKI glass, Chiba, Japan; fetal bovine serum (FBS) from PAA Laboratories, Exton, PA; and rhodamine-conjugated phalloidin from Molecular Probes, Eugene, OR. Water used in this study was purified by a Milli-Q system (Nihon Millipore Co., Tokyo, Japan) to have a specific conductivity of less than 0.1 $\mu S\,cm^{-1}$.

Synthesis of acetal (or methoxy)-PEG/PLA block copolymers: α-Acetal (or methoxy)-PEG/PLA was synthesized by a one-pot anionic ring-opening polymerization of EO followed by LA initiated with potassium 3,3-diethoxypropanolate (PDP) at room temperature under argon.[25] The molecular weights of PEG and PLA segments were determined to be 5604 and 10260, respectively. The obtained α-acetal-PEG/PLA was treated with aqueous media adjusted to pH 2 to transform an acetal group at the PEG-chain end into an aldehyde end group. Further, *p*-aminophenyl-β-D-lactopyranoside was successfully treated with an aldehyde group at the distal PEG-chain end through a reductive amination reaction to introduce the lactose moiety (Lac).[26]

Microfabrication: Preparation of α-lactosyl-PEG/PLA-coated micropatterned surfaces for cell culture: The glass substrates, cleaned by a Piranha etch, were treated with (3-(methacryloyl-oxy)-propyl)trimethoxysilane to obtain the hydrophobic silanized substrates. The PEG-brushed layer was then constructed on this silanized glass surface by spin coating with a 4% (*w*/*v*) solution of PLA/toluene, followed by a 2% (*w*/*v*) solution of α-lactosyl-PEG/PLA/toluene. Photoresist patterns were defined by a photolithography technique. Round, 100 μm diameter holes separated by 100 μm (edge-to-edge distance) spacing were used to mask a N_2+H_2 plasma etch, forming the patterned α-lactosyl (or methoxy)-PEG/PLA surface. Patterned substrate surfaces were visualized by fluorescence microscopy; the circular glass domain was confirmed by the adsorption of FN from an aqueous solution, immunostained in situ with rabbit anti-bovine FN polyclonal antibody, followed by a rhodamine-conjugated goat antibody against rabbit IgG. The α-lactosyl groups were also confirmed by staining with FITC, treated with a secondary amine of *p*-aminophenyl-β-D-lactopyranoside, tethered at the PEG-chain end.

Coculture of hepatocytes and endothelial cells: Bovine aortic endothelial cells at passage 13 were purchased from the Japanese Collection of Research Bioresources cell bank and cultured with DMEM that was supplemented with 10% FBS, penicillin (100 units per mL), and streptomycin (100 $\mu g\,mL^{-1}$) at 37 °C under humidified atmosphere with 5% CO_2. Rat primary hepatocytes were obtained from five-week-old male Wister rats as previously described by using collagenase for cell dissociation.[32] The primary culture was plated in DMEM, supplemented with 10% FBS, penicillin (100 units per mL), streptomycin (100 $\mu g\,mL^{-1}$), EGF (10 $ng\,mL^{-1}$), nicotinamide (10 $mmol\,L^{-1}$), L-ascorbic acid 2-phosphate (0.2 $mmol\,L^{-1}$), and 1% dimethylsulfoxide, and incubated at 37 °C in a humidified atmosphere with 5% CO_2.[32] Bovine aortic endothelial cells were seeded onto patterned dishes at a cell density of 1×10^6 per 20 mm square dish at 37 °C. After 24 h of culture at 37 °C, unattached cells were washed away twice with the culture medium, then rat primary hepatocytes were seeded into these dishes at the same cell density and cocultured at 37 °C with a hepatocyte medium in a humidified atmosphere with 5% CO_2. Cell morphology was monitored under a phase-contrast microscope (ET300, Nikon, Tokyo, Japan). For fluorescence microscopy observation, cultured cells were fixed at 37 °C with prewarmed 4% paraformaldehyde in Dulbecco's phosphate buffered saline (PBS) for 20 min. Fixed surfaces were washed with PBS and permeabilized with 0.5%

Triton X-100 in PBS for 2 min. Surfaces were then blocked with 0.1% BSA in PBS for 90 min, and albumin synthesis was observed by immunocytochemistry with a rabbit anti-rat albumin antibody at a 1:500 dilution overnight at 4°C. Following three washes with 0.1% BSA in PBS, the surfaces were incubated for 2 h with a 1:500 dilution of FITC-conjugated goat antibody against rabbit immunoglobulin G (IgG). To examine F-actin, fixed cells were co-stained with a 1:100 dilution of rhodamine-conjugated phalloidin. The stained surfaces were mounted and observed under a microscope with fluorescence equipment (ET300, Nikon, Tokyo, Japan) or under a confocal-laser scanning microscope (LSM510, Zeiss, Germany). Fixed surfaces were also observed by interference reflection microscopy with a confocal-laser scanning microscope (emission wavelength = 543 nm with a band pass filter from 515 to 565 nm).

Cell-viability assay: The cell viability of the hepatocyte spheroid was assessed with the LIVE/DEAD viability/cytotoxicity assay kit, based on a simultaneous determination of living and dead cells with two probes, calcein AM for intracellular esterase activity and ethidium homodimer-1 (EthD-1) for plasma-membrane integrity. Briefly, the cultured cells were washed with PBS, and the working solution (2 μM of calcein AM and 5 μM of EthD-1 in PBS) was added directly to these cells. After 30 min incubation at 37°C, the stained cells were observed under a fluorescence microscope. For nuclear staining, the cells were stained with a DNA-binding dye (Hoechst 33342).

Measurement of hepatocytes function: A time-course of albumin secretion by hepatocyte heterospheroids, underlaid with endothelial cells was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) by using rabbit anti-rat albumin antibody. The secondary antibody was peroxidase-conjugated sheep anti-rat albumin. 3,3′5,5′-tetramethylbenzidine solution mixed with H_2O_2 in citrate-phosphate buffer, pH 5.0 was used as a substrate of peroxidase. The reaction was terminated with 2 N sulfuric acid, and the absorption was measured at 490 nm with a microplate reader (WAKO SPECTRA MAX250, Osaka, Japan).

Animal care was in accordance with the policies of the University of Tokyo School of Medicine.

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Keywords: biosensors · endothelial cells · hepatocytes · high-throughput screening · microarrays

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