J Mater Sci: Mater Med (2011) 22:2357–2363
DOI 10.1007/s10856-011-4414-4

Induced albumin secretion from HepG2 spheroids prepared using poly(ethylene glycol) derivative with oleyl groups

Tetsushi Taguchi · Zhi Rao · Michiko Ito · Miyuki Matsuda

Received: 3 September 2010 / Accepted: 3 August 2011 / Published online: 13 August 2011

Abstract We developed a poly(ethylene glycol) (PEG) derivative with oleyl groups, so-called "cell adhesive", for the promotion of human hepatocellular carcinoma HepG2 cell spheroids. Our approach was based on crosslinking of the cell membrane with a cell adhesive via a hydrophobic interaction. A cell adhesive, PEG derivative with hydrophobic oleyl groups at both ends was synthesized and characterized. HepG2 spheroids formed when the adhesive was added to cell suspensions. The size of the spheroids increased with time in culture. In addition, Ammonia elimination of HepG2 spheroid with cell adhesive was 3.4 times higher than that without cell adhesive. Furthermore, albumin secretion from HeG2 spheroids grown with the cell adhesive for 7 days was 3.3 times that from HepG2 spheroids grown without cell adhesive. Fluorescence microscopy showed greater albumin staining in spheroids grown with cell adhesive compared with spheroids grown without adhesive. This cell adhesive may be useful not only for single type of cells but also for multi types of cells to form artificial organs. This cell adhesive will be a key material for liver tissue engineering when it will apply to primary hepatocytes.

T. Taguchi (✉) · M. Ito
Biomaterials Center, National Institute for Materials Science, Namiki 1-1, Tsukuba, Ibaraki 305-0044, Japan
e-mail: TAGUCHI.Tetsushi@nims.go.jp

T. Taguchi
International Center for Materials Nanoarchitectonics (MANA), National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan

T. Taguchi · Z. Rao · M. Matsuda
Graduate School of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan

1 Introduction

The regeneration of organs is considered the next step in the field of tissue engineering. In vitro and in vivo tissue engineering techniques have been used to construct connective tissues containing large amounts of extracellular matrix (ECM) [1–3], and regenerated tissues have been applied clinically. However, the regeneration of organs such as the liver and pancreas, which have complex cellular structures, are highly vascularized, and have few ECM components, presents many difficulties. One of the key factors in the regeneration of organs is control of cell–cell interactions; there are significant functional differences between a flat layer of cells and 3-dimensional tissue [4–8]. In general, cell functions in 3-dimensional culture result from enhanced cell–cell interactions. Cellular spheroids, which are small, 3-dimensional aggregates of cells, are an attractive model for organ regeneration. Materials and techniques, such as bioreactors [9–11], spheroid plates [12], and microfabricated surface-controlled cell adhesion [13, 14], have been used to study the formation of spheroids in vitro. However, these can be time-consuming and expensive. The development of convenient methods and inexpensive materials for the formation of spheroids will be helpful with respect to clinical applications of spheroids as well as cell transplantation.

We recently prepared a polymeric cell adhesive, poly(ethylene glycol) (PEG) derivative with hydrophobic oleyl groups at both ends [15]. This cell adhesive interconnects cells by anchoring to the cell membrane via a hydrophobic interaction. We used this adhesive to induce spheroid formation in the RIN pancreatic β-cell line. We also showed enhanced insulin secretion and mRNA expression of the cell adhesion protein E-cadherin in pancreatic β-cell spheroids grown with the adhesive compared to those cultured without the adhesive. However, there are

no researches on the hepatic cell spheroid formation by addition of cell adhesive.

In the present study, we assessed the effect of the cell adhesive on human hepatic cell. For the fundamental study of spheroid formation of hepatic cells, we employed HepG2 and performed biochemical characterization of resulting HepG2 spheroids.

2 Materials and methods

2.1 Materials

Oleyl-*O*-PEG-succinyl *N*-hydroxy-succinimidyl ester (SUNBRIGHT® OE-080CS; $M_W = 8{,}525$ Da, purity >95%, polydispersity 1.02) and PEG diamine (SUNBRIGHT® DE-350PA; $M_W = 33{,}652$ Da) were purchased from NOF Corporation (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and were used without further purification.

2.2 Synthesis of cell adhesive

Synthesis of cell adhesive was performed by the method previously reported [15]. Briefly, a mixture of oleyl-*O*-PEG-succinyl *N*-hydroxy-succinimidyl ester (1.84 g, 216 μmol, 2.2 eq) and PEG diamine (3.30 g, 98.1 μmol, 1.0 eq) in *N*,*N*′-dimethylformamide (DMF)/tetrahydrofuran (THF) (70 ml; 1/1 [v/v]) was stirred at room temperature overnight. The solvent was then removed by evaporation under reduced pressure, and the residue was purified via dialysis against water. The resulting solution was lyophilized by freeze-drying to give a product (4.93 g, 86% yield) in the form of a white, fluffy material. The product was characterized by gel permeation chromatography (GPC) with an HLC-8220GPC system equipped with RI and TSK gel G4000H_{HR} and TSK gel G3000H_{HR} columns (Tosoh Corp., Tokyo, Japan) in DMF (with 10 mM LiCl additive) and by Fourier-transform infrared spectroscopy (FTIR) using an FTIR-8400S system (Shimadzu Corp., Kyoto, Japan). For GPC, $M_n = 55{,}880$ and $M_W = 61{,}021$. For FTIR, KBr values (cm^{-1}) were, 2889 (C–H for alkane), 1736 (C=O for ester), 1647 (C=O for amide), 1543 (N–H for amide), 1470 (C–H for –CH_2O–), 1342 (C–H for alkane), 1281 (C–H for alkane), 1099 (C–C for alkane, C–O for ether). The critical micelle concentrations in phosphate-buffered saline (PBS), measured by the pyrene method, were 1.84 and 1.78 μM at 25 and 37°C, respectively.

2.3 Cell culture

The human HepG2 hepatocellular carcinoma cell line was used for the evaluation of spheroid formation. Cells were grown in Dulbecco modified Eagle medium (DMEM; plus 4500 mg glucose/l; Sigma-Aldrich, St. Louis, MO, USA) containing 2% penicillin–streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA). Spheroid formation was induced as follows: 100 μl of HepG2 cells (1×10^6 cells/ml) was suspended in medium without serum in a 1.5-ml microtube. A volume of 100 μl of the cell adhesive (0.1 mM; sterilized under ultraviolet light for 15 min) dissolved in phosphate buffer was added to the microtube, and the mixture was stirred with a micromixer for 2 min and placed into a well of a 48-well culture dish. The mixture was cultured in a humidified incubator (ESPEC Corp., Osaka, Japan) at 37°C in a 5% CO_2 atmosphere. Half of the culture medium was replaced every 2 days with DMEM containing 10% FBS. The number of cells cultured with or without cell adhesive was determined by Cell counting kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

2.4 Biochemical characterization

For the determination of ammonia elimination ability of spheroid, HepG2 spheroids were first cultured with 1 mM NH_4Cl in buffer solution for 2 h at 37°C in a 5% CO_2 atmosphere. Then, the ammonia concentration was measured with an ammonia test kit (BioVision, CA, USA) according to the manufacturer's instructions. In order to determine albumin secretion from HepG2 spheroids, they were cultured in DMEM without serum for 1 h, after which albumin concentration was quantified using a Human Albumin Enzyme-Linked Immunosorbent Assay (ELISA) Quantitation Kit (Bethyl Laboratories, Inc., Montgomery, TX, USA), according to the manufacturer's instructions. For fluorescence staining of albumin, HepG2 cells cultured with or without cell adhesive were fixed in 4% paraformaldehyde for 5 min. Samples were then permeabilized with 0.2% Triton X-100 in PBS for 1 h and washed 3 times with 0.05% Tween 20 in PBS. Spheroids were subsequently treated with 10% pig serum for 1 h at 37°C to block nonspecific adsorption and then subjected to immunofluorescence staining with polyclonal rabbit anti-human albumin antibody (DakoCytomation Denmark A/S, Glostrup, Denmark) dissolved in PBS containing 1% bovine serum albumin (BSA) at 4°C for 16 h, followed by 3 washes with 0.05% Tween 20 in PBS. Samples were incubated in fluorescein isothiocyanate (FITC)-labeled polyclonal swine anti-rabbit immunoglobulin (DakoCytomation Denmark A/S) at 4°C for 3 h and washed with PBS 3 times. Nuclei were stained by incubating spheroids with 300 μl of 20 μg/ml propidium iodide in PBS at room temperature for 20 min, followed by 3 washes with PBS. After sealing the spheroids with mounting medium, images

were acquired with a fluorescence microscope (IX-71; Olympus, Tokyo, Japan).

2.5 Statistical analysis

Results are expressed as mean ± standard deviation (SD). Data were analyzed by Student's *t*-test with the statistical function of Microsoft Excel. All *P* values were compared to a value of 0.05 to determine significance.

3 Results and discussion

The cell plasma membrane is composed of an amphiphilic phospholipid bilayer. Our approach was based on physical crosslinking of the cell membrane over time via a hydrophobic interaction using a cell adhesive. Hydrophobic units, such as lipids, cholesterol, and oleyl groups, can anchor to the phospholipid bilayer of membranes of liposomes and cells [16–26]. Via a hydrophobic interaction, cell membranes can be anchored without causing damage. Our cell adhesive consists of 2 distinct units, a hydrophobic unit that anchors to the phospholipid bilayer of the cell membrane, and a hydrophilic polymer unit that promotes solubility in aqueous media. An oleyl group was used as the hydrophobic unit, and a PEG chain was used as the hydrophilic unit (Fig. 1a). Oleyl group-bearing polymers have been shown to anchor to the cell membrane without causing damage [16]. As in our previous study [15], in which we used the RIN pancreatic β-cell line, we hypothesized that the adhesive induces physical crosslinking among cells via a hydrophobic interaction (Fig. 1b). The effect of addition of the cell adhesive (0.05 mM) on the formation of hepatic cell spheroids is shown in Fig. 2. We employed HepG2 cells for the spheroid formation because there are no researches on the spheroid formation by the addition of cell adhesive. Also, it is reported that albumin secretion from HepG2 spheroid significantly increased as compared with flat cultured cells. In the absence of the cell adhesive, cells were distributed over the surface of the tissue culture flask after 7 days (Fig. 2a–d). In the presence of the cell adhesive, small spheroids were observed after 1 day (Fig. 2e), increasing in size over time (Fig. 2e–h). We also performed experiments with other concentrations of the cell adhesive; however, no spheroid formation was observed. This may be due to the fact that the cell surface is covered with hydrophilic PEG units at concentrations greater than 0.05 mM. Only a few hepatic spheroids formed at concentrations less than 0.05 mM, which may be explained by a lower crosslinking density. It is known that the cell membrane can flexibly interact with molecules dissolved in culture medium, even under static culture conditions. To enhance the anchoring effect of the cell adhesive with the cell membrane, we stirred the HepG2 cells in the presence of cell adhesive. We obtained HepG2 spheroids in quite a short period of time (less than 10 min; data not shown), indicating that spheroids may be useful in cell transplantation.

After different culture periods, we determined the number of cells using Cell Counting Kit-8. No significant differences between cells cultured with or without cell adhesive. On the other hand, the number of cells cultured with cell adhesive decreased compared with cells without adhesive after the culture for 3 days. Similar behavior was observed when the culture period was 7 days. Cell adhesive used in this study is amphiphilic polymer. Critical micelle concentration of employed cell adhesive was 1.78 μM at 37°C. While, the concentration of cell adhesive added to HepG2 was 0.05 mM, therefore, cell adhesive formed micelle in cell culture medium. At this condition, it is supposed that cell adhesive will show some kinds of toxicity to HepG2, however, significant decrease of cell

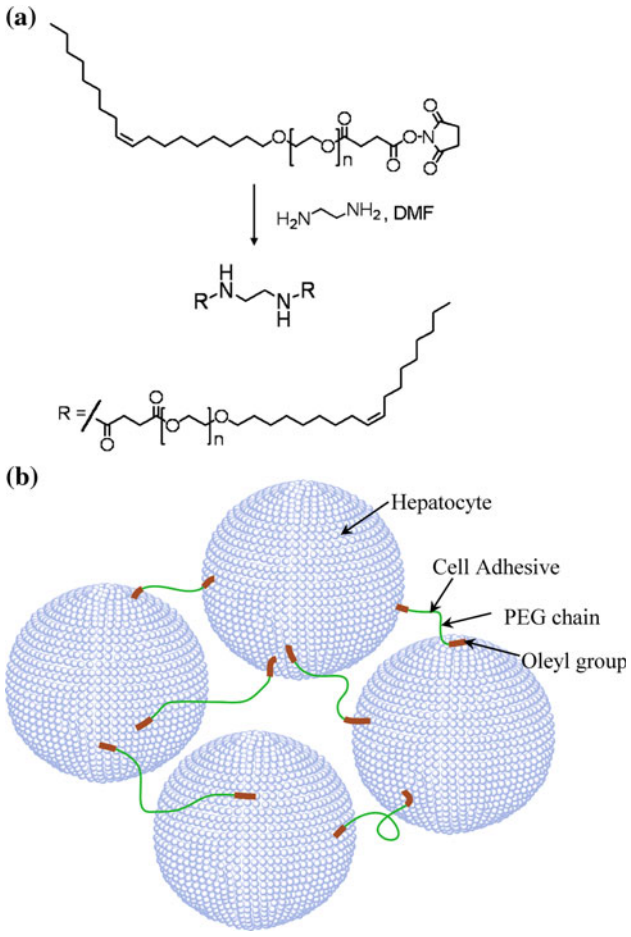

Fig. 1 Cell adhesive used for spheroid formation. (**a**) Schematic of synthesis of cell adhesive. (**b**) Schematic of crosslinked HepG2 cells in culture after the addition of cell adhesive

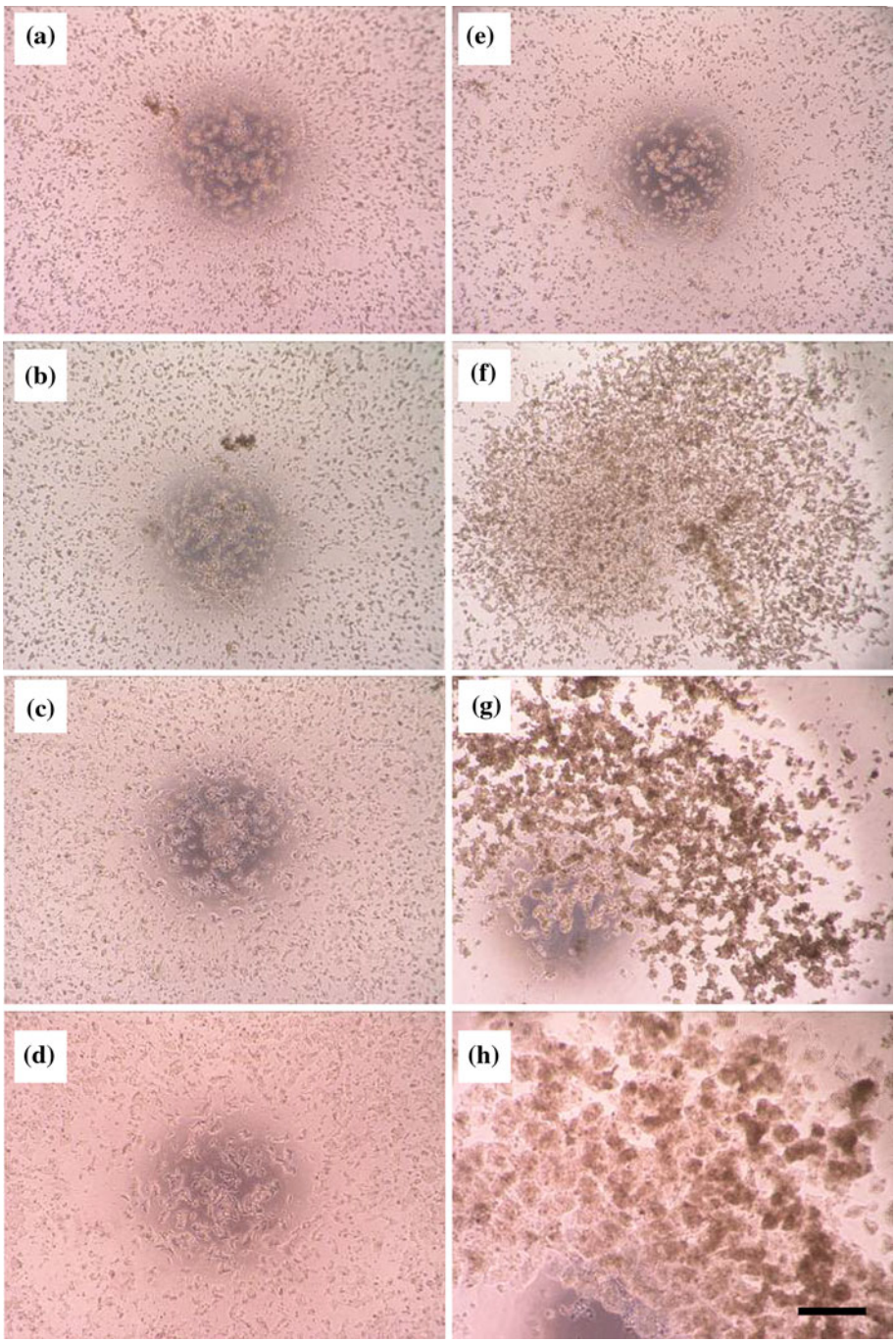

Fig. 2 HepG2 spheroid formation in the absence (**a**–**d**) or presence (**e**–**h**) of cell adhesive. The culture time was 10 min (**a**, **e**), 1 day (**b**, **f**), 3 days (**c**, **g**), and 7 days (**d**, **h**). *Scale bar* 500 μm

number was not observed during the culture periods. This may be due to the cytocompatibility of PEG chain Fig. 3

In order to determine the function of resulting HepG2 spheroid, we evaluated capability of ammonia elimination because metabolism of ammonia by hepatocyte is one of the typical functions. Figure 4 shows the ammonia elimination of HepG2 spheroids cultured for 7 days in the presence or absence of cell adhesive. It is clear that HepG2 spheroid prepared using cell adhesive had significant capability of ammonia elimination compared with spheroids without cell adhesive; Ammonia elimination of HepG2 spheroid with cell adhesive was 3.4 times higher than that without cell adhesive. Comparing this value with the reported data [27], ammonia elimination of HepG2 cultured in the presence of cell adhesive was lower than that of primary hepatocyte cultured in alginate/galactosylated chitosan scaffold. This was due to the difference of activity and seeding density of hepatocytes. We also analyzed albumin secretion from HepG2 spheroid in order to clarify the efficiency of cell adhesive. Albumin is known as one of markers of hepatocyte differentiation. Figure 5 shows the albumin secretion from spheroids over time. In

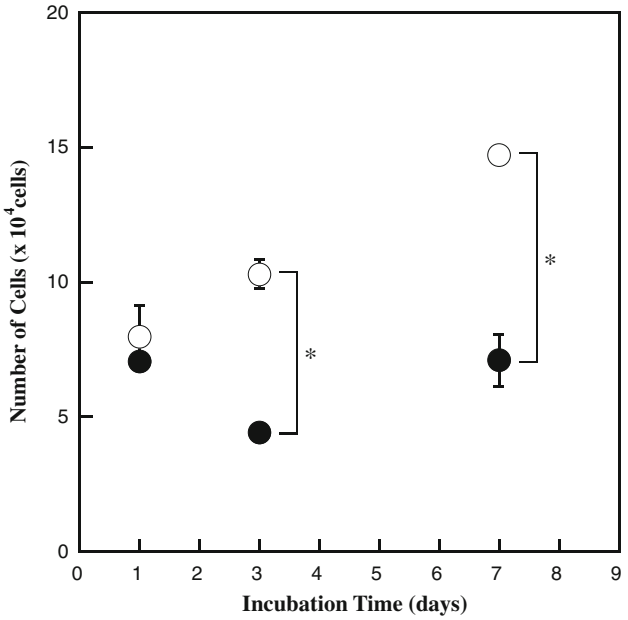

Fig. 3 Number of HepG2 cells cultured for various periods in the presence (*filled circle*) or absence (*open circle*) of cell adhesive. *Error bars* denote standard deviation; $n = 3$ ($^*P < 0.001$)

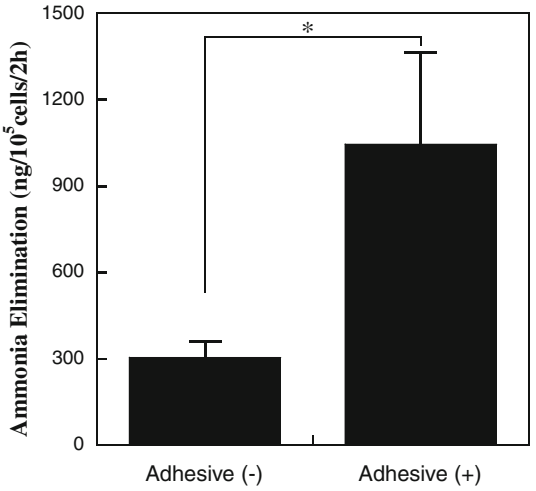

Fig. 4 Ammonia elimination of HepG2 spheroids cultured for 7 days in the presence or absence of cell adhesive. *Error bars* denote standard deviation; $n = 3$ ($^*P < 0.05$)

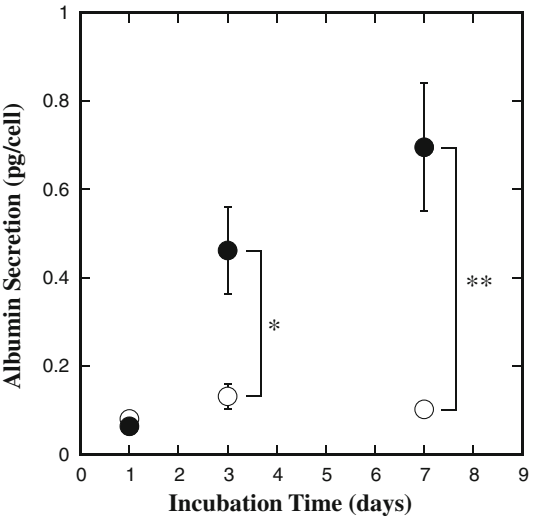

Fig. 5 Albumin secretion from HepG2 spheroids in the presence (*filled circle*) or absence (*open circle*) of cell adhesive. *Error bars* denote standard deviation; $n = 3$ ($^*P < 0.05$, $^{**}P < 0.005$)

the absence of the cell adhesive, albumin secretion increased with increasing incubation time up to 3 days and then reached a plateau. Albumin secretion in the presence of the cell adhesive was initially slightly lower than that in the absence of the cell adhesive. However, albumin secretion was significantly enhanced after culture for 3 days, and even more so (3.3 times greater) after 7 days. Hepatocyte spheroid formation has been done using substrates or various techniques just like micro-patterned surface [28], biodegradable scaffold [27, 29–31] and ultrasound trap [32]. Albumin secretion from resulting hepatocytes has been also reported. Comparing our data with the values reported by these literatures, our obtained value was about half of albumin secretion from spheroid prepared using the reported methodology. This indicated that the optimization of hydrophobicity was needed to promote cell viability.

To confirm albumin secretion from HepG2 spheroids with the cell adhesive, albumin was immunostained and assessed by fluorescence microscopy. A low level of albumin staining was observed in HepG2 spheroids cultured in the absence of the cell adhesive (Fig. 6a). No notable increase was observed even after 7 days (Fig. 6b). In the presence of the cell adhesive, albumin staining was observed after culture for 3 days, with increased staining after culture for 7 days, along with an increase in spheroid size. These results showed that the cell adhesive effectively induced spheroid formation and enhanced albumin secretion. We are currently investigating the mechanism underlying this effect; however, we previously reported insulin secretion and mRNA expression of the cell adhesion protein E-cadherin in response to the addition of the cell adhesive to pancreatic β cells [15], suggesting that the cell adhesive interconnects cells until they form cell–cell interactions of their own. Similar to pancreatic β-cells, the cell adhesive may induce activation and expression of cell adhesion proteins by HepG2 cells, resulting in the promotion of adhesion and albumin secretion.

4 Conclusion

We reported phospholipid-anchorable hydrophobic groups and water-soluble groups to form HepG2 spheroids.

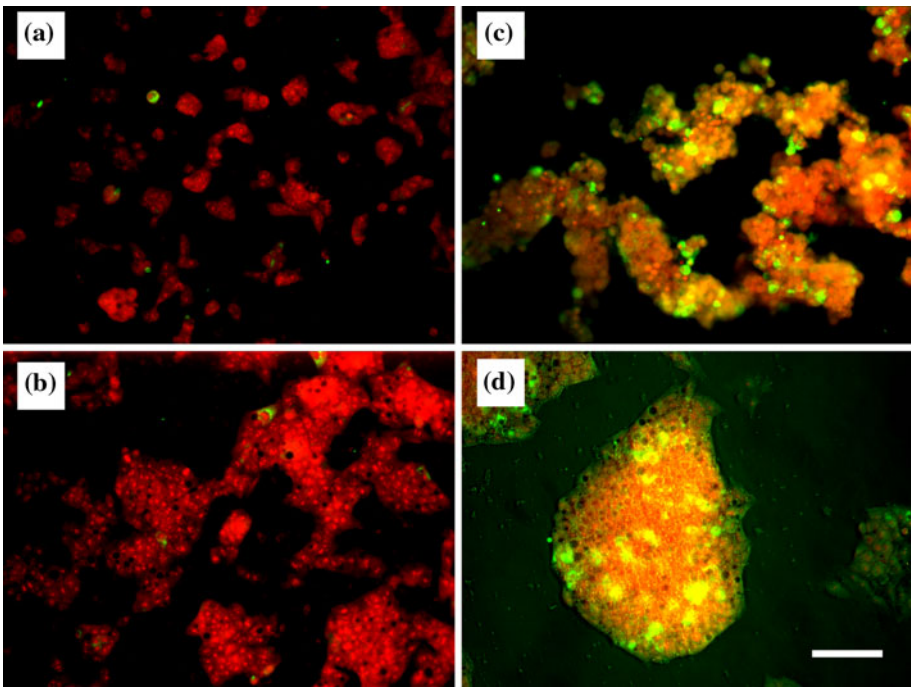

Fig. 6 Fluorescence images of HepG2 spheroids in the absence (**a**, **b**) or presence (**c**, **d**) of cell adhesive after the culture for 3 days (**a**, **c**) or 7 days (**b**, **d**). *Scale bar* 100 μm

Spheroid formation, ammonia elimination and albumin secretion were promoted when HepG2 was cultured in the presence of the cell adhesive. This cell adhesive can anchor to many cell types because it can interact with phospholipids. This cell adhesive therefore provides a key material for cell spheroid formation as well as a tool for the generation of organs composed of various cell types. We will plan to apply this cell adhesive for primary hepatocytes for the regeneration of liver.

Acknowledgments We thank Ms. M. Morita of National Institute for Materials Science for technical support. This study was financially supported in part by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (Grant-in-Aid for Exploratory Research No. 21659074), the Japan Society for the Promotion of Science (JSPS) through the "Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program)," initiated by the Council for Science and Technology Policy (CSTP), MANA and the World Premier International Research Center (WPI) Initiative on Materials Nanoarchitectonics, MEXT, Japan.

References

1. Langer R, Vacanti JP. Tissue engineering. Science. 1993;260:920–6.
2. Griffith LG, Naughton G. Tissue engineering-current challenges and expanding opportunities. Science. 2002;295:1009–14.
3. Drury JL, Mooney DJ. Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterials. 2003;24:4337–51.
4. Abbott A. Biology's new dimension. Nature. 2003;424:870–2.
5. Service RF. Molecular scaffolding helps raise a crop of neurons. Science. 2003;302:46–7.
6. Seo SJ, Kim IY, Choi YJ, Akaike T, Cho CS. Enhanced liver functions of hepatocytes cocultured with NIH 3T3 in the alginate/galactosylated chitosan scaffold. Biomaterials. 2006;27:1487–95.
7. Kinoshita N, Echigo Y, Shinohara S, Gu Y, Miyazaki J, Inoue K, Imamura M. Regulation of cell proliferation using tissue engineering in MIN6 cells. Cell Transplant. 2001;10:473–7.
8. Nagaki M, Shidoji Y, Yamada Y, Sugiyama A, Tanaka M, Akaike T, Ohnishi H, Moriwaki H, Muto Y. Regulation of hepatic genes and liver transcription factors in rat hepatocytes by extracellular-matrix. Biochem Biophys Res Commun. 1995;210:38–43.
9. Martin Y, Vermette P. Bioreactors for tissue mass culture: design, characterization, and recent advances. Biomaterials. 2005;26:7481–503.
10. Brown LA, Arterburn LM, Miller AP, Cowger NL, Hartley SM, Andrews A, Silber PM, Li AP. Maintenance of liver functions in rat hepatocytes cultured as spheroids in a rotating wall vessel. In Vitro Cell Dev Biol Anim. 2003;39:13–20.
11. Nishikawa M, Ohgushi H, Tamai N, Osuga K, Uemura M, Yoshikawa H, Myoui A. The effect of simulated microgravity by three-dimensional clinostat on bone tissue engineering. Cell Transplant. 2005;14:829–35.
12. Yamauchi N, Yamada O, Takahashi T, Hashizume K. Spheroid formation of bovine endometrial stromal cells with non-adherent culture plate. J Reprod Develop. 2001;47:165–71.
13. Otsuka H, Hirano A, Nagasaki Y, Okano T, Horiike Y, Kataoka K. Two-dimensional multiarray formation of hepatocyte spheroid on a microfabricated PEG-brush surface. ChemBioChem. 2004;5:850–5.
14. Tamura T, Sakai Y, Nakazawa K. Two-dimensional microarray of HepG2 spheroids using collagen/polyethylene glycol micropatterned chip. J Mater Sci Mat Med. 2008;19:2071–7.
15. Ito M, Taguchi T. Enhanced insulin secretion of physically crosslinked pancreatic β-cells by using a poly(ethylene glycol) derivative with oleyl groups. Acta Biomater. 2009;5:2945–52.
16. Kato K, Umezawa K, Funeriu DP, Miyake M, Miyake J, Nagamune T. Immobilized culture of nonadherent cells on an oleyl

poly(ethylene glycol) ether-modified surface. Biotechniques. 2003;35:1014–22.
17. Kato K, Itoh C, Yasukouchi T, Nagamune T. Rapid protein anchoring into the membranes of mammalian cells using oleyl chain and poly(ethylene glycol) derivatives. Biotechnol Prog. 2004;20:897–904.
18. Wang T, Leventis R, Silvius JR. Artificially lipid-anchored proteins can elicit clustering-induced intracellular signaling events in Jurkat T-lymphocytes. Independent of lipid Raft association. J Biol Chem. 2005;24:22839–46.
19. Miura S, Teramura Y, Iwata H. Encapsulation of islets with ultrathin polyion complex membrane through poly(ethylene glycol)-phospholipids anchored to cell membrane. Biomaterials. 2006;27:5828–35.
20. Meier W, Hotz J, Günther-Ausborn S. Vesicle and cell networks: interconnecting cells by synthetic polymers. Langmuir. 1996;12:5028–32.
21. Boonyarattanakalin S, Athavankar S, Sun Q, Peterson BR. Synthesis of an artificial cell surface receptor that enables oligohistidine affinity tags to function as metal-dependent cell-penetrating peptides. J Am Chem Soc. 2006;128:386–7.
22. Mart RJ, Liem KP, Wang X, Webb SJ. The effect of receptor clustering on vesicle–vesicle adhesion. J Am Chem Soc. 2006;128:14462–3.
23. Menger FM, Zhang H. Self-adhesion among phospholipid vesicles. J Am Chem Soc. 2006;128:1414–5.
24. Yoshimoto N, Yoshimoto M, Yasuhara K, Shimanouchi T, Umakoshi H, Kuboi R. Evaluation of temperature and guanidine hydrochloride-induced protein-liposome interactions by using immobilized liposome chromatography. Biochem Eng J. 2006;29:174–81.
25. Thongborisute J, Takeuchi H, Yamamoto H, Kawashima Y. Properties of liposomes coated with hydrophobically modified chitosan in oral liposomal drug delivery. Pharmazie. 2006;61:106–11.
26. Teramura Y, Kaneda Y, Totani T, Iwata H. Behavior of synthetic polymers immobilized on a cell membrane. Biomaterials. 2008;29:1345–55.
27. Seo SJ, Kim IY, Choi YJ, Akaike T, Cho CS. Enhanced liver functions of hepatocytes cocultured with NIH 3T3 in the alginate/galactosylated chitosan scaffold. Biomaterials. 2006;27:1487–95.
28. Fukuda J, Khademhosseini A, Yeo Y, Yang X, Yeh J, Eng G, Blumling J, Wang CF, Kohane DS, Langer R. Micromolding of photocrosslinkable chitosan hydrogel for spheroid microarray and co-cultures. Biomaterials. 2006;27:5259–67.
29. Verma P, Verma V, Ray P, Ray AR. Formation and characterization of three dimensional human hepatocyte cell line spheroids on chitosan matrix for in vitro tissue engineering applications. In Vitro Cell Dev Biol Anim. 2007;43:328–37.
30. Verma P, Verma V, Ray P, Ray AR. Agar-gelatin hybrid sponge-induced three-dimensional in vitro 'liver-like' HepG2 spheroids for the evaluation of drug cytotoxicity. J Tissue Eng Regen Med. 2009;3:368–76.
31. Khalil M, Shariat-Panahi A, Tootle R, Ryder T, McCloskey P, Roberts E, Hodgson H, Selden C. Human hepatocyte cell lines proliferating as cohesive spheroid colonies in alginate markedly upregulate both synthetic and detoxicatory liver function. J Hepatol. 2001;297:68–77.
32. Liu J, Kuznetsova LA, Edwards GO, Xu J, Ma M, Purcell WM, Jackson SK, Coakley WT. Functional three-dimensional HepG2 aggregate cultures generated from an ultrasound trap: comparison with HepG2 spheroids. J Cell Biochem. 2007;102:1180–9.