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Hepatocyte spheroid formation on a titanium dioxide gel surface and hepatocyte long-term culture

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Abstract The cell morphology and expression of differentiated functions of primary rat hepatocytes on a titanium dioxide (TiO_2) gel surface were investigated. Polystyrene culture dishes were coated with TiO_2 gel by spin-coating an ethanol solution of titanium n-butoxide, hydrolyzing in a humidity chamber and drying with nitrogen gas. The TiO_2 gel layer formed on the polystyrene dishes was transparent and robust, and its surface was quite flat. Rat hepatocytes inoculated on the TiO_2 gel-coated polystyrene dishes gradually accumulated with increasing culture time, and then spontaneously formed many hepatocyte spheroids at $90 \pm 21\ \mu$m diameter from about 3 days of culture. The diameter of the spheroids increased during the culture, and was $151 \pm 43\ \mu$m at 14 days of culture. Ammonia removal and albumin secretion by hepatocytes on the TiO_2 gel-coated polystyrene dishes were maintained at a high level for at least 14 days of culture compared with on a type I collagen-coated dish and a non-coated polystyrene dish. These results indicate that TiO_2 gel is an adequate material for hepatocyte spheroid formation and long-term culture of spheroids.

1. Introduction

Primary hepatocyte culture has attracted attention for many applications, such as a bioartificial liver, tissue engineering of liver and the study of drug metabolism. For the success of such applications, the hepatocytes have to express differentiated functions at a high level and accomplish long-term maintenance of these functions.

Expression of differentiated functions in the hepatocytes has a close relation to the *in vitro* cell configuration, which depends on the surface property of the culture substratum. That is, designing the surface of the culture substratum for useful cell culture is important. Generally, hepatocytes have a spreading morphology, which is a monolayer cell configuration, on collagen, fibronectin or laminin-coated surfaces [1–3]. The differentiated functions of hepatocytes in such a monolayer decrease or are lost in a few days. In contrast, a cell aggregate that accumulates dispersed hepatocytes, or spheroid (spherical multicellular aggregate) derived from rearrangement and compaction of a cell aggregate and that has a three-dimensional cell configuration, are formed by some extracellular matrices or synthetic substrata, such as proteoglycan [3], matrigel [4], poly(N-p-vinylbenzyl-4-o-β-D-galactopyranosyl-D-gulconamide) [5], galastosylated materials [6], poly-(2-hydroxyethyl methacrylate) [7], poly-N-isopropyl acrylamide [8], polyurethane foam [9] and rutile titanium dioxide ceramic [10, 11]. They can maintain differentiated functions at a high level for several weeks. Although the mechanism by which a cell aggregate or spheroid maintain superior functions is not fully understood, the mechanism seems to depend on the tissue-like structure of the cell aggregate or spheroid that have abundant cytoplasmic organelles, close cell-cell contact with junctional complexes, such as desmosomes, and an extensive internal network of microvilli-lined bile canalicular-like channels, similar to that of living tissue [3, 12].

We recently developed a novel method to prepare ultrathin layers of metal oxide gels by sequential chemisorption and activation [13–15]. This surface sol-gel process gives

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rise to metal oxide gel films of nanometer thickness, and is capable of incorporating various organic molecules in the inorganic layer. Furthermore, this process is simple, and can apply to a surface coating of organic or inorganic templates having various shapes. We have demonstrated that these ultrathin films are very useful as matrices of molecular imprinting [13–15]. In particular, TiO_2 gel layers have a strong affinity for biomolecules, such as amino acids, proteins, and an extracellular matrix. As far as we know, there has not been a report on cell culture using amorphous TiO_2 gel matrices.

In this study, we investigated the amorphous TiO_2 gel matrix as a new culture substratum for hepatocytes. A culture substratum with TiO_2 gel surface was prepared by spin-coating an ethanol solution of titanium n-butoxide, hydrolyzing in a humidity chamber and drying with nitrogen gas. Hepatocyte morphology and its functions on TiO_2 gel-coated surface were characterized, and compared with non-coated polystyrene or collagen-coated surfaces. The goal was to investigate the hepatocyte properties on the amorphous TiO_2 gel surface.

2. Materials and methods

2.1. Substratum preparation

Figure 1 shows the preparation process of a TiO_2 gel-coated culture substratum. Conventional polystyrene culture dishes (1008, Falcon, Franklin Lake, NJ), 35-mm diameter, were used as a template for TiO_2 gel coating. Titanium n-butoxide (Kishida Chem., Japan) dissolved in ethanol at concentrations 10, 20, 30, 40 and 50% (v/v) was coated on the polystyrene dishes by using a spin coater (K-359SD-1, Kyowa Riken, Japan) at 6000 rpm for 30 sec. Then, the dishes were kept in a humidity chamber (above 90% humidity) for 24 hours to promote hydrolysis and dried by flushing with nitrogen gas. Subsequently, the TiO_2 gel-coated polystyrene dishes were disinfected by submerging them in 70% ethanol solution before the cell culture. All data were analyzed using three dishes from three independent experiments.

To confirm the surface and cross section of the obtained TiO_2 gel layers, the spin-coating was also carried out on glass plates. Scanning electron micrographs were obtained with a Hitachi S-5200 instrument (FE-SEM) at an acceleration voltage of 15 kV. The samples were coated with 30 Å thick Pt by use of an ion coater (Hitachi E-1030 ion sputter, 10mA/10Pa) under argon atmosphere to prevent charge up.

2.2. Hepatocyte culture

Hepatocytes were isolated from a whole liver of an adult Wistar rat (male, 7–8 weeks old, weighing about 200 g) by using liver perfusion with 0.05% collagenase (Wako Pure Chemical Industries, Osaka, Japan). The cell viability was measured by using the trypan blue exclusion method, and cells with over 85% viability were used for the culture. Isolated hepatocytes (5×10^5 cells) were inoculated on the TiO_2 gel-coated polystyrene dish in 2 ml of culture medium comprising Dulbecco's modified Eagle medium (Gibco BRL, Grand Island, NY) supplemented with 10 μg/ml insulin (Sigma, St. Louis, MO), 7.5 mg/ml hydrocortisone (Wako Pure Chemical Industries), 50 ng/ml epidermal growth factor (Biomedical Technologies Inc., Stoughton, MA), 60 mg/l proline (Wako Pure Chemical Industries), 50 μg/ml linoleic acid (Sigma), 0.1 μM $CuSO_4 \cdot 5\ H_2O$, 3 μg/ml H_2SeO_3, 50 pM $ZnSO_4 \cdot 7\ H_2O$, 58.8 μg/ml penicillin, and 100 μg/ml streptomycin. The hepatocytes were cultured at 37°C in an atmosphere of 5% CO_2 and 95% air. For control culture, hepatocytes (5×10^5 cells) were inoculated on a 35-mm type I collagen-coated dish (Asahi Techno Glass, Japan) or a 35-mm non-coated polystyrene dish (1008, Falcon, Franklin Lake, NJ) in 2 ml of the same culture medium under the same conditions. The medium was changed at 4 hours and 24 hours after inoculation and then at one-day intervals.

2.3. Spheroid diameter measurement

To evaluate the distribution of spheroids of different diameters in each culture, pictures of 200 spheroids that formed on a TiO_2 gel-coated polystyrene dish or on a non-coated polystyrene dish were taken using a phase-contrast microscope at 3, 7, 10, and 14 days of culture, and the diameters were measured using a Windows computer with a image

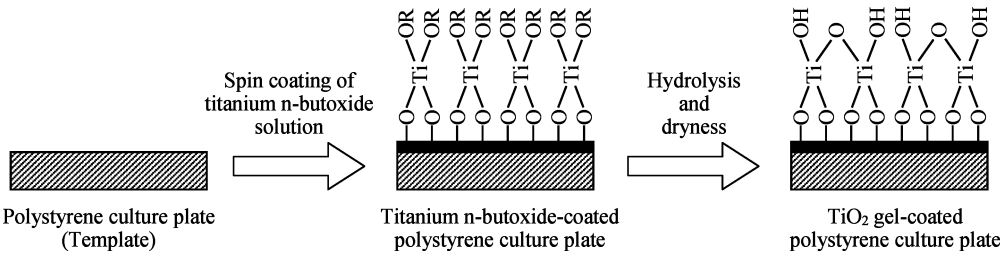

Fig. 1 Preparation process of a TiO_2 gel-coated culture substratum. Polystyrene culture dishes with a TiO_2 gel surface were prepared by spin-coating an ethanol solution of titanium n-butoxide, hydrolyzing in a humidity chamber and drying with nitrogen gas.

analysis program (Win ROOF, Mitani Co., Japan). The diameter of spheroids without a round shape was calculated by converting the spheroid area into an equivalent circle diameter. Single cells and small aggregates with diameters less than 40 μm were excluded from the analysis.

2.4. Hepatocyte-differentiated functions and cell number

To evaluate ammonia removal by the cells, the culture medium was replaced by fresh culture medium supplemented with 1 mM ammonium chloride. The ammonia concentration was measured using a commercial kit (Wako Pure Chemical Industries). The ammonia removal rate was calculated from the decrease in ammonia concentration during 24 hours of culture after the culture medium was changed. The concentration of albumin secreted during 24 hours into the culture medium was measured using an enzyme-linked immunosorbent assay.

The number of nuclei in the cultured cells was counted by using the method of van Wezel [16]. In brief, the cultured hepatocytes were suspended in 0.1 M citric acid solution containing 0.1% crystal violet. After incubation for 24 hours at 37 °C, the stained nuclei released from the cells were counted with a hemocytometer. In a preliminary study, the ratio of nucleus to cell was 1.45: 1. On the basis of this ratio, the nucleus number was converted to a cell number in this study. Ammonia removal and albumin secretion were evaluated at 1, 3, 7, 10, and 14 days of culture, and were normalized by the cell number at each culture time.

2.5. Statistical analysis

Data represented the mean ± standard derivation of nine experiments from three independent cell preparation. Statistical significances were determined by the *t*-test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Surface characterization of TiO_2 gel

The state of TiO_2 gel layer on glass plate was observed by SEM. Figures 2a and b show scanning electron micrographs of the surface and cross section of the TiO_2 gel layer prepared with a 20% titanium n-butoxide solution in ethanol. The TiO_2 gel surface was clear, uniformly thick and flat. In addition, the thickness of the TiO_2 gel layer increased with increasing concentration of titanium n-butoxide: the thickness at 10, 20, 30, 40 and 50% (v/v) concentration was about 40, 114, 156, 236 and 324 nm, respectively.

3.2. Hepatocyte morphology

Rat hepatocytes were spread on the collagen-coated surface (Fig. 3a) in a monolayer configuration, which remained in this condition during the culture (Figs. 3b,c). Rat hepatocytes weakly adhered to the non-coated polystyrene surface or to the TiO_2 gel surface in the initial stage of culture, and then gradually accumulated with increase in culture time, and spontaneously began to form hepatocyte spheroids from about 3 days of culture (Figs. 3d,g). Although the hepatocytes on the non-coated polystyrene surface showed different morphologies, such as spheroids, cell aggregates and spreading cells (Figs. 3e,f), most hepatocytes on the TiO_2 gel surface formed spheroids (Figs. 3h,i). Some spheroids on the non-coated polystyrene surface and on the TiO_2 gel surface detached from the surface, and floated in the culture medium. The morphology of hepatocytes on TiO_2 gels formed at

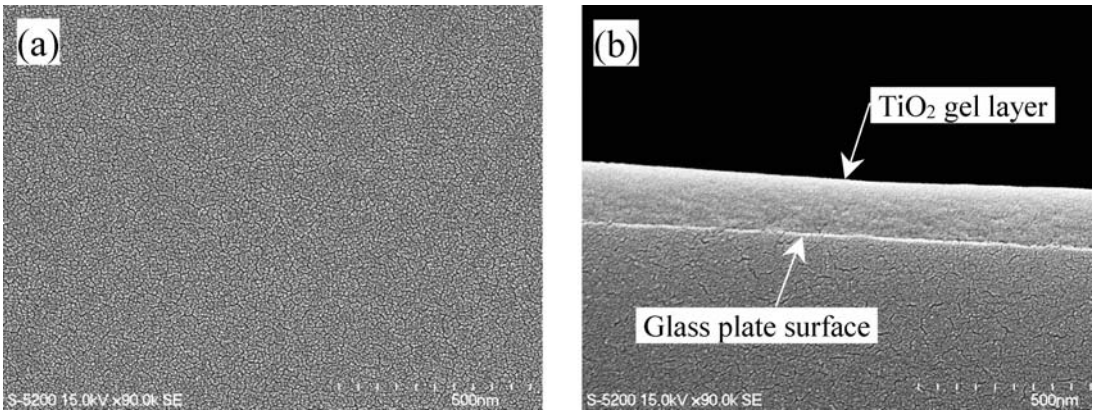

Fig. 2 Scanning electron micrographs of the surface (a) and cross section (b) of the TiO_2 gel layer prepared with a 20% titanium n-butoxide solution in ethanol. The TiO_2 gel surface was clear and robust on the template.

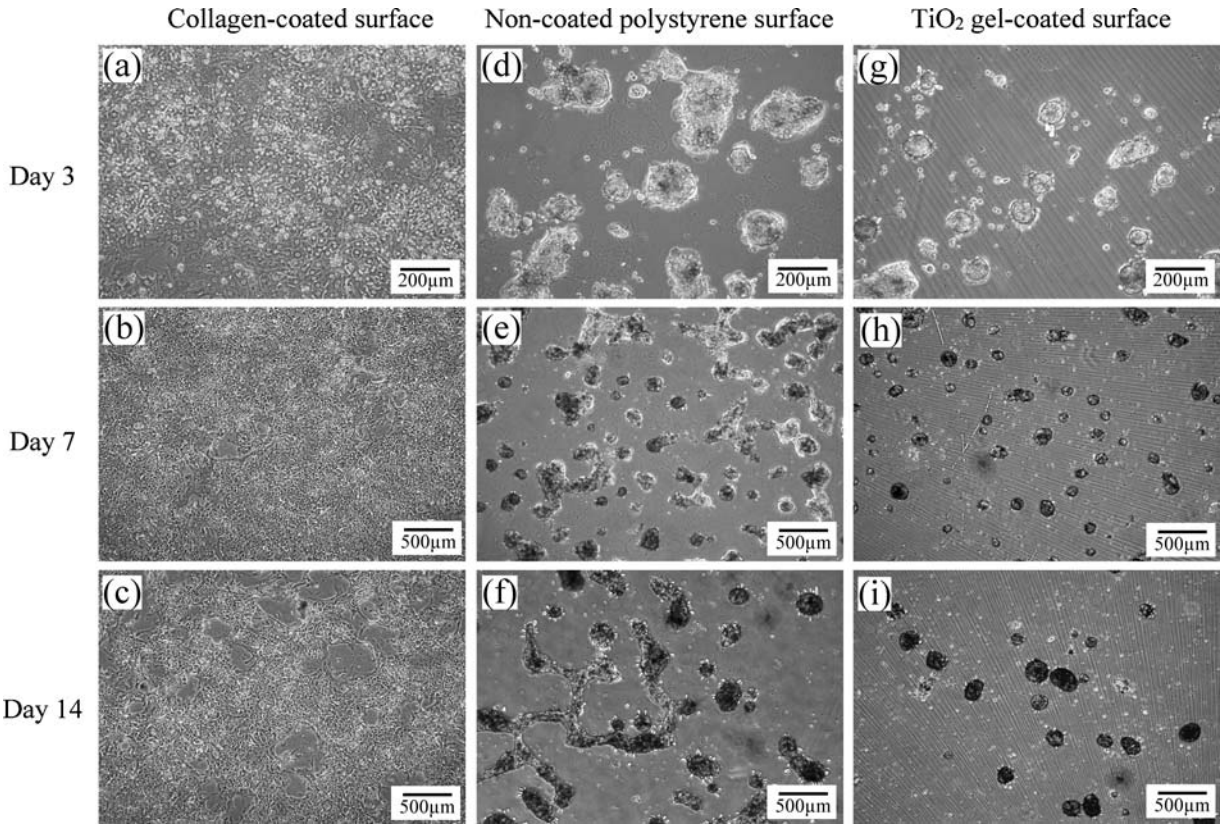

Fig. 3 Phase-contrast micrograph of hepatocyte configurations on a collagen-coated surface (a, b, c), a non-coated polystyrene surface (d, e, f) and a TiO_2 gel surface (g, h, i). (a) (d) (g): 3 day of culture, (b) (e) (h): 7 days of culture and (c) (f) (i): 14 days of culture.

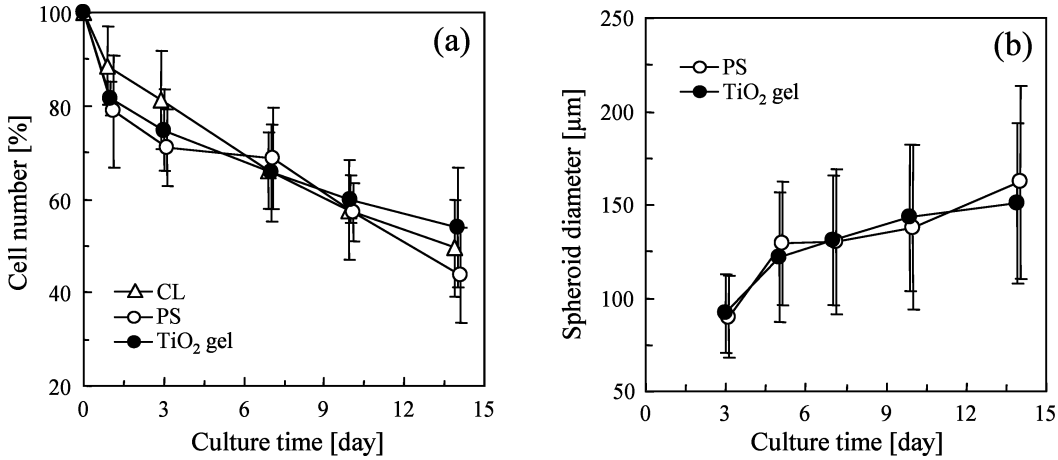

Fig. 4 Time courses of cell number (a) and spheroid diameter (b). CL ( ), PS (○) and TiO_2 gel (●) represent collagen-coated surface, non-coated polystyrene surface and TiO_2 gel surface, respectively. Error bars are SD.

different concentrations of titanium n-butoxide, were nearly the same.

3.3. Time courses of cell number and spheroid diameter

Figure 4a shows the time course of the cell number in collagen, non-coated polystyrene and TiO_2 gel. The hepatocyte number in all conditions gradually decreased during the culture, and were about half of the initial cell number at 14 days of culture. The cell numbers in the three conditions were not markedly different. The time course of spheroid diameters was evaluated on the non-coated polystyrene surface and on the TiO_2 gel surface (Fig. 4b). Both conditions showed similar trends of diameter: diameters of spheroids formed at 3 and 14 days of culture were $90 \pm 22\,\mu$m and $162 \pm 52\,\mu$m, respectively, in the non-coated polystyrene surface

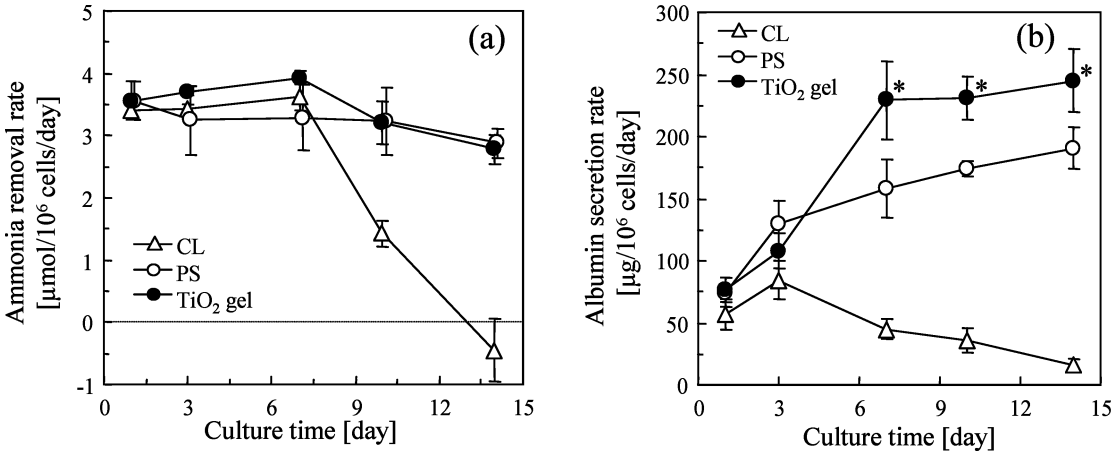

Fig. 5 Time courses of ammonia removal function (a) and albumin secretion function (b). CL ( ), PS (○) and TiO_2 gel (●) represent collagen-coated surface, non-coated polystyrene surface and TiO_2 gel surface, respectively. Error bars are SD. $^*p < 0.05$ compared with each value of PS.

and were 92 ± 21 μm and 151 ± 43 μm, respectively, in the TiO_2 gel surface. The larger spheroids formed by fusion of small spheroids.

3.4. Hepatocyte-differentiated functions

Hepatocyte-differentiated functions were evaluated by ammonia removal and albumin secretion (Figs. 5a,b), which are markers of detoxification and synthesis by the liver cells. Hepatocytes in the collagen monolayer culture decreased ammonia removal until the ammonia removal stopped at 14 days of culture. Hepatocytes on the non-coated polystyrene surface and on the TiO_2 gel surface continued to remove ammonia at levels similar to initial levels for at least 14 days of culture. Albumin secretion decreased in the collagen monolayer culture during the culture, but increased in hepatocytes on the non-coated polystyrene and TiO_2 gel surfaces with spheroid formation, and then was maintained at a high level for at least 14 days of culture. The albumin secretion activity of hepatocytes on the TiO_2 gel surface was higher than on the non-coated polystyrene surface.

4. Discussion

Hepatocyte spheroids are one of the most attractive culture techniques for many applications, because they have an *in vivo*-like structure and can maintain hepatocyte functions for a long time. This study showed that TiO_2 gel promotes hepatocyte spheroid formation without an extracellular matrix. Spheroid formation occurs only if cell-cell interaction is stronger than cell-substratum interaction [17, 18], and is promoted by a positively charged surface [3] or hydrophilic surface [7, 19] that seem to affect weak cell adherent environments in the case of synthetic substrata, but not with extracellular matrices including a derived matrix. Although hepatocytes adhered to the TiO_2 gel surface at the initial stage of culture, the cell adherence to the surface was weak and subsequent intercellular attachment accelerated markedly (Fig. 3). Because such a process of spheroid formation on the TiO_2 gel surface is similar to that on positively charged or hydrophilic surfaces, the TiO_2 gel may also promote hepatocyte spheroid formation by the same mechanism as on these other surfaces. Rutile TiO_2 ceramic sintered at about 1400°C has a weak adhesion for hepatocytes and seems to affect cell aggregation, but not spheroid formation that is derived from rearrangement and compaction of cell aggregates [10, 11]. Although further studies are needed to understand the surface properties and cell-substratum interaction, probably, the cell adhesion of TiO_2 gel appears weaker than that of rutile TiO_2 ceramic, when comparing results. Some hepatocyte spheroids that easily detached from the TiO_2 gel surface and then became floating spheroids may support this view.

In this study hepatocytes were cultured on various TiO_2 gels formed by different concentrations of titanium n-butoxide. Hepatocytes that spontaneously formed spheroids were the same, indicating that the surface properties of TiO_2 gel layers are almost the same in all prepared conditions, except for different thicknesses of the TiO_2 gel layer. However, the optimum condition to prepare a stable TiO_2 gel was a concentration of titanium n-butoxide at 10% to 40%; the TiO_2 gel formed by more than 50% titanium n-butoxide solution had an uneven, cracked surface on the template (data not shown).

Hepatocytes forming spheroids on the non-coated polystyrene surface and on the TiO_2 gel surface maintained ammonia removal and albumin at high level (Figs. 5a, b). Albumin secretion on the TiO_2 gel surface was higher than on the non-coated polystyrene surface, this may be because hepatocytes on the non-coated polystyrene surface formed different cell morphologies, such as spheroids, cell

aggregates and spreading cells (Figs. 3e,f), and most hepatocytes on the TiO_2 gel surface formed spheroids (Figs. 3h,i). However, ammonia removal showed no marked difference with either surface condition. This result agrees with previous reports that albumin secretion is upregulated by spheroid formation and even if hepatocytes form spheroids, ammonia removal is unvaried [3, 20–22]. On the other hand, spheroid formation seems to affect the long-term maintenance of ammonia removal. The level and trend of expressing functions on the TiO_2 gel surface were almost the same as in previous spheroid studies [3, 20–22]. These results indicate that the TiO_2 gel has no cytotoxicity and is effective for long-term maintenance of the differentiated functions.

The cell number on the TiO_2 gel surface gradually decreased during the culture, owing to the detachment of hepatocyte spheroids from the surface (Fig. 4a), and the diameter of spheroids increased with culture time by fusion of floating spheroids (Fig. 4b). Cell detachment from the surface and the different spheroid diameters cannot be controlled on the scaffold that has a flat surface such as in this study. However, TiO_2 gel layer can apply to a surface coating of organic or inorganic scaffolds having various shapes, and consequently the surface of scaffolds may come to promote the spheroid formation, even if original scaffolds can not. Therefore, the control of spheroid diameter and the spheroid immobilization in a scaffold may be able to realize by the TiO_2 gel coating of scaffold that has a three-dimensional structure, such as porosity, cavity and hollow fiber.

5. Conclusions

As a new culture substratum, a TiO_2 gel surface was prepared by using the sequence of spin coating a titanium n-butoxide solution, hydrolyzing under saturation with water vapor and drying by flushing with nitrogen gas. This promoted spheroid formation of rat hepatocytes and their differentiated functions were maintained in long-term culture. These results suggest TiO_2 gel is a promising material for hepatocyte culture.

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