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Blood-Compatible Polymer for Hepatocyte Culture with High Hepatocyte-Specific Functions toward Bioartificial Liver Development

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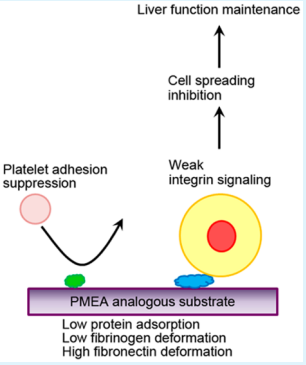
Supporting Information

ABSTRACT: The development of bioartificial liver (BAL) is expected because of the shortage of donor liver for transplantation. The substrates for BAL require the following criteria: (a) blood compatibility, (b) hepatocyte adhesiveness, and (c) the ability to maintain hepatocyte-specific functions. Here, we examined blood-compatible poly(2-methoxyethyl acrylate) (PMEA) and poly(tetrahydrofurfuryl acrylate) (PTHFA) (PTHFA) as the substrates for BAL. HepG2, a human hepatocyte model, could adhere on PMEA and PTHFA substrates. The spreading of HepG2 cells was suppressed on PMEA substrates because integrin contribution to cell adhesion on PMEA substrate was low and integrin signaling was not sufficiently activated. Hepatocyte-specific gene expression in HepG2 cells increased on PMEA substrate, whereas the expression decreased on PTHFA substrates due to the nuclear localization of Yes-associated protein (YAP). These results indicate that blood-compatible PMEA is suitable for BAL substrate. Also, PMEA is expected to be used to regulate cell functions for blood-contacting tissue engineering.



KEYWORDS: *hepatocyte, bioartificial liver, blood compatibility, protein adsorption, cell spreading*

1. INTRODUCTION

Donor shortage is a substantial barrier for liver transplantation, although transplantation is a standard therapy for severe liver failure such as fulminant hepatitis, liver fibrosis, cancer, Wilson's disease, and Bud−Chiari syndrome. Instead of using a donor liver, a bioartificial liver (BAL) that is constructed with hepatocytes is expected to be developed to overcome this shortage.[1] A BAL requires a connection to the circulatory system, and the substrate of the BAL for hepatocyte adhesion requires blood compatibility to prevent clotting and to maintain blood circulation. Moreover, hepatocytes lose their specific functions in an in vitro culture with conventional substrates.[2] Therefore, the criteria for a BAL substrate include (a) blood compatibility, (b) hepatocyte adhesiveness, and (c) the ability to maintain hepatocyte-specific functions.

In general, the cells adhere to polymer substrates through the interaction with proteins adsorbed on the substrates. Therefore, conventional blood-compatible substrates, such as polyethylene glycol (PEG) and poly(2-methacryloyloxyethyl phosphorylcholine) (MPC), are designed to suppress the adsorption of proteins including fibrinogen that promotes platelet adhesion.[3,4] On these polymer substrates, the adsorption of proteins such as fibronectin that promotes hepatocyte adhesion is also suppressed.[3,4] Hepatocytes cannot adhere to conventional blood-compatible polymers, and nonadherent hepatocytes undergo apoptosis.[5]

Additionally, hepatocytes rapidly lose their specific functions in an in vitro culture with conventional substrates.[5] To prevent the loss of hepatocyte-specific functions, various cell culture systems using the substrates were proposed.[5−7] The substrates composed of extracellular matrix proteins (e.g., Matrigel) are frequently used to maintain hepatocyte-specific functions,[6] although the blood compatibility of Matrigel is low. Galactose-carrying polystyrene, poly(*N*-*p*-vinylbenzyl-4-*O*-*β*-D-galactopyranosyl-D-gluconamide) (PVLA), is also capable of maintaining hepatocyte-specific functions and of exhibiting blood compatibility.[5,7] However, nonparenchymal cells cannot adhere to PVLA substrate to construct a coculture system for BAL development.[7]

We have previously reported that the substrates coated with poly(2-methoxyethyl acrylate) (PMEA) and its analogous polymer, poly(tetrahydrofurfuryl acrylate) (PTHFA), showed blood compatibility.[8,9] We have recently reported that cancerous and normal cells can adhere to PMEA and PTHFA substrates.[10] Moreover, we showed that protein adsorption was suppressed on PMEA substrates, reducing the integrin contribution to cell adhesion on a PMEA substrate.[10] It is well-known that integrin-dependent adhesion and the

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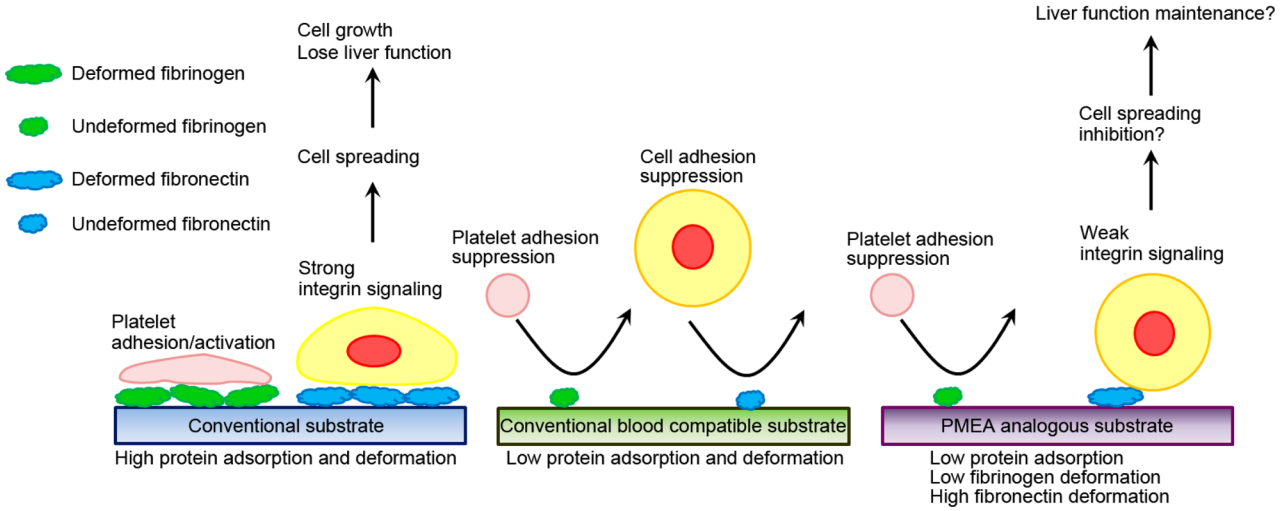

Figure 1. Study concept. Regulation of cell shape to maintain hepatocyte-specific functions through the suppression of protein adsorption on a blood-compatible PMEA substrate.

following activation of integrin signaling promote cell spreading.[11] In the case of hepatocytes, hepatocyte spreading leads to the loss of hepatocyte-specific functions.[12] Therefore, we assumed that hepatocytes can adhere and be cultured on a blood-compatible PMEA substrate and can inhibit hepatocyte spreading due to the low contribution of integrin to cell adhesion, which leads to the maintenance of hepatocyte-specific functions (Figure 1). Moreover, it is expected that the coculture with nonparenchymal cells can be constructed on PMEA substrate through the interaction with integrins.

HepG2 cells have been used as a primary hepatocyte model.[13−15] Moreover, HepG2 has been expected to be a cell source for BAL development because the proliferation of primary hepatocytes is strictly limited.[16,17] In this study, HepG2 cells were used as a human hepatocyte model. The cells were cultured on the polyethylene terephatalate (PET) substrates coated with the blood-compatible polymers PMEA, PTHFA, poly(2-hydroxyethyl methacrylate) (PHEMA), and the copolymer of MPC and *n*-butyl acrylate (PMPC) as well as bare PET substrates. Moreover, we compared the expression levels of hepatocyte-specific genes on these substrates to assess the possibility of PMEA substrates for BAL development.

2. MATERIALS AND METHODS

2.1. Preparation of Polymer-Coated Substrates. PMEA and PTHFA were synthesized according to previous reports.[8,9] The copolymer of MPC and butyl methacrylate (30:70 mol %, PMPC) was kindly gifted by the NOF Corp. (Tokyo, Japan). PHEMA was purchased from Scientific Polymer Products (Ontario, NY). The chemical structures of these polymers were shown in Figure S1. These polymers were coated on PET discs (ϕ = 14 mm, thickness = 125 μm, Mitsubishi Plastics, Tokyo, Japan) and tissue culture polystyrene (TCPS) with spin-coating and polymer casting methods, respectively.[10] The prepared substrates were exposed to UV for 2 h to sterilize and stored at 4 °C until use. Fibronectin (Calbiochem, Darmstadt, Germany)-coated substrates were also prepared according to a previous report.[8]

2.2. Adsorbed Protein Quantification. Adsorbed protein amount was quantified with the method reported previously.[10] Briefly, polymer-coated 96-well TCPS plates were immersed in PBS for 1 h at 37 °C. Subsequently, 100 μL of 10% FBS containing DMEM/F-12 medium was added to each well, and the plate was incubated for 1 h at 37 °C. The adsorbed proteins were extracted by incubating the plate with a 5% sodium dodecyl sulfate (SDS) solution and 0.1 N NaOH for 60 min at room temperature. The extracted proteins were assessed with a microBCA assay (Thermo Scientific, Rockford, IL).

2.3. Evaluation of Deformed Fibronectin by Enzyme-Linked Immunosorbent Assay (ELISA). Deformed fibronectin was evaluated by the method reported previously.[10] Briefly, polymer-coated 96-well TCPS plates were immersed in PBS for 1 h at 37 °C. After incubation with PBS, human fibronectin (5 μg/mL, 50 μL/well, Sigma) was added to the plate and the plate was incubated for 1 h at 37 °C. Deformed fibronectin was evaluated by colorimetric ELISA using HFN7.1 Ab (Abcam, Cambridge, UK) as a primary Ab and peroxidase-conjugated antimouse IgG Ab as a secondary Ab. Deformed fibronectin was detected with 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid ammonium salt) (ABTS) substrate (Roche Diagnostics). The absorbance was measured at a wavelength of 405 nm.

2.4. HepG2 Cell Culture. HepG2 cells were obtained from Health Science Research Resources Bank (Osaka, Japan), and the cells were maintained in Dulbecco's Modified Eagle/Nutrient Mixture F-12 (DMEM/F-12, Gibco, Carlsbad, CA), containing 10% fetal bovine serum (FBS, Equitech-Bio, Kerrville, TX). Prior to the experiments, the cells were detached from the TCPS dish (IWAKI, Chiba, Japan) with a 0.25% trypsin/EDTA solution (Gibco).

2.5. Cell Adhesion Assay. Prior to cell culture, the polymer substrates were immersed in 10% FBS containing DMEM/F-12 for 1 h at 37 °C. The HepG2 cells were seeded on the polymer substrates at a density of 5 × 10^4 cells/cm^2 and were allowed to adhere to the substrates in 10% FBS containing DMEM/F-12 for 3 h. The nonadherent cells were removed from the culture by washing twice with PBS. The adherent cells were fixed with 0.1% glutaraldehyde overnight at room temperature. The cells were visualized by crystal violet staining. After staining, the adherent cells in three randomly selected fields were counted using an optical microscope.

For the inhibition assay, the cells were treated with 5 mM EDTA (Sigma) for 10 min at 37 °C before cell seeding. After treatment, the cell adhesion assay was performed as described above.

Cell adhesion assay in serum-free medium was performed as described below. Prior to cell culture, the polymer substrates were immersed in serum-free DMEM/F-12 for 1 h at 37 °C. The HepG2 cells were seeded on the polymer substrates at a density of 5 × 10^4 cells/cm^2 and were allowed to adhere to the substrates in serum-free DMEM/F-12 for 3 h. The adherent cells were counted as described above.

2.6. Evaluation of Cell Shape. The HepG2 cells were cultured on the polymer substrates at a density of 1 × 10^4 cells/cm^2 in 10% FBS containing DMEM/F-12. After 1 day of culture, the cells were visualized by crystal violet staining. Each cell shape was traced using a graphics tablet (Bamboo Fun, Wacom, Saitama, Japan). After tracing,

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the projected cell area was measured using the GNU Image Manipulation Program (GIMP) and imageJ software programs.

2.7. Immunocytochemical Analysis. Immunocytochemical analysis was performed as reported previously.[10] Antivinculin antibody (Ab) (Millipore, Billerica, MA) and anti-YAP Ab (Cell Signaling Technology, Danvers, MA) were used as primary Abs. Alexa Fluor 568-conjugated antimouse IgG Ab and Alexa Fluor 488-conjugated antirabbit IgG Ab were used as corresponding secondary Abs.

2.8. Gene Expression Analysis. Total RNA was extracted from the cells using the Sepasol-RNA I Super reagent according to the manufacturer's instructions (Nacalai Tesque). Total RNA (1 μg) was used as a first-strand reaction that included random hexamer primers and ReverTra Ace-α reverse transcriptase (TOYOBO, Osaka, Japan). Real-time PCR was amplified for genes coding glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), albumin (*ALB*), and hepatocyte nuclear factor 4α (*HNF4A*) using TaqMan Gene Expression Assays (*GAPDH*, Hs02758991; *ALB*, Hs00910225_m1; *HNF4A*, Hs00230853_m1). The reaction was performed with 10 ng of cDNA, TaqMan Expression Assays, and Premix Ex Taq (Probe qPCR) (TaKaRa, Shiga, Japan), according to the manufacturer's instruction. The gene expression levels relative to *GAPDH* were calculated using the comparative Ct method.

2.9. Statistical Analysis. All of the data are expressed as the means ± SD. The significance of the differences between two samples was determined through an unpaired Student's *t* test using Microsoft Excel 2010. The statistical analyses used to analyze the differences between three or more samples were performed using R, a language and environment for statistical computing. The significance of the differences was determined using analysis of variance (ANOVA). Tukey's multiple comparison test was applied as a posthoc test. Differences with *P* values less than 0.05 were considered statistically significant.

3. RESULTS

3.1. Proteins Adsorption on Polymer-Coated Substrates. As the first step, we confirmed whether protein adsorption was altered on PMEA and PTHFA substrates. We measured the amount of serum proteins adsorbed on the substrates after 1 h (Figure 2A). The amount of serum proteins adsorbed on the PMEA, PHEMA, and PMPC substrates was approximately 58% or less as compared to that on tissue culture polystyrene (TCPS). In contrast, the amount of the proteins adsorbed on PTHFA substrates was similar to that on TCPS. In addition to protein adsorption, the deformation of proteins adsorbed from serum (i.e., fibrinogen and fibronectin (FN)) is necessary for cell adhesion.[18,19]

It has already been reported that fibrinogen deformation to allow platelet adhesion is suppressed on PMEA and PTHFA substrates, leading to the exhibition of blood compatibility.[8] In contrast, fibronectin gave rise to deformations that allowed nonblood cell adhesion on PMEA and PTHFA substrates (Figure 2B). Therefore, it is possible that nonblood cells, especially hepatocytes, can adhere to blood-compatible PMEA and PTHFA substrates at least via an interaction with fibronectin. These results are consistent with our previous report.[10] In addition to fibronectin, vitronectin can interact with cells to allow cell adhesion.[20] In this study, the deformation of vitronectin is unclear. Cells can adhere to polymer substrates by interacting with vitronectin instead of fibronectin.

3.2. Cell Adhesion and Morphology on Polymer-Coated Substrates. There is the possibility that cell adhesion decreases on the substrates due to the suppression of protein adsorption. Therefore, we checked whether HepG2 cells could adhere to polymer substrates even when protein adsorption was suppressed. The adherent cell numbers were counted on these substrates after 3 h (Figure 3A). HepG2 cells adhered to blood-compatible PMEA and PTHFA substrates as well as PET substrate, as a positive control, after 3 h. This result indicates that HepG2 cells can adhere to blood-compatible PMEA and PTHFA substrates. On the other hand, HepG2 cells hardly adhered to PHEMA and PMPC substrates, which were conventional blood-compatible substrates.

As a next step, the cell shapes were observed after 1 day (Figure S2). Projected cell areas were measured after 1 day to

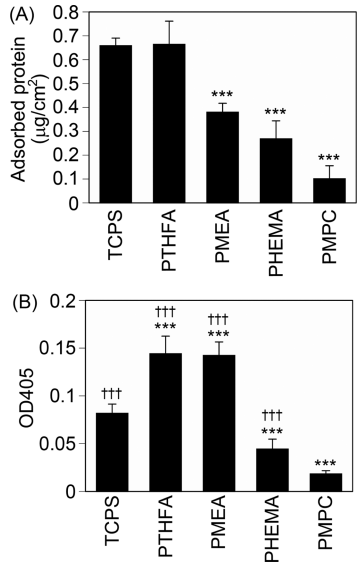

Figure 2. Protein adsorption on polymer substrates. (A) Adsorbed protein amount on polymer-coated TCPS or bare TCPS after a 1-h incubation in 10% FBS-containing DMEM/F-12 medium. Data represent the means ± SD (n = 5). ***: P < 0.005 vs TCPS. (B) Fibronectin deformation on polymer substrates. Fibronectin was adsorbed on polymer substrates for 1 h. Deformed fibronectin levels were evaluated by ELISA using conformation-specific antibody. Data represent the means ± SD (n = 5). ***: P < 0.005 vs TCPS. †††: P < 0.005 vs PMPC.

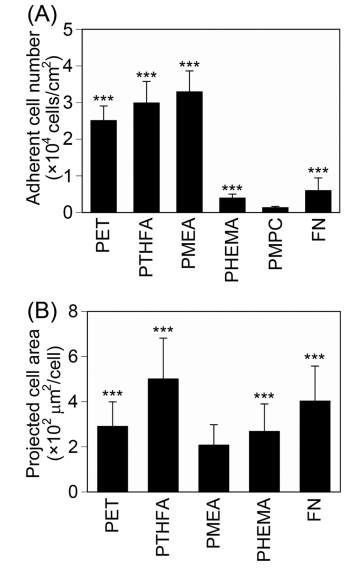

Figure 3. Cell adhesion and shapes on polymer substrates. (A) Adherent cell numbers on the substrates after 3 h. Data represent the means ± SD (n = 3). ***: P < 0.005 vs PMPC. (B) Projected cell area on the substrate after 1 day. Data represent the means ± SD (n = 60). ***: P < 0.005 vs PMEA.

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compare the cell shape on polymer substrates (Figure 3B). The projected cell areas were in the order of PTHFA, FN > PET > PHEMA > PMEA. The projected cell areas on PMEA, especially, were the lowest among the substrates. This result indicates that HepG2 cell spreading was suppressed on PMEA substrates.

3.3. Cell Adhesion Mechanisms on the Polymer-Coated Substrates. To clarify the mechanism that suppressed HepG2 cell spreading on PMEA substrates, we focused on integrin-dependent adhesion because integrin activates intracellular signaling to promote cell spreading.[11] For this purpose, we counted the adherent HepG2 cells on polymer substrates in the presence of EDTA, which inhibits integrin-dependent adhesion (Figure 4A).[21] Cell adhesion was significantly inhibited on the PET, PTHFA, PMEA, PHEMA, and FN substrates. Adherent cell numbers on the PET, PTHFA, PHEMA, and FN substrates were similar to the PMPC substrate in the presence of EDTA, suggesting that cells adhered to these substrates via an integrin-dependent mechanism. On the other hand, the adherent cell numbers were significantly higher on the PMEA substrate than the PMPC substrate, even in the presence of EDTA, suggesting that cells adhered to the PMEA substrate via integrin-dependent and -independent mechanisms.

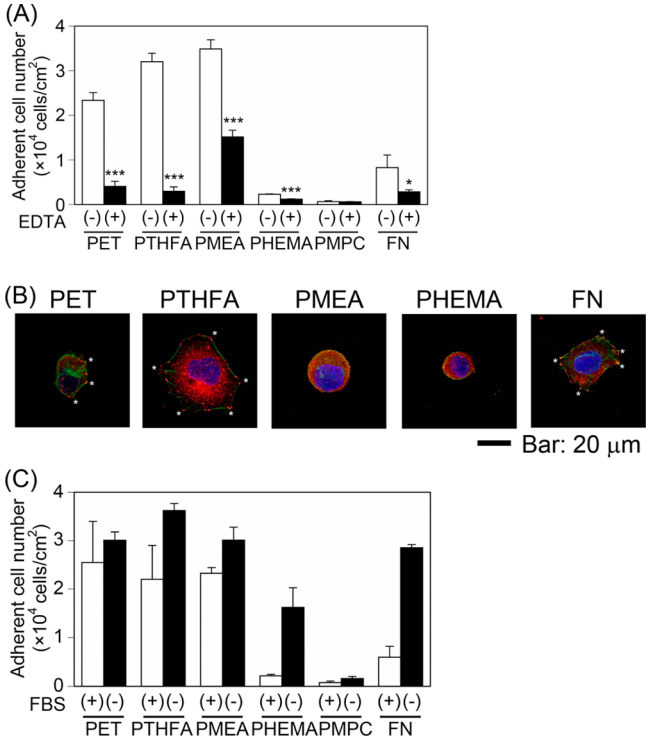

Figure 4. Cell adhesion mechanisms on polymer substrates. (A) Inhibitory effect of EDTA on cell adhesion. Adherent cell numbers were counted in the presence or absence of 5 mM EDTA after 3 h. White and black bars indicate the absence (−) or presence (+) of EDTA. Data represent the means ± SD (n = 3). *: $P < 0.05$. ***: $P < 0.005$ vs EDTA (−). (B) Focal adhesion formation. Cell nuclei (blue), F-actin (green), and vinculin (red) were observed after 1 day. Asterisk indicates focal adhesion. Bar = 20 μm. (C) HepG2 cell adhesion to polymer substrates in the presence or absence of serum proteins. White and black bars indicate the presence (+) or absence (−) of FBS in the medium. Data represent the means ± SD (n = 3).

For further investigation, vinculin localization was observed as an indicator of focal adhesion, which is formed by integrin-dependent adhesion and activates integrin signaling (Figure 4B).[11,22] Few focal adhesions were observed on PMEA and PHEMA substrates after 1 day, whereas evident focal adhesions were observed on PET, PTHFA, and FN substrates on which cell adhesion was completely inhibited by EDTA. These results indicate that HepG2 cells adhere on PMEA substrate via both integrin-dependent and -independent mechanisms, whereas the cells adhere on PET, PTHFA, PHEMA, and FN substrates. These results also suggest that integrin signaling is strongly activated on PET, PTHFA, and FN substrates.

HepG2 cells adhered on PMEA substrate via both integrin-dependent and -independent mechanisms. On the PMEA substrate, protein adsorption was suppressed and the substrate surface was exposed to the cells. The cells may interact with the substrates directly. Indeed, HepG2 cells can adhere to PMEA substrates without serum proteins (Figure 4C). Therefore, it appeared that HepG2 cells adhered to the PMEA substrate via an integrin-dependent mechanism and direct interaction with polymer substrates.

3.4. Hepatocyte-Specific Gene Expression on the Polymer-Coated Substrates. Finally, we examined hepatocyte-specific gene expression on the PMEA and PTHFA substrates as well as TCPS as an experimental control. We examined the expression levels of genes encoding albumin (*ALB*) and hepatocyte nuclear factor 4α (*HNF4A*) as markers of hepatocyte-specific functions. Especially, it is well-known that HNF4α is a master regulator of hepatocyte-specific gene expression.[23] The functions on the PHEMA and PMPC substrates were not evaluated because not enough HepG2 cell adhesion was observed. Additionally, the functions on FN were not evaluated because FN is not blood compatible. The expression levels of *ALB* and *HNF4A* on the PMEA substrate were the highest among the substrates (Figure 5A and B). *ALB* expression levels on PMEA substrates were 1.3−2.0 times higher than those on TCPS within 4 days (Figure 5A). *HNF4A* expression levels on PMEA substrates were 2.0−2.4 times higher than those on TCPS within 4 days (Figure 5B). These results suggested that HepG2 cells possess higher liver function on the PMEA substrate than PTHFA substrates and TCPS.

It is known that hepatocyte functions are strongly related to their shape when cultured.[12] Recently, it has been reported that hepatocyte dedifferentiation is led by nuclear localization of the Yes-associated protein (YAP), which is regulated by actin filament-dependent cell shape alteration.[24,25] Therefore, we observed intracellular localization of YAP on the substrates after 1 day to clarify the reason why hepatocyte-specific gene expression was higher on the PMEA substrate than on the PTHFA substrate and TCPS (Figure 5C). YAP was localized in the cytosol on the PMEA substrate, whereas it was colocalized with cell nuclei on the PTHFA substrate and TCPS.

4. DISCUSSION

In this study, we tried to regulate hepatocyte-specific gene expression through the change of protein adsorption on PMEA analogous polymer substrates. FBS protein adsorption was significantly suppressed on PMEA substrate (Figure 2A). We have previously pointed out that intermediate water, one of the water structures in hydrated polymers, acted as a barrier to protect the interaction with polymer.[10,26−28] Therefore, FBS protein adsorption might be suppressed on the substrate coated with PMEA possessing intermediate water. Although PTHFA

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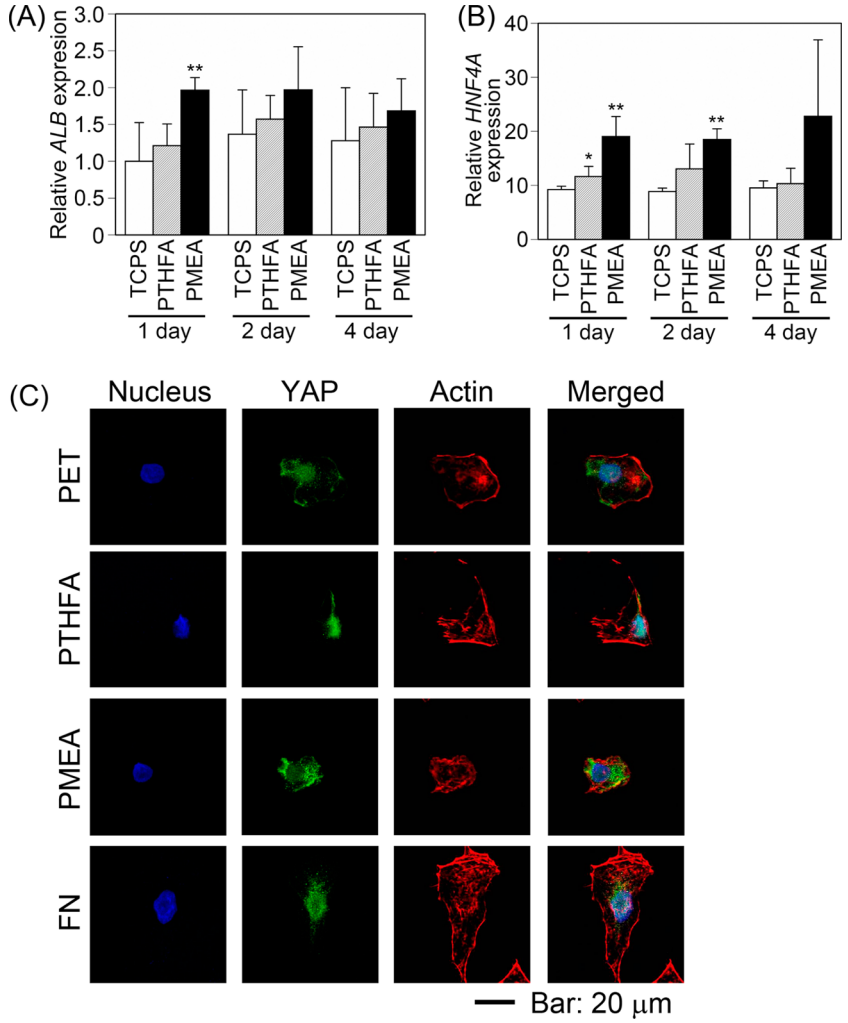

Figure 5. HepG2 cell functions on polymer substrates. (A) *ALB* expression levels in HepG2 cells on the substrates. Data represent the means ± SD (n = 3). **: P < 0.01 vs TCPS. (B) *HNF4A* expression levels in HepG2 cells on the substrates. Data represent the means ± SD (n = 3). *: P < 0.05. **: P < 0.01 vs TCPS. (C) Intracellular localization of YAP on polymer substrates. Cell nuclei (blue), YAP (green), and F-actin (red) were observed after 1 day. Bar 20 μm.

possesses intermediate water, FBS protein adsorption was not suppressed on PTHFA substrate (Figure 2A). Recently, we reported that intermediate water content influenced the amount of protein adsorption.[29] We reported that increasing intermediate water content decreased protein adsorption amounts. As compared to intermediate water content in hydrated PMEA, the contents in hydrated PTHFA are lower.[9] It seems that intermediate water content in hydrated PTHFA is not sufficient for the suppression of FBS protein adsorption.

We showed that HepG2 could adhere on blood-compatible PMEA and PTHFA substrates. Generally, platelets adhere on polymer substrates via the interaction between integrin αIIbβ3 and fibrinogen, which was adsorbed and deformed. It has been already reported that fibrinogen was not deformed on PMEA and PTHFA substrates.[8] Therefore, PMEA and PTHFA substrates exhibit blood compatibility. In contrast to platelet adhesion, nonblood cells generally adhere on the polymer substrates via the interaction with corresponding integrins to fibronectin and vitronectin.[20] Moreover, HepG2 adhered on PMEA substrate via an integrin-independent mechanism, which is speculated as direct interaction with the substrates. Therefore, HepG2 adhered on blood-compatible PMEA and PTHFA substrates via these mechanisms. However, HepG2 cells weakly adhered to fibronectin (FN), an extracellular matrix protein, because HepG2 cells express a receptor against FN, integrin α5β1, at a low level.[30] HepG2 adhesion via integrin−FN interaction might be weak within 3 h.

Although fibronectin led to the adsorption-induced deformation for cell adhesion to the PMEA substrate as well as the PTHFA substrate (Figure 2B), the contribution of integrin to cell adhesion on the PMEA substrate decreased. Protein adsorption on the PMEA substrate was lower than that on the PTHFA substrate. This low protein adsorption may create space to allow for a more mobile fibronectin cell adhesion site, leading to an unstable integrin−fibronectin interaction.[31] Therefore, the integrin contribution to cell adhesion to the PMEA substrate was lower than that to the PTHFA substrate.

As shown in Figure 4B, obvious focal adhesions were observed on PET, PTHFA, and FN substrates, whereas focal adhesions were hardly observed on PMEA and PHEMA substrates. These results indicate that integrin weakly contributes to HepG2 cell adhesion and that integrin signaling is weak on PMEA substrates. Moreover, it suggests that weak integrin signaling cannot promote cell spreading and that HepG2 cell spreading is suppressed on PMEA substrates. On the PHEMA substrate, HepG2 cell spreading was also weak. It

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seems that HepG2 cells loosely adhere to the PHEMA substrate and that this loose adhesion cannot allow for the HepG2 cells to spread.

It has been reported that YAP localization is regulated through actin filament formation regulated by Rho signaling.[24] Rho signaling is generally activated by integrin-dependent adhesion and promotes cell spreading.[11] Evident focal adhesions were observed on the PTHFA substrate and TCPS (or PET), indicating strong activation of integrin signaling. Moreover, HepG2 were well-spread on PTHFA substrate and TCPS (or PET). Therefore, Rho signaling might be activated by strong integrin signaling on the PTHFA substrate and TCPS, and this Rho signaling led to YAP localization in cell nuclei. It has been reported that nuclear localization of YAP led to hepatocyte dedifferentiation.[25] Therefore, the nuclear localization of YAP suppressed the expression of *HNF4A* and *ALB* on PTHFA substrate and TCPS. In contrast, few focal adhesions and the suppression of cell spreading were observed on PMEA, indicating that integrin signaling is weak. Rho signaling might not be activated on PMEA substrates. Therefore, YAP did not localize to cell nuclei, leading to *HNF4A* and *ALB* expression. The putative regulation mechanism of hepatocyte-specific gene expression on PMEA analogous polymer substrates was summarized in Figure 6. In addition to YAP nuclear localization, we cannot exclude other possible mechanisms to increase hepatocyte-specific gene expression.

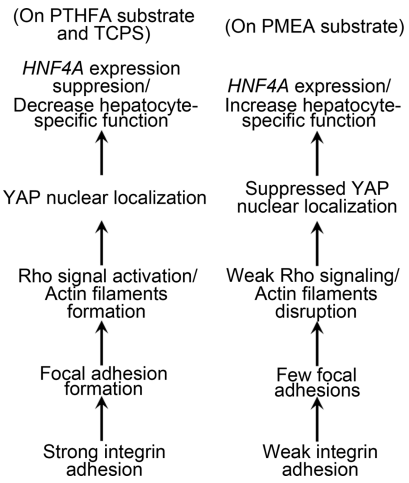

Figure 6. Putative intracellular signaling on PMEA and PTHFA substrates and TCPS.

The expression levels of *HNF4A* and *ALB* gradually decreased at 4 days of the culture. It seemed that protein adsorption occurred even on PMEA to lead to cell spreading. Therefore, HepG2 cells gradually lost their specific function at 4 days of the culture. To avoid this loss of liver-specific functions, PMEA analogous polymers that can strongly suppress protein adsorption should be selected for BAL development. We have already reported that other PMEA analogous polymers can strongly suppress protein adsorption[29] and allowed for the cells to adhere.[32] The optimization of PMEA analogous polymers should be required for BAL development in the future. Additionally, the expression levels of liver functions at protein level should be checked.

HepG2 cells were used as a model of human primary hepatocytes in this study. HepG2 cells have been useful as a model of primary hepatocytes, although HepG2 cells are derived from hepatic carcinoma cells and partially lose their specific functions.[13−15] Despite these features, HepG2 cells are expected to be an important proliferative cell source for BAL development because primary hepatocytes hardly proliferate under in vitro condition.[16,17] Therefore, HepG2 cells were used as a suitable hepatocyte model in this study.

Further future studies are required for the development of BAL using our polymers. One of the problems to be solved is optimal cell density in the BAL. Hepatocytes increase their specific functions by the cell−cell interaction via E-cadherins, suggesting that higher cell density is better for the expression of liver functions.[33] On the other hand, higher cell density culture gives rise to hepatic cell death.[34] Therefore, the optimal cell density should be examined carefully. Additionally, over cell growth and protein synthesis should be checked after the culture for longer period. Another problem to be solved is optimal cell culture system for BAL. Especially, it has been reported that fluidic bioreactor system can improve liver-specific functions.[35] To examine whether our polymers can be adapted to this system, we should examine cell adhesion strength to the polymer substrates and we should optimize the fluidic rate in the bioreactors.

The expression levels of *ALB* and *HNF4A* were measured as markers of hepatocyte-specific gene expression. Especially, HNF4α is a master regulator of the expression of hepatocyte-specific genes such as cytochrome P450s, coagulation factors, and HNF1α, 1β, and 6.[23] Therefore, it is expected that many other hepatocyte-specific functions might increase on PMEA substrate through the expression of HNF4α, although hepatocytes exhibit many specific functions.

5. CONCLUSION

In this study, we showed that HepG2 cells, a human hepatocyte model, can adhere to a blood-compatible PMEA substrate. Also, hepatocyte spreading was inhibited to increase hepatocyte-specific gene expression, suggesting that PMEA meets the criteria of the substrate for a BAL. Similar to HepG2 cells, primary hepatocytes maintain their specific functions when their spreading is inhibited.[12] In addition to primary hepatocytes, stem cell-derived hepatocytes might maintain their specific functions on the PMEA substrate. Therefore, PMEA is expected to be a suitable substrate for BAL development using primary hepatocytes and stem cell-derived hepatocytes. Future studies using primary hepatocytes, stem cell-derived hepatocytes, or other hepatic cell lines are required to improve the performance of BAL using PMEA substrate.

There is a requirement for the regulation of cell functions, such as tissue-specific functions, for tissue engineering under blood contacting conditions (e.g., bioartificial pancreas). Adsorbed proteins on substrates strongly influence the cell shapes of a wide variety of cells.[36,37] Cell shape strongly relates to the functions of a wide variety of the cells.[12,38] In this study, we demonstrated that HepG2 cells exhibit round and spreading forms on PMEA and PTHFA substrates, respectively. It is possible that cell functions can be regulated on these blood-compatible polymer substrates through the regulation of cell shape under blood contacting conditions. Therefore, PMEA and its analogous polymer, PTHFA, are expected to be applied to tissue engineering using a wide variety of cells under blood contacting conditions.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b05210.

Chemical structure of the polymers used in this study; shapes of HepG2 cells (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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