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Immobilization of Lactobionic Acid on Polyurethane Films and Their Interaction with Hepatocytes

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Abstract: Polyurethanes containing z-lysine segments in the main chain (PULL) were synthesized from 4,4′-diphenylmethyl diisocyanate, poly(tetramethylene glycol), and z-lysine oligomer as a chain extender. The PULL film was treated first with a 10% HBr-acetic acid solution and subsequently with a saturated sodium bicarbonate aqueous solution to produce a primary amine group on the surface (PULL-N). Lactobionic acid (LA)–immobilized PULL (PULL-L) was prepared by the coupling reaction of the PULL surface amine groups and the LA carboxylic acid groups. The surface-modified PULLs were then characterized by attenuated total reflection-Fourier transform infrared spectroscopy, electron spectroscopy for chemical analysis, atomic force microscopy, and contact angle goniometry. In the hepatocytes adhesion experiment, the cells poorly adhered to the PULL surface, although they adhered moderately well to the PULL-N surface. On the other hand, the cells adhered well to the PULL-L surface, suggesting the good affinity of the surface β-galactose moieties for hepatocytes. When hepatocytes were cultured in the presence of epidermal growth factor for 48 h, the cells rapidly aggregated on the PULL-L surface, whereas they aggregated only slowly on the other surfaces. The PULL prepared in this study has the potential to be used as a coating material for the enhancement of hepatocyte adhesion.

Keywords: films, polyurethanes, surfaces, hepatocytes.

Introduction

Segmented polyurethane (PU), consisting of hard and soft segments, shows not only good physical properties but also a relatively good biocompatibility compared to other synthetic polymers.[1] Therefore, PU is widely used in such blood-contacting devices as catheters,[2] heart assist pumps,[3] and artificial hearts.[4] However, the biocompatibility of PUs needs to be improved for greater biomedical applications. To fulfill this requirement, the surface of the PU membrane has been modified with biological macromolecules such as insulin, heparin, and collagen.[5,6] Several methods to introduce functional groups that can couple with biological macromolecules to the surface of PU membrane have been reported. Ito *et al.*[7] and Kang *et al.*[8] prepared polyurethanes containing ester groups in the side chains and introduced carboxyl groups on the PU surfaces by treatment with sodium hydroxide and subsequently citric acid. They immobilized heparins on the carboxyl groups of the PU surfaces and reported that the immobilized heparins showed good antithrombogenicity. Han *et al.*[9] and Park *et al.*[10] have also studied the surface modification of polyurethanes for long term biomedical application using poly(ethylene oxide) (PEO) grafting and/or heparin immobilization. Hyun *et al.*[11] and Meng *et al.*[12] prepared polyurethanes containing γ-benzyl L-glutamate and z-lysine segments in the main chain, respectively. They reported that carboxylic acid and primary

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amine groups could be introduced to the surface of PU films by hydrolysis reaction using alkaline aqueous solution and acidic aqueous solution, respectively.

It has been shown that the existence of cell specific ligands or extracellular signaling molecules can facilitate the interaction of cells with biomaterials and control the growth and differentiation of cells in culture.[13,14] The β-galactose moiety can be recognized by asialoglycoprotein receptors (ASGP-R) on the hepatocyte membrane and can promote cell adhesion.[15,16] The β-galactose moiety can also better facilitate the maintenance of differentiated functions with promoting spheroid formation compared to collagen and fibronectin extracellular matrices (ECM).[17,18] Previously,[17] we prepared *N-p*-vinylbenzyl-4-O-β-*D*-galactopyranosyl-*D*-gluconamide (VLA)-grafted polystyrene dish (PS-VLA) by the treatment of a PS dish with oxygen plasma glow discharge followed by graft polymerization of VLAs, and revealed that the hepatocytes slowly adhered to PS-VLA but not to the polystyrene dish coated with PVLA during the first 2 h incubation.

In the current study, polyurethane containing z-lysine segments in the main chain (PULL) were synthesized according to our previously reported method.[12] Lactobionic acid-immobilized PULL films were prepared by treatment with HBr-acetic acid followed by washing with citric acid. Subsequently, this caused a reaction with lactobionic acid. The surface-modified PULLs were characterized by an attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR), an electron spectroscopy for chemical analysis (ESCA), an atomic force microscope (AFM), and a contact angle goniometer. Hepatocyte behaviors on surface-modified PULLs were also examined.

Experimental

Materials. Poly(tetramethylene glycol) (PTMG) with 1,000 molecular weight was purchased from Polyscience and L-lysine hydrochloride was purchased from TCI. 4,4,-Diphenylmethane diisocyanate (MDI) and benzyloxy carbonyl chloride (z-Cl) were purchased from Wako.

Synthesis of PULL. A solution of L-lysine hydrochloride (75.3 g, 410 mmol) and sodium hydroxide (80 g, 2 mol) dissolved in distilled water (500 mL) was cooled to 0 °C and z-Cl (196 g, 1.14 mol) was added drop by drop, followed by vigorously stirring. After 2 h, the solution was extracted with diethyl ether, the unused z-Cl was removed, and the aqueous layer was acidified. The oil was isolated with diethyl ether. N^{α},N^{ε}-Dicarbobenzoxy-L-lysine (z-Lys (z)) was extracted from the reaction solution using benzene and evaporated to produce white crystal. The crystal mixture was dried under reduced pressure.[18]

z-Lysine *N*-carboxyanhydride (z-LysNCA) was synthesized using phosphorus pentachloride. Briefly, z-lysine (z) (20 g) was mixed with diethyl ether (100 mL) in a flask and the mixture was suspended using a magnetic stirrer. Phosphorus pentachloride (12 g) was then added to the mixed solution, which was stored at 0 °C for 30 min. The reaction solution was precipitated in *n*-hexane (300 mL) and kept at 0 °C. The precipitates were filtrated using a glass filter and dried at room temperature for 24 h. The samples were then recrystallized using ethyl acetate to produce z-LysNCA and dried at room temperature under reduced pressure. The z-lysine oligomer was synthesized by ring opening polymerization. Briefly, z-LysNCA (14.5 g, 0.0475 mol) was dissolved in dimethylformamide (DMF, 10 mL) and mixed with ethylene diamine (0.33 mL, 0.0049 mol). The solution was kept at room temperature for 2 h to produce primary amine-terminated z-lysine oligomers.

The PU prepolymer was prepared by reacting MDI with PTMG at a molar ratio of 1.15 : 1. z-Lysine oligomers dissolved in DMF (20 mL) were then added drop by drop to a flask containing PU prepolymer to produce polyurethanes containing z-lysine segments in the main chain (PULL), as shown in Figure 1. The reaction solution was diluted using DMF (10 mL) when its viscosity increased. Finally, butylamine diluted by DMF (10 mL) was added to the reaction solution to stop further reaction with the terminal isocyanate group. The reaction solution was then precipitated in distilled water and the precipitates were washed with methanol three times and dried under reduced pressure at 60 °C for 24 h.

Preparation of PULL Film. A 10 wt% dimethylformamide solution of PULL was used to prepare the sample film.

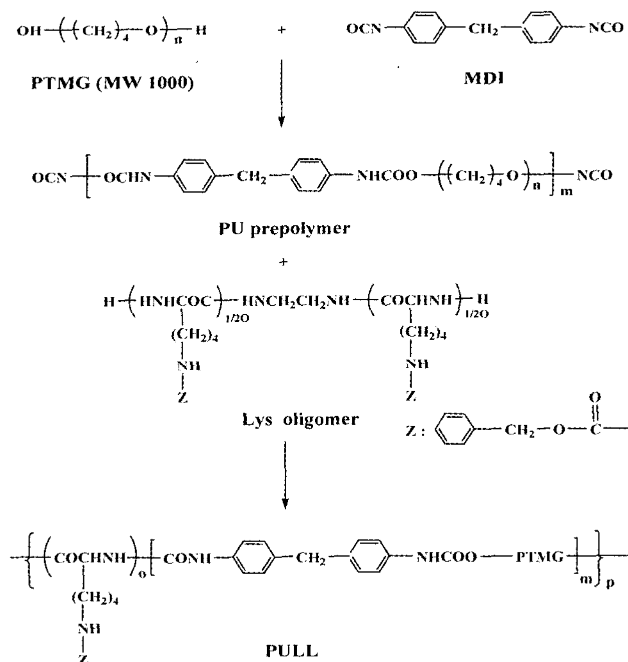

Figure 1. Synthesis of polyurethane containing L-lysine segments in the main chain (PULL).

The solution was spread on a glass plate (6×6 cm^2) and the solvent was evaporated at room temperature. The concentrated PULL solution was then irradiated by an infrared lamp to evaporate the residual solvent. The glass plate was immersed in distilled water to separate the film from the plate. Finally the film was dried under reduced pressure at room temperature for 48 h.

Immobilization of Lactobionic Acid. To introduce amine groups to the surfaces, the PULL film was immersed in a 10% HBr-acetic acid solution for 30 sec at room temperature and subsequently washed with diethyl ether, ethanol, saturated sodium bicarbonate solution, and deionized water, as shown in Figure 2. The concentration of amine groups introduced to the PULL film surface was 2.1 μmolcm^{-2} as determined using the modified fluorescamin interaction method.[19] The film was then washed with 0.01 wt% Triton X-100 aqueous solution, next with distilled water, and finally dried under reduced pressure for 24 h at 30 °C. An aqueous solution containing 1-ethyl-3-dimethylamino propyl carbodiimide (WSC) was mixed with lactobionic acid (LA) and kept at 4 °C for 5 h to activate the carboxylic acid groups of LA. In this solution, the PULL film containing primary amine groups on the surface (PULL-N) was immersed and incubated at 4 °C for 24 h to produce a lactobionic acid-grafted PULL film (PULL-L). After grafting, the film was washed with distilled water and with 0.1 wt% Triton X-100 aqueous solution in an ultrasonic cleaner filled with distilled water, and finally dried under reduced pressure for 12 h at room temperature. After immobilization of lactobionic acid on the PULL-N, the non-reacted amine groups (1.5 μmolcm^{-2}) on PULL-L film surface was determined by fluorescamine method. As the result, the concentration of immobilized lactobionic acid on the PULL film was 0.6 μmolcm^{-2}.

Surface Characterization. The surface-modified PULLs were analyzed using ESCA (ESCALAB MKII, V.G. Scientific Co., East Grinstead, UK) equipped with AlKα at 1487 eV and 300 W power at the anode. Survey scan spectra were taken and surface elemental compositions relative to carbon were calculated from peak heights with a correction for atomic sensitivity.[20] Surfaces of modified PULLs were investigated using a Midac spectrophotometer equipped with a ZnSe reflection element. The water contact angles of the modified surfaces were also measured at room temperature using a contact angle goniometer (model 100-0, Rame-Hart, NJ, USA) to evaluate the wettability of the samples.[21] Topographic images of surface-modified PULL films were examined using an atomic force microscope (AFM, Nanoscope IIIa controller, Digital instrument, Santa Barbara, CA) combined with an optical microscope.[22] The tapping mode of the AFM was employed to observe the modified surfaces. In the tapping mode, the tip oscillates with a high frequency close to its resonant frequency. In the vicinity of the surface, weak interactions can significantly change the amplitude of tip oscillations (amplitude detection) and lead to a phase shift (phase imaging). Silicon nitride cantilevers with a spring constant of 20~100 Nm^{-1} and K-type head (200×200 μm scanner) were used in air at room temperature. The parameters were chosen as follows: scan size, 0.5 μm; scan rate, 0.9 Hz; driven frequency, 270 kHz; and driven amplitude, 146~212 mV.

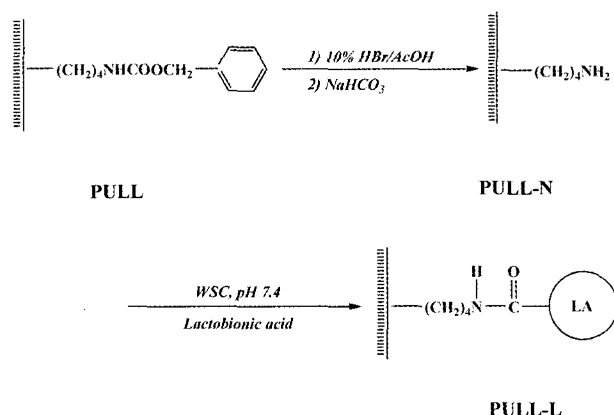

Figure 2. Schematic diagram showing surface hydrolysis and lactobionic acid grafting.

Preparation and Culture of Hepatocytes. Hepatocytes were isolated from the livers of male Sprague Dawley rats (5-6 weeks old, male, Daehan Biolink Co., Korea) by the modified collagenase perfusion technique of Seglen.[14] The viability of the isolated hepatocytes was determined by the trypan blue exclusion method. Hepatocytes with higher than 90% viability were used for the following experiments. The cell density was adjusted to 1.5×10^5/mL in Hanks' solution containing 5,000 units/mL of penicillin and 100 mg/mL streptomysin with or without 2.5% fetal bovine serum (FBS). An aliquot was seeded onto the PULL, PULL-N, and PULL-L, and maintained at 37 °C in a humidified air/CO_2 incubator (95/5 vol%). The morphology of adhered cells was observed using an inverted microscope (Nikon ECLIPSE TS100, Tokyo Japan) with a phase contrast optic.

Adhesion of Hepatocytes. In order to examine the effect of calcium ions on cell adhesion, ethylenediaminetetraacetic acid (EDTA) was added to the Hanks' balanced salt solution (HBSS, 2.5% FBS) at a concentration of 0.02 wt% and subjected to the experiments. Polymer films were placed in 24-well dishes and kept submerged by a glass ring. The cell suspension was added to each of them, shaken gently for a few seconds, and incubated in a humidified air/CO_2 incubator at 37 °C in the presence or absence of serum for 6 h. The adhesion of hepatocytes to the surface-modified PULLs was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.[23] Briefly, after culturing the hepatocytes on the modified PULLs for a requisite time, the supernatant in each dish was removed by pipetting and washed twice with phosphate buffered saline. MTT was

then added to the dish at a final concentration of 5 mg/mL, and the cells were incubated for 4 h at 37 °C. Two mililiter of acidic isopropyl alcohol were then added to each dish, and the solution was vigorously mixed to solubilize the reacted dye. The absorbance of each dish at 570 nm was measured using a micro-plate reader, Multiskan MS (Labsystems, Helsinki, Finland). The experiment was carried out in triplicate and a mean value taken. In order to measure the galactose competitive property, lactobionic acid was added in culture medium (1.5 $mgmL^{-1}$) and the morphology of hepatocytes adhered to PULL-L film surface was observed using a phase contrast optical microscope.

Results and Discussion

Surface Characterization. Figure 3 shows the attenuated total reflection-Fourier transform infrared spectra of PULL-N and PULL-L. In the case of PULL-N, a broad absorption at 3100~3400 cm^{-1} appeared possibly due to the primary and secondary amine of the film surfaces. After lactobionic acid grafting, a strong absorption appeared at around 3300 cm^{-1} due to the hydrogen-bonded hydroxyl groups of the grafted lactonates. Changes in the chemical structure of surface-modified PULLs were investigated using ESCA. Figure 4 shows ESCA survey scans of the (a) PULL, (b) PULL-N, and (c) PULL-L surfaces. As expected, the surface-modified PULLs show three peaks corresponding to C1s (binding energy, 285 eV), N1s (binding energy, 400 eV), and O1s (binding energy, 532 eV). The chemical compositions of the surface-modified PULLs calculated from the ESCA survey scan spectra are shown in Table I. The oxygen content (24.8%) of the PULL surface slightly increased after hydrolysis (29.0%), probably because of the chain rearrangement on the surfaces. On the other hand, the oxygen content of PULL-N was increased while the nitrogen content of PULL-N was slightly decreased, indicating successful lactobionic acid grafting.

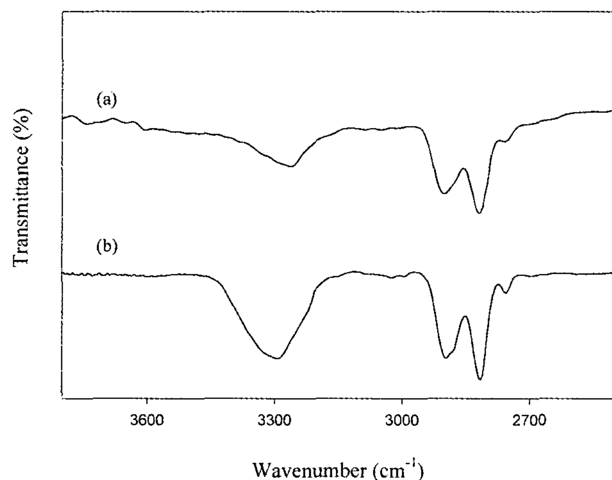

Figure 3. Attenuated total reflection-Fourier transform infrared spectra of ; (a) PULL-N and (b) PULL-L films.

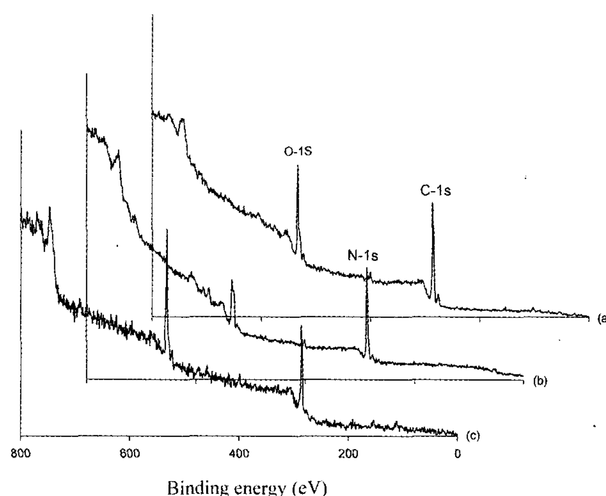

Figure 4. Electron spectroscopy for chemical analysis survey scan spectra of ; (a) PULL, (b) PULL-N, and (c) PULL-L films.

Table I. Chemical Composotion of Surface-Modified PULLs Calculated from ESCA Survey Scan Spectra

Sample	Atomic Percentage (%)		
	C	O	N
PULL	72.4	24.8	2.8
PULL-N	69.1	29.0	1.9
PULL-L	67.8	30.5	1.7

The water contact angles on the surface-modified polyurethanes in air and in water were determined and are summarized in Table II. The water contact angle of PULL film in air (69°) was largely decreased by hydrolysis reaction (48°) and further decreased by LA grafting (42°). The water contact angles of PULLs in air were slightly greater than those in water. To study the surface morphologies of PULL, PULL-N, and PULL-L, an atomic force microscope image was examined using a tapping mode and expressed as phase images. On the PULL surface, a relatively homogeneous pattern was observed as shown in Figure 5. On the PULL-N

Table II. Water Contact Angle of Surface-Modified PULL Films[a]

Sample	Water Contact Angle (°)	
	in water[a]	in air[b]
PULL	65 ± 3	69 ± 3
PULL-N	42 ± 5	48 ± 4
PULL-L	37 ± 3	42 ± 3

[a]Measured by air captive bubble method. [b]Measured by water droplet method.

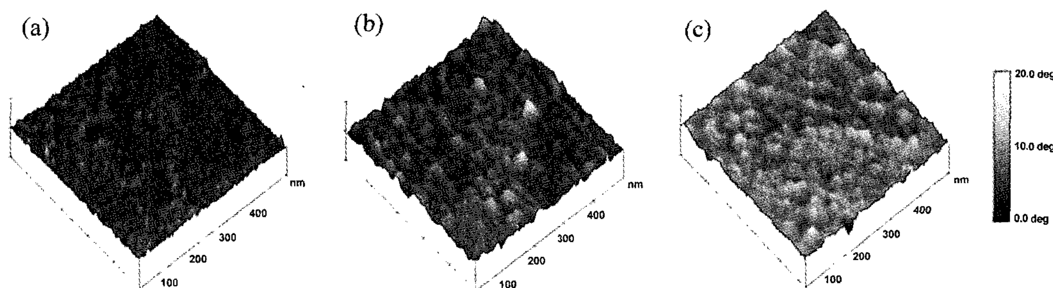

Figure 5. Atomic force microscopic images of ; (a) PULL, (b) PULL-N, and (c) PULL-L films represented by phase mode.

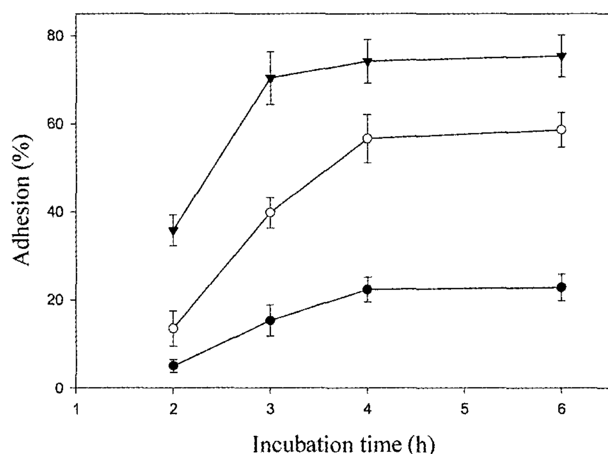

Figure 6. Adhesion of rat hepatocytes to the surface of PULL (●), PULL-N (○), and PULL-L (▼) films in the absence of serum as a function of incubation time.

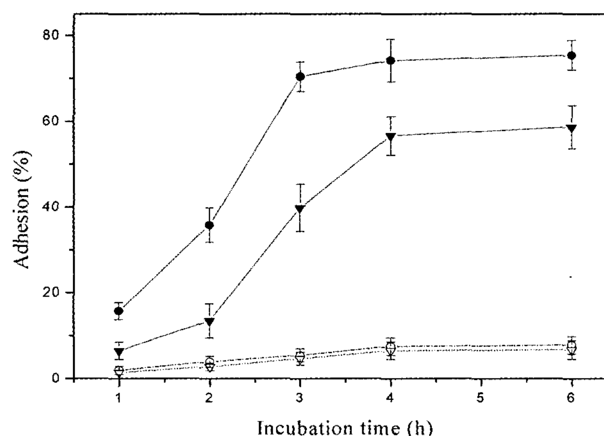

Figure 7. Effect of calcium ion on the adhesion of hepatocytes to surface of PULL-N (with ▼ , without ▽) and PULL-L (with ●, without ○) Films.

(Figure 5(b)), a phase-separated structure appeared, showing the aggregation of poly(L-lysine) segments on the surface. On the other hand, after LA grafting, a large part of the surface was covered by LA and the surface roughness decreased, as shown in Figure 5(c).

Adhesion of Hepatocytes. Figure 6 shows the experimental results of hepatocyte adhesion to surface-modified PULLs in the absence of serum. The ordinate represents the ratio of cells adhered relative to the cell number in an aliquot of the cell suspension. Cell adhesion to the modified PULLs increased gradually up to 4 h incubation and, thereafter, almost maintained same value. Hepatocytes were largely adhered to the LA-grafted PULL while poorly adhered to the PULL. On the other hand, hepatocytes were intermediately adhered to the PULL-N surface. In order to examine the effect of calcium ions on cell adhesion, hepatocytes were cultured on surface-modified PULLs for 1~6 h in the absence or presence of EDTA. Cell adhesion was highly suppressed in the absence of calcium, irrespective of the kind of substrate (PULL-L, ○; PULL-N, ▽), as shown in Figure 7. However, in the presence of calcium ions, hepatocytes were adhered to the PULL-N and further adhered to the PULL-L. The morphology of the cells adhered to surface-modified PULLs for 6 h in the presence of serum was observed using a scanning electron microscope and the results are shown in Figure 8. As shown in Figure 8, cells did not much adhere well to the PULL (a) and only intermediately adhered to the positively charged surface of PULL-N (b). On the other hand, cells were densely adhered to the surface of LA-grafted PULL, showing a specific interaction of the β-galactose moieties on the surface and the asialoglycoprotein receptors of hepatocyte membrane. To examine the characteristics of cell aggregation, hepatocytes were cultured on surface-modified PULLs in the presence of serum and epidermal growth factor for 48 h and the results are shown in Figure 9. The cells were partially aggregated on the surface of PULL and PULL-N while the cells were greatly aggregated on the surface of PULL-L. The effective aggregation of hepatocytes on the PULL-L seemed due to the mediation by β-galactose moieties grafted on the surfaces. In order to measure the galactose competitive character, hepatocytes were cultured on PULL-L film for 6 h after the addition of lactobionic acid and their results were shown in Figure 10. As shown in Figure 10, the adhesion of hepatocytes was highly suppressed in the presence of lactobionic acid. This is because the asialoglycoprotein receptors of hepatocyte membrane were blocked by β-galactose moieties.

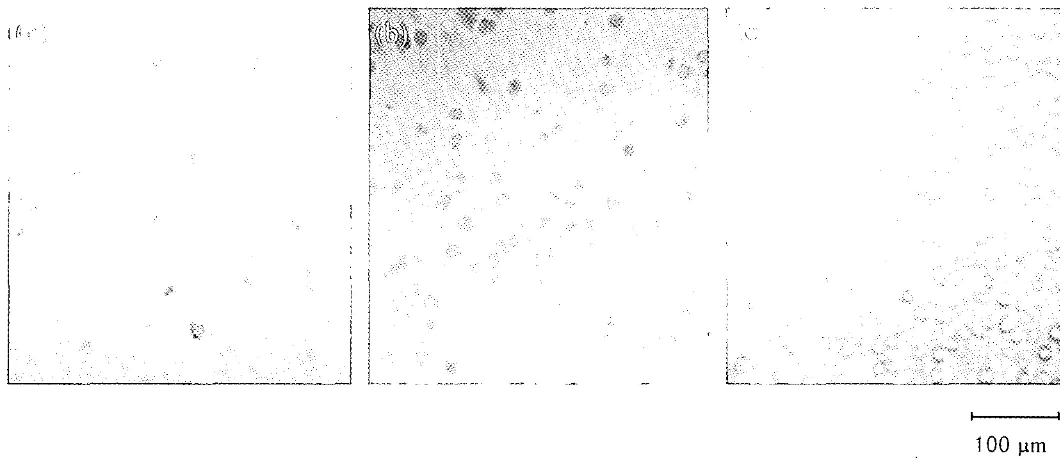

Figure 8. Phase contrast micrographs of hepatocytes cultured for 6 h in the presence of fetal calf serum ; (a) PULL, (b) PULL-N, and (c) PULL-L.

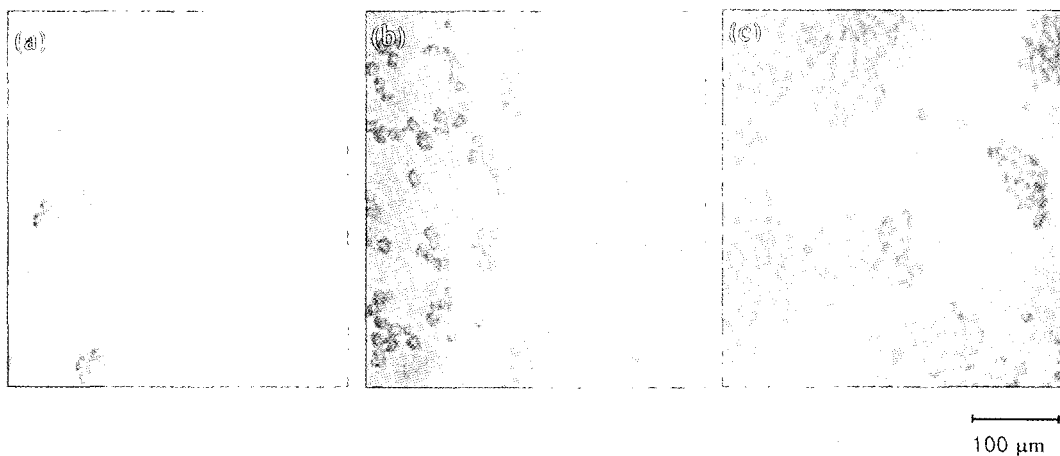

Figure 9. Phase contrast micrographs of hepatocytes cultured for 48 h in the presence of epidermal growth factor; (a) PULL , (b) PULL-N, and (c) PULL-L.

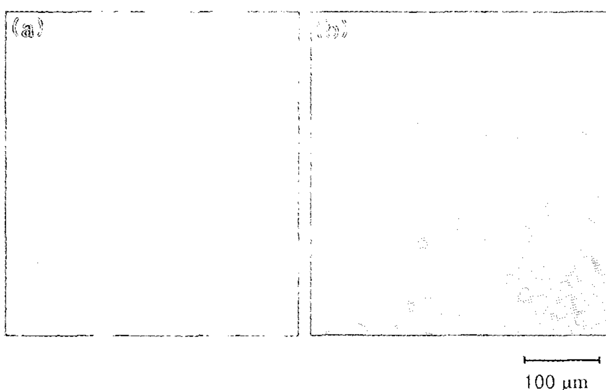

Figure 10. Adhesion of hepatocytes to PULL-L surface for 6h in the presence (a) and absence (b) of lactobionic acid.

Hepatocytes adhere to culture dishes coated with adhesive proteins of the ECM such as fibronectin (Fn)[22] and collagen (Coll),[23] and with glycopolymer such as poly(*N*-*p*-vinylbenzyl-4-O-*β*-*D*-galactopyranosyl-*D*-gluconamide) (PVLA)[24] and poly(*N*-*p*-vinylbenzyl-1,2-*D*-glucuronamide)(PV6Gna).[14] Cells exhibit different morphology, growth, and differentiated function depending on the matrix conditions. For example, cells do not spread on a low density of Fn or Coll and the round morphology is associated with a differentiated phenotype as measured by the secretion of liver specific proteins such as albumin and fibrinogen. Kobayashi *et al.*[25] investigated the effect of the surface density of PVLA substratum on the differentiation and proliferation of hepatocytes, and reported that at low PVLA densities (0.07 μgcm^{-2}) the adhered hepatocytes were flat and expressed high levels of ^{3}H-thymidine uptake and low levels of bile acid secretion, while at high PVLA densities (1.08 μgcm^{-2}) the cells were round and expressed a low level of ^{3}H-thymidine uptake and a high level of bile acid secretion.

Since the *β*-galactose moieties were found to be a ligand for

hepatocyte adhesion, several attempts to immobilize them on synthetic polymers such as polystyrene,[26] poly(lactic-*co*-glycolic acid)(PLGA),[27] and poly(allylamine)[28] or natural polymers such as alginate[29] and chitosan[30] have been performed. Many oligosaccharide-carrying styrene homopolymers, in which each vinylbenzyl main chain is bound to an oligosaccharide moiety through an amide linkage, have been reported by Kobayashi *et al.*[26] In our previous study, we prepared photosensitive poly(allylamine) containing β-galactose moieties in the side chain ($LPAN_3$) and examined co-culture of hepatocytes and fibroblasts by micropatterned immobilization of β-galactose derivatives. As a result, hepatocytes and fibroblasts adhered to the $LPAN_3$ and PMMA lane, respectively.

Polyurethane has been frequently used as a substrate for high density culture of hepatocyte in the form of nonwoven[31] or interconnected foam.[32,33] To improve the specific interaction of hepatocytes with polyurethane substrates, the surface needs to be modified with cell attachment factors or cell specific ligands. Sato *et al.*[34] coated PVLA on the surface of reticulated polyurethane (RPU) and reported that the cells were attached to the surface of the PVLA-RPU and formed multicellular spheroids in the reticulated pores. Yang *et al.*[35] prepared gelatin-immobilized polyurethane foams and cultured rat hepatocytes on their surfaces. The results showed that the amount of hepatocytes seeded on PUFG1, prepared by using a 1% aqueous gelatin solution, was higher than that on other PUFGs. In the current study, lactobionic acids (LA) were covalently coupled with primary amine groups generated on the PULL surface. As shown in Figure 6, hepatocytes were significantly adhered to LA-grafted PULL surface compared to positively charged PULL surface and PULL control, indicating a specific interaction between β-galactose moiety on the surface and asialoglycoprotein receptor (ASGPR) of hepatocyte membrane.

The morphology of hepatocytes cultured on films and in non-woven fabrics of hyaluronic acid esters has been reported.[36] Cells adhering to the film made of the benzyl ester of Hyal, 100% degree of substitution, spread, flattened to a large extent, and exhibited more protrusions than those on the ethylester of Hyal, 100% degree of substitution. Cell-cell interaction and cell morphology play important roles in highly differentiated tissues.[37] Cell aggregation may permit reconstruction of the cell-cell contact and communication that is observed in the original tissue.[38] Guo *et al.*[39] prepared the encapsulated hepatocytes with sodium alginate and cultured them in a CO_2 incubator. They reported that cell aggregation was accelerated by the addition of galactosylated chitosan as a synthetic extracellular matrix. In experiments involving hepatocytes adhesion using polyurethane containing lactose groups, it was found that the cells specifically recognized β-galactose moieties, thus leading to good adhesion on the surfaces, as shown in Figure 8(c). Moreover, the cells were more aggregated on the LA-grafted surface than on the other surfaces (Figure 8). These results suggest that polyurethanes containing L-lysine segments in the main chain (PULL) are a good candidate as a coating material for improving hepatocytes adhesion.

Conclusions

Polyurethanes containing z-lysine segments in the main chain (PULL) were synthesized from 4,4′-diphenylmethyl diisocyanate, poly(tetramethylene glycol), and z-lysine oligomer as a chain extender. Lactobionic acid-immobilized PULL (PULL-L) film was prepared by a hydrolysis reaction of the film with a hydrobromide-acetic acid solution, followed by a coupling reaction with lactobionic acid. Rat hepatocytes highly adhered to PULL-L and were effectively aggregated on the surface in the presence of epidermal growth factor, suggesting a good affinity of the surface β-galactose moieties for hepatocytes.

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