Grafting of lactose-carrying styrene onto polystrene dishes using plasma glow discharge and their interaction with hepatocytes

INN-KYU KANG*, DONG WOO LEE, SEUNG KYUNG LEE
Department of Polymer Science, Kyungpook National University, Taegu 702–701, Korea
TOSHIHIRO AKAIKE
Department of Biomolecular Engineering, Tokyo Institute of Technology, Yokohama 227, Japan
E-mail: ikkang@bh.knu.ac.kr

Lactose-carrying styrene (VLA)-grafted polystyrene (PS) dish (PS-VLA) was prepared by treatment of PS dish with oxygen plasma glow discharge followed by the graft polymerization of VLA. The surface topology and hepatocytes behavior on PS-VLA were examined by comparison with those on a PVLA-coated PS dish (PS-PVLA). According to the results of surface topologies obtained by a phase mode of atomic force microscope (AFM), it was found that PS-VLA exhibits a pointed texture image similar to forest while PS-PVLA exhibits a phase-separated, cloud-like image. In an experiment involving hepatocytes adhesion, the cells more slowly adhered to PS-VLA than to PS-PVLA during the first 2 h incubation. According to topological data, it may be suggested that lactose density on the air side surface of PS-VLA is lower than that of PS-PVLA, thus leading to the slow adhesion of hepatocytes to PS-VLA.

1. Introduction

Proliferation and differentiation of hepatocytes are regulated not only by growth factors [1, 2] such as insulin, epidermal growth factors, and hepatocyte growth factors, but also by extracellular matrices [3, 4] such as collagen, laminin, and fibronectin. Among extracellular matrices, collagen has been most frequently used for the control of spreading, migration, adhesion, and proliferation of hepatocytes. Collagen is the most abundant protein in the animal kingdom and may constitute 25% of the total protein of a mammal. However, it is of limited use as a coating material for polymeric surfaces because of poor solubility.

On the other hand, Kobayashi *et al*. [5] have synthesized poly[*N*-*p*-vinylbenzyl-4-*O*-β-D-galactopyranosyl-D-gluconamide (PVLA), which is soluble in water as well as organic solvents such as dimethylsulfoxide, and have reported that PVLA shows a good affinity for hepatocytes. They have also investigated the effect of the surface density of PVLA substratum on the differentiation and proliferation of hepatocytes, and reported that, at low PVLA densities ($0.07\,\mu g\,cm^{-2}$), 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 ($1.08\,\mu g\,cm^{-2}$), the cells were round and expressed a low level of ^{3}H-thymidine uptake and a high level of bile acid secretion [6]. Cho *et al*. [7] studied the orientation effect of galactose ligand on hepatocyte adhesion using PVLA as a model ligand of asialoglycoprotein. They transferred PVLA onto poly(γ-benzyl L-glutamate) (PBLG) or PBLG/poly(ethylene glycol) (PEG) Langmuir–Blodgett (LB) films and reported that hepatocytes recognized well-oriented galactose moieties of the transferred PVLAs through asialoglycoprotein receptors.

Plasma can create radicals on polymeric surfaces without any remarkable defect. These radicals are easily converted into peroxides when exposed to air. The peroxides generated on the surfaces can be used as the initiating sites for the graft polymerization of functional monomers such as acrylic acid, acrylamide, and glycidylmethacrylate [8, 9].

In this study, an *N*-*p*-vinylbenzyl-4-*O*-β-D-galactopyranosyl-D-gluconamide (VLA)-grafted polystyrene dish (PS-VLA) was prepared by the treatment of PS dish with oxygen plasma glow discharge followed by graft polymerization of VLAs. The surface characteristics of PS-VLA were compared with those of PVLA-coated PS (PS-PVLA) using an electron spectroscope for chemical analysis (ESCA), an atomic force microscope (AFM),

*Author to whom all correspondence should be addressed.

and a water contact angle goniometer. Hepatocyte behaviors on both surfaces of PS-VLA and PS-PVLA were also examined.

2. Experimental

2.1. Materials

A non-treated polystyrene (PS) dish with a 35 mm diameter was purchased from Iwaki Company in Japan and used for the experiments of surface treatment. VLA used in this study was synthesized according to a previously reported method [10]. Briefly, lactonolactone (15.4 g, 45 mmol) was dissolved in refluxing methanol and mixed with a *p*-vinylbenzylamine (6.0 g, 45 mmol) solution in methanol. The mixed solution was refluxed with magnetic stirring for 2 h and allowed to stand at room temperature to yield a white crystal. The crystal was filtrated, washed with a small amount of cold methanol, and dried in a vacuum. Finally, it was purified by recrystallization from methanol.

2.2. Treatment of oxygen plasma glow discharge

A plasma reactor consists of a stainless-steel chamber (ca. 8l volume) with a pair of stainless-steel discharge electrodes as reported previously [11]. The upper electrode (diameter: 12 cm) was connected to a 13.56 MHz radio frequency generator via an impedance matching circuit and the lower electrode (diameter: 12 cm) was grounded. The system pressure before discharge was monitored by a Hoyt thermocouple vacuum gauge (model PT2500) connected to the downstream of the reactor. Oxygen flow rate was measured using a mass flow controller (Stec Inc., Kyoto, Japan, Model EC-400 MK3). Oxygen plasma treatment of the PS dish was carried out according to the previous report [11].

2.3. Grafting of VLA and collagen

A schematic diagram showing grafting of VLA and collagen on PS surface is illustrated in Fig. 1. VLA aqueous solution (10 mg/2 ml) was poured into a vial and kept for 30 min in a nitrogen atmosphere. Sodiumpyrosulfate, $Na_2S_2O_5$ (2 mg), was then dissolved in the same vial and subsequently transferred to the oxygen plasma-treated PS dish and incubated at 25 °C for 24 h to obtain VLA-grafted PS dish (PS-VLA). After graft polymerization, the dish was washed with distilled water and with 0.1 wt % Triton X-100 (Sigma, St. Louis, MO, USA) aqueous solution in an ultrasonic cleaner for 2 min to remove any free homopolymers (PVLAs) that had been formed. The dish was further washed using an ultrasonic cleaner filled with distilled water, and finally dried under reduced pressure for 12 h at room temperature.

A collagen-grafted PS dish was prepared as follows (Fig. 1). Ten weight percent aqueous acrylic acid (3 ml) containing sodiumpyrosulfate (3 mg) was degassed with nitrogen, transferred to the oxygen plasma-treated PS dish, and incubated at 25 °C for 24 h. The dish was then washed using the same method as described for the

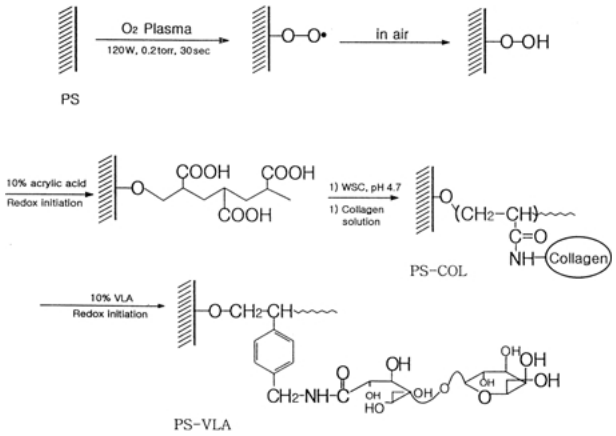

Figure 1 Schematic diagram showing the formation of collagen-immobilized (PS-COL) and VLA-grafted polystyrene (PS-VLA) dishes.

preparation of PS-VLA. An aqueous solution containing 1-ethyl-3-dimethylamido propyl carbodiimide (WSC) was added to the acrylic acid-grafted PS dish (PS-C) and kept at 4 °C for 5 h to activate the carboxylic acid groups on the surface. The PS dish was then washed gently with double distilled water, subsequently contacted with a sodium citrate aqueous solution (2 ml) containing 2 mg of collagen (Type III, acid soluble), and incubated at 4 °C for 24 h to produce a collagen-grafted PS dish (PS-COL). After grafting, the dish was washed using the same method as described for the preparation of PS-VLA.

To obtain PVLA, a VLA monomer (2.37 g) and azobisisobutyronitrile (4 mg) were mixed in a glass ampoule containing dimethylsulfoxide (5 ml). The temperature of the mixed solution was lowered below − 40 °C and degassed using a rotary pump. The solution was then incubated at 60 ± 1 °C for 6 h. The final product was poured into methanol (50 ml) to precipitate PVLA, filtrated by a glass filter, and dried under vacuum. Two milliliters of PVLA aqueous solution (50 μg/ml) was added to a PS dish (diameter: 35 mm) and kept for 1 h at room temperature to prepare the PVLA-coated PS dish (PS-PVLA) [6]. Unused PVLA solution remaining in the dish was removed by pipetting and washed with distilled water twice.

2.4. Surface characterization

Chemical compositions of surface-modified PSs were examined using electron spectroscopy for chemical analysis (ESCA, VG Microtech. MT500/1 etc., UK) equipped with AlKα at 1487 eV and a 300 W power at the anode. A survey scan spectrum was taken and the surface elemental compositions relative to carbon-1s were calculated form the peak height with a correction for atomic sensitivity [12]. To evaluate surface wettability, the water contact angles of the surface-modified PS dishes were measured at room temperature using a contact angle goniometer (model G-I, Erma Inc., Tokyo) [13]. Topographic images of surface-modified PS dishes were examined using an atomic force microscope (AFM, Nanoscope IIIa controller, Digital instrument, Santa Barbara, CA) combined with an optical microscope [7].

Tapping mode of 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 $20 \sim 100\,\mathrm{N\,m^{-1}}$ and K-type head ($200 \times 200\,\mu\mathrm{m}$ 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; driven amplitude, $146 \sim 212$ mV.

2.5. Preparation and culture of hepatocytes

Hepatocytes were isolated from the livers of male Sprague Dawley rats (150–200 g, 4–6-weeks-old) 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 Williams' E medium (Sigma) containing 5000 units/mL of penicillin and 100 mg/mL streptomycin (Sigma) with or without 2.5% fetal bovine serum (Sigma). An aliquot was seeded onto the PS-VLA, PS-PVLA and PS-COL, and maintained at 37 °C in a humidified air/CO_2 incubator (95/5 vol %). The morphology of adhered cells was observed with a phase contrast microscope (Olympus IM).

2.6. 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) at a concentration of 0.02 wt % and subjected to the experiments. The primary hepatocytes were treated with the medium in a humidified air/CO_2 incubator at 37 °C for 60 min and then plated and cultured on PS-VLA, PS-PVLA and bovine serum albumin-blocked PS dishes (diameter: 35 mm) for $0.5 \sim 4$ h. The adhesion of hepatocytes to the surface-modified PS dishes was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [15]. Briefly, after the culture of hepatocytes on modified PS dishes for a requisite time, the supernatant in each dish was removed by pipetting and washed with phosphate buffered saline twice. 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 ml of acidic isopropyl alcohol was 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 duplicate and a mean value taken.

3. Results

3.1. Surface characterization

The changes in chemical structure of surface-modified PSs were examined using ESCA. Fig. 2 shows ESCA survey scans of the PS and surface-modified PSs. The modified PSs showed three peaks corresponding to C1s (binding energy, 285 eV), N1s (binding energy, 400 eV), and O1s (binding energy, 532 eV). Table I represents the atomic percent of modified PS surfaces calculated from ESCA survey scan spectra. The oxygen content of PS dish surface (6.6%) was largely increased by the grafting of acrylic acid (16.7%), collagen (15.4%) and VLA (14.8%), and further increased by coating with PVLA (21.9%). The nitrogen content of the surface of PS-COL, PS-VLA and PS-PVLA was 9, 1.6 and 1.9%, respectively. The high nitrogen content of PS-COL surface is due to the peptide bond (–CONH–) of collagen.

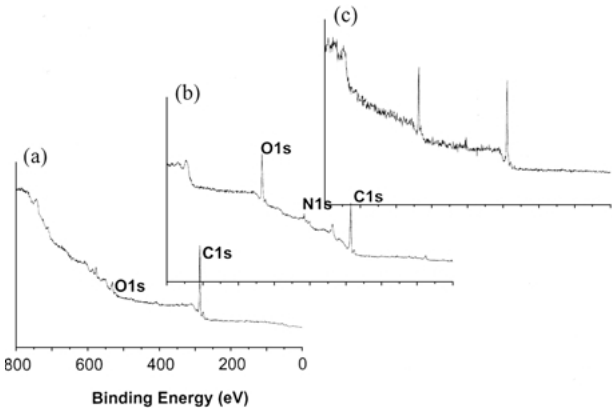

Figure 2 ESCA survey scan spectra of modified polystyrene dishes: (a), PS; (b) PS-VLA; (c) PS-PVLA.

The water contact angle of surface-modified PSs is shown in Table II. The water contact angle of PS (85°) was largely decreased by the introduction of acrylic acid (PS-C, 50°), collagen (PS-COL, 35°), VLA (PS-VLA, 42°) and PVLA (PS-PVLA, 41°). This result indicates that the PS dish became more wettable after surface modification [16].

TABLE I Chemical composition of surface modified PSs calculated from ESCA survey scan spectra

Substrate	Atomic (%)		
	C	O	N
PS	93.4	6.6	
PS-C	83.3	16.7	
PS-COL	75.6	15.4	9
PS-VLA	83.6	14.8	1.6
PS-PVLA	79.2	21.9	1.9

TABLE II Water contact angle of surface modified PS dishes

Substrate	Water contact angle (°)
PS	85 ± 1
PS-C	50 ± 2
PS-COL	35 ± 3
PS-VLA	42 ± 2
PS-PVLA	41 ± 1

3.2. Cell adhesion

In order to examine the effect of calcium ions on cell adhesion, hepatocytes were cultured on surface-modified PSs for 0.5–4 h in the absence and presence of EDTA. Cell adhesion was highly suppressed in the absence of calcium, irrespective of the kind of substrate (PS, ○; PS-VLA, △; PS-PVLA, □), as shown in Fig. 3. In the presence of calcium ions, hepatocytes were much adhered to the PS-VLA (▲) and PS-PVLA (■) surfaces, and were less adhered to the PS (●) surface. During 30 min culture, about 23% of the cells adhered to the PS-VLA and, thereafter, gradually increased, reaching 84% after 4 h. In the case of PS-PVLA, cell adhesion was about 69% after 30 min culture, reaching 88% after 4 h. The different pattern of cell adhesion during the initial stage of culture seems attributable to the fact that the density and morphology of β-galactose moieties introduced to the surface of PS-VLA differ from those of PS-PVLA. On the other hand, the amount of cells adhered to the PS dish blocked with bovine serum albumin was below 22%, showing no specific interaction of the cells with the surface.

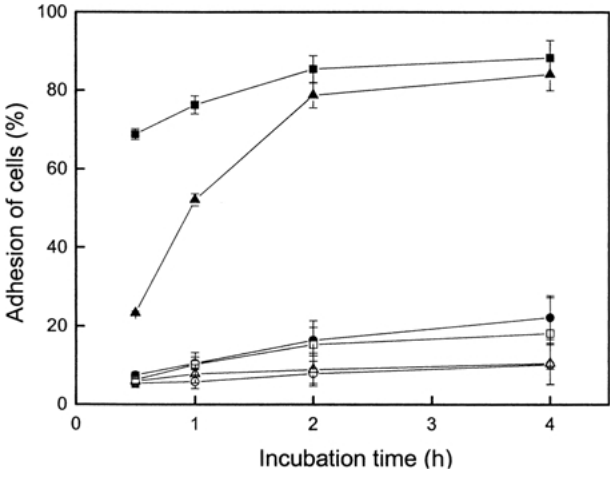

Figure 3 Adhesion of mouse hepatocytes to the surface of PS (○), PS-VLA (△) and PS-PVLA (□) in the absence of calcium ion and PS (●), PS-VLA (▲) and PS-PVLA (■) in the presence of calcium ion as a function of incubation time. Data expressed as mean ± S.D. ($n = 2$).

Fig. 4 shows the morphology of adherent hepatocytes for 4 h culture onto the surface-modified PS dishes with a phase-contrast microscope. Hepatocytes cultured on the PVLA-coated dish (PS-PVLA) were observed as round shapes while those on the VLA- and collagen-grafted PS dishes (PS-VLA, PS-COL) were observed as spreading ones. Similar results has been reported by Akaike *et al.* [6]

To study the surface morphologies of PS-VLA and PS-PVLA, an atomic force microscope (AFM) image was examined using a tapping mode and expressed as phase images. On the PS surface, a homogeneous pattern was observed as shown in Fig. 5. On the PS-VLA, a pointed texture similar to a forest was observed. This result suggests that VLA monomers were perpendicularly grown on the PS surface treated with oxygen plasma glow discharge. On the other hand, on the PS-PVLA surface, a phase-separated and cloud-like structure appeared, showing an aggregation of PVLA on the surface during solvent evaporation.

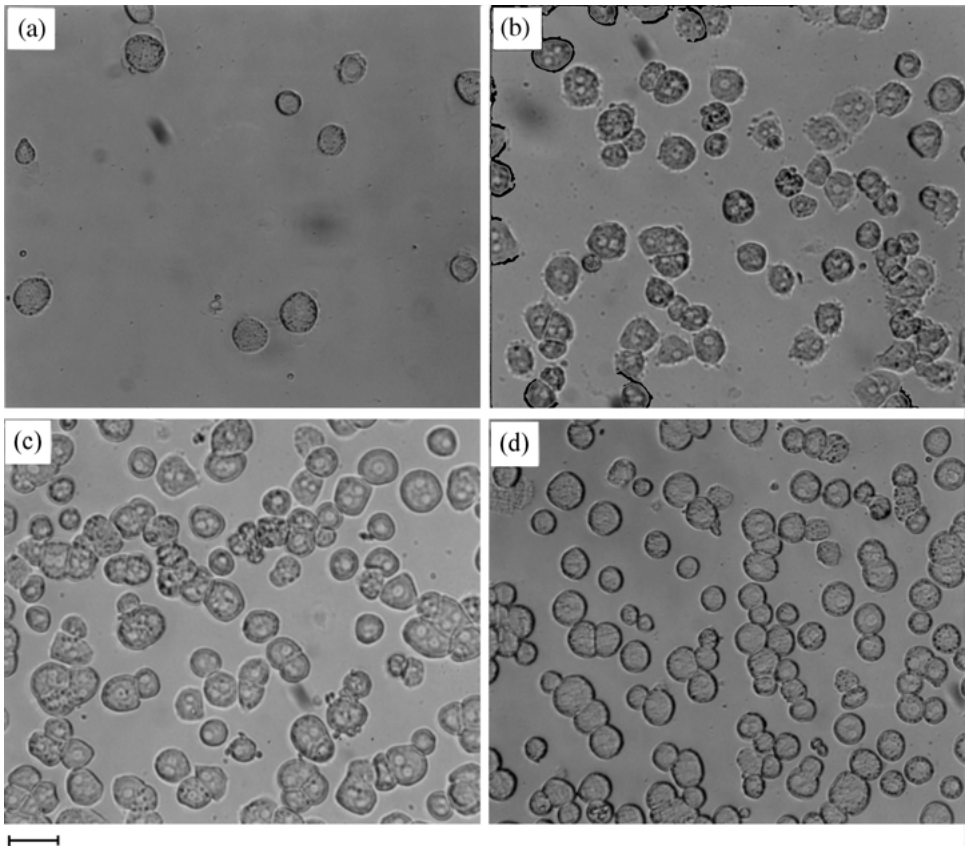

Figure 4 Phase-contrast micro photographs of mouse hepatocytes cultured for 4 h: (a) PS; (b) PS-COL; (c) PS-VLA; (d) PS-VLA (bar = 20 μm).

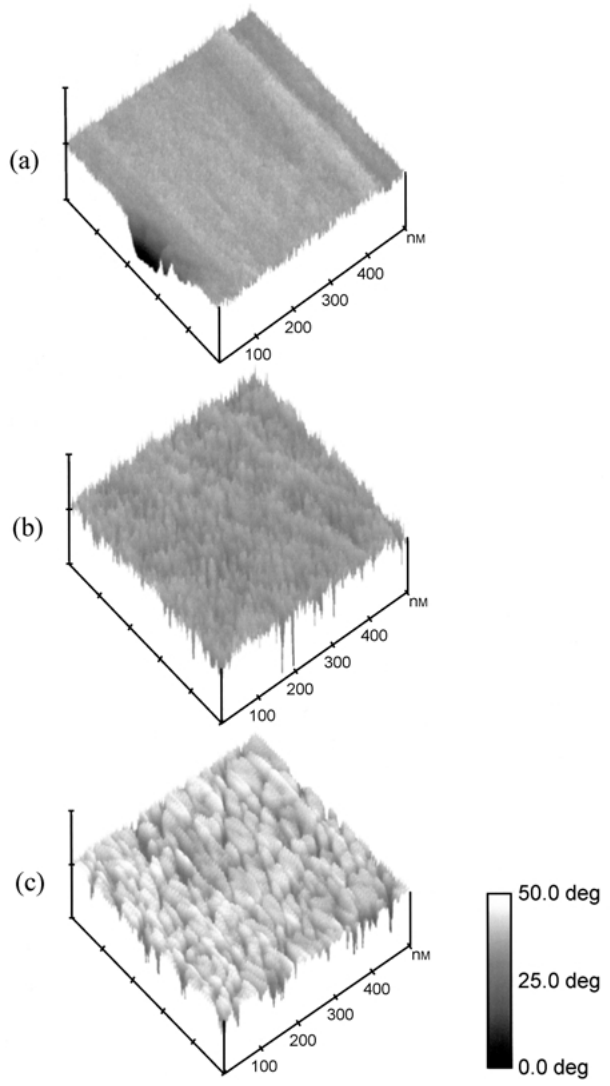

Figure 5 AFM images represented by phase mode: (a) PS; (b) PS-VLA; (c) PS-PVLA.

4. Discussion

In this study, VLA-grafted PS dishes were prepared by graft polymerization of VLAs. We examined their surface properties and interaction with hepatocytes in comparison with PVLA-coated PS dishes. It was found, from the ESCA result (Table I), that the PS-PVLA surface showed a higher oxgen content (21.9%) than the PS-VLA (14.8%). This result indicates that PS-PVLA has higher β-galactose density on the surface than PS-VLA. Akaike *et al*. [17] reported that PVLA is an artificial cellular matrix for hepatocyte culture. They proposed that adhesion is mediated by β-galactose-specific interactions between hepatocytes and PVLA [5]. The specific interaction between hepatocytes and β-galactose moieties on the polymer surface are greatly affected by ligand properties such as ligand density [17], ligand distribution [18], and ligand orientation [7]. Adachi *et al*. [19] prepared particles coated with different ligand densities using a mixture of PVLA and poly(vinylbenzyl-D-gluconamide) (PVGA), and reported that particles with low ligand densities associated more with hepatocytes than did high ligand density particles. In their results, most of the particles bearing high ligand densities were found inside the cells, whereas particles with low ligand densities were found on the plasma membrane surface of the hepatocytes.

As shown in Fig. 3, in the absence of calcium ions, hepatocytes have no specific interaction with PS-VLA and PS-PVLA during culture time. This result is consistent with the report by Kim *et al*. [20] that the adhesion of hepatocytes is dependent on Ca^{2+}. On the contrary, in the presence of calcium ions, the adhesion of cells to PS-VLA and PS-PVLA for 4 h was over 80%. However, cell adhesion to the PS dish was below 23%, independent of the presence of calcium ions. These results indicate that hepatocytes were largely adhered to the PS-VLA and PS-PVLA via the bio-specific interaction of asialoglycoprotein receptors of the membrane and β-galactose moieties of the PS surface. On the other hand, hepatocytes more rapidly adhered to the PS-PVLA surface than to the PS-VLA (Fig. 3). The different adhesion pattern between the two surfaces seems to originate from the difference in surface topology and β-galactose density. As described in Table I, the PS-PVLA surface has higher oxygen content (21.9%) than PS-VLA (14.8%). This result indicates that β-galactose density on PS-PVLA is higher than on PS-PVLA. Kobayashi *et al*. [6] prepared PS dishes with different PVLA densities (0–1.08 $\mu g\,cm^{-2}$) by changing the concentration of the PVLA coating solution. In their results, PVLA molecules were adsorbed in dispersed clusters on a low density surface and uniformly concentrated over the dish on a high density surface. Wataoka *et al*. [21] studied the conformation of PVLAs by combining the small-angle X-ray scattering and the molecular simulation, and reported that PVLA was found to have the shape of a molecular bottlebrush in aqueous solution, composed of a large pseudo helix of polystyrene backbone. They also proposed that the backbone conformation seems to be determined not by the excluded volume effect of side chains, but by the amphiphilic character of the backbone (hydrophobic) and side chains (hydrophilic).

As revealed by the AFM results (Fig. 5), the PS-PVLA surface showed pointed texture images while PS-PVLA showed bumpy, cloud-like structures. Fig. 6 represents a

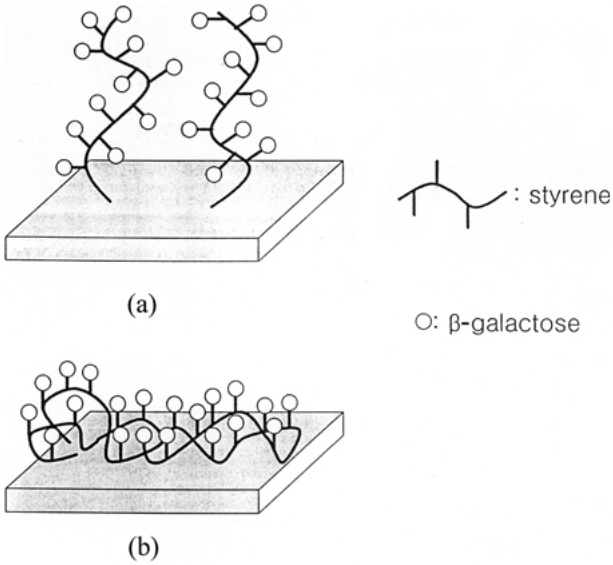

Figure 6 Proposed models showing the chain arrangement of PVLA on the surfaces: (a) PS-VLA; (b) PS-PVLA.

model scheme showing the spatial arrangements of the PS-VLA and PS-PVLA, which was constructed based on the AFM image of the surfaces. It is considered that VLA monomers are grown perpendicularly via initiating sites on the PS surface during gaft polymerization. Meanwhile, styrene moieties of PVLAs are preferentially adsorbed on the PS dish surface while lactose moieties are aggregated and exposed to the air surface during PVLA coating. Therefore, the slow adhesion of hepatocytes to PS-VLA during first 2 h incubation seems due to the low density of β-galactose on the air side, as illustrated in Fig. 6.

Acknowledgment

This work was partially supported by the KOSEF through the Biomolecular Engineering Center at Kyungpook National University.

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Received 13 June
and accepted 17 October 2002