In vitro blood compatibility of heparin-immobilized polyurethane containing ester groups in the side chain

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In a previous study, we reported on the synthesis of heparin-immobilized polyetherurethanes containing ester groups in the side chain. In this study, the blood compatibility of heparin-immobilized polyurethanes was investigated using *in vitro* plasma recalcification time (PRT), activated partial thromboplastin time (APTT), platelet adhesion and activation and peripheral blood mononuclear cell (PBMC) adhesion and activation. In the experiment with plasma proteins, the PRT of the polyurethane (PU) surface was prolonged by polyethylene oxide (PEO) grafting and further prolonged by heparin immobilization. The APTT was prolonged on the PU–C–H and PU–P–H, suggesting the binding of immobilized heparin to the antithrombin III. The percentage of platelet adhesion on the PU was almost the same as that on carboxylic acid-introduced PU (PU–C), but was slightly decreased by PEO grafting and further decreased by heparin immobilization. The release of serotonin from the adhering platelets was slightly suppressed on the PEO-grafted PU yet significantly suppressed on the heparin-immobilized PUs. In the PBMC experiments, the adhesion and activation of the cells were significantly suppressed on the heparin-immobilized PUs, and the amount of interleukin-6 (IL-6) released from the PBMCs stimulated with the surface-modified PUs decreased with a decrease in the PBMC adhesion.

1. Introduction

Segmented polyurethane (PU) consisting of hard and soft segments shows not only relatively good flexibility, fast recovery and fatigue resistance, but also relatively good *in vitro* blood compatibility when compared to other synthetic polymer films [1]. Accordingly, PU has often been used as the basic material of blood-contacting devices such as catheters [2], heart assisting pumps [3] and artificial hearts [4]. The blood compatibility of PU, however, needs to be substantially improved to enable a wider biomedical application. The most efficient method to improve the blood compatibility of the PU membrane is heparinization. The immobilization of heparin on the PU membranes can be carried out in three ways; physical adsorption [5], ionic [6, 7] and covalent bonding [8, 9]. Barbucci *et al*. [6] have developed a method of grafting poly(amidoamine) on to PUs using hexamethylene diisocyanate (HMDI) as the spacer, thereby providing ionically heparinizable surfaces. Many investigators have focussed on the covalent bonding of heparin using functional groups. Ito *et al*. [9] and Kang *et al*. [10] prepared PU containing hydrolysable ester groups in the side chains, introduced carboxyl groups to the PU surfaces by aqueous alkaline treatment, and then coupled them with heparin. Han *et al*. [11] and Park *et al*. [12] also studied the surface modification of PUs for long-term biomedical application and used polyethylene oxide (PEO) grafting and/or heparin immobilization.

Although the blood compatibility of PU has been improved to a certain extent by heparin, one limitation still remains, which is the restricted coupling for heparin immobilization. Kim *et al*. [13] have attempted a novel method to increase the surface concentration for anti-coagulating activity. They grafted a polyfunctional compound, polyethylene imine, on a PU surface through diisocyanate groups, introduced a PEO spacer on to the grafted surface, and then covalently immobilized heparin on to the end group of the grafted PEO. The plasma glow

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discharge treatment is also one of the methods to increase the surface concentration of an immobilizing site. Kang *et al*. [14] introduced a large amount of carboxylic acid groups to the PU surfaces using an oxygen plasma treatment followed by a graft polymerization of acrylic acid, and noted that the carboxylic acid groups, which were introduced, were useful in their reaction with biological molecules such as heparin and insulin.

When blood comes into contact with a foreign material, the plasma proteins are quickly adsorbed onto the material surface, followed by the activation of the platelets, and finally the formation of a fibrin network, that is, thrombus formation [15]. The interaction of the adhered cells with the implanted foreign materials are believed to affect the overall biocompatibility and thereby determine the long-term effectiveness of the implant [16]. The host response to an implant results in acute and chronic inflammatory responses. Cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6), and the tumor necrosis factor (TNF) can be released from the peripheral blood mononuclear cells (PBMCs) when the cells are stimulated with foreign materials [17]. These cytokines have often been used as a measure to evaluate the activation of PBMCs.

The aim of this study was to examine the interaction of blood components with the synthesized PEO-grafted and heparin-immobilized PUs [10]. The blood interaction with the surface-modified PUs was investigated using plasma recalcification time (PRT), activated partial thromboplastin time (APPT), platelet adhesion and activation, and PBMC adhesion and activation.

2. Materials and methods

2.1. Surface modification of PU containing ester groups in the side chain

The methods for the preparation of the PEO-grafted and heparin-immobilized PUs have been previously described in detail [10]. Briefly, the PU was synthesized from 4,4′-diphenylmethanediisocyanate (MDI), poly(tetramethylene glycol), and diethyl bis(hydroxymethyl)malonates (DBM) as a chain extender in a mole ratio of 2 : 1 : 1 as shown in Fig. 1. The film was prepared using the solvent cast method with a 10 wt % dimethylformamide solution of the PU. Carboxyl group-introduced PU (PU–C) was prepared by immersing the PU film in a mixture of 4 N NaOH aqueous solution and methanol (1 : 2 v/v) followed by washing with a mixture of a 10% citric acid aqueous solution and methanol (1 : 2 v/v) (Fig. 2). The PEO-grafted PU (PU–P) was prepared by the reaction of amino-terminated PEO (MW: 600, Nippon Oil and Fats Co., Tokyo, Japan) with the PU–C film, which was previously activated with 1-ethyl-3-(dimethylamidopropyl)carbodiimide (WSC). Heparin-immobilized PU–C (PU–C–H) was prepared by activating the PU–C film with WSC followed by immersing the activated film in an aqueous solution containing

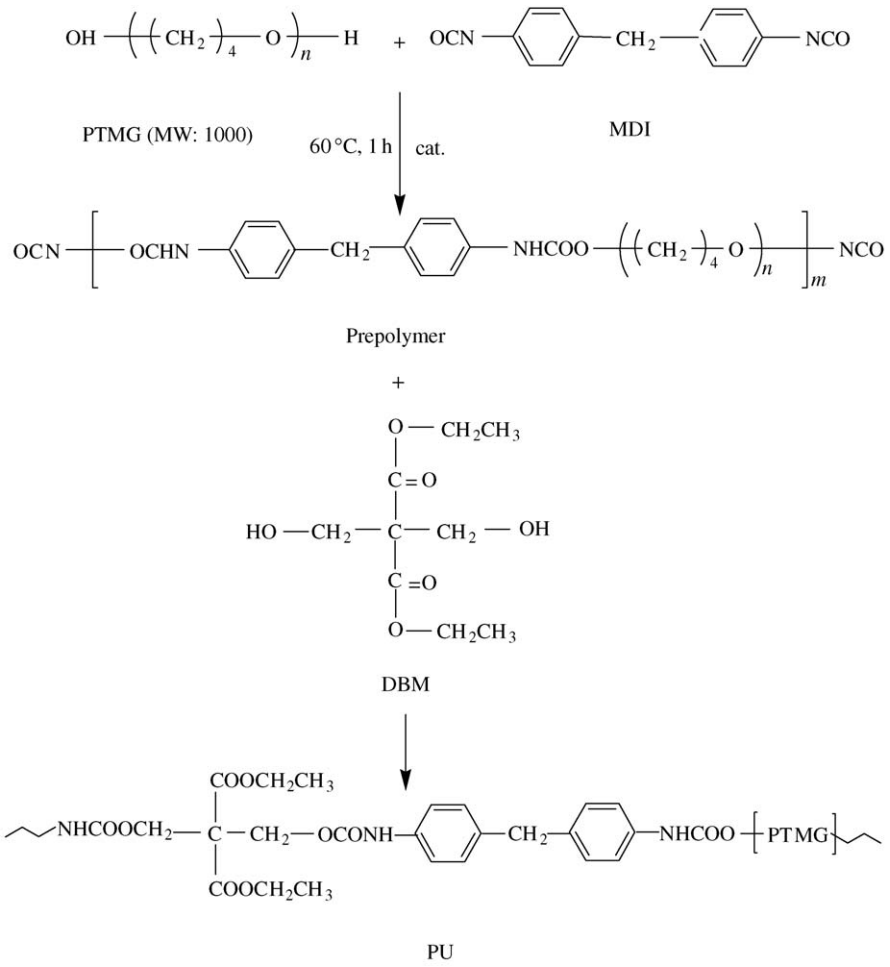

Figure 1 Schematic diagram showing the synthesis of PU containing diester groups in the side chains.

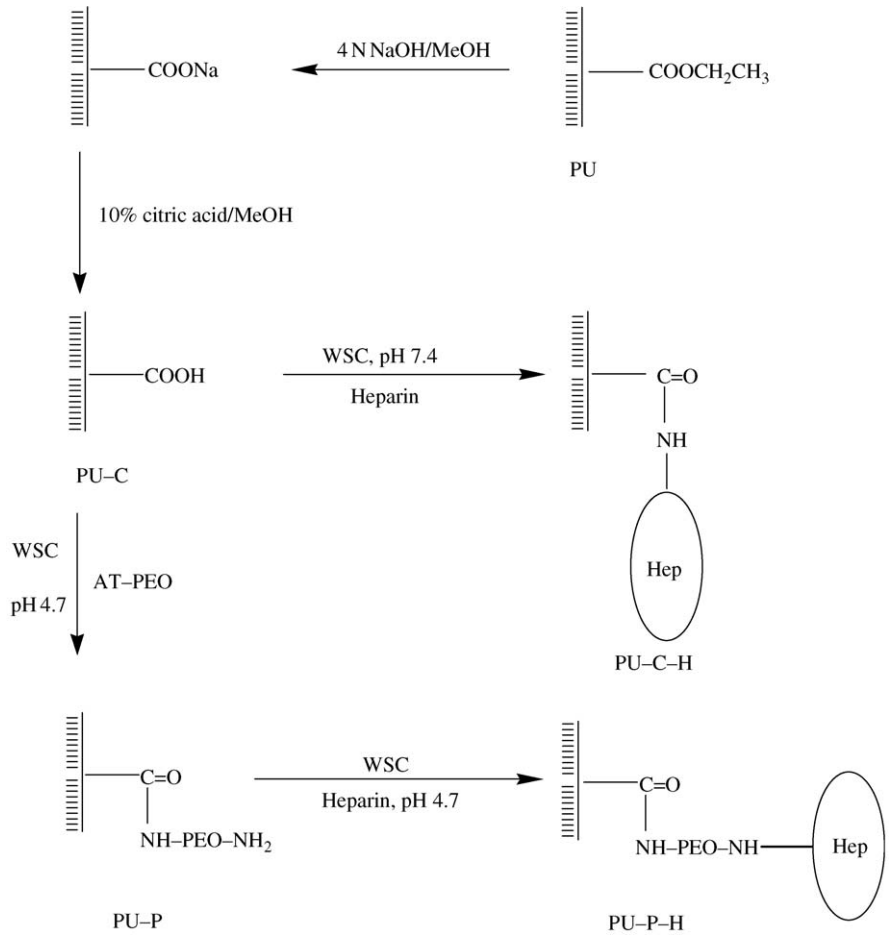

Figure 2 Schematic diagram showing the immobilization of heparin on the surfaces.

heparin (non-fractionated heparin sodium salt, 174 IU mg^{-1}, Sigma Chem. Co.).

2.2. Plasma recalcification time

Human whole blood (30 ml) from a healthy volunteer was collected and mixed with an aqueous solution containing anhydrous D-glucose (0.136 M), sodium citrate hydrate (0.075 M) and citric acid monohydrate (0.0004 M) (ACD, 3 ml). The whole blood was then centrifuged at 3000 × *g* for 10 min to separate the blood cells and the resulting platelet-poor plasma (PPP) was used for the PRT experiments. The PPP (300 µl) was placed on the sample film (sample number = 4, sample size = $3 \times 3\,cm^2$) attached to a watch glass (diameter: 4 cm) and incubated statically at 37 °C. Thereafter, a 0.025 M $CaCl_2$ aqueous solution (300 µl) was added to the PPP and the plasma solution was monitored for clotting by manually dipping a stainless-steel wire hook coated with silicone into the solution to detect any fibrin threads. Clotting times were recorded at the first sign of fibrin formation on the hook [18]. The experiment was repeated three times using the same blood sample and a mean value was taken.

2.3. Activated partial thromboplastin time

The sample film ($3 \times 3\,cm^2$) was attached to a glass (diameter 4 cm, height 1.5 cm), and pre-swelled with distilled water (2 ml). The plasma solution (100 µl) and an actin-activated cephaloplastin reagent (100 µl, Baxter Diagnostics Inc., Deerfield, IL, USA) were then added to the sample film, followed by the addition of a 0.025 M $CaCl_2$ solution (100 µl) after 5 min of incubation [19]. The clotting time of the plasma solution was monitored as described in the PRT experiment. The experiment was repeated three times using the same blood sample and a mean value taken.

2.4. Adhesion and activation of platelets

Human ACD-blood was centrifuged at 180 × *g* for 10 min to obtain platelet-rich plasma (PRP). The PRP (100 µl) was then placed on the surface-modified PUs ($3 \times 3\,cm^2$) at 37 °C for 30 and 60 min, respectively. Phosphate-buffered saline (PBS, 6 ml) was then added to the PRP to stop further platelet adhesion, and was left for 1 min. The number of platelets adhered to the samples was determined by measuring the lactate dehydrogenase (LDH) activity of the cells lysed with Triton X-100. A linear relationship was thereby obtained between the LDH activity of the aliquots of the cell suspension and the number of platelets counted with a haemocytometer. The LDH activity was determined by measuring the initial rate of nicotinamide adenine dinucleotide hydride (NADH) oxidation in the presence of Triton X-100 (0.02 wt %). According to a Shimadzu UV-2100 spectrophotometer, the NADH oxidation was followed by a decrease in absorbance at 340 nm [20]. The experiment was carried out in triplicate and a mean value taken.

The serotonin released from the adhering platelets was measured as follows: To examine reabsorption of the

released serotonin, the experiment was carried out with (4 μg/ml) and without imipramine. Surface-modified PUs (diameter 1.5 cm) was attached to a 24-well plate and kept at 37 °C. The PRP (500 μl) was then placed on the sample. After 30 or 60 min of incubation, 0.02 M ethylenediamine tetraacetic acid (EDTA, 500 μl) was added to stop any further release reaction of the platelets. The platelet and plasma layers were then transferred to different containers after centrifugation at 12000 rpm. Thereafter, 200% trichloroacetic acid (TCA, 200 μl) was added to both the platelet and plasma layers to aggregate the proteins. The proteins aggregated by the TCA were removed by centrifugation, and the resulting protein-free samples were transferred to a glass tube containing 2 ml of an o-phthalaldehyde solution (10 ml of 0.5% o-phthalaldehyde ethanol + 100 ml of 8 N hydrochloric acid) and subsequently incubated at 100 °C for 10 min. The excess TCA was extracted with chloroform (2 ml) and the fluorescent intensity of the aqueous layer phase at 475 nm was measured using a Shimadzu RF-5000 spectrophotometer. The amount of serotonin remaining in both the platelet and plasma layers was calculated from a standard calibration curve, constructed according to the relationship between the fluorescent intensity and the known concentration of serotonin creatinine sulphate [19]. The experiment was repeated three times using the same blood sample and a mean value taken.

2.5. Adhesion and activation of PBMCs

The PBMCs from a healthy human volunteer were prepared using ACD-blood centrifugation ($400 \times g$, 30 min) on a Ficoll-Hypaque (Sigma Co.). The cells were washed three times and then resuspended at a concentration of 2.6×10^6 cells ml^{-1} with an RPMI-1640 medium (Gibco, NY, USA) supplemented with 1% bovine serum albumin. The cells were placed on surface-modified PUs which had been prewetted with RPMI-1640 in 5% CO_2 at 37 °C, and then the number of cells adhered on to each PU after 6 and 24 h of incubation was examined using the LDH method [20].

Interleukin 6 (IL-6) was measured using an enzyme-linked immunosorbent assay (ELISA IL6 kit, Predicta Co.) as follows [21]: A PBMC solution (200 μl, 2.6×10^6 cells ml^{-1}) was incubated on each surface-modified PU in both the presence (2–20 μg ml^{-1}) and absence of lipopolysaccharide (LPS) for 6, 18, 24 and 48 h. The supernatant (100 μl) was then lifted, transferred into a tube, and centrifuged at 3500 rpm for 10 min to precipitate the PBMCs from the medium. The resulting supernatant (100 μl) was then added to the multiplate wells precoated with IL-6 monoclonal antibodies and kept at 37 °C for 30 min. Subsequently, the supernatant was discarded and the wells were washed vigorously with Hank's balanced salt solution (HBSS). A biotinylated antibody solution (100 μl) was then added to the well and incubated for another 30 min. Finally, avidin peroxidase and substrates were added to the well, incubated for 10 min, and measured by UV absorbance at 450 nm using an ELISA reader (Labsystems Multiscan MS). The experiment was repeated three times using the same blood sample and a mean value taken.

2.6. Scanning electron microscopy (SEM) analysis

The blood cells that adhered to the surface-modified PUs were dipped into a 0.1% glutaraldehyde aqueous solution (pH 7.2) at 37 °C for 30 min. The films were then dehydrated in a graded series of ethanol, dried in a Hitachi model HCP-2 critical point drier using liquid CO_2 as the transition liquid, and finally sputter-coated with platinum. These samples were observed with a Hitachi S-510 SEM.

2.7. Statistical analysis

Experiments were run in triplicate per sample. All data were expressed as means ± standard deviation (SD) for $n = 4$. Differences between the PU control and the surface-modified PUs were analyzed statistically using the *t*-test from the two samples. The differences observed between samples were considered significant for *p*-values lower than 0.05.

3. Results

The characteristics of the surface of modified PUs used in this study are shown in Table I. Carboxylic acid groups were introduced to the surface of PU at the concentration of 61 nmol/cm^2 when the PU film was hydrolyzed with an alkaline aqueous solution. The concentration of amino-terminated PEO (MW; 600) grafted to the surface of the PU–C was 4.1 μg/cm^2. This means that 11% of carboxylic acid groups were coupled with the PEO. The amount of heparin grafted to the surface of the PU–C (0.92 μg/cm^2) was slightly higher than that of the PU–P (0.81 μg/cm^2). On the other hand, the water contact angle of the PU surface (78°) decreased by the introduction of carboxylic acid groups (61°), PEO (59°) and heparin (55–57°). The characteristics of the surface-modified PUs were previously reported in detail [8]. To examine the blood compatibility of the surface-modified PUs, the interaction of the materials with blood components such as plasma proteins, platelets and PBMCs were carried out.

3.1. Activation of plasma proteins

The PRT of the surface-modified PUs is shown in Fig. 3. The PRT of the PU control (308 s) was slightly shortened by the introduction of carboxylic acids (PU–C, 263 s). It

TABLE I Characteristics of surface-modified PU films

Sample	Concentration of carboxyl group (nmol/cm^2)[a]	Amount of PEO (μg/cm^2)[b]	Amount of heparin (μg/cm^2)[c]	Water contact angle (°)[d]
PU				78 ± 2
PU–C	61			61 ± 1
PU–P		4.1		59 ± 1
PU–C–H			0.92	57 ± 2
PU–P–H			0.81	55 ± 1

[a]Determined by rhodamine 6G method.
[b]Determined by ninhydrin method.
[c]Measured by toluidine blue method.
[d]Measured by sessile droplet method.

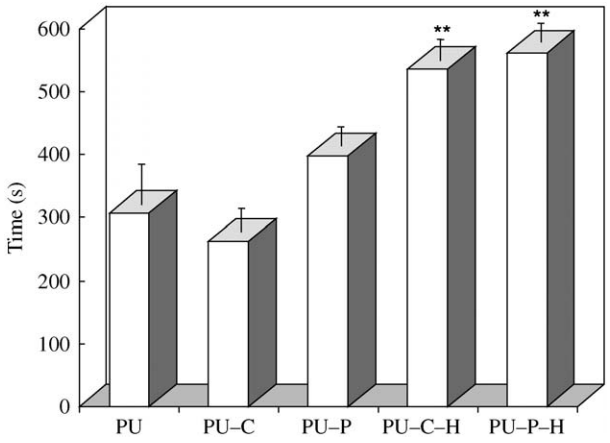

Figure 3 PRT of surface-modified PU films. Data is presented as the mean ± standard deviation of three different experiments. $^{**}p < 0.05$ (compared to PU control).

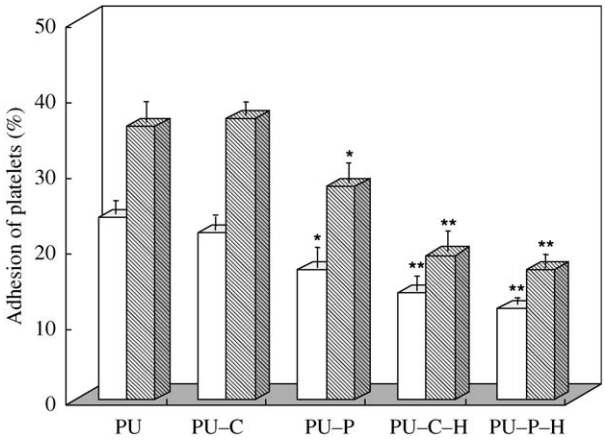

Figure 4 Adhesion of platelets on surface-modified PU films after 30 (□) and 60 (▨) min of incubation. Data is presented as the mean ± standard deviation of three different experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ (compared to PU control).

TABLE II APTT of PU and surface-modified PUs

Sample	APTT (s)
Glass	28 ± 2
PU	33 ± 1
PU–C	34 ± 2
PU–P	37 ± 1
PU–C–H	42 ± 3
PU–P–H	45 ± 2

was prolonged, however, by the introduction of the PEO (PU–P, 398 s) and further prolonged by heparin immobilization (PU–C–H, 536 s; PU–P–H, 563 s). The activation of plasma clotting factors is generally accelerated by the addition of activated partial thromboplastin [22]. The APTT is presented in Table II. The APTT of PU (33 s) was barely prolonged by the introduction of carboxylic acid (PU–C, 34 s), and slightly prolonged by the PEO grafting (PU–P, 37 s). The APTT, however, was significantly prolonged by heparin immobilization (PU–C–H, 42 s; PU–P–H, 45 s, $p < 0.008$). This result suggests that antithrombin III (AT III) was bound to the immobilized heparin, thus leading to a suppression of the thrombin activity [23].

3.2. Adhesion and activation of platelets

Fig. 4 shows the amount of platelets adhered to various modified PUs after 30 and 60 min of incubation. The number of platelets adhered to the PU–C after 60 min of incubation was almost the same as that adhered to the PU control. The platelet adhesion, however, was slightly suppressed by the PEO grafting (PU–P, $p < 0.05$) and further suppressed by heparin immobilization ($p < 0.01$). The number of platelets adhered to the surface-modified PUs after 60 min of incubation was higher than that after 30 min of incubation, independent of the kind of sample. Table III shows the amount of serotonin released from the platelets adhered to the surface-modified PUs. As expected, the serotonin released after 60 min of incubation was more than that after 30 min of incubation, irrespective of the kind of sample. The percentage of serotonin released from the

TABLE III Release of serotonin from platelets adhered on surface-modified PU films[a]

Sample	Without imipramin (%)		With imipramin (%)	
	30 min	60 min	30 min	60 min
PU	34 ± 2	52 ± 6	31 ± 3	49 ± 5
PU–C	36 ± 5	56 ± 7	33 ± 4	53 ± 6
PU–P	29 ± 2	41 ± 5	27 ± 1	37 ± 4
PU–C–H	21 ± 4	30 ± 4	18 ± 3	29 ± 1
PU–P–H	17 ± 3	28 ± 2	18 ± 4	26 ± 5

[a]Average ± SD ($n = 3$).

platelets adhered to the PU (52%) after 60 min of incubation was no different from that adhered to the PU--C (56%) within a standard deviation. However, this percentage slightly decreased on the PU–P (41%, $p < 0.05$) and further decreased on the PU–C–H (30%) and PU–P–H (28%, $p < 0.01$). As shown in Table III, the amount of released serotonin obtained in the presence of imipramin was almost the same as that in the absence of imipramin. This result indicates that serotonin release from the platelets that adhered to the surface-modified PUs is not influenced by the presence of imipramin. Fig. 5 shows a scanned electron micrograph of the platelets, that adhered to the surface-modified PUs after 1 h of incubation. As seen in Fig. 5, the platelets were partially aggregated on the surfaces of the PU and PU–C. However, the platelets were less aggregated on the heparin-immobilized PU (PU–C–H, PU–P–H).

3.3. Adhesion and activation of PBMCs

Fig. 6 indicates the amount of PBMCs adhered to the surface-modified PUs after 6 and 24 h of incubation. The cell adhesion increased with an increase in the incubation time, irrespective of the kind of sample. The amount of cells adhered to the PU–C (55%) for 24 h incubation was almost the same as that of the PU control (53%). However, the cell adhesion on the PU decreased with the introduction of PEO (PU–P, 38%) and further decreased by heparin immobilization (PU–C–H, 29%; PU–P–H, 26%). These adhesion patterns of the PBMCs on the surface-modified PUs were similar to those obtained in

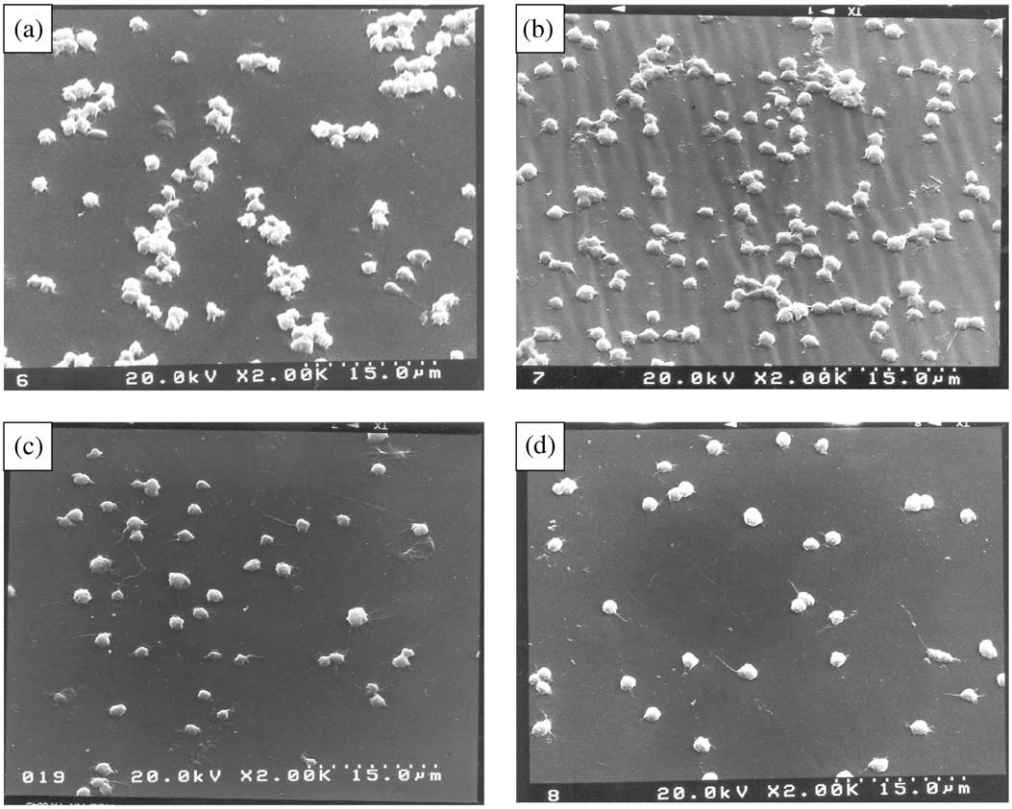

Figure 5 Scanned electron micrographs of platelets adhered to surface-modified PU films after 90 min of incubation: (a) PU; (b) PU–C; (c) PU–C–H; (d) PU–P–H.

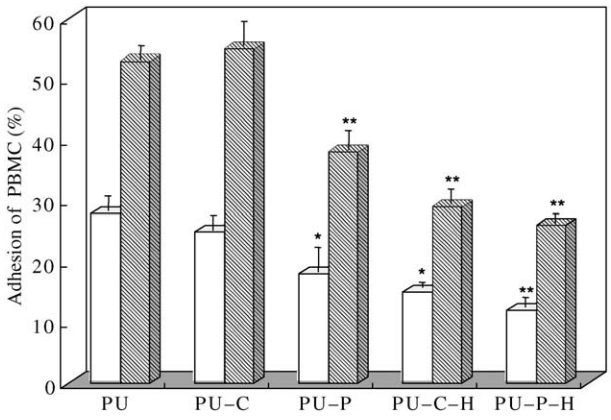

Figure 6 Adhesion of PBMCs on surface-modified PUs after 6 h (□) and 24 h (▨) of incubation. The initial cell concentration was taken as 100. Data is presented as the mean ± standard deviation of three different experiments. $*p < 0.05$; $**p < 0.01$ (compared to PU control).

the platelet adhesion experiment (Fig. 4). The SEM photographs of the adhering PBMCs on the surface-modified PUs are shown in Fig. 7. The PBMCs were highly aggregated on the PU and PU–C while the cells did not adhere and aggregate as well on the heparin-immobilized PUs (PU–C–H, PU–P–H). These results suggest that the activation of the PBMCs may be generated by their contact with surface-modified PUs and change depending on the physicochemical properties of the surfaces of these materials.

To study the activation of the PBMCs stimulated with surface-modified PUs, the amount of IL-6 was measured using an ELISA method [21]. Fig. 8 shows the amount of IL-6 released from the PBMCs stimulated with surface-modified PUs as a function of the incubation time. The release of IL-6 rapidly increased up to 24 h of incubation and thereafter, remained basically unchanged. Therefore, the incubation time of the cells was fixed at 24 h in order to measure the amount of IL-6 released relative to the kind of surface of the functional group. The PBMCs were incubated on the surface-modified PUs for 24 h in the presence ($10\,\mu g\,ml^{-1}$) and absence of lipopolysaccharide (LPS) and the amount of IL-6 released is shown in Fig. 9. The amount of IL-6 released in the presence of LPS was slightly higher than that released in the absence of LPS, irrespective of the kind of sample. The IL-6 release on the PU in the presence of LPS slightly decreased by the introduction of PEO having a molecular weight of 600 (PU–P, 1940 pg/ml, $p < 0.01$). On the other hand, the IL-6 release was significantly lower on the heparin-immobilized PUs (PU–C–H, 1480 pg/ml, $p < 0.01$; PU–P–H, 1580 pg/ml, $p < 0.01$).

4. Discussion

One of the methods to improve blood compatibility of polymeric materials is the immobilization of biomolecules such as heparin and urokinase on the surfaces. Many researchers have focused on the preparation of PUs containing reactive groups in the main chain or side chain, which could be a site for the reaction with heparins [24, 25]. Barbucci *et al.* [6] immobilized heparins ionically on the surface of poly(amidoamine)-grafted PU. Han *et al.* [26] have also studied the surface modification of PUs by heparin-immobilization for long-term biomedical applications. Although the blood compatibility of the PU has been improved to a certain

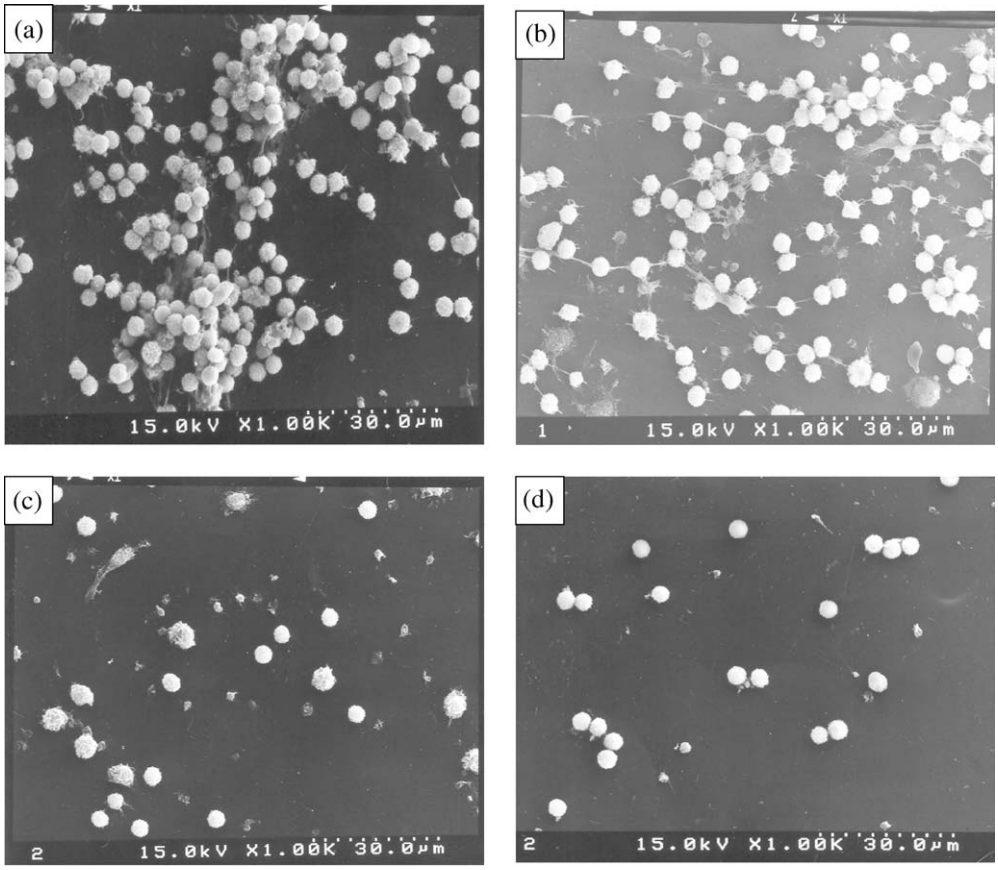

Figure 7 Scanned electron micrographs of PBMCs adhered to surface-modified PU films after 6 h of incubation: (a) PU; (b) PU–C; (c) PU–C–H; (d) PU–P–H.

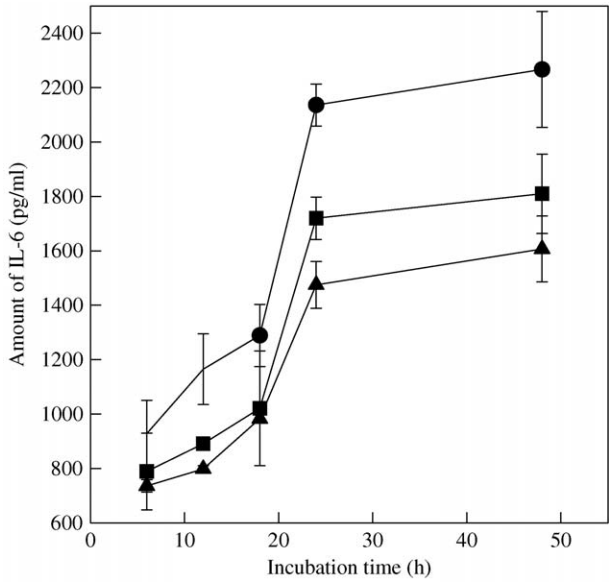

Figure 8 Release of IL-6 from PBMCs adhered to PU (●), PU–P (■), and PU–P–H (▲) as a function of incubation time. Data is presented as the mean ± standard deviation of two different experiments.

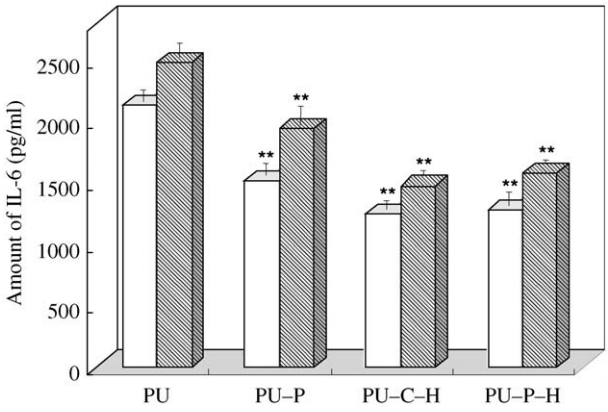

Figure 9 Amount of IL-6 released from PBMCs adhered to surface-modified PUs in the absence (□) and presence (▨) of LPS after 24 h of incubation. Data is presented as the mean ± standard deviation of three different experiments. $^{**}p < 0.01$ (compared to PU control).

extent by the introduction of heparin, one limitation that still remains is the restricted coupling for heparin-immobilization. Park *et al*. [12] introduced polyethylene imine to the PU surface to increase the immobilizing site. Kang *et al*. [27] also introduced polyacrylic acid to the PU surface by plasma glow discharge treatment to enhance the immobilizing site. In a previous study [10], we synthesized poly(ether urethanes) containing diester groups in the side chains using 4,4′-diphenyl diisocyanate, polytetramethylene glycol, and diethyl bis(hydroxymethyl)malonate as a chain extender and introduced carboxylic acid groups to the surface by treating the film with an alkaline aqueous solution.

Poly(ethylene oxide) (PEO) has been frequently used as a surface modifier or spacer in the design and synthesis of blood compatible PUs [28, 29]. Han *et al*. [26] synthesized PUs grafted with PEO with molecular weights ranging from 200 to 2000 and reported that a PEO with a molecular weight of 2000 is most effective for the improvement of blood compatibility. Using an *in vivo* canine shunt system, they reported that a PU valve coated with a sulphonated PEO graft was less thrombo-

genic and more calcification-resistant than unmodified PU [30]. Cooper *et al*. [31] evaluated the bulk, surface and blood-contacting properties of polyether PUs based on PEO (Mw = 1450), polytetramethylene oxide (PTMO) (Mw = 1000), and PEO/PTMO soft segments. They reported that the higher PEO-containing polymers were more thrombogenic than the pure PTMO-based PU. From the above results, it could be considered that the grafted PEO is more effective than the PEO incorporated in the main chain of PU for the attainment of blood compatibility. In this study, heparin was immobilized on the PU surfaces by two methods. In the first method, heparin was directly coupled with surface carboxyl groups (PU–C–H). In the second method, heparin was coupled with surface carboxyl groups through the PEO spacer with a molecular weight of 600 (PU–P–H). As a result, the amount of heparin immobilized on the PU–C–H (0.92 $\mu g/cm^2$) was not significantly different from that on the PU–P–H (0.81 $\mu g/cm^2$). Moreover, platelet activation on both surfaces (Table III) was almost the same within a standard deviation.

Heparin has often been used as an immobilizing biomolecule in the design of new antithrombogenic materials or modification of the surfaces of polymeric materials for the purpose of achieving high blood compatibility [32, 33]. Rhodes and Williams [34] studied the intrinsic coagulation pathway by bringing a variety of biomaterials into contact with a plasma aliquot and observing the rate of clotting diminish by virtue of factor XII activation and reported that heparinized materials could be distinguished from non-heparinized materials and a non-activated plasma control. The activation of plasma proteins was largely suppressed by heparin immobilization (PU–C–H, PU–P–H) when compared to the non-heparinized surfaces (PU, PU–C, PU–P), as seen in Table III and Fig. 3. Similar results were found in the experiments of platelet adhesion (Fig. 4) and activation (Table III). It was also found, from the results of the APTT (Table II), that the activation of plasma proteins was more suppressed on the PU–C–H and PU–P–H when compared to other substrates. This result suggests that antithrombin III (ATIII) was bound to the immobilized heparin, thereby leading to a suppression of thrombin activity [23, 35]. Reports on the effect of immobilized heparin on platelet adhesion and thromboresistance are very diverse. In some reports, platelet adhesion was suppressed [36] or not influenced [37] by heparinization. Wilson [38] emphasized that the dependence of thromboresistance on heparin leaching, the covalent versus ionic bonding of heparin and the effect of heparin on platelet adhesion, are important factors. On the other hand, Basmadjian and Sefton [39] studied the relationship of the releasing rate and the surface concentration of heparin using a mathematical model and reached the conclusion that the covalently immobilized heparin-poly(vinyl alcohol) retains its biological activity after immobilization. Rosenberg and Damus [40] reported the overall mechanism by which thrombin is activated by ATIII. They showed that ATIII neutrizes thrombin by forming a 1 : 1 stoichiometric complex via a reactive site (arginine)-active center (serine) interaction. The complex formation occurs at a relatively slow rate in the absence of heparin. However, heparin binds to lysine residues on ATIII, thereby accelerating the inhibition of thrombin [35].

It is considered that the amount of released cytokine can be used as a measure for evaluating the activation of the PBMCs when in contact with foreign materials [41]. Among the numerous cytokines which are involved in the induction and regulation of the host responses in inflammation, interleukin-6 (IL-6) and interleukin-1 (IL-1) seem to play central roles in the inflammatory reaction [42, 43]. These cytokines show several overlapping effects with each other and with a tumor-necrosis factor (TNF) [44]. In a previous study, the cytokine mRNA level from the PBMCs stimulated by surface-modified PUs after 5 h of incubation was measured using the reverse transcription polymerase chain reaction (RT-PCR) technique [19]. The results indicated that the production level of TNF mRNA from the cells stimulated by heparin-immobilized PU was smaller than that of the PU control and functional group-grafted PUs (PU–COOH, PU–NH_2). This study focussed on the influence of the surface-modified PUs on the production of the proinflammatory mediator IL-6. As shown in Fig. 8, the IL-6 release patterns were similar to those obtained from the experiments with plasma proteins (Table II, Fig. 3) and platelets (Table III). Namely, the fewer the plasma proteins and platelets activated by the materials, the less IL-6 is released from the PBMCs by these same materials. These results suggest that the activation of PBMCs can be influenced by the degree of the activation of plasma proteins and platelets. LPS is known as an activator to promote the activation of immune cells [45]. Cardona *et al*. [46] have shown that biomedical polymers can stimulate monocytes to produce significant amounts of IL-1β in the presence of LPS, yet the TNF production has not been determined. Takahashi *et al*. [47] detected the IL-6 protein mainly in the fibroblasts, endothelial cells, and macrophages in all the inflamed gingival tissues examined, yet none in any of the healthy gingival tissue. Furthermore, the LPS from several oral inflammatory pathogens is capable of amplifying the local immune response and promoting periodontal tissue inflammation and damage by stimulating gingival fibroblasts and periodontal ligament cells to secrete IL-6 [48]. As seen in Fig. 9, the amount of IL-6 released from the PBMCs increased with the addition of LPS at a concentration of 10 $\mu g ml^{-1}$, irrespective of the kind of sample.

To conclude, the *in vitro* blood compatibility of heparin-immobilized PU containing ester groups in the side chain was examined using plasma protein, platelets and PBMCs. The APTT was prolonged on the PU–C–H and PU–P–H, suggesting the binding of immobilized heparin to the antithrombin III. The release of serotonin from the adhering platelets and the release of interleukin-6 from the adhering PBMCs are significantly suppressed on the heparin-immobilized PUs when compared to the PU control, suggesting good *in vitro* blood compatibility.

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