Anticoagulant Surface Prepared by the Heparinization of Ionic Polyurethane Film

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ABSTRACT: We present a new method for heparinization on the surface of polyurethane. The segmented polyurethane was first modified with an epoxide monomer and followed by a ring-opening reaction with diethanolamine to introduce sufficient hydroxyl groups on the surface of cast film. On this film surface, a cationic monomer was grafted by using tetravalent Cerium salt as an initiator. Heparin was immobilized in high efficiency on the ionized surface through static interactions in aqueous solution. The structure of ionized and heparinized surfaces were characterized by attenuated total reflectance infrared spectroscopy (ATR–FTIR) and electron spectroscopy for chemical analysis (ESCA) spectra. The platelet-rich plasma (PRP) contacting test and the platelet-poor plasma (PPP) clotting time measurements showed that the immobilized heparin retained its strong anticoagulant property. The release of heparin from film into salt solution was also studied, and it was found that only a small portion of heparin (10–20%) was released over a period as long as 10 h. It is expected that this new method for surface heparinization can be used to prepare antithrombogenic materials with long-term stability. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 76: 382–390, 2000

Key words: segmented polyurethane; graft polymerization; immobilized heparin; blood compatibility

INTRODUCTION

A number of approaches have been investigated to develop nonthrombogenic materials. The most popular method is heparinization; that is, to immobilize heparin onto a polymer surface.[1–3] Heparin is a very powerful anticoagulant molecule as it potentiates the action of antithrombin III, the enzyme that is responsible for the inactivation and breakdown of thrombin. Immobilized heparin can also act as an anticoagulant for blood and an inhibitor of the complement system at the place, where it is required and avoid the disadvantages of systemic administration.[4]

The linkage between heparin and material surface can be either covalent or ionic. Many techniques have been developed to couple heparin covalently to biomaterials.[5,6] Some of them are based on a two-step procedure, whereby the biomaterial is first activated by a bi- or trifunctional agent, after which heparin is added to form the heparin–material linkage. In order to avoid the possible crosslinking of biomaterial when it is treated with multifunctional agent in the first step. Engbers developed an alternative procedure for heparinization of cuprophan hemodialysis membranes.[7,8] Heparin is first reacted with *N,N′*-

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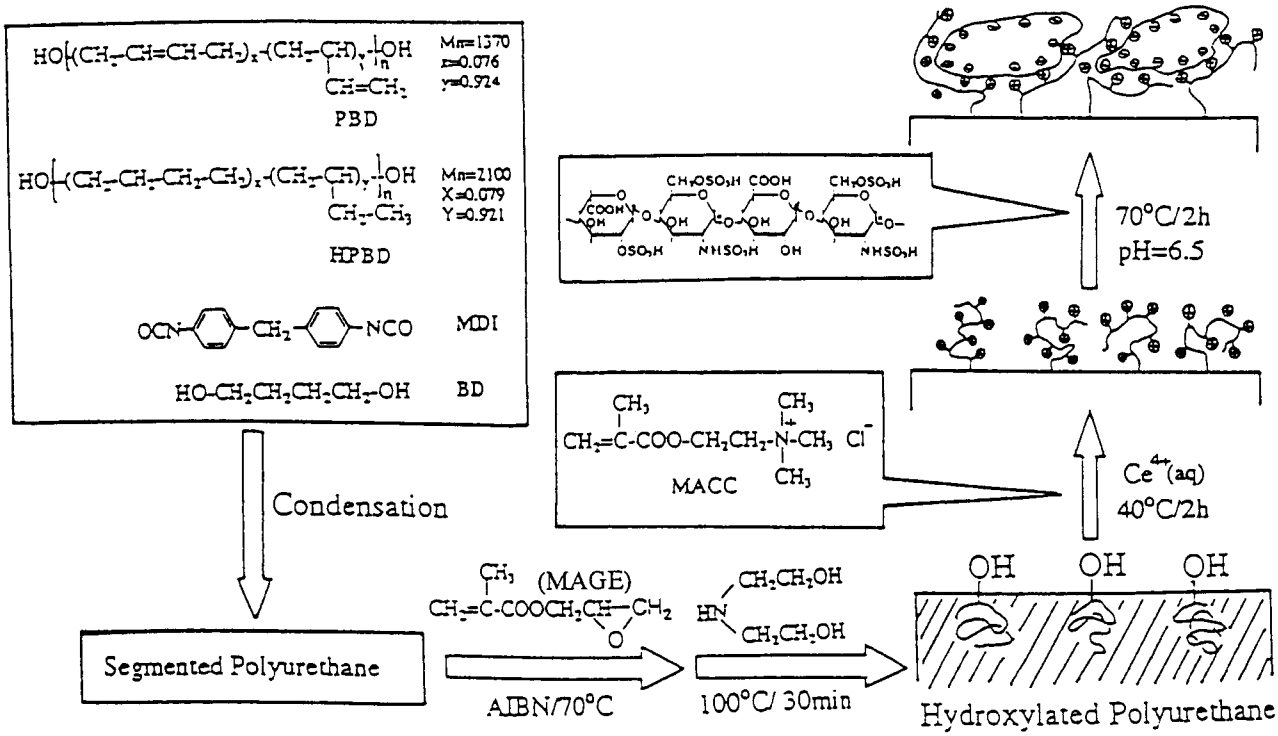

Figure 1 Scheme for the heparinization of polymer surface.

carbonyldiimidazole (CDI), and the activated product is subsequently contacted with the membrane by which a covalent coupling between heparin and membrane is established. Kim et al.[9,10] incorporated a long-chain hydrophilic poly(ethylene oxide) as spacer group into the heparin–material linkage, which is expected to increase the bioactivity of heparin to prevent fibrin formation as well as to reduce protein adsorption and platelet adhesion. Due to the strong anionic nature of heparin, heparinization of a surface can therefore be realized through static attraction for which a cationized surface should be prepared before contacting with heparin aqueous solution.[11] Imanishi et al. reported the synthesis of poly(etherurethaneurea) bearing tertiary amino groups in the main and side chains.[12,13] After quaternization, the cast film interacted with heparin in aqueous solution, and a polymer/heparin complex was formed at the polymer surface, which showed an in vitro antithrombogenicity. However, immobilized heparin through ionic binding was found to exhibit release from the polymer surface, which may gradually reduce the blood compatibility of the biomaterial.[14] We believe that the strength of ionic interaction depends upon the cation density on the polymer surface. As far as cast film of ionized polyurethane is concerned, the cation density at the film surface cannot be so high as expected for the formation of complex with heparin. The interaction between polymer and heparin is not only through static attractions but also interactions, such as hydrophilic interactions, hydrophibic interactions, and hydrogen bonding.

In the present study, we aim to immobilize heparin onto polyurethane surface that had been ionized in high density. For this purpose, a cationic monomer methacrylic choline chloride (MACC) was grafted onto polymer surface by means of surface graft copolymerization using Ce(IV) as an initiator.[15] In order to reach a high graft density, the segmented polyurethane must be chemically modified to introduce functional groups that can react with Ce(IV) in aqueous media to generate free radicals at the film surface. Therefore, we designed the following reaction scheme (see Fig. 1): The segmented polyurethane was first prepared according to the method reported in our previous work,[16–20] involving the condensation of 4,4′-methylenediphenyl diisocyanate (MDI) with poly(butadiene) (PBD), hydrogenated poly(butadiene) (HPBD) diols, and butanediol (BD) as a chain extender. Then, by use of

double bonds on PBD soft segment, the obtained segmented polyurethane was grafted with a vinyl monomer methacrylic acid glycidyl ester (MAGE) in solution in the presence of 2,2-azobis(isobutyronitrile) (AIBN) as initiator. Finally, the grafted polyurethane containing epoxide groups reacted with diethanolamine to produce enough hydroxyl groups to undergo a ring-opening reaction. The reacted product is now a hydroxylated polyurethane, which was fabricated into cast films that can be used for surface graft copolymerization.

The structure of the polymer surface before and after heparinization was characterized by attenuated total reflection–Fourier transform infrared (ATR–FTIR) and electron spectroscopy for chemical analysis (ESCA) spectroscopies. The blood compatibility was evaluated through the platelet-rich plasma PRP contacting test and the platelet-poor plasma PPP clotting time measurements. The stability of immobilized heparine was also studied by incubating heparinized film in salt solution for varying times.

EXPERIMENTAL

Materials

1,4-Dioxane, toluene, *N,N*-dimethyl acetamide (DMAc), methanol, and acetone were of reagent grade and distilled before use. 1,4-Butanediol (BD), 4,4′-methylenediphenyl diisocyanate (MDI), and cerium diammonium nitrate were purchased from Nacalai Tesque, Inc., Japan, and used without further purification; poly(butadiene) glycol (PBD) and hydrogenated poly(butadiene) glycol (HPBD) were purchased from Nisso Chemical Co., Ltd. Methacrylic acid glycidyl ester (MAGE) was freed of inhibitor by distillation. Methacroylcholine chloride (MACC) (80% aqueous solution) was purchased from Tokyo Kasei Organic Chemicals Co., Ltd. Heparin isolated from porcine intestinal mucosa, which has an anticoagulant activity of 150–160 IU mg, was purchased from Nacalai Tesque Co., Ltd.

Synthesis of Segmented Polyurethane

PBD (13.7 g, 0.01 mol) and HPBD (21.0 g, 0.01 mol) were dissolved in 60 mL of a mixture solvent (DMAc/toluene, 1 : 4). To this solution, MDI (10.0 g, 0.04 mol), dissolved in 30 mL of the same mixture solvent, was added under a dry nitrogen atmosphere with vigorous stirring. The reaction was carried out at 70–75°C for 1 h. After that, a solution of 1.8 g of BD in 10 mL DMAc was added dropwise to the reaction mixture over a period of 10 min, followed by raising the reaction temperature up to 100–110°C for another 1 h. Finally, the reaction mixture was cooled to room temperature and then poured into a large excess of methanol. The white solid was filtered, washed with methanol for several times, and then dried in a vacuum oven at 70°C for at least 48 h. The weight-average molecular weight was 41,000 with a polydispersity of 1.83 measured by gel permeation chromatography (GPC) in THF using polystyrene standard sample.

Modification of Polyurethane

Segmented polyurethane (5.0 g) was dissolved in 40 mL of dioxane. MAGE (3 mL), and 50 mg of AIBN were added to this solution, followed by saturating with nitrogen. The reaction mixture was maintained at 70°C in a water bath for 2 h and finally poured into a large excess of methanol. The obtained polymer was filtered, washed with acetone to remove all ungrafted monomer and homopolymer, then dried in vacuum at 60°C for 2 days. The grafting percentage was 18.4% calculated according to elemental analysis (N%: 3.26, and 2.66 before and after grafting, respectively). In the second step, the grafted polyurethane was dissolved in dioxane and reacted with an excess of diethanolamine at 100°C for 1 h with vigorous stirring. After precipitation from methanol, the hydroxylated polyurethane was washed with methanol and dried under vacuum at 60°C for 1 day.

Preparation of Cast Film

The hydroxylated polyurethane was dissolved in the mixture of dioxane–methanol (8 : 2) to form a clear solution. A small amount of benzene was added to remove the foam in solution. Then the solution was casted onto a glass plate followed by drying at room temperature for 24 h and then at 70°C for 10 h.

Surface Graft Copolymerization

The cast film was completely dried and weighed before immersion in aqueous solution. The concentration of Ce(IV) was 0.01*M,* and that of methacroylcholine chloride was 0.50*M*. Under nitrogen, the reaction system was maintained at 40°C for 2 h. During the process of surface graft-

ing, a thin white layer of polymer was formed at the film surface, and the grafted surface became slippery. After washing with pure water several times, the film was dried in a vacuum at 60°C for 24 h and weighed. The graft density was 1.85 mg cm^2, as calculated by the following equation:

$$\text{Graft density (mg cm}^2) = (W_f - W_i)/A$$

where A is the surface area of cast film, and W_f and W_i are the final and initial weights of cast film, respectively.

Heparinization

Surface grafted film was immersed in 1 wt % aqueous sodium heparinate solution at 70°C for 5 h. At the end of this process, the film was taken out of the heparin solution and then washed with distilled water until the rinse solution was free of heparin tested by using toluidine blue, which showed that heparin was not released from polymer surface. The film was then dried in vacuum at room temperature for 2 days. We weighed the film before and after heparinization and calculated the content of immobilized heparin to be $\approx$ 2.16 mg cm^2.

General Methods

The infrared (IR) spectra of polymers were taken on thin films casted on a KBr single crystal plate using a Jasco A202 spectrometer. ATR–FTIR spectroscopy was performed on the surface of cast films. The spectra were collected at 4-cm^{-1} resolution using a Jasco Micro FT/IR-200 microsampling spectrometer over 50 scans. The sampling area was 25 μm^2, coupled with an ATR accessory and 45° KRS-5 crystal. ESCA spectra were obtained on a Shimadzu ESCA 750 spectrometer using Mg Kα radiation. The takeoff angle of the photoelectron was kept at 60°. The peak area was calculated using the standard Shimadzu ESPAC 100 software. The binding energy was referenced by setting the C1s hydrocarbon peak to be 285 eV. GPC measurements were carried on an HLC802UR GPC instrument with G4000H8 + G2000H8 columns, and the samples were dissolved in THF using polystyrene as standard. Elemental analysis was performed by Osaka Gas Co. Ltd (Osaka, Japan).

Evaluation of the Blood Compatibility

The methods and procedures of the PRP contacting test and the PPP clotting time measurement have been described previously.[16–20] The morphology of PRP contacted surface was observed using a JEOL Hitachi S-2500 SEM operated at an accelerating voltage of 20 kV. Clotting time was measured using cow platelet-poor plasma containing sodium citrate. The plasma was first dropped onto the film surface and incubated at 37°C for 10 min. Clotting time was recorded as the point when plasma clotted with the addition of 0.025M $CaCl_2$.

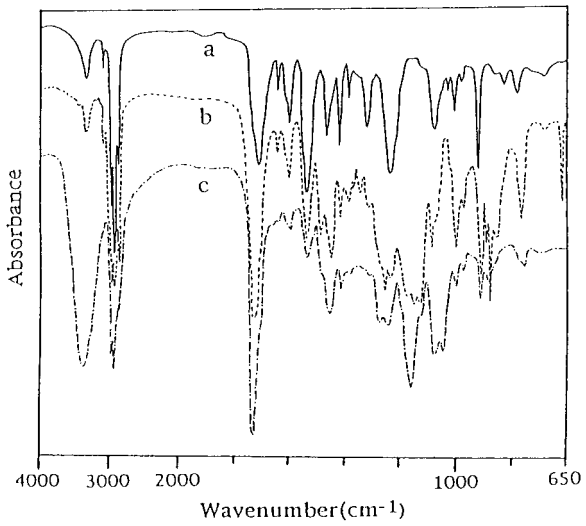

Figure 2 IR spectra of (a) pure polyurethane, (b) grafted polyurethane, and (c) hydroxylated polyurethane.

RESULTS AND DISCUSSION

Characterization of Bulk and Surface Structure

Figure 2 shows the IR spectra of polyurethane together with the grafted and hydroxylated polyurethane. Characteristic peaks on curve (a) in Figure 2 correspond to the structure of segmented polyurethane, as follows: absorptions at 3300 cm^{-1} (N—H *str* and hydrogen bond between aggregated —OH), 1710 cm^{-1} (C═O *str* from urethane group), 1605 cm^{-1} (aromatic C═C *str* from MDI hard segment), and 910 cm^{-1} (C—H *def* in 1,2-addition CH═CH_2 from PBD soft segment). For the IR spectrum of MAGE-grafted polyurethane [Fig. 2(b)], the strong peak at 1720 cm^{-1} is the overlap of absorption of C═O from urethane and from the ester group in the grafted MAGE moiety. The new peaks at 1268 and 1135 cm^{-1} were ascribed to the stretching vibration of the

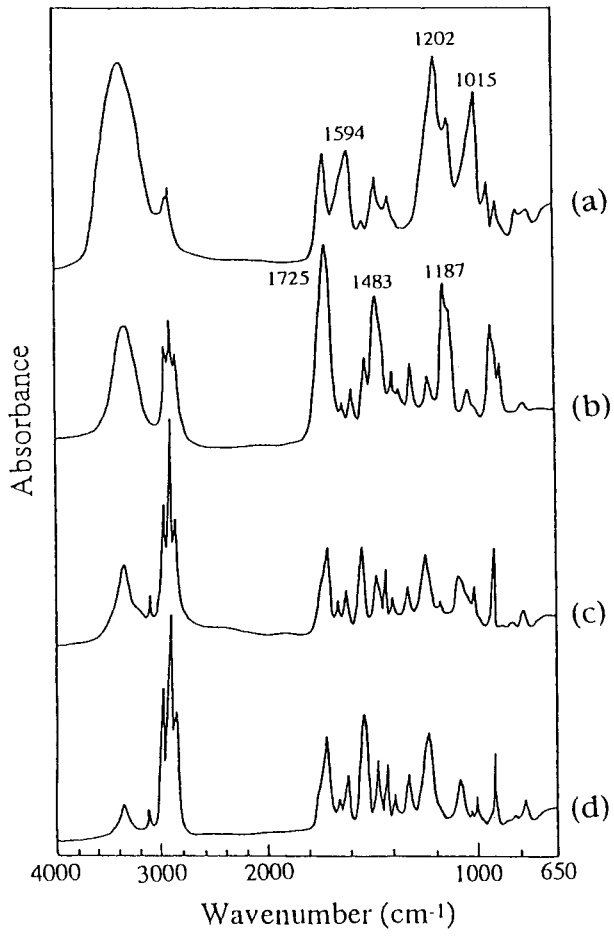

Figure 3 ATR–FTIR spectra obtained from (a) polyurethane, (b) hydroxylated, (c) ionized, and (d) heparinized surfaces.

C—O bond from epoxide and ester groups, respectively. The hydroxyl group produced from the ring-opening reaction of epoxide with diethanolamine shows a strong absorption at 3300 cm^{-1} on curve (c) in Figure 2. Also, the absorption of epoxide at 1268 cm^{-1} is remarkably reduced after the ring-opening reaction.

Figure 3 is the ATR–FTIR spectra recorded on the four kinds of polymer surfaces. The ATR–FTIR spectrum of pure polyurethane [Fig. 3(a)] and hydroxylated polyurethane [Fig. 3(b)] are quite similar to their general IR spectra shown in Figure 1. Curve (b) in Figure 3 has a more stronger absorption at 3300 cm^{-1} than curve (a), (also in Fig. 3), which indicates that the hydroxyl groups in the hydroxylated polyurethane enrich the surface and reacted with Ce(IV) ion in aqueous solution. Curve (c) (in Fig. 3) is the spectrum of the ionized surface with a MACC graft density of 1.8 mg cm^2. Three characteristic peaks are observed at 1725, 1483, and 1187 cm^{-1}, corresponding to the stretching vibration of C═O, N—CH_3, and C—O on MACC units, respectively. The peak at 3380 cm^{-1} corresponds to the absorption of water bound by quaternary ammonium groups. Upon the heparinization of ionized surface, the following new peaks appear in the ATR–FTIR spectrum: the strong absorption of hydroxyl groups at 3350 cm^{-1}, characteristic absorption of COO^- at 1594 cm^{-1}, and that of SO_3^- at 1202 cm^{-1} and 1015 cm^{-1}. The results indicate that heparin was adsorbed on the ionized surface through static interactions as a driving force.

ESCA spectra (Fig. 4) provide ample evidence for the determination of surface structure. In order to make a comparison, we put two classes of spectra together in Figure 4, the bottom and the top are spectra obtained from film surface before and after heparinization, respectively. In the C1s spectrum of ionized surface, the signal is composed of the following three peaks: the peak at 285.75 eV results from the hydrocarbon backbone in PBD and HPBD soft segments, the peak at 287.12 eV corresponds to —CH_2—O— from both urethane and grafted polymer, and finally the peak at 289.50 eV belongs to carboxide groups of the grafted polymer. After heparinization, the C1s spectrum became a broadened and unresolvable peak as a result of overlapping of signals from many different carbon sources. The O1s spectra of both surfaces are broadened peaks near 532 eV, providing little information concerning the surface structure. The N1s spectra contains useful details for the clarification of structures of both surfaces. Before heparinization, two peaks can be detected, the peak at 399.87 eV from the urethane structure, and the peak at 402.76 eV from quaternary ammonium groups in the grafted polymer. After heparinization, the spectrum is resolved into three peaks, as follows: two of them at 399.87 and 402.75 eV are the same as mentioned above; the new peak at 398.48 eV results from the $NHSO_3$—groups of the immobilized heparin. The negative charge gives a slight low energy shift. The ion exchange behavior during heparinization can be seen from Cl2p spectrum. Before interaction with heparin, Cl^- anion exists in two states, with one binding state corresponding to the peak at 198.64 eV, and the other relatively free state corresponding to the peak at 197.05 eV. After interaction with heparin in aqueous solution, most Cl^- anions are exchanged by the heparin anion. Therefore, in the Cl2p spec-

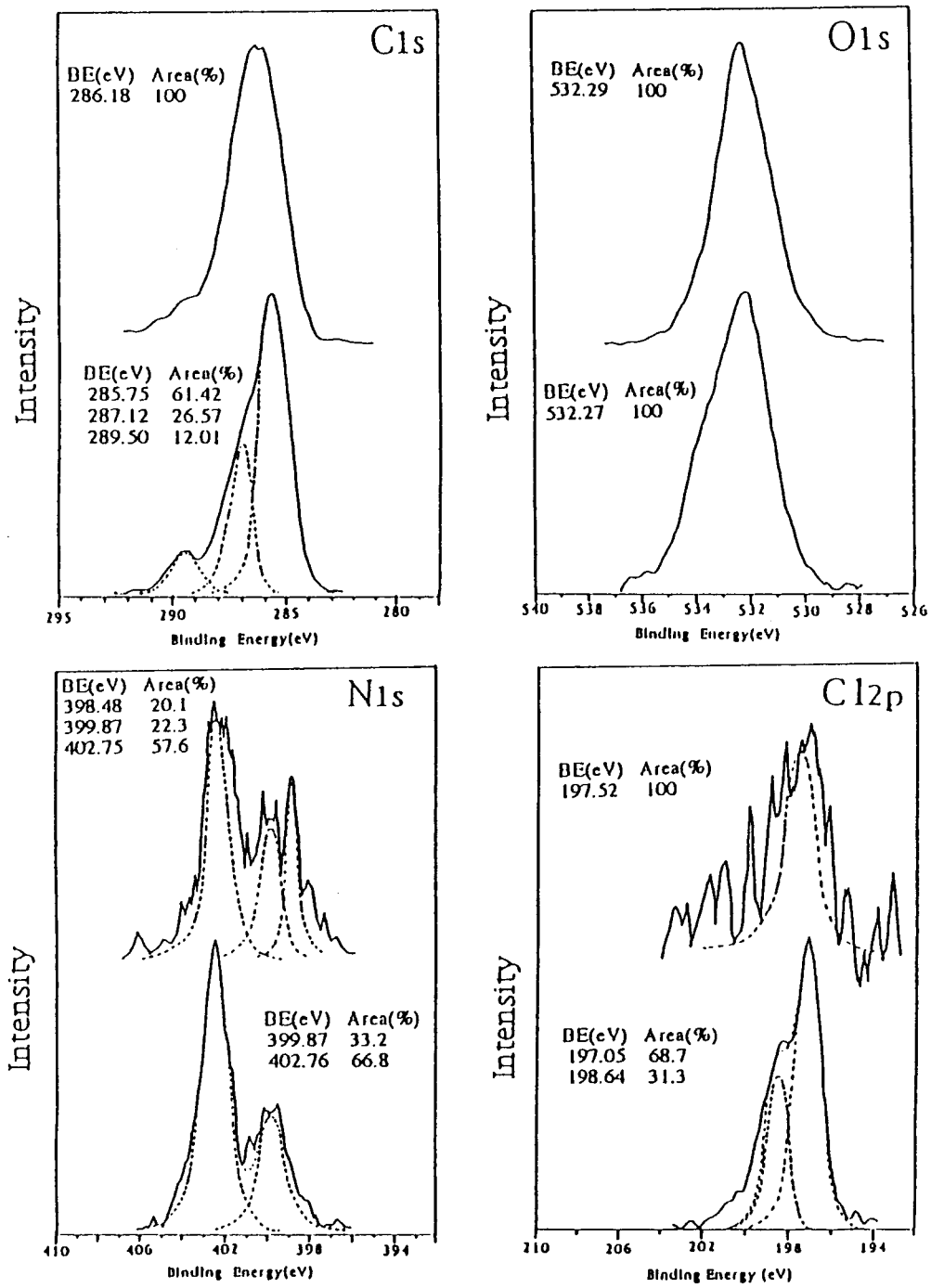

Figure 4 ESCA spectra of the ionized (bottom) and heparinized (top) surfaces.

trum, the remaining Cl^- anions show a very weak signal with a composition percentage of 0.65%. The S2p spectrum shows a strong peak at 168.68 eV, which indicates that the heparinization of this ionized surface was complete. Table I listed the elemental composition of four surfaces. The ionized and heparinized surfaces show lower carbon content and higher oxygen content as compared with the polyurethane surface, which is why ionized and heparinized surfaces have high wettability. In addition, the high sulfuric content by 6.15% indicates that heparin was immobilized on the film surface in a high efficiency.

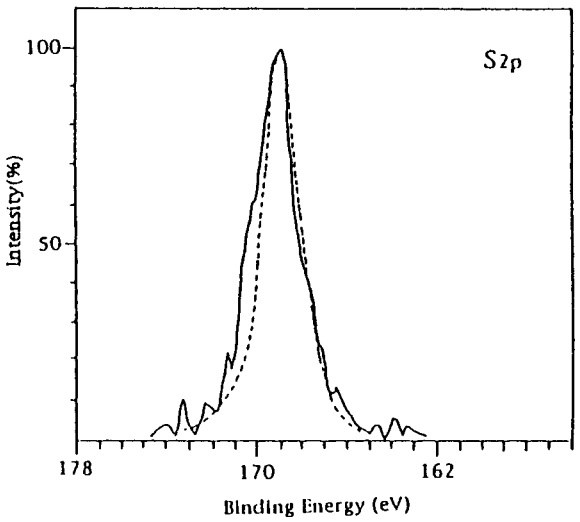

Figure 4 (*Continued from the previous page*)

Blood Compatibility and Anticoagulant Property

The blood compatibility of polymer surface was evaluated in terms of the number and differentiation of platelets adhered on the film surface after contacting with platelet-rich plasma at 37°C for 1 h. Figure 5 shows the morphologies of PRP-contacted surfaces under the same magnification (×2000). Figure 5(a) is the surface of pure polyurethane, on which a substantial number of platelets adhered and differentiated to some extent. As for the surface of hydroxylated polyurethane [Fig. 5(b)], the platelet adhesion is reduced, and the aggregation is not controlled. Figure 5(c) describes the ionized surface, showing a unique morphology, in which a thin layer of graft-on polymer with a porous structure covered on the background of hydroxylated polyurethane. The formation of small pores on the surface might result from the uneven distribution of graft density. The graft density is in proportion to the local concentration of hydroxyl group on film surface. Microphase separation of the polymer material lead to a concentration of hydroxyl groups in some areas and depletion in the other part of surface. On the other hand, the blood compatibility of this surface is good because both platelet adhesion and differentiation were inhibited, as seen from the surface morphology. The heparinized surface [Fig. 5(d)] has a morphology quite different from the grafted surface. A continual domain structure can be observed, but no small holes remained after heparinization. Although a number of platelets adhered on the surface, the aggregation or differentiation of adhered platelet has been successfully inhibited. The size of adhered platelet is about 0.2–0.5 μm in diameter. The immobilized heparin exhibited affinity to platelet but has a strong anticoagulant ability to inhibit the aggregation and differentiation of adhered platelet.

The anticoagulant property of immobilized heparin has also been examined by clotting time measurements using two commercial polyurethanes, Tecoflex and Pellethane, as references. Clotting time is defined by the time of platelet-poor plasma clotted at glass surface as unit, in which Tecoflex, Pellethane, and heparinized surface are 2.4, 2.1, and 6.8, respectively. Obviously, the heparinized surface has a much longer clotting time than commercial polyurethane, which is in good agreement with the result of PRP contacting test. It has been reported that the water content of heparinized polymer plays an important role for achieving excellent anticoagulant function.[13] The complex of heparin and grafted polycation is a typical polymer electrolyte with a strong ability to absorb water. The water content of this heparinized surface was measured to be $\approx$ 14 mg cm^2. Therefore, the excellent anticoagulant property of film surface should be attributed to both the activity of heparin and high water content of surface complex.

Table I Surface Elemental Composition from ESCA

	Elemental Composition (%)				
Sample	C	N	O	Cl	S
Pure polyurethane	83.67	2.85	13.48	0	0
Hydroxylated polyurethane	81.38	3.02	15.60	0	0
Cationalized surface	73.30	7.11	15.63	3.97	0
Heparinized surface	63.98	6.07	23.15	0.65	6.15

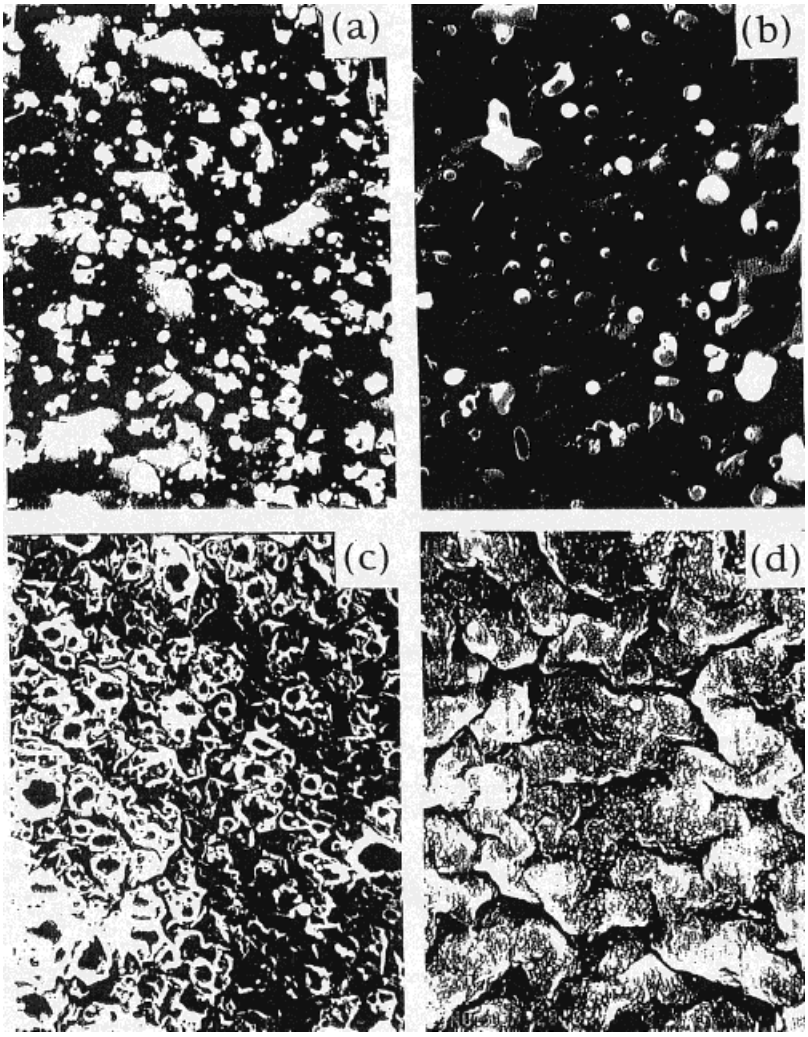

Figure 5 SEM photographs for the morphologies of PRP contacted surfaces (×2000). (a) pure polyurethane; (b) hydroxylated surface; (c) cationalized surface; (d) heparinized surface.

Stability of Immobilized Heparin

There have been two points of view regarding the contribution of antithrombogenicity of the heparinized material. One is that the antithrombogenicity of heparinized polymer is attributed to the contribution from the microenvironment of released heparin,[21–22] and the other is attributed to the bound heparin.[23,24] As far as the current heparin–polymer complex formed through static interactions is concerned, there is difference between bound heparin and free heparin because the binding and releasing are reversible processes in an equilibrium state. It can retain its anticoagulant activity well. The high water content in the surface complex can provide an environment in which heparin is free from steric hindrance. Therefore, the stability of immobilized heparin was studied by incubating heparinized film in physiological salt solution and 1*N* aqueous NaCl solution at 37°C up to 10 h. The release of heparin from the film surface was measured by weighing the incubated films after completely dried in vacuum. Figure 6 is plotted the percent of residual heparin as a function of incubation time. It was found that heparin released faster into solution containing higher salt concentrations. A higher concentration of salt produced solutions with higher ion strengths and greatly reduces the static interactions between cation and anion. After 10 h of incubation, about 10 and 20% of heparin was released into physiological salt solution and 1*N* aqueous NaCl solution, respectively. The

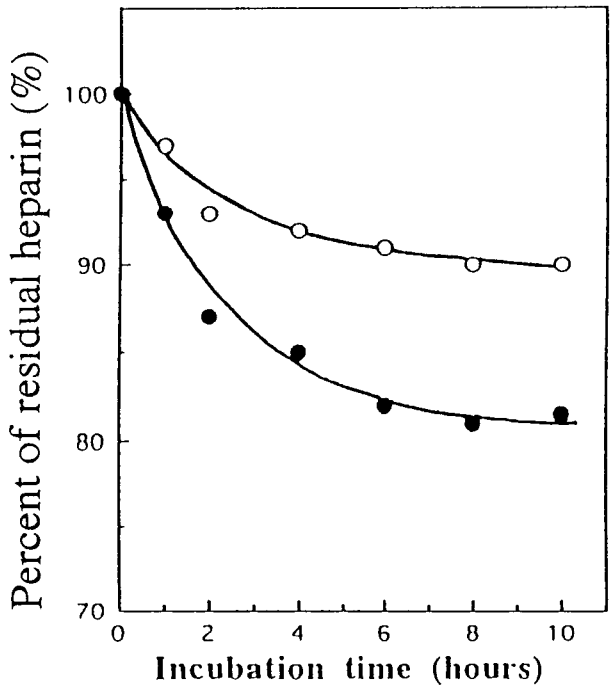

Figure 6 Release of heparin from heparinized surface into physiological salt solution (○) and in 1N NaCl solution (○) at 37°C.

majority of heparin was still immobilized on the film surface, which means that the complex of heparin and grafted cationic polymer can be stable after reaching an equilibrium state. The high density of cation on the surface plays an important role in preventing heparin release.

CONCLUSIONS

A new methodology has been developed to immobilize heparin onto the ionized polyurethane surface. The cationalization of polyurethane film surface was carried out through a surface graft copolymerization using a cationic monomer and Ce(IV) as an effective initiator. The structure of ionized and heparinized surfaces were characterized by ATR–FTIR and ESCA spectra. About 2.16 mg cm^2 of heparin was immobilized onto a cationic surface with a graft density of 1.85 mg cm^2. The PRP contacting test and the PPP clotting time measurements showed that the immobilized heparin retained its strong anticoagulant ability. The immobilized heparin was stable, only 10% of heparin released from the film surface into physiological salt solution over a period of 10 h.

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