Surface characterization and platelet adhesion studies on polyethylene surface with hirudin immobilization

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Hirudin, a protein composed of 65 or 66 amino acid, is a newly risen anticoagulant agent and has been considered as the most potent thrombin inhibitor. Hirudin can block the active site of thrombin by means of its carboxylic acid reaction with the active regime of thrombin, and becomes a tightly bound complex, and thus controls the formation of thrombus. Hirudin was covalently immobilized onto the water-soluble carbodiimide preactivated and chromic acid oxidized PE surface. Surface chemistry analysis indicated that a certain amount of carboxylic acid groups was generated on the polyethylene surface after oxidation with chromic acid solution. The amount of carboxylic acid functional group increased with the oxidation time. In addition, polyethylene surface was etched by chromic acid solution, and a rougher surface was created. The morphology of oxidized polyethylene surface was similar to each other among the samples with oxidation time from 1 to 8 min. ESCA results indicated the number of hirudin molecules immobilized was constant at the reaction time studied. *In vitro* platelet adhesion assay indicated the number of adhered platelets on the oxidized polyethylene surface increased significantly after oxidation. In contrast, surface with hirudin immobilization showed a reduction in adhered platelet density than the chromic acid oxidized counterpart due to the decrease of platelet-activating capability by the hirudin–thrombin complex and the differences in the adsorbed protein composition.

1. Introduction

Despite decades of extensive research in synthesis of blood compatible artificial biomaterials, thrombus formation is still the major complication as blood contacts the foreign surface. Hence, anticoagulants are needed after major surgery in order to dissolve this thrombus. Heparin, a sulfated glycosaminoglycan, is the major anticoagulant used clinically. Its anticoagulant effect is mediated by the antithrombin III, a physiological inhibitor of coagulation, through forming a heparin-AT III complex. The major drawback of using heparin is that it may cause hemorrhage if the dosage is too high. In addition, the anticoagulation capability of heparin may not function properly if the patient is antithrombin III (AT III)-deficient. Moreover, heparin-resistance is noticed in patients with high concentrations of platelet factor 4, histidin-rich glycoprotein, or vitronectin [1].

Hirudin, a protein made up by 65 or 66 amino acid residuals, has been under clinical evaluation as a new anticoagulant agent [2]. Studies have indicated hirudin is the most potent natural thrombin inhibitor. Hirudin can block the active site of thrombin by forming a tightly bound hirudin-thrombin complex through the carboxylic acid reaction with the active regime of thrombin. As thrombin complexes with hirudin in 1 : 1 ratio, it will lose its ability to activate fibrinogen and platelet [2] and thus controls the thrombus formation. Moreover, the risk of thrombocytopenia is decreased significantly since hirudin does not interact directly with platelets and endothelial cells [1].

Different antithrombogenic or fibrinolytic biomolecules, such as streptokinase [3], lumbrokinase [4], and heparin [5, 6] have been immobilized onto material's surface with an attempt to improve the blood compatibility of the substrate. However, the results are not satisfactory. In the present study, hirudin was immobilized onto the polyethylene (PE) surface through a simple and less expansive two-step reaction scheme, surface oxidation and subsequent carbodiimide activation techniques (Fig. 1). Results from various surface characterization techniques on different modified PE substrates will be discussed. In addition, blood compatibility study using *in vitro* platelet adhesion assay will also be presented.

2. Materials and methods

Polyethylene (PE) film, kindly provided by Formosa Plastic Co., was extensively cleaned with various solvent

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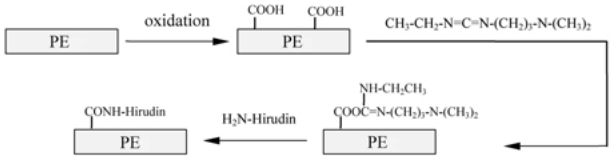

Figure 1 Reaction scheme for hirudin immobilization onto polyethylene surface.

extractions to remove the additives. PE film was first oxidized with chromic acid solution ($CrO_3/H_2O/H_2SO_4 = 29/42/29$ by weight percentage) at 72 °C. After different time duration, the oxidized PE films were removed and cleaned with copious distilled water. These chromic acid solution oxidized films were then immersed into 70% nitric acid solution at 50 °C for 15 min to remove the residual chromic metal compounds. The oxidized PE surface was extensively cleaned with 50 °C distilled water and dried for subsequent reactions.

1 - ethyl - 3 - (3 - dimethylaminopropyl) - carbodiimide (water-soluble carbodiimide, from Sigma) was used to activate the oxidized PE surface [7] for subsequent hirudin immobilization. The oxidized film was immersed in the solution composed of 0.03 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 0.05 M, pH = 4.8, phosphate buffer. The activation reaction was 1 h and the reaction temperature was set at 4 °C. After the activation reaction, the surface was cleaned with 0.05 M, pH = 7.5 phosphate buffer twice to remove the unreacted residues.

Hirudin (from Leeches, Cat. # H7016, Sigma) was first dissolved in 0.05 M, pH = 7.5 phosphate buffer saline (PBS) to a final concentration of 143 ATU/ml. The hirudin-PBS solution was then snap-frozen and stored at − 18 °C for later immobilization reaction.

The carbodiimide activated oxidized-PE surface was incubated with hirudin-PBS solution (hirudin concentration for immobilization reaction is further diluted to 23.8 ATU/ml) at 4 °C for 6 h or 12 h. After immobilization reaction, the surface was cleaned with PBS three times to remove the physically adsorbed hirudin. Hirudin was immobilized onto the PE surface via its primary amine functional group.

ESCA (ESCA-210, VG Scientific Ltd. UK) and ATR-FTIR (Nicolet 550, Magna Series II, USA) were used to determine the surface chemical functionality. Captive bubble contact angle measurement (Model CA-DT A, Face, Japan) was carried out for surface hydrophilicity assessment.

In vitro platelet adhesion assay [8], using human platelet concentrates from Tainan Blood Donation Center instead of washed platelets as Ko *et al*. [8], was performed to evaluate the blood compatibility of hirudin immobilized surfaces. Samples were first equilibrated with Hepes-Tyrodes buffer for 1 h prior to platelet adhesion experiments. These samples were then incubated with platelet concentrates at 37 °C, 5% CO_2 incubator for 20 min. After incubation, the platelet concentrate was removed and the samples were then incubated with Hepes-Tyrodes buffer for another 40 min for further development in adhered platelets. These samples were fixed by 2% glutaraldehyde and critical point dried before SEM analysis. The surface density and the morphology of adhered platelets were determined with SEM (S-4100, Hitachi, Japan) pictures. At least three different positions per sample were evaluated.

3. Results and discussion

The captive contact angle values of PE samples after different chromic acid oxidation time were shown in Fig. 2. The contact angle value decreased significantly after 1-min oxidation time and gradually leveled off after 8 min. This indicates the hydrophilic functional groups, such as carbonyl or carboxylic acid group, were formed on the PE surface shortly after oxidation with the chromic acid solution. SEM pictures shown in Fig. 3 indicated the PE surface was etched in short contact with the oxidation solution for 1 min. However, the surface morphology of oxidized samples remained similar even with longer oxidation time.

ATR-FTIR results of virgin PE and oxidized PE samples were shown in Fig. 4. A new peak at 1710 cm^{-1} was clearly noticed on all oxidized PE surfaces. This peak can be attributed to the formation of –COOH, –C = O and –HC = O functional groups on the oxidized surface. In order to confirm the formation of –COOH group that will be used for subsequent hirudin immobilization reaction, the oxidized surface was immersed into 0.1 N. NaOH solution using the method suggested by Whitesides *et al*. [9]. The peak height at 1710 cm^{-1} was decreased and a new peak at 1560 cm^{-1} was found after contact with NaOH solution (Fig. 4c). This new peak at 1560 cm^{-1} can be assigned to the formation of $-COO^-Na^+$ group on surface. This implies the formation of –COOH group did occur on the chromic acid oxidized surface. Furthermore, X_{IR}, defined in the following equation, was used to assess the fraction of –COOH group formed on the oxidized PE surface. Fig. 5 indicates –COOH fraction, X_{IR}, on the oxidized surface did not increase with the oxidation time.

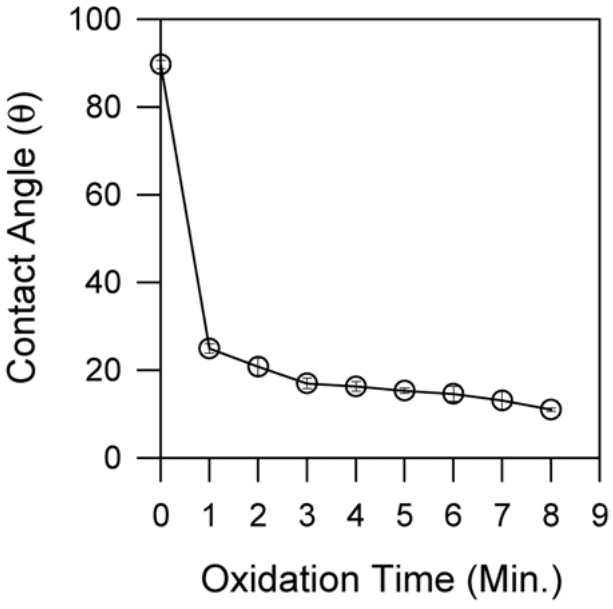

Figure 2 The captive contact angle values of oxidized PE samples at different chromic acid solution contact time.

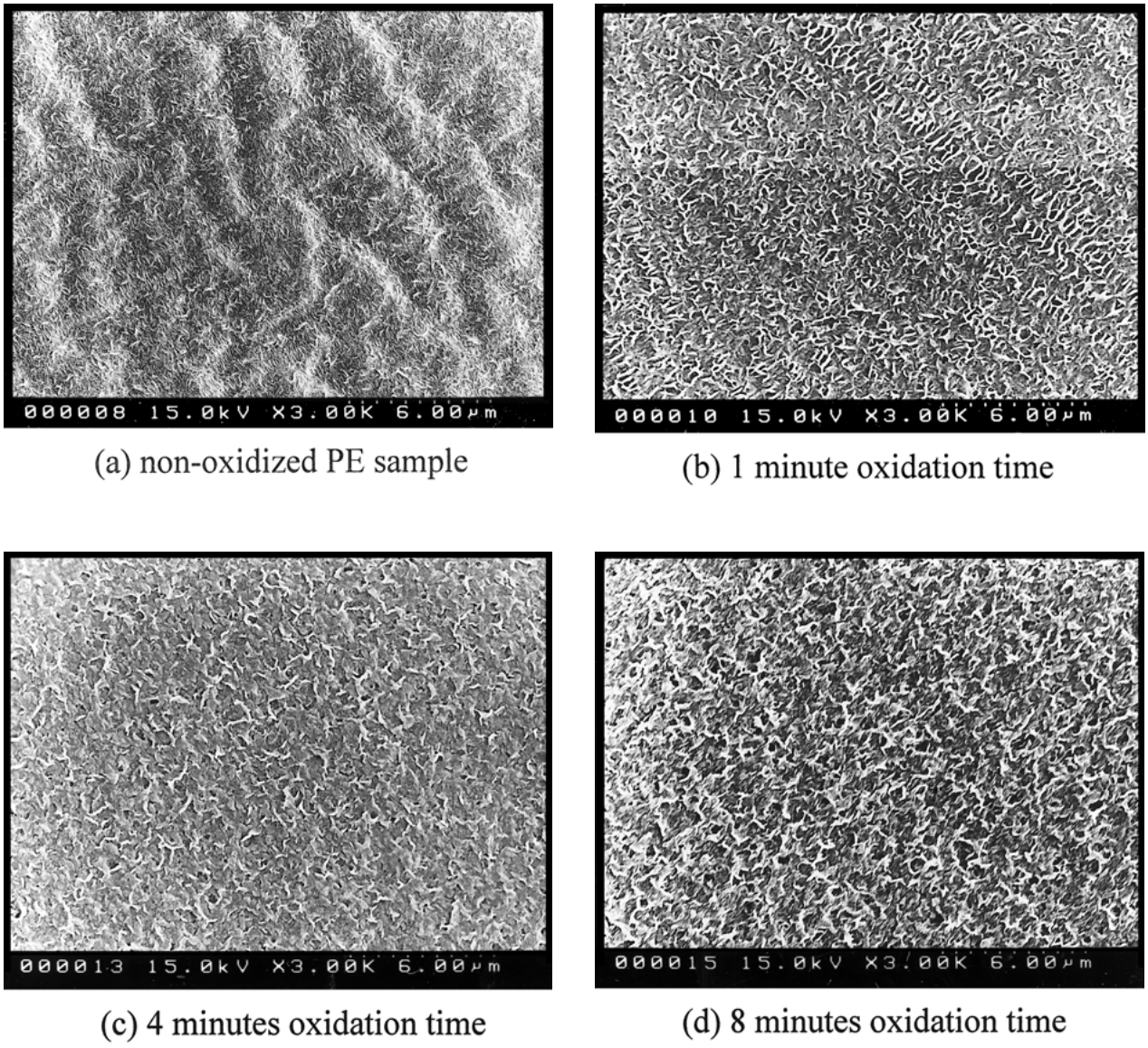

Figure 3 SEM micrographs of PE samples at different oxidation time (a) 0 min (b) 1 min (c) 4 min (d) 8 min.

$$X_{IR} = \frac{A_{1560}}{A_{1710} + A_{1560}}$$

where A_{1560} is the absorbance at 1560 cm^{-1} ($-COO^-$) in NaOH treated sample, A_{1710} is the absorbance at 1710 cm^{-1} (–C=O and –HC=O) in NaOH treated sample.

The surface chemistry of chromic acid solution oxidized PE samples was also examined by the ESCA technique. Oxygen atoms were found on these oxidized surfaces, indicating the oxygen-containing functional groups, such as carbonyl or carboxyl groups, were formed once the PE samples were contacted with oxidizing solution. Further analysis using C1s deconvolution technique demonstrated –COOH functionality (peak A, binding energy = 289 eV as shown in Fig. 6) was formed after oxidation reaction. In addition, the peak area ratio of C1s at 289 eV (–COOH) to C1s at 286.2 eV (–C=O and –HC=O) increased initially but leveled off at longer oxidation time. However, the total area for oxygen-containing functional groups (C1s peaks at 289 eV→286.2 eV) is constant (Table I). It indicated more –C=O and –HC=O functional groups were formed on the top 100Å layer of oxidized surface initially. These carbonyl groups (–C=O and –HC=O) were replaced by the carboxylic acid groups while keeping the total amount of oxygen-containing functional groups constant as the oxidation time increased. The difference between the ratio of carboxylic acid to carbonyl groups probed by ESCA (Table I) and by ATR-FTIR (Fig. 5) can be attributed to the difference in penetration depth of these two techniques ($\sim$ 100 Å for ESCA and $\sim$ 1 µm for ATR-FTIR). In addition, the surface roughness of the oxidized PE may contribute some effects, which are not determined yet, on these differences.

ESCA results have indicated hirudin was immobilized onto the oxidized PE surface as indicated by the appearance of S2p peak on the spectrum (Table II). These sulfur atoms suggested the disulfide linkages and SO_3 functionality within the hirudin molecule are located on the top 100 Å surface layer. ESCA analysis also showed there is no difference in the amount of hirudin immobilized on the surface between 6 and 12 h immobilization reaction time (data are not shown).

The surface morphology of adhered platelets on different PE samples was shown in Fig. 7. There is no

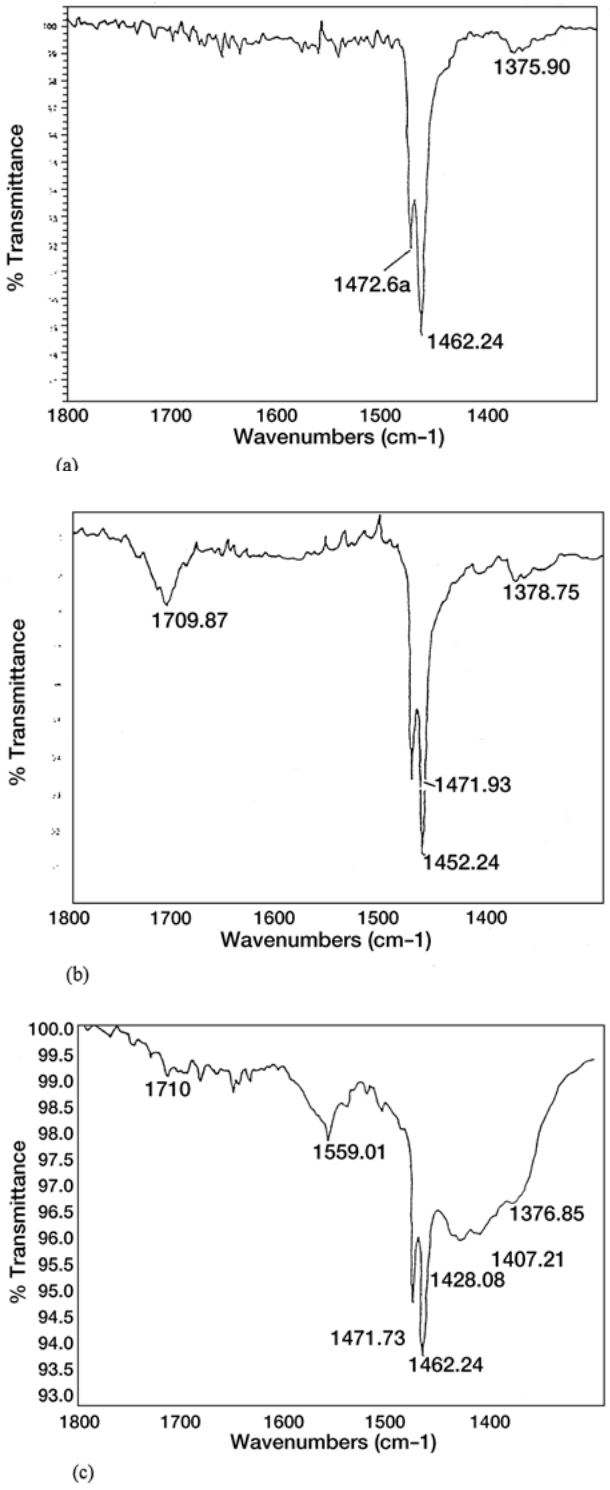

Figure 4 ATR-FTIR spectra of different PE samples (a) nontreated PE, (b) PE with 1 min oxidation time, (c) NaOH treated oxidized PE with 1 min oxidation time.

significant difference in the shape of adhered platelets among these modified PE samples. However, the surface density of adhered platelets varied noticeably. More platelets were adhered onto the oxidized surfaces than the nontreated control due to the rougher surface morphology and the formation of hydrophilic, carboxylic acid and carbonyl functional groups on the oxidized surface [8, 12]. Moreover, the density of adhered platelets increased with the oxidation time among various oxidized PE samples (Fig. 8). This indicates the amount of platelet adherent onto the oxidized PE

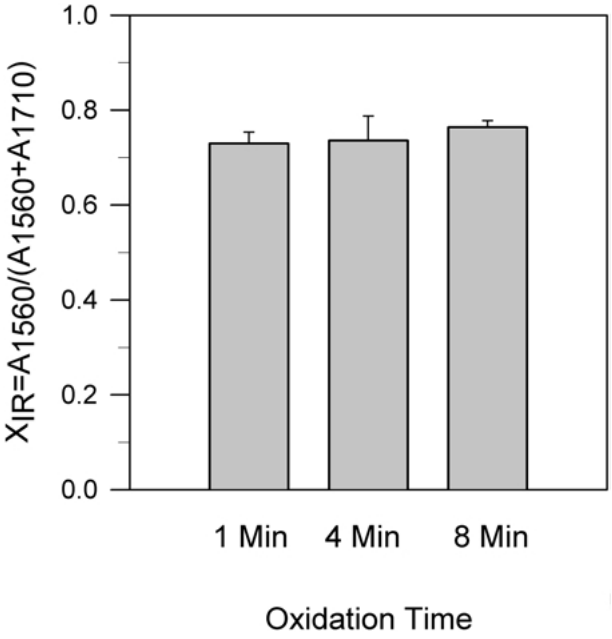

Figure 5 X_{IR} for oxidized PE samples at different oxidation time.

surface is highly related to the amount of carboxylic acid functional group existed on the top layer, i.e. the more carboxylic acid functional group on the surface is, the higher platelet adhesion density is noticed on the oxidized PE.

In contrast, fewer platelets were adhered onto the hirudin-immobilized oxidized surface than the oxidized

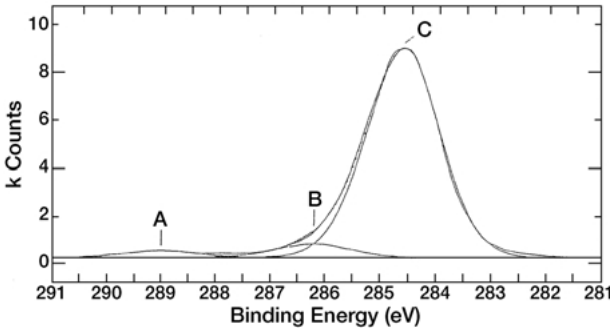

Figure 6 ESCA C1s spectrum of the oxidized PE surface. A: 289 eV, –COOH; B: 286.2 eV (–C = O and –HC = O); C: 284.5 eV, –C–C–.

TABLE I ESCA C1s deconvolution analysis of peak area percentages for PE samples at different oxidation time

Oxidation time (min)	C1s at 284.6 eV, –C–C (%)	C1s at 286.2 eV, –C = O and –HC = O (%)	C1s at 289 eV, –COOH (%)	C1s, 289 eV / C1s, 286.2 eV
0	100	0	0	—
1	92.12	6.00	1.88	0.127
4	92.32	5.45	2.23	0.290
8	91.44	5.97	2.59	0.301

TABLE II ESCA analysis for hirudin-immobilized oxidized PE surface (carbodiimide activation time: 2 h, hirudin immobilization reaction time: 12 h)

Oxidation time (min)	S2p/C1s	N1s/C1s
0	0.0000	0.0000
1	0.0049	0.0461
4	0.0047	0.0423
8	0.0022	0.0311

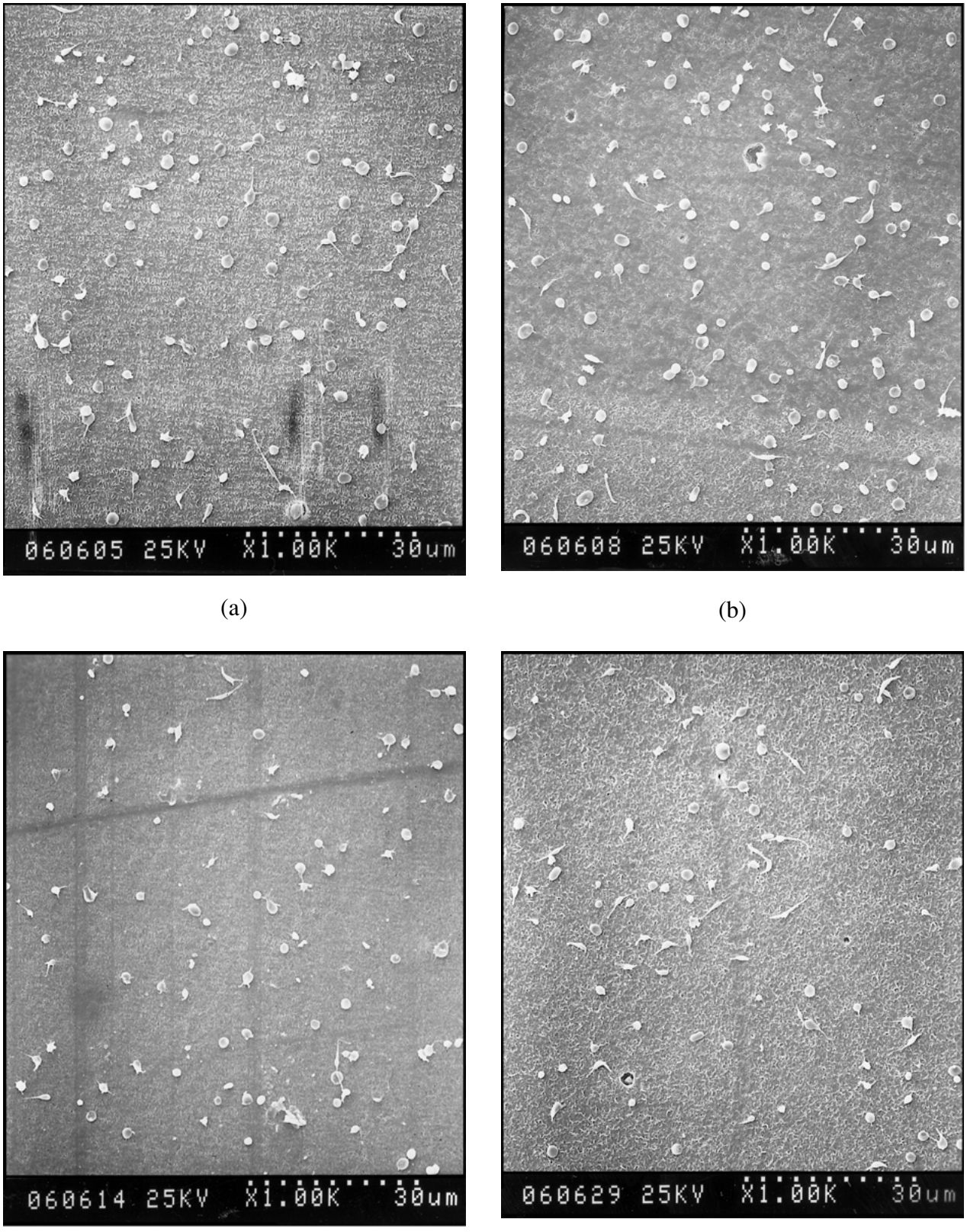

Figure 7 SEM micrographs of the adhered platelets on: (a) oxidized PE after 1 min oxidation reaction; (b) oxidized PE after 4 min oxidation reaction; (c) hirudin immobilized oxidized PE (1 min oxidation + 12 h immobilization reaction); (d) hirudin-immobilized oxidized PE (4 min oxidation + 12 h immobilization reaction).

counterpart. The density of adherent platelet on hirudin-immobilized oxidized PE surface was 30–35% less than the value on the oxidized PE counterpart. This can be attributed to the loss of platelet activation capability by thrombin, which is resulted from the formation of thrombin–hirudin complex. Seifer *et al*. has also reported a decrease in platelet adhesion and activation on the hirudin-immobilized polylactide-polyglycolide surface [1]. In addition, Phaneuf *et al*. and Ku *et al*. [10, 11] have also noticed the covalent linked hirudin still maintained its ability to complex with thrombin thereby inhibiting thrombin catalyzed blood coagulation cascade. This

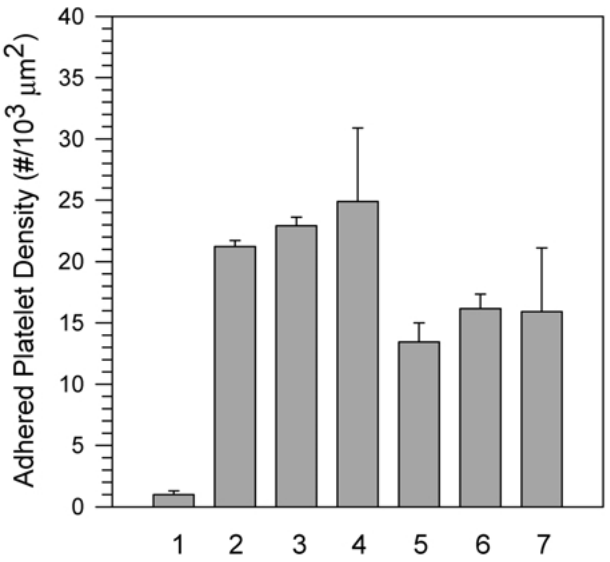

Figure 8 Adhered platelet density on different polyethylene surfaces, 1. nontreated control, 2. oxidized PE with 1 min oxidation reaction, 3. oxidized PE with 4 min oxidation reaction, 4. oxidized PE with 8 min oxidation reaction, 5. hirudin immobilized oxidized PE with 1 min oxidation reaction and 12 h immobilization reaction, 6. hirudin-immobilized oxidized PE with 4 min oxidation reaction and 12 h immobilization reaction, 7. hirudin-immobilized oxidized PE with 8 min oxidation reaction and 12 h immobilization reaction

further indicated the covalently immobilized hirudin using the reaction scheme here could retain its anticoagulation capability on the oxidized PE surface. Moreover, the differences in the protein adsorption from the platelet concentrates onto these hirudin-immobilized oxidized PE surface could also lead to the improvement in platelet reactivity as well.

However, these hirudin-immobilized oxidized PE surfaces exhibited more platelets adhered onto the surface than the nontreated PE control. This can be explained by the platelet-activating effects caused by the hydrophilic functional groups, such as aldehyde and ketone, remained after the hirudin immobilization reaction, due to the much lower concentration of hirudin used than previous studies [1, 10, 11]. Ko *et al*. have noticed similar phenomenon that the carbon dioxide plasma modified PE surface, containing carbonyl and carboxyl groups, is more platelet reactive than the nontreated control [8]. In addition, the rougher surface texture found on the hirudin modified PE might activate platelets easier than the smooth one on the nontreated control [12]. A study, currently under investigation, utilizing oxygen plasma surface preactivation technique might be able to avoid such surface etching/roughing effects brought by the chemical oxidation method.

4. Summary

A simple two-step surface preactivation scheme is explored for hirudin immobilization. Polyethylene surface was oxidized by chromic acid solution. More carbonyl functional groups, such as aldehyde and ketone, were formed in short oxidation time than carboxylic acid functional groups. As oxidation time was increased, the amount of carboxylic acid functional group was also increased while the total amount of oxygen-containing functional groups remained constant in the top 100Å surface layer. Surface carboxylic acid functional groups were activated by water-soluble carbodiimide for subsequent hirudin immobilization. ESCA results indicated the number of hirudin molecules immobilized was constant at the reaction time studied. *In vitro* blood compatibility assessment using platelet adhesion assay demonstrated that less platelet were adhered onto the hirudin-immobilized surface than onto the oxidized ones, resulting from the decrease of platelet-activating capability of thrombin by the formation of hirudin-thrombin complex, and the differences in the adsorbed protein composition. This indicated these covalent-bound hirudin molecules could maintain their anti-platelet-activating capability after immobilization scheme used in this study.

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