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Enhancement of Antithrombogenicity by Urokinase Immobilization on the Gelatin-Adsorbed Polyurethane Surface

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=Abstract=The porous polyurethane (PU) sheet was impregnated with 5% (w/v) gelatin solution and the fibrinolytic enzyme to promote the antithrombogenicity of artificial ventricle's surface. Urokinase was immobilized on the gelatin-treated surface with 0.45% (w/v) buffered glutaraldehyde solution (pH 7.4). The coagulation time was delayed to 1.2 times in the gelatin-urokinase co-immobilized surface than the nonporous PU control by APTT measurement.

By urokinase immobilization, the maximal activities of the enzyme were elongated (from 2 hour to 4 hour). In vitro study shows that the antithrombogenicity was improved after gelatin adsorption and urokinase immobilization on the polyurethane.

Key words: ***Urokinase immobilization, Gelatin, Antithrombogenicity***

INTRODUCTION

Exposure of blood to artificial surfaces leads to adsorption of plasma proteins within a matter of seconds. The composition of this early protein layer is known to be variable according to the surface characteristics of artificial surfaces. After this adsorption, there may be subsequent deposition of platelets and leukocytes, leading to blood coagulation on the surface of the biomaterial, a phenomenon known as thrombosis (Graevelink *et al.* 1986; Niewiarowski and Varma 1982).

Blood coagulation results from the conversion of a soluble circulating plasma protein, fibrinogen, into insoluble fibrin. As antithrombogenic agent, plsminogen is present as fibrinolytic enzyme in plasma at concentration of 200 to 495 μg/m1. The conversion of plasminogen to plasmin is caused by plasminogen activator, and plasmin lyses fibrin polymer and a number of other plasma proteins, such as Factor V, VII, and XII. Thus plasmin is a nonspecific proteolytic enzyme. In this study, we performed immobilization of gelatin and urokinase on the porous polyurethane (PU) sheet.

Gelatin is hydrophilic and inert to blood, so gelatin is known to retard the adsorption of hydrophobic materials, such as platelets, which lead to blood coagulation pathway. Urokinase is a fibrinolytic enzyme and it activates plasminogen to plasmin. In the present study, we developed a new technique of gelatin adsorption and urokinase immobilization to enhance the antithrombogenicity of the segmented polyurethane polymer material, which is widely used in the artificial ventricular sacs.

MATERIALS AND METHODS

Preparation of porous PU sheet and gelatin adsorption: PU sheet (Pellathane 2363-80A, Dow Chemical, U.S.A.) was made with 12% (w/v) PU solution (12g PU/100 ml N, N-dimethylacetqmide, DMAc) through solvent exchange between water and DMAc. A five percent (w/v) gelatin solution (5g gelatin, Type A: porcine skin,

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Table 1. Pump biolization procedure

Assemble pump housing, diaphragm and actuator
↓
Dip the assembly in 5% gelatin solution
↓
Reduce pressure for 30 min
↓
Return to 1 atm for 5 min
↓
Reduce pressure for 5 min
↓
Return to 1 atm
↓
Place the assembly in high humidity chamber at 4°C for 15 min
↓
Dip in gelatin solution at 30°C for a few seconds
↓
Place in high humidity chamber at 4°C for 15 min
↓
Submerge in 0.45% glutaraldehyde solution at 4°C for 10 hrs
↓
Store in 4% formaldehyde solution at room temperature until used

Sigma, U.S.A./100 ml distilled water) was degassed in vacuum oven (60°C, −25 - −28 mmHg) for about 30 minutes. Then gelatin solution was adsorbed by the method listed in Table 1 (Kambic *et al.* 1983).

Surface roughness vs. gelatin concentration: Gelatin solutions of different concentrations (1%, 3%, 5% (w/v)) were adsorbed on the porous PU sheets to observe what concentration of gelatin solution revealed the most smooth surface. Then, they were compared with the surface of nonporous and porous PU sheets as control group. Surfaces were observed with a scanning electron microscope (SEM, 2000X).

***In vitro* test for blood compatibility:**

1) Prothrombin time (PT) and activated partial thromboplastin time (APTT)

The PU sheets (1×2 cm) treated with gelatin of different concentrations were immersed in 200 μl of control plasma (AHS delcaribe, Inc.) and incubated at room temperature for 1 hour. After incubation, APTT was measured by fibrometer method and PT was measured by one-stage prothrombin time (Miale 1972).

2) Platelet adsorption test

The degree of platelet adsorption was examined using following method (Kim *et al.* 1987). Platelet rich plasma (PRP) was obtained by centrifugation of citrated human blood. Sheets were immersed in PRP at 37°C for 1 hour and washed with phosphate buffered saline. The adsorbed platelets on each sheet surface were immobilized with 2% (w/v) glutaraldehyde buffer (pH 7.4) for 2 hours. After they were dehydrated and freeze-dryed, the adsorbed platelets were observed wih SEM (2000x).

Urokinase immobilization method on gelatin-adsorbed surface: Urokinase was immobilied on the gelatin-adsorbed porous PU sheet with 0.45% (w/v) glutaraldehyde buffer (pH 7.4). Then urokinase of different concentrations (0.0001, 0.001, 0.01, 0.1, 1, 2, 5, 10, 20 IU) was immobilized for duration of 1, 2, 3, 4, 5 hours to examine the optimal concentration of urokinase and immobilization time for enzyme activity. And the ratio of the immobilizd urokinase activity to the nonimmobilized urokinase activity was examined by measurement of urokinase activity of each concentration to a control group.

Immobilized urokinase activity test:

1) Materials

The following solutions were used to perform the immobilized urokinase activity test.

-Acto-Med (1M Tris pH 8.1 100 ml+ Plasminogen free serum 50 ml + 1.5 M NaN_3 (10%) 6.7 ml/1000 ml Distilled water)

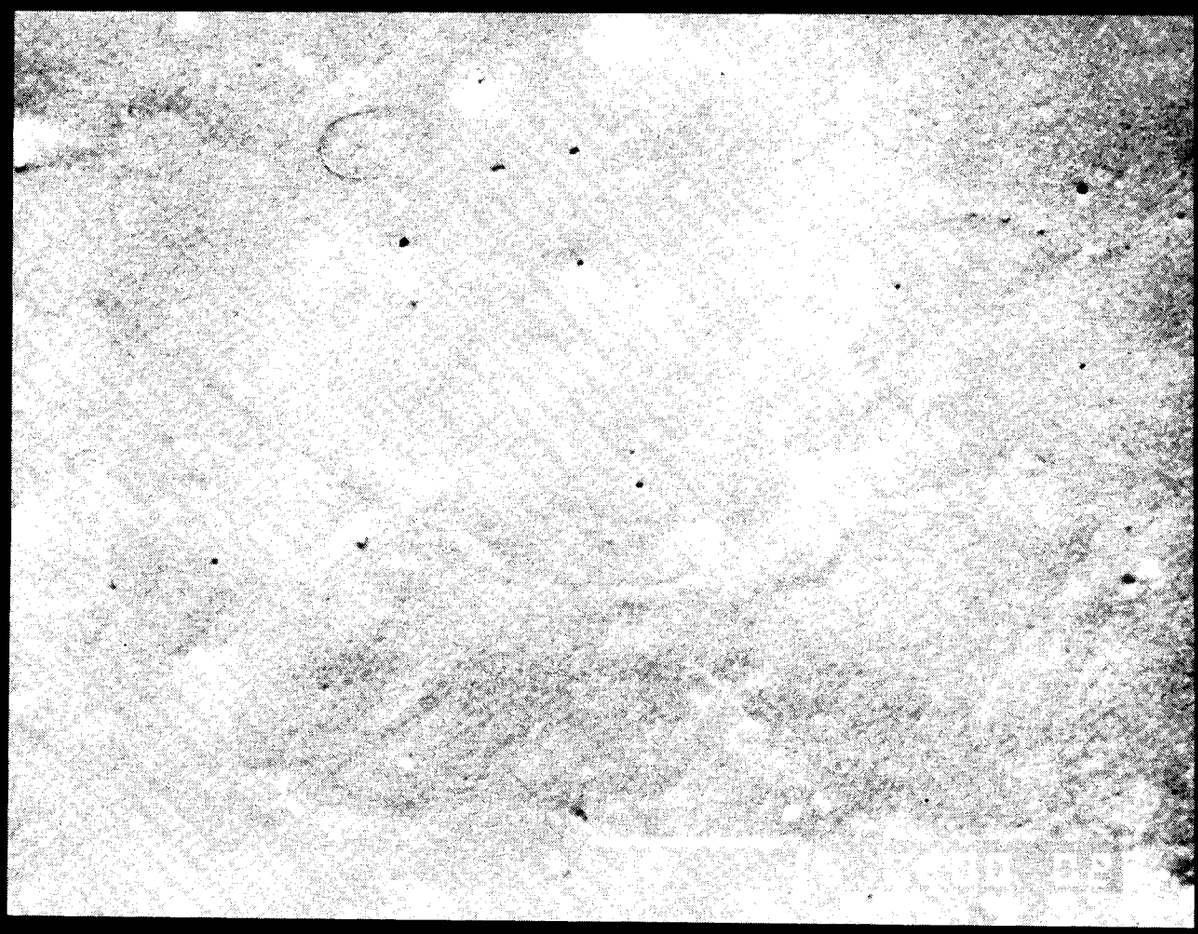
-PT buffer (1M NaH_2PO_4 50 ml + 100% Tx-100 10 ml/500 ml Distilled water)

-PA buffer (1M Tris pH 8.1 100 ml + Gelatin 1 g + 100% Tx-100 50 ml/1000 ml Distilled water)

2) Analysis method

125-Iodine labeled 24-well fibrin plate was made by Chloramine-T method. Fibrin plate was activated by addition of 200 μl of Acto-Med per well and incubation at 37°C for 1 hour. Then the plate was washed three times with 10 mM Tris (pH 8.1). Enzyme rection was initiated by adding urokinase 200 μl, PA buffer 750 μl, and plasminogen 50 μl (80 μl/ml).

(a)



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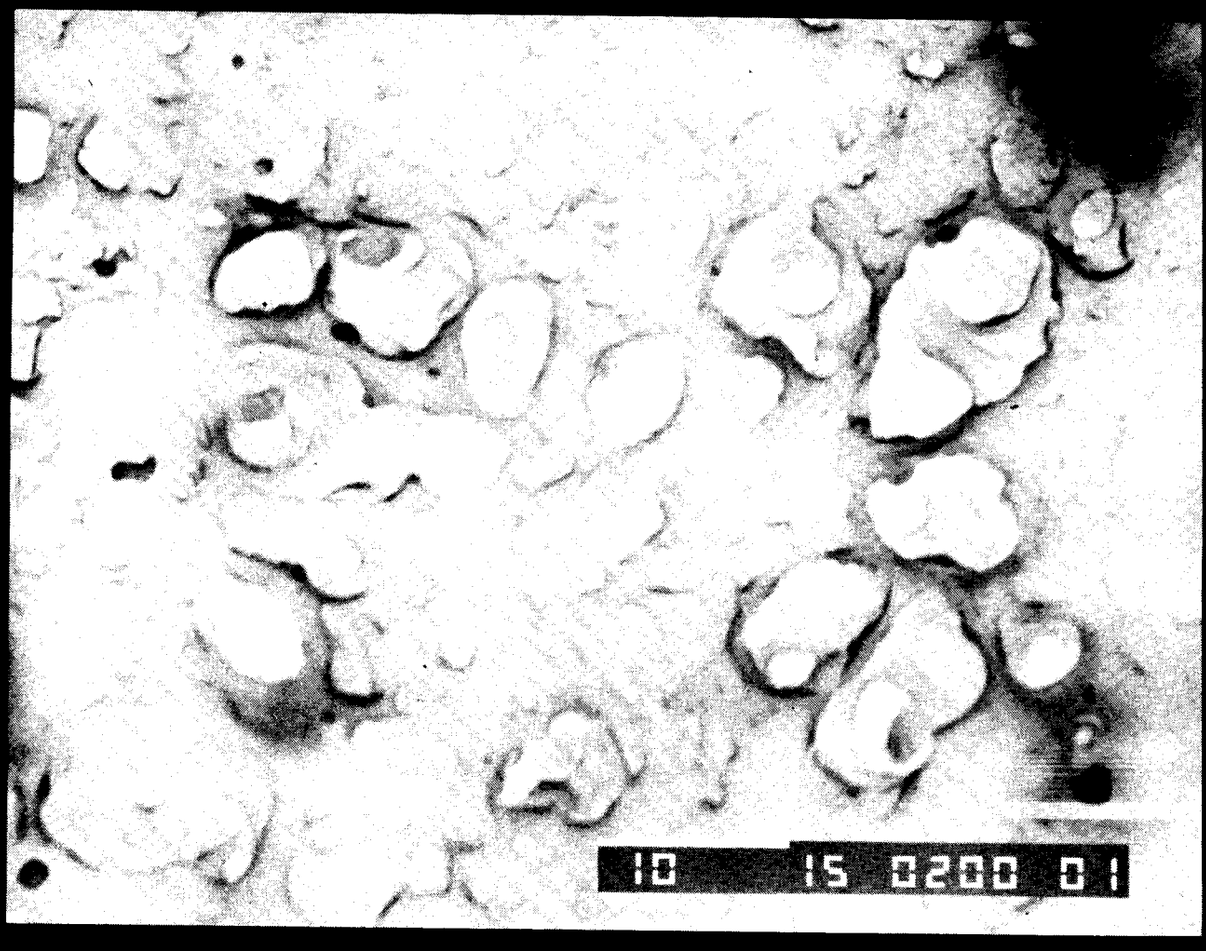

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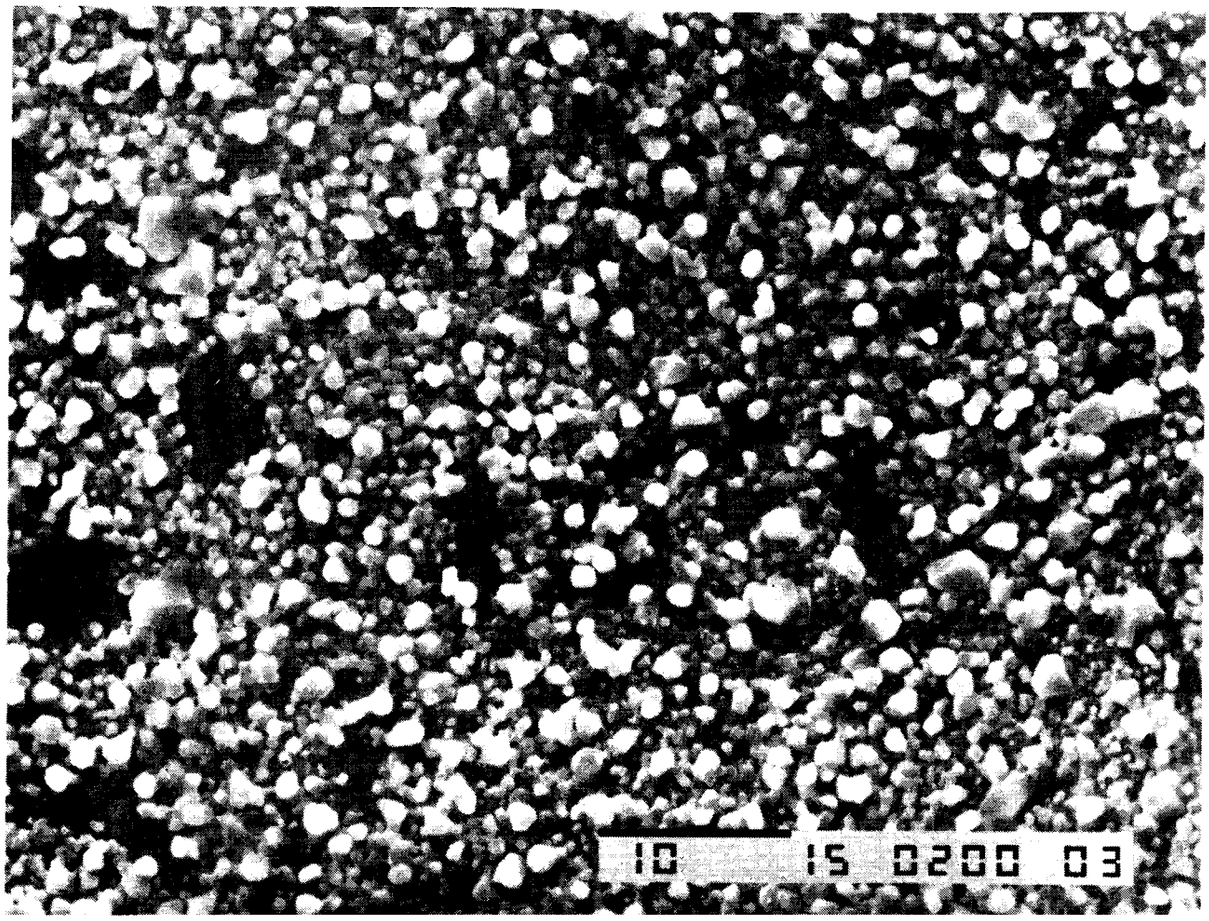

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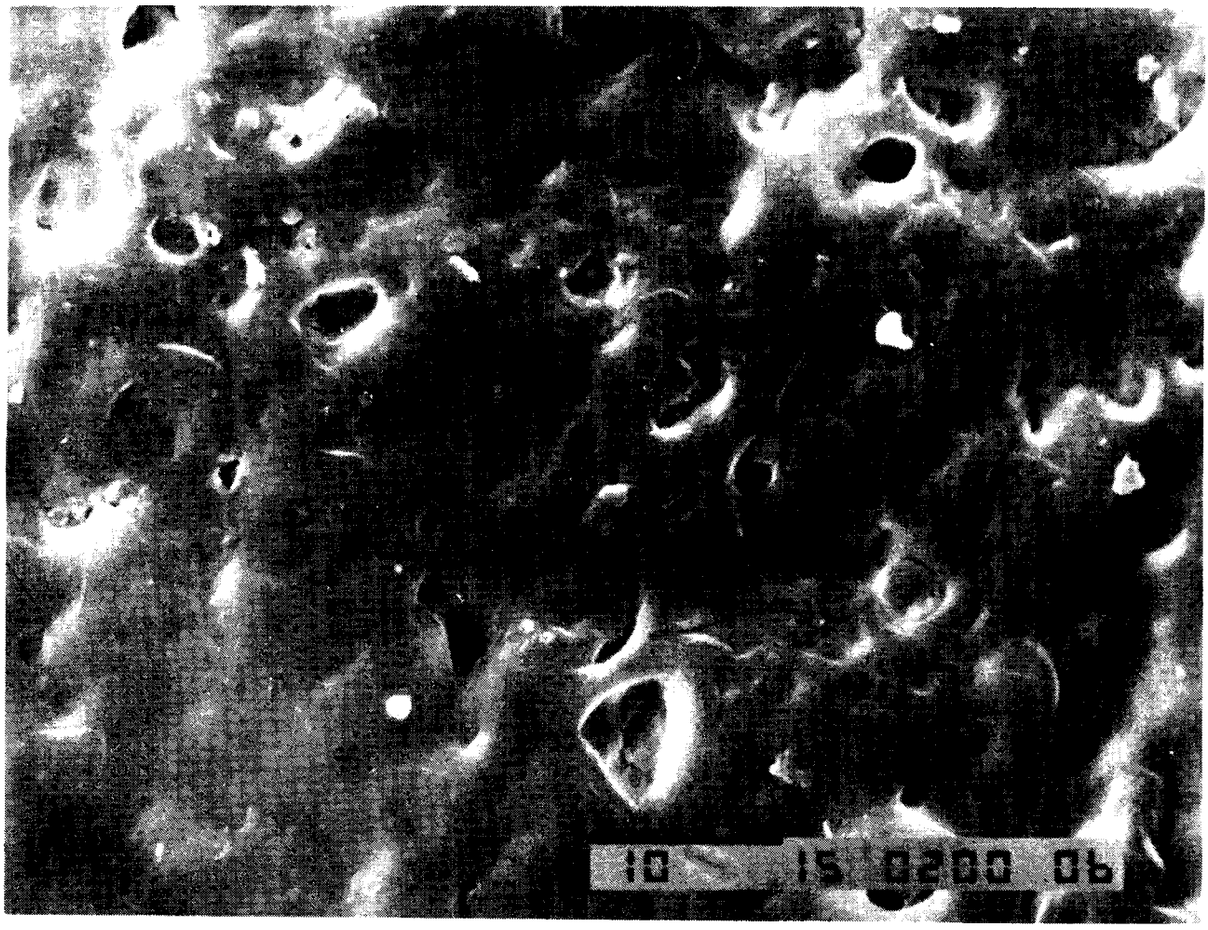


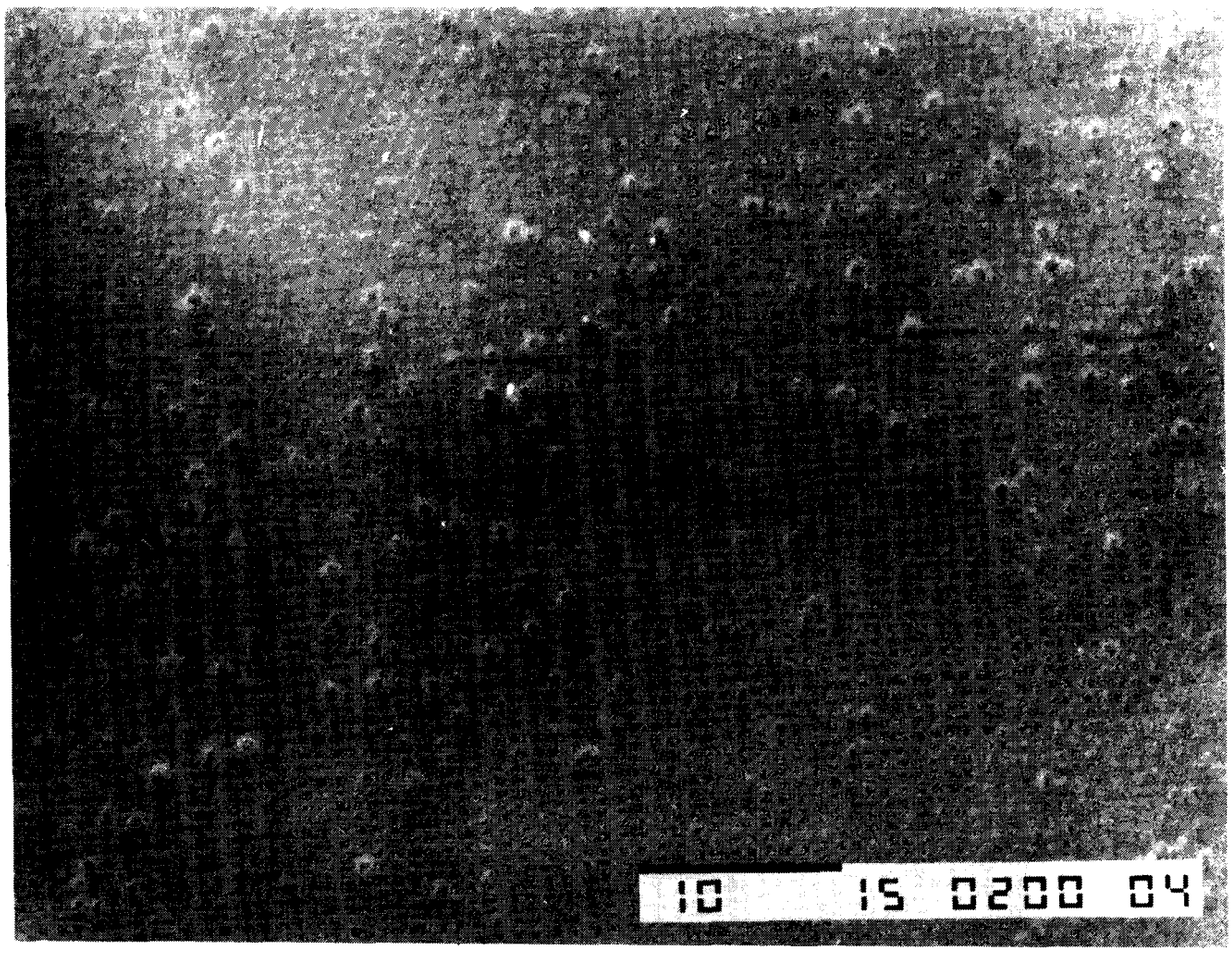


Fig. 1. The surface appearances of gelatin crosslinked with 0.45% (w/v) glutaraldehyde.

The fibrin plate was incubated at 37°C in a high humidity chamber. After incubation, radioactivity (i.e. enzyme activity) was measured by gamma-specrometer (United technologies Packard Auto-gamma 500c/800c, U.S.A.).

RESULTS

Surface roughness vs. gelatin concentration: Fig 1. shows the SEM pictures of the surface at various gelatin concentrations. Nonporous PU sheet showed smooth surface (Fig 1 (a)). The porous PU sheet had many pores and the average size of them was about $5.2 \times 6.5\ \mu m^2$ (shortest diameter × longest diameter) (Fig 1 (b)).

The above two sheets were used as control group. The gelatin adsorbed sheets of 1% and 3% (w/v) of concentration showed rough surface (Fig 1(c), (d)).

But the 5% (w/v) gelatin-adsorbed sheet showed smooth surface, almost similar to the nonporous PU sheet (Fig 1(e)).

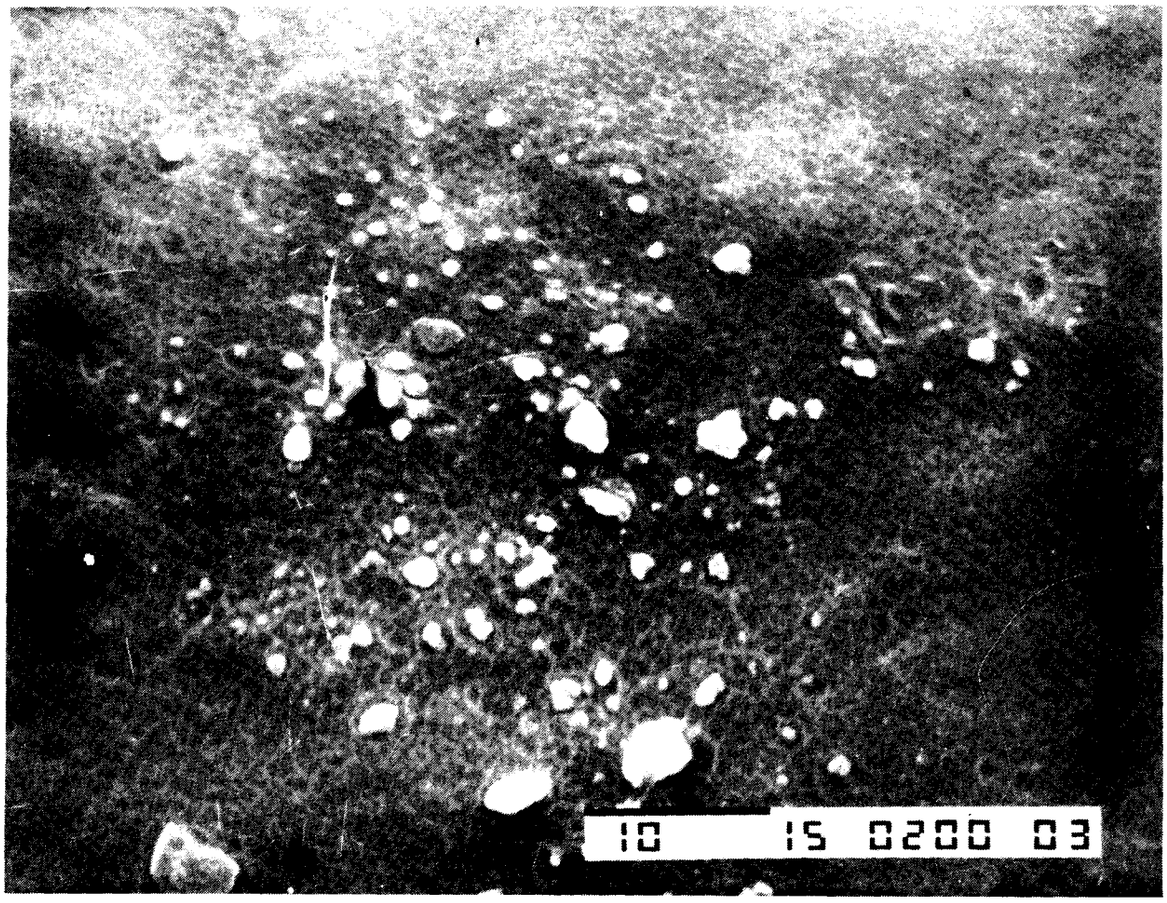
From the result of Fig 1, it was shown that 5% (w/v) sheet has the most smooth surface after gelatin-coating.

In vitro tests for blood compatibility

PT and APTT: Antithrombogenic properties were studied for each sheet using PT and APTT measurements (Table 2). In the result of APTT measurements the coagulation time was delayed by 1.2 times in the gelatin-adsorbed surface and 1.6 times in the gelatin-urokinase (2U, immobilized for 1 hour) coimmobilized surface compared to the nonporous PU control. This result suggests that hydrophilicity of gelatin and urokinase immobilization may contribute to antithromobgenicity. In case of PT, the coagulation times were also extended in gelatin-adsorbed and gelatin-urokinase co-immobilized surface.

Platelet adsorption test: Nonporous, porous, and gelatin-adsorbed porous PU sheets were incubated at 37°C for 1 hour and observed by SEM (Fig 2). Platelets were broken and adsorbed on the

(a)



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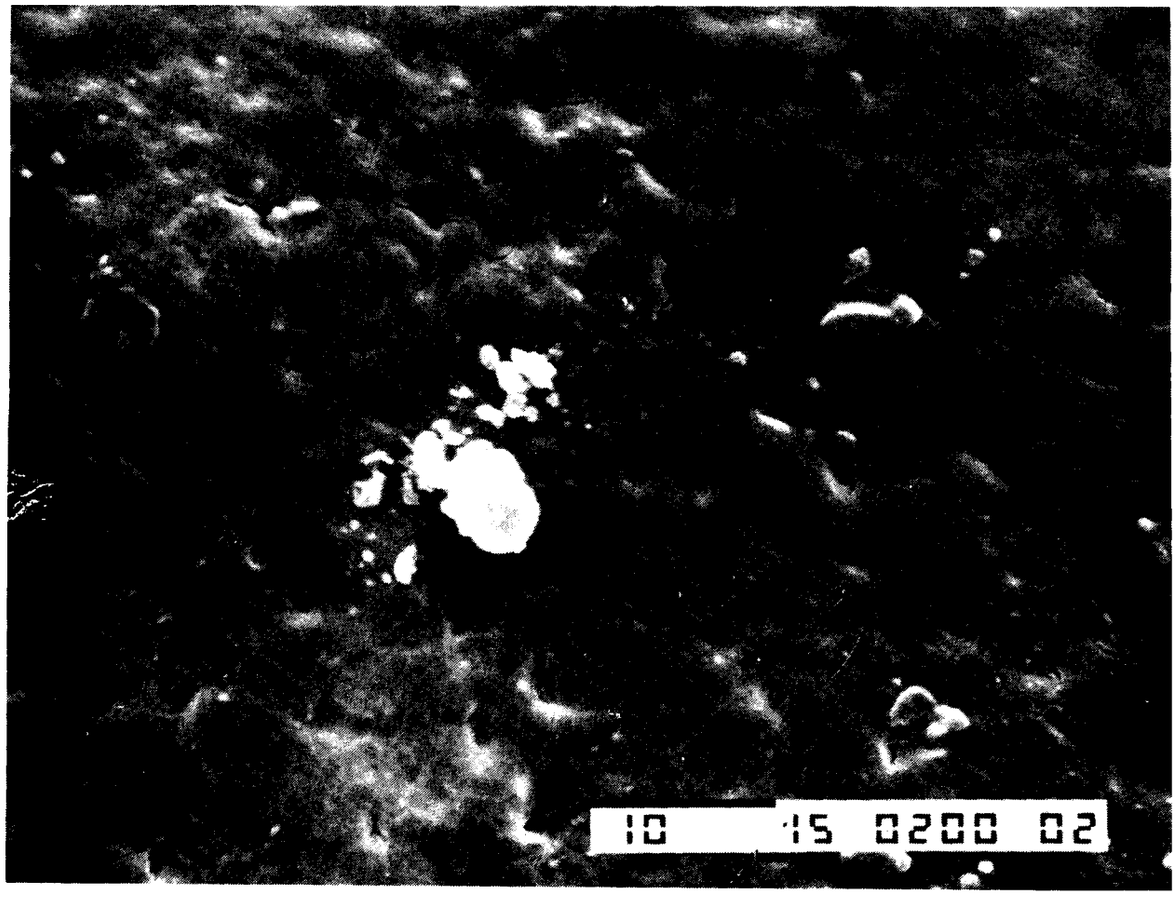

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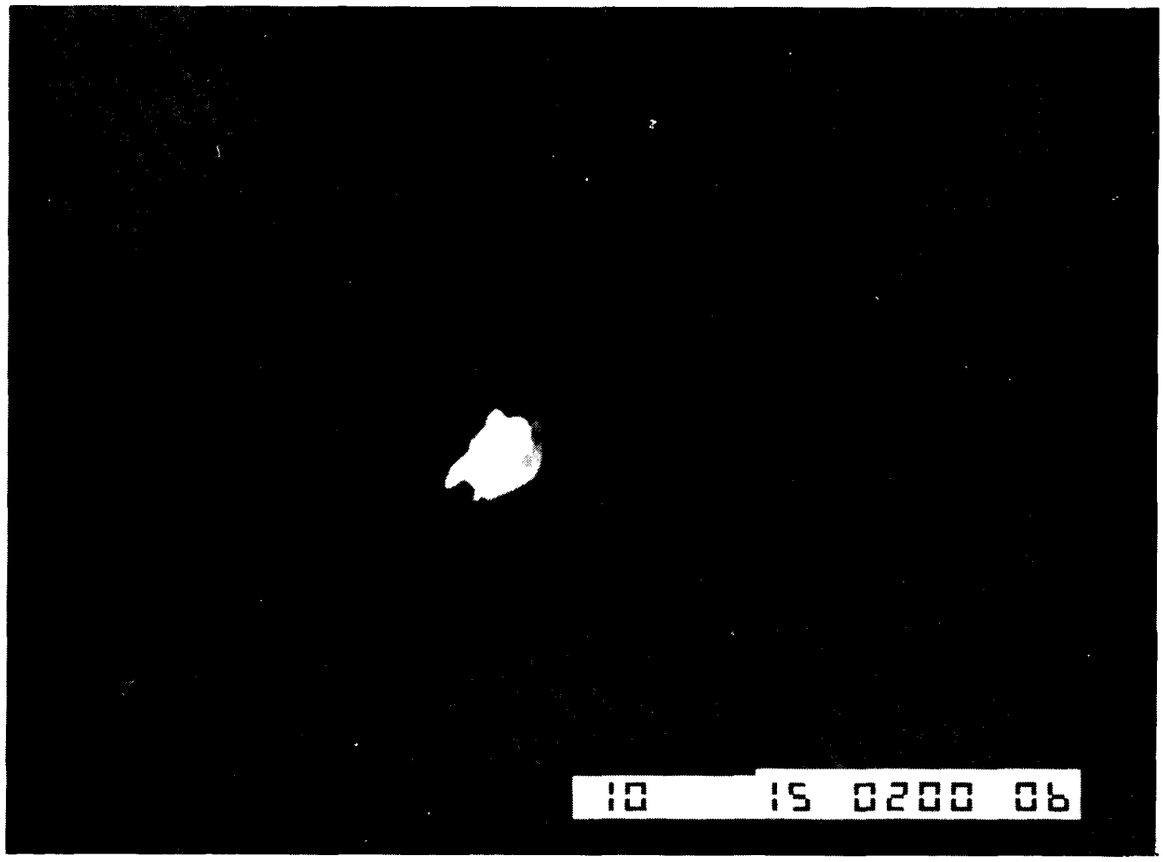

Fig. 2. Figure of platelet adsorption on various surfaces.

Table 2. PT and APTT results of gelatin and urokinase immobilized PU surfaces

	control plasma	Nonporous PU control	Gelatin-coated surface	Gelatin-UK* coimmobilized surface
PT(sec)	13.9	13.1	14.8	19.6
APTT(sec)	28.9	28.9	36.0	45.8

*UK:urokinase (2U, immobilized for 1 hr)

nonporous and the porous sheets (Fig 2(a), (b)).

But a very small number of platelets were adsorbed on the gelatin-adsorbed sheet and nearly all the adsorbed platelets maintained their original forms (Fig 2(c)).

Urokinase activity: Table 3 and Fig 3 show the experimental data of the time variation of nonimmobilized urokinase activity on fibrin plate at various concentrations of urokinase. As shown in Fig 3, the urokinase activity was saturated at the concentration range of 1 to 2U.

Thus 1U and 2U of urokinase concentration were adopted for immobilization. Immobilization time with glutaraldehyde was for duration of 1 hour, 2 hours, and 3 hours.

The comparison of urokinase activity for different crosslinking time with glutaraldehyde was listed in Table 4. The numbers in parentheses of Table 4 shows the value of the nonimmobilized urokinase activity. The best immobilized urokinase activity was obtained with 1 hour reaction with glutaraldehyde. Fig 4 shows the activity of urokinase which was immobilized for 1 hour. In comparison with Fig 3, the maximal activities of Fig 4 were shown to occur at longer effective time duration (from 2 hour to 4 hour).

Table 3. Nonimmobilized urokinase activity on 125-I fibrin plate as control group

Urokinase activity (cpm*)					
Time(hr)	0.0001U	0.001U	0.01U	0.1U	1U
0	0	0	0	0	0
1	83	514	1906	3241	4771
2	348	1710	3597	5498	6853
2.5	506	2074	4269	5906	7065
8.5	1430	2098	2089	2126	4486

Urokinase activity (cpm*)				
Time(hr)	2U	5U	10U	20U
0	0	0	0	0
1	4758	5021	5157	5310
2	6067	6468	6208	5310
2.5	6361	6799	6263	6497
8.5	1045	1399	912	1120

Note:*; count per minute

The activity ratio of immobilized urokinase to the control group (nonimmobilized, glutaraldehyde-nontreated group) is listed in Table 5, and shows the saturation of activity at 1U of urokinase concentration.

DISCUSSION

The blood compatibility of any material depends upon the characteristics of the contact surface in interaction with plasma proteins and it should demonstrate the capacity to remain free of thrombus. Though polyurethane is one of the widely used biomaterials for its better antithrombogenicity than other materials, thrombus formation usually occurs due to the hydrophobicity of polyurethane surface. Thus in this study, we adsorbed gelatin, a hydrophilic protein, on polyurethane surface.

Adsorbing gelatin on porous PU sheet, the critical problem was its viscosity, thus the 5% (w/v) gelatin showed the most smooth surface and was adsorbed well in the pores of sheet under vacuum condition. Gelatin-adsorbed sheet also showed better antithrombogenicity than the control group in platelet adsorption test, PT and APTT tests. The antithrombogenicity was shown to be further improved after gelatin adsorption and urokinase immobilization on the gelatin surface by covalent bond. Glutaraldehyde (0.45% (w/v)) was used as a crosslinking agent as in the case of gelatin-adsorption. As shown in Fig. 3, one unit of urokinase showed the maximum activity among various concentrations and these saturated phenomenon of activity was observed in all concentrations.

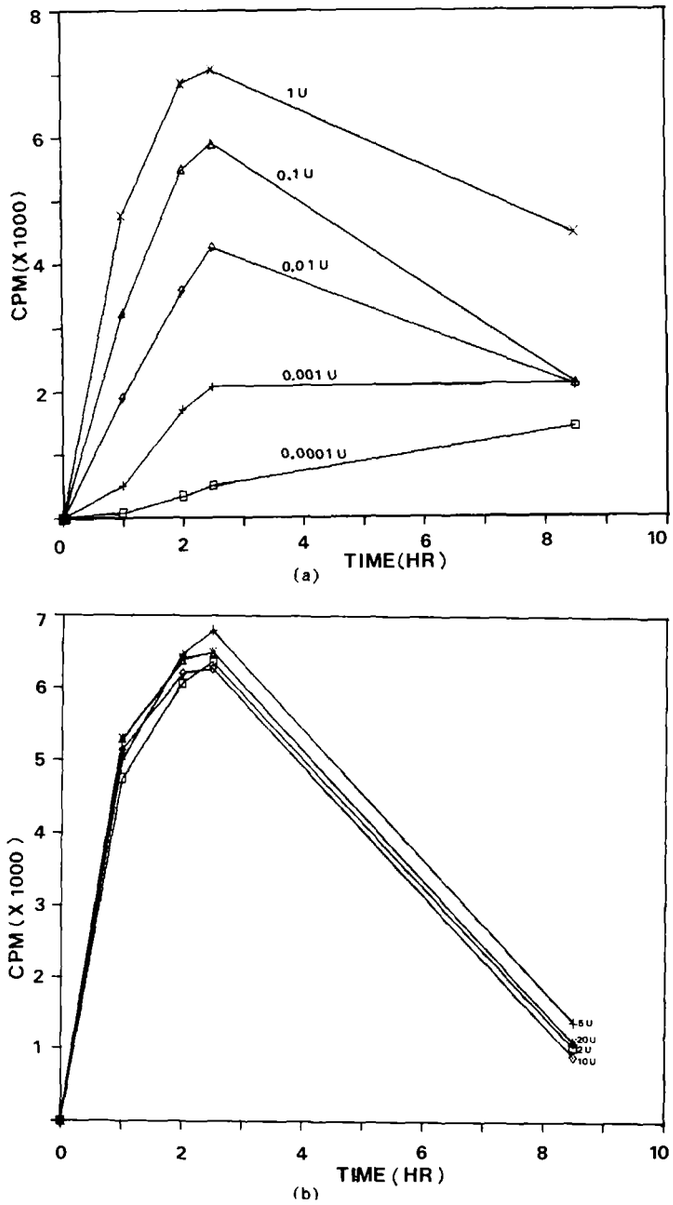

Fig. 3. Urokinase activity of control group.

The range of a linear relationship between enzyme concentration and its activity at 2.5 hours of incubation time were shown in Fig. 5. As shown in Table 5, the relative activity of immobilizd urokinase increased with reaction time. This phenomenon was due to the late occurrence of the maximum activity on the urokinase-immoilized sheet. The absolute value of activity was

Table 4. Comparison of Immobilized Urokinase activity on 125I-fibrin plate for different incubation time and crosslinking time with glutaraldehyde

Incubation Time(hr)	1U Urokinase activity, cpm(*)		
	1hr reaction with GA**	2hr reaction with GA	3hr reaction with GA
0	0	0	0
1	113(161)	31(31)	44(61)
2	594(606)	107(148)	
3	1594(1595)		
4	2399(2399)		
5	2360(3124)		

Incubation Time(hr)	2U Urokinase activity(cpm)		
	1hr reaction with GA	2hr reaction with GA	3hr reaction with GA
0	0	0	0
1	314(370)	99(99)	118(137)
2	1045(1123)	311(311)	
3	2403(2403)		
4	3923(3923)		
5			

Note

*:Numbers in parentheses shows the cpm value of nonimmobilized glutaraldehyde treated urokinase activity

**:Glutaraldehvde

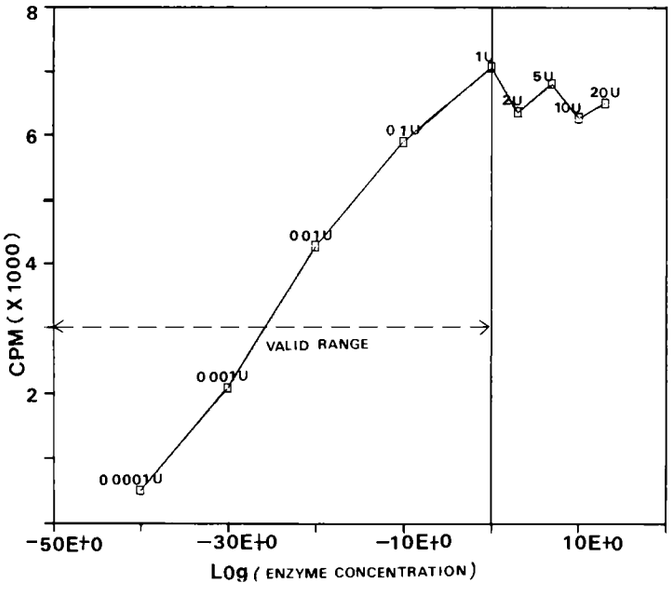

Fig. 5. Relationship of enyme activity to enzyme concentration after 2.5 hours incubation.

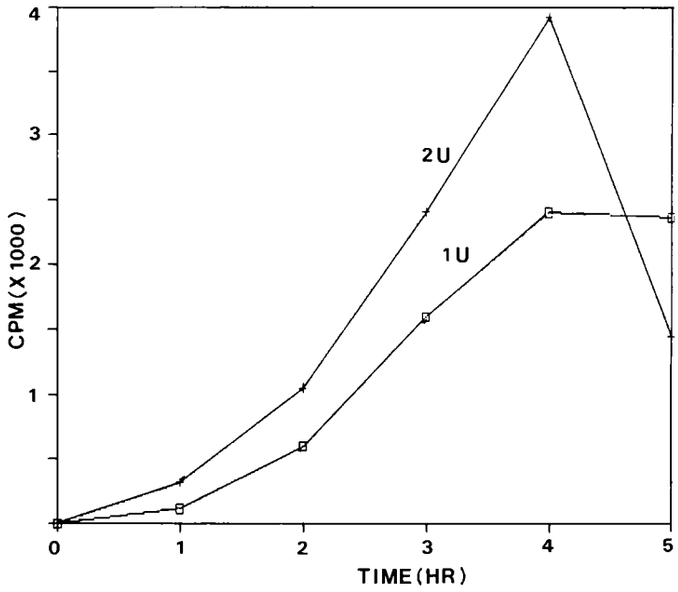

Fig. 4. Immobilized urokinase activity.

Table 5. Relative activity ratio of immobilized urokinase of control group

Incubation Time(hr)	Relative activity of 1U Urokinase(%)		
	1hr reaction with GA	2hr reaction with GA	3hr reaction with GA
0	0	0	0
1	2	0.65	0.9
2	9	1.6	
3	23.3		
4	37.5		
5	39.3		

Incubation Time(hr)	Relative activity of 2U urokinase(%)		
	1hr reaction with GA	2hr reaction with GA	3hr reaction with GA
0	0	0	0
1	6.6	2.1	2.5
2	17.2	5.1	
3	40.4		
4	77.7		
5	32.2		

lower in immobilized urokinase than that of nonimmobilized urokinase by the ratio of 39.3% after five hours of incubation time.

These results suggest that the further study be required to increase the immobilization yield

of urokinase and maintain the activity of immoilized urokinase for longer duration. And this diminution of activity may be due to the enzyme's tertiary structural chang causing immobilization in the wrong direction in the interaction with the substrate (fibrin clot). Glutaraldehyde is a strong crosslinking agent, for stable protein immobilization. However it may also damage enzyme activity. Also it was reported that glutaraldehyde had antigenicity. Evaluation of other cross-linking agents was under researches by some investigators (Habeeb 1969).

There we need further study to find specific crosslinking agent which reacts with a specific side chain of urokinase irrelevant of active site, to minimze the loss of activity and immune reaction. Immobilized urokinase is known to be less affected by antiplasmin and antibody in blood because fibrnolysis occurs by direct contact on urokinase immobilized surface. This phenomenon is also similar in the case of the endothelialized surface of blood vessel.

In conclusion, we showed that gelatin adsorption and urokinase immobilization on polyurethane can enhance antithrombogenicity. Since the polyurethane has very desirable characteristics of mechanical strength and compliance, our present study of further improving its antithrombogencity may contribute to the development of artificial organs such as artificial heart and artificial blood vessel.

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=국문초록=

항혈전성 향상을 위한 폴리우레탄표면에 대한 젤라틴과 유로키나제의 고정화

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류규하 · 박선양* · 조한익** · 민병구

인공 심실 표면의 항혈전성을 향상시키기 위해서 다공성 폴리우레탄층 위에 5%(W/V)의 젤라틴 용액을 침투시키고 이 위에 섬유소 분해효소인 유로키나제를 0.45%(W/V)의 글루타르알데히드 (pH 7.4)로 고정시켰다.

APTT테스트 결과 무공성 폴리우레탄층 대조군에 비하여 젤라틴을 흡착시킨 표면에서는 응고시간이 약 1.2배, 젤라틴과 유로키나제를 함께 고정시킨 표면에서는 응고 시간이 약 1.6배 연장되었다. 고정화된 유로키나제의 최대 활성도에 도달하는 시간은 비고정화된 유로키나제의 2시간에 비해 4시간으로 연장 되었다. 따라서 최근에 실시한 체외실험에 의한 연구 결과 폴리우레탄층 위에 젤라틴을 흡착시키고 유로키나제를 고정화시키게 되면 항혈전성이 향상됨을 알 수 있었다.