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# γ-벤질 L-글루타메이트/프로필렌 옥사이드 블록 공중합체의 항혈전성에 관한 in vitro와 in vivo에서의 연구

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# In Vitro and in Vivo Studies on Antithrombogenicity of γ-Benzyl L-Glutamate-Propylene Oxide Block Copolymer

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요 약 : 폴리 γ-벤질 L-글루타메이트/폴리 프로필렌 옥사이드 블록 공중합체의 항혈전성에 대하여 in vitro와 in vivo에서 행해졌다. in vitro에서의 혈소판 점착실험은 미립자칼럼방법이 채택되었고, in vivo에서의 항혈전성은 블록공중합체로 코팅된 1.66mm의 작은 직경의 catheter을 개의 정맥혈관에 삽입한 후 일정한 시간에서 형성된 혈전량을 가지고 평가하였다. in vitro 실험결과 혈소판의 점착은 미크로상분리구조와 2차 구조를 갖는 블록공중합체에서 억제되었다. in vivo 실험결과에서도 블록공중합체에서 폴리우레탄에 비해 월등한 항혈전성을 나타내었다.

**Abstract** : In vitro and in vivo antithrombogenicity of poly(γ-benzyl L-glutamate)/poly(propylene oxide) block copolymer was studied. For in vitro evaluation of platelet adhesion on block copolymer surfaces, a microsphere column method was used. For in vivo evaluation of the antithrombogenicity of copolymer surfaces, small diameter(I.D. : 1.66mm) catheter precoated with copolymer was implanted in dogs to determine the mean thrombus weight. In vitro test showed that platelet adhesion was suppressed on block copolymers having microphase-separated structure. In vivo test showed that block copolymer surface exhibited excellent antithrombogenicity as compared with polyurethane catheter.

## INTRODUCTION

Biomaterial thrombogenicity remains the most important concern preventing even more widespread application of artificial organs. Researchers have typically taken one of two approaches in attempts to eliminate this problem : the surface modification approach,[1,2] in which formed polymers are modified or new polymers synthesized with surface properties deemed blood compatible, or the pharmaceutical approaches,[3,4] in which anticoagulant and antiplatelet agents are employed along the polymer.

It has been reported that the heterogenicity of the synthetic polymer surface plays an important role in blood compatibility.[5] Okano et al.[6] proposed that the polymeric hydrophilic-hydrophobic microseparated structure is a key parameter for controlling antithrombogenic activity of polymers due to its apparent inhibition of platelet aggregation.

The thrombogenic properties A-B-A triblock copolymers composed poly(γ-benzyl L-glutamate) (PBLG) and random copolymer of butadiene/acrylonitrile were investigated taking notice of morphological order and macromolecular motions of polymer surfaces by Anderson et al.[7] The microheterophase structure and biocompatible properties of A-B-A block copolymers consiting of PBLG as the A component and polybutadiene as the B component were performed by Nakajima et al.[8] Thrombus formation on the A-B-A block copolymers consisting of PBLG and poly(propylene oxide)(PPO) from an in vitro test using canine blood was minimum on a block copolymer containing a PPO segment of 50 mol% by Imanishi et al..[9]

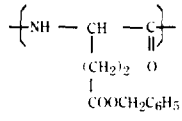
In a previous study,[10] we have studied antithrombogenicity of A-B-A type block copolymers composed of γ-benzyl L-glutamate as the A component and poly(ethylene oxide)(PEO) as the B component in relation to the secondary structure of the block copolymers. Throughout these studies, we found out that platelet adhesion was minimized at the highest content of α-helix in the block copolymer.

In another paper,[11] we have synthesized and characterized A-B-A type block copolymers consisting of PBLG as the A component and poly(propylene oxide)(PPO) as the B component(abbreviated GPG). It was found that the helical content of the GPG block copolymers decreases with increasing PPO content.

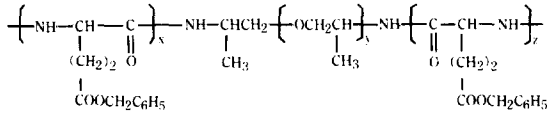
The present study was aimed to clarify further relationship between the antithrombogenicity of GPG block copolymers and the secondary structure of the block copolymers. For this purpose, in vitro evaluation of thrombosis on these block copolymer surfaces and implantation of these block copolymer tubings in dog were performed.

## METHODS AND MATERIALS

PBLG and three kinds of PBLG/PPO(GPG) block copolymers with different content of PPO were used in this study. The structural formulae of these polymers are :



PBLG homopolymer



GPG block copolymer

The synthesis and structure study of the GPG block copolymers have been reported[11] and their characteristics and secondary structure were briefly summarized in Table 1 and 2, respectively.

Contact angles measurement : Chloroform solutions containing about 5 wt% of the polymers were prepared. Films were cast from these solutions on clean microscope cover glass by evaporating the solvent slowly at room temperature.

**Table 1.** List of Samples Prepared

| Sample | $[\eta]$, dl/g | $\bar{M}_w \times 10^{-4}$ | Propylene Glycol | |
|---|---|---|---|---|
| | | | mol.(%) | wt.(%) |
| PBLG | 1.49 | 27.3 | 0.0 | 0.0 |
| GPG-1 | 0.60 | 9.6 | 17.0 | 5.1 |
| GPG-2 | 0.40 | 6.0 | 26.0 | 8.5 |
| GPG-3 | 0.10 | 1.2 | 60.0 | 28.4 |

**Table 2.** $-[\theta]_{222}$ Values of Samples in 1,2-Dichloroethane(EDC) at 25℃

| Sample | G, mol.%[a] | $-[\theta]_{222}$ | $[\theta]^c_{222}/[\theta]^o_{222}$[b] |
|---|---|---|---|
| PBLG | 100.0 | 32,400 | 1.00 |
| GPG-1 | 83.0 | 29,800 | 0.92 |
| GPG-2 | 74.0 | 28,100 | 0.87 |
| GPG-3 | 40.0 | 15,800 | 0.49 |

[a] G : content of $\gamma$-benzyl *L*-glutamate unit in copolymer samples.

[b] $[\theta]^c_{222}$ : ellipticity of the block copolymers, $[\theta]^o_{222}$ : ellipticity of the PBLG homopolymer.

After drying, the advancing contact angle exhibited by a sessile drop of water or organic liquids such as glycol, formamide, 1.2-diethanediol, diethylene glycol, and oleic acid was measured at 5 different sites of a sample by a contact angle goniometer(Erma goniometer G-1 type). The contact angle was determined by averaging these values. The critical surface tension($\gamma_c$) was obtained by Zisman's method.[12]

Transmission electron microscopy : A solution of the block copolymer in chloroform(0.05 wt%) was cast on ion-etched carbon sputtered on collodion-coated copper meshes, and thin films were prepared by evaporation at room temperature. After vaccum drying at room temperature for 24hr, stained with $RuO_4$, and observed by a transmission electron microscopy(JEOL JEM-7).

**In vitro Evaluation of the Antithrombogenicity on Polymer Surface**

The coating of polymer on glass beads was carried out by solvent evaporation technique : the glass beads (15-35 meshes) were immersed in about 2 wt% polymer solution in dichloromethane for 30min, and the glass beads were separated from the solution. The glass beads were then dried in vacuo at room temperature for 24hr. 1g of copolymer-precoated glass beads was closely packed in a glass column(inner diameter : 3mm, length : 10cm) equipped with stop-cock. The packed column was subjected to the following platelet adhesion test : 4.0$cm^3$ of fresh whole blood was collected from a healthy person with a disposable syringe without using any anticoagulant and was immediately passed through the column for 1 min at a flow rate of 3$cm^3 min^{-1}$ using infusion pump (Sage Instruments Model 351). The eluted blood was collected in a sampling bottle containing 0.1$cm^3$ of EDTA as an anticoagulant. Platelet counts in the eluted blood were done with platelet counter (Coulter Counter Model S-plus). Platelet-rich plasma(PRP) was prepared from the citrated blood of a healthy person. 1.00$cm^3$ of fresh blood was collected in a disposable syrings containing 10$cm^3$ of 3.8 wt% aqueous solution of sodium citrate. The citrated blood was centrifused at 4℃ for 15 min at 700 revolution per minute to obtain PRP. The platelet concentration of PRP was adjusted to $2 \times 10^8$ cell/$cm^3$ by dilution with platelet-poor plasma, which was prepared from the citrated blood by centrifugation at 4℃ for 15 min at 3500 rev $min^{-1}$. 1g of copolymer-precoated glass beads was poured into the above PRP suspension and incubated at 37℃ for 4hr. Platelet counts in the eluted PRP were done in the same way as the whole blood.

**Effect of $Ca^{++}$ Ion on Platelet Adhesion on Polymer Surface**

$Ca^{++}$ ion was re-added into PRP suspension containing 1g of polymer-precoated glass beads and PRP suspension was incubated at 37℃ according to the time. The final concentration of re-added $Ca^{++}$ ion was 2.5 mM/L.

**In Vivo Evalution of the Antithrombogenicity on Polymer Surfaces**

The catheter of polyether urethane(Pellethane) (outer diameter : 1.66mm, length : 20cm) was coated on its internal and external surface with the GPG-1 block copolymer by solvent evaporation

technique with 2 wt% polymer solution in dichloromethane. The catheter was dried at room temperature overnight, followed by sterilization with gaseous ethylene oxide for 4 hr. The polymer-coated catheter was implanted in mongrel dogs of mixed sex (10-15 kg) and anesthetized with pentobarbital.

The catheter was rinsed with PBS and, following surgical cutdown, positioned in external jugular and femoral veins in the direction of blood flow.[4] Bare PU catheter was used as contralateral control.

Following 1 hr and 48 hr of implantation, the animal was systematically heparinized(200~300V/kg) and terminated(conc. KCI) by syringe. Vessels were exposed, ligated, and remaining blood was gently flushed with PBS, followed by in situ fixation of thrombi with 1.25% glutaraldehyde in PBS solution(pH 7.4).

After removal and fixation in fresh solution, vessels were opened longitudinally. After gross examination, the surfaces was observed by scanning electron microscope (SEM) (Jeol, Model TSM-35). The weight of difference between 3cm section of implanted and nonimplanted catheters determined the thrombus weight.

## RESULTS AND DISCUSSION

### Surface Properties of Block Copolymers

Contact angles and critical surface tension($\gamma_c$) of block copolymers by varous liquids were determined, which were affected by the copolymer composition. As is shown in Table 3, the contact angles of the block copolymers decrease and the critical surface tension of the polymers increases with increasing PPO content in the block copolymers. These results indicate that the surface properties of the block copolymer films are influenced by hydrophilic component in the blocks.

### Microstructure of the Block Copolymers

The morphology of the block copolymer film is shown in Fig. 1. The dark and light regions of the micrographs corresponded to the crystalline PBLG and amorphous PPO phases, respectively. Each block copolymer shows microphase-separated structures and the micro-structures of them are dependent on the content of PPO. GPG-1 shows a cylindrical or spheric structure whereas GPG-2 and GPG-3 show lamellar ones. With increasing PPO content in the block copolymers, the light regions increase.

**Table 3.** Contact Angle and Critical Surface Tension ($\gamma_c$) of the PBLG Homopolymer and GPG Block Copolymer

| Solvent | Sample | | | |
|---|---|---|---|---|
| | PBLG | GPG-1 | GPG-2 | GPG-3 |
| Water | 72.3 | 47.0 | 43.0 | 31.0 |
| Glycol | 61.7 | 43.0 | 32.0 | 27.0 |
| Formamide | 59.0 | 30.0 | 27.2 | 21.7 |
| 1,2 di-Ethanediol | 57.0 | 27.0 | 26.0 | 19.0 |
| Diethylene Glycol | 21.0 | 25.0 | 23.0 | 17.7 |
| Oleic Acid | 11.0 | 12.3 | 12.0 | 12.0 |
| $\gamma_c$ | 25.0 | 35.0 | 39.0 | 42.0 |

Comparing the two micrographs leading to lamella structure, it was found that bright and dark images with sizes of ca. 0.1~1 μm are seen in two cases because of a difference in thickness, i. e., a macroscopic phase separation formed by the casting of the dilute solution in $CHCl_3$. By a closer observation, very fine striations with an average thickness of 0.05 μm for GPG-2 and 0.03 μm for GPG-3 were seen in Fig. 1(b) and Fig. 1(c), repectively.

### Adhesion Behavior of Blood Platelets on the Surfaces of GPG Block Copolymers

Adhesion behavior of blood platelets on the block copolymer surfaces was examined by a microsphere column method. Fig. 2(a) and 2(b) shows platelet adhesion from whole blood and PRP, respectively, on the polymer surfaces. These results indicate that less platelets are adhered on the block copolymer surface than on PBLG homopolymer.

Fig. 3 shows the relationship between platelet

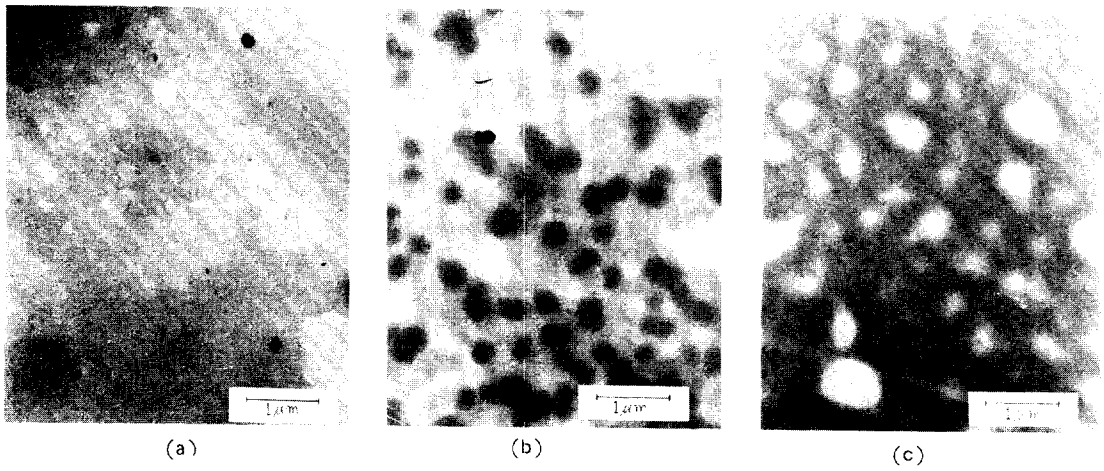



**Fig. 1.** Transmission electron micrographs of polymer films : (a)GPG-1 block copolymer ; (b)GPG-2 block copolymer ; (c)GPG-3 block copolymer.

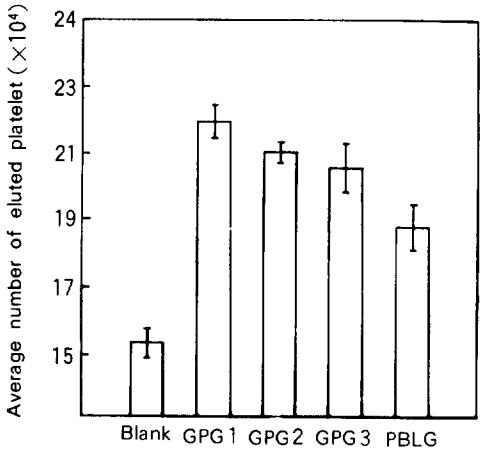



**Fig. 2(a).** Average number of eluted platelet from whole blood through polymer-coated glass beads column.

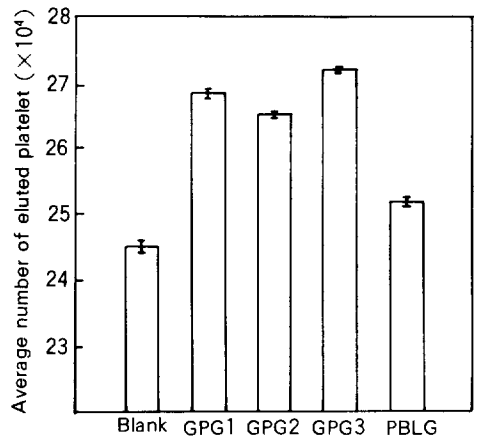



**Fig. 2(b).** Average number of eluted platelet from PRP suspension in the polymer-coated glass beads by depositing system.

adhesion from whole blood and PPO content in the block copolymer. However, there was not found a certain linear relationship between platelet adhesion and PPO content as PRP, which was different from platelet adhesion of PBLG-PEO block colopolymer.[10] The suppression of platelet adhesion on the block copolymers was attributed to the microphase-separated structures of them. These results may be regarded that protein structure may be disrupted mildly after adsorption onto the block copolymer which has the microphase-separated structure.[13] But the relationship between antithrombogenicity and secondary structure of adsorbed protein should be clarified in more detail. Yui et al.[14,15] reported that platelet adhesion has the close relationship between the crystalline-amorphous microstructure of the copolymers. Okano et al.[16] reported that platelet adhesion was suppressed at the lamellar microstructure of the poly(hydroxy ethylmethacrylate)/polystyrene block co-

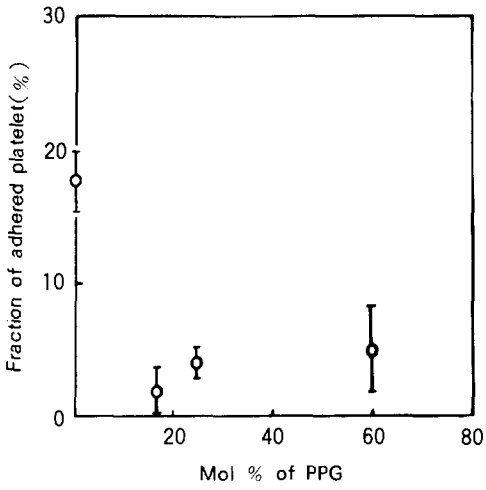



**Fig. 3.** Platelet adhesion on the surfaces of GPG block copolymer according to the content of PPO.

polymer surface. But the relationship between microstructure of the our block copolymers and platelet adhesion was not obtained by this time.

Fig. 4 shows the time dependence of the number of eluted platelets from the surfaces of homopolymer and GPG-1 block copolymer, respectively, in the presence of calcium ions. These results indicate that the presence of $Ca^{++}$ enhances the platelet adhesion because calcium ions are required for the platelet activation and aggregation accompanied with energy metabolism as well as the process of cascade reaction of coagulation factors.[17] Also, in the case of the homopolymer, the number of eluted platelets rapidly decreased with increasing incubation. On the other hand, the number of eluted platelets from the block copolymer surfaces decreased gently. These results demonstrate that platelets adhering to the surfaces of the block copolymers are less activated.

**In Vivo Evaluation of the Antithrombogenicity of Polymer Surface**

The antithrombogenicity of our synthesized block copolymer surface was examined by implanting the block copolymer coated catheter of 1.66 mm outer diameter into the canine external jugular and femoral veins, where the antithrombogenicity was evaluated by mean thrombus weights on

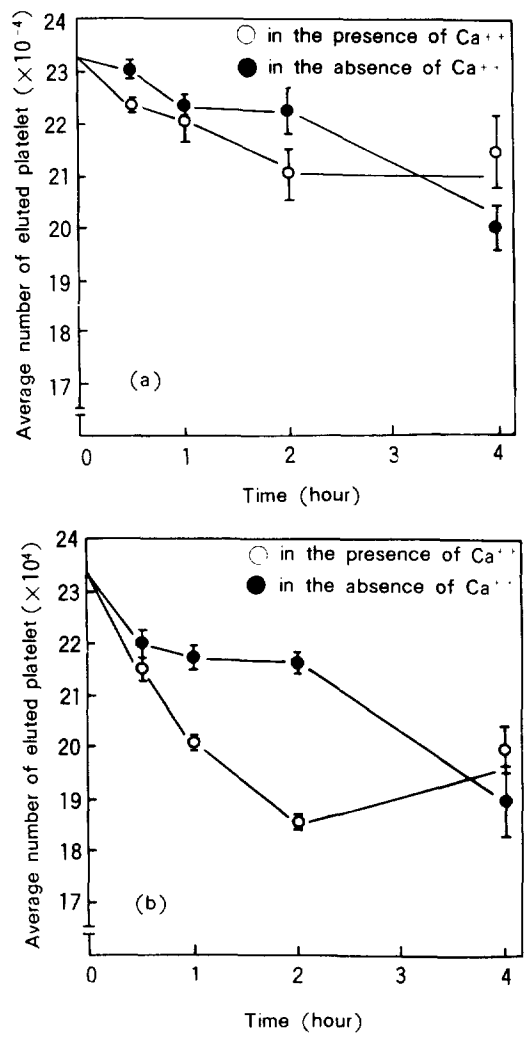



**Fig. 4.** Effect of calcium ion on platelet adhesion on the surfaces of GPG-1 block copolymer(a) and PBLG homopolymer(b) deposited in the PRP suspension with time.

3cm sections. Extenal jugular and femoral veins proved to be readily accessible, the latter via the medical saphenous vein, but were tremendously variable in size. Three experiments were run for 1 hr and two experiments were run for 48 hr. The results of in vivo experiments for control and test catheters are shown in Table 4, where the mean values are demonstrated. These results indicated that excellent improvement of antithrombogenicity

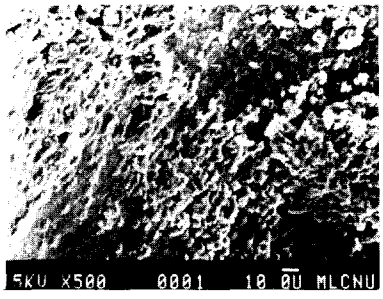
was found in the block copolymer as compared with control PU catheter. Blood compatibility was expected to be superior, in jugular veins, with their consistently greater diameters and, therefore, reduced contact between catheter and vascular epithelium. However, significant differences in thrombus formation at the two sites were not observed.

**Table 4.** Thrombus Weight on GPG-1 Coated Polyurethane(PU) and PU Catheters

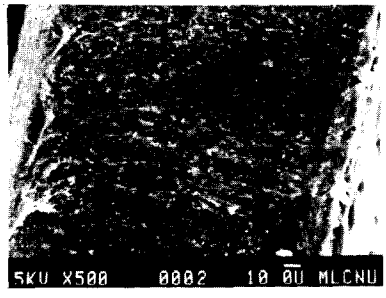
| Sample | Site | Venous Thrombus (mg/3cm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | One Hour | | | | Two Days | | |
| | | 1st | 2nd | 3rd | av. | 1st | 2nd | av. |
| GPG-1 | Jugular | 1.6 | 1.1 | – | 1.4 | 5.1 | 10.7 | 7.9 |
| PU | 〃 | 3.9 | 9.1 | – | 6.5 | 34.6 | 42.5 | 38.6 |
| GPG-1 | femoral | 1.3 | 3.2 | 2.3 | 2.3 | – | – | – |
| PU | 〃 | 6.8 | 8.2 | 11.4 | 8.8 | – | – | – |

Scanning electron micrographs(SEM) of middle section of catheter surfaces implanted for 1 hr and 48 hr are shown in Fig. 5 and 6, respectively. The surface of PU catheter was totally covered with thrombus while less and thinner thrombus was adhered to the block copolymer surface. GPG-1 coated catheter was superior in mean thrombus weight to PU catheter, which is known to be of relatively good antithrombogenicity. Therefore, in vitro and in vivo experiments suggest the block copolymer with microphase-separated and secondary structure exhibited excellent antithrobogenicity.

In conclusion, the block copolymers having microphase-separated structure may be a promising concept for the excellent antithrombogenicity materials.

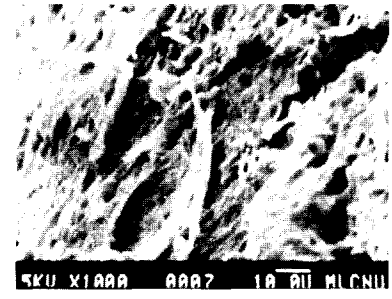



(a)

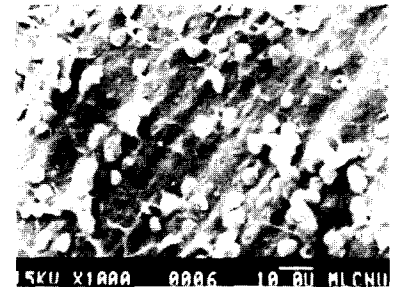



(b)

**Fig. 5.** Scanning electron micrographs of thrombus formation on the surfaces of PU(a) and GPG-1 block copolymer(b) implanted for one hour in canine femoral vein.




(a)




(b)

**Fig. 6.** Scanning electron micrographs of thrombus formation on the surfaces of PU(a) and GPG-1 block copolymer(b) implanted for two days in canine jugular vein.

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