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Synthesis, Blood Compatibility, and Gas Permeability of Block Copolymers Consisting of Polyoxypropylene and Poly(γ-benzyl L-glutamate)

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ABSTRACT: A-B-A-type block copolymers were prepared by the polymerization of γ-benzyl L-glutamate NCA (A segment) using polyoxypropylene (POP, B segment) with amino groups at both ends of a chain as an initiator. Thrombus formation on the block copolymer films was minimum on a block copolymer containing a POP segment of 50 mol%. It was found that on the block copolymer, denaturation of plasma proteins such as albumin and fibrinogen upon adsorption was sufficiently low not to activate adhered platelets, leading to a low degree of thrombogenesis. The oxygen permeation coefficients (P) of block copolymer films in water were about twice as large as that of poly(γ-benzyl L-glutamate) (PBLG) film. The increased permeation was ascribed to an increased diffusion coefficient (D) of dissolved oxygen through the block copolymer films. Arrhenius plots of P and D showed a break point in the temperature range of 20—30°C. At lower temperatures than the break temperature, the activation energy for oxygen permeation of the block copolymer film was lower than that of PBLG film.

KEY WORDS Block Copolymer / Poly(γ-benzyl L-glutamate) / Polyoxypropylene / Thrombogenesis / Oxygen Permeation / Plasma Protein / Platelet /

It has been reported that the surface of a synthetic polymer film with a microphase-separated structure is very antithrombogenic,[1-3] since the microphase-separated structure in its heterogeneous morphology resembles that of endothelium of the vascular wall.[4] We have synthesized block and graft copolymers consisting of crystalline poly(α-amino acid) and hydrophobic and gas-permeable polydimethylsiloxane (PDMS), and found that copolymers with a specific composition were highly antithrombogenic.[2] We explain the excellent antithrombogenicity in terms of the inertness of polymer surfaces covered with native plasma proteins toward the activation of adhered platelets.[5]

Recently, the antithrombogenicity of synthetic polymers containing polyethers as component has become the subject of research. For example, the adhesion of lymphocytes to block and graft copolymer films consisting of polyoxyethylene (POE) and poly(γ-benzyl L-glutamate) (PBLG) was dependent on the copolymer composition and the chain lengths of individual components.[6] The number of platelets adhered to block copolymer films consisting of poly(2-hydroxyethyl methacrylate) (PHEMA) and POE or polyoxypropylene (POP) was minimum for a certain composition of copolymers.[7] Yui *et al.*[8] reported that the amount of adhering platelets was minimum for the surfaces of multiblock POP-segmented co-

polymers having a crystallite thickness of 6.0—6.5 nm and a long period of 12—13 nm.

Artificial lung materials should have high biocompatibility, in particular blood compatibility, and a high biofunctionality, in particular oxygen and carbon dioxide permeability. The present authors aimed at the design and synthesis of antithrombogenic and gas-permeable materials. In the present investigation, A-B-A-type block copolymers, in which A and B represent PBLG and POP block, respectively, were synthesized. The POP segment is nonpolar and as flexible as a PDMS segment. Polymers containing the flexible POP segment are expected to have a high gas-permeability. On the other hand, the PBLG segment is polar and rigid. Block copolymers consisting of nonpolar and polar segments have been reported to be antithrombogenic.[1-3] The relationship between the conformation of adsorbed plasma proteins and the adhesion of platelets was investigated. The permeation of dissolved oxygen across the block copolymer film was also investigated.

EXPERIMENTAL

Synthesis of Block Copolymers

Block copolymers were synthesized by the polymerization of Glu(OBzl) NCA using α,ω-diaminopolyoxypropylene as an initiator as shown in Scheme 1. The initiator diamine was purchased from Texaco Co., U.S.A. and the amine content of the compound having a molecular weight of 2,000 was 90%, is determined by the titration with 0.01 M perchloric acid using thymol blue as an indicator in dioxane solution. The polymerization was carried out in dichloromethane. After the characteristic infrared absorption of NCA disappeared, the solution was concentrated and poured into ether to precipitate the product and remove the remaining POP initiators. The reaction product was dissolved in a small amount of chloroform and the solution was poured into an excess of benzene–acetone (1 : 1, v/v) mixture to separate soluble block copolymers from insoluble PBLG. There was, however, very little formation of PBLG.

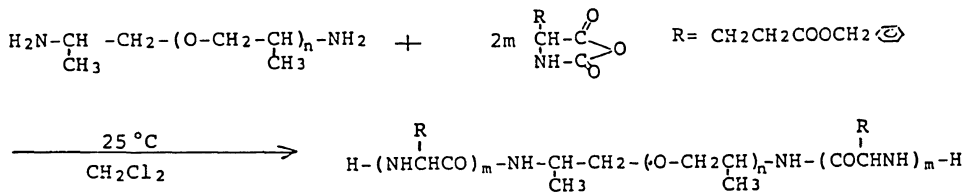
The block copolymers produced were dissolved in trifluoroacetic acid (TFA)/$CDCl_3$ mixture and the solution was subjected to ^1H nuclear magnetic resonance (NMR) spectroscopy. An example is shown in Figure 1. The degree of polymerization (m) of PBLG block was determined by the integrated peak ratio of the methyl proton signal of the POP segment (*ca.* 1.6 ppm) against the phenyl proton signal of the PBLG segment (*ca.* 7.2 ppm).

Casting of Block Copolymer Film

Three wt% *N*,*N*-dimethylformamide (DMF) solution (1 ml) of block copolymer was spread on a glass plate (2 cm × 2 cm) and the solvent was evaporated off under the irradiation by infrared lamp (*ca.* 70°C). The polymer film coated on a glass plate was dried under vacuum and subjected to contact angle measurement. It was separated from the glass plate by immersion in distilled water for the observation by transmission electron microscope and the measurement of gas permeation.

Surface Morphology Observed by Transmission Electron Microscope

The polymer film separated from the glass



Scheme 1. Synthesis of block copolymer.

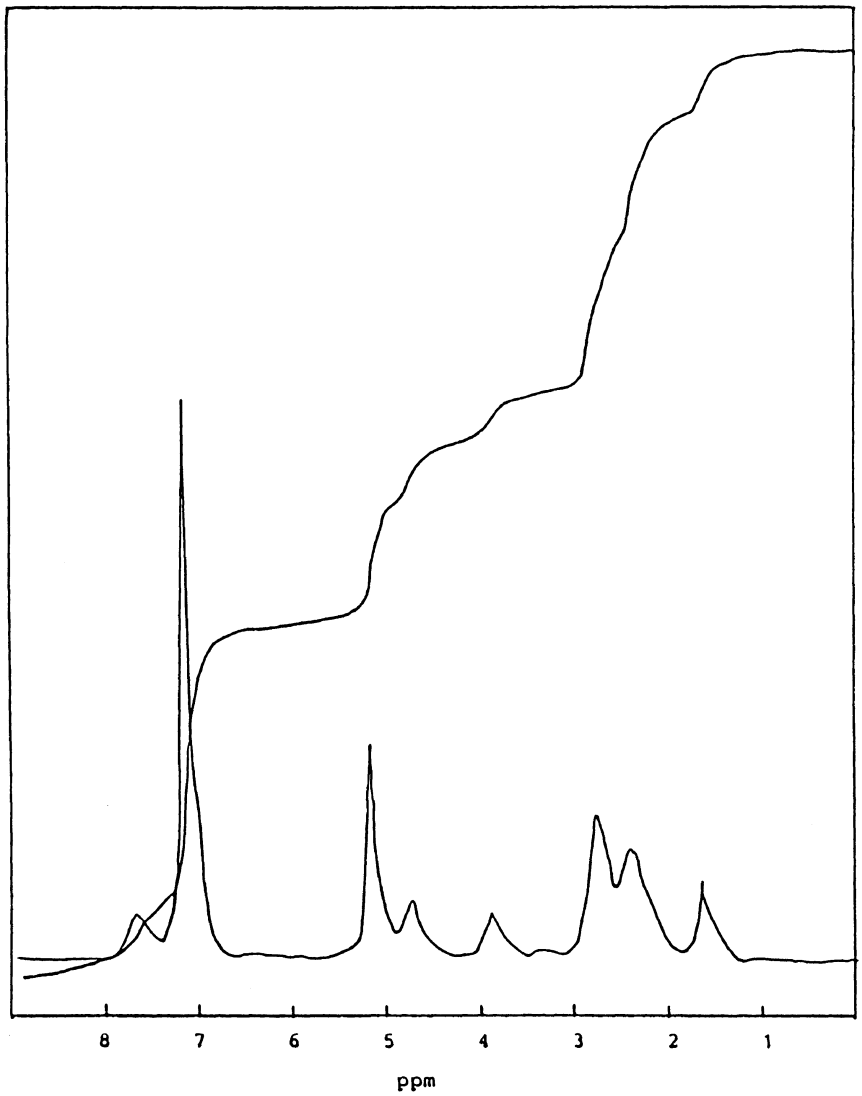


Figure 1. ^{1}H NMR spectrum of a block copolymer $(BLG)_{16}$–$(OP)_{33}$–$(BLG)_{16}$ in $CDCl_3$/TFA.

plate was vacuum-dried for 2 days, stained with aqueous osmic acid solution, and examined on Hitachi H 600 transmission electron microscope.[9]

Measurement of Contact Angles of Film Surface

The measurement of contact angles was carried out by the method previously reported.[5] A drop of water or organic liquid such as formamide, *n*-tetradecene, diiodomethane, β-thiodiglycol, and 1-bromonaphthalene was placed on a polymer film, and the contact angles were measured at 10 different sites of a sample. The contact angle was determined by averaging these values. The surface free energy was calculated from the contact angle by using the equation reported by Kitazaki and Hata.[10]

In Vitro Blood Clotting Test

DMF solution (1.2 wt%) of block copolymer (1.5 ml) was spread on a watch glass with a diameter of 7.5 cm. The solvent was evaporated off under irradiation by infrared lamp for 5 h and the film was dried under vacuum overnight. Canine blood was obtained from a femoral vein of an adult dog by gravity through a 19-gauge scalp vein needle into polypropylene bag containing one part acid-citrate-dextrose (ACD) for 9 parts of blood. The ACD blood was brought into contact with a polymer film and the rate of thrombus formed was determined according to the method reported by Imai and Nosé.[11] Four sheets of films were prepared for each block copolymer and tested for blood coagulation using different samples of canine blood. The rate of thrombus formation was determined by averaging the experimental values.

Adsorption of Plasma Proteins

DMF solution (0.1 wt%) of block copolymer (1 ml) was spread on a CaF_2 plate (diameter of 2.4 cm) and the solvent was evaporated off by irradiation of infrared lamp for 4 h. The film was dried under vacuum overnight. An aqueous solution of a single kind of plasma protein such as bovine serum albumin (BSA, Sigma Co., No. A6003 fraction V), bovine γ-globulin (BγG, Sigma Co., No. G3500, cohn fraction II), or bovine plasma fibrinogen (BPF, Sigma Co., No. F4758, type IV, 96% clottable) was prepared without purification (0.01 M Tris–HCl, 0.9% NaCl, pH 7.4). The concentration of the protein solution was adjusted to the physiological concentration of each protein (BSA, 4.5 wt%; BγG, 1.6 wt%; BPF, 0.3 wt%). The CaF_2 plate coated with polymer was immersed in the protein solution for 30 min at 37°C, and dipped in 0.9 wt% saline buffered with Tris–HCl for 5 min at 37°C, and then vacuum-dried overnight. It has been observed that desorption of adsorbed proteins is serious if the dipping procedure is continued for less than one min, but that it becomes equilibrated if the dipping procedure is continued for more than 5 min.[5] After washing with the buffer solution for 5 min at 37°C and vacuum drying, the amount and conformation of adsorbed proteins were measured with a transmission Fourier-transform (FT) infrared (IR) spectroscopy. The calibration curve for determination of adsorbed proteins and the details of the FT–IR measurement have been reported.[12]

Adhesion and Activation of Platelets

The ACD blood was centrifuged at $180 \times g$ for 10 min to obtain platelet-rich plasma (PRP). The supernatant was centrifuged at $2000 \times g$ for 5 min to obtain platelet poor plasma (PPP). To a part of PRP, $Na_2{}^{51}CrO_4$ was added, and to the other part of PRP, ^{3}H-serotonin was added. They were incubated for 1 h to label the cell membrane or cytoplasma of platelets in PRP with ^{51}Cr or to label serotonin of platelets in PRP with ^{3}H. After centrifugation at $3000 \times g$ for 5 min, phosphate-buffered saline (PBS) containing 0.01 M EDTA (20 ml) was added. The suspension was again centrifuged at $3000 \times g$ for 5 min to remove unreacted radioisotope. Labeled PRP was obtained by adding PPP to the labeled platelets. Suspension of labeled washed platelets (WP) was obtained by adding PBS to the labeled platelets.

DMF solution (1.2 wt%) of block copolymer (0.5 ml) was put in a borosilicate glass tube of 13 mm × 100 mm in size (Corning Ltd., U.S.A.), and vacuum-dried for 24 h at 70°C to cast polymer film on the wall of the glass tube. Labeled PRP or labeled WP was put in the glass tube and brought into contact with the polymer film for 10 min at 37°C. After decantation, the polymer film was washed with PBS. The number of adhering platelets was determined by γ-counting, and serotonin remaining in adhered platelets was determined by β-counting. The details of these procedures have been reported.[13]

Permeation of Oxygen in Water

The permeation of dissolved oxygen through the block copolymer film was measured by the previously reported method.[14,15] A polymer film was attached to the top edge of an electrode consisting of silver tube (anode) and platinum plate (cathode). A reduction current of oxygen, which diffused across the polymer film, was input to a microcomputer through an A/D converter. The permeation coefficient (P) was determined from the steady-state current and the diffusion coefficient (D) from the nonsteady-state current.

RESULTS

Synthesis, Morphology, and Surface Properties of Block Copolymers

In Table I, the experimental results of block copolymerization are summarized. The yield of ether-insoluble products was about 80%. The experimental degree of polymerization of a single A chain agreed well with the calculated one on the basis of the molar ratio of NCA against amine concentration. This agreement indicates that the polymerization of Glu(OBzl) NCA by α,ω-diaminopolyoxypropylene proceeded *via* the nucleophilic addition mechanism. A-B-A-type block copolymers having three different compositions were synthesized.

The morphology of the block copolymer film is shown in Figure 2. Each block copolymer shows a lamellar structure due to a microphase separation, in which POP segments constitute a continuous phase on PBLG matrices. With increasing PBLG content in the block copolymers, the thickness of PBLG phase increased.

In order to get information on the surface properties of block copolymers, contact angles by liquid were determined and surface free energies were calculated, which were almost unaffected by the copolymer composition as shown in Table II. Microphase-separated structure was dependent on copolymer composition as shown in Figure 2, but it did not affect the wettability of the whole surface.

Whole Blood Clotting Time

In Figure 3, the experimental results of *in vitro* blood clotting tests on PBLG and block copolymers are shown. The rates of thrombus formation on various polymer films under the same conditions are shown, taking the thrombus formed on glass during 20 min contact with blood being as 100. The rate of thrombus formation on the block copolymer PB-1, which contained POP segment of about 50 mol%, was about 40% as much as that on glass. However, with increasing peptide content in the block copolymers, their rates of thrombus formation increased and approached that of PBLG.

Protein Adsorption

IR spectra of plasma proteins adsorbed on the block copolymer PB-1 are shown in Figure

Table I. Block copolymerization of γ-benzyl L-glutamate NCA with α,ω-diaminopoly(oxypropylene) at 25°C in CH_2Cl_2

Sample	Glu(OBzl) NCA	POP		Yield	Block copolymer, m[c]	
	mol $l^{-1} \times 10^3$	mol $l^{-1} \times 10^4$[a]	n	%[b]	Calcd	Obsd by NMR
PB-1	7.623	1.540	33	81	25	16
PB-2	7.730	0.775	33	80	50	65
PB-3	7.984	0.494	33	76	81	124

[a] Concentration of amino group.
[b] Ether-insoluble product.
[c] Degree of polymerization of A segment.

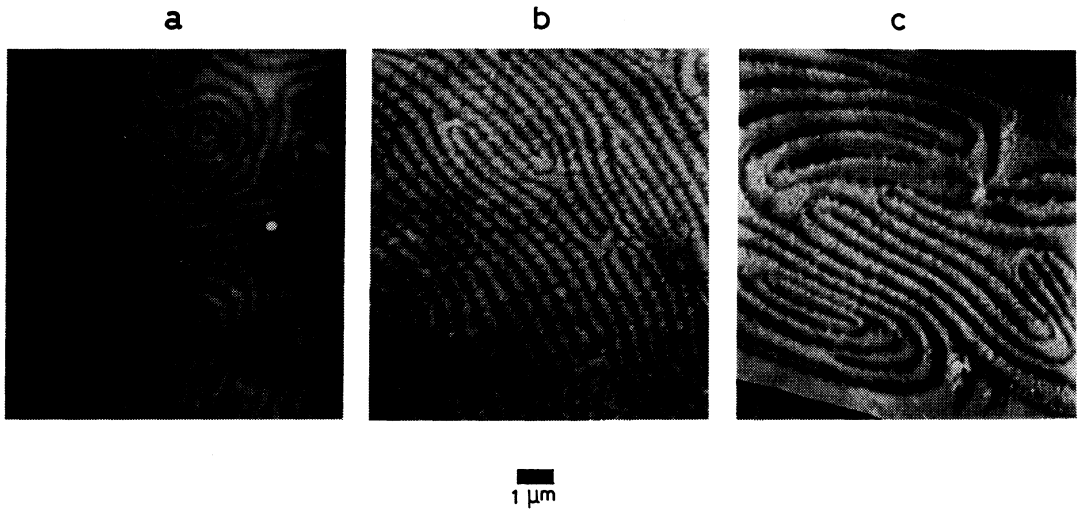


Figure 2. Transmission electron micrograph of polymer film cast from a DMF solution: a, block copolymer PB-1; b, block copolymer PB-2; c, block copolymer PB-3.

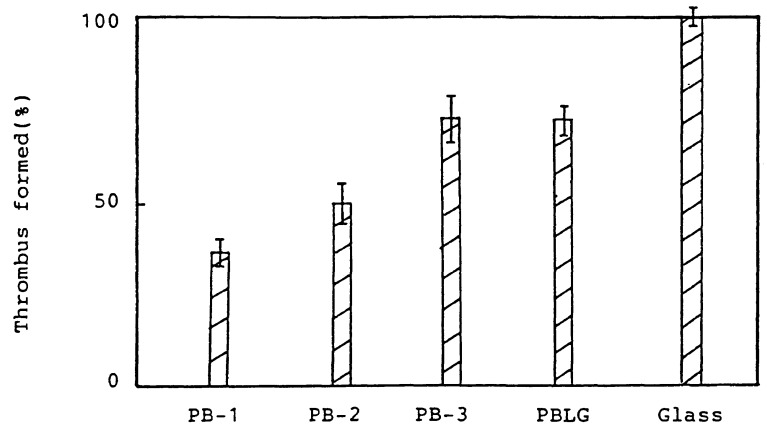


Figure 3. Thrombus formation on block copolymer and PBLG films.

Table II. Surface free energy of block copolymers and P[Glu(OBzl)] at 25°C

Sample	POP / mol%	Surface free energy[a] / dyn cm^{-1}			γ_s^d/γ_s^p
		γ_s^d	γ_s^p	γ_s	
PB-1	51	32.3	13.7	46	2.35
PB-2	20	31.7	12.8	44	2.47
PB-3	12	31.2	11.8	43	2.64
PBLG	0	32.8	16.2	49	2.02

[a] Standard deviation is within ±3.4 (dyn cm^{-1}).

4. The use of transmission FT-IR spectroscopy, which has been reported previously,[5] enabled us to overcome the difficulty in determining adsorbed proteins with attenuated total reflection FT-IR spectroscopy in the presence of amide absorptions due to matrix polymer. As is seen in Figure 4, the IR spectra (a) of protein-adsorbed polymers are very similar to that (b) of the matrix polymer. Subtraction of (b) from (a), however, gave the spectra (c) of adsorbed proteins. It seems that the different absorption behavior of carbonyl

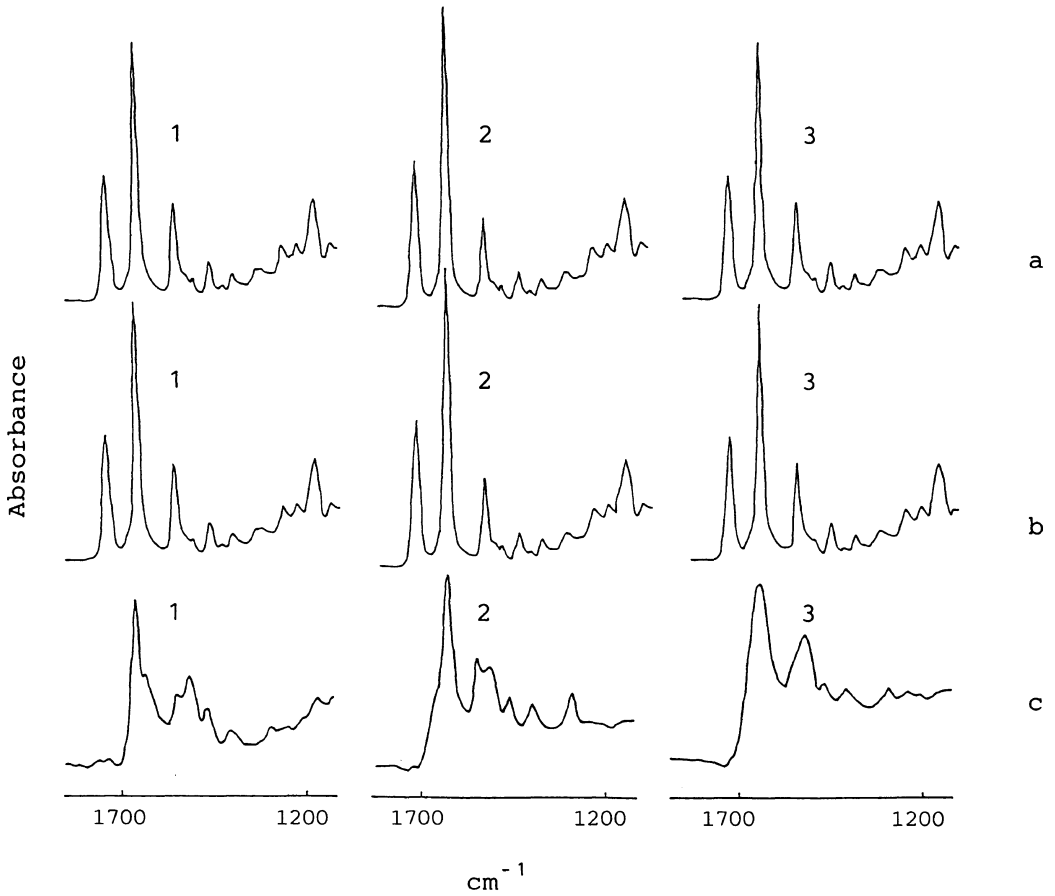


Figure 4. IR spectra of single plasma proteins adsorbed on the block copolymer PB-1: a, adsorbed proteins + block copolymer; b, block copolymer; c, adsorbed proteins; 1, BSA; 2, BγG; 3, BPF.

Table III. Conformation[a] of plasma protein adsorbed to block copolymer and PBLG films

Sample	POP	BSA				BγG				BPF			
	mol%	N	D	T	D%	N	D	T	D%	N	D	T	D%
PB-1	51	3.8	1.0	4.8	20	0	2.7	2.7	100	9.1	0	9.1	0
PB-2	20	6.7	6.8	13.5	50	0	2.1	2.1	100	6.7	1.8	8.5	21
PB-3	12	1.9	5.0	6.9	72	0	3.9	3.9	100	6.7	1.0	7.7	12
PBLG	0	0	11.2	11.2	100	0	1.3	1.3	100	0	3.8	3.8	100

[a] N, native ($\mu g cm^{-2}$); D, denatured ($\mu g cm^{-2}$); D%, degree of denaturation; T, total amount of adsorption ($\mu g cm^{-2}$).

groups in the matrix polymer from that in adsorbed proteins led to the successful subtraction spectroscopy. The measurement was repeated three times for each protein and the experimental values were averaged, the error of the determination being $\pm 15.3\%$.

In Table III, the experimental results of *in vitro* protein adsorption onto block copolymer films are summarized. No particular relationship was found between the amount of protein adsorbed and the copolymer composition. BγG was denatured on adsorption onto all block copolymers studied. BSA and BPF were denatured to a lesser extent upon adsorption onto block copolymers with a high POP content. But the denaturation of BSA and BPF on adsorption was enhanced with decreasing content of POP segment. The relatively high antithrombogenicity of a block copolymer PB-1 as shown in Figure 3, which contains a high rate of POP segment, is considered to be due to the low degree of denaturation of adsorbed BSA and BPF.

Adhesion of Platelet from PRP

In Table IV, the experimental results of platelet adhesion onto PBLG and block copolymers, which were not precoated with plasma proteins, from ^{51}Cr-labeled PRP are summarized. The number of adhered platelets was independent of polymer compositions within experimental error. On the other hand, the rate of serotonin remaining in adhered platelets was high on block copolymers PB-1 and PB-2, which had high content of the POP segment. In other words, on these block copolymers, adhering platelets were little activated. This experimental fact should be related with the relatively high antithrombogenicity of the block copolymers PB-1 and PB-2 (Figure 3).

Table IV. Platelet adhesion and serotonin remained in platelets adhered to block copolymer and PBLG homopolymer films

Sample	POP	Radioactivity due to adhered platelets[a]	Remained serotonin
	mol%	cpm	%
PB-1	51	1492±112	89±7
PB-2	20	1438±108	86±6
PB-3	12	1400±192	54±4
PBLG	0	1336±155	49±5

[a] Radioactivity of platelet rich plasma: 52654 cpm per 0.1 ml.

Permeation of Oxygen in Water

In Table V, the permeation coefficient (P), the diffusion coefficient (D), and the solubility coefficient (S) of dissolved oxygen through POP/PBLG block copolymer film are summarized. With increasing POP content, P became about twice that of PBLG. It was found that the increase of P was ascribed mainly to the increase of D.

In Figure 5, Arrhenius plots of P of PBLG and a block copolymer are shown. At low temperatures, the activation energy for permeation across PBLG film is a little higher than that across a block copolymer film, but at high temperatures, the difference is small. In the Arrhenius plots of P of PBLG and a block copolymer film, a break was observed in the temperature range of 20—30°C.

Arrhenius plots of D are shown in Figure 6. As was observed with the Arrhenius plots of P in Figure 5, the difference of activation en-

Table V. Permeabilities, diffusion and solubility coefficients at 25°C for block copolymer and PBLG films

Sample	POP	$P\times10^{10}$	$D\times10^{7}$	$S\times10^{3}$
	mol%	$cm^3(stp)\cdot cm\,cm^{-2}\cdot s^{-1}\cdot cmHg^{-1}$	$cm^2\,s^{-1}$	$cm^3(stp)\,cm^{-3}\cdot cmHg^{-1}$
PB-1	51	0.98	2.31	0.42
PB-2	20	0.84	2.62	0.32
PB-3	12	0.74	2.49	0.30
PBLG	0	0.45	1.26	0.35

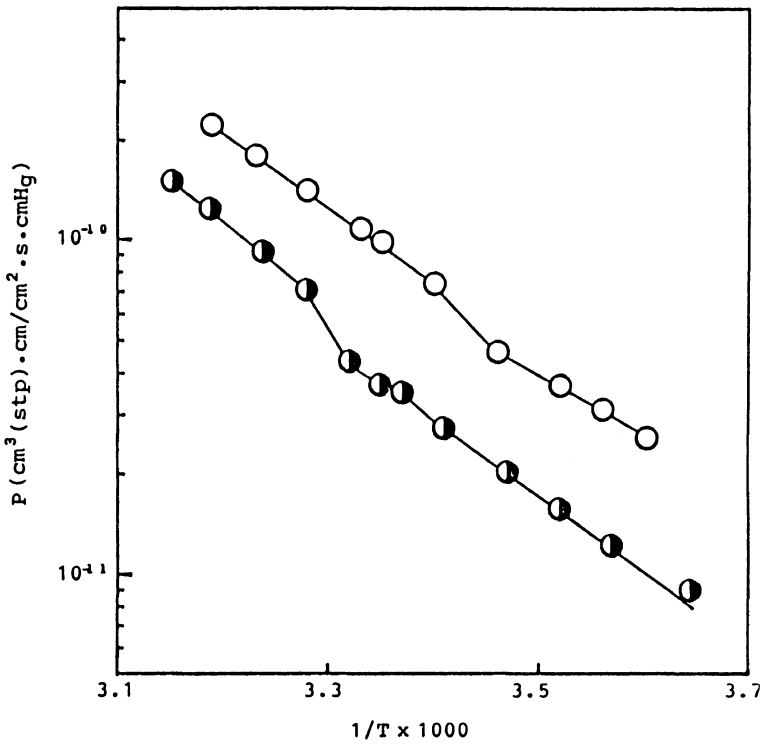


Figure 5. Temperature dependence of permeability coefficients for dissolved oxygen in water: ○, block copolymer PB-1; ◑, PBLG.

ergies is large between the two kinds of polymers at low temperatures and a break was observed in the temperature range of 20—30°C.

DISCUSSION

In the present investigation, the development of antithrombogenic and oxygen-permeable materials was attempted by connecting a nonpolar and flexible polyoxypropylene block and polar and rigid poly(α-amino acid) blocks.

Surface Properties and Antithrombogenicity

As is seen in Table II, the dependence of the surface free energy (γ_s) on the composition of the block copolymers is relatively small. γ_s values of the block copolymer films are in a narrow range of 43—46 dyn cm^{-1}. Since the γ_s values of PBLG and POP are 49 dyn cm^{-1} and 32 dyn cm^{-1},[16] respectively, the values of the block copolymer films are much closer to that of PBLG than to that of POP. This observation indicates that the surface free energy of the block copolymer films is strongly influenced by polar and hydrophilic blocks. This finding is in a sharp contrast to that described in the previous report on the block copolymers of PBLG and PDMS.[5] In these block copolymers, PDMS segments tended to appear on the air-side surface during film casting on a glass plate. On the contrary, in the present block copolymers of PBLG and POP, PBLG segments tended to appear on the air-side surface. The different behavior of the two kinds of block copolymers is probably attributable to the relatively short chain of the POP segment ($n=33$) and the low POP content.

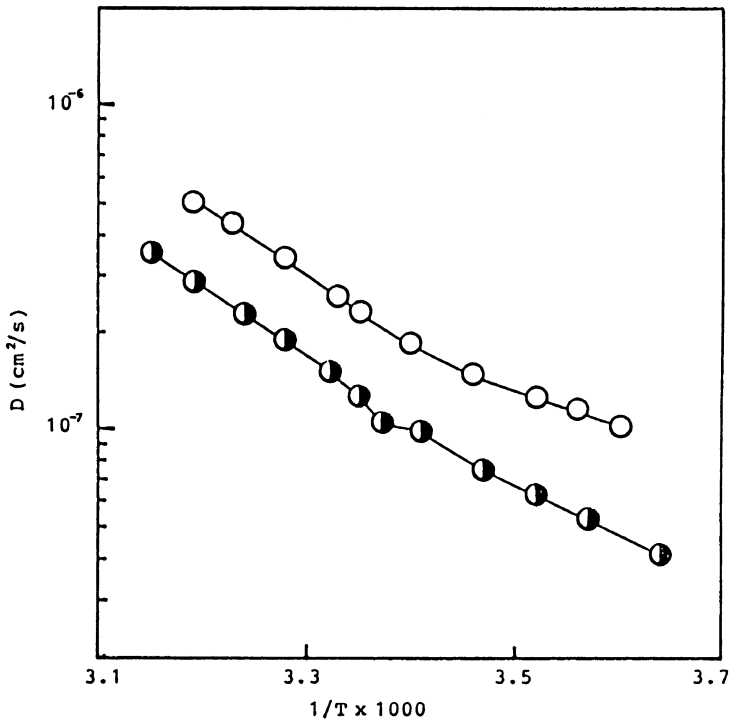


Figure 6. Diffusion coefficient as a function of temperature for dissolved oxygen in water: ○, block copolymer PB-1; ◑, PBLG.

The improved antithrombogenicity of a polypeptide, PBLG, by combination with a different kind of oligomer has been observed with the introduction of PDMS.[2,5] In the present investigation, the same effect was observed on the combination of POP with PBLG. In the case of the block copolymers of PBLG with PDMS[2,5] and POP, the rate of thrombus formation was only 40% as much as that on glass. In the case of the PBLG/PDMS block copolymers an adsorption layer of specific proteins is formed on the material surface, which weakens the interaction with platelets and leads to improved antithrombogenicity.[5] In the present PBLG/POP block copolymers, it is considered that BSA and BPF, which are adsorbed onto the material surface without denaturation, suppress platelet activation, *i.e.*, serotonin release, leading to high antithrombogenicity. It is particularly interesting with the present PBLG/POP block copolymers that the interaction of biocomponents with the material surface varied extensively by copolymer composition, keeping the surface wettability unchanged. Using similar polymer films, different adhesiveness of lymphocytes[6] and platelets[7] according to the composition has been reported.

Permeation of Oxygen in Water

By the block copolymerization of POP, the permeability of dissolved oxygen across PBLG film increased. It was found that the increased permeability was due to increased diffusion. Since the wettability of PBLG film was influenced very little by the incorporation of POP, it is considered that the solubility of oxygen at the film/water interface should be unaffected by incorporation of the POP segment. In the Arrhenius plot of the diffusion

and permeation coefficient of the block copolymer PB-1, a break point was shown at 20—30°C. The break point has been observed in the Arrhenius plot of permeability coefficients of PBLG/PDMS block copolymers[14] and various copolypeptides,[15] and it was concluded that the occurrence of break point in Arrhenius plot should be ascribed to the change of molecular motions of side chains at the transition temperature.[15] The introduction of the amorphous POP segment into crystalline PBLG chain seems to have gas molecules diffusing preferably through the amorphous POP region, leading to the two fold increase of the diffusion coefficient D and consequently the increase of the permeation coefficient P.

At low temperatures, the activation energies of P and D were different for PBLG and for a block copolymer. Taking into account that the transition temperatures for PBLG are *ca.* 120°C with the main chain and 30—40°C with the side chain[17] and that for POP is -60—-70°C,[8] it seems that POP domains generated in the block copolymer with a high mobility facilitate the permeation and diffusion of gases at low temperatures.

To conclude, by generation of amorphous domains in a polypeptide by block copolymerization of polyoxypropylene, a characteristic antithrombogenicity of the block copolymer was attained and gas permeability was improved.

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