Preparation of Cellulose Graft Copolymers Having Polypeptide Side Chains and Their Blood Compatibility

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Synopsis

Blood compatibility of cellulose graft copolymers with poly(γ-benzyl-L-glutamate) and poly(N^5-2-hydroxyethyl-L-glutamine) (Cell-*g*-PBLG and Cell-*g*-PHEG) was examined *in vivo* blood tests. For this purpose, Cell-*g*-PBLG graft copolymers with PBLG contents ranging from 7 to 60 mol % were prepared by polymerizing *N*-carboxy-γ-benzyl-L-glutamate(γ-BLG NCA) using aminoethyl cellulose (AE–Cell) with degree of substitution of 0.05 as macroinitiator. Graft copolymerization was carried out under a variety of conditions at 20°C in dimethylsulfoxide. Monomer conversions higher than 60% were obtained for all the polymerization runs. The solubility tests revealed that all of the AE–Cell and the polypeptides formed were grafted. The Cell-*g*-PHEG graft copolymers were prepared by treating Cell-*g*-PBLG graft copolymers with 2-amino-1-ethanol. Characterization of these graft copolymers were carried out by IR spectroscopy, DSC, and water content measurement. Tests for blood compatibility, *in vivo*, were made by a method of peripheral vein indwelling suture which was developed by one of the authors. The coating of graft copolymers on the polyester suture was made by casting either from formic acid solution or LiCl/dimethylacetamide solutions using water as the regenerating medium, and the polymer-coated sutures were implanted into a jugular and femoral vein of a dog. The results showed that the graft copolymers examined have excellent antithrombogenic properties.

INTRODUCTION

Block and graft copolymers composed of polysaccharide and polypeptide are very aattractive composite materials from both a structural and a biological point of view.[1,2] Expecially, the block and graft copolymers composed of cellulose or its derivative and polypeptide are of practical interest as candidates of biomedical materials. With regard to such copolymers, however, only a few studies by Avny et al.[3-5] have been reported on the preparation of graft copolymers by polymerizing *N*-carboxyanhydrides (NCAs) of amino acids using cellulosic alcoxide derivatives as macroinitiator. Very little has been investigated on the characteristics of these copolymers as a biomaterial.

Recently, we have been engaged in the preparation of various types of cellulose block and graft copolymers with polypeptides in order to investigate their functional properties, especially the characteristics of these copolymers as a biomaterial. One of the effective methods for introducing polypeptide into cellulose or its derivatives is the polymerization of NCA

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by cellulose derivatives having primary amino groups either at the chain end or as the side chain.[6,7] In the present work, we attempted to prepare the cellulose graft copolymers having poly(γ-benzyl-L-glutamate) (PBLG) side chains (Cell-*g*-PBLG). To this end, aminoethyl cellulose (AE–Cell) and aminopropyl cellulose (AP–Cell) were used as macroinitiator of *N*-carboxy-γ-benzyl-L-glutamate (γ-BLG·NCA). We found that the Cell-*g*-PBLG graft copolymers could be prepared with high yields using dimethyl sulfoxide (DMSO) as polymerization media and that the graft copolymers obtained exhibited excellent antithrombogenic properties.

This paper deals with the preparation and characterization of Cell-*g*-PBLG graft copolymers and their blood compatibility. PBLG is not only a representative hydrophobic polypeptide as known to adopt a helical conformation in many solvents, but also can be easily converted into poly(N^5-2-hydroxylethyl-L-glutamine) (PHEG) which is a hydrophilic polypeptide.[8,9] We also prepared the cellulose graft copolymers with PHEG (Cell-*g*-PHEG) from Cell-*g*-PBLG graft copolymers. Tests for blood compatibility, *in vivo*, were made by a method of peripheral vein indwelling multifilament.[10]

EXPERIMENTAL

Materials

DMSO was dried over calcium hydride and then distilled under reduced nitrogen atmosphere immediately before use. Deionized water was used throughout the experiments. The other reagents used as solvents were refined, respectively, according to the relevant procedures.

The monomer, γ-BLG·NCA, was prepared from γ-benzyl-L-glutamate according to the method of Blout and Karlson[11] and purified by repeated recrystallization from ethyl acetate solution with the addition of petroleum ether.

Cellulose Derivatives

An AE–Cell sample was purchased from Serva Chemical Co. The degree of substitution (DS) was 0.05. The sample was treated with aqueous ammonia and thoroughly washed with deionized water. A sample of AP–Cell was prepared from regenerated cellulose according to the method of Daly and Munir.[12] The value of DS was found to be 0.3. The regenerated cellulose was prepared from cellulose acetate by treatment with aqueous ammonia.

The content of NH_2 groups per anhydroglucose (AHG) unit, i.e., the DS value of the sample derivatives was estimated according to the usual method. The sample (ca. 100 mg) was transferred in a flask containing 10 mL of 0.1*N* HCl at room temperature. After 24 h, the solution was titrated with 0.1*N* NaOH, phenolphthalein being used as indicator. The DS values obtaiend were consistent with those by elemental amalysis.

Graft Copolymerization

Graft copolymerization was carried out for 48 h under nitrogen in DMSO at room temperature. The graft products were recovered from the reaction mixture by pouring it into a tenfold excess of cold methanol and dried *in*

vacuo.[13] The respective amount of γ-BLG·NCA and AE–Cell or AP–Cell was calculated to obtain the desired degree of polymerization (DP) of the polypeptide side chains by DP = [NCA]/[NH_2], where [NCA] and [NH_2] are the mole concentration of γ-BLG·NCA and amino groups in substrate, respectively.

Extraction of Homopolypeptides

The homo-PBLG was removed at room temperature for 48 h by successive extraction of the graft products with dioxane, which is a selective solvent for PBLG. However, the graft products contained no material extractable with dioxane, showing that all the polypeptides formed are grafted.

Preparation of Cell-g-PHEG Graft Copolymers

Cell-*g*-PHEG graft copolymers were prepared by aminolysis of Cell-*g*-PBLG graft copolymers. The method of Lupu-Lotan et al.[8] was modified so that degradation of the polypeptide might be minimized: The reaction with 2-amino-1-ethanol was carried out at 56°C for 24 h in dioxane, and care was taken to avoid a rise in temperature during the reaction.[14] The complete aminolysis was confirmed by IR spectroscopy using the C=O band around 1730 cm^{-1}.

Characterization of Graft Copolymers

The characterization of graft copolymers was made by infrared (IR) spectroscopy, differential scanning calorimetry (DSC), viscometry, and water content measurements.

IR spectra were obtained with a Perkin-Elmer Model 521 Spectrophotometer. The sample films were prepared by casting either from trifluoroacetic acid (TFA) solution or from LiCl/dimethyl acetamide (DMAc) solution[15,16] using water as the regenerating medium. In the latter case, 10% LiCl/DMAc solutions which contained 8–10% of the graft copolymer sample were used. The films were cast with a doctor's knife on a glass plate and then regenerated with water.

DSC thermograms were recorded on a Rigakudenki Model DSC-8230 thermal analyzer. The sample sizes were on the order of 1–2 mg. The experiments were carried out at a heating rate of 10°C/min.

Intrinsic viscosities [η] were determined in TFA at 25°C. All the experiments were carried out immediately after the preparation of solutions to avoid the possible esterification and/or degradation of cellulose backbone and the hydrolysis of PBLG side chains.

The water content was determined in the standard manner, by blotting the superficial aqueous solution and weighing the specimens both in their swollen state and after drying to constant weight.

Blood Compatibility Tests

The test, *in vivo,* for blood compatibility was performed according to a method of peripheral vein indwelling multifilament which was developed by one of the authors (Y. N.).[10,17] In this test, a piece of polyester suture coated with a desired polymer was implanted into a jugular and femoral

vein of an anesthesized mongrel dog. After an an adequate time (in this work, 1 day), the suture was examined by naked eyes.

The coating of sample polymer on the polyester suture was carried out in a similar manner as described in the preparation of the films for IR spectra. Polymer-coated sutures were washed thoroughly with deionized water and stored in deionized water containing 20 wt % ethanol. For comparison, PBLG-coated sutures were also prepared from chloroform solution.

RESULTS AND DISCUSSION

Preparation and Characterization of Graft Copolymers

Four preliminary polymerizations runs of γ-BLG·NCA were made at 20°C for 48 h using AE–Cell and AP–Cell as macroinitiator. Table I summarizes the polymerization data. γ-BLG·NCA and PBLG are soluble in DMSO used as polymerization media, while the cellulose derivatives employed in the present work are not, but swell considerably. Therefore, the reaction mixture was heterogeneous during the whole polymerization process, but the total conversions of γ-BLG·NCA monomer to polymer were very high for both AE–Cell and AP–Cell. In addition, it was found that the polymerization was not accompanied by the formation of homopolypeptides, showing that the graft efficiencies were approximately 100%, as described later.

The DS value of AE–Cell used as substrate is relatively low (DS = 0.05); every AE–Cell molecule possesses one possible grafting site per 20 AHG units. This is suitable for the preparation of the graft copolymers with a relatively low degree of grafting substitution. In the present work, we attempted to prepare the cellulose graft copolymers with different PBLG contents using AE–Cell as substrate. The results of polymerization under various conditions are summarized in Table II. It should be noted that all graft products obtained were not contaminated with homo-PBLG, as proved by selective extraction of the graft products with dioxane which dissolves homo-PBLG.

It is known that two types of initiation mechanism of NCA coexist even in the case of macroinitiator with primary amine functions: One is the primary amine type leading to the block or graft copolymers, and the other is the tertiary amine type leading to a homopolypeptide. The priority in the two mechanisms is determined not only by a balance between the nucleophilicity and basicity of the amine[6,7,18] but also by the nature of the polymerization solvent employed.[6,7] The results obtained here show that

TABLE I
Polymerization Data of γ-BLG·NCA by AE–Cell and AP–Cell in DMSO at 20°C

Run	Substrate (g)	DMSO, (mL)	γ-BLG·NCA (g)	Reaction time (h)	Conversion[a] (%)
1	AE–Cell (1)	100	1	48	90
2	AE–Cell (1)	100	5	48	85
3	AP–Cell (1)	100	1	48	95
4	AP–Cell (1)	100	5	48	90

[a] Total conversion of γ-BLG·NCA to polymer.

TABLE II
Polymerization and Characterization Data of Cell-*g*-PBLG Graft Copolymers[a]

Sample code	NCA/Substrate[b] (wt ratio)	Conv. of NCA (%)	PBLG content (mol %)	Dioxane extract (%)	DP of grafted PBLG[c]	$[\eta]$[d] (dL/g)
AE–Cell	—	—	—	—	—	3.90
Cell–PBLG-10	1/5	60	7	Nil	1–2	3.83
Cell–PBLG-20	1/2	70	20	Nil	4	3.75
Cell–PBLG-30	1/2	80	27	Nil	5–6	3.55
Cell–PBLG-40	1/1	70	35	Nil	7	3.23
Cell–PBLG-60	2/1	90	58	Nil	18	2.52

[a] Polymerization conditions: total weight of (AE–Cell + NCA) = 2 g/100 mL DMSO; reaction time = 48 h; temperature = 20°C.
[b] AE–Cell with a DS value of 0.05.
[c] Apparent degree of polymerization estimated from DS value of AE–Cell and average composition of graft copolymer.
[d] In trifluoroacetic acid at 25°C.

the primary amine type initiation is predominant in the polymerization of γ-BLG·NCA by AE–Cell in DMSO.

The solubility test of the graft products also revealed that the graft products were completely soluble in dichloroacetic acid (DCA). Since DCA is a selective solvent for PBLG, this test has proved that all of the AE-Cell were grafted. Table III shows the solubility of the graft copolymers. As can be seen from this table, the solubility behavior of the graft copolymers is mainly determined by that of cellulose, reflecting that the degree of grafting substitution is relatively low.

In the present experiments, we used DMSO as polymerization media. It may be considered that the rate of initiation is lower than that of polymerization, because the initiation is performed in heterogeneous phase and the polymerization in homogeneous phase (γ-BLG·NCA and PBLG are soluble in DMSO, whereas AE–Cell swells). Consequently, the molecular weight distribution of grafted PBLG may be considered to be rather broad. The initiation efficiency of amino groups in AE–Cell also comes into question. In order to isolate the grafted PBLG, we attempted to degradate the cellulose backbone in the grafted copolymers selectively with cellulase. Unfortunately, these attempts were unsuccessful. However, the sufficiently high initiation efficiency of amino groups was confirmed from DSC thermograms of the graft copolymers, as discussed below.

Traces of the DSC thermograms obtained from both the graft copolymers and the blends of AE–Cell and homo-PBLG are shown in Figure 1. A single endotherm is observed at approximately 280°C for AE–Cell and 320°C for PBLG, respectively. Two endotherms can be observed for the graft copolymers and blends with higher PBLG contents than 30 mol %. The peak of PBLG (320°C) is considered to be due to the dissolution of the helical molecules of PBLG. This endotherm at around 320°C becomes only a shoulder for the graft copolymers with lower PBLG contents than 20 mol %, showing that the helix content of these graft copolymers are very low. In the case of blends, on the other hand, two endotherms can be clearly observed even for the sample with a PBLG content of 20 mol %. It is known that oligo-PBLG molecules with lower DP values than 5–6 can not assume a helical

TABLE III
Solubility of Cellulose, PBLG, and Cell-*g*-PBLG Graft Copolymers[a]

Solvent[b]	Regenerated cellulose	PBLG	Cell-*g*-PBLG
DMF	–	+	–
DMSO	–	+	–
DMAc	–	+	–
LiCl/DMAc	+	+	+
DCA	–	+	+
TFA	+	+	+
FA	+[c]	–	+[d]

[a] + , soluble; – , insoluble.
[b] DMF = dimethylformamide; FA = formic acid.
[c] Dissolved as cellulose formate.
[d] Graft copolymers with PBLG contents higher than 30 mol % are insoluble.

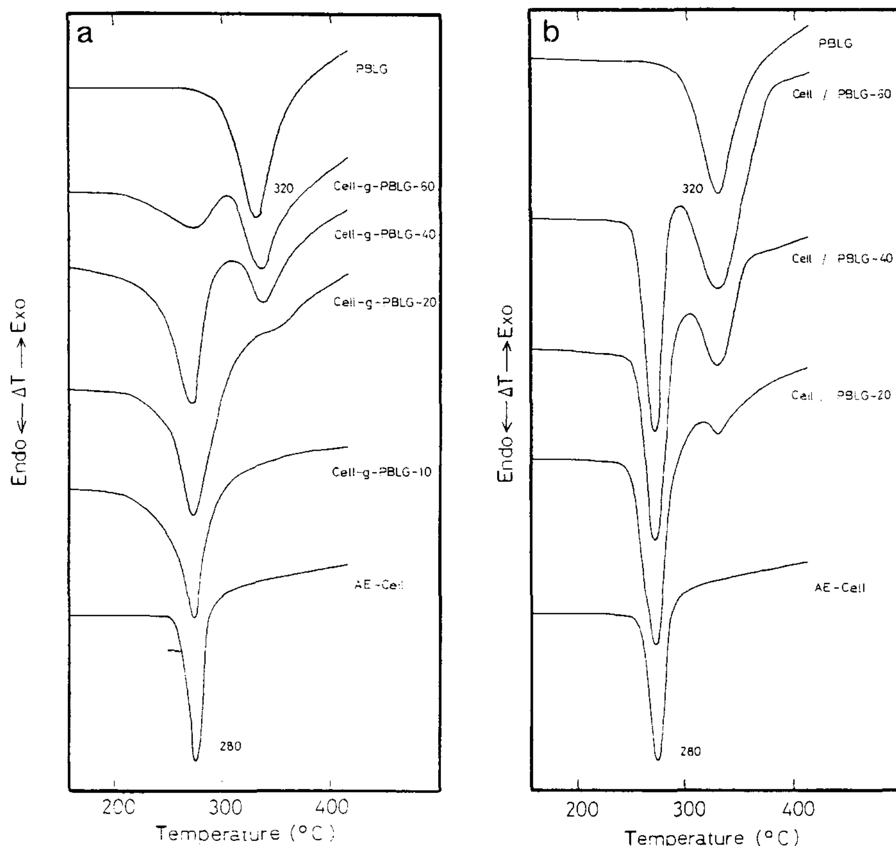

Fig. 1. DSC thermograms of Cell-*g*-PBLG graft copolymers (a) and various blends of AE-Cell and PBLG (b). Heating rate 10°C/min.

conformation. The results obtained here suggest that the average molecular weights of grafted PBLG are less than 5–6 in the samples Cell-*g*-PBLG-10 and Cell-*g*-PBLG-20; in other words, the chemical structure of the graft copolymers obtained will be close to that calculated from the feed ratio in the reaction mixture (see Table II).

The IR spectra have demonstrated the presence of both polymer segments. For reference, the IR spectra in the region 2000–500 cm^{-1} of unoriented solid films of Cell-*g*-PBLG graft copolymers and PBLG homopolymer are shown in Figure 2. The amide I, II, and V bands of the graft copolymers appear at 1650, 1550, and 620 cm^{-1}, respectively, at just the same wave number for PBLG. No other useful information can be obtained from the spectra of unoriented films.

Figure 3 shows the IR spectra of the samples Cell-*g*-PBLG-60 and Cell-*g*-PHEG-60. The latter was obtained by aminolysis of the former. The spectrum of the former shows a characteristic absorption band at 1730 cm^{-1}, which may be assignable to the carbonyl group in PBLG, while the carbonyl band is not found in that of the latter, proving that the deesterification was practically complete. The problem is the degradation of the polypeptide during the aminolysis. It has been proved that the degradation of polypep-

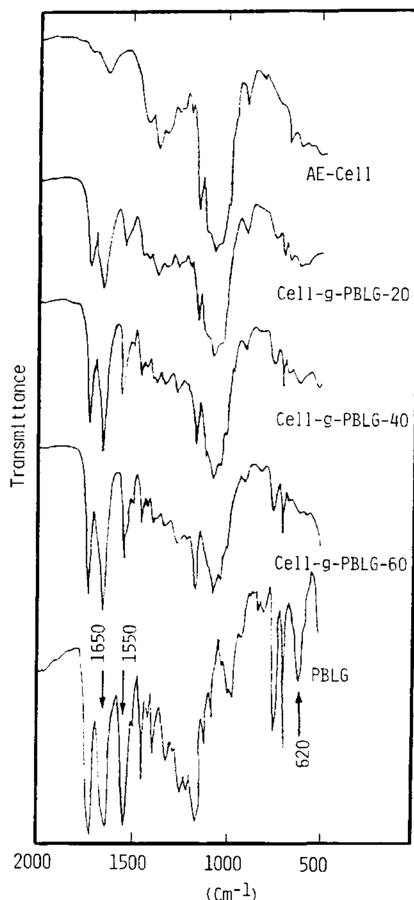

Fig. 2. Infrared spectra of unoriented solid films of Cell-*g*-PBLG graft copolymers cast from TFA solution.

tide is negligible under the reaction conditions mentioned in the Experimental section.[14] In fact, this was also confirmed by an elemental analysis; the polypeptide content of Cell-*g*-PHEG graft copolymers was in agreement with that of the corresponding Cell-*g*-PBLG graft copolymers within experimental error.

The water contents of unoriented solid films prepared from graft copolymers are listed in Table IV. The values for cellophane, viscose rayon, and PBLG are included for comparison. The films were prepared by casting from TFA solutions. Regenerated cellulose was considerably higher water content than normal cellulose samples. This presumably reflects a lower crystallinity in the cast film from TFA solution. An increase in PBLG content of Cell-*g*-PBLG graft copolymers caused a decrease in water content due to the hydrophobic nature of PBLG. On the other hand, it is of interest to note that the water content of Cell-*g*-PHEG graft copolymers is independent of PHEG content, in spite of PHEG being a water-soluble polypeptide.

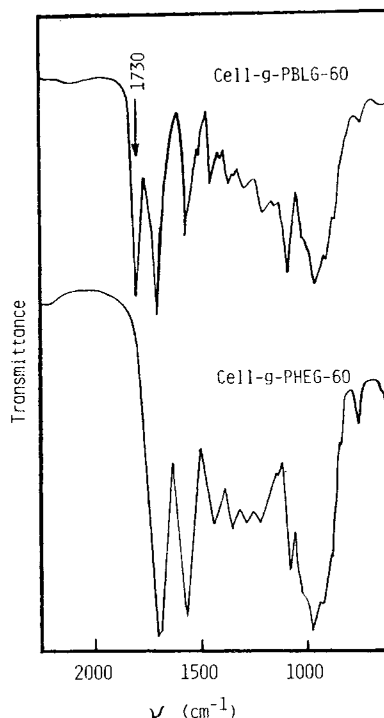

Fig. 3. Infrared spectra of unoriented solid films of Cell-*g*-PBLG-6 and Cell-*g*-PHEG-60 samples cast from TFA solution.

Blood Compatibility

The results on the blood compatibility of the graft copolymers with PBLG are shown in Tables V and VI. The symbols used to evaluate the results have following significance: The + symbols indicate the relative degree of the thrombus formation of the samples in three steps where + + + denotes

TABLE IV
Water Absorption by Cellulose, PBLG, Cell-*g*-PBLG, and Cell-*g*-PHEG Graft Copolymers

Sample code	Peptide content (mol %)	Water absorption: Weight increase (%)	Water absorption: Content[a] (%)
Regenerated cellulose	0	240	70
Viscose rayon	0	130	55
Cellophane	0	130	55
Cell-PBLG-20	20	80	45
Cell-PBLG-40	35	65	40
Cell-PBLG-60	58	55	35
PBLG	100	10	10
Cell-PHEG-20	20	170	65
Cell-PHEG-40	35	200	67
Cell-PHEG-60	58	240	70

[a] (Wet wt − dry wt)/ wet wt × 100.

TABLE V
In Vivo Results on Blood Compatibility of Cell-*g*-PBLG Graft Copolymer Films Prepared from LiCl/DMAc Solution Using Water as Regenerating Media

Sample code	Solvent for film preparation	Thrombus formation[a]
Regenerated cellulose	Formic acid[b]	+
Cell-PBLG-10	LiCl/DMAc	–
Cell-PBLG-30	LiCl/DMAc	–
Cell-PBLG-40	LiCl/DMAc	–
Cell-PBLG-60	LiCl/DMAc	–
PBLG	Chloroform[b]	++~+++

[a] See text for symbols.
[b] Cast by evaporation of solvents.

the thrombus formation along the entire length of the coated surface. The – indicates the case where no thrombus formation had occurred. It can be seen that the graft copolymers examined here have an excellent blood compatibility. However, the results also indicate that the blood compatibility depends not only on the PBLG content of the graft copolymers but also on the coating conditions of the sample. The samples with polypeptide contents less than 20 mol % are not graft copolymers in a strict sense but cellulose derivatives with amino acids and/or oligopeptides as substituents, because such peptide sequences cannot assume a helical conformation characteristic of PBLG and PHEG. It should be noted that these copolymers with low polypeptide contents exhibited excellent antithrombogenic properties, independent of the casting conditions of the sample. Figure 4(a) shows a macroscopic view of a suture with Cell-*g*-PBLG graft copolymer. No thrombus is observed around the suture (S). For comparison, the result on PBLG homopolymer, which was evaluated as + + +, is shown in Figure 4(b). Table VI also shows the results on the blood compatibility of Cell-*g*-PHEG graft copolymers. The test sutures were coated with the sample copolymers by casting from formic acid solutions. It can be seen that these copolymers also exhibited an excellent blood compatibility.

The implantation time in this work was relatively short, i.e., 24 h. To elucidate the biocompatibility of these graft copolymers, it is necessary to

TABLE VI
In Vivo Results on Blood Compatibility of Cell-*g*-PBLG and Cell-*g*-PHEC Graft Copolymer Films Cast from Formic Acid Solutions

Sample code	Thrombus formation[a]
Regenerated cellulose	+
Cell-PBLG-10	–
Cell-PBLG-20	–
Cell-PBLG-30	±
Cell-PHEG-10	–
Cell-PHEG-30	–

[a] See text for symbols.

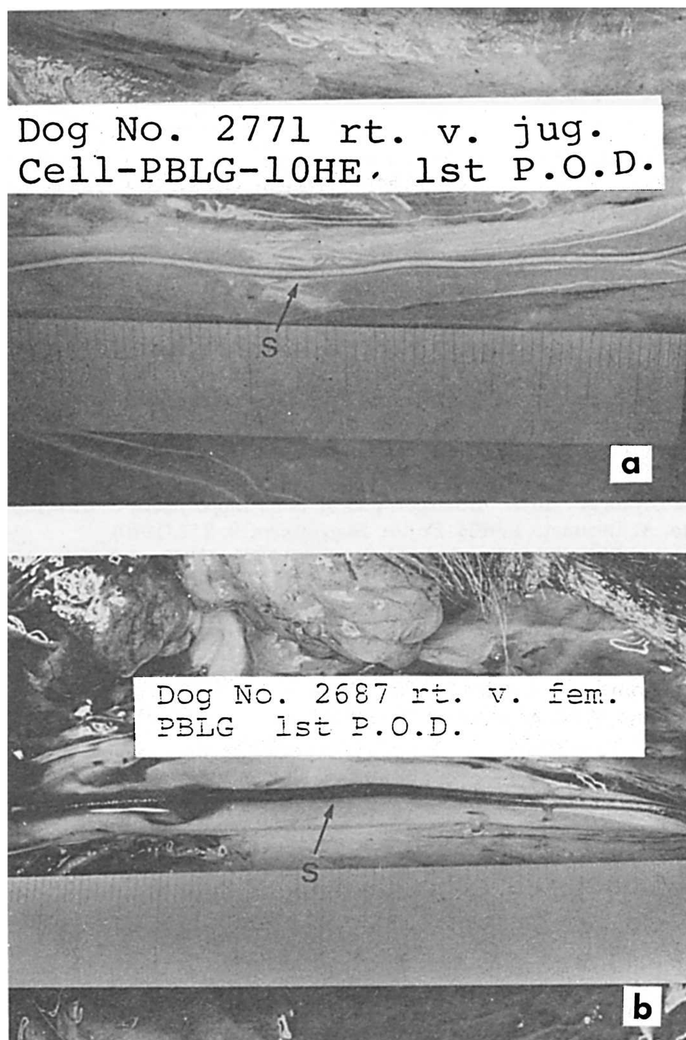

Fig. 4. Macroscopic views of polyester sutures coated with Cell-*g*-PBLG graft copolymer (a) and PBLG homopolymer (b) after inplantation in the jugular vein of dog for 1 day: (a) no thrombus was observed around the suture (S): (b) in this case, the blood compatibility was evaluated as + + +.

observe the blood compatibility at various time intervals after implantation. At the same time, the experiments for the foreign body reaction and absorbability of these graft copolymers must be carried out. Such experiments are currently under investigation. From the preliminary experiments on the absorption property, it was found that about 25% of the sample copolymers was absorbed without 4 weeks for all the graft copolymers prepared here, showing that the cellulose graft copolymers with polypeptides are absorbable. The test for this purpose was carried out as follows. The sample copolymers were coated on the cloth of polyester fibers, and implanted subcutaneously in dogs for 4 weeks. The degree of absorption was

evaluated by observing microscopically the sample copolymer.[17] The details of these results and the results on the foreign body reaction will be reported in a subsequent paper.[19]

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