In Vivo and *In Vitro* Blood Compatibility of Polyelectrolyte Complexes Formed Between Cellulose Derivatives

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Synopsis

The blood compatibility of cellulosic polyelectrolyte complexes (PECs) and the effect of excess charge in PEC on the blood compatibility were examined in detail by both *in vivo* and *in vitro* blood tests. For this purpose, two types of quaternary ammonium cellulose derivatives were prepared by treating cellulose or hydroxyethyl cellulose with glycidyl trimethylammonium chloride. *In vivo* blood tests were made by implanting the polymer-coated suture into a jugular and femoral vein of a dog. *In vitro* blood tests include the measurement of whole blood coagulation time on polymer-coated glass tubes, platelet adhesion measurements using a column packed with polymer-coated glass beads, and a measurement of activation of the intrinsic coagulation system. It was found that among the PECs examined, the PECs containing quaternary ammonium derivatives as polycation components have an excellent blood compatibility. The experiments on the effect of excess charge in PEC revealed that (i) the relative coagulation time of whole blood is almost independent of the mole ratio of polycation to polyanion component within the mole ratios examined, being in good agreement with those by *in vivo* blood tests, but (ii) platelet adhesion increases with increasing the mole ratio of polycation/polyanion in the PEC, and (iii) the activation of the intrinsic coagulation system increases with decreasing the mole ratio.

INTRODUCTION

A number of studies have been reported on *in vivo* and *in vitro* test methods for evaluating the blood compatibility of polymeric materials.[1] A representative *in vitro* test method is a so-called Lee-White method,[2] in which the coagulation time of whole blood is measured. However, it is well known that the adhesion, aggregation, and deformation of blood platelets on the polymer surface play an important role in the process of thrombus formation.[1,3-5] The thrombus formation is also triggered by the activation of coagulation system.[1] The initiation reaction of the coagulation system is surface mediated and is called contact-phase activation.[6] Therefore, in order to elucidate the blood compatibility of polymeric materials, it is important to compare the results between *in vivo* and *in vitro* test methods and/or between different *in vitro* test systems. However, very little study has been reported on the comparison of *in vivo* and *in vitro* test results on the blood compatibility of the sample polymer.[1]

Recently, we found from *in vivo* experiments that by the criteria of the

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test method employed, the polyelectrolyte complexes (PECs) formed between cellulose derivatives have an excellent blood compatibility.[7] We also reported that the formation of PECs between cellulose derivatives, in general, does not follow a stoichiometric reaction, but quaternary ammonium derivatives of hydroxyethyl cellulose(Q-HEC) react stoichiometrically with carboxymethyl cellulose(CMC) and cellulose sulfate(CS). These systems are convenient for investigating the effect of the excess charge in PEC on the blood compatibility.[3,8]

In the present study, the blood compatibility of various types of cellulosic PECs was examined by *in vivo* and *in vitro* test methods. Furthermore, the effect of the excess charge in PEC on the blood compatibility was also examined in detail using the system of Q-HEC and CMC. To this end, in addition to *in vivo* and Lee-White blood tests, which determine the overall thrombolytic potential, the hemocompatibility tests differentiating the contributions of platelet and coagulation systems were performed; The behavior of blood platelets on the polymer surface was examined by the use of a column packed with polymer-coated glass beads[3-5] and the degree of contact-phase activation in plasma (the initiation reaction of plasma coagulation cascade) was evaluated by a test method using a synthetic substrate specific to plasma kallikrein as developed recently by Matsuda et al.[8,9]

EXPERIMENTAL

Materials

Deionized water was used throughout the experiments. All other solvents and reagents were of highest purity available and used without further purification.

Cellulose Derivatives

Two types of quaternary ammonium cellulose ether derivatives, Q-HEC and Q-Cell, were used as polycation components. A quaternary ammonium derivative of hydroxyethyl cellulose(HEC), Q-HEC, which was prepared by treating HEC with molar substitution of ca. 1.8 with glycidyl trimethylammonium chloride(GMAC), was obtained from Union Carbide Co. (US). The degree of substitution (DS) was estimated as 0.4 by elementary analysis. A

TABLE I
Characteristics of Cellulose Derivatives Employed

Sample code	Substituent	Degree of substitution
Q-Cell	$-CH_2CH(OH)CH_2N^+CH_3)_3{}^-Cl$	0.41
Q-HEC	$-(CH_2CH_2O)_n-CH_2CH(OH)CH_2N^+(CH_3)_3{}^-Cl$	0.40
AEC	$-CH_2CH_2NH_2$	0.05[a]
DAC	$-CH_2CH_2N(C_2H_5)_2$	0.18[a]
CMC	$-CH_2COOH$	0.32, 0.85
CS	$-SO_3H$	0.54

[a] Insoluble in water.

quaternary ammonium derivative of cellulose, Q-Cell, was prepared by homogeneous etherification of cellulose with GMAC in a 10% LiCl/dimethylacetamide solvent.[10] The details of the preparation of Q-Cell are described elsewhere.[7] Aminoethyl cellulose (AEC) and diethylminoethyl cellulose (DAC) were purchased from Serva Chemical Co. (US), CMC-Na samples with different DS values and a CS-Na sample with a DS value of 0.54 were kindly supplied by Dr. T. Shibata, Daicel Chem. Ind., Japan. The chemical structure and the DS values of the sample derivatives are summarized in Table I.

Blood Compatibility Tests

In Vivo Test

The *in vivo* test for blood compatibility was performed according to a peripheral vein suture method developed by one of the authors (Y.N.).[11,12] The specimen was prepared as follows. A polyester suture No. 1-0 (USP) with length of 10 cm was coated with a desired PEC. In the animal experiment (in this work, dog), an 18-gauge needle was inserted into the peripheral vein (jugular and femoral vein) under general anesthesia. The suture was introduced through the needle into the lumen of the vessel. After the needle was withdrawn, the edge of the suture was ligated to the connective tissue near the puncture site of the vessel. After an adequate time (in this work, one day), the dog was sacrificed by acute exsanguination from the aorta under general anesthesia with the administration of heparin (2–3 mg/kg). The vein in which the suture had been inserted was opened gently and the suture was examined visually.

The sample polymer was coated on the polyester suture by casting from formic acid solution containing polycation and polyanion components at the weight ratio in the complex formed at pH 7.4. The polymer-coated sutures were washed thoroughly with deionized water and stored in deionized water containing 20 wt% ethanol.

Lee-White Test[2]

Dogs' whole blood was introduced into the test tubes, the interior walls of which were coated with sample polymers, and the coagulation time of blood was measured under every one minute tilting of test tube after the first four minutes incubation at 37°C. The coagulation time is expressed in terms of a ratio to an untreated glass tube.

Blood Platelet Adhesion test

Coating of the sample polymers on the glass beads with a 40–60 mesh was carried out using a solvent evaporation method. The evaluation was made using fresh blood collected from the jugular vein of a dog according to the method of Kataoka et al.[3,4] The number of platelets in the eluted blood was counted according to the method of Brecher and Cronkite.[13] The number of platelets in whole blood was found to be in the range of 2.7×10^5 to 3.1×10^5 counts/mm^3. The column packing of PEC-coated glass beads and the elution conditions were similar to those by Kataoka et al.[3,4]

Contact Activation Test

The degree of contact-phase activation of the intrinsic pathway of blood coagulation was measured by using fluorogenic oligopeptidyl substrate (Z-Phe-Arg-MAC) highly specific to the activated form (kallikrein) of prekallikrein,[14] which is simultaneously generated upon activation. The principle and the detailed procedure of this test method have been reported elsewhere.[8,9]

RESULTS AND DISCUSSION

Comparison Between *In Vivo* and *In Vitro* Test Methods

In general, anionic and cationic cellulose derivatives are water soluble if their DS value is sufficiently high. All the cellulose derivatives employed were water soluble except for AEC and DAC samples. As reported previously,[7] water-insoluble PECs can be obtained by mixing oppositely charged cellulose derivatives in aqueous solution, but the cellulosic PECs obtained are soluble in formic acid. Formic acid is a convenient casting solvent for coating cellulosic PECs on the interior walls of glass tubes or on the polyester sutures. In the present study, the formic acid solutions of the sample polymers were prepared by dissolving the water-insoluble PECs formed at pH 7.4. The AEC and DAC samples employed in the present study were insoluble in water, because of their low DS values, but soluble in formic acid. It was possible to prepare the PECs containing these derivatives as polycation components. This is one of the advantages of formic acid as casting solvent of cellulosic PECs. In these cases, the stoichiometric reaction was assumed to reckon the necessary amount of polyanion to be admixed. It was found that this procedure led to satisfactory formation of PEC.

The *in vivo* and *in vitro* test results on the blood compatibility of cellulosic PECs are summarized in Table II. The sample derivatives are designated by attaching the number of DS $\times$ 10 to the code of each sample. The whole blood coagulation data are mean values of five experiments. To obtain reliable results, other experiments were carried out at least twice. The symbols evaluate the *in vivo* results as follows: The + symbols indicate the relative degree of the thrombus formation of the sample in 3 steps where +++ denotes the thrombus formation along the entire length of the coated surface. – indicates the case where no thrombus was observed. Figure 1(a) shows an example of macroscopic view of a PEC-coated suture, around which no thrombus was observed (the degree of thrombus formation was evaluated as minus). For the sake of comparison, a macroscopic view of a suture coated with wool keratin derivative, which was evaluated as ++,[12] is shown in Figure 1(b).

The Lee-White test showed that these PECs have high relative coagulation times (2.7 – 5.8) compared with glass as a control. In this connection, it should be mentioned that most of synthetic polymers studied so far fell into the range of 1.5 – 3.0.[15] It should be noted also that the results on the coagulation time of whole blood by Lee-White tests are in good agreement with those by *in vivo* experiments. The results by other *in vitro* experiments

TABLE II
In Vivo and *In Vitro* Results on Blood Compatibility of Cellulosic PECs

		In vitro		
	In vivo[a]	Lee-White test	Platelet adhesion[b]	Contact activation
Sample code	Thrombus formation	Relative clotting time (Glass=1)	Relative number of adhered platelets (Glass=1)	Relative value (Glass=100)
Q-HEC-4/CMC-8	-	5.8 ± 0.8	0.28	1.1 (1.5)[c]
Q-HEC-4/CS-5	-	4.9 ± 0.3	—	— (0)
Q-Cell-4/CMC-8	-	4.7 ± 0.3	—	— (0)
AEC-0.5/CMC-3	+	2.9 ± 0.3	1.05	3.7
DAC-2/CS-2	+~++	2.4 ± 0.3	0.73	7.7
Polyester suture	+++			
Glass		1.0	1.00	100 (100)

[a] See text for symbols.
[b] Testing conditions: weight of beads, 0.5 g; flow rate, 1.7 mL/min.; contact time, 60 s.
[c] Values obtained with human plasma.

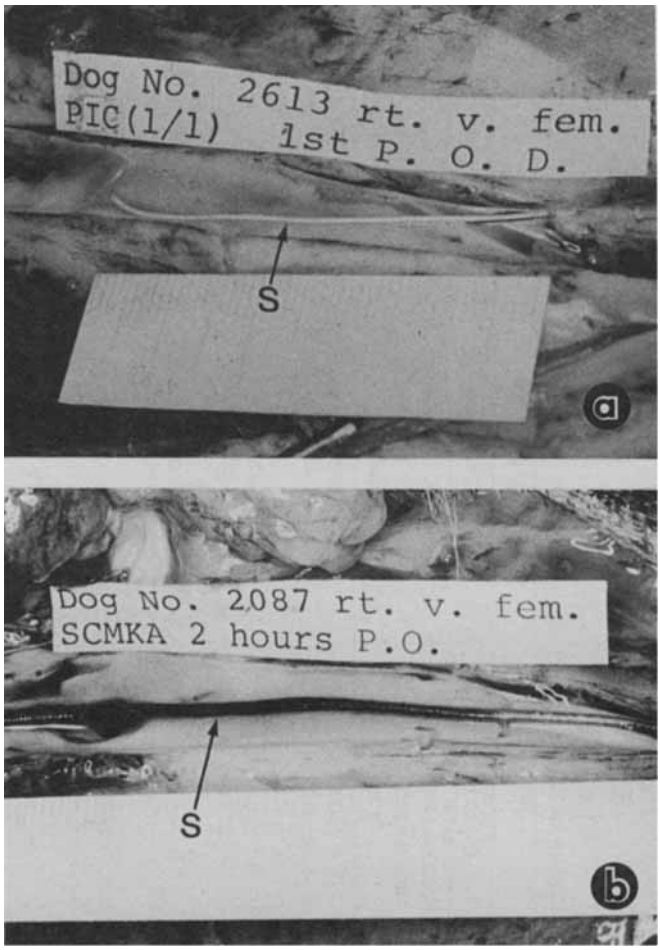

Fig. 1. Macroscopic views of polyester sutures coated with cellulosic PEC (a) and wool keratin derivatives (b) after implantation in the femoral vein of dog for 24 h and 2 h, respectively. (a) No thrombus was observed around the suture (S); (b) in this case, the blood compatibility was evaluated as ++.

were also consistent with those by *in vivo* experiments: The relative number of adhered platelets is in the order DAC-2/CS-5 $\simeq$ AEC-0.5/CMC-3 > Q-HEC-4/CMC-8 and the degree of contact phase activation is DAC-2/CS-5 > AEC-0.5/CMC-3 > Q-HEC-4/CMC-8. Among the PECs examined, the PECs containing quaternary ammonium cellulose derivatives as polycation components showed excellent blood compatibility. However, the DS values were considerably different among the cationic derivatives employed. In order to discuss the effect of the chemical structure of cellulosic PECs on the blood compatibility, a further investigation must be carried out to clarify the effect of DS value of the component derivatives on the blood compatibility. Such a study is in progress in collaboration with our three laboratories.

In connection with the above problem, it should be considered also that the blood compatibility will be more or less affected by the amount and the

distribution of non-neutralized domains, which are composed of unbound polycation and polyanion segments, on the surface coated with these PECs.[4] In addition to the geometric hindrance brought about by the semirigid glucopyranose ring, commercial AEC and DAC have been prepared from cellulose by heterogeneous reaction, so that the distribution of substituents along the cellulose chain in these samples is not uniform as compared with those in Q-HEC and Q-Cell prepared by homogeneous reaction.[7,16] Consequently, the non-neutralized domains may be present in the PECs containing AEC and DAC as polycation component. In any event, however, the emphasis placed here is that the blood compatibility of these PECs are relatively good.

Effect of Excess Charge in PEC

As already mentioned, the complex formation of Q-Cell with CMC does not follow a stoichiometric reaction, but Q-HEC reacts stoichiometrically with CMC. In the case of Q-HEC, the quaternary ammonium groups are not attached directly to semirigid glucopyranose rings but indirectly through flexible poly(oxyethylene) spacers (see Table I), causing the difference between the reaction schemes of Q-HEC and Q-Cell. Therefore, the system of Q-HEC and CMC is suitable for investigating the effect of excess charge in PEC on the blood compatibility. In this section, such an effect was examined in detail by *in vivo* and three different *in vitro* test methods.

The results by *in vivo* and Lee-White test methods are given in Table III. The results by three different *in vitro* test methods are shown in Figure 2. Experimental conditions for platelet adhesion test were a little different from those used in previous sections: weight of beads, 0.5 g; flow rate, 0.45 mL/min; contact time, 30 s. The mole ratio of polycation to polyanion component was limited in the range of 1.5/1 to 1/1.5, since either component became soluble in water beyond this range. It can be seen that the relative coagulation time of blood is almost independent of the mole ratio of polycation to polyanion component within the mole ratios examined, but the platelet adhesion increases with increasing the mole ratio of polycation/polyanion in the PEC. On the other hand, the degree of contact phase activation in plasma increases with decreasing the mole ratio. In general,

TABLE III
In Vivo and Lee-White Test Results on Blood Compatibility of PECs composed of Q-HEC-4 and CMC-8

	In vivo[a]	Lee-White test
Mole ratio (cation/anion)	Thrombus formation	Relative clotting time (Glass=1.0)
1.5/1.0	–	4.2 ± 0.7
1.25/1.0		4.1 ± 0.2
1.0/1.0	–	4.0 ± 0.2
1.0/1.25		4.1 ± 0.5
1.0/1.5	–	4.1 ± 0.3
Glass		1.0

[a] See text for symbols.

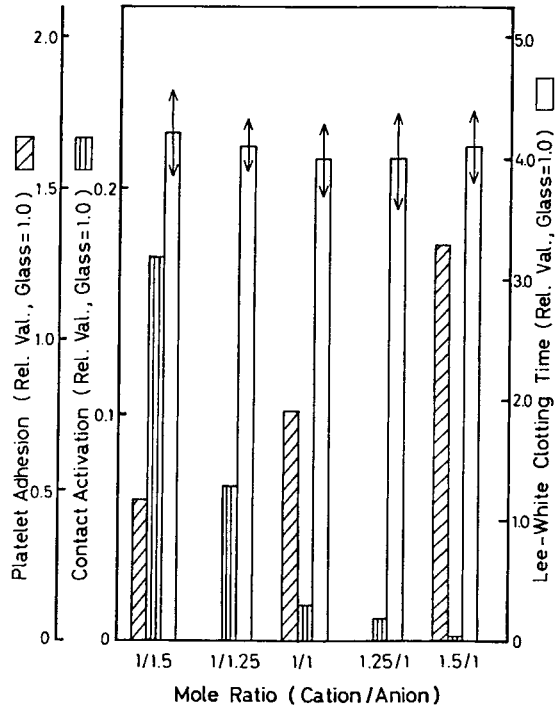

Fig. 2. Effect of the mole ratio in Q-HEC-4/CMC-8 on the clotting time, platelet adhesion and contact phase activation. (□) Clotting time; (▨) platelet adhesion; (▤) contact phase activation.

anionically charged surfaces tend to suppress the platelet adhesion, whereas the cationically charged ones greatly enhances it. The reverse tendency is observed for the contact activation of the plasma coagulation system, showing that the ionic charge balance of the PEC film surface is one of the important factors determining blood compatibility. The results obtained here are consistent with those observed previously by Kataoka et al.[3,4] and Matsuda et al.,[8] respectively. This strongly suggests that polyelectrolytes with well-distributed negatively and positively charged groups would lead to a new type of molecular-designed blood-compatible polymer.

The coating of the sample PECs was carried out not from aqueous solutions but from formic acid solutions. Therefore, as mentioned in the previous section, the degree of neutralization should be taken into account in the discussion of the results. However, the PEC samples obtained from formic acid solutions were no longer soluble in deionized water and physiological salt solution similarly to those prepared from aqueous solutions. It may be considered that such an effect has no serious influence on the results obtained here.

In connection with the interesting results of Figure 2, last to be mentioned is that the exposure time to blood for measuring biological responses are different among the test methods. That is, the measurements of the coagulation time by Lee-White test and the degree of contact activation are in the order of minutes, usually 5 – 50 min, while the measurements by platelet

adhesion test are in the order of seconds (usually within 60 s). Therefore, to understand the results of Figure 2, it is necessary to examine the time dependence of platelet adhesion and that of the decrease in hemocoagulation parameters. Very recently, one of the authors has developed a test method for this purpose, called "Quenched Lee-White Test"[17] in which the degree of platelet adhesion and the change in hemocoagulation parameters during Lee-White test are measured by quenching the coagulation with sodium citrate. Experiments with this "Quenched Lee-White" method are currently under investigation. The results will be reported in the near future.

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References

1. See, e.g., D. L. Coleman, D. E. Gregonis, and J. D. Andrade, *J. Biomed. Mater Res.*, **16**, 381 (1982).

2. R. I. Lee and P. D. White, *Am. J. Med. Sci.*, **145**, 495 (1913).

3. K. Kataoka, T. Akaike, Y. Sakurai, and T. Tsuruta, *Makromol. Chem.*, **179**, 1121 (1978).

4. K. Kataoka, T. Tsuruta, T. Akaike, and Y. Sakurai, *Makromol. Chem.*, **181**, 1363 (1980).

5. N. Ogata, K. Sanui, H. Tanaka, Y. Takahashi, Y. Sakurai, T. Akaike, T. Okano, and K. Kataoka, *J. Appl. Polym. Sci.*, **26**, 2293 (1981).

6. R. I. Heimark, K. Kurachi, K. Fujikawa, and E. W. Davie, *Nature*, **286**, 456 (1980).

7. H. Ito, T. Miyamoto, H. Inagaki, and Y. Noishiki, in press.

8. T. Matsuda, K. Uchida, H. Takano, Y. Taenaka, K. Hayashi, M. Umezu, and T. Nakamura, paper presented at the 11th Symposium on Biomedical Polymers, Tokyo, November 1981.

9. T. Matsuda, K. Uchida, H. Takano, Y. Taenaka, M. Umezu, T. Nakamura, and T. Akutsu, Proceedings of 28th IUPAC, 353 (1982).

10. A. El-Kafrawy, *J. Appl. Polym. Sci.*, **27**, 2435 (1982).

11. Y. Noishiki, *Jinko-Zoki*(Jpn. J. Artif. Organs) **11**, 794 (1982).

12. H. Ito, T. Miyamoto, H. Inagaki, and Y. Noishiki, *Kobunshi Ronbunshu*, **39**, 249 (1982).

13. G. Brecher and E. P. Cronkite, *J. Appl. Physiol.*, **3**, 365 (1950).

14. T. Morita, H. Kato, S. Iwanaga, K. Takada, T. Kimura, and S. Sakakibara, *J. Biochem.*, **82**, 1495 (1977).

15. T. Matsuda, unpublished data.

16. T. Miyamoto, Y. Sato, H. Shibata, M. Tanahashi, and H. Inagaki, *J. Polym. Sci. Polym. Chem. Ed.*, **23**, 1373 (1985).

17. T. Matsuda, Y. Taenaka, H. Hatano, K. Hayashi, M. Umezu, T. Nakamura, T. Nakatani, S. Takatani, and T. Akutsu, Proceedings of 3rd ISAO (1983).

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