Self-Organization of Amphiphilic Poly[2-(methacryloyloxy)ethyl phosphorylcholine] Carrying Tocopheryl Moieties as Terminal Groups

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ABSTRACT: Poly[2-(methacryloyloxy)ethyl phosphorylcholine](PMPC) with one pendant tocopheryl moiety at the polymer terminus (PMPC-Toco) was prepared by the radical polymerization of 2-(methacryloyloxy)ethyl phosphorylcholine (MPC) initiated with 4,4′-azobis[(3-tocopheryl)-4-cyanopentanoate] in the presence of 2-mercaptoethanol as a chain transfer reagent. The self-organization of PMPC-Toco was analyzed with fluorescence and ^{1}H-NMR measurements. The critical micelle concentrations of PMPC-Toco with [η] = 0.25, 0.13, 0.10, and 0.05 dL g^{-1} were found to be 200, 100, 100, and 90 mg L^{-1}, respectively. The blood compatibility of PMPC-Toco was evaluated from the Michaelis constant (K_m) for the enzymatic reaction of thrombin and a synthetic substrate, *S*-2238, in the presence of PMPC-Toco. The K_m values were 0.21, 0.23, 0.36, and 0.21 for PMPC-Toco-1, 2, 3, and PMPC ([η] = 0.56 dL g^{-1}), respectively. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 113: 959–965, 2009

Key words: poly[2-(methacryloyloxy)ethyl phosphorylcholine]; tocopherol; amphiphiles; self-organization; radical polymerization

INTRODUCTION

Considerable attention has been paid to the incorporation of the biocompatible phosphorylcholine moiety into the sidechains of polymers, because the phosphorylcholine moiety exists on the extracellular surface of the lipid bilayer of the cell membrane. Polymeric materials modified with the phosphorylcholine moiety are useful for the optimization of performance as blood compatible polymers. It is well known that polymer materials, including the 2-(methacryloyloxy)ethyl phosphorylcholine (MPC) moiety, show excellent blood compatibility and biocompatibility. The MPC polymer effectively suppressed clot formation following platelet adhesion and activation when human whole blood came in contact with the MPC polymer without the aid of anticoagulants.[1] The blood compatibility of various polymer materials[2–5] has been improved by modifications with an MPC polymer. In previous articles, the surface of poly(ethylene terephthalate)[6] and polyethylene[7] films modified with poly[2-(methacryloyloxy)ethyl phosphorylcholine] (MPC) segments also showed activity for the inhibition of fibrin formation without the adhesion of mouse fibroblasts. However, there has been a growing interest in the self-organization phenomena of water-soluble polymers containing a few hydrophobic substituents per chain and hydrophobic groups attached to the polymer ends.[8–11] Because these amphiphilic polymers exhibit unique properties in dilute solutions, they have found application as feasible multi-functionalized drug carriers.[8,9]

As mentioned previously, we have made an effort to modify the surface of polymer substrates with hydrogel-forming PMPCs. These hydrogels have physical properties similar to human tissue and exhibit excellent biocompatibility.[6,7,12] We have reported that PMPC[13] and poly[*N*-(2-hydroxypropyl)-methacrylamide]s with either a single pendant cholesteryl moiety at the polymer end or two cholesteryl moieties at both polymer ends can self-organize in water solution and exhibit a critical micelle concentration (CMC).[14] Our goal is to develop a new polymer having superior biocompatibility than polymers containing terminal cholesteryl groups. This article describes the self-organization phenomena and blood compatibility of PMPC modified with one tocopheryl moiety at the polymer terminus, providing basic information about novel DDS carrier macromolecules with improved thrombo-resistance via intravenous injection.

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EXPERIMENTAL

Reagents

4,4′-Azobis(4-cyanopentanoic acid) was kindly supplied by Wako Pure Chemicals (Japan) and purified by recrystallization from methanol. 4,4′-Azobis (4-cyanopentanoic acid chloride) was obtained from the reaction of 4,4′-azobis(4-cyanopentanoic acid) and phosphorus pentachloride in benzene according to the method of Smith.[15] 4,4′-Azobis[(3-tocopheryl)-4-cyanopentanate] (VA-Toco) was prepared from the reaction of 4,4′-azobis(4-cyanopentanoic acid chloride) and tocopherol according to the method described in the previous paper.[13] ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.84–0.87 (t, 24H, —CH_3), 1.23 (s, 6H, —CH_3), 1.08–1.56 (m, 21H, —CH_2CH_2CH—), 1.72–1.82 (d, 12H, —CH_3), 1.72–1.82 (m, 4H, —CH_2—), 1.96–2.00 (d, 6H, —CH_3), 2.07–2.17 (d, 6H, —CH_3), 2.50–2.60 (m, 4H, —CH_2—), 2.60–2.88 (m, 8H, —C—CH_2CH_2COO—). UV: $\lambda_{max} = 346$ (ε18). Anal. $C_{70}H_{112}O_6N_4$ = (1105.683), Calcd. C : H : N = 77.04% : 10.21% : 5.07%, Found C : H : N = 76.09% : 10.09% : 5.05%. VA-Toco has an mp of 109.0–112.5°C (dec).MPC was prepared according to the method described in the literature.[16] Tocopherol (SIGMA) was column chromatographed over silica gel using a mixed solvent (hexane : ethanol = 90 : 10 vol %). 2-Mercaptoethanol (Tokyokasei, Japan) was redistilled before use. 1-(6-Dimethylamino-2-naphthyl) dodecanone (DMAND: Molecular Probes) was used as obtained. Human thrombin was purchased from Mochida Pharmacy Co., Ltd. (Tokyo, Japan), whereas the synthetic chromogenic substrate *S*-2238 was purchased from Dai-ichi Chemicals (Tokyo, Japan) and used without further purification. Distilled deionized water was used throughout the experiments.

Polymerization procedure

The MPC monomer was radically polymerized with VA-Toco as an initiator in the presence of 2-mercaptoethanol as a chain transfer reagent in the mixed solvent of ethanol and tetrahydrofuran (70 : 40 vol %) at 60°C for 12 h according to the method described in the literature.[13] The yield was calculated from the dry polymer obtained. The intrinsic viscosity of PMPC-Toco (1.0 g dL^{-1}) was determined in ethanol at 25°C using an Ubbelohde viscometer.

^{1}H-NMR measurements

The ^{1}H-NMR spectra of PMPC-Toco dissolved in a mixed solvent of CD_3OD and D_2O were recorded with a Jeol NMR EX-400 spectrometer. The concentration of CD_3OD : D_2O was varied continuously from 100 : 0, 75 : 25, 50 : 50, 25 : 75, to 0 : 100 vol %.

CMC measurements

The fluorescence spectra of the aqueous PMPC-Toco solutions were recorded with a Shimadzu RF-1500 fluorophotometer. DMAND with a 364 nm excitation wavelength was used as a fluorescent probe for hydrophobicity. The concentration of DMAND was adjusted to 1.0 μmol L^{-1} by the addition of 20 μL of a stock solution containing 500 μmol L^{-1} DMAND in methanol to 10 mL of each amphiphilic PMPC-Toco aqueous solution. The concentration of PMPC-Toco was changed in the range of 20–1.0 × 10^4 mg L^{-1}. The CMC was determined by the break point in the plot of the fluorescence intensity and PMPC-Toco concentration.

Enzymatic reaction of thrombin and *S*-2238

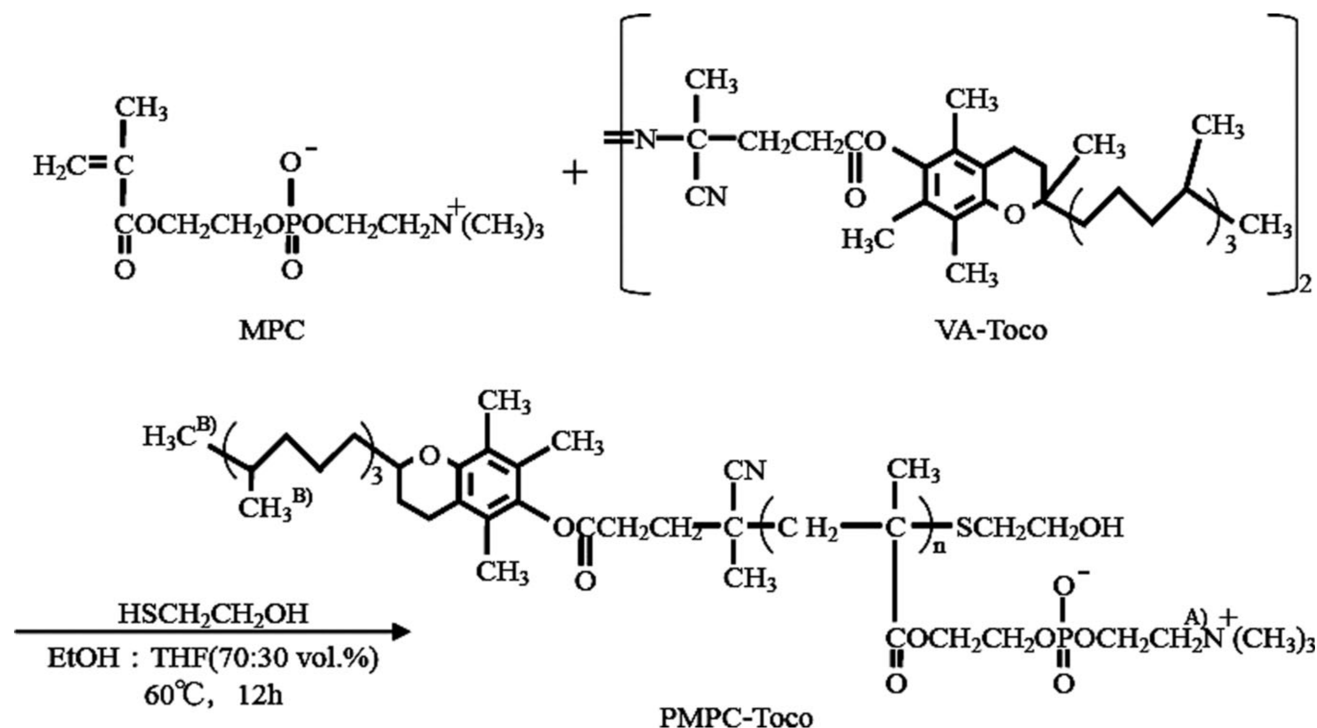
Seventy microliters of 2.0 mg mL^{-1} PMPC-Toco in a Tris buffer solution (pH 7.4) and 20 μL of 10 units mL^{-1} thrombin in a Tris buffer solution were poured into the 6.4 mm diameter cells of a 96-well multiplate at 37°C; this was followed by shaking for 10 min and standing for 2 min. Then, 100 μL of a defined concentration of *S*-2238 (0.1–1.0 mmol) was added to the system at 37°C and the multiplate was shaken for 2 h. The initial rate of enzymatic reaction was determined by spectrophotometry; the optical density at 405 nm of free *p*-nitroaniline derived from *S*-2238 was measured with a Bio-Tek Instruments ELx808IU microplate reader. The reaction was repeated three times. The reaction rate represents the amount of *p*-nitroaniline produced by 1 unit of thrombin for 1 min.

RESULTS AND DISCUSSION

Preparation of PMPC-Toco

Amphiphilic macromolecule PMPC-Toco with a hydrophobic tocopherol group attached to the end of the water-soluble PMPC was prepared from the polymerization of MPC initiated with a novel lipophilic radical initiator, VA-Toco, in the presence of 2-mercaptoethanol as depicted in Scheme 1.

For the radical polymerization of MPC, the recombination and disproportionation of growing radicals cannot be expected to occur as a bimolecular termination event in the presence of an excellent chain-transfer agent. PMPC-Toco with a single pendant cholesteryl moiety was obtained by the radical polymerization of MPC initiated with VA-Toco in the presence of 2-mercaptoethanol, because of the large chain transfer constant $C_T = 17$ for 2-mercaptoethanol, whereas for example, the small propagation constant $k_p = 1.0 \times 10^{-5}$ for methyl methacrylate was used as a model for MPC. PMPC-Toco with a single pendant tocopheryl moiety was, therefore, obtained by the radical polymerization of MPC



Scheme 1 Polymerization of MPC initiated with VA-Toco in the presence of 2-mercaptoethanol.

initiated with VA-Toco in the presence of 2-mercaptoethanol. Table I shows the results of the polymerization and characterization of the resulting polymers.

These MPC polymers were obtained in high yield. The intrinsic viscosity [η] decreased from 0.25 to 0.05 dL g^{-1} as the amount of 2-mercaptoethanol increased from 0.11 to 1.58 mmol. An increase in the amount of the chain transfer reagent resulted in a lowering of the [η].

Self-organization

The self-organization of PMPC-Toco-2 was first confirmed with ^{1}H-NMR measurements. The spectra of PMPC-Toco-2 in CD_3OD and D_2O, used as typical examples, are shown in Figure 1. The proton signal of the tocopheryl moiety (H_B) that appeared in CD_3OD disappeared completely in D_2O, whereas the signal of H_A in the MPC moiety did not show any change. The half-widths of the two independent peaks as a function of the D_2O content in CD_3OD are shown in Figure 2. The half-width of H_B ($\delta = 0.85$ ppm) gradually broadened with an increase in the D_2O content and the peak completely disappeared in 50% (vol %) of D_2O. However, the half-width of the peak of H_A ($\delta =$ 3.8 ppm) did not change with a change in the water content. The line broadening of the proton signals of the tocopheryl group at the polymer end in aqueous medium was attributed to the restricted molecular

TABLE I
Preparation of PMPC-Toco[a]

PMPC-Toco	2-ME (mmol)	Yield (%)	$[\eta]^b$ (dL g^{-1})	CMC (mg L^{-1})	K_m[c,d] (mM)	Vmax (mM min^{-1})	k_{cat}/K_m[c,d] (mM^{-1} min^{-1})
PMPC-Toco-1	0.11	98.0	0.25	200	0.21	2.3×10^{-2}	12.0×10^{-1}
-2	0.55	95.0	0.13	100	0.23	2.3×10^{-2}	10.9×10^{-1}
-3	1.10	93.7	0.10	100	0.36	3.1×10^{-2}	8.9×10^{-1}
-4	1.58	91.3	0.05	90	0.38	3.2×10^{-2}	8.5×10^{-1}
PMPC-Chol	0.11	83.1	0.14	250	0.12	2.5×10^{-2}	25.2×10^{-1}

[a] Condition: MPC, 10.5 mmol; VA-Toco, 0.11 mmol; ethanol: tetrahydrofuran (70 : 30 vol.%), 10 mL, 60°C, 12 h. 2-ME represents 2-mercaptoethanol.
[b] Measured at concentration of 1.0 g dL^{-1} in ethanol at 25°C.
[c] K_m and k_{cat}/K_m of PMPC homopolymer were 0.21 mM and 8.8×10^{-4} mM^{-1} min^{-1}, respectively.
[d] In the case of control (in the absence of PMPC-Toco), K_m and k_{cat}/K_m were 0.21 mM and 10.6×10^{-4} mM^{-1} min^{-1}, respectively.

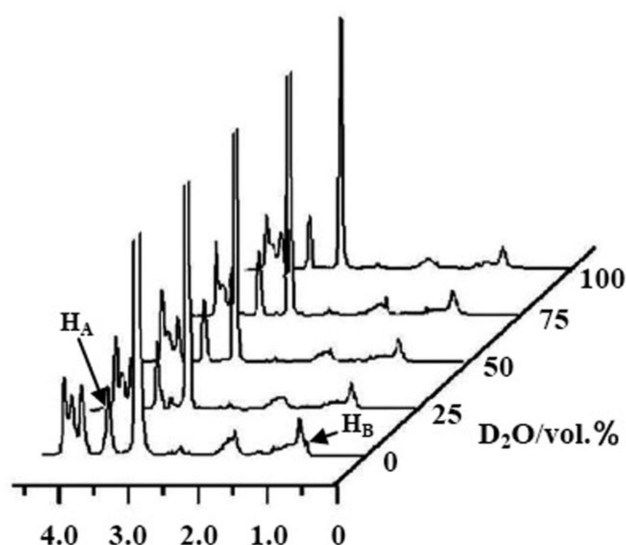


Figure 1 ^{1}H-NMR spectra of PMPC-Toco-2 in the mixed solvent of D_2O and CD_3OD.

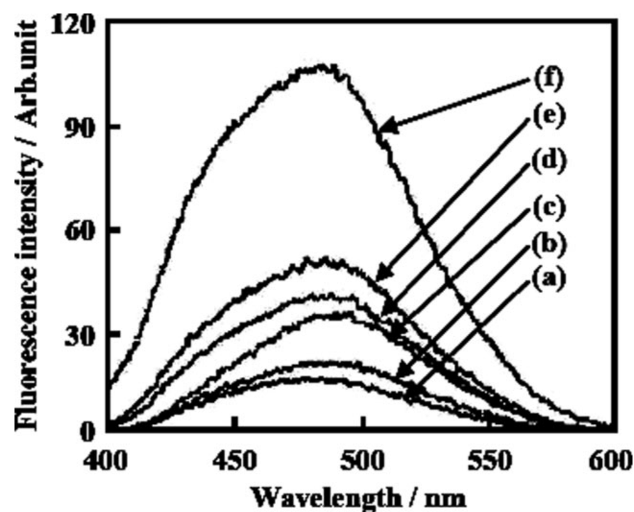


Figure 3 Fluorescence spectra of PMPC-Toco-2 in water. [DMAND] = 1.0 μmol L^{-1}. λ_{EX} = 364 nm. [PMPC-Toco-2] was varied : (a) 78, (b) 160, (c) 310, (d) 630, (e) 1300, (f) 5000 mg L^{-1}

motion of the terminal tocopheryl groups upon self-aggregation.[11,17] Nevertheless, the mobility of the hydrophilic main chain of PMPC-Toco remained steady as the solvent polarity increased. These data suggest that the PMPC-Toco self-aggregate had microdomains provided by both a rigid core of hydrophobic tocopheryl groups and a mobile shell of a hydrophilic PMPC main chain.

Figure 3 shows typical fluorescence spectra at different concentrations for an aqueous solution of PMPC-Toco-2 with [η] = 0.13 dL g^{-1} in the presence of DMAND as a probe. No fluorescence emission was observed in the DMAND aqueous solution without PMPC-Toco-2 or at a polymer concentration below 78 mg L^{-1}. When the polymer concentration

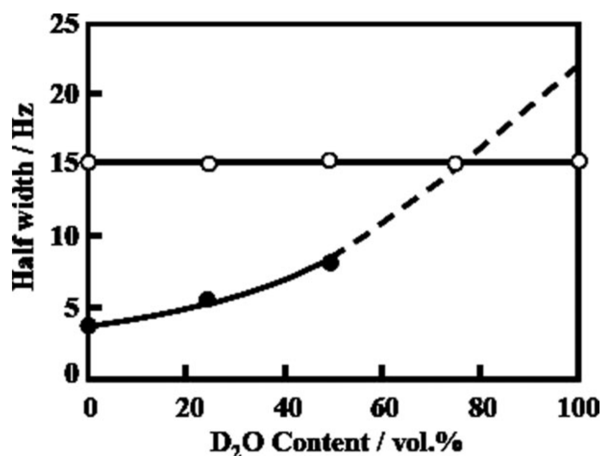


Figure 2 Change of the half-width of ^{1}H-NMR proton signals of H_A and H_B for PMPC-Toco-2 as a function of D_2O content in CD_3OD.

increased, the fluorescence intensity at 482 nm of the DMAND solution became stronger. This was due to the assembly of hydrophobic tocopheryl moieties of PMPC and the formation of hydrophobic domains in water by the hydrophobic interaction of the tocopheryl groups at the polymer ends. Although PMPC homopolymer shows no fluorescence emission,[13] PMPC-Toco-1,3,4 gave fluorescence spectra similar to those of PMPC-Toco-2. The tocopheryl moiety is fluorescent, but its intensity is negligible, because the hydrophobic tocopheryl moiety attached to the hydrophilic PMPC end can self organize to form the core part in the core-shell type of micelle in water. Therefore, the fluorescence of the tocopheryl moiety cannot exit the shell. Considering also that the self-organization of PMPC-Toco was confirmed with ^{1}H-NMR spectroscopy, the fluorescence intensity of DMAND is not influenced by that of the tocopheryl moiety. DMAND is considered to exist at the boundary of the hydrophilic shell and hydrophobic core of the micelle. The critical micelle concentration (CMC) due to the association of PMPC-Toco in an aqueous solution was deduced from the concentration at which there was an abrupt increase in the fluorescence intensity of DMAND in water with a gradual increase in the concentration of PMPC-Toco.

Figure 4 illustrates the concentration dependence on the fluorescence intensity of the aqueous PMPC-Toco-2 solution. The breaks at 100 mg L^{-1} are interpreted to be the CMC values for the aqueous solutions of PMPC-Toco-2. The CMC values for other PMPC-Toco solutions were also obtained in a similar manner and are summarized in Table I. Because of the decrease in the CMC of PMPC-Toco at decreasing [η],

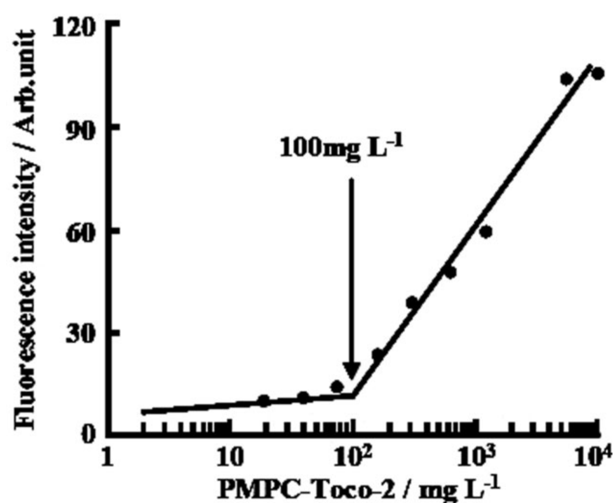
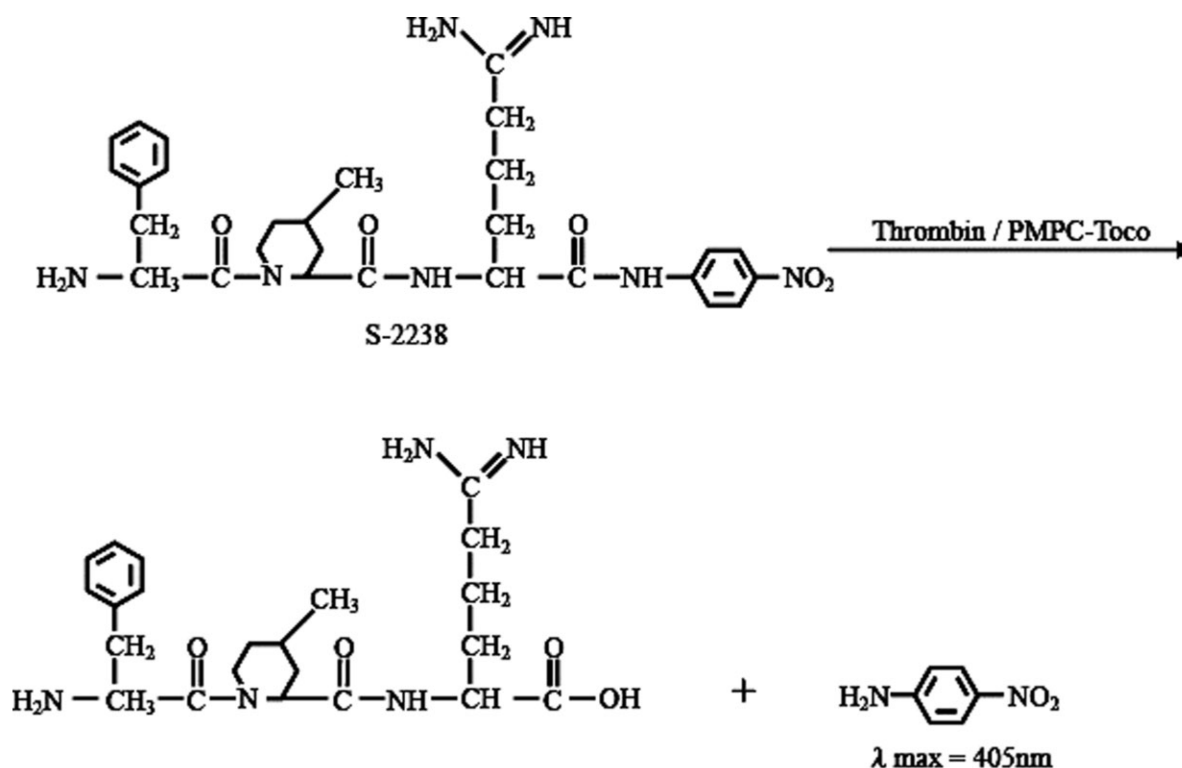


Figure 4 Relationship between fluorescence intensity and concentration of PMPC-Toco-2. [DMAND] = 1.0 μmol L^{-1}. λ_{EX} = 364 nm.

the smaller degree of polymerization (related to the [η] value) of the water-soluble PMPC resulted in easier micelle formation, whereas the disruptive motion of a larger degree of polymerization of PMPC prevents the tocopheryl group from micelle formation. The P_n of PMPC-Toco can be estimated from the relationship between [η] and P_n. In the case of PMPC-Chol, it was found that [η] = 0.14 dL g^{-1} and [η] = 0.02 dL g^{-1} correspond to the degrees of polymerization, $P_n = 91$ and $P_n = 34$, respectively. PMPC-Toco-2 with [η] = 0.13 dL g^{-1} and PMPC-Toco-4 with [η] = 0.05 dL g^{-1} were then estimated to have $P_n = 91$ and $P_n = 34$, respectively. Consequently, it can be said that an appropriate hydrophobic-hydrophilic balance of PMPC-Toco is considered an important factor in forming a core-shell type of polymer micelle.

Enzymatic reaction of thrombin and *S*-2238 in the presence of PMPC-Toco

The blood compatibility of polymer materials is evaluated in several ways, including the adsorption of plasma protein,[18] the inhibition activity of fibrin formation with the activated partial thromboplastion reagent,[6] the aggregation of platelets by the addition of adenosine 5-diphosphate sodium salt,[17] the enzymatic reaction of thrombin and synthetic chromogenic substrate *S*-2238[13,19] and the adhesion and activation of platelets.[18,20] In the previous article,[7] the enzymatic method was found to be the most suitable way of evaluating water-soluble polymers such as PMPC, poly[2-(glucosyloxy)ethyl methacrylate], polyoxyethylene, and poly(*N*-isopropylacrylamide). The blood compatibility of PMPC-Toco was then evaluated from the degree of inhibition of the thrombin activity by the addition of *S*-2238 in the presence of PMPC-Toco in a Tris buffer solution at 37°C, with a concentration of *S*-2238 valued from 0.1 to 1.0 mmol L^{-1}.



Scheme 2 Enzymatic reaction of thrombin and *S*-2238 in the presence of PMPC-Toco.

It has been shown that thrombin catalyzes the hydrolytic scission of coagulation factor I (fibrinogen), VII, VIII, or XIII at the carboxyl end of an arginine (Arg) residue adjacent to hydrophobic amino acid residues,[21] i.e., thrombin accelerates the conversion of fibrinogen to fibrin monomer. In the case of *S*-2238, thrombin catalyzed hydrolytic scission at the amide bond of Arg; this released *p*-nitroaniline through thrombin, as shown in Scheme II.

In a typical example, the rate of reaction was determined by spectrophotometry; the change in the optical density at 405 nm of *p*-nitroaniline derived from *S*-2238 was measured over time during the reaction of thrombin and *S*-2238 in the presence of PMPC-Toco in a Tris buffer solution of pH 7.4. Figure 5 shows the Lineweaver-Burk plots for the reaction of thrombin and *S*-2238 in the presence and absence of PMPC-Toco (as a control), along with PMPC homopolymer. V and S represent the amount of *p*-nitroaniline produced by 1 unit of thrombin for 1 min and the initial concentration of *S*-2238, along with PMPC without the terminal tocopherol group. In the case of polyoxyethylene ($M_n = 4 \times 10^3$) as a reference sample in a Tris buffer solution (pH 8.4) and 20 μL of 3 units thrombin, K_m and k_{cat}/K_m were found to be 0.53 and 72, respectively.[13] On the basis of the Michaelis–Menten equation, the Michaelis constant (K_m) and the catalytic efficiency of thrombin (k_{cat}/K_m) were calculated as shown in Table I, together with the data from PMPC-Chol. As K_m is a characteristic of the affinity of an enzyme and substrate, a higher K_m value represents a lower affinity of thrombin to *S*-2238, i.e., the higher inhibiting effect of thrombin activity. As the K_m of PMPC homopolymer was 0.21 (0.56 for the case of 3 units of thrombin[13]), there was no acceleration of the rate for the enzymatic reaction of thrombin and *S*-2238 in the presence of PMPC.

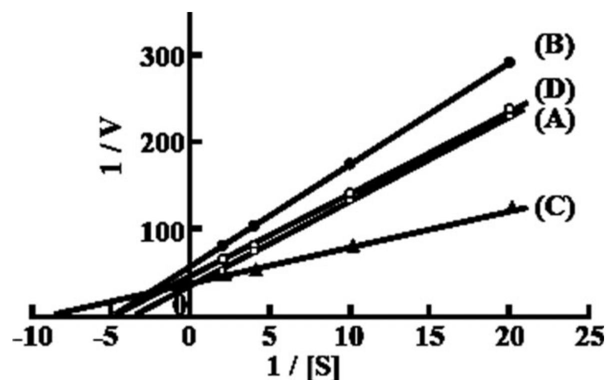


Figure 5 Lineweaver-Burk plots for the reaction of thrombin and *S*-2238 in the presence or in the absence of PMPC-Toco in Tris buffer solution (pH 7.4). [PMPC-Toco]:0.7 mg mL^{-1}. [Thrombin]:1 unit mL^{-1}. [*S*-2238] was varied from 0.05 to 0.5 m*M*. (A) PMPC-Toco-2, (B) PMPC homopolymer, (C) PMPC-Chol, (D) control.

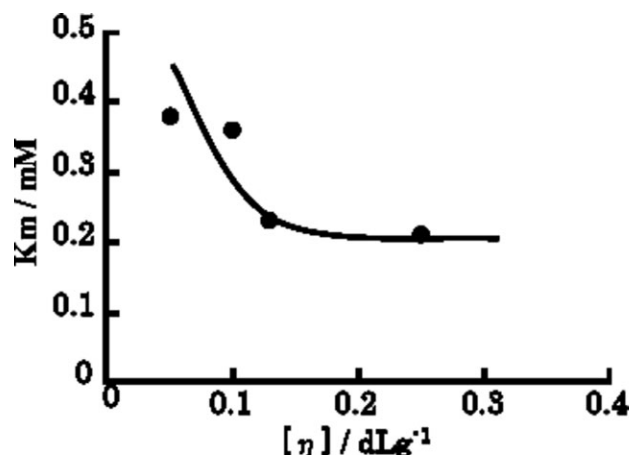


Figure 6 Effect of [η] on K_m for the reaction of thrombin and *S*-2238 in the presence of PMPC-Toco in Tris buffer solution (pH 7.4) at 37°C. [PMPC-Toco] = 0.7 mg mL^{-1}, [Thrombin] = 1 units mL^{-1}, [*S*-2238] = 0.05–0.5 m*M*.

However, PMPC-Toco exhibits a lower blood compatibility than PMPC homopolymer. It was also found that PMPC-Toco does not accelerate the activation of thrombin, whereas PMPC-Chol accelerates that of thrombin. It was thought that the length of the PMPC chain in the PMPC-Toco was insufficient to show excellent blood compatibility. To determine the effect of the molecular weight of PMPC on the K_m and k_{cat}/K_m, we carried out the enzymatic reaction of thrombin and *S*-2238 in the presence of PMPC-Toco with different [η]. Figure 6 illustrates the effect of [η] of PMPC on the K_m and k_{cat}/K_m for the enzymatic reaction in the presence of PMPC-Toco. K_m decreased with an increase in [η] of PMPC-Toco. This means the lower molecular weight of the PMPC-Toco will result in a higher blood compatibility, i.e., easier micelle forming PMPC-Toco-4 has a better blood compatibility than other PMPC-Toco polymers and blood compatibility is expected to improve when a suitably long PMPC chain is introduced.

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