

Blood Compatibility of Amphiphilic Poly(N- α -acrylamide-L-lysine-*b*-dimethylsiloxane) Block Copolymers

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ABSTRACT

Amphiphilic block copolymers poly(LysAA-*b*-DMS) consisting of a hydrophilic poly(N- α -acrylamide-L-lysine) [poly(LysAA)] segment with different molecular weights and a hydrophobic polydimethylsiloxane (PDMS) segment were prepared as follows. The precursor copolymer poly(Boc-LysAA-OtBu-*b*-PDMS) was obtained from radical polymerization of N- α -acrylamide-N- ϵ -tert-butoxycarbonyl-L-lysine-tert-butylester (Boc-LysAA-OtBu) initiated with 4,4'-azobis(polydimethylsiloxane 4-cyanopentanoate) (azo-PDMS) with the molecular weight of PDMS $M_w = 4.3 \times 10^3$ in the presence of 2-mercaptoethanol (2-ME) as a chain-transfer agent. Removal of the protecting groups of the precursor copolymer was carried out in 80% trifluoroacetic acid aqueous solution to give poly(LysAA-*b*-DMS)-1-3. The weight average molecular weight of poly(LysAA-*b*-DMS)-1-3 was $M_w = 1.02 \times 10^4 - 2.52 \times 10^4$. From the ¹H-NMR and fluorescence spectra measurements, poly(LysAA-*b*-DMS)-1-3 was determined to self-organize and form core-shell micelles in water. The critical micelle concentration (CMC) increased to 1000 - 4000 mg·L⁻¹ with increasing molar ratio of the poly(LysAA) segment from 0.42 to 0.65. From morphological analysis with a scanning probe microscope (SPM), poly(LysAA-*b*-DMS) has microphase-separated structures made up of hydrophilic and hydrophobic regions with the domain size ranging from several tens to several hundreds of nanometers. Inhibition of thrombin activity of poly(LysAA-*b*-DMS) was evaluated from the Michaelis constant (K_M) and catalytic activity (k_{cat}) for the enzymatic reaction of thrombin and synthetic substrate S-2238 in the presence of poly(LysAA-*b*-DMS). The K_M and k_{cat} were 0.10 - 0.11 mM and $4.04 \times 10^5 - 4.26 \times 10^5 \text{ min}^{-1}$, respectively. Fibrinolytic activity was also verified from the transformation of plasminogen to plasmin by tissue plasminogen activator (t-PA) using synthetic substrate S-2251 in the presence of poly(LysAA-*b*-DMS). The K_M and k_{cat} were 0.07 mM and $5.73 \times 10^6 - 5.95 \times 10^6 \text{ min}^{-1}$, respectively.

Keywords: Poly(N- α -acrylamide-L-lysine), Polydimethylsiloxane, Block Copolymer, Molecular Assembly, Blood Compatibility, S-2238/S-2251, Biomedical Polymer Material

1. Introduction

Some graft and block copolymers containing the polydimethylsiloxane (PDMS) segment have been synthesized in order to improve the mechanical properties and biocompatibility of silicone rubber as a useful biomedical material [1-3]. In poly(etherurethaneurea)s including various molar ratios of the tetramethyldisiloxane moiety in the main chain, the siloxane moiety was located on the surface. As the hydrophobicity was increased with increasing siloxane content, the surface was able to adsorb bovine serum albumin [4]. On the other hand, a series of block copolymers consisting of PDMS and hydrophilic

polymethacrylates, such as poly(2-hydroxyethyl methacrylate), poly(2,3-dihydroxypropyl methacrylate), and poly(2,3,4,5,6-pentahydroxyhexyl methacrylate) suppressed the adsorption of albumin and γ -globulin as well as platelets, to a level less than PDMS [5].

The microphase-separated structure of a polymer is considered to exhibit effective suppression of the adsorption of fibrinogen, an important blood coagulation factor, as well as inhibition of adhesion and activation of human platelets. Plasma protein adsorption, which is the initial event in blood-material interaction, influences subsequent platelet adhesion and activation. The amphiphilic poly(MPC-*b*-PDMS) obtained from introduction of the

2-(methacryloyloxy)ethyl phosphorylcholine (MPC) moiety into PDMS has a microphase-separated structure and shows no adhesion or activation of human platelets [6,7].

In our work on biocompatible polymeric materials [4-9], we found that incorporation of *L*-serine or *L*-lysine residues into polymers as side groups, for example, poly(*O*-methacryloyl-*L*-serine) [poly(SerMA)] and poly(*N*- α -methacrylamide-*L*-lysine) [poly(LysMA)] [10], was useful for creating blood compatible materials. The amphiphilic block copolymer poly(LysMA-*b*-DMS) consisting of poly(LysMA) as the hydrophilic segment and a PDMS segment with almost monodispersed molecular weight as the hydrophobic segment has also been reported [11]. The poly(LysMA-*b*-DMS) also exhibits antithrombotic properties. Blood compatibility of poly(LysMA) and poly(LysMA-*b*-DMS) may arise from the strong interaction of the *L*-lysine group in the polymer and the *L*-lysine-binding sites (LBS) of plasminogen, the precursor of plasmin [12]. Plasminogen is converted to the fibrinolytic active plasmin by the tissue plasminogen activator (t-PA), thus showing increased fibrinolytic activities.

It is well known that differences in the glass transition temperatures (T_g) of polymers reflect the flexibility of the main chain and side chain. For instance, the micro-brownian motion of poly(methyl acrylate) and poly(*N*-*tert*-butylacrylamide) begins at $T_g = 10^\circ\text{C}$ and $T_g = 128^\circ\text{C}$, respectively, whereas that of poly(methyl methacrylate) and poly(*N*-*tert*-butylmethacrylamide) begins at $T_g = 105^\circ\text{C}$ and $T_g = 160^\circ\text{C}$, respectively [13]. The former polymers are more flexible due to the difference in the substituent group (H or methyl) at the α -position of the monomer units in the vinyl polymers. The more flexible main chain is therefore expected to be the origin of the stronger interaction between the polymer *L*-lysine moiety and the LBS of plasminogen. In the course of our study on biomedical polymeric materials, our interest has been focused on the preparation of amphiphilic block copolymers poly(LysAA-*b*-DMS) consisting of the hydrophilic poly(*N*- α -acrylamide-*L*-lysine) [poly(LysAA)] segment with different molecular weights and a hydrophobic PDMS segment. Poly(LysAA-*b*-DMS) is expected to exhibit stronger fibrinolytic activity than poly(LysMA-*b*-DMS).

There has also been 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 end [8,9], as well as amphiphilic block copolymers consisting of hydrophilic and hydrophobic segments [11,14-17]. These amphiphilic polymers and copolymers are expected to play a role as carrier molecules for drug delivery systems (DDS), because they form a core-shell polymer micelle

composed of a hydrophobic core and a hydrophilic shell, making it possible to stably hold hydrophobic medicines in the core when placed in water.

In this article, it is confirmed that poly(LysAA-*b*-DMS) can self-assemble to form a core-shell polymer micelle composed of a hydrophobic core and a hydrophilic shell in water, which can be used for the intravenous injection of DDS carrier macromolecules. It is also confirmed that its polymer film has a microphase-separated structure useful for biomedical materials such as artificial veins. The blood compatibility of poly(LysAA-*b*-DMS) was evaluated by the Michaelis constant (K_M) and catalytic activity (k_{cat}) for the enzymatic reaction of thrombin and a synthetic substrate S-2238, as well as by the transformation of plasminogen to plasmin by tissue plasminogen activator (t-PA) using synthetic substrate S-2251 in the presence of poly(LysAA-*b*-DMS).

2. Materials and Methods

2.1. Materials

4,4'-Azobis(4-cyanopentanoic acid) was kindly supplied by Wako Pure Chemical Co. (Japan) and purified by recrystallization from methanol. 4,4'-Azobis(4-cyanopentanoic acid chloride) was prepared according to the method of Smith [18]. Reactive silicone oil (PDMS-OH; X-22-170BX, OH value: 21.0 KOH mg·g⁻¹, $M_w = 4.3 \times 10^3$, $M_n = 3.9 \times 10^3$, $M_w/M_n = 1.09$) was used as obtained from Shinetsu Chemicals Ind. (Tokyo, Japan). 1-(6-Dimethylamino-2-naphthyl)-1-dodecanone (DMAND; Molecular ProbesTM, invitrogen) was used as obtained. Human thrombin was purchased from Mochida Pharmaceutical CO., LTD. (Tokyo, Japan), whereas the synthetic chromogenic substrates S-2238 and S-2251 were purchased from Sekisui medical CO. LTD. (Tokyo, Japan), and used without any purification. Distilled and deionized water was used for all experiments.

2.2. Synthesis of

4,4'-Azobis(polydimethyl-siloxane 4-cyanopentanoate) (azo-PDMS)

To a CHCl₃ solution (150 mL) of reactive silicone oil (PDMS-OH: 19 mmol) and triethylamine (10 mmol) was added dropwise a dichloromethane solution (30 mL) of 4,4'-azobis(4-cyanopentanoic acid chloride) (9.5 mmol) at 25°C. The reaction mixture was washed with water, and then the solution was evaporated under reduced pressure. The crude azo-PDMS was purified by fractional precipitation with a solvent system of tetrahydrofuran-methanol. The molecular weight distribution was found to be $M_w/M_n = 1.12$ by means of gel permeation chromatography analysis conducted in CHCl₃ solution with TSK gel (column: TOSOH G4000H_{XL}, G3000H_{XL},

G2000H_{XL}), using TOSOH LC-8020 GPC. Yield: 68.3%. ¹H-NMR (CDCl₃, TMS): δ = 0.14 [s, 420H, -Si(CH₃)₂], 0.79 - 0.82 [m, 6H, -CH₃], 1.23 - 1.72 [m, 12H, -CH₂-], 2.32 - 2.52 [m, 4H, -CH₂-], 3.34 - 3.39 [m, 4H, -OCH₂-], 3.55 - 3.58 [m, 4H, -OCH₂-], 4.18 - 4.20 [t, 4H, -COOCH₂-]. IR(KBr) ν (cm⁻¹): 1255 (Si-CH₃), 1735 (-COOR), 2250 (-CN).

2.3. Synthesis of N- α -Acrylamide-N- ϵ -*tert*-butoxycarbonyl-L-lysine-*tert*-butylester (Boc-LysAA-OtBu)

To a CHCl₃ solution (150 mL) of N- ϵ -*tert*-butoxycarbonyl-L-lysine-*tert*-butyl ester (15 mmol) and triethylamine (23 mmol) was added dropwise a CHCl₃ solution (30 mL) of acrylic acid chloride (18 mmol) at 4°C. The reaction mixture was washed with 1 M HCl and water, and then the solution was evaporated under reduced pressure. The crude Boc-LysAA-OtBu was purified by passage through a basic alumina column with the mixed solvent of dichloromethane and hexane (50/50 vol%). Yield: 64.1%; mp: 62.0°C - 62.5°C. $[\alpha]_D^{20} = +20.1^\circ$.

¹H-NMR (CDCl₃, TMS): δ = 1.24 - 1.47(m, 2H, >CH-CH₂-CH₂-CH₂-CH₂-), 1.43(s, 9H, -C(CH₃)₃), 1.47(s, 9H, -C(CH₃)₃), 1.63 - 1.82 (m, 2H, >CH-CH₂-CH₂-CH₂-CH₂-), 1.81 - 2.05 (m, 1H, >CH-CH₂-CH₂-CH₂-CH₂-), 2.99 - 3.24 (m, 2H, >CH-CH₂-CH₂-CH₂-CH₂-), 4.56 - 4.60 (m, 2H, >CH-CH₂-CH₂-CH₂-CH₂-), 5.63 - 5.79(m, 1H, H-CH=CH-), 4.58 (br, 1H, -NH-), 6.12 - 6.29 (m, 1H, H-CH=CH-), 6.24 (br, 1H, -NH-), 6.28 - 6.47 (m, 1H, CH₂=CH-). ¹³C-NMR (CDCl₃, TMS): δ = 22.27 [-CH₂-], 28.01 [-C(CH₃)₃], 28.43 [-C(CH₃)₃], 29.59 [-CH₂-], 32.34 [-CH₂], 40.12 [-CH₂-], 52.44 [-CH], 79.02 [>C<], 82.21 [>C<], 126.77 [=CH₂], 130.43 [=CH], 155.94 [-CONH], 164.95 [-COO-], 171.51 [-COO-]. Elemental Anal.; Calcd for C₁₈H₃₂N₂ 356.461: C, 60.65%; H, 9.05%; N, 7.86%, Found: C, 60.46%; H, 9.06%; N, 8.08%.

2.4. Synthesis of Poly(LysAA-*b*-DMS)

A mixed solution of tetrahydrofuran and ethanol (40:60 vol%, 15 mL) containing Boc-LysAA-OtBu (10 mM), azo-PDMS (10 mM) as an initiator, and 2-mercaptoethanol (2-ME: 0.10 - 1.0 mM) as a chain transfer agent in a glass tube was degassed by the freeze-thaw technique with a liquid nitrogen bath and was sealed in a vacuum. After polymerization at 60°C for 20 h, the contents of the tube were poured into a large quantity of hexane to precipitate the precursor block copolymer, poly(Boc-LysAA-OtBu-*b*-DMS)-1-3. The number-average molecular weight (*M*_n) was calculated from gel permeation chromatography (GPC) with a polystyrene standard. GPC analysis was conducted in a dimethylformamide solution with a TSK gel column (TOSOH H_{HR} 6000, 5000, 4000, 3000, and 2000) with a TOSOH LC-

8020 GPC apparatus. The composition ratio of the Boc-LysAA-OtBu polymer segment (p) and PDMS segment (q) of poly(Boc-LysAA-OtBu-*b*-DMS) was calculated using the N content of elemental analysis performed with a Silber Hegner elemental analysensysteme GmbH. The removal of the *tert*-butoxycarbonyl (Boc) and *tert*-Bu (*t*-Bu) protecting groups in the Boc-LysAA-OtBu polymer segment was carried out as follows. Poly (Boc-LysAA-OtBu-*b*-DMS)-1-3 was dissolved in tetrahydrofuran, 80% trifluoroacetic acid was added, and the mixture was stirred at 25°C for 48 h. After removing the solvent under reduced pressure and washing with diethyl ether, the resulting copolymer was neutralized with 0.1 M sodium hydroxide, and then purified by dialysis against water using a seamless cellulose membrane (Cell-SepT1™, Spectra/Por™ Biotech, USA; MWCO 3500). Poly (LysAA-*Zb*-DMS)-1-3 was obtained from the aqueous solution by freeze drying.

2.5. Measurements of Critical Micelle Concentration (CMC)

The fluorescence spectra of an artificial blood plasma solution of poly(LysAA-*b*-DMS) were recorded with a Shimadzu RF-1500 fluorophotometer [19]. DMAND with a 364 nm excitation wavelength (λ_{EX}) was used as a fluorescent probe for hydrophobicity. The concentration of DMAND was adjusted to 1 μ M by the addition of 20 μ L of a solution containing 500 μ M DMAND in methanol to 10 mL of each amphiphilic poly(LysAA-*b*-DMS) solution. The concentration of poly(LysAA-*b*-DMS) was varied in the range of 34 - 1.0×10^4 mg·L⁻¹. The CMC was determined by the break point in the plot of the fluorescence intensity and poly(LysAA-*b*-DMS) concentration.

2.6. Measurements with Scanning Probe Microscope (SPM)

SPM images of poly(LysAA-*b*-DMS) film were taken by means of a Shimadzu SPM-9500J3 with Budget sensors BS-Tap 300AI (Innovative Solutions Bulgaria, Ltd., USA). The copolymer film for SPM measurements was prepared by casting 20 mL of an aqueous solution containing a known amount of poly(LysAA-*b*-DMS) on a mercury bath of 6.0 cm diameter. The solvent was evaporated by the freeze-dry method and a film with a thickness of 0.06 - 0.08 mm was obtained.

2.7. Measurements of Degree of Thrombin Activity Inhibition

Twenty microliters of thrombin (10 unit mL⁻¹) dissolved in a Tris buffer solution (pH 7.4), and 70 μ L of poly(LysAA-*b*-DMS) (2 mg·mL⁻¹) were placed in a 96-well microplate of diameter ϕ = 6.4 mm (Iwaki brand, Scitech

division, Asahi Techno Glass Corporation, Japan) and shaken for 10 min. Next, 10 μL of calcium chloride aqueous solution ($300 \text{ mg}\cdot\text{L}^{-1}$) and 100 μL of S-2238 ($0.1 - 1.0 \text{ mM}$), which specifically reacts to thrombin, were added, and the mixture was shaken at 37°C for 2 h. The degree of inhibition of thrombin activity was evaluated from the changes in the maximum absorption strength (λ_{max}) of *p*-nitroaniline, which was derived from S-2238, with a microplate reader (BIO-TEC Instruments, Inc., Elx808, USA). Kinetics parameters, Michaelis constant (K_M) and catalyst rate constant (k_{cat}) for the reaction of thrombin and S-2238 in the presence of poly(LysAA-*b*-DMS) were calculated from a Lineweaver-Burk plot.

2.8. Measurements of Degree of Plasminogen Activation

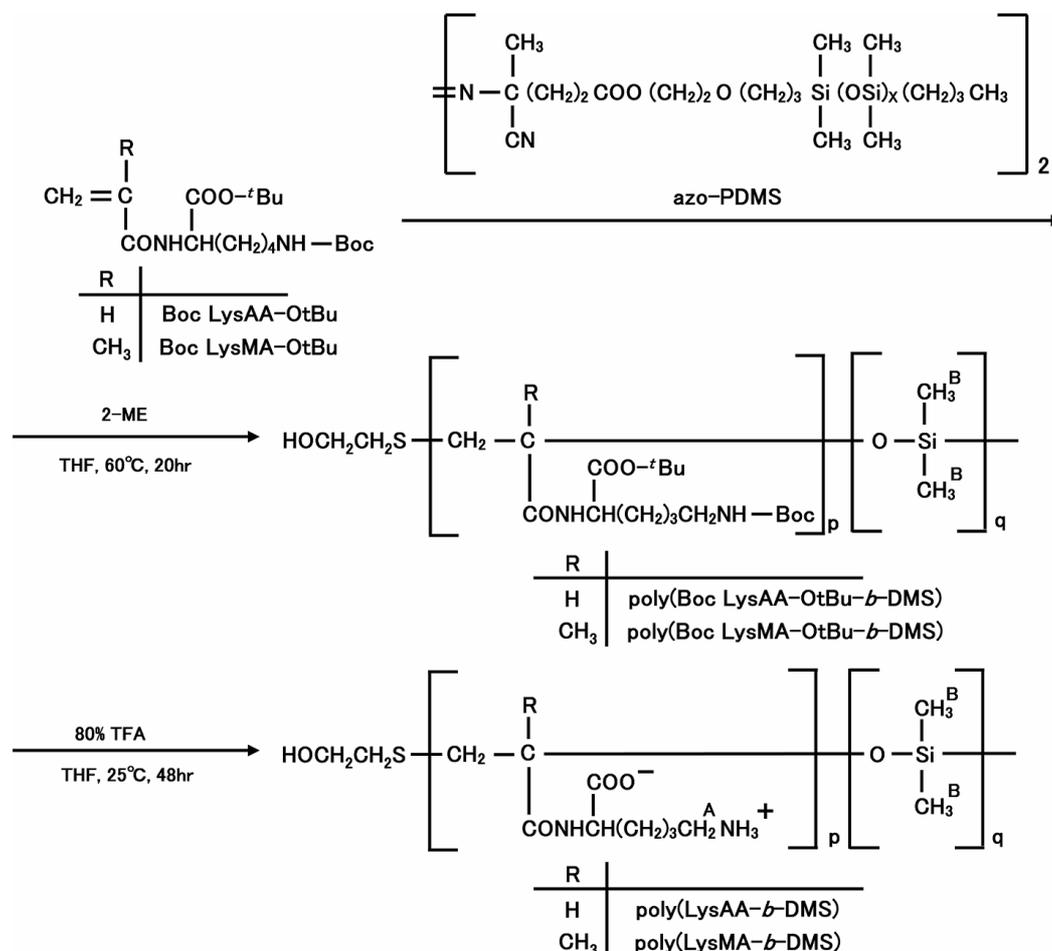
Twenty microliters of plasminogen ($34 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$) dissolved in a Tris buffer solution (pH 7.4), and 70 μL of poly(LysAA-*b*-DMS) ($2 \text{ mg}\cdot\text{mL}^{-1}$) were placed in a 96-well microplate of diameter $\phi = 6.4 \text{ mm}$, and shaken for

10 min. Next, 70 μL of S-2251 ($0.1 - 1.0 \text{ mM}$) solution and 40 μL of t-PA ($0.25 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$) solution were added and the mixture was shaken at 37°C for 2 h. The degree of plasminogen activity by t-PA in the presence of poly(LysAA-*b*-DMS) was evaluated based on the amount of *p*-nitroaniline, which was derived from S-2251, with a microplate reader (BIO-TEC Instruments, Inc., Elx808, USA). K_M and k_{cat} for the enzymatic reaction in the presence of poly(LysAA-*b*-DMS) were determined according to the method mentioned above.

3. Results and Discussion

3.1. Characterization of Block Copolymers

Amphiphilic block copolymers consisting of hydrophilic and hydrophobic segments can be prepared by the polymerization of hydrophilic monomers initiated with a polymer initiator such as azo-PDMS. A series of amphiphilic block copolymers poly(LysAA-*b*-DMS)-1-3 with different molar ratios of poly(LysAA) to PDMS segment (p:q) were synthesized according to **Scheme 1**. First, pre-



Scheme 1. Preparation of amphiphilic block copolymers.

cursor block copolymers, poly(Boc-LysAA-OtBu-*b*-DMS)-1-3, were obtained by radical polymerization of Boc-LysAA-OtBu, in which amino and carbonyl groups of the L-lysine moieties were protected with *tert*-butoxycarbonyl (Boc) and *tert*-butyl (*t*-Bu) groups, respectively, initiated with azo-PDMS in the presence of chain transfer agent 2-ME, and varying the molar ratio of 2-ME to Boc-LysAA-OtBu in the feed from 10:0.1, 10:0.5, and 10:1. For the higher concentration of 2-ME, the smaller molecular weight poly(Boc-LysAA-OtBu) segment was obtained based on the larger chain-transfer constant of 2-ME. Second, the removal of the protecting groups of poly(Boc-LysAA-OtBu-*b*-DMS)-1-3 was carried out in 80% trifluoroacetic acid aqueous solution, and then purification by dialysis against water to give poly(LysAA-*b*-DMS)-1-3. Block copolymer, poly(Boc-LysAA-OtBu-*b*-DMS) with differing chain lengths of the LysAA polymer segment, was obtained as tabulated in **Table 1**, along with the results for poly(Boc-LysMA-OtBu-*b*-DMS) [11], where Boc-LysMA-OtBu represents N- α -methacrylamide-N- ϵ -*tert*-butoxycarbonyl-L-lysine-*tert*-butyl ester. The composition ratio of poly(Boc-LysAA-OtBu) (p) : PDMS (q) was then altered to p:q = 0.35:0.65 - 0.58:0.42. With an increase in the concentration of 2-ME, the number-average molecular weight (M_n) of the poly(Boc-LysAA-OtBu) segment decreased from $M_n = 1.56 \times 10^4$ to 0.88×10^4 and the weight-average molecular weight (M_w) decreased to $M_w = 2.52 \times 10^4 - 1.02 \times 10^4$, whereas the molecular weight of the PDMS segment derived from azo-PDMS had a constant molecular weight of $M_n = 3.9 \times 10^3$. Comparing the molecular weight of poly(Boc-LysAA-OtBu-*b*-DMS) and poly(Boc-LysMA-OtBu-*b*-DMS), it can be seen that Boc-LysAA-OtBu without the methyl moiety at the α -position of the monomer causes a somewhat smaller molecular weight and larger degree of polydispersion (M_w/M_n) than those of Boc-LysMA-OtBu, because the chain transfer constant of Boc-LysAA-OtBu to 2-ME is expected to be larger than that of Boc-LysMA-OtBu based

on the higher monomer reactivity of Boc-LysAA-OtBu. The propagating rate constant (k_p) of Boc-LysAA-OtBu is also expected to be higher than that of Boc-LysMA-OtBu, though the k_p is not known yet. For example, the k_p of methyl acrylate and methyl methacrylate are 2090 and 734 L·mol⁻¹·sec⁻¹, respectively [13]. The protecting groups in the Boc-LysAA-OtBu polymer segment were then hydrolyzed in 80% trifluoroacetic acid, converting it to a hydrophilic LysAA polymer segment, and poly(LysAA-*b*-DMS)-1-3 was obtained. The removal of the protecting groups ($\delta = 1.42$ ppm, 1.46 ppm) was confirmed by ¹H-NMR spectroscopy. The removal of the protecting group of Boc-LysAA-OtBu resulted in a change in its solubility. Poly(Boc-LysAA-OtBu-*b*-DMS) is insoluble in water but soluble in acetone, diethyl ether and tetrahydrofuran, yet poly(LysAA-*b*-DMS) is soluble in water but insoluble in the organic solvents mentioned above.

3.2. Self-Organization

The self-organization of poly(LysAA-*b*-DMS) was first confirmed by ¹H-NMR measurements. The spectra of poly(LysAA-*b*-DMS)-1 in CD₃OD and D₂O, used as typical examples, are shown in **Figure 1**. The proton signal (H_B) of the methyl moiety in PDMS that appeared in CD₃OD weakened gradually in D₂O, whereas the signal of H_A in the LysAA moiety sharpened gradually with increasing water content. The half-width of the two independent peaks as a function of the D₂O content in CD₃OD is shown in **Figure 2**. The half-width of H_B ($\delta = 0$ ppm) broadened gradually with an increase in the D₂O content.

However, the half-width of H_A ($\delta = 2.8$ ppm) became narrower with an increase in the water content. The line broadening of the proton signals of the methyl group in PDMS in aqueous medium is ascribed to the restricted molecular motion of the PDMS chains upon self-organization [20,21]. Nevertheless, the mobility of the hydrophilic segments increased when the solvent polarity in-

Table 1. Preparation^{a)} and Characterization of poly(BocLysAA-OtBu-*b*-DMS).

Copolymer	2-ME mmol	Yield %	Elemental analysis			P:q ^b ×10 ⁻⁴	Mn ^c ×10 ⁻⁴	Mw ^c	Mw/Mn	
			C(%)	H(%)	N(%)					
poly(Boc LysAA-OtBu- <i>b</i> -DMS)	1	0.10	60.1	58.21	8.56	7.07	0.65:0.35	1.56	2.52	1.62
	2	0.50	58.7	55.33	8.48	6.54	0.51:0.49	1.29	1.69	1.31
	3	1.00	50.2	54.28	8.41	6.12	0.42:0.58	0.88	1.02	1.16
poly(Boc LysAA-OtBu- <i>b</i> -DMS)	1 ^d	0.10	66.4	58.52	9.02	6.63	0.59:0.41	2.32	3.41	1.47
	2	0.50	63.0	56.84	8.95	6.27	0.49:0.51	1.52	1.91	1.26
	3	1.00	58.7	54.83	8.63	5.66	0.38:0.62	1.25	1.39	1.11

a) Reaction condition: Boc LysAA-OtBu or Boc LysMA-OtBu: 10.0 mmol, DMS: 100mmol, 2-ME: 0.10 - 1.0 mmol, Mixed solvent of tetrahydrofuran-ethanol(40:60 vol %) 15 ML, 60°C, 20 hr; b) Molar ratio (p:q) in block copolymer was calculated from nitrogen content obtained by elemental analysis; c) Mn and Mw of block copolymers were estimated from GPC; d) Ref.12.

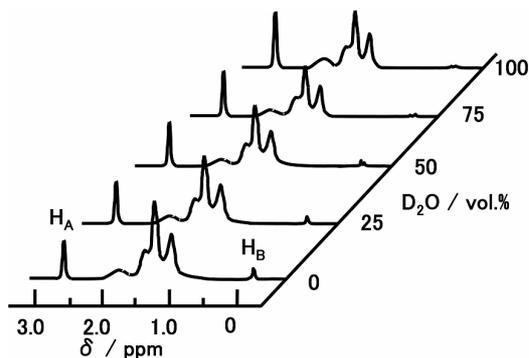


Figure 1. $^1\text{H-NMR}$ spectra of poly(LysAA-*b*-DMS)-1 in a mixed solvent of D_2O and CD_3OD , $[\text{poly}(\text{LysAA-}b\text{-DMS)-1}] = 1.0 \times 10^{-4} \text{ mg}\cdot\text{L}^{-1}$.

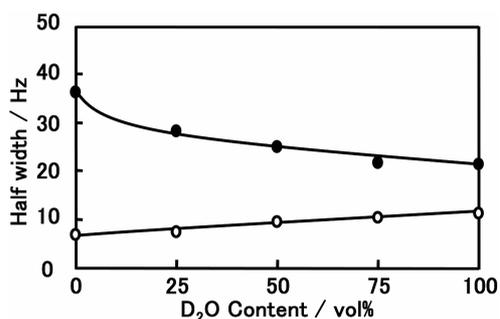


Figure 2. Effect of D_2O on the half-width of the $^1\text{H-NMR}$ proton signal of poly(LysAA-*b*-DMS)-1, ●: H_A , ○: H_B , see Scheme 1.

creased. These data suggest that poly(LysAA-*b*-DMS) self-aggregates have microdomains provided by both a core of hydrophobic PDMS segments and a mobile shell of hydrophilic poly(LysAA) segments. The self-organization ability of poly(LysAA-*b*-DMS)-1-3 was also verified in a solution of artificial blood plasma by a fluorescent probe method using DMAND at 20°C [8,17]. The concentration of poly(LysAA-*b*-DMS)-3 increased, as did the fluorescent intensity from DMAND, thus showing that the self-organization of the PDMS segment due to hydrophobic interaction created a hydrophobic core that absorbed DMAND. Note that poly(LysAA-*b*-DMS)-3 is not itself fluorescent. By plotting the largest value of fluorescent intensity and concentration of poly(LysAA-*b*-DMS)-3, we can see the breaking point at which the fluorescent intensity begins to suddenly increase ($1000 \text{ mg}\cdot\text{L}^{-1}$), as shown in **Figure 3**, and we take this as the critical micelle concentration (CMC) for self-organization. The CMCs of poly(LysAA-*b*-DMS)-1-3 with different composition ratios of the copolymer are summarized in **Table 2**. While the molecular weight of the PDMS segment is a constant, CMC decreases from 4000 to $1000 \text{ mg}\cdot\text{L}^{-1}$ as the amount of the hydrophilic LysAA polymer segment decreases.

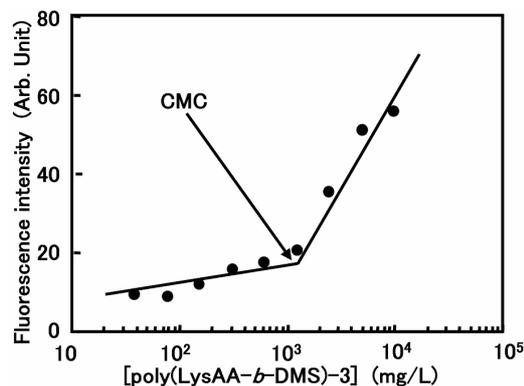


Figure 3. Relationship between the fluorescence of DMAND and concentration of poly(LysAA-*b*-DMS)-3, $[\text{poly}(\text{LysAA-}b\text{-DMS)-3}]$ was varied: 34 to $1.0 \times 10^4 \text{ mg}\cdot\text{L}^{-1}$ $[\text{DMAND}] = 1.0 \mu\text{mol}\cdot\text{L}^{-1}$, $\lambda_{\text{EX}} = 364 \text{ nm}$, $\text{CMC} = 1000 \text{ mg}\cdot\text{L}^{-1}$.

While the particle sizes of the poly(LysAA-*b*-DMS)-1-3 micelles have not yet been measured, it has been reported that in the case of poly(LysMA-*b*-DMS)-1-3, the diameters of micelles were from 220 to 270 nm as measured by light scattering [11]. It is presumed that poly(LysAA-*b*-DMS) self-assembles to form nanometersized polymer micelles in water, similar to particles of poly(LysMA-*b*-DMS). Considering the core-shell micelle, this means that hydrophobic interactions of the PDMS segment in water play a dominant role with the decrease in the length of the LysAA polymer segment in the shell, allowing a hydrophobic core to form even when the concentration of poly(LysAA-*b*-DMS)-3 is low [13]. The self-assembled poly(LysAA-*b*-DMS) is expected to play a role as a carrier molecule for DDS, making it possible to stably hold hydrophobic medicines in the core when placed in water.

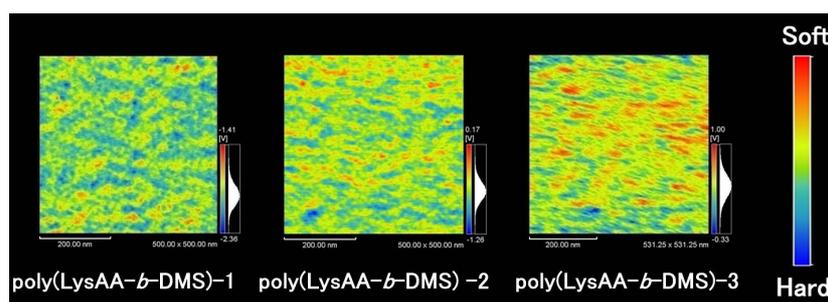
3.3. Microphase-Separated Structure

As amphiphilic poly(LysAA-*b*-DMS) is expected to be applied in biomedical materials such as artificial veins, its film manufactured by freeze drying was morphologically analyzed with a scanning probe microscope (SPM) in order to confirm its microphase-separated structure. The poly(LysAA-*b*-DMS) was found to have a microphase-separated structure with a domain size ranging from several tens to several hundreds of nanometers, as shown in **Figure 4**. The red and yellow areas of the phase image represent the soft domain from PDMS, whereas the blue areas represent the hard domain from poly(LysAA). In the case of poly(LysAA-*b*-DMS)-1, based on the composition ratio $p:q = 0.65:0.35$, the blue areas of the poly(LysAA) segment are more numerous than the yellow areas representing PDMS. On the other hand, with poly(LysAA-*b*-DMS)-3, based on the composition ratio $p:q = 0.42:0.58$, the red and yellow areas

Table 2. Kinetics Parameters for the Reaction of Thrombin and S-2238 and for the Reaction of t-PA and S-2251 in the Presence of poly(LysAA-*b*-DMS).

Copolymer	p:q ^c	S-2238 ^a						S-2238 ^a			
		CMC mg·L ⁻¹	V_{max} $\times 10^{-2}$ mM·min ⁻¹	K_M mM	k_{cat} $\times 10^{-5}$ min ⁻¹	k_{cat}/K_M $\times 10^{-5}$ mM·min ⁻¹	V_{max} $\times 10^{-3}$ mM·min ⁻¹	K_M mM	k_{cat} $\times 10^{-6}$ min ⁻¹	k_{cat}/K_M $\times 10^{-5}$ mM·min ⁻¹	
poly(LysAA- <i>b</i> -DMS)	1	0.65:0.35	4000	2.13	0.11	4.26	3.87	8.63	0.07	5.94	8.50
	2	0.51:0.49	3000	2.11	0.11	4.22	3.84	8.49	0.07	5.83	8.37
	3	0.42:0.58	1000	2.02	0.10	4.04	4.04	8.31	0.07	5.71	8.19
poly(LysAA- <i>b</i> -DMS)	1	0.59:0.41	2000	1.96	0.17	3.92	2.31	6.86	0.09	4.93	5.26
	2	0.49:0.51	1000	1.86	0.16	3.72	2.33	6.31	0.09	4.35	4.83
	3	0.38:0.62	500	1.82	0.15	3.64	2.43	6.03	0.09	4.02	4.62
Control	-	-	-	0.14	1.62	2.68	1.91	0.09	4.01	1.28	1.72

a) [Thrombin] = 10 unit·mL⁻¹, [poly(LysAA-*b*-DMS)] = 2 mg·mL⁻¹, [S-2238] = 0.1 - 1.0 mM, Ph 7.4, 37°C, 2 h; b) [plasminogen] = 34 μ g·mL⁻¹, [t-PA] = 0.25 μ g·mL⁻¹, [poly(LysAA-*b*-DMS)] = 2 mg·mL⁻¹, [S-2251] = 0.1~1.0 mM pH 7.4, 37°C, 2 h; c) p:q represents the molar ratio of poly(LysAA) and PDMS segments; d) Ref.12.

**Figure 4.** SPM images of poly(LysAA-*b*-DMS) films.

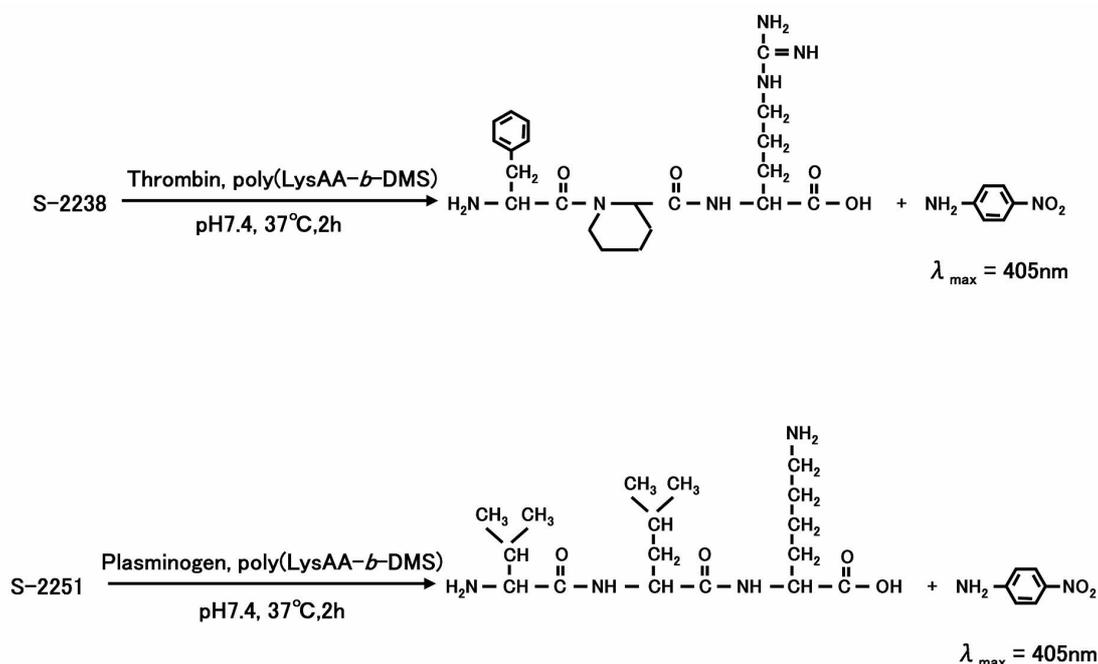
outnumber the blue. The ratio of poly(LysAA) segment (p) in the copolymer decreased with increasing amounts of 2-ME due to lowering of the molecular weight of poly(LysAA) based on the chain transfer reaction. In this manner, we can see that poly(LysAA-*b*-DMS) possesses a sea-island structure, made up of domains corresponding to the composition ratio.

3.4. Evaluation of Inhibition of Thrombin Activity

In order to use poly(LysAA-*b*-DMS) in biomedical materials such as DDS carrier molecules for intravenous injection and artificial blood vessels, it must have a high blood compatibility. To confirm this, the inhibition of thrombin activity was evaluated. Thrombus formation includes a process whereby fibrinogen is converted to fibrin by thrombin. Furthermore, the fibrin forms a cross-linked structure by transglutaminase XIIIa and completes the blood clotting. The level of antithrombotic activity can be verified by the enzymatic reaction of thrombin and a synthetic substrate S-2238 in the presence of poly(LysAA-*b*-DMS)-1-3 as shown in **Scheme 2** [22]. The reaction in the absence of poly(LysAA-*b*-DMS) was used as a control. The reaction rate [V] is monitored by

the absorption change at 405 nm of *p*-nitroaniline derived from S-2238 over time when thrombin causes the C-terminal of the residual arginine to hydrolyze [16].

A Lineweaver-Burk plot of this reaction is shown in **Figure 5**. [V] is the rate in terms of the amount of *p*-nitroaniline produced by one unit of thrombin in one minute, while [S] represents the initial concentration of S-2238. By the slope of the line (K_M/V_{max}) and the intercept ($1/V_{max}$), kinetics parameters such as the maximum rate of enzymatic reaction (V_{max}), Michaelis constant (K_M) and the catalyst constant of enzyme (k_{cat}) in the presence of poly(LysAA-*b*-DMS)-3 were calculated as summarized in **Table 2**, together with those data obtained in the presence of poly(LysMA-*b*-DMS). The V_{max} was 2.02×10^2 - 2.13×10^2 mM·min⁻¹ and that of the control was 0.14×10^2 mM·min⁻¹. With increasing molar ratio of the poly(LysAA) segment, k_{cat} increased from 4.04×10^5 - 4.26×10^5 min⁻¹, while K_M remained almost constant. k_{cat}/K_M decreased from 4.04×10^5 - 3.87×10^5 mM⁻¹·min⁻¹ with increasing molar ratio of the poly(LysAA) segment. K_M , k_{cat} , and k_{cat}/K_M were 0.06 - 0.07, 1.51 - 1.59, and 2.12 - 2.03 times those of the control, respectively. The effect of the molecular weight of the poly(LysAA) segment on the kinetics data cannot be



Scheme 2. Enzymatic reaction of thrombin/S-2238 and plasminogen /S-2251 in the presence of poly(LysAA-*b*-DMS).

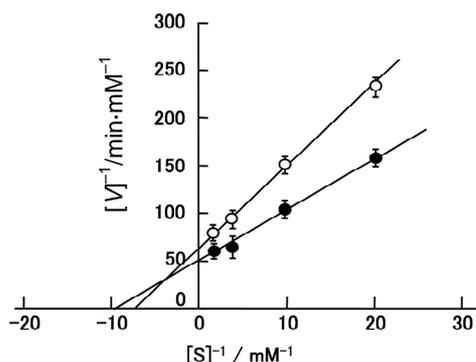


Figure 5. Lineweaver-Burk plots for the reaction of thrombin and S-2238 in the absence (\circ :control) and in the presence of poly(LysAA-*b*-DMS)-3 (\bullet) in a Tris buffer solution (pH = 7.4) at 37°C, [Thrombin] = 1 unit, [S-2238] was varied from 0.05 to 0.5 mM. [poly(LysAA-*b*-DMS)-3] = 0.7 $\text{mg}\cdot\text{L}^{-1}$.

regarded as significant. Comparing poly(LysAA-*b*-DMS) and poly(LysMA-*b*-DMS), the former shows larger k_{cat} and $k_{\text{cat}}/K_{\text{M}}$ than the latter with k_{cat} (3.64×10^5 - $3.92 \times 10^5 \text{ min}^{-1}$) and $k_{\text{cat}}/K_{\text{M}}$ (2.43×10^6 - $2.31 \times 10^6 \text{ mM}^{-1}\cdot\text{min}^{-1}$). From the difference in $k_{\text{cat}}/K_{\text{M}}$ of the enzymatic reaction in the presence of the two different copolymers, poly(LysAA-*b*-DMS) shows a somewhat stronger tendency to promote thrombin activity than poly(LysMA-*b*-DMS). The poly(LysAA) segment can interact with thrombin and promote the enzymatic reaction more efficiently than the poly(LysMA) segment, due to the differences in their flexibility. From the kinetics parameters of the enzymatic reaction, it was found that neither of the two copolymers

could inhibit the thrombin activity.

3.5. Evaluation of Activation of Tissue Plasminogen Activator (t-PA)

Plasmin, which converts fibrinogen to a fibrin net, exists as a precursor called plasminogen in the blood. Plasminogen is activated by t-PA, becoming the fibrinolytic agent plasmin, and dissolving the fibrin net. At this point, the L-lysine-binding sites (LBS) of the plasminogen or t-PA interact with the L-lysine group of fibrin. The degree of activation can be verified using the enzymatic reaction of plasmin and a synthetic substrate S-2251 [23]. It is known that polymeric materials with surfaces modified by L-lysine groups exhibit strong interaction with plasminogen, increasing the fibrinolytic activity [24-26].

We have also reported that plasminogen activation by t-PA using S-2251 in the presence of poly(LysMA) increases fibrinolytic characteristics compared to a control in the absence of poly(LysMA) [12].

Based on this finding, fibrinolytic activity was verified by enzymatic reaction of plasminogen and t-PA using S-2251 in the presence of poly(LysMA-*b*-DMS) as shown in Scheme 2. A Lineweaver-Burk plot of this enzymatic reaction is shown in Figure 6. Kinetic parameters in the presence of poly(LysAA-*b*-DMS)-3 were calculated as summarized in Table 2, together with those parameters obtained in the presence of poly(LysMA-*b*-DMS). V_{max} , K_{M} , and k_{cat} increased with increasing molar ratio of the poly(LysAA) segment. The V_{max} of the transformation of plasminogen to plasmin by t-PA in

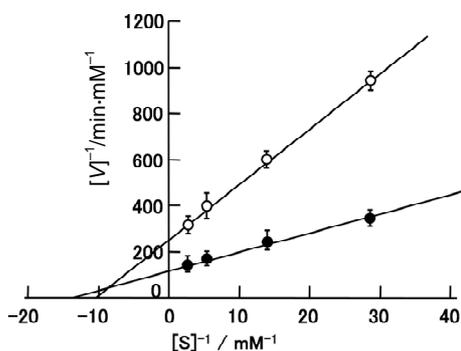


Figure 6. Lineweaver-Burk plots for the reaction of plasminogen by t-PA and S-2251 in the absence (\circ : control) and in the presence of poly(LysAA-*b*-DMS)-3 (\bullet) in a Tris buffer solution (pH=7.4) at 37°C, [PIg] = 34 μL^{-1} , [S-2251] was varied from 0.035 to 3.5 mM, [poly(LysAA-*b*-DMS)-3] = 0.7 $\text{mg}\cdot\text{mL}^{-1}$, [t-PA] = 0.2 $\mu\text{g}\cdot\text{mL}^{-1}$.

the presence of poly(LysAA-*b*-DMS)-1-3 using S-2251 was found to be 92 - 96 times faster than the control, proving its increased fibrinolytic activities. K_M was 0.017 times that of the control, while k_{cat} was 4.46 - 4.64 times that of the control. It is suggested that the blood compatibility of the L-lysine group in poly(LysAA-*b*-DMS) is due to the strong interaction of the L-lysine residue and the L-lysine-binding sites (LBS) of the plasminogen [12]. With increasing molar ratio of the poly(LysAA) segment, V_{max} , k_{cat} , and k_{cat}/K_M increased from 8.31×10^3 - 8.63×10^3 $\text{mM}\cdot\text{min}^{-1}$, 5.71×10^6 - 5.95×10^6 min^{-1} , and 8.19×10^5 - 8.50×10^5 $\text{M}^{-1}\cdot\text{min}^{-1}$, respectively, while K_M was constant. Comparing poly(LysAA-*b*-DMS) and poly(LysMA-*b*-DMS), the former shows larger k_{cat} and k_{cat}/K_M than the latter with k_{cat} (4.02×10^6 - 4.93×10^6 min^{-1}) and k_{cat}/K_M (4.62×10^5 - 5.26×10^5 $\text{mM}^{-1}\cdot\text{min}^{-1}$). These data indicate that poly(LysAA-*b*-DMS) shows a stronger tendency to promote fibrinolytic activity than poly(LysMA-*b*-DMS). It is explained that the L-lysine residue of poly(LysAA) segment can interact with t-PA easier and accelerate the fibrinolysis better than the poly(LysMA) segment can based on the difference in their flexibilities as mentioned above. It should be emphasized from the difference in k_{cat}/K_M of the enzymatic reaction in the presence of different block copolymers that poly(LysAA-*b*-DMS) exhibits much more tendency to promote fibrinolysis than poly(LysMA-*b*-DMS) does.

3.6. Surface Plasmon Resonance Measurements

From the surface plasmon resonance measurements on the polymer immobilized sensor surface, the association equilibrium constants (K_A) for the enzymatic reaction of plasminogen with poly(α -LysAA) and poly(α -LysMA) were determined to be 389×10^9 and 0.281×10^9 , respectively. K_A for the reaction of t-PA with poly(α -LysAA) and poly(α -LysMA) were found to be 155×10^9 and

6.24×10^9 , respectively [27]. This means that poly(α -LysAA) binds more strongly to plasminogen and t-PA than poly(α -LysMA). Based on these results, it was found that poly(LysAA-*b*-DMS) promotes thrombin activity and is a better enhancer of fibrinolytic activity than poly(LysMA-*b*-DMS) in promoting the conversion of plasminogen into plasmin.

4. Conclusions

From the results obtained here, the following conclusions can be drawn:

- 1) Because the nanometer-sized micelles derived from self-assembly of poly(LysAA-*b*-DMS) had microdomains consisting of a hydrophobic core of PDMS segments and a mobile shell of hydrophilic poly(LysAA) segments, lipophilic drugs could be included in the hydrophobic core to form a novel intravenous injection DDS.
- 2) A film of amphiphilic copolymer poly(LysAA-*b*-DMS) adopts a microphase-separated structure. The copolymer is expected to be applied in biomedical polymeric materials for artificial organs such as blood vessels.
- 3) The inhibition of thrombin activity is promoted by the presence of poly(LysAA-*b*-DMS) in the enzymatic reaction system more than poly(LysMA-*b*-DMS).
- 4) Because it can promote the conversion of plasminogen into plasmin by t-PA, poly(LysAA-*b*-DMS) enhances the fibrinolytic activity of t-PA, thus increasing blood compatibility.
- 5) Both activities of the inhibition of thrombin and fibrinolysis are influenced by the differences in the molar ratio of poly(LysAA) and the flexibility of the main chain hydrophilic polymer segment. The kinetic parameters of the enzymatic reaction in the presence of poly(LysAA-*b*-DMS) containing the flexible poly(LysAA) segment showed larger K_M and k_{cat} than poly(LysMA-*b*-DMS).

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